AWARD NUMBER: W81XWH-17-2-0026

TITLE: Collaborative Research to Optimize Warfighter Nutrition (CROWN)III

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REPORT DATE: November 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2022	Final	15Jul2017 – 14Jul2022

4. TITLE AND SUBTITLE

Collaborative Research to Optimize Warfighter Nutrition (CROWN)III

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-2-0026

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Louisiana State University System
Pennington Biomedical Research Center
6400 Perkins Rd
Baton Rouge, LA 70808-4124

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Collaborative Research to Optimize Warfighter Nutrition (CROWN) III project is a series of 5 projects that used nutrition, metabolism and human physiology to address objectives relevant to the nutritional health of military personnel. Scientists at Pennington Biomedical Research Center and the Military Nutrition Division at the U.S. Army Research Institute of Environmental Medicine collaborated on the design, execution, and analysis of the 5 research projects that fall within three topic areas: Operational Stress and Nutritional Requirements; Nutrition, Inflammation and Resiliency; and Healthy Eating and Behavior. The 5 projects were as follows:
1. Determine the physiological and psychological effects of testosterone during severe energy deficit and recovery
2. Determine the substrate utilization, exercise performance, and skeletal muscle response to energy deficit and altitude acclimatization
3. Effects of energy balance and energy deficit on inflammatory response
4. Go for Green (GFG) Effectiveness
5. Development of a valid and military-appropriate survey tool for the assessment of Soldier eating behaviors
The outcomes provided the scientific basis to develop novel nutritional programs, products, and strategies that promote Warfighter health, performance and resilience to operational stress.

15. SUBJECT TERMS
Metabolism, nutrition, energy expenditure, readiness, performance, warfighter, resilience, operational stress

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	487	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	4
3. Accomplishments	5
4. Impact	27
5. Changes/Problems	30
6. Products	31
7. Participants & Other Collaborating Organizations	38
8. Special Reporting Requirements	42
9. Appendices	42

1. INTRODUCTION:

The Collaborative Research to Optimize Warfighter Nutrition (CROWN) III project is a series of projects that used nutrition, metabolism, and human physiology research to address objectives relevant to the nutritional health of personnel in all branches of the military as well as the American public. Scientists at Pennington Biomedical Research Center (PBRC) and the Military Nutrition Division at the U.S. Army Research Institute of Environmental Medicine (USARIEM) collaborated on the design, execution, and analysis of the research. The outcomes provide the scientific basis to develop novel nutritional programs, products, and strategies that promote Warfighter health, performance, and resilience to operational stress. This project enables advances in our understanding of operational stressors on nutritional requirements; examines effectiveness of nutritional approaches for combating inflammation, sustaining gut health, and promoting metabolic recovery; develops better tools for assessing eating behaviors; and establishes methods that lead to healthier food choices and overall diet quality. The body of work conducted by PBRC provides an efficient and cost-effective approach for achieving the DOD objective of a healthy and fit fighting troop base, ready for deployment and resilient to the stressors of duty. In order to achieve our objective, we conducted a series of projects that fall within the following persisting gap (topic) areas:

1. Operational Stress and Nutritional Requirements
2. Nutrition, Inflammation, and Resiliency
3. Healthy Eating and Behavior

We conducted 5 projects that are listed below:

1. Determine the physiological and psychological effects of testosterone during severe energy deficit and recovery
2. Determine the substrate utilization, exercise performance, and skeletal muscle response to energy deficit and altitude acclimatization
3. Effects of energy balance and energy deficit on inflammatory response
4. Go for Green (GFG) Effectiveness
5. Development of a valid and military-appropriate survey tool for the assessment of Soldier eating behaviors.

2. KEYWORDS:

Metabolism, nutrition, energy expenditure, readiness, performance, warfighter, resilience, operational stress

3. ACCOMPLISHMENTS:

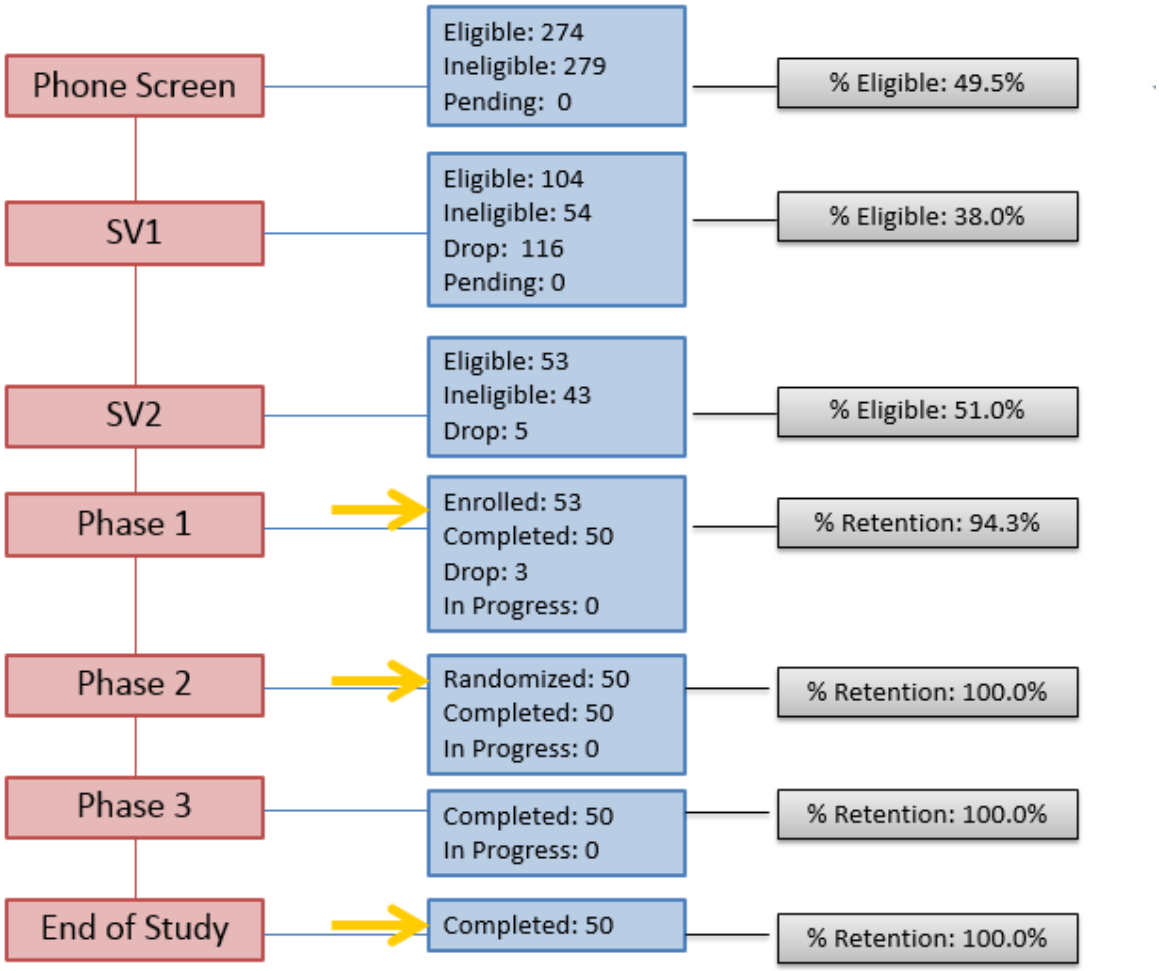
The major goals as defined in the statement of work involve 5 individual projects. Each of those projects is described below along with milestones and dates of completion.

Project 1: Determine the physiological and psychological effects of testosterone during severe energy deficit and recovery

Specific Aim: Clinical study to assess the effects of testosterone during severe energy deficit and recovery

Major Task 1: Conduct the clinical study (months 1-12)

This task was completed in September 2017. The recruitment and enrollment numbers are shown below.



This project was a continuation from a previous award and as such the Pennington Biomedical Research Center Institutional Review Board (IRB) and Human Research Protection Office (HRPO) approvals were obtained under the previous award. However, I have listed the IRB and HRPO numbers below:

"Physiological and Psychological Effects of Testosterone During Severe Energy Deficit and Recovery: A Randomized, Placebo Controlled Trial," Submitted by Jennifer C. Rood, PhD, Pennington Biomedical Research Center, Baton Rouge, Louisiana, in Support of the Proposal "Collaborative Research to Optimize Warfighter Nutrition II," Proposal Log Number 13210003, Award Number W81XWH-14-1-0335, HRPO Log Number A-18080.2, Pennington Biomedical Research Center IRB #2015-063-PBRC OPS

This trial is registered on ClinicalTrials.gov as NCT02734238.

I received continuing review approval for this project from the PBRC IRB on September 20, 2017. All data was collected and I requested that the IRB status be changed from "active" to "data analysis only." I received the approval on January 31, 2018. I notified HRPO of this status change to the project on February 22, 2018. Upon completion of data analysis, I asked the PBRC IRB to close the study, which they did on May 9, 2022. I reported this to HRPO on June 13, 2022, and they closed the file.

Major Task 2: Data Analysis (months 6-24)

Data analysis for this project began in Year 1 and continued through Year 5.

Biospecimens (blood, urine, saliva, muscle tissue, and fecal samples) were analyzed in Years 1-2 for basic biochemistry markers, markers of endocrine function, gut hormones, inflammatory markers, and measures of metabolism. Data analysis was completed and reviewed in Year 3.

We completed blood analysis on the following analytes: glucose, creatinine, potassium, uric acid, albumin, calcium, magnesium, CPK, ALT, alkaline phosphatase, iron, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, complete blood count with differential, cortisol, insulin, total testosterone, free testosterone, FSH, LH, estradiol, SHBG, lipoprotein binding protein, DHEAs, ferritin, IGF-1, leptin, LpA, prolactin, prostate specific antigen, soluble transferrin receptor, hepcidin, active ghrelin, Interleukin 6, amino acids, erythroferrone, erythropoietin, glucagon-like peptide-1, des-acyl ghrelin, and peptide YY (PYY). We also completed insulin, leptin, and lipoprotein binding protein.

We also completed total nitrogen and creatinine on all of the urine samples collected during the study. All results were sent to the PI via the secure website https://safe.amrdec.army.mil/safe/.

De-identified fecal samples were shipped to USARIEM for short-chain fatty acid concentration analysis.

Analysis of questionnaires, including 3-day food records, cognitive tests, and physical activity logs, was completed.

Analysis of body composition data from dual energy X-ray absorptiometry (DXA) and brain imaging/function from functional magnetic resonance imaging (fMRI) was completed in Year 4. In total, there are 132 body composition variables that were measured and/or calculated during the DXA test. The primary outcome from DXA is lean body mass and fat mass and the distribution of these two compartments throughout the body.

Exercise and strength testing data from VO2 max and Biodex testing was compiled. The three main variables are strength, total endurance, and peak torque.

Major Task 3: Data Interpretation and Publishing of Results (months 6-12)
Milestone #1: Co-author initial manuscript on findings from clinical study (months 6-12)

The initial manuscript for this project was prepared in Year 1. It was submitted for publication, reviewed, revised, and resubmitted in Year 2. This milestone was met when the first manuscript was published in Year 3. Additional manuscripts were published in Years 3, 4, and 5. Copies of the published manuscripts are included in the appendices:

1. Pasiakos SM, Berryman CE, Karl JP, Lieberman HR, Orr JS, Margolis LM, Caldwell JA, Young AJ, Montano MA, Evans WJ, Vartanian O, Carmichael OT, Gadde KM, Johannsen NM, Beyl RA, Harris MN, Rood JC. Effects of testosterone supplementation on body composition and lower-body muscle function during severe exercise- and diet-induced energy deficit: A proof-of-concept, single centre, randomised, double-blind, controlled trial. EBioMedicine. 2019 Aug;46:411-422. doi: 10.1016/j.ebiom.2019.07.059. Epub 2019 Jul 27. PMID: 31358477. PMCID: PMC6711889.

2. Hennigar SR, Berryman CE, Harris MN, Karl JP, Lieberman HR, McClung JP, Rood JC, Pasiakos SM. Testosterone Administration During Energy Deficit Suppresses Hepcidin and Increases Iron Availability for Erythropoiesis. J Clin Endocrinol Metab. 2020 Apr 1;105(4). doi: 10.1210/clinem/dgz316. Epub 2020 Jan 2. PMID: 31894236.

3. Karl JP, Berryman CE, Harris MN, Lieberman HR, Gadde KM, Rood JC, Pasiakos SM. Effects of testosterone supplementation on ghrelin and appetite during and after severe energy deficit in healthy men. J. Endocr. Soc. 2020 Apr 1;4(4):bvaa024. doi: 10.1210/jendso/bvaa024. eCollection 2020 Apr 1. PMID: 32258956. PMCID: PMC7101089.

4. Howard EE, Margolis LM, Berryman CE, Lieberman HR, Karl JP, Young AJ, Montano MA, Evans WJ, Rodriguez NR, Johannsen NM, Gadde KM, Harris MN, Rood JC, Pasiakos SM. Testosterone supplementation up-regulates androgen receptor expression and translational capacity during severe energy deficit. Am J Physiol Endocrinol Metab. 2020 Oct 1;319(4):E678-E688. doi: 10.1152/ajpendo.00157.2020. Epub 2020 Aug 10. PubMed PMID: 32776828. PMCID: PMC7750513.

5. Carmichael OT, Pillai SR, Murray K, Shankapal P, Caldwell J, Vartanian O, Berryman CE, Karl JP, Harris M, Rood JC, Pasiakos SM, Lieberman HR. Effects of testosterone administration on fMRI responses to executive function, aggressive behavior, and emotion processing tasks during severe exercise- and diet-induced energy deficit.

Neuroimage. 2021 Aug 20;243:118496. doi: 10.1016/j.neuroimage.2021.118496. Online ahead of print. PMID: 34425226.

6. Howard EE, Shankaran M, Evans WJ, Berryman CE, Margolis LM, Lieberman HR, Karl JP, Young AJ, Montano MA, Matthews MD, Bizieff A, Nyangao E, Mohammed H, Harris MN, Hellerstein MK, Rood JC, Pasiakos SM. Effects of testosterone on mixed-muscle protein synthesis and proteome dynamics during energy deficit. J Clin Endocrinol Metab. 2022 May 9:dgac295. doi: 10.1210/clinem/dgac295. Online ahead of print. PMID: 35532889.

Another manuscript for Project 1 was accepted by an international conference and published as a book chapter:

Ramakrishnapillai S. et al. (2021) Constrained Learning of Task-Related and Spatially-Coherent Dictionaries from Task fMRI Data. In: Abdulkadir A. et al. (eds) Machine Learning in Clinical Neuroimaging. MLCN 2021. Lecture Notes in Computer Science, vol 13001. Springer, Cham. https://doi.org/10.1007/978-3-030-87586-2_17.

Four abstracts were presented at national conferences. Copies of the abstracts are included in the appendices:

1. Hennigar S, Berryman C, Harris M, McClung J, Rood J, Pasiakos S. Testosterone Administration During Energy Deficit Suppresses Hepcidin Concentrations and Increases Iron Utilization in Healthy Males. American Society for Nutrition, June 2019, Baltimore, MD. Curr Dev Nutr. 2019 Jun 13;3(Suppl 1). pii: nzz044.OR15-08-19. doi: 10.1093/cdn/nzz044.OR15-08-19. eCollection 2019 Jun. PMID: 31224847 PMCID: PMC6575034.

2. Howard EE, Margolis LM, Berryman CE, Lieberman HR, Karl JP, Young AJ, Montano MA, Evans WJ, Rodriguez NR, Johannsen NM, Gadde KM, Harris MN, Rood JC, Pasiakos SM. Testosterone supplementation upregulates myogenesis and attenuates proteolytic gene expression during severe energy deficit. American College of Sports Medicine, May 27, 2020.

3. Pasiakos S, Shankaran M, Berryman CE, Margolis LM, Lieberman HR, Karl JP, Young AJ, Montano MA, Matthews MD, Bizieff AA, Nyangau E, Mohammed HA, Harris MN, Evans WJ, Hellerstein MK, Rood JC. Effects of Testosterone on Mixed Muscle Protein Synthesis and Proteome Dynamics During Energy Deficit. American Society for Nutrition, May 2020.

4. Berryman C, Shankaran M, Nyangau E, Evans W, Hellerstein M, Rood J, Pasiakos S. Longitudinal Comparison of Lean Body Mass by Dual Energy X-Ray Absorptiometry and Muscle Mass by Creatine (methyl-d3) Dilution in Response to a 28-d Severe Energy Deficit. American Society for Nutrition, 29 May 2020. Current Developments in Nutrition, Volume 4, Issue Supplement_2, June 2020, Page 614, https://doi.org/10.1093/cdn/nzaa049_007.

This study aimed to determine whether preventing hypogonadism would mitigate declines in physical and mental function that typically occur during prolonged energy deficit, and the efficacy of testosterone replacement on recovery from severe underfeeding. Fifty physically active men participated in a three-phase, randomized, placebo-controlled study. After completing a 14-day, energy-adequate, diet acclimation phase (protein: 1.6g·kg-1·d-1; fat: 30% total energy intake), participants were randomized to undergo a 28-day, 55% energy deficit phase with (DEF+TEST: 200mg testosterone enanthate per week) or without (DEF) exogenous testosterone. Diet and physical activity were rigorously controlled. Recovery from the energy deficit (ad libitum diet, no testosterone) was assessed until body mass was recovered within ±2.5% of initial body mass. Body composition, stable isotope methodologies, proteomics, muscle biopsies, whole-room calorimetry, molecular biology, activity/sleep monitoring, personality and cognitive function assessments, functional MRI, and comprehensive biochemistries were used to assess physiological and psychological responses to energy restriction and recovery feeding while volunteers were in an expected hypogonadal versus eugonadal state.

The major finding in this trial was that subjects in the placebo group lost more body weight than those receiving testosterone. Fat mass decreased similarly in both groups while lean mass increased in the testosterone group compared to the placebo group. Bone mineral content did not differ between the two groups. Lower body muscle function declined in both groups and there was no preservation or improvement of muscle function in the testosterone group.

Hepcidin declined by 41%, indicators of iron turnover increased, and functional iron stores were reduced with testosterone administration during energy deficit. Testosterone administration during energy deficit increased circulating concentrations of erythropoietin and maintained erythropoiesis, as indicated by an attenuation in the decline in hemoglobin and hematocrit with placebo. Erythroferrone did not differ between groups, suggesting that the reduction in hepcidin with testosterone occurs through an erythroferrone-independent mechanism.

Testosterone supplementation during severe energy deficit prevented deficit-induced increases in fasting acyl ghrelin concentrations, and in post-prandial acyl and des-acyl ghrelin concentrations in nonobese, healthy men, but these effects did not blunt energy deficit-induced increases in appetite, slow recovery of total body mass, or impact changes in fat mass. These findings confirm and extend evidence supporting an inhibitory effect of testosterone supplementation on circulating ghrelin in healthy men, but suggest this effect is not sufficient to suppress appetite during energy deficit or subsequent body mass recovery.

This study presents novel data reflecting skeletal muscle molecular adaptations to supplemental testosterone during a severe exercise- and diet-induced energy deficit. Physically active, nonobese men who received 200 mg testosterone enanthate per week (TEST) and those who received a placebo (PLA) had similar molecular responses to an exercise bout and protein-containing mixed meal. Lean mass differences appear predominantly driven by adaptations under resting fasted conditions. Resting androgen receptor (AR) protein content was higher and fibroblast growth factor-inducible 14 (Fn14), IL-6 receptor (IL-6R), and muscle ring-finger protein-1 gene expression was lower in TEST versus PLA during 28 days of an exercise- and diet-induced 55% energy deficit (ED) relative to 14 prior days of weight maintenance (WM) ($P < 0.05$). Changes in inflammatory, myogenic, and proteolytic gene expression did not differ between groups after exercise and recovery feeding. Mechanistic target of rapamycin (mTOR)

signaling (i.e., translational efficiency) was also similar between groups at rest and after exercise and the mixed meal. Muscle total RNA content (i.e., translational capacity) increased more during ED in TEST than PLA ($P < 0.05$). These findings indicate that attenuated proteolysis at rest, possibly downstream of AR, Fn14, and IL-6R signaling, and increased translational capacity, not efficiency, may drive lean mass accretion with testosterone administration during energy deficit. Additionally, mTOR pathway activation did not differ between groups at rest or in response to exercise; however, a more positive change in skeletal muscle total RNA content at rest in TEST compared with PLA suggests increased translational capacity, not efficiency, drives lean mass accretion in response to testosterone supplementation during ED.

This study found that in a group of healthy young men subjected to 28 days of severe energy deficit, testosterone administration was associated with differences in behavior and brain functioning at the end of the administration period, as well as differences in brain functioning at a post-recovery time point. First, those receiving testosterone experienced changes in fMRI-assessed brain function over the treatment period during two of five cognitive tasks administered, compared to those receiving placebo. Second, those who were treated with testosterone exhibited greater self-reported levels of anger during a provoked aggression task shortly after testosterone was discontinued and testosterone concentrations were still elevated, compared to those who received placebo. Third, overall patterns of change over time in brain functioning and task performance in this cohort of energy-deficient individuals were largely congruent with those observed previously in energy-balanced individuals. Overall, these findings suggests that 28 days of sustained-release testosterone treatment has significant behavioral and brain functional effects on energy-deficient young men. However, some effects of testosterone observed in other studies were not observed, possibly due to differing participant characteristics, different energy status, and especially different dose, time course, and formulation of testosterone treatment. These results point to the need for additional research on the neurocognitive effects of chronic testosterone administration in diverse realistic settings.

The data demonstrates that energy deficit has a strong suppressive effect on mixed-muscle protein synthesis (MPS), which was markedly lower at the middle and end of the 28-day energy deficit. Mixed-MPS did not differ between treatment groups; however, testosterone supplementation did result in a high proportion of proteins across the muscle proteome with numerically greater synthesis rates than placebo during the second half of the energy deficit. Muscle mass measured by D_3-creatine dilution did not change during the energy deficit phase but was greater in TEST than PLA with ad libitum recovery feeding. The divergent values of lean body mass and muscle mass are consistent with DXA-based lean mass not being an accurate surrogate measure of muscle mass. These findings collectively reveal a stimulatory effect of supplemental testosterone on synthesis rates across the muscle proteome during prolonged energy deficit that results in muscle mass accretion with subsequent periods of positive energy balance.

Project 2: Determine the substrate utilization, exercise performance, and skeletal muscle response to energy deficit and altitude acclimatization (study completed prior to award)

Specific Aim: Clinical study to assess the response to energy deficit and altitude acclimatization

This clinical study and data collection was completed under a previous award. However, some of the data was analyzed and published under this award.

Major Task 1: Data Interpretation and Publishing of Results (months 1-12)
Milestone #2: Co-author initial manuscript on findings from clinical study (months 1-12)

This milestone was achieved in Year 1, when the first five manuscripts from this project were published. Additional manuscripts were published in Years 2, 3, and 4. Copies of the published manuscripts are included in the appendices:

1. Berryman CE, Young AJ, Karl JP, Kenefick RW, Margolis LM, Cole RE, Carbone JW, Lieberman HR, Kim IY, Ferrando AA, Pasiakos SM. Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. FASEB J. 2018 Feb;32(2):894-905. doi: 10.1096/fj.201700915R. Epub 2018 Jan 3. PMID: 29066613.

2. Karl JP, Cole RE, Berryman CE, Finlayson G, Radcliffe PN, Kominsky MT, Murphy NE, Carbone JW, Rood JC, Young AJ, and Pasiakos SM. Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high altitude, but are not affected by protein intake. High Alt Med Biol. 2018 Jun;19(2):156-169. doi: 10.1089/ham.2017.0155. Epub 2018 Feb 12. PMID: 29431471. PMCID: PMC6014054.

3. Margolis LM, Carbone JW, Berryman CE, Carrigan CT, Murphy NE, Ferrando AA, Young AJ, Pasiakos SM. Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. FASEB J. 2018 Jun 7:fj201800163RR. doi: 10.1096/fj.201800163RR. Epub ahead of print. PMID: 29878853.

4. Pasiakos SM, Berryman CE, Carbone JW, Murphy NE, Carrigan CT, Bamman MM, Ferrando AA, Young AJ, Margolis LM. Muscle Fn14 gene expression is associated with fat-free mass retention during energy deficit at high altitude. Physiol Rep. 2018 Jul;6(14):e13801. doi: 10.14814/phy2.13801. PMID: 30009538. PMCID: PMC6046641.

5. Young AJ, Berryman CE, Kenefick RW, Derosier AN, Margolis LM, Wilson MA, Carrigan CT, Murphy NE, Carbone JW, Rood JC, Pasiakos SM. Altitude acclimatization alleviates the hypoxia-induced suppression of exogenous glucose oxidation during steady-state aerobic exercise. Front Physiol. 2018 Jul 9;9:830. doi: 10.3389/fphys.2018.00830. eCollection 2018. PMID: 30038576. PMCID: PMC6046468.

6. Karl JP, Berryman CE, Young AJ, Radcliffe PN, Branck TA, Pantoja-Feliciana IG, Rood JC, Pasiakos SM. Associations between the gut microbiota and host responses to high

altitude. Am J Physiol Gastrointest Liver Physiol. 2018 Dec 1;315(6):G1003-G1015. doi: 10.1152/ajpgi.00253.2018. Epub 2018 Sep 13. PMID: 30212253. PMCID: PMC6336946.

7. Margolis LM, Berryman CE, Murphy NE, Carrigan CT, Young AJ, Carbone JW, Pasiakos SM. PI3K-AKT-FOXO1 pathway targeted by skeletal muscle microRNA to suppress proteolytic gene expression in response to carbohydrate intake during aerobic exercise. Physiol Rep. 2018 Dec;6(23):e13931. doi: 10.14814/phy2.13931. PMID: 30548426. PMCID: PMC6289907.

8. Young AJ, Karl JP, Berryman CE, Montain SJ, Beidleman BA, Pasiakos SM. Variability in human plasma volume responses during high-altitude sojourn. Physiol Rep. 2019 Mar;7(6):e14051. doi: 10.14814/phy2.14051. PMID: 30920186. PMCID: PMC6437695.

9. Margolis LM, Wilson MA, Whitney CC, Carrigan CT, Murphy NE, Hatch AM, Montain SJ, Pasiakos SM. Exercising with low muscle glycogen content increases fat oxidation and decreases endogenous, but not exogenous carbohydrate oxidation. Metabolism. 2019 Aug;97:1-8. doi: 10.1016/j.metabol.2019.05.003. Epub 2019 May 13. PMID: 31095946.

10. Bradbury KE, Berryman CE, Wilson MA, Luippold AJ, Kenefick RW, Young AJ, Pasiakos SM. Effects of carbohydrate supplementation on aerobic exercise performance during acute high altitude exposure and after 22 days of acclimatization and energy deficit. J Int Soc Sports Nutr. 2020 Jan 9;17(1):4. doi:10.1186/s12970-020-0335-2. PMID: 31918720. PMCID: PMC6953153.

11. Hennigar SR, Berryman CE, Kelley AM, Anderson BJ, Young AJ, McClung JP, Pasiakos SM. High-Altitude Acclimatization Suppresses Hepcidin Expression During Severe Energy Deficit. High Alt Med Biol. 2020 Apr 21. doi: 10.1089/ham.2019.0109. Epub ahead of print. PMID: 32316799.

12. Margolis LM, Karl JP, Wilson MA, Coleman JL, Ferrando AA, Young AJ, Pasiakos SM. Metabolomic profiles are reflective of hypoxia-induced insulin resistance during exercise in healthy young adult males. Am J Physiol Regul Integr Comp Physiol. 2021 Jul 1;321(1):R1-R11. doi: 10.1152/ajpregu.00076.2021. Epub 2021 May 5. PMID: 33949213. PMCID: PMC8321788.

This was a two-phase, randomized controlled study to examine whether consuming a higher-protein (HP) diet would attenuate fat-free mass (FFM) loss during energy deficit (ED) at high altitude (HA) in healthy males. During phase 1 at sea level (SL), participants consumed a eucaloric diet providing standard protein (SP; 1.0 g protein/kg) for 21 days. During phase 2, participants resided at HA for 22 days and were randomly assigned to either an SP or HP (2.0 g protein/kg) hypocaloric diet and engaged in prescribed physical activity designed to elicit a 40% ED. Body composition, substrate oxidation, and postabsorptive whole-body protein kinetics were measured. Participants were weight stable during SL and lost 7.9 ± 1.9 kg (P<0.01) during HA, regardless of dietary protein intake. Decrements in whole-body FFM (3.6 ± 2.4 kg) and fat mass (3.6 ± 1.3 kg) were not different between SP and HP. HP oxidized 0.95 ± 0.32 g protein/kg per day more than SP, and whole-body net protein balance was more negative for HP than for SP

(P<0.01). Based on changes in body energy stores, the overall ED was 70% (-1849 ± 511 kcal/day, no group differences).

To our knowledge, this is the first study to evaluate the effects of an SP versus an HP diet on changes in body composition, specifically FFM, during a 21-day ED at HA. This study demonstrated that: 1) within the context of severe ED (~70%), an HP diet did not spare FFM, in part, because of the induction of greater protein oxidation; 2) body composition changes were highly variable and independent of diet group; participants who lost predominantly FFM had lower aerobic fitness and testosterone concentrations and more FM before HA than individuals who lost predominantly FM (>50% of TBM loss); and 3) the total ED, indicated by changes in energy stores during 21 days at HA, could not be entirely accounted for by measured energy intake, estimated energy expenditure of prescribed daily physical activity, or elevated RMR. Incorporating increased aerobic training into pre-ascent preparations may provide added benefit (i.e., beyond the improved cardiovascular responses to exercise) in protection against FFM loss during ED at HA.

Anorexia and unintentional body weight loss are common during high altitude sojourn. This study aimed to determine the effects of a hypocaloric higher-protein diet on perceived appetite and food preferences during HA sojourn and to examine longitudinal changes in perceived appetite, appetite mediating hormones, and food preferences during acclimatization and weight loss at HA. Appetite, food preferences, and appetite-mediating hormones were measured at SL and at the beginning and end of HA. Diet composition had no effect on any outcome. To our knowledge, this study is the first to examine differential effects of controlled standard- and higher-protein hypocaloric diets on appetite regulation at HA. Dietary protein intake did not impact appetite during energy deficit at HA. However, acute HA exposure was associated with appetite suppression concomitant to elevated concentrations of the anorexigenic hormones insulin, leptin, and CCK, depressed concentrations of the orexigenic hormone acylated ghrelin, and preferences for sweet and low-protein foods. Although anorexigenic hormone concentrations did not remain elevated following acclimatization and weight loss, appetite and acylated ghrelin concentrations remained depressed while food preferences shifted toward high-fat foods. Findings suggest that appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at HA. Although dietary protein intake did not impact appetite, the possible incongruence with food preferences at HA warrants consideration when developing nutritional strategies for HA sojourn.

Muscle loss at high altitude is attributable to energy deficit and a potential dysregulation of anabolic signaling. Exercise and protein ingestion can attenuate the effects of energy deficit on muscle at SL. Muscle obtained from lowlanders at SL, acute HA (3 hours, 4300 m), and chronic HA (21 days, -1766 kcal/day energy balance) before [baseline (Base)] and after 80 minutes of aerobic exercise followed by a 2-mile time trial [postexercise (Post)] and 3 hours into recovery (Rec) after ingesting whey protein (25 g) were analyzed using standard molecular techniques. Both acute HA exposure and chronic HA exposure, coupled with a severe energy deficit, inhibited p-mechanistic target of rapamycin (mTOR) complex 1 (C1) signaling at Post. Moreover, combined chronic HA exposure and energy deficit inhibited the anabolic sensitivity of muscle to consuming 25 g of whey protein. Reductions in mTORC1 signaling, despite lower peripheral oxygen saturation (SpO_2) at acute and chronic HA compared with SL, seem to be

independent of purported hypoxia-mediated mTORC1 inhibition, as neither regulated in development and DNA damage response 1 (REDD1) mRNA expression, nor its coupling to 14-3-3 differed between study phases (SL, acute HA, and chronic HA). Markers of muscle protein breakdown were not indicative of elevated proteolysis at acute or chronic HA, which suggests a negligible effect of proteolysis in the modulation of skeletal muscle mass in response to chronic HA exposure and severe energy deficit in healthy individuals. These findings suggest that HA and severe energy deficit induces mTORC1-mediated anabolic resistance and may account, in part, for muscle loss during periods of sustained HA exposure.

To explore whether muscle inflammation modulates FFM loss reportedly developing during HA sojourns, muscle inflammation, myogenesis, and proteolysis were assessed in men at SL and following 21 days of energy deficit (-1862 ± 525 kcal/day) at HA. TBM, FFM, and FM were assessed using dual energy X-ray absorptiometry (DXA). Gene expression and proteolytic enzymatic activities were assessed in muscle samples collected at rest at SL and HA. Participants lost 7.2 ± 1.8 kg TBM ($P < 0.05$); 43 ± 30% and 57 ± 30% of the TBM lost was FFM and FM, respectively. Fn14, TWEAK, TNF alpha-receptor (TNFα-R), TNFα, MYOGENIN, and paired box protein-7 (PAX7) were upregulated ($P < 0.05$) at HA compared to SL. Stepwise linear regression identified that Fn14 explained the highest percentage of variance in FFM loss ($r^2 = 0.511$, $P < 0.05$). Dichotomization of volunteers into HIGH and LOW Fn14 gene expression indicated HIGH lost less FFM and more FM (28 ± 28% and 72 ± 28%, respectively) as a proportion of TBM loss than LOW (58 ± 26% and 42 ± 26%; $P < 0.05$) at HA. MYOGENIN gene expression was also greater for HIGH versus LOW ($P < 0.05$). The findings show that, in response to 21 days of severe energy deficit at HA: (1) muscle inflammation and myogenic regulatory factor gene expression were upregulated, with no measureable change in ubiquitin-mediated proteolytic activity; and (2) an increase in the inflammatory response, particularly Fn14, was associated with the upregulation of MYOGENIN and attenuation of FFM loss. These findings suggest that, contrary to our hypothesis, heightened Fn14 gene expression was not catabolic and may protect FFM during energy deficit at HA by upregulating myogenesis.

This study also investigated the effects of hypoxia, before and after development of altitude acclimatization, on oxidation of exogenous glucose ingested during steady-state exercise in healthy, young SL residents sojourning at HA while in negative energy balance. During exercise, participants consumed carbohydrate (CHO) or placebo (PLA). Total carbohydrate oxidation was determined by indirect calorimetry and exogenous glucose oxidation by tracer technique with ^{13}C. Participants lost (P ± 0.05, mean ± SD) 7.9 ± 1.9 kg body mass during the HA acclimatization and ED period. In CHO, total exogenous glucose oxidized during the final 40 minutes of exercise was lower ($P < 0.01$) at acute HA (7.4 ± 3.7 g) than SL (15.3 ± 2.2 g) and chronic HA (12.4 ± 2.3 g), but there were no differences between SL and chronic HA. Blood glucose and insulin increased ($P \leq 0.05$) during the first 20 minutes of exercise in CHO, but not PLA. In CHO, glucose declined to pre-exercise concentrations as exercise continued at SL, but remained elevated ($P \leq 0.05$) throughout exercise at acute and chronic HA. Insulin increased during exercise in CHO, but the increase was greater ($P \leq 0.05$) at acute HA than at SL and chronic HA, which did not differ. We demonstrated that exogenous glucose oxidation during metabolically matched exercise is suppressed by acute hypoxia compared to normoxic conditions, and that 21 days of HA acclimatization alleviates this hypoxic-suppression. Furthermore, the effects of acute and chronic hypoxia on blood glucose and insulin responses to

exercise and carbohydrate ingestion that we observed raise the possibility that the concomitant changes in exogenous glucose oxidation may be related to adjustments in glycemic regulation. The finding that the hypoxic suppression of exogenous glucose oxidation was alleviated following 21 days of HA acclimatization, and exogenous glucose oxidation rates had returned to levels not different from those measured during similar exercise at SL, has to our knowledge not been previously reported.

This study is the first to examine interactions among diet, the gut microbiota, and host responses to weight loss at HA. We aimed to determine the effect of increasing the dietary protein:fat ratio on gut microbiota composition, gut microbiota-derived metabolites, gastrointestinal (GI) barrier function, and GI symptoms during weight loss at HA. Results demonstrated that protein:fat ratio had little impact on fecal microbiota composition and only transient effects on fecal short-chain fatty acid (SCFA) and plasma LPS-binding protein (LBP) concentrations. These effects did not appear clinically meaningful as no between-group differences in intestinal permeability, GI symptoms, incidence of moderate-to-severe acute mountain sickness (AMS), or inflammation were observed. Another aim was to identify associations among the gut microbiota, weight loss at HA, and the host response to HA as measured by AMS severity. Notably, AMS severity and weight loss at HA were not affected by diet but demonstrated associations with fecal microbiota composition and, in the case of AMS severity, GI symptoms, providing preliminary evidence of a possible role for the gut microbiota in individual responses to HA. Findings demonstrated several novel associations between the gut microbiota and host responses to HA that were independent of dietary protein:fat ratio. Intriguingly, the ratio of *Bacteroides* to *Prevotella* in the gut microbiota, a well-established driver of interindividual variability in human gut microbiota composition, was associated with interindividual variability in host responses at HA, and increased gut microbiota diversity, considered a marker of a healthy and resilient microbiota, was elevated after HA exposure in individuals who experienced a more severe stress response at HA as measured by AMS symptomology. Although the study design precluded establishing a causal role for the gut microbiota in host responses to HA, findings provide preliminary evidence for a potential role, and support the need for additional research designed to determine if the gut microbiota can be leveraged to improve physiologic responses to HA.

Ingesting protein and carbohydrate together during aerobic exercise suppresses the expression of specific skeletal muscle microRNA and promotes muscle hypertrophy. This study determined skeletal muscle microRNA responses to aerobic exercise with or without carbohydrate, and recovery whey protein (WP). Males were randomized to consume carbohydrate (CHO; 145 g) or non-nutritive control (CON) beverages during exercise. Muscle was collected before (BASE) and after 80 minutes of steady-state exercise followed by a 2-mile time trial (POST), and three hours into recovery after consuming WP (25 g; REC). RT-qPCR was used to determine microRNA and mRNA expression. Bioinformatics analysis was conducted using the mir-Path software. Western blotting was used to assess protein signaling. We found that miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p were more highly expressed in human skeletal muscle following aerobic exercise in CHO compared to CON. Bioinformatics analysis determined that all six of these miRNA target the PI3K-AKT pathway, ubiquitin proteasome, FOXO, and mTOR signaling pathways. Examination of these pathways identified group differences in markers of proteolysis, but not anabolism. These findings suggest carbohydrate ingestion during aerobic exercise alters miRNA that inhibit skeletal muscle

proteolysis with no impact on anabolism. These data provide a new level of understanding of the mechanistic role of miRNA to facilitate skeletal muscle adaptations to exercise with carbohydrate and protein feeding.

When SL residents rapidly ascend to HA, plasma volume (PV) decreases. A quantitative model for predicting individual %ΔPV over the first seven days at HA has recently been developed from the measurements of %ΔPV in 393 HA sojourners. We compared the measured %ΔPV with the %ΔPV predicted by the model in SL natives living 21 days at HA. Fasting hematocrit (Hct), hemoglobin (Hb), and total circulating protein (TCP) concentrations at SL and on days 2, 7, 13, and 19 at HA were used to calculate %ΔTCP and %ΔPV. Mean [95%CI] measured %ΔPV on HA2, 7, 13, and 19 was -2.5 [-8.2, 3.1], -11.0 [-16.6, -5.5], -11.7 [-15.9, -7.4], and -16.8 [-22.2, -11.3], respectively. %ΔPV and %ΔTCP were positively correlated ($P < 0.001$) at HA2, 7, 13, and 19 ($r^2 = 0.77, 0.88, 0.78, 0.89$, respectively). The model overpredicted mean [95% CI] decrease in %ΔPV on HA2 (-12.5 [-13.9, -11.1]) and HA7 (-21.5 [-23.9, -19.1]), accurately predicted the mean decrease on HA13 (-14.3, [-20.0, -8.7]), and predicted a mean increase in %ΔPV on HA19 (12.4 [-5.0, 29.8]). On HA2, 7, 13, and 19 only 2, 2, 6, and 1, respectively, of 17 individual measures of %ΔPV were within 95% CI for predicted %ΔPV. These observations indicate that PV responses to HA are largely oncotically mediated, vary considerably among individuals, and available quantitative models require refinement to predict %ΔPV exhibited by individual sojourners. These findings indicate that the model does not reliably predict the PV responses of individual HA sojourners, either during the first seven days at altitude, or for longer sojourns, suggesting that the model does not account for all factors modulating ΔPV in response to hypoxia.

Initiating aerobic exercise with low muscle glycogen content promotes greater fat and less endogenous carbohydrate oxidation during exercise. We aimed to determine the effects of muscle glycogen content at the onset of exercise on whole-body and muscle substrate metabolism. Men completed two cycle ergometry glycogen depletion trials separated by seven days, followed by a 24-hour refeeding to elicit low (LOW; 1.5 g/kg carbohydrate, 3.0 g/kg fat) or adequate (AD; 6.0 g/kg carbohydrate, 1.0 g/kg fat) glycogen stores. Participants then performed 80 minutes of steady-state cycle ergometry while consuming a carbohydrate drink (95 g glucose+51 g fructose; 1.8 g/min). Substrate oxidation (g/min) was determined by indirect calorimetry and ^{13}C. Muscle glycogen (mmol/kg dry weight), pyruvate dehydrogenase (PDH) activity, and gene expression were assessed in muscle. Initiating steady-state exercise with LOW (217 ± 103) or AD (396 ± 70; $P < 0.05$) muscle glycogen did not alter exogenous carbohydrate oxidation (LOW: 0.84 ± 0.14, AD: 0.87 ± 0.16; $P > 0.05$) during exercise. Endogenous carbohydrate oxidation was lower and fat oxidation was higher in LOW (0.75 ± 0.29 and 0.55 ± 0.10) than AD (1.17 ± 0.29 and 0.38 ± 0.13; all $P < 0.05$). Before and after exercise PDH activity was lower ($P < 0.05$) and transcriptional regulation of fat metabolism (FAT, FABP, CPT1a, HADHA) was higher ($P < 0.05$) in LOW than AD. We found that initiating steady-state aerobic exercise with low versus adequate muscle glycogen content does not impair exogenous carbohydrate oxidative capacity. To compensate for lower endogenous carbohydrate oxidation and PDH activity, the primary adaptations to exercising with low muscle glycogen content appear to be enhanced regulation of whole-body and skeletal muscle fat oxidation. Results from this study indicate when muscle glycogen content is low, consuming carbohydrate during

exercise can be used as a fueling strategy to prolong submaximal exercise without impairing adaptations in whole-body and skeletal muscle fat metabolism.

This study also examined the effects of consuming supplemental carbohydrate on aerobic exercise performance in recreationally active, healthy, young lowlanders at high altitude, before and following 22 days of acclimatization while in a constant state of negative energy balance. We demonstrated that consuming supplemental carbohydrate during steady-state exercise had no performance-enhancing effects on time trial performance before or after high altitude acclimatization. As expected, time trial performance was approximately 55% slower at acute high altitude compared to sea level. Acclimatization partially restored exercise performance, but performance at chronic high altitude was still 34% slower than at sea level. Carbohydrate supplementation had no effect on perceived effort during any of the performance trials. These data suggest that carbohydrate supplementation does not enhance aerobic exercise performance of lowlanders at high altitude.

Data also suggest that increased erythropoietic activity with high altitude acclimatization suppresses hepcidin expression to increase iron availability during severe energy deficit. The decline in ferritin and increase in soluble transferrin receptor suggest a reduction in body iron stores with hypoxia and energy deficit even when dietary iron intake was held constant. The increase in transferrin saturation was a result of increased serum iron and total iron-binding capacity. Collectively, these data indicate that hypoxia is a strong signal for iron mobilization to support iron transfer to the erythropoietic compartment. Importantly, dietary iron and iron recycling from macrophages cannot match the increased requirements for iron at high altitude.

Results from this study show differences in circulating metabolite profiles during exercise under acute HA compared with sea level conditions, indicating increased glycolysis and tricarboxylic acid cycle activity, amino acid breakdown, oxidative stress, and fatty acid storage, and decreased fatty acid mobilization. Increased concentrations and inverse associations of metabolites within branched-chain amino acids and oxidative stress pathways with exogenous glucose oxidation, glucose rate of disappearance, and metabolic clearance rate suggest that changes in metabolite profiles under acute HA conditions may be reflective of hypoxia-induced insulin resistance. These data provide new insight into the potential underlying alterations in metabolic pathways that govern metabolic dysregulation in substrate oxidation under acute HA exposure.

Project 3: Effects of energy balance and energy deficit on inflammatory response

Specific Aim: Clinical study to assess the effects of severe negative energy balance on inflammation, iron absorption, nutritional status, skeletal muscle and whole-body metabolic homeostasis, cognitive, and physical performance during a 96 h simulated sustained operations (SUSOPS)

Major Task 1: Conduct the clinical study (months 1-24)
Milestone #3: Regulatory approval received (months 1-12)

At the time of the award, Project 3 was not completely detailed. The protocol was drafted in Year 1. Milestone #3 was achieved when the Headquarters, US Army Medical Research and Materiel Command Institutional Review Board (HQ USAMRMC IRB) approved the protocol on April 27, 2018. The Statement of Work (SOW) was updated and submitted for approval in May 2018 and accepted in August 2018. Pennington Biomedical Research Center's IRB determined Dr. Jennifer Rood's role in this project was "not human subjects research" as defined by 45 CFR Part 46 and did not require an additional IRB submission.

USARIEM conducted a controlled laboratory study in Years 2 and 3 that simulated the physiological stressors imposed during sustained operations (SUSOPS) to determine if the IL-6 inflammatory response is exacerbated by underfeeding. Male, non-obese (body mass index 19.9-29.9 kg/m^2), active duty military personnel (aged 18-39 years) were recruited to participate in a 32-day, longitudinal study. The study was comprised of four sequential phases: 1) a 96-hour SUSOPS, 2) a 7-day recovery period (Recovery 1), 3) a second, 96-hour SUSOPS, and 4) a 14-day recovery period (Recovery 2). During SUSOPS, subjects were randomized to consume either sufficient food (combat rations) to maintain energy balance within ± 10% of estimated total daily energy expenditure (SUSOPS BAL) or were provided only enough food to match 45% of total daily energy expenditure to elicit severe negative energy balance (SUSOPS NEG BAL). Testing procedures and primary outcome measures included dual energy X-ray absorptiometry (DXA), maximal and sub-maximal aerobic exercise testing, load carriage exercise, controlled feeding, stable isotope assessments of energy expenditure, fractional iron absorption and whole-body protein turnover, percutaneous muscle biopsies to assess intramuscular IL-6, glycogen status, and regulators of muscle remodeling and substrate metabolism, intestinal permeability, gut microbiome composition and function, and blood sampling to assess circulating concentrations of IL-6 and other biomarkers of inflammation, muscle damage, nutritional, metabolic stress, and immune responses to SUSOPS BAL and NEG BAL. Physical performance was assessed before and after each SUSOPS and during each recovery period using the vertical jump test. Self-reported mood, sustained attention, and cognitive performance were assessed twice daily using the Profile of Mood States, the Psychomotor Vigilance Task, the Evaluation of Risk Questionnaire, the Match-to-Sample task, the Grammatical Reasoning test, the Visuomotor Integration task, and a 3D tracking task. Overall vigilance and nighttime sleep quality were assessed via the wrist-worn USARIEM Vigilance Monitoring System. This design tested the hypothesis that maintaining energy balance will attenuate the IL-6 response to SUSOPS, improve iron absorption, and attenuate the potential negative effects of excessive inflammation on whole-body metabolic homeostasis. Risks included those associated with venous catheterization, venipuncture, muscle biopsies, DXA, exercise, and the discomfort of severe underfeeding.

PBRC provided stable isotopes (doubly labeled water) for dosing participants and shipped barcoded biospecimen collection tubes and storage vials to the field collection site. USARIEM investigators collected biospecimens (blood and urine) and shipped them to PBRC for analysis.

Major Task 2: Data Analysis (months 24-48)

PBRC analyzed biospecimens (blood, urine) for basic biochemistry markers, markers of inflammation, gut hormones, markers of iron status, and markers of energy expenditure.

In Year 2, 546 results were sent to the PI via the secure website https://safe.amrdec.army.mil/safe/. Biomarkers that were assayed included the following:

1. Urine nitrogen and creatinine to calculate nitrogen balance
2. Interleukin-6 and hepcidin to assess inflammatory response

In Year 3, PBRC performed the following assays:

Test	Number of Samples
Zonulin	100
S100B	100
Pancreatic polypeptide	120
Osmolality	160
Lipoprotein Binding protein	100
GLP-1	120
Active ghrelin	120
Active PYY	120
I-FABP	80
Erythroferrone	350
IL-6	105
hsCRP	100
Erythropoietin	40
Hepcidin	105
Percent iron saturation	170
Total iron binding capacity	170
Iron	380
Ferritin	310
Soluble transferrin receptor	170
Erythroferrone	350
Leptin	80
Cortisol	100
Creatine kinase	100
DHEA-s	100
LDH	100
LH	100
Myoglobin	100
SHBG	100
Testosterone	100
BHBA	140
Free fatty acids	140
Glucose	140
Glycerol	140
Insulin	140
Sucralose	60
Erythritol	60

We also determined Total Daily Energy Expenditure and Total Body Water on 9 subjects at 2 time periods.

All data was transmitted to the Principal Investigator at USARIEM via a secure file transfer. Data analysis was completed and reviewed in Year 3.

Major Task 3: Data Interpretation and Publishing of Results (months 24-36)
Milestone #4: Co-author initial manuscript on findings from clinical study (months 24-36)

This was completed when the first two manuscripts for Project 3 were published in Year 4. A third manuscript was accepted for publication after this award's period of performance. Copies of the published manuscripts are included in the appendices:

1. Hennigar SR, McClung JP, Hatch-McChesney A, Allen JT, Wilson MA, Carrigan CT, Murphy NE, Teien HK, Martini S, Gwin JA, Karl JP, Margolis LM, Pasiakos SM. Energy deficit increases hepcidin and exacerbates declines in dietary iron absorption following strenuous physical activity: a randomized-controlled cross-over trial. Am J Clin Nutr. 2020 Nov 12:nqaa289. doi: 10.1093/ajcn/nqaa289. Online ahead of print. PMID: 33184627.

2. Karl JP, Hatch-McChesney A, Allen JT, Fagnant HS, Radcliffe PN, Finlayson G, Gwin JA, Margolis LM, Hennigar SR, McClung JP, Pasiakos SM. Effects of energy balance on appetite and physiological mediators of appetite during strenuous physical activity: secondary analysis of a randomised crossover trial. Br J Nutr. 2021 Jan 14:1-40. doi: 10.1017/S0007114521000131. Online ahead of print. PMID: 33441218.

3. Margolis LM, Hatch-McChesney A, Allen JT, DiBella MN, Carrigan CT, Murphy NE, Karl JP, Gwin JA, Hennigar SR, McClung JP, Pasiakos SM. Circulating and skeletal muscle microRNA profiles are more sensitive to sustained aerobic exercise than energy balance in males. J Physiol. July 2022. (Accepted)

This was a randomized, crossover, controlled-feeding trial in healthy men with sufficient iron status. Each trial consisted of a 72-hour simulated sustained military operation (SUSOPS) designed to elicit high energy expenditure, glycogen depletion, and inflammation, followed by a 7-day recovery period. Two SUSOPS trials were performed, during which participants were either in energy deficit or energy balance. The order in which participants completed the trials was randomly assigned and balanced.

A major finding from this study was that 72 hours of strenuous physical activity decreased dietary iron absorption compared with rest in nonanemic individuals with sufficient iron status. Findings indicate that energy deficit during strenuous physical activity increased hepcidin and diminished iron absorption compared with energy balance. The increase in hepcidin with energy deficit suggests that the peroxisome proliferator-activated receptor gamma coactivator 1-alpha/cyclic adenosine monophosphate response element binding protein-H pathway of hepcidin activation may contribute to the observed increase in hepcidin with physical activity. While most markers of inflammation and muscle damage increased with physical activity regardless of

energy status, slightly higher concentrations of C-reactive protein with energy deficit suggest a greater inflammatory response compared with energy balance. Likewise, the increase in ferritin with physical activity during energy deficit, but not energy balance, likely indicates a heightened acute phase response and not an improvement in iron stores, as changes in ferritin tend to reflect changes in inflammatory status and C-reactive protein.

Project 4: Go for Green (GFG) Effectiveness

Specific Aim: Determine the effectiveness of the Go for Green Program for changing dietary intake and attitudes toward nutrition for performance

Major Task 1: Conduct the clinical study (months 1-24)
Milestone #5: Regulatory approval received (months 1-12)

Regulatory approval was granted and recruitment began in the first quarter of Year 1. Dining facility (DFAC) staff participated in focus group sessions, and DFAC patrons were recruited, consented, and enrolled for baseline data collection in Years 1, 2, and 3. The G4G project was closed by USARIEM and at PBRC in Year 3.

"Go for Green(r) Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance among Service Members," Submitted by Catherine Champagne, PhD, RD, Pennington Biomedical Research Center (PBRC), Baton Rouge, LA, in Support of the Proposal, "Collaborative Research to Optimize Warfighter Nutrition (CROWN) III," Submitted by Jennifer Rood, PhD, PBRC, Baton Rouge, LA, Proposal Log Number JW160039, Award Number W81XWH-17-2-0026, HRPO Log Number A-20006.2b, Pennington Biomedical Research Center IRB #2017-019.

This project employed a time series, multisite (Fort Hood and Fort Carson), noncontrolled intervention with data collected from participants at pre- and post-G4G program implementation using food photography and surveys. The program has eight requirements, including a traffic light, color-coded labeling system, menus that offer tasty and nutrient-dense (Green-coded) foods, strategic placement of Green-coded foods to maximize visibility, DFAC staff training, and a robust marketing campaign. Participants freely selected meals from food choices served at the DFAC. Digital food photography captured diners' food selections and analyzed nutrient composition and Healthy Eating Index (HEI) scores to measure meal quality. Surveys gathered demographic, lifestyle, and diner satisfaction data and nutrition knowledge. Data on food cost and program fidelity also were collected. Participants were active-duty Soldiers with food intake data from at least one breakfast, lunch, and dinner meal at the intervention DFAC.

Major Task 2: Data Analysis (months 24-36)

Photo captures of Soldiers' trays, estimation of tray contents, and data compilation was completed in Year 3, and data was sent to Nicholes Armstrong at USARIEM.

Major Task 3: Data Interpretation and Publishing of Results (months 36-48)

The initial manuscript from this study was submitted for publication in Year 4 and published in Year 5:

> Kleinberger CA, Bukhari AS, Kirkpatrick KM, Moylan EM, Billington JL, Armstrong NJ, Cole RE, Deuster PA. Go For Green® Nutrition Program: Translating Evidence into Practice. Military Medicine, 24 Feb 2022, usab503, https://doi.org/10.1093/milmed/usab503.

A technical report was drafted in Year 5 and submitted to LTC Bukhari. A copy is included in the appendices:

> Bukhari AS, Champagne CM, Armstrong NJ, McGraw SM, Allen R, Kirkpatrick KM, Moylan EM, Kleinberger CA, Billington JL, Deuster PA, Cole RE. Effectiveness of Go For Green® Nutrition Program to Improve Warfighter Meal Quality and Nutrition Knowledge. USARIEM Technical Report T22-11, Military Nutrition Division, 21 Apr 2022.

Eleven abstracts for Project 4 were presented at national conferences in Years 2, 3, 4, and 5 and are included in the appendices:

1. Champagne CM, Allen HR, Cole RE, Armstrong NJ, Miketinas DC. Using the HEI-2010 vs HEI-2015 to determine measures of diet quality – where are the differences? American Society for Nutrition Annual Conference, June 2018, Boston.

2. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Diet Adequacy of Army Soldiers Supports the Need for a Planned Dining Facility Intervention. Society for Nutrition Education and Behavior Annual Conference, July 2018, Minneapolis, MN.

3. Serrano EE, Armstrong NJ, Champagne CM, Hatch A, McGraw SM, Cole RE. A Nutrition Knowledge Comparison of Army Culinary Staff and the Soldiers They Feed. Society for Nutrition Education and Behavior Annual Conference, July 2018, Minneapolis, MN.

4. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Dietary Intakes of Army Soldiers Prior to Go For Green® 2.0 Dining Facility Dietary Intervention. Military Health System Research Symposium, August 2018, Kissimmee, FL.

5. Serrano EE, Armstrong NJ, Champagne CM, Hatch A, McGraw S, Cole RE. A nutrition knowledge comparison of Army culinary staff and the Soldiers they feed. Military Health System Research Symposium, August 2018, Kissimmee, FL.

6. Gideon S, Bustillos B, Armstrong NJ, Cole R. Military Dining Facility Staff Perspectives on the Implementation Barriers and Facilitators of Go for Green 2.0, a Department of

Defense Nutrition Initiative. Society for Nutrition Education and Behavior Annual Conference, July 2019, Orlando, FL.

7. Champagne CM, Miketinas DC, Allen HR, Cole RE, McGraw SM, Moylan E, Armstrong N. Does Go For Green 2.0 Joint Service Performance-Nutrition Initiative Improve Dietary Intake of Soldiers? ObesityWeek, Nov 2019, Las Vegas, NV.

8. Bukhari AS, Cole RE, Champagne CM, McGraw SM, Moylan E, Armstrong N. Nutrition Interventions in Military Dining Facilities Can Enhance Diet Quality and Meal Satisfaction. Food and Nutrition Conference and Expo, Oct 2020, virtual event.

9. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition knowledge deficit identified among military dining facility patrons. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

10. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Go for Green® program evaluation finds nutrition knowledge gaps among foodservice staff. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

11. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Moylan EM, Kleinberger CA, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition interventions in military dining facilities can enhance diet quality and meal satisfaction. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

Also, Catherine Champagne was approved to present an oral presentation after this award's period of performance:

Bukhari AS, Champagne CM, Armstrong NJ, McGraw SM, Moylan EM, Kleinberger CA, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition interventions in military dining facilities can enhance meal quality while promoting meal satisfaction. Military Health System Research Symposium, Sep 2022, Kissimmee, FL. (Accepted)

Diet quality (pre/post HEI scores, median ± IQR) improved significantly from 55.0 ± 12.8 to 64.1 ± 12.04, $p<0.001$. Significant improvements were noted in selection of whole grains, seafood and plant proteins, fewer refined grains, and improved trends for other food groups. More diners agreed that main dishes were nutritious/performance-based (32% pre; 57% post; $p<0.01$) and reported using color-coded labels to choose performance foods (38% pre; 54% post; $p<0.04$). The intervention resulted in an 11% increase in plate cost. The overall nutrition knowledge scores of diners was below 60% post program implementation. Nevertheless, a greater proportion of correct responses to nutrition knowledge questions was observed for those participants exposed to the program who received at least one hour of nutrition education. Program fidelity assessment indicated successful implementation of six of the eight program requirements.

The G4G program is feasible and efficacious, and it improved meal quality and satisfaction of diners. Implementing the program requirements provides a greater amount and variety of strategically placed Green-coded (high-performance) menu items to create a more supportive nutrition environment. This program has the potential to improve performance nutrition knowledge, which could translate to overall improved nutrition-related behaviors both within and outside the DFAC. Improving opportunities for service members to fuel with nutritious and tasty food choices along with strategic nutrition education messaging is crucial for optimal nutritional fitness and military readiness.

Project 5: Develop a military-specific eating behavior survey

Specific Aim: Develop a military-specific eating behavior survey using input from subject matter experts and face-to-face interviews

Major Task 1: Develop a draft survey (months 1-24)

The draft survey was developed and IRB approval was obtained in Year 1.

Major Task 2: Validation of new survey (months 24-36)
Milestone #6: Development of the new/validated survey (months 24-36)

Initial pretests were conducted in soldier populations at Fort Jackson, SC; Fort Campbell, KY; Fort Riley, KS; Joint Base San Antonio, TX; Fort Carson, CO; Joint Base Lewis-McChord, WA; and Joint Base Elmendorf-Richardson, AK, and analysis and refinement of the survey began in Year 1. In Year 2, initial testing of the survey was completed, and validation and factor analysis began. The protocol for the project was submitted for scientific/IRB review in Year 2. Validation of the new survey was completed in Year 3. Ninety-eight subjects were enrolled in the study in February 2020 and data collection was completed in early July 2020. Factor analysis was used to finalize the survey, and this milestone was met in Year 3.

Major Task 3: Data Interpretation and Publishing of Results (months 36-48)
Milestone #7: Co-author initial manuscript on findings from clinical study (months 36-48)

This milestone was met when the initial manuscript was published in Year 3. Additional manuscripts were published in Years 4 and 5, and copies of the published manuscripts are included in the appendices:

1. Jayne JM, Ayala R, Karl JP, Deschamps BA, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Body Weight Status, Perceived Stress, and Emotional Eating among US Army Soldiers: A Mediator Model. Eat Behav. 2020 Jan;36:101367. doi: 10.1016/j.eatbeh.2020.101367. Epub 2020 Jan 16. PMID: 32018191.

2. Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Eating behaviors are associated with physical fitness and body composition among US Army Soldiers. J Nutr Educ Behav. 2021 Jun;53(6):480-488. doi: 10.1016/j.jneb.2021.01.013. Epub 2021 Mar 3. PMID: 33674236.

3. Stukenborg MJ, Deschamps BA, Jayne JM, Karl JP, McGraw SM, DiChiara AJ, Cole RE. Exceeding body composition standards is associated with a more negative body image and increased weight cycling in active duty U.S. soldiers. Eat Behav. 2021 May 24;42:101532. doi: 10.1016/j.eatbeh.2021.101532. Online ahead of print. PMID: 34120036.

4. Cole RE, Jayne JM, O'Connor K, McGraw SM, Beyl R, DiChiara AJ, Karl JP. Development and Validation of the Military Eating Behavior Survey. J Nutr Educ Behav. 2021 Jun 29;S1499-4046(21)00608-4. doi: 10.1016/j.jneb.2021.04.467. Online ahead of print. PMID: 34215517.

5. Allen JT, Jayne J, Karl JP, McGraw SM, O'Connor K, DiChiara A, Cole RE. Weight management behaviours mediate the relationship between weight cycling, BMI and diet quality among US Army Soldiers. Br J Nutr. 2021 Sep 2:1-8. doi: 10.1017/S000711452100338X. Online ahead of print. PMID: 34470676.

6. Dyal RN, Deschamps BA, McGraw SM, Jayne JM, Karl JP, Cole RE. Healthy Eating Score-7 as a Measure of Diet Quality in a Military Population. J Nutr Educ Behav. 2022 Jan 6:S1499-4046(21)00860-5. doi:10.1016/j.jneb.2021.09.019. Epub ahead of print. PMID: 35000832.

Eight abstracts were prepared using data from the study. The seven that have been presented are included in the appendices:

1. O'Connor KL, Karl JP, McGraw SM, DiChiara AJ, Cole RE. Soldiers Who Have Failed U.S. Army Body Composition Standards Report a Faster Eating Rate than Soldiers Who Have Not. American Society of Nutrition Annual Meeting, June 2018, Boston, MA.

2. Cole RE, McGraw SM, O'Connor KL, Karl JP, DiChiara AJ. Soldier Body Satisfaction is Associated with Undesirable Weight Management Behaviors and Stress in Meeting Body Composition Standards. Military Healthy System Research Symposium, August 2018, Kissimmee, FL.

3. Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Armstrong NJ, Fagnant H, Cole RE. Eating Behaviors are Associated with Body Composition and Physical Fitness Standards Failure among US Army Soldiers. American Society of Nutrition Annual Meeting, June 2019, Baltimore, MD.

4. Dyal RN, Deschamps BA, McGraw SM, Jayne JM, Karl JP, Cole RE. Using the Healthy Eating Score-5 as a measure of diet quality in an Active Duty Military population. Military Healthy System Research Symposium, August 2019, Kissimmee, FL.

5. Jayne JM, Ayala R, Karl JP, Deschamps BA, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Emotional Eating Behavior Mediates the Association between Perceived Stress and Body Weight Status. Military Healthy System Research Symposium, August 2019, Kissimmee, FL.

6. Stukenborg MJ, Deschamps BA, McGraw SM, Jayne JM, Karl JP, Cole RE. The association between military weight status and body image in a sample of active duty Soldiers. Military Healthy System Research Symposium, August 2019, Kissimmee, FL.

7. Allen JT, Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Weight Management Behaviors Mediate the Relationship between Weight Cycling, Body Mass Index, and Diet Quality among US Army Soldiers. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

8. Jayne JM, Karl JP, McGraw SM, Cole RE. Analyzing Eating Behavior Within the Military Environment and Culture: Development, Use, and Results of the Military Eating Behavior Survey. Military Health System Research Symposium, Sep 2022, Kissimmee, FL. (Accepted)

Also, Drs. Julianna Jayne and Renee Cole delivered a symposium lecture moderated by Dr. J. Phillip Karl in Year 5:

> Jayne JM, Cole RE. Eating Behavior within the Military Environment and Culture: Development, Results, and Utilization of the Military Eating Behavior Survey. Academy of Nutrition and Dietetics Food & Nutrition Conference and Expo, Oct 2021.

This study examined the relationship between perceived stress, body mass index (BMI), failing body composition (BC) standards and gender among US Army Soldiers to determine if emotional eating behaviors may mediate associations between perceived stress and outcomes of BMI and BC failure. Soldiers who reported emotional eating behaviors “sometimes” were nearly two times more likely to fail BC standards and were 3.25 pounds heavier (0.65 BMI points) than those who did not report emotional eating behaviors. Those who reported emotional eating behaviors “often” were two times more likely to fail BC standards and were 6.0 pounds heavier (1.19 BMI points) than those who did not report emotional eating behaviors. Although gender differences in the frequency of emotional eating behaviors were observed, they did not moderate the relationship with BMI or BC failure.

This project led to the development of the Military Eating Behavior Survey, which is a validated tool that can now be used by scientists to study the relationships between military environments and changes in eating behavior in relation to health outcomes. This survey has now been used in multiple projects to assess the role of eating behavior and its association with body composition, physical fitness, body image, and weight change patterns.

What opportunities for training and professional development has the project provided?
The data obtained from the Project 5 survey has been used for masters thesis projects for three MS/RD students enrolled in the Military-Baylor Graduate Program in Human Nutrition, and in a PhD dissertation for a US Army RD who obtained a PhD in Nutrition from Michigan State University.

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Major findings from this project include the following:

Project 1
The major finding in this trial was that subjects in the placebo group lost more body weight than those receiving testosterone. Fat mass decreased similarly in both groups while lean mass increased in the testosterone group compared to the placebo group. Bone mineral content did not differ between the two groups. Lower body muscle function declined in both groups and there was no preservation or improvement of muscle function in the testosterone group.

This study's findings indicate that attenuated proteolysis at rest, possibly downstream of androgen receptor, fibroblast growth factor-inducible 14, and IL-6 receptor signaling, and increased translational capacity, not efficiency, may drive lean mass accretion with testosterone administration during energy deficit. Additionally, mechanistic target of rapamycin pathway activation did not differ between groups at rest or in response to exercise; however, a more positive change in skeletal muscle total RNA content at rest in the testosterone group compared with placebo suggests increased translational capacity, not efficiency, drives lean mass accretion in response to testosterone supplementation during energy deficit.

Project 2
Fat free mass declines during periods of underfeeding for 21 days at high altitude despite increasing protein intake to twice the recommended dietary intake. Appetite is suppressed during underfeeding at high altitude, and this is associated with appetite-mediating hormones. Dietary protein did not impact appetite.

Underfeeding at high altitude inhibits skeletal muscle signaling pathways, which leads to a loss of muscle. Gene expression of the Fn14 gene may protect against the loss of muscle during underfeeding at high altitude.

Initial exposure to high altitude is associated with an increase in glucose oxidation, but this is alleviated after 21 days of high altitude acclimatization.

These findings have enhanced our knowledge base related to operational stressors (particularly underfeeding at high altitude) and the role nutrition may play in helping to mitigate the negative consequences of the operational stress.

We conducted the first study to examine the interactions between diet, gut flora, and response to weight loss at high altitude. We showed that the gut microbiota may contribute to the variability in host response to high altitude. Additional research is needed to determine if gut microbiota can be manipulated to improve physiologic responses to high altitude.

Certain microRNA (miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p) are more highly expressed in skeletal muscle after exercise when carbohydrate is consumed compared to after exercise when no carbohydrate is consumed. These findings suggest that adaptations in the skeletal muscle response to exercise occur by inhibiting markers of proteolysis.

Plasma volume decreases when people rapidly ascend to high altitude; however, this varies greatly among individuals. These changes are likely mediated by changes in oncotic pressure. Predictive models for plasma volume changes at high altitude need further refinement due to the large variability in response.

Initiating exercise with low muscle glycogen does not impair exogenous carbohydrate oxidative capacity, rather, to compensate for lower endogenous carbohydrate oxidation acute adaptations lead to increased whole-body and skeletal muscle fat oxidation.

We demonstrated that consuming supplemental carbohydrate during steady-state exercise had no performance-enhancing effects on time trial performance before or after high altitude acclimatization. Acclimatization partially restored exercise performance, but performance at chronic high altitude was still 34% slower than at sea level. Carbohydrate supplementation had no effect on perceived effort during any of the performance trials. These data suggest that carbohydrate supplementation does not enhance aerobic exercise performance of lowlanders at high altitude.

Data also suggest that increased erythropoietic activity with high altitude acclimatization suppresses hepcidin expression to increase iron availability during severe energy deficit. Our data indicate that hypoxia is a strong signal for iron mobilization to support iron transfer to the erythropoietic compartment. Importantly, dietary iron and iron recycling from macrophages cannot match the increased requirements for iron at high altitude.

This study provided novel insight into the changes of metabolomics profiles while consuming carbohydrate during aerobic exercise under acute high altitude (HA) exposure. While changes in metabolomics profiles during HA are reflective of changes in substrate oxidation and mirror profiles of insulin resistant populations, causality cannot be determined. The information obtained from this analysis can be used to identify potential interventions for future investigation to overcome changes in metabolite profiles to improve glucose tolerance to support physical performance at HA. Based on the present study findings, short-term use of insulin sensitizing drugs, such as metformin or pioglitazone, may be appropriate with unacclimatized HA exposure to efficiently metabolize dietary carbohydrate. Alternatively, potential nutrition interventions such as antioxidant supplement L-carnitine or choline supplementation to reduce oxidative stress and enhance fat oxidation may improve metabolic dysregulation by increasing reliance on fatty acids for fuel during exercise, minimizing oxidative stress associated with insulin resistance.

Project 3

The results of this study suggest that interventions to maintain energy balance may be an effective strategy to prevent the decline in iron status with physical activity. These findings may be important for designing and implementing policies to prevent and treat iron deficiency in

military personnel, endurance athletes, and potentially other populations that experience negative energy balance, such as in areas where malnutrition and infection are common.

Project 4
Dining facility nutrition interventions are needed to improve food offerings and Soldiers' healthy eating scores at dining facilities. The G4G program successfully improved access to healthy/performance-based foods and improved the patrons' satisfaction and meal quality. The G4G program enabled patrons to select performance-based food choices. Strategies to overcome program barriers can further improve patrons' diet quality. Military dining facilities are important avenues to improve diet quality and nutrition-related behaviors to optimize warfighters' fueling requirements.

Project 5
The Military Eating Behavior Survey (MEBS), which is available in both paper and electronic formats, is a valuable research tool for holistically examining Warfighter eating behaviors and mediators of these behaviors. Several studies have identified behaviors that may be modifiable, are relevant to key readiness metrics, and may be used by military health professionals to guide public health initiatives and patient education materials. Additionally, the MEBS is a validated tool enabling the collection of nutrition metrics that may be useful for evaluating the impact of Department of Defense policies and programs on Warfighter eating behaviors, performance, and adherence to military standards.

Several main themes have emerged from analyses of data collected during MEBS validation: (1) Where, what, and how Soldiers eat is related to performance and meeting body composition and physical fitness standards. For example, eating most meals at a dining facility lowered the odds of body composition failure by 2.27 times (OR: 0.44; 95% CI, 0.26−0.73), skipping breakfast lowered odds of high physical performance by 2.43 times (OR: 0.41; 95% CI, 0.23−0.74), and eating at a fast rate raised odds of failing body composition standards by 1.51 times (95% CI, 1.05−2.17); (2) Stress and emotional eating influenced body composition in some Soldiers such that Soldiers with more stress reported more emotional eating, which raised odds of body composition failure by 2.29 times (95% CI, 1.43, 3.65); (3) Pressure to meet body composition standards may influence Soldiers to resort to undesirable weight management behaviors such as dehydration methods (11.3%), laxative use (4.7%), or supplement use (13%); (4) Exposure to body composition testing may contribute to a more negative body image, such that failing body composition standards was associated with increased concern with conforming to military image ($p < 0.01$); and (5) Repeated bouts of weight loss and regain are common among Soldiers and associated with a higher BMI ($p < 0.001$). As eating behaviors may be modifiable, these findings could identify opportunities for improving the specificity of Army health promotion and education programs aimed at increasing force readiness and performance.

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The contract was modified in November 2017 to incorporate the revised Statement of Work (SOW) dated 16 Nov 2017. This revision eliminated a milestone that was achieved under a separate award and updated the subject numbers and Gantt chart for Project 4, Go for Green (GFG) Effectiveness, to match the protocol approved by the Institutional Review Board and Human Research Protection Office. Regulatory approval to increase the maximum number of participants was sought based on a lower than anticipated completion rate in the first iteration. More participants were recruited in order to attain the budgeted sample size of participants completing the study.

At the time of the award, Project 3 was not completely detailed. The project was clearly defined in a second revision of the SOW that was submitted in May 2018 and approved in August 2018. USARIEM investigators recruited, consented and enrolled 5 additional participants for Project 3 in Year 3. The protocol required 15 subjects to complete testing to provide 80% power to detect significant differences in circulating hepcidin responses between SUSOPS 1 and SUSOPS 2.

LTC Asma Bukhari replaced Nicholas Armstrong as Principal Investigator for Project 4: Go for Green (GFG) Effectiveness in Year 3.

Dr. James McClung was appointed Chief of the Military Nutrition Division in Year 2.

Actual or anticipated problems or delays and actions or plans to resolve them

In Year 1, Nicholes Armstrong assumed leadership of Project 4, Go for Green (GFG) Effectiveness, in place of LTC Renee Cole, who was transferred to San Antonio. Data collection was delayed due to the transfer, delays in implementing the protocol within the dining facility, and deployment schedules of soldiers. The data collection schedule for Year 2 was adjusted. USARIEM investigators determined Fort Carson was not feasible for continued data collection for Project 4 due to poor recruitment and identified Fort Hood, Killeen, TX, as an alternate study site. Plans were made to recruit, consent and enroll patrons and staff at this site and collect data.

PBRC suspended on-site operations (including specimen analysis) from mid-March to mid-May 2020 due to the COVID-19 pandemic. Laboratory staff returned to work and resumed specimen analysis on May 18, 2020.

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Berryman CE, Young AJ, Karl JP, Kenefick RW, Margolis LM, Cole RE, Carbone JW, Lieberman HR, Kim IY, Ferrando AA, Pasiakos SM. Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. FASEB J. 2018 Feb;32(2):894-905. doi: 10.1096/fj.201700915R. Epub 2018 Jan 3. PMID: 29066613.

2. Karl JP, Cole RE, Berryman CE, Finlayson G, Radcliffe PN, Kominsky MT, Murphy NE, Carbone JW, Rood JC, Young AJ, and Pasiakos SM. Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high altitude, but are not affected by protein intake. High Alt Med Biol. 2018 Jun;19(2):156-169. doi: 10.1089/ham.2017.0155. Epub 2018 Feb 12. PMID: 29431471. PMCID: PMC6014054.

3. Margolis LM, Carbone JW, Berryman CE, Carrigan CT, Murphy NE, Ferrando AA, Young AJ, Pasiakos SM. Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. FASEB J. 2018 Jun 7:fj201800163RR. doi: 10.1096/fj.201800163RR. Epub ahead of print. PMID: 29878853.

4. Pasiakos SM, Berryman CE, Carbone JW, Murphy NE, Carrigan CT, Bamman MM, Ferrando AA, Young AJ, Margolis LM. Muscle Fn14 gene expression is associated with fat-free mass retention during energy deficit at high altitude. Physiol Rep. 2018 Jul;6(14):e13801. doi: 10.14814/phy2.13801. PMID: 30009538. PMCID: PMC6046641.

5. Young AJ, Berryman CE, Kenefick RW, Derosier AN, Margolis LM, Wilson MA, Carrigan CT, Murphy NE, Carbone JW, Rood JC, Pasiakos SM. Altitude acclimatization

alleviates the hypoxia-induced suppression of exogenous glucose oxidation during steady-state aerobic exercise. Front Physiol. 2018 Jul 9;9:830. doi: 10.3389/fphys.2018.00830. eCollection 2018. PMID: 30038576. PMCID: PMC6046468.

6. Karl JP, Berryman CE, Young AJ, Radcliffe PN, Branck TA, Pantoja-Feliciana IG, Rood JC, Pasiakos SM. Associations between the gut microbiota and host responses to high altitude. Am J Physiol Gastrointest Liver Physiol. 2018 Dec 1;315(6):G1003-G1015. doi: 10.1152/ajpgi.00253.2018. Epub 2018 Sep 13. PMID: 30212253. PMCID: PMC6336946.

7. Margolis LM, Berryman CE, Murphy NE, Carrigan CT, Young AJ, Carbone JW, Pasiakos SM. PI3K-AKT-FOXO1 pathway targeted by skeletal muscle microRNA to suppress proteolytic gene expression in response to carbohydrate intake during aerobic exercise. Physiol Rep. 2018 Dec;6(23):e13931. doi: 10.14814/phy2.13931. PMID: 30548426. PMCID: PMC6289907.

8. Young AJ, Karl JP, Berryman CE, Montain SJ, Beidleman BA, Pasiakos SM. Variability in human plasma volume responses during high-altitude sojourn. Physiol Rep. 2019 Mar;7(6):e14051. doi: 10.14814/phy2.14051. PMID: 30920186. PMCID: PMC6437695.

9. Margolis LM, Wilson MA, Whitney CC, Carrigan CT, Murphy NE, Hatch AM, Montain SJ, Pasiakos SM. Exercising with low muscle glycogen content increases fat oxidation and decreases endogenous, but not exogenous carbohydrate oxidation. Metabolism. 2019 Aug;97:1-8. doi: 10.1016/j.metabol.2019.05.003. Epub 2019 May 13. PMID: 31095946.

10. Pasiakos SM, Berryman CE, Karl JP, Lieberman HR, Orr JS, Margolis LM, Caldwell JA, Young AJ, Montano MA, Evans WJ, Vartanian O, Carmichael OT, Gadde KM, Johannsen NM, Beyl RA, Harris MN, Rood JC. Effects of testosterone supplementation on body composition and lower-body muscle function during severe exercise- and diet-induced energy deficit: A proof-of-concept, single centre, randomised, double-blind, controlled trial. EBioMedicine. 2019 Aug;46:411-422. doi: 10.1016/j.ebiom.2019.07.059. Epub 2019 Jul 27. PMID: 31358477. PMCID: PMC6711889.

11. Hennigar SR, Berryman CE, Harris MN, Karl JP, Lieberman HR, McClung JP, Rood JC, Pasiakos SM. Testosterone Administration During Energy Deficit Suppresses Hepcidin and Increases Iron Availability for Erythropoiesis. J Clin Endocrinol Metab. 2020 Apr 1;105(4). doi: 10.1210/clinem/dgz316. Epub 2020 Jan 2. PMID: 31894236.

12. Bradbury KE, Berryman CE, Wilson MA, Luippold AJ, Kenefick RW, Young AJ, Pasiakos SM. Effects of carbohydrate supplementation on aerobic exercise performance during acute high altitude exposure and after 22 days of acclimatization and energy deficit. J Int Soc Sports Nutr. 2020 Jan 9;17(1):4. doi:10.1186/s12970-020-0335-2. PMID: 31918720. PMCID: PMC6953153.

13. Jayne JM, Ayala R, Karl JP, Deschamps BA, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Body weight status, perceived stress, and emotional eating among US Army Soldiers: A mediator model. Eat Behav. 2020 Jan;36:101367. doi: 10.1016/j.eatbeh.2020.101367. Epub 2020 Jan 16. PMID: 32018191.

14. Karl JP, Berryman CE, Harris MN, Lieberman HR, Gadde KM, Rood JC, Pasiakos SM. Effects of testosterone supplementation on ghrelin and appetite during and after severe energy deficit in healthy men. J. Endocr. Soc. 2020 Apr 1;4(4):bvaa024. doi: 10.1210/jendso/bvaa024. eCollection 2020 Apr 1. PMID: 32258956. PMCID: PMC7101089.

15. Hennigar SR, Berryman CE, Kelley AM, Anderson BJ, Young AJ, McClung JP, Pasiakos SM. High-Altitude Acclimatization Suppresses Hepcidin Expression During Severe Energy Deficit. High Alt Med Biol. 2020 Apr 21. doi: 10.1089/ham.2019.0109. Epub ahead of print. PMID: 32316799.

16. Howard EE, Margolis LM, Berryman CE, Lieberman HR, Karl JP, Young AJ, Montano MA, Evans WJ, Rodriguez NR, Johannsen NM, Gadde KM, Harris MN, Rood JC, Pasiakos SM. Testosterone supplementation up-regulates androgen receptor expression and translational capacity during severe energy deficit. Am J Physiol Endocrinol Metab. 2020 Oct 1;319(4):E678-E688. doi: 10.1152/ajpendo.00157.2020. Epub 2020 Aug 10. PubMed PMID: 32776828. PMCID: PMC7750513.

17. Hennigar SR, McClung JP, Hatch-McChesney A, Allen JT, Wilson MA, Carrigan CT, Murphy NE, Teien HK, Martini S, Gwin JA, Karl JP, Margolis LM, Pasiakos SM. Energy deficit increases hepcidin and exacerbates declines in dietary iron absorption following strenuous physical activity: a randomized-controlled cross-over trial. Am J Clin Nutr. 2020 Nov 12:nqaa289. doi: 10.1093/ajcn/nqaa289. Online ahead of print. PMID: 33184627.

18. Karl JP, Hatch-McChesney A, Allen JT, Fagnant HS, Radcliffe PN, Finlayson G, Gwin JA, Margolis LM, Hennigar SR, McClung JP, Pasiakos SM. Effects of energy balance on appetite and physiological mediators of appetite during strenuous physical activity: secondary analysis of a randomised crossover trial. Br J Nutr. 2021 Jan 14:1-40. doi: 10.1017/S0007114521000131. Online ahead of print. PMID: 33441218.

19. Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Eating behaviors are associated with physical fitness and body composition among US Army Soldiers. J Nutr Educ Behav. 2021 Jun;53(6):480-488. doi: 10.1016/j.jneb.2021.01.013. Epub 2021 Mar 3. PMID: 33674236.

20. Margolis LM, Karl JP, Wilson MA, Coleman JL, Ferrando AA, Young AJ, Pasiakos SM. Metabolomic profiles are reflective of hypoxia-induced insulin resistance during exercise in healthy young adult males. Am J Physiol Regul Integr Comp Physiol. 2021 Jul 1;321(1):R1-R11. doi: 10.1152/ajpregu.00076.2021. Epub 2021 May 5. PMID: 33949213. PMCID: PMC8321788.

21. Stukenborg MJ, Deschamps BA, Jayne JM, Karl JP, McGraw SM, DiChiara AJ, Cole RE. Exceeding body composition standards is associated with a more negative body image and increased weight cycling in active duty U.S. soldiers. Eat Behav. 2021 May

24;42:101532. doi: 10.1016/j.eatbeh.2021.101532. Online ahead of print. PMID: 34120036.

22. Cole RE, Jayne JM, O'Connor K, McGraw SM, Beyl R, DiChiara AJ, Karl JP. Development and Validation of the Military Eating Behavior Survey. J Nutr Educ Behav. 2021 Jun 29;S1499-4046(21)00608-4. doi: 10.1016/j.jneb.2021.04.467. Online ahead of print. PMID: 34215517.

23. Carmichael OT, Pillai SR, Murray K, Shankapal P, Caldwell J, Vartanian O, Berryman CE, Karl JP, Harris M, Rood JC, Pasiakos SM, Lieberman HR. Effects of testosterone administration on fMRI responses to executive function, aggressive behavior, and emotion processing tasks during severe exercise- and diet-induced energy deficit. Neuroimage. 2021 Aug 20;243:118496. doi: 10.1016/j.neuroimage.2021.118496. Online ahead of print. PMID: 34425226.

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25. Dyal RN, Deschamps BA, McGraw SM, Jayne JM, Karl JP, Cole RE. Healthy Eating Score-7 as a Measure of Diet Quality in a Military Population. J Nutr Educ Behav. 2022 Jan 6:S1499-4046(21)00860-5. doi:10.1016/j.jneb.2021.09.019. Epub ahead of print. PMID: 35000832.

26. Kleinberger CA, Bukhari AS, Kirkpatrick KM, Moylan EM, Billington JL, Armstrong NJ, Cole RE, Deuster PA. Go For Green® Nutrition Program: Translating Evidence into Practice. Military Medicine, 24 Feb 2022, usab503, https://doi.org/10.1093/milmed/usab503.

27. Howard EE, Shankaran M, Evans WJ, Berryman CE, Margolis LM, Lieberman HR, Karl JP, Young AJ, Montano MA, Matthews MD, Bizieff A, Nyangao E, Mohammed H, Harris MN, Hellerstein MK, Rood JC, Pasiakos SM. Effects of testosterone on mixed-muscle protein synthesis and proteome dynamics during energy deficit. J Clin Endocrinol Metab. 2022 May 9:dgac295. doi: 10.1210/clinem/dgac295. Online ahead of print. PMID: 35532889.

28. Margolis LM, Hatch-McChesney A, Allen JT, DiBella MN, Carrigan CT, Murphy NE, Karl JP, Gwin JA, Hennigar SR, McClung JP, Pasiakos SM. Circulating and skeletal muscle microRNA profiles are more sensitive to sustained aerobic exercise than energy balance in males. J Physiol. July 2022. (Accepted)

Books or other non-periodical, one-time publications.

1. Ramakrishnapillai S. et al. (2021) Constrained Learning of Task-Related and Spatially-Coherent Dictionaries from Task fMRI Data. In: Abdulkadir A. et al. (eds) Machine

Learning in Clinical Neuroimaging. MLCN 2021. Lecture Notes in Computer Science, vol 13001. Springer, Cham. https://doi.org/10.1007/978-3-030-87586-2_17.

2. Bukhari AS, Champagne CM, Armstrong NJ, McGraw SM, Allen R, Kirkpatrick KM, Moylan EM, Kleinberger CA, Billington JL, Deuster PA, Cole RE. Effectiveness of Go For Green® Nutrition Program to Improve Warfighter Meal Quality and Nutrition Knowledge. USARIEM Technical Report T22-11, Military Nutrition Division, 21 Apr 2022.

Other publications, conference papers and presentations.
(indicates presentation produced a manuscript)*

1. Champagne CM, Allen HR, Cole RE, Armstrong NJ, Miketinas DC. Using the HEI-2010 vs HEI-2015 to determine measures of diet quality – where are the differences? American Society for Nutrition Annual Conference, June 2018, Boston.

2. O'Connor KL, Karl JP, McGraw SM, DiChiara AJ, Cole RE. Soldiers Who Have Failed U.S. Army Body Composition Standards Report a Faster Eating Rate than Soldiers Who Have Not. American Society of Nutrition Annual Meeting, June 2018, Boston, MA.

3. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Diet Adequacy of Army Soldiers Supports the Need for a Planned Dining Facility Intervention. Society for Nutrition Education and Behavior Annual Conference, July 2018, Minneapolis, MN.

4. Serrano EE, Armstrong NJ, Champagne CM, Hatch A, McGraw SM, Cole RE. A Nutrition Knowledge Comparison of Army Culinary Staff and the Soldiers They Feed. Society for Nutrition Education and Behavior Annual Conference, July 2018, Minneapolis, MN.

5. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Dietary Intakes of Army Soldiers Prior to Go For Green® 2.0 Dining Facility Dietary Intervention. Military Health System Research Symposium, August 2018, Kissimmee, FL.

6. Serrano EE, Armstrong NJ, Champagne CM, Hatch A, McGraw S, Cole RE. A nutrition knowledge comparison of Army culinary staff and the Soldiers they feed. Military Health System Research Symposium, August 2018, Kissimmee, FL.

7. Cole RE, McGraw SM, O'Connor KL, Karl JP, DiChiara AJ. Soldier Body Satisfaction is Associated with Undesirable Weight Management Behaviors and Stress in Meeting Body Composition Standards. Military Healthy System Research Symposium, August 2018, Kissimmee, FL.

8. * Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Armstrong NJ, Fagnant H, Cole RE. Eating Behaviors are Associated with Body Composition and Physical Fitness

Standards Failure among US Army Soldiers. American Society of Nutrition Annual Meeting, June 2019, Baltimore, MD.

9. * Hennigar S, Berryman C, Harris M, McClung J, Rood J, Pasiakos S. Testosterone Administration During Energy Deficit Suppresses Hepcidin Concentrations and Increases Iron Utilization in Healthy Males. American Society for Nutrition, June 2019, Baltimore, MD. Curr Dev Nutr. 2019 Jun 13;3(Suppl 1). pii: nzz044.OR15-08-19. doi: 10.1093/cdn/nzz044.OR15-08-19. eCollection 2019 Jun. PMID: 31224847 PMCID: PMC6575034.

10. Gideon S, Bustillos B, Armstrong NJ, Cole R. Military Dining Facility Staff Perspectives on the Implementation Barriers and Facilitators of Go for Green 2.0, a Department of Defense Nutrition Initiative. Society for Nutrition Education and Behavior Annual Conference, July 2019, Orlando, FL.

11. Dyal RN, Deschamps BA, McGraw SM, Jayne JM, Karl JP, Cole RE. Using the Healthy Eating Score-5 as a measure of diet quality in an Active Duty Military population. Military Healthy System Research Symposium, August 2019, Kissimmee, FL.

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and Muscle Mass by Creatine (methyl-d3) Dilution in Response to a 28-d Severe Energy Deficit. American Society for Nutrition, 29 May 2020. Current Developments in Nutrition, Volume 4, Issue Supplement_2, June 2020, Page 614, https://doi.org/10.1093/cdn/nzaa049_007.

18. Bukhari AS, Cole RE, Champagne CM, McGraw SM, Moylan E, Armstrong N. Nutrition Interventions in Military Dining Facilities Can Enhance Diet Quality and Meal Satisfaction. Food and Nutrition Conference and Expo, Oct 2020, virtual event.

19. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition knowledge deficit identified among military dining facility patrons. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

20. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Go for Green® program evaluation finds nutrition knowledge gaps among foodservice staff. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

21. Bukhari AS, Armstrong NJ, Champagne CM, McGraw SM, Moylan EM, Kleinberger CA, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition interventions in military dining facilities can enhance diet quality and meal satisfaction. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

22. * Allen JT, Jayne JM, Karl JP, McGraw SM, O'Connor K, DiChiara AJ, Cole RE. Weight Management Behaviors Mediate the Relationship between Weight Cycling, Body Mass Index, and Diet Quality among US Army Soldiers. Military Health System Research Symposium, Aug 2021, Kissimmee, FL.

23. Jayne JM, Cole RE. Eating Behavior within the Military Environment and Culture: Development, Results, and Utilization of the Military Eating Behavior Survey. Academy of Nutrition and Dietetics Food & Nutrition Conference and Expo, Oct 2021.

24. Bukhari AS, Champagne CM, Armstrong NJ, McGraw SM, Moylan EM, Kleinberger CA, Kirkpatrick KM, Billington JL, Deuster PA, Cole RE. Nutrition interventions in military dining facilities can enhance meal quality while promoting meal satisfaction. Military Health System Research Symposium, Sep 2022, Kissimmee, FL. (Accepted)

25. Jayne JM, Karl JP, McGraw SM, Cole RE. Analyzing Eating Behavior Within the Military Environment and Culture: Development, Use, and Results of the Military Eating Behavior Survey. Military Health System Research Symposium, Sep 2022, Kissimmee, FL. (Accepted)

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**
  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

The projects in this award were carried out in collaboration with scientists from the U.S. Army Research Institute of Environmental Medicine; however, funds from this award covered personnel costs at Pennington Biomedical Research Center only. I have detailed the contributions of Pennington Biomedical Research Center employees during the period of performance:

Name: Jennifer Rood
Project Role: Principal Investigator
ORCID ID: 0000-0001-5848-2987
Nearest Person Month Worked: 32.4
Contribution to Project: She oversaw/managed/coordinated the award, acted as the point of contact for interactions with USARIEM scientists, and oversaw all regulatory processes. She also served as PI for project 1.

Name: Catherine Champagne
Project Role: Co-investigator
ORCID ID: 0000-0001-6127-1072
Nearest Person Month Worked: 11.1
Contribution to Project: She served as the investigator for project 4.

Name: Tiffany Stewart
Project Role: Co-investigator
Nearest Person Month Worked: 8.1
Contribution to Project: She oversaw project 5 and worked on the development of the military-specific eating behavior survey.

Name: Ray Allen
Project Role: Computer support
Nearest Person Month Worked: 11.8
Contribution to Project: He provided computer support for project 4.

Name: Bridget Conner
Project Role: Medical Technologist
Nearest Person Month Worked: 38.3
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1, 2, and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Danielle Geis
Project Role: research project assistant
Nearest Person Month Worked: 3.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for project 3. She prepared specimen collection containers and processed specimens.

Name: Margaret Graves
Project Role: medical technologist
Nearest Person Month Worked: 9.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1 and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Melissa Harris
Project Role: project manager
ORCID ID: 0000-0001-9113-1695
Nearest Person Month Worked: 5.4
Contribution to Project: She served as the project manager for project 1.

Name: Alexandria Henderson
Project Role: research project assistant
Nearest Person Month Worked: 3.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for project 3. She prepared specimen collection containers and processed specimens.

Name: Laura Holloway
Project Role: medical technologist
Nearest Person Month Worked: 9.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1 and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Valery Hymel
Project Role: research associate
Nearest Person Month Worked: 38.3
Contribution to Project: She provided analytical support in the Mass Spectrometry Laboratory. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Jessica Landry
Project Role: research specialist
Nearest Person Month Worked: 10.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for project 3. She prepared specimen collection containers and processed specimens.

Name: Steve Lee
Project Role: medical technologist
Nearest Person Month Worked: 38.3
Contribution to Project: He provided analytical support in the Clinical Chemistry laboratory for projects 1, 2, and 3. He prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Stacey Roussel
Project Role: medical technologist
Nearest Person Month Worked: 38.3
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1, 2, and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Connor Sanford
Project Role: research project assistant
Nearest Person Month Worked: 12.0
Contribution to Project: He provided analytical support in the Clinical Chemistry laboratory for project 3. He prepared specimen collection containers and processed specimens.

Name: Jonathan Savoie
Project Role: research associate
Nearest Person Month Worked: 44.3
Contribution to Project: He provided analytical support in the Mass Spectrometry Laboratory. He prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Christy Tucker
Project Role: medical technologist
Nearest Person Month Worked: 3.0
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1 and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Jamie Tuminello
Project Role: medical technologist
Nearest Person Month Worked: 59.7
Contribution to Project: She provided analytical support in the Clinical Chemistry laboratory for projects 1, 2, and 3. She prepared specimen collection containers, processed specimens, analyzed biological samples, and reported results.

Name: Dawn Turner
Project Role: research associate
Nearest Person Month Worked: 7.8
Contribution to Project: She provided visual estimation and food photography support for project 4.

Name: Edie White
Project Role: administrative assistant
Nearest Person Month Worked: 29.9
Contribution to Project: She provided administrative support for the entire award.

Name: Dachuan Zhang
Project Role: biostatistician
Nearest Person Month Worked: 12.8
Contribution to Project: He provided data analysis for project 1.

Total Expenditures to Date:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: United States Army Research Institute of Environmental Medicine, Military Nutrition Division
Location of Organization: Natick, MA
Partner's contribution to the project: Collaboration (as detailed in the Statement of Work)

8. SPECIAL REPORTING REQUIREMENTS:

Collaborative Research to Optimize Warfighter Nutrition (CROWN) III

Proposal Log #: JW160039

W81XWH-17-2-0026

PI: Jennifer Rood **Org:** Pennington Biomedical Research Center **Award Amount:** $6,665,000.00

Study/Product Aim(s)

The aim of CROWN III is to conduct research in nutrition, metabolism and human physiology to discover novel nutritional interventions, field feeding programs, and food products that promote Warfighter resilience, improve Warfighter combat readiness, and assure optimal Warfighter performance. Investigators from PBRC and USARIEM collaborated on 5 projects:

1) Determine the physiological and psychological effects of testosterone during severe energy deficit and recovery
2) Determine the substrate utilization, exercise performance, and skeletal muscle response to energy deficit and altitude acclimatization
3) Determine the effects of energy balance and energy deficit on inflammatory response
4) Quantify the effectiveness of the Go For Green (GFG) military dining facility intervention
5) Development of a valid and military-appropriate survey tool for the assessment of Soldier eating behaviors



Accomplishment: This project has provided the scientific basis to develop novel nutritional programs, products, and strategies to promote Warfighter health, performance, and resilience to operational stress.

Timeline and Cost

Activities FY	17	18	19	20	21
Protocol Development/ Approval					
Data Analysis					
Publication of Results					
Budget (M)	$2.165	$1.5	$1.5	$1.5	$0

Updated: 07/14/2022

Goals/Milestones:

FY17 – Completed the clinical study for project 1, published initial manuscript on findings from project 2, obtained regulatory approval for projects 3 and 4, developed a draft survey for project 5

FY18 – Continued data analysis and interpretation for project 1, published additional manuscripts for project 2, conducted the clinical study for project 3, conducted the study and analyzed data for project 4, began survey validation and data interpretation for project 5

FY19 – Continued data analysis and interpretation for projects 1, 3 and 4, validated survey for project 5, published findings from projects 1, 2 and 5

FY20-21 – Published additional findings from projects 1-5

Budget Expenditures to Date: $6,665,000.00

9. APPENDICES:

A. Journal publications

B. Books or other non-periodical, one-time publications

C. Other publications, conference papers and presentations

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Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial

Claire E. Berryman,[*,†] Andrew J. Young,[*,†] J. Philip Karl,[*] Robert W. Kenefick,[‡] Lee M. Margolis,[*,†] Renee E. Cole,[*] John W. Carbone,[†,§] Harris R. Lieberman,[*] Il-Young Kim,[¶] Arny A. Ferrando,[¶] and Stefan M. Pasiakos[*,1]

[*]Military Nutrition Division and [‡]Thermal Mountain Medicine Division, U.S. Army Research Institute of Environmental Medicine (USARIEM), Natick, Massachusetts, USA; [†]Oak Ridge Institute for Science and Education, Belcamp, Maryland, USA; [§]School of Health Sciences, Eastern Michigan University, Ypsilanti, Michigan, USA; and [¶]Department of Geriatrics, Center for Translational Research in Aging and Longevity, Donald W. Reynolds Institute on Aging, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

ABSTRACT: In this 2-phase randomized controlled study, we examined whether consuming a higher-protein (HP) diet would attenuate fat-free mass (FFM) loss during energy deficit (ED) at high altitude (HA) in 17 healthy males (mean ± SD: 23 ± 6 yr; 82 ± 14 kg). During phase 1 at sea level (SL, 55 m), participants consumed a eucaloric diet providing standard protein (SP; 1.0 g protein/kg,) for 21 d. During phase 2, participants resided at HA (4300 m) for 22 d and were randomly assigned to either an SP or HP (2.0 g protein/kg) diet designed to elicit a 40% ED. Body composition, substrate oxidation, and postabsorptive whole-body protein kinetics were measured. Participants were weight stable during SL and lost 7.9 ± 1.9 kg ($P < 0.01$) during HA, regardless of dietary protein intake. Decrements in whole-body FFM (3.6 ± 2.4 kg) and fat mass (3.6 ± 1.3 kg) were not different between SP and HP. HP oxidized 0.95 ± 0.32 g protein/kg per day more than SP and whole-body net protein balance was more negative for HP than for SP ($P < 0.01$). Based on changes in body energy stores, the overall ED was 70% (−1849 ± 511 kcal/d, no group differences). Consuming an HP diet did not protect FFM during severe ED at HA.—Berryman, C. E., Young, A. J., Karl, J. P., Kenefick, R. W., Margolis, L. M., Cole, R. E., Carbone, J. W., Lieberman, H. R., Kim, I.-Y., Ferrando, A. A., Pasiakos, S. M. Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. FASEB J. 32, 894–905 (2018). www.fasebj.org

KEY WORDS: hypobaric hypoxia · weight loss · metabolic rate · muscle · substrate oxidation

For lowlanders sojourning at high altitude (HA >1500 m), maintaining energy balance can be challenging because of an increase in resting metabolic rate (RMR) (1, 2) and a decrease in appetite (3) caused by physiologic adaptations to hypoxia. The effects of hypoxia on energy balance are frequently compounded by limited access to food and high physical activity levels while at HA. Negative energy balance usually develops at HA and typically leads to weight loss (4). Barnholt *et al.* (4) showed that individuals sustaining a 40% energy deficit (ED) for 21 d at HA lost more body weight than individuals adhering to the same controlled diet and physical activity at sea level (SL). Furthermore, some studies (5–8) have observed >50% of total body mass (TBM) loss as fat-free mass (FFM) during prolonged ED at HA, which is greater than the 25–35% typically observed at SL (9–11), suggesting protein turnover kinetics and substrate utilization during weight loss at HA may differ from that at SL.

At SL, protein intake above the Recommended Dietary Allowance (RDA, 0.8 g protein/kg per day) limits FFM loss during moderate negative energy balance (~40% ED) in healthy, normal weight adults (12–14). We have shown that consuming a diet containing 1.6 or 2.4 g protein/kg per day (2 and 3 times the RDA, respectively) for 21 d, within the context of a 40% ED, attenuates FFM loss as a percentage of TBM loss (30 ± 7 and 36 ± 5%, respectively) compared to an isocaloric diet containing 0.8 g protein/kg per day (58 ± 5%) (12). Similarly, Longland *et al.* (13) showed that combining a higher-protein (HP)

ABBREVIATIONS: DEXA, dual-energy X-ray absorptiometry; ED, energy deficit; EIEE, exercise-induced energy expenditure; ΔES, change in body energy stores; FFM, fat-free mass; FM, fat mass; HA, high altitude; HP, higher protein; MRE, Meal, Ready-to-Eat; NB, net balance; PB, protein breakdown; PS, protein synthesis; R_a, rate of appearance; RDA, Recommended Dietary Allowance; RMR, resting metabolic rate; SHBG, sex hormone-binding globulin; SL, sea level; SP, standard protein; TBM, total body mass; TDEE, total daily energy expenditure; TTR, tracer-to-tracee ratio; $\dot{V}_{CO_2}$, carbon dioxide production; $\dot{V}_{O_2}$, oxygen consumption; $\dot{V}_{O_{2peak}}$, peak oxygen uptake

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doi: 10.1096/fj.201700915R

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diet (2.4 g protein/kg/d) with resistance-type exercise (6 d/wk) increases lean body mass compared to a standard protein (SP) diet (1.2 g protein/kg/d) and resistance exercise (1.2 ± 1.0 *vs.* 0.1 ± 1.0 kg) during a 28 d moderate ED (40%). Increased protein consumption (1.6–2.4 g protein/kg) also has been shown to preserve protein balance during a moderate ED (12, 15), suggesting that the availability of substrate to sustain protein turnover is a mechanism by which HP diets preserve FFM during ED at SL. However, whether the FFM-sparing effect of consuming HP diets during ED at SL extend to ED experienced under extreme environmental conditions, such as HA, is unknown.

Military personnel often endure sustained periods of ED during training and combat operations resulting in TBM and FFM loss (16–18). Current protein recommendations to minimize FFM loss during military operations that elicit an ED do not account for potential environmental stressors, including HA, that may exacerbate negative energy balance and FFM loss. Therefore, dietary protein intakes demonstrated to be efficacious for sparing FFM during ED at SL may not be as efficacious during ED at HA. The objective of this study was to determine whether consuming dietary protein at amounts consistent with current recommendations for metabolically demanding military operations (2.0 g protein/kg) would attenuate FFM loss during prolonged ED at HA (19). This study was designed to extend on our previous work showing a benefit of HP diets on FFM during a 21 d, 40% ED at SL (12). We hypothesized that consuming 2.0 g protein/kg per day would preserve FFM during a 21 d ED at HA compared with an isocaloric diet providing 1.0 g protein/kg per day.

MATERIALS AND METHODS

The study was conducted from May 2016 through August 2016. Details regarding participant recruitment, randomization, and attrition are presented in **Fig. 1**. In total, 21 participants were randomized and 17 volunteers completed the study and were included in the final analyses. All volunteers provided written informed consent before participation. This study was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (USARIEM) and registered at ClinicalTrials.gov (*www.clinical trials.gov*) as NCT02731066. Investigators adhered to the policies for protection of human subjects as prescribed in the U.S. Department of Defense Instruction 3216.02, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Participants

Recreationally active (programmed physical activity 2–4 d/wk) men (n = 17), aged 18–42 yr, who were not acclimatized to HA (*i.e.*, born at altitudes <2100 m, currently residing at altitudes <1200 m, and who had not traveled to altitudes >1200 m for 5 d or more within 2 mo before beginning the study) were recruited. Participants were not taking any medications or were willing to refrain from all medication use for 4 wk before and throughout the entire study period, unless provided or approved by the study's medical oversight. Participants agreed to refrain from alcohol, smoking any nicotine product, using electronic cigarettes (vaping), chewing tobacco, caffeine, and dietary supplement use throughout the entire study period. Additional exclusion criteria included metabolic diseases (*e.g.*, diabetes and cardiovascular disease); injuries that would compromise exercise capability; gastrointestinal disorders; evidence of apnea or other sleeping disorders; prior HA pulmonary edema or HA cerebral edema diagnosis; allergies or intolerance to foods; vegetarian practices; history of complications with lidocaine; evidence of any physical, mental, or medical condition that would make participation more hazardous; present condition of alcoholism, anabolic steroid use, or other substance abuse; anemia; sickle cell anemia or trait; abnormal blood clotting; blood donation within 8 wk of beginning the study; any use of antibiotics, except topical antibiotics, within 3 mo of study participation; colonoscopy within 3 mo of study participation; use of laxatives, stool softeners, or antidiarrheal medications more than once a week.

Experimental design

This randomized, controlled trial included 2 phases conducted over 43 consecutive days. In phase 1 of the study (SL d 1–21, 21 d) participants were free-living and resided at SL (55 m, Natick, MA, USA). On d 21, participants were flown to Denver, CO, and transported by car to Colorado Springs, CO, where they stayed overnight under supervision in a local hotel. The following morning (HA d 1, ~3 AM), participants were transported by car to the USARIEM Maher Memorial Altitude Laboratory on the summit of Pikes Peak, CO (4300 m). Participants breathed supplemental oxygen (portable O_2 concentrators, FreeStyle 5; AirSep, Buffalo, NY, USA or EverGo, Respironics, Murrysville, PA, USA) after landing in Denver until they arrived at the summit to maintain normoxic oxygen saturation (means ± SD for SpO_2 measured hourly from arrival until 10 PM and every 30 min beginning at 3 AM was 97.0 ± 0.9%, range 95–99%) in an effort to normalize exposure across volunteers and limit nonstudy hypoxic exposure. During phase 2 (HA d 1–22, 22 d), participants were randomized (computer generated, with 1:1 allocation for the 2 parallel groups) to a measured and controlled 40% ED diet (see Diet Intervention) and resided at the Maher Memorial Altitude Laboratory.

Assessment of energy intake and physical activity at SL

Volunteers completed three 24 h diet and activity records (2 weekdays, 1 weekend day) at baseline (before d 1) following instructions provided by a registered dietitian. The data collected were used to more accurately predict weight maintenance energy intake requirements and total daily energy expenditure (TDEE). Body weight was measured daily, and three 24 h diet and activity records (2 weekdays, 1 weekend day) were completed weekly during phase 1 (SL d 1–7, 8–14, and 15–21) and reviewed by registered dietitians to verify compliance with dietary intake and activity guidelines and to provide corrective instructions as needed.

Diet intervention

During phase 1, registered dietitians instructed participants to follow a weight-maintaining diet providing 1.0 g protein/kg per day, 45–65% total energy from carbohydrate, and 20–35% total energy from fat. The protein intake level for SP was chosen based on mean *ad libitum* protein intakes in active-duty military personnel (20). TDEE was estimated from the sum of RMR (multiplied by 1.3 to account for activities of daily living and diet-induced thermogenesis) and exercise-induced energy expenditure (EIEE) estimated from corresponding metabolic equivalents of activities reported in the 3 d physical activity log (21). Those TDEE estimates were then averaged with

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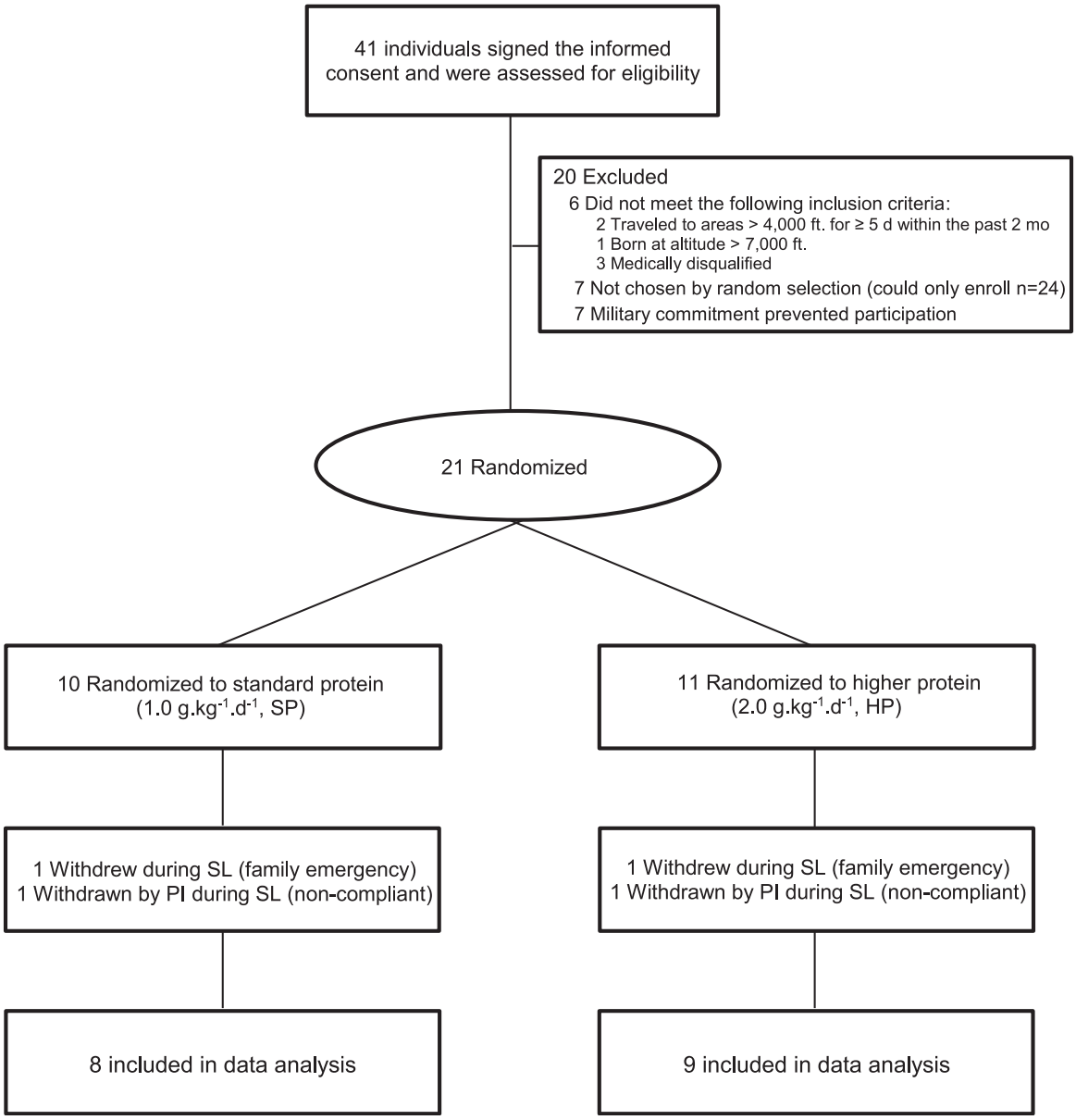


Figure 1. Volunteer selection chart.

estimated TDEE requirements calculated using the Harris-Benedict equation (22). Participants were asked to maintain their self-reported exercise routine for the duration of phase 1.

During phase 2, energy intake was individualized for each participant. The controlled diet began on HA d 2 (HA d 2 to 22; 21 d diet intervention), after the stable isotope infusion protocol on HA d 1 (see Whole-Body Protein Turnover). To achieve the 40% ED, daily physical activity was increased by 10% (see Physical Activity Prescription) and daily energy intake was decreased by 30% relative to TDEE estimated at SL during energy balance. Registered dietitians developed individualized daily menus using the Combat Rations Database (U.S. Army Natick Soldier Research, Development, and Engineering Center and USARIEM) and Food Processor SQL (v.10.14; ESHA Research, Salem, OR, USA). Dietary carbohydrate provided 45% of total energy. Dietary protein provided either 1.0 ± 0.2 (SP) or 2.0 ± 0.2 (HP) g/kg per day. Fat provided the remainder of energy in the diets. The rationale for manipulating fat, instead of carbohydrate, to compensate for the 2 levels of protein intake was to avoid compromising a secondary study objective investigating carbohydrate metabolism during exercise at SL and HA. Most of the foods in these diets were components of the military combat ration, Meal, Ready-to-Eat (MRE; menu 34; Ameriqual, Evansville, IN, USA). The remainder of energy came from non-MRE foods (*e.g.*, fruits, vegetables, olive oil, and ranch dressing) and whey protein beverages (*i.e.*, Isopure Zero Carb; Isopure, Hauppauge, NY, USA) to achieve individualized macronutrient requirements that could not be met using MRE rations alone. All foods and caloric beverages were weighed to the nearest 0.1 g by trained research staff. Meals and snacks were supervised, and participants were instructed to consume all provided foods and beverages. Any uneaten foods or beverages were weighed and documented. Noncaloric, noncaffeinated beverages (*e.g.*, water and carbonated drinks) were allowed *ad libitum*.

Physical activity prescription

During phase 2, participants completed low-to-moderate intensity endurance exercise to increase their daily EIEE by 10%

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above phase 1 TDEE [*i.e.*, HA EIEE = SL EIEE + (0.1 × SL TDEE)]. Exercise sessions included treadmill walking, running, and outdoor hiking, all supervised and documented by research personnel to ensure safety and compliance with the exercise prescription. The amount of physical activity necessary to elicit the desired daily EIEE was individually prescribed, and determined daily by the Pandolf equation for level and uphill walking (23), adjusted as necessary for weight loss, terrain factors, and downhill walking (24).

Anthropometric measurements

Height was measured to the nearest 0.1 cm with a stadiometer (Seca; Creative Health Products, Plymouth, MI, USA) at baseline. Body weight was measured with participants in undergarments, upon waking (overnight fast, ≥8 h), and after voiding, with a calibrated digital scale (Model PS6600; Befour Inc., Saukville, WI, USA) to the nearest 0.1 kg at baseline and daily during phases 1 and 2. FFM and fat mass (FM) were determined at baseline, before ascent to HA (SL d 20), and immediately after descent from HA (~0630–0800 on HA d 23) using dual energy X-ray absorptiometry (DEXA; DPX-IQ; GE Lunar Corp., Madison, WI, USA). In this study, FFM refers to TBM minus both FM and bone mass. DEXA scans were performed under fasted conditions by trained personnel. In addition to whole-body composition, leg and trunk composition are presented because of potential differences in region-specific FFM and FM; for example, skeletal muscle mass comprises a greater proportion of FFM in the appendicular region than in the trunk region (25). FFM loss as a percentage of TBM loss was used to divide participants into groups (<50 or >50% TBM loss as FFM) to elucidate factors responsible for the variability in body composition responses to ED.

Changes in body energy stores (ΔES) were used to estimate the total ED (26) (Eq. 1):

$$\Delta ES = \Big[\Delta FM \times 9.51\ \text{kcal/g} + \Delta FFM \times (1 - \text{FFM hydration}) \times 4.40\ \text{kcal/g}\Big] \div 21\ \text{d} \quad (1)$$

with FFM hydration representing the aqueous fraction of FFM, estimated as 0.73 (27).

Determination of peak oxygen uptake

Peak oxygen uptake ($\dot{V}O_{2peak}$) was assessed at baseline with an indirect, open circuit metabolic measurement system (True Max 2400; Parvo Medics, Sandy, UT, USA) on a treadmill. Volunteers fasted overnight (≥8 h) before testing. After a 3 min self-paced warm-up, participants ran for 4 min at a pace predetermined to be comfortable at a 0% grade. At 4 min, the grade was increased to 4%, followed by an additional 2% increase every 2 min thereafter until volitional exhaustion.

Assessment of resting metabolic rate

RMR was measured at baseline with a True Max 2400 system (Parvo Medics) to determine diet prescriptions (28). Throughout phases 1 and 2, RMR was measured with a Vmax 229 (CareFusion, Yorba Linda, CA, USA) because of technical difficulties with the True Max 2400 system. All measures were collected in the morning upon waking (between 6 and 8 AM). RMR measurements were collected once at SL (d 7) and 9 times at HA (d 1, 2, 4, 6, 8, 11, 14, 20, and 22). Daily change in RMR from SL was calculated by assigning measured RMR values to subsequent days when measures were not taken (*e.g.*, the HA change in RMR on d 2 was also assigned to HA d 3). Thus, the 21 d at HA that coincided with the diet intervention (i.e., HA d 2 to HA d 22) were assigned an RMR value to allow estimation of the cumulative contribution of RMR to the total ED.

Estimation of substrate oxidation

Urine was collected for 24 h on HA d 7, 13, and 19. Collection began after the first morning void and ended on the subsequent day, immediately after RMR testing (*i.e.*, HA d 8, 14, and 20). Urine volume was measured and aliquots stored at −20°C until analyzed. Urinary nitrogen was measured from a single, pooled 24 h sample and determined using pyrochemiluminescence (Model 9000; Antek, Houston, TX, USA). Substrate oxidation was estimated according to Eqs. 2–4, developed by Frayn (29):

$$\text{Protein oxidation} = (6.25 \times \text{urinary nitrogen})/1440\ \text{min}(i.e.,\ 24\ \text{h}) \quad (2)$$

$$\text{Glucose oxidation} = 4.55 \times \dot{V}co_2 - 3.21 \times \dot{V}o_2 - 2.87 \times \text{urinary nitrogen} \quad (3)$$

$$\text{Fat oxidation} = 1.67 \times \dot{V}o_2 - 1.67 \times \dot{V}co_2 - 1.92 \times \text{urinary nitrogen} \quad (4)$$

where protein, glucose, and fat oxidation and urinary nitrogen (Eq. 3 and 4) are expressed in grams per minute, and urinary nitrogen is expressed in grams (Eq. 2). Substrate oxidation values are presented as the overall mean at HA, based on measures collected on HA d 8, 14, and 20.

Metabolic and endocrine biomarkers

Blood samples (10 h fast, sitting upright and resting in a chair for 30 min) were collected on SL d 12 and HA d 3, 8, 14, and 20 by antecubital venipuncture. Serum and plasma were isolated, frozen at −80°C, and shipped on dry ice to the Pennington Biomedical Research Center (Baton Rouge, LA, USA) for analysis of serum glucose, free-fatty acids, and glycerol measured in duplicate using enzymatic and colorimetric assays (DXC 600 Pro; Beckman Coulter, Brea, CA, USA). Serum insulin, total testosterone, sex hormone–binding globulin (SHBG), and cortisol were measured in duplicate with an advanced automated immunoassay instrument (Immulite 2000; Siemens Healthcare Diagnostic, Deerfield, IL, USA). Free testosterone was calculated as described in Vermeulen *et al*. (30) with measured total testosterone and SHBG and assuming a concentration of 4.3 g/dl for albumin.

TABLE 1. *Prestudy characteristics*

Characteristic	SP	HP
Age (yr)	23.1 ± 3.3	23.7 ± 7.3
Height (cm)	177 ± 7	176 ± 8
Weight (kg)	85.0 ± 15.1	79.1 ± 13.1
BMI (kg/m^2)	27.0 ± 4.0	25.5 ± 3.1
Body fat (%)	22.8 ± 7.0	22.6 ± 5.5
$\dot{V}O_{2peak}$ (L/min)	4.13 ± 0.61	4.21 ± 0.73
TDEE (kcal/d)	2744 ± 264	2635 ± 344
EIEE (kcal/d)	379 ± 168	388 ± 179
Protein (g/kg/d)	1.6 ± 0.8	1.4 ± 0.6

Independent Student's *t* tests were used to compare means in the SP (n = 8) and HP (n = 9) protein groups. No differences were observed between groups. BMI, body mass index.

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TABLE 2. *Dietary intake in the SP and HP diet groups during weight maintenance at SL and ED at HA*

	Weight maintenance, SL		Energy deficit, HA	
Dietary intake	SP	HP	SP	HP
Absolute intake (kcal/d or g/d)				
Energy	2366 ± 277	2418 ± 542	1950 ± 186	1885 ± 269
Protein	94 ± 20	83 ± 15	88 ± 14	154 ± 25*
Carbohydrate	300 ± 62	327 ± 80	223 ± 24	221 ± 35
Fat	89 ± 9	88 ± 27	84 ± 6	47 ± 7*
Relative intake (kcal/kg/d or g/kg/d)				
Energy	28.5 ± 6.5	30.9 ± 6.2	24.5 ± 3.1	25.5 ± 2.0
Protein	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.0	2.1 ± 0.0*
Carbohydrate	3.6 ± 1.1	4.2 ± 1.0	2.8 ± 0.4	3.0 ± 0.2
Fat	1.1 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	0.7 ± 0.1*
Energy intake (%)				
Protein	16 ± 3	14 ± 2	18 ± 2	33 ± 2*
Carbohydrate	50 ± 6	54 ± 5	46 ± 1	47 ± 1
Fat	34 ± 4	32 ± 4	39 ± 2	23 ± 3*

Independent Student's *t* tests were used to compare means in the SP ($n = 8$) and HP ($n = 9$) groups at SL and HA. *$P < 0.05$.

Whole-body protein turnover

On SL d 7 and HA d 1 (acute HA) and 22 (chronic HA), intravenous catheters were placed in each arm, one for the infusion of stable isotopes (Cambridge Isotope Laboratories, Andover, MA, USA) and the other for blood sampling. Immediately before isotope tracer infusion (0 min), baseline blood was collected to determine background isotopic enrichments. For determination of whole-body protein synthesis (PS), protein breakdown (PB), and net balance (NB), 4.5 h primed continuous infusions of L-[ring-2H5]-phenylalanine (prime, 4.6 μmol/kg; rate, 3.92 μmol/kg/h) and L-[3,3-2H2]tyrosine (prime, 0.95 μmol/kg; rate, 1.57 μmol/kg/h) were performed. To appropriately reach isotopic equilibrium of L-[ring-2H4]-tyrosine enrichment derived from L-[ring-2H5]phenylalanine tracer, a priming dose of L-[ring-2H4]-tyrosine was injected (prime: 0.33 μmol/kg) at 0 min. Postabsorptive, whole-body protein turnover on SL d 7 and HA d 1 were conducted before the diet intervention (*i.e.*, weight maintenance phase), whereas HA d 22 measurements were collected after the ED and dietary protein intervention.

Blood sample processing for isotope enrichment

Blood samples were mixed with 125 μl of 10% sulfosalicylic acid and centrifuged. Free amino acids were extracted from 300 μl supernatant fluid by cation exchange chromatography and dried in a Speed Vac (Savant Instruments, Farmingdale, NY, USA). Phenylalanine and tyrosine enrichments were measured on the *tert*-butyldimethylsilyl derivative using gas chromatography-mass spectrometry (7890A/5975; Agilent Technologies, Santa Clara, CA, USA) (31, 32). Ions of mass-to-charge ratio of 234, 235, and 239 for phenylalanine, and 466, 467, 468, and 470 for tyrosine were monitored with electron impact ionization and selective ion monitoring. Blood isotopic enrichment analyses were conducted by the Center for Translational Research in Aging and Longevity at the University of Arkansas for Medical Sciences (Little Rock, AR, USA).

Whole-body protein turnover calculations

Whole-body PS and PB rates were calculated based on the determinations of the rate of appearance (R_a) into the plasma of phenylalanine and tyrosine and the fractional R_a of endogenous tyrosine converted from phenylalanine (33). Plasma enrichments of phenylalanine and tyrosine tracers were curve fitted with a cubic spline method by using Prism 5 (GraphPad Software, La Jolla, CA, USA). Whole-body protein turnover was calculated by dividing kinetic values of phenylalanine by its fractional contribution to protein. For calculations of whole-body PB rate, contribution from tracers infused were subtracted from total R_a. Eqs. 5–10 were used for calculations of whole-body protein kinetics:

$$\text{Total plasma } R_a = F/E \tag{5}$$

$$\text{Fractional } R_a \text{ of Tyr from Phe} = E_{\text{Tyr M+4}}/E_{\text{Phe M+5}} \tag{6}$$

$$\text{Phe hydroxylation} = \text{fractional } R_a \text{ of Tyr from Phe} \times R_{a|\text{Tyr}} \tag{7}$$

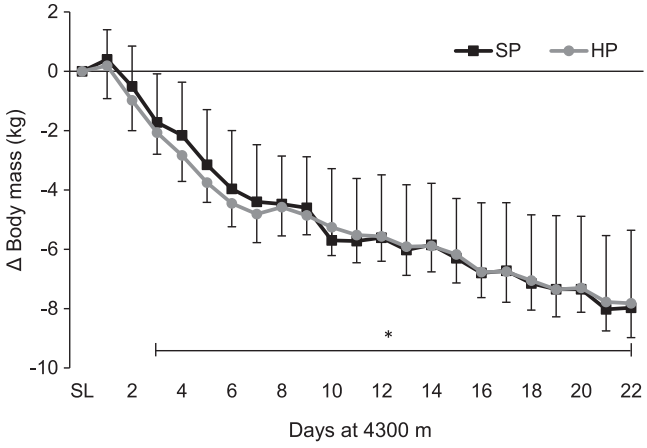


Figure 2. Mean change in body mass with consumption of an SP ($n = 8$) *vs.* an HP ($n = 9$) diet during ED at HA. Data were analyzed with linear mixed models with volunteer as the random effect and diet, time, and their interaction as fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni correction. Main effect of time, $P < 0.0001$; *$P < 0.05$ *vs.* SL.

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TABLE 3. *Total and regional body mass and composition assessed by DEXA in the SP and HP diet groups before and after 21 d of ED at HA*

Region	SP			HP			*P*		
	SL	HA	Δ	SL	HA	Δ	Diet	Time	Interaction
Body mass (kg)									
Total	86.6 ± 16.3	79.4 ± 14.3	−7.2 ± 2.4	80.0 ± 12.5	72.9 ± 12.3	−7.0 ± 0.9	0.34	<0.0001	0.88
Fat free	63.1 ± 10.7	59.1 ± 8.3	−4.0 ± 3.3	59.1 ± 7.7	55.8 ± 7.3	−3.2 ± 1.5	0.39	<0.0001	0.53
Nonaqueous[a]	17.0 ± 2.9	16.0 ± 2.3	−1.1 ± 0.9	16.0 ± 2.1	15.1 ± 2.0	−0.9 ± 0.4	0.39	<0.0001	0.53
Aqueous[b]	46.1 ± 7.8	43.2 ± 6.1	−2.9 ± 2.4	43.1 ± 5.6	40.8 ± 5.4	−2.4 ± 1.1	0.39	<0.0001	0.53
Fat	20.0 ± 8.3	16.7 ± 9.6	−3.3 ± 1.8	17.8 ± 6.1	13.8 ± 6.4	−3.9 ± 0.8	0.50	<0.0001	0.37
Leg mass (kg)									
Total	29.3 ± 6.0	26.5 ± 4.8	−2.8 ± 1.7	28.0 ± 5.2	25.2 ± 4.8	−2.8 ± 0.9	0.61	<0.0001	0.96
Fat free	21.7 ± 4.3	19.8 ± 3.2	−2.0 ± 1.9	20.6 ± 3.1	19.0 ± 2.7	−1.7 ± 1.1	0.56	0.0001	0.67
Fat	6.3 ± 2.1	5.4 ± 2.4	−0.9 ± 0.5	6.2 ± 2.2	5.0 ± 2.3	−1.1 ± 0.4	0.82	<0.0001	0.23
Trunk mass (kg)									
Total	40.4 ± 8.3	37.8 ± 7.4	−2.6 ± 1.8	36.8 ± 5.7	33.8 ± 5.8	−3.1 ± 0.5	0.27	<0.0001	0.48
Fat free	28.8 ± 4.2	27.4 ± 3.3	−1.4 ± 1.6	27.1 ± 3.8	25.8 ± 3.6	−1.3 ± 0.6	0.36	0.0002	0.89
Fat	10.5 ± 5.6	9.2 ± 6.1	−1.3 ± 1.2	8.8 ± 3.5	7.0 ± 3.6	−1.8 ± 0.6	0.40	<0.0001	0.31

Linear mixed models were used to determine the main effects of diet: SP (n = 8) *vs.* HP (n = 9); time: SL d 20 *vs.* HA d 23, and their interaction. [a]Nonaqueous FFM was estimated as 27% of total FFM (27). [b]Aqueous FFM was estimated as 73% of total FFM (27).

$$\mathrm{PS} = \left[\left(R_{\mathrm{a|Phe}} - \text{Phe hydroxylation}\right) \times 25\right] \quad (8)$$

$$\mathrm{PB} = \left[\left(R_{\mathrm{a|Phe}} - F_{\mathrm{Phe}}\right) \times 25\right] \quad (9)$$

$$\mathrm{NB} = \mathrm{PS} - \mathrm{PB} \quad (10)$$

where E is enrichment of respective tracers at plateau and is expressed as tracer-to-tracee ratio (TTR) for calculation of PB: TTR/(TTR + 1); F is the respective tracer infusion rate into a venous site: F_{Phe} for phenylalanine tracer; and $E_{\mathrm{Tyr\ M+4}}$ and $E_{\mathrm{Phe\ M+5}}$ are plasma enrichments of tyrosine and phenylalanine tracers at M+4 and M+5, relative to M+0, respectively. The correction factor of 25 is for conversion of phenylalanine values to total protein based on the assumption that the contribution of phenylalanine to a standard protein is 4% (100/4 = 25) (34). Phenylalanine hydroxylation is the R_{a} of tyrosine derived from phenylalanine through hydroxylation.

Statistical analysis

Statistical analyses were performed with SAS (v.9.3; SAS Institute, Cary, NC, USA). Normality was assessed by using univariate analysis to evaluate skewness quantitatively and inspect box and probability plots visually. A paired Student's t test was used to compare mean body weight on SL d 1 and 20. Independent Student's t tests were used to compare prestudy characteristics, SL diet, HA diet, and EIEE, and HA substrate oxidation between the SP and HP diets. Independent Student's t tests also were used to compare SL and HA characteristics between participants who lost greater than and <50% of TBM as FFM. Linear mixed models, with volunteer treated as a random effect, were used to determine the effects of diet and time and their interaction (fixed effects) on body mass and composition; RMR; and metabolic and endocrine biomarkers. Linear mixed models, with volunteer treated as a random effect, were used to determine effects of time (fixed effect) on whole-body PS, PB, and NB. In addition, ANCOVA, adjusted for acute HA (*i.e.*, HA d 1), was used to further assess the effects of diet on postabsorptive whole-body protein turnover at chronic HA (*i.e.*, HA d 22). If main or interaction effects were significant, Bonferroni *post hoc* comparisons were evaluated. Significance was set at $P < 0.05$, and data are presented as means ± SD. Statistical power and sample size were based on those in previous studies (4, 12), and an expected mean difference of 1.0 kg in FFM loss between groups, an SD of 0.7 kg, and an α of 0.05, to provide more than 80% power to detect differences with 8 per group.

RESULTS

Participants (n = 17; **Table 1**) remained weight stable during SL (d 1: 82.1 ± 14.1 and d 20: 82.9 ± 14.5 kg; $P >$ 0.05), while consuming 2394 ± 426 kcal/d and 1.1 ± 0.3 g protein/kg per day (no group differences at SL; **Table 2**).

During HA, participants lost 7.9 ± 1.9 kg (main effect of time, $P < 0.01$) regardless of protein group (**Fig. 2**). Similarly, whole-body, leg, and trunk FFM (aqueous and nonaqueous) and FM decreased at HA (main effect of time, $P < 0.01$), but were not significantly different between HP and SP (**Table 3**) dietary groups. HP oxidized 75% more protein than SP (2.21 ± 0.41 *vs.* 1.26 ± 0.21 g protein/kg/d; $P < 0.01$; **Fig. 3**). However, carbohydrate (2.63 ± 1.38 compared to 3.38 ± 1.34 g carbohydrate/kg/d) and fat

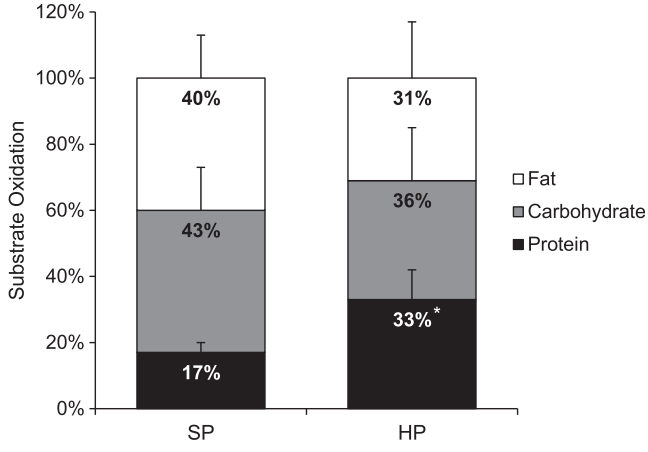


Figure 3. Substrate oxidation with consumption of an SP (n = 8) *vs.* HP (n = 8) diet during ED at HA. Independent Student's t tests were used to compare means between diet groups for each macronutrient. *$P < 0.05$.

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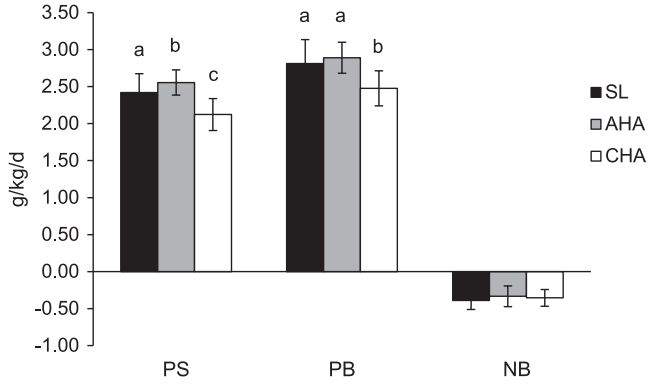


Figure 4. Postabsorptive whole-body protein turnover at SL, acute HA (AHA), and chronic HA (CHA). Linear mixed models, with volunteer treated as a random effect, were used to determine effects of time (fixed effect). Different lowercase letters within a variable indicate a significant difference. $P < 0.05$.

(1.00 ± 0.64 compared to 1.37 ± 0.55 g fat/kg/d) oxidation were not significantly different between HP and SP. Postabsorptive whole-body PS was increased at acute HA compared to SL and decreased at chronic HA compared to both SL and acute HA (main effect of time, $P < 0.01$; **Fig. 4**). PB was lower at chronic HA compared to both SL and acute HA (main effect of time, $P < 0.01$). NB did not differ by time ($P = 0.40$). To investigate diet-specific effects on whole-body protein turnover after chronic HA exposure, PS, PB, and NB were examined, with adjustment for acute HA measures. HP and SP PS (2.08 ± 0.23 *vs.* 2.17 ± 0.21 g/kg/d; $P = 0.17$) and PB (2.52 ± 0.24 *vs.* 2.43 ± 0.24 g/kg/d; $P = 0.79$) were not significantly different; however, NB was more negative for the HP than the SP diet (−0.44 ± 0.08 *vs.* −0.26 ± 0.06 g/kg/d; $P < 0.01$).

Glucose and insulin concentrations decreased during prolonged ED at HA, where free fatty acid concentrations increased (main effect of time, $P < 0.01$; **Fig. 5*A–C***), regardless of protein group. Glycerol concentrations increased acutely, but returned to SL values by HA d 20 (main effect of time, $P < 0.01$; no group differences; Fig. 5*D*). There was an interaction effect (diet × time; $P = 0.02$) for total testosterone concentrations; however, no *post hoc* comparisons were significant, and the change from SL to HA on d 20 was not different by diet group (**Fig. 6*A***). Free testosterone decreased during prolonged ED at HA and SHBG and cortisol concentrations increased (main effect of time, $P < 0.01$; Fig. 6*B–D*).

Based on ΔES, the ED was 1729 ± 685 kcal/d (actual, 64% ED from SL TDEE) in SP and 1956 ± 293 kcal/d (actual, 75% ED from SL TDEE) in HP (no group differences), which was greater than the prescribed ED [SP: 1080 ± 160 kcal/d (diet: 794 ± 103 kcal/d and exercise: 286 ± 64 kcal/d) and HP: 1015 ± 124 kcal/d (diet: 750 ± 98 kcal/d and exercise: 265 ± 41 kcal/d); no group differences]. The greater than prescribed ED experienced by participants was attributable, in part, to an increase in

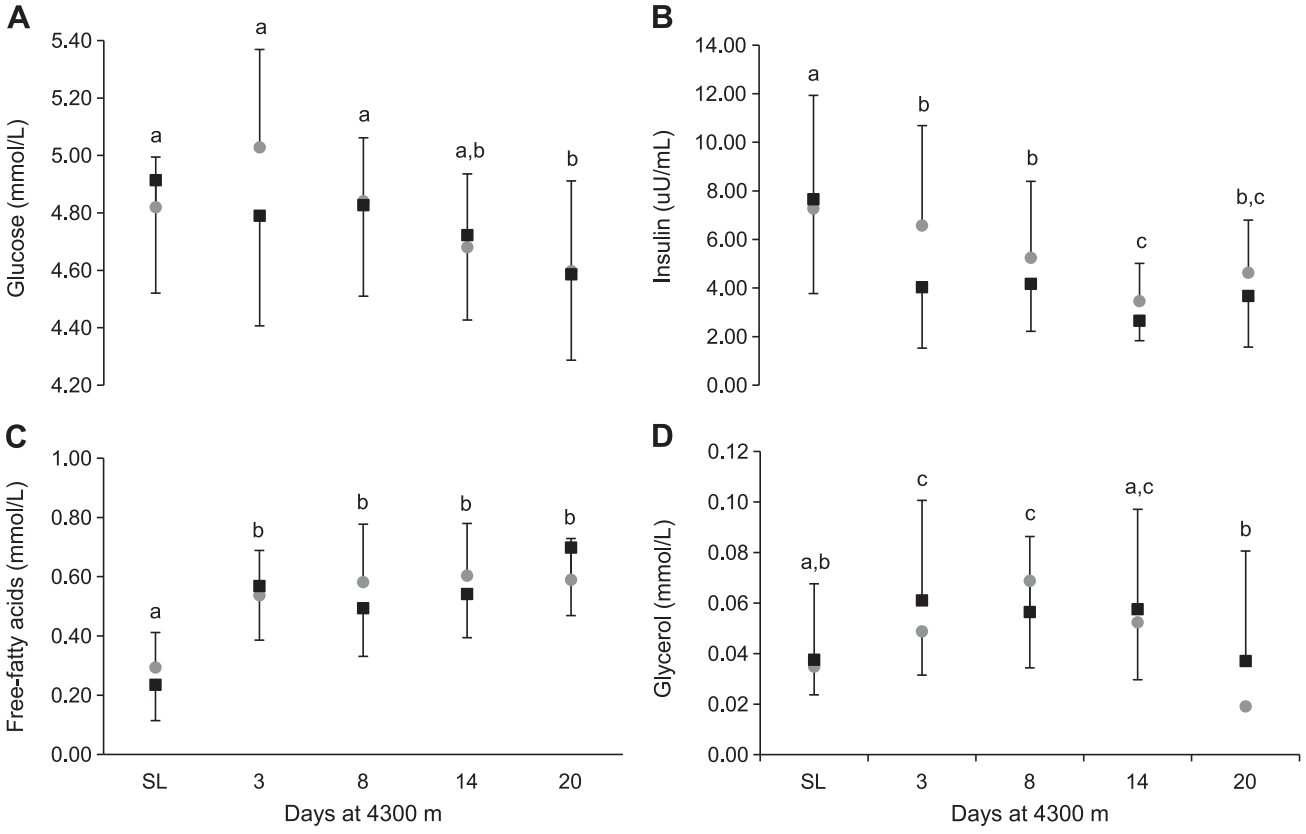


Figure 5. Longitudinal effects of a SP ($n = 8$) *vs.* HP ($n = 9$) diet on glucose (*A*), insulin (*B*), free-fatty acids (*C*), and glycerol (*D*) during energy deficit at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni correction. Main effect of time, $P < 0.001$ for all. Days not sharing a lowercase letter are significantly different. $P < 0.05$.

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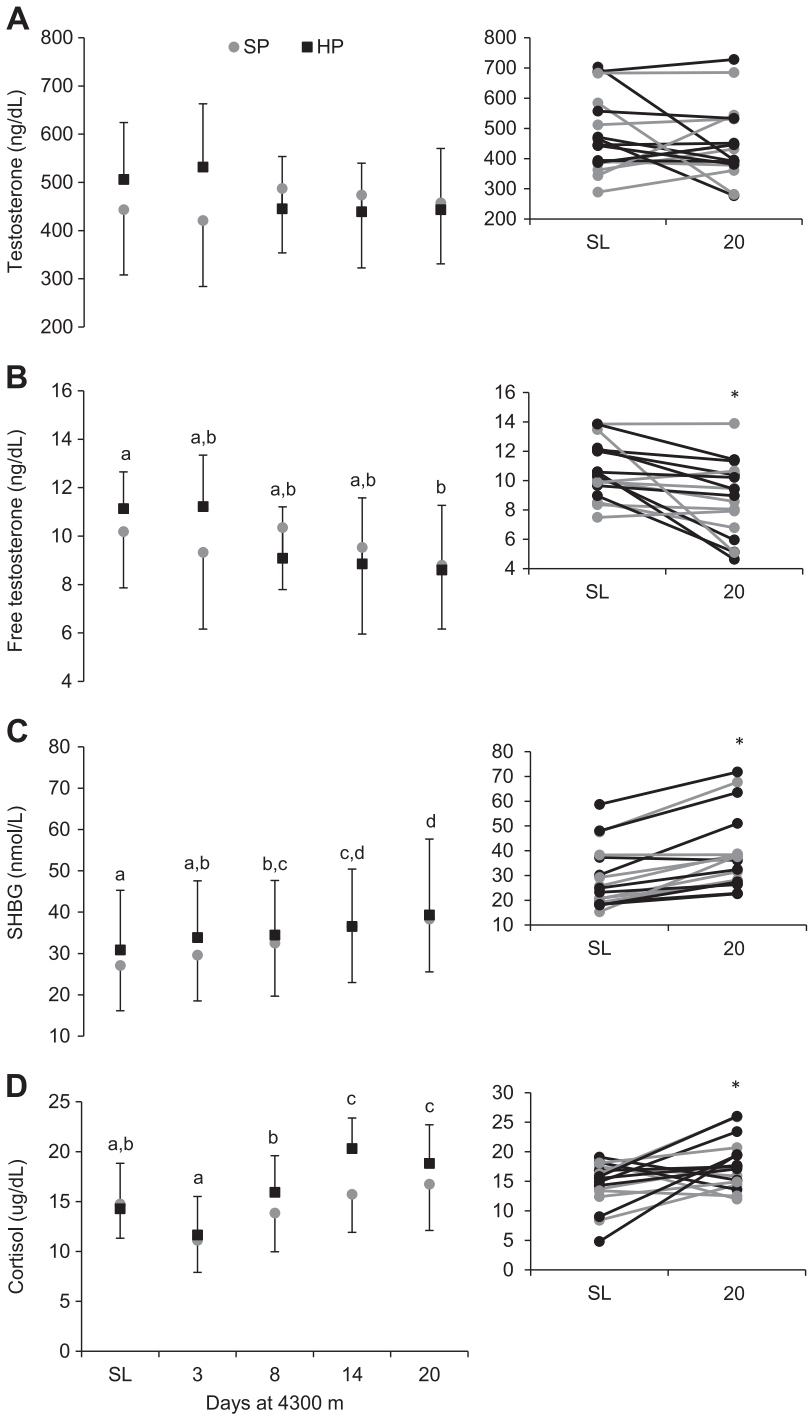


Figure 6. Longitudinal effects of an SP *vs.* HP protein diet on total testosterone (*A*), free testosterone (*B*), sex hormone-binding globulin (*C*), and cortisol (*D*) during ED at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni correction. Diet × time interaction effect; $P = 0.02$ (*A*). Main effect of time, $P < 0.01$ (*B–D*). Days not sharing a lowercase letter are significantly different, $P < 0.05$. Individual participant responses are represented at the right (*A–D*). *$P < 0.05$ *vs. SL*.

RMR, which was 30% greater than SL (d 7) on HA d 1 and remained ≥22% above that at SL until HA d 8 (main effect of time, $P < 0.01$; **Fig. 7**) and contributed 380 ± 200 kcal/d in SP and 288 ± 243 kcal/d in HP (no group differences) to the ED. However, 269 ± 687 kcal/d in SP and 652 ± 412 kcal/d in HP (no group differences) of the ED could not be explained by measured energy intake, estimated energy expenditure of prescribed daily physical activity, or elevated RMR.

Participants who lost less FFM (<50% of TBM loss; $n = 10$) had lower TBM and FM and higher aerobic fitness and total and free testosterone concentrations at SL than participants who lost more FFM (>50% of TBM loss; $n = 7$; **Table 4**). In addition, at HA, participants who lost less FFM had greater ED as measured by ΔES, despite consuming significantly greater energy and carbohydrates per kilogram body weight than participants who lost predominantly FFM. Metabolic parameters and whole-body protein turnover measures at SL and substrate oxidation (absolute, relative, and percentage) at HA did not differ by FFM loss group (data not shown).

DISCUSSION

To our knowledge, this is the first study to evaluate the effects of an SP *vs.* an HP diet on changes in body

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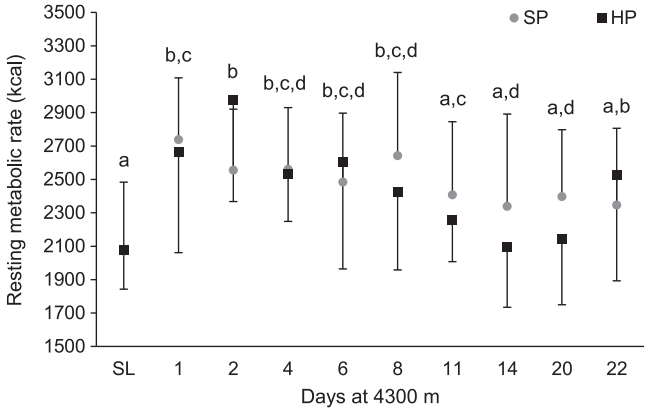


Figure 7. Longitudinal effects of an SP *vs.* HP diet on resting metabolic rate during energy deficit at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as the fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni correction. Main effect of time, $P < 0.0001$. Days that do not share a lowercase letter are significantly different. $P < 0.05$.

composition, specifically FFM, during a 21 d ED at HA. This study demonstrated that: *1*) within the context of severe ED (~70%), an HP diet did not spare FFM, in part, because of the induction of greater protein oxidation; *2*) body composition changes were highly variable and independent of diet group; participants who lost predominantly FFM had lower aerobic fitness and testosterone concentrations and more FM before HA than individuals who lost predominantly FM (>50% of TBM loss); and *3*) the total ED, indicated by changes in energy stores during 21 d at HA, could not be entirely accounted for by measured energy intake, estimated energy expenditure of prescribed daily physical activity, or elevated RMR.

When energy intake is sufficient to match energy expenditure, whole-body protein balance is maintained with dietary protein intake consistent with the RDA (35). For example, during energy balance, consuming 0.8 g protein/kg per day, or ~5–7% of total energy as protein maintains whole-body protein balance; whereas, during even minimal ED (*i.e.*, 15%) this amount of protein is inadequate, resulting in a negative nitrogen balance (36, 37). Previous reports have demonstrated that consuming HP diets (1.6–2.4 g protein/kg) at SL can minimize negative nitrogen balance and spare FFM during weight loss induced by moderate ED (~40%) (12, 13, 38, 39). In the current study, an HP diet (2.0 g protein/kg) was ineffective in sparing FFM during an ED at HA that was much more severe (~70%) than the 40% ED used in SL studies. Our prior work (40) showed an inability to restore protein and nitrogen balance at SL during ED >40% with dietary protein manipulation alone. The inability of an HP diet to spare FFM in the current study was probably related to the severity of the ED.

Postabsorptive NB was more negative with an HP than with an SP diet at chronic HA, despite no significant differences in PS or PB. In another study, we demonstrated this effect at SL using ^{13}C-leucine whole-body protein turnover kinetics (41), showing that postabsorptive net protein balance was more negative with increased protein intakes (0.8, 1.6, or 2.4 g protein/kg) because of a dose-dependent increase in whole-body protein oxidation. Similarly, in the current study, HP oxidized 75% more protein than SP. However, in the context of a 40% ED, HP diets have been shown to maintain an anabolic stimulus to feeding similar to that observed during weight maintenance, suggesting that although greater overall amounts of dietary protein are oxidized with increasing protein intakes, sufficient amino acid precursors remain to sustain anabolic sensitivity to protein-containing meals (12). In the current study, the additional protein provided to the HP group was almost completely oxidized (0.95 ± 0.32 g protein/kg/d more than in the SP group), precluding a sustained anabolic response to protein-containing meals and negating the FFM-sparing effects of the HP diet. The increase in whole-body PS at acute HA compared to SL is consistent with other studies showing increased PS in response to acute stress (17, 42, 43), which may be related to increased synthesis of acute phase proteins at HA (44). The observed suppression of both PS and PB at chronic HA is consistent with that in previous reports of whole-body protein kinetics during prolonged ED at SL (41, 45, 46), which indicates that protein and energy sparing are adaptive responses to sustained negative energy balance.

Participants who lost predominantly FFM (>50% of TBM loss) during exposure to HA were less aerobically fit and had lower initial testosterone concentrations and more FM before HA than individuals who lost predominantly FM (>50% of TBM loss). Therefore, aerobic training before an HA sojourn may provide a potential strategy to attenuate FFM loss at HA. Aerobic training enhances the capacity for fat oxidation during and immediately after exercise (47, 48) and could be the mechanism by which fitter individuals lose less FFM at HA. If this is the case, substrate use during and immediately after bouts of physical activity probably influences overall changes in body composition more than resting substrate metabolism, because resting substrate oxidation was not different between participants who lost <50% FFM (fat, 34 ± 18%; protein, 24 ± 10%, and carbohydrate, 42 ± 19%) or those who lost >50% FFM (fat, 36 ± 13%; protein, 27 ± 13%; and carbohydrate, 36 ± 8%; $P > 0.05$). In addition, circulating testosterone concentrations have been shown to be dose-dependently and positively associated with FFM accretion and FM loss in healthy young males consuming a eucaloric diet at SL (49). Therefore, in the current study, greater circulating testosterone at SL may have slowed subsequent FFM loss by delaying, at least initially, the catabolic effects of ED on FFM at HA.

In this study, energy intake was not adjusted for increases in RMR, to better resemble real-world HA sojourns and military operations, as sojourners often do not adequately increase food intake to compensate for a greater RMR. As a consequence of this study design choice, the actual ED (~70%) was 80% greater than the prescribed ED (~40%). Other studies have reported anywhere from a 6 to 28% increase in basal metabolic rate upon initial ascent to HA (1, 50–53). In the current study, we observed an initial 30–33% increase in RMR during the first 2 d at HA and a sustained 22–23% elevation for the next 6 d. For the

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TABLE 4. *Characteristics of participants losing less than or greater than 50% of TBM as FFM after 21 d of energy deficit at HA*

Characteristic	>50% of TBM loss as FFM	<50% of TBM loss as FFM
SL body mass [kg (%)]		
Total	92.3 ± 9.9	76.6 ± 13.7*
Fat free	65.3 ± 8.0 (71 ± 6)	58.0 ± 9.1 (76 ± 5)
Fat	23.7 ± 6.3 (26 ± 6)	15.4 ± 5.6* (20 ± 5)*
SL $V_{O_{2peak}}$ (ml/kg/m)	46.5 ± 7.1	55.2 ± 5.2*
SL TDEE (kcal/d)	2802 ± 187	2606 ± 352
RMR	1849 ± 97	1713 ± 246
EIEE	370 ± 200	394 ± 154
SL endocrine parameters		
Total testosterone (ng/dl)	382 ± 60	543 ± 120*
Free testosterone (ng/dl)	9.6 ± 1.5	11.4 ± 1.9*
Sex hormone-binding globulin (nM/L)	22.1 ± 7.4	33.9 ± 13.6
Body mass loss [kg (%)]		
Total	8.2 ± 1.4 (8.9 ± 1.3)	6.4 ± 1.5* (8.4 ± 1.8)
Fat free	6.0 ± 1.4 (73 ± 16)	1.9 ± 1.4* (26 ± 23)*
Fat	2.4 ± 1.2 (29 ± 15)	4.5 ± 0.4* (75 ± 22)*
Energy store loss (kcal/d)	1431 ± 537	2141 ± 206*
Reduced energy intake	827 ± 74	731 ± 99*
Increased EIEE	303 ± 43	256 ± 52
Increased RMR	288 ± 245	363 ± 212
Unknown loss	14 ± 532	792 ± 348*
Total ED from SL TDEE (%)	51 ± 19	83 ± 10*
HA absolute intake (kcal/d or g/d)		
Energy	1975 ± 132	1875 ± 278
Protein	125 ± 34	122 ± 45
Carbohydrate	225 ± 17	219 ± 37
Fat	69 ± 20	62 ± 20
HA relative intake (kcal/kg/d or g/kg/d)		
Energy	23.1 ± 1.5	26.4 ± 2.2*
Protein	1.5 ± 0.5	1.7 ± 0.5
Carbohydrate	2.6 ± 0.2	3.1 ± 0.2*
Fat	0.8 ± 0.2	0.9 ± 0.3
HA percent intake (%)		
Protein	25 ± 8	26 ± 8
Carbohydrate	46 ± 1	47 ± 2
Fat	31 ± 8	30 ± 9

Independent Student's *t* tests were used to compare means in participants losing greater than ($n = 7$) and less than ($n = 10$) 50% of TBM as FFM. *$P < 0.05$.

remainder of exposure to HA, RMR was elevated 7–18%, but was not significantly different from SL. Another study showed that elevations in RMR (≥17%) can persist for up to 21 d after initial HA exposure (1). Earlier attenuation of RMR in this study may be partially explained by adaptation to an ED diet.

The prescribed ED and elevated RMR accounted for 74% of the total deficit. Studies have indicated a similar inability to account entirely for the apparent ED indicated by TBM loss at HA (3, 4, 50, 54). The thermic effect of food is ~10% of that measured for RMR, but may be greater when dietary protein intake is increased (55, 56). For example, those on the HP diet consumed ~65 g more protein than those on the SP diet, and if the thermic effect of metabolizing protein is 20–35% (compared to 5–15% for fat and carbohydrate), then the HP group may have expended 30–70 kcal/d more than the SP group. The unexplained deficit was numerically but not significantly greater in those consuming the HP diet, which is consistent with the greater energy cost associated with metabolizing protein, as compared to carbohydrate and fat (57, 58). The Pandolf equation (23) has been shown to adequately predict EIEE at HA in young healthy males walking on a level or positive grade with oxygen uptake levels below 2.1 L/min (59), but a more recent study (60) evaluating energy expenditure during level walking in both males and females of a broader age, weight, and height range, suggests that the Pandolf equation (23) underestimates energy expenditure. Our findings are consistent with this underestimation, which may explain some of the unaccounted for energy expenditure. Finally, grade and terrain, 2 factors in the Pandolf equation (23, 61), were estimated from elevation maps and terrain coefficients (62), respectively, and errors in these estimations may also have contributed to an underestimation of EIEE.

A limitation of the current study is the lack of a direct measure of total body water and TDEE. In the current study, FFM hydration was estimated as 73% of total FFM measured by DEXA (27). In a study by Macdonald and others (6), in which total body water was measured by deuterium oxide dilution, FFM hydration was consistently measured as 73% during 21 d of moderate weight loss at

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HA. Moreover, Krzywicki and colleagues (63) described the effects of acute HA exposure on water metabolism, reporting a 2.25 kg decrease in total body water in healthy adults exposed to HA for 3 d. Our estimated findings were similar, with participants losing 2.6 ± 1.8 kg of aqueous FFM (no group differences); however, the current findings were recorded over a 21 d period. Evidence has shown that diuresis occurs within the first several days at HA and subsides thereafter (63). Applying a broad constant to estimate the contribution of total body water to DEXA-measured FFM likely oversimplifies body water kinetics and omits interindividual variation. Using the doubly labeled water method would have provided a direct measure of TDEE and avoided the need for assumptions related to total body water and ΔES.

In summary, consuming an HP diet was ineffective in sparing FFM during severe ED at HA. However, individuals with greater aerobic fitness before HA ascent experienced attenuated FFM loss. Therefore, incorporating increased aerobic training into pre-ascent preparations may provide added benefit (*i.e.*, beyond the improved cardiovascular responses to exercise) in protection against FFM loss during ED at HA. FJ

ACKNOWLEDGMENTS

The authors thank the volunteers who participated in this research; the medical oversight team, and Dr. Stephen Muza for their support; and Allyson Derosier, Nancy Murphy, Katelyn Guerriere, Karleigh Bradbury, Adrienne Hatch, Ryanne Haskell, Laura Lutz, Alyssa Kelley, Marques Wilson, Christopher Carrigan, Adam Luippold, Bradley Anderson, Grant Holmes, Anthony Karis, Matthew Kominsky, Aaron Stein, Dr. Stephen R. Hennigar, Dr. Taylor Roth, Dr. James P. McClung, and Dr. Scott J. Montain (U.S. Army Research Institute of Environmental Medicine) for their contributions to the project. This work was supported, in part, by the U.S. Army Medical Research and Materiel Command and through fellowship appointments to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education (to C.E.B., A.J.Y., L.M.M., and J.W.C.) through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Materiel Command. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Army or the U.S. Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official U.S. Department of the Army endorsement or approval of the products or services of these organizations. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

C. E. Berryman, A. J. Young, and S. M. Pasiakos designed the research; C. E. Berryman, A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, R. E. Cole, J. W. Carbone, I.-Y. Kim, A. A. Ferrando, and S. M. Pasiakos performed the research; C. E. Berryman and S. M. Pasiakos analyzed the data; C. E. Berryman, A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, J. W. Carbone, I.-Y. Kim, A. A. Ferrando, and S. M. Pasiakos interpreted the results; C. E. Berryman and S. M. Pasiakos prepared the figures; C. E. Berryman and S. M. Pasiakos drafted the manuscript; and C. E. Berryman, A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, R. E. Cole, J. W. Carbone, H. R. Lieberman, I.-Y. Kim, A. A. Ferrando, and S. M. Pasiakos edited and revised the article and approved the final version.

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Received for publication August 30, 2017.
Accepted for publication October 2, 2017.

Downloaded from www.fasebj.org by Louisiana State Univ Troy H Middleton Library (198.62.89.27) on April 05, 2018. The FASEB Journal Vol. 32, No. 2, pp. 894-905.

HIGH ALTITUDE MEDICINE & BIOLOGY
Volume 00, Number 00, 2018
Mary Ann Liebert, Inc.
DOI: 10.1089/ham.2017.0155

Scientific Article

Appetite Suppression and Altered Food Preferences Coincide with Changes in Appetite-Mediating Hormones During Energy Deficit at High Altitude, But Are Not Affected by Protein Intake

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Abstract

Karl, J. Philip, Renee E. Cole, Claire E. Berryman, Graham Finlayson, Patrick N. Radcliffe, Matthew T. Kominsky, Nancy E. Murphy, John W. Carbone, Jennifer C. Rood, Andrew J. Young, and Stefan M. Pasiakos. Appetite Suppression and Altered Food Preferences Coincide with Changes in Appetite-Mediating Hormones During Energy Deficit at High Altitude, But Are Not Affected by Protein Intake. *High Alt Med Biol.* 00:000–000, 2018.—Anorexia and unintentional body weight loss are common during high altitude (HA) sojourn, but underlying mechanisms are not fully characterized, and the impact of dietary macronutrient composition on appetite regulation at HA is unknown. This study aimed to determine the effects of a hypocaloric higher protein diet on perceived appetite and food preferences during HA sojourn and to examine longitudinal changes in perceived appetite, appetite mediating hormones, and food preferences during acclimatization and weight loss at HA. Following a 21-day level (SL) period, 17 unacclimatized males ascended to and resided at HA (4300 m) for 22 days. At HA, participants were randomized to consume measured standard-protein (1.0 g protein/kg/d) or higher protein (2.0 g/kg/d) hypocaloric diets (45% carbohydrate, 30% energy restriction) and engaged in prescribed physical activity to induce an estimated 40% energy deficit. Appetite, food preferences, and appetite-mediating hormones were measured at SL and at the beginning and end of HA. Diet composition had no effect on any outcome. Relative to SL, appetite was lower during acute HA (days 0 and 1), but not different after acclimatization and weight loss (HA day 18), and food preferences indicated an increased preference for sweet- and low-protein foods during acute HA, but for high-fat foods after acclimatization and weight loss. Insulin, leptin, and cholecystokinin concentrations were elevated during acute HA, but not after acclimatization and weight loss, whereas acylated ghrelin concentrations were suppressed throughout HA. Findings suggest that appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at HA. Although dietary protein intake did not impact appetite, the possible incongruence with food preferences at HA warrants consideration when developing nutritional strategies for HA sojourn.

Keywords: ingestive behavior, hypoxia, energy restriction, hunger, satiety, craving

Introduction

Lowlanders sojourning at high altitude (HA; 2500–5000 m) commonly experience unintentional weight loss (Westerterp and Kayser, 2006; Pasiakos et al., 2017). The etiology is multifactorial, but reduced energy intake is a contributing factor (Hamad and Travis, 2006; Westerterp and Kayser, 2006), and coincides with a loss of appetite putatively characterized by a rapid reduction in hunger during meals and early satiation (Westerterp-Plantenga et al., 1999).

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Some evidence suggests that this HA anorexia may be related to hypoxia-induced increases in the anorexigenic hormones, leptin (Tschop et al., 1998; Shukla et al., 2005; Barnholt et al., 2006; Sierra-Johnson et al., 2008; Snyder et al., 2008) and cholecystokinin (CCK) (Bailey et al., 2000), and suppression of the orexigenic hormone ghrelin (Shukla et al., 2005; Riepl et al., 2012; Wasse et al., 2012; Matu et al., 2017a, 2017b). However, findings are not consistent across all studies (Zaccaria et al., 2004; Benso et al., 2007; Vats et al., 2007; Riepl et al., 2012; Aeberli et al., 2013; Debevec et al., 2014, 2016; Mekjavic et al., 2016; Morishima and Goto, 2016). These inconsistencies and heterogeneous study designs have impeded reaching consensus on the role of appetite-mediating hormones in the development and persistence of HA anorexia (Hamad and Travis, 2006; Westerterp and Kayser, 2006; Raff et al., 2008; Sierra-Johnson et al., 2008; Debevec, 2017). An improved understanding of the contribution of appetite-mediating hormones to HA anorexia, and how that impact evolves with acclimatization, could help develop optimized strategies for mitigating HA anorexia.

Higher protein (>1.2 g protein/kg body weight/d) diets preserve fat-free mass during weight loss (Wycherley et al., 2012; Leidy et al., 2015; Pasiakos et al., 2015a, 2015b) which may be beneficial for maintaining physical function and health. These benefits underpin recommendations supporting higher protein diets for athletes (Thomas et al., 2016) and military personnel (Pasiakos et al., 2013a, 2013b, 2015a, 2015b) and have stimulated recent interest in studying the effectiveness of higher protein diets for mitigating fat-free mass loss during HA sojourn (Pasiakos et al., 2017). However, protein increases fullness (Dhillon et al., 2016) and is more satiating than carbohydrate or fat (Leidy et al., 2015). These effects may be potentiated at HA due to the higher thermogenic effect of protein relative to fat (Veldhorst et al., 2008) which could worsen hypoxia by increasing postprandial oxygen consumption (Westerterp and Kayser, 2006; Veldhorst et al., 2008). Higher protein diets may also be incompatible with food preferences at HA, which have favored carbohydrate in some (Boyer and Blume, 1984; Kayser et al., 1993; Westerterp-Plantenga et al., 1999; Bailey et al., 2004; Hamad and Travis, 2006; Matu et al., 2017a, 2017b), but not all (Rose et al., 1988; Reynolds et al., 1998; Aeberli et al., 2013), studies. Collectively, these effects could prove counterproductive to retaining fat-free mass during HA-induced weight loss by exacerbating HA anorexia and further reducing energy intake. Although these possibilities have been acknowledged (Westerterp and Kayser, 2006; Pasiakos et al., 2017), to date, the effects of higher protein diets on appetite and food preferences during HA sojourn are undetermined.

We recently reported that consuming a controlled higher protein hypocaloric diet relative to a controlled standard-protein hypocaloric diet did not protect fat-free mass during weight loss over 22 days at HA (Berryman et al., 2017). This report details changes in perceived appetite, appetite-mediating hormones, and food preferences measured as secondary outcomes during that study. The objectives associated with these outcomes were: (1) to determine the effects of a higher protein hypocaloric diet relative to a standard-protein hypocaloric diet on perceived appetite and food preferences during HA sojourn and (2) to examine longitudinal changes in perceived appetite, appetite-mediating hormones, and food preferences during acclimatization and weight loss at HA.

Materials and Methods

Participants

Twenty-one men gave their free and informed voluntary consent to participate in this randomized controlled trial. Participants were 18–42 years, exercised ≥2 days/week, were born at altitudes <2100 m, had not traveled to altitudes >1200 m for ≥5 days within 2 month of participation, and were not taking any medications. Exclusion criteria included musculoskeletal injury, metabolic, cardiovascular, and gastrointestinal abnormalities or disorders, diseases affecting macronutrient metabolism or ability to participate in strenuous exercise, sleeping disorders or apnea, history of HA pulmonary or cerebral edema, alcoholism, substance abuse, anabolic steroid use, anemia, sickle cell trait, and elevated prothrombin time. Participants were asked to abstain from alcohol, caffeine, dietary supplements, and nicotine products throughout the study. The study was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (Natick, MA) and conducted May-Aug 2016. Investigators adhered to the policies regarding the protection of human subjects as prescribed in Army Regulation 70–25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. The trial was registered on www.clinicaltrials.gov as NCT02731066.

Study design

The 43-day study included a 21-day sea level (SL) phase (50 m; Natick, MA), which was immediately followed by 22 days at HA (4300 m; Pikes Peak, CO) (Berryman et al., 2017) (Fig. 1). Upon enrollment, investigators randomized participants using computer-generated randomization to consume a standard-protein or higher protein diet at HA. During SL, participants were free living but visited the laboratory daily and maintained habitual exercise. Compliance with diet instructions and body weight maintenance was verified by diet records and daily body weight monitoring.

On SL day 21 participants were flown from Boston, MA to Denver, CO where they were placed on supplemental oxygen until ascent. In the early morning after arrival in Colorado (HA day 0), participants ascended to 4300 m by car where they resided for 22 days in a temperature-controlled indoor environment at the U.S. Army Research Institute of Environmental Medicine Maher Memorial Laboratory.

During HA, participants consumed a measured higher protein or standard-protein diet designed to provide 70% of SL weight maintenance energy requirements. To induce a 40% total energy deficit, supervised low-to-moderate intensity exercise was conducted to increase physical activity expenditure by 10% of weight-maintenance energy needs. The duration and magnitude of the energy deficit were designed to replicate those used in a recent study from our group investigating the effects of varying protein intake on body composition during weight loss at SL (Pasiakos et al., 2013a, 2013b) and was in accord with the primary study objective of determining the efficacy of a higher protein diet for fat-free mass retention during weight loss at HA (Berryman et al., 2017). This magnitude of energy deficit was within ranges reported during HA sojourns in which dietary intake was *ad libitum* (Hoyt et al., 1994; Westerterp et al., 1994) and was consistent with previous studies investigating endocrine responses at HA (Barnholt et al., 2006).

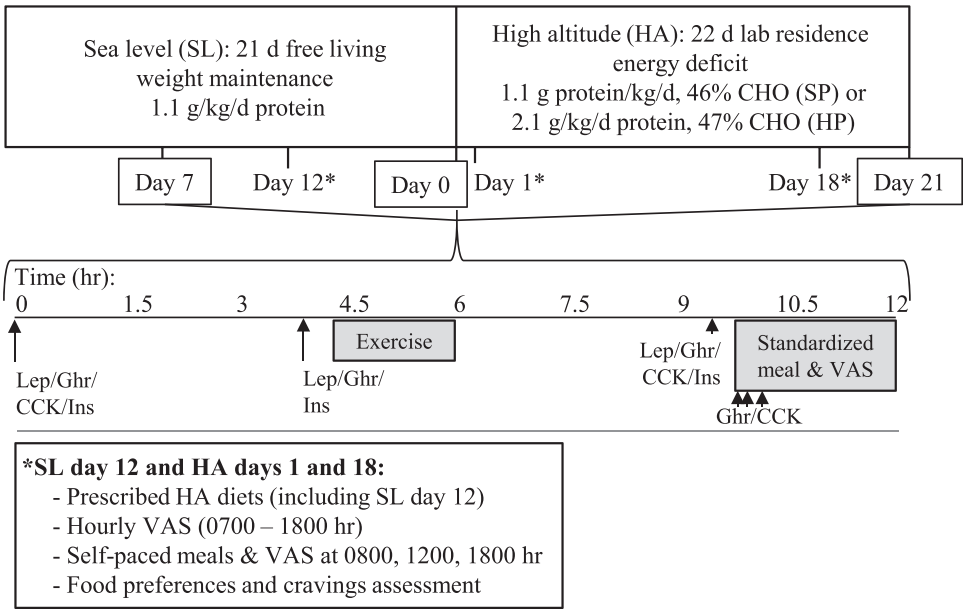


FIG. 1. Study design. Volunteers were followed for 21 days at SL, then for 22 days while living at HA (4300 m) and consuming standard-protein or higher protein hypocaloric diets. CCK, cholecystokinin; CHO, carbohydrate; Ghr, acylated ghrelin; Ins, insulin; Lep, leptin; VAS, visual analog scale measuring appetite; SL, sea level; HA, high altitude.

The shortened version of the Environmental Symptoms Questionnaire was administered at SL and daily during HA to assess the incidence of acute mountain sickness, which was defined as a Lake Louise score of >0.7 (Beidleman et al., 2007). Oxygen saturation was measured at SL and daily during HA by finger pulse oximetry (Nonin Model 7500 or 8600; Plymouth, MN).

Study diets

During SL, all participants consumed a self-selected weight maintaining diet. Registered dietitians provided instruction on consuming a diet containing 1.0 g protein/kg/d, 45%–65% energy from carbohydrate, and 20%–35% energy from fat. Twenty-four hour food records were completed and reviewed every 2–3 days, and additional education was provided as needed to promote adherence. During SL, most participants consumed meals from the local cafeteria or local restaurants.

During HA, all food and caloric beverages consumed were strictly controlled, weighed and measured, and provided by study staff. Meals and snacks were supervised, and participants were instructed to consume all provided food and beverages. Any uneaten foods were weighed and documented. Diets were individualized, followed a five-day rotating menu, and were designed to provide a standard (SP; 1.0 ± 02 g/kg/d) or higher (HP; 2.0 ± 0.2 g/kg/d) amount of protein, and 45% of total energy as carbohydrate each day. Fat intake was reduced in HP to accommodate the higher protein intake (Table 1). Most of the foods were entrées, sides, and snack items included in U.S. military Meals Ready-to-Eat rations. These items were supplemented with fresh fruits and vegetables, fruit snacks, olive oil, and ranch-flavored salad dressing. Protein intake was also manipulated using a whey-protein beverage (Isopure® Zero Carb; Isopure Co., Hauppauge, NY). Although the diets differed in appearance, participants were not told to which dietary group they were assigned. Whether participants could correctly identify their group assignment was not assessed. Water and noncaffeinated calorie-free sodas were allowed *ad libitum*. Dietary intake was assessed using daily food records, the Combat Rations Database (U.S. Army Natick Soldier Research Development and Engineering Center and USARIEM), and Food Processor SQL v.10.14 (ESHA Research, Salem, OR).

Effects of protein intake on appetite and food preferences during energy deficit at HA (objective 1)

Appetite testing. To determine the effects of protein intake on appetite at HA, perceived appetite was measured using visual analog scales administered hourly, and immediately before and after meals on SL day 12 and HA days 1 and 18. Participants consumed the same individualized menu

Table 1. Baseline Participant Characteristics and Actual Dietary Intakes Over 22 Days at High Altitude (4300 m)

	Standard protein (n = *8*)	*Higher protein* (n = *9*)
Age, *year*	23 ± 3	24 ± 7
BMI, *kg/m*^{2}	27.0 ± 4.0	25.5 ± 3.1
Body fat,%	22.8 ± 7.0	22.6 ± 5.5
TDEE, *kcal/d*	2744 ± 264	2635 ± 344
Energy intake, *kcal/d*	1950 ± 186	1885 ± 269
Protein intake, *g/kg/d*	1.1 ± 0.0	2.1 ± 0.0*
Protein intake,%	18 ± 2	33 ± 2*
Carbohydrate intake, %	46 ± 1	47 ± 1
Fat intake,%	39 ± 2	23 ± 3*

Values are mean ± SD. TDEE, total daily energy expenditure during weight maintenance at sea level. Adapted from Berryman et al. (2017).

*Independent samples *t*-test, $p < 0.05$.

on all three test days and ate all meals and snacks at the same times each day. On SL day 12 the prescribed meals matched the HA energy prescription and diet assignment to eliminate any confounding resulting from differences in energy intake between SL and HA. Diets were constant within participants across test days, but because the diets were individualized, the types of foods and the volume of food and beverages consumed differed between participants. Water was allowed *ad libitum* between meals.

Food preferences and cravings. Questionnaires were administered on SL day 12 and HA days 1 and 18 to determine the effects of protein intake on food preferences and cravings at HA. Food preferences were measured using the Leeds Food Preference Questionnaire (LFPQ), which was administered before and after lunch. The LFPQ is a computerized platform that uses pictures of individual food items selected from a validated database of common foods to measure different components of food preferences and hedonics (Finlayson et al., 2007). Pictures for each test varied in two separate dimensions; fat (low and high) and taste (sweet and savory) or protein (low and high) and taste (sweet and savory). Four pictures from each food type were selected resulting in 16 separate pictures being used within each test (Supplementary Table S1; Supplementary materials are available online at http://www.liebertpub.com/ham).

The LFPQ measures three constructs as follows: explicit liking (i.e., perceived hedonic impact of the food), explicit wanting (i.e., conscious desire to consume a food), and implicit wanting (i.e., subconscious motivation for a food). To measure explicit liking, the participant was shown each picture in random order and asked to respond to the question ''how pleasant would you find the taste of this food right now'' using a visual analog scale. To measure explicit wanting, the participant was shown each picture in random order and asked to respond to the question ''how much do you want to eat this food right now'' using a visual analog scale. Implicit wanting was measured using forced choice methodology in which pairs of food images from separate food categories were presented in randomized order on the computer screen. Participants were asked to as quickly as possible select the food that they most wanted to eat at that moment.

Both frequencies of selections within each food category and response time (i.e., relative preference) were recorded. The preference for different food types was computed for each metric by subtracting the mean scores (visual analog scale scores or frequency of selection and reaction time) from a comparator group (low fat/protein and savory) relative to the matched reference group (high fat/protein and sweet, respectively). Food cravings were assessed before lunch using the Food Cravings Inventory (White and Grilo, 2005) and Control of Eating Questionnaire (Dalton et al., 2015).

Longitudinal changes in appetite and appetite-mediating hormones during acclimatization and weight loss at HA (objective 2)

Longitudinal changes in appetite and related hormones were examined on SL day 7 and HA days 0 and 21 by measuring circulating hormone concentrations throughout the day and by measuring appetite, CCK, and acylated ghrelin responses to a standardized meal. On these days participants did not consume their prescribed diets to conduct testing for unrelated study objectives which have been (Berryman et al., 2017) or will be reported elsewhere. Hormone concentrations were not measured on SL day 12 and HA days 1 and 18 (i.e., full-day appetite testing) for logistical reasons. Therefore, because prescribed SP and HP diets were not consumed until after HA day 0, and because diet habituation has been reported not to influence fasting hormone concentrations independent of body weight changes (Ellis et al., 2012), we did not expect hormone concentrations to differ by diet group unless differential changes in body composition were observed. As such, determining the effects of protein intake on appetite-mediating hormones was not a study objective. However, these study days provided an opportunity to examine appetite-mediating hormone responses to acute HA exposure and whether those responses persisted after acclimatization and weight loss. In addition, these days allowed us to examine whether the rates of change of postprandial appetite responses, as was previously reported (Westerterp-Plantenga et al., 1999), and related hormones differed between HA and SL.

Fasting blood samples were collected shortly after waking following a >10-hour fast on SL day 7 and HA days 0 and 21. Additional blood samples were collected from an indwelling venous catheter 4 hours and 9.5 hours later and during a standardized meal test beginning 10 hours after the fasting blood draw (Fig. 1). No food was consumed after dinner the evening prior until the standardized meal test. However, participants consumed beverages containing 145 g ($n=9$) or 0 g ($n=8$) of carbohydrate during an 80-minute submaximal aerobic exercise bout 3.5–5 hours before the meal test, and all participants drank 25 g of whey-protein (Isopure® Zero Carb) 3 hours before the meal test. The rationale for providing these beverages was to conduct testing for unrelated study objectives (Berryman et al., 2017). Potential confounding due to carbohydrate intake was addressed by including this variable in statistical models.

Standardized meal tests occurred between 1530 and 1630 hours. For each meal test, participants were provided a fixed-portion meal to consume at a controlled eating rate and 240 g water to drink. The meal was macaroni and cheese with added butter and provided 20% of individual weight maintenance energy needs determined during SL (41%, 49%, and 10% energy from carbohydrate, fat, and protein, respectively). Participants were instructed to consume all of the food and water provided in 12 minutes while eating at a constant rate (Karl et al., 2011). Over the subsequent 105 minutes, water intake was prescribed, and no additional food or beverage was allowed. Blood samples were collected approximately 30 minutes before the meal and at 7.5, 15, and 30 minutes after starting the meal. Perceived appetite was measured before, during, and periodically for 105 minutes after completing the meal using visual analog scales.

Serum insulin and leptin and plasma acylated ghrelin and CCK were measured throughout SL day 7 and HA days 0 and 21. However, only acylated ghrelin and CCK responses were measured during the standardized meal test. For serum measures, blood was collected into Monovettes containing clotting activator (Sarstedt, Newton, NC) and stored at −20°C to −80°C until analysis. Serum insulin was determined using the IMMULITE® 2000 (Siemens Healthcare Diagnostics, Deerfield, IL). Serum leptin was determined by RIA (EMD Millipore, St. Charles, MO) according to manufacturer's instructions. For plasma measures, blood was collected into

chilled Monovettes containing EDTA-K_3 and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (1 mg/mL whole blood) for acylated ghrelin determination or protease inhibitor cocktail (40 μL/mL whole blood; cOmplete, EDTA-free) for CCK determination. Acylated ghrelin aliquots were combined with 50 μL 1N HCl/mL plasma, and all samples were stored at −20°C to −80°C until analysis. Plasma acylated ghrelin was determined by RIA (EMD Millipore) and plasma CCK by ELISA (Cusabio, College Park, MD) according to manufacturer's instructions.

Visual analog scales

Four separate visual analog scales were used to measure self-perceived appetite (Blundell et al., 2010). Participants rated their levels of hunger and fullness, their desire to eat, and the amount of food they thought they could eat ''right now'' (i.e., prospective consumption) by marking anywhere on a 10-cm scale anchored by phrases representing opposite extremes of a spectrum (e.g., ''not at all hungry'' and ''extremely hungry''). A total appetite score was computed as follows:

$$\begin{aligned}\text{Overall appetite} = (&\text{hunger} + \text{desire to eat}\\ &+ \text{prospective consumption}\\ &+ (10 - \text{fullness}))/4\end{aligned}$$

Statistical analyses

Sample size estimates based on the primary study outcome, fat-free mass, indicated $n = 8$/grp were required to detect a 1 kg between-group difference in fat-free mass loss (Berryman et al., 2017). This sample size was estimated to allow detection of a 25% difference in mean between-group appetite ratings at $\alpha = 0.05$ and power = 0.80 based on variability measured in our previous work (Karl et al., 2016). Although a 25% difference in appetite ratings is larger than the 8%–10% difference deemed to be of practical relevance (Blundell et al., 2010), >25% differences have been reported in previous studies comparing the impact of higher and lower protein meals on appetite (Dhillon et al., 2016). Statistical analyses were completed using SPSS v.21 (IBM Analytics; Armonk, NY). All data were checked to verify adherence to model assumptions and $\log_{10}$-transformed when necessary. Data are presented as mean ± SD unless otherwise noted, and statistical significance was set at $p \le 0.05$.

The effects of study day, diet (SP and HP), and their interaction on outcomes measured on SL day 12 and HA days 1 and 18 were tested using linear mixed models, generalized linear mixed models, and repeated measures ANOVA. Appetite ratings measured hourly on SL day 12 and HA days 1 and 18 were analyzed using area under the curve calculated from ground (AUC; where ground is y = 0).

The effects of study day, diet, carbohydrate ingestion during exercise (145 g and 0 g carbohydrate), time, and their interactions on appetite ratings and hormone concentrations measured on SL day 7 and HA days 0 and 21 were tested using linear mixed models. All possible interactions except any between dietary protein group and carbohydrate supplement group were included in these models. Appetite ratings measured during and after the standardized meal test were analyzed using the metrics AUC, rate of change in appetite ratings during the meal, and rate of change in appetite ratings after the meal, and therefore, models did not include time as a variable. To calculate rates of change, slopes were extracted from linear regression models run separately for each individual using the appetite ratings measured from 0 to 15 minutes during the meal and from 15 to 120 minutes after the meal. To aid in interpretation of hormone analyses, models were reduced by removing effects that did not significantly contribute to the overall model fit until the most parsimonious model was reached. As expected, dietary protein group did not impact outcomes measured on SL day 7 and HA days 0 and 21, and therefore, results are presented with groups combined for outcomes measured on those days. In addition, hormones were analyzed both as measured and after adjusting for plasma volume changes (Dill and Costill, 1974). Plasma volume adjustment did not affect interpretation of the results, and only unadjusted values are reported. For all models, when a significant interaction or main effect was observed, *t*-tests with Bonferroni corrections were used to identify pairwise differences.

Results

During SL testing, one individual from each diet group withdrew, and one individual from each diet group was withdrawn for nonadherence with study procedures. As such, 17 of 21 enrolled participants completed both the SL and HA phases (Table 1). Primary study outcomes are reported elsewhere (Berryman et al., 2017). Briefly, participants were weight stable during SL (mean weight change 0.7 kg [95% CI: −0.3 to 1.7], $p = 0.21$). Total weight loss during HA (SP: 7.2 ± 2.4 kg vs. HP: 7.0 ± 0.9 kg; diet-by-day interaction, $p = 0.88$) and fat-free mass loss during HA as measured by dual energy X-ray absorptiometry (SP: 4.0 ± 3.3 kg vs HP: 3.2 ± 1.5 kg; diet-by-day interaction, $p = 0.53$) did not differ by dietary protein group.

Lake Louise scores were elevated on HA days 0–3 relative to SL, but not thereafter (main effect of day, $p < 0.01$), and did not differ by diet group (data not shown). On HA days 1 and 18 (i.e., full-day appetite testing) the incidence of acute mountain sickness was higher relative to SL (main effect of day, $p < 0.001$), but did not differ by diet group (Table 2). Oxygen saturation decreased upon arrival at HA and remained below SL values throughout HA (main effect of time, $p < 0.01$) and did not differ by diet group (data not shown). However, when only SL day 12 and HA days 1 and 18 were included in the model, oxygen saturation was lower in HP relative to SP on HA day 1 (diet-by-day interaction, $p = 0.02$; Table 2).

Effects of protein intake on appetite and food preferences during energy deficit at HA (SL day 12 and HA days 1 and 18)

Appetite ratings measured on SL day 12 and HA days 1 and 18 indicated changes in appetite over time that were independent of dietary protein group (Fig. 2). Specifically, hunger, prospective consumption, desire to eat, and overall appetite AUC were lower, whereas fullness AUC was higher on HA day 1 relative to SL (main effect of day, $p < 0.01$). On HA day 18 none of these measures differed from SL. Of note, overall appetite AUC was 19% higher in SP relative to HP on HA day 18, but this difference was not statistically significant. Self-selected meal duration ranged from 6.1 ± 1.4 minutes at SL to 7.8 ± 5.3 minutes on HA day 18 and did not differ by day ($p = 0.36$) or by dietary protein group ($p = 0.80$).

Table 2. Acute Mountain Sickness, Oxygen Saturation, Actual Dietary Intakes, and Meal Acceptability Measured During Appetite Testing Days Conducted at Sea Level and While Living at High Altitude (4300 m)

	Standard protein (n = *8*)			*Higher protein* (n = *9*)		
	SL day 12	*HA day 1*	*HA day 18*	*SL day 12*	*HA day 1*	*HA day 18*
AMS, *%n*[1,2]	0	50	12	0	78	44
Nausea, *%n*[1,3]	0	37	25	0	44	44
O_2 saturation, %[4]	96.6 ± 1.1[a]	83.0 ± 1.4[b]	87.0 ± 1.9[c]	97.1 ± 1.1[a]	79.0 ± 3.6[b]*	87.2 ± 1.9[c]
Energy intake, *kcal/d*[4]	1981 ± 185	2019 ± 193	1966 ± 243	1966 ± 274[a]	1775 ± 387[b]	1968 ± 267[a]
Protein intake, *g/d*[5]	93 ± 15	93 ± 14	91 ± 16	166 ± 26	148 ± 37	163 ± 26
Carbohydrate intake, *g/d*[4]	214 ± 23	219 ± 23	211 ± 30	215 ± 32[a]	199 ± 34[b]	216 ± 32[a]
Fat intake, *g/d*[5]	90 ± 7	92 ± 7	90 ± 9	55 ± 9	48 ± 15	56 ± 9
Meal acceptability[6,7]	7 ± 2	6 ± 2	5 ± 3	7 ± 2	6 ± 2	5 ± 3

Values are *%n* or mean ± SD.
[1]Generalized linear mixed model with Bonferroni corrections; subject included as a random factor, and diet, day, and their interaction as fixed factors. Main effect of day ($p < 0.001$); incidence higher on HA days 1 and 18 relative to SL.
[2]Acute mountain sickness (AMS) was defined as a Lake Louise score >0.7 on the shortened version of the Environmental Symptoms Questionnaire (Beidleman et al., 2007).
[3]Incidence of nausea defined as slight to extreme nausea reported on the Environmental Symptoms Questionnaire.
[4–6]Linear mixed model with Bonferroni corrections; subject included as a random factor, and diet, day, and their interaction as fixed factors. [4]Diet-by-day interaction, $p < 0.05$. [a–c]Values within a dietary protein group sharing a superscript letter are not significantly different within that group. [5]Main effect of group, $p < 0.001$. [6]Main effect of day ($p < 0.001$); lower acceptability on HA days 1 and 18 relative to SL.
[7]Overall meal acceptability rated from 1 (dislike extremely) to 9 (like extremely). Values are the mean of ratings at breakfast, lunch, and dinner.
*Significantly different from the same day in the standard-protein group, $p < 0.05$.

Overall meal acceptability was lower on HA days 1 and 18 relative to SL (main effect of day, $p < 0.001$) independent of dietary protein group (Table 2).

Responses to the LFPQ indicated changes in food preferences over time independent of dietary protein group (Fig. 3). Specifically, an increased preference for sweet over savory foods across all domains of the LFPQ and a conscious preference for low-protein over high-protein foods was observed on HA day 1 (main effect of day, $p < 0.05$), but not HA day 18, relative to SL. An increased preference for high-fat over low-fat food across all domains of the LFPQ was observed on HA day 18 relative to SL day 12 and HA day 1 (main effect of day, $p < 0.05$).

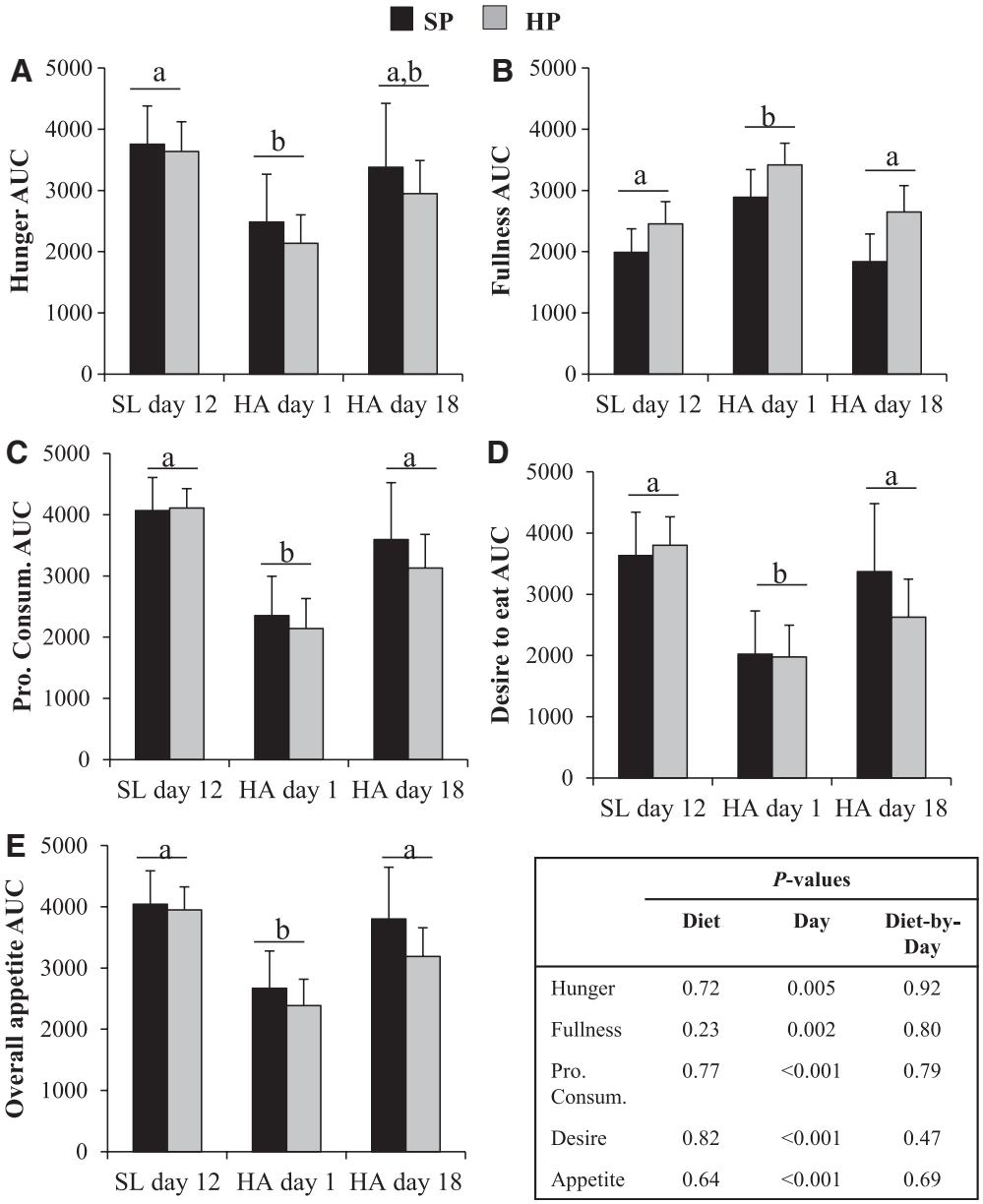
Scores on the Control of Eating Questionnaire indicated that the overall strength and frequency of food cravings were higher during the final week of HA relative to the final week at SL (main effect of day, $p = 0.03$) (Supplementary Table S2). Similarly, scores on the Food Craving Inventory indicated higher total food cravings during HA relative to the final week at SL (main effect of day, $p = 0.03$) which was attributable to an increase in cravings for high fat and fast foods (main effect of day, $p \leq 0.02$) (Supplementary Table S2). No food craving metric differed between dietary protein groups.

Longitudinal changes in appetite and appetite-mediating hormones during acclimatization and weight loss at HA (SL day 7 and HA days 0 and 21)

Appetite responses to a standardized test meal. Appetite responses to the standardized meal test administered on SL day 7 and HA days 0 and 21 indicated changes in appetite responses over time that were independent of both dietary protein group and carbohydrate supplementation during exercise. Specifically, hunger AUC was lower on HA day 0, but not HA day 21, relative to SL, whereas fullness AUC was higher on HA day 21, but not HA day 0, relative to SL (main effect of day, $p \leq 0.03$; Fig. 4A, B). Overall appetite, prospective consumption, and desire to eat AUC were lower on HA days 0 and 21 relative to SL (main effect of day, $p \leq 0.01$; Fig. 4C–E). These differences were attributable to a combination of differences in premeal appetite ratings (main effect of day, $p < 0.05$) and differences in the rate of change in appetite ratings during the postprandial period (main effect of day, $p \leq 0.01$) across study days (Fig. 4). No differences between days in the rate of change in any appetite rating measured during the meal (0–12 minutes) were observed (main effect of day, $p > 0.05$). The overall acceptability of the meal was rated as ''moderately liked'' (mean score = 7.1 ± 1.6), which did not change over time (main effect of day, $p = 0.10$) or differ between dietary protein groups (main effect of diet, $p = 1.00$).

Appetite-mediating hormones. Insulin, leptin, acylated ghrelin, and CCK concentrations measured before the standardized meal test indicated changes over time that were independent of dietary protein group. Specifically, fasting insulin concentrations were higher on SL day 7 and HA day 0 relative to HA day 21 and decreased during the day on SL day 7 and HA day 21, but not on HA day 0 (Fig. 5A). Leptin concentrations decreased during the day at SL but not during HA days 0 and 21 and were higher at all time points on SL day 7 and HA day 0 relative to HA day 21 (Fig. 5B).

Acylated ghrelin concentrations measured during the day were affected by carbohydrate supplementation during exercise (Fig. 5C). In the carbohydrate group, mean acylated ghrelin concentrations were lower during HA days 0 and 21 relative to SL. In the placebo group, ghrelin concentrations did not differ across study days. A diet-by-day interaction was observed for aclyated ghrelin concentrations measured before the fixed meal ($p = 0.01$). However, *post hoc* testing indicated that the interaction was due to higher acylated ghrelin concentrations at HA day 0 in HP relative to SP ($p = 0.03$). As consumption of the intervention diets did not start until HA day 1, this result was considered unrelated to



	P-values		
	Diet	**Day**	**Diet-by-Day**
Hunger	0.72	0.005	0.92
Fullness	0.23	0.002	0.80
Pro. Consum.	0.77	<0.001	0.79
Desire	0.82	<0.001	0.47
Appetite	0.64	<0.001	0.69

FIG. 2. Appetite at SL and HA (4300 m) while consuming hypocaloric standard-protein (SP, 1.1 g/kg/d, $n = 8$) or higher protein (HP; 2.1 g/kg/d, $n = 9$) diets. AUC for perceived hunger **(A)**, fullness **(B)**, prospective consumption (Pro. Consum.,) **(C)**, and desire to eat **(D)** measured at SL and after 1 and 18 days of living at HA. Metrics measured by visual analog scales administered hourly (0700–1800) and after meals. Overall appetite **(E)** was computed from hunger, fullness, prospective consumption, and desire to eat ratings. **(A–E)** Bars are mean ± SEM. Effects of diet, day, and their interaction tested using linear mixed models with subject included as a random factor and Bonferroni corrections. Superscripts indicate main effect of day, $p < 0.05$. Bars not sharing a superscript letter are significantly different, $p < 0.05$. AUC, area under the curve calculated from ground.

the study diets. CCK concentrations decreased during the day on SL day 7 and HA day 21, but not HA day 0 (Fig. 5D).

During the standardized meal test, CCK concentrations increased and were higher throughout the test during HA day 0 relative to SL day 7 and HA day 21 (Fig. 6A). Acylated ghrelin concentrations decreased following the standardized meal test and were higher at SL relative to HA (Fig. 6B).

Discussion

The primary finding of this study was that dietary protein intake did not impact appetite during energy deficit at HA. However, acute HA exposure was associated with appetite suppression concomitant to elevated concentrations of the anorexigenic hormones insulin, leptin, and CCK, depressed concentrations of the orexigenic hormone acylated ghrelin, and preferences for sweet and low-protein foods. Although anorexigenic hormone concentrations did not remain elevated following acclimatization and weight loss, appetite and acylated ghrelin concentrations remained depressed while food preferences shifted toward high fat foods.

To our knowledge, this study is the first to examine differential effects of controlled standard- and higher protein hypocaloric diets on appetite regulation at HA. Contrary to

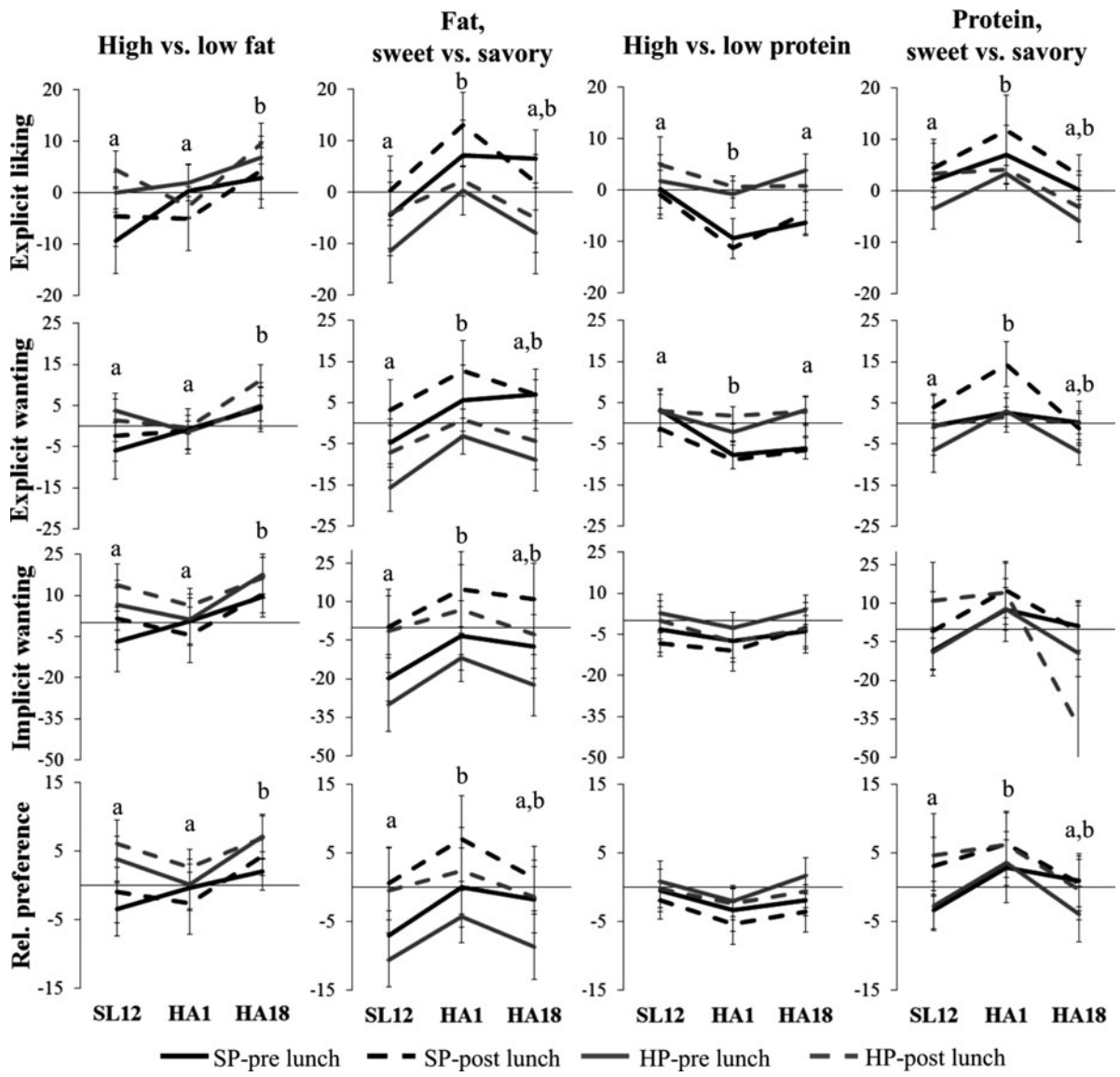


FIG. 3. Food preferences at SL and HA (4300 m) while consuming hypocaloric standard-protein (SP, 1.1 g/kg/d, $n = 8$) or higher protein (HP; 2.1 g/kg/d, $n = 9$) diets. Food preferences were measured before and after lunch on SL day 12 and after 1 and 18 days of living at HA using the Leeds Food Preference Questionnaire. Positive values indicate preference for high fat/protein and sweet foods, negative values indicate preference for low fat/protein and savory foods. Values are mean ± SEM. Effects of diet, day, and their interaction tested using repeated measures ANOVA. Superscripts indicate main effect of day, $p < 0.05$. Values not sharing a superscript letter are significantly different, $p < 0.05$.

our hypothesis, protein intake did not significantly impact appetite at SL or during HA exposure (Fig. 2). Of note, between-group differences in overall appetite following acclimatization and weight loss were sufficiently large to be considered of practical relevance if statistically significant (Blundell et al., 2010). Unfortunately, logistical constraints prevented obtaining a larger sample size, and one limitation of the study is limited statistical power due to the small sample sizes within dietary protein groups.

Additional factors may also have decreased the ability to detect significant effects of protein intake on appetite. First, between-group differences in protein intake ranged from 8 to 19 g/meal, which is less than the 30 g/meal difference others have suggested as a threshold for observing satiety-enhancing effects of higher protein meals (Paddon-Jones and Leidy, 2014). Second, the proportion of total energy intake derived from liquids was higher in HP relative to SP as a result of the beverage used to increase protein intake, and liquids have been shown to elicit weaker satiety effects than solid foods (Leidy et al., 2011). Given these limitations, the hypothesis that higher protein diets could be counterproductive for maintaining fat-free mass at HA by exacerbating HA anorexia cannot be conclusively refuted and warrants further investigation.

A second aim of this study was to explore mechanisms underlying acute HA anorexia and their persistence. Several recent studies have likewise aimed to characterize appetite-mediating hormone responses to acute and chronic hypoxia (Debevec et al., 2014, 2016; Mekjavic et al., 2016), but were conducted at lower simulated altitudes in normobaric hypoxia. That environment may elicit different physiological responses than the hypobaric hypoxia at 4300 m studied in

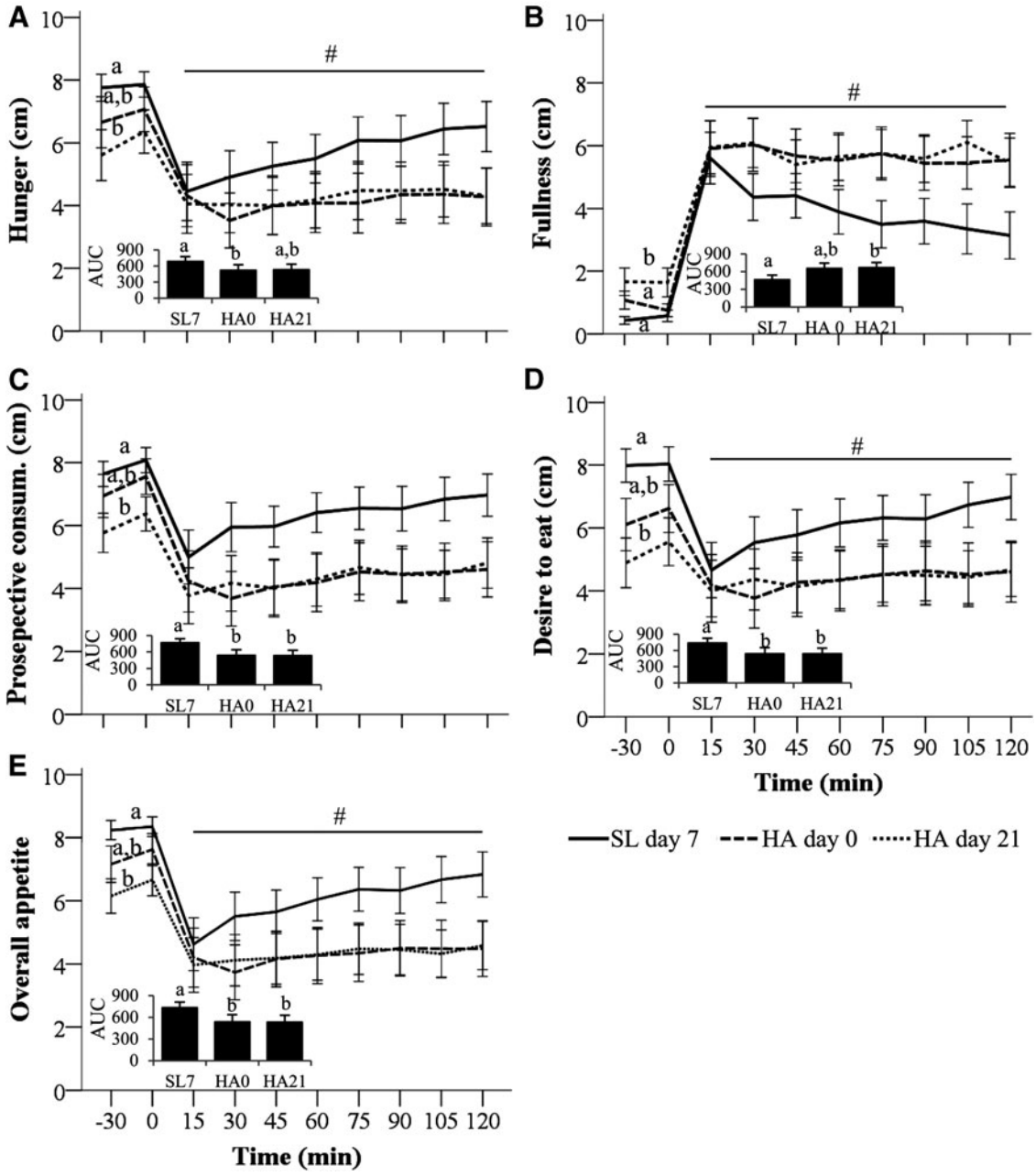


FIG. 4. Appetite response to a standardized meal at SL and HA (4300 m). Perceived hunger **(A)**, fullness **(B)**, prospective consumption **(C)**, and desire to eat **(D)** measured during a standardized meal test at sea level (SL7), on the first day of HA exposure (HA0) and after 21 days of living at HA (HA21). Overall appetite **(E)** was computed from hunger, fullness, prospective consumption, and desire to eat ratings. Values and bars (AUC) are mean ± SEM. Effects of diet (standard- or higher protein), carbohydrate group (carbohydrate beverage or placebo), day, and their interactions tested using linear mixed models with subject included as a random factor and Bonferroni corrections. Diet and carbohydrate groups had no effects on any outcome. Premeal values (0 minutes) and bars not sharing a superscript are significantly different (main effect of day, $p < 0.05$). #Slopes from 15 to 120 minutes on SL day 7 are significantly different from those on HA days 0 and 21 (main effect of day, $p \leq 0.01$).

this investigation (Millet et al., 2012). Furthermore, in the present study, we tightly controlled physical activity, climate, and diet, factors which may have confounded several previous field studies and contributed to inconsistencies in observations (Sierra-Johnson et al., 2008; Debevec, 2017). However, the absence of a SL control group is a limitation of the present study that prevents establishing causal effects of HA on study outcomes. Although this limitation is less likely to impact study outcomes measured during acute HA, it does preclude definitively separating the impact of acclimatization to HA on study outcomes from factors such as weight loss, diet monotony, and living in a confined environment. Potential confounding due to weight loss is particularly important to consider because weight loss independently influences appetite, food preferences, and appetite-mediating hormones (Maclean et al., 2011; Sumithran and Proietto,

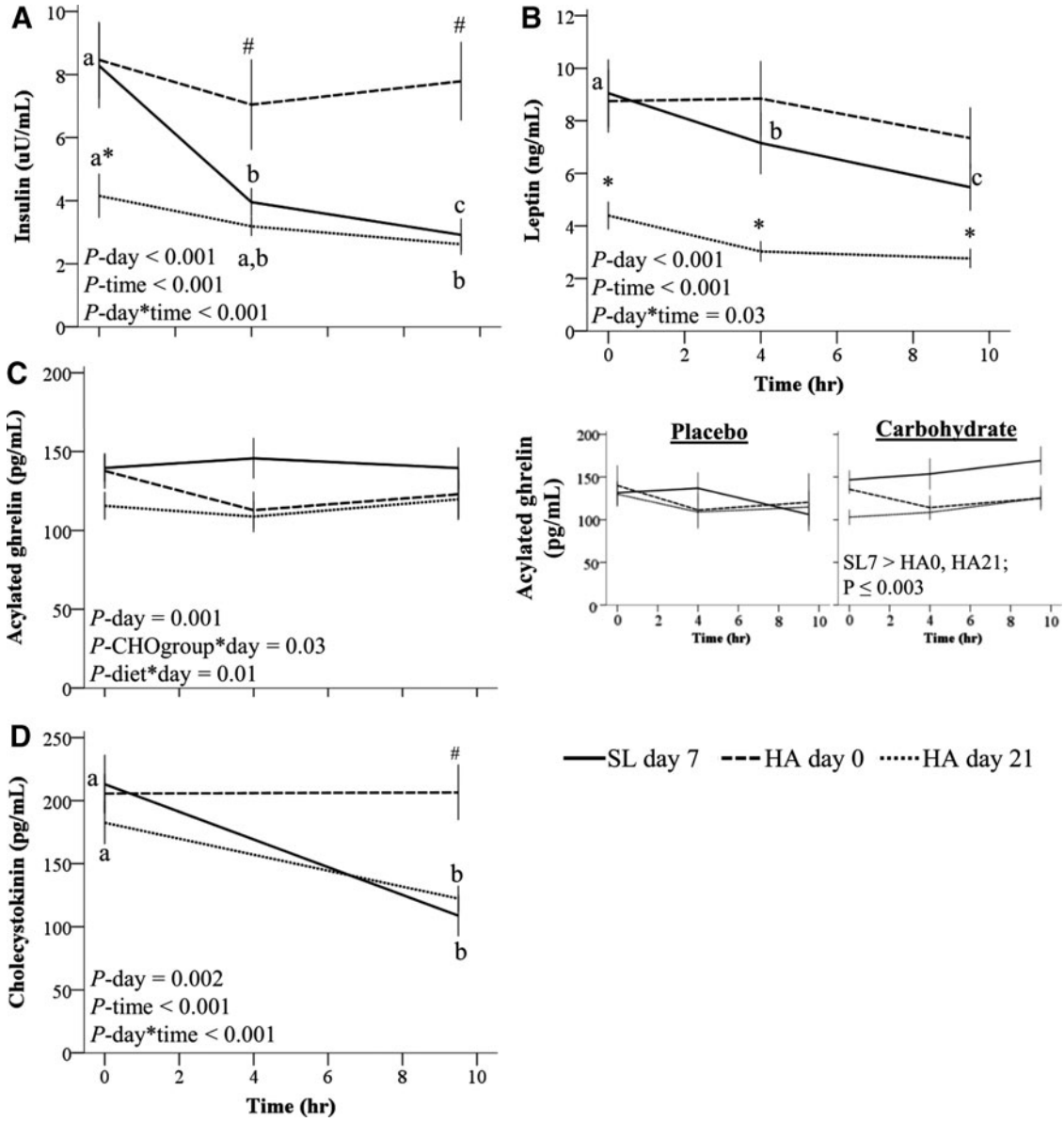


FIG. 5. Appetite-mediating hormones at HA (4300 m). **(A)** Insulin ($n = 17$; $\log_{10}$-transformed for analysis), **(B)** leptin ($n = 16$), **(C)** acylated ghrelin ($n = 17$), and **(D)** cholecystokinin ($n = 17$) measured at SL, on the first day of HA exposure and after 21 days of living at HA. Volunteers consumed a hypocaloric standard-protein (1.1 g/kg/d, $n = 8$) or higher protein (2.1 g/kg/d, $n = 9$) diet while living at HA. Values are mean ± SEM. Effects of diet group (standard- or higher protein), carbohydrate group (carbohydrate beverage or placebo), day, time, and all interactions not including those between diet group and carbohydrate group were tested using linear mixed models with subject included as a random factor and Bonferroni corrections. Only significant effects ($p < 0.05$) from the parsimonious model are listed. Within a study day, values not sharing a superscript letter are significantly different, $p < 0.05$. *Different from SL day 7 and HA day 0, $p \leq 0.03$. #Different from SL day 7 and HA day 21, $p \leq 0.03$. CHOgroup, carbohydrate supplementation during exercise at 4.5 hours (145 g or 0 g).

2013). As such, observed changes following acclimatization must be interpreted cautiously and within the context of the known effects of weight loss on study outcomes.

Appetite suppression during acute HA exposure is a well-established response (Hamad and Travis, 2006; Westerterp and Kayser, 2006) and was supported in the present study (Figs. 2 and 4). While previous studies report that appetite remains suppressed at very high altitudes (>5000 m) for several weeks (Rose et al., 1988; Westerterp-Plantenga et al., 1999), others suggest that appetite suppression may be alleviated during chronic (>7 days) exposure to altitudes below 5000 m, at least under normobaric hypoxic conditions [3000–4000 m (Debevec et al., 2014, 2016; Mekjavic et al., 2016)]. In the present study, appetite was lower during the meal test on HA day 21 relative to SL and did not differ from SL on HA day 18 despite substantial weight loss. It is well established that weight loss stimulates compensatory mechanisms which act to stimulate appetite and restore body weight (Maclean et al., 2011; Sumithran and Proietto, 2013). Therefore, as we are not aware of any reports demonstrating weight regain in people sojourning at >4000 m for ≥3 weeks, these findings suggest that normal physiologic adaptations to weight loss

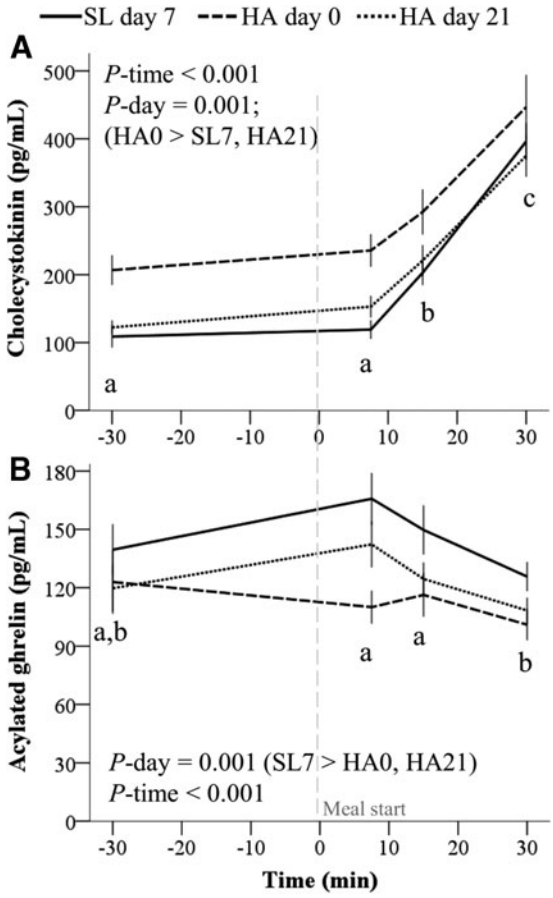


FIG. 6. Appetite-mediating hormone responses to a standardized meal at HA (4300 m). **(A)** Cholecystokinin ($n = 17$) and **(B)** acylated ghrelin ($n = 17$) responses to a fixed portion meal measured at SL, on the first day of HA exposure and after 21 days of living at HA. Volunteers consumed a hypocaloric standard-protein (1.1 g/kg/d, $n = 8$) or higher protein (2.1 g/kg/d, $n = 9$) diet while living at HA. Values are mean ± SEM. Effects of diet group (standard- or higher protein), carbohydrate group (carbohydrate beverage or placebo), day, time, and all interactions not including those between diet group and carbohydrate group were tested using linear mixed models with subject included as a random factor and Bonferroni corrections. Only significant effects ($p < 0.05$) from the parsimonious model are listed. Within a study day, values not sharing a superscript letter are significantly different, $p < 0.05$.

that stimulate weight regain may be blunted and persistent appetite suppression may be a relevant concern during sojourn at ≥4300 m for several weeks. Studies with appropriate control groups are needed to verify this hypothesis.

Our findings suggest, but do not prove, that the orexigenic hormone ghrelin may be one factor in the persistence of HA anorexia. Previous studies have reported that total ghrelin concentrations are transiently suppressed in response to hypoxia (Shukla et al., 2005; Riepl et al., 2012; Debevec et al., 2014, 2016; Mekjavic et al., 2016). However, the relevance of these findings to appetite regulation is unclear because the appetite-stimulating effects of ghrelin require posttranslational acylation (Lim et al., 2010), and acylated ghrelin comprises only a small variable fraction of total ghrelin concentrations (Liu et al., 2008). Previous studies measuring acylated ghrelin responses to HA have reported suppressed concentrations during 5–7 hours of exposure to normobaric hypoxia simulating altitudes of 3500–4000 m (Wasse et al., 2012; Matu et al., 2017a, 2017b), but not <3000 m (Morishima and Goto, 2016), and suppressed concentrations during a 12-day trek to 5140 m (Matu et al., 2017a, 2017b). Our findings are consistent with and extend those results by demonstrating that the suppression of acylated ghrelin concentrations upon acute HA exposure may be sustained for up to 21 days (Figs. 5C and 6B). Importantly, this observation is not likely confounded by weight loss as weight loss at SL is associated with increased acylated ghrelin concentrations (Martins et al., 2010).

Whether the 12%–22% lower mean acylated ghrelin concentrations observed at HA relative to SL in our study would be sufficient to cause changes in energy intake had volunteers been given free access to food was not assessed. However, Wasse et al. (2012) reported an 18% reduction in acylated ghrelin concentrations and 31% reduction in voluntary energy intake in response to 7-hour exposure to hypoxia simulating 4000 m altitude, while Matu et al. (2017a, 2017b) recently reported a ∼25% reduction in both fasting acylated ghrelin concentrations and in voluntary energy intake during a 12-day trek to 5140 m. Although appetite regulation at HA is undoubtedly influenced by multiple factors, collectively these studies suggest, but do not prove, a role for acylated ghrelin in HA anorexia. Additional studies with appropriate controls are warranted to confirm the hypothesis that HA suppresses acylated ghrelin concentrations and that this effect meaningfully impacts energy intake.

Our findings suggest that elevations in insulin, leptin, and CCK occur concomitant to appetite suppression during acute HA exposure. Elevated insulin concentrations during initial HA exposure are consistent with previous reports of transient hypoxia-induced impairments in insulin sensitivity and glucose homeostasis (Barnholt et al., 2006; Woolcott et al., 2015). Leptin concentrations also reportedly increase during the initial 24 hours of hypoxia (Tschop et al., 1998; Snyder et al., 2008). However, whether this effect persists is controversial because of inconsistent findings across multiple studies (Zaccaria et al., 2004; Shukla et al., 2005; Barnholt et al., 2006; Vats et al., 2007; Debevec et al., 2014, 2016; Mekjavic et al., 2016) attributable to differences in the control of confounding factors (Raff et al., 2008; Sierra-Johnson et al., 2008; Debevec, 2017). In particular, differences in the timing of measurements may be critical as reductions in food intake due to HA anorexia would provide a strong stimulus to suppress leptin concentrations within 48–72 hours (Karl et al., 2016).

Although results from the present study support a transient elevation in insulin and leptin concentrations during HA sojourn (Fig. 5), both hormones decrease with fat mass loss. As such, similar to most previous studies (Zaccaria et al., 2004; Shukla et al., 2005; Vats et al., 2007; Riepl et al., 2012; Debevec et al., 2014, 2016; Mekjavic et al., 2016), the lack of a weight stable control group precludes isolating the independent effect of acclimatization to HA on insulin and leptin.

CCK is secreted during meals and limits meal size by stimulating satiation (Woods, 2009). We hypothesized that hypoxia may induce a faster rise in CCK concentrations during meals thereby accounting for the more rapid onset of satiation previously observed at HA (Westerterp-Plantenga et al., 1999). Although the data did not support a faster rise in CCK at HA (Fig. 6A), mean CCK concentrations were higher

relative to SL after 10 hours of exposure to HA (Fig. 6A, D). However, this difference did not occur concomitant to increased fullness immediately following the standard test meal where the effects of CCK on satiation would be expected to occur. That observation, in combination with previous studies reporting decreases [3454 m, <72 hours; (Riepl et al., 2012)], no changes [4559 m, two and four days; (Aeberli et al., 2013)], and increases [20-day trek to 5100 m (Bailey et al., 2000)] in CCK concentrations at HA, indicates that additional research is needed to clarify the role of CCK, if any, in HA anorexia.

A potential strategy for overriding physiologic signals contributing to HA anorexia may be to provide highly palatable foods that exploit hedonic (i.e., pleasure and reward) pathways impacting eating behavior (Berthoud, 2006). Presently, the effects of hypoxia on food hedonics and subsequent food intake are unclear as previous reports on changes in taste perception and food hedonics (Premavalli et al., 2009; Aeberli et al., 2013) and self-selected macronutrient intake (Boyer and Blume, 1984; Rose et al., 1988; Reynolds et al., 1998; Westerterp-Plantenga et al., 1999; Bailey et al., 2004; Aeberli et al., 2013) at HA are inconsistent.

In the present study, HA exposure was acutely associated with a preference for sweet over savory foods and liking of low-protein over higher protein foods suggesting that sweet, low-protein foods may encourage eating during acute HA. The subsequent transition to a preference for high-fat foods suggests that a high-fat diet may have greater utility for promoting food intake following acclimatization. However, these hypotheses require confirmation as the design of this study precludes determining whether observed changes would have influenced *ad libitum* food intake, and factors such as diet monotony, limited food variety, and weight loss may have also impacted results. Nonetheless, our findings extend those of Aeberli et al. (2013) who used the same food preference assessment method to demonstrate that perceived liking of high-fat foods increased and was positively associated with *ad libitum* energy intake during a four day sojourn at HA (4559 m). One implication of these findings is that diet recommendations for HA sojourn may differ according to the duration of sojourn if the aim is to leverage hedonic mechanisms to promote energy intake and minimize weight loss.

Finally, while the present findings suggest that high protein and savory foods may not be preferred upon initial HA exposure, dietary protein intake within the levels studied did not appear to differentially influence food preferences. Using the same food preference assessment methodology, Griffioen-Roose et al. (2012) reported that a 14-day low-protein (0.5 g/kg/d) diet stimulated a compensatory response characterized by an increased drive to consume protein and protein intake. That food preferences were not influenced by dietary protein intake in the present study suggests that the protein content of the standard-protein diet was sufficient to prevent this response. It is also acknowledged that an increased drive to consume protein when protein intake is low, known as the protein leverage hypothesis, has not been observed in all studies (Martens et al., 2013).

Conclusion

Taken together, these findings suggest that maximizing the effectiveness of dietary strategies for mitigating weight loss at HA may require overriding physiologic signals that suppress appetite, accommodating food preferences, and adjusting diets to both the duration and elevation of the sojourn. These observations in combination with the absence of a SL control group highlight the need for additional research with appropriate controls to establish causal effects of prolonged HA exposure on appetite, appetite-mediating hormones, and food preferences. Contrary to our hypothesis, a higher protein diet did not exacerbate HA anorexia in the present study. However, study limitations preclude definitive conclusions, and the potential for the satiating properties of protein to reduce *ad libitum* food intake at HA and the possible incongruence of higher protein diets with food preferences at HA warrant further investigation.

Acknowledgments

The authors thank the study volunteers, our medical oversight team, and Dr. Stephen Muza for their support and the Pennington Biomedical Research Center Clinical Laboratory staff, Marques Wilson, Christopher Carrigan, Adrienne Hatch, Laura Lutz, Adam Luippold, Bradley Anderson, Grant Holmes, Anthony Karis, Karleigh Bradbury, Alyssa Kelley, Dr. Lee Margolis, Dr. Stephen Hennigar, Dr. Robert Kenefick, and Dr. Scott Montain for their significant contributions.

This work was supported by the U.S. Army Medical Research and Materiel Command and the U.S. Department of Defense, Defense Health Program.

Disclaimers

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Author Disclosure Statement

No authors report a conflict of interest.

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Received December 11, 2017;
accepted in final form January 3, 2018.

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Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity

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ABSTRACT: Muscle loss at high altitude (HA) is attributable to energy deficit and a potential dysregulation of anabolic signaling. Exercise and protein ingestion can attenuate the effects of energy deficit on muscle at sea level (SL). Whether these effects are observed when energy deficit occurs at HA is unknown. To address this, muscle obtained from lowlanders (n = 8 males) at SL, acute HA (3 h, 4300 m), and chronic HA (21 d, −1766 kcal/d energy balance) before [baseline (Base)] and after 80 min of aerobic exercise followed by a 2-mile time trial [postexercise (Post)] and 3 h into recovery (Rec) after ingesting whey protein (25 g) were analyzed using standard molecular techniques. At SL, Post, and REC, p-mechanistic target of rapamycin (mTOR)^{Ser2448}, p-p70 ribosomal protein S6 kinase (p70S6K)$^{Ser424/421}$, and p-ribosomal protein S6 (rpS6)$^{Ser235/236}$ were similar and higher ($P < 0.05$) than Base. At acute HA, Post p-mTOR^{Ser2448} and Post and REC p-p70S6K$^{Ser424/421}$ were not different from Base and lower than SL ($P < 0.05$). At chronic HA, Post and Rec p-mTOR^{Ser2448} and p-p70S6K$^{Ser424/421}$ were not different from Base and lower than SL, and, independent of time, p-rpS6$^{Ser235/236}$ was lower than SL ($P < 0.05$). Post proteasome activity was lower ($P < 0.05$) than Base and Rec, independent of phase. Our findings suggest that HA exposure induces muscle anabolic resistance that is exacerbated by energy deficit during acclimatization, with no change in proteolysis.—Margolis, L. M., Carbone, J. W., Berryman, C. E., Carrigan, C. T., Murphy, N. E., Ferrando, A. A., Young, A. J., Pasiakos, S. M. Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: hypoxia · rpS6 · proteolysis · REDD1 · weight loss

Lowlanders who sojourn at high altitude (HA; hypobaric hypoxia) often lose skeletal muscle mass (1–4). Dysregulated mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling and up-regulated ubiquitin proteasome-mediated proteolysis may regulate this muscle loss and modulate the extent to which muscle mass degrades under hypoxic conditions (5). *In vitro* models (6–8) have demonstrated that up-regulated hypoxia-inducible factor-1α (HIF-1α) inhibits mTORC1 signaling by triggering regulated in development and DNA damage response 1 (REDD1) coupling with the tuberous sclerosis complex-2 (TSC2) inhibitor, 14-3-3. In rat models, increased REDD1/14-3-3 coupling is associated with mTORC1 inhibition and reduction in muscle mass (9). Muscle ubiquitin expression, protein ubiquitylation, and 26S proteasome activity in *ad libitum*–fed rats are also up-regulated over 14 d of acclimatization to HA, which contributes to muscle atrophy (10). To our knowledge, there have been no studies that have collectively examined the skeletal muscle anabolic signaling and ubiquitin proteasome-mediated proteolytic responses during both acute and chronic HA exposure in humans.

Unaccustomed exposure to HA often results in negative energy balance, as individuals are typically unable to consume enough food to match their daily

ABBREVIATIONS: Base, baseline; EIEE, exercise-induced energy expenditure; FFM, fat-free mass; FOXO1, forkhead box O1; HA, high altitude; HIF-1α, hypoxia-inducible factor-1α; HSP90, heat-shock protein 90; mTOR, mammalian target of rapamycin; mTORC1, mechanistic target of rapamycin complex 1; MuRF1, muscle ring-finger protein-1; p70S6K, p70 ribosomal protein S6 kinase; PGPH, peptidyl-glutamyl peptide hydrolyzing; Post, postexercise; Rec, recovery; REDD1, regulated in development and DNA damage response 1; rpS6, ribosomal protein S6; SL, sea level; TSC2, tuberous sclerosis complex-2; USARIEM, U.S. Army Research Institute of Environmental Medicine

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doi: 10.1096/fj.201800163RR

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energy expenditures (11, 12). Negative energy balance at HA may exacerbate the overall catabolic effect of hypoxia on muscle mass. Energy deficits at sea level (SL) suppress basal (13–17) and postprandial (17, 18) muscle anabolism (*i.e.*, protein synthesis) by as much as 30% compared with energy balance. *In vitro* (19) and rat studies (20, 21) additionally demonstrate that reductions in translation and phosphorylation of mTORC1 signaling proteins occur after underfeeding. Anabolic resistance that results from negative energy balance at SL does so without consistent and appreciable changes in muscle proteolysis (15, 22–24). These findings add to the theory that the modulation of protein synthesis exerts a much more pronounced regulatory effect on muscle mass in healthy muscle than alterations in proteolysis (23, 25); however, the extent to which muscle anabolism and proteolysis are modulated in healthy individuals who are exposed to the combined environmental and physiologic stresses of hypoxia and negative energy balance has not been documented.

Synergistic effects of exercise and high-quality protein ingestion can preserve skeletal muscle anabolic sensitivity and attenuate muscle loss during moderate energy deficit in normoxic conditions (15, 16). During energy deficit, whey protein consumption immediately after resistance exercise restores protein synthesis to rates above those observed during energy balance in the basal (*i.e.*, rested/postabsorptive) state (15, 16). Moreover, during energy deficit at SL, high-quality protein intake alone or in recovery from exercise seems to suppress or maintain proteolytic activity compared with rates observed when in energy balance. Stimulating anabolism while suppressing or maintaining proteolysis provides the underlying conditions for a net improvement in protein balance (15, 23, 24) and the attenuation of muscle loss (5, 26).

The objective of the current study was to examine the impact of acute HA (4300 m) exposure and 21 d of HA acclimatization coupled with severe energy deficit (~70% energy deficit) on mTORC1-meidated anabolic signaling and ubiquitin proteasome-mediated proteolysis in unacclimatized SL natives. Anabolic and proteolytic signaling was examined at rest and in response to aerobic exercise and recovery after whey protein ingestion. We hypothesized that acute HA exposure while in energy balance, and chronic HA exposure concomitant with severe energy deficit would blunt mTORC1 signaling and up-regulate proteolysis at rest and immediately postexercise and diminish the anabolic and antiproteolytic effects of ingesting whey protein during recovery. The objective of our study, which was not to delineate between the independent or synergistic effects of hypoxia and energy deficit, but rather to study those effects in concert, has practical relevance for various populations that sojourn at HA and experience energy deficit and decrements in skeletal muscle mass, particularly military personnel (27, 28). Under those conditions, it is unknown whether exercise and protein intake can alter molecular regulation of skeletal muscle mass.

MATERIALS AND METHODS

Participants

Participants were part of a larger randomized, controlled study that compared the effects of consuming higher-protein (2.0 g/kg/d) *vs.* standard-protein (1.0 g/kg/d) diets on body composition responses with 21 d of energy deficit at HA (29). Participant eligibility and recruitment details are reported elsewhere (29). In brief, 17 recreationally active (programmed physical activity 2–4 d/wk) men, age 18–42 yr, who were not acclimatized to HA completed the parent study (29). For experiments described in this report, data were obtained from 8 of those 17 participants who were not subjected to other nutritional interventions that had the potential to confound the anabolic and proteolytic signaling responses we were investigating. This study was approved by the institutional review board at the U.S. Army Research Institute of Environmental Medicine (USARIEM; Natick, MA, USA) and conducted from May to August 2016. All participants provided written, informed consent before participation. (ClinicalTrials.gov; ID: NCT02731066; *www.clinicaltrials.gov*).

Study design

This 43-consecutive-day, controlled study included 21 d of energy balance at SL (SL, 55 m, d 1–21) and 21 d of energy deficit at HA (chronic, HA, 4300 m, d 2–22; **Fig. 1**) (29). Muscle anabolic signaling and proteolytic responses to exercise and recovery protein ingestion were conducted at SL d 7, acute HA d 1, and chronic HA d 22. During SL, participants were free living but visited the USARIEM laboratory in Natick, MA, USA, daily. After receiving instructions by study registered dietitians, participants followed a weight maintenance diet and consumed 1.0 g/kg/d of protein, 45–65% total energy from carbohydrate, and 20–35% total energy from fat, and maintained their habitual exercise routines. Energy requirements to maintain body mass were determined using the Harris-Benedict equation, resting metabolic rate measured by indirect calorimetry (True Max 2400; Parvomedics, Sandy, UT, USA), and intake and activity derived from 3-d records (2692 ± 78 kcal/d). Compliance with diet instructions was verified using 24-h diet records and by measuring body mass daily to the nearest 0.1 kg using a calibrated digital scale (Befour Model PS6600; Befour, Saukville, WI, USA). As previously reported (29), participants were weight stable during SL (d 1, 80.6 ± 5.6 kg; d 21, 81.6 ± 5.9 kg).

On d 21, after completing the SL phase, participants were flown from Boston, MA, USA, to Denver, CO, USA, where they stayed overnight under supervision in a local hotel. The next morning (HA d 1, ~3 AM MDT), participants were transported by car to the USARIEM Maher Memorial Laboratory on the summit of Pikes Peak, CO, USA (4300 m). Participants breathed supplemental oxygen to maintain normoxic oxygen saturations and to control the duration of hypoxic exposure after landing in Denver, CO, USA until arriving at the summit the next morning and commencing acute HA testing. Starting on HA d 2, participants were randomly assigned and consumed either a standard- (1.0 g/kg/d) or higher- (2.0 g/kg/d) protein diet for the next 21 d (HA d 2–22). Diets provided ~45% of total energy from carbohydrate and the remainder of the energy from fat. For HA, registered dietitians created and provided diets on the basis of individualized energy requirements that consisted primarily of components of the military combat ration, Meal, Ready-to-Eat (Menu 34; AmeriQual, Evansville, IN, USA). The remainder of

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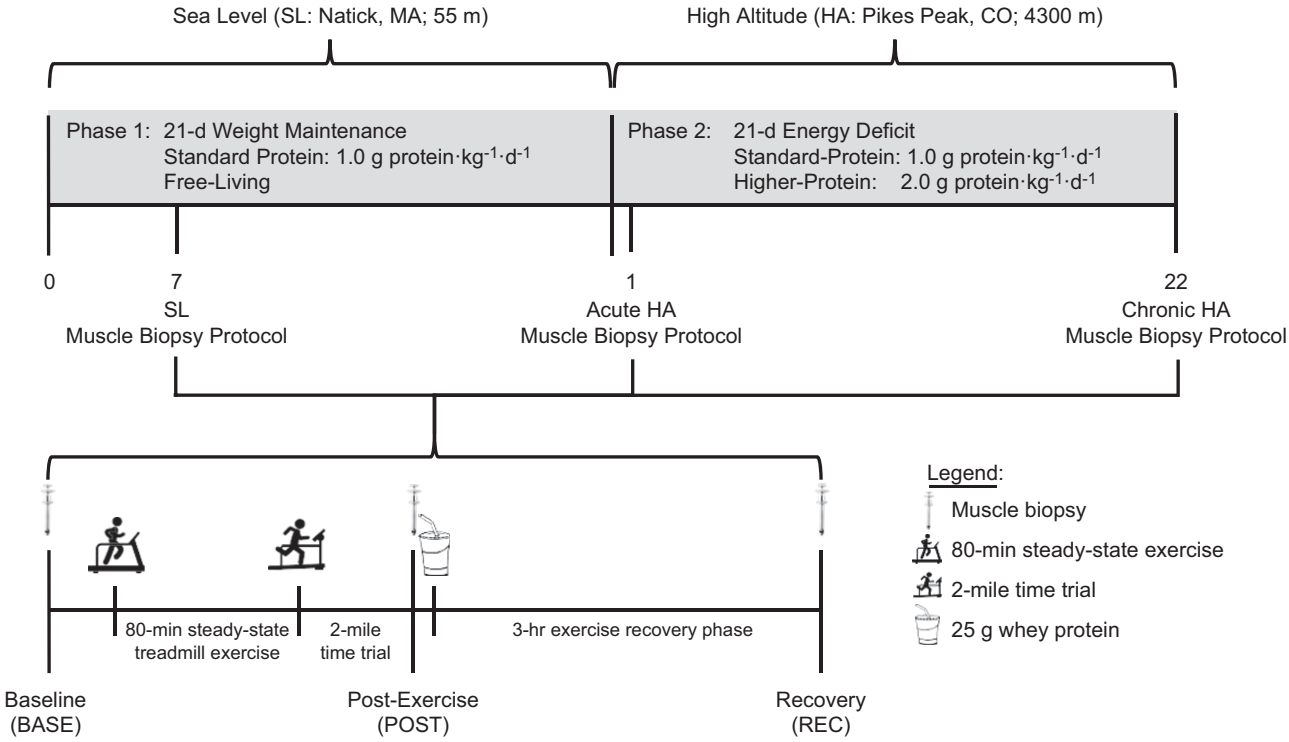


Figure 1. Study design. Energy deficit was designed to be 40% of total energy needs; however, on the basis of changes in fat mass and FFM, the actual deficit was closer to 70%. The current analysis was part of a larger study that assessed the impact of dietary protein intake on changes in body composition after 21 d of HA exposure coupled with energy deficit. Habitual protein intake did not influence end point measures from this analysis, so diet groups were collapsed. Steady-state exercise tests were metabolically matched ($\dot{V}O_2$, 1.63 ± 0.05 L/min; metabolic rate, 653 ± 20 kcal).

the energy was derived from perishable foods and whey protein drinks (Isopure Zero Carb; Isopure, Hauppauge, NY, USA), as previously described (29). At SL, there was no difference in self-reported dietary intake, with all participants (n = 8) consuming 2253 ± 119 kcal/d, 1.0 ± 0.1 g protein/kg/d, 3.8 ± 0.4 g carbohydrate/kg/d, and 1.1 ± 0.1 g fat/kg/d. At HA, participants who were assigned to the standard-protein group (n = 4) consumed 1921 ± 247 kcal/d, 1.1 ± 0.1 g protein/kg/d (19% total kcal), 2.6 ± 0.1 g carbohydrate/kg/d (43% total kcal), and 1.0 ± 0.1 g fat/kg/d (38% total kcal), whereas participants assigned to the higher-protein group (n = 4) consumed 1715 ± 138 kcal/d, 2.0 ± 0.1 g protein/kg/d (32% total kcal), 2.9 ± 0.1 g carbohydrate/kg/d (44% total kcal), and 0.7 ± 0.1 g fat/kg/d (24% total kcal).

To elicit negative energy balance at HA, exercise-induced energy expenditure (EIEE) was increased by 10% more than estimated SL daily energy expenditures [*i.e.*, HA EIEE = SL EIEE + (0.1 X SL total daily energy expenditure)], and daily energy intake was decreased by 30% relative to total daily energy expenditure estimated at SL to maintain body mass. The 40% energy deficit was chosen to be consistent with our previous controlled (18) and observational studies (30). Physical activity at HA consisted of supervised and documented aerobic-type exercise bouts (*e.g.*, treadmill walking, running, and outdoor hiking), prescribed using the Pandolf equation for level and uphill walking (31), adjusted as necessary for body mass loss, terrain factors, and downhill walking (32). Body mass was measured daily at HA, and body composition was determined at baseline, before ascent to HA (SL d 20), and immediately after descent from HA (~0630–0800 on HA d 23) using dual-energy X-ray absorptiometry (DPX-IQ; GE Lunar Corp., Madison, WI, USA). As previously reported (29), total body mass loss was beyond expected for an estimated energy deficit of 40%. As such, changes in body energy stores were used to better estimate the total daily energy deficit (29, 33) using Eq. 1:

$$\Delta\ \mathrm{ES(kcal/d)} = [\Delta\mathrm{FM\ (g)} \times 9.51\ \mathrm{kcal/g} + \Delta\mathrm{FFM\ (g)} \times (1 - \mathrm{FFM\ hydration}) \times 4.40\ \mathrm{kcal/g}]/21\ \mathrm{d} \quad (1)$$

where ΔES is the change in energy stores, FM is fat mass, FFM is fat-free mass, and FFM hydration representing the aqueous fraction of FFM, estimated as 0.73 (34). Estimated energy deficit on the basis of changes in body energy stores was 69 ± 7% (−1766 ± 145 kcal/d), which we attributed, in part, to elevated resting metabolic rate in our previous publication (29); however, like others (35, 36), 26% of the energy deficit could not be accounted for.

Aerobic exercise and muscle biopsy studies

Aerobic exercise and muscle biopsy studies were conducted at SL after 7 d of following the weight maintenance diet and again on d 1 (acute HA) and d 22 (chronic HA) at HA. Aerobic exercise was chosen because it represents the primary exercise modality performed by military personnel during strenuous operations (37, 38), particularly during missions at HA (27, 28). This exercise type, coupled with protein feeding, is sufficient to stimulate mTORC1-mediated anabolic signaling at SL (39–42). A biopsy was collected from the vastus lateralis to capture the basal state at baseline (Base; *i.e.*, rested/postabsorptive). Participants then completed 80 min of steady-state treadmill exercise, walking at a fixed speed and grade. Steady-state exercise tests were metabolically matched ($\dot{V}O_2$, 1.63 ± 0.05 L/min; metabolic rate, 653 ± 20 kcal). Indirect calorimetry was performed to ensure that $\dot{V}O_2$ was within ± 5% of target $\dot{V}O_2$. If necessary, speed and/or grade adjustments were made during the initial 20 min of exercise. After completion of steady-state exercise, participants were given a 15 min rest before performing a self-paced 2-mile

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treadmill time trial. Peripheral oxygen saturation (SpO_2; finger pulse oximetry, Model 9560; Nonin, Plymouth, MN, USA) was assessed throughout the exercise bout to document normoxic and hypoxic exposure. As expected, mean SpO_2 was different ($P < 0.05$) between all 3 study phases (SL, 96 ± 1%; acute HA, 76 ± 2%; chronic HA, 84 ± 1%). In terms of intensity, participants averaged ~85% of SL $V_{O_{2peak}}$ and between ~75 and 85% of HA $V_{O_{2peak}}$ during 2-mile time trials. Immediately after the time trial, a postexercise (Post) muscle biopsy was obtained. Participants then consumed a bolus, 25 g of whey protein drink (Isopure Zero Carb) and rested in a chair for 3 h. After 3 h of recovery (Rec), a final muscle biopsy was obtained. Muscle biopsies were taken from 2 incisions on 1 leg per biopsy protocol day. BASE biopsies were taken from 1 incision, generally distal to the midpoint of the vastus lateralis. Post and REC biopsies were taken from the second incision that was proximal to the initial incision by ~7 cm. The angle of the biopsy needle was changed to limit excessive trauma and inflammation to any one area. Legs were alternated between SL and acute and chronic HA, and new incisions were made for each protocol day.

Muscle protein content

Total protein content in muscle was estimated at SL and chronic HA as an indirect maker of anabolism in response to the combined environmental and metabolic stress (20, 43, 44). Muscle samples were homogenized in ice-cold homogenization buffer (1:10 w/v) that contained 50 mM Tris–HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton X-100, 1 mM DTT, 1 mM benz-amidine, 1 mM PMSF, 10 μg/ml trypsin inhibitor, and 2 μg/ml aprotinin. Samples were homogenized using a TissueLyser II with a 5-mm steel bead (Qiagen, Valencia, CA, USA). Protein concentrations were determined using the 660 nm Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). Homogenate total protein concentration was expressed relative to dual-energy X-ray absorptiometry–derived leg FFM to provide an estimate of muscle protein content (43, 44). In the current study, bone mass was subtracted from FFM values (29). Muscle protein content was calculated using Eq. 2:

$$\text{Muscle protein content (g)} = \text{protein concentration (mg/ml)} \times \text{homogenate vol (ml)/muscle wt (mg)} \times \text{Leg FFM (g)} \quad (2)$$

Intracellular signaling

Phosphorylation status and total protein expression of molecular markers in mTORC1-mediated anabolic signaling and ubiquitin proteasome-mediated proteolysis were determined from Western blot analysis. Homogenates used to estimate muscle protein content were centrifuged for 15 min at 10,000 *g* at 4°C. Supernatant (lysate) was collected and protein concentrations were analyzed (Thermo Fisher Scientific). Muscle lysates were solubilized in Laemmli buffer, and equal amounts of total protein (15 μg) were separated by SDS-PAGE using precast Tris-HCl gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred to PVDF membranes and exposed to commercially available primary Abs that were specific to Akt, p-Akt^{Ser473}, AMPK, p-AMPK^{Thr172}, TSC2, p-TSC2^{Thr1462}, mTOR, p-mTOR^{Ser2448}, p70 ribosomal protein S6 kinase (p70S6K), p-p70S6K$^{Ser424/Thr421}$, ribosomal protein S6 (rpS6), p-rpS6$^{Ser235/236}$, forkhead box O1 (FOXO1), p-FOXO1^{Thr24}, and ubiquitin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Secondary Ab (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology) and chemiluminescent reagent (Pierce, Rockford, IL, USA) were then applied. Blots were quantified using ChemiDoc XRS (Bio-Rad) and Image Lab software (Bio-Rad). Heat-shock protein 90 (HSP90) was used to confirm that equal amounts of protein were loaded per well. Base phosphorylation was normalized to its total protein. Base total protein was normalized to HSP90. For time course analysis, phase-by-time, phosphorylation status was normalized to HSP90. Total protein ubiquitylation was expressed relative to free ubiquitin. To examine the differences in Base across study phases, Base acute HA and chronic HA were expressed relative to Base SL. To examine the effects of exercise and Rec protein feeding within and across study phases, Post and Rec data were expressed relative to Base within each study phase.

26S proteasome enzymatic activity

Proteolytic activity of the 3 catalytic sites of 26S proteasome [chymotrypsin-like, 26S β_5; trypsin-like, 26S β_2; peptidylglutamyl peptide hydrolyzing (PGPH) –like, 26S β_1] were assessed using fluorescence-based assays and amino acid–conjugated fluorophores (24). In brief, 25 mg of muscle were homogenized in chilled lysis buffer (1:7 w/v) that contained 20 mM Tris-HCl (pH 7.2), 0.1 mM EDTA, 20% glycerol, and 0.04% NP-40 substitute with ATP (5 mM), and 2-ME (1 mM) was added immediately before use. Homogenates were centrifuged at 13,000 *g* for 15 min at 4°C, and protein content was determined (Bradford Protein Assay; Bio-Rad). Each assay tube contained 10 (PGPH-like), 6 (trypsin-like), or 25 (chymotrypsin-like) μg of total protein in 50 mM HEPES/5 mM EGTA buffer. An identical assay tube that included the appropriate protease inhibitor was prepared for each sample. Samples were then incubated at 37°C on an orbital shaker for 30 min. Proteasome subunit-specific, amino acid–conjugated fluorophores were added to each tube and incubated for an additional 60 min at 37°C. Enzyme activities were measured in duplicate in black, clear bottom, polystyrene, 96-well plates. Fluorescence was measured with 380 nm excitation and 460 nm emission wavelengths on a SpectraMax 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Duplicates were averaged, and values from inhibited samples were subtracted from corresponding uninhibited samples to account for background fluorescence. This difference represents the relative activity of the proteasome subunits.

mRNA expression

Total RNA was isolated from 20 mg of muscle using a Trizol/ethanol precipitation method. Quantity and quality of RNA was determined using a NanDrop ND-2000 (NanoDrop, Wilmington, DE, USA). Equal amounts (500 ng) of total RNA were synthesized into cDNA (iScript Advanced cDNA Synthesis Kit; Bio-Rad) and reverse-transcribed in a T100 Thermal Cycler (Bio-Rad). Samples were run in 10-μl reactions in duplicate using iTaq Universal SYBR in a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). mRNA expression of HIF-1α, REDD1, and the muscle-specific ubiquitin ligases, MuRF1 (muscle ring-finger protein-1) and Atrogin-1, were assessed using commercially available primers (Bio-Rad). Data were normalized to the geometric mean of β-actin, glyceraldehyde 3-phosphate dehydrogenase, and β_2-microglobulin mRNA, and fold changes were calculated using the $\Delta\Delta C_t$ method (45). To examine the differences in Base across study phases, Base acute HA and chronic HA were expressed relative to Base SL. To examine the effects of exercise and Rec protein feeding within and across study phases, Post and Rec data were expressed relative to Base within each study phase.

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REDD1/14-3-3 coupling

On the basis of initial findings from Western blot analysis, additional analyses were conducted to examine the influence of REDD1 on mTORC1 inhibition. One hundred fifty micrograms of protein from muscle lysates that were generated for Western blotting were incubated in 1.5 μl of pan–14-3-3 primary antibody (EMD Millipore, Billerica, MA, USA) overnight at 4°C. Immunocomplexes were then precipitated for 1 h at 4°C using Agarose A/G beads and washed 3 times before proteins were eluted from the beads using a commercially available kit (Pierce Classic IP Kit; Thermo Fisher Scientific). Western blot analysis was then performed on the samples and probed for REDD1 and 14-3-3 (Cell Signaling Technology) according to procedures described in the Intracellular signaling section, and REDD1 was quantified relative to 14-3-3. To examine the differences in Base across study phases, Base acute HA and chronic HA were expressed relative to Base SL. To examine the effects of exercise and Rec protein feeding within and across study phases, Post and Rec data were expressed relative to Base within each study phase.

Statistical analysis

On the basis of recent publications (15, 18, 46) and the lack of an effect of dietary protein on minimizing FFM lost in the parent study (29), we did not expect the protein group (standard and higher protein) to affect anabolic and proteolytic responses to HA (SL and acute HA were not subject to dietary protein intervention). We confirmed this using an independent Student's *t* test to assess the differences of Base chronic HA between standard- and higher-protein groups and using a mixed-model repeated measures ANOVA that assessed dietary protein group (standard and higher protein) by time at chronic HA (Base, Post, and Rec). As anticipated, there were no effects of dietary protein group on any outcome at chronic HA; therefore, statistical models were run that excluded dietary protein group from the final statistical analysis.

Paired Student's *t* tests were used to compare changes in body mass, composition, and muscle protein content from SL to chronic HA. Mixed-model repeated measure ANOVA with participant ID as a random effect (deemed appropriate by Akaike's information criteria) was used to assess the influence of phase (SL, acute HA, and chronic HA) on Base measurements of phosphorylation and total protein content of anabolic and proteolytic signaling markers and mRNA expression, as well as the impact of phase (SL, acute HA, and chronic HA) and time (Base, Post, and Rec) on change in phosphorylation status of anabolic and proteolytic signaling markers, mRNA expression, and ubiquitin proteasome activity. Bonferroni adjustments were performed for multiple comparisons if significant main effects of phase or interactions were observed. All data are presented as means ± SEM. The α level for significance was set at $P < 0.05$. Data were analyzed using SPSS Statistics for Windows (v.22.0; SPSS, Chicago, IL, USA).

RESULTS

Participants lost 7.6 ± 0.8 kg (4.3 ± 1.0 and 3.4 ± 0.4 kg from FFM and fat mass, respectively; $P < 0.05$) after 21 d of HA exposure and energy deficit compared with SL. Similarly, leg FFM (−2.1 ± 0.5 kg; $P < 0.05$) and estimated muscle protein content (780 ± 337 g; $P = 0.05$) was lower at chronic HA compared with SL (**Table 1**).

Base phosphorylation status and total protein content of AKT, AMPK, TSC2, mTOR, p70S6K, and rpS6 were not different between study phases (**Fig. 2**). Independent of phase, Rec p-AKT^{Ser473} was lower than Post ($P < 0.05$, time main effect; **Fig. 3*A***). No main effects of study phase or time point were observed for p-AMPK^{Thr172} or p-TSC2^{Thr1462} (Fig. 3*B, C*). At SL, Post and Rec p-mTOR^{Ser2448} and p-p70S6K$^{Ser424/421}$ were higher than Base ($P < 0.05$, phase-by-time interaction; Fig. 3*D, E*). Independent of phase, Post and Rec p-rpS6$^{Ser235/236}$ were higher than Base ($P < 0.05$, time main effect; Fig. 3*F*). At acute HA, Post p-mTOR^{Ser2448}, and Post and Rec p-p70S6K$^{Ser424/421}$ were not different from Base and lower than SL ($P < 0.05$, phase-by-time interaction; Fig. 3*D, E*). At acute HA, Rec p-mTOR^{Ser2448} was higher than Base ($P < 0.05$, phase-by-time interaction; Fig. 3*D*), but not different from SL. At chronic HA, Post and Rec p-mTOR^{Ser2448} and p-p70S6K$^{Ser424/421}$ were not different from Base and were lower than SL ($P < 0.05$, phase-by-time interaction; Fig. 3*D, E*). At chronic HA, independent of time, p-rpS6$^{Ser235/236}$ was lower ($P < 0.05$, phase main effect; Fig. 3*F*) than SL.

Base HIF-1α mRNA was not different between phases (**Fig. 4*A***). At SL, Base and Post HIF-1α were lower than Rec ($P < 0.05$, phase-by-time interaction; Fig. 4*B*). At acute and chronic HA, Rec HIF-1α was lower than SL ($P < 0.05$, phase-by-time interaction; Fig. 4*B*). No significant effects of study phase or time point were observed in REDD1 mRNA (Fig. 4*C, D*). Base REDD1/14-3-3 coupling was not different across study phases (Fig. 4*E*). Independent of study phase, Rec REDD1/14-3-3 coupling was higher than Post ($P < 0.05$, time main effect; Fig. 4*F*).

At chronic HA, Base FOXO1 total protein content was higher ($P < 0.05$, phase main effect) than acute HA (**Fig. 5*A***). No differences were observed between study phases in BASE p-FOXO1^{Thr24} (Fig. 5*B*). Base MuRF1 and Atrogin-1 mRNA were not different between study phases (Fig. 5*C, D*). Base ubiquitylation was not different between study phases (data not shown). Independent of study phase, p-FOXO1^{Thr24} was lower ($P < 0.05$, time main effect) at Rec than Base and Post (**Fig. 6*A***). At SL, Post protein ubiquitylation was higher than Base and Rec ($P < 0.05$,

TABLE 1. *Muscle protein content*

Variable	SL	Chronic HA	*P*
Protein concentration (mg/ml)	13.4 ± 1.0	11.3 ± 0.4	0.14
Muscle sample mass (mg)	20.0 ± 1.6	20.7 ± 2.4	0.83
Homogenate volume (ml)	0.21 ± 0.02	0.21 ± 0.03	0.86
Leg FFM (g)	20663 ± 3674	18595 ± 988	0.01
Muscle protein content (g)	2942 ± 390	2161 ± 82	0.05

Values are means ± SEM. Muscle protein content = protein concentration (mg/ml) × homogenate vol (ml)/muscle wt (mg) × leg FFM (g).

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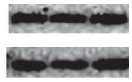
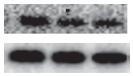
	SL	Acute HA	Chronic HA	*P* Value	Representative Blots
p-AKT^{Ser473}	1.00±0.36	0.62±0.09	0.87±0.31	0.62	
Total AKT	1.00±0.28	0.95±0.25	1.32±0.25	0.60	
p-AMPK^{Thr172}	1.00±0.15	0.58±0.12	0.73±0.12	0.09	
Total AMPK	1.00±0.10	1.31±0.35	1.56±0.37	0.43	
p-TSC2^{Thr1462}	1.00±0.11	1.13±0.10	0.79±0.10	0.12	
Total TSC2	1.00±0.16	0.83±0.16	0.87±0.16	0.77	
p-mTOR^{Ser2448}	1.00±0.41	0.76±0.19	0.91±0.32	0.87	
Total mTOR	1.00±0.20	0.79±0.25	0.92±0.30	0.83	
p-p70S6K$^{Ser424/Thr421}$	1.00±0.41	1.68±0.79	2.94±1.23	0.30	
Total p70S6K	1.00±0.20	1.04±0.25	1.03±0.19	0.99	
p-rpS6$^{Ser235/236}$	1.00±0.50	0.71±0.12	1.04±0.27	0.77	
Total rpS6	1.00±0.18	0.94±0.24	1.08±0.13	0.87	
HSP90[1]	-	-	-	-	

Figure 2. Values are presented means ± SEM. Base phosphorylation status normalized total protein content. Base total protein content normalized to HSP90. [1]Normalization protein used as a loading control per gel. No significant differences between phases.

phase-by-time interaction; Fig. 6*B*). In addition, at acute and chronic HA, Post ubiquitylation was lower than SL ($P < 0.05$, phase-by-time interaction; Fig. 6*B*). Independent of study phase, Rec MuRF1 mRNA was higher than Base and Post ($P < 0.05$, time main effect; Fig. 6*C*). Independent of time point, MuRF1 mRNA at chronic HA was lower than SL ($P < 0.05$, phase main effect). Independent of phase, Post and Rec Atrogin-1 mRNA was lower than Base ($P < 0.05$, time main effect; Fig. 6*D*). POST chymotrypsin-like and PGPH-like enzymatic activities were lower ($P < 0.05$; time main effect) compared with Base and Rec, regardless of phase (Fig. 6*E*). No study phase or time point effects were observed for trypsin-like activity.

DISCUSSION

The primary observation of this investigation is that both acute HA exposure and chronic (21 d) HA exposure, coupled with a severe energy deficit, inhibited mTORC1 signaling at Post. Moreover, combined chronic HA exposure and energy deficit inhibited the anabolic sensitivity of muscle to consuming 25 g of whey protein. Reductions in mTORC1 signaling, despite lower SpO_2 at acute and chronic HA compared with SL, seem to be independent of purported hypoxia-mediated mTORC1 inhibition, as neither REDD1 mRNA expression, nor its coupling to 14-3-3 differed between study phases (SL, acute HA, and chronic HA). Markers of muscle protein breakdown were not indicative of elevated proteolysis at acute or chronic HA, which suggests a negligible effect of proteolysis in the modulation of skeletal muscle mass in response to chronic HA exposure and severe energy deficit in healthy individuals. These findings suggest that HA and severe energy deficit induces mTORC1-mediated anabolic resistance and may account, in part, for muscle loss during periods of sustained HA exposure.

Exercise-induced activation of p-mTOR^{Ser2448} and p-p70S6K$^{Thr424/421}$ was inhibited after acute hypoxia exposure at HA relative to normoxia at SL. Yet no marker of hypoxia-mediated inhibition of mTORC1 was upregulated at acute HA compared with SL in the current study. These findings, to an extent, are in agreement with those reported by Etheridge *et al.* (47), who demonstrated that 3.5 h of hypoxic exposure equivalent to 4300 m suppressed muscle protein synthetic responses to the mechanical stimulus of resistance exercise. Likewise, past human studies reported that HIF-1α and REDD1 do not blunt acute mTORC1 signaling after exercise (47, 48) or consumption of a mixed meal (49) in response to the stress of acute hypoxia. These data suggest that hypoxia-mediated modulation of mTORC1 signaling in humans, particularly in response to exercise, may be independent of HIF-1α and REDD1/14-3-3 coupling. Anabolic sensitivity to whey protein feeding was largely maintained at acute HA in the current study. D'Hulst *et al.* (49) similarly reported that mTORC1 signaling was not inhibited during exposure to hypoxia equivalent to 5180 m after the consumption of a protein-containing (30–40 g) mixed meal. This apparent maintenance of mTORC1-mediated anabolic signaling highlights the sensitivity of muscle to high-quality protein independent of acute hypoxia.

Chronic HA exposure coupled with severe energy deficit was accompanied by overt anabolic resistance. By d 21, chronic HA exposure while being severely underfed manifested in a complete suppression of mTORC1-mediated anabolic signaling in response to both exercise and protein ingestion. Two previous human investigations (50, 51) reported little or no change in mTORC1-mediated anabolic signaling under basal conditions after 7–15 d of altitude exposure at 3200–4600 m, but, to the best of our knowledge, no human study has examined the acute anabolic signaling response to exercise and protein feeding after chronic HA exposure combined with severe energy deficit. Several investigations, using stable isotope

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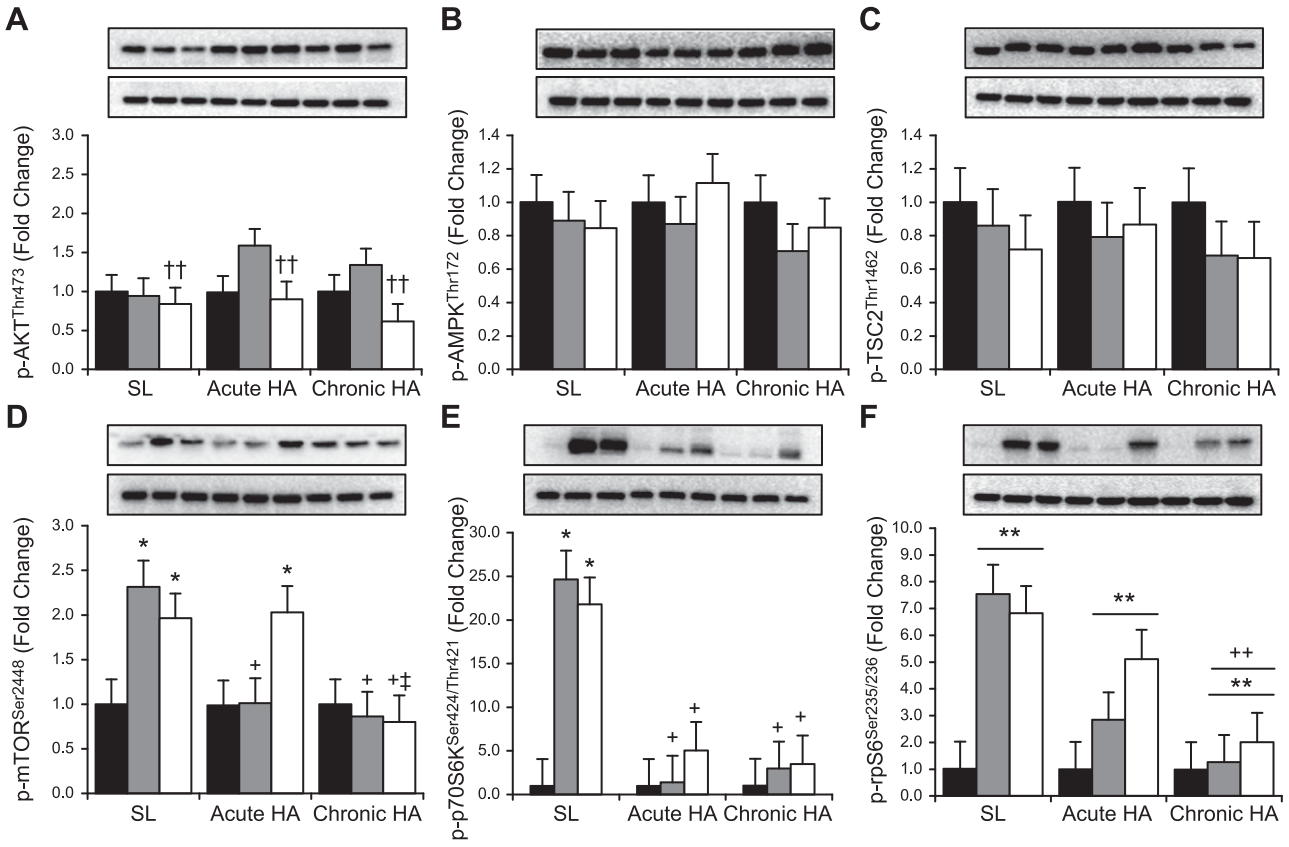


Figure 3. Values are presented as means ± SEM (n = 8). Within [Base (■), Post (■), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and whey protein ingestion for p-AKT^{Thr473} (*A*), p-AMPK^{Thr172} (*B*), p-TSC2^{Thr1462} (*C*), p-mTOR^{Ser2448} (*D*), p-p70S6K$^{Ser424/421}$ (*E*), and p-rpS6$^{Ser235/236}$ (*F*). Top image is a representative band for the target phosphorylation site listed on the *y* axis. Bottom image is a representative band for the loading control, HSP90. $^{++}$Different from SL, phase main effect, $P < 0.05$; **different from Base, time main effect, $P < 0.05$; ††different from Post, time main effect, $P < 0.05$; $^{+}$different from SL, phase-by-time interaction, $P < 0.05$; ‡different from acute HA, phase-by-time interaction, $P < 0.05$; *different from Base, phase-by-time interaction, $P < 0.05$.

methodologies, have consistently demonstrated that short-term (<21 d) moderate energy deficit (~20–40%) reduces rates of basal skeletal muscle protein synthesis (13–17) and blunts synthetic response to a protein-containing meal (17, 18) at SL. Although this diminished rate of skeletal muscle protein synthesis has been a reproducible finding, the exact mechanism that attenuates protein synthesis has not been reported. Previous work from our group (18) demonstrated that 21 d of a 40% energy deficit elicited a lower anabolic, albeit still significant, response to a protein-containing (20 g) mixed meal relative to weight maintenance. In the current study, 21 d of severe energy deficit at HA eliminated an mTORC1-mediated anabolic response to exercise and protein feeding observed at SL. As such, coupling of HA exposure with severe energy deficit creates a more profound resistance of mTORC1-meidated anabolic signaling compared with acute HA exposure or moderate energy deficit alone. Despite the magnitude of the energy deficit, there was no change in AMPK, a key regulator of energy utilization. The apparent maintenance of AMPK in the presence of inhibited mTORC1-mediated anabolic signaling is in agreement with several previous studies (20, 21, 52). Taken together, these data suggest that pathways that are necessary for preserving cellular function are maintained at the expense of anabolism.

Acute HA exposure blunted the Post protein ubiquitylation response compared with SL, but did not affect proteasome enzymatic activity. After 21 d of severe energy deficit, the total protein content of FOXO1 was greater at chronic HA compared with acute HA, but ubiquitin ligases MuRF1 and Atrogin-1 mRNA expressions were unchanged compared with SL. This lack of an effect of FOXO1 on the regulation of its downstream targets is likely attributable to the maintenance of relative FOXO1 phosphorylation status across study phases. FOXO1 activation at chronic HA was not different from SL or acute HA, despite higher total FOXO1 expression. There were also no differences between phases in the enzymatic activities of the catalytic subunits of the 26S proteasome, an additional indication that muscle proteolysis was not significantly influenced by HA or the combination of chronic HA and severe energy deficit. In agreement with findings from our study, MuRF1 mRNA expression was lower in humans who were exposed to HA at 3200 m for 15 d compared with SL, with no other changes in the markers of proteolysis between phases (50). Similarly, D'Hulst *et al.* previously reported lower postprandial 26S proteasome activity under acute HA conditions compared with SL (49). Likewise,

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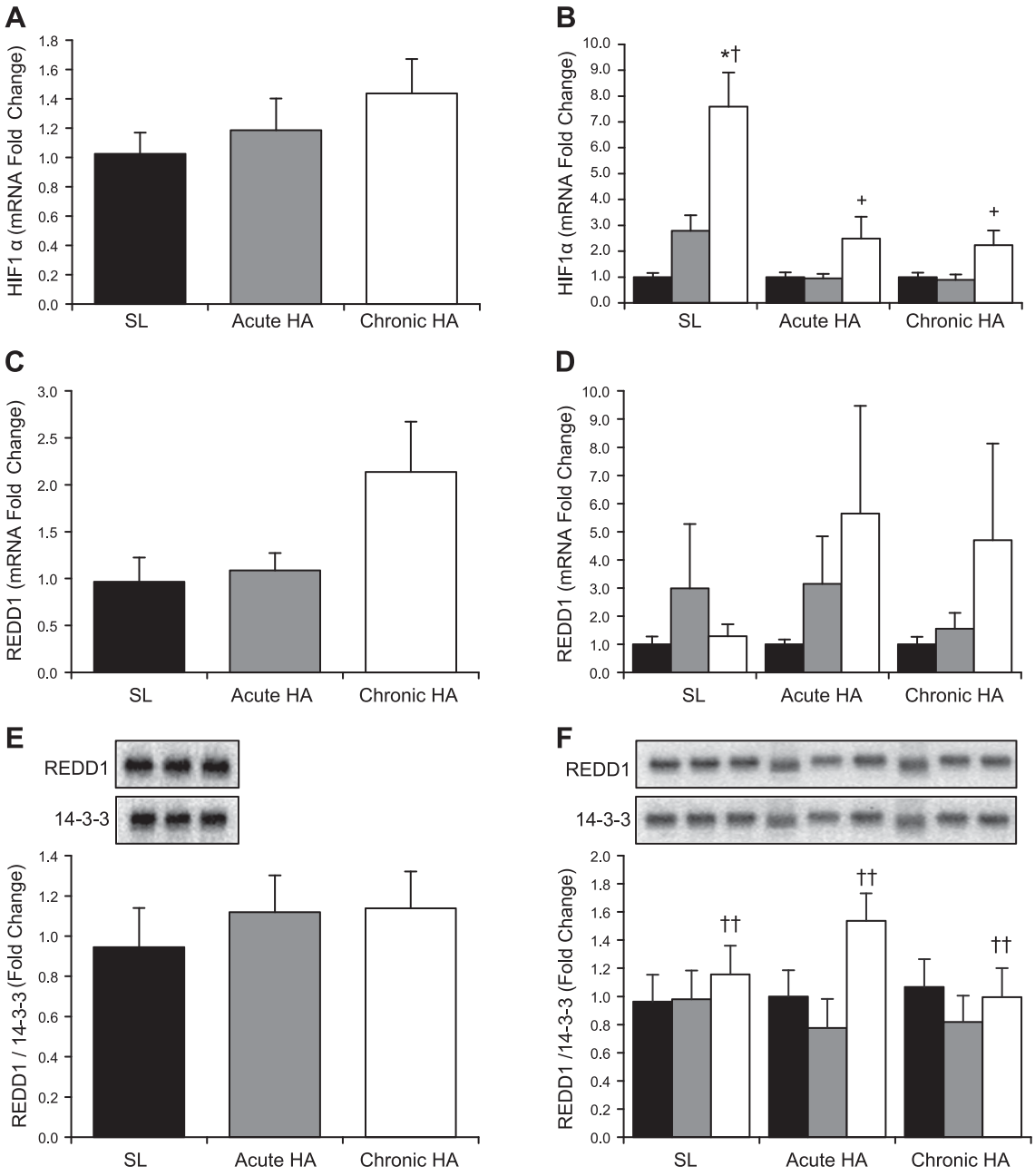


Figure 4. Values are presented as means ± SEM ($n = 8$). *A*, *C*, *E*) Base HIF-1α (*A*) and REDD1 mRNA expression (*C*), and REDD1/14-3-3 coupling (*E*) between study phases. *B*, *D*, *F*) Within [Base (■), Post (■), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and whey protein ingestion for HIF-1α (*B*) and REDD1 mRNA expression (*D*) and REDD1/14-3-3 coupling (*F*). ††Different from Post, time main effect, $P < 0.05$; †different from SL, phase-by-time interaction, $P < 0.05$; †different from Post, phase-by-time interaction, $P < 0.05$.

several recent studies have shown little to no alteration in muscle proteolysis after short-term energy deficit (15, 23, 24). Our group previously reported that 21 d of consuming a 40% energy deficient diet at SL did not alter basal or postprandial ubiquitin proteasome subunit activities relative to weight maintenance values (24), and Hector *et al.* (15) reported no changes in muscle protein breakdown in response to consuming a 40% energy deficient diet for 10 d. Furthermore, Smiles *et al.* (23) reported no difference in the intramuscular markers of proteolysis after 5 d of ~30% energy deficit compared with weight maintenance. The lack of increased muscle proteolysis with acute HA exposure or after chronic HA exposure, in combination with severe energy deficit, suggests that muscle protein breakdown is not a major contributor to muscle loss during HA sojourns.

A strength of the current study is that exercise and diet were tightly controlled, and the exercise stimulus was metabolically matched at the same absolute oxygen uptake to allow for phase comparisons between SL and HA. To our knowledge, this study is the first to comprehensively assess changes in muscle signaling pathways that regulate anabolism and proteolysis, both acutely and after 21 d of HA exposure with concomitant

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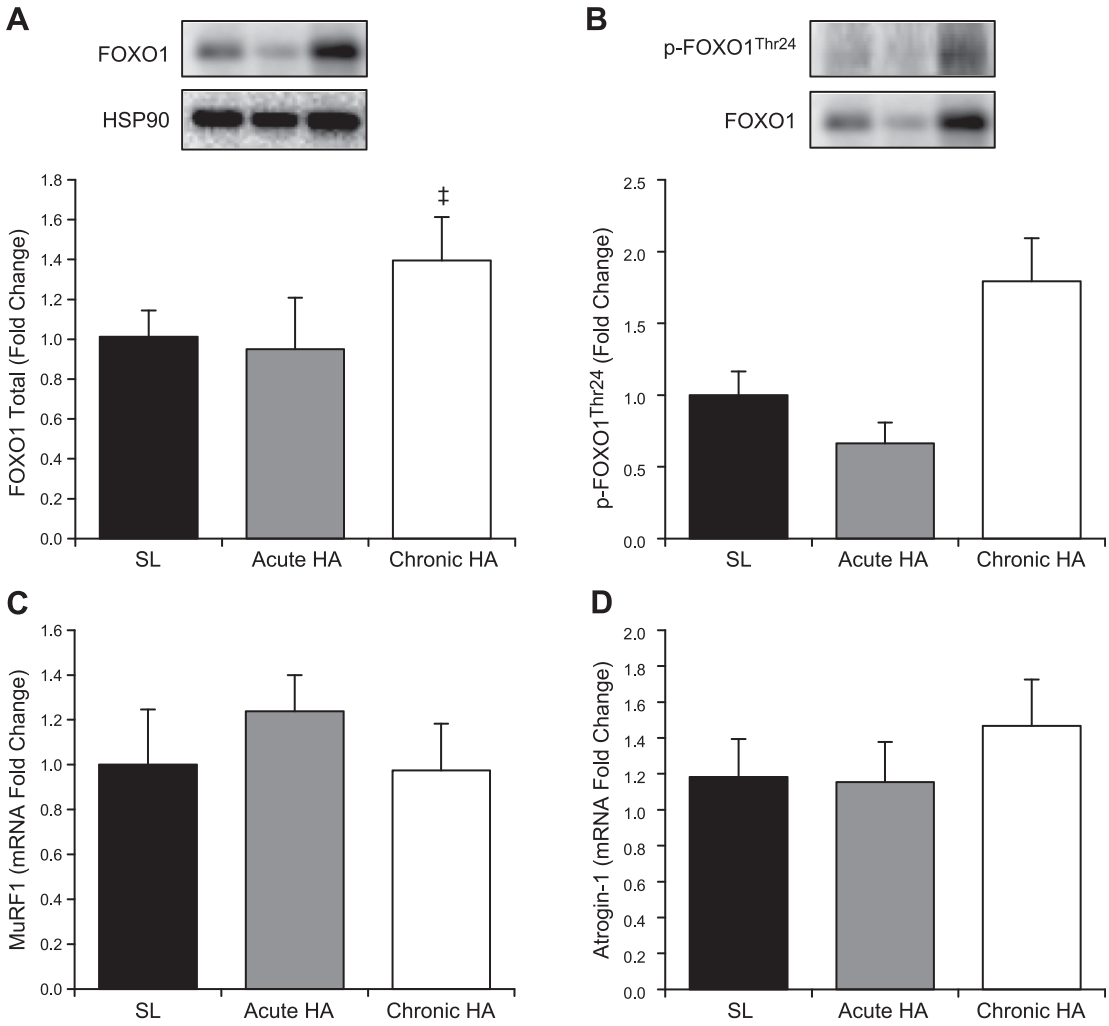


Figure 5. Values are presented as means ± SEM ($n = 8$). Base total FOXO1 (*A*) and p-FOXO1^{Thr24} (*B*), MuFR1 (*C*), and Atrogin-1 (*D*) mRNA expression at SL, acute HA, and chronic HA. ‡Different from acute HA, time-by-phase interaction, $P < 0.05$.

energy deficit. However, there are several limitations. Despite observing robust changes in mTORC1-mediated anabolic signaling, we are unable to determine whether the signaling changes we observed manifested in tangible reductions in muscle protein synthesis. As such, we acknowledge that including stable isotope–derived estimates of muscle protein synthesis (and breakdown) would have strengthened our data and the interpretation of our findings; however, as a result of unexplainable analytical issues, those data are not available. Nevertheless, reductions in FFM (whole body and leg) and muscle protein content, which may be considered an indirect estimate of protein anabolism, suggest that muscle protein was lost in response to the 21 d of HA exposure and severe energy deficit. We also acknowledge that we are unable to determine the mechanism of inhibited mTORC1-meidated anabolic signaling in response to exercise at acute HA. Furthermore, the study design precluded us from determining whether the inhibition of mTORC1-mediated anabolic signaling at chronic HA was the result of prolonged exposure to HA, severe energy deficit, or a combination of both; however, our study design was not intended to isolate the independent and synergistic effects of hypoxia and energy deficit on the molecular regulation of muscle mass. Our objective was to assess the environmental and physiologic stressors in concert, just as they would be experienced by military Service members (and other HA sojourners) performing strenuous work at HA. Observations made in the current study provide the scientific basis for future, more targeted, studies to delineate the role of hypoxia and underfeeding on the regulation of muscle mass.

We report that both acutely and after 21 d of HA (4300 m) exposure with severe energy deficit, phosphorylation of mTORC1-mediated anabolic signaling is inhibited after exercise and whey protein consumption. The proteolytic pathway was minimally impacted, as markers of proteolysis were either maintained or down-regulated at HA. Inhibited mTORC1 signaling in the absence of increased proteolysis suggests skeletal muscle anabolic resistance is the primary regulatory mechanism that contributes to skeletal muscle loss with underfeeding, particularly during sojourns at HA. FJ

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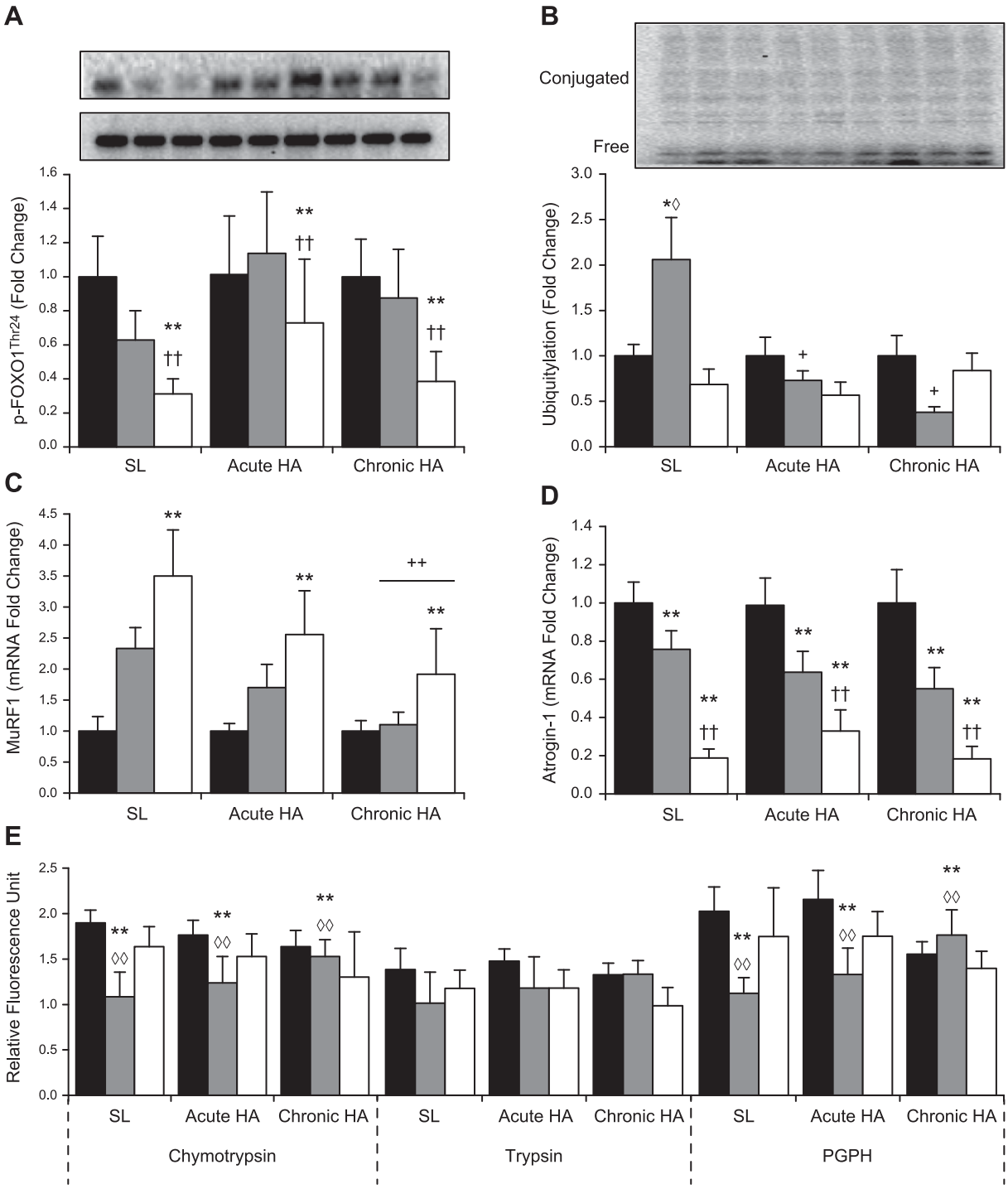


Figure 6. Values are presented as means ± SEM ($n = 8$). Within [Base (■), Post (■), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and whey protein ingestion for p-FOXO1^{Thr24} (*A*), protein ubiquitylation (*B*), MuRF1 (*C*), and Atrogin-1 (*D*) mRNA expression, and chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) –like enzymatic activities (*E*). Top image is a representative band for the target phosphorylation site listed on *y* axis. Bottom image is a representative band for the loading control, HSP90. $^{++}$Different from SL, phase main effect, $P < 0.05$; **different from Base, time main effect, $P < 0.05$; ††different from Post, time main effect, $P < 0.05$, $^{◊◊}$different from Rec, time main effect, $P < 0.05$; $^{+}$different from SL, phase-by-time interaction, $P < 0.05$; *different from Base, phase-by-time interaction, $P < 0.05$; $^{◊}$different from Rec, phase-by-time interaction, $P < 0.05$.

ACKNOWLEDGMENTS

The authors thank the volunteers who participated in this research experiment. The authors acknowledge the medical oversight team and Dr. Stephen Muza (U.S. Army Research Institute of Environmental Medicine) for support of this study. Most importantly, the authors sincerely thank Patrick Radcliffe, Adam Luippold, Bradley Anderson, Grant Holmes, Anthony Karis, Matthew Kominsky, Katelyn Guerriere, Karleigh Bradbury, Adrienne Hatch, Laura Lutz, and Drs. Stephen

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R. Hennigar, Robert W. Kenefick, Taylor Roth, J. Philip Karl, Scott J. Montain, and LTC Renee Cole (U.S. Army Research Institute of Environmental Medicine) for significant contributions to the project. This work was supported, in part, by the U.S. Army Medical Research and Materiel Command and appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education (to L.M.M., J.W.C., C.E.B., and A.J.Y.) through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Materiel Command. The investigators adhered to the policies for protection of human subjects as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

C. E. Berryman, A. J. Young, and S. M. Pasiakos designed research; L. M. Margolis, J. W. Carbone, C. E. Berryman, C. T. Carrigan, N. E. Murphy, A. A. Ferrando, A. J. Young, and S. M. Pasiakos performed research; L. M. Margolis, J. W. Carbone, C. T. Carrigan, N. E. Murphy, and S. M. Pasiakos analyzed data; L. M. Margolis, J. W. Carbone, and S. M. Pasiakos interpreted results; L. M. Margolis, C. T. Carrigan, N. E. Murphy, and S. M. Pasiakos prepared figures; L. M. Margolis, J. W. Carbone, and S. M. Pasiakos drafted the manuscript; and L. M. Margolis, J. W. Carbone, C. E. Berryman, C. T. Carrigan, N. E. Murphy, A. A. Ferrando, A. J. Young, and S. M. Pasiakos approved the final version.

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Received for publication January 26, 2018.
Accepted for publication April 30, 2018.

fasebj.org by Louisiana State Univ Troy H Middleton Library (198.62.89.27) on August 10, 2018. The FASEB Journal Vol. ${article.issue.getVolume()}, No. ${article.issue.getIssueNu

Physiological Reports

Open Access

Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Muscle Fn14 gene expression is associated with fat-free mass retention during energy deficit at high altitude

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Keywords
Hypoxia, interleukin-6, myogenin, negative energy balance, TWEAK.

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Funding information
Supported in part by the U.S. Army Medical Research and Material Command and appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education (to C. E. B., A. J. Y., J. W. C., L. M. M.) through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Materiel Command.

Received: 25 June 2018; Accepted: 28 June 2018

doi: 10.14814/phy2.13801

Physiol Rep, 6 (14), 2018, e13801, https://doi.org/10.14814/phy2.13801

Abstract

Intramuscular factors that modulate fat-free mass (FFM) loss in lowlanders exposed to energy deficit during high-altitude (HA) sojourns remain unclear. Muscle inflammation may contribute to FFM loss at HA by inducing atrophy and inhibiting myogenesis via the tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor-inducible protein 14 (Fn14). To explore whether muscle inflammation modulates FFM loss reportedly developing during HA sojourns, muscle inflammation, myogenesis, and proteolysis were assessed in 16 men at sea level (SL) and following 21 days of energy deficit (−1862 ± 525 kcal/days) at high altitude (HA, 4300 m). Total body mass (TBM), FFM, and fat mass (FM) were assessed using DEXA. Gene expression and proteolytic enzymatic activities were assessed in muscle samples collected at rest at SL and HA. Participants lost 7.2 ± 1.8 kg TBM ($P < 0.05$); 43 ± 30% and 57 ± 30% of the TBM lost was FFM and FM, respectively. Fn14, TWEAK, TNF alpha-receptor (TNFα-R), TNFα, MYOGENIN, and paired box protein-7 (PAX7) were upregulated ($P < 0.05$) at HA compared to SL. Stepwise linear regression identified that Fn14 explained the highest percentage of variance in FFM loss ($r^2 = 0.511$, $P < 0.05$). Dichotomization of volunteers into HIGH and LOW Fn14 gene expression indicated HIGH lost less FFM and more FM (28 ± 28% and 72 ± 28%, respectively) as a proportion of TBM loss than LOW (58 ± 26% and 42 ± 26%; $P < 0.05$) at HA. MYOGENIN gene expression was also greater for HIGH versus LOW ($P < 0.05$). These data suggest that heightened Fn14 gene expression is not catabolic and may protect FFM during HA sojourns.

Introduction

Hypoxia exposure during high-altitude (HA) sojourns elicits a persistent systemic inflammatory response (Mazzeo et al. 1985; Hartmann et al. 2000; Lundby and Steensberg 2004). This response represents a homeostatic adaptation to hypoxia that may promote acute-phase protein synthesis, angiogenesis, and erythropoiesis (Mazzeo et al. 1985; Klausen et al. 1997; Mazzeo 2005). Sojourners at HA are also subject to additional pro-inflammatory stressors, such as high physical activity levels (Hoppeler et al. 1990; Hoyt et al. 1994) and energy deficits

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(Butterfield et al. 1985; Rose et al. 1985), that may exacerbate inflammation and/or modulate its downstream effects. Strenuous military operations at sea level (SL) induce an inflammatory response that upregulates the hepatic release of hepcidin, a regulator of iron status, associated, at least in part, with failure to match total daily energy expenditure with sufficient energy intake (McClung et al. 2013; Pasiakos et al. 2016). The muscle inflammatory response associated with energy deficits may be due to a reduction in muscle energy availability (Hennigar et al. 2017). The magnitude and consequences of the inflammation caused during military operations have received only limited attention (McClung et al. 2013; Pasiakos et al. 2016), particularly for operations at HA (Hagobian et al. 2006). The skeletal muscle inflammatory response might be a mechanism modulating loss of muscle mass often experienced by lowlanders sojourning at HA (Pasiakos et al. 2017).

Muscle inflammation can promote muscle loss (Dogra et al. 2006; Mittal et al. 2010; Merritt et al. 2013). This generally occurs in response to injury and pathogenic conditions of muscle wasting, as chronic elevations and/or exceedingly high muscle inflammation can inhibit muscle regeneration (Merritt et al. 1985; Dogra et al. 2007a), blunt muscle protein synthesis (Bamman et al. 2015), and contribute to muscle atrophy (Kumar et al. 2012; Merritt et al. 2012, 2013). This catabolic state is modulated, in part, by TNF alpha (TNFα), the TNF-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor-inducible 14 (Fn14), which activate nuclear factor-κB (NF-κB), subsequent atrophic gene expression, and inhibition of muscle regeneration (Dogra et al. 2006, 2007a). We suspect that because FFM is often markedly degraded when lowlanders are chronically underfed when sojourning at HA that muscle inflammation will be upregulated, triggering downstream atrophic gene expression, and inhibition of muscle regeneration. However, to the best of our knowledge, no study has investigated the TWEAK-Fn14 axis response or examined associated downstream effects on muscle mass (i.e., fat-free mass; FFM) in lowlanders exposed to the combined stress of HA and energy deficit.

The objective of this study was to determine the combined effects of HA exposure and energy deficit on muscle inflammation and examine associations between markers of inflammation and muscle mass lost (Berryman et al. 2018). This secondary analysis was pursued to explore potential intramuscular explanations for the interindividual variability and magnitude of FFM lost we observed in lowlanders exposed to 21 days of energy deficit at HA as part of the parent study (Berryman et al. 2018). We hypothesized that muscle inflammation would increase in response to the combined stress of hypoxia and energy deficit, and the magnitude of this response would be directly associated with the magnitude of FFM loss experienced by individuals exposed to the stress of hypoxic exposure and energy deficit.

Methods

Participants

Participant eligibility, recruitment details, and results of the primary study objective have been previously reported elsewhere (Berryman et al. 2018). Due to limited quantity of muscle sample for one volunteer, data from 16 of the original 17 male lowlanders that completed the parent study are included in this report (Berryman et al. 2018). All volunteers provided written informed consent prior to participation. This study was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA) and registered at www.clinical trials.gov as NCT02731066. Investigators adhered to the policies for protection of human subjects as described in the US Department of Defense Instruction 3216.02, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Experimental design

This diet and physical activity controlled study consisted of 21 days at SL (Natick, MA; 55 m; Fig. 1) followed immediately by 22 days at HA (Pikes Peak, CO; 4300 m) (Berryman et al. 2018). Participants maintained their habitual exercise routines and adhered to strict dietary instruction at SL, and were weight stable (Berryman et al. 2018). At HA, additional exercise was prescribed daily and the participants were fed an energy deficient diet. Skeletal muscle inflammation and downstream myogenic regulatory factors were assessed in vastus lateralis biopsy samples collected under fasted, rested conditions on day 7 at SL and on day 22 at HA. The SL muscle biopsies were performed on day 7 in order to provide sufficient time to pack and transport laboratory equipment from our laboratory in Natick, MA, to our laboratory on Pikes Peak, CO.

After completing the SL phase, participants were flown from Boston, MA to Denver, CO and driven to Colorado Springs, CO, where they stayed overnight under supervision in a local hotel. The following morning (HA day 1, ~3 AM), participants were transported by car to the USARIEM Maher Memorial Laboratory on the summit of Pikes Peak, CO and underwent additional experimental procedures reported elsewhere (Berryman et al. 2018). Starting on HA day 2, participants were randomized to a

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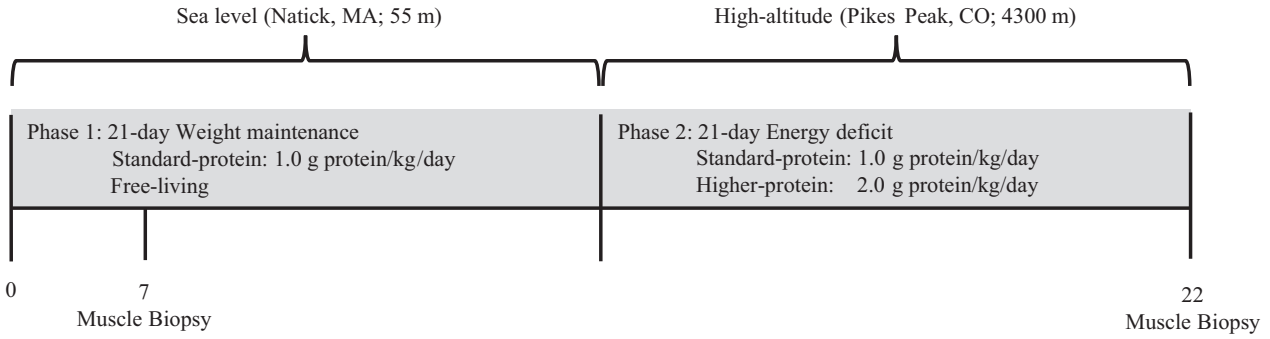


Figure 1. Study design. Energy deficit was designed to be 40% of total energy needs; however, on the basis of changes in body energy stores, the actual deficit was closer to 70% (−1862 ± 525 kcal/day). The current analysis was part of a larger study that assessed the impact of dietary protein intake on changes in body composition in response to 21 days of energy deficit at high altitude (4300 m). Habitual protein intake did not influence end point measures in this analysis, so diet groups were collapsed.

controlled diet with either standard- (mean ± SD, 1.1 ± 0.0 g protein·kg^{-1} day^{-1}, 2.8 ± 0.4 g carbohydrate·kg^{-1} day^{-1}, 1.1 ± 0.1 g fat·kg^{-1} day^{-1}, and 1950 ± 186 kcal day^{-1}) or higher- (2.1 ± 0.0 g protein kg^{-1} day^{-1}, 3.0 ± 0.2 g kg^{-1} day^{-1}, 0.7 ± 0.1 g fat kg^{-1} day^{-1}, and 1894 ± 286 kcal day^{-1}) protein for the next 21 days (Berryman et al. 2018).

To induce weight loss, exercise-induced energy expenditure (EIEE) was increased by 10% above estimated SL total daily energy expenditures (TDEE) [i.e., HA EIEE = SL EIEE + (0.1 × SL TDEE)], and daily energy intake was decreased by 30% relative to TDEE estimated at SL to maintain body mass to induce an energy deficit level consistent with our previous work (McClung et al. 2013; Pasiakos et al. 2013). As previously described (Berryman et al. 2018), SL TDEE was estimated from the sum of resting metabolic rate measured by indirect calorimetry (True Max 2400, Parvo Medics, Sandy, UT, USA; multiplied by 1.3 to account for activities of daily living and diet induced thermogenesis) and EIEE estimated from corresponding metabolic equivalents of activities reported in 3 days physical activity logs. These data were then averaged with TDEE derived from the Harris-Benedict equation (Roza and Shizgal 1984), and activity derived from 3-days records. Exercise was performed daily at HA, with the exception of day 7, 14, and 21, the day prior to the muscle biopsy procedure on day 22 at HA (exercise was also restricted for the 24 h preceding the SL biopsy). Exercise was supervised and consisted of treadmill walking, running, and outdoor hiking, with the energy cost estimated using the Pandolf et al. (1977) Equation, with adjustments for body mass loss, terrain factors, and downhill walking (Pimental and Pandolf 1979).

Body mass was measured daily at HA and body composition (total body mass, TBM; FFM; and fat mass, FM) was determined at baseline, before ascent to HA (SL day 20), and immediately after descent from HA (~0630–0800 on HA day 23) using dual-energy X-ray absorptiometry (DEXA; iDEXA, GE Lunar Corporation, Madison, WI, USA) (Berryman et al. 2018). In this study, FFM refers to TBM minus both FM and bone mass. As previously reported (Berryman et al. 2018), TBM loss was more than expected for an estimated, prescribed energy deficit of 40%. As such, changes in body energy stores were used to better estimate the total daily energy deficit (Hoyt et al. 2006; Berryman et al. 2018). Those estimates are presented in this paper for descriptive purposes and to explore the role of energy balance on modulating muscle inflammation.

Muscle inflammatory and myogenic mRNA

Muscle interleukin 6 (IL-6), IL-6 receptor (IL-6R), TNFα, TNFα-receptor (TNFα-R), TWEAK, Fn14, paired box protein-7 (PAX7), MYOD, MYOGENIN, MuRF1, and ATROGIN-1 gene expression were determined using primers from Bio-Rad Laboratories (Hercules, CA, USA). TRIzol (ThermoFisher, Waltham, MA, USA) was used to isolate total RNA from 20 mg of muscle followed by quantity and quality assessments using a Nanodrop ND-2000 spectrophotometer (ThermoFisher). cDNA was then synthesized from 500 ng of total RNA using iScriptTM Advanced cDNA Synthesis Kits from Bio-Rad Laboratories and a T100™ Thermal Cycler (Bio-Rad). cDNA amplifications were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Samples were run in 20 μL reactions in duplicate using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). mRNA data were normalized to the geometric mean of β-actin, GAPDH, and B2M mRNA, and fold changes (HA vs. SL) were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). The MURF1 and ATROGIN-1 gene expression responses to the HA and severe energy deficit have been reported previously (Margolis et al. 2018), but are reported briefly in this paper to explore the potential

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role of muscle inflammation in mediating MURF1 and ATROGIN-1 gene expression.

NF-κB phosphorylation and total protein expression

Phosphorylation status and total protein content of NF-κB p65 were determined using standard homogenization and Western blot methodologies, as described in detail elsewhere (Margolis et al. 2016). In brief, after 15 μg of total protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes, primary antibodies for p-NF-κB $p65^{Ser468}$ and total NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA) were applied, and membranes were incubated overnight at 4°C. Secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology) and chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA) were applied the next morning, and blots were quantified using the ChemiDoc XRS and Image Lab software from Bio-Rad Laboratories. Heat shock protein 90 (HSP90) was used to confirm equal protein loading across wells. Phosphorylation and total protein content of NF-κB p65 were normalized to HSP90, the ratio of phosphorylation-to-total protein was determined, and HA data were expressed as fold change relative to SL.

26S proteasome enzymatic activity

26S proteasome responses to HA and severe energy deficit have been reported previously (Margolis et al. 2018), but included in this paper to explore the potential role of muscle inflammation in mediating this response. Extended methodological details for assessing 26S β_5 (chymotrypsin-like), 26S β_2 (trypsin-like), and 26S β_1 (peptidyl-glutamyl peptide hydrolyzing [PGPH]-like) enzymatic activities have been reported (Carbone et al. 2013). HA proteolytic activity data were expressed as fold change relative to SL.

Statistical analysis

This study was a secondary objective of a larger randomized controlled trial powered to detect differences in FFM between standard- and higher-protein diets following HA exposure and concomitant energy deficit (Berryman et al. 2018). As reported, dietary protein intake had no effect on FFM in response to the combined physiological and environmental stress (Berryman et al. 2018). Therefore, we did not expect dietary protein group to affect skeletal muscle inflammation, regeneration, and proteasome enzymatic activities after 21 days of exposure to HA and energy deficit. After testing for normality (Shapiro-Wilk), and $\log_2$ transformation of skewed data (all mRNA endpoints), we examined the effects of dietary protein on study outcomes at HA relative to SL using repeated measures ANOVA with phase (SL and HA) and dietary protein group (standard- and higher-protein) as within- and between-subjects factors, respectively. As anticipated, there were no effects of dietary protein group on any study outcome.

The data were then re-analyzed, excluding dietary protein group as a between-subjects factor, using paired *t*-tests (HA vs. SL). To be consistent with Bamman et al. (2015), stepwise linear regression was used to explore the associations between skeletal muscle inflammatory markers and FFM loss, expressed as the proportion of TBM loss in response to HA and severe energy deficit. Participants were then dichotomized as HIGH and LOW based on median gene expression at HA relative to SL for the inflammatory marker (i.e., Fn14) most strongly associated with FFM loss. Body composition and myogenic gene expression responses to HA exposure and severe energy deficit were then analyzed by HIGH and LOW group using unpaired *t*-tests. Within the text, data are presented as mean ± SD; in the figures, data are presented as mean ± SD and as individual data points to demonstrate intersubject variability. The α level for significance was two-tailed and set at $P < 0.05$, and data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp. Armonk, NY, USA).

Results

Participants ($n = 16$) lost 7.2 ± 1.8 kg TBM (SL, 79.8 ± 14.4 kg; HA, 72.6 ± 13.1 kg) and 43 ± 30% (3.5 ± 2.5 kg) and 57 ± 30% (3.7 ± 1.4 kg) of the TBM lost was FFM and FM, respectively. Daily exercise contributed 671 ± 172 kcal/day (108 ± 23 min/day) and the estimated daily energy deficit based on changes in body energy stores was 1862 ± 525 kcal/day.

Muscle IL-6 and IL-6R gene expressions were not significantly different at HA relative to SL (Fig. 2A). TNFα, TNFα-R, TWEAK, and Fn14 gene expressions were upregulated at HA relative to SL (Fig. 2B–C, $P < 0.05$), while p-NF-κB $p65^{Ser468}$ or NF-κB p65 total protein content at HA was not significantly different from SL. PAX7 and MYOGENIN gene expression were upregulated at HA relative to SL (Fig. 3A–B, $P < 0.05$), but MYOD gene expression was not significantly different between HA and SL (Fig. 3C). MuRF1 and ATROGIN-1 gene expression were not significantly different at HA relative to SL. Likewise, there were no significant differences in chymotrypsin-like, trypsin-like, and PGPH-like enzymatic activities at HA compared to SL.

Stepwise linear regression analyses showed increased IL-6 ($r = -0.605$), TNFα ($r = -0.496$), TWEAK

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($r = -0.513$), and Fn14 ($r = -0.715$) gene expression were associated ($P < 0.05$) with less FFM loss. Based on these results, participants were dichotomized into HIGH and LOW according to the median Fn14 gene expression at HA, respectively (Fig. 4A). HIGH lost less FFM and more FM (28 ± 28% and 72 ± 28%), as a proportion of TBM loss, than LOW (58 ± 26% and 42 ± 26%; Fig. 4B, $P < 0.05$). MYOGENIN gene expression after HA exposure and severe energy deficit was also greater for HIGH versus LOW (Fig. 4C, $P < 0.05$). MYOGENIN gene expression changes in response to HA and severe energy deficit were associated ($r = 0.530$, $P < 0.05$) with the

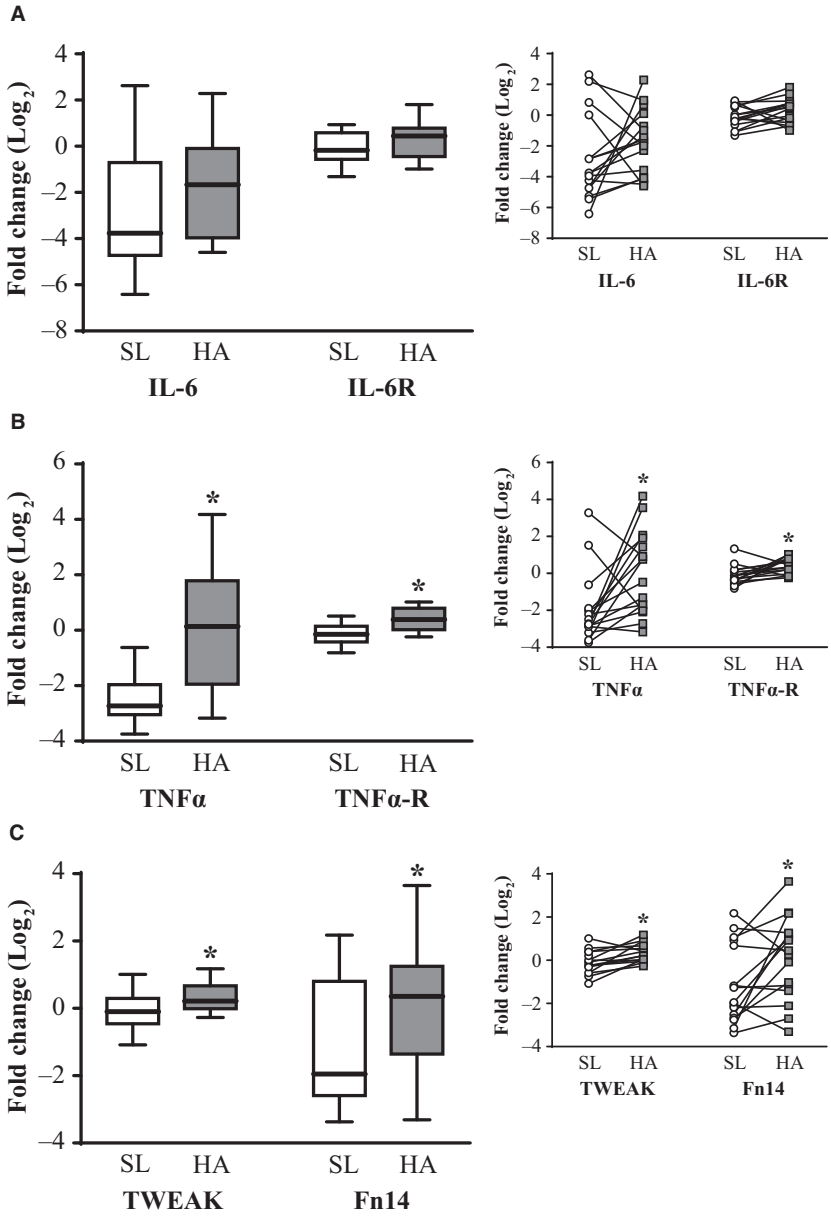


Figure 2. Skeletal muscle IL-6 and IL-6R (A), TNFα and TNFα-R (B), and TWEAK and Fn14 (C) gene expression in male ($n = 16$) lowlanders exposed to 21 days of energy deficit at high altitude (4300 m). Data were normalized to the geometric mean of β-actin, GAPDH, and B2M gene expression, and fold changes were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). HA data were expressed as fold change relative to SL to examine differences using paired *t*-tests after data were $\log_2$ transformed. Values are presented as mean ± SD and individually per participant. *HA different than SL, $P < 0.05$. IL-6, interleukin 6; IL-6R, interleukin 6 receptor; TNFα, tumor necrosis factor alpha; TNFα-R, tumor necrosis factor alpha-receptor; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; Fn14, fibroblast growth factor-inducible 14; SL, day 7 at sea level; and HA, day 22 at high altitude.

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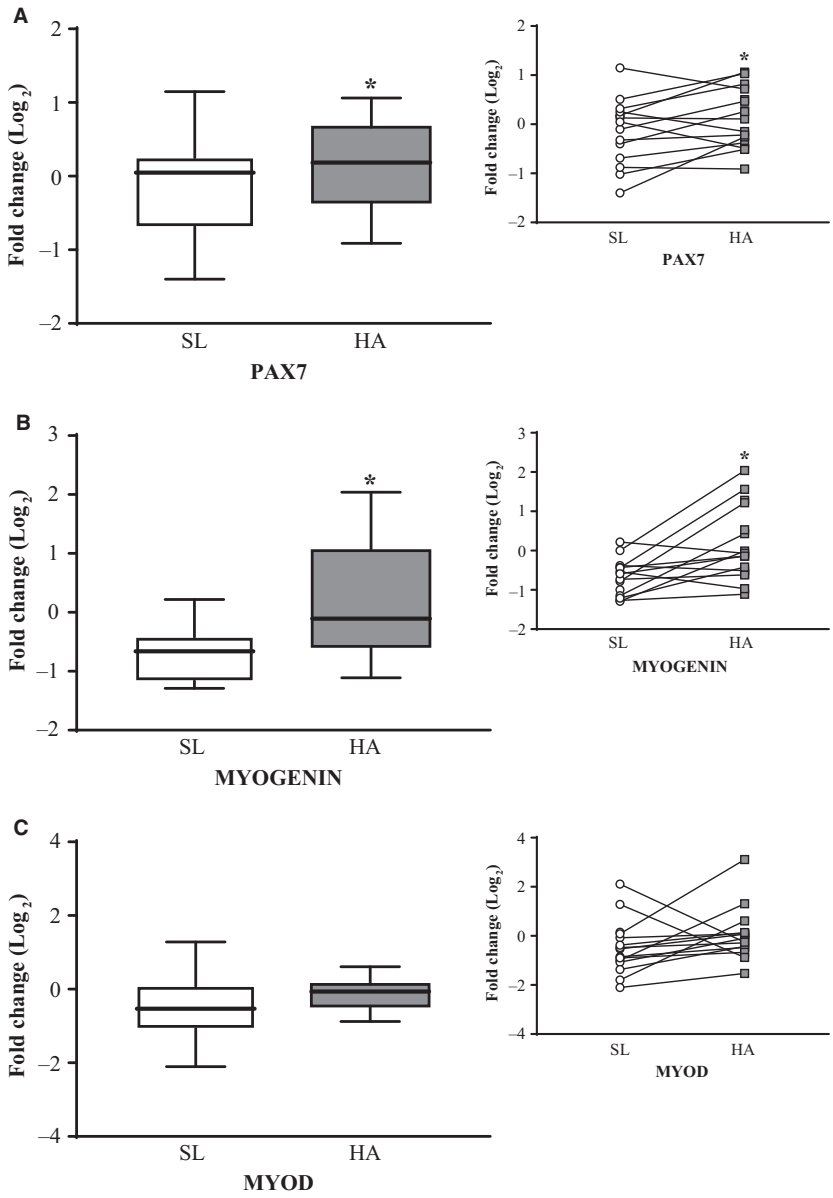


Figure 3. Skeletal muscle PAX7 (A), MYOGENIN (B), and MYOD (C) gene expression in male (n = 16) lowlanders exposed to 21 days of energy deficit at high altitude (4300 m). Data were normalized to the geometric mean of β-actin, GAPDH, and B2M gene expression, and fold changes were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). HA data were expressed as fold change relative to SL to examine differences using paired t-tests after data were $\log_2$ transformed. Values are presented as mean ± SD and individually per participant. *HA different than SL, $P < 0.05$. PAX7, paired box protein-7; SL, day 7 at sea level; and HA, day 22 at high altitude.

change in Fn14. There were no significant differences in MuRF1 or ATROGIN-1 gene expression, or chymotrypsin-like, trypsin-like, and PGPH-like enzymatic activities between HIGH and LOW. No significant differences were observed in EIEE between the two groups (HIGH: 686 ± 156 kcal day^{-1}, LOW: 655 ± 196 kcal day^{-1}, $P > 0.05$) or in the estimated energy deficit (HIGH: -2021 ± 228 kcal day^{-1}, LOW: −1702 ± 693 kcal day^{-1}, $P > 0.05$).

Discussion

The current study explored muscle inflammation, myogenic regulatory factor gene expression, and ubiquitin-mediated proteolytic responses to the 9% loss of TBM induced by the combined stress of chronic HA exposure and concomitant energy deficit. The primary findings show that, in response to 21 days of severe energy deficit at HA: (1) muscle inflammation and myogenic regulatory factor gene expression were

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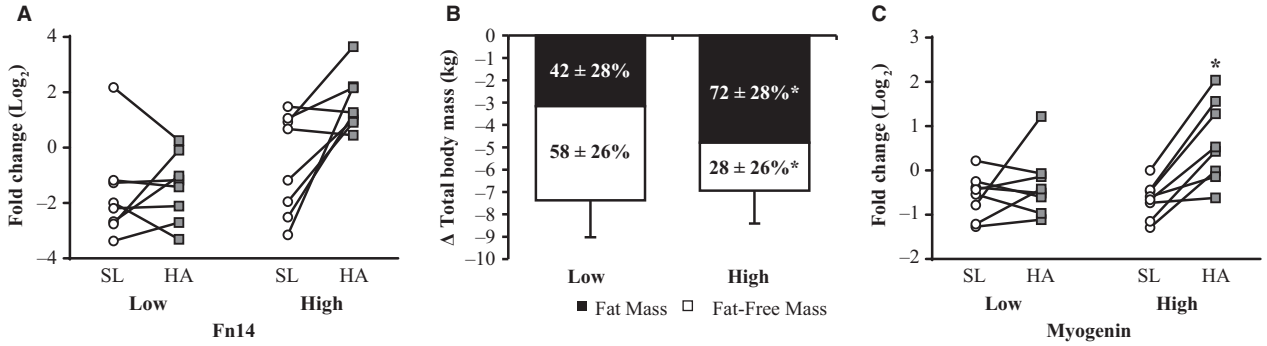


Figure 4. Fn14 (A), FM and FM loss as a proportion (%) of total body mass loss (B) and MYOGENIN gene expression (C) in male (n = 16) lowlanders dichotomized based on median Fn14 gene expression at HA relative to SL into LOW and HIGH after being exposed to 21 days of energy deficit at high altitude (4300 m). FFM and FM differences between HIGH and LOW were determined using unpaired t-tests. MYOGENIN gene expression data were normalized to the geometric mean of β-actin, GAPDH, and B2M gene expression, and fold changes were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). HA data were expressed as fold change relative to SL to examine differences between HIGH and LOW using unpaired t-tests after mRNA data were $\log_2$ transformed. Values are presented as mean ± SD and individually per participant. *HIGH significantly different from LOW, $P < 0.05$. FFM, fat-free mass; FM, fat mass; SL, day 7 at sea level; and HA, day 22 at high altitude.

upregulated, with no measureable change in ubiquitin-mediated proteolytic activity; and (2) an increase in the inflammatory response, particularly Fn14, was associated with the upregulation of MYOGENIN and attenuation of FFM loss. These findings suggest that, contrary to our hypothesis, heightened Fn14 gene expression was not catabolic and may protect FFM during energy deficit at HA by upregulating myogenesis.

Certain experimental issues must be acknowledged to appropriately interpret our findings and their potential implications. First, our study design did not include a SL control group that experienced the energy deficit and a HA control group that experienced energy balance. This precludes us from delineating the independent effects of the sustained underfeeding and stress of HA on muscle inflammatory gene expression and its associated effects. Also, while our analytical approach was modeled after recent studies exploring the role of muscle inflammation on indices of muscle anabolism, wasting, and function (Merritt et al. 1985, 2013; Bamman et al. 2015; Yarar-Fisher et al. 2016), the observational and correlative nature of our analysis may be considered limitations. However, we show muscle inflammation, particularly Fn14, is upregulated and associated with greater MYOGENIN and reduced FFM loss following 21 days of energy deficit at HA, which are all novel findings that may advance our current understanding of how healthy muscle adapts to energy deficit and the environmental stress of unaccustomed HA exposure.

Muscle inflammation and its potential downstream effects on proteolytic activity in healthy individuals exposed to chronic HA and concomitant underfeeding, to our knowledge, has not been explored. We observed modest increases in muscle TNFα, TNFα-R, TWEAK, and Fn14 gene expression in individuals that lost 3.5 ± 2.5 kg of FFM (43 ± 30% of TBM lost). Upregulated TNF-α and TWEAK gene expression may indirectly induce skeletal muscle atrophy by activating NF-κB signaling and subsequent transcription of the ubiquitin ligases, ATROGIN-1 and MuRF1 (Cai et al. 2004; Dogra et al. 2006, 2007a). However, despite increases in TNF-α and TWEAK gene expression, NF-κB signaling, and downstream ATROGIN-1 and MuRF1 gene expression were not different after 21 days of energy deficit at HA. More importantly, there were no changes in proteolytic activity, as 26S proteasome chymotrypsin-like, trypsin-like, and PGPH-like enzymatic activities after chronic HA exposure and energy deficit were not different than SL. Overall, these findings suggest that regardless of the observed muscle inflammatory response, healthy muscle is relatively protected against excessive muscle protein catabolism in response to chronic HA exposure and concomitant energy deficit.

Although NF-κB signaling and subsequent proteolysis were not affected by the combined stress of HA and energy deficit, myogenesis was, as PAX7 and MYOGENIN were upregulated following 21 days of energy deficit at HA. Although inflammation is generally associated with muscle degradation, transient elevation in muscle inflammation following routine exercise is a normal physiological response to contractile-mediated muscle damage that triggers muscle remodeling. Specifically, elevations in muscle inflammation in response to acute exercise stress occur with parallel increases in myogenic regulatory factor gene expression to aid in the regeneration of damaged muscle fibers (Raue et al. 1985a,b; Chen et al. 2007). TWEAK and its receptor, Fn14, may be the link between muscle inflammation and regeneration (Girgenrath et al. 2006), whereby TWEAK stimulates myoblast proliferation

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and Fn14 is required for myoblast differentiation into myofibers (Girgenrath et al. 2006; Dogra et al. 2007b). Adult muscle regeneration that is mediated by myogenic regulatory factors, a form of nonhypertrophic remodeling, results in the repair or replacement of damaged muscle fibers (Tedesco et al. 2010; Burd and De Lisio 2017). This process of regeneration is critical for the maintenance of muscle mass. Raue et al. (1985a) showed that a 30 min treadmill run at 75% of max $\dot{V}O_2$ (i.e., ~3.4 L/min, ~506 kcal/30 min) elicits an 8.5 to 12-fold increase in Fn14 gene expression 12–24 h postexercise. In the current study, exercise was prohibited for at least 36 h prior to the muscle biopsy, but on the other days at HA participants were required to complete multiple bouts (Bamman et al. 2015; Burd and De Lisio 2017; Berryman et al. 2018) of aerobic-type exercise to elicit an EIEE of approximately 670 kcal/day. The observed increase in Fn14 and associated myogenic regulatory factors following 21 day of energy deficit at HA was likely a residual and/or cumulative response to repeated exercise bouts performed at least 36 h before muscle samples were collected. Our findings suggest that magnitude of muscle inflammation observed, whether due to chronic exposure to HA and severe underfeeding or multiple bouts of daily aerobic exercise, may benefit muscle maintenance by favoring regeneration.

Based on a previous study by Bamman et al. (2015), we used regression analysis and subsequent dichotomization to explore whether the interindividual variability in muscle inflammation dictated the magnitude of FFM loss in response to energy deficit at HA. In the Bamman study (Bamman et al. 2015), patients undergoing elective total hip arthroplasty were dichotomized based on perioperative Fn14 gene expression in the gluteal muscle of the surgical limb, and deemed either susceptible (high Fn14 expression relative to controls) or not susceptible (low Fn14 gene expression relative to controls) to excessive muscle inflammation. Patients with high Fn14 gene expression had lower muscle protein synthesis rates and higher NF-κB activity and MURF1 gene expression compared to patients with lower Fn14 gene expression (Bamman et al. 2015). Bamman et al. (2015) concluded that elevated Fn14 gene expression may be a prognostic indicator of poor recovery from surgery. Accordingly, we expected that the muscle inflammatory response to 21 days of energy deficit at HA would be detrimental, such that those with an elevated muscle inflammatory response would lose more FFM than participants exhibiting minimal changes in muscle inflammation. After regression analysis confirmed Fn14 was the strongest predictor of FFM loss, participant dichotomization into HIGH and LOW Fn14 demonstrated that those classified as HIGH lost significantly less FFM and more FM than those classified as LOW. We suspect that the apparent beneficial effect of increased muscle inflammation, particularly Fn14, in the current study was due to the upregulation in myogenic regulatory factor gene expression. This is highlighted by the finding that MYOGENIN gene expression in response to chronic HA and concomitant energy deficit was greater in HIGH than LOW. These results, although in contrast to our original hypothesis, are in accordance with studies that show muscle inflammation in response to routine exercise is not overtly catabolic and aids in the repair, remodeling, and maintenance of healthy muscle tissue (Raue et al. 1985a,b; Warren et al. 2002; Karalaki et al. 2009).

In conclusion, 21 days of HA exposure with concomitant energy deficit, achieved with high levels of aerobic-type activity and dietary restriction, upregulated muscle inflammation. The modest upregulation in muscle inflammation occurred without any change in muscle proteolysis. However, myogenic regulatory factor gene expression was upregulated. Participants that exhibited a greater increase in Fn14 gene expression during chronic HA and energy deficit exposure had greater expression of the myogenic regulatory factor MYOGENIN and lost less FFM than participants who had a less robust inflammatory response. These data suggest a heightened muscle inflammatory response to physiological (i.e., exercise and diet restriction) and environmental stress promotes a myogenic response that may protect muscle mass by aiding in muscle regeneration.

Acknowledgments

The authors thank the volunteers that participated in this study. The authors acknowledge our medical oversight team and Dr. Stephen Muza for their support of this study. Most importantly, we wish to sincerely thank Mr. Adam Luippold, Mr. Bradley Anderson, Mr. Grant Holmes, Mr. Anthony Karis, Mr. Matthew Kominsky, Ms. Katelyn Guerriere, Ms. Karleigh Bradbury, Ms. Adrienne Hatch, Ms. Laura Lutz, Drs. Stephen R. Hennigar, Taylor Roth, J. Philip Karl, Scott J Montain, Robert W. Kenefick, and LTC Renee Cole from the U.S. Army Research Institute of Environmental Medicine for their significant contributions to the project.

Conflict of Interest

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these

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organizations. The authors declare that they have no conflicts of interest.

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ORIGINAL RESEARCH
published: 09 July 2018
doi: 10.3389/fphys.2018.00830

Altitude Acclimatization Alleviates the Hypoxia-Induced Suppression of Exogenous Glucose Oxidation During Steady-State Aerobic Exercise

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Edited by:
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Laval University, Canada

Reviewed by:
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *15 February 2018*
Accepted: *12 June 2018*
Published: *09 July 2018*

Citation:
Young AJ, Berryman CE, Kenefick RW, Derosier AN, Margolis LM, Wilson MA, Carrigan CT, Murphy NE, Carbone JW, Rood JC and Pasiakos SM (2018) Altitude Acclimatization Alleviates the Hypoxia-Induced Suppression of Exogenous Glucose Oxidation During Steady-State Aerobic Exercise. Front. Physiol. 9:830. doi: 10.3389/fphys.2018.00830

This study investigated how high-altitude (HA, 4300 m) acclimatization affected exogenous glucose oxidation during aerobic exercise. Sea-level (SL) residents (n = 14 men) performed 80-min, metabolically matched exercise ($\dot{V}O_2 \sim 1.7$ L/min) at SL and at HA < 5 h after arrival (acute HA, AHA) and following 22-d of HA acclimatization (chronic HA, CHA). During HA acclimatization, participants sustained a controlled negative energy balance (−40%) to simulate the "real world" conditions that lowlanders typically experience during HA sojourns. During exercise, participants consumed carbohydrate (CHO, n = 8, 65.25 g fructose + 79.75 g glucose, 1.8 g carbohydrate/min) or placebo (PLA, n = 6). Total carbohydrate oxidation was determined by indirect calorimetry and exogenous glucose oxidation by tracer technique with ^{13}C. Participants lost ($P \leq 0.05$, mean ± SD) 7.9 ± 1.9 kg body mass during the HA acclimatization and energy deficit period. In CHO, total exogenous glucose oxidized during the final 40 min of exercise was lower ($P < 0.01$) at AHA (7.4 ± 3.7 g) than SL (15.3 ± 2.2 g) and CHA (12.4 ± 2.3 g), but there were no differences between SL and CHA. Blood glucose and insulin increased ($P \leq 0.05$) during the first 20 min of exercise in CHO, but not PLA. In CHO, glucose declined to pre-exercise concentrations as exercise continued at SL, but remained elevated ($P \leq 0.05$) throughout exercise at AHA and CHA. Insulin increased during exercise in CHO, but the increase was greater ($P \leq 0.05$) at AHA than at SL and CHA, which did not differ. Thus, while acute hypoxia suppressed exogenous glucose oxidation during steady-state aerobic exercise, that hypoxic suppression is alleviated following altitude acclimatization and concomitant negative energy balance.

Keywords: high-altitude, substrate metabolism, carbohydrate supplementation, aerobic exercise, insulin

INTRODUCTION

Ingesting carbohydrate during endurance exercise at sea level (SL) increases exogenous carbohydrate oxidation and limits endogenous carbohydrate utilization (Jeukendrup et al., 1997; Jeukendrup, 2004, 2008). Only two studies have investigated how hypoxia affects oxidation of exogenous carbohydrate ingested during exercise, and the observations reported from those studies differ (Peronnet et al., 2006; O'Hara et al., 2017). When carbohydrate was ingested during exercise at 77% $\dot{V}O_{2max}$ in normoxia and hypoxia equivalent to 4,300 m, exogenous carbohydrate oxidation was the same (Peronnet et al., 2006). In contrast, when carbohydrate was ingested during lower intensity exercise (55% W_{max}, ∼55–60% $\dot{V}O_{2max}$), exogenous carbohydrate oxidation was lower at high-altitude (HA; 3,375 m) than normoxia (O'Hara et al., 2017). The reasons for the discrepant effects of hypoxia observed in these studies on exogenous carbohydrate oxidation during exercise are not clear, but might reflect differences in the exercise intensities employed.

Hypoxia reduces $\dot{V}O_{2max}$ (Young et al., 1985; Young and Reeves, 1998), so in the experiments described above (Peronnet et al., 2006; O'Hara et al., 2017), exercise was performed at lower absolute intensity and metabolic rate in hypoxia so as to maintain relative exercise intensity (i.e., % $\dot{V}O_{2max}$) the same as it was in normoxia. However, from a scientific (Brooks et al., 1991; Roberts et al., 1996) and practical perspective (the metabolic rate during physical activities is the same at SL and HA, notwithstanding differences in relative intensity, i.e., % $\dot{V}O_{2max}$), we believe that differences in substrate metabolism during exercise at HA and SL should be investigated at the same absolute intensity and metabolic rate in both conditions. In one of the aforementioned studies, Peronnet et al. (2006) also compared exogenous carbohydrate oxidation during exercise performed at the same absolute intensity and metabolic rate (i.e., $\dot{V}O_2$ = ∼2.2 L/min, 11.6 kcal/min) in normoxia (54% $\dot{V}O_{2max}$) and hypoxia equivalent to 4,300 m (78% $\dot{V}O_{2max}$), and reported that exogenous carbohydrate oxidation was again the same in hypoxia as normoxia, despite increases in both total and endogenous carbohydrate oxidation in hypoxia. Maximal exogenous carbohydrate oxidation rates are achieved between 51 and 64% of $\dot{V}O_{2max}$ (Pirnay et al., 1982). Therefore, in that experiment (Peronnet et al., 2006), it is possible that exogenous carbohydrate oxidation was already maximal during the normoxic exercise trials and could not increase any further during the hypoxic exercise. Whether oxidation rate of exogenous carbohydrate ingested would differ during exercise performed at the same absolute intensity in normoxic and hypoxic conditions at exercise intensities eliciting less than maximal exogenous carbohydrate oxidation rates at HA has not been determined. In addition, previous investigations of the effect of hypoxia on exogenous carbohydrate oxidation during exercise have studied unacclimatized lowlanders (Peronnet et al., 2006; O'Hara et al., 2017), and how altitude acclimatization affects exogenous carbohydrate oxidation during exercise is not known.

Sojourners at HA experience both anorexia and increased total daily energy expenditure (TDEE) (Westerterp et al., 1994; Butterfield, 1999). The resultant negative energy balance and associated loss of body mass during HA sojourns lasting longer than several days (Butterfield et al., 1992), could also influence changes in substrate metabolism developing during HA acclimatization. While imposing strict dietary controls can attenuate daily energy deficits and partially mitigate weight loss (Butterfield et al., 1992), most individuals fail to consume sufficient food to maintain energy balance during HA sojourns and, thus, experience weight loss (Rose et al., 1988; Hoyt et al., 1994). As such, the metabolic effects of putative nutrition interventions for HA sojourns should be evaluated in response to the "real world" conditions that lowlanders typically experience during prolonged HA sojourns (i.e., negative energy balance).

Thus, the objective this study was to examine how the oxidation of exogenous carbohydrate by lowlanders performing metabolically matched, steady-state aerobic exercise was affected by acute HA (AHA) exposure (<5 h) and 22 days of HA acclimatization and negative energy balance. In our experiments, exercise was performed at an intensity selected to elicit less than maximal rates of exogenous carbohydrate oxidation (Pirnay et al., 1982). We hypothesized that exogenous carbohydrate oxidation in lowlanders consuming carbohydrate during exercise in hypoxic conditions would be the same or lower during acute hypoxic exposure compared to exercise at SL, and that exogenous carbohydrate oxidation would increase with HA acclimatization.

MATERIALS AND METHODS

Participants

Participants in this study were part of a larger randomized, controlled study that assessed the effects of standard (1.0 g/kg/d) versus higher (2.0 g/kg/d) protein diets on body composition in response to 21 days of negative energy balance at HA (Berryman et al., 2018). In brief, 17 recreationally active (programmed physical activity 2–4 d/week) men, aged 18–42 years who were not acclimatized to HA (i.e., born at altitudes < 2,100 m, currently residing at altitudes < 1,200 m, and who had not traveled to altitudes > 1,200 m for 5 days or more within 2 months before beginning the study) completed that parent study (Berryman et al., 2018). To address whether AHA exposure, and acclimatization to HA, affected exogenous glucose oxidation during aerobic steady-state exercise, participants assigned to the standard (n = 8) and higher (n = 9) protein diets were further randomized to groups provided equal volumes of flavor-matched carbohydrate (CHO; n = 9, 4 standard and 5 higher protein) and placebo (PLA; n = 8, 4 standard and 4 higher protein) drinks during exercise. During data analysis, it was discovered that ambient conditions were inaccurately entered for one of the metabolic carts used for testing three participants during one of the substrate oxidation experiments, so all data for those participants were excluded from the analysis. Data from the remaining 14 participants (CHO; n = 8, 3 standard and 5 higher protein; and PLA; n = 6, 4 standard and 2 higher protein) are presented in this report. All volunteers provided written informed consent prior to participation. This study was approved by the Institutional Review Board at

the United States Army Research Institute of Environmental Medicine (USARIEM, Natick, MA, United States) and registered at www.clinicaltrials.gov as NCT02731066. Investigators adhered to the policies for protection of human subjects as prescribed in the US Department of Defense Instruction 3216.02, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Experimental Design and Diet

This 43 consecutive day controlled study included three distinct phases; 21 days of energy balance at sea level (SL, 1–21), 1 day of AHA exposure (day 1), and 21 days of energy deficit at HA (days 2–22) (**Figure 1A**). Substrate oxidation responses to CHO and PLA ingestion during steady-state exercise were assessed beginning at 10:15–11:15 h at SL day 7, AHA, and on day 22 of chronic HA exposure (CHA). During SL, Registered Dietitians instructed (i.e., what foods to eat, how much, what foods to avoid, etc.) participants to follow a weight-maintaining diet providing 1.0 g protein/kg/d, 45–65% total energy from carbohydrate, and 20–35% total energy from fat. The protein intake level was chosen based on mean *ad libitum* protein intakes in active duty military personnel (Margolis et al., 2012). TDEE was estimated from the sum of (a) measured resting metabolic rate (multiplied by 1.3 to account for activities of daily living and diet-induced thermogenesis) and (b) exercise-induced energy expenditure (EIEE) estimated from corresponding metabolic equivalents of activities reported in the 3 days physical activity log (Ainsworth et al., 2011; Berryman et al., 2018). Those TDEE estimates were then averaged with estimated TDEE requirements calculated using the Harris–Benedict equation (Roza and Shizgal, 1984). Participants were asked to maintain their self-reported exercise routine for the duration of SL testing, although participants were prohibited from performing any exercise within 24 h of the substrate oxidation experiments. Compliance with diet and activity instructions were verified using 24 h diet records, activity recalls, and by measuring body mass daily, upon waking (overnight fast, $\geq$8 h), and after voiding, to the nearest 0.1 kg using a calibrated digital scale (Befour model PS6600; Befour Inc., Saukville, WI, United States).

On day 21, participants were flown to Denver, CO (1,630 m) and transported by research staff to Colorado Springs, CO (1,840 m), where they stayed overnight, under supervision, in a local hotel before being transported ($\sim$03:00 h) to the USARIEM Maher Memorial Altitude Laboratory on the summit of Pikes Peak, CO (4,300 m, day 22). Participants breathed supplemental oxygen (portable O_2 concentrators, FreeStyle 5, AirSep, Buffalo, NY, United States or EverGo, Respironics, Murrysville, PA, United States) after landing in Denver until they arrived at the summit about 13-h later, so as to maintain normoxic oxygen saturation (mean $\pm$ SD for peripheral oxygen saturation [finger pulse oximetry, Nonin, Model 9560, Plymouth, MN, United States] measured hourly from arrival until 22:00 h and every 30 min beginning at 03:00 h was 97.0 $\pm$ 0.9%, range 95–99). This allowed the initiation of hypoxic exposure before experimental testing began to be standardized among all of the participants ($\sim$315 min). Participants did not breathe supplemental oxygen until they arrived in Denver, and thus experienced a mild, 4-h hypoxic exposure equivalent to $\sim$1,900 m (Hampson et al., 2013) during the 4-h flight from Boston. Starting the morning following the AHA experiment, participants were randomized and consumed either the standard or higher protein [amount consistent with recommendations for strenuous military operations, (Pasiakos et al., 2013a)] diet for the next 21 days (HA days 2–22). The carbohydrate content of the diets was fixed at 45% of total energy consumed so as to avoid confounding effects of variations in daily carbohydrate intake on substrate oxidation during exercise during the CHA trial. The remainder of the energy consumed was derived from fat. For HA, Registered Dietitians created diets based on individualized energy requirements to maintain body mass at SL, consisting primarily of components of the military combat ration, Meal, Ready-to-Eat (MRE; menu 34; AmeriQual, Evansville, IN, United States). The remainder of the energy was derived from perishable foods and whey protein drinks (Isopure® Zero Carb), as previously described (Berryman et al., 2018). All foods and caloric drinks were weighed to the nearest 0.1 g, and preparation and consumption of all meals and snacks were supervised by trained research staff. Non-caloric, non-caffeinated drinks were allowed *ad libitum*.

Daily energy intake during the HA phase was reduced by 30% relative to estimated TDEE required to maintain stable body mass at SL, and EIEE was increased 10% above SL TDEE [i.e., HA EIEE = SL EIEE + (0.1 $\times$ SL TDEE)] (Berryman et al., 2018). This was done to elicit an energy deficit consistent with our previous work (Pasiakos et al., 2013b, 2014) and equivalent to the deficit commonly experienced by Soldiers subsisting solely on military rations during extended-duration combat operations and training exercises (Tharion et al., 2005; Margolis et al., 2014). Exercise sessions included low to moderate intensity treadmill walking, running, and outdoor hiking, all supervised and documented by research personnel to ensure safety and compliance with the exercise prescription. The amount of physical activity necessary to elicit the desired daily EIEE during altitude acclimatization was individually prescribed, and determined using the Pandolf Equation for level and uphill walking (Pandolf et al., 1977), adjusted as necessary for downhill walking (Santee et al., 2003). Changes in body mass and composition during HA acclimatization phase are presented and discussed in detail separately (Berryman et al., 2018). In this report, only body mass changes will be presented so as to provide perspective for the controlled energy deficit.

Peak Oxygen Uptake

Peak oxygen uptake ($\dot{V}O_{2peak}$) was assessed during a progressive-intensity, treadmill (Trackmaster TMX425C, Newton, KS, United States) running exercise test on day 0 at SL using an indirect, open circuit respiratory system (True Max 2400, Parvo Medics, Sandy, UT, United States, calibrated to ambient pressure, temperature and humidity prevailing at each $\dot{V}O_{2peak}$ test). The $\dot{V}O_{2peak}$ test also was done once at HA (day 26, fourth full day at HA) to evaluate changes in $\dot{V}O_{2peak}$ from SL. Participants fasted overnight ($\geq$8 h) before testing. For each participant, the same metabolic cart was used for respiratory exchange measurements during both $\dot{V}O_{2peak}$ tests.

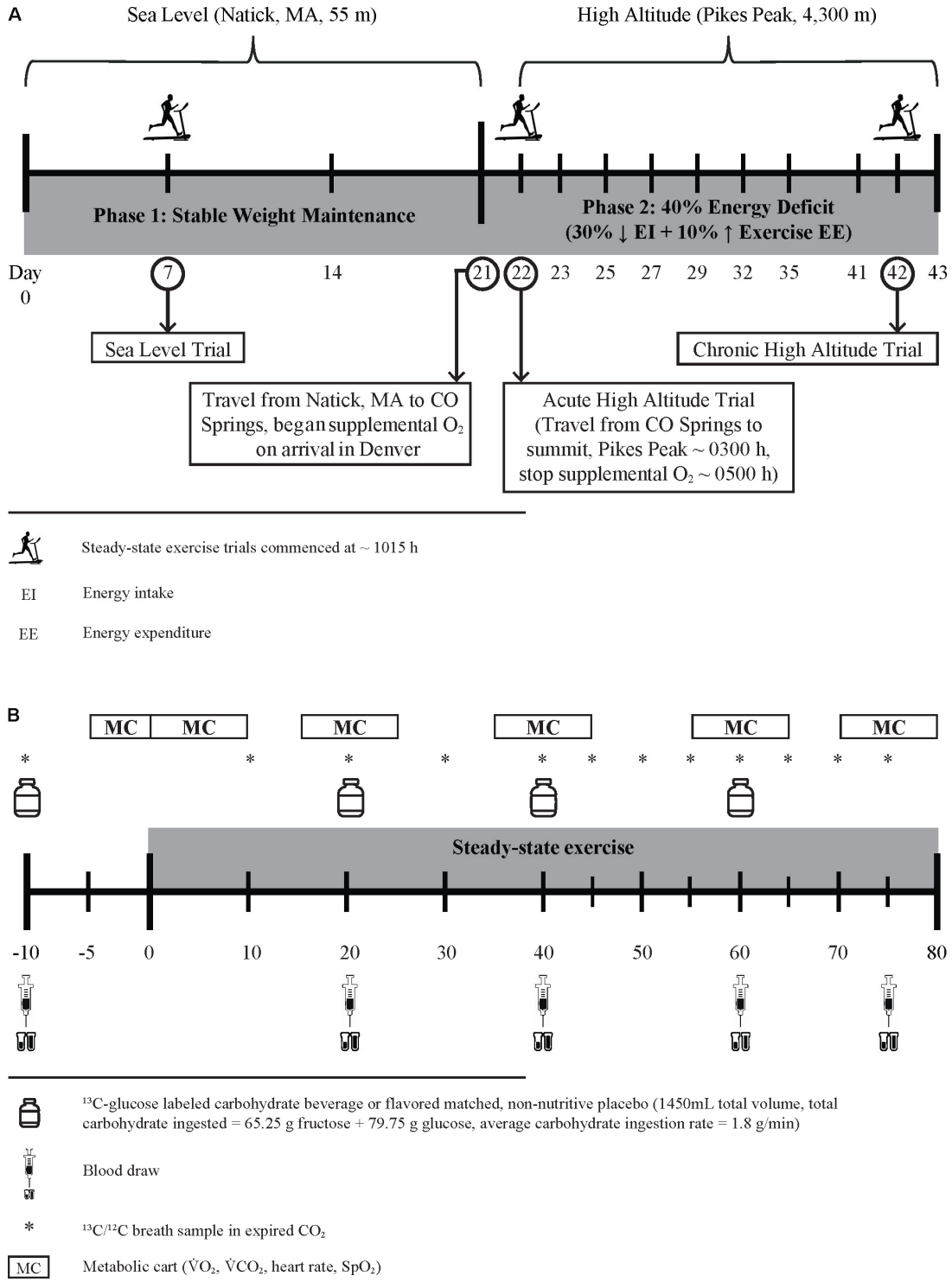


FIGURE 1 | Experimental design schematic **(A)** and protocol for assessment of substrate oxidation and blood metabolite changes during steady-state exercise **(B)**.

Steady-State Exercise Protocol

Substrate oxidation during steady-state exercise (treadmill walking) was assessed following the same protocol (**Figure 1B**) at SL, AHA, and CHA. Participants completed all three of the steady-state exercise tests following a 15-h fast, and all three exercise tests were performed at the same absolute $\dot{V}O_2$. The absolute $\dot{V}O_2$ (i.e., L/min) for the exercise experiments was selected to correspond to ∼55% of each individual's absolute

$\dot{V}O_{2peak}$ at 4,300 m. Since the study schedule/design precluded measuring absolute $\dot{V}O_{2peak}$ at 4,300 m before the participants completed the steady-state exercise test at SL, the absolute $\dot{V}O_{2peak}$ at SL was used to establish the desired absolute $\dot{V}O_2$ at which participants would exercise during the three steady-state exercise experiments. To calculate the desired absolute $\dot{V}O_2$ for these tests, we assumed that absolute $\dot{V}O_{2peak}$ at 4,300 m would be 27% lower than absolute $\dot{V}O_{2peak}$ at SL (Young et al., 1985), so the target absolute $\dot{V}O_2$ for the steady-state exercise experiments was calculated as follows: target absolute $\dot{V}O_2$ = 0.55 × (0.73 × absolute $\dot{V}O_{2peak}$ at SL). The absolute $\dot{V}O_{2peak}$ measured on the fourth full day at HA was used to confirm the % of absolute $\dot{V}O_{2peak}$ at which each participant exercised during the steady-state exercise experiments at AHA and CHA. The ACSM equations (American College of Sports Medicine, 2014) were used to predict the treadmill speed likely to elicit that target $\dot{V}O_2$ while walking at a 2% grade. During the first week of phase 1 (SL days 1–6) and twice during phase 2 (HA days 7 and 14), participants completed familiarization trials using the same steady-state exercise protocol described below, except that blood and breath samples were not collected. Initially, they walked at a 2% grade and at the speed that had been predicted to elicit the target absolute $\dot{V}O_2$. Respiratory exchange measurements during these walks were examined to determine whether the absolute $\dot{V}O_2$ during walking was within ±5% of the target absolute $\dot{V}O_2$, and if necessary, speed and/or grade adjustments were made during the first 20 min of exercise to ensure the desired absolute $\dot{V}O_2$ was elicited.

Participants arrived at the laboratory at 05:00 h, voided, were weighed and had catheters placed in superficial forearm veins. They then sat semi-recumbent for ∼315 min while being infused with stable-isotope labeled amino acids for assessment of post-absorptive whole-body protein turnover in conjunction with other concurrent studies described elsewhere (Berryman et al., 2018). Approximately 20 min before the steady-state exercise experiment was scheduled to begin, the participants arose, voided their bladder (which started the timed urine collection for measurement of nitrogen excretion), and then stood quietly on the treadmill. Resting blood and breath samples were obtained 10 min before exercise, and then participants ingested 550 mL of the CHO or PLA drink. The participants then began walking for 80 min at a speed and grade selected to elicit the target $\dot{V}O_2$. Participants consumed 300 mL of the carbohydrate or placebo drink after 20, 40, and 60 min of walking. Blood samples were obtained at 20, 40, 60, and 75 min of walking. Breath samples were collected every 10 min during the first 40 min of walking, and every 5 min during the last 40 min. Respiratory gas exchange (indirect calorimetry, True Max 2400, Parvo Medics, Sandy, UT, United States, calibrated to ambient pressure, temperature and humidity prevailing at each steady-state exercise experiment) was measured during the 5 min before the participants began walking, during the first 5 min of walking, and for 5 min at 20, 40, 60, and 75 min of walking. For each participant, the same metabolic cart was used for respiratory exchange measurements during all three of the steady-state exercise experiments. Rate of energy expenditure (kcal/min) was calculated from respiratory exchange measurements as described by Jeukendrup and Wallis (2005):

$$\text{Energy expenditure rate} = (0.575 \times \dot{V}CO_2) + (4.435 \times \dot{V}O_2),$$

where $\dot{V}CO_2$ and $\dot{V}O_2$ are in L/min. Immediately after completing the 80th min of steady-state exercise, participants voided their bladder (end timed urine collection period).

The carbohydrate drink consumed by CHO during the steady-state exercise trials contained corn-derived, crystalline fructose (KRYSTAR® 300, Tate and Lyle Sugars, London, United Kingdom) and glucose (STALEYDEX® 333, Tate and Lyle Sugars). During the 80 min steady-state exercise bouts, 1,450 mL total volume and 145 g total carbohydrate (62.25 g fructose + 79.75 g glucose for a 0.8 fructose-to-glucose ratio) were ingested, achieving an average carbohydrate ingestion rate of 1.8 g/min. We chose this beverage formulation so as to be consistent with current recommendations for the consumption of multiple transportable carbohydrates during endurance exercise (Jeukendrup, 2014). The natural ^{13}C enrichment of the combined fructose-glucose drink was −11.03‰ ^{13}C versus the internationally accepted standard for carbon isotope measures, Vienna Pee Dee Belemnite (VPDB), respectively. The carbohydrate drink was enriched with 200 mg U-^{13}C-glucose (Cambridge Isotope Laboratories Inc., Tewksbury, MA, United States) to achieve a final enrichment of the glucose in the beverage of 213.5‰ δ ^{13}C versus VPDB. This high ^{13}C enrichment of the ingested glucose provides a strong signal in expired CO_2, which negates the potential confounding effects of relatively small changes in background enrichment of CO_2 observed from rest to exercise based on the experimental, Western-based diet (Gautier et al., 1993), and the relatively small contribution of the ingested naturally enriched fructose that is oxidized. Due to regulatory constraints on use of radioactive compounds in the Pikes Peak laboratory (located in a US National Park), we could not label one of the carbohydrates with ^{13}C and the other with a radio isotope (i.e., ^{14}C) to independently quantify glucose and fructose oxidation during the HA sojourn. Therefore, our measurements of $^{13}CO_2$ in expired air only reflect the oxidation of the ingested glucose, and our data are reported as exogenous glucose oxidation rate.

Measurement of Substrate Oxidation During Steady-State Exercise

Carbohydrate, fat, and protein oxidation rates were calculated from $\dot{V}O_2$ (L/min), $\dot{V}CO_2$ (L/min), and urinary nitrogen excretion rate (g/min) during the 80-min exercise bout as described by Jeukendrup and Wallis (2005):

$$\text{Carbohydrate (g/min)} = (4.344 \times \dot{V}CO_2) - (3.061 \times \dot{V}O_2) - 0.40 \times \text{urinary nitrogen};$$

$$\text{Fat (g/min)} = (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2) - 1.77 \times \text{urinary nitrogen};$$

$$\text{Protein (g/min)} = 6.25 \times [\text{urine nitrogen excreted (g)}/\text{urine collection duration}].$$

Calculation of protein oxidation assumed that nitrogen lost as sweat urea was negligible. The natural enrichment of the fructose-glucose ingested and the $^{13}C/^{12}C$ in expired gas samples were analyzed using isotope-ratio mass spectroscopy (Metabolic Solutions, Inc., Nashua, NH, United States). The isotopic enrichments were expressed in ‰ difference by comparison with VPDB (see **Figure 2** for expired $\delta\ ^{13}CO_2$ versus VPDB during each endurance exercise substrate utilization protocol) and used to calculate exogenous glucose oxidation rate as follows:

$$\text{Exogenous glucose oxidation (g/min)} = \dot{V}CO_2\,[(R_{exp} - R_{ref})/(R_{exo} - R_{ref})]/k,$$

where $\dot{V}CO_2$ is in L/min, R_{exp} is the observed isotopic composition of expired CO_2, R_{ref} is the isotopic composition of expired CO_2 at rest before ingestion of the first dose of ^{13}C-glucose, R_{exo} is the isotopic composition of the exogenous glucose ingested. The volume of CO_2 provided by the complete oxidation of glucose is 0.747 L/g (k) (Trimmer et al., 2001; Ruzzin et al., 2003). Subtracting exogenous glucose oxidation from total carbohydrate oxidation yielded the amount of "other" carbohydrates oxidized. For PLA, the "other" carbohydrate oxidized was entirely comprised of endogenous carbohydrate (i.e., glucose provided by muscle and liver glycogen stores) oxidation. For CHO, the "other" carbohydrate oxidized was comprised of endogenous carbohydrate oxidation and exogenous fructose oxidation, the latter of which was not captured by the tracer techniques employed in the CHO group. These calculations assume that, in response to exercise, ^{13}C labeled glucose is not irreversibly lost in pools of tricarboxylic acid cycle intermediates (Ruzzin et al., 2003) and/or bicarbonate (Trimmer et al., 2001) allowing for near complete $^{13}CO_2$ recovery in expired gases. Due to the slow equilibration of the $^{13}C/^{12}C$ in expired CO_2 (i.e., mouth) with $^{13}C/^{12}C$ in tissue derived CO_2 (Pallikarakis et al., 1991), and to account for transient effects of hyperventilation (Jones and Jurkowski, 1979), only data and samples from the final 40 min of the steady-state exercise test were used to calculate expenditure and substrate oxidation (rates, g/min, and total amounts in 40 min, g).

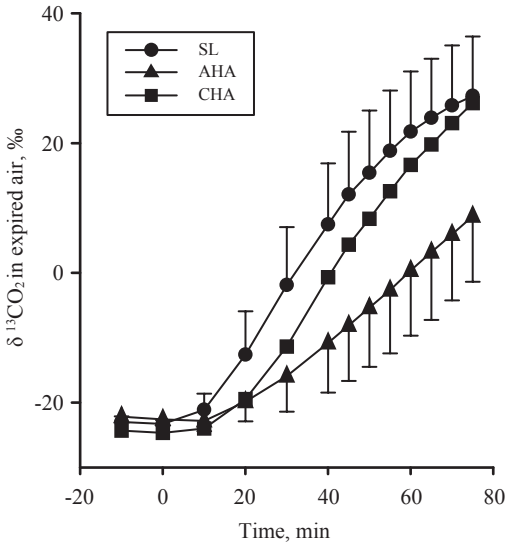


FIGURE 2 | The expired $\delta\ ^{13}CO_2$ versus VPDB in participants provided 1.8 g/min carbohydrate (CHO, *n* = 8, 65.25 g fructose + 79.75 g glucose, closed symbols, during 80-min steady-state exercise (treadmill walking) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA).

Blood and Urine Analyses

Serum glucose, free-fatty acids, glycerol, and plasma lactate concentrations were determined using enzymatic and colorimetric measurements (Beckman Coulter DXC 600 Pro, Beckman Coulter, Brea, CA, United States). Serum insulin concentrations were determined using an advanced automated immunoassay instrument (Immulite® 2000: Siemens Healthcare Diagnostic, Deerfield, IL, United States). Glucose and insulin concentrations measured in the blood sample before the participants ingested the initial bolus of carbohydrate or placebo beverages and began exercising were used to calculate homeostasis model assessment of insulin resistance (HOMA-IR). Total urine volume produced during the timed collection was measured, and aliquots were frozen and stored at −20°C until analyzed. Nitrogen concentration of the urine was determined in triplicate using pyrochemiluminescence (Antek 9000, Houston, TX, United States), and nitrogen excretion was calculated by multiplying nitrogen concentration by volume of urine produced divided by collection duration.

Statistical Analyses

As mentioned previously, this study was part of a larger investigation (Berryman et al., 2018) that was powered to detect body composition differences between standard and higher protein diets during concomitant altitude acclimatization and negative energy balance. However, eight participants per drink group (CHO vs. PLA) provided 80% power to detect differences in the rate of exogenous glucose oxidation with an expected mean difference of 0.25 g/min between treatment groups and between phases, assuming an alpha of 0.05 and a standard deviation of 0.17 g/min (Jeukendrup et al., 2006). We did not expect dietary protein level to affect substrate oxidation and blood measures at CHA (neither SL nor AHA were subject to the standard versus higher protein diet intervention). We confirmed that this was the case using linear mixed models with subject ID as a random effect, and dietary protein group, treatment (CHO, PLA), phase (SL, AHA, CHA), exercise time point, and their interactions as fixed effects. No significant effects of dietary protein group were found for any outcome except plasma lactate, for which a significant diet-by-treatment interaction was observed; however, the diet-by-treatment-by-phase interaction was not significant. Thus, the differences between dietary protein groups persisted across all phases, not just CHA, and as such are not a consequence of the dietary protein intervention, *per se*. Therefore, effects of the

dietary protein intervention are not presented in this report, and the data were re-analyzed without dietary protein group in the model.

Linear mixed models were used to test the effects of treatment, phase, exercise time point, and their interactions on substrate oxidation and blood measures. Subject ID was treated as a random effect, and treatment, phase and time as fixed effects as appropriate per study outcome. These outcomes were modeled as doubly repeated measures with unstructured by unstructured or unstructured by autoregressive symmetry (based on lowest Bayesian information criterion) for phase and time point, respectively. Linear mixed models were also used to test the effects of treatment, phase and their interactions on cumulative substrate oxidation measures (i.e., no time effects). In this model, subject ID was treated as a random effect and treatment and phase as fixed effects. *Post hoc* analyses were adjusted for multiple comparisons using Bonferroni corrections. The alpha level for statistical significance is set at $P \leq 0.05$. Data is reported as means ± SD unless otherwise specified. Statistical analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Participant Characteristics

The participants were recreationally active (average $\dot{V}O_{2peak}$ = 51.7 ± 7.4 mL/min/kg), non-obese (BMI = 26 ± 3; body mass = 82.8 ± 14.0 kg), young (age = 24 ± 6 years) men (**Table 1**). Dietary intakes during the sea level and high-altitude phases did not differ between groups (**Table 2**), with the exception of relative carbohydrate intake (CHO: 3.1 ± 0.3; PLA: 2.7 ± 0.2 g/kg/d, $P < 0.05$) and contribution of total energy derived from carbohydrate (CHO: 48 ± 1; PLA: 45 ± 1%, $P < 0.05$), which was attributable to ingestion of the carbohydrate beverage (145 g carbohydrate) during the CHA substrate oxidation experiment on day 22 at HA, and two additional practice trials (no substrate oxidation or blood measures) on days 7 and 14 at HA. Daily EIEE associated with individually prescribed exercise during the HA phase were the same for both groups, averaging 690 ± 178 kcal/d for the CHO and 695 ± 185 kcal/d for PLA.

TABLE 1 | Detailed listing of physical characteristics of participants.

	CHO (n = 8)	PLA (n = 6)
Characteristic		
Age (years)	22.2 ± 2.0	26.6 ± 8.5
Height (cm)	178 ± 9	176 ± 6
Weight (kg)	82.3 ± 12.8	82.9 ± 16.6
BMI (kg/m^2)	25.9 ± 3.0	26.7 ± 4.1
Body fat (%)	22.4 ± 5.4	25.0 ± 7.0
$\dot{V}O_{2peak}$ (L/min) at SL	4.34 ± 0.74	4.06 ± 0.70
$\dot{V}O_{2peak}$ (L/min) at HA	2.81 ± 0.38	2.75 ± 0.62

Values are mean ± SD; peak O_2 uptake ($\dot{V}O_{2peak}$) at sea level (SL) and 4,300 m (HA). CHO participants ingested 1.8 g carbohydrate/min (65.25 g fructose + 79.75 g glucose) and PLA participants ingested placebo during steady-state exercise trials.

TABLE 2 | Detailed dietary intake for participants.

	SL, weight maintenance		HA, energy deficit	
	CHO	PLA	CHO	PLA
Absolute intake (kcal/d or g/d)				
Energy	2535 ± 485	2295 ± 333	2002 ± 230	1856 ± 231
Protein	92 ± 18	83 ± 18	134 ± 48	108 ± 31
Carbohydrate	339 ± 80	293 ± 62	238 ± 28	207 ± 26
Fat	92 ± 23	90 ± 17	62 ± 20	71 ± 20
Relative intake (kcal/kg body mass/d or g/kg body mass/d)				
Energy	31.5 ± 7.1	28.4 ± 6.5	26.1 ± 2.4	23.9 ± 2.3
Protein	1.2 ± 0.4	1.0 ± 0.1	1.7 ± 0.5	1.4 ± 0.5
Carbohydrate	4.2 ± 1.1	3.7 ± 0.3	3.1 ± 0.3	2.7 ± 0.2*
Fat	1.1 ± 0.3	1.1 ± 0.2	0.8 ± 0.3	0.9 ± 0.2
Percent energy intake (%)				
Protein	15 ± 2	15 ± 4	27 ± 9	23 ± 7
Carbohydrate	53 ± 5	51 ± 7	48 ± 1	45 ± 1*
Fat	32 ± 3	35 ± 4	28 ± 9	34 ± 8

*Values are mean ± SD for absolute (kcal/d, g/d), relative (kcal/kg body mass/d, g/kg body mass/d), and percent (%) energy intake; CHO participants (n = 8) ingested 1.8 g carbohydrate/min (65.25 g fructose + 79.75 g glucose) and PLA participants (n = 6) ingested placebo during steady-state exercise trials at sea level (SL) and high-altitude (HA). Participants in the CHO and PLA groups were further randomized to standard (1.0 g/kg/d) and higher (2.0 g/kg/d) protein diets beginning on day 2 at HA. Protein intake did not influence any study outcome; thus, dietary data are presented for CHO and PLA only. *Different ($P < 0.05$) from CHO during HA.*

Participants were weight stable during SL (day 1: 82.8 ± 14.0 kg and day 20: 83.3 ± 14.5 kg, ns), and lost ($P < 0.05$) 7.9 ± 1.9 kg of total body mass over the 22-day period of HA acclimatization and energy deficit. The participants exhibited an average 34% decrease in $\dot{V}O_{2peak}$ at HA compared to SL. No differences were observed between treatments.

Respiratory Gas Exchange During Steady-State Exercise

Resting peripheral blood oxygen saturation averaged 98.2 ± 1.3%, 85.3 ± 8.3%, and 89.5 ± 3.0% before exercise at SL, AHA, and CHA, respectively. There were no differences in $\dot{V}O_2$, $\dot{V}CO_2$ or energy expenditure measurements at the 40, 60, and 75 min of walking during any of the trials, indicating that the participants were in steady-state. There were no differences in $\dot{V}O_2$ or energy expenditure (**Table 3**) between treatment groups or phases. Average $\dot{V}O_2$ during the last 40 min of walking corresponded to 40% of $\dot{V}O_{2peak}$ at SL and 60% of $\dot{V}O_{2peak}$ at AHA and CHA. There were no differences in $\dot{V}CO_2$ (**Table 3**) between CHO and PLA, but $\dot{V}CO_2$ was lower at CHA than SL ($P < 0.05$).

Substrate Oxidation During Steady-State Exercise

Total carbohydrate oxidation was greater ($P < 0.05$) in CHO than PLA for all three trials (**Table 4**). Total carbohydrate oxidation (g/40 min) was lower ($P < 0.05$) at CHA than at SL and AHA. For CHO, total exogenous glucose oxidation was less at AHA

TABLE 3 | Ambient testing conditions during steady-state experiments, exercise intensity, respiratory gas exchange and energy expended during final 40 min of steady-state exercise.

	SL		AHA		CHA	
	CHO	**PLA**	**CHO**	**PLA**	**CHO**	**PLA**
Pressure* (mmHg)	761 ± 3	759 ± 2	459 ± 3	458 ± 2	459 ± 2	458 ± 2
Temperature (°C)	21.8 ± 0.9	21.8 ± 1.2	21.5 ± 2.3	19.8 ± 2.9	22.4 ± 2.0	21.8 ± 1.2
Humidity* (%)	50.3 ± 9.2	45.0 ± 7.8	34.4 ± 9.2	38.4 ± 5.0	32.9 ± 6.9	35.6 ± 5.1
Speed* (m/s)	1.8 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.2
Grade (%)	2.1 ± 0.4	1.8 ± 0.4	1.7 ± 0.8	1.8 ± 0.4	1.8 ± 0.5	1.9 ± 0.5
$\dot{V}O_2$ (L/min)	1.77 ± 0.27	1.63 ± 0.28	1.68 ± 0.30	1.62 ± 0.25	1.71 ± 0.23	1.63 ± 0.27
$\dot{V}CO_2$* (L/min)	1.64 ± 0.28	1.42 ± 0.29	1.53 ± 0.31	1.39 ± 0.26	1.47 ± 0.20	1.31 ± 0.22
RER*†	0.93 ± 0.05	0.86 ± 0.06	0.91 ± 0.04	0.85 ± 0.04	0.86 ± 0.4	0.81 ± 0.08
Energy (kcal)	353 ± 51	322 ± 54	342 ± 62	324 ± 47	343 ± 46	318 ± 50

*Values are mean ± SD of ambient pressure, temperature, and humidity during experimental trials, walking speed, oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), respiratory exchange ratio (RER), and total energy expenditure (energy) during final 40 min of 80 min steady-state exercise (treadmill walking) performed at sea level (SL, 55 m), at 4,300 m altitude ∼5 h after arrival (acute high-altitude, AHA), and on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA) of participants who ingested 1.8 g carbohydrate/min (CHO, n = 8, 65.25 g fructose + 79.75 g glucose) or non-nutritive placebo (PLA, n = 6) during exercise. *Main effect (P < 0.05) of phase for pressure (SL > AHA and CHA), humidity (SL > AHA and CHA), walking speed (AHA < SL), $\dot{V}CO_2$ (CHA < SL), and RER (CHA < SL). †Main effect of treatment for RER (CHO > PLA).*

TABLE 4 | Total substrate oxidized during final 40 min of steady-state exercise.

	SL		AHA		CHA	
	CHO	**PLA**	**CHO**	**PLA**	**CHO**	**PLA**
Carbohydrate (g) glucose						
Total*†	68.3 ± 19.8	45.9 ± 21.4	59.7 ± 18.9	42.9 ± 15.7	45.5 ± 13.3	28.2 ± 17.9
Exogenous glucose†	15.3 ± 2.2	–	7.4 ± 3.7	–	12.4 ± 2.3	–
Other†	53.0 ± 20.1	45.9 ± 21.4	52.3 ± 16.9	42.9 ± 15.7	33.0 ± 12.6	28.2 ± 17.9
Fat*† (g)	7.5 ± 3.5	13.6 ± 6.6	9.1 ± 3.9	14.7 ± 3.5	15.2 ± 6.2	20.5 ± 8.5
Protein* (g)	3.1 ± 0.3	2.6 ± 0.8	3.1 ± 1.2	2.2 ± 0.3	3.4 ± 1.4	2.5 ± 0.4

*Values are mean ± SD of total (g) substrate oxidized during final 40 min of 80 min steady-state exercise (treadmill walking) performed at sea level (SL), at 4,300 m altitude ∼5 h after arrival (acute high-altitude, AHA), and on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA) of participants who ingested 1.8 g carbohydrate/min (CHO, n = 8, 65.25 g fructose + 79.75 g glucose) or placebo (PLA, n = 6) during exercise. "Other" carbohydrate oxidation was calculated by subtracting exogenous glucose oxidation from total carbohydrate oxidation, and for PLA, was entirely comprised of endogenous carbohydrate (i.e., glucose provided by muscle and liver glycogen stores) oxidation, but for CHO, was comprised of endogenous carbohydrate oxidation and exogenous fructose oxidation, the latter of which was not captured by the tracer techniques employed in the CHO group. *Main effect (P < 0.05) of treatment on total carbohydrate (CHO > PLA), fat (CHO < PLA) and protein (CHO > PLA) oxidation; †main effect (P < 0.05) of phase on total (CHA < SL, AHA) and other carbohydrate oxidation (CHA < SL, AHA), exogenous glucose oxidation (AHA < SL, CHA), and fat oxidation (CHA > SL, AHA).*

than at SL and CHA. The rate of exogenous glucose oxidation (g/min) in CHO (**Figure 3**) increased similarly over the final 40 min of each exercise trial across phases (main effect of time, $P < 0.05$). Exogenous glucose oxidation rate for CHO differed between all phases, being highest at SL, lowest at AHA and intermediate at CHA (main effect of phase, $P < 0.05$). Total other (as previously defined in Section "Materials and Methods") carbohydrate oxidation (g/40 min, **Table 4**) was not different between CHO and PLA, but was lower ($P < 0.05$) at CHA than SL and AHA. Fat oxidation (g/40 min, **Table 4**) was greater ($P < 0.05$) in PLA than CHO during all three phases, and was greater at CHA than SL or AHA ($P < 0.05$). Protein oxidation was greater ($P < 0.05$) in CHO than PLA, but did not differ between SL, AHA, and CHA. No other treatment or phase effects were observed.

The contribution of protein oxidation to total energy production during exercise did not differ between groups or phases and averaged 3.4 ± 0.9%, overall (**Figure 4**). Total carbohydrate oxidation accounted for a greater fraction of total energy production in CHO than PLA for all phases ($P < 0.05$), and carbohydrate oxidation contributed less to total energy production at CHA than SL ($P < 0.05$). In the CHO, exogenous glucose oxidation accounted for 18 ± 4% of total energy production at SL, but at AHA exogenous glucose oxidation accounted for much less ($P < 0.05$), only 9 ± 4% of total energy production. At CHA, the contribution of exogenous glucose oxidation to total energy production in CHO was 15 ± 3%, which was greater than at AHA ($P < 0.05$) and not different from SL. The contribution from oxidation of other carbohydrates to total energy production did not differ between groups, but was less during exercise at CHA than for both SL and AHA ($P < 0.05$). Total fat oxidation accounted for a greater fraction of total energy production in PLA than CHO for all phases ($P < 0.05$), and fat oxidation

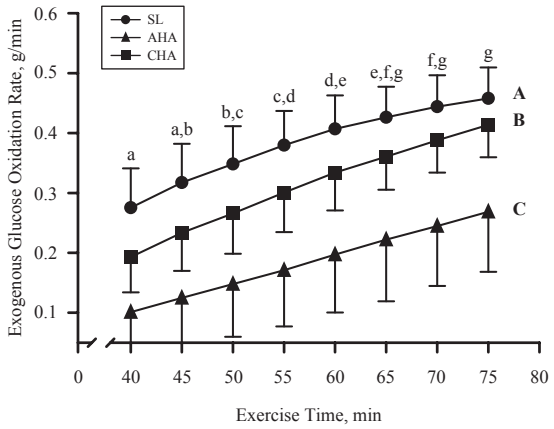


FIGURE 3 | Mean ± SD of exogenous glucose oxidation rate (g/min) during last 40 min of 80-min steady-state exercise (treadmill walking) in participants provided 1.8 g/min carbohydrate (CHO, n = 8, 65.25 g fructose + 79.75 g glucose) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA). Points not sharing same letter are different (lower case = main effect of time, $P < 0.05$; upper case = main effect of phase, $P < 0.05$).

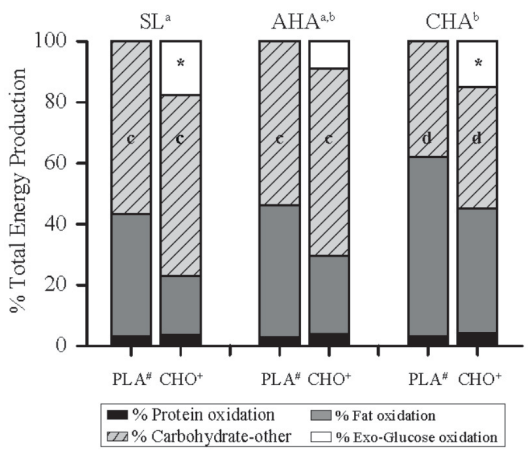


FIGURE 4 | The percentage contribution from oxidation of different energy substrates to total energy production during the last 40 min of 80-min steady-state exercise (treadmill walking) in participants provided 1.8 g/min carbohydrate (CHO, n = 8, 65.25 g fructose + 79.75 g glucose) or non-nutritive placebo (PLA, n = 6) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA). For PLA, "other" carbohydrate oxidation is entirely endogenous carbohydrate (i.e., glucose provided by muscle and liver glycogen) oxidation, but for CHO, "other" carbohydrate is endogenous carbohydrate oxidation and exogenous fructose oxidation, the latter of which was not captured by the tracer techniques employed in the CHO group. [a,b]Contribution from oxidation of fat was greater and from carbohydrate (exogenous glucose + other) less at CHA than at SL, but neither differed from AHA (main effect of phase, $P < 0.05$). *In CHO, contribution of exogenous carbohydrate oxidation was less at AHA than at SL or CHA, which did not differ (main effect of phase, $P < 0.05$). [c,d]Contribution from oxidation other carbohydrates at SL and AHA did not differ, but was greater than at CHA. #Contribution of fat oxidation greater in PLA than CHO during all trials (main effect of treatment, $P < 0.05$). +Contribution of carbohydrate (exogenous glucose + other carbohydrate) oxidation was greater in CHO than PLA during all trials (main effect of treatment, $P < 0.05$).

contributed more to total energy production at CHA than SL ($P < 0.05$).

Changes in Blood Metabolites During Steady-State Exercise

Serum glucose concentrations (**Figures 5A,B**) before exercise and beverage consumption did not differ between groups or phases. In PLA, glucose concentrations remained unchanged throughout the exercise bouts, and did not differ between phases. In contrast, glucose concentrations in CHO increased ($P < 0.05$) during the first 20 min of exercise in all three phases. By 60 min of exercise at SL, glucose concentrations in CHO had returned to pre-exercise levels, whereas glucose concentrations remained higher ($P < 0.05$) than pre-exercise levels throughout exercise at AHA and CHA. Serum insulin concentrations (**Figures 5C,D**) before exercise and beverage consumption were lower ($P < 0.05$) at CHA compared to AHA in CHO but not PLA. Insulin levels remained unchanged during exercise in PLA. The increase in insulin during exercise in CHO was more pronounced at AHA than at SL or CHA, with the differences achieving significance ($P < 0.05$) by 60 min. HOMA-IR did not differ between groups at any phase, and averaged (all participants) 1.04 ± 0.73 at SL, increasing ($P < 0.05$) to 1.82 ± 1.46 at AHA and then decreasing ($P < 0.05$) to 0.70 ± 0.34, which was not different from SL.

Serum free fatty acid concentrations (**Figures 5E,F**) before exercise and beverage consumption did not differ between groups or phases. However, during exercise, free fatty acid concentrations progressively increased ($P < 0.05$) in PLA, but progressively decreased ($P < 0.05$) in CHO. By 40 min of exercise and thereafter, free fatty acid concentrations were higher ($P < 0.05$) in PLA than CHO. In both CHO and PLA, free fatty acid concentrations were higher at AHA than SL, ($P < 0.05$) and higher at CHA than AHA ($P < 0.05$). Serum glycerol concentrations (**Figures 5G,H**) before exercise and beverage consumption were on average higher (main effect of treatment, $P < 0.05$) in CHO than PLA. Glycerol concentrations in PLA rose progressively through 60 min of exercise, and remained plateaued thereafter, but glycerol concentrations in CHO remained unchanged throughout exercise. In both groups, glycerol concentrations during exercise were highest at CHA, intermediate at AHA, and lowest at SL ($P < 0.05$).

Plasma lactate concentrations (**Figures 6A,B**) before exercise and beverage consumption did not differ between groups, and were higher at AHA than SL ($P < 0.05$). Lactate concentrations increased in both groups during the first 20 min of exercise; in CHO, lactate concentrations remained higher than the resting value for the remainder of the exercise, but in PLA, lactate concentrations had declined after 40 min and were no longer different from resting values. In both groups, lactate concentrations rose higher during exercise at AHA than at SL or CHA. Lactate concentrations during exercise at CHA were higher than at SL after 60 min of exercise until the end of the bout.

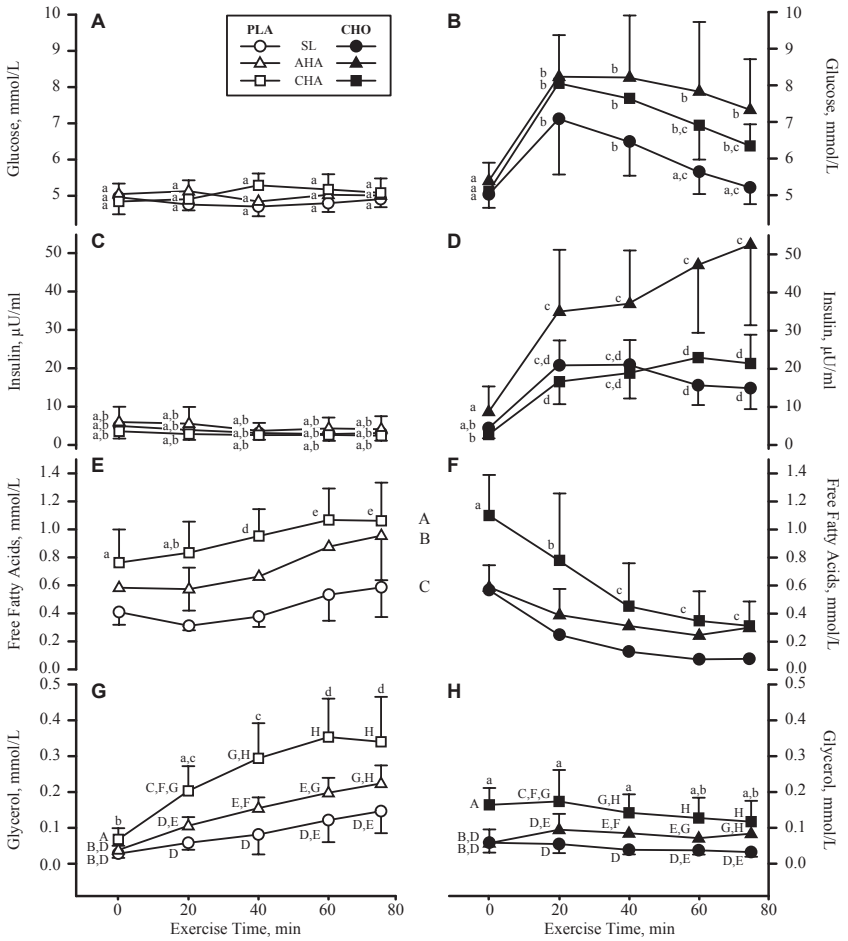


FIGURE 5 | Mean + SD of serum concentrations of glucose (mmol/L, **A,B**), insulin (μU/mL, **C,D**), free fatty acids (mmol/L, **E,F**) and glycerol (mmol/L, **G,H**) concentrations in participants provided non-nutritive placebo (PLA, $n = 6$, open symbols, left column of panels) or 1.8 g/min carbohydrate (CHO, $n = 8$, 65.25 g fructose + 79.75 g glucose, closed symbols, right column of panels) during 80-min steady-state exercise (treadmill walking) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA). For glucose and insulin, points not sharing the same lower case letter are different (treatment by phase by time interaction, $P < 0.05$ for both metabolites). For free fatty acids, time points not sharing the same lower case letter are different (treatment by time interaction, $P < 0.05$), and phases not sharing same upper case letter are different (main effect of phase, $P < 0.05$), no interactions). For glycerol, time points not sharing the same lower case letter are different (treatment by time interaction, $P < 0.05$) and time points not sharing the same upper case letter are different (time by phase interaction, $P < 0.05$).

DISCUSSION

This study investigated the effects of hypoxia, before and after development of altitude acclimatization, on oxidation of exogenous glucose ingested during steady-state exercise in healthy, young SL residents sojourning at HA while in negative energy balance. We demonstrated that exogenous glucose oxidation during metabolically matched exercise is suppressed by acute hypoxia compared to normoxic conditions, and that 21 days of HA acclimatization alleviates this hypoxic-suppression. Furthermore, the effects of acute and chronic hypoxia on blood glucose and insulin responses to exercise and carbohydrate ingestion that we observed raise the possibility that the concomitant changes in exogenous glucose oxidation may be related to adjustments in glycemic regulation.

In our CHO participants, 15.3 g of exogenous glucose were oxidized over the final 40 min of treadmill exercise at SL, but during exercise at same absolute metabolic rate at AHA 7.4 g of exogenous glucose was oxidized, over 52% less than at SL. Following 21 days of HA acclimatization, this hypoxic suppression of exogenous glucose oxidation was alleviated, and 12.4 g of exogenous glucose was oxidized, not different from that measured during metabolically matched exercise at SL. If we assume that fructose oxidation rate was 48% of the measured glucose oxidation rate (Jentjens et al., 2004; Rowlands et al., 2008), a reasonable estimate of exogenous carbohydrate oxidization in final 40 min of SL exercise would be 23 g. That is less than the exogenous carbohydrate oxidation rate measured by O'Hara et al. (2017) in participants exercising at SL and consuming a similar glucose and fructose mixture as the one our participants consumed. However, participants in that other

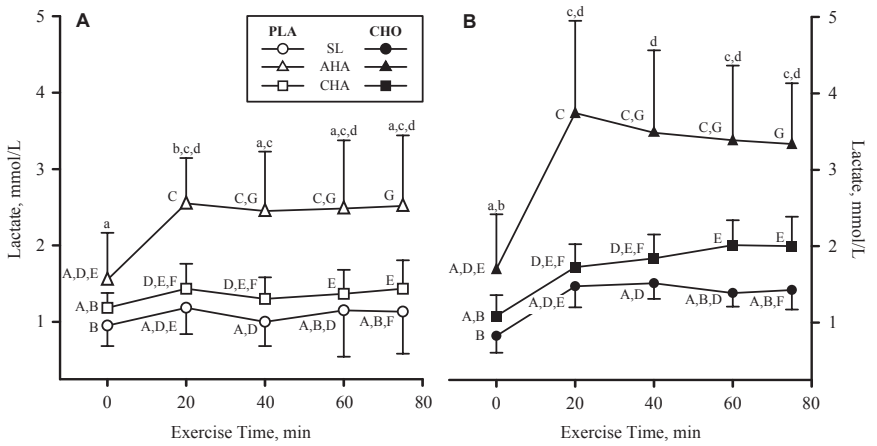


FIGURE 6 | Mean + SD of plasma lactate concentrations in participants provided non-nutritive placebo (PLA, $n = 6$, open symbols, **(A)** or 1.8 g/min carbohydrate (CHO, $n = 8$, 65.25 g fructose + 79.75 g glucose, closed symbols, **(B)** during 80-min steady-state exercise (treadmill walking) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHA). Time points not sharing the same lower case letter are different (treatment by time interaction, $P < 0.05$) and time points not sharing the same upper case letter are different (time by phase interaction, $P < 0.05$).

study exercised at a substantially higher metabolic rate, 13 kcal/m, than our participants, who exercised at 9 kcal/min. The higher exercise intensity (Pirnay et al., 1982) used in that investigation would account, at least in part, for the higher exogenous carbohydrate oxidation rates compared to our study. Regardless of those differences, the finding that the hypoxic suppression of exogenous glucose oxidation was alleviated following 21 days of HA acclimatization, and exogenous glucose oxidation rates had returned to levels not different from those measured during similar exercise at SL, has to our knowledge not been previously reported.

It was surprising that total amount of carbohydrate oxidized was the same at AHA as SL in both groups, given that several previous studies of altitude effects on substrate utilization during exercise (Brooks et al., 1991; Roberts et al., 1996; Lundby and Van Hall, 2002; O'Hara et al., 2017) have reported that carbohydrate utilization increases during exercise at HA compared to exercise at SL. We speculate that the differences between our observations and previous reports may not only reflect differences in exercise intensity, but also differences in the exercise mode. Previous studies that observed increased carbohydrate utilization at HA compared to sea level employed cycling exercise, whereas in our study, participants walked on a treadmill. Even in the studies in which cycling exercise was performed at very similar rates of whole-body energy expenditure as in ours (Brooks et al., 1991; Roberts et al., 1996; Lundby and Van Hall, 2002), that energy expenditure is distributed over a smaller mass of muscle during cycling exercise than during walking. The higher metabolic rate maintained by the smaller volume of actively contracting muscle fibers during cycling exercise as compared to walking is likely to elicit higher rates of carbohydrate oxidation during exercise, and that effect is likely to be even greater at HA when the relative intensity of exercise ($\dot{V}O_{2max}$) is increased compared to at SL.

Acute hypoxia alters glycemic regulation, in particular insulin sensitivity is decreased (Larsen et al., 1997; Braun et al., 2001; Oltmanns et al., 2004; Peltonen et al., 2012), whereas chronic hypoxia (altitude acclimatization) at least partially alleviates that effect (Brooks et al., 1991; Larsen et al., 1997; Braun et al., 1998), and those changes in insulin sensitivity modulate changes in blood glucose homeostasis. The changes in HOMA-IR exhibited by both PLA and CHO during altitude sojourn, and the blood glucose and insulin responses observed in CHO and PLA groups during hypoxic exercise before and after HA acclimatization are consistent with those reported effects of hypoxia on glycemic regulation.

Ingestion of exogenous carbohydrate is well documented to increase the contribution of carbohydrate oxidation and decrease the contribution of fat oxidation to total energy production during exercise at SL (Jentjens et al., 2004), and our observations during exercise at HA are consistent with that. However, since fat oxidation contributed more to total energy production during exercise at AHA in PLA than CHA, the question might arise as to why the suppression of exogenous glucose oxidation in CHO during exercise at AHA was not offset by an increased contribution of fat oxidation. During SL exercise, hyperglycemia and hyperinsulinemia have been shown to inhibit lipolysis and mobilization of fatty acids, as well as oxidation of intramuscular fatty acids and triglycerides (Coyle et al., 1997). Our observations suggest a similar effect at HA, in that circulating free fatty acids concentrations in CHO declined during exercise both at SL and at HA, but increased in PLA. Thus, during exercise under conditions of acute hypoxia, ingesting exogenous carbohydrate provides less of an advantage in terms of sparing endogenous glucose stores from oxidation, since exogenous glucose oxidation is suppressed. Further, the resulting high levels of blood glucose and insulin likely inhibit fat oxidation, forcing energy requirements to be achieved with greater reliance

on endogenous carbohydrate oxidation. During exercise at CHA, not only was the suppression of exogenous glucose oxidation alleviated, but the lower blood glucose and insulin concentrations during exercise appears to have enabled a greater contribution of fat oxidation to total energy production in CHO as well as PLA, compared to SL and AHA.

Certain aspects of our experimental approach should be considered to appropriately interpret our findings in the context of previous research. Our sample size, albeit sufficient to test our hypothesis, can be considered small, which may limit the extension of our findings. Further, our participants maintained negative energy balance and lost ∼8 kg body mass over the 21-day HA acclimatization phase. Since the SL and AHA experiments had already been completed before negative energy balance began, those experiments were not affected by the energy deficit. Including fully fed, energy balanced control groups in the design was logistically unfeasible and outside the scope of our study (i.e., test effects of AHA exposure and energy deficit during HA acclimatization), so we cannot rule out the possibility that body mass and fat loss affected the metabolic responses observed during the CHA exercise tests. Thus, our conclusion that altitude acclimatization alleviates the blunting of exogenous glucose oxidation produced by acute hypoxia requires confirmation in fully fed participants maintaining stable body weight during HA acclimatization. Another consideration is that we enriched the carbohydrate beverage with ^{13}C-glucose, but did not enrich the fructose component. Therefore, we could not measure total exogenous carbohydrate oxidation, but only exogenous glucose oxidation.

In summary, our study demonstrated that exogenous glucose oxidation during exercise is suppressed by acute hypoxia, but after 21 days of altitude acclimatization and energy deficit, that suppression is alleviated. Whether altitude acclimatization alleviates hypoxic suppression of exogenous glucose oxidation in fully fed sojourners maintaining stable body weight remains to be demonstrated. From a practical perspective, our observations suggest that traditional recommendations for consuming carbohydrate during exercise may not have the same effects on substrate oxidation during exercise on the first few days of a HA sojourn as during exercise at SL, but following a period of HA acclimatization, metabolic effects of consuming exogenous carbohydrate during exercise may normalize.

AUTHOR CONTRIBUTIONS

AY, CB, and SP conceived the study question and designed the experiments. AY, CB, RK, AD, LM, MW, CC, NM, JC, JR, and SP performed the experiments. CB, AD, and CC analyzed the data. AY, CB, RK, and SP interpreted the data. AY, CB, and SP prepared the figures and drafted the manuscript. All authors edited and revised the manuscript and approved the final version.

FUNDING

This study was funded by the United States Army Medical Research and Materiel Command.

ACKNOWLEDGMENTS

We thank the volunteers that participated in this research experiment. We acknowledge our medical oversight team and Dr. Stephen Muza for their support of this study. Most importantly, we wish to sincerely thank Mr. Bradley Anderson, Ms. Karleigh Bradbury, Ms. Katelyn Guerriere, Mr. Grant Holmes, Mr. Anthony Karis, Mr. Matthew Kominsky, Ms. Adrienne Hatch, Mr. Adam Luippold, Ms. Laura Lutz, Mr. Phillip Niro, Drs. Steven R. Hennigar, Taylor Roth, Arny A. Ferrando, J. Philip Karl, Scott J. Montain, and LTC Renee Cole from the United States Army Research Institute of Environmental Medicine for their significant contributions to the project.

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Am J Physiol Gastrointest Liver Physiol 315: G1003–G1015, 2018.
First published September 13, 2018; doi:10.1152/ajpgi.00253.2018.

RESEARCH ARTICLE | *Microbiome and Host Interactions*

Associations between the gut microbiota and host responses to high altitude

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[1]*Military Nutrition Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts;* [2]*Oak Ridge Institute for Science and Education, Belcamp, Maryland;* [3]*Soldier Performance Optimization Directorate, Natick Soldier Research, Development and Engineering Center, Natick, Massachusetts; and* [4]*Pennington Biomedical Research Center, Baton Rouge, Louisiana*

Submitted 20 July 2018; accepted in final form 8 September 2018

Karl JP, Berryman CE, Young AJ, Radcliffe PN, Branck TA, Pantoja-Feliciano IG, Rood JC, Pasiakos SM. Associations between the gut microbiota and host responses to high altitude. *Am J Physiol Gastrointest Liver Physiol* 315: G1003–G1015, 2018. First published September 13, 2018; doi:10.1152/ajpgi.00253.2018.—Hypobaric hypoxia and dietary protein and fat intakes have been independently associated with an altered gastrointestinal (GI) environment and gut microbiota, but little is known regarding host-gut microbiota interactions at high altitude (HA) and the impact of diet macronutrient composition. This study aimed to determine the effect of dietary protein:fat ratio manipulation on the gut microbiota and GI barrier function during weight loss at high altitude (HA) and to identify associations between the gut microbiota and host responses to HA. Following sea-level (SL) testing, 17 healthy males were transported to HA (4,300 m) and randomly assigned to consume provided standard protein (SP; 1.1 $g \cdot kg^{-1} \cdot day^{-1}$, 39% fat) or higher protein (HP; 2.1 $g \cdot kg^{-1} \cdot day^{-1}$, 23% fat) carbohydrate-matched hypocaloric diets for 22 days. Fecal microbiota composition and metabolites, GI barrier function, GI symptoms, and acute mountain sickness (AMS) severity were measured. Macronutrient intake did not impact fecal microbiota composition, had only transient effects on microbiota metabolites, and had no effect on increases in small intestinal permeability, GI symptoms, and inflammation observed at HA. AMS severity was also unaffected by diet but in exploratory analyses was associated with higher SL-relative abundance of *Prevotella*, a known driver of interindividual variability in human gut microbiota composition, and greater microbiota diversity after AMS onset. Findings suggest that the gut microbiota may contribute to variability in host responses to HA independent of the dietary protein:fat ratio but should be considered preliminary and hypothesis generating due to the small sample size and exploratory nature of analyses associating the fecal microbiota and host responses to HA.

NEW & NOTEWORTHY This study is the first to examine interactions among diet, the gut microbiota, and host responses to weight loss at high altitude (HA). Observed associations among the gut microbiota, weight loss at HA, and acute mountain sickness provide evidence that the microbiota may contribute to variability in host responses to HA. In contrast, dietary protein:fat ratio had only minimal, transient effects on gut microbiota composition and bacterial metabolites which were likely not of clinical consequence.

gut barrier; hypoxia; macronutrient; microbiome; weight loss

INTRODUCTION

The human host and its gut microbiota coexist in a dynamic mutualistic relationship, with the host providing a favorable environment for microbes that, in turn, modulate gastrointestinal (GI) health, GI barrier integrity, immunity, and inflammation (13, 35, 36). However, this relationship can be perturbed by exposure to environmental and physiologic stressors that alter the GI environment, the gut microbiota, or both (38, 49). Consequences can include degradation of GI barrier integrity leading to GI distress, translocation of bacterial antigens (e.g., LPS) from the gut lumen into circulation, systemic inflammation, and increased susceptibility to illness and infection (73, 74).

Hypobaric hypoxia is a physiologic stressor which characterizes high-altitude (HA; >2,500-m elevation) environments. Exposure to hypobaric hypoxia is associated with increased inflammation (34), increased risk of illness and infection (41, 54), and increased acute mountain sickness (AMS) (7, 55), a constellation of symptoms that includes several GI maladies (4). Rodent studies suggest that host-gut microbiota dynamics could contribute to these responses, demonstrating that exposure to hypobaric hypoxia increases GI inflammation, oxidative stress, and GI permeability concomitant to changes in gut microbiota composition and activity (1, 82, 86, 88). Although few human studies have attempted to corroborate those findings, transient increases in GI permeability (21) and increases in the abundance of proinflammatory gut bacteria (2, 42) in association with inflammation (42) have been reported during mountaineering expeditions. These observations collectively suggest that the gut microbiota may both be affected by and contribute to host responses at HA, but greater characterization of these relationships is needed.

Gut microbiota composition and activity are also modulated by dietary macronutrient intake (19, 28, 57, 80). It is well established that carbohydrate fermentation by the gut microbiota promotes the growth of beneficial bacteria and production of the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate (48, 75), whereas amino acid fermentation produces multiple byproducts including SCFAs, branched short-chain fatty acids (BCFAs; e.g., isovalerate and isobutyrate), and ammonia (48, 75). While SCFAs, and butyrate in particular, are beneficial to GI health, several amino acid fermentation metabolites (e.g., ammonia) may harm the GI barrier (75), suggesting that protein fermentation could be deleterious to GI health (83). At sea level (SL), consuming higher protein diets

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that are also high in fat (>40% total energy), low in carbohydrate (<20% total energy), and low in fiber has been associated with reductions in beneficial gut bacteria and fecal SCFAs and increased fecal concentrations of amino acid fermentation metabolites (12, 19, 24, 25, 57, 65). When macronutrient intakes have been more consistent with dietary recommendations and fiber intakes have been matched between groups, higher protein diets have been shown to increase fecal concentrations of amino acid metabolites without impacting fecal microbiota composition (5, 79) or markers of GI health (5, 8, 79). Thus protein fermentation does not appear to acutely degrade GI barrier function when fiber intake is controlled. However, none of these studies were conducted in environments such as HA that may render the GI barrier more sensitive to gut microbes and their metabolites.

Recent interest in studying higher protein, moderate carbohydrate diets at HA (59) has been driven by the knowledge that unintentional fat-free mass loss is common during HA sojourn (33, 78), and that higher protein diets preserve fat-free mass during weight loss at SL (60, 81). However, the effects of these diets on host-gut microbiota dynamics at HA are unknown. This study aimed to both address that gap and the need for greater characterization of host-gut microbiota dynamics at HA by *1*) determining the effect of altering the dietary protein:fat ratio on GI barrier function, GI symptoms, and gut microbiota composition and metabolites during weight loss at HA; and *2*) identifying associations among the gut microbiota, weight loss at HA, and the host response to HA as measured by AMS severity.

METHODS

Participants and study design. The analyses reported herein were included as secondary objectives in a randomized, controlled feeding study designed to examine the efficacy of a higher protein diet for preserving fat-free mass during HA sojourn (9). Participants were 17 healthy, unacclimatized, physically active men. Although study enrollment was open to women, none volunteered. Participants had no GI abnormalities or disorders, had not taken oral antibiotics or had a colonoscopy in the previous 3 mo, and did not regularly use laxatives, stool softeners, or antidiarrheal medications. The study was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (Natick, MA) and conducted May–Aug 2016. Investigators adhered to the policies for the protection of human participants as prescribed by Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. (The trial is registered on https://clinicaltrials.gov/ as NCT02731066.)

Study methods have been previously reported in detail (9). Briefly, the study was a randomized, controlled trial consisting of two phases conducted over 43 consecutive days. During the 21-day first phase (SL), participants resided at SL, consumed a self-selected weight-maintaining diet, maintained habitual exercise routines, and were free living but visited the laboratory daily. On SL *day 21*, participants were flown from Boston, MA to Denver, CO, where they were placed on supplemental oxygen until being driven to the summit of Pike's Peak, CO (4,300 m) the following morning (HA *day 0*) where they resided for the next 22 days at the U.S. Army Research Institute of Environmental Medicine Maher Memorial Laboratory (*phase 2*; HA). During HA, participants were under constant supervision, consumed a controlled and measured diet, and engaged in prescribed physical activity. Diets contained either a standard or higher amount of protein, and were designed to induce weight loss, which is common during HA sojourn (33). The estimated energy deficit at HA was 70% or 1,849 kcal/day (SD 511) (9).

Study diets. Beginning on HA1, the first full day of residence at 4,300 m, and continuing until they completed the HA phase of the study, participants consumed a controlled diet. Participants were randomized by study staff using computer-generated randomization to consume either a standard protein [SP; 1.1 $g \cdot kg^{-1} \cdot day^{-1}$ (SD 0.2)] or higher protein [HP; 2.1 $g \cdot kg^{-1} \cdot day^{-1}$ (SD 0.2)] diet during HA. Both diets were designed to provide 45% of energy as carbohydrate, while fat intake was reduced in HP to accommodate the higher protein intake (Table 1). Diets were primarily comprised of entrées, sides, and snack items included in U.S. military Meals Ready-to-Eat rations and were supplemented with fresh fruits and vegetables, fruit snacks, olive oil, and ranch-flavored salad dressing. A whey-protein beverage (Isopure Zero Carb; Isopure, Hauppauge, NY) was also provided as appropriate to manipulate protein intake. Water and noncaffeinated sodas were allowed ad libitum.

Questionnaires. Modified versions of the Irritable Bowel Syndrome-Symptom Severity Score (IBS-SSS) Questionnaire (30) and the Gastrointestinal Quality of Life Index (GIQLI) Questionnaire (27)

Table 1. *Baseline participant characteristics, weight loss, and dietary intakes at sea level and over 22 days at high altitude*

	SP		HP	
	SL	HA	SL	HA
Age, yr	23 ± 3		24 ± 7	
Body mass index, kg/m^2	27.0 ± 4.0		25.5 ± 3.1	
Body fat, %	22.8 ± 7.0		22.6 ± 5.5	
$\dot{V}O_{2peak}$, $ml \cdot kg^{-1} \cdot min^{-1}$	49.2 ± 7.0		53.8 ± 7.2	
ΔWeight at HA, ‡ kg		−8.0 ± 2.6		−7.8 ± 1.2
ΔFFM at HA, ‡ kg		−4.0 ± 3.3		−3.2 ± 1.5
Energy intake, † kcal/day	2,366 ± 277	1,950 ± 186	2,418 ± 542	1,885 ± 269
Protein intake, %	16 ± 3	18 ± 2	14 ± 2	33 ± 2*
Protein intake, g/day	94 ± 20	88 ± 14	83 ± 15	154 ± 25*
Carbohydrate intake, %	50 ± 6	46 ± 1	54 ± 5	47 ± 1
Carbohydrate intake, g/day	300 ± 62	223 ± 24	327 ± 80	221 ± 35
Fat intake, %	34 ± 4	39 ± 2	32 ± 4	23 ± 3*
Fat intake, g/day	89 ± 9	84 ± 6	88 ± 7	47 ± 7*
Saturated fat intake, g/day	31 ± 4	26 ± 2	26 ± 7	15 ± 2*
Fiber intake, g/day	18 ± 7	21 ± 3	19 ± 10	18 ± 2*

Values are means ± SD; $n = 8$ from standard-protein diet group (SP) and 9 from higher-protein diet group (HP). SL, sea level (weight maintenance); FFM, fat-free mass; HA, high altitude (4,300 m; energy deficit). Adapted from Berryman et al. (9). *Different from SP during HA, $P < 0.05$. †For all dietary variables independent samples *t*-test were used to compare groups at SL (ad libitum diet) and HA (provided diet); no significant differences during SL. ‡Linear mixed model, main effect of time, $P < 0.001$.

were administered weekly throughout the study. The GIQLI Questionnaire asked participants to subjectively rate the frequency of several GI-related symptoms (e.g., flatulence, constipation, loose stool, cramping, etc.), which were then used to compute an overall GIQLI score (27). AMS was assessed by Lake Louise score calculated from the shortened version of the Environmental Symptoms Questionnaire (6) and categorized as mild ($\geq$0.7 and $<$1.53), moderate ($\geq$1.53 and $<$2.63), and severe ($\geq$2.63) (7).

Blood biochemistries. Blood samples were collected by venipuncture and following a $>$10 h fast to measure markers of gut barrier function, stress, and inflammation. All samples were separated into serum or plasma and stored at -20 to -80°C until analysis. Plasma LPS-binding protein (LBP) was measured on SL *day 7* and HA *days 0*, *7*, and *21* by ELISA according to manufacturer's instructions (Abonva, Taipei, Taiwan). Serum glucagon-like peptide-2 (GLP-2), a gastrointestinal hormone shown to modulate effects of diet-gut microbe interactions on GI permeability (14), was measured on SL *day 7* and HA *days 0*, *7*, and *21* by ELISA according to manufacturer's instructions (EMD Millipore, St. Charles, MO). Serum IL-6 concentrations were measured on SL *day 0* and HA *days 0* and *19* by multiplex assay (Milliplex MAP; EMD Millipore). Serum cortisol concentrations were measured on SL *day 12* and HA *days 2*, *7*, *13*, and *19* by autoimmunoassay (Immulite 2000; Siemens Healthcare Diagnostics, Tarrytown, NY).

Small intestinal permeability. Small intestinal permeability was assessed by quantifying the urinary excretion of orally ingested sugar substitutes (45, 53) on SL *day 12* and HA *days 1* and *18*. Measurements began in the morning with fasted participants consuming 5 g lactulose and 4 g mannitol dissolved in 180 ml of water. All urine produced over the next 5 h was collected. During the 5-h period, participants consumed the same individualized diet (breakfast and morning snack) on all 3 test days, with water allowed ad libitum. Urine aliquots were immediately frozen and stored at -20 to -80°C until analysis. Urine lactulose and mannitol concentrations were measured by HPLC with refractive index detection (Agilent 1100 HPLC, Santa Clara, CA) as previously described (51). Fractional excretion was calculated by multiplying the measured concentration of each probe by the total volume of urine collected and dividing by the dose administered. Small intestinal permeability was then calculated as the ratio of the fractional excretions of lactulose and mannitol (LM ratio) (45, 53).

Fecal sample collection and analysis. Participants provided a single fecal sample during five separate time periods of the study; SL *days 0–4* (SL1) and *16–20* (SL2) and HA *days 1–2* (HA1), *8–11* (HA2), and *18–21* (HA3), to assess fecal microbiota composition and fecal concentrations of SCFAs, BCFAs, and ammonia. All samples were collected into plastic collection containers, immediately refrigerated, and processed within 3 h (SD 4; range: 5 min – 21 h) of production. Aliquots were immediately frozen and stored at -20 to -80°C until analysis.

Fecal metabolites. Fecal SCFA and BCFA concentrations were measured as previously described (61, 87) with minor modifications. Fecal aliquots were thawed immediately before extraction, homogenized in distilled water (1:4 wt/vol), and centrifuged. Samples were then acidified using 50% H_2SO_4 (1:2 wt/vol), and fatty acids were extracted using diethyl ether (2:5 wt/vol). After incubating on ice for 2 min, samples were centrifuged, the organic layer was removed, and ethyl butyric acid was added as an internal standard. Samples were then stored at -80°C until analysis. Acetic acid, propionic acid, butyric acid, isobutyric acid, and isovaleric acid were quantified using an Agilent 7890A GC system with Flame Ionization Detection (60 m $\times$ 250 μm $\times$ 0.25 μm; DB-FFAP, Agilent J&W). Samples (1 μl) were injected by autosampler in triplicate using a split ratio of 10:1. The temperature program started with an initial temperature of 110°C for 2 min, increased 10°C/min up to 180°C, and was then maintained at 180°C for 5 min. The carrier gas was nitrogen with a constant flow of 1 ml/min. Calibration standards were included for each fatty acid, and used for peak identification and quantification.

Fecal ammonia concentrations were measured using a colorimetric assay according to manufacturer instructions (Abcam, Cambridge, MA).

Fecal microbiota composition. DNA was extracted from fecal samples using the MoBio PowerFecal DNA isolation kit (Qiagen, Germantown, MD). Primers designed to amplify the V3-V4 region of the 16S rRNA gene were used for PCR amplification, and all samples were sequenced in triplicate on the Illumina MiSeq platform (Illumina, San Diego, CA). Sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME) v.1.9.1 (16). Read quality assessment, filtering, barcode trimming, and chimera detection were performed on demultiplexed sequences using Trimmomatic (11). Reads were joined in QIIME using a minimum overlap of 32 bp and a maximum percent difference within the overlap of 20%. Operational taxonomic units (OTUs) were assigned by clustering sequence reads at 97% similarity and aligned against the Greengenes database core set v.13_8 (52) using PyNAST (15). Taxonomic assignment was completed using the RDP classifier v.2.2 (77).

Read counts averaged 80,024 reads/sample (SD 61,030; range: 31,267–417,621), and were grouped into 12,966 unique OTUs, which could be assigned to 134 unique genera and 13 unique phyla. For genus-level analyses, any OTUs that could not be assigned to a genus were grouped at the next lowest level of classification possible (e.g., family or order).

Diversity metrics were calculated after rarefaction at 31,267 reads/sample. Within-sample diversity (α-diversity) was calculated in QIIME using the Shannon and observed OTUs diversity metrics. Between-sample diversity (β-diversity) was measured using Bray-Curtis distances calculated using the R packages stats v.3.4.3 and phyloseq v.1.16.2. Ordinations of β-diversity metrics were then completed using principal coordinates analyses (PCoA) and average hierarchical clustering within the R package ape.

Statistical analysis. Data were checked to verify adherence to model assumptions and transformed when necessary to meet model assumptions. Unless otherwise noted, statistical analyses were completed using SPSS v.21, data are presented as mean (SD), statistical significance was set at $P \leq 0.05$, and P values between 0.05 and 0.10 were considered evidence of a trend for an effect.

All study outcomes except for genus-level read counts were analyzed by linear mixed models, generalized linear mixed models, or marginal models as appropriate (see table and figure legends for model specifications). All models accounted for the within-subject correlation and included diet group, time, and their interaction as fixed factors and age and baseline body mass index as covariates. The baseline value of the dependent variable was also included as a covariate in models where the dependent variable was measured at multiple SL time points (questionnaires and fecal outcomes) or if the first measurement at HA was completed immediately after ascent (plasma LBP and serum GLP-2). In all models, if a significant interaction or main effect was observed, post hoc comparisons were conducted using *t*-tests, and *P*-values were adjusted using Bonferroni corrections. Correlations were assessed using Pearson's or Spearman's correlation as appropriate or by including time-varying covariates in linear models.

Bray-Curtis dissimilarities in gut microbiota composition were analyzed using distance-based redundancy analysis in the R package vegan. Unrarefied genus-level read counts were analyzed using DESeq2 v.1.16.1 (47) and R v.3.4.2 to test for effects of diet, time, and their interaction on changes in gut microbiota composition while controlling for individual effects. For these analyses, likelihood ratio tests were used to test for diet-by-time interactions, and, if no interactions were observed, main effects of time. False discovery rate (FDR) was controlled by adjusting P values obtained for diet-by-time interactions and main effects of time using the Benjamini-Hochberg correction. If a significant interaction or main effect of time was

observed (FDR ≤ 0.20), pairwise contrasts were examined for those taxa to identify between group differences or differences over time within the full cohort. *P*-values for pairwise contrasts were adjusted for the number of contrasts using Bonferroni corrections.

To address exploratory aims, participants were dichotomized as "responders" and "nonresponders" to the physiologic stress of HA using Lake Louise scores measured within the first 48 h of ascent (peak scores for all participants occurred within 48 h of ascent as expected; Ref. 7). Moderate or severe AMS (responder) was defined as a peak Lake Louise score of $\geq$1.53, and no or mild AMS (nonresponder) was defined as a peak Lake Louise score of $<$1.53 (6). The AMS-responder group was included in separate marginal or mixed models as an independent variable to examine changes in physiologic outcomes. All models accounted for the within-subject correlation and included diet group, time, and responder group as fixed factors, the responder group-by-time interaction, and age, baseline body mass index, and baseline value of the dependent variable as covariates. For analysis of changes in genus-level read counts over time, the AMS-responder group was substituted for diet group in the DESeq2 models.

Finally, linear discriminant analysis of effect size (LEfSe) (67) was used to identify taxa associated with AMS severity. For these analyses the AMS-responder group was used as the grouping factor (i.e., "class"), time point was used as a subgroup factor (i.e., "subclass"), and relative abundances of taxa measured at SL were included in the analysis. Default analysis parameters were used with the exceptions of reducing the significance threshold to $P < 0.01$ due to the small sample size and conducting pairwise between-group comparisons only within the same time points. As such, discriminant taxa were defined as those with an effect size $>$2.0 between groups ($P < 0.01$) at both SL time points.

RESULTS

Primary and secondary results have been previously reported (9, 37, 50, 58, 84). Total body mass and fat-free mass losses during HA did not differ between diet groups (Table 1). During HA, protein intake was higher in HP relative to SP while fat intake was lower, and total fiber intake was marginally lower (Table 1).

Effects of diet and time on GI symptoms and small intestinal permeability. GIQLI scores were lower (indicating worse symptomology) throughout HA relative to SL, and did not differ by diet group (Fig. 1*A*). IBS symptom severity scores did not differ over time (main effect of time, $P = 0.69$) or by diet group (diet-by-week interaction, $P = 0.15$) (data not shown).

Lactulose excretion was lower on HA *day 18* relative to SL and HA *day 1* while mannitol excretion was higher at SL relative to HA *days 1* and *18* (main effect of time, $P \leq 0.01$, Table 2). The resulting LM ratio was 71% (SD 73) higher on HA *day 1* and 67% (SD 77) higher on HA *day 18* relative to SL (main effect of time, $P < 0.001$) indicating increased small intestinal permeability. Diet had no impact on the LM ratio (Table 2). When urine volume was included as a covariate in the model, the main effect of time for lactulose was no longer significant whereas results for mannitol excretion and the LM ratio were unchanged, the latter observation remaining consistent with increased small intestinal permeability at HA.

Diet had no significant effect on serum IL-6 concentrations; however, an upwards trend was observed over time at HA (Fig. 1*B*). Changes in plasma LBP concentrations over time differed by diet with concentrations initially increasing at HA in HP but then dropping to a concentration 24% lower than that measured in SP on HA *day 21* ($P = 0.02$; Fig. 1*C*). Diet had no effect on serum GLP-2 concentrations (Fig. 1*D*). However, in the full cohort, GLP-2 concentrations were lower on HA *day 7* relative

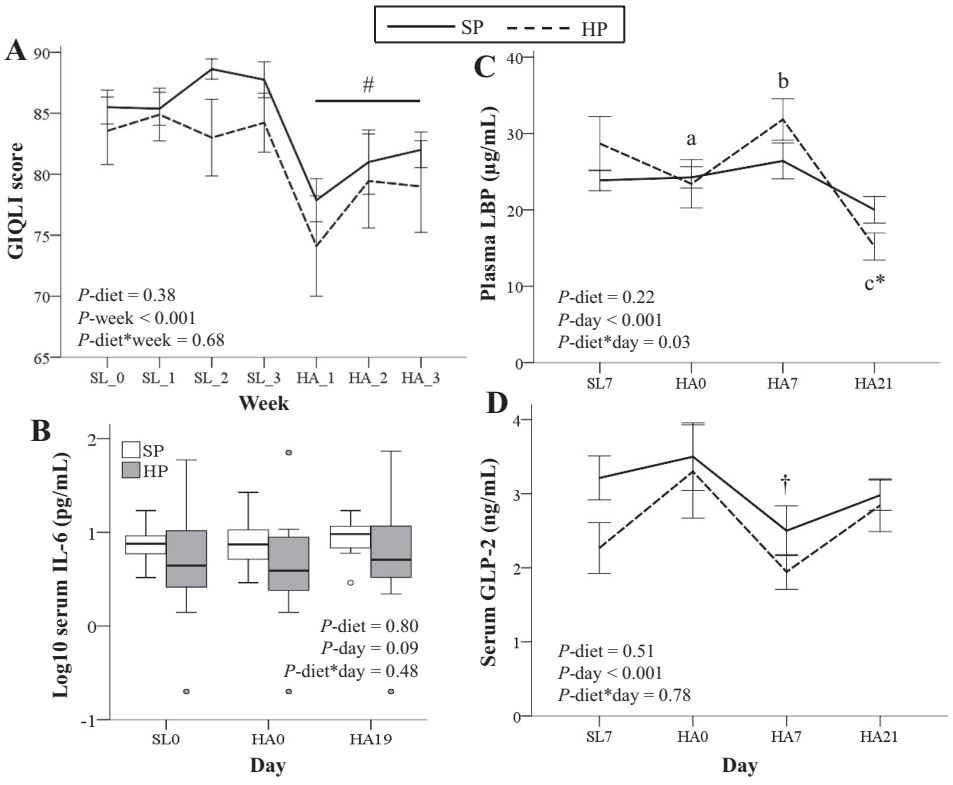


Fig. 1. Changes in gastrointestinal (GI) symptomology, GI function, and inflammation at high altitude (HA; 4,300 m) are largely independent of dietary protein:fat ratio. GI quality of life (GIQLI; lower scores indicate worse symptoms; *A*) and fasting serum IL-6 (*B*), plasma LPS binding protein (LBP; *C*), and serum glucagon-like peptide (GLP)-2 (*D*) concentrations. Data are analyzed by marginal models controlling for age, body mass index, and baseline value. *B* and *C*: data were $\log_{10}$-transformed for analysis. HP, higher protein diet group; SL, sea level; SP, standard-protein diet group. HP, $n = 9$; SP, $n = 8$. Values are means ± SE. Time points not sharing a superscript letter are significantly different within a diet group ($P \leq 0.05$). #Significantly different from SL ($P < 0.05$). *Significantly different from SP ($P = 0.02$). †Significantly different from HA *days 0* and *21* ($P \leq 0.01$).

Table 2. *Small intestinal permeability measured at sea level and high altitude*

			P Value‡		
	SP	HP	Diet	Time	Diet × time
Lactulose,§ %			0.96	0.01	0.35
SL *day 12*	0.15 ± 0.07	0.13 ± 0.04			
HA *day 1*	0.16 ± 0.10	0.15 ± 0.05			
HA *day 18**	0.11 ± 0.10	0.12 ± 0.05			
Mannitol, %			0.41	<0.001	0.76
SL *day 12**	16.2 ± 4.4	17.6 ± 3.0			
HA *day 1*	10.5 ± 2.6	12.8 ± 5.1			
HA *day 18*†	8.6 ± 5.4	9.2 ± 2.6			
LM ratio§			0.47	<0.001	0.55
SL *day 12**	0.010 ± 0.005	0.007 ± 0.002			
HA *day 1*	0.014 ± 0.007	0.013 ± 0.005			
HA *day 18*	0.013 ± 0.007	0.013 ± 0.005			

Values are means ± SD; $n = 8$ from standard-protein diet group (SP) and 9 from higher-protein diet group (HP). SL, sea level (weight maintenance); HA, high altitude (4,300 m; energy deficit). LM, lactulose:mannitol. *Significantly different from the other days ($P < 0.05$). †Trend for significant difference relative to HA *day 1* ($P = 0.08$). ‡Linear mixed model controlling for age and body mass index with Bonferroni corrections. §Log_{10} transformed for analysis.

to HA *days 0* and *21* and were inversely correlated with LBP concentrations over time [β: −2.3 μg/ml (SE 0.9), $P = 0.01$].

Effects of diet and time on fecal metabolite concentrations. Total fecal SCFA concentrations were 39% lower during the second week of HA in HP relative to SP due to lower acetate, propionate and butyrate concentrations in HP but did not differ before or after (Table 3). Total BCFA concentrations trended toward being higher in SP relative to HP independent of diet (Table 3) due to higher mean concentrations of isovalerate (main effect of diet, $P = 0.07$) in SP throughout the study (data not shown). Fecal ammonia concentrations did not differ by diet but were higher during the second and third weeks at HA relative to SL (Table 3).

Effects of diet and time on fecal microbiota composition. At the community level, no metric of α-diversity changed over time or differed by diet (Fig. 2*A*). PCoA (Fig. 2*B*) and hierarchical clustering (data not shown) of Bray-Curtis dissimilarities did not reveal any clustering by diet (diet-by-time interaction, $P = 0.91$). The effects of diet on genus-level fecal microbiota relative abundances were observed for only two taxa. Both changes in the relative abundances of *Holdemania* (phyla: Firmicutes) and unclassified taxa within the *Christensenellaceae* family (phyla: Firmicutes) at HA were greater in HP relative to SP (diet-by-time interaction, $P \leq 0.003$; FDR < 0.20; Fig. 2*C*). However, both taxa demonstrated a trend toward being less abundant in HP relative to SP at SL (LEfSe effect size ≤ 2.6, $P < 0.02$), which implicated regression to the mean rather than an effect of diet. Finally, no between group difference was noted when LEfSe was used to examine differences in fecal microbiota composition measured during the final week of HA ($P > 0.01$; data not shown). Taken together these findings indicated that diet had little impact on fecal microbiota community composition.

PCoA of Bray-Curtis dissimilarities did show evidence of clustering over time (Fig. 2*B*, main effect of time, $P = 0.02$). In support, several genera demonstrated significant changes over time independent of diet (main effect of time, FDR <0.20). Specifically, relative to SL, *Lachnospira* relative abundance was lower on HA days 1–2 [log_2 fold change = −1.5 (SE 0.5); $P = 0.005$] but not thereafter, *Turicibacter* relative abundance was lower during only the second week at HA (Fig. 2*D*), and *Bacteroides* relative abundance trended toward being higher during only the second week at HA [log_2 fold change = 0.9 (SE 0.4); $P = 0.06$]. *Lactococcus, Streptococcus*, and *Lactobacillus* (Fig. 2*D*) relative abundances were all lower relative to SL during the second and third weeks of HA (range

Table 3. *Fecal short-chain fatty acid, branched-chain fatty acid, and ammonia concentrations measured at sea level and high altitude*

			P Value[a]		
	SP	HP	Diet	Time	Diet × time
Acetate, μmol/g wet wt			0.42	0.18	0.08
SL1	35.0 ± 12.1	29.7 ± 14.9			
SL2	37.1 ± 26.5	24.5 ± 8.6			
HA1	26.2 ± 6.9	23.7 ± 6.7			
HA2	28.2 ± 8.9	18.6 ± 10.0[b]			
HA3	20.2 ± 8.7	22.3 ± 2.3			
Propionate, μmol/g wet wt			0.79	0.14	0.03
SL1	15.4 ± 8.1	16.9 ± 9.5			
SL2	14.7 ± 12.8	13.6 ± 5.2			
HA1	12.5 ± 4.9	10.1 ± 4.1			
HA2	14.1 ± 7.9	8.3 ± 4.5[b]			
HA3	7.2 ± 3.7[c]	10.6 ± 3.5			
Butyrate, μmol/g wet wt			0.29	0.07	0.02
SL1	13.6 ± 6.2	13.6 ± 9.2			
SL2	14.1 ± 12.2	9.7 ± 4.2			
HA1	13.0 ± 3.9	11.7 ± 2.3			
HA2	12.8 ± 4.2	6.7 ± 4.8[b]			
HA3	10.4 ± 4.7	10.9 ± 3.4			
Total SCFA, μmol/g wet wt			0.55	0.19	0.04
SL1	64.1 ± 23.8	60.2 ± 30.3			
SL2	66.0 ± 51.0	47.8 ± 16.2			
HA1	51.6 ± 13.4	45.6 ± 9.5			
HA2	55.1 ± 19.3	33.6 ± 18.7[b]			
HA3	37.9 ± 15.2	43.8 ± 6.2			
BCFA, μmol/g wet wt			0.07	0.32	0.29
SL1	3.2 ± 2.2	2.6 ± 1.4			
SL2	3.1 ± 2.7	1.6 ± 1.6			
HA1	3.7 ± 2.9	2.2 ± 1.9			
HA2	3.8 ± 1.7	1.9 ± 1.0			
HA3	1.8 ± 1.2	2.1 ± 1.8			
Ammonia, μmol/g wet wt			0.38	0.001	0.42
SL1	38.0 ± 23.9	24.2 ± 17.9			
SL2	16.1 ± 7.4	18.6 ± 12.3			
HA1	34.2 ± 21.1	23.9 ± 15.1			
HA2[d]	59.1 ± 27.3	29.5 ± 15.5			
HA3[e]	28.9 ± 14.1	28.4 ± 17.0			

Values are means ± SD; $n = 8$ from standard-protein diet group (SP) and 9 from higher protein diet group (HP). SL, sea level (weight maintenance); HA, high altitude (4,300 m; energy deficit); BCFA, branched chain fatty acid (isobutyrate and isovalerate); SCFA, short-chain fatty acid (acetate, propionate, and butyrate). Samples collected on study *days 0–4* (SL1) and *16–20* (SL2) at sea level and *days 1–2* (HA1), *8–11* (HA2), and *18–21* (HA3) at high altitude. [a]Linear mixed model with Bonferroni corrections controlling for age, body mass index, and concentration on SL day 0–4. All concentrations were log_{10}- transformed for analysis. [b]Significantly different from SP ($P \leq 0.04$). [c]Significantly different from HA2 within SP ($P = 0.05$). [d]Significantly different from SL2 ($P < 0.001$). [e]Trend for difference from SL2 ($P = 0.06$).

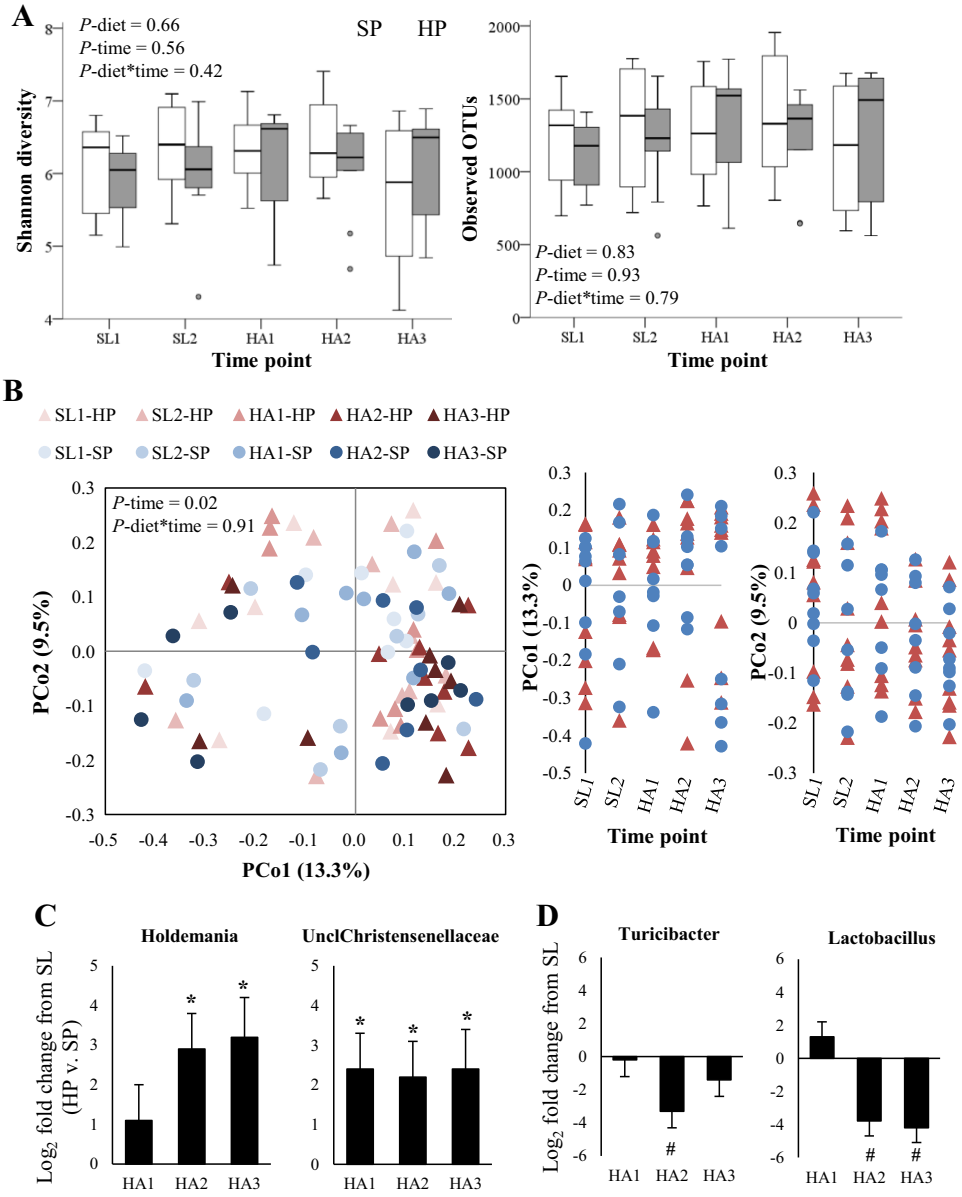


Fig. 2. Changes in fecal microbiota composition during weight loss at high altitude (HA 4,300 m) are largely independent of dietary protein:fat ratio. *A*: α-diversity [Shannon diversity and observed operational taxonomic units (OTUs)] analyzed by marginal models controlling for age, body mass index, and baseline diversity (i.e., SL1). *B*: principle coordinates (PCo) analysis of Bray-Curtis dissimilarities. Individual data points represent the entire fecal microbiota community of a single individual at one point in time. Samples closer together are more similar than samples farther apart. *C*: $\log_2$-fold change in relative abundance from SL in HP relative to SP for taxa demonstrating significant diet-by-time interactions (false discovery rate <0.20). *Change from SL significantly different in HP vs. SP ($P \leq 0.02$). *D*: $\log_2$-fold change in relative abundance from SL in genera demonstrating a significant main effect of time (false discovery rate <0.20). #Significant change from SL ($P \leq 0.005$). HA, high altitude; HP, higher protein diet group; SL, sea level; SP, standard protein diet group; Uncl, unassigned genus level taxonomy. HP, $n = 9$; SP, $n = 8$.

of $\log_2$ fold changes = 2.2–4.9; $P \leq 0.005$). Of note, all three of these taxa include strains that are commonly used as starter cultures in dairy products (*Streptococcus thermophilus* and *Lactobacillus* spp. in yogurt and *Lactococcus* spp. in cheese). Therefore, SL food records were used to estimate yogurt consumption during SL (consumption of *Lactococcus*-containing cheeses during SL could not be accurately estimated). Yogurt consumption during SL was correlated with mean *Streptococcus* relative abundance during SL (Spearman's $\rho = 0.68$, $P = 0.003$) and the change in *Streptococcus* relative abundance from SL to the third week at HA (Spearman's $\rho = -0.71$, $P = 0.002$). In contrast, SL yogurt consumption was not correlated with mean *Lactobacillus* relative abundance during SL (Spearman's $\rho = 0.08$, $P = 0.75$) or the change in *Lactobacillus* relative abundance from SL to the third week at HA (Spearman's $\rho = -0.03$, $P = 0.89$). These observations suggest that the decrease in *Streptococcus*, but not *Lactobacillus*, relative abundance at HA was attributable to reduced yogurt consumption. Of note, neither mean dietary fiber intake at SL nor the change in mean fiber intake from SL to HA was correlated with changes in the relative abundance of any of these six taxa ($P \geq 0.24$ for all).

Responders and nonresponders: AMS severity. AMS severity as measured by the Lake Louise score represents an overall cognitive and physical response phenotype resulting from the physiologic stress induced by hypobaric hypoxia. Therefore, in additional exploratory analyses, AMS severity was considered an indicator of the cumulative stress response to HA, and associations between gut microbiota-related factors and both the development of and response to AMS were explored.

Eleven participants reported moderate or severe AMS (responders) at ≥1 time points during the first 48 h at HA (Table 4). Lake Louise scores were consistently higher during the first 6 days of sojourn in AMS responders relative to those who experienced no or mild AMS (nonresponders) (Fig. 3*A*). In support of AMS severity reflecting the cumulative stress re-

Table 4. *Participant characteristics categorized by peak acute mountain sickness severity measured within 48 h of ascent to high altitude*

	AMS Severity		
	None or mild	Moderate or severe	*P* value
SP/HP, *n*	3/3	5/6	0.86
Age, yr	23 ± 4	24 ± 6	1.00
Body mass index, kg/m^2	24.7 ± 3.7	27.0 ± 3.3	0.20
Body fat, %	20.6 ± 6.7	23.8 ± 5.7	0.30
$\dot{V}O_{2peak}$, ml·kg^{-1}·min^{-1}	51.8 ± 6.3	51.5 ± 8.1	0.96
LM ratio	0.009 [0.005]	0.008 [0.004]	0.72
α-Diversity			
Shannon	6.2 ± 0.5	6.0 ± 0.7	0.32
Observed OTU	1,313 ± 269	1,154 ± 364	0.15
Bacteroides:Prevotella ratio	234 [2,219]	1 [37]	0.07

Data were measured at sea level and are means ± SD or median [interquartile range]. Frequencies were measured using χ^2-tests, and means by independent samples *t*-test. Marginal models were used to analyze gut microbiota metrics, which include 2 measurements at sea level. Acute mountain sickness was measured by Lake Louise score. HP, higher protein diet group; LM ratio, lactulose:mannitol ratio measurement of small intestinal permeability; OTU, operational taxonomic unit; SP, standard-protein diet group; SL, sea level (weight maintenance); HA, high altitude (4,300 m; energy deficit).

sponse, mean serum cortisol concentrations were slightly higher throughout HA in AMS responders relative to nonresponders (Fig. 3*B*). Finally, GIQLI scores were lower during the first 2 wk of sojourn in AMS responders relative to nonresponders (Fig. 3*C*), consistent with GI issues contributing to AMS symptomology. No differences in baseline characteristics between responder groups were noted (Table 4) nor were any relationships observed with changes in the LM ratio (Fig. 3*D*), plasma LBP concentrations (data not shown), or fecal metabolite concentrations (data not shown). Lake Louise scores were higher in SP relative to HP on HA *day 0* [before the prescribed diets were administered; mean difference = 0.6 (95% confidence interval: 0.2, 0.9), $P = 0.004$] and on HA *day 2* [mean difference = 0.7 (95% confidence interval: 0.3, 1.1), $P = 0.001$] but did not differ other days (diet-by-day interaction, $P = 0.02$). In contrast, AMS severity did not differ between diet groups on any day (diet-by-day interaction, $P = 1.0$), and frequency of AMS responder/nonresponder classification did not differ by diet group (Table 4), indicating that the incidence of moderate-to-severe AMS was not affected by dietary protein:fat ratio.

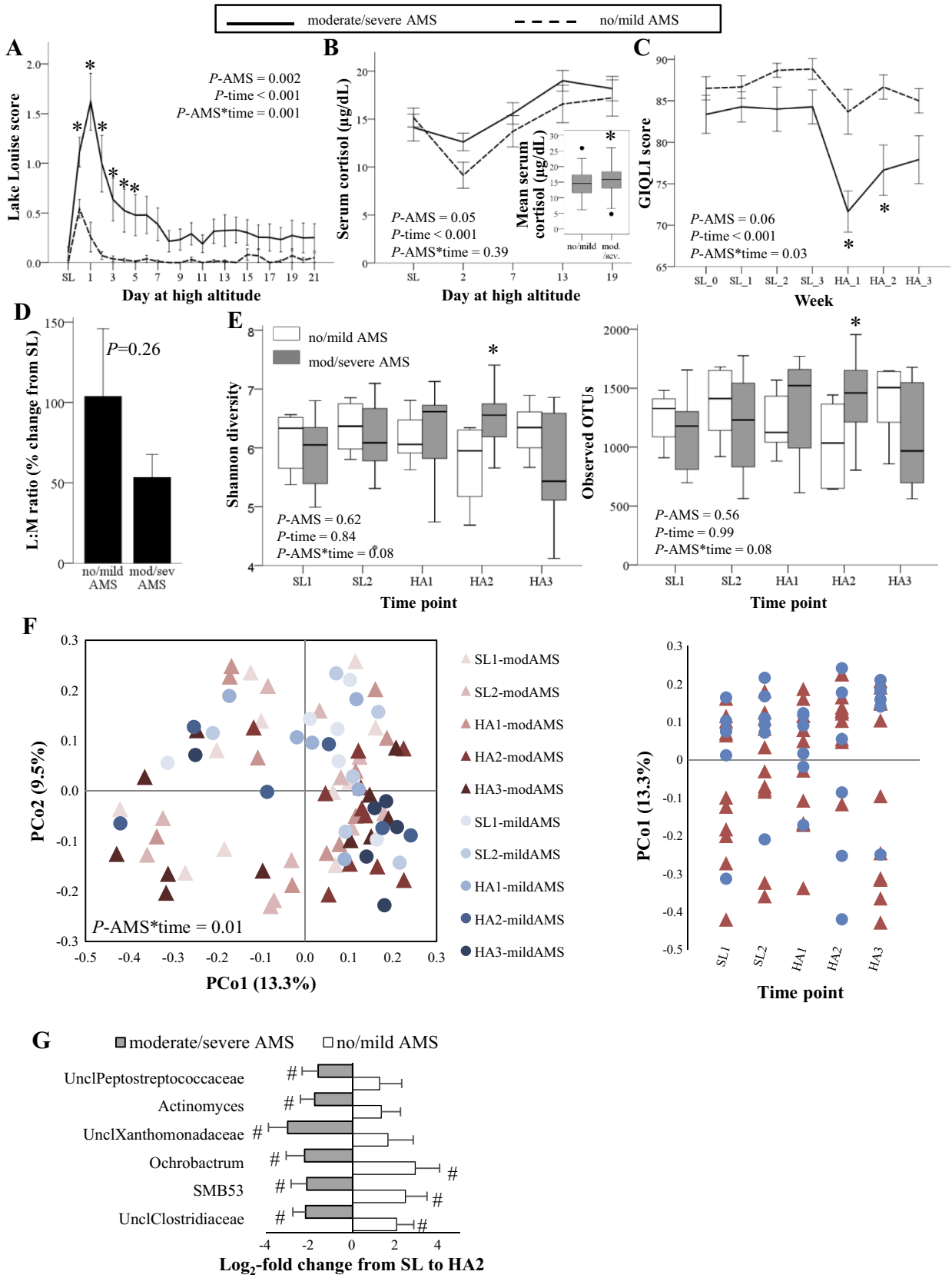
Differences in fecal microbiota composition between AMS responder groups were observed before ascent to HA. Specifically, LEfSe analyses of SL gut microbiota composition indicated that *Prevotella* (phyla: Bacteroidetes) relative abundance at SL was higher in AMS responders relative to nonresponders (effect size = 4.2, $P = 0.006$). Furthermore, the *Bacteroides:Prevotella* ratio, which is known to drive interindividual variation in human gut microbiota composition (18), trended toward being lower at SL in the AMS responders (Table 4). We previously reported that the proportion of total body mass loss at HA attributable to fat-free mass also demonstrated substantial interindividual variability in this cohort with 7 participants losing predominantly fat-free mass and 10 losing predominantly fat mass (9). Interestingly, the mean *Bacteroides:Prevotella* ratio at SL was lower in individuals whose total body mass loss at HA was >50% fat-free mass relative to those whose total body mass loss at HA was >50% fat mass [median (interquartile range): 1 (1) vs. 203 (1,317), $P = 0.01$]. *Prevotella* was also the most discriminant taxa between these two groups, being enriched in the SL microbiota of individuals whose weight loss at HA comprised >50% fat-free mass (LEfSe effect size = 4.4, $P = 0.0001$).

Differences in fecal microbiota composition between AMS responder groups were also observed after ascent to HA. A trend for an AMS responder group-by-time interaction ($P = 0.08$) was observed for both the Shannon and observed OTUs α-diversity metrics (Fig. 3*E*). Post hoc testing indicated differences were present only during the second week at HA during which Shannon diversity was 15% higher and observed OTUs were 45% higher in AMS responders relative to nonresponders ($P = 0.03$ for both). These results were unchanged after excluding two individuals from the moderate/severe AMS group who reported constipation during HA2, and bowel movement frequency during HA2 did not differ between AMS responder groups [median: nonresponder = 5 per week (interquartile range 8) vs. responder = 5 per week (interquartile range 5), $P = 0.56$]. These observations indicated that increased GI transit time, which has been associated with greater α-diversity (63), likely did not explain between group differences. Furthermore, Bray-Curtis dissimilarities demonstrated clustering over time as a function of AMS responder group (Fig. 3*F*; group-by-time interaction, $P = 0.01$). Responder group-by-time interactions (FDR <0.20) were also observed for six taxa, which were all within the top third of the most abundant taxa, and all decreased in relative abundance from SL to HA2 in AMS responders relative to nonresponders (Fig. 3*G*).

DISCUSSION

The first aim of this study was to determine the effect of increasing the dietary protein:fat ratio on gut microbiota composition, gut microbiota-derived metabolites, GI barrier function, and GI symptoms during weight loss at HA. Results demonstrated that protein:fat ratio had little impact on fecal microbiota composition and only transient effects on fecal SCFA and plasma LBP concentrations. These effects did not appear clinically meaningful as no between-group differences in intestinal permeability, GI symptoms, incidence of moderate-to-severe AMS, or inflammation were observed. Although previous studies have reported unfavorable effects of higher

Fig. 3. Acute mountain sickness (AMS) severity is associated with gastrointestinal (GI) symptoms, and changes in fecal microbiota composition. Lake Louise scores (*A*), serum cortisol (*B*), GI symptomology (*C*), lactulose:mannitol ratio (L:M; *D*), and α-diversity (*E*) by AMS severity group (no/mild vs. moderate/severe). *A–D*: data are means ± SE and analyzed by marginal models or mixed models controlling for diet group, age, body mass index, and baseline value. *Different from no/mild AMS ($P < 0.05$). *F*: principle coordinates analysis of Bray-Curtis dissimilarities. Individual data points represent the entire fecal microbiota community of a single individual at one point in time. Samples closer together are more similar than samples farther apart. *G*: $\log_2$-fold change in relative abundance from SL to HA2 in taxa demonstrating significant AMS group-by-time interactions (false discovery rate <0.20). #Significant within group difference from SL. HA, high altitude; SL, sea level; Uncl, unassigned genus level taxonomy. No/mild AMS, $n = 6$; moderate/severe AMS, $n = 11$.


moderate/severe AMS
no/mild AMS
A
Lake Louise score
P-AMS = 0.002
P-time < 0.001
P-AMS*time = 0.001
Day at high altitude
B
Serum cortisol (µg/dL)
P-AMS = 0.05
P-time < 0.001
P-AMS*time = 0.39
Mean serum cortisol (µg/dL)
Day at high altitude
C
GIQLI score
P-AMS = 0.06
P-time < 0.001
P-AMS*time = 0.03
Week
D
L:M ratio (% change from SL)
P=0.26
no/mild AMS
mod/sev AMS
E
no/mild AMS
mod/severe AMS
Shannon diversity
P-AMS = 0.62
P-time = 0.84
P-AMS*time = 0.08
Time point
Observed OTUs
P-AMS = 0.56
P-time = 0.99
P-AMS*time = 0.08
Time point
F
PCo2 (9.5%)
P-AMS*time = 0.01
PCo1 (13.3%)
SL1-modAMS
SL2-modAMS
HA1-modAMS
HA2-modAMS
HA3-modAMS
SL1-mildAMS
SL2-mildAMS
HA1-mildAMS
HA2-mildAMS
HA3-mildAMS
PCo1 (13.3%)
Time point
G
moderate/severe AMS
no/mild AMS
UnclPeptostreptococcaceae
Actinomyces
UnclXanthomonadaceae
Ochrobactrum
SMB53
UnclClostridiaceae
Log2-fold change from SL to HA2

protein diets on fecal microbiota composition and metabolites, those results were more likely attributable to reductions in fiber intake because the taxa most consistently affected were saccharolytic (12, 19, 24, 25, 65) and decreased in proportion to reductions in fiber intake (24, 25, 65). Our findings are more consistent with experiments conducted under normal environmental conditions in which dietary macronutrient composition was manipulated within recommended intake ranges and fiber intake was matched between groups (5, 79). Those studies also did not demonstrate detrimental effects of higher protein diets on markers of GI health despite evidence of increased protein fermentation and/or decreased carbohydrate fermentation (5, 8, 79). As such, any impact of gut microbes and their metabolites on GI barrier function at HA is likely to be independent of dietary protein:fat ratio within the macronutrient intake ranges and time period studied.

The second aim of this study was to identify associations among the gut microbiota, weight loss at HA, and the host response to HA as measured by AMS severity. Notably, AMS severity and weight loss at HA were not affected by diet but demonstrated associations with fecal microbiota composition and, in the case of AMS severity, GI symptoms, providing preliminary evidence of a possible role for the gut microbiota in individual responses to HA.

Recent evidence indicates that the gut microbiota warrants consideration in studies aiming to explain interindividual variance in human phenotypes in response to different exposures (43, 85), and it has been established that the *Bacteroides:Prevotella* ratio is a predominant driver of interindividual variability within the human gut microbiota (18). Intriguingly, AMS severity, a phenotype known to demonstrate large unexplained interindividual variability (7), and body composition changes during weight loss at HA, which also demonstrated large interindividual variability (9), were both associated with an enrichment of *Prevotella* at SL and a correspondingly lower *Bacteroides:Prevotella* ratio. These results suggest that the ratio of *Bacteroides* to *Prevotella* in the gut microbiota, a well-established driver of interindividual variability in human gut microbiota composition, may contribute to the interindividual variability in host responses at HA.

That higher *Prevotella* abundance was associated with worse AMS symptomology and greater fat-free mass losses at HA was somewhat unexpected. A higher ratio of *Prevotella* to *Bacteroides* is generally considered a marker of a healthy high-fiber, plant-rich diet (18, 32, 80), *Prevotella* are known SCFA producers (48), and certain *Prevotella* species have been associated with improved glucose homeostasis (43), which could be beneficial for mitigating impairments in glucose tolerance common during HA sojourn (84). However, *Prevotella* can also be detrimental depending on the environment (44). For example, recent studies suggest that *Prevotella* may thrive during oxidative stress, promoting intestinal mucus barrier dysfunction and inflammation (26, 66), and some species can act as opportunistic pathogens (29). *Prevotella*-dominated microbiota are also specialized in the degradation of plant fibers and have decreased lipolytic and proteolytic fermentation potential (76). This could have altered the availability of substrates from the low plant-fiber diets used in the present study thereby impacting body composition. Of note, the *Bacteroides:Prevotella* ratio has been correlated with long-term habitual dietary patterns but appears to be relatively stable over short time frames, even in response to substantial dietary changes (18, 80). As such, to what extent the *Bacteroides:Prevotella* ratio provides a useful intervention target for improving host responses to HA is unclear, especially as current knowledge suggests that diets designed to increase the *Bacteroides:Prevotella* ratio would likely be high in animal fat and protein and low in fiber (80). Rather, these preliminary findings suggest that the *Bacteroides:Prevotella* ratio could serve as a possible marker for identifying individuals who may be more susceptible to the effects of HA.

AMS severity was also associated with higher fecal microbiota diversity a week after AMS onset. Of note, our group recently documented an increase in gut microbiota diversity in association with larger increases in GI permeability in soldiers engaged in a multiple-stressor military training exercise conducted at low altitude (39). These observations, which correlate increased diversity with worse responses to stress, contrast with animal studies reporting decreased microbiota diversity following exposure to various psychological and physiologic stressors (10, 71, 72). The discrepancy could reflect differential impacts of separate stressors on the gut microbiota (38) and/or the recognized difficulty in extrapolating animal microbiota studies to humans (56). Interpreting our findings is also complicated by an inability to separate cause and effect. For example, as higher diversity is generally considered a marker of a healthy and resilient microbiota (62), the increase in diversity in individuals with a more severe stress response observed in our human studies could reflect a beneficial adaptation to stress. This hypothesis would be consistent with murine studies of cold stress in which changes in the gut microbiota facilitate host acclimatization to the cold (17, 89). Alternately, our observations may simply reflect greater, transient changes in the colonic environment in individuals with a more severe stress response. Future studies are needed to separate cause and effect in the relationship between the human gut microbiota and host stress response, while also determining the extent to which any stress-induced restructuring of the gut microbiota is sustained.

Independent of AMS symptomology, weight loss at HA was associated with reduced relative abundance of both *Lactobacillus* and *Turicibacter*. The observed decrease in *Lactobacillus* relative abundance at HA was of particular interest as this SCFA-producing genus promotes GI barrier integrity, immune function, and resistance against enteric pathogens (31). The transient decrease in *Turicibacter* relative abundance during HA was also notable as *Turicibacter* depletion has been reported in rodent models of immunodeficiency (20, 40) and inflammatory bowel disease (3, 64) in association with increased inflammation (46). Our findings therefore raise the possibility that the physiologic effects of weight loss at HA may include *Lactobacillus* and *Turicibacter* depletion, which could in turn contribute to reduced GI barrier integrity, inflammation, altered immune function, and heightened susceptibility to bacterial pathogens, which have been reported at HA (41, 54). On the other hand, previous studies have not reported a reduction in *Lactobacillus* during HA sojourn (*Turicibacter* was not measured) (2, 42) or reductions in the relative abundances of these taxa in individuals exposed to normobaric hypoxia simulating ~4,000-m altitude (68–70). The latter observation implies that sustained hypoxia per se is likely not the mechanism underpinning observed changes in relative abun-

dance and that some combination of hypobaria, hypoxia, weight loss, increased physical activity, and dietary change is responsible. Of note, all of these factors are inevitable or common during HA sojourn (33).

This combination of factors was also associated with a physiologic stress response and increased inflammation, which is consistent with previous reports (34, 41), and could drive changes in gut microbiota composition. In addition, a rapid and sustained increase in small intestinal permeability was observed. To our knowledge, only one previous study has directly measured intestinal permeability during HA sojourn. In that observational study of mountaineers, Dinmore et al. (21) reported increased small intestinal permeability at 5,570 m but attributed the response to residual effects of GI infection. However, rodent studies have shown that intestinal permeability increases upon exposure to hypobaric hypoxia (82, 88), attributing the response to inflammation, oxidative stress, atrophy, and villous collapse within the GI epithelium (1, 82, 86, 88). Although the present study did not directly address those mechanisms, the progressive decrease in mannitol excretion is consistent with the findings of Dinmore et al. (21) and suggests that small intestinal absorptive capacity and/or surface area was reduced during weight loss at HA, which could reflect villous atrophy.

Our data suggest that the pleiotropic hormone GLP-2 could be one factor impacting GI permeability at HA. GLP-2 is secreted from intestinal enteroendocrine cells and is thought to benefit GI function in part by stimulating blood flow, reducing inflammation, and enhancing GI barrier integrity (14, 22, 23). Furthermore, elevated GLP-2 concentrations following intestinal injury and in response to fasting have been shown to facilitate GI growth and repair (22). We hypothesize that a reduction in enteroendocrine cell density resulting from diminished intestinal surface area, as suggested by the decrease in mannitol excretion, could underpin the observed transient decrease in GLP-2 concentrations, which in turn could contribute to an increase in intestinal permeability (14). The subsequent GLP-2 rebound may reflect an adaptive response to intestinal injury and underfeeding to promote GI growth and repair. The inverse correlation with plasma LBP further implicates GLP-2 as having influenced GI barrier function, although the sustained increase in intestinal permeability indicates that other unmeasured factors also contributed.

Study findings should be interpreted within the context of several limitations. First, the sample size was small, and some between-group differences were likely not detected. As such, the absence of effects of diet composition on many study outcomes warrants cautious interpretation and requires replication. Second, the absence of a weight-maintenance control group at HA precludes determining whether temporal changes in study outcomes were attributable to hypobaric hypoxia and acclimatization, weight loss, changes in diet, other factors, or their combination. Third, study outcomes were not all assessed at the same time points which prevented examination of or complicated interpretation of associations between outcomes. Fourth, functional assessment of GI permeability was limited to the small intestine, and it cannot be assumed that small intestinal permeability mirrors that of the large intestine where gut microbes and their metabolites are more abundant. Additionally, intestinal permeability was measured only once at SL which prevented assessing whether any stress related to research study participation was associated with an increase in intestinal permeability at SL. However, even if such an effect were present, it did not mask the increases in permeability observed at HA. Finally, the findings related to weight loss at HA, AMS severity, and the gut microbiota must be considered preliminary and hypothesis generating due to the small sample size and exploratory and correlative nature of the analysis.

In summary, this tightly controlled study integrated stress-induced changes in physiology with temporal changes in the gut microbiota and its metabolites, and objective and subjective measures of GI health and function thereby allowing for an analysis of multiple steps along the pathway linking environment, stress, the gut microbiota, and host health. Findings demonstrated several novel associations between the gut microbiota and host responses to HA that were independent of dietary protein:fat ratio. Intriguingly, the ratio of *Bacteroides* to *Prevotella* in the gut microbiota, a well-established driver of interindividual variability in human gut microbiota composition, was associated with interindividual variability in host responses at HA, and increased gut microbiota diversity, considered a marker of a healthy and resilient microbiota, was elevated after HA exposure in individuals who experienced a more severe stress response at HA as measured by AMS symptomology. Although the study design precluded establishing a causal role for the gut microbiota in host responses to HA, findings provide preliminary evidence for a potential role, and support the need for additional research designed to determine if the gut microbiota can be leveraged to improve physiologic responses to HA.

ACKNOWLEDGMENTS

We thank the study volunteers, our medical oversight team, and Dr. Stephen Muza for support and the Pennington Biomedical Research Center Clinical Laboratory staff, Robert Player, Jason Soares, Katherine Kensil, Karen Conca, Nancy Murphy, Marques Wilson, Christopher Carrigan, Adrienne Hatch, Laura Lutz, Matthew Kominsky, Adam Luippold, Bradley Anderson, Grant Holmes, Anthony Karis, Karleigh Bradbury, Alyssa Kelley, Katakyie Sarpong, Alfonzo Patino, Dr. Renee Cole, Dr. John Carbone, Dr. Lee Margolis, Dr. Stephen Hennigar, Dr. Robert Kenefick, and Dr. Scott Montain for significant contributions.

GRANTS

This work was supported by the U.S. Army Medical Research and Materiel Command and the U.S. Department of Defense, Defense Health Program.

DISCLAIMERS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Approved for public release; distribution is unlimited.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.P.K., C.E.B., A.J.Y., J.C.R., and S.M.P. conceived and designed research; J.P.K., C.E.B., A.J.Y., P.N.R., and S.M.P. performed experiments; J.P.K., C.E.B., T.A.B., and I.G.P.-F. analyzed data; J.P.K., C.E.B., A.J.Y., I.G.P.-F., and S.M.P. interpreted results of experiments; J.P.K. prepared figures; J.P.K. drafted manuscript; J.P.K., C.E.B., A.J.Y., P.N.R., T.A.B., I.G.P.-F., J.C.R., and S.M.P. edited and revised manuscript; J.P.K., C.E.B., A.J.Y., P.N.R., T.A.B., I.G.P.-F., J.C.R., and S.M.P. approved final version of manuscript.

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Physiological Reports

Open Access

Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

PI3K-AKT-FOXO1 pathway targeted by skeletal muscle microRNA to suppress proteolytic gene expression in response to carbohydrate intake during aerobic exercise

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Keywords
miR-206, miR-486, muscle protein breakdown, muscle protein synthesis, myomiR, whey protein.

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Received: 5 November 2018; Revised: 6 November 2018;

doi: 10.14814/phy2.13931

Physiol Rep, 6 (23), 2018, e13931, https://doi.org/10.14814/phy2.13931

Abstract

Ingesting protein and carbohydrate together during aerobic exercise suppresses the expression of specific skeletal muscle microRNA and promotes muscle hypertrophy. Determining whether there are independent effects of carbohydrate and protein on microRNA will allow for a clearer understanding of the mechanistic role microRNA serve in regulating skeletal muscle protein synthetic and proteolytic responses to nutrition and exercise. This study determined skeletal muscle microRNA responses to aerobic exercise with or without carbohydrate, and recovery whey protein (WP). Seventeen males were randomized to consume carbohydrate (CHO; 145 g; $n = 9$) or non-nutritive control (CON; $n = 8$) beverages during exercise. Muscle was collected before (BASE) and after 80 min of steady-state exercise (1.7 ± 0.3 $\dot{V}O_2$ L·min^{-1}) followed by a 2-mile time trial (17.9 ± 3.5 min; POST), and 3-h into recovery after consuming WP (25 g; REC). RT-qPCR was used to determine microRNA and mRNA expression. Bioinformatics analysis was conducted using the mirPath software. Western blotting was used to assess protein signaling. The expression of six microRNA (miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p) were higher ($P < 0.05$) in CHO compared to CON, all of which target the PI3K-AKT, ubiquitin proteasome, FOXO, and mTORC1 pathways. p-AKT^{Thr473} and p-FOXO1^{Thr24} were higher ($P < 0.05$) in POST CHO compared to CON. The expression of *PTEN* was lower ($P < 0.05$) in REC CHO than CON, while *MURF1* was lower ($P < 0.05$) POST CHO than CON. These findings suggest the mechanism by which microRNA facilitate skeletal muscle adaptations in response to exercise with carbohydrate and protein feeding is by inhibiting markers of proteolysis.

Introduction

Protein and carbohydrate ingestion modulate cellular mechanisms regulating skeletal muscle mass in response to exercise (Churchward-Venne et al. 2012; Pasiakos 2012; Stokes et al. 2018). Consuming high-quality protein, such as whey, increases activation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway, eliciting a greater muscle protein synthetic response compared to exercise alone (Kimball and Jefferson 2004, 2006). Carbohydrate ingestion may contribute to muscle accretion through antiproteolytic effects, reducing muscle protein breakdown by inhibiting the forkhead box O 1 (FOXO1) pathway through an insulin-dependent mechanism (Roy et al. 1997; Biolo et al. 1999; Greenhaff et al. 2008; Abdulla et al. 2016). Several investigations have reported that the expression of specific microRNA (miRNA) linked to governing mTORC1 are also acutely altered in response to exercise (Drummond et al. 2008; Rivas et al. 2014; Zacharewicz et al. 2014; Fyfe et al. 2016;

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D'Souza et al. 2017b) and protein feeding (Drummond et al. 2009; Camera et al. 2016; Margolis et al. 2017). However, few studies have been able to capture downstream modifications in biological processes targeted by altered skeletal muscle miRNA in humans. Our laboratory (Margolis et al. 2017) recently reported that specific miRNA (miR-1, miR-206, miR-208, and miR-499) are diminished and inversely associated with muscle protein synthesis after aerobic exercise when essential amino acids and carbohydrate are consumed together during exercise.

miRNA control biological processes through inhibition, binding to target mRNA to repress protein translation (Kong et al. 2008). The posttranscriptional control that miRNA exert on target mRNA make them prospective regulators of exercise and diet-induced alterations in skeletal muscle. Rodent and cell culture models have shown that the overexpression of miR-1, miR-99a, miR-99b, miR-100-5p, and miR-133 blunt rates of protein synthesis by directly or indirectly inhibiting mTORC1, resulting in lower muscle fiber size and mass (McCarthy and Esser 2007; Elia et al. 2009; Hua et al. 2012; Jin et al. 2013; Wei et al. 2013). Conversely, the overexpression of miR-221, miR-222, and miR-486 inhibit phosphatase and tensin homolog (PTEN), allowing for increased AKT and diminished FOXO1, inhibiting proteolysis and resulting in muscle growth (Chun-Zhi et al. 2010; Small et al. 2010). However, the function of these miRNA in humans is not well defined. Particularly, much remains unknown regarding independent effects of protein and carbohydrate intake on modulation of miRNA responses to exercise in humans. Understanding how protein and carbohydrate alter miRNA and their downstream targets would offer a new level insight into the mechanism by which manipulation of nutrient intake promotes muscle growth.

The objective of this study was to examine the effects of aerobic exercise with or without carbohydrate ingestion during exercise, and whey protein (WP) feeding during postexercise recovery, on skeletal muscle miRNA. Additionally, this study sought to identify the pathways that differentially expressed miRNA targets in response to carbohydrate supplementation. We hypothesized, independent of carbohydrate intake, that miRNA associated with the regulation of mTORC1 would be downregulated, and that miRNA differentially expressed in response to carbohydrate intake would target proteolysis via the FOXO1 pathway.

Materials and Methods

Participants

Seventeen free-living, recreationally active (programmed physical activity 2–4 days/week) males completed this randomized, parallel, single-blinded, placebo-controlled study. Participants were in good health and refrained from consuming alcohol, caffeine, or dietary supplements, and nicotine products during the study. Data presented in this manuscript were collected as part of a larger study examining the impact of high-altitude exposure and underfeeding on skeletal muscle mass, intramuscular regulators of muscle anabolism and proteolysis, and substrate metabolism (Berryman et al. 2018; Margolis et al. 2018; Young et al. 2018). This manuscript reports observations from only the sea-level experiments, in which acute miRNA expression and their targets were measured in response to aerobic exercise, with or without carbohydrate, and recovery protein feeding. This study was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA). This study was conducted from May 2016 through August 2016. All testing was conducted at Natick, MA. All participants provided written informed consent before participation. Forty-one individuals signed the informed consent. The trial was registered at www.clinicaltrials.gov as NCT02731066.

After obtaining informed consent, participants were randomly assigned to consume either a carbohydrate (CHO; $n = 9$) or flavor-matched non-nutritive control (CON; $n = 8$) drink during 80 min of steady-state treadmill exercise (Young et al. 2018). Randomization was computer generated, with 1:1 allocation for the parallel groups. Height was measured to the nearest 0.1 cm with a stadiometer (Seca; Creative Health Products, Plymouth, MI), and body mass was determined to the nearest 0.1 kg using a calibrated digital scale (Model PS6600; Befour Inc., Saukville, WI). Body composition was measured by dual energy X-ray absorptiometry (DEXA; DPX-IQ; GE Lunar Corp., Madison, WI). For 7 days before testing, participants followed dietary instructions for a weight maintenance diet, consuming 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ protein, with 45–65% total energy from carbohydrate, and 20–35% total energy from fat, which was verified by study research dietitians using 24 h recalls. There were no differences in participant characteristics or energy and macronutrient intake between groups (Table 1).

Experimental design

Following a 10-h overnight fast, a biopsy was collected from the vastus lateralis to capture rested/postabsorptive muscle basal state (BASE; Fig. 1). Participants then completed 80 min of steady-state treadmill exercise, walking at a fixed speed and grade that elicited 40% of VO_2peak. After completing 80 min of walking, participants were allowed 5-min standing rest, and then they completed a 2-mile time trial in which they attempted to run or walk at the fastest

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pace that they could maintain. Participants consumed either a carbohydrate (CHO; $n = 9$) or flavor-matched non-nutritive control (CON; $n = 8$) drink at 0-, 20-, 40-, and 60-min during the steady-state portion of the exercise bout (Young et al. 2018). The carbohydrate drink contained 145 g of fructose and glucose (62.25 g fructose + 79.75 g glucose; 0.8 fructose-to-glucose ratio) to achieve an average ingestion rate of 1.8 g min^{-1}. Indirect calorimetry (True Max 2400, Parvo Medics, Sandy, UT) was performed during the steady-state exercise to ensure $\dot{V}O_2$ was within ±5% of the target $\dot{V}O_2$. Steady-state exercise was metabolically matched between the groups (CHO: 1.78 ± 0.22 $\dot{V}O_2$ L·min^{-1}; 713 ± 86 kcal, CON: 1.63 ± 0.26 $\dot{V}O_2$ L·min^{-1}; 651 ± 104 kcal; $P > 0.05$).

Table 1. Volunteer characteristics*

Characteristic	CHO ($n = 9$)	CON ($n = 8$)
Age (years)	22 ± 2	25 ± 8
Height (cm)	178 ± 9	175 ± 6
Weight (kg)	84 ± 13	80 ± 16
Body mass index (kg·m^{-2})	27 ± 3	26 ± 4
Fat-free mass (kg)	63 ± 8	59 ± 10
Fat mass (kg)	19 ± 6	18 ± 8
Body fat (%)	23 ± 5	23 ± 7
VO_{2peak} (L·min^{-1})	4.3 ± 0.7	4.0 ± 0.6
†Energy (kcal·d^{-1})	2491 ± 525	2414 ± 591
†Protein (g·kg^{-1}·d^{-1})	1.2 ± 0.4	1.3 ± 0.4
†Carbohydrate (g·kg^{-1}·d^{-1})	3.9 ± 0.9	4.0 ± 1.1
†Fat (g·kg^{-1}·d^{-1})	1.1 ± 0.3	1.1 ± 0.4

CHO, carbohydrate; CON, control.
*Values are means ± SD.
†Energy and macronutrient intake determined using 24 h food recalls.

There was no difference between the groups in the time required to complete the 2-mile time trial (CHO: 17.8 ± 3.9 min, CON: 18.0 ± 3.3 min; $P > 0.05$). Immediately after the time trial, a postexercise (POST) muscle biopsy was obtained. Participants then consumed a 25 g bolus of whey protein drink (Isopure® Zero Carb; Isopure Co., Hauppauge, NY). After a 3-h recovery period, a final muscle biopsy was obtained (REC).

microRNA and mRNA expression

Total RNA was isolated from 20 mg muscle samples using TRIzol reagent (ThermoFisher, Waltham, MA). RNA quantity and quality were assessed using a Nanodrop ND-2000 spectrophotometer (Nanodrop, Wilmington, DE). For miRNA analysis, equal amounts of total RNA (250 ng) were reverse-transcribed using the TaqMan® microRNA RT kit (Applied Biosystems, Foster City, CA) with the miRNA-specific stem-loop reverse transcript (RT) primers pooled in 1X Tris-EDTA (TE) buffer for a final dilution of 0.05× for each miRNA RT primer. For mRNA, equal amounts (500 ng) of total RNA were reverse-transcribed using a iScript™ Advanced cDNA Synthesis Kit (Bio-Rad). Both mRNA and miRNA reverse transcription was conducted using a T100™ Thermal Cycler (Bio-Rad). Amplifications were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). Samples were run in 15 μL reactions in duplicate using TaqMan® Universal PCR MasterMix (2X), no UNG (Applied Biosystems) and commercially available miRNA probes (miR-1-3p, miR-26a-5p, miR-29a-3p, miR-99a-5p, miR-99b-5p, miR-100-5p, miR-103, miR-133a-3p, miR-133b, miR-206, miR-208b, miR-221-5p, miR-222-3p, miR-324-3p,

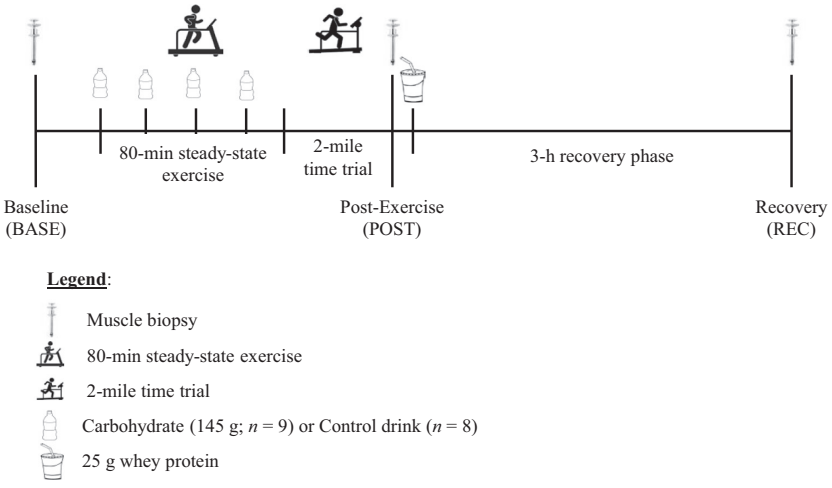


Figure 1. Study design.

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miR-378-5p, miR-451a, miR-486-5p, and miR-499-3p; Applied Biosystems), and 10 μL reactions using iTaq™ Universal SYBR® and commercially available primers for mRNA (*PTEN, AKT1*, *FOXO1, MURF1*; Bio-Rad). miRNA included in the present analysis were chosen based on their potential to regulate pathways governing skeletal muscle protein synthesis and proteolysis. All miRNA were normalized to RNU44, and all mRNA were normalized to the geometric mean of *B2M* and *β-ACTIN*. Fold changes for miRNA and mRNA were calculated using the ΔΔ cycle threshold (ΔΔCT) method (Pfaffl 2001) and expressed as fold change relative to BASE for each group. *MURF1* data from the CON group was previously published (Margolis et al. 2018) to assess changes in response to exercise and protein feeding at sea level, acute high altitude (3 h), and chronic high altitude (21 days). No comparison of the CHO and CON groups has been published for the measures included in the current manuscript.

Bioinformatics analysis

miRNA that were differentially expressed between CHO and CON were uploaded to DNA Intelligent Analysis (DIANA)-miRPath 3.0 (Alexander Fleming Biological Sciences Research Center [BSRC], Athens, Greece; http://diana.cslab.ece.ntua.gr) to determine common molecular pathways that these miRNA regulate. Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways were identified using only experimentally verified targets from TarBase 7.0 (Alexander Fleming BSRC). Pathways specific to disease were excluded from results.

Intracellular signaling

Phosphorylation status were determined from western blots as previously described (Margolis et al. 2018). Muscle samples (~20 mg) were homogenized in ice-cold homogenization buffer. Homogenates were centrifuged for 15 min at 10,000***g*** at 4°C. Supernatant (lysate) was collected and protein concentrations were determined using 660 nm Protein Assay (ThermoFisher). Muscle lysates were solubilized in Laemmli buffer, with equal amounts of total protein (15 μg) and separated by SDS-PAGE using precast Tris-HCl gels (Bio-Rad). Proteins were transferred to polyvinylidene fluoride membranes and exposed to commercially available primary antibodies specific to p-mTOR^{Ser2448}, p-rpS6$^{240/244}$, p-Akt^{Ser473}, p-FOXO1^{Thr24} (Cell Signaling Technology, Danvers, MA) overnight at 4°C. Secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology) and chemiluminescent reagent (Pierce Biotechnology, Rockford, IL) were applied to label primary antibodies. Blots were quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). Heat shock protein 90 (HSP90) was used to confirm equal amounts of protein were loaded per well. All phosphorylation data were presented as fold change relative to BASE phosphorylation for each group. Data from the CON group were previously published (Margolis et al. 2018) to assess changes in response to exercise and protein feeding at sea level, acute high altitude (3 h), and chronic high altitude (21 days). No comparison of the CHO and CON groups has been published for the measures included in the current manuscript.

Statistical analysis

Mixed model repeated measured ANOVA was used to determine impact of time (BASE, POST, REC) and supplement (CHO and CON) on miRNA and mRNA expression and phosphorylation status of intramuscular signaling markers. Bonferroni adjustments for multiple comparisons were performed if significant interactions were observed. All data are presented as mean ± SD. The α level for significances was set at $P < 0.05$. Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp. Armonk, NY).

Results

miR-1-3p, miR-26a-5p, miR-29a-3p, miR-133b, miR-206, and miR-378-5p, were lower ($P < 0.05$) at POST and REC compared to BASE, regardless of group (Fig. 2A–F). Additionally, miR-1-3p, miR-133b, and miR-378-5p were lower ($P < 0.05$) at REC compared to POST, independent of group (Fig. 2A,D and F). Overall, miR-19b-3p, miR-222-3p, and miR-324-3p were lower ($P < 0.05$) in CON compared to CHO, with no effect of time (Fig. 3A,D and E). Time-by-group interactions were observed for miR-99a-5p, miR-100-5p, and miR-486-5p (Fig. 3B,C and F). Specifically, miR-99a-5p POST was higher ($P < 0.05$) than BASE and REC in CHO (Fig. 3B). miR-100-5p was lower ($P < 0.05$) at REC compared BASE and POST in CHO (Fig. 3C). Both miR-99a-5p and miR-100-5p were lower ($P < 0.05$) at POST in CON versus CHO (Fig. 3B and C). miR-486-5p REC was lower ($P < 0.05$) than BASE in CON, and CON was lower ($P < 0.05$) than CHO at REC (Fig. 3F). No impact of time or group was observed for miR-99b-5p, miR-103, miR-133a-3p, miR-451a, and miR-499-3p. miR-221-5p was measured but did not cross the cycle threshold during the 40 cycle run, so no data were generated for this miRNA.

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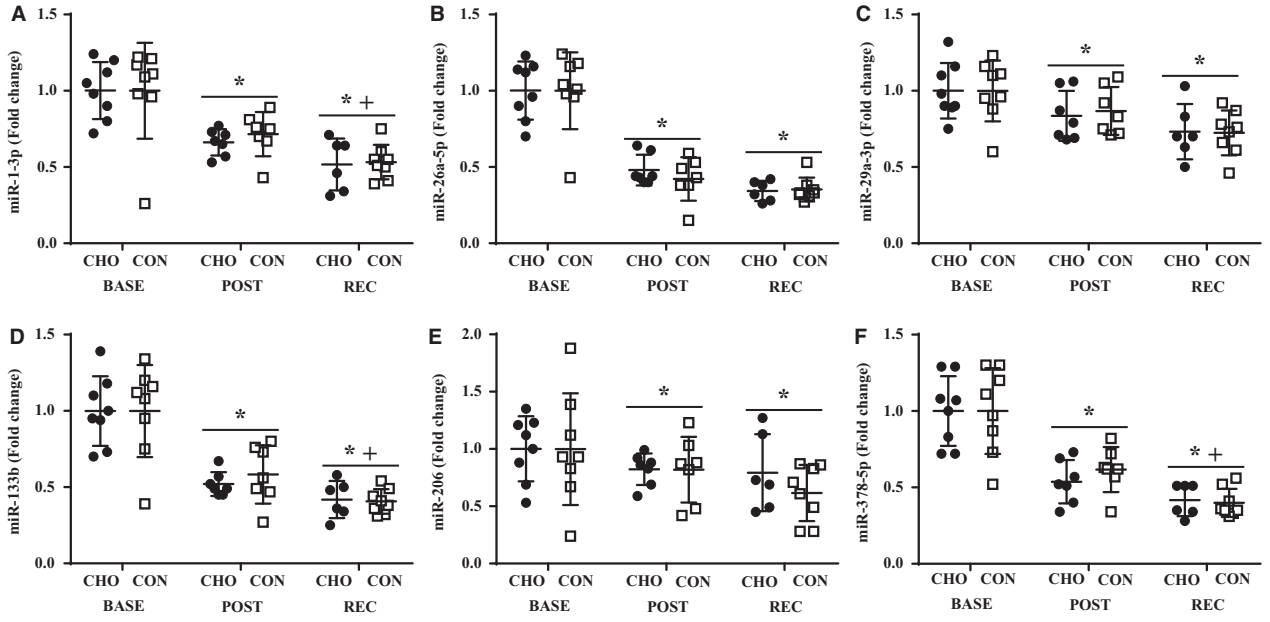


Figure 2. Expression miR-1-3p (A), miR-26a-5p (B), miR-29a-5p (C), miR-133b (D), miR-206 (E), miR-206 (D), and miR-378-5p (F). *Different from BASE, $P < 0.05$. ⁺Different from POST, $P < 0.05$. BASE, baseline; POST, postexercise; REC, recovery; CHO, carbohydrate; CON, control.

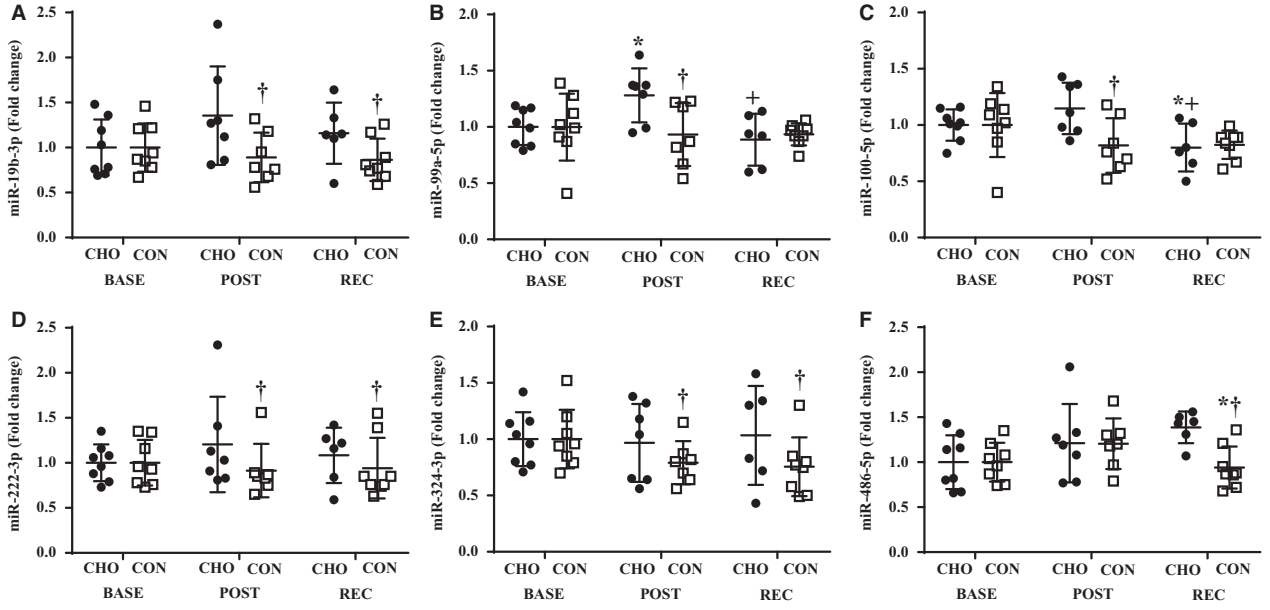


Figure 3. Expression miR-19b-3p (A), miR-99a-5p (B), miR-100-5p (C), miR-222-3p (D), miR-324-3p (E), and miR-486-5p (F) at BASE, POST, and REC. *Different from BASE, $P < 0.05$. ⁺Different from POST, $P < 0.05$. †Different from CHO, $P < 0.05$. BASE, baseline; POST, post exercise; REC, recovery; CHO, carbohydrate; CON, control.

Bioinformatics analysis indicated that miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p all interact with the PI3K-AKT signaling pathway (Fig. 4). Downstream targets of PI3K-AKT, ubiquitin-mediated proteolysis, FOXO signaling, and mTOR signaling were also identified as pathways targeted by all six miRNA that were differentially expressed between CHO and CON.

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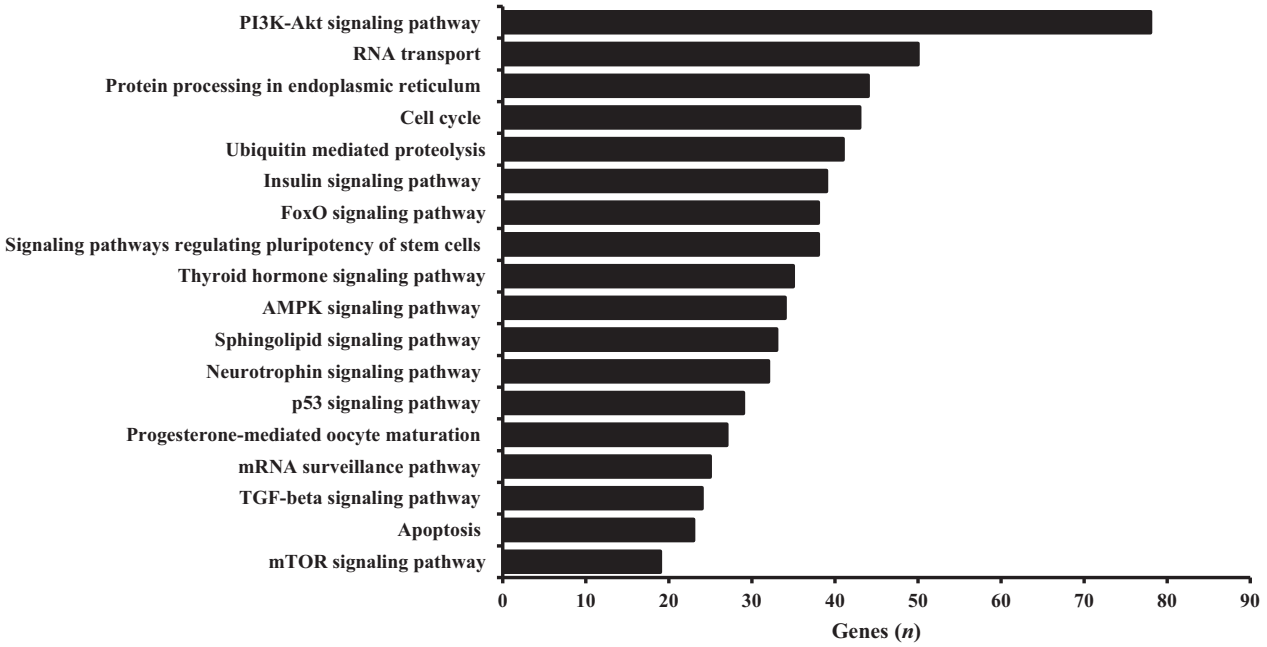


Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways experimentally validated to be targeted by all six miRNA (miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p) differentially expressed between carbohydrate (CHO) and control (CON).

Independent of group, p-mTOR^{Ser2448} and p-rpS6$^{Ser240/244}$ were higher ($P < 0.05$) at POST and REC than BASE (Fig. 5A and B). Time-by-group interactions were present in both p-AKT^{Thr473} and p-FOXO1^{Thr24}, where POST was higher ($P < 0.05$) than BASE in CHO, and CON was lower ($P < 0.05$) than CHO at POST (Fig. 5C and D). Time-by-group interactions were observed for *PTEN*, with REC being higher ($P < 0.05$) than BASE and POST in CON, and CON was greater ($P < 0.05$) than CHO at REC (Fig. 6A). There was no effect of time or group for *AKT1* and *FOXO1* (Fig. 6B and C). Time-by-group interactions were observed for *MURF1*, with POST and REC being higher than BASE in CON, and CON was greater than CHO at POST (Fig. 6D).

Discussion

The main outcome of this study was that miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p were more highly expressed in the skeletal muscle following aerobic exercise in CHO compared to CON. Bioinformatics analysis determined that all six of these miRNA target the PI3K-AKT pathway, ubiquitin proteasome, FOXO, and mTOR signaling pathways. Examination of these pathways identified group differences in markers of proteolysis, but not anabolism. These findings suggest carbohydrate ingestion during aerobic exercise alters miRNA that inhibit skeletal muscle proteolysis with no impact on anabolism.

Of the six miRNA expressed differently in CHO and CON, miR-19b-3p, miR-222-3p, and miR-486-5p have been experimentally validated to inhibit *PTEN* (Schiaffino and Mammucari 2011). In vitro studies demonstrate that overexpression of miR-19b-3p, miR-222-3p, and miR-486-5p diminishes PTEN protein content and increases the phosphorylation status and total protein content of AKT (Chun-Zhi et al. 2010; Small et al. 2010; Alexander et al. 2011; Xu et al. 2016). Additionally, overexpression of miR-100-5p and miR-324-3p in cell culture experiments have been found to result in elevated AKT protein content (Jin et al. 2013; Xiao et al. 2017). Increased activation of AKT inhibits FOXO1, a transcription factor mediating expression of the ubiquitin ligases *MURF1* and *ATROGIN-1* (Bodine et al. 2001; Sandri et al. 2004; Leger et al. 2006). As such, increased AKT total protein content through altered miRNA expression, particularly miR-486-5p, in cell culture and rodent studies (Small et al. 2010; Alexander et al. 2011; Xu et al. 2012) results in reduced transcription of these atrophy genes, diminished muscle proteolysis, and increased muscle fiber size (Xu et al. 2012). However, there has been little translational work to determine if there are similar alterations with miRNA and their target pathways in humans. Analysis of the proteolytic pathway in the current study is largely in agreement with past findings in animal and cell culture models

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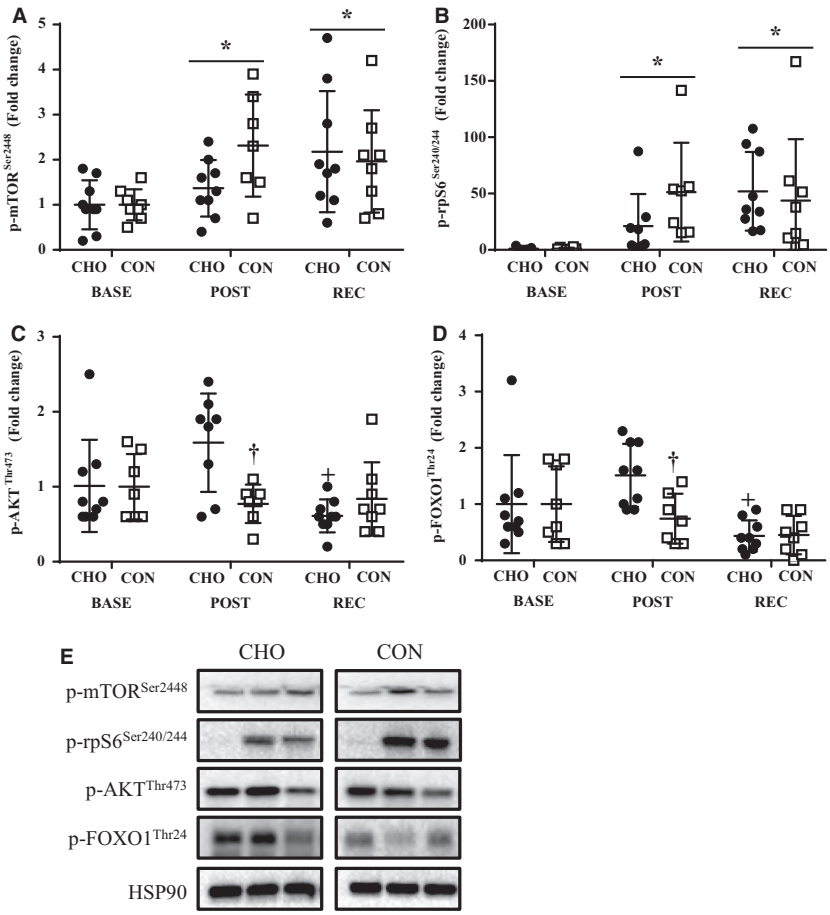


Figure 5. Phosphorylation status p-mTOR^{Ser2448} (A), p-rpS6$^{Ser240/244}$ (B), p-AKT^{Thr473} (C), and p-FOXO1^{Thr24} (D). Representative bands (E). *Different from BASE, $P < 0.05$. $^{+}$Different from POST, $P < 0.05$. †Different from CHO, $P < 0.05$. BASE, baseline; POST, postexercise; REC, recovery; CHO, carbohydrate; CON, control.

(Chun-Zhi et al. 2010; Small et al. 2010; Xu et al. 2012, 2016). When miR-19b-3p, miR-222-3p, miR-324-3p, and miR-486-5p expression is higher in the muscle, there is lower *PTEN* expression, increased activity of p-AKT^{Thr473}, and lower activity of p-FOXO1^{Thr24} and *MURF1* expression. Higher expression of miRNA that target markers of proteolysis, such as miR-486-5p, in the recovery phase of exercise reported here and in previous studies (Camera et al. 2016; Fyfe et al. 2016; D'Souza et al. 2017b), may be favorable to facilitate remodeling, repair, and synthesis of new muscle proteins with exercise.

Independent of carbohydrate, following aerobic exercise with recovery whey protein feeding miR-1-3p, miR-133b, and miR-206 were lower compared to resting measures. Reductions in these miRNA occurred concurrent with increased mTORC1 signaling. These results are in agreement with previous findings from our laboratory (Margolis et al. 2017) and others (McCarthy and Esser 2007; Drummond et al. 2008; Elia et al. 2009; Rivas et al. 2014; Zacharewicz et al. 2014), indicating that when muscle anabolism is upregulated in response to exercise and protein feeding, miRNA that inhibit the mTORC1 pathway are diminished. Despite findings from the current study corroborating past results from our group and others, conflicting responses in miRNA expression following exercise have been reported (Drummond et al. 2009; Nielsen et al. 2010; Russell et al. 2013; Camera et al. 2016; Fyfe et al. 2016). Discordant results can primarily be attributed to nutrient intake. Specifically, aerobic exercise without nutritional intervention results in increased expression of miR-1, miR-133a, miR-133b, and miR-206 1 to 3-hour postexercise (Nielsen et al. 2010; Russell et al. 2013). These differences in miRNA expressions indicate that inclusion of protein feeding immediately after aerobic exercise elicits a unique miRNA expression profile that may potentiate the muscle protein synthetic response compared to aerobic exercise alone.

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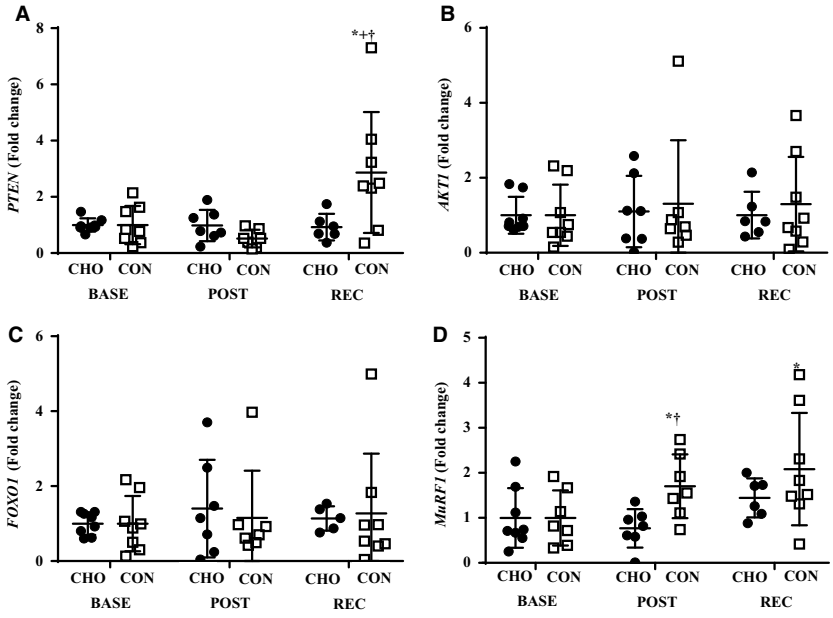


Figure 6. Expression of *PTEN* (A), *AKT1* (B), *FOXO1* (C), and *MURF1* (D). *Different from BASE, $P < 0.05$. $^{+}$Different from POST, $P < 0.05$. †Different from CHO, $P < 0.05$. BASE, baseline; POST, post exercise; REC, recovery; CHO, carbohydrate; CON, control.

Overall, results from the current study are consistent with previous investigations showing muscle protein synthetic response is unaffected by carbohydrate ingestion during exercise (Borsheim et al. 2004b; Koopman et al. 2008; Kato et al. 2016), while proteolysis is lower compared to a non-nutritive control (Roy et al. 1997; Borsheim et al. 2004a; Greenhaff et al. 2008; Glynn et al. 2010). However, to the best of our knowledge, this is the first report in which differences in acute protein synthetic and proteolytic responses to carbohydrate feeding during exercise extend to the miRNA that regulate these pathways. These findings provide new insight into the impact of protein and carbohydrate supplementation on acute alterations in the expression of skeletal muscle miRNA following exercise and their potential regulation of pathways governing muscle accretion. While several investigations have focused on the impact of exercise on skeletal muscle miRNA (Nielsen et al. 2010; Russell et al. 2013; Rivas et al. 2014; Zacharewicz et al. 2014; Fyfe et al. 2016; D'Souza et al. 2017a, 2017b), only a few have explored the role of protein and/or carbohydrate supplementation on modulation of miRNA (Drummond et al. 2009; Camera et al. 2016; Margolis et al. 2017; D'Souza et al. 2018). While the current study shows divergent responses in specific miRNA with or without carbohydrate supplementation, the mechanism that caused these differences is unclear. It is likely that higher expressions in CHO compared to CON were driven by alterations in insulin or glucose concentrations. However, the present study is limited in making this mechanistic distinction. Given the limited information available on nutrient manipulation and miRNA expression, future investigation is warranted to expand our understanding of the mechanistic impact that miRNA may have on the regulation of human skeletal muscle mass.

In conclusion, results from this study with human research participants expands upon previous findings from cell culture and animal research. Specifically, the findings from this study demonstrate that miR-19b-3p, miR-99a-5p, miR-100-5p, miR-222-3p, miR-324-3p, and miR-486-5p were more highly expressed in human skeletal muscle after aerobic exercise during which carbohydrate was consumed compared to after exercise during which no carbohydrate was consumed. These six miRNA target the PI3K-AKT-FOXO pathway. Alterations in markers of these pathways were confirmed by western blotting and RT-qPCR analysis. These data provide a new level of understanding of the mechanistic role of miRNA to facilitate skeletal muscle adaptations to exercise with carbohydrate and protein feeding.

Acknowledgments

The authors thank the volunteers that participated in this research experiment. The authors acknowledge our medical oversight team. Most importantly, we sincerely thank Mr. Adam Luippold, Allyson Derosier, Mr. Bradley Anderson, Mr. Grant Holmes, Mr. Anthony Karis, Ms. Katelyn Guerriere, Ms. Karleigh Bradbury, Ms. Laura

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Lutz, Drs. Stephen R. Hennigar, and Robert W Kenefick from the US Army Research Institute of Environmental Medicine for their significant contributions to the project.

Conflict of Interest

The authors have no conflicts of interests. The investigators adhered to the policies for protection of human subjects as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR part 219. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. This study was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine.

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Physiological Reports

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Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Variability in human plasma volume responses during high-altitude sojourn

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Keywords
Acclimatization, hemoconcentration, hypoxia, intravascular volume.

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Funding Information
Funding support for the study was provided by the US Army Medical Research and Materiel Command.

Received: 9 March 2019; Accepted: 13 March 2019

doi: 10.14814/phy2.14051

Physiol Rep, 7 (6), 2019, e14051, https://doi.org/10.14814/phy2.14051

Abstract

When sea-level (SL) residents rapidly ascend to high altitude (HA), plasma volume (PV) decreases. A quantitative model for predicting individual %ΔPV over the first 7 days at HA has recently been developed from the measurements of %ΔPV in 393 HA sojourners. We compared the measured %ΔPV with the %ΔPV predicted by the model in 17 SL natives living 21 days at HA (4300 m). Fasting hematocrit (Hct), hemoglobin (Hb) and total circulating protein (TCP) concentrations at SL and on days 2, 7, 13, and 19 at HA were used to calculate %ΔTCP and %ΔPV. Mean [95%CI] measured %ΔPV on HA2, 7, 13 and 19 was −2.5 [−8.2, 3.1], −11.0 [−16.6, −5.5], −11.7 [−15.9, −7.4], and −16.8 [−22.2, −11.3], respectively. %ΔPV and %ΔTCP were positively correlated ($P < 0.001$) at HA2, 7, 13, and 19 ($r^2 = 0.77, 0.88, 0.78, 0.89$, respectively). The model overpredicted mean [95% CI] decrease in %ΔPV on HA2 (−12.5 [−13.9, −11.1]) and HA7 (−21.5 [−23.9, −19.1]), accurately predicted the mean decrease on HA13 (−14.3, [−20.0, −8.7]), and predicted a mean increase in %ΔPV on HA19 (12.4 [−5.0, 29.8]). On HA2, 7, 13, and 19 only 2, 2, 6, and 1, respectively, of 17 individual measures of %ΔPV were within 95% CI for predicted %ΔPV. These observations indicate that PV responses to HA are largely oncotically mediated, vary considerably among individuals, and available quantitative models require refinement to predict %ΔPV exhibited by individual sojourners.

Introduction

Soon after people who live at sea level (SL) ascend to high altitude (HA), their plasma volume (PV) begins to decrease, causing hematocrit (Hct) and hemoglobin concentration (Hb) to increase. This hemoconcentration increases arterial oxygen carrying capacity (Sawka et al. 2000) thereby satisfying muscle oxygen requirements during submaximal aerobic exercise with a lower cardiac output (and less cardiovascular strain) that would be possible without this physiological adjustment. Collectively, these physiological adjustments developing early during altitude acclimatization contribute to the improvement in aerobic exercise endurance observed during the 1st or 2nd week of sojourns at HA (Maher et al. 1974; Horstman et al. 1980; Pandolf et al. 1998). However, these hematological adjustments also have the potential to influence the parameters used to generate the Athlete Biological Passport (Sottas et al. 2011) for athletes incorporating HA training in their conditioning program, such that a positive indication for blood doping might be calculated. Therefore, there is a need to reliably predict what an individual's "normal" PV response would be during sojourn at HA. Further, since PV changes and other fluid shifts between body compartments may influence performance (Robertson et al. 1982) at HA and/or susceptibility to HA illnesses (Gatterer et al. 2013), this prediction capability may be applicable and has utility for military personnel and others sojourning at altitude.

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A quantitative model has recently been described (Beidleman et al. 2017) for predicting the magnitude of PV changes (%ΔPV) that a HA sojourner would experience. The model was developed by regressing parameters describing the altitude ascended, duration of altitude exposure, age and sex of the altitude sojourner, and the interactions among those parameters on the %ΔPV observed in 393 unacclimatized, well-nourished men and women who had rapidly ascended to altitudes ranging from 2500 to 4500 m and remained for periods of 2 h to 7 days (most for 2–36 h) (Beidleman et al. 2017). The developers intended that the model would be used to describe the "normal" PV responses exhibited by SL athletes while training at HA, thus allowing identification of abnormal or unexpected responses that might have implications for performance or health during HA sojourns, and/or possibly indicating the use of banned blood doping practices (Beidleman et al. 2017). However, while that model was internally cross-validated using a boot-strapping resampling process, it has not been externally validated against %ΔPV measured in an independent sample of HA sojourners. Further, real-world sojourns at HA often last longer than 7 days, and sojourners often experience energy deficit and body mass losses (Rose et al. 1988; Butterfield 1996; Pasiakos et al. 2017). If the model could be shown reliable for predicting responses to altitude under those conditions, its utility would be extended.

The model for predicting %ΔPV during HA sojourns (Beidleman et al. 2017) does not include a parameter accounting for changes in total circulating protein (ΔTCP), but considerable evidence indicates that the PV reduction during HA sojourns is largely oncotically mediated. In SL residents who ascend to HA, total circulating protein (TCP) decreases, and the magnitude of the ΔTCP strongly correlates with the magnitude of the concomitant ΔPV (Sawka et al. 1996). The decrease in TCP results from protein translocation from intravascular to extravascular space (Surks 1966; Westergaard et al. 1970). Changes in the rate of breakdown of some plasma protein have also been suggested to contribute to the decrease in TCP (Westergaard et al. 1970).

Recently, we conducted a study investigating the effects of varied levels of dietary protein intake on body composition changes experienced by 17 healthy SL residents who ascended to 4300 m and remained living for 21 consecutive days at that altitude in negative energy balance (Berryman et al. 2018). Our study participants (Berryman et al. 2018) had similar characteristics to the population used to develop the recently published quantitative model (Beidleman et al. 2017), providing an opportunity to compare the %ΔPV between SL and HA measured in an independent population of participants to the %ΔPV predicted for these same participants using the model (Beidleman et al. 2017). Thus, the aim of this study was to determine how accurately the recently published quantitative model (Beidleman et al. 2017) for predicting changes in PV of well-nourished SL residents during the first 7 days after ascent to HA would predict changes in PV exhibited by SL residents during a 21-days high-altitude sojourn, under real-world conditions of energy deficit and weight loss. Since our participants were all men and they resided at a single altitude, effects of variations of those parameters on model predictions were avoided. We hypothesized that %ΔPV measured in our participants during the first 7 days at HA would be accurately predicted by the model, but changes measured after 7 days at altitude would not be as well predicted, as the effects of altitude acclimatization and negative energy balance on factors modulating Hb, Hct, and PV at altitude accumulated in our participants.

Methods

Participants and study design

The analyses reported herein were included as secondary objectives in a randomized, controlled feeding study that examined the effects of manipulating dietary protein on fat-free mass during HA sojourn (Berryman et al. 2018). Participants were 17 young, healthy, physically active male SL natives (Table 1). The study was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (Natick, MA), conducted May–Aug 2016, and registered on www.clinicaltrials.gov as NCT02731066. Investigators adhered to the policies for the protection of human participants as prescribed by Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

The study design has been reported in detail, previously (Berryman et al. 2018; Young et al. 2018). Briefly, the study consisted of two phases conducted over 43 consecutive days. Body mass was measured in undergarments, upon waking (overnight fast, ≥8 h), and after voiding, using a calibrated digital scale (Befour model PS6600;

Table 1. Prestudy characteristics of participants.

Characteristic	Mean ± SD
Age, y	23.4 ± 5.6
Height, cm	177 ± 7
Weight, kg	81.9 ± 13.9
Body mass index, kg/m^2	26.2 ± 3.6
Body fat, %	22.7 ± 6.1
$\dot{V}O_2$peak, L/min	4.17 ± 0.65

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Befour Inc., Saukville, WI) to the nearest 0.1 kg daily, throughout the study. During the first 21 days (phase 1, SL), participants resided at SL, consumed a self-selected, weight-maintaining diet, maintained habitual exercise routines, and were free living but visited the laboratory daily. Body composition was determined at SL (day 0) using dual energy X-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI). On SL day 21, participants were flown from Boston, MA to Denver, CO where they were placed on supplemental oxygen until being driven to the summit of Pikes Peak, CO (4300 m) the following morning (HA day 0) where they resided for the next 22 days at the U.S. Army Research Institute of Environmental Medicine Maher Memorial Laboratory (phase 2; HA). During HA, participants were under constant supervision, consumed a controlled and measured diet, and engaged in prescribed physical activity.

Study diets

The diets have been described in detail, previously (Berryman et al. 2018). Briefly, beginning on HA1, the first full day of residence at 4300 m, and continuing until they completed the HA phase of the study, participants consumed a controlled diet designed to induce weight loss which is common during HA sojourn (Pasiakos et al. 2017). Computer-generated randomization was used to assign participants to consume a diet containing either a standard-protein (SP; 1.1 ± 0.2 g/kg/d) or higher-protein (HP; 2.1 ± 0.2 g/kg/d), carbohydrate-matched diet during HA. Water and noncaloric, noncaffeinated sodas were allowed ad libitum.

Blood biochemistries

Blood samples were collected at SL (SL day 6), and on the 2nd, 7th, 13th and 19th full day of residence at 4300 m (HA2, HA7, HA13, and HA19, respectively). All blood samples were obtained by venipuncture after ≥ 20 min of seated rest, and following a >10 h fast. Hct and Hb concentrations were determined using an automated analyzer (i-STAT™, Abbott Point of Care Inc., Princeton, NJ). Serum protein and osmolality were determined using an automated analyzer (Beckman Coulter DXC 600 Pro, Brea, CA), with osmolality calculated from sodium (Na), glucose (glu) and blood urea nitrogen (BUN) concentrations [Osm = (1.86*Na) + (Glu/18) + (BUN/2.8) + 9] (Dorwart and Chalmers 1975).

Calculation of vascular volume changes

Each participant's lean body mass measured on SL day 0 was used to estimate their total blood volume at SL using the equation developed by Sawka et al. (1992). The Hct measured on SL day 6 was used to calculate the SL erythrocyte volume, and the corresponding PV was calculated as the difference between total blood and erythrocyte volumes. The % ΔPV on HA2, HA7, HA13, and HA19 relative to PV at SL was estimated from the differences in Hct and Hb concentrations using the Dill and Costill equation (Dill and Costill 1974). The absolute PV (and the absolute ΔPV) at each of the test days at HA was then calculated using the calculated absolute PV at SL, adjusted for %ΔPV on that test day. TCP was calculated as the product of serum protein concentration and absolute PV for each test day, and %ΔTCP was calculated as the difference between TCP at SL and TCP at each test day at HA, expressed at a % of the SL TCP.

Data analyses and statistical tests

Statistical analyses were performed using SPSS v.24. All data were checked for adherence to model assumptions and for outliers. All tests were two-sided and statistical significance was set at $P \leq 0.05$. A general linear model (i.e., repeated measures ANOVA) without covariates was used to examine main effects of time, diet, and their interactions on Hct, Hb, TCP, osmolality, and the vascular volumes. No main or interaction effects of dietary protein level were found on any parameter, so diet was removed as a factor and the analyses rerun. When significant main effects of time were observed, post hoc comparisons were conducted and *P*-values were adjusted using the Bonferoni correction.

The model developed by Beidleman et al. (2017) predicts %ΔPV as follows:

$$\%\Delta PV = 0.05 - 1.93(T) + 0.27(T)^2 - 2.82(A) - 0.58(A)(T) - 0.82(S) + 0.73(S)(T) + 0.064(A)(Y),$$

where T is time at altitude (days), A is altitude (km), S is sex (male = 0, female = 1), and Y is age (years). The predicted % ΔPV was calculated for each of our participants using the same value for altitude, 4.04 km [that is the "physiological" altitude at the Pikes Peak Laboratory based on usual barometric pressures recorded, and this was the altitude used by the model developers (Beidleman et al. 2017) for data collected at the Pikes Peak laboratory], and each individual participant's age and sex. We ran the prediction four times, varying the time at altitude value as appropriate to predict % ΔPV on HA2 ($T = 2$), HA7 ($T = 7$), HA13 ($T = 13$), and HA19 ($T = 19$). The upper and lower limits of the 95% confidence interval were also calculated for the model predictions at each time point, over the age range that encompassed our

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participants. Finally, the model was used to predict % ΔPV at each of those times for the participant group as a whole, using mean age of the participant group as the age value. The individual and group mean values of the measured % ΔPV were then compared to the upper and lower limits of the 95% confidence interval for the predicted % ΔPV, to determine whether they fell within those limits.

Results

Both Hct and Hb increased (main effect of time, $P \leq 0.001$) during the altitude sojourn (Table 2). The pairwise differences between SL and HA2 were not significant, but values at HA7, HA13, and HA19 were all greater ($P < 0.05$) than at SL. Plasma protein concentration (Fig. 1A) increased (main effect of time, $P \leq 0.001$) with ascent from SL to HA, with the difference significant at HA2 and thereafter, but protein concentrations did not differ among HA2, HA7, HA13, and HA19. Plasma osmolality (Fig. 1B) did not differ between trials (main effect of time, $P = 0.51$). Blood, erythrocyte, and PV at SL were 4.97 ± 0.65 L, 2.20 ± 0.32 L, and 2.78 ± 0.39 L, respectively (Table 3). PV (Fig. 2A) and TCP (Fig. 2B) both decreased after ascent from SL to HA (Fig. 2, main effect, $P \leq 0.01$) with the difference becoming larger as the sojourn continued. As indicated by the large standard deviations, there was considerable variability in these responses among participants.

The variability in the %ΔPV among the participants was not significantly correlated with absolute plasma volume at sea level (data not shown), but was strongly correlated with the variability in the %ΔTCP (Fig. 3) at HA2, 7, 13 & 19 ($r^2 = 0.77$, 0.88, 0.78, 0.89, respectively; $P \leq 0.01$). As reported in detail elsewhere (Berryman et al. 2018), participants total body mass loss was 7.9 ± 1.9 kg during the 21-day residence at HA, but the absolute changes in body mass (relative to SL) on HA2, HA7, HA13, and HA19 were not significantly correlated with the corresponding %ΔPV ($P \geq 0.13$) or %ΔTCP ($P \geq 0.21$) on those days.

The model overpredicted mean [95% CI] decrease in %ΔPV on HA2 (−12.5 [−13.9, −11.1]) and HA7 (−21.5 [−23.9, −19.1]), accurately predicted the mean decrease on HA13 (−14.3, [−20.0, −8.7]), and predicted an increase % ΔPV on HA19 (12.4 [−5.0, 29.8]), when mean measured % ΔPV still indicated a decrease in PV relative to SL (Fig. 4). Further, only 2, 2, 6, and 1 of 17 individual measures of % ΔPV on HA2, 7, 13 and 19, respectively, were within 95% CI for predicted %ΔPV.

Discussion

The primary aim of this study was to determine the extent to which a recently published quantitative model (Beidleman et al. 2017) for predicting changes in PV of well-nourished SL residents during the first 7 days following ascent to HA accurately predicted the changes in PV exhibited by SL residents on the 2nd, 7th, 15th and 19th days of a high-altitude sojourn, under real-world conditions of progressively accumulating energy deficit and weight loss (Butterfield 1996). Our primary findings indicate that the PV changes experienced by our participants exhibited much greater inter-individual variability than was predicted by the model, and the variability in the PV responses at HA appears to be mediated by changes in oncotic pressure associated with changes in TCP. These findings indicate that the model does not reliably predict the PV responses of individual HA sojourners, either during the first 7 days at altitude, or for longer sojourns,

Table 2. Hematocrit (Hct) and hemoglobin (Hb) concentrations at sea level (SL) and high altitude (HA).

			95% Confidence interval for mean		
	Day	Mean	Lower bound	Upper bound	Standard deviation
Hct, %	SL6	44.12[a]	42.74	45.50	2.69
	HA2	44.88[a]	43.46	46.30	2.76
	HA7	47.18[b]	45.75	48.60	2.77
	HA13	47.29[b]	46.12	48.47	2.28
	HA19	48.88[b]	47.48	50.29	2.74
Hb, g/100 mL	SL	15.00[a]	14.52	15.48	0.93
	HA2	15.26[a]	14.78	15.75	0.90
	HA7	16.05[b]	15.56	16.53	0.90
	HA13	16.08[b]	15.68	16.48	0.61
	HA19	16.61[b]	16.13	17.08	0.87

Hematocrit (Hct) and hemoglobin (Hb) at SL and HA (4300 m) over a 19 day sojourn (main effect of day, $P \leq 0.01$). Means not sharing the same superscript are significantly different (pairwise comparisons, $P \leq 0.04$).

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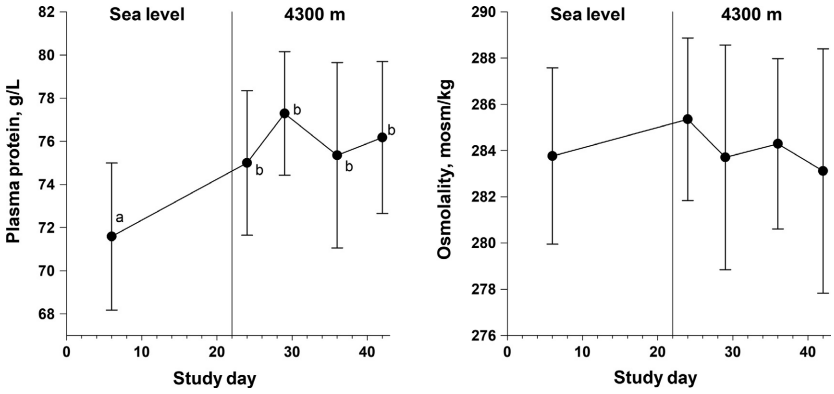


Figure 1. Mean ± SD of plasma protein concentration (Panel A) and osmolality (Panel B) measured in 17 male sea-level residents at sea level and after 2, 7, 13, and 19 days of continuous residence at high altitude (4300 m). Means not sharing the same superscript are significantly different (main effect of day, $P \leq 0.001$ and pairwise comparisons, all $P \leq 0.008$).

Table 3. Vascular volumes at sea level.

Participant	Lean mass[1], kg	Blood[2], L	Hematocrit[3], %	Erythrocyte[4], L	Plasma[5], L
1	51.4	4.28	41.00	1.76	2.53
2	70.9	5.69	42.00	2.39	3.30
3	78.5	6.24	45.00	2.81	3.43
4	60.1	4.91	44.00	2.16	2.75
5	62.8	5.11	45.00	2.30	2.81
6	59.2	4.85	45.00	2.18	2.67
7	57.8	4.75	47.00	2.23	2.52
8	52.0	4.33	43.00	1.86	2.47
9	51.2	4.27	43.00	1.84	2.44
10	65.8	5.32	40.00	2.13	3.19
11	48.2	4.06	47.00	1.91	2.15
12	56.9	4.68	49.00	2.29	2.39
13	70.4	5.65	46.00	2.60	3.05
14	50.6	4.22	41.00	1.73	2.49
15	59.3	4.85	45.00	2.18	2.67
16	72.8	5.83	47.00	2.74	3.09
17	68.3	5.50	40.00	2.20	3.30

[1]Lean mass determined by DEXA on sea level day 0; [2]total blood volume = 0.072 (lean mass) + 0.584 (Sawka et al. 1992); [3]hematocrit measured on sea level day 6; [4]erythrocyte volume = blood volume (hematocrit/100); [5]plasma volume = blood volume–erythrocyte volume.

suggesting that the model does not account for all factors modulating ΔPV in response to hypoxia.

The model (Beidleman et al. 2017) did not reliably predict PV responses of individual sojourners who had been at HA longer than 7 days, the maximum sojourn duration for subjects whose data were used to develop the model. The individual %ΔPV values of 11 of 17 and 16 of 17 participants in our study fell outside the 95% confidence interval for the predicted values on HA13 and HA19, respectively. This was not too surprising given that the model's developers had acknowledged that the quadratic equation used in their model was applicable to only the first 7 days at HA (Beidleman et al. 2017). However contrary to our hypothesis, the model (Beidleman et al. 2017) also did not reliably predict PV changes exhibited by our participants after 2 and 7 days of HA sojourn either, durations that are within the ranges of exposure durations for the participants whose data were used to develop the model. On both HA2 and HA7, individual % ΔPV values of 15 of 17 participants in our study fell outside the 95% confidence interval for the predicted values. The lack of agreement between observed and predicted changes in PV at these shorter exposure durations may reflect the same limitation as the discrepancy observed and predicted changes at the longer exposure durations. Specifically, hypoxic exposure durations were only 2–36 h

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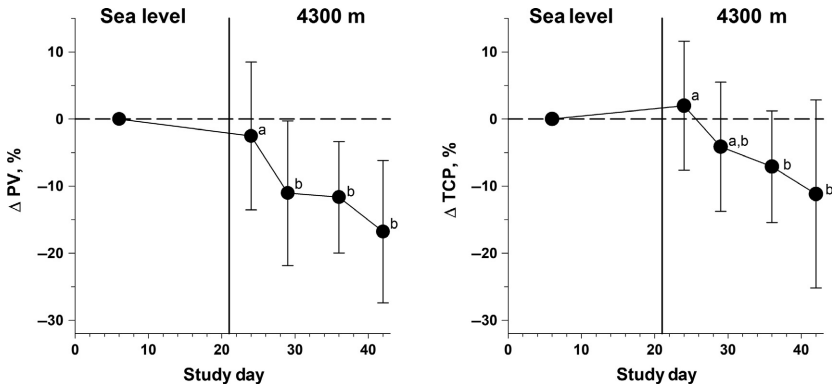


Figure 2. Mean ± SD of change in plasma volume (%ΔPV) and total circulating protein (%ΔTCP) measured in 17 male sea-level residents after 2, 7, 13, and 19 days of continuous residence at high altitude (4300 m). Means not sharing the same superscript are significantly different (main effect of day, $P \leq$ main effect of day 0.001 and pairwise comparisons all $P \leq 0.045$).

for well over half of the subjects whose data were used to develop the prediction model (Beidleman et al. 2017). Therefore, the parameter estimates developed for the model equation (Beidleman et al. 2017) may be more influenced by responses to relatively short hypoxic exposures (i.e., ≤36 h), making the model inherently less reliable for exposures ≥36 h). These findings indicate that the model needs to be refined by incorporating more data from longer altitude sojourns.

In agreement with previously reported findings (Sawka et al. 1996), we observed that at in all four trials during the 19-day sojourn at HA, the change in PV (relative to SL) in our participants was highly correlated with the concomitant change in TCP. The r^2 values for the relationship indicated that 80–90% of the variation in the change in PV between SL and HA were accounted for by variation in the TCP content. The exact mechanism by which hypoxic exposure mediates changes in TCP is not entirely clear. However, pulmonary vasculature is known to become more permeable to proteins at HA (Bartsch et al. 2005), so probably other vascular regions do as well. Further, Westergaard et al. (1970) observed that plasma albumin synthesis and breakdown rates remained unchanged in SL residents living at 3450 m for 8 days, but albumin content shifted from intravascular to extravascular space, while at the same time the rate of IgG breakdown was increased at HA. More recently, we have observed that whole body protein synthesis is decreased during chronic HA exposure (Berryman et al. 2018). Thus, both decreased synthesis of plasma protein and leakage of plasma protein into the extravascular space could be contributing to a decrease in TCP during hypoxic exposure. If these mechanisms could be better characterized and quantified, perhaps a parameter describing that aspect of the PV response to altitude exposure could be devised and added to model to improve its reliability for predicting individual PV changes during altitude sojourns.

The possibility that certain elements of our research approach influenced our findings needs to be considered. Our participants were part of a larger study evaluating the effects of high versus moderate levels of dietary protein content during energy deficit while sojourning at HA. Therefore, the possibility that the energy deficit or variation in dietary protein intake may have influenced the regulation of PV during the altitude sojourn cannot be entirely ruled out. However, such an effect seems unlikely since, as described earlier, there were no effects of dietary protein on any measured or derived parameter, and no correlations were observed between changes in body mass (compared to SL) at any of the four altitude trials and the changes in PV and TCP. Further, the dietary manipulations began on HA1, so any undetected effects of diet on our results would likely have been minimal on HA2. Also, while we encouraged participants to drink frequently and provided easy access to ample amounts of flavored beverages and water, we did not measure changes in total body water during the altitude sojourn. Nevertheless, plasma osmolality remained unchanged from SL values throughout the altitude sojourn, suggesting that the participants were not dehydrated. Finally, the changes in PV, from which the changes in TCP were derived, were calculated using the Dill-Costill equation (Dill and Costill 1974). This approach, which is similar to the approach used to assess plasma volume changes in the altitude sojourners studied by the developers of the model we were evaluating, assumes that total erythrocyte volume remains constant throughout the study. We have previously demonstrated that assumption is valid for at least the first 13 days of altitude acclimatization at 4300 m (Sawka et al. 1996). Moreover, since the maximal rate of erythrocyte volume

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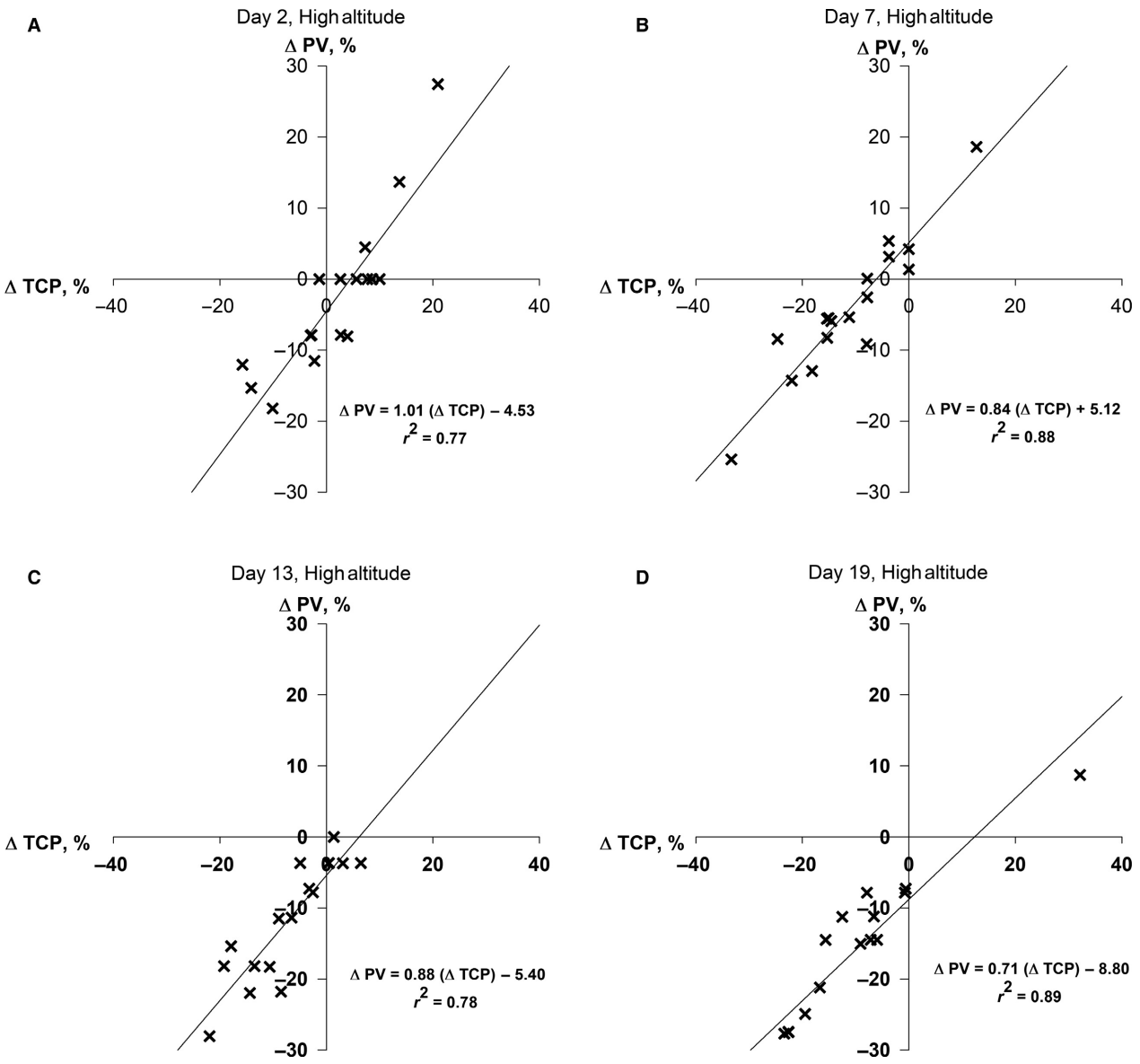


Figure 3. Individual changes in plasma volume (%ΔPV) plotted as a function of the concomitant changes in total circulating protein (%ΔTCP) measured in 17 male sea-level residents after 2, 7, 13, and 19 days (Panels A, B, C and D, respectively) of continuous residence at high altitude (4300 m).

expansion is 50 mL/week (Berglund and Ekblom 1991), erythrocyte volume in our participants (Table 3) could have increased no more than about 2% per week in our participants. Expansion of red cell mass of that magnitude would result in even smaller decreases in plasma volume on HA7, HA13, and HA19 than indicated by use of Dill-Costill equation, and the agreement between observed and model--predicted plasma volume changes would not be improved. Therefore, we believe use of the Dill-Costill equation (Dill and Costill 1974) does provide valid estimates of PV changes during our study.

The model that we investigated was developed to predict the "normal" PV response to HA (Beidleman et al. 2017). Therefore, it is appropriate to consider to what degree the PV responses that we observed reflect "normal" HA responses. Recently, Siebenmann et al. (2017) reviewed the reduction in PV observed at HA in 21 studies. The mean PV reductions exhibited by our group of participants on HA7, HA13, and HA19 (11–17%) appear similar in magnitude to studies of similar exposure durations at altitudes >4000 m [Fig. 1A in Siebenmann et al. (2017)]. On the other hand, the mean reduction in PV

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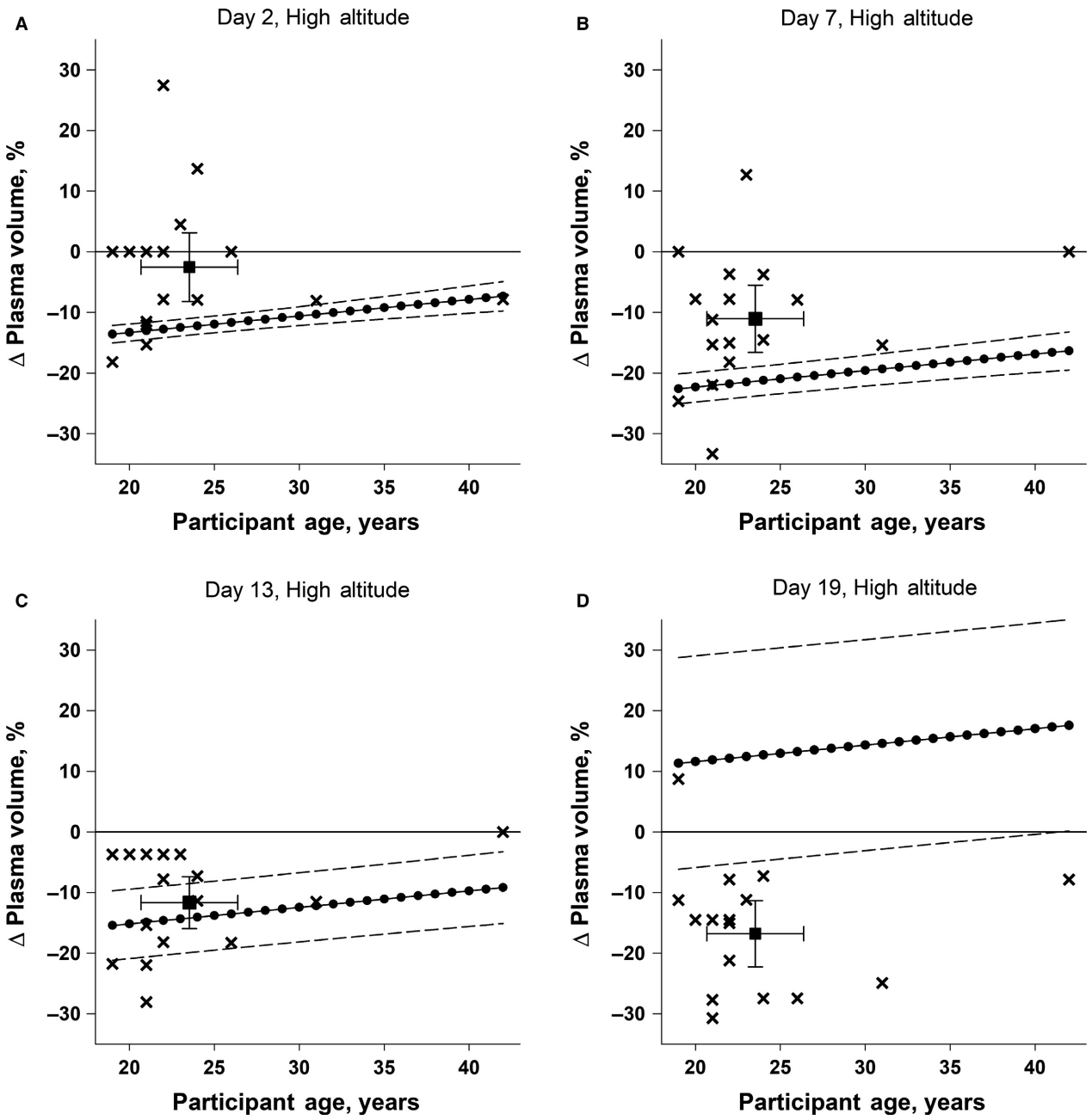


Figure 4. Individual (X) and mean ± 95% CI (filled square) of the % changes in plasma volume measured in 17 male sea-level residents after 2, 7, 13, and 19 days (Panels A, B, C, and D, respectively) of continuous residence at high altitude (4300 m) plotted as a function of the participants' ages. For each year of age across the age range of the participants, the model-predicted (Beidleman et al. 2017) change in plasma volume is also plotted (filled circles), with upper and lower bounds of 95% CI of the model predictions indicated (dashed lines).

exhibited by our participants on HA2 (2.5%) is somewhat less than observed in some, but not all of the studies reviewed by Siebenmann et al. (2017) that reported changes during the first 1–2 days at altitudes >4000 m. This might raise the question of whether our participant pool might have included a high prevalence of abnormal responders. However, we do not believe this is likely, since the mean PV changes (~8%) observed on the 1st day at Pikes Peak in control participants in a previous study conducted by Sawka et al. (1996) were within the 95% confidence interval of the mean PV reduction observed in the current study. Further, Sawka et al.

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(1996) observed that five of 16 participants exhibited no change in PV on the 1st day after arriving at Pikes Peak, whereas in our study six of 17 participants exhibited no change on the 2nd day at Pikes Peak. One factor that might account for the somewhat smaller PV reductions observed by Sawka et al. (1996) and in the current study is the physical activity of the subjects. Physical activity levels of participants in both the study by Sawka et al. (1996) and our current study (Berryman et al. 2018) were higher at HA than their habitual physical activity levels at SL. As discussed by Siebenmann et al. (2017), two factors appear to modulate the reduction in plasma volume at high altitude: (1) changes in oncotic pressure (as a result of changes in TCP) and (2) a hormonally mediated diuresis that increases loss of body water. Siebenmann et al. (2017) suggest that in HA sojourners who maintain high levels of physical activity, exercise may activate the renin-angiotensin-aldosterone and ADH systems such that diuresis is prevented or at least limited. In that case, only changes in oncotic pressure would be acting to reduce plasma volume, and the magnitude of the reduction might not be as pronounced as when hormonally mediated diuresis is contributing. Therefore, the variation in PV responses exhibited by our physically active participants at HA is likely to represent normal variation.

In conclusion, PV changes exhibited by sea-level residents during sojourn at HA vary considerably between individuals. The changes in PV experienced by physically active people during high-altitude sojourns are likely to be mediated primarily by changes in oncotic pressure, since the majority of the variability in the PV response to HA was accounted for by variability in the concomitant changes in TCP. Quantitative models currently available for predicting individual PV changes at HA (Beidleman et al. 2017) require further refinement to improve capability for identifying whether an individual's response is "normal" or indicative of the use of banned blood doping practices.

Acknowledgments

The authors thank the volunteers who participated in this research experiment. The authors acknowledge our medical oversight team and Stephen Muza for their support of this study. We wish to sincerely thank Allyson Derosier, Nancy Murphy, Katelyn Guerriere, Karleigh Bradbury, Adrienne Hatch, Ryanne Haskell, Laura Lutz, Alyssa Kelley, Marques Wilson, Christopher Carrigan, Adam Luippold, Bradley Anderson, Grant Holmes, Anthony Karis, Matthew Kominsky, Aaron Stein, Stephen R. Hennigar, Lee M. Margolis, John W. Carbone, Taylor Roth, and James P. McClung for their contributions to the project.

Conflict of Interest

The investigators adhered to the policies for protection of human subjects as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR part 219. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. The authors declare that they have no conflicts of interest.

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Metabolism Clinical and Experimental 97 (2019) 1–8

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Clinical Science

Exercising with low muscle glycogen content increases fat oxidation and decreases endogenous, but not exogenous carbohydrate oxidation

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ARTICLE INFO

Article history:
Received 6 March 2019
Accepted 10 May 2019

Keywords:
Fat oxidation
Fatty acid binding protein
Carnitine Palmitoyl transferase 1a
Pyruvate dehydrogenase activity
Peroxisome proliferator-activated receptors

ABSTRACT

Background: Initiating aerobic exercise with low muscle glycogen content promotes greater fat and less endogenous carbohydrate oxidation during exercise. However, the extent exogenous carbohydrate oxidation increases when exercise is initiated with low muscle glycogen is unclear.
Purpose: Determine the effects of muscle glycogen content at the onset of exercise on whole-body and muscle substrate metabolism.
Methods: Using a randomized, crossover design, 12 men (mean ± SD, age: 21 ± 4 y; body mass: 83 ± 11 kg; VO_{2peak}: 44 ± 3 mL/kg/min) completed 2 cycle ergometry glycogen depletion trials separated by 7-d, followed by a 24-h refeeding to elicit low (LOW; 1.5 g/kg carbohydrate, 3.0 g/kg fat) or adequate (AD; 6.0 g/kg carbohydrate, 1.0 g/kg fat) glycogen stores. Participants then performed 80 min of steady-state cycle ergometry (64 ± 3% VO_{2peak}) while consuming a carbohydrate drink (95 g glucose +51 g fructose; 1.8 g/min). Substrate oxidation (g/min) was determined by indirect calorimetry and ^{13}C. Muscle glycogen (mmol/kg dry weight), pyruvate dehydrogenase (PDH) activity, and gene expression were assessed in muscle.
Results: Initiating steady-state exercise with LOW (217 ± 103) or AD (396 ± 70; $P < 0.05$) muscle glycogen did not alter exogenous carbohydrate oxidation (LOW: 0.84 ± 0.14, AD: 0.87 ± 0.16; $P > 0.05$) during exercise. Endogenous carbohydrate oxidation was lower and fat oxidation was higher in LOW (0.75 ± 0.29 and 0.55 ± 0.10) than AD (1.17 ± 0.29 and 0.38 ± 0.13; all $P < 0.05$). Before and after exercise PDH activity was lower ($P < 0.05$) and transcriptional regulation of fat metabolism (FAT, FABP, CPT1a, HADHA) was higher ($P < 0.05$) in LOW than AD.
Conclusion: Initiating exercise with low muscle glycogen does not impair exogenous carbohydrate oxidative capacity, rather, to compensate for lower endogenous carbohydrate oxidation acute adaptations lead to increased whole-body and skeletal muscle fat oxidation.

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1. Introduction

The practice of initiating aerobic exercise with low muscle glycogen content has grown in popularity over recent years [1–5]. Lowering muscle glycogen through prolonged aerobic exercise and high fat, low carbohydrate feeding increases reliance on fat as a fuel source, while sparing endogenous carbohydrate during subsequent exercise bouts [6–11]. While this strategy optimizes fat oxidation, common nutritional guidance is to consume carbohydrate during prolonged exercise, particularly when muscle glycogen content is low, to sustain submaximal performance [12]. The limited number of studies [13–15] assessing the impact of pre-exercise muscle glycogen on exogenous carbohydrate oxidation have yielded conflicting results, reporting an increase [13], decrease [14], or no change [15] in exogenous carbohydrate oxidation. Given the inconsistencies in the current literature, as well as limited sample size ($n \leq 7$) and lack of direct muscle glycogen measurements in past studies [13–15], it is unclear if initiating exercise with low muscle glycogen impairs exogenous carbohydrate oxidative capacity. As carbohydrate intake is an important factor sustaining performance during exercise, understanding how pre-exercise muscle glycogen affects

Abbreviations: FAT, fatty acid translocase; HADHA, hydroxyacyl-CoA dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; PDH, pyruvate dehydrogenase; PGC-1α, Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1α; DEXA, dual energy x-ray absorptiometry; BL, baseline; GD, glycogen depletion; LOW, low glycogen; AD, adequate glycogen; PRE, pre steady-state exercise; POST, post steady-state exercise; VO_{2peak}, peak oxygen consumption; MRE, meal, ready-to-eat; VPDB, Vienna Pee Dee Belemnite; B2M, beta-2-microglobulin; FABP, fatty acid binding protein; CPT1a, Carnitine Palmitoyl transferase 1a; ACOX1, Acyl-CoA Oxidase 1; PPARδ, Peroxisome Proliferator-Activated Receptor-δ; PPARγ, Peroxisome Proliferator-Activated Receptor-γ; GSK3α, Glycogen Synthase Kinase-3α; GLUT4, Glucose Transporter type 4; HK2, Hexokinase 2.

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https://doi.org/10.1016/j.metabol.2019.05.003
0026-0495/Published by Elsevier Inc.

exogenous carbohydrate oxidation is integral to make appropriate fueling recommendations.

Skeletal muscle molecular adaptations are the primary mechanism regulating shifts in whole-body substrate oxidation when aerobic exercise is performed with low muscle glycogen [16–18]. To reduce carbohydrate oxidation when muscle glycogen is low, upregulation of pyruvate dehydrogenase kinase 4 (PDK4) [19] reduces glycolytic flux into the tricarboxylic acid cycle by suppressing pyruvate dehydrogenase (PDH) activity [20,21]. To compensate for lower carbohydrate oxidation there is an upregulation in transcription factors peroxisome proliferator activated receptors (PPARs) and PPAR gamma coactivator 1α (PGC-1α), which are central regulators of mitochondrial function and oxidative capacity [22,23]. These factors target and upregulate the transcription of fatty acid translocase (FAT), carnitine palmitoyl transferase 1a (CPT1a) and hydroxyacyl-CoA dehydrogenase (HADHA) when muscle glycogen content is low [19,24–26]. Alterations in transcriptional regulation of fatty acid uptake, transport, and oxidation contribute to greater whole-body fat oxidation [24]. However, when carbohydrate is consumed after aerobic exercise that produces low muscle glycogen content, the acute molecular adaptations governing substrate oxidation are suppressed compared to no or minimal carbohydrate intake [25,26]. Whether acute molecular adaptations that spare endogenous carbohydrate and enhance fat oxidation are maintained when carbohydrate is consumed during exercise initiated with low muscle glycogen content is undetermined.

Discordant results on exogenous carbohydrate oxidation from past studies [13–15], make it difficult to provide recommendations on carbohydrate fueling to sustain physical performance when muscle glycogen is low at the onset of exercise. Additionally, lack of direct measurement of muscle glycogen and acute molecular adaptations limit the interpretation of results from these previous studies. As such, the purpose of this study was to determine if there is a difference in exogenous carbohydrate oxidation when steady-state aerobic exercise is initiated with low or adequate muscle glycogen concentrations. We hypothesized that exogenous carbohydrate oxidation would not be different, and exogenous carbohydrate oxidation would be lower when exercise is performed with low versus adequate glycogen content. Furthermore, the current study sought to examine molecular adaptations to link mechanistic alterations within skeletal muscle to changes in whole-body substrate oxidation.

2. Methods

2.1. Participants

Participants in this study were part of a larger investigation assessing the impact of the influence of muscle glycogen content on microRNA expression. However, analysis of exogenous carbohydrate oxidation was set as a separate objective from microRNA analysis prior to data collection (www.clinicaltrials.gov; NCT03250234). Twelve healthy, nonobese, recreationally active men between the ages of 18–39 years were recruited to participate in this randomized, crossover study. Individuals were excluded from analysis if they were not in good health, refused to abstain from use of alcohol, nicotine, and dietary supplement use during the study, had musculoskeletal injury that compromised ability to exercise, or donated blood with 8 weeks of beginning the study. One participant identified as using tobacco products prior to the start of the study. Information was not captured on the type, frequency, or amount of tobacco product use. While cessation of smoking can impact substrate metabolism [27], data from this participant did not appear as an outlier and directional changes in their data were consistent with change in overall group mean differences between LOW and AD treatments. After providing written informed consent, height was measured to the nearest 0.1 cm using a stadiometer (Seritex, Inc., Carlstadt, NJ, USA), and body mass was measured after a 10-hr overnight fast, using a calibrated digital scale (WB-110A, Tanita, Tokyo, Japan) to the nearest 0.1 kg. Body composition was determined using dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI, USA). These data were used for participant descriptive characteristics (age: 21 ± 4 y; height: 1.8 ± 0.1 m; body mass: 83 ± 11 kg; body mass index: 26 ± 2 kg/m^2; fat mass: 19 ± 10 kg; fat-free mass: 63 ± 9 kg; VO_{2peak}: 44 ± 3 mL/kg/min). All data collection took place at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA), between August 2017 to May 2018. This study was approved by the Institutional Review Board at the US Army Medical Research and Materiel Command (MRMC, Fort Detrick, MD).

2.2. Study Design

To normalize glycogen content, 48 h prior to testing, participants completed a glycogen depletion protocol by cycling at various intensities until failure [28], followed by ingestion of a controlled diet to replete glycogen content (Fig. 1). At the conclusion of the repletion phase, a baseline (BL) muscle biopsy was obtained from the vastus lateralis under local anesthesia (lidocaine) after a 10-h overnight fast. Participants then completed the same glycogen depletion (GD) protocol, immediately followed by a muscle biopsy. Participants then consumed a controlled diet designed to elicit low (LOW) or adequate (AD) glycogen stores for 24 h. Participants returned to the laboratory the following morning after a 10-h overnight fast, and performed 80 min of steady-state cycle ergometry (64 ± 3% VO_{2peak}) while ingesting 146 g of carbohydrate (95 g glucose +51 g fructose; 1.8 g/min). Substrate oxidation during exercise was determined by indirect calorimetry and tracer techniques (^{13}C-glucose and ^{13}C-fructose). Muscle biopsies were obtained before (PRE) and after (POST) steady-state exercise. Following a minimum 7-d washout period, participants returned to the laboratory to complete the second arm of the investigation. Treatment (LOW vs. AD) order was randomized using a random numbers generator to avoid order bias.

2.3. Glycogen Depletion Protocol

Exercise intensities were based on peak oxygen uptake (VO_{2peak}) determined using a progressive-intensity cycle ergometer (Lode, BV, Netherlands) test and an indirect, open circuit respiratory system (True Max 2400, Parvomedics, Sandy, Utah, USA). To deplete muscle glycogen, participants completed 2 min of high-intensity cycling (work period) at 90% VO_{2peak}, followed by a 2-min recovery period cycling at 50% VO_{2peak} [28]. This work-to-recovery ratio was maintained until the participant was no longer able to complete 2 min of cycling at 90% VO_{2peak}. Cycling intensity during the work period was progressively lowered to 80%, 70%, and 60% VO_{2peak} when the participant was unable to complete 2 min of cycling at the given workload. Once the participant could not complete 2 min of cycling at 60% VO_{2peak}, exercise was stopped. The recovery period was maintained at 50% VO_{2peak}. Participants performed two practice sessions to ensure they were familiar with the protocol before testing.

2.4. Study Diets

Dietary intake was controlled for 48 h before testing began, to ensure participants presented for both trials with similar glycogen stores. Participants were provided all food and beverages (except water), consuming on average 5.7 ± 0.6 g/kg/d carbohydrate, 1.2 ± 0.1 g/kg/d protein, and 1.0 ± 0.1 g/kg/d fat in both study arms during the normalization phase. On glycogen depletion protocol days, participants consumed a high fat or high carbohydrate diet for 24 h to elicit LOW or AD glycogen stores at the start of steady-state exercise (Table 1). Study dietitians prepared and administered all meals, derived from Meal, Ready-to-Eat (MRE; Ameriqual, Evansville, IN, USA) combat rations and commercially available food items. Participants returned all

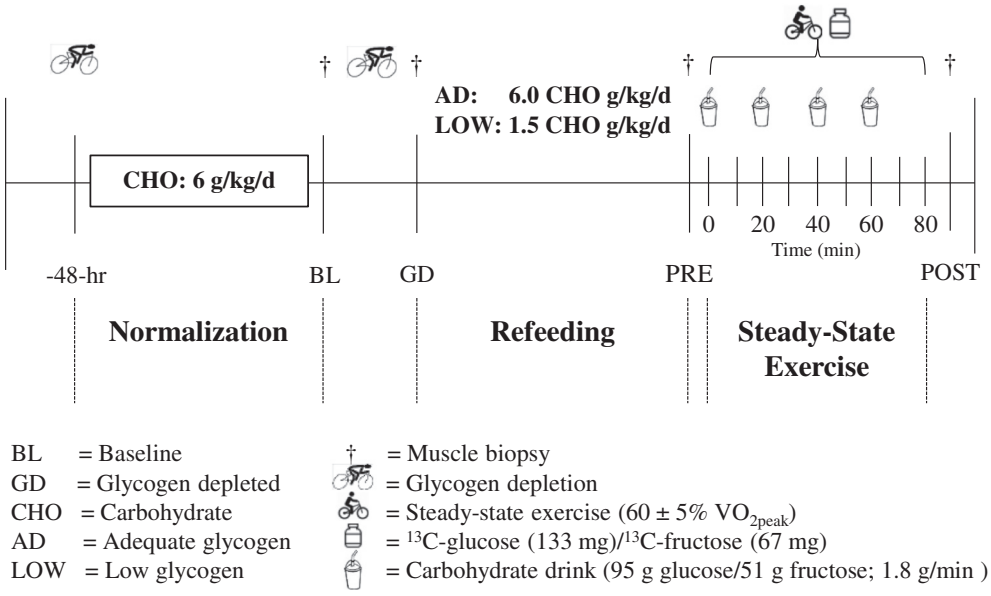


Fig. 1. Study design.

wrappers to dietitians to confirm intake. Participants abstained from nicotine and alcohol use during the study.

2.5. Steady-State Exercise Protocol

Participants ingested 146 g total carbohydrate (95 g glucose +51 g fructose), dissolved in 1450 mL of water, at an average ingestion rate of 1.8 g/min. The natural enrichment of the glucose-fructose drink was −11.10‰ δ ^{13}C versus internationally accepted standard for carbon isotope measures, Vienna Pee Dee Belemnite (VPDB). The carbohydrate drink was enriched with 200 mg of ^{13}C-glucose (133 mg) and ^{13}C-fructose (67 mg; Cambridge Isotope Laboratory, Andover, MA, USA) to match the ratio of glucose and fructose in the drink to achieve a final enrichment of 104.92‰ δ ^{13}C versus VPDB [29]. Isotope enrichment was used to ensure a significant increase in isotopic enrichment above natural abundance levels to allow for optimal measurement of exogenous carbohydrate oxidation. Immediately before starting the exercise bout, participants ingested 550 mL of the carbohydrate drink. Participants then began cycling for 80 min at their target VO_2 (64 ± 3% VO_{2peak}). Participants consumed 300 mL of the carbohydrate drink at 20, 40, and 60 min during exercise. The carbohydrate drink was prepared by the Combat Feeding Directorate (Natick, MA, USA) contained corn-derived crystalline fructose (KRYSTAR® 300, Tate and Lyle Sugars, London, UK), maltodextrin (MALTRIN QD® M500, Grain Processing Corporation, Muscatine, IA, USA) and dextrose (CERELOSE®, Ingredion, Westchester, IL, USA). Nutrient content was confirmed before use using gas chromatography (Covance Laboratories, Inc., Madison, WI, USA).

Respiratory gas exchange was measured during steady-state exercise using indirect calorimetry (Parvo Medics). Measurements were collected at 0, 15, 30, 45, 60, and 75 min during exercise. Breath samples were collected at 0, 20, 40, 50, 60, 65, 70, 75, and 80 min during exercise to assess expired ^{13}C (Quin-Tron Instrument Company, Milwaukee, WI, USA). Heart rate was monitored throughout the exercise bout (Polar Electro, Inc., Oulu, Finland).

2.6. Substrate Metabolism

Total carbohydrate and fat oxidation were calculated as [30]:

Fat oxidation (g/min) = $1.695 \times VO_2$ (L/min) − $1.701 \times VCO_2$ (L/min).

Total carbohydrate oxidation (g/min) = $4.585 \times VCO_2$ (L/min) − $3.226 \times VO_2$ (L/min).

Measurement of $^{13}C/^{12}C$ in expired CO_2 were measured using isotope-ratio mass spectroscopy (Metabolic Solutions, Inc., Nashua,

Table 1
Diet intervention to induce low (LOW) or adequate (AD) muscle glycogen.

	LOW	AD	*P* value
Absolute			
Energy (kcal/d)	3081 ± 374	3086 ± 347	0.77
Carbohydrate (g/d)	125 ± 18	498 ± 56	< 0.01
Protein (g/d)	106 ± 13	98 ± 11	< 0.01
Fat (g/d)	256 ± 30	86 ± 11	< 0.01
Relative			
Energy (kcal/kg/d)	37.1 ± 0.6	37.1 ± 0.7	0.87
Carbohydrate (g/kg/d)	1.5 ± 0.1	6.0 ± 0.2	< 0.01
Protein (g/kg/d)	1.3 ± 0.5	1.2 ± 0.5	< 0.01
Fat (g/kg/d)	3.0 ± 0.5	1.0 ± 0.5	< 0.01
Percent Total Energy			
Carbohydrate (%)	16 ± 1	65 ± 1	< 0.01
Protein (%)	14 ± 1	13 ± 1	< 0.01
Fat (%)	72 ± 1	25 ± 1	< 0.01

Values mean ± SD, n = 12.

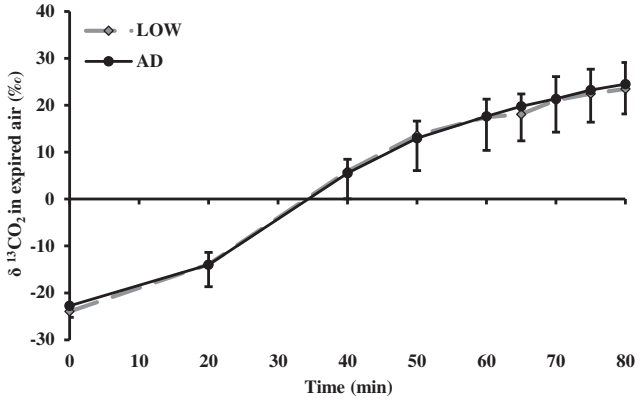


Fig. 2. Expired δ ^{13}C versus VPDB (Vienna Pee Dee Belemnite) during steady-state cycling, while consuming 1.8 g/min carbohydrate (95 g glucose +51 g fructose) enriched with ^{13}C-glucose (133 mg) and ^{13}C-fructose (67 mg) with low (LOW) or adequate (AD) glycogen stores. Values mean ± SD, *n* = 12.

NH, USA). The isotopic enrichments were expressed as ‰ δ versus VPDB (Fig. 2). Exogenous carbohydrate oxidation was calculated as:

$$\text{Exogenous carbohydrate (g/ min)} = VCO_2\,[(Rexp - Rref)/(Rexo - Rref)]/k$$

where VCO_2 is in L/min, Rexp is the observed isotopic composition of expired CO_2, Rref is the isotopic composition of expired CO_2 at rest before ingestion of the first dose of ^{13}C-glucose and ^{13}C-fructose, Rexo is the isotopic composition of the exogenous glucose ingested, and k (0.747 L/g) is the volume of CO_2 provided by the complete oxidation of glucose. Endogenous carbohydrate oxidation was calculated by subtracting exogenous carbohydrate oxidation from total carbohydrate oxidation. The first 40 min of steady-state exercise were used to allow for equilibration between the $^{13}C/^{12}C$ in expired CO_2 and the $^{13}C/^{12}C$ in CO_2 produced in tissues [31].

2.7. Muscle Analysis

Glycogen concentration was determined in ~3 mg (dry weight) freeze dried muscle homogenized in water using a TissueLyser II with a 5-mm steel bead (Qiagen, Valencia, CA, USA). Homogenates were boiled at 100 °C for 5 min and centrifuged at 13,000×*g* for 5 min at room temperature. Supernatant was removed and muscle glycogen concentrations were assessed using an endpoint colorimetric assay (Cat# MAK016; Sigma-Aldrich, St. Louis, MO, USA).

Pyruvate dehydrogenase (PDH) activity was determined in ~20 mg muscle samples homogenized in ice-cold homogenization buffer (1:10 *w*/*v*) [32]. Homogenates were centrifuged at 10,000×*g* for 15 min at 4 °C. Supernatant (lysate) was collected and protein concentrations were determined using the 660 nm Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). PDH activity was assessed in lysates using a kinetic colorimetric assay (Cat# MAK183; Sigma-Aldrich). Plates were run at 37 °C with reads every 5 min for 60 min or until activity plateaued. PDH activity was normalized to time of run and sample protein concentrations for final analysis.

Expression of genes regulating carbohydrate and fat oxidation were determined from total RNA isolated in ~20 mg muscle samples using TRIzol reagent (Thermo Fisher). RNA quantity and quality were assessed using a Nanodrop ND-2000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA (500 ng) were reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) conducted in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplifications were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). Samples were run in 10 μL reactions in duplicates using TaqMan® fast advanced master mix and commercially available TaqMan® probes (Applied Biosystems; **Supplemental Table 1**). All mRNA were normalized to B2M. Fold changes for PRE and POST steady-state exercise were calculated using the ΔΔ cycle threshold (ΔΔCT) method [33] and expressed as fold change relative to individual BL values.

2.8. Statistical Analysis

Statistical power and sample size were determined based on the primary outcome of this investigation, influence of carbohydrate availability on microRNA expression. A sample size of 9 was determined using a mean and standard deviation of 1.5 ± 1 fold change based on recent publications assessing change in microRNA expression in skeletal muscle [34–36], with an alpha of 0.05 and 80% power. A total of 12 participants were requested to account for potential inter-individual variance. Based an effect size of 1.1 ($n = 6$) for differences in exogenous carbohydrate oxidation from Pérronet et al. [13], a samples size of 12 provided ~95% power to detect differences between treatments in the current study.

Paired *t*-tests were used to assess differences in energy and macronutrient intake, exercise intensity for glycogen depletion, and steady-state exercise between treatments (LOW vs. AD). Mixed-model repeated measures ANOVA was used to assess substrate oxidation, glycogen, PDH activity and mRNA expression for effects of time, treatment, and their interactions. Bonferroni adjustments for multiple comparisons were performed if significant interactions were observed. All data are presented as mean ± SD. The α level for significance was set at $P < 0.05$. Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp. Armonk, NY, USA).

3. Results

3.1. Glycogen Depletion and Steady-State Exercise Intensity

There were no differences in total exercise time (LOW: 84 ± 25, AD: 88 ± 24 min), mean power (LOW: 164 ± 26, AD: 161 ± 25 watts), and heart rate (LOW: 159 ± 14, AD: 159 ± 13 BPM) between treatments for glycogen depletion cycle ergometry. There was no differences in percent VO_{2peak} (LOW: 65 ± 4, AD 64 ± 3%), power (LOW: 138 ± 19, AD: 135 ± 21 watts), and heart rate (LOW: 147 ± 8, AD: 147 ± 12 BPM) between treatments during steady-state cycle ergometry.

3.2. Muscle Glycogen

Muscle glycogen concentrations were not different at BL and declined ($P < 0.05$) similarly at GD in both LOW and AD (Fig. 3). Following 24 h refeeding, PRE steady-state glycogen was lower ($P < 0.05$) in LOW than AD. POST steady-state glycogen in AD was lower ($P < 0.05$) than PRE but remained higher ($P < 0.05$) than LOW.

3.3. Substrate Oxidation

Fat oxidation was higher ($P < 0.05$) in LOW (0.55 ± 0.10 g/min) than AD (0.38 ± 0.13 g/min; Fig. 4A), independent of time. Total carbohydrate oxidation was lower ($P < 0.05$) in LOW (1.59 ± 0.40 g/min) than AD (2.03 ± 0.36 g/min; Fig. 4B), regardless of time. Exogenous carbohydrate oxidation was not different between LOW (0.84 ± 0.14 g/min) or AD (0.87 ± 0.16 g/min; $P > 0.05$), and increased similarly in both treatments over time during steady-state exercise (Fig. 4C). Independent of time, endogenous carbohydrate oxidation was lower in LOW (0.75 ± 0.29 g/min) than AD (1.17 ± 0.29 g/min; $P < 0.05$), but decreased ($P < 0.05$) similarly in both treatments over time during steady-

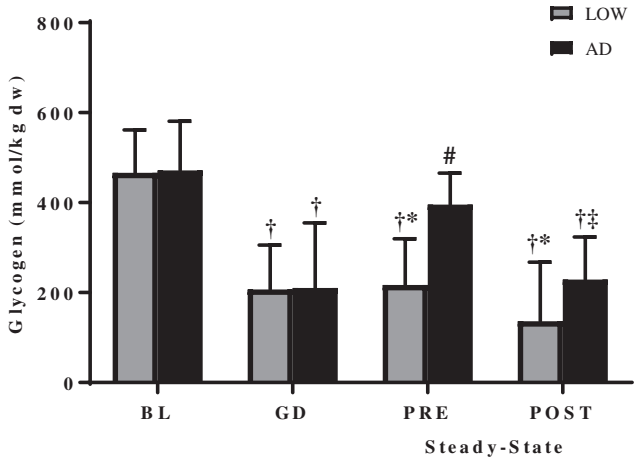


Fig. 3. Glycogen concentration at baseline (BL), after glycogen depletion (GD) and, before (PRE) and after (POST) steady-state cycling following 24-hr refeeding designed to elicit low (LOW) or adequate (AD) glycogen stores. Values mean ± SD, $n = 11$. †Different than PRE glycogen depletion, $P < 0.05$. ‡Different than PRE steady-state, $P < 0.05$. #Different than POST glycogen depletion, $P < 0.05$. *Different than AD, $P < 0.05$.

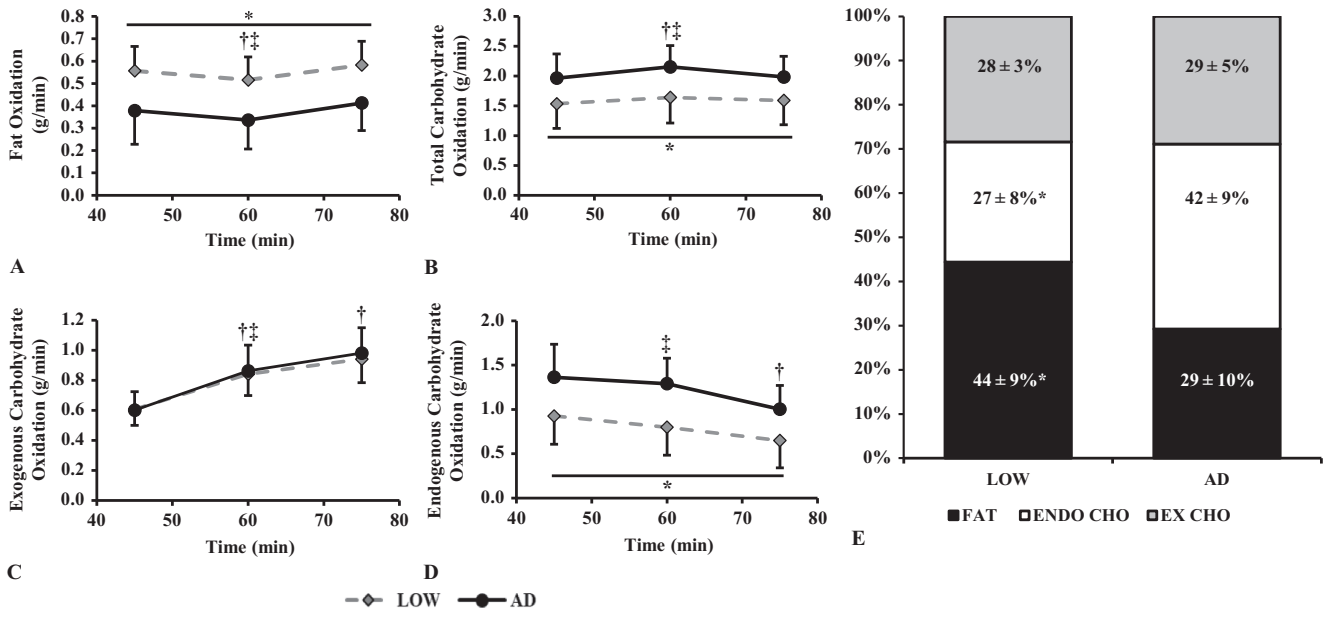


Fig. 4. Oxidation rate for fat (A), total carbohydrate (B), exogenous carbohydrate (C), and endogenous carbohydrate (D) at 45, 60, and 75 min during steady-state cycling with low (LOW) or adequate (AD) glycogen stores. Percent contribution of fat (FAT), endogenous (ENDO), and exogenous (EXO) carbohydrate (CHO) to total energy expenditure during the last 40 min of exercise. Values mean ± SD, n = 12. †Different than 45 min, $P < 0.05$. ‡Different than 75 min, $P < 0.05$.*Different than AD, $P < 0.05$.

state exercise (Fig. 4D). As a percentage of exercise-induced energy expenditure, fat constituted a higher percentage and endogenous carbohydrate a lower percentage in LOW than AD, with no difference in contribution of exogenous carbohydrate (Fig. 4E).

3.4. Acute Molecular Adaptations

Regardless of time, PDH activity was lower ($P < 0.05$) in LOW (PRE: 171 ± 102, POST 235 ± 216 nmol/μg protein/min) compared to AD (PRE: 366 ± 145, POST: 447 ± 347 nmol/μg protein/min) for steady-state exercise. PRE steady-state GSK3α was lower ($P < 0.05$) in LOW than AD (Fig. 5A). PRE and POST steady-state GSK3α were higher ($P < 0.05$) than BL in AD, while there was no difference in time in LOW. PRE and POST steady-state FAT were higher ($P < 0.05$) in LOW than AD (Fig. 5B). PRE and POST steady-state FAT were higher ($P < 0.05$) than BL in LOW, with no difference in time in AD. Independent of time, FABP, CPT1a, and HADHA were higher ($P < 0.05$) in LOW than AD (Fig. 5C-E). PRE and POST steady-state FABP, CPT1a, and HADHA were higher ($P < 0.05$) than BL, regardless of treatment. POST steady-state PPARδ was higher ($P < 0.05$) than BL and PRE in LOW, while POST steady-state PPARδ was higher ($P < 0.05$) than BL in AD, but not different than PRE (Fig. 5F). Regardless of treatment, PRE and POST HK2, PGC-1α, and ACOX1 were higher ($P < 0.05$) than BL (Table 2). There were no differences in treatment or time for GLUT4 and PPARγ.

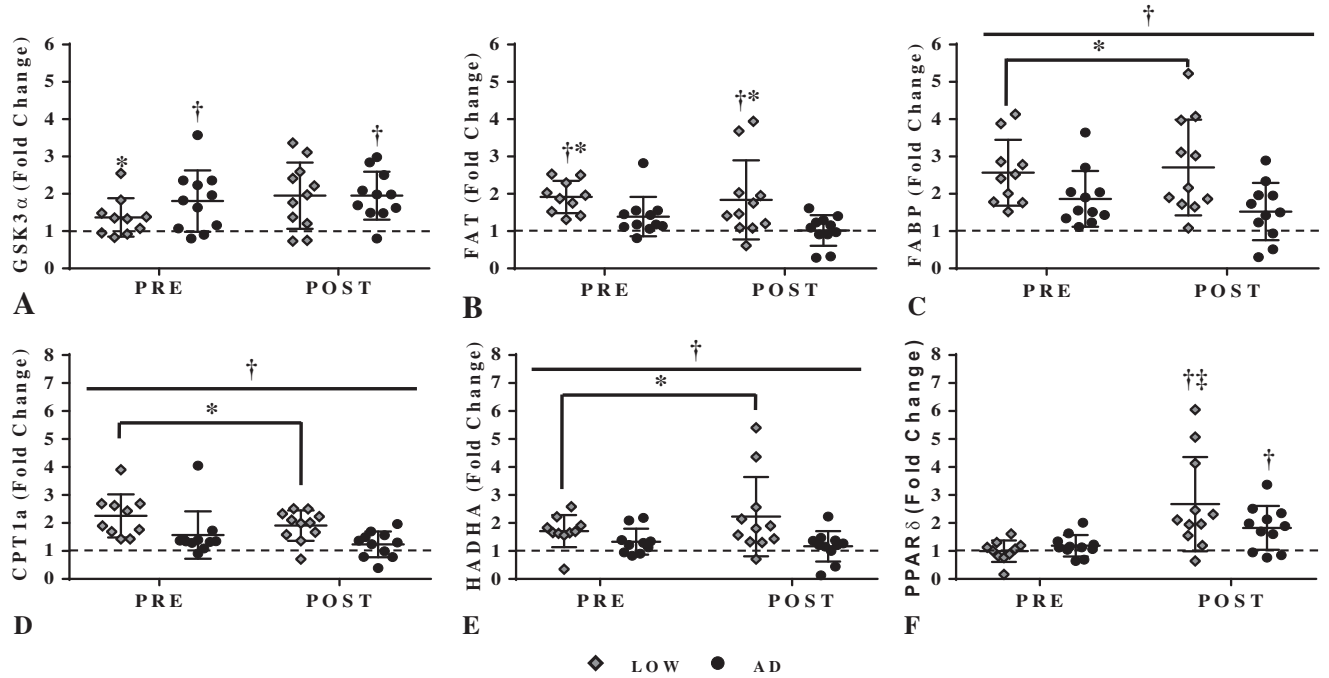


Fig. 5. Fold change before (PRE) and after (POST) steady-state for GSK3α (A) FAT (B), FABP (C), CPT1a (D), HADHA (E), and PPARδ (F) relative to baseline values (dotted line) with low (LOW) or adequate (AD) glycogen stores. Values mean ± SD, n = 11. †Different than baseline, $P < 0.05$. ‡Different than PRE, $P < 0.05$. *Different than AD, $P < 0.05$.

Table 2
Gene expression.

Gene	LOW		AD		*P* value		
(Fold Change)	PRE	POST	PRE	POST	Time	Treatment	T × T
GLUT4	1.40 ± 0.55	1.18 ± 0.56	1.27 ± 0.51	1.05 ± 0.58	0.24	0.67	0.77
HK2	2.62 ± 1.46	4.37 ± 2.99	3.01 ± 1.75	4.37 ± 2.97	< 0.01	0.94	0.59
PGC-1α	1.79 ± 0.75	2.01 ± 1.08	1.38 ± 0.51	1.71 ± 1.44	< 0.01	0.30	0.45
PPARγ	1.23 ± 0.51	1.54 ± 0.98	1.57 ± 0.64	1.47 ± 0.56	0.06	0.38	0.27
ACOX1	1.70 ± 0.33	1.37 ± 0.29	1.66 ± 0.57	1.30 ± 0.45	< 0.01	0.73	0.97

Values mean ± SD, n = 12. PRE and POST steady-state exercise data presented as fold change relative to baseline (BL). Time main effects, both PRE and POST were different than BL ($\underline{P} < 0.05$) but not different from one another. T × T, time-by-treatment interaction.

4. Discussion

The primary outcome from this study was that initiating steady-state aerobic exercise with low versus adequate muscle glycogen content did not hinder exogenous carbohydrate oxidative capacity, but did result in reduced reliance on endogenous carbohydrate, while increasing utilization of fat as a fuel source during exercise. Alterations in whole-body oxidation were driven by acute molecular adaptations resulting in reduction in PDH activity and an upregulation in skeletal muscle transcriptional control of fatty acid uptake, transport, and oxidation. These findings show that when muscle glycogen content is low, consuming carbohydrate may be used as a fueling strategy during to prolong submaximal exercise without impairing adaptations to enhance whole-body and skeletal muscle fat metabolism.

Results from the current investigation show that rates of exogenous carbohydrate oxidation are not dependent on muscle glycogen content. Three past studies assessing the influence of muscle glycogen content of exogenous carbohydrate oxidation during exercise had conflicting results, reporting an increase [13], decrease [14], or no change [15] in oxidation. Discrepancies between studies could likely be attributed to differences in exercise intensity (> or <60% VO_{2peak}), timing of carbohydrate intake (before or during exercise), participants training status (recreational versus highly trained) and method to reduce muscle glycogen content (exercise plus diet manipulation or exercise alone). Of these works, the current investigation most resembles the design used by Péronnet et al. [13], which manipulated glycogen content with exercise followed by 2 days of high carbohydrate or high fat feeding. Participants in the Péronnet study then performed 120 min of steady-state exercise at 64% VO_{2max} while consuming 200 g (1.7 g/min) of carbohydrate [13]. Primary result was reported that oxidation of exogenous carbohydrate was increased by 8% when participants performed steady-state exercise with low muscle glycogen content [13]. However, alteration in exogenous carbohydrate oxidation were driven by differences observed between 40 and 80 min, with no treatment effect reported from 80 to 120 min in the exercise bout [13]. Moreover, the overall contribution of exogenous carbohydrate as a percent of exercise-induced energy expenditure was not different between the treatments, constituting ~18% and ~19% of total energy following the high carbohydrate or high fat diets, respectively [13]. Though some disparities exist, the overall results of this past work are in agreement with our study, suggesting exogenous carbohydrate oxidation is not influenced by muscle glycogen content at the onset of exercise.

Though not a direct measurement of carbohydrate oxidation, several past studies [6,7,37–40] have reported that initiating steady-state aerobic exercise with lower muscle glycogen content did not increase glucose rates of disappearance (uptake) and plasma glucose oxidation using stable isotope kinetic methodologies (6,6-$^2H_2$-glucose) in healthy participants. These past and present results indicate lower total carbohydrate oxidation in the current study are the result of lower muscle glycogen oxidation, due to lower glycogen content at the onset of exercise. To increase glucose rates of disappearance or plasma glucose oxidation during exercise, it appears that fat oxidation needs to be impaired. Zderic et al. [41] blunted fat oxidation by having participants consume a β-adrenergic receptor block [42] and complete 60 min of steady-state aerobic exercise with low or normal muscle glycogen content. Impairment of fat oxidation with the β-adrenergic receptor block resulted in an increased rate of glucose disappearance when muscle glycogen content was low compared to normal glycogen. Together, past and present findings indicate that healthy individuals possess the metabolic flexibility to primarily adapt to use fat as a fuel source when initiating steady-state aerobic exercise with low muscle glycogen, rather than increase glucose uptake into the cell to be used for fuel.

Corroborating metabolic shifts in whole-body substrate oxidation were reductions in skeletal muscle PDH activity and increased FAT, FABP, CPT1a, HADHA, and PPARδ expressions with low muscle glycogen, even when carbohydrate was consumed during exercise in the present study. Though there were marked increases in transcriptional regulators of fatty acid uptake, transport, and oxidation, upstream targets PGC-1α and PPARγ were not different between treatments. Overall, decreased PDH activity and increased transcriptional regulation of fat oxidation indicate these acute intramuscular adaptations were the primary mechanism altering whole-body substrate oxidation in our study. In agreement with findings from the current study, previous studies assessing the impact of changes in resting and post-exercise muscle glycogen on the regulation of fat oxidation demonstrated reductions in PDH activity [20] and increased FAT, CPT1a, and HADHA expression [19,24–26]. Contrary to results from the current study, past studies have shown that when carbohydrate is consumed during exercise, transcriptional regulators of fat oxidations are lower compared to a non-nutritive placebo [43–45]. The discrepancies between past and present findings are likely attributed to pre-exercise muscle glycogen content. Unlike the current study, in these previous studies [43–45], exercise was initiated with similar glycogen content between treatments. The fact that muscle glycogen content was lower at the onset of exercise, and remained lower post-exercise in LOW compared to AD in our study, likely accounted for the sustained increase in transcriptional regulation of fatty acid uptake, transport, and oxidation when carbohydrate was consumed during exercise.

While results from this investigation show agreement in treatment effects in both whole-body substrate oxidation and acute molecular adaptations in skeletal muscle, our study is not without limitations. Primarily, the lack of serial blood draws during exercise precludes analysis of changes in circulating glucose, insulin, lactate, glycerol, and free fatty acids over time and between treatments. Data on circulating substrates and hormones would have allowed for a more complete description of alterations in substrate metabolism.

In conclusion, initiating steady-state aerobic exercise with low versus adequate muscle glycogen content does not impair exogenous carbohydrate oxidative capacity. To compensate for lower endogenous carbohydrate oxidation and PDH activity, the primary adaptations to exercising with low muscle glycogen content appear to be enhanced regulation of whole-body and skeletal muscle fat oxidation. Results from this study indicate when muscle glycogen content is low, consuming carbohydrate during exercise can be used as a fueling strategy to

prolong submaximal exercise without impairing adaptations in whole-body and skeletal muscle fat metabolism.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.05.003.

Statement of Competing Interest

The investigators adhered to the policies for protection of human subjects as prescribed in Army Regulation 70–25, and the research was conducted in adherence with the provisions of 32 CFR part 219. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. The authors declare that they have no conflicts of interest.

Acknowledgements

The authors thank the volunteers that participated in this research experiment. The authors acknowledge our medical oversight team for their support of this study. Most importantly, we wish to sincerely thank Dr. Nicholas Barringer and Mr. Anthony Karis from the US Army Research Institute of Environmental Medicine, and Ms. Danielle Anderson from the Combat Feeding Directorate for their contributions to the project.

Funding

Supported in part by the US Army Medical Research and Materiel Command and appointment to the US Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education (to L.M.M.) through an interagency agreement between the US Department of Energy and the US Army Medical Research and Materiel Command.

Author Contributions

L.M.M., S.J.M., and S.M.P. designed research; L.M.M., M.A.W., C.C.W., C.T.C., N.E.M., and A.M.D. performed research; L.M.M., M.A.W., C.C.W., C.T.C., C.T.C., and N.E.M. analyzed data; L.M.M., S.J.M., and S.M.P. interpreted results; L.M.M., M.A.W., C.C.W., and S.M.P. prepared tables and figures; L.M.M. drafted the manuscript; and L.M.M., M.A.W., C.C.W., C.T.C., N.E.M., A.M.D., S.J.M. and S.M.P. approved final version.

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EBioMedicine 46 (2019) 411–422

Contents lists available at ScienceDirect

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Published by THE LANCET

Effects of testosterone supplementation on body composition and lower-body muscle function during severe exercise- and diet-induced energy deficit: A proof-of-concept, single centre, randomised, double-blind, controlled trial

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ARTICLE INFO

Article history:
Received 3 June 2019
Received in revised form 23 July 2019
Accepted 23 July 2019
Available online 27 July 2019

Keywords:
Hypothalamic-pituitary-gonadal axis
Hypogonadism
Military operational stress
Semi-starvation
Anabolism
Muscle mass
Lower-body muscle function

ABSTRACT

Background: Severe energy deficits during military operations, produced by significant increases in exercise and limited dietary intake, result in conditions that degrade lean body mass and lower-body muscle function, which may be mediated by concomitant reductions in circulating testosterone.
Methods: We conducted a three-phase, proof-of-concept, single centre, randomised, double-blind, placebo-controlled trial (CinicalTrials.gov, NCT02734238) of non-obese men: 14-d run-in, free-living, eucaloric diet phase; 28-d live-in, 55% exercise- and diet-induced energy deficit phase with (200 mg testosterone enanthate per week, Testosterone, n = 24) or without (Placebo, n = 26) exogenous testosterone; and 14-d recovery, free-living, ad libitum diet phase. Body composition was the primary end point; secondary endpoints included lower-body muscle function and health-related biomarkers.
Findings: Following energy deficit, lean body mass increased in Testosterone and remained stable in Placebo, such that lean body mass significantly differed between groups [mean difference between groups (95% CI), 2.5 kg (3.3, 1.6); P < .0001]. Fat mass decreased similarly in both treatment groups [0.2 (−0.4, 0.7), P = 1]. Change in lean body mass was associated with change in total testosterone (r = 0.71, P < .0001). Supplemental testosterone had no effect on lower-body muscle function or health-related biomarkers.
Interpretation: Findings suggest that supplemental testosterone may increase lean body mass during short-term severe energy deficit in non-obese, young men, but it does not appear to attenuate lower-body functional decline.
Funding: Collaborative Research to Optimize Warfighter Nutrition projects I and II, Joint Program Committee-5, funded by the US Department of Defence.
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1. Introduction

US military personnel conducting strenuous training and combat operations commonly experience sustained periods of severe, unavoidable energy deficit, the effects of which resemble the pathophysiology of

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https://doi.org/10.1016/j.ebiom.2019.07.059
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Research in context

Evidence before this study

PubMed was searched for clinical trials published in English up to August 1, 2018, using search terms that included "weight loss", "starvation", "testosterone", "hypogonadism", "young men", "non-obese", "energy deficit", "military", and "muscle mass" alone or in combination. There have been no studies that have assessed whether restoring testosterone minimizes the catabolic effects of severe exercise- and diet-induced energy deficit in non-obese, young men reflective of military personnel participating in strenuous military operations.

Added value of this study

This proof-of-concept, single centre, randomised, double-blind, placebo controlled trial is the first, to our knowledge, assessing the efficacy of supplemental testosterone on body composition, muscle function, and clinical health biomarkers in healthy, non-obese, young men exposed to 28 d of severe exercise- and diet-induced energy deficit representative of strenuous military training and combat operations. Our study was rigorously controlled, particularly during the 28 d intervention phase, as diet and physical activity were individualized and continuously supervised on the inpatient unit at the Pennington Biomedical Research Centre. Despite the 28 d severe exercise- and diet-induced energy deficit, individuals receiving weekly injections of supplemental testosterone experienced an increase in lean mass and an attenuation in total weight loss compared to controls, with no difference in lower-body muscle functional decline or clinical health biomarkers.

Implications of all available evidence

Short-term (4 wk) use of supplemental testosterone minimizes weight loss and promotes lean mass gain in non-obese, young men exposed to military-relevant exercise- and diet-induced energy deficit, although the lean mass gain did not translate to improvements in muscle function. These data support further exploration of various pharmacologic interventions to mitigate the loss of muscle experienced by military personnel during periods of unavoidable, severe energy deficit, particularly those that may also safely enhance performance.

semi-starvation [1–3]. Energy deficits produced during strenuous operations are due, in large part, to increases in aerobic-type physical activity and restricted dietary intake, which often result in conditions that degrade both lean body mass and lower-body muscle function [4]. Further, many military personnel experience these severe and unavoidable periods of exercise- and diet-induced energy deficit and associated loss of lean body mass repeatedly over their military career, raising concerns about the potential accumulated health effects of those energy deficits. The US military has sponsored considerable research to develop nutritional countermeasures to mitigate lean body mass loss under these conditions; however, dietary interventions, including those attempting to leverage the anabolic potential of increased protein intake, have been ineffective during severe energy deficit [5–7].

The extent to which lean body mass is lost in men during severe energy deficit may, in part, be attributable to reductions in testosterone. Energy deficits inhibit the hypothalamic-pituitary-gonadal axis by preventing luteinising hormone release and testosterone synthesis [8]. Reductions in testosterone are accompanied by muscle atrophy and decreased muscle strength [9]. Testosterone status is markedly degraded during sustained military operations that involve prolonged, strenuous physical work, reduced energy intake, altered sleep, and psychological stress. Most notably, Friedl et al. [1] demonstrated a 50–65% decline in testosterone in male Soldiers during the first 28 d of US Army Ranger School. Therefore, exogenous testosterone administration may be an effective biomedical strategy to counter declines in testosterone, lean body mass, and physical function that result from severe energy deficit.

In a recent study, supplementing obese men who had low testosterone concentrations (≤12.15 nmol/L) with exogenous testosterone during 56-wk of underfeeding resulted in significantly more fat mass and less lean body mass loss than those receiving placebo injections [10]. However, no study, to our knowledge, has attempted to induce testosterone decrements by replicating the stressors responsible for energy deficits observed during military operations or test the subsequent efficacy and safety of supplemental testosterone for maintaining lean body mass and lower-body muscle function in non-obese males exposed to those military-relevant stressors. The purpose of this study was to evaluate the effects of supplemental testosterone on body composition and lower-body muscle function in non-obese, young adult males, representative of US military personnel, exposed to severe exercise- and diet-induced energy deficit for 28 d. We hypothesized that the group receiving supplemental testosterone during energy deficit would lose more fat mass, less lean body mass, and an equivalent amount of total body mass and have attenuated lower-body muscle functional declines compared to placebo. In addition, we hypothesized that testosterone supplementation during the energy deficit, compared to placebo, would result in more lean body mass accretion and less fat mass gain during recovery from exercise- and diet-induced energy deficit [2,11].

2. Materials and methods

2.1. Participants

Participants were recruited from the Baton Rouge, LA community. Procedures regarding recruitment, randomisation, and follow-up of this study are provided in Fig. 1. Participants were healthy (no cardiometabolic disorders), physically active (≥2-d/wk aerobic and/or resistance exercise) men aged 18–39 y, with total testosterone concentrations within normal physiological range [10.4–34.7 nmol/L, based on clinical practice guidelines at the time the study was conceived [12]]. Participants met age-specific US Army body composition standards [13]. Participants with prostate-specific antigen concentrations > 3 μg/L, haematocrit > 50%, a positive urine drug screen, or reporting anabolic steroid, human growth hormone, or nutritional testosterone precursor-like supplement use within the past 6-mo were excluded. Additional participant inclusion and exclusion criteria as well as details of the recruitment process are published elsewhere [14]. This study was approved by the Pennington Biomedical Research Centre Institutional Review Board and the Human Research Protection Office of the US Army Medical Research and Materiel Command. A data safety monitoring board, consisting of a biostatistician, an exercise physiologist, a clinician, and a layperson, oversaw the safety of participants, monitored recruitment and adherence, and reviewed adverse events, proposed modifications, and reports of related studies as appropriate. The data safety monitoring board was blinded to the study treatments, received quarterly reports, met annually, and provided written documentation of their assessments and recommendations for study continuation. Extended details of the roles and responsibilities of the data and safety monitoring board are provided as Supplementary Material. Participants provided written informed consent. Participants were studied between April 12, 2016 and September 15, 2017. This study is registered at www.clinicaltrials.gov as NCT02734238.

2.2. Study design and intervention

The 3-phase, proof-of-concept, single centre, randomised, double-blind, placebo-controlled, Optimizing Performance for Soldiers trial was conducted at Pennington Biomedical Research Centre to assess

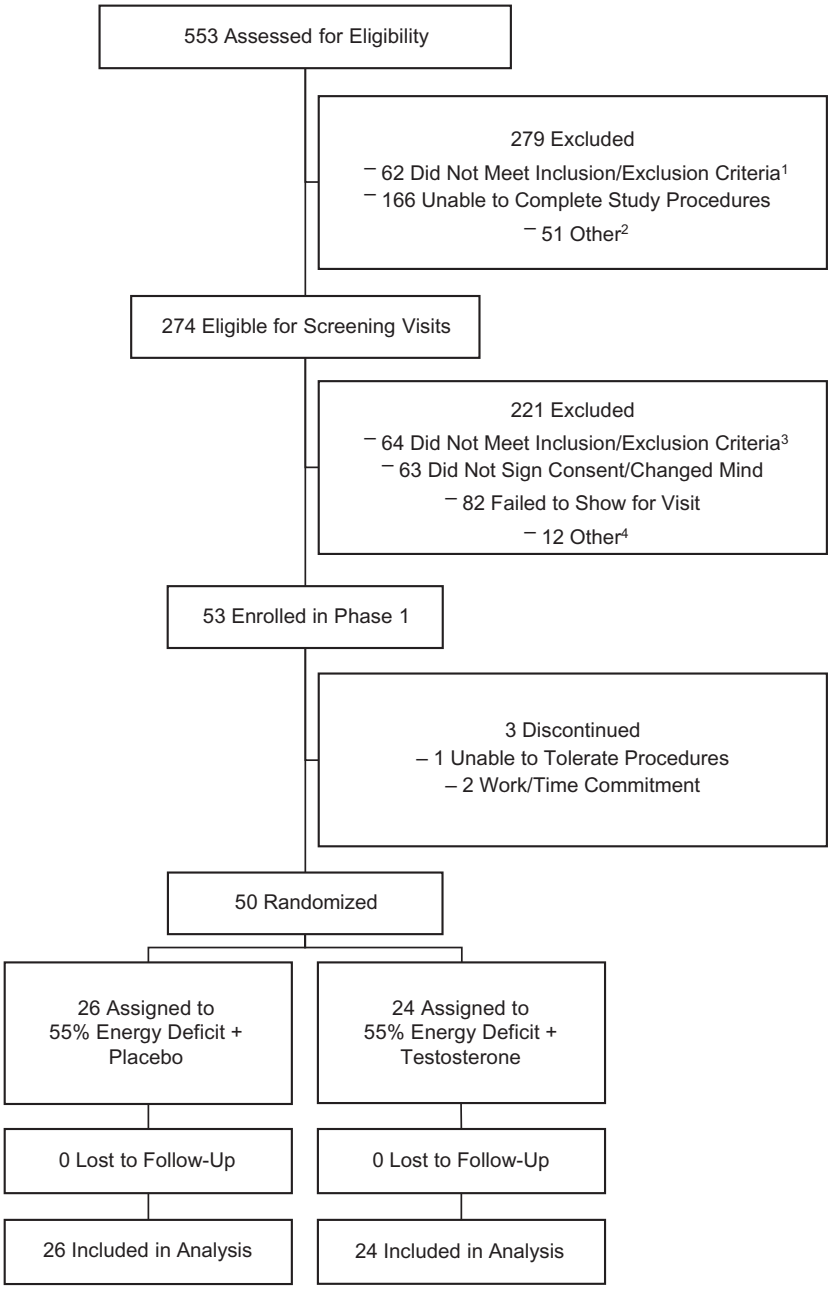


Fig. 1. Participant flow chart. 553 individuals were assessed for eligibility, 279 were excluded, 274 were found eligible for screening, 221 were excluded following screening, 53 were enrolled, 3 discontinued participation prior to phase 2 (i.e., were not randomised), 50 were randomised and completed the intervention (24 into Testosterone and 26 into Placebo). [1]Taking medications or supplements (n = 11), irremovable metal (n = 16), allergies or food intolerance (n = 5), not physically active (n = 2), age (n = 5), body mass index (n = 9), did not meet >1 inclusion/exclusion criteria (n = 14); [2]Recruitment complete/full (n = 28), could not contact (n = 17), not a citizen (n = 5), unknown (n = 1); [3]Medical history or lab results (n = 18), smoking/drug use (n = 12), body mass index (n = 20), dietary limitations (n = 3), non-compliant with screening procedures (n = 9), not willing to receive testosterone injections (n = 1), head circumference too large for MRI machine (n = 1); [4]Recruitment complete/full (n = 4), schedule conflict (n = 8).

the effects of exogenous testosterone administration on body composition changes during severe energy deficit and recovery (Fig. 2). Phase 1 was a 14-d (days 1–14), free-living, eucaloric diet period. Total daily energy expenditure for diet prescriptions during Phase 1 was determined using the Mifflin St. Jeor Equation with an activity factor of 1.3 to account for activities of daily living, and in combination with results from 7-d accelerometer data and 3-d activity logs collected during screening visits [14]. Diets provided 1.6 ± 0.2 g protein/kg/d distributed equally across meals, 30% of total daily energy requirements from fat, with the remaining energy derived from carbohydrate (Supplementary Table 1). Participants maintained their habitual (documented by pre-study, survey questionnaire) physical activity levels during Phase 1. Diet and physical activity adherence were verified by research dietitians, accelerometry, and by ensuring body mass was maintained within ±2%.

Participants were admitted to the inpatient unit after completing Phase 1 and underwent a 28-d (Phase 2; days 15–42), highly controlled exercise- and diet-induced energy deficit equal to 55% of Phase 2 total daily energy expenditure. This 55% energy deficit during Phase 2 involved increasing exercise-induced energy expenditure to elevate total daily energy expenditure by 50% above Phase 1 total daily energy expenditure, and restricting energy intake to 45% of this elevated total daily energy expenditure (Supplementary Table 2). The macronutrient composition of the Phase 2 diet was consistent with Phase 1. All meals were provided and consumed under supervision.

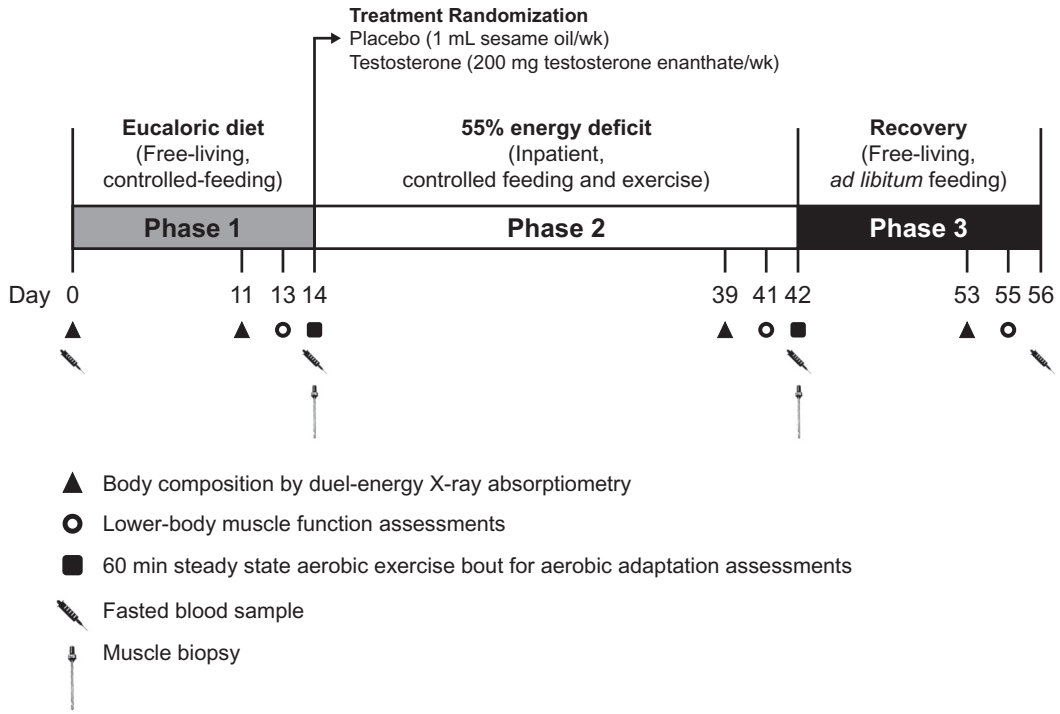


Fig. 2. Experimental design. Adapted from Pasiakos et al. [14].

To increase Phase 2 total daily energy expenditure, exercise consisted of varied, low-, moderate-, and high-intensity (40–85% of predetermined peak rate of oxygen uptake) aerobic-type exercise, including treadmill and/or outdoor walking and/or running, elliptical, stationary bike and weighted backpack (carrying 30% of body mass) walking (Supplementary Table 3). Steady-state, aerobic-type exercise was chosen as the primary means by which total daily energy expenditure was elevated during Phase 2 to reflect the aerobic-type physical work that occurs during sustained, strenuous military operations. High energy flux and limited dietary intake were the principal military-relevant stressors imposed in our study. Participants were not psychologically stressed, nor was sleep restricted in anyway during Phase 2. On average, participants performed 3.5 exercise sessions per day, all directly supervised by research staff. The physical activity prescription was verified biweekly and adjusted as needed to achieve the desired energy expenditure using open circuit indirect calorimetry (ParvoMedics TruOne 2400, East Sandy, UT). Light calisthenics were incorporated into the exercise routine every 3–4 d [14]. Adherence to the prescribed exercise was ≥89% and not different between groups (Supplementary Table 3).

After completing Phase 2, participants were released from the inpatient unit and instructed to return to their pre-study, ad libitum, habitual diet and physical activity routines; body mass and composition were assessed during this recovery period (Phase 3, days 43–56). Participants completed 3-d food records (1 weekend day, 2 weekdays) the week prior to day 56 that were reviewed by research dietitians.

2.3. Treatment randomisation and masking

At the start of Phase 2, participants were randomised according to a 1:1 ratio to receive either intramuscular injections of testosterone enanthate (200 mg/wk, Testosterone) or placebo (1 mL sesame oil, Placebo) on days 15, 21, 28, and 35 by a biostatistician unaffiliated with the study. The testosterone dosage was the same for each participant and chosen to maintain normal testosterone concentrations during severe energy deficit [14]. A computer-generated randomisation plan (SAS Institute, Cary, NC, USA, version 9.4) using the permuted-block method (n = 4/block) and age stratification (<29 or ≥29 y) was developed to allocate participants. The randomisation plan was delivered via a secured network to the study pharmacist who prepared the testosterone or placebo injections. The pharmacist was the only individual affiliated with the study that had access to the randomisation plan. The plan was kept in a password-protected file and accessed only when needed to allocate new participants to a group. Participants and all remaining study staff were blinded to group allocation until study completion.

2.4. Outcome measures

Body composition (total body mass, lean body mass, fat mass, and bone mineral content) measured on days 0, 11, 39, and 53 after an overnight fast and morning void by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, Madison, WI, extended details on scanner positioning standards are provided as Supplementary Materials), was the primary study end point [14]. In this study, lean body mass is defined as total body mass minus fat mass and bone mineral content. All other outcomes described are considered secondary end points.

Semi-nude body weight was measured after an overnight fast and morning void by calibrated digital scale (GSE Inc. Model 450, GSE Scale Systems, Novi, MI) during each screening visit and daily throughout Phase 1 and Phase 2. During Phase 3, participants weighed themselves daily (semi-nude, after an overnight fast) using a calibrated scale provided by staff (Body Trace, Inc. Model BT003, New York, NY).

Percutaneous muscle biopsies of the vastus lateralis were collected in the rested, fasted state under local anaesthesia (1% lidocaine) using a 5 mm Bergstrom needle with manual suction on days 14 and 42 [15]. Muscle biopsy samples were used to characterize cross-sectional area and percent distribution of myosin heavy chain myofibres delineated as type II fast-twitch and type I slow-twitch at the end of Phase 1 and Phase 2. Muscle biopsy samples were also used to assess nuclear peroxisomal proliferator–activated receptor γ coactivator 1α percentage. Extended methodological details for these assessments are provided as Supplementary Material.

Lower-body muscular strength and endurance were assessed using isometric and isokinetic knee extension tests (Biodex Medical Systems, Shirley, NY) on days 13, 41, and 55. Knee extensor dynamometry was chosen to assess muscle function due to its reliability in assessing muscle strength in response to changes in lean mass [16]. Isometric peak torque was measured at 75° knee flexion (3 repetitions, separated by 30 s rest). Isokinetic peak torque was determined from six maximal knee extensions at 60° per second, and isokinetic total work was determined from 20 repeated maximal knee extensions at 180° per second. Muscle strength and endurance data are presented as absolute values and relative to lean body mass of the tested leg.

Participants also performed 60 min steady-state aerobic exercise bouts on days 14 and 42 to characterize submaximal aerobic adaptations (e.g., heart rate, ratings of perceived exertion, power output, energy expenditure, and substrate oxidation) to testosterone and severe energy deficit. Exercise intensity was matched within participants based on power output and total work performed to limit the confounding effects of weight loss on relative exercise intensity [14].

2.5. Sample analyses

Fasted blood samples were collected on days 0, 14, 42, and 56 between 06:00 h and 09:00 h to limit the potential confounding effects of circadian rhythm on total testosterone in young men [17]. Blood samples were analysed for total testosterone, follicle-stimulating hormone, estradiol, sex hormone-binding globulin, luteinising hormone, insulin, cortisol, and prostate-specific antigen (Siemens Immulite 2000, Llanberis, UK). Free testosterone was determined by calculation [18]. Insulin-like growth factor-1 was analysed using an enzyme-linked immunoassay (ALPCO, Salem, NH). Glucose, total cholesterol, HDL-cholesterol, triglycerides, and alanine aminotransferase were analysed on a Beckman DXC 600 Pro (Brea, CA). LDL-cholesterol was determined by calculation [19]. Complete blood counts were analysed on a Beckman DxH (Brea, CA). Systolic and diastolic blood pressure were measured manually on days 0, 15 (prior to the first treatment injection), and 42.

2.6. Sample size determination

Relevant data (means ± standard deviations) demonstrating the effects of moderate-to-severe energy deficit on lean body mass were used to determine statistical power and sample size [20,21]. Based on these estimates, with 90% statistical power, 5% type I error rate, and according to a 2-sided test, 22 males per group were needed (n = 44) [14]. Based on the variability in lean body mass loss in response to moderate-to-severe energy deficit, the sample size was increased to n = 50.

2.7. Data collection and management

Participants were assigned unique identification numbers that were used on all data collection forms, questionnaires, biological specimen tubes, and computer records. The master list linking participant names and identification numbers were kept in a password-protected computer file with access only granted to the principal investigator and study manager. Biological samples moved off-site for analysis (i.e., MyoSyntax performed the muscle cross-sectional area and nuclear peroxisomal proliferator–activated receptor γ coactivator 1α analyses) did not contain any personal identifiable information, nor did they have access to the master list at any time.

Data collection forms were kept under lock and key, or in password-protected files, and under the control of the principal investigator and project coordinator. The Pennington Biomedical Research Centre has a fully integrated, campus wide, automated Central Database management system that is fully validated and continually vetted for quality assurance. Data from self-report or questionnaires were also collected and managed using the REDCap electronic data capture tools hosted at the Pennington Biomedical Research Centre [22]. Data are backed up daily, and the Research Computing Core at the Pennington Biomedical Research Centre oversees all data management.

2.8. Statistical analysis

All analyses are considered 2-tailed, with $\alpha = .05$ considered statistically significant. All primary analyses were intention-to-treat. When luteinising hormone (n = 15), follicle stimulating hormone (n = 27), estradiol (n = 3), and prostate-specific antigen (n = 2) values were below the level of detection, values for data analyses were imputed at one quarter of the detection value. A single testosterone observation was above 55.5 nmol/L and was imputed as 55.5 nmol/L. SAS (SAS Institute, Cary, NC, USA), version 9.4, was used for analyses.

Between group comparisons for Phase 1, Phase 2, and Phase 3 dietary intake and exercise-induced energy expenditure data were assessed using two sample Student's *t*-tests. Baseline and day 14 body weights were compared using a paired t-test to determine whether weight was maintained during Phase 1. Primary analysis of body composition, clinical, muscular function, and muscle fibre cross-sectional area, nuclear peroxisomal proliferator–activated receptor γ coactivator 1α, and aerobic adaptation parameters were performed using a mixed effect linear model. Models include the final measurement day of each phase as the outcome. Treatment (Testosterone and Placebo), phase (Phases 1–3), phase-by-treatment interaction, age, and pre-study values (only for body composition and clinical parameters, Table 1) were considered fixed effects covariates in the model. The random effect included an unstructured covariance matrix to account for the correlation within-subjects over time. Least squares means from the model estimated interaction effects. For outcomes with significant phase-by-treatment interactions, within group comparisons across phases (n = 6) and between group comparisons within each phase (n = 3) were made using two sample Student's *t*-tests based on the least squares means and adjusted using the Bonferroni correction (maximum of 9 comparisons). Unadjusted data are reported in Supplementary Table 4.

3. Results

3.1. Inclusion

Non-obese, young men were enrolled (n = 53), randomised (n = 50), and completed (n = 50) this double-blinded, placebo-controlled trial (Fig. 1, Table 1). Throughout the study, adverse events (Table 2) were not different between Testosterone and Placebo.

3.2. Characteristics and effects during phase 1

During Phase 1, exercise-induced energy expenditure (mean ± standard deviation, Testosterone, 331 ± 387 kcal/d and Placebo, 360 ± 345 kcal/d) and dietary intake did not differ between groups ($P > .05$, Supplementary Table 1). Participants in both groups remained weight stable [Testosterone, d 0: 81 kg (77, 85) and d 14: 80 kg (76, 85), $P = .38$; Placebo, d 0: 77 kg (72, 81) and d 14: 76 kg (72, 81), $P = .62$].

3.3. Characteristics of phase 2

During Phase 2, total daily energy expenditure, exercise-induced energy expenditure, percent and absolute energy deficit, and modality-specific exercise did not differ ($P > .05$) between groups (Supplementary Tables 2–3). Except for relative protein intake, energy and macronutrient intake did not differ between treatments (Supplementary Table 1).

Table 1
Pre-study participant characteristics in the intention-to-treat population.

	Testosterone (n = 24)	Placebo (n = 26)
Race and ethnicity, n (%)		
Non-Hispanic Black	6 (25)	7 (27)
Non-Hispanic White	14 (58)	16 (61)
Hispanic	3 (13)	0 (0)
Other	1 (4)	3 (12)
Age, y	25 (5)	25 (5)
Height, cm	178 (9)	178 (6)
Weight, kg	81 (14)	77 (10)
Body mass index, kg/m^2	25 (3)	24 (3)
Systolic blood pressure, mmHg	119 (8)	117 (10)
Diastolic blood pressure, mmHg	73 (8)	74 (6)
Total testosterone, nmol/L	17 (5)	15 (3)†
Free testosterone, nmol/L	0.34 (0.10)	0.31 (0.10)
Follicle stimulating hormone, IU/L	3.3 (1.9)	3.8 (2.2)
Estradiol, pmol/L	157 (52)	155 (53)
Sex-hormone binding globulin, nmol/L	37 (18)	32 (11)
Luteinising hormone, IU/L	3.7 (1.3)	4.4 (2.0)
Insulin-like growth factor-1, nmol/L	37 (9)	36 (12)
Glucose, mmol/L	4.9 (0.4)	4.9 (0.3)
Insulin, pmol/L	68 (106)	55 (51)
Cortisol, nmol/L	371 (107)	411 (141)
Total cholesterol, mmol/L	4.2 (0.7)	4.3 (0.9)
High-density lipoprotein cholesterol, mmol/L	1.5 (0.3)	1.4 (0.3)
Low-density lipoprotein cholesterol, mmol/L	2.4 (0.6)	2.6 (0.7)
Triglycerides, mmol/L	0.81 (0.41)	0.87 (0.50)
Haemoglobin, g/L	150 (10)	150 (11)
Haematocrit, %	45 (2)	44 (3)
Prostate-specific antigen, μg/L	0.78 (0.61)	0.67 (0.34)
Alanine aminotransferase, μkat/L	0.39 (0.14)	0.35 (0.13)
Body mass composition, kg		
Total	81.0 (13.7)	76.5 (10.3)
Lean	60.7 (10.1)	56.0 (5.4)*
Fat	17.0 (6.8)	17.4 (6.6)
Peak oxygen uptake, L/min	3.6 (0.8)	3.4 (0.6)
Absolute intake, kcal/d or g/d		
Energy	2432 (777)	2478 (950)
Carbohydrate	258 (99)	281 (114)
Protein	118 (39)	113 (40)
Fat	106 (38)	103 (49)
Relative intake, kcal/kg body mass/d or g/kg body mass/d		
Energy	32 (14)	33 (13)
Carbohydrate	3.4 (1.7)	3.8 (1.6)
Protein	1.5 (0.6)	1.5 (0.6)
Fat	1.4 (0.6)	1.4 (0.7)

Values are mean (standard deviation). Testosterone = 55% energy deficit + 200 mg testosterone enanthate per week during Phase 2, Placebo = 55% energy deficit + 1 mL sesame seed oil placebo per week during Phase 2.

Table 2
Incidence of adverse events in the intention-to-treat population.

	Testosterone (n = 24)			Placebo (n = 26)		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Death	0	0	0	0	0	0
Serious adverse event						
Low absolute neutrophil count	0	0	0	1	0	0
Elevated alanine aminotransferase	0	0	3	0	0	0
Biopsy infection, cellulitis	0	1	0	0	0	0
Adverse event-related withdrawals	0	0	0	0	0	0
Adverse event						
Biopsy pain	20	20	0	17	23	2
Blister/chafing	0	8	1	0	7	1
Foot pain	0	7	0	0	7	0
Joint/muscle soreness	3	28	0	2	26	0
Gastrointestinal	1	5	1	0	5	1
Dermatological	1	5	1	3	10	0
Insomnia	0	9	0	0	7	0
Headache	2	4	3	0	2	3
Allergy/eye irritation/ear pain	3	2	3	4	5	0
Low haemoglobin, haematocrit, and mean cell volume	0	1	0	0	0	0
Iron deficiency anaemia	0	0	0	0	1	0
Other[1]	1	4	0	2	2	1

Chi square tests were used to compare adverse event incidence in Testosterone (n = 24) and Placebo (n = 26) within each phase. There were no between group differences during any phase (P > .05). Percutaneous muscle biopsies of the vastus lateralis were obtained during P1 and P2. P1 = phase 1, P2 = phase 2, P3 = phase 3, Testosterone = 55% energy deficit + 200 mg testosterone enanthate per week during Phase 2, Placebo = 55% energy deficit + 1 mL sesame seed oil placebo per week during Phase 2. [1]Other: Testosterone; P1, vasovagal/syncope (n = 1); P2, vasovagal/syncope (n = 1), fatigue (n = 1), blurred vision (n = 1), herpes simplex (n = 1); Placebo; P1, chest pain (n = 1), vertigo (n = 1); P2, vertigo (n = 1), dysuria (n = 1); P3, visual disturbance (n = 1).

3.4. Effects of testosterone supplementation on circulating biomarkers during phase 2

Following energy deficit in Phase 2, concentrations of free testosterone decreased, total testosterone remained unchanged, and sex-hormone binding globulin increased in Placebo; whereas, total testosterone and free testosterone increased and sex-hormone binding globulin was unchanged in Testosterone, such that, total testosterone [all bracketed data are mean difference between groups (95% CI), 24.4 nmol/L (21.8, 27.0), P < .0001] and free testosterone [0.69 nmol/L (0.61, 0.77), P < .0001] were greater and sex-hormone binding globulin concentrations [15.9 (11.4, 20.4), P < .0001] were lower in Testosterone than Placebo (Table 3). Estradiol [116 pmol/L (91, 140), P < .0001], follicle stimulating hormone [2.6 IU/L (2.0, 3.2), P < .0001], and luteinising hormone [2.5 IU/L 1.7, 3.4, P < .0001] concentrations were different for Testosterone compared to Placebo (Table 3). Furthermore, Testosterone had higher haemoglobin [6.7 g/L (3.0, 10.3), P < .0048] and haematocrit [2.1% (0.9, 3.2), P < .0053] than Placebo (Table 3).

3.5. Effects of testosterone supplementation on body composition during phase 2

During Phase 2, Placebo lost more body weight than Testosterone (P < .0001, Fig. 3A). Fat mass decreased similarly in both treatment groups (Fig. 3B, Table 3). Bone mineral content did not differ between treatment groups (Table 3). Lean body mass increased in Testosterone, whereas lean body mass remained stable in Placebo, such that lean body mass significantly differed between groups at the end of Phase 2 [2.5 kg (3.3, 1.6); P < .0001]. The change in total testosterone was positively associated with change in lean body mass (r = 0.71, r^2 = 0.50, P < .0001; Supplementary Fig. 1). Similarly, free testosterone was associated with the change in lean body mass (r = 0.69, r^2 = 0.48, P < .001).

3.6. Effects of testosterone supplementation on muscle function, myofibre cross-sectional area, and aerobic adaptations during phase 2

Lower-body muscle function declined similarly for both groups during Phase 2, despite no loss of lean body mass in either group (Table 3). Type I slow-twitch myofibre cross-sectional area and percent myofibre distribution increased, whereas type II fast-twitch myofibre cross-sectional area and percent distribution decreased during Phase 2 as compared to Phase 1, independent of treatment (Fig. 4A–H, all P < .05). Independent of treatment, submaximal aerobic exercise economy was markedly improved for a given matched workload (i.e., greater reliance on fat oxidation and lower perceived effort, heart rate, energy expenditure, and oxygen uptake, all P < .05, Supplementary Table 5). There were no effects of energy deficit or treatment on basal (fasted, rested) nuclear percentage of peroxisomal proliferator–activated receptor γ coactivator 1α [Testosterone, d 14: 42.6% (33.4, 51.8) and d 42: 48.2% (38.1, 58.2); Placebo, d 14: 50.7% (41.9, 59.6) and d 42: 52.4% (42.8, 62.1), P-interaction = 0.42].

Table 3
Adjusted least squares means for body composition, clinical biomarkers, and lower-body muscular strength and endurance in the intention-to-treat population.

	Testosterone (n = 24)			Placebo (n = 26)			P-value		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3	Phase	Treat	Phase × Treat
Body composition									
Body mass, kg									
Total	78.0 (77.4, 78.7)[a]	75.8 (75.1, 76.5)[b,*]	79.3 (78.6, 79.9)[a,*]	78.3 (77.7, 78.9)[a]	73.3 (72.7, 74.0)[b]	76.5 (75.8, 77.1)[c]	<0.0001	<0.0001	<0.0001
Lean	57.9 (57.3, 58.5)[a]	60.4 (59.8, 61.0)[b,*]	63.1 (62.5, 63.7)[c,*]	58.3 (57.7, 58.9)[a]	58.0 (57.4, 58.6)[a]	60.5 (60.0, 61.1)[b]	<0.0001	<0.0001	<0.0001
Fat[1]	16.8 (16.3, 17.2)	12.0 (11.6, 12.4)	12.8 (12.4, 13.2)	16.8 (16.4, 17.2)	12.2 (11.8, 12.6)	12.8 (12.4, 13.2)	<0.0001	0.78	0.79
Bone mineral content, kg	3.27 (3.26, 3.28)[a]	3.27 (3.26, 3.28)[a]	3.26 (3.25,3.27)[a]	3.27 (3.26, 3.28)[a]	3.25 (3.24, 3.27)[a]	3.24 (3.23, 3.25)[b,*]	<0.0001	0.016	0.035
Leg mass, kg									
Total	26.7 (26.4, 27.0)[a]	25.8 (25.5, 26.1)[b]	26.9 (26.6, 27.2)[a,*]	26.8 (26.5, 27.1)[a]	25.2 (24.9, 25.5)[b]	26.1 (25.8, 26.4)[c]	<0.0001	0.0089	0.012
Lean	19.5 (19.3, 19.8)[a]	20.1 (19.8, 20.4)[b,*]	21.2 (20.9, 21.4)[c,*]	19.6 (19.3, 19.8)[a]	19.3 (19.0, 19.6)[a]	20.2 (19.9, 20.4)[b]	<0.0001	0.0001	<0.0001
Fat	6.0 (5.8, 6.1)[a]	4.4 (4.3, 4.6)[b]	4.5 (4.4, 4.6)[b]	6.0 (5.8, 6.1)[a]	4.7 (4.6, 4.8)[b]	4.7 (4.6, 4.9)[b]	<0.0001	0.039	0.015
Trunk mass, kg									
Total	35.7 (35.3, 36.1)[a]	34.8 (34.3, 35.2)[b,*]	36.7 (36.3, 37.1)[c*]	35.9 (35.5, 36.2)[a]	33.4 (33.0, 33.8)[b]	35.1 (34.7, 35.5)[a]	<0.0001	<0.0001	<0.0001
Lean	26.7 (26.3, 27.1)[a]	28.5 (28.1, 28.8)[b,*]	29.8 (29.5, 30.2)[c,*]	27.0 (26.6, 27.3)[a]	27.4 (27.1, 27.8)[a]	28.7 (28.4, 29.1)[b]	<0.0001	0.0018	<0.0001
Fat[1]	8.0 (7.7, 8.3)	5.3 (5.0, 5.6)	5.9 (5.6, 6.2)	8.0 (7.7, 8.3)	5.1 (4.8, 5.4)	5.6 (5.3, 5.8)	<0.0001	0.28	0.39
Clinical health-biomarkers									
Total testosterone, nmol/L	15.5 (13.7, 17.4)[a]	36.2 (34.3, 38.0)[b,*]	7.8 (5.9, 9.8)[c,*]	14.6 (12.9, 16.4)[a]	11.8 (10.0, 13.5)[a]	14.6 (12.8, 16.3)[a]	<0.0001	<0.0001	<0.0001
Free testosterone, nmol/L	0.33 (0.27, 0.38)[a]	0.87 (0.81, 0.92)[b,*]	0.15 (0.10, 0.21)[c,*]	0.31 (0.26, 0.36)[a]	0.18 (0.13, 0.23)[b]	0.29 (0.23, 0.34)[a,b]	<0.0001	<0.0001	<0.0001
Follicle stimulating hormone, IU/L	3.1 (2.7, 3.6)[a]	0.3 (−0.2, 0.7)[b,*]	2.2 (1.7, 2.7)[c,*]	3.5 (3.0, 3.9)[a]	2.8 (2.4, 3.3)[a]	3.5 (3.0, 3.9)[a]	<0.0001	<0.0001	<0.0001
Estradiol, pmol/L	148 (130, 166)[a]	247 (229, 265)[b,*]	144 (125, 162)[a]	142 (124, 159)[a]	131 (114, 148)[a]	158 (141, 175)[a]	<0.0001	<0.0001	<0.0001
Sex-hormone binding globulin, nmol/L	31.6 (28.3, 34.8)[a,b]	36.5 (33.2, 39.7)[a,*]	30.6 (27.2, 34.0)[b]	31.1 (27.9, 34.2)[a]	52.4 (49.2, 55.5)[b]	35.7 (32.6, 38.8)[a]	<0.0001	0.0001	<0.0001
Luteinizing hormone, IU/L	3.3 (2.6, 3.9)[a]	0.3 (−0.4, 0.9)[b,*]	2.2 (1.6, 2.9)[a,*]	3.8 (3.2, 4.3)[a,b]	2.8 (2.2, 3.4)[a]	4.1 (3.5, 4.7)[b]	<0.0001	<0.0001	0.0006
Insulin-like growth factor-1, nmol/L[2]	38.5 (35.6, 41.5)	25.0 (22.0, 27.9)	36.6 (33.5, 39.7)	38.2 (35.3, 41.0)	25.8 (22.9, 28.6)	37.5 (34.7, 40.4)	<0.0001	0.73	0.89
Glucose, mmol/L[1]	4.71 (4.53, 4.88)	4.14 (3.96, 4.32)	4.93 (4.74, 5.11)	4.63 (4.46, 4.81)	4.19 (4.02, 4.36)	4.91 (4.74, 5.08)	<0.0001	0.88	0.76
Insulin, pmol/L[2]	59 (35, 83)	15 (−9, 39)	80 (55, 105)	54 (31, 77)	11 (−12, 34)	87 (64, 110)	<0.0001	0.93	0.86
Cortisol, nmol/L[2]	397 (356, 438)	512 (472, 553)	388 (345, 430)	434 (395, 473)	551 (512, 590)	378 (339, 417)	<0.0001	0.73	0.89
Total cholesterol, mmol/L[1]	3.85 (3.59, 4.11)	4.39 (4.13, 4.65)	4.97 (4.70, 5.24)	3.86 (3.61, 4.11)	4.12 (3.87, 4.37)	4.98 (4.73, 5.23)	<0.0001	0.54	0.22
High-density lipoprotein cholesterol, mmol/L	1.22 (1.11, 1.32)[a]	1.64 (1.54, 1.75)[b]	1.85 (1.74, 1.96)[c]	1.22 (1.12, 1.32)[a]	1.75 (1.65, 1.85)[b]	1.74 (1.64, 1.84)[b]	<0.0001	0.98	0.008
Low-density lipoprotein cholesterol, mmol/L	2.23 (2.03, 2.43)	2.44 (2.24, 2.63)	2.74 (2.53, 2.95)	2.29 (2.10, 2.48)	2.14 (1.95, 2.34)	2.78 (2.59, 2.98)	<0.0001	0.53	0.054
Triglyceride, mmol/L	0.84 (0.69, 0.99)[a]	0.65 (0.49, 0.80)[a]	0.78 (0.62, 0.94)[a]	0.80 (0.66, 0.95)[a,b]	0.52 (0.37, 0.67)[a]	1.03 (0.88, 1.17)[b]	0.0001	0.63	0.038
Haemoglobin, g/L	145 (143, 148) [a]	145 (142, 148)[a,*]	145 (142, 147)[a]	144 (142, 147)[a]	138 (136, 141)[b]	139 (137, 142)[b]	0.012	0.002	0.035
Haematocrit, %	42.9 (42.1, 43.7)[a]	43.6 (42.7, 44.4)[a,*]	43.2 (42.3, 44.0)[a]	42.8 (42.0, 43.6)[a]	41.5 (40.7, 42.3)[a]	41.5 (40.7, 42.3)[a]	0.45	0.003	0.020
Alanine aminotransferase, μkat/L[3]	0.32 (−0.20, 0.83)	0.42 (−0.09, 0.94)	2.65 (2.11, 3.18)	0.38 (−0.12, 0.87)	0.49 (0.00, 0.98)	1.68 (1.19, 2.18)	<0.0001	0.19	0.074
Prostate-specific antigen, μg/L[1]	0.77 (0.56, 0.98)	0.68 (0.47, 0.89)	—	0.59 (0.39, 0.79)	0.43 (0.23, 0.63)	—	<0.0001	0.14	0.15
Systolic blood pressure, mm Hg[1]	104 (99, 108)	99 (94, 103)	—	103 (98, 107)	98 (94, 102)	—	0.0002	0.80	0.75
Diastolic blood pressure, mm Hg[1]	63 (60, 66)	58 (55, 60)	—	64 (61, 67)	58 (55, 60)	—	<0.0001	0.73	0.66
Lower-body muscular strength and endurance									
Isometric torque									
Peak, Nm[4]	245 (221, 269)	219 (195, 243)	214 (190, 238)	243 (220, 266)	219 (196, 243)	229 (205, 252)	<0.0001	0.78	0.37
Peak, Nm/kg lean body mass[4]	24 (22, 26)	21 (19, 23)	20 (18, 22)	26 (24, 28)	24 (22, 26)	24 (22, 26)	<0.0001	0.014	0.31
Isokinetic torque									
Peak, Nm[4]	200 (181, 219)	164 (145, 183)	174 (155, 193)	195 (177, 213)	163 (145, 181)	168 (150, 186)	<0.0001	0.72	0.82
Peak, Nm/kg lean body mass[4]	20 (18, 21)	16 (14, 17)	16 (14, 17)	21 (19, 22)	17 (16, 19)	17 (16, 19)	<0.0001	0.12	0.72
Total work, J[1]	3339 (3024, 3653)	2800 (2485, 3115)	3180 (2865, 3494)	3423 (3121, 3726)	2843 (2541, 3146)	2985 (2683, 3288)	<0.0001	0.91	0.26
Total work, J/kg lean body mass[4]	327 (303, 352)	266 (242, 290)	291 (267, 316)	362 (339, 385)	306 (282, 329)	307 (284, 331)	<0.0001	0.033	0.33

Values are least squares mean (95% confidence interval). Data were analysed using linear mixed models, adjusted for age (all parameters) and pre-study value (only for body composition and clinical parameters). Bonferroni corrections were used for post hoc comparisons. Data not sharing the same letter superscript within a treatment are different by phase and *indicates a between group difference at a particular phase (phase-by-treat interaction). Main effect of phase, [1]all phases are different, [2]Phase 1 and Phase 3 are different than Phase 2, [3]Phase 1 and Phase 2 are different than Phase 3, [4]Phase 1 is different than Phase 2 and Phase 3. $P < .05$ considered statistically significant. Testosterone = 55% energy deficit + 200 mg testosterone enanthate per week during Phase 2, Placebo = 55% energy deficit + 1 mL sesame seed oil placebo per week during Phase 2.

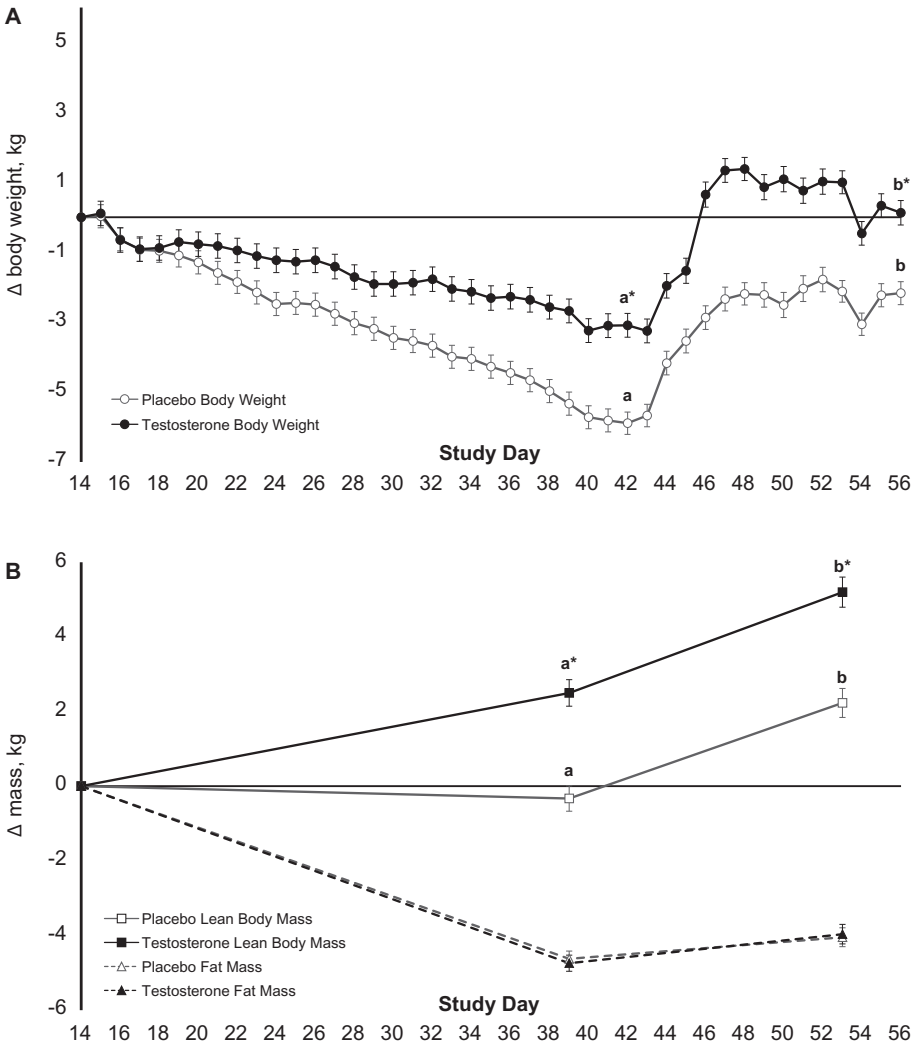


Fig. 3. Change in body weight and composition during energy deficit and recovery. Least squares mean ± standard error change in body weight (A) and composition (B) during Phase 2 (55% energy deficit) and Phase 3 (recovery/weight regain) relative to the final body weight and composition measured during Phase 1 (eucaloric diet) for Testosterone (n = 24), 55% energy deficit + 200 mg testosterone enanthate per week during Phase 2 (n = 24) and Placebo, 55% energy deficit + 1 mL sesame seed oil placebo per week during Phase 2 (n = 26). Data were analysed using linear mixed models, adjusting for age and pre-study value. Bonferroni corrections were used for post hoc comparisons. Data not sharing the same letter superscript within a treatment are different; and *indicates a between group difference (phase-by-treatment interaction).

3.7. Characteristics and effects during phase 3

During Phase 3, ad-libitum dietary intake was not different between groups (Supplementary Table 1). Total testosterone [6.7 nmol/L (4.1, 9.4), P < .0001], free testosterone [0.13 nmol/L (0.05, 0.21), P < .013], and follicle stimulating hormone [1.3 IU/L (0.6, 1.9), P < .0011] concentrations were lower for Testosterone than Placebo (Table 3), but no other health-related biomarkers differed by group. Both groups regained body weight; however, body weight remained greater in Testosterone than Placebo (P < .0001; Fig. 3A). Both groups also gained lean body mass, but lean body mass remained greater in Testosterone than Placebo [2.6 kg (1.8, 3.4), P < .0001; Fig. 3B, Table 3]. Bone mineral content decreased in Placebo and remained stable in Testosterone, such that bone mineral content significantly differed between groups at the end of Phase 3 [21 g [7,36]; P < .043]. Fasting insulin concentrations were greater following recovery compared to the energy deficit, with no difference between groups. Muscle function measures remained lower during Phase 3 than Phase 1, regardless of treatment allocation (P < .0001).

4. Discussion

In the current proof-of-concept study, we report that during 28-d of severe exercise- and diet-induced energy deficit, non-obese, young-adult males receiving weekly intramuscular injections of testosterone (200 mg testosterone enanthate/wk) gained lean body mass and lost less total mass compared to those receiving placebo injections. Testosterone fully recovered their body weight and were 2.8 kg heavier at the end of the 14-d recovery period than Placebo, due almost entirely to lean body mass. Testosterone did not differ from Placebo with respect to lower-body muscular strength and endurance, incidence of adverse events, systolic or diastolic blood pressure, or glucose, insulin, cortisol, lipid, lipoprotein, insulin-like growth factor 1, alanine aminotransferase, or prostate-specific antigen concentrations during energy deficit or recovery.

Suppression of the hypothalamic-pituitary-gonadal axis in energy deficient, non-obese men is well-documented, particularly during sustained, strenuous military operations [1,23,24]. However, no study, to our knowledge, has attempted to replicate these testosterone

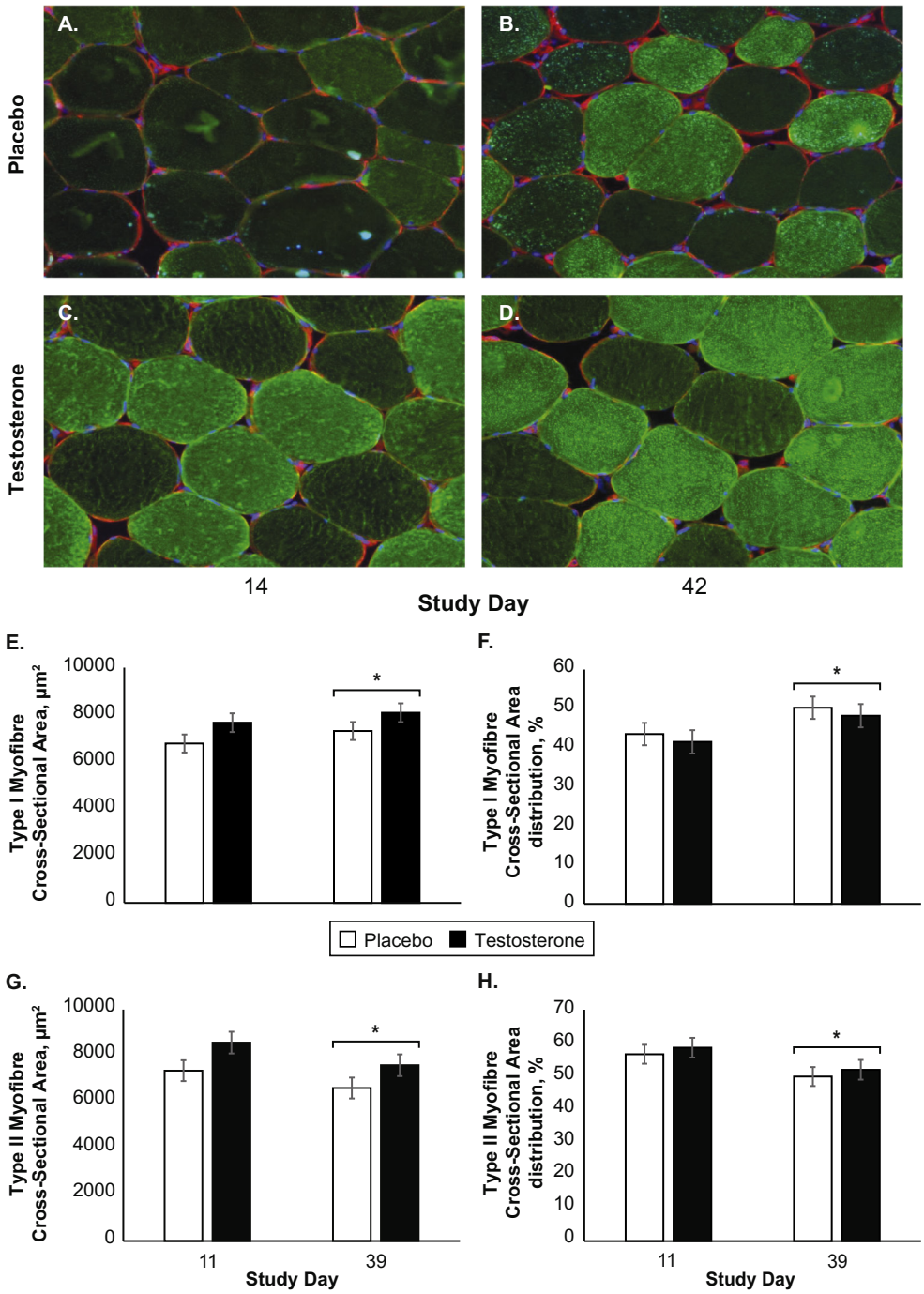


Fig. 4. Muscle fibre type and percent distribution during energy balance and energy deficit. Representative images of serial cross-sections from vastus lateralis samples collected at rest, under fasted conditions during phase 1 (eucaloric phase, day 14) and phase 2 (55% energy deficit, day 42) for Placebo (A–B), 55% energy deficit + 1 mL sesame seed oil placebo per week during Phase 2 (n = 26) and Testosterone (C–D), 55% energy deficit + 200 mg testosterone enanthate per week during Phase 2 (n = 24). Images were taken with the 20× objective lens in a 15 × 10 grid. Laminin signals were stitched into one image to cover the entire specimen and analysed using Cell Profiler. Myosin heavy chain myofibres with a median intensity < 0.3 and those ≥0.3 were delineated as type II fast-twitch myofibres and type I slow-twitch myofibres, respectively. Least squares mean ± standard error in type 1 slow-twitch myofibre cross-sectional area (E), type 1 slow-twitch myofibre percent distribution (F), type II fast-twitch myofibre cross-sectional area (G), and type II fast-twitch myofibre percent distribution (H). Data were analysed using linear mixed models, adjusting for age. *Indicates a between phase difference (phase main effect).

decrements in a tightly controlled clinical trial using military-relevant exercise and diet stressors. The inability of our severe exercise- and diet-induced energy deficit to reduce total testosterone concentrations to levels measured during US Army Ranger School [1], the basis for the design of the current study, provides 1) controlled scientific evidence to counter several of the points made in the paper by Friedl and colleagues [1], and 2) valuable information regarding the design of future trials attempting to induce non-pharmacologic decrements in endogenous testosterone status in a clinical setting.

In the current study, the 28-d exercise- and diet-induced energy deficit intervention was modelled to reflect the exercise- and diet-induced stress experienced by US Army Soldiers participating in US Army Ranger School [1], an example of severe military operational stress. Friedl and colleagues [1] suggested that the energy deficit alone, caused by increased energy expenditure and restricted dietary intake, was the primary determinant for the decline in total testosterone. This reasoning was based on the observed restoration of eugonadal status in Soldiers fed adequate energy following the first 28 d of Army Ranger School,

but still exposed to the remaining stressors (i.e., prolonged and strenuous physical work, sleep deprivation, and psychological stress) [1]. The authors concluded that the energy deficit (~1100 kcal/d) alone, not the means by which the deficit was produced, was the primary determinant of the 50–65% decline in total testosterone observed during the first 28-d of US Army Ranger School. Therefore, the current study, which induced a greater daily energy deficit (~1950 kcal), was expected to elicit a similar decline in total testosterone and lean body mass.

This discrepancy between studies may suggest that, in addition to energy restriction and strenuous physical work, other factors, such as sleep deprivation, psychological stress, and the type of physical work employed meaningfully contribute to declines in testosterone status observed during strenuous military training. During real-world military operations, recovery is minimal and military personnel are often sleep deprived. In the current study, psychological stress was not imposed, and sleep was not restricted. Sleep restriction (5 h/night) has been shown to suppress total testosterone by as much as 15% [25]. In a study that involved strenuous military training and produced a substantial energy deficit, continuous sleep (3 h/d), compared to non-continuous sleep, attenuated reductions in total testosterone [24]. Furthermore, discrete aerobic-type exercise bouts were used to increase energy expenditure (3.5 sessions per day, averaging 2.5 h/d) during the 28-d energy deficit, followed by periods of rest. Energy deficits during real-world military operations often result from low-to-moderate increases in metabolic demand and are sustained for long periods, as physical/aerobic exercise-type activity can exceed 10 h/d. As such, the stressors applied in the current study may not have replicated the multi-faceted stress experienced during real-world military operations, which generally produce marked suppression of the hypothalamic-pituitary-gonadal axis and catabolism of lean body mass [6,7,26]. These realizations are critical for the design of future interventional studies of military operational stress and add important evidence to the literature regarding endogenous testosterone decrements that occur during physiologically and psychologically stressful events.

Finally, while the magnitude of the total testosterone decline (19%, NS) in the current study was less than that reported by others [1,27], the significance of a 42% decline in free testosterone – i.e., the bioactive form of testosterone – should be highlighted. Recent guidance from the US Endocrine Society recommends that free testosterone be relied upon for diagnosis of low testosterone in conditions where sex-hormone binding globulin is altered and total testosterone concentrations are borderline low [28,29]. For Placebo, sex-hormone binding globulin increased by 68% during Phase 2 compared to Phase 1 and total testosterone concentrations were borderline low [<13.9 nmol/L, [28]] at the end of Phase 2. Importantly, free testosterone concentrations [0.18 (0.13, 0.23) nmol/L] were below the lower-end of the normal range estimated using the Vermeulen equation [0.25–0.785 nmol/L, [30]] following Phase 2. Therefore, a more holistic examination of the data (i.e., total and free testosterone and sex-hormone binding globulin) suggests testosterone status was degraded by the severe exercise- and diet-induced energy deficit, albeit not to the same degree as during US Army Ranger School.

The hypertrophic effects of supplemental testosterone are well-described [31]. Bhasin et al. [32] reported a 5% (2.5 kg) and 9% (5.5 kg) increase in lean mass in non-obese, young men receiving weekly injections of 125 mg and 300 mg, respectively, of testosterone enanthate while consuming a eucaloric diet for 20-wk. We report a similar increase in lean body mass (~4%, 2.5 kg) after 4-wk in young men receiving 200 mg/wk. of testosterone enanthate while being underfed. The hypertrophic effects of testosterone, despite energy deficit, may be due to enhanced commitment of satellite cells to myogenesis [33] or androgen receptor-mediated cell signalling [34]. Given the magnitude of energy deficit in the current study, preservation of lean body mass in Placebo was unexpected. However, the combination of higher-protein feeding and high-volume exercise may, in part, explain this finding, due to the synergistic restorative effects of dietary protein and mechanical stress on muscle protein synthesis during energy deficit [20,35–37], providing the conditions by which lean body mass may be maintained [38]. The addition of supplemental testosterone may have provided an anabolic stimulus that was additive to the stimulus provided by protein and exercise, explaining, in part, the gain in lean body mass in Testosterone. This hypothesis is supported by studies showing that testosterone administration enhances muscle protein synthetic efficiency, meaning, for a given amount of intracellular essential amino acids, a greater proportion is routed towards protein synthesis than towards oxidative metabolism, thus conserving lean body mass [39,40].

Lower-body muscle function deteriorated similarly between treatments during the 28-d exercise- and diet-induced energy deficit. The decline in muscle function may be attributable to neuromuscular fatigue, secondary to multiple daily submaximal aerobic exercise sessions for 28 d [41]. Reduced motivation over time may also be a factor contributing to the observed decline in muscular strength and endurance [42,43]. However, the incongruence between the lean body mass change and muscle function in our study is not a unique observation. A similar inability to detect differences in muscle function with testosterone supplementation, despite greater lean mass, has also been demonstrated in young men receiving exogenous testosterone during 28 d of bed rest [44], in middle-aged, obese males receiving exogenous testosterone for 56 wk [10], and in older, overweight males receiving testosterone supplementation for 6 mo [45]. However, the lack of a functional benefit should not discount the potential advantage that may be gained by increasing or maintaining lean body mass in response to severe energy deficit. Accrual or preservation of muscle protein mass may afford greater physiological resiliency during severe military operational stress, particularly when repeated bouts of stress are unavoidable, and recovery is limited. For example, stress-mediated lean body mass losses approaching 10% have been associated with impaired immune function [46]. Lean body mass can be viewed as an amino acid reservoir, and increased protein mass, even to a limited extent, may prolong physiological homeostasis during periods of severe energy deficit.

The lack of a quantitative functional benefit for Testosterone despite gains in lean body mass may also raise methodological concerns about the use of DXA for body composition measurements. DXA is the most commonly employed body composition measurement tool in biomedical research [47], yet it provides estimates for only three body compartments (i.e., bone, fat, and lean soft tissue), and does not discern skeletal muscle mass from body water. If gains in lean body mass in Testosterone were due, at least in part, to an increase in extracellular water [48] and muscle glycogen content [49], and not solely to an increase in protein, those changes would not be delineated by DXA. Assessing total body water in combination with DXA (i.e., four compartment model) may have mitigated those concerns. If lean mass hydration is conservatively estimated to be 75%, total body protein accretion in TEST would be ~625 g. Gains in protein mass, without gains in muscle function, may be a reflection of the specific myofibres (i.e., protein fraction) affected by the intervention. More specifically, the reduction in type II fast-twitch myofibre cross-sectional area would support the functional declines observed with the isokinetic and isometric functional measures. The increase in type I slow-twitch myofibre cross-sectional area is consistent with the observed improvement in submaximal aerobic exercise economy. These findings, in addition to the possibility that gains in lean mass were due to fluid or non-contractile protein, may help explain why lean body mass accrual in Testosterone had no functional benefit.

5. Limitations

While the findings from the current study are strengthened by 100% retention and ≥89% adherence with diet and exercise prescriptions during the energy deficit phase, there are limitations to acknowledge, in addition to those already discussed with regards to DXA methodology and muscle function measures. This study was designed to provide

testosterone supplementation at a dose that maintained total and free testosterone concentrations during a severe energy deficit, in order to attenuate expected lean body mass loss. However, following testosterone supplementation in Testosterone, total and free testosterone concentrations were 134% and 164% higher, respectively, than concentrations prior to supplementation. In addition, following Phase 2, total testosterone concentrations in Testosterone were approximately 14% above the upper-end of the harmonized reference range for testosterone (9.2–31.8 nmol/L) recently established by the Endocrine Society in 2018 [28]. Free testosterone concentrations were 11% above the upper-end of the reference range for free testosterone (0.25–0.785 nmol/L) established by Vermeulen and colleagues [18,30]. The increases in total and free testosterone above basal concentrations and the upper-end of the normal reference range were likely due to the administration of a greater dosage than the recommended initial dose for testosterone replacement therapy [28] and, possibly, the discrepancy between actual endogenous testosterone suppression produced by the severe energy deficit and the anticipated suppression, on which the chosen dosage was based [32]. As discussed previously, the anticipated suppression was based on military studies demonstrating marked reductions in total testosterone during demanding military operations that produced severe energy deficits and lean body mass loss [1,27,50]. Instead, we observed lean body mass gains and maintenance in Testosterone and Placebo, respectively; whereas, both groups lost the same amount of body fat. Administering a lower testosterone dose, implementing more demanding stressors that better mimic the multi-stressor environment of real-world military operations, or a combination of both may have facilitated greater changes in testosterone status and lean body mass.

The dose and duration of exogenous supplementation dictates the recovery of endogenous testosterone production [28]. In the current study, total testosterone concentrations in Testosterone were ~78% and ~50% lower following recovery compared to the energy deficit and run-in phases, respectively. Similarly, free testosterone concentrations in Testosterone were ~83% and ~55% lower following Phase 3 compared to Phases 1 and 2, respectively. Despite lower circulating total and free testosterone concentrations in Testosterone compared to Placebo, lean body mass remained greater in Testosterone than Placebo. However, muscle function measures remained lower in recovery than during the run-in phase and similar to the measures taken at the end of Phase 2, regardless of treatment group. Total and free testosterone concentrations were; however, restored to normal levels within 28 d ($n = 13$), 90 d ($n = 8$), and 11 mo ($n = 1$) after recovery (2 participants did not return for follow-up) [14]. The potential physiological and psychological consequences of supplemental testosterone-induced hypogonadism and subsequent endogenous and functional recovery require consideration. Finally, the benefits and risks of using supplemental testosterone to aid in total body mass and lean body mass retention and muscle function during and in recovery from severe energy deficit in non-obese, young-adult males beyond the 4 wk energy deficit and 2 wk recovery period studied in this trial are unknown.

6. Conclusions

Exogenous testosterone, to our knowledge, has not been administered as an intervention to counter anticipated reductions in testosterone status, lean body mass, and muscle function due to military operational stress. Findings from this proof-of-concept study show testosterone supplementation increases total and free testosterone, and lean body mass but has no effect on lower-body muscle function, type I slow-twitch and type II fast-twitch myofibre cross-sectional area, whole-body and muscle aerobic adaptations, circulating health biomarkers, or blood pressure during a tightly controlled clinical study resembling military operational stress that is severe, but less so than US Army Ranger School. This indicates testosterone supplementation is likely not warranted for operational scenarios that do not markedly decrease testosterone status and, when designing future studies, more sensitive outcomes to assess muscle function and physical performance (e.g., anaerobic/aerobic capacity, occupational and tactical military tasks, including timed load carriage marches, casualty drag, litter carries, and marksmanship) should be included. The primary findings from this study provide rationale for further exploration of practical pharmacologic interventions to mitigate losses of lean body mass experienced by military personnel during periods of unavoidable, severe energy deficit, particularly those that may enhance muscle function.

Contributors

Conceptualization, S.M.P., C.E.B., J.P.K., H.R.L., J.S.O., L.M.M., J.A.C., A.J.Y., M.A.M., W.J.E., O.V., O.T.C., K.M.G., and J.C.R.; Formal Analysis, S.M.P., C.E.B., R.A.B., and J.C.R.; Investigation, S.M.P., C.E.B., N.M.J., M.N.H., and J.C.R.; Writing – Original Draft, S.M.P., C.E.B., and J.C.R.; Writing – Reviewing & Editing, J.P.K., H.R.L., J.S.O., L.M.M., J.A.C., A.J.Y., M.A.M., W.J.E., O.V., O.T.C., K.M.G., N.M.J., R.A.B., and M.N.H.; Supervision,; Project Administration, S.M.P., C.E.B., K.M.G., M.N.H., and J.C.R.; Funding Acquisition, S.M.P. and J.C.R.

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Declaration of Competing Interest

J.C.W., O.T.C., and K.M.G. reported that their institution received funding from the US Department of Defence for work associated with this publication. H.R.L. reported receiving personal fees from Pfizer, Inc., for work outside this publication. All remaining authors declare no competing interests. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defence. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

Acknowledgements

The research reported in this publication is supported by the Collaborative Research to Optimize Warfighter Nutrition II and III projects and the Joint Program Committee-5, funded by the US Department of Defence. The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. The full trial protocol can be accessed as online Supplementary material. The authors wish to thank the participants who consented and participated in this research experiment. The authors would like to thank Dr. Shalender Bhasin for sharing his expertise and assisting the research team on the design of the testosterone intervention. We would also like to thank the study team at Pennington Biomedical Research Centre for their significant contributions to study management and conduct, data collection, and analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.059.

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Testosterone Administration During Energy Deficit Suppresses Hepcidin and Increases Iron Availability for Erythropoiesis

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Context: Severe energy deprivation markedly inhibits erythropoiesis by restricting iron availability for hemoglobin synthesis.

Objective: The objective of this study was to determine whether testosterone supplementation during energy deficit increased indicators of iron turnover and attenuated the decline in erythropoiesis compared to placebo.

Design: This was a 3-phase, randomized, double-blind, placebo-controlled trial.

Setting: The study was conducted at the Pennington Biomedical Research Center.

Patients or Other Participants: Fifty healthy young males.

Intervention(s): Phase 1 was a 14-day free-living eucaloric controlled-feeding phase; phase 2 was a 28-day inpatient phase where participants were randomized to 200 mg testosterone enanthate/week or an isovolumetric placebo/week during an energy deficit of 55% of total daily energy expenditure; phase 3 was a 14-day free-living, ad libitum recovery period.

Main Outcome Measure(s): Indices of erythropoiesis, iron status, and hepcidin and erythroferrone were determined.

Results: Hepcidin declined by 41%, indicators of iron turnover increased, and functional iron stores were reduced with testosterone administration during energy deficit compared to placebo. Testosterone administration during energy deficit increased circulating concentrations of erythropoietin and maintained erythropoiesis, as indicated by an attenuation in the decline in hemoglobin and hematocrit with placebo. Erythroferrone did not differ between groups, suggesting that the reduction in hepcidin with testosterone occurs through an erythroferrone-independent mechanism.

Conclusion: These findings indicate that testosterone suppresses hepcidin, through either direct or indirect mechanisms, to increase iron turnover and maintain erythropoiesis during severe energy deficit. This trial was registered at www.clinicaltrials.gov as #NCT02734238. **(*J Clin Endocrinol Metab* 105: e1316–e1321, 2020)**

Key Words: testosterone, erythropoiesis, hepcidin, iron

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA
Published by Oxford University Press on behalf of the Endocrine Society 2020. This work is written by (a) US Government employee(s) and is in the public domain in the US.
Received 19 October 2019. Accepted 30 December 2019.
First Published Online 2 January 2020.
Corrected and Typeset 11 March 2020.

Steady-state erythropoiesis in healthy human adults produces ~200 billion new erythrocytes each day, a process that requires 20 mg to 25 mg of iron daily (1). Erythropoiesis is inhibited during severe energy

deprivation (2–5), which can occur during military training and operations (6). Diminished erythropoiesis as a result of energy deficit is accompanied by decreases in erythroid progenitors in the bone marrow to preserve iron stores for vital functions. This response underlies the pathogenesis of iron-restricted anemias, such as iron deficiency anemia and the anemia of chronic disease and inflammation (7).

Testosterone is a potent stimulator of erythropoiesis (8–12). In fact, prior to the advent of recombinant erythropoietin (EPO), androgens were used to treat iron deficiency anemia and the anemia of chronic disease and inflammation (8). However, the impact and mechanisms by which testosterone affects erythropoiesis in humans during severe energy deficit remains to be determined. Energy-restricted mice injected with testosterone have increased ^{59}Fe incorporation into newly formed erythrocytes, an indirect measure of erythropoiesis, compared to energy restriction alone (13). Although this suggests that testosterone administration may be an effective strategy to maintain erythropoiesis during energy deficit in mice, to our knowledge, this has not been tested in humans. The objective of this study was to determine the effects of testosterone supplementation, compared to placebo, on erythropoiesis and iron homeostasis in healthy young males during energy deficit.

Materials and Methods

This study addresses secondary objectives of the Optimizing Performance for Soldiers study (14, 15). The study conformed to the principles in the Declaration of Helsinki and was approved by the Institutional Review Board of the Pennington Biomedical Research Center and the U.S. Army Human Research Protections Office. Participants provided written and voluntary informed consent (ClinicalTrails.gov #NCT02734238).

Participants

Healthy, physically active males with normal total testosterone concentrations (10.4–34.7 nmol/L) were recruited. Additional inclusion/exclusion criteria are reported elsewhere (14). Fifty-three participants were enrolled, 50 randomized, and 50 completed the study (15).

Study design and procedures

This study was a 3-phase, randomized, double-blind, placebo-controlled trial. Prestudy characteristics of participants are shown in Table 1. Phase 1 was a 2-week (days 1–14), free-living, controlled-feeding phase. Phase 1 diets were designed to maintain body mass and provide 1.6 g protein/kg/day and 30% fat. Average iron content of the diets during phase 1 were (mean ± SD) 18.8 ± 7.9 mg/day and 19.6 ± 13.4 mg/day for the placebo and testosterone groups, respectively (P = 0.81).

Table 1. Prestudy characteristics of study participants

	Placebo (n = 26)	Testosterone (n = 24)
Race and ethnicity, n (%)		
Non-Hispanic Black	7 (27)	6 (25)
Non-Hispanic White	16 (61)	14 (58)
Hispanic	0 (0)	3 (13)
Other	3 (12)	1 (4)
Age, y	25 ± 1	25 ± 1
Weight, kg	76 ± 2	81 ± 3
BMI, kg/m^2	24 ± 1	25 ± 1
Fat mass, %	17.4 ± 1.3	17.0 ± 1.4
Lean mass, %	56.0 ± 1.1	60.6 ± 2.1*
VO_{2peak}, L/min	3.4 ± 0.6	3.6 ± 0.8
Dietary iron, mg/d	18.8 ± 7.9	19.1 ± 13.3
Energy, kcal/d	2478 ± 950	2432 ± 777

Differences at baseline (day 0) were analyzed using Student's t-tests. Values are means ± SD or n (%). *P < 0.05 compared to placebo.
Abbreviation: BMI, body mass index; VO_2, volume of oxygen uptake.

Phase 2 was a 4-week (days 15–42), inpatient, controlled exercise and feeding phase. At the start of phase 2, participants were randomized to receive either intramuscular injection of testosterone enanthate (200 mg/week) or placebo (1 mL sesame oil/week) on days 15, 21, 28, and 35. Participants were provided a diet and exercise prescription that resulted in a 55% energy deficit during phase 2. Dietary energy intake was restricted to 45% and total daily energy expenditure was increased by 50% of phase 1 total daily energy expenditure. The macronutrient composition of the phase 2 diet was consistent with phase 1 and did not differ between groups. Average iron content of the diets during phase 2 were 17.1 ± 3.5 mg/day and 17.8 ± 4.0 mg/day for the placebo and testosterone groups, respectively (P = 0.52). To increase phase 2 total daily energy expenditure, participants performed low-, moderate-, and high-intensity (40–85% of predetermined peak rate of oxygen uptake) aerobic-type exercise (eg, treadmill and/or outdoor walking and/or running, elliptical, stationary bike, and walking with a weighted backpack containing 30% of body mass). Steady-state, aerobic-type exercise was chosen as the primary means by which total daily energy expenditure was elevated during phase 2 to reflect the aerobic-type physical work that occurs during sustained, strenuous military operations. On average, participants performed 3.5 exercise sessions per day. The physical activity prescription was verified biweekly and adjusted as needed to achieve the desired energy expenditure using open circuit indirect calorimetry (TruOne 2400, ParvoMedics, Salt Lake City, UT). Light calisthenics were incorporated into the exercise routine every 3 to 4 days. Adherence to the prescribed exercise was ≥89% and not different between groups.

Phase 3 (days 43–56) was a free-living, ad libitum recovery period. Participants were instructed to return to their prestudy diet and physical activity routines. Average iron content of the diets during phase 3 were 19.3 ± 7.0 mg/day and 31.4 ± 24.7 mg/day for the placebo and testosterone groups, respectively (P = 0.03).

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Submaximal exercise test

To assess submaximal aerobic adaptations in response to testosterone and energy deficit, participants performed 60 minutes of steady-state aerobic exercise on a cycle ergometer on days 14 and 42. Exercise intensity was matched within participants based on power output and total work performed to limit the confounding effects of weight loss on relative exercise intensity.

Blood collection

Fasting blood samples were collected on days 0, 14, 42, and 56 between 6:00 AM and 9:00 AM. Total testosterone, estradiol, and serum ferritin were measured using an automated immunoassay instrument (Siemens Medical Solutions USA Inc., Malvern, PA). Free testosterone was calculated (16). Hemoglobin was measured using the Beckman Coulter DxH (Beckman Coulter, Brea, CA). Hematocrit was calculated by multiplying red blood cell count by mean cell volume. Erythropoietin and soluble transferrin receptor (sTfR) (R&D Systems Inc., Minneapolis, MN), serum hepcidin (high-sensitivity, DRG International, Springfield, NJ) and erythroferrone (ERFE) (Instrinsic Life Sciences, La Jolla, CA) were determined using enzyme-linked immunoassays. Interleukin-6 (IL-6) was determined using a kit (Millipore, Burlington, MA) and read on a Luminex instrument. Intra- and interassay coefficient of variation for enzyme-linked immunoassays were EPO (5.2% and 8.3%), sTfR (6.4% and 7.1%), hepcidin (4.1% and 14.4%), ERFE (11.0% and 15.0%), and IL-6 (5.0% and 20.0%), respectively. All assays were conducted by Pennington Biomedical Research Center. Pennington Biomedical Research Center follows good clinical practices and is accredited by the College of American Pathologists. All assays were run with standards and appropriate quality control material. In addition, Pennington Biomedical Research Center runs external proficiency samples and results are compared with other laboratories across the country.

Statistical analysis

Data were analyzed using SAS, version 9.4. Differences at baseline (day 0) were analyzed using Student's t-tests. Time-by-treatment effects were analyzed using linear mixed models adjusted for age and baseline values. Bonferroni corrections were used for post hoc comparisons. $P < 0.05$ was considered statistically significant.

Results

Adjusted least square means for clinical biomarkers following each study phase are shown in Table 2. As reported previously (15), total and free testosterone concentrations increased by 134% and 155%, respectively, with testosterone administration during energy deficit, whereas concentrations remained stable or declined with placebo administration. Testosterone administration during energy deficit attenuated the decline in body weight that occurred with placebo.

Hepcidin concentrations declined by 41% with testosterone and remained stable with placebo during energy deficit (P-interaction = 0.0003). Serum ferritin

Table 2. Adjusted least square means for clinical biomarkers for placebo and testosterone following each study phase

	Placebo (n = 26)			Testosterone (n = 24)			*P*		
	Day 14	Day 42	Day 56	Day 14	Day 42	Day 56	Treatment	Day	Interaction
Weight, kg	78.3 (77.7, 78.9)[a]	73.3 (72.7, 74.0)[b]	76.5 (75.8, 77.1)[c]	78.0 (77.4, 78.7)[a]	75.8 (75.1, 76.5)[b*]	79.3 (78.6, 79.9)[a*]	<0.0001	<0.0001	<0.0001
TT, nmol/L	14.6 (12.9, 16.4)[a]	11.8 (10.0, 13.5)[a]	14.6 (12.8, 16.3)[a]	15.5 (13.7, 17.4)[a]	36.2 (34.3, 38.0)[b*]	7.8 (5.9. 9.8)[c*]	<0.001	<0.001	<0.0001
FT, nmol/L	0.31 (0.26, 0.36)[a]	0.18 (0.13, 0.23)[b]	0.29 (0.23, 0.34)[a,b]	0.33 (0.27, 0.38)[a]	0.87 (0.81, 0.92)[b*]	0.15 (0.10, 0.21)[c*]	<0.001	<0.001	<0.0001
Estradiol, pmol/L	142 (124, 159)[a]	131 (114, 148)[a]	158 (141, 175)[a]	148 (130, 166)[a]	247 (229, 265)[b*]	144 (125, 162)[a]	<0.001	<0.001	<0.0001
Hemoglobin, g/L	144 (142, 147)[a]	138 (136, 141)[b]	139 (137, 142)[b]	145 (143, 148)[a]	145 (142, 148)[a*]	145 (142, 147)[a]	0.002	0.012	0.035
Hematocrit, %	42.8 (42.0, 43.6)[a]	41.5 (40.7, 42.3)[a]	41.5 (40.7, 42.3)[a]	42.9 (42.1, 43.7)[a]	43.6 (42.7, 44.4)[a*]	43.2 (42.3, 44.0)[a]	0.003	0.450	0.020
EPO, mIU/mL	8.8 (7.6, 9.9)[a]	8.5 (7.1, 9.8)[a]	14.1 (12.0, 16.2)[b]	8.4 (7.2, 9.6)[a]	11.3 (9.9, 12.8)[b]	8.8 (6.5, 11.1)[a,b*]	0.218	0.0003	0.0002
Hepcidin, ng/mL	8.1 (6.6, 9.8)[a,b]	10.1 (8.5, 11.6)[a]	6.7 (5.1, 8.3)[b]	8.6 (7.0, 10.3)[a]	5.1 (3.4, 6.7)[b*]	6.4 (4.7, 8.2)[a,b]	0.044	0.076	0.0003
Ferritin, ng/mL	92.6 (78.7, 106.5)[a,b]	107.9 (94.2, 121.6)[a]	76.3 (62.6, 90.0)[b]	91.0 (76.8, 105.2)[a]	59.1 (44.6, 73.5)[b*]	47.2 (32.1, 62.2)[b*]	0.001	<0.0001	0.0001
sTfR, nmol/L	17.0 (15.4, 18.6)[a]	18.3 (16.7, 19.9)[a,b]	20.6 (19.1, 22.3)[b]	17.7 (16.1, 19.4)[a]	20.6 (18.9, 22.2)[b]	24.4 (22.6, 26.1)[c*]	0.021	<0.0001	0.139
sTfR/$\log_{10}$ ferritin	9.5 (8.9, 10.1)[a]	10.1 (8.8, 11.5)[a,b]	11.8 (10.1, 13.6)[b]	9.7 (9.1, 10.3)[a]	12.5 (11.1, 13.9)[b]	15.5 (13.6, 17.5)[c]	0.005	<0.0001	0.003
IL-6, pg/mL	8.5 (6.2, 10.8)	10.2 (7.9, 12.5)	11.1 (8.9, 13.4)	9.2 (6.9, 11.6)	8.5 (6.1, 10.9)	11.9 (9.4, 14.5)	0.971	0.054	0.401
ERFE, ng/mL	3.0 (2.2, 3.9)	3.6 (2.8, 4.5)	2.9 (2.1, 3.8)	3.4 (2.6, 4.3)	3.0 (2.1, 3.8)	3.4 (2.5, 4.3)	0.860	0.953	0.165

Values are adjusted least square means (95% confidence interval). Time-by-treatment effects were analyzed using linear mixed models, adjusted for age and baseline values. Bonferroni corrections were used for post hoc comparisons. $P < 0.05$ was considered statistically significant. Data not sharing the same letter superscript within a treatment are different by day. Asterisk indicates a between group difference at a particular day.

Abbreviations: EPO, erythropoietin; ERFE, erythroferrone; FT, free testosterone; IL-6, interleukin-6; sTfR, soluble transferrin receptor; TT, total testosterone.

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concentrations declined by 35% (P-interaction = 0.0001) and sTfR increased by 16% (P-treatment = 0.021) with testosterone and was not affected with placebo during energy deficit. Likewise, the sTfR/$\log_{10}$ ferritin ratio, an index of iron-dependent erythropoietic activity, increased by ~30% with testosterone but remained unchanged with placebo during energy deficit (P-interaction = 0.0026).

Testosterone prevented the decline in hemoglobin (P-interaction = 0.035) and hematocrit (P-interaction = 0.020) that occurred with placebo during energy deficit, such that hemoglobin and hematocrit were 5% greater at the end of energy deficit with testosterone compared to placebo. Erythropoietin increased by 34% with testosterone during energy deficit, whereas energy deficit alone did not affect EPO concentrations (P-interaction = 0.0002). Erythroferrone and IL-6 were not affected by energy deficit or testosterone.

As reported previously, lower-body muscle function declined in both groups with energy deficit (15). Submaximal aerobic adaptations in response to testosterone and energy deficit are shown in Table 3. Total energy, heart rate, perceived exertion, and measures of respiratory gas exchange improved and there was a greater reliance on fat oxidation during phase 2 regardless of treatment.

Discussion

The majority of iron required to support erythropoiesis is provided by reticuloendothelial macrophages, which recycle approximately 20 mg to 25 mg iron per day from senescent red blood cells. Iron availability is regulated by the liver-produced hormone, hepcidin. Hepcidin binds to ferroportin, which is expressed by macrophages, enterocytes, and hepatocytes, signaling for its internalization and degradation, thereby limiting iron availability for erythropoiesis. In the current study, hepcidin concentrations were markedly suppressed with testosterone compared to placebo during energy deficit.

Concomitant with the decline in hepcidin was a decline in serum ferritin concentration and an increase in sTfR with testosterone compared to placebo during energy deficit. The transferrin receptor is shed from the plasma membrane of erythrocytes during maturation and circulates as sTfR. Thus, circulating sTfR concentrations are a measure of total erythroid activity and reflect plasma iron turnover and erythroid transferrin uptake if iron deficiency is not present (17). Along with the increase in sTfR/$\log_{10}$ ferritin ratio, an index of iron-dependent erythropoietic activity (17, 18), these findings suggest that suppressed hepcidin and a decline

Table 3. Exercise intensity, perceived exertion, respiratory gas exchange, and substrate oxidation during 60 minutes of steady-state aerobic exercise during phases 1 and 2

	Placebo (n = 26)		Testosterone (n = 24)		*P*		
	Day 14	Day 42	Day 14	Day 42	Treatment	Day	Interaction
Total work, kJ	451 (412, 490)	451 (412, 490)	462 (422, 502)	462 (421, 502)	0.89	0.70	0.58
Power output, W	125 (114, 136)	125 (114, 136)	128 (117, 139)	128 (117, 139)	0.81	0.70	0.40
Total energy, kcals	616 (571, 661)	540 (501, 579)	640 (593, 687)	573 (533, 614)	<0.0001	0.31	0.65
Heart rate, bpm	162 (157, 167)	131 (126, 136)	158 (153, 163)	131 (125, 136)	<0.0001	0.45	0.40
Rating of perceived exertion	15 (15, 16)	13 (13, 14)	15 (14, 16)	13 (12, 14)	<0.0001	0.80	0.98
Respiratory gas exchange							
VO_2, L/min	2.09 (1.93, 2.24)	1.88 (1.75, 2.01)	2.16 (2.00, 2.31)	1.98 (1.85, 2.12)	<0.0001	0.36	0.63
VO_2 peak, %	61.0 (58.8, 63.2)	55.2 (52.6, 57.8)	60.3 (58.0, 62.6)	55.7 (53.1, 58.4)	<0.0001	0.96	0.51
VCO_2, L/min	1.88 (1.74, 2.02)	1.48 (1.37, 1.60)	1.99 (1.84, 2.13)	1.61 (1.49, 1.73)	<0.0001	0.18	0.78
RER	0.90 (0.89, 0.91)	0.79 (0.77, 0.80)	0.92 (0.91, 0.93)	0.81 (0.79, 0.82)	<0.0001	0.01	0.96
Substrate oxidation, total g							
Carbohydrate, total g	104 (95, 114)	40 (32, 48)	119 (109, 129)	53 (45, 62)	<0.0001	0.01	0.81
Fat, total g	22.0 (18.7, 25.4)	42.1 (38.5, 45.7)	18.2 (14.7, 21.6)	40.0 (36.2, 43.7)	<0.0001	0.13	0.58

Values are adjusted least square means (95% confidence interval). Time-by-treatment effects were analyzed using linear mixed models and adjusted for age. Adapted from (15).
Abbreviations: RER, respiratory exchange ratio; VCO_2, volume of exhaled carbon dioxide; VO_2, volume of oxygen uptake.

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in functional iron stores support the increase in erythropoiesis with testosterone during energy deficit.

It is unclear whether testosterone directly suppresses hepcidin or indirectly suppresses hepcidin through its effects on EPO. Erythropoiesis is stimulated by EPO, a renal hormone that rapidly increases the expression of transferrin receptor on the plasma membrane of erythroid precursors. Transferrin receptor can then take up transferrin-bound iron in circulation to produce heme for incorporation into hemoglobin. During steady-state erythropoiesis in healthy human adults, this process translates to 20 mL of blood production each day, containing 6 g of hemoglobin and 20 mg of iron. A study in older men receiving 10 g testosterone gel or placebo daily demonstrated that testosterone stimulates the secretion of EPO (12). The response appears to be transient, as the increase in EPO was observed at 1 and 3 months but returned to placebo levels at 6 months. In the current study, EPO increased by 34% compared to baseline values with 1 month of testosterone administration during energy deficit, whereas energy deficit alone did not affect EPO concentrations. The increase in EPO with testosterone during energy deficit is consistent with animal studies that demonstrate an exponential increase in ^{59}Fe incorporation into erythrocytes in starved rats receiving graded doses of EPO (19).

Recent studies have demonstrated that EPO stimulates the secretion of ERFE from erythroid precursors (20). Erythropoietin-stimulated ERFE suppresses hepcidin levels, resulting in an increase in circulating levels of iron and iron incorporation into erythrocytes. However, in the current study, ERFE was not affected by energy deficit or testosterone, suggesting that the decrease in hepcidin with testosterone occurs through an ERFE-independent mechanism. Alternatively, a biphasic effect of EPO on hepcidin expression was recently proposed: an ERFE-independent repression in hepcidin where the immediate and transient decrease in serum iron with EPO inhibits the BMP-SMAD pathway and an ERFE-dependent repression whereby hepcidin is repressed until increased iron needs are met (21). Other possibilities include a direct effect of testosterone on bone morphogenetic protein (BMP)–sons of mother against decapentaplegic (SMAD) signaling (11) and/or the aromatization of testosterone to estradiol, which suppresses hepcidin transcription (22, 23). The lack of change in IL-6 during energy deficit in the current study excluded the possibility of a mediating role of the IL-6–induced Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) signaling pathway on hepcidin transcription.

Findings from the current study indicate that testosterone supplementation suppresses hepcidin in healthy human adults, thereby increasing iron utilization and maintaining erythropoiesis during energy deficit. Despite these findings, submaximal aerobic adaptations improved in both groups regardless of treatment. This is likely due to the aerobic-type exercise performed during phase 2 to increase total daily energy expenditure. Future work should investigate whether attenuations in weight loss and maintained erythropoiesis with testosterone supplementation during severe energy deficit lead to improvements in performance outcomes. Furthermore, rodent models may be necessary to determine the mechanism by which testosterone suppresses hepcidin during severe energy deficit. These findings suggest that testosterone administration may be an effective therapy to maintain erythropoiesis during energy deficit, which may be particularly relevant for military training or operations and in certain types of anemias of chronic disease (24, 25).

Acknowledgments

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations or commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. Supported by the Collaborative Research to Optimize Warfighter Nutrition II and III projects, the Defense Health Program Joint Program Committee-5, and appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Materiel Command.

Clinical Trial Information: Registered at www.clinicaltrials.gov as #NCT02734238.

Additional Information

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in the References section.

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Bradbury et al. Journal of the International Society of Sports Nutrition (2020) 17:4
https://doi.org/10.1186/s12970-020-0335-2

Journal of the International Society of Sports Nutrition

SHORT REPORT Open Access

Effects of carbohydrate supplementation on aerobic exercise performance during acute high altitude exposure and after 22 days of acclimatization and energy deficit

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Abstract

Background: The ergogenic effects of supplemental carbohydrate on aerobic exercise performance at high altitude (HA) may be modulated by acclimatization status. Longitudinal evaluation of potential performance benefits of carbohydrate supplementation in the same volunteers before and after acclimatization to HA have not been reported.

Purpose: This study examined how consuming carbohydrate affected 2-mile time trial performance in lowlanders at HA (4300 m) before and after acclimatization.

Methods: Fourteen unacclimatized men performed 80 min of metabolically-matched (~ 1.7 L/min) treadmill walking at sea level (SL), after ~ 5 h of acute HA exposure, and after 22 days of HA acclimatization and concomitant 40% energy deficit (chronic HA). Before, and every 20 min during walking, participants consumed either carbohydrate (CHO, $n = 8$; 65.25 g fructose + 79.75 g glucose, 1.8 g carbohydrate/min) or flavor-matched placebo (PLA, $n = 6$) beverages. A self-paced 2-mile treadmill time trial was performed immediately after completing the 80-min walk.

Results: There were no differences ($P > 0.05$) in time trial duration between CHO and PLA at SL, acute HA, or chronic HA. Time trial duration was longer ($P < 0.05$) at acute HA (mean ± SD; 27.3 ± 6.3 min) compared to chronic HA (23.6 ± 4.5 min) and SL (17.6 ± 3.6 min); however, time trial duration at chronic HA was still longer than SL ($P < 0.05$).

Conclusion: These data suggest that carbohydrate supplementation does not enhance aerobic exercise performance in lowlanders acutely exposed or acclimatized to HA.

Trial registration: NCT, NCT02731066, Registered March 292,016

Keywords: Hypoxia, Time trial, Ergogenic aid

Background

Reduced arterial oxygen content degrades aerobic exercise performance in lowlanders initially exposed to high altitude (HA) [1]. After several weeks of HA exposure, acclimatization occurs, and aerobic exercise performance partially improves [1–3]. Another factor that may impact the performance degradation seen at altitude are changes in substrate oxidation [4], as endogenous carbohydrate oxidation during exercise may be higher with acute HA exposure compared to absolute VO_2-matched exercise at sea level (SL) [5]. After acclimatization, muscle glucose uptake during exercise is also increased relative to SL [6]. Collectively, these data suggest that consuming supplemental carbohydrate during exercise at HA might be an effective strategy to attenuate performance decrements by sparing endogenous carbohydrate stores and meeting the apparent increase in carbohydrate requirements during exercise.

Carbohydrate supplementation during exercise at SL spares endogenous carbohydrate stores and delays the onset of fatigue [7, 8]. Effects of carbohydrate supplementation on

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exercise performance at HA are not well described. In unacclimatized men, Fulco et al. [9] demonstrated that carbohydrate supplementation during exercise enhanced time trial performance compared to placebo after 3 days of HA (4300 m) exposure concomitant with a 30% energy deficit (negative energy balance is largely unavoidable and commonly experienced by lowlanders sojourning at HA) [10]. However, carbohydrate supplementation had no further performance benefit after 10 days of acclimatization and negative energy balance. In a follow-up study, Fulco et al. [11] found no benefit of carbohydrate supplementation on time trial performance on the first and third day of HA exposure in previously HA acclimatized men in a state of energy balance. These discordant results from vastly different experimental designs (i.e., energy and acclimatization status of participants) suggest that the potential ergogenic effects of supplemental carbohydrate on exercise performance at HA may, in part, be modulated by acclimatization. To extend these findings [9, 11], we examined the effects of carbohydrate supplementation on aerobic exercise performance in unacclimatized men after 5 h of HA (4300 m) exposure and following 22 days of HA acclimatization and concomitant 40% energy deficit. Based on the studies by Fulco and colleagues [9, 11], we hypothesized that carbohydrate supplementation would enhance exercise performance during acute HA exposure, but not after HA acclimatization.

Methods

Experimental design

Data included in this Short Report were secondary analyses from a controlled feeding and exercise study that assessed the effects of high protein diets on body composition during sustained energy deficit at HA [12]. This study (clinical trials.gov: NCT02731066) was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA) and participants provided written informed consent. Characteristics of the 14 unacclimatized, young men included in this report were detailed by Young et al. [13].

The experimental design has been reported extensively [12–16]. In brief, the study was conducted over 43 consecutive days. During the first 21 days (SL), participants consumed a self-selected, weight-maintaining diet, maintained habitual exercise levels, and were free-living but visited the laboratory daily. On SL day 21, participants were flown from Boston, MA to Denver, CO where they were placed on supplemental oxygen until being driven to the summit of Pikes Peak, CO (4300 m) the following morning where they resided at the USARIEM Maher Memorial Altitude Laboratory for the next 22 days at HA. During HA, participants were under constant supervision, performed daily exercise, and consumed either standard protein (mean ± SD; 1.1 ± 0.2 g/kg/d) or high protein (2.1 ± 0.2 g/kg/d), carbohydrate-matched, energy deficient diets (40%; 30% by energy restriction and 10% by exercise). Fat was the primary manipulated macronutrient during the energy deficit, such that the standard protein group consumed 1.1 ± 0.2 g/kg/d fat, and the high protein group consumed 0.7 ± 0.1 g/kg/d fat [12]. The diet intervention resulted in a 7.9 ± 1.9 kg loss of total body mass [13].

Exercise and 2-mile time trial

Participants were randomized to groups and were provided equal volumes of flavor-matched carbohydrate (CHO; 65.25 g fructose + 79.75 g glucose ingested at 1.8 g/min, $n = 8$, 3 standard and 5 high protein) and placebo (PLA; $n = 6$, 4 standard and 2 high protein) beverages during 80 min of metabolically-matched, steady-state treadmill walking at SL (day 7), 5 h after arriving at HA (acute HA), and after 22 days of acclimatization and energy deficit (chronic HA, day 42). As previously reported [13] treadmill speed, grade, absolute oxygen uptake (L/min), and metabolic rate were not different across study phases. All time trials were completed at the same time of day (between 1130 and 1230) and participants were fasted prior to the start of exercise. Participants were given a 5 min rest after completing the steady-state exercise before performing a self-paced, 2-mile treadmill time trial. Peripheral oxygen saturation (SpO_2; finger pulse oximetry, Model 9560; Nonin, Plymouth, MN, USA) and heart rate were assessed at baseline and in half mile intervals, and Ratings of Perceived Exertion (RPE) [17] were recorded at baseline and immediately after completing the 2-mile time trial. Participants were familiarized to the test procedures (80 min steady-state and 2-mile time trial) on three occasions before completing the SL trial on day 7. The coefficient of variation of the three familiarization time trials was 5.06%, indicating that the test-retest variation in performance was small.

Statistical analyses

As mentioned, this study was part of a larger investigation [12], powered to test the effects of dietary protein on body composition during altitude acclimatization and concomitant energy deficit. We did not expect dietary protein level to affect time trial performance at chronic HA (neither SL or acute HA were subject to the dietary protein intervention), which we confirmed using linear mixed models with dietary protein level, treatment (CHO, PLA), phase (SL, acute and chronic HA), exercise time point (for SpO_2 and heart rate), and their interactions as fixed effects. Therefore, the effects of the dietary protein intervention are not presented in this report, and the data were re-analyzed using the same linear mixed-model without dietary protein in the model. Tukey's HSD tests were used for multiple comparisons if significant main effects of interaction effects were

observed. Freidman's test was used to assess differences in RPE (baseline, end of the time trial) across study phases. Statistical significance was accepted at $P < 0.05$. Data were analyzed using SPSS (v.22.0; Chicago, IL, USA).

Results

There was no effect ($P > 0.05$) of CHO on time trial duration, oxygen saturation, heart rate, or RPE across study phases. Time trial duration was longer ($P < 0.05$) at acute HA (27.3 ± 6.3 min) compared to chronic HA (23.6 ± 4.5 min) and SL (17.6 ± 3.6 min); however, time trial duration at chronic HA was still longer than SL (phase main effect, $P < 0.05$, Fig. 1). Mean heart rate and SpO_2 during the time trials were lower at acute and chronic HA compared to SL, and SpO_2 at chronic HA was higher than acute HA (phase main effect, $P < 0.05$, Table 1). RPE was similar across study phases.

Discussion

This study examined the effects of consuming supplemental carbohydrate on aerobic exercise performance in recreationally-active, healthy, young lowlanders at HA, before and following 22 days of acclimatization while in a constant state of negative energy balance. We demonstrated that consuming supplemental carbohydrate during steady-state exercise had no performance-enhancing effects on time trial performance before or after HA acclimatization. As expected, time trial performance was approximately 55% slower at acute HA compared to SL. Acclimatization partially restored exercise performance relative to acute HA; however, time trial performance at chronic HA was still 34% slower than SL. Carbohydrate supplementation had no effect on perceived effort during any of the performance trials. These data, which were derived from a highly controlled exercise and diet intervention study, suggest that carbohydrate supplementation does not enhance aerobic exercise performance of lowlanders at HA.

In our study, supplemental carbohydrate did not enhance exercise performance in lowlanders acutely exposed to HA (5 h), which conflicts with data reported by Fulco et al. [9], who demonstrated that lowlanders consuming carbohydrate on the third day of continuous residence at 4300 m performed a 720 kJ cycle ergometry time trial faster than participants consuming the placebo. We suspect the discrepancy between our data and those by Fulco et al. [9] are due, in part, to the apparent inability to effectively oxidize exogenous carbohydrate upon initial exposure to HA. In the same study, we demonstrated a 52% reduction in exogenous carbohydrate oxidation and a corresponding increase in endogenous carbohydrate oxidation (i.e., blood glucose, muscle and liver glycogen) during the 80 min steady-state exercise bout with acute HA exposure compared to SL[13]. The mechanisms accounting for the reduction in exogenous carbohydrate oxidation during acute HA exposure are not known, but may be attributable to adjustments in glycemic regulation, as participants were markedly hyperinsulinemic, which inhibited lipolysis and likely accelerated glycogenic flux. The inability to effectively oxidize exogenous carbohydrate during acute HA exposure was alleviated after 22 days of acclimatization [13]. It is possible that the adaptations that improve exogenous carbohydrate oxidation with acclimatization develop within only a few days of HA exposure, which would account for the performance enhancing effects observed by Fulco et al. [9] after 3 days of acclimatization. That, combined with the longer duration time trial (greater reliance on carbohydrates) in the Fulco study [9] (720 kJ cycle ergometry time trial), compared to our 2-mile time trial, may explain why they observed improvements in time trial performance and we did not.

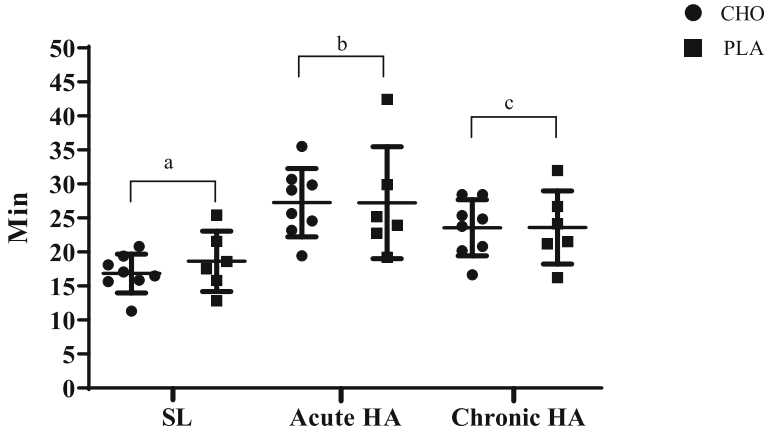


Fig. 1 2-mile time trial duration (min) at sea level, acute and chronic HA for CHO and PLA. Values are presented as mean ± SD. Points not sharing the same letter are different (phase main effect, P < 0.05). SL, sea level; HA, high altitude; CHO, carbohydrate; PLA, placebo

Table 1 Heart rate, peripheral oxygen saturation, and ratings of perceived exertion during the 2-mile time trial[a]

	SL		Acute HA		Chronic HA	
	CHO	PLA	CHO	PLA	CHO	PLA
HR (bpm)[2]	181 ± 10	175 ± 9	159 ± 9	168 ± 14	166 ± 13	161 ± 18
SpO_2 (%)[2,3]	93 ± 2	95 ± 3	71 ± 4	73 ± 4	77 ± 3	81 ± 4
RPE	17 (14–19)	17 (16–18)	16 (13–20)	18 (15–20)	18 (14–19)	19 (16–19)

[a]Values presented as mean ± SD, except for RPE which is presented as median (range)
[2]Phase main effect; acute and chronic HA different than SL, $P < 0.05$
[3]Phase main effect; chronic HA different than acute HA, $P < 0.05$.
SL Sea level, *HA* High altitude, *HR* Heart rate, *CHO* Carbohydrate, *PLA* Placebo, *SpO_2* Peripheral oxygen saturation, *RPE* Rating of Perceived Exertion

Our chronic HA results are in line with what has been previously reported about the influence of carbohydrate supplementation on time trial performance after acclimatization [9, 11]. Although we demonstrated that ability to oxidize exogenous carbohydrates was restored with acclimatization to HA [13], carbohydrate supplementation still did not improve performance. However, exercise performance in both carbohydrate and placebo following 22 days of acclimatization and energy deficit did improve relative to acute HA as a result of the normal adaptive responses that occur with acclimatization. Since muscle glycogen utilization is related to relative exercise intensity ($\%VO_{2peak}$) [9, 11, 18], it may be that the exercise intensity during the time trials was not high enough to cause decreases in muscle glycogen that would cause the body to rely on exogenous sources of carbohydrate to complete the exercise task.

The practical implications of our findings must be interpreted in the context of certain experimental limitations. For example, it may be that carbohydrate supplementation did not improve performance due to the potential that such high carbohydrate intakes, particularly at altitude, may have reduced gastric emptying and intestinal carbohydrate absorption [19]. However, those measures were not included in our study. Likewise, our applied performance outcomes may have been strengthened if additional mechanistic measures oxygen transport capacity were included. Our design also does not allow us to completely rule out the potential effects of sustained energy deficit on time trial performance after 22 days of acclimatization. However, including fully fed, energy balance control groups was impractical and outside the scope of our study. We also contend that our findings, which show that carbohydrate supplementation failed to enhance performance following 22 days of acclimatization and concomitant energy deficit, are not only consistent with Fulco et al. [9], but are actually strengthened by our experimental design. More specifically, rather than experimentally enforcing energy balance, we assessed the putative performance enhancing effect of supplemental carbohydrate in response to the real world conditions (i.e., negative energy balance) that lowlanders typically experience during prolonged HA sojourns [10]. Most importantly, considering carbohydrate supplementation did not affect performance, and reliance on endogenous carbohydrate to fuel steady-state exercise appears to be higher during acute HA exposure than it is for absolute intensity-matched exercise at sea level [20], provides practical evidence to recommend that individuals planning to sojourn at HA to complete physically demanding work or exercise should prioritize carbohydrate intake at SL to optimize glycogen stores before ascending to HA.

Conclusion

We conclude that carbohydrate supplementation during steady-state exercise does not enhance exercise performance in lowlanders acutely exposed to hypoxia or sojourning at HA for 22 days.

Abbreviations

CHO: Carbohydrate; HA: High altitude; PLA: Placebo; RPE: Rating of Perceived Exertion; SL: Sea level; SpO_2: Oxygen saturation; USARIEM: US Army Research Institute of Environmental Medicine

Acknowledgements

We thank the volunteers that participated in the study. We acknowledge our medical oversight team and Dr. Stephen Muza for their support of this study. Importantly, we sincerely thank Mr. Bradley Anderson, Ms. Katelyn Guerriere, Mr. Grant Holmes, Mr. Anthony Karis, Mr. Matthew Kominsky, Ms. Allyson Derosier, Ms. Adrienne Hatch, Ms. Laura Lutz, Mr. Phillip Niro, Ms. Nancy Murphy, Drs. Steven Hennigar, Taylor Roth, Arny Ferrando, J. Phillip Karl, Lee Margolis, Scott Montain, John Carbone, James McClung, and LTC Renee Cole for their significant contributions to the study.

Declarations

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

Authors' contributions

CEB, RWK, AJY, and SMP designed the research; KEB, CEB, MAW, AJL, RWK, AJY, and SMP performed the research. KEB and AJL analyzed the data. KEB and SMP prepared tables, figures, and drafted the manuscript. All authors edited and revised the manuscript and approved the final version.

Funding

This work was supported by the US Army Medical Research and Development Command.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine (USARIEM, Natick, MA) and participants provided written informed consent prior to any participation in the study. The research was performed in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 9 October 2019 Accepted: 3 January 2020
Published online: 09 January 2020

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Eating Behaviors 36 (2020) 101367

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Body weight status, perceived stress, and emotional eating among US Army Soldiers: A mediator model

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ARTICLE INFO

Keywords:
Stress
Emotional eating
Body mass index
Body composition
Military
Mediation analysis

ABSTRACT

Background: In civilians, overweight and obesity are associated with emotional eating behaviors such as eating in response to stress, but this association has not been examined in Soldiers, a population with unique stressors. This cross-sectional study examined the relationship between perceived stress (PS), emotional eating (EE), and outcomes of body mass index (BMI), and failing Army body composition (BC) standards among Soldiers.

Methods: Soldiers (N = 1460, 83% male, 23.5 ± 5.2 years old) completed validated surveys on PS, EE, and adherence with military BC standards. Conditional process models and mediation models tested gender as a moderator and EE as a mediator of associations between PS and BMI and PS and BC failure, respectively.

Results: Higher PS was associated with more frequent self-reported EE behaviors ($p < 0.001$), higher BMI ($p < 0.001$), and BC failure ($p < 0.001$). BMI significantly increased with frequency of reported EE behaviors ($p < 0.001$). Gender was not a statistically significant moderator in the relationship between PS, EE, and, BMI ($p = 0.83$) or BC failure ($p = 0.57$). PS appears to affect BMI indirectly through EE behaviors ($c' = 0.03$, 95% CI: 0.02, 0.04). PS may affect BC failure directly ($c' = 1.04$, 95% CI: 1.01, 1.08) and indirectly ($ab = 1.02$, 95% CI: 1.01, 1.03) through EE as a mediator.

Conclusions: EE behaviors may mediate the positive association between PS, BMI, and BC failure. Prospective investigation is warranted to better understand the role of EE in health-related outcomes among Soldiers and populations in high stress professions.

1. Introduction

Overweight and obesity among Soldiers is a major concern for the US Army. Although Soldiers are required to meet body weight and body composition standards, the prevalence of overweight and obesity has risen such that in 2017, over 17% were considered obese based on a body mass index (BMI) of ≥30 kg/m^2 (https://phc.amedd.army.mil/topics/campaigns/hof/Pages/default.aspx, 2017). Soldiers with a higher BMI have an increased risk of injury and illness (Cowan, Bedno, Urban, Yi, & Niebuhr, 2011; Hruby, Bulathsinhala, McKinnon, et al., 2016; Knapik, Jones, Darakjy, et al., 2007), which can limit their ability to meet job requirements and causes a significant financial burden to the military health care system (Dall, Zhang, Chen, et al., 2007). Understanding the underlying factors contributing to overweight and obesity among Soldiers is critical to informing the development of healthy weight management interventions and, ultimately, improving military readiness (Hauret, Jones, Bullock, Canham-Chervak, & Canada, 2010; McNulty, 2009).

One potential underlying factor is stress. Similar to other public service professionals (e.g. first responders), Soldiers commit to a highly stressful and often hazardous lifestyle, and must deal with unique military-specific stressors such as physically demanding job duties, upheavals resulting from deployments, and routine relocations. Stress negatively impacts health through psychological, physiological, and behavioral pathways (Hammen, 2005; Steptoe, 1991), and is known to contribute to a variety of health issues, including overweight/obesity (Lagraauw, Kuiper, & Bot, 2015). For example, in civilian populations, higher stress levels have been associated with elevated BMI (Laugero,

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https://doi.org/10.1016/j.eatbeh.2020.101367
Received 1 May 2019; Received in revised form 13 January 2020; Accepted 14 January 2020
Available online 16 January 2020
1471-0153/ Published by Elsevier Ltd.

Falcon, & Tucker, 2011; Mouchacca, Abbott, & Ball, 2013), increased visceral adipose tissue (Adam & Epel, 2007; Mouchacca et al., 2013; Nastaskin & Fiocco, 2015), and weight gain (Torres & Nowson, 2007).

Relationships between stress and overweight/obesity are thought to be mediated, in part, by the influence of stress on eating behaviors (Koenders & van Strien, 2011; Wiedemann, Ivezaj, & Barnes, 2018). For example, stress has been associated with increased energy intake due to higher consumption of palatable foods, specifically fats and sugars (Born et al., 2011; Laugero et al., 2011; Nastaskin & Fiocco, 2015). Research in civilian populations has shown that individuals overeat to reduce stress, anxiety, fear, or negative emotions (Greeno & Wing, 1994), and eating in response to emotional stimuli such as stress, or emotional eating (EE), has been associated with weight gain (Bongers & Jansen, 2016). Further, stress alone has been associated with increased energy intake due to higher consumption of palatable foods, specifically fats and sugars (Born et al., 2011; Greeno & Wing, 1994; Laugero et al., 2011; Nastaskin & Fiocco, 2015).

In civilian studies, variations in the extent to which eating behaviors are affected by stress have been observed. For example, EE appears to be more prevalent among women, who may choose higher fat and sugar foods when under stress than men (Adam & Epel, 2007; Peneau, Menard, Mejean, Bellisle, & Hercberg, 2013). Women, who often have additional sources of stress than men as primary caregivers and caretakers (Wahl & Rundall, 1996), are more prone to increase food consumption when under stress (Beydoun, 2014; Nevanpera et al., 2012; Peneau et al., 2013). Eating in response to emotional stimuli such as stress may therefore contribute to overweight and obesity. The effect of EE may be stronger among populations in high stress professions and may be of greater concern among women in these professions.

While stress inherent to military or public service is to some extent unavoidable, an improved understanding of the relationship between stress and undesirable health outcomes, such as overweight and obesity, is needed to inform the development of interventions aimed at minimizing the adverse health effects of stress. To date, no study has examined the extent to which EE mediates associations between stress and body weight outcomes in Soldiers. Therefore, this cross-sectional study in Soldiers examined gender as a moderator and EE as a mediator of associations between perceived stress (PS), BMI, and failing body composition (BC) standards, respectively. We hypothesized that, in Soldiers, frequency of EE behaviors would be greater among female Soldiers, and would mediate associations between PS and BMI and PS and BC failure, respectively.

2. Methods

2.1. Study population

From 2016 to 2017, 1544 Soldiers from Army installations at Fort Jackson, SC, Joint-Base Lewis McChord, WA, Fort Riley, KS, Fort Sam Houston, TX, Fort Carson, CO, Joint Base Elmendorf-Richardson, AK, and Fort Campbell, KY were briefed in person and a total of 1460 (95%) completed surveys. Soldiers completing the survey ranged in age from 18 to 58 years (mean ± SD 23.5 ± 5.2). Soldiers were surveyed to obtain information on multiple topics conceptually related to eating behaviors or mediators of eating behaviors to include PS, EE, adherence with Army body composition (BC) standards, and sociodemographic information (military status, i.e., initial trainee or not a trainee), age, race/ethnicity, level of education, marital status, use of tobacco, and physical activity. All active-duty Soldiers 18 years of age and older were eligible to participate. A *post-hoc* power analysis using G*Power was conducted for regression models (Faul, Erdfelder, Lang, & Buchner, 2007). The sample size was more than sufficient for regression models with ten predictors to detect an R^2 as small as 0.02 or an odds ratio of 1.2 with 80% probability at a significance level of 0.05. The study was approved by the Institutional Review Board of the US Army Medical Research and Development Command, Fort Detrick, Maryland. Investigators adhered to the policies regarding the protection of human subjects as prescribed in Army Regulation 70–25, the research was conducted in adherence with the provisions of 32 CFR Part 219, all participants provided written informed consent prior to participation, and all participants were assured that their responses would be kept confidential.

2.2. Dependent variables

2.2.1. Body mass index and body composition failure

BMI was calculated from self-reported height and weight. Data were screened for biologically implausible values and outliers. Visual inspection and residual statistics during assumption testing yielded two BMI measures above the 99th percentile with a residual > 3. These two observations were demonstrated to be influential outliers when removing these observations significantly changed the estimate of coefficients. The Army assesses compliance with body composition standards twice yearly through BMI-based screening, and for those who exceed the maximum allowable screening weight, a circumference-based body fat assessment commonly referred to as the "tape test" is conducted (https://armypubs.army.mil/epubs/DR_pubs/DR_a/pdf/web/ARN7779_AR600-9_FINAL.pdf, 2019). Female Soldiers are measured at three circumference sites: neck, waist, hip, and male Soldiers are measured at two sites: neck and waist (https://armypubs.army.mil/epubs/DR_pubs/DR_a/pdf/web/ARN7779_AR600-9_FINAL.pdf, 2019). Soldiers who exceed maximum allowable body fat percent are counseled and provided with nutrition education (https://armypubs.army.mil/epubs/DR_pubs/DR_a/pdf/web/ARN7779_AR600-9_FINAL.pdf, 2019). In this study, BC failure was determined by the question "Have you ever failed the body fat tape test?" with possible responses being "yes" or "no".

2.3. Independent variables

2.3.1. Perceived stress

PS scores were assessed using Cohen's ten-item Perceived Stress Scale (PSS-10). The PSS-10 is a validated measure ($\alpha = 0.91$) (Cohen & Janicki-Deverts, 2012) where respondents rate how unpredictable, uncontrollable, or overloaded they perceive their life has been in the past 30 days on a 5-point Likert-type scale (Cohen, 1988). The PSS-10 has been used widely in diverse populations since its inception (Cohen & Janicki-Deverts, 2012). Each Soldier's PS score was based on the sum of their responses with a possible range of 0–40. Scores ranging from 0 to 13 are considered low perceived stress. Scores ranging from 14 to 26 are considered moderate perceived stress. Scores ranging from 27 to 40 are considered high perceived stress (Cohen, 1988).

2.4. Mediator

2.4.1. Emotional eating

EE was assessed by two questions taken from the Intuitive Eating Scale-2 (IES-2): "I found myself eating when I felt emotional (e.g., anxious, depressed, sad), even when I was not physically hungry" and "I found myself eating when I was stressed out, even when I was not physically hungry" on a 5-point Likert-type scale. The IES-2 is a validated tool ($\alpha = 0.87$) measuring tendency towards following internal cues guiding eating behaviors (Tylka & Kroon Van Diest, 2013). These questions from the IES-2 were chosen because they appeared to represent eating in response to external cues, such as in response to stress. The IES-2 prompts ask about behaviors in the present tense, but in this study, the response prompt was changed to ask about the last 30 days to match the timeframe of the PSS-10. Responses to these questions were combined to create a variable that grouped frequency of EE behaviors responses into three categories: "never" for those who selected never or rarely, "sometimes" for those who selected sometimes, or "often" for those who selected often or always.

2.5. Statistical analysis

Data were analyzed using Stata, v.15.1 (StataCorp, College Station, TX) or SPSS, v.26 (IMB Statistics). There was missing data among 72 (4.9%) Soldiers, therefore data was screened for patterns in missing data among variables. For the main variables of interest, 10 Soldiers had missing PS responses (0.7%), there were 39 missing EE responses (2.7%), 9 missing BMI responses (0.6%), and 13 missing BC failure responses (0.9%). No difference in the distribution of the variables between missing and non-missing was found. The data were also screened for violations of assumptions prior to analysis. BMI skewness and kurtosis was 0.32 and 2.84, respectively. Preliminary models using the natural log of BMI did not appreciably change model outcomes, therefore to aid interpretation, BMI was used without transformation (Jayne, Blake, Frongillo, et al., 2019). No other violations were found. Cronbach's alpha was used to assess scale reliability. The characteristics of Soldiers by frequency of EE behaviors was examined using chi square and ANOVA statistics to include post-hoc tests to show between group differences.

Linear regression analysis was implemented using the Stata *sem* command with full-information maximum likelihood estimation using the *mlvm* option to examine the effect of frequency of emotional eating behaviors on BMI. This estimation procedure assumes missing data among independent variables is missing at random, which makes use of all participants not missing on the dependent variable. Logistic regression analysis using the Stata *gsem* command was implemented for BC failure. Soldiers who had not been in the Army long enough to have been screened for BC were excluded from logistic regression models.

A conditional process model (also known as moderated mediation) as described by Hayes (Hayes, 2018) was implemented using the *process* command in SPSS to test the relationship between PS and BMI, also known as the direct effect, and examine if EE acts as a mediator between PS and BMI, also known as the indirect effect, with gender as a moderator. An additional model was implemented to test the same moderator-mediator relationships with the outcome of BC failure. The *process* command assumes complete data and excludes participants that have missing data on any of the variables included in the models. To evaluate gender as a moderator, interaction terms were examined for significance. Based on the moderation results, a simple mediation model, also implemented with the *process* command in SPSS, was generated for both outcomes of BMI and BC failure as either a multivariate linear regression or a logistic regression for the binary outcome of BC failure (Hayes, 2019). All models controlled for a Soldiers' military status (initial trainee or not a trainee), age, race/ethnicity, level of education, marital status, tobacco use, and physical activity. Training status was controlled for because Soldiers in training status have less choice in eating behaviors. Age was included as a control variable because of the association between BMI and age. Race/ethnicity, education level, and marital status were used as control variables to reduce confounding between the independent and dependent variables. We controlled for tobacco use since tobacco is often used for stress relief and for physical activity because of its relationship to BMI. Bootstrap confidence intervals were generated based on 10,000 bootstrap samples ensuring the normality of the sampling distribution and to assess the indirect effect of PS on BMI and BC, through EE. EE was considered to be a mediator if the bootstrap 95% CI for the indirect effect was above zero.

3. Results

Reliability testing of the PSS-10 ($\alpha = 0.93$) and IES-2 items ($\alpha = 0.91$) showed good internal consistency among the sample population. BMI was significantly different based on frequency of reported EE behaviors (Table 1). Among those who failed BC standards, a greater proportion reported more frequent EE behaviors. Compared to those who never reported EE behaviors, Soldiers who reported EE behaviors "often" also reported higher PS levels. Mean ± SD PS score was 17.5 ± 4.7 and 17.7 ± 4.4 for men and women, respectively ($p = 0.46$) (Cohen, 1988). These scores are higher than those measured in a similar aged cohort of 18–25 year old civilians (mean ± SD PS of 16.8 ± 6.8; $p = 0.02$) (Cohen & Janicki-Deverts, 2012). Gender differences in Soldiers reporting EE behaviors sometimes or often were significantly different.

Table 1
Characteristics of soldiers by frequency of emotional eating behaviors (N = 1460).

Sociodemographic variables, mean (SD) or %	Self-reported emotional eating behavior			
	Never	Sometimes	Often	*p*
Body mass index (kg/m^2)	25.5(4.5)	**25.8(3.7)**	**26.6(4.1)**	< 0.001
Failed body composition standards	14.5	**20.2**	**29.1**	< 0.001
Mean perceived stress score	16.7(4.5)	**18.6(4.3)**	**20.1(4.6)**	< 0.001
Perceived stress level				
Low (score: 0–13)	85.7	**9.4**	**4.9**	< 0.001
Moderate (score: 14–26)	65.3	**22.3**	**12.4**	< 0.001
High (score: 27–40)	36.1	36.1	**27.8**	< 0.001
Gender				
Male	70.7	**18.9**	**10.4**	< 0.001
Female	56.4	**26.9**	**16.7**	< 0.001
Age, years	23.5(5.2)	23.3(4.7)	24.1(6.1)	0.25
Rank categories				0.45
Enlisted	68.8	20.0	11.2	
Officer	62.6	23.1	14.3	
Marital status				0.31
Single	67.2	21.4	11.4	
Married	70.5	18.1	11.4	
Education level				0.03
High school/GED	71.5	19.2	**9.3**	
Some college	66.9	19.7	13.4	
Degree	61.4	25.5	13.1	
Race/ethnicity				0.17
Non-Hispanic White	67.5	21.5	11.0	
Non-Hispanic Black	71.3	19.4	9.3	
Non-Hispanic other	66.0	23.6	10.4	
Hispanic	55.1	17.1	27.8	
Recruit status				0.18
Trainee	63.9	23.4	12.7	
Not a trainee	69.6	19.4	11.0	
Tobacco use				0.27
No	67.8	20.0	12.2	
Yes	69.7	21.1	9.2	

Note: Referent group is emotional eating: never; numbers in bold indicate differences from the referent group significant at the $p < 0.05$ level.

In adjusted regression models of BMI or BC failure, those who reported EE behaviors "sometimes" or "often" had BMIs that were significantly higher and they had higher odds of failing BC standards than those who did not report emotional eating behaviors (Table 2).

The conditional process analysis models did not reveal gender as a statistically significant moderator in the relationship between PS, EE, and, BMI ($p = 0.83$) or BC failure ($p = 0.57$), therefore these results are not reported. In simple mediation models, PS indirectly influenced BMI and BC failure through its effect on emotional eating behaviors (Table 3), thus emotional eating appeared to mediate the relationship between PS and BMI or BC failure. Statistical tests did not show evidence that PS influenced BMI independently of its effect on EE behaviors. For BC failure, both the direct effect and indirect effect was statistically significant demonstrating that PS may affect BC failure directly and through EE as a mediator.

4. Discussion

This study examined the relationship between PS, BMI, BC failure, and gender among US Army Soldiers to determine if EE behaviors may mediate associations between PS and outcomes of BMI and BC failure.

Table 2
Results of regression models of body mass index or body composition failure by perceived stress and frequency of emotional eating among Soldiers.

	Body mass index N = 1,451[a]		Body composition failure N = 1,183[b]	
	b	*p*	OR	95% CI
Predictor/mediator				
Perceived stress score	0.03	0.095	1.04	[1.00, 1.08]
Emotional eating: sometimes	0.65	0.004	1.65	[1.11, 2.46]
Emotional eating: often	1.19	< 0.001	2.29	[1.43, 3.65]
Control variables				
Women	−2.09	< 0.001	1.48	[0.95, 2.30]
Recruit status: not a trainee	1.10	< 0.001	0.95	[0.48, 1.90]
Mean age	0.14	< 0.001	1.07	[1.03, 1.10]
Non-Hispanic Black	0.72	< 0.001	1.28	[0.82, 2.00]
Non-Hispanic other	0.01	0.960	0.94	[0.54, 1.62]
Hispanic	0.64	0.005	1.46	[0.97, 2.20]
Some college	0.37	0.061	0.83	[0.58, 1.20]
Degree	−0.11	0.727	0.40	[0.21, 0.77]
Married	0.54	0.006	1.35	[0.95, 1.93]
Use tobacco	0.25	0.209	1.27	[0.90, 1.80]
Physical activity (min/week)	0.19	0.025	0.99	[0.85, 1.15]
Constant	19.80	< 0.001	0.16	[0.005, 0.06]

Note: Referent group was no emotional eating, men, trainees, non-Hispanic White, high school education, single, no tobacco. Level of significance: $p < 0.05$.
[a] Model excluded 9 Soldiers with a missing BMI observation.
[b] Model excluded 277 Soldiers who had never been tested for body composition.

Table 3
Mediation effect of emotional eating on the association between perceived stress and outcomes of body mass index or body composition failure among Soldiers.

	Model		Model	
	Body Mass Index N = 1,333[a]		Body Comp. Failure N = 1,096[b]	
Paths	*b*	*p*	OR	95% CI
Perceived stress score to emotional eating	0.03	0.12	1.04	1.01, 1.08
Emotional eating to BMI or body comp. failure	0.72	< 0.001	1.53	1.21, 1.65
Effects	***b***	**95% CI**	**OR**	**95% CI**
Direct effect (*c'*)	0.03	-0.01, 0.07	1.04	1.01, 1.08
Indirect effect (*ab*)	0.03	0.02, 0.04	1.02	1.01, 1.03

Note: *b*, unstandardized regression coefficient; 95% CIs generated from 10,000 bootstrap samples. Models adjusted for training status (initial trainee or not a trainee), gender, age, race/ethnicity, level of education, marital status, tobacco use, and physical activity. Level of significance: $p < 0.05$.
[a] Model excluded 127 Soldiers with missing data.
[b] Model excluded 364 Soldiers (277 had not taken the BC test), 87 had missing data.

The results supported the mediational hypothesis, such that a positive relationship between PS and study outcomes was mediated by the frequency of EE behaviors. Although gender differences in the frequency of EE were observed, gender differences did not moderate the relationship between EE and BMI or BC failure.

The mean PS score of Soldiers in this sample was in the moderate range which is likely attributable to the multiple, unique sources of stress faced by many Soldiers. Military service routinely ranks as one of the most stressful professions (https://www.careercast.com/jobs-rated/-most-stressful-jobs, 2018), nonetheless, stress is an understudied, but important health-modifying factor among military populations. The results of this study support this assertion, demonstrating a relationship between PS, and BMI and BC failure, which are critically related to military service requirements (https://armypubs.army.mil/epubs/DR_pubs/DR_a/pdf/web/ARN7779_AR600-9_FINAL.pdf, 2019).

The association between PS and BMI and BC failure was mediated by EE. This finding emphasizes the potential importance of addressing EE given that Soldiers must maintain a body composition that is compliant with Army standards for career success. In this study, Soldiers who reported EE behaviors "sometimes," were nearly two times more likely to fail body composition standards and were 3.25 pounds heavier (0.65 BMI points) than those who did not report EE behaviors. Those who reported EE behaviors "often," were two times more likely to fail body composition standards and weighed 6.0 pounds heavier (1.19 BMI points) than those who did not report EE behaviors. The Army's BMI-based screening tables allow for variation in weight, but with an estimated 50% of Soldiers currently exceeding maximum allowed weight (https://phc.amedd.army.mil/topics/campaigns/hof/Pages/default.aspx, 2017), and the link between obesity and elevated injury risk (Knapik et al., 2007), the need to address behaviors that affect Soldiers' body weight status is increasingly critical.

These findings add to a growing body of literature examining modifiable behaviors affecting body weight and composition in military populations. Recent studies on the effects of intuitive eating characteristics (Cole, Clark, Heileson, DeMay, & Smith, 2016) or affiliation with a healthy eating identity (Jayne et al., 2018) have shown that understanding and leveraging individual motivations to eat may help the Army meet goals for reducing obesity among Soldiers. Like health promotion messaging aimed at decreasing obesity nationally (Roberto, Swinburn, Hawkes, et al., 2015), the Army uses campaign such as the Total Force Fitness Framework which encompasses a variety of programs aimed at improving nutrition, among other health behaviors (https://www.hprc-online.org/page/total-force-fitness, 2018). Prospective research is needed to examine the ways that food choice behaviors, such as EE, developing a healthy eating identity, and mindful eating may be related to health outcomes of importance to the Army. With the emphasis on increased readiness and improved physical performance among the force (Lacdan, 2017), further research on behavioral mediators that may influence dietary intake could support obesity reduction and performance objectives.

The results of this study are consistent with research showing EE behaviors to be related to weight status in a variety of populations (Ozier et al., 2008; Solomon, 2001; Torres & Nowson, 2007). No studies have examined this association in military personnel, and few have done so in other like service professionals who have high chronic stress (Cole et al., 2016). Studies on this topic have focused on the effects of PTSD or traumatic experiences on weight status (Dorflinger & Masheb, 2018; Jacobson, Smith, Smith, et al., 2009; Mattocks et al., 2012), rather than on the influence of perceived stress on eating behaviors. This study is consistent with literature showing women to be more likely to engage in EE behaviors (Cole et al., 2016; Torres & Nowson, 2007). Male and female Soldiers reported similar PS levels, but female Soldiers reported more EE behaviors, suggesting that, like civilian populations (Hawks, Merrill, Gast, & Hawks, 2004), there are gender differences in how Soldiers cope with stress and that some military women's eating behaviors may be negatively influenced by their military experience (Breland, Donalson, Nevedal, Dinh, & Maguen, 2017). While interactions between EE and gender were not found, over 80% of study participants were men, and, consequently, this study may have been underpowered to detect gender differences in the effect of EE on study outcomes. There remains a need to determine whether gender differences exist in how Soldiers and other public service professionals for whom stress is an unavoidable occupational hazard cope with stress.

Several studies have documented the need for nutrition to be improved among Soldiers (Purvis, Lentino, Jackson, Murphy, & Deuster, 2013; Smith, Dotson, Young, et al., 2013), but few studies have

identified specific factors underlying food choice. This study did not examine the longitudinal effects of PS on body weight status, but it is plausible that habitual EE increases the risk of higher BMI and failing BC standards over time. A limitation of this study was that BC failure was self-reported, that the timing of BC failure was not known, and that EE was measured by a two-item scale that has not been validated as a measure of EE. It is also acknowledged that some individuals respond to stress by reducing energy intake, and that the effects of stress in body weight status may be different in those individuals. For example, in this study the EE variable used in preliminary models included a third question that asked how frequently participants ate *less* when stressed. When that question was included in the EE variable, models showed a smaller effect size for the association between PS and body weight status, but the association remained significant, indicating that PS may have a powerful effect on body weight status, especially among those for which EE is a coping method. Additionally, the study population was of primarily young, healthy adults and results may not be generalizable to other groups.

5. Conclusion

This study found relationships between PS, EE, and body weight status among Soldiers. The results emphasize the need for stress to be considered a harmful influence on force readiness, and identified EE as a behavior that may mediate the relationship between stress and body weight. Since food choice behaviors are often modifiable, emotional eating behaviors should be researched further to better understand if there is a causal relationship with health outcomes and to investigate how reduction of less healthy food choice behaviors can be targeted by health promotion interventions aiming to reduce obesity in both military and civilian populations.

CRediT authorship contribution statement

Julianna M. Jayne: Conceptualization, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Rachel Ayala:** Conceptualization, Writing - original draft, Writing - review & editing. **J. Philip Karl:** Conceptualization, Funding acquisition, Writing - review & editing. **Bethany A. Deschamps:** Conceptualization, Writing - review & editing. **Susan M. McGraw:** Data curation, Writing - review & editing. **Kristie O'Connor:** Writing - review & editing. **Adam J. DiChiara:** Writing - review & editing. **Renee E. Cole** Conceptualization, Funding acquisition, Methodology, Writing - review & editing.

Acknowledgements

The Soldiers who participated in this study are gratefully acknowledged along with Dr. Andrew Young for providing editorial assistance.

Role of funding source

Funding for this study was provided by the US Army Medical Research and Development Command (MRDC). The MRDC did not influence the study design, collection, analysis or interpretation of the data, writing the manuscript, or the decision to submit the paper for publication.

Disclaimer

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the US Government.

Declaration of competing interest

The authors declare there is no conflict of interest.

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ISSN 2472-1972

Effects of Testosterone Supplementation on Ghrelin and Appetite During and After Severe Energy Deficit in Healthy Men

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Background: Severe energy deficits cause interrelated reductions in testosterone and fat free mass. Testosterone supplementation may mitigate those decrements, but could also reduce circulating concentrations of the orexigenic hormone ghrelin, thereby exacerbating energy deficit by suppressing appetite.

Objective: To determine whether testosterone supplementation during severe energy deficit influences fasting and postprandial ghrelin concentrations and appetite.

Design and methods: Secondary analysis of a randomized, double-blind trial that determined the effects of testosterone supplementation on body composition changes during and following severe energy deficit in nonobese, eugonadal men. Phase 1 (PRE-ED): 14-day run-in; phase 2: 28 days, 55% energy deficit with 200 mg testosterone enanthate weekly (TEST; n = 24) or placebo (PLA; n = 26); phase 3: free-living until body mass recovered (end-of-study; EOS). Fasting and postprandial acyl ghrelin and des-acyl ghrelin concentrations and appetite were secondary outcomes measured during the final week of each phase.

Results: Fasting acyl ghrelin concentrations, and postprandial acyl and des-acyl ghrelin concentrations increased in PLA during energy deficit then returned to PRE-ED values by EOS, but did not change in TEST (phase-by-group, $P < 0.05$). Correlations between changes in free testosterone and changes in fasting acyl ghrelin concentrations during energy deficit ($\rho = -0.42$, $P = 0.003$) and body mass recovery ($\rho = -0.38$; $P = 0.01$) were not mediated by changes in body mass or body composition. Transient increases in appetite during energy deficit were not affected by testosterone treatment.

Conclusions: Testosterone supplementation during short-term, severe energy deficit in healthy men prevents deficit-induced increases in circulating ghrelin without blunting concomitant increases in appetite.

Clinical Trials Registration: www.clinicaltrials.gov NCT02734238 (registered 12 April 2016).

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Keywords: hypothalamic-pituitary-gonadal axis, hypogonadism, androgen, military, weight cycling

Abbreviations: AUC, area under the curve; CI, confidence interval; ED, energy deficit; EOS, end-of-study time point; FM, fat mass; FT, free testosterone; LBM, lean body mass; PLA, placebo group; POST-ED, posttreatment time point; PRE-ED, pretreatment time point; TBM, total body mass; TEST, testosterone treatment group; TT, total testosterone

Received 13 December 2019
Accepted 26 February 2020
First Published Online 3 March 2020
Corrected and Typeset 27 March 2020

Severe energy deficits (~50%-100% of total daily energy expenditure) are common in military personnel during training [1, 2]. These deficits result from an inability or unwillingness to increase energy intake to match energy expenditures ranging from 4000 to 7000 kcal/d [3–5]. Consequences include total body mass (TBM) and fat free mass losses [6–10], and decreased physical performance [1]. Substantial reductions in circulating testosterone likely contribute to those decrements [6, 11–14]. Although body mass is regained and testosterone concentrations are restored by refeeding [6, 15], the amount of body mass regained has been reported to exceed the amount of TBM lost and be disproportionately comprised of fat mass (FM) [6, 16–18]. This "weight and fat overshoot" is thought to be caused, in part, by a physiologic drive to fully recover body protein stores [18–20], which may suggest reduced testosterone is a contributing factor.

Energy deficits also elicit adaptive responses in peripheral and central regulators of energy balance that stimulate appetite and promote TBM regain [21, 22]. One potential endocrine mediator of this response is ghrelin [23], a peptide hormone secreted primarily from the gastric mucosa in an acylated form that is rapidly converted into an unacylated (des-acyl) form that composes the majority of ghrelin in circulation [24, 25]. Acyl ghrelin exerts multiple physiologic effects [26], which include modulating glucose homeostasis and stimulating appetite and adipogenesis [27–31]. Des-acyl ghrelin also appears to regulate these outcomes, although effects may either reinforce or antagonize those of acyl ghrelin depending on the experimental context [32]. Nonetheless, circulating total (acyl + des-acyl) ghrelin increases in response to energy deficit [23, 33], and may contribute to a drive to eat that promotes regain of TBM and FM [21].

Circulating ghrelin concentrations may also be regulated by testosterone. Ghrelin is expressed in human testes [34, 35], and testosterone supplementation suppresses circulating total ghrelin in prepubertal boys and weight-stable, nonobese, eugonadal men [36, 37]. This effect, if present in nonobese men during severe energy deficit, may have the unintended and undesirable consequence of blunting energy deficit-induced increases in ghrelin and, subsequently, appetite. This could, in turn, exacerbate energy deficit. Recovery of TBM and FM during refeeding might also be slowed. Although effects of testosterone and other androgens on appetite regulation are poorly characterized [38], those responses would be consistent with studies reporting that testosterone supplementation is associated with gradual reductions in FM [39], and in some cases TBM [40]. However, testosterone supplementation has also been reported to increase circulating total ghrelin during energy balance [41] and have no effect during energy deficit [42] in men with overweight or obesity and low testosterone. The effect of testosterone supplementation on circulating ghrelin therefore remains uncertain and may depend on population demographics and/or be influenced by energy balance. Whether testosterone supplementation affects acyl and des-acyl ghrelin concentrations differently, and if any effects influence appetite and eating behavior has not been examined.

Our group recently published the results of a study designed to determine the effects of testosterone supplementation on changes in body mass and body composition during and following 28 days of severe energy deficit in healthy, nonobese, eugonadal young men [43]. Study results demonstrated that supplemental testosterone increased lean body mass during energy deficit. Herein, we report secondary outcomes included in the design of that study that addressed tertiary study objectives. Those objectives were to determine the effects of testosterone supplementation on circulating acyl and des-acyl ghrelin concentrations and appetite during and after severe energy deficit, and to explore relationships between changes in testosterone, ghrelin, appetite, TBM, and body composition during severe energy deficit and TBM recovery [44].

1. Materials and Methods

This study addressed tertiary objectives included in the Optimizing Performance for Soldiers study [43–45]. All outcomes presented herein were defined in the original study protocol.

The study was approved by the Pennington Biomedical Research Center (PBRC) institutional review board and the Human Research Protection Office of the US Army Medical Research and Materiel Command. Participants provided written informed consent, and the study was registered at www.clinicaltrials.gov as NCT02734238.

A. Subjects

Healthy, physically active men aged 18–39 years (mean ± SD = 25 ± 1 year), with total testosterone concentrations within normal physiological range (300 – 1000 ng/dL), and who met age-specific US Army body composition standards were recruited. Full inclusion and exclusion criteria and details of the recruitment process are published elsewhere [44].

B. Study Design

The study used a randomized, double-blind, placebo-controlled design. Full details of the Optimizing Performance for Soldiers study are published elsewhere [44], and only described in brief here.

The study consisted of a screening period followed by 3 separate study phases. During screening, volunteers recorded dietary intake for 3 consecutive days to assess usual energy intake. During phase 1 (study days 1–14), participants were free-living, but were provided a TBM-maintaining diet [44]. Immediately after completing phase 1, participants were admitted to the PBRC inpatient unit where they resided for 28 days during phase 2 of the study (study days 15–42). Participants were randomly assigned to one of 2 treatment groups for phase 2, testosterone (TEST), or placebo (PLA) [44]. Participants randomized to PLA received IM injections of 1 mL sesame oil on study days 15, 21, 28, and 35. Participants randomized to TEST received IM injections of 200 mg testosterone enanthate on the same days [44]. The only person not blinded to treatment allocation was the study pharmacist who prepared the treatments but did not interact with participants. During phase 2, dietary intake and physical activity were strictly monitored and controlled as described elsewhere [44], and prescribed to elicit a 55% energy deficit. Percent energy deficit did not differ between PLA (53 ± 4%) and TEST (52 ± 5%) [43].

Phase 3 (study day 43 to end-of-study [EOS]) began immediately upon completion of phase 2. Participants were released from the inpatient unit following testing on day 43, and instructed to return to their prestudy diet and exercise habits. All participants were followed for a minimum of 14 days (i.e., to study day 56). If, by day 56, a participant had returned to ±2.5% of his or her TBM measured on day 14 (as determined by average TBM over 3 consecutive days), EOS measurements were initiated and conducted over consecutive days (EOS, EOS + 1 day, EOS + 2 days). Otherwise, participants were followed until meeting the ±2.5% threshold, or until study day 84, whichever occurred first. These participants visited the laboratory weekly from day 57 to EOS for TBM measurement in addition to conducting daily TBM measurements at home. Therefore, the duration of phase 3 varied by participant and ranged from 14 to 42 days. Dietary intake during phase 3 was measured over 3 days the week before day 56 using the same methods used during screening. Study outcomes were measured during the last week of phase 1 (PRE-ED) and phase 2 (POST-ED), and at EOS as described below.

C. Appetite and Ghrelin Testing (Study Days 7, 43, and EOS + 1)

During the final week of each phase, on study days 7, 43, and EOS + 1, an experiment was conducted to determine the effects of testosterone supplementation on postprandial ghrelin and appetite responses during and after severe energy deficit (Fig. 1) [44]. Testing began at 07:00 hours on days 7 and 43, and at 07:00 hours (n = 27), 08:00 hours (n = 17), or 09:00 hours (n = 6) on EOS + 1. On each testing day, fasted participants consumed a standardized breakfast that provided energy equivalent to 20% of their individual total daily energy

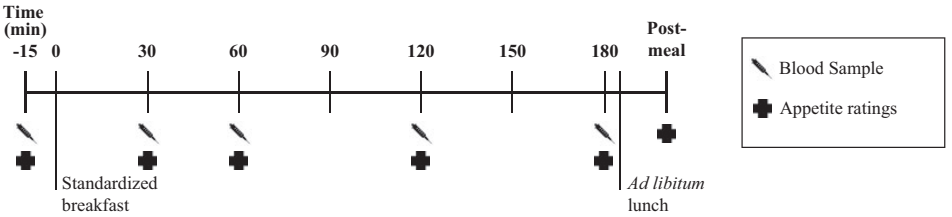


Figure 1. Study design. Appetite and ghrelin measurements were included as secondary outcomes in the Optimizing Performance for Soldiers study [43, 44]. On study days 7, 43, and EOS + 1, appetite ratings and fasted blood samples were collected 15 minutes before a standardized breakfast, and every 30–60 minutes after starting breakfast for 180 minutes. Appetite was also measured by energy intake during a lunch meal consumed ad libitum (185 minutes). Blood samples were collected to measure fasting insulin, fasting leptin, and fasting and postprandial glucose, acyl ghrelin, and des-acyl ghrelin. EOS, end of study.

expenditure determined during phase 1 (PLA: 515 ± 61 kcal, TEST: 542 ± 75 kcal; $P = 0.18$; percent energy from carbohydrate/fat/protein = 60/25/14). After 185 minutes, a lunch meal was served (percent energy from carbohydrate/fat/protein = 41/36/23) in a portion equivalent to ≥75% of each participant's total daily energy expenditure determined during phase 1. Participants were instructed to eat ad libitum until comfortably full, and the amount consumed was recorded. Blood samples were collected via intravenous catheter, and appetite was measured using visual analog scales 15 minutes before breakfast (served at time 0 minutes), and again 30, 60, 120, and 180 minutes after starting breakfast (Fig. 1). Appetite was measured again after lunch was finished. Visual analog scales were 100 mm in length and asked participants to rate their level of fullness, hunger, desire to eat, and the amount of food they thought they could eat (i.e., prospective consumption) at that moment [46]. Insulin, glucose, leptin, acyl ghrelin, and des-acyl ghrelin were measured at the -15 minute time point (i.e., fasting). Glucose, acyl-ghrelin, and des-acyl ghrelin concentrations were also measured at the 30, 60, 120, and 180 minute time points.

D. Biochemistries

Fasted blood samples were drawn on study days 0, 14, 42, and EOS between 06:00 hours and 09:00 hours (all day 42 measurements at 06:00 hours). Total testosterone (TT) was measured by automated immunoassay (Siemens Immulite 2000, Llanberis, UK) [47], and free testosterone (FT) was calculated according to the method of Vermeulen et al [48].

Blood samples were also drawn on appetite testing days (study days 7, 43, and EOS + 1) for measurement of fasting insulin and leptin, and fasting and postprandial glucose, acyl-ghrelin, and des-acyl ghrelin concentrations. Insulin was measured by automated immunoassay (Siemens Immulite 2000) [49]. Leptin was measured by radioimmunoassay (EMD Millipore Corporation, St Louis, MO) [50]. Glucose was measured using the Beckman DXC 600 Pro (Brea, CA). Acyl ghrelin was measured by radioimmunoassay (EMD Millipore Corporation) [51], and des-acyl ghrelin by ELISA (SPI Bio, Montigny le Bretonneux, France) [52]. All blood samples collected for measurement of acyl ghrelin were collected into chilled tubes containing 1 mg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and were kept on ice until processing. After centrifugation, plasma acyl ghrelin aliquots were acidified using HCl to a final concentration of 0.05N. Des-acyl ghrelin was collected into chilled EDTA tubes and kept on ice until centrifugation. Samples were stored at -80°C until analysis. Total ghrelin concentration was calculated as the sum of acyl and des-acyl ghrelin.

E. Anthropometrics and Body Composition

Semi-nude, fasted, morning TBM was measured during laboratory visits using a calibrated digital scale (GSE Inc. Model 450, GSE Scale Systems, Novi, MI). Participants weighed themselves daily during phase 3 when they did not visit the laboratory using a calibrated

scaled provided by research staff (Body Trace, Inc. Model BT003, New York, NY). TBM, FM, and bone mass were determined by dual-energy X-ray absorptiometry (Lunar iDXA, GE Healthcare, Madison, WI) on study days 0, 11, 39, and EOS after an overnight fast. Lean body mass (LBM) was defined as TBM minus FM and bone mass.

F. Statistical Analysis

As described elsewhere [43, 44], sample size estimates were based on the primary study outcome, LBM, and indicated that 22 participants/group would provide 90% power to detect an expected 25% attenuation in LBM loss with testosterone supplementation. Sample size estimates for postprandial ghrelin area under the curve (AUC) were based on an expected 25% between-group difference following testosterone supplementation [36]. Using means and standard deviations from previous work by our laboratory conducted in a similar population [53], we calculated that an effect of that magnitude could be detected with 19 participants/group with 80% power and $\alpha = 0.05$.

All data were examined quantitatively and graphically, and logarithmic transformations were applied when necessary to meet model assumptions. Between-group comparisons of preintervention measurements were conducted using Student *t*-test or the Mann-Whitney *U*-test as appropriate. Linear mixed models with participant included as a random factor were used to examine effects of treatment, study day, and their interaction on fasting appetite ratings, glucose and hormone concentrations, body composition, and ad libitum energy intake at lunch. Models for testosterone and body composition were adjusted for day 0 values of each variable. The AUC with respect to ground (y = 0) was calculated for postprandial appetite ratings, and postprandial glucose and ghrelin concentrations using the trapezoidal method [54]. AUC was analyzed using linear mixed models as described above. Significant main effects and interactions were investigated further using between-group and within-group pairwise comparisons. Bonferroni corrections were applied to adjust for multiple comparisons. Because of evidence for between group differences in PRE-ED ghrelin concentrations (see Results), mixed models with PRE-ED values included as a covariate were also used to analyze between-group differences in fasting ghrelin concentrations and postprandial ghrelin AUC. Finally, in exploratory analyses, correlations between study outcomes were assessed using Pearson or Spearman rank correlations as appropriate, and multiple linear regression was used to assess relationships between variables while controlling for covariates.

Statistical analyses were completed using SPSS v24.0 (IBM, Armonk, NY). Tests were 2-sided and considered statistically significant at $P \leq 0.05$. Results are presented as mean ± SD or adjusted least square mean difference (95% confidence interval [CI]) unless otherwise noted.

2. Results

Of the 53 men who enrolled, 50 were randomized (60% non-Hispanic white, 26% non-Hispanic black, 6% Hispanic, 8% other) and all 50 completed the study [43]. Changes in testosterone concentrations and body composition measured between study days 0 to 56 have been previously reported [43]. To provide context for this secondary analysis, these changes are presented in Table 1 and briefly summarized here. EOS values have not been previously reported.

Within PLA, FT and TT concentrations were stable or declined from PRE-ED to POST-ED, then returned to PRE-ED concentrations by EOS; whereas, within TEST, FT and TT concentrations increased from PRE-ED to POST-ED, then decreased to below PRE-ED concentrations by EOS. Relative to PLA, concentrations of both TT and FT were higher in TEST at POST-ED but lower at EOS (Table 1). Changes in FM did not differ between groups (Table 1). In contrast, TBM loss from PRE-ED to POST-ED was attenuated in TEST relative to PLA because of an increase in LBM in TEST (Table 1). Participants

Table 1. Body Composition and Fasting Testosterone Concentrations

	PLA (n = 26)			TEST (n = 24)			P Value		
	PRE-ED (Day 11 or 14)	POST-ED (Day 39 or 42)	EOS	PRE-ED (Day 11 or 14)	POST-ED (Day 39 or 42)	EOS	Phase	Group	P × G
Body mass (kg)[1]	76.3 ± 10.2	71.3 ± 9.8$^{\#}$	74.9 ± 9.0$^{\#‡}$	80.2 ± 13.5	78.0 ± 12.3$^{\#*}$	81.0 ± 12.6‡*	<0.001	<0.001	<0.001
Fat mass (kg)[1§]	17.0 ± 6.7	12.3 ± 6.6	14.0 ± 5.4	16.6 ± 6.9	11.8 ± 6.1	13.7 ± 5.3	<0.001	0.67	0.93
Lean mass (kg)[1]	56.1 ± 5.1	55.8 ± 5.0	57.7 ± 5.1$^{\#‡}$	60.3 ± 10.1	62.8 ± 9.5$^{\#*}$	64.0 ± 10.2$^{\#‡*}$	<0.001	<0.001	<0.001
Total testosterone (ng/dL)[2]	402 ± 101	319 ± 131	424 ± 120‡	472 ± 153	1016 ± 246$^{\#*}$	324 ± 135$^{\#‡*}$	<0.001	<0.001	<0.001
Free testosterone (ng/dL)[2,3]	8.8 ± 2.3	5.0 ± 2.5$^{\#}$	8.8 ± 2.7‡	9.7 ± 2.4	24.4 ± 8.0$^{\#*}$	6.4 ± 2.7$^{\#‡*}$	<0.001	<0.001	<0.001

ED, energy deficit; EOS, end of study (return to initial body mass); PLA, placebo treatment group; TEST, testosterone treatment group.
Values are mean ± SD. Analyzed by linear mixed models with Bonferroni corrections, and adjusted using day 0 values.
*Significant difference from PLA at same time point ($P < 0.05$).
$^{\#}$Significant difference from PRE-ED within the same treatment group ($P < 0.05$).
‡Significant difference from POST-ED within the same treatment group ($P < 0.05$).
§Main effect of phase, all phases are significantly different ($P < 0.05$).
[1]Measured on study days 11 (phase 1), 39 (phase 2) and EOS.
[2]Measured on study days 14 (phase 1), 42 (phase 2) and EOS.
[3]Log_{10}-transformed for analysis.

in TEST (median [interquartile range] = 2 days [3]) then returned to their initial TBM earlier than those in PLA (11 days [16], $P = 0.001$). Five participants in PLA and 1 in TEST (Pearson $\chi^2 = 2.7$, $P = 0.10$) did not return to initial TBM within 42 days of ad libitum eating, which was the predetermined EOS for participants who did not fully recover lost TBM. As a result, EOS TBM in PLA remained 1.4 kg (95% CI: 0.4-2.5) lower than initial TBM ($P = 0.005$), but did not differ between the same time point in TEST (0.8 kg [95% CI: -0.3 to 1.9] $P = 0.22$).

A. Glucose and Hormone Concentrations

Testosterone supplementation had no effect on fasting leptin and insulin concentrations which were both lower at POST-ED relative to other time points (Table 2). Fasting glucose concentrations decreased in both groups from PRE-ED to POST-ED and were higher at EOS relative to PRE-ED in TEST but not PLA (Table 2). Testosterone supplementation had no effect on postprandial glucose AUC (data not shown).

Fasting acyl ghrelin concentrations were not statistically different between groups before treatment (i.e., at PRE-ED; $P = 0.16$). However, statistical adjustment for PRE-ED acyl ghrelin affected model results. When PRE-ED concentrations were not included in the model, a phase-by-group interaction was observed ($P = 0.05$). Fasting acyl ghrelin was found to increase from PRE-ED to POST-ED and then return to PRE-ED values at EOS in PLA but did not change over time in TEST (Table 2). In contrast, after statistically adjusting for PRE-ED concentrations, no significant treatment effects were observed (phase-by-group interaction, $P = 0.11$).

Postprandial acyl ghrelin AUC was higher in TEST relative to PLA before treatment ($P = 0.05$; Fig. 2C). However, statistical adjustment for PRE-ED acyl ghrelin AUC did not affect model results. When PRE-ED AUC was not included in the model, a phase-by-group interaction was observed ($P = 0.004$). Acyl ghrelin AUC increased from PRE-ED to POST-ED and then returned to PRE-ED values at EOS in PLA, but did not change over time in TEST (Fig. 2A-C). The phase-by-time interaction remained significant after adjusting for PRE-ED acyl ghrelin AUC (phase-by-group interaction, $P = 0.02$). Following the adjustment, POST-ED acyl ghrelin AUC was 21% higher in PLA relative to TEST (adjusted mean difference = 4189 pg × min/mL [95% CI: 1679-6699] $P = 0.001$), and no between group difference was observed at EOS ($P = 0.75$). Finally, when postprandial AUC was recalculated as a change from fasting concentrations, no effect of testosterone was observed (data not shown; phase-by-group interaction, $P = 0.58$), indicating that the effect of testosterone on acyl ghrelin AUC was primarily attributable to a change in fasting concentrations rather than a change in the magnitude of the postprandial response.

Fasting des-acyl ghrelin concentrations trended toward being statistically different between groups before treatment ($P = 0.06$). In the full cohort, fasting des-acyl ghrelin concentrations increased from PRE-ED to POST-ED and then decreased and did not differ from PRE-ED concentrations at EOS (Table 2). No effect of testosterone supplementation was observed whether or not PRE-ED des-acyl ghrelin was included in the model.

Postprandial des-acyl ghrelin AUC was higher in TEST relative to PLA before treatment ($P = 0.01$). However, statistical adjustment for PRE-ED des-acyl ghrelin AUC did not affect model results. When PRE-ED AUC was not included in the model, a phase-by-group interaction was observed ($P = 0.02$). Des-acyl ghrelin AUC increased from PRE-ED to POST-ED and then returned to PRE-ED values at EOS in PLA but did not change over time in TEST (Fig. 2D-F). The phase-by-time interaction remained significant after adjusting for the PRE-ED differences in des-acyl ghrelin AUC (phase-by-group interaction, $P = 0.05$). The ratio of the geometric mean of POST-ED des-acyl ghrelin AUC in PLA relative to that in TEST was 1.15 (95% CI: 1.03, 1.29; $P = 0.01$), and no between-group difference was observed at EOS ($P = 0.98$; phase-by-group interaction, $P = 0.02$). When postprandial AUC was recalculated as a change from fasting des-acyl ghrelin concentrations, no effect of testosterone was observed (data not shown; phase-by-group interaction, $P = 0.97$), suggesting

Table 2. Fasting Glucose, Insulin, Leptin and Ghrelin Concentrations, Fasting Appetite Ratings, and Energy Intake During an ad libitum Lunch Meal

	PLA (n = 26)			TEST (n = 24)			P Value		
	PRE-ED (Day 7)	POST-ED (Day 43)	EOS + 1 day	PRE-ED (Day 7)	POST-ED (Day 43)	EOS + 1 day	Phase	Group	P × G
Glucose (mg/dL)	86 ± 7	79 ± 6[#]	86 ± 7[‡]	85 ± 7	76 ± 6[#]	90 ± 6[#‡]	<0.001	0.98	0.01
Insulin (μIU/mL)[1†]	4.5 [6.3]	0.2 [2.7]	5.7 [6.4]	4.6 [3.8]	0.2 [2.1]	6.3 [5.9]	<0.001	0.56	0.97
Leptin (ng/mL)[1†]	6.2 [6.1]	3.2 [1.7]	5.8 [6]	5.4 [7.9]	3.2 [1.2]	4.8 [3.1]	<0.001	0.65	0.77
Acyl ghrelin (pg/mL)	118 ± 44	145 ± 59[#]	130 ± 49	137 ± 53	132 ± 59	141 ± 52	0.27	0.65	0.05
Des-acyl ghrelin (pg/mL)[1†]	163 [108]	204 [121]	199 [102]	228 [103]	244 [177]	228 [172]	<0.001	0.38	0.68
Total ghrelin (pg/mL)[1¶]	287 [168]	326 [233]	310 [160]	350 [180]	353 [273]	357 [276]	0.002	0.49	0.15
Acyl/des-acyl ghrelin ratio	0.65 ± 0.20	0.64 ± 0.25	0.71 ± 0.36	0.64 ± 0.30	0.49 ± 0.22	0.59 ± 0.21	0.08	0.49	0.15
Fullness (mm)[1†]	13 [33]	9 [23]	12 [34]	15 [39]	10 [32]	17 [34]	0.01	0.91	0.60
Hunger (mm)[¥]	59 ± 24	73 ± 16	68 ± 22	62 ± 21	75 ± 18	73 ± 15	<0.001	0.46	0.85
PC (mm)	66 ± 19	74 ± 20	70 ± 21	69 ± 19	75 ± 17	73 ± 19	0.12	0.75	0.81
DtE (mm)[†]	62 ± 26	76 ± 20	68 ± 23	66 ± 25	77 ± 19	70 ± 22	0.001	0.64	0.86
Energy intake at lunch meal (kcal)[†]	1211 ± 438	1418 ± 560	1108 ± 455	1145 ± 366	1581 ± 544	1241 ± 468	<0.001	0.52	0.11

DtE, Desire to eat; ED, energy deficit; EOS, end of study (return to initial body mass); PLA, placebo treatment group; PC, prospective consumption; TEST, testosterone treatment group.

Values are mean ± SD or median [IQR]. Analyzed by linear mixed models with Bonferroni corrections.

*Significant difference from PLA at same time point ($P < 0.05$).

[#]Significant difference from PRE-ED within the same treatment group ($P < 0.05$).

[‡]Significant difference from POST-ED within the same treatment group ($P < 0.05$).

[†]Main effect of phase, POST-ED differs from PRE-ED and EOS.

[¶]Main effect of phase, POST-ED differs from PRE-ED only.

[¥]Main effect of phase, PRE-ED differs from POST-ED and EOS.

[1]Log_{10}-transformed for analysis.

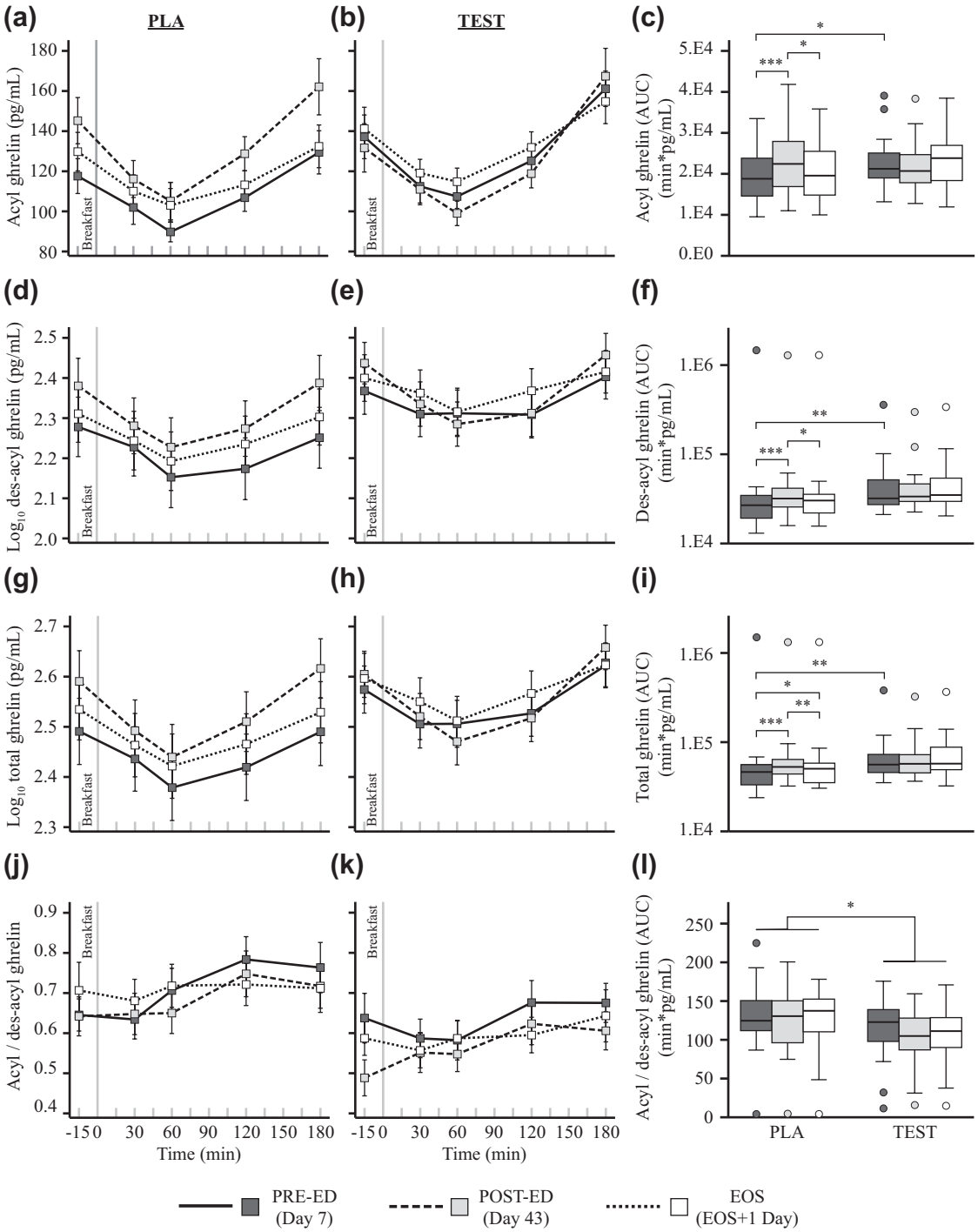


Figure 2. Testosterone supplementation alters postprandial ghrelin responses during energy deficit. Fasting and postprandial acyl ghrelin (A-C), des-acyl ghrelin (D-F), total ghrelin (G-I), and the acyl ghrelin/des-acyl ghrelin ratio (J-L) measured during a standardized meal challenge (0–180 minutes) administered before (PRE; study day 7) and after (POST; study day 43) severe energy deficit (ED), and 1 day after body mass recovery (EOS). (A-B, D-E, G-H, J-K) Individual data points are mean ± SEM. (C, F, I, L) Boxes span 25th to 75th percentile with the median value represented by a solid line inside the box. Whiskers extend to minimum and maximum values or to 1.5 times the interquartile range if any values outside that range (represented by circles). Main effects of group, study phase, and their interaction on area under the postprandial response curve (AUC) analyzed by linear mixed models with Bonferroni corrections. (C, F, I) Group-by-phase interaction, $P < 0.05$. (L) Main effect of group, $P = 0.05$. (C, F, I, L) * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. PLA, placebo treatment group; TEST, testosterone treatment group.

that the effect of testosterone on des-acyl ghrelin AUC was attributable to a combination of changes in fasting concentrations and in the magnitude of the postprandial response.

Results for fasting total ghrelin and total ghrelin AUC largely mirrored those for des-acyl ghrelin which was the predominant form in circulation (Table 2 **and** Fig. 2G-I). Testosterone supplementation had no effect on the ratio of acyl to des-acyl ghrelin under either fasting or postprandial conditions (Table 2 **and** Fig. 2J-L).

B. Appetite

Fasting and postprandial appetite ratings demonstrated an increase in hunger, desire to eat, prospective consumption, and a decrease in fullness from PRE-ED to POST-ED that was fully or partially resolved by EOS and not affected by testosterone supplementation (Table 2, Fig. 3). Changes in energy intake during the ad libitum lunch mirrored that response and did not differ by study group (Table 2). The difference in self-reported energy intake measured during the first week of phase 3 relative to that measured during screening also did not differ between groups (PLA: 347 kcal/d [95% CI: -171 to 864] vs. TEST: 900 kcal/d [95% CI: 432-1368], $P = 0.11$). However, the median [IQR] rate of TBM regain from POST-ED until a participant was considered returned to their initial TBM was higher in TEST (1.7 kg/d [1.9]) relative to PLA (0.4 kg/d [1.1]; $P = 0.03$).

C. Associations Between Changes in Testosterone, Ghrelin, and Appetite

In the full cohort, changes in TT and FT concentrations were inversely correlated with changes in fasting acyl ghrelin, but not fasting des-acyl ghrelin, concentrations (Table 3). The correlations between changes in FT concentrations and changes in fasting acyl ghrelin concentrations remained statistically significant after adjusting for changes in FM, LBM, and TBM (Table 4).

Changes in postprandial acyl ghrelin, des-acyl ghrelin, total ghrelin, and the acyl/des-acyl ghrelin ratio AUCs from PRE-ED to POST-ED were not correlated with changes in postprandial hunger AUC during the same period, or with changes in ad libitum energy intake as measured by: 1) the difference in self-reported energy intake during the first week of phase 3 relative to screening; 2) the difference in lunch meal ad libitum energy intake at POST-ED relative to PRE-ED; or 3) the rate of TBM regain from POST-ED until initial TBM was reached (data not shown).

3. Discussion

In this secondary analysis, testosterone supplementation during severe energy deficit prevented deficit-induced increases in fasting acyl ghrelin concentrations, and in postprandial acyl and des-acyl ghrelin concentrations in nonobese, healthy men. Despite an established role for ghrelin in energy balance regulation, these effects did not blunt energy deficit-induced increases in appetite, slow TBM recovery, or impact changes in FM. These findings confirm and extend evidence supporting an inhibitory effect of testosterone supplementation on circulating ghrelin in healthy men, but suggest this effect is not sufficient to suppress appetite during energy deficit or subsequent body mass recovery.

Whereas previous studies have examined associations between testosterone supplementation and fasting total ghrelin concentrations [36, 37, 41, 42], to our knowledge, this study is the first to examine relationships between testosterone and both fasting and postprandial acyl and des-acyl ghrelin forms. This is a strength of the study, and important to developing a complete understanding of any effects of testosterone supplementation on ghrelin because: 1) acyl ghrelin is considered the more biologically active ghrelin form [26]; 2) acyl and des-acyl ghrelin may have independent, and, in some cases, opposing effects [32]; and 3) most individuals spend the majority of the day in a postprandial state [55, 56]. Using this comprehensive approach, this study extends the findings of Gambineri et al [36], who demonstrated

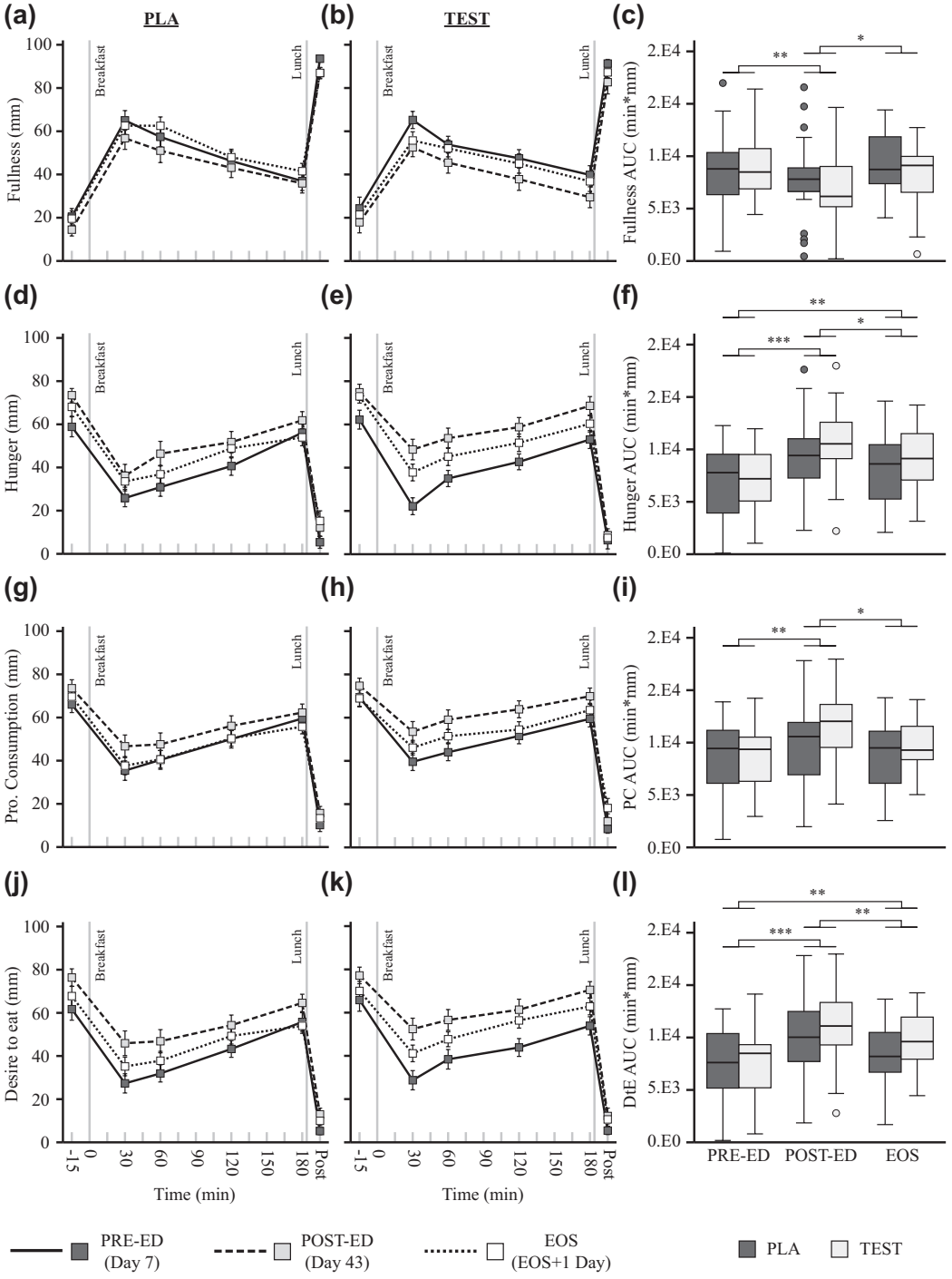


Figure 3. Testosterone supplementation does not affect appetite during energy deficit. Fasting and postprandial fullness (A-C), hunger (D-F), prospective consumption (Pro. Consumption, G-I), and desire to eat (J-L) measured during a standardized breakfast (0–180 minutes) followed by an ad libitum lunch meal (served at 185 minutes) administered before (PRE; study day 7) and after (POST; study day 43) severe energy deficit (ED), and one day after body mass recovery (EOS). (A-B, D-E, G-H, J-K) Individual data points are mean ± SEM. (C, F, I, L) Boxes span 25th to 75th percentile with the median value represented by a solid line inside the box. Whiskers extend to minimum and maximum values or to 1.5 times the interquartile range if any values outside that range (represented by circles). Main effects of group, study phase, and their interaction on area under the postprandial response curve (AUC) analyzed by linear mixed models with Bonferroni corrections. (C, F, I, L) Main effect of phase, $P < 0.05$. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. PLA, placebo treatment group; TEST, testosterone treatment group.

Table 3. Correlations Between Changes in Fasting Ghrelin Concentrations and Changes in Testosterone and Body Mass Outcomes

	ΔdaG	ΔtotG	ΔaG/daG	ΔTT	ΔFT	ΔFM	ΔLBM	ΔTBM
ΔPRE-ED to POST-ED								
Δacyl ghrelin	0.46***	0.69***	0.40**	-0.37**	-0.42**	0.12	-0.12	-0.04
Δdes-acyl ghrelin		0.88***	-0.48***	-0.03	-0.04	0.16	-0.05	0.01
Δtotal ghrelin			-0.12	-0.23	-0.23	0.20	-0.11	-0.03
Δacyl/des-acyl ghrelin				-0.26	-0.34*	-0.03	-0.11	-0.13
ΔPOST-ED to EOS								
Δacyl ghrelin	0.41**	0.75***	0.45***	-0.39**	-0.38**	-0.42**	-0.15	-0.31*
Δdes-acyl ghrelin		0.86***	-0.45***	-0.14	-0.12	-0.36*	0.01	-0.18
Δtotal ghrelin			-0.04	-0.31*	-0.30*	-0.41**	-0.06	-0.27
Δacyl/des-acyl ghrelin				-0.17	-0.22	-0.09	-0.13	-0.15

n = 49–50. Δchange; aG, acyl ghrelin; daG; des-acyl ghrelin; FM, fat mass; FT, free testosterone; LBM, lean body mass; TBM, total body mass; TT, total testosterone. Values are Pearson's correlation coefficients or Spearman's rho. All correlations were computed using change scores calculated over the same time period for the independent and dependent variables (ie, PRE-ED to POST-ED and POST-ED to EOS).
*$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

that supplementing testosterone to supraphysiologic concentrations (~1400ng/dL; ~60% increase from baseline) resulted in a ~25% decrease in fasting total ghrelin concentrations over 8wk in normal-weight men in whom endogenous testosterone production was suppressed by oral progestin administration. The present study demonstrates that those results may be driven more by changes in acyl ghrelin than des-acyl ghrelin, and that the effects on acyl ghrelin concentrations can be produced at testosterone concentrations that span the lower to upper limits of the normal physiologic range (300-1000 ng/dL [57]; 115% increase from baseline). Further, these findings demonstrate that testosterone supplementation does not appear to measurably impact the magnitude of the postprandial acyl or des-acyl ghrelin response independent of changes in fasting concentrations (Table 2 and Fig. 2).

Changes in TBM, FM, and LBM did not mediate associations between testosterone and fasting acyl ghrelin concentrations (Table 4). This suggests that the observed effects of testosterone on acyl ghrelin were independent of changes in TBM and body composition, both of which have previously been associated with ghrelin concentrations [26]. The result contrasts with a recent study in which decreased TBM and FM were hypothesized to override any effect of testosterone treatment on fasting total ghrelin concentrations in men with obesity and low-normal testosterone concentrations who were consuming a very low energy diet [42]. Whether the discrepancy is due to the absence of acyl ghrelin measurements in that study, or due to differences in the study populations, the magnitude of TBM and FM loss or the method used to induce energy deficit (increased energy expenditure vs. very low energy diet) is unclear. Additionally, it has been postulated that any effect of testosterone on ghrelin concentrations may be indirect and mediated through changes in insulin or leptin concentrations [58]. However, neither insulin nor leptin was affected by testosterone supplementation in this study. Of note, multiple endocrine, neural, and nutritive factors collectively regulate basal and periprandial ghrelin concentrations, and the combinations of those factors underpinning ghrelin responses to body mass loss are not fully characterized [26, 58]. Study findings strengthen evidence that testosterone may be 1 of those factors. Conversely, ghrelin has been shown to suppress hypothalamic-pituitary-gonadal axis activity [59, 60], and an energy deficit-induced increase in ghrelin is thought to be 1 mechanism by which reproductive function is suppressed when energy availability is low [61, 62]. Taken together, these observations add support for a bidirectional interaction between testosterone and ghrelin linking energy homeostasis and male reproductive function.

Despite preventing energy deficit-induced increases in acyl ghrelin, testosterone supplementation did not blunt deficit-induced increases in appetite, or slow recovery of TBM

Table 4. Association Between Changes in Fasting Acyl Ghrelin Concentrations and Changes in Free Testosterone (FT) Concentrations are not Mediated by Changes in Body Composition or Body Mass

	Model 1[a]		Model 2		Model 3		Model 4	
	β [95% CI]	P	β [95% CI]	P	β [95% CI]	P	β [95% CI]	P
ΔPRE-ED to POST-ED								
ΔFT (ng/dL)	-1.7 [-3.0, -0.4]	0.01	-1.7 [-3.1, -0.4]	0.01	-2.6 [-4.6, -0.6]	0.01	-2.7 [-4.6, -0.8]	0.01
Age (yr)	-2.5 [-5.6, 0.7]	0.12	-2.4 [-5.6, 0.7]	0.13	-2.3 [-5.5, 0.8]	0.14	-2.3 [-5.4, 0.9]	0.15
Baseline BF (%)	0.3 [-2.1, 2.7]	0.78	0.7 [-2.2, 3.7]	0.61	0.2 [-2.2, 2.6]	0.84	0.9 [-1.6, 3.5]	0.45
ΔFM (kg)	-		3.9 [-12.0, 20.0]	0.62	-		-	
ΔLBM (kg)	-		-		6.3 [-4.4, 1.7]	0.24	-	
ΔTBM (kg)	-		-		-		6.8 [-2.9, 16.5]	0.16
Constant	74.3 [-2.8, 151.5]	0.06	83.2 [-2.5, 168.9]	0.06	70.8 [-6.2, 147.9]	0.07	85.8 [7.7, 163.8]	0.03
R^2	0.20	0.02	0.20	0.04	0.22	0.02	0.23	0.02
ΔPOST-ED to EOS								
ΔFT (ng/dL)	-1.5 [-2.6, -0.3]	0.01	-1.4 [-2.5, -0.2]	0.02	-1.6 [-2.9, -0.4]	0.01	-1.5 [-2.7, -0.2]	0.02
Age (yr)	0.8 [-2.3, 3.9]	0.59	0.2 [-3.1, 3.5]	0.90	0.7 [-2.5, 3.9]	0.67	0.8 [-2.3, 4.0]	0.59
Baseline BF (%)	3.0 [0.6, 5.3]	0.01	2.0 [-0.9, 4.8]	0.16	3.1 [0.7, 5.6]	0.01	2.9 [0.3, 5.6]	0.03
ΔFM (kg)	-		9.7 [-2.6, 6.4]	0.23	-		-	
ΔLBM (kg)	-		-		2.5 [-6.4, 1.1]	0.57	-	
ΔTBM (kg)	-		-		-		-0.2 [-7.1, 6.7]	0.95
Constant	-97.1 [-173.3, -20.9]	0.01	-42.7 [-160.6, 75.3]	0.47	-101.5 [-179.9, -23.1]	0.01	-95.6 [-187.8, -3.3]	0.04
R^2	0.28	0.002	0.30	0.003	0.29	0.004	0.28	0.005

n = 49. Δ, change; BF, body fat; FM, fat mass; LBM, lean body mass; TBM, total body mass.

[a]All models are multiple linear regression analyses. Dependent variable is change in fasting acyl ghrelin concentration over the time period indicated in the table.

and FM during refeeding. These results are incongruent with acyl ghrelin's putative role as an orexigenic and adipogenic hormone. However, clinical studies demonstrating acute orexigenic effects of ghrelin have generally used IV infusions resulting in supraphysiologic acyl and total ghrelin concentrations [63]. Studies conducted in normal-weight adults using lower dose infusions that produce more physiologically relevant ghrelin concentrations have not reported statistically significant increases in appetite despite 1.6- to 2.4-fold increases in circulating acyl and total ghrelin [64, 65]. Further, some [66–68], but not all [30], rodent studies suggest that des-acyl ghrelin may antagonize the appetite-stimulating effects of acyl ghrelin. We therefore posit that the ~1.25-fold between-group difference in changes in fasting acyl-ghrelin concentrations in this study were likely not sufficient to result in between-group differences in hunger, energy intake or changes in FM in the absence of differences in the acyl:des-acyl ghrelin ratio. Additionally, eating behavior is influenced by myriad interactions among endocrine, neural, and other factors [69] that are sensitive to energy deficit, and promote an appetitive drive to regain lost body mass [22]. Responses of those factors to energy deficit may have overwhelmed any influence of testosterone-mediated differences in ghrelin on appetite.

Strengths of this study include the lack of attrition, strict control of energy intake and expenditure, assessment of postprandial acyl and des-acyl ghrelin dynamics, and concomitant measurements of appetite. However, results should be interpreted within the context of several limitations. First, as ghrelin and appetite were secondary outcomes, the trial was not designed specifically to examine interactions between those outcomes and testosterone. Second, despite randomization, between-group differences in acyl ghrelin and des-acyl ghrelin AUC were noted before testosterone treatment. However, when statistical adjustment for the pretreatment differences was used, both acyl ghrelin AUC and des-acyl ghrelin AUC were higher at the POST-ED time point in PLA relative to TEST. Further, in confirmatory analysis of covariance analyses adjusting for PRE-ED ghrelin AUC, changes in acyl and des-acyl ghrelin AUCs from PRE-ED to POST-ED remained greater in PLA relative to TEST (data not presented). Taken together, these results are consistent with a testosterone-mediated suppression of ghrelin. Because no statistically significant between-group differences in changes in fasting ghrelin concentrations were observed, these findings also highlight the value of obtaining serial measurements for appetite-associated hormones that deviate from fasting concentrations throughout much of the day.

An additional study limitation is that mean TT concentrations in PLA did not fall as precipitously as has been reported in studies of military personnel experiencing severe energy deficit, and the substantial LBM losses reported in those studies were not reproduced [6, 12]. The presence of additional physiologic stressors in previous studies, such as sleep deprivation, likely explain these differences. However, we previously reported that PLA did experience reductions in luteinizing hormone in addition to reductions in FT, and increases in sex-hormone binding globulin during energy deficit [43]. Those responses are consistent with hypothalamic-pituitary-gonadal axis suppression [57]. Therefore, study findings can to some extent be extrapolated to the real-life conditions the study was intended to mimic. Results may also be generalizable to physiologic adaptations experienced in men in whom energy imbalances result from intentional or unintentional restriction of energy intake and increases in energy expenditure during athletic training [61]. Finally, mean POST-ED testosterone concentrations in TEST were at the high end of the normal physiologic range for young men and substantially higher than PRE-ED concentrations. This allowed an examination of relationships between testosterone, ghrelin, and appetite across the full range of normal physiologic concentrations. However, whether similar effects would have been observed had testosterone concentrations been maintained closer to the normal basal range in this population cannot be determined.

4. Conclusions

Testosterone supplementation blunted energy deficit-induced increases in ghrelin, associations between changes in testosterone and ghrelin were not mediated by changes in

body composition, and effects of testosterone supplementation on ghrelin did not suppress appetite. Testosterone supplementation therefore appears unlikely to appreciably influence appetite or energy intake in nonobese young men, to include military personnel, during and following periods of unavoidable severe energy deficit. However, observations strengthen evidence of testosterone-ghrelin interactions, and provide additional insight into endocrine factors potentially linking energy homeostasis and male reproductive function.

Acknowledgments

We thank the study participants who participated in the study, the study team at the PBRC for significant contributions to study management and data collection, Dr. Shalender Bhasin for assistance in designing the testosterone intervention, and Philip Niro for assistance in designing the figures.

Financial Support: This work was funded by the US Department of Defense under the Collaborative Research to Optimize Warfighter Nutrition II and III projects and the Joint Program Committee-5. C.E.B. was supported in part by an appointment to the Department of Defense (DoD) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DoE) and the DoD. ORISE is managed by the Oak Ridge Associated Universities (ORAU) under DoE contract number DE-SC0014664.

Additional Information

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HIGH ALTITUDE MEDICINE & BIOLOGY
Volume 00, Number 00, 2020
© Mary Ann Liebert, Inc.
DOI: 10.1089/ham.2019.0109

High-Altitude Acclimatization Suppresses Hepcidin Expression During Severe Energy Deficit

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Abstract

Hennigar, Stephen R., Claire E. Berryman, Alyssa M. Kelley, Bradley J. Anderson, Andrew J. Young, James P. McClung, and Stefan M. Pasiakos. High-altitude acclimatization suppresses hepcidin expression during severe energy deficit. *High Alt Med Biol.* 00:000–000, 2020.

Background: The erythropoietic cells in the bone marrow require iron to synthesize heme for incorporation into hemoglobin. Exposure to hypoxic conditions, such as extended sojourns to high altitude (HA), results in increased erythropoiesis and an increased physiological requirement for iron. In addition to increasing iron requirements, hypoxic conditions suppress appetite and often lead to decreased energy intake. The objective of this study was to determine the combined effects of severe energy deficit and hypoxia on hepcidin and measures of iron status in lowlanders sojourning to HA.

Methods: Iron status indicators and hepcidin were determined in 17 healthy male volunteers (mean ± standard deviation, age 23 ± 6 years, body mass index 27 ± 4 kg/m^2) fed a controlled diet (12 ± 1.2 mg iron/day) during a 20-day sojourn to 4300 m above sea level.

Results: Chronic exposure to HA during severe energy deficit increased hematocrit by 12% ($p < 0.01$) and decreased serum hepcidin by 37% ($p < 0.01$) compared with baseline. Ferritin declined by 18% ($p = 0.02$) and transferrin saturation and soluble transferrin receptor increased by 55% and 83%, respectively ($p < 0.01$ for both) compared with baseline.

Conclusions: HA acclimatization suppresses hepcidin expression to increase iron availability during severe energy deficit. Registered at ClinicalTrials.gov as NCT02731066.

Keywords: erythropoiesis; hypoxia; iron

Introduction

Steady-state erythropoiesis in healthy human adults produces ~200 billion new erythrocytes each day, a process that requires 20–25 mg of iron daily (Muckenthaler et al., 2017). The physiological demand for iron increases by an estimated 8 mg iron per day to support the increased rate of erythropoiesis with exposure to hypoxic conditions, assuming the average increase in rate in red blood cell volume across all altitudes is ~50 mL per week (Sawka et al., 2000). The increased physiological demand for iron is thought to be facilitated, in part, by a suppression in the iron regulatory hormone hepcidin. Hepcidin is a 25-amino acid peptide hormone produced by the liver in response to iron loading, inflammation, and infection (Collins et al., 2008). Hepcidin signals for the internalization and degradation of ferroportin, the primary cellular iron export protein, thereby limiting iron efflux into circulation. Conversely, hepatic hepcidin mRNA is suppressed in mouse models of anemia and hypoxia (Nicolas et al., 2002).

Recent studies have observed significant declines in circulating concentrations of hepcidin after exposure to high altitude (HA) (3400–5400 m) (Piperno et al., 2011; Talbot et al., 2012). Declines in hepcidin are thought to facilitate iron availability for enhanced erythropoiesis with hypoxia. A major hurdle for Warfighters conducting dismounted operations or training in HA regions, particularly for extended periods, is maintaining energy balance. Sojourns to HA are

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also accompanied by a decrease in appetite (Butterfield, 1999), which is compounded by an increase in resting metabolic rate (Butterfield et al., 1992) and, oftentimes, increased physical activity. Prolonged energy deficit at HA results in greater weight loss with a higher percentage of total body mass loss coming from fat-free mass compared with that at sea level. Recently, we conducted a randomized controlled trial to determine the effects of dietary protein intake during energy deficit at HA on fat-free mass preservation (Berryman et al., 2017). We found that energy deficit during 21 days at 4300 m decreased fat-free mass regardless of protein intake. The objective of this secondary analysis was to determine the combined effects of severe energy deficit and hypoxia on hepcidin and measures of iron status in lowlanders during 20 days of controlled feeding at 4300 m above sea level.

Methods

The study conformed to the principles of the Declaration of Helsinki and was approved by the institutional review board at the U.S. Army Research Institute of Environmental Medicine (USARIEM) and registered at ClinicalTrials.gov as NCT02731066. Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to DoD Instruction 3216.02 and 32 CFR 219 on the use of human subjects in research.

Participants

Males, age 18–42 years, who were born at altitudes <2100 m and were physically active (programmed physical activity 2–4 day/week), were eligible to volunteer. Exclusion criteria included living or traveled to areas >1200 m for ≥5 days within the past 2 months, metabolic or cardiovascular abnormalities, anemia (hematocrit <38% and hemoglobin <12.5 g/dL), and blood donation within 8 weeks of beginning the study. Twenty-one participants were randomized and 17 participants completed the study and were included in the final analyses (Fig. 1). No participants displayed signs of excessive erythrocytosis during the study period.

Study design and procedures

Participants lived at sea level (Natick, MA; 50 m) for 21 days before traveling to HA. During this free-living and weight maintenance phase, participants followed dietary prescriptions administered by registered dietitians for energy and protein intake. On day 21, participants were flown to Denver, Colorado, and were provided supplemental oxygen to maintain normoxic oxygen saturation until they arrived at the USARIEM Maher Memorial Altitude Laboratory at the summit of Pikes Peak (4300 m) the next morning (peripheral oxygen saturation measured hourly from arrival to 2200 h and every 30 minutes beginning at 0300 h was 97.0% ± 0.9%). For the next 20 days, participants were provided a controlled diet containing either a standard [1.1 ± 0.0 g protein/kg per day ($n = 8$)] or higher amount of protein [2.1 ± 0.0 g protein/kg per day ($n = 9$)]. The standard protein level was chosen based on mean *ad libitum* protein intakes in active-duty military personnel (Margolis et al., 2012), and the higher protein level has been shown to attenuate fat-free mass loss during energy deficit at sea level (Pasiakos et al., 2013). Dietary protein had no effect on any outcome measure in this study (data not shown) and, therefore, marginal means for time are shown.

Blood collection and circulating biomarkers of iron homeostasis

Fasting blood samples were collected 30 minutes after ceasing supplemental oxygen (day 1) and after 20 days at

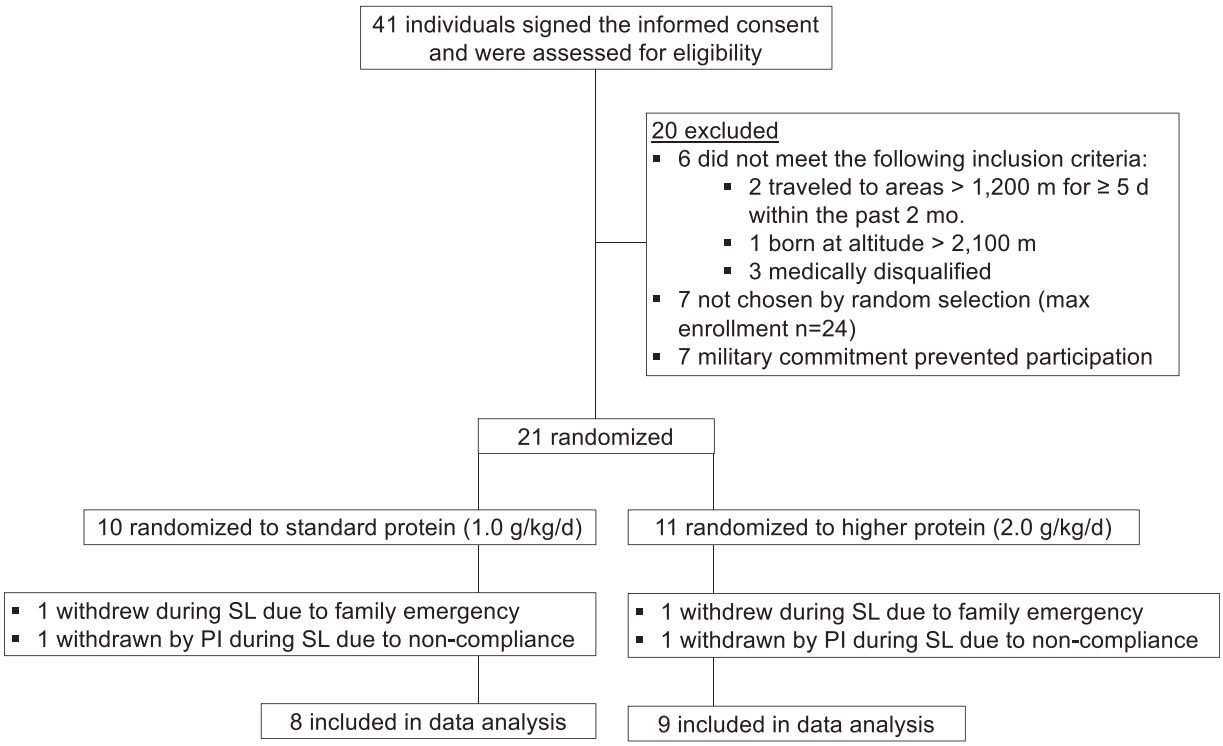


FIG. 1. Volunteer selection chart. PI, principal investigator; SL, sea level.

Table 1. Participant Characteristics at Sea Level and High Altitude

	Sea level	*HA*	p
Age, years	23.4 ± 5.6	—	
Weight, kg	82.9 ± 14.5	75.0 ± 13.2	<0.0001
BMI, kg/m^2	26.5 ± 3.7	24.0 ± 3.4	<0.0001
Body fat, %	23.0 ± 6.1	20.0 ± 8.3	0.0003
SpO_2, %	97.0 ± 0.9	87.0 ± 1.5	<0.0001
Dietary iron, mg/day	11.6 ± 4.1	12.0 ± 1.2	0.67
Energy, kcal/day	2394 ± 426	1916 ± 229	<0.0001

Values are means ± standard deviation. Average dietary iron and energy intakes for the 3-week period at sea level and HA are shown; all other values represent the final day at sea level or HA, respectively.

BMI, body mass index; HA, high altitude; SpO_2, peripheral capillary oxygen saturation.

HA (day 20). Twenty days was selected as the data point as hepcidin expression is lowest with chronic altitude exposure (i.e., >10 days) compared with acute altitude exposure (Piperno et al., 2011). Serum ferritin was measured using an automated immunoassay instrument (Siemens Medical Solutions USA, Inc.). Soluble transferrin receptor (sTfR) was determined using a Quantikine IVD ELISA Kit (R&D Systems Inc.). Serum iron and total iron-binding capacity (TIBC) were measured using the Beckman Coulter DxC 600 Pro System (Beckman Coulter), and transferrin saturation was calculated by dividing serum iron by TIBC. Hematocrit was determined in whole heparinized blood using a handheld iSTAT® point-of-care device and Chem8+ Cartridges (Abbott Laboratories). Serum hepcidin was assessed using enzyme-linked immunoassays (high sensitivity; DRG International).

Statistical analysis

Data are reported as means ± standard deviation. Comparisons between days 1 and 20 were conducted using paired Student's *t*-tests. Pearson correlations were conducted when testing for linear associations. Differences were considered significant if $p < 0.05$. Data were analyzed using SPSS version 21 (IBM Corp.) and graphed using GraphPad Prism 5.04 (GraphPad Software Inc.).

Results

Participants' demographics and dietary information after sea level and HA are given in Table 1. As reported previously, total energy deficit at HA was −1849 ± 511 kcal/day, inducing an overall weight loss of 7.9 ± 1.9 kg (Berryman et al., 2017). Dietary iron intake remained constant from sea level to HA. Twenty days at HA resulted in a 12% increase in hematocrit (Fig. 2A; $p < 0.01$) and a 37% decline in serum hepcidin concentrations (Fig. 2B; $p < 0.01$) compared to day 1. Serum iron (65.4 ± 16.5 and 104.7 ± 34.2 μg/dL; $p < 0.01$) and TIBC (317.2 ± 37.2 and 332.5 ± 30.0 μg/dL; $p < 0.01$) increased, resulting in a 55% increase in transferrin saturation (Fig. 2C; $p < 0.01$) after 20 days at HA compared to day 1. Ferritin concentrations declined by 18% (Fig. 2D; $p = 0.02$), sTfR increased by 83% (Fig. 2E; $p < 0.01$), and the sTfR to $\log_{10}$ ferritin ratio, an index of iron-dependent erythropoietic activity (Punnonen et al., 1997; Beguin, 2003), increased by 99% (Fig. 2F; $p < 0.0001$) on day 20 compared to day 1. Circulating concentrations of interleukin-6 were not different between days 1 and 20 at HA (11.0 ± 16.6 and 11.9 ± 16.7 pg/mL; $p = 0.31$).

Discussion

In this study, we report suppressed hepcidin concentrations and increased iron availability in lowlanders experiencing

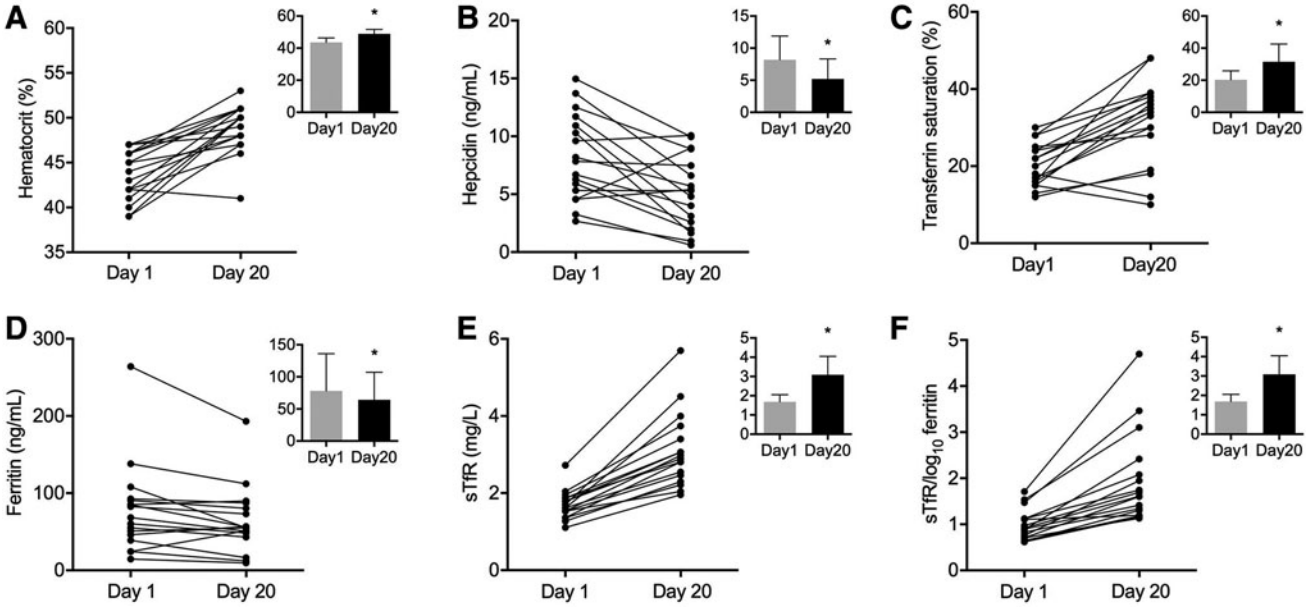


FIG. 2. Changes in hematocrit, hepcidin, and indicators of iron status during energy deficit at HA. Line graphs for each participant ($n = 17$) and means ± standard deviations are shown for **(A)** hematocrit and **(B)** hepcidin, **(C)** transferrin saturation, **(D)** ferritin, **(E)** sTfR, and **(F)** sTfR/$\log_{10}$ ferritin. An *asterisk* denotes a significant difference ($p < 0.05$). HA, high altitude; sTfR, soluble transferrin receptor.

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severe energy deficit during a 20-day sojourn to 4300 m above sea level. These data suggest that increased erythropoietic activity with HA acclimatization suppresses hepcidin expression to increase iron availability during severe energy deficit.

Dietary iron intake and body iron stores decline with exposure to hypoxia (Richalet et al., 1994; Robach et al., 2004). The former is due to a decrease in overall food intake, which may be explained by decreased appetite, nausea, and vomiting from acute mountain sickness, or a decreased availability of food (Butterfield, 1999). The latter may be explained by a reduction in dietary iron intake and/or iron mobilization to the erythropoietic compartment. The suppression in hepcidin signals for iron release from storage organs including macrophages and enhanced absorption of dietary iron in the intestine (Gassmann and Muckenthaler, 2015). In this study, the decline in ferritin and increase in sTfR suggest a reduction in body iron stores with hypoxia and energy deficit even when dietary iron intake was held constant. The increase in transferrin saturation was a result of increased serum iron and TIBC. In one study, after spending 3 days at 3400 m, participants trekked for a period of 5 days to 5400 m where they remained for 11 days (Piperno et al., 2011). Transferrin saturation decreased compared with that at sea level after 20 days at >3400 m, which was consistent with iron mobilization and transfer to the erythropoietic compartment. These seemingly contradictory findings may be due to the opposing effects of energy deficit and hypoxia on erythropoiesis and iron metabolism. Severe energy deprivation inhibits erythropoiesis (Fried et al., 1957; McCarthy et al., 1959; Donati et al., 1964; Fruhman, 1966), which is accompanied by decreases in erythroid progenitors in the bone marrow to preserve iron stores for vital functions. Regardless, these data indicate a strong overall iron mobilization and further support the concept of increased iron transfer to the erythropoietic compartment during energy deficit at HA.

Recent evidence in humans (Troutt et al., 2012) and rodent models (Mirciov et al., 2018) indicates that food deprivation increases hepcidin production. A time-dependent increase in serum hepcidin was observed in men and women during an 18-, 42-, and 66-hour fast compared with baseline (Troutt et al., 2012). Interestingly, hepcidin declined slightly, but remained elevated compared with baseline, when participants were refed with a small amount of food (500 kcals) in the 24 hours after fasting. Previous studies that have measured circulating concentrations of hepcidin at HA report a >80% reduction in hepcidin with exposure to HA (Piperno et al., 2011; Talbot et al., 2012; Goetze et al., 2013; Lundgrin et al., 2013). Although this study did not test the independent effects of energy deficit and hypoxia on hepcidin, the reduction in hepcidin with HA during energy deficit in this study was only 37% (i.e., >50% less than the other reports). Findings from this study suggest that the magnitude of the reduction in hepcidin at HA may be reduced with energy deficit, but that the increased erythropoietic activity with hypoxia may override the stimulatory effects of energy deficit on hepcidin expression.

The regulation of hepcidin in the context of hypoxia remains unclear. Piperno et al. (2011) observed a strong correlation between serum ferritin and hepcidin at HA and hypothesized that iron or the kinetics of iron use in response to hypoxia may signal for the downregulation in hepcidin (Piperno et al., 2011). Consistent with their findings, the percentage change in hepcidin during HA acclimation and energy deficit in this study was strongly correlated with the change in ferritin ($r = 0.894$, $p < 0.01$). However, Talbot et al. (2012) supplied volunteers with intravenous infusion of either 200 mg iron or saline placebo immediately before HA exposure and found that the suppression in hepcidin by hypoxia was too rapid to be due to a reduction in iron availability (Talbot et al., 2012), suggesting that a signal released during erythropoiesis and/or hypoxia may signal for the downregulation in hepcidin.

In this study, HA and energy deficit had no effect on circulating concentrations of interleukin-6, a known inflammatory regulator of hepcidin expression (Nemeth et al., 2004). Erythroferrone (ERFE) is a recently identified hormone encoded by the *FAM132B* gene that mediates hepcidin suppression during stress erythropoiesis (Kautz et al., 2014). Kautz et al. (2014) demonstrated that erythropoietin stimulates expression of ERFE in the bone marrow through Jak2-Stat5 signaling and that ERFE is secreted by (pro)erythroblasts to suppress hepcidin expression in mouse liver (Kautz et al., 2014). A validated commercially available kit to measure serum ERFE was not available at the time of this study; however, recently, Talbot et al. indicated no relationship between hepcidin and ERFE in HA natives undergoing venesection for chronic mountain sickness (Talbot et al., 2016). Rodents exposed to acute hypoxia (6–15 hours) showed a persistent downregulation in hepcidin and a transient increase in ERFE in the bone marrow (Ravasi et al., 2018), indicating that alternative mediators may be involved in maintaining the downregulation in hepcidin with hypoxia. Further studies are needed to clarify the role of ERFE in hypoxia-induced inhibition of hepcidin.

Collectively, these data indicate that hypoxia is a strong signal for iron mobilization to support iron transfer to the erythropoietic compartment. Importantly, dietary iron and iron recycling from macrophages cannot match the increased requirements for iron at HA. These findings are relevant to military personnel, who often endure sustained periods of energy restriction during training and combat operations in HA regions, for mountaineering expeditions and those trekking to HA, and may be clinically relevant to the hypoxemia and energy restriction observed in critically ill patients.

Authors' Contributions

S.R.H., C.E.B., A.J.Y., J.P.M., and S.M.P. conceived the study. S.R.H., C.E.B., B.J.A., A.J.Y., J.P.M., and S.M.P. conducted the study and A.M.K. and B.J.A. performed the experiments. S.R.H. analyzed the data and wrote the article with input from all authors. All authors have reviewed and approved the article.

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Author Disclosure Statement

The authors declare no competing financial interests.

Funding Information

This study was supported, in part, by the U.S. Army Medical Research and Material Command and appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Material Command.

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Received September 16, 2019;
accepted in final form March 11, 2020.

Am J Physiol Endocrinol Metab 319: E678–E688, 2020.
First published August 10, 2020; doi:10.1152/ajpendo.00157.2020.

RESEARCH ARTICLE

Testosterone supplementation upregulates androgen receptor expression and translational capacity during severe energy deficit

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Submitted 16 April 2020; accepted in final form 3 August 2020

Howard EE, Margolis LM, Berryman CE, Lieberman HR, Karl JP, Young AJ, Montano MA, Evans WJ, Rodriguez NR, Johannsen NM, Gadde KM, Harris MN, Rood JC, Pasiakos SM. Testosterone supplementation upregulates androgen receptor expression and translational capacity during severe energy deficit. *Am J Physiol Endocrinol Metab* 319: E678–E688, 2020. First published August 10, 2020; doi:10.1152/ajpendo.00157.2020.—Testosterone supplementation during energy deficit promotes whole body lean mass accretion, but the mechanisms underlying that effect remain unclear. To elucidate those mechanisms, skeletal muscle molecular adaptations were assessed from muscle biopsies collected before, 1 h, and 6 h after exercise and a mixed meal (40 g protein, 1 h postexercise) following 14 days of weight maintenance (WM) and 28 days of an exercise- and diet-induced 55% energy deficit (ED) in 50 physically active nonobese men treated with 200 mg testosterone enanthate/wk (TEST) or placebo (PLA) during the ED. Participants (n = 10/group) exhibiting substantial increases in leg lean mass and total testosterone (TEST) were compared with those exhibiting decreases in both of these measures (PLA). Resting androgen receptor (AR) protein content was higher and fibroblast growth factor-inducible 14 (Fn14), IL-6 receptor (IL-6R), and muscle ring-finger protein-1 gene expression was lower in TEST vs. PLA during ED relative to WM ($P < 0.05$). Changes in inflammatory, myogenic, and proteolytic gene expression did not differ between groups after exercise and recovery feeding. Mechanistic target of rapamycin signaling (i.e., translational efficiency) was also similar between groups at rest and after exercise and the mixed meal. Muscle total RNA content (i.e., translational capacity) increased more during ED in TEST than PLA ($P < 0.05$). These findings indicate that attenuated proteolysis at rest, possibly downstream of AR, Fn14, and IL-6R signaling, and increased translational capacity, not efficiency, may drive lean mass accretion with testosterone administration during energy deficit.

androgen receptor; inflammation; muscle mass; myonuclear accretion; negative energy balance; translational capacity

INTRODUCTION

The effects of testosterone administration on lean body mass accretion are well documented (7–9) and suggest testosterone supplementation is a viable strategy for preserving muscle mass in populations exposed to extreme stress. United States military personnel, in particular, endure high physical demands, sleep deprivation, and sustained periods of severe unavoidable energy deficit during training and combat operations. Those stressors, especially energy deficit, may alter molecular regulation of muscle mass [i.e., mechanistic target of rapamycin (mTOR)-mediated anabolic signaling; see Ref. 35], attenuate skeletal muscle and whole body anabolism and increase catabolism (12), and result in lean body mass losses (4, 36, 37). Loss of lean mass under these conditions is likely accelerated by the concomitant suppression of endogenous testosterone synthesis (25, 29) and may therefore be attenuated with supplemental testosterone (42, 43).

The anabolic effects of supplemental testosterone have been attributed to androgen receptor (AR)-dependent and -independent regulation of muscle protein synthesis and breakdown (47), and satellite cell and muscle pluripotent stem cell commitment and differentiation (49, 51). Testosterone treatment in older men was shown to increase skeletal muscle AR expression (23, 27). Upregulation of anabolic signaling through the mTOR pathway (i.e., translational efficiency; see Refs. 3 and 58) and AR-dependent downregulation of ubiquitin-mediated proteolysis (64) has also been observed in vitro following testosterone administration in cultured rodent muscle cells. A persistent increase in muscle protein synthesis and decrease in muscle protein breakdown would lead to muscle mass accrual over time. Furthermore, testosterone-related increases in the fusion of activated satellite cells to existing muscle fibers has been hypothesized to contribute to greater muscle volume (13, 52). Activation, proliferation, and differentiation of normally quiescent muscle satellite cells and pluripotent stem cells occur with the sequential expression of specific myogenic regulatory factors [i.e., myogenic differentiation-1 (MyoD), paired box 7 (Pax7), myogenic factor 5 (Myf5), myogenic factor 6 (Myf6), and myogenin]. Testosterone supplementation in humans increases the number of proliferating satellite cells and expression of myogenin, indicating testosterone promotes cell cycle entry and later stages of myogenesis (51). Testosterone-mediated increases in satellite cell number and their fusion with existing fibers may be regulated through nongenomic AR-independent pathways (26) and

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AR-dependent signaling (32, 49, 50). Additionally, the potential anti-inflammatory effect of exogenous testosterone administration (1, 15, 54) may promote myogenesis and decrease proteolysis by attenuating excessive inflammation (10, 18, 31).

To our knowledge, the molecular effects of exogenous testosterone administration during exercise- and diet-induced energy deficits remain unexplored. Whether testosterone supplementation alters intramuscular signaling at rest or after exercise and recovery feeding is also unclear. Understanding the effect of testosterone on intracellular signaling pathways regulating muscle mass under these conditions may facilitate the development of androgen-targeted therapies for mitigating muscle loss during situations of severe energy deficit. Given the well-established intramuscular anabolic, proteolytic, myogenic, and inflammatory signaling responses to exercise and feeding (33, 57, 61), the possibility exists that testosterone administration promotes lean mass accretion by modulating these pathways under postexercise and postfeeding conditions. For example, testosterone may potentiate the synergistic effect of high-protein feeding and exercise on stimulating rates of muscle protein synthesis by improving the reutilization of amino acids (24). Testosterone-mediated increases in myogenesis may also enhance the repair, replacement, and remodeling of mechanical stress-induced muscle fiber damage during postexercise recovery to facilitate muscle mass maintenance (11). Therefore, the objective of this study was to determine the effect of testosterone (200 mg testosterone enanthate/wk, TEST) or placebo (PLA) supplementation during 28 days of a severe exercise- and diet-induced energy deficit (ED; ~55% deficit) on AR protein content, mTOR-mediated anabolic signaling (translational efficiency), muscle total RNA content (translational capacity), ubiquitin-mediated proteolysis, myogenesis, and muscle inflammation relative to a period of weight maintenance (WM) under fasted rested conditions and in recovery from exercise and a protein-containing meal. We hypothesized that TEST vs. PLA supplementation would increase resting AR expression and attenuate intramuscular inflammation, resulting in greater myogenesis, enhanced efficiency of anabolic signaling, and lower proteolysis at rest and after exercise and recovery feeding during ED relative to WM.

MATERIALS AND METHODS

Participants. Participants were part of a larger proof-of-concept, single center, randomized, double-blind, placebo-controlled trial that assessed the effects of exogenous testosterone administration during 28 days of a severe exercise- and diet-induced energy deficit on changes in body composition (43). Participant eligibility and recruitment details have been reported previously (42). Briefly, 50 young (18–39 yr) physically active (≥2 days/wk aerobic and/or resistance exercise) men who met age-specific U.S. Army body composition standards (55) and had total testosterone concentrations within the normal physiological range (300–1,000 ng/dL) were recruited locally from the Baton Rouge, LA, area. Data in this manuscript are presented for 20 of those 50 participants who were dichotomized into two groups characterized by increases (TEST, $n = 10$) or decreases (PLA, $n = 10$) in both leg lean mass and total testosterone during energy deficit. This study was approved by the Institutional Review Board at the Pennington Biomedical Research Center (PBRC, Baton Rouge, LA) and by the Human Research Protection Office of the U.S. Army Medical Research and Development Command (Ft. Detrick, Fredericksburg, MD). All participants provided written informed consent and the study is registered with ClinicalTrials.gov as NCT02734238.

Experimental design. This study involved 14 days of weight maintenance (WM) followed by 28 days of a highly controlled exercise- and diet-induced energy deficit (ED) (Fig. 1). Skeletal muscle inflammatory, myogenic, synthetic, and proteolytic signaling pathways were assessed in vastus lateralis biopsies following WM (*day 14*) and ED (*day 42*) at rest and after exercise and a protein-containing mixed meal. Before WM, participants wore an accelerometer for 7 days, recorded physical activity for 3 days, and completed a 3-day food log to assess habitual diet and physical activity patterns. Participants subsequently maintained their habitual physical activity during the 14-day free-living WM phase as verified by daily activity records and accelerometry.

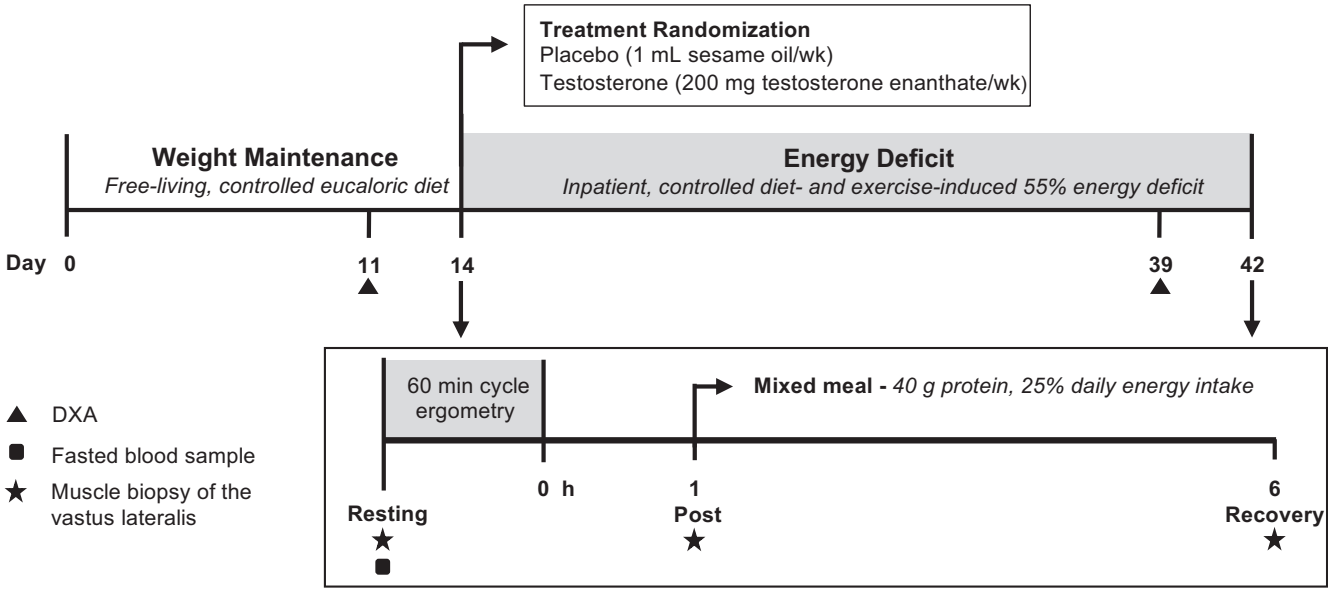


Fig. 1. Experimental design. The current analysis was part of a larger study assessing the effects of exogenous testosterone administration on changes in body composition after a 28-day exercise- and diet-induced energy deficit (ED) designed to be 45% of total energy needs (43). Biopsies were collected at the end of weight maintenance (WM) and ED before (Resting) and 1 (Post) and 6 (Recovery) h after exercise (1 h cycle ergometry), with a mixed meal (40 g protein) consumed following the first postexercise biopsy. Steady-state aerobic exercise bouts were matched between WM and ED for each participant based on power output (124 ± 22 W) and total work performed (448 ± 77 kJ). DXA, dual-energy X-ray absorptiometry.

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Participants also began a controlled eucaloric diet providing 1.6 g protein·kg^{-1}·day^{-1}, 30% energy intake from fat, and remaining calories from carbohydrates during WM. This macronutrient distribution was maintained throughout the study. Energy requirements for WM were individualized using the Mifflin St. Jeor Equation with an activity factor of 1.3, as well as the 7-day accelerometer and 3-day activity log data obtained during screening visits (42, 43). Compliance with diet instructions during WM was verified by research dietitians and by measuring seminude body weight daily with a calibrated digital scale (GSE Inc. model 450; GSE Scale Systems, Novi, MI) after an overnight fast and morning void (43). Caloric adjustments were made if necessary to maintain body mass within ±2%.

Participants were admitted to an inpatient unit at PBRC at the end of WM to begin the 28-day (*days 15–42*) exercise- and diet-induced ED. They were randomized (1:1 ratio) at the beginning of this phase to receive weekly intramuscular injections of 200 mg of testosterone enanthate (TEST) or 1 mL of sesame oil placebo (PLA). Participants were allocated to groups using a computer-generated randomization plan (version 9.4; SAS Institute, Cary, NC) with a permuted-block method (n = 4/block) and age stratification (<29 or ≥29 yr; see Ref. 43). The dose of testosterone administered on *days 15*, *21*, *28*, and *35* was chosen based on previous dose-response studies (9, 14, 30) to maintain normal testosterone concentrations during the severe energy deficit while minimizing the potential for secondary health effects (42). As previously described (43), participants performed ~3.5 sessions of varied-intensity (40–85% of predetermined $\dot{V}o_{2peak}$) aerobic-type exercise per day to increase exercise-induced energy expenditure (EIEE) from habitual levels and elevate total daily energy expenditure (TDEE) by 50% from WM [i.e., ED EIEE = WM EIEE + (0.5 × WM TDEE)]. The 55% energy deficit was established by setting energy intake at 45% of the elevated TDEE. TDEE was increased during ED using discrete bouts of steady-state aerobic-type exercise in an effort to reflect the aerobic-type physical work performed during strenuous military training and operations. Exercise modalities used to increase EIEE have been reported previously (43) and included elliptical, stationary bike, and treadmill or outdoor walking, running, and load carriage (weighted backpack ~30% of body mass). Exercise intensity was verified biweekly using open circuit indirect calorimetry (ParvoMedics TruOne 2400, East Sandy, UT) and adjusted when needed to maintain the prescribed EIEE (43). Light calisthenics were also incorporated every 3–4 days during ED to decrease the monotony of the prescribed aerobic exercise and better simulate field operations. Calisthenics were not performed within 48 h of exercise testing and muscle biopsies and integrated into individual exercise prescriptions to meet target energy expenditure.

Body composition. Body composition was determined using a three-compartment model (lean body mass, fat mass, bone mineral content) derived from dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, Madison, WI). Scans were analyzed using the Lunar Encore software (version 13.6). Total body mass, lean body mass, and fat mass were primary outcomes of the parent study (43) and are reported in this manuscript for the involved participants as a change from WM to ED. Lean body mass was calculated as total body mass minus fat mass and bone mineral content. DXA scans were conducted by trained personnel on *days 11* and *39* after an overnight fast and morning void using the participant positioning standards reported previously (43). Water intake was monitored, and conditions for the DXA were controlled across time and participants.

Endocrine profile. Endocrine profiles were analyzed from fasted blood samples collected before the first muscle biopsy procedure on *days 14* and *42* and have been presented previously for all 50 participants (43). In brief, an Immulite 2000 system (Siemens, Llanberis, UK) was used to analyze blood samples for follicle-stimulating hormone [FSH, analytical range of 0.1–170 mIU/mL, and intra-assay coefficient of variation (CV) of 3.2%], luteinizing hormone (LH, 0.05–200 mIU/mL and 3.2%), total testosterone (20–1,600 ng/dL and 8.3%), estradiol (E_2; 20–2,000 pg/mL and 6.4%), sex-hormone binding globulin (SHBG; 0.02–180 nmol/L and 2.5%), and insulin (2–150 μIU/mL and 3.8%). Glucose (10–600 mg/dL and 2.0%) was analyzed on a Beckman DXC 600 Pro (Brea, CA). An enzyme-linked immunoassay (ALPCO, Salem, NH) with an analytical sensitivity of 0.09 ng/mL and intra-assay CV of 6.65% was used to analyze IGF-I. All samples were analyzed in duplicate. Blood was sampled between 0600 and 0900 h given the diurnal variation of total testosterone in young men (56). Hormone data in the current manuscript are presented for the involved participants as a change from WM to ED.

Participant stratification. Changes in leg lean mass and total testosterone from WM to ED were positively associated for all 50 participants (r = 0.541, P < 0.001, Fig. 2). These changes remained close to zero for several individuals in both groups, however, suggesting intramuscular adaptations in TEST participants with substantial increases in both total testosterone and leg lean mass, and PLA participants whose total testosterone and leg lean mass markedly decreased, may best explain the mechanisms driving differences in lean mass with testosterone vs. placebo administration during energy deficit (43). Therefore, muscle biopsies from a subset of participants exhibiting substantial increases in leg lean mass and total testosterone (TEST, n = 10) or decreases in both of these measures (PLA, n = 10) were assayed to assess phosphorylation status, total protein and gene expression. This method of participant stratification allowed exclusion of individuals who, for example, had large increases in total testosterone but no change in leg lean mass during the intervention (i.e., nonresponders), as well as those with minimal changes in both of these parameters. This approach has been implemented previously by studies investigating differences in high vs. low responders to resistance exercise (16, 39, 41), which allocate participants who fall into extremes for study outcomes (i.e., greatest increase or decrease in muscle cross-sectional area, muscle mass, etc.) into subsets and evaluate group differences. Available muscle tissue was also a consideration when selecting individuals for analysis. A TEST participant with large increases in total testosterone (474 ng/dL) and leg lean mass (0.70 kg) was excluded, since there was insufficient muscle tissue for multiple muscle biopsy time points.

Experimental exercise bout and muscle biopsies. Percutaneous muscle biopsies of the vastus lateralis were collected at rest and after a steady-state aerobic exercise bout on *day 14* of WM and following the 28-day ED on *day 42*. The exercise bout included 60 min of cycle ergometry (Lode Excalibur Sport, Lode B.V., Groningen, the Netherlands) with exercise intensity matched between ED and WM for each participant based on power output (124 ± 22 W) and total work performed (448 ± 77 kJ). An absolute intensity was used to limit the confounding effects of weight loss on relative exercise intensity and standardize the absolute stress. Workloads for the experimental exercise bouts were determined during the 1st wk of WM using intermittent indirect

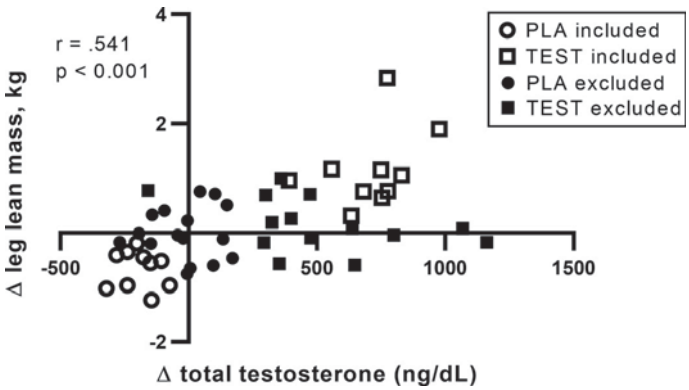


Fig. 2. Participant stratification according to leg lean mass and total testosterone. Changes in leg lean mass (kg) and total testosterone (ng/dL) were positively associated for all 50 participants. A subset of individuals exhibiting marked increases (TEST, n = 10) or decreases (PLA, n = 10) in both leg lean mass and total testosterone were included in all analyses.

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calorimetry assessment of oxygen kinetics throughout a familiarization ride on the cycle ergometer. A total of three muscle biopsies were collected from one incision on one leg per biopsy protocol day using a 5-mm Bergström needle with manual suction (22) and under local anesthesia (1% lidocaine). The biopsy needle was inserted at different angles to separate sample sites by ~5 cm and limit excessive trauma or inflammation. Muscle biopsies were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Muscle biopsies were collected under fasted rested conditions (Resting) and again after the cycle ergometry bout at 1 h (Post) and 6 h (Recovery) postexercise. Participants also consumed a standardized meal after the 1-h postexercise biopsy providing 25% of daily energy intake, 40 g of protein from animal sources, and 30% of kcal from fat (Table 1). The high protein content of the meal (~40 g) was chosen to ensure maximal stimulation of the postexercise synthetic response (59), especially since a portion of dietary protein may be oxidized for fuel rather than support protein synthesis under energy deficit conditions (5).

mRNA expression and total RNA content. Total RNA was extracted from ~15 mg of muscle using a TRIzol/ethanol precipitation method. The resulting RNA pellet was resuspended in 50 μL of nuclease-free water and assessed for quality and quantity using a Nanodrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE). The muscle total RNA concentration (μg RNA/mg muscle) was assessed to provide insights on muscle translational capacity, since ribosomal RNA comprises the majority of cellular RNA (>85%; see Ref. 63). This was calculated based on the total RNA yield and the weight of the analyzed muscle sample [RNA concentration (μg/μL) × solution volume (50 μL) × muscle weight (mg)$^{-1}$]. Equal amounts of total RNA (500 μg) were reverse-transcribed into cDNA using High-Capacity cDNA RT Kits (Applied Biosystems, Foster City, CA) and a T100 Thermal Cycler (Bio-Rad, Hercules, CA). Transcript levels of select genes linked to skeletal muscle inflammation [IL-6, IL-6 receptor (IL-6R), TNF-α, TNF-α receptor (TNFα-R), TNF-like weak inducer of apoptosis (TWEAK), and fibroblast growth factor-inducible 14 (Fn14)], myogenesis [MyoD, myogenin, Pax7, Myf5, and Myf6], and protein breakdown [muscle atrophy F-box (MAFbx) and muscle ring-finger protein-1 (MuRF1)] were determined using commercially available TaqMan probes (Applied Biosystems). Samples were run in 10-μL reactions in duplicate using TaqMan fast advanced master mix with a Step One Plus Real-Time PCR system (Applied Biosystems). Data were normalized to the geometric mean of glucuronidase-beta (GUSB) and tubulin beta class I (TUBB) mRNA, and fold changes were calculated using the $\Delta\Delta C_T$ method (45). Resting gene expression during energy deficit was expressed as a fold change relative to WM for TEST and PLA. Gene data were also expressed as a fold change from resting values within each treatment (TEST and PLA) and phase (WM and ED) to evaluate the response to exercise and feeding. There was insufficient sample for one PLA participant at Post during WM and one TEST participant at Resting and one PLA participant at Post during ED ($n = 9$ for these time points). One Fn14 and IL-6 data point for a PLA participant at Post and Recovery during WM, and at Resting during ED, were considered outliers and removed given their values were greater than 3 SDs from the mean.

Table 1. *Macronutrient composition of the postexercise meal in TEST vs. PLA at weight maintenance and energy deficit*

	WM			ED		
Absolute Intake	TEST	PLA	*P* Value	TEST	PLA	*P* Value
Energy, kcal	675 ± 122	640 ± 101	0.498	390 ± 58	376 ± 45	0.545
Carbohydrate, g	81 ± 23	75 ± 19	0.511	29 ± 11	26 ± 8	0.533
Protein, g	40 ± 0	40 ± 0	0.821	40 ± 0	40 ± 0	0.780
Fat, g	23 ± 4	21 ± 3	0.483	13 ± 2	13 ± 1	0.522

Values are means ± SD. Unpaired *t* tests were used to compare testosterone (TEST, $n = 10$) and placebo (PLA, $n = 10$) within weight maintenance (WM) and energy deficit (ED) phases.

Intracellular signaling. Total protein content of AR and the relative abundance and phosphorylation state of proteins involved in mTOR-mediated anabolic signaling were determined using standard SDS-PAGE and Western blot analysis. Approximately 15 mg of muscle were homogenized in ice-cold lysis buffer with protease and phosphatase inhibitors. Homogenized samples were snap-frozen in liquid nitrogen, thawed on ice, and centrifuged for 15 min at 10,000 *g* (4°C). Supernatant (lysate) was subsequently collected, and protein concentrations were determined using a 660-nm Protein Assay (ThermoScientific, Rockford, IL). Muscle lysates were solubilized in Laemmli buffer and loaded in equal amounts (i.e., 15 μg/lane) in precast Tris·HCl gels (Bio-Rad). Proteins were then separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad) that were incubated overnight at 4°C with commercially available primary antibodies specific to total ribosomal protein S6 (rpS6; Abcam, Cambridge, MA), p-rpS6$^{Ser240/244}$, total mTOR, p-mTOR^{Ser2448}, total p70 ribosomal protein S6 kinase (p70S6K), p-p70S6K$^{Ser424/Thr421}$, and total AR (Cell Signaling Technology, Danvers, MA). Labeling was performed using horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), and signals were detected using a ChemiDoc XRS system (Bio-Rad) with Image Laboratory software (Bio-Rad) following application of chemiluminescent reagent (Pierce, Rockford, IL). Heat-shock protein 90 (HSP90) was used to confirm that equal amounts of protein were loaded per well. Phosphorylation status was expressed relative to totals of each protein, and total protein content was expressed relative to HSP90. Resting phosphorylation status and total protein content during ED are displayed as a fold change from WM for TEST and PLA. Protein phosphorylation status was also expressed as a fold change from resting values within each treatment (TEST and PLA) and phase (WM and ED) to evaluate the response to exercise and feeding. There was insufficient sample for one TEST participant at Recovery during WM and at Resting during ED ($n = 9$), one PLA participant at Resting and Post during WM ($n = 9$), and two PLA participants at Post during ED ($n = 8$).

Statistical analysis. Differences between TEST and PLA in the composition of the postexercise meal and dietary intake during WM and ED, change (ED − WM) in body composition and endocrine profile, and participant characteristics were analyzed using unpaired *t* tests. We were also interested in the intramuscular molecular response to exercise and recovery feeding in TEST vs. PLA within WM and ED phases. Mixed-model repeated-measures ANOVA was therefore used to examine changes in phosphorylation status and mRNA expression following exercise and a high-protein mixed meal (Resting, Post, and Recovery) within each treatment (TEST and PLA) and phase (WM and ED). Bonferroni adjustments were performed for multiple comparisons if significant main effects or interactions were observed. This analysis did not examine the effect of treatment on molecular outcomes at rest, since the Resting time points were used as the control within each phase. Therefore, differences between TEST and PLA for fold change relative to WM in phosphorylation status, total protein content, and gene expression were evaluated at Resting time points using unpaired *t* tests. We have used similar methods previously to separately examine molecular adaptations at rest and changes in the response to exercise and recovery feeding (35). Associations between changes in Resting AR total protein content, total RNA, Fn14, IL-6R, and MuRF1 gene expression were examined using Pearson's correlation. Normality was assessed using Shapiro-Wilk tests for dependent variables, and mRNA data were $\log_2$ transformed given several mRNA end points were not normally distributed. These data were presented as fold change means ± SD in Figs. 3 and 4 and Table 3 for clarity. Gene and protein data in correlations were $\log_2$ transformed and presented as such, since negative fold change means are on a scale of 0–1 while positive data are >1, resulting in uneven scales. All data within text, Figs. 1–5 and Tables 1–3 are presented as means ± SD. The α level of significance for all statistical tests was two-tailed and set at $P < 0.05$. Data were

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analyzed using IBM SPSS Statistics for Windows version 26 (IBM, Armonk, NY).

RESULTS

Participants in the TEST and PLA groups did not differ in age (22 ± 3 vs. 25 ± 6 y), body mass index (25 ± 3 vs. 23 ± 2 kg/m^2), or dietary intake during WM [energy (33.5 ± 3.1 vs. 36.5 ± 5.3 kcal·kg^{-1}·day^{-1}), carbohydrate (4.4 ± 0.5 vs. 4.9 ± 0.9 g·kg^{-1}·day^{-1}), protein (1.7 ± 0.1 vs. 1.7 ± 0.1 g·kg^{-1}·day^{-1}), and fat (1.1 ± 0.1 vs. 1.2 ± 0.1 g·kg^{-1}·day^{-1})] and ED [energy (25.0 ± 4.0 vs. 24.0 ± 4.0 kcal·kg^{-1}·day^{-1}), carbohydrate (2.7 ± 0.5 vs. 2.6 ± 0.6 g·kg^{-1}·day^{-1}), protein (1.8 ± 0.3 vs. 1.6 ± 0.2 g·kg^{-1}·day^{-1}), and fat (0.8 ± 0.1 vs. 0.8 ± 0.1 g·kg^{-1}·day^{-1})] (TEST vs. PLA, $P > 0.05$). Absolute energy and macronutrient content of the postexercise meal were also similar for TEST and PLA within WM and ED phases (Table 1). Adverse events were reported previously and not different between treatment groups (43).

Body composition and endocrine profile changes during the 28-day ED differed between groups (Table 2). Loss of total body mass and total leg mass was less for TEST than PLA ($P < 0.05$). Lean body mass and leg lean mass changes were more positive for TEST than PLA ($P < 0.05$). Changes in total testosterone, free testosterone, and E_2 were more positive and changes in FSH and LH were more negative in TEST than PLA ($P < 0.05$). Change in SHBG was also lower for TEST than PLA following ED relative to WM ($P < 0.05$).

Table 2. *Body composition and endocrine profile changes in TEST and PLA following energy deficit*

	ED – WM, Δ		
	TEST	PLA	*P* Value
Body composition			
Body mass, kg			
Total	−0.9 ± 1.9	−5.1 ± 1.4	<0.0001
Lean	3.8 ± 1.2	−0.9 ± 1.0	<0.0001
Fat	−4.7 ± 1.6	−4.1 ± 1.2	0.348
Leg mass, kg			
Total	−0.3 ± 0.7	−1.8 ± 0.6	<0.0001
Lean	1.2 ± 0.7	−0.7 ± 0.4	<0.0001
Fat	−1.5 ± 0.4	−1.3 ± 0.3	0.063
Trunk mass, kg			
Total	−0.3 ± 1.4	−2.2 ± 0.9	0.003
Lean	2.4 ± 0.8	0.4 ± 0.7	<0.0001
Fat	−2.7 ± 1.1	−2.6 ± 1.0	0.798
Endocrine profile			
TT, ng/dL	711.9 ± 159.3	−193.4 ± 77.8	<0.0001
FT, ng/dL	19.0 ± 3.7	−6.0 ± 1.7	<0.0001
FSH, mIU/mL	−3.2 ± 1.2	−0.6 ± 1.0	0.007
E_2, pg/mL	39.7 ± 17	−9.1 ± 8.8	<0.0001
SHBG, μg/mL	3.7 ± 9.3	18.3 ± 9.7	0.003
LH, mIU/L	−2.8 ± 1.0	−1.1 ± 1.5	0.009
IGF-I, ng/mL	−82.7 ± 85.6	−120.8 ± 77.3	0.310
Glucose, mg/dL	−10.2 ± 8.8	−8.5 ± 14.7	0.757
Insulin, μIU/mL	−7.4 ± 8.5	−7.0 ± 11.3	0.925
Cortisol, μg/dL	4.7 ± 4.3	6.0 ± 4.5	0.494

Values are means ± SD. Data were expressed as energy deficit (ED) minus weight maintenance (WM), and differences between subjects receiving 200 mg testosterone enanthate/wk (TEST, $n = 10$) or placebo (PLA, $n = 10$) were analyzed using unpaired *t* tests. TT, total testosterone; FT, free testosterone; FSH, follicle-stimulating hormone; E_2, estradiol; SHBG, sex-hormone binding globulin; LH, luteinizing hormone.

Resting AR total protein content was higher for TEST than PLA during ED relative to WM ($P < 0.05$, Fig. 3*A*). Change in Resting total RNA was higher for TEST than PLA ($P < 0.05$, Fig. 3*B*). Resting Fn14, IL-6R, and MuRF1 gene expression was lower for TEST than PLA during ED relative to WM ($P < 0.05$, Fig. 3, *C–E*). Additional markers of inflammation, myogenesis, and proteolysis were not different between groups at rest (Table 3). Changes in Resting AR total protein were positively associated with changes in Resting total RNA ($r = 0.467$, $P < 0.05$, Fig. 3*F*), and negatively associated with Fn14 and IL-6R ($r = -0.643$ and -0.616, $P < 0.05$, Fig. 3, *G* and *H*). Changes in Resting IL-6R and MuRF1 were also positively associated ($r = 0.838$, $P < 0.05$, Fig. 3*I*).

Fn14 was greater than Resting at Post and Recovery independent of phase in both groups, with differences between Post and Recovery evident only in PLA ($P < 0.05$, time-by-treatment interaction; Fig. 4*A*). TWEAK and MAFbx were lower than Resting and Post at Recovery independent of phase and treatment ($P < 0.05$, time main effect, Fig. 4, *B* and *M*). TNFα-R, IL-6R, MyoD, and Myf6 were greater than Resting at Post and greater than Resting and Post at Recovery independent of phase and treatment ($P < 0.05$, time main effect, Fig. 4, *C, E,* and *G*, and *K*). IL-6R expression was also greater during ED in TEST vs. PLA independent of time ($P < 0.05$, phase-by-treatment interaction, Fig. 4*E*). Myf5 was greater during ED vs. WM independent of time and treatment ($P < 0.05$, phase main effect), greater than Resting at Post, and lower than Resting and Post at Recovery independent of phase and treatment ($P < 0.05$, time main effect, Fig. 4*J*). TNF-α was lower during ED than WM independent of time and treatment ($P < 0.05$, phase main effect) and greater than Resting at Post and Recovery independent of treatment and phase ($P < 0.05$, time main effect, Fig. 4*D*). IL-6 was greater than Resting at Post and Recovery and greater in TEST vs. PLA at Post independent of phase ($P < 0.05$, time-by-treatment interaction, Fig. 4*F*). MuRF1 was greater than Resting at Post and Recovery in both groups, with lower expression at Recovery than Post in TEST, and greater expression at Post in TEST vs. PLA independent of phase ($P < 0.05$, time-by-treatment interaction, Fig. 4*L*). Pax7 was lower than Resting at Post during WM and lower than Resting and Post at Recovery during WM and ED independent of treatment ($P < 0.05$, time-by-phase interaction, Fig. 4*I*). Myogenin was not different at any time point (Fig. 4*H*).

Phosphorylation status and total protein of mTOR, p70S6K, and rpS6 were not different during ED relative to WM under fasted rested conditions (Fig. 3). Post and Recovery p-mTOR^{Ser2448} and p-rpS6$^{Ser240/244}$ were greater than Resting independent of treatment and phase ($P < 0.05$, time main effect; Fig. 5, *A* and *C*), whereas p-p70S6K$^{Ser424/Thr421}$ was similar at all time points (Fig. 5*B*).

DISCUSSION

The primary observation of this study is that AR protein content was higher and Fn14, IL-6R, and MuRF1 gene expression was lower at rest in TEST compared with PLA during ED relative to WM. Levels of mTOR-mediated anabolic signaling (i.e., translational efficiency) did not differ between groups at any time point; however, the greater increase in muscle total RNA content for TEST than PLA during ED supports an enhanced translational capacity. Molecular responses to an exercise bout

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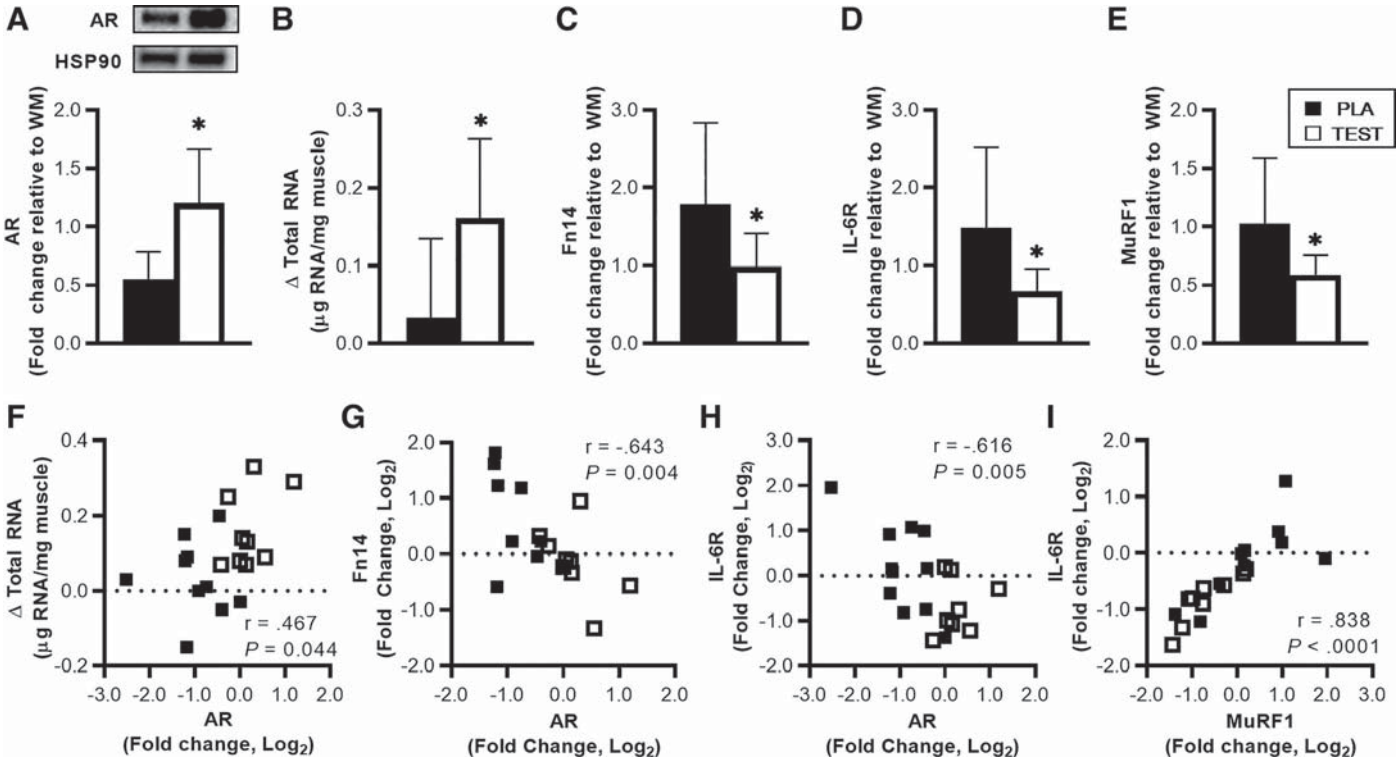


Fig. 3. Resting androgen receptor (AR) protein content (*A*), muscle total RNA content (*B*), fibroblast growth factor-inducible 14 (Fn14, *C*), IL-6 receptor (IL-6R, *D*), and muscle ring-finger protein-1 (MuRF1, *E*) gene expression during energy deficit (ED) relative to weight maintenance (WM) and associations between AR, total RNA, Fn14, IL-6R, and MuRF1 changes relative to WM (*F–I*) in subjects receiving 200 mg testosterone enanthate/wk (TEST) or placebo (PLA). Total AR was normalized to heat shock protein 90 (HSP90), and muscle total RNA concentrations (μg RNA/mg muscle) were calculated based on muscle sample total RNA yield relative to muscle weight. Gene data were normalized to the geometric mean of GUSB and TUBB, and fold changes were calculated using the $\Delta\Delta C_T$ method (45). Differences between TEST and PLA were examined at each time point using unpaired *t* tests, and associations were examined using Pearson's correlation. Values are means ± SD [TEST, $n = 9$ and PLA, $n = 10$ ($n = 9$ for Fn14)]. *TEST different from PLA, $P < 0.05$.

and high-protein mixed meal were also similar in TEST and PLA. These novel findings suggest that, in addition to altered translational capacity, testosterone administration during a severe exercise- and diet-induced energy deficit attenuates proteolytic gene expression at rest, possibly via upstream AR, Fn14, and IL-6R signaling.

The anabolic effect of supplemental testosterone during energy deficit may be mediated by AR signaling and its downstream effect on proteolytic activity. Higher AR total protein content was observed at rest in TEST vs. PLA during ED relative to WM. This is consistent with previous work showing increases in skeletal muscle AR gene expression and protein abundance with testosterone administration in older men (23, 27). Likewise, castration-induced testosterone deficiency was shown to increase muscle proteolytic gene expression in mice (48, 58), whereas testosterone administration in C_2C_{12} cells represses MAFbx expression through AR-dependent signaling (64). Recent work from Muta et al. (40) also showed that a selective AR agonist decreased expression of MAFbx and MuRF1 in cultured C_2C_{12} myotubes. Although MAFbx did not differ between groups at rest, these findings are consistent with lower abundance of Resting MuRF1 in TEST than PLA during ED relative to WM, which may result from upstream changes in AR abundance.

Lower expression of the inflammatory markers Fn14 and IL-6R at rest in TEST vs. PLA during ED relative to WM may also mediate changes in proteolytic activity. Levels of Fn14 are generally low in healthy tissues, and therefore the induction of Fn14 expression is tied to TWEAK/Fn14 pathway activity (21). Fn14 expression is substantially increased under several catabolic conditions in mice (i.e., denervation, starvation), leading to muscle loss through downstream activation of MuRF1 (38, 44). Heightened activation of this pathway following TWEAK administration to cultured myotubes similarly increased MuRF1 and MAFbx expression (19). Likewise, inhibiting IL-6R decreased MuRF1 but not MAFbx expression and prevented disuse-induced muscle atrophy in mice subjected to hindlimb unloading (60). This potential relationship between IL-6R and MuRF1 is consistent with the highly associated changes in IL-6R and MuRF1 expression during ED relative to WM in the current study ($r = 0.838$, $P < 0.0001$). These data collectively indicate that greater AR protein content and lower Fn14 and IL-6R expression at rest in TEST vs. PLA during ED relative to WM may be tied to decreased MuRF1 expression in these individuals.

Lower Resting Fn14 expression in TEST vs. PLA during ED relative to WM may be regulated by AR activity. Yin et al. (62) reported an inverse relationship between Fn14 expression and AR signaling output (i.e., mRNA signature of AR target genes) in a microarray data set composed of 131 primary and 19 metastatic prostate cancer samples. Predicting androgen response elements in Fn14 promoter regions and subsequent analyses revealed that AR binding to the Fn14 enhancer decreased its expression (62). An inverse association between Fn14 expression and AR protein content in the current study supports a similar mechanism of action in human skeletal muscle. Participants with greater

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Table 3. *Inflammatory, myogenic, proteolytic, and anabolic signaling under fasted rested conditions during ED relative to WM*

	PLA	TEST	*P* Value
TNF-α	1.21 ± 1.78	1.13 ± 0.36	0.303
TNFα-R	1.00 ± 0.90	0.80 ± 0.34	0.550
TWEAK	0.98 ± 0.26	0.99 ± 0.33	0.993
IL-6	0.87 ± 0.59	1.21 ± 0.78	0.413
Myf5	0.66 ± 0.24	0.72 ± 0.41	0.963
Myf6	0.99 ± 0.47	0.68 ± 0.26	0.125
Myogenin	0.98 ± 0.41	0.75 ± 0.25	0.173
Pax7	0.54 ± 0.21	0.57 ± 0.18	0.519
MAFbx	0.92 ± 0.47	0.75 ± 0.29	0.911
p-mTOR^{Ser2448}	1.09 ± 0.80	1.27 ± 0.77	0.613
Total mTOR	0.96 ± 0.51	1.41 ± 0.46	0.062
p-p70S6K$^{Ser424/Thr421}$	0.74 ± 0.55	1.17 ± 0.68	0.146
Total p70S6K	0.83 ± 0.33	0.88 ± 0.39	0.780
p-rpS6$^{Ser240/244}$	0.89 ± 1.09	0.73 ± 0.29	0.655
Total rpS6	1.12 ± 0.48	1.40 ± 0.88	0.402

Values are fold change means ± SD; $n = 9$ for testosterone (TEST) and $n = 10$ for placebo (PLA) ($n = 9$ for PLA IL-6). ED, energy deficit; MAFbx, muscle atrophy F-box; mTOR, mechanistic target of rapamycin; Myf5, myogenic factor 5; Myf6, myogenic factor 6; Pax7, paired box 7; p70S6K, p70 ribosomal protein S6 kinase; TNFα-R, TNF-α-receptor; TWEAK, TNF-like weak inducer of apoptosis; rpS6, ribosomal protein S6; WM, weight maintenance. ED data are expressed as a fold change relative to WM under fasted rested conditions (Resting) in TEST and PLA. Gene data were normalized to the geometric mean of GUSB and TUBB, and fold changes were calculated using the $\Delta\Delta C_T$ method (45). Protein phosphorylation status expressed relative to totals of each protein and total protein content normalized to heat shock protein 90. Differences between TEST and PLA were examined at each time point using unpaired *t* tests.

changes in AR protein content also had lower IL-6R. Although a potential mechanism underlying this relationship is less clear, Maggio et al. (34) similarly reported an inverse association between total testosterone and soluble IL-6R in a population of older men, suggesting increases in testosterone may act to suppress production of the receptor. The possibility exists that AR-induced attenuation of Fn14 expression and decreases in IL-6R expression downstream of AR signaling mediate the lower MuRF1 expression under resting fasted conditions with testosterone supplementation during ED relative to WM. These findings suggest testosterone supplementation may protect muscle mass by attenuating proteolysis at rest. It must be noted, however, that changes in proteolytic gene expression do not always translate to changes in protein abundance or rates of muscle protein breakdown (28), suggesting dynamic measures of proteolysis (i.e., stable isotope methodology) may be necessary to confirm this effect.

Markers of myogenesis were not different between TEST and PLA at rest or following exercise and recovery feeding despite the hypothesized effect of testosterone administration on the myogenesis. While exogenous testosterone administration in vitro and in humans enhanced proliferation and differentiation of myogenic progenitor cells (26, 51) and increased commitment of muscle pluripotent stem cells to the myogenic lineage (49), Englund et al. (20) recently challenged whether the consequent fusion of activated satellite cells to existing muscle fibers (i.e., myonuclear accretion) drives testosterone-induced skeletal muscle hypertrophy. Muscle fiber cross-sectional area was increased with testosterone administration in satellite cell-depleted mice, suggesting muscle hypertrophy following testosterone administration does not require an increase in satellite cell abundance or myonuclear accretion (20). Although it is unclear if these findings extend to humans, our work suggests increases in muscle mass with testosterone supplementation during energy deficit also occur independent of changes in the myogenesis.

Interestingly, we observed no differences in mTOR signaling between groups at rest or in response to exercise, suggesting changes in translation initiation had a limited role, at the selected sampling time points, in mediating the anabolic effect of supplemental testosterone during ED. These results are contrary to in vitro studies implicating the mTOR pathway in testosterone-induced increases in myotube hypertrophy (3, 58), as well as literature reporting altered synthetic rates with testosterone administration and androgen withdrawal in humans and mice, respectively (24, 53, 58). This apparent discrepancy may be attributed to energy status (i.e., energy balance vs. energy deficit) and differences in timing of biopsies and metabolic state (i.e., fed vs. fasted). The 6-h postexercise biopsy (5 h postfeeding) may have occurred too late to determine whether maximal activation of mTOR signaling during ED differed in TEST vs. PLA relative to WM, since peak stimulation of this pathway has been observed 1–2 h after feeding (2, 57). Additionally, while testosterone-mediated changes in protein synthetic rates have been observed in humans in the postabsorptive state (24, 53), the role of mTOR signaling in regulating protein synthesis under these conditions remains unclear (17). Reidy et al. (46) found that increased postabsorptive muscle protein synthesis following resistance exercise occurred with concomitant increases in translational capacity, whereas mTOR-dependent and -independent regulation of translation initiation (i.e., translational efficiency) did not change. We therefore measured total RNA content of skeletal muscle (μg RNA/mg muscle) during ED relative to WM to examine changes in translational capacity, since ribosomal RNA comprises the majority of cellular RNA (>85%; see Ref. 63). A more positive change in total RNA from WM to ED was observed in TEST vs. PLA. These findings are consistent with work in older men demonstrating increases in muscle total RNA content with testosterone vs. placebo administration during a resistance exercise training program (27). Positive associations between changes in total RNA and AR protein content suggest alterations in translational capacity may occur downstream of AR activity.

Testosterone treatment during ED had limited effects on changes in molecular markers of inflammation, myogenesis, and proteolysis following exercise and recovery feeding. Although MuRF1 and IL-6 were greater in TEST than PLA at Post, and Fn14 was greater at Recovery than Post in PLA but not TEST, these treatment effects occurred independent of phase (WM or ED) and are likely the result of baseline (WM) participant differences in the molecular response to exercise that persisted during ED rather than an effect of the TEST vs. PLA treatment. Increases in IL-6R expression with exercise and recovery feeding were also greater in TEST vs. PLA during ED. However, the physiological relevance of this finding is unclear, since there do not appear to be related effects on other markers of inflammation, myogenesis, or proteolysis (i.e., TWEAK, TNFα-R, TNF-α, MyoD, myogenin, Pax7, Myf5, Myf6, and MAFbx). Greater increases in IL-6R expression may also be a compensatory response resulting from lower baseline IL-6R mRNA in TEST vs. PLA. These findings collectively suggest that, despite the hypothesized effect of testosterone on the molecular response to

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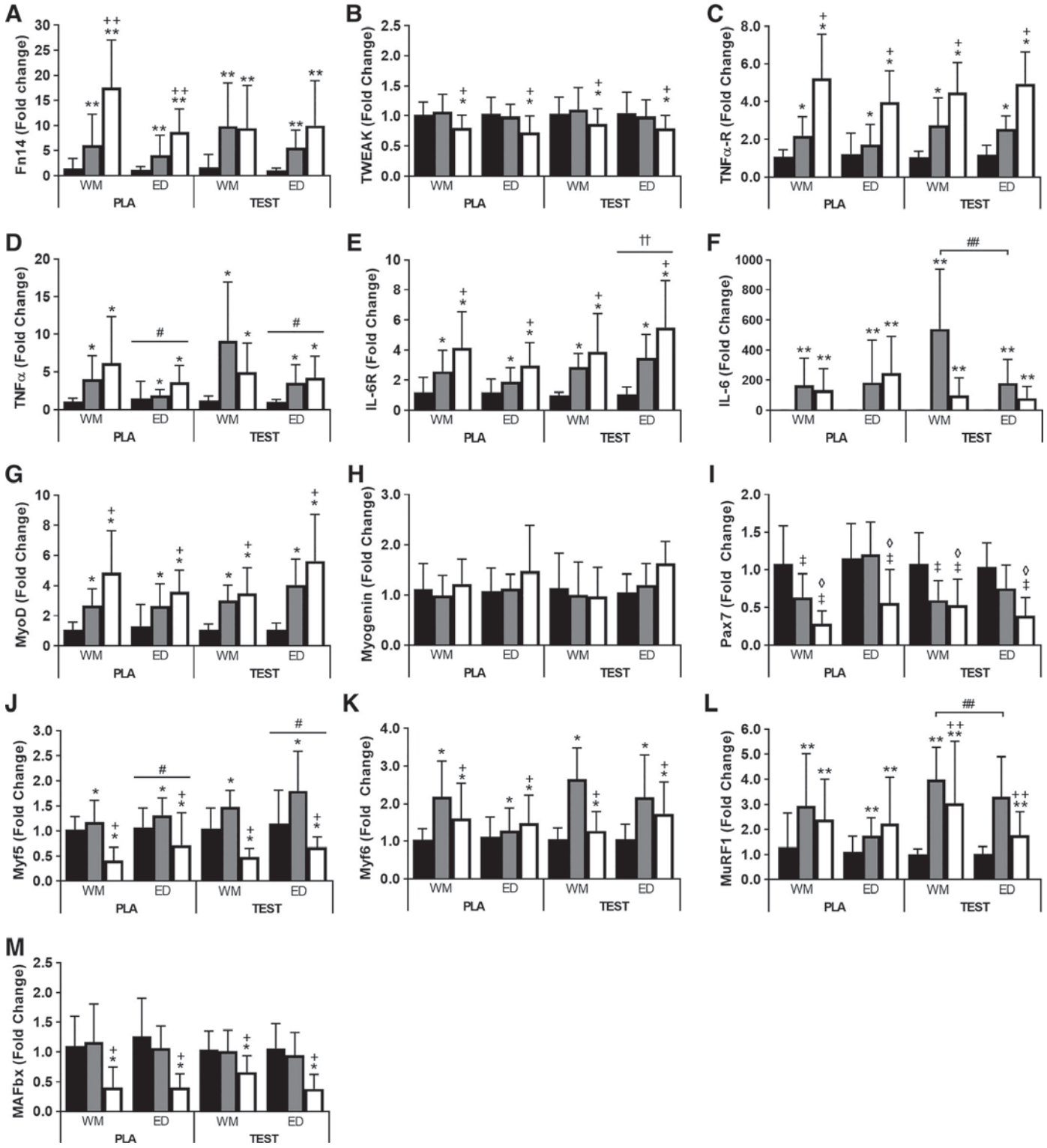


Fig. 4. Within [Resting (black bars), Post (gray bars), and Recovery (white bars)]- and between [weight maintenance (WM) and energy deficit (ED)]-study phase responses to exercise and a high-protein mixed meal for fibroblast growth factor-inducible 14 (Fn14, *A*), TNF-like weak inducer of apoptosis (TWEAK, *B*), TNF-α receptor (TNFα-R, *C*), TNF-α (*D*), IL-6 receptor (IL-6R, *E*), IL-6 (*F*), myogenic differentiation-1 (MyoD, *G*), myogenin (*H*), paired box 7 (Pax7, *I*), myogenic factor 5 (Myf5, *J*), myogenic factor 6 (Myf6, *K*), muscle ring-finger protein-1 (MuRF1, *L*), and muscle atrophy F-box (MAFbx, *M*) in subjects receiving 200 mg testosterone enanthate/wk (TEST) or placebo (PLA). Data were normalized to the geometric mean of GUSB and TUBB, and fold changes were calculated using the $\Delta\Delta C_T$ method (45). Changes in gene expression were evaluated using mixed-model repeated-measure ANOVA [$n = 10$ except Post for PLA during WM and ED ($n = 9$) and Resting for TEST during ED ($n = 9$); Fn14 and IL-6 Post for PLA during WM ($n = 8$), Recovery for PLA during WM ($n = 9$) and Resting for PLA during ED ($n = 9$). *Different from Resting, time main effect, $P < 0.05$. $^{+}$Different from Post, time main effect, $P < 0.05$. **Different from Resting, time-by-treatment interaction, $P < 0.05$. $^{++}$Different from Post, time-by-treatment interaction, $P < 0.05$. #Different from WM, phase main effect, $P < 0.05$. ††Different from PLA, phase-by-treatment interaction, $P < 0.05$. ##Different from PLA, time-by-treatment interaction, $P < 0.05$. ‡Different from Resting, time-by-phase interaction, $P < 0.05$. ◊Different from Post, time-by-phase interaction, $P < 0.05$.

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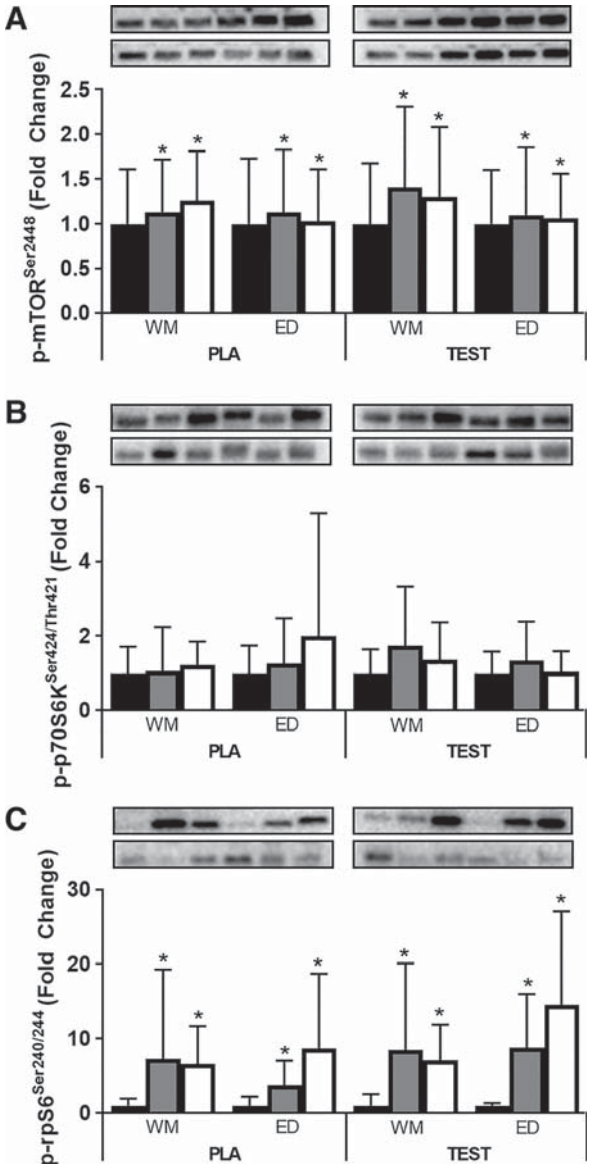


Fig. 5. Within [Resting (black bars), Post (gray bars), and Recovery (white bars)]- and between [weight maintenance (WM) and energy deficit (ED)]-study phase responses to exercise and a high-protein mixed meal for phosphorylated (p) mechanistic target of rapamycin (mTOR)^{Ser2448} (*A*), p-p70 ribosomal protein S6 kinase (p70S6K)$^{Ser424/Thr421}$ (*B*), and p-ribosomal protein S6 (rpS6)$^{Ser240/244}$ (*C*) in subjects receiving 200 mg testosterone enanthate/wk (TEST) or placebo (PLA). For *A–C*, a representative band for the target phosphorylation site is on *top* and a representative band for total protein is on *bottom*. Protein phosphorylation status was expressed relative to totals of each protein, and changes were examined using mixed-model repeated-measure ANOVA. [n = 10 except WM Recovery (n = 9) and ED Resting (n = 9) for TEST and WM Resting (n = 9), WM Post (n = 9), and ED Post (n = 8) for PLA]. *Different from Resting, time main effect, $P < 0.05$.

an exercise bout and high-protein mixed meal, lean mass differences in TEST vs. PLA appear predominantly driven by adaptations under resting conditions rather than changes in the acute response to exercise and recovery feeding.

Some limitations must be acknowledged when interpreting these findings and their potential implications. First is the dichotomization of individuals into groups characterized by substantial increases in leg lean mass and total testosterone (TEST) or decreases in both of these measures (PLA). The analysis was conducted in this manner given observed differences in the anabolic response to supplemental testosterone during energy deficit (i.e., no change in leg lean mass or total testosterone for some individuals) and to understand the influence of molecular adaptations in two distinct groups of volunteers. The possibility exists that molecular adaptations (i.e., changes in resting AR, total RNA content, Fn14, IL-6R, and MuRF1) during ED relative to WM may have been similar in all individuals receiving testosterone regardless of whether they were included or excluded from the analysis. However, excluding individuals whose leg lean mass or total testosterone did not change was preferable to optimally evaluate potential mechanisms of testosterone-mediated lean mass accretion during energy deficit. Similar approaches have been effectively used to identify intracellular mechanisms underlying low vs. high muscle hypertrophic responses to resistance exercise training (16, 39, 41). Our study design and muscle biopsy time points also precluded us from examining nongenomic actions of testosterone in muscle. This signaling occurs within seconds to minutes of testosterone administration in vitro, although the physiological relevance is humans has not been fully elucidated. Nongenomic actions of testosterone may influence gene expression and cellular processes, as testosterone promotes myogenesis independent of AR via G protein-coupled receptors in L6 cells (26). Whether nongenomic actions of testosterone contributed to changes in gene expression observed in the current study is unknown and should be an area of future work.

In conclusion, the current study presents novel data reflecting skeletal muscle molecular adaptations to supplemental testosterone during a severe exercise- and diet-induced energy deficit. Given the similar molecular responses to an exercise bout and protein-containing mixed meal, lean mass differences in TEST vs. PLA appear predominantly driven by adaptations under resting fasted conditions. Testosterone vs. placebo administration during ED increased AR protein content and attenuated Fn14, IL-6R, and MuRF1 gene expression at rest. These findings suggest that AR, Fn14, and IL-6R signaling and subsequent alterations in downstream proteolytic activity may contribute to changes in lean mass. Additionally, mTOR pathway activation did not differ between groups at rest or in response to exercise; however, a more positive change in skeletal muscle total RNA content at rest in TEST compared with PLA suggests increased translational capacity, not efficiency, drives lean mass accretion in response to testosterone supplementation during ED.

GRANTS

This work was supported by the Collaborative Research to Optimize Warfighter Nutrition II and III projects, the Defense Health Program Joint Program Committee-5, and appointment to the U.S. Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Army Medical Research and Development Command.

DISCLAIMERS

The views and assertions expressed herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organization and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

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DISCLOSURES

J.C.R. and K.M.G. reported that their institution received funding from the US Department of Defense for work associated with this publication. H.R.L. reported receiving personal fees from Pfizer, Inc., for work outside this publication. All remaining authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

S.M.P., C.E.B., J.P.K., H.R.L., L.M.M., A.J.Y., M.A.M., W.J.E., K.M.G., and J.C.R. conceived and designed research; E.E.H., N.M.J., M.N.H., J.C.R., S.M.P., and L.M.M. performed experiments; E.E.H., S.M.P., L.M.M., and N.R.R. analyzed data; E.E.H., S.M.P., and L.M.M. interpreted results of experiments; E.E.H. prepared figures; E.E.H., S.M.P., and L.M.M. drafted manuscript; E.E.H., N.M.J., K.M.G., M.N.H., J.C.R., S.M.P., L.M.M., C.E.B., H.R.L., J.P.K., A.J.Y., M.A.M., W.J.E., and N.R.R. edited and revised manuscript; E.E.H., N.M.J., K.M.G., M.N.H., J.C.R., S.M.P., L.M.M., C.E.B., H.R.L., J.P.K., A.J.Y., M.A.M., W.J.E., and N.R.R. approved final version of manuscript.

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Energy deficit increases hepcidin and exacerbates declines in dietary iron absorption following strenuous physical activity: a randomized-controlled cross-over trial

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ABSTRACT

Background: Strenuous physical activity promotes inflammation and depletes muscle glycogen, which may increase the iron regulatory hormone hepcidin. Hepcidin reduces dietary iron absorption and may contribute to declines in iron status frequently observed following strenuous physical activity.

Objectives: To determine the effects of strenuous physical activity on hepcidin and dietary iron absorption and whether energy deficit compared with energy balance modifies those effects.

Methods: This was a randomized, cross-over, controlled-feeding trial in healthy male subjects ($n = 10$, mean ± SD age: 22.4 ± 5.4 y, weight: 87.3 ± 10.9 kg) with sufficient iron status (serum ferritin 77.0 ± 36.7 ng/mL). Rest measurements were collected before participants began a 72-h simulated sustained military operation (SUSOPS), designed to elicit high energy expenditure, glycogen depletion, and inflammation, followed by a 7-d recovery period. Two 72-h SUSOPS trials were performed where participants were randomly assigned to consume either energy matched (±10%) to their individual estimated total daily energy expenditure (BAL) or energy at 45% of total daily energy expenditure to induce energy deficit (DEF). On the rest day and at the completion of BAL and DEF, participants consumed a beverage containing 3.8 mg of a stable iron isotope, and plasma isotope appearance was measured over 6 h.

Results: Muscle glycogen declined during DEF and was preserved during BAL (−188 ± 179 mmol/kg, P-adjusted < 0.01). Despite similar increases in interleukin-6, plasma hepcidin increased during DEF but not BAL, such that hepcidin was 108% greater during DEF compared with BAL (7.8 ± 12.2 ng/mL, P-adjusted < 0.0001). Peak plasma isotope appearance at 120 min was 74% lower with DEF (59 ± 38% change from 0 min) and 49% lower with BAL (117 ± 81%) compared with rest (230 ± 97%, P-adjusted < 0.01 for all comparisons).

Conclusions: Strenuous physical activity decreases dietary iron absorption compared with rest. Energy deficit exacerbates both the hepcidin response to physical activity and declines in dietary iron absorption compared with energy balance. This trial was registered at clinicaltrials.gov as NCT03524690. *Am J Clin Nutr* 2020;00:1–11.

Keywords: energy balance, exercise, hepcidin, inflammation, iron

Introduction

Hepcidin is a 25–amino acid peptide hormone that functions to reduce circulating iron concentrations by binding and signaling for the degradation of the cellular iron exporter ferroportin (1).

Supported in part by the US Army Medical Research and Development Command and appointment to the US Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Army Medical Research and Development Command. Approved for public release; distribution is unlimited. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations or commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

Data Availability: Data described in the manuscript and analytic code will be made available upon request.

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SRH and JPM contributed equally to this work.

Abbreviations used: BAL, energy balance; CPK, creatine phosphokinase; CREBH, cyclic adenosine monophosphate response element binding protein-H; CRP, C-reactive protein; DEF, energy deficit; DLW, doubly labeled water; EPO, erythropoietin; IL-6, interleukin-6; Jak, Janus kinase; LDH, lactate dehydrogenase; PBRC, Pennington Biomedical Research Center; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Stat, signal transducer and activator of transcription; sTfR, soluble transferrin receptor; SUSOPS, sustained military operations; TIBC, total iron binding capacity; VO_{2peak}, peak oxygen uptake

Received May 22, 2020. Accepted for publication September 22, 2020.

First published online 0, 2020; doi: https://doi.org/10.1093/ajcn/nqaa289.

Ferroportin is highly expressed in cells and tissues associated with iron transport, including reticuloendothelial macrophages and absorptive epithelial cells in the small intestine (2, 3). Therefore, hepcidin-mediated declines in ferroportin inhibit iron recycling and limit entry of dietary iron into portal circulation. Shortly after the discovery of hepcidin, Roecker et al. reported increased hepcidin concentrations in athletes following a marathon (4). Recent investigations have sought to determine whether the increase in hepcidin with strenuous physical activity is responsible for the declines in iron status that are frequently observed in physically active populations, such as manual laborers, military personnel, and endurance athletes (5).

Most studies have proposed that the increase in hepcidin with strenuous physical activity is due to an inflammatory response to repeated muscular contractions. These studies point to interleukin-6 (IL-6), as IL-6 is required for hepcidin induction and hypoferremia during inflammation through the Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway (6). The inflammation–hepcidin axis is thought to be an evolutionary adaptation of the host to restrict iron availability to pathogens (7–9). Glycogen depletion may also contribute to hepcidin activation through IL-6–dependent and -independent mechanisms. For example, IL-6 is synthesized and released by contracting skeletal muscle during prolonged physical activity in response to reductions in intramuscular glycogen (10–12). In addition, hepcidin is transcriptionally upregulated by gluconeogenic signals through the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A)/cyclic adenosine monophosphate response element binding protein-H (CREBH) signaling pathway (13). The PPARGC1A/CREBH pathway of hepcidin activation may be important in situations such as prolonged fasting and vigorous physical activity where glycogen stores are depleted and glucose must be synthesized de novo from nonhexose precursors in order to maintain blood glucose concentrations.

Military personnel experience periods of energy deficit during sustained combat and training operations (SUSOPS) due to increased energy expenditure and limited dietary intake. Such energy deficits during military operations reduce glycogen stores and activate gluconeogenesis (14). A previous study from our laboratory reported 245% and 33% increases in IL-6 and hepcidin, respectively, in volunteers participating in a 96-h SUSOPS that produced high energy expenditures (~6000 kcal/d) and energy deficits of ~50% total energy expenditure (15). Hepcidin concentrations post-SUSOPS were positively associated with total daily energy expenditure and the magnitude of energy deficit and negatively associated with energy intake. The primary objectives of this study were to determine the effects of SUSOPS on IL-6, hepcidin, and dietary iron absorption and whether energy deficit compared with energy balance modifies those effects. We hypothesized that SUSOPS would increase circulating concentrations of IL-6 and hepcidin and decrease iron absorption, and that energy deficit would exacerbate the increase in circulating concentrations of IL-6 and hepcidin and declines in iron absorption compared with energy balance.

Methods

The study conformed to the principles in the Declaration of Helsinki and was approved by the Institutional Review Board at the US Army Human Research Protections Office (Ft. Detrick). Participants provided written and voluntary informed consent (clinicaltrails.gov NCT03524690).

Participants

Healthy, recreationally active adult men (age 18–39 y, active duty military personnel) were recruited to participate in this study in April and September 2019. Inclusion criteria included no evidence of chronic illness, medication use, or musculoskeletal injury, and willingness to refrain from the following: *1*) pain relievers, nonsteroidal anti-inflammatory drugs, or other aspirin-containing products for 10 d before starting and for the duration of the study, *2*) alcohol and nicotine for the duration of the study, and *3*) vitamin and mineral supplements for at least 2 wk before starting and for the duration of the study. Exclusion criteria included musculoskeletal injuries, metabolic or cardiovascular abnormalities, history of any disease or abnormality of the gastrointestinal tract, anemia (hemoglobin $<$ 13 g/dL), blood donation within 4 mo of beginning the study, C-reactive protein (CRP) $>$ 5 mg/dL, abnormal prothrombin time/partial thromboplastin time test or problems with blood clotting, and history of complications with lidocaine.

Study design and procedures

This study was a 22-d, 2-trial, randomized cross-over study (**Figure 1**). Each trial consisted of a 72-h SUSOPS during which participants were either in energy deficit (DEF) or energy balance (BAL) followed by a 7-d recovery period. All aspects of the 2 trials were the same, including the timing of measures. The order in which participants completed the BAL and DEF trials was randomly assigned and balanced. Treatment order for each participant was randomly assigned using the random number generator feature in Microsoft Excel.

Prestudy baseline testing

Participants completed a 3-d diet record and a 3-d activity log during prestudy testing days. Peak oxygen uptake (VO_{2peak}) on a cycle ergometer and resting metabolic rate were measured during the prestudy testing period using standardized techniques and an indirect, open circuit respiratory system (True Max 2400, ParvoMedics).

Glycogen normalization

Participants completed a muscle glycogen normalization protocol prior to both SUSOPS trials to limit the potential influence of baseline differences in muscle glycogen on the IL-6 and hepcidin response. Following an overnight fast, participants performed a 5-min warmup on a cycle ergometer at 50% of peak oxygen uptake (VO_{2peak}). After the warmup, participants completed repeated periods of 2 min of work at a mean ± SD 80 ± 5% VO_{2peak} followed by 2 min of recovery at 50 ± 5% VO_{2peak} for 50 min (i.e., 12 cycles). After completing the glycogen depletion protocol and until the beginning of each SUSOPS trial (days 1–2 and 11–12), participants were fed a controlled diet prescribed to maintain energy balance and providing ≥60% of total energy from carbohydrate to ensure adequate glycogen

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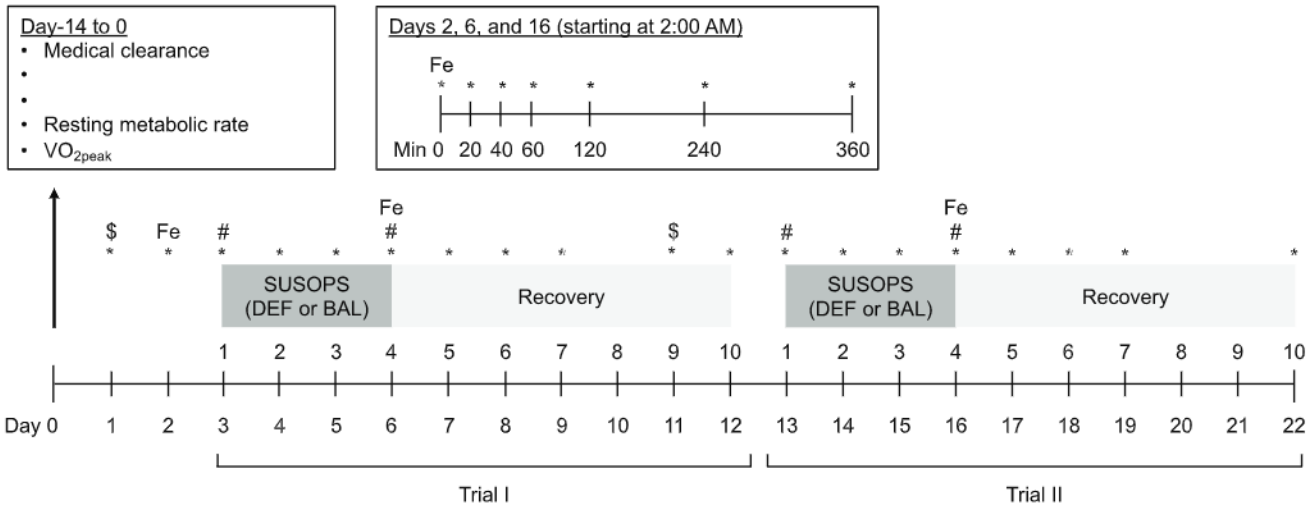


FIGURE 1 Overview of study design. Participants were randomly assigned to complete the SUSOPS trials in DEF or BAL. *blood draw; #muscle biopsy; $start of glycogen normalization and refeeding; Fe consumption of a beverage containing a stable iron isotope. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations; VO_{2peak}, peak oxygen uptake.

repletion and homogeneous glycogen concentrations within and between participants during both SUSOPS trials (DEF and BAL). Food and beverages were prepared and provided by study dietitians and consisted of commercial items.

SUSOPS DEF and BAL

The SUSOPS comprised a variety of military tasks designed to elicit high energy expenditure, glycogen depletion, and inflammation. Resting metabolic rate was multiplied by a factor of 1.3 to estimate energy expenditures for activities of daily living. Physical activity was prescribed at levels to expend ~5000–6000 total kcal/d using the American College of Sports Medicine metabolic equations for steady-state exercise and the compendium of metabolic equivalents for physical activities (16). Total daily energy expenditure prescriptions were individualized to each participant's requirements and were held constant between SUSOPS DEF and SUSOPS BAL. Low-to-moderate intensity (30–65% VO_{2peak}) steady-state endurance-type exercise was the primary exercise modality. Participants performed 3 prolonged steady-state exercise bouts per day. Two of the 3 exercise bouts were ~60–120-min load carriage exercise sessions, whereas the third was unloaded. A 120-min loaded steady-state road march was performed immediately prior to the end of the SUSOPS at 0000 on the evenings of day 5 and 15. The total distance covered was dictated by individual exercise prescriptions. The load carried was 33.5 ± 0.2 kg and comprised the basic uniform (~5.3 kg), weapon and tactical equipment (~11.2 kg), and rucksack (~15 kg). During the remainder of each day, participants performed a number of military tasks to increase energy expenditure and simulate operational tasks. Sleep was restricted to 4 h/d beginning the evenings of days 2 and 12 and ending the evenings of days 5 and 15.

Plasma iron isotope appearance

To assess dietary iron absorption, plasma isotope appearance was determined on the day before the first trial (referred to as the "rest" day) and at the completion of each SUSOPS period (Figure 1). Participants consumed a stable iron isotope in the morning (~02:00) after an 8-h fast. The timing was chosen such that the isotope was absorbed when hepcidin concentrations were expected to be the greatest (i.e., ~3 h after completion of the final SUSOPS event (17), a 2-h loaded road march). An indwelling intravenous catheter was placed in the antecubital fossa (or distally) and a baseline blood sample was drawn before consuming the iron isotope (0 min). Participants then consumed a 300-mL drink containing 3.8 mg iron (representative of dietary iron in an iron-rich meal) as isotopically labeled $^{54}FeSO_4$ or $^{57}FeSO_4$. Venous blood samples were collected 20, 40, 60, 120, 240, and 360 min and 24, 48, and 72 h later to assess plasma isotope appearance.

Iron isotope preparation and sample analysis

Iron stable isotopes (^{57}Fe, 92.88% enrichment and ^{54}Fe, 98.37% enrichment) were purchased as iron (III) sulfate powder from Oak Ridge National Laboratory. The powder was dissolved in doubly distilled water and Fe^{3+} was reduced to Fe^{2+} by adding ascorbic acid at a molar ratio of 2:1 (ascorbic acid: iron) prior to use. Stable isotope concentrations were determined by inductively coupled plasma mass spectrometry (XSERIES II, Thermo Fisher Scientific). Plasma iron isotope appearance was calculated using isotope dilution as described previously (18, 19). Briefly, the amount of absorbed iron circulating in blood was calculated based on the amount of stable isotope administered, the amount of stable isotope detected in the blood, hemoglobin concentration, and blood volume, which were estimated based on participant height and weight.

Diets during SUSOPS DEF and BAL

Registered dietitians developed individualized daily menus for SUSOPS using Food Processor SQL (ESHA Research, Version 10.14). The diets during SUSOPS were derived primarily from components of US Military Meals Ready-to-Eat and

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supplemental commercial food items to achieve prescribed macronutrient proportions. To limit the potential confounding effect of differing iron intakes, supplemental iron (ferrous sulfate drops, RxChoice) was added to an entree item with each of the meals during SUSOPS DEF to match total iron consumed during SUSOPS BAL. Water was allowed ad libitum. Participants received instructions from study dietitians on how to consume an ad libitum diet with consistent macronutrient distribution during the 2 recovery periods. Diet records were completed during the recovery periods (days 6–8 and 16–18).

Total daily energy expenditure

Doubly labeled water (DLW) was used to determine actual total daily energy expenditure and to verify the accuracy of estimated total daily energy expenditure during SUSOPS (DEF and BAL). Immediately before drinking the DLW, participants provided a urine sample to determine the natural abundance of ^{2}H and ^{18}O. A total of 120 g of DLW containing 10% $H_2{}^{18}O$ (~0.285 g $H_2{}^{18}O$/kg total body water) and 99% $^{2}H_2O$ (~0.15 g $^{2}H_2O$/kg total body water; Sigma-Aldrich) was administered on day 2 (~0000 after an 8-h fast). Urine samples were collected ~4 and 6 h after the DLW dosing for initial total body water determinations. One participant was randomly chosen to consume only locally available drinking water to control for natural changes in ^{2}H and ^{18}O abundance, and local water was analyzed to determine isotopic enrichments. The rate of disappearance of ^{2}H and ^{18}O for participants dosed with DLW was corrected for mean changes in background enrichments based on controls. Morning urine samples were collected daily during each SUSOPS period to determine elimination rates over time. Total body water was calculated by determining the regression line for the elimination of ^{2}H and ^{18}O and extrapolated to a maximum enrichment. Enrichments of ^{2}H and ^{18}O were determined using isotope ratio mass spectrometry (Finnigan Mat 252, Thermo Fisher Scientific). The ^{2}H and ^{18}O isotope elimination rates (k_H and k_O) were calculated by linear regression using the isotopic disappearance rates during each SUSOPS period. Thermic effect of food was estimated as 10% of DLW total daily energy expenditure (thermic effect of food = total daily energy expenditure × 0.1). Activity-induced energy expenditure was estimated by subtracting measured resting metabolic rate and thermic effect of food from total daily energy expenditure [activity-induced energy expenditure = total daily energy expenditure − (resting metabolic rate + thermic effect of food)].

Muscle biopsies

Percutaneous muscle biopsies were obtained from the vastus lateralis using a 5-mm Bergstrom needle with manual suction while the participant was under local anesthesia (1% lidocaine). The biopsy procedures were performed after an 8-h fast immediately before starting and within 30 min after completing each SUSOPS period (days 3, 6, 13, and 16). Glycogen concentration was determined in ~3 mg (dry weight) freeze dried muscle. Tissue was broken apart and visible connective tissue was removed. The tissue was then homogenized in water using a TissueLyser II with a 5-mm steel bead (Qiagen). Homogenates were boiled at 100°C for 5 min and centrifuged at 13,000 × *g* for 5 min at room temperature. Supernatants were removed and muscle glycogen concentrations were assessed using an endpoint colorimetric assay (Sigma-Aldrich).

Blood collection

Participants fasted ≥8 h before all blood draws. With the exception of blood collected for plasma iron isotope appearance, which started at ~2:00 AM, all blood was collected between 05:00 and 08:00. Hemoglobin was measured in whole, heparinized blood using a handheld iSTAT® point-of-care device and Chem8 + Cartridges (Abbott Point of Care; reference range: 12–17 g/dL). Serum insulin (reference range: 6–27 mIU/mL), testosterone (reference range: 160–726 ng/dL), CRP (high sensitivity; reference range: 0.2–11.0 mg/L), and serum ferritin (reference range: 28–365 ng/mL) were determined using an advanced automated immunoassay instrument (ImmuliteR 2000; Siemens Healthcare Diagnostic). Serum glucose (reference range: 70–110 mg/dL), free fatty acids (reference range: 0.1–0.6 mmol/L), glycerol (reference range: 0.03–0.19 mmol/L), myoglobin (reference range: 0–70 ng/mL), creatine phosphokinase (CPK; reference range: 38–333 IU/L), lactate dehydrogenase (LDH; reference range: 82–195 IU/L), serum iron (reference range: 50–160 μg/dL), and total iron-binding capacity (TIBC; reference range: 255–450 μg/dL) were determined using enzymatic and colorimetric measurements (Beckman Coulter DXC 600 Pro, Beckman Coulter). Transferrin saturation was calculated by dividing serum iron by TIBC. Erythroferrone (ERFE; Intrinsic Life Sciences; reference range: 0.16–10 ng/mL), erythropoietin (EPO; R&D Systems Inc.; reference range: 1.1–523 mIU/mL), serum hepcidin (high sensitivity, DRG International; reference range: 0.153–81 ng/mL), IL-6 (R&D Systems Inc.; reference range: 3.1–300 pg/mL), and soluble transferrin receptor (sTfR) (R&D Systems Inc.; reference range: 3.0–80 nmol/L) were determined using ELISAs. All assays were conducted by Pennington Biomedical Research Center (PBRC). PBRC follows good clinical practices and is accredited by the College of American Pathologists. All assays were run with standards and appropriate quality control material. In addition, PBRC runs external proficiency samples and results are compared with other laboratories across the country.

Statistical analysis

The primary outcomes for this study were IL-6, hepcidin, and dietary iron absorption. All other variables measured were secondary outcomes. Sample size calculations were derived from a previous study that found significant increases in hepcidin in male soldiers following a 7-d military training exercise (20). Using baseline hepcidin concentrations of 6.5 ± 3.5 ng/mL and an estimated increase in hepcidin of ~50% with training, it was estimated that 9 participants were sufficient to detect an increase in hepcidin with training at $\alpha = 0.05$ and power = 0.80. This sample size provides sufficient statistical power to detect large trial effects (power = 0.80, $\alpha = 0.05$, $d \geq 1.0$; GPOWER 3.1.9.7). To account for potential attrition, 13 participants were enrolled in the study. Statistical analyses were performed using SPSS version 25 (IBM Corp.). Data are presented as means ± SDs.

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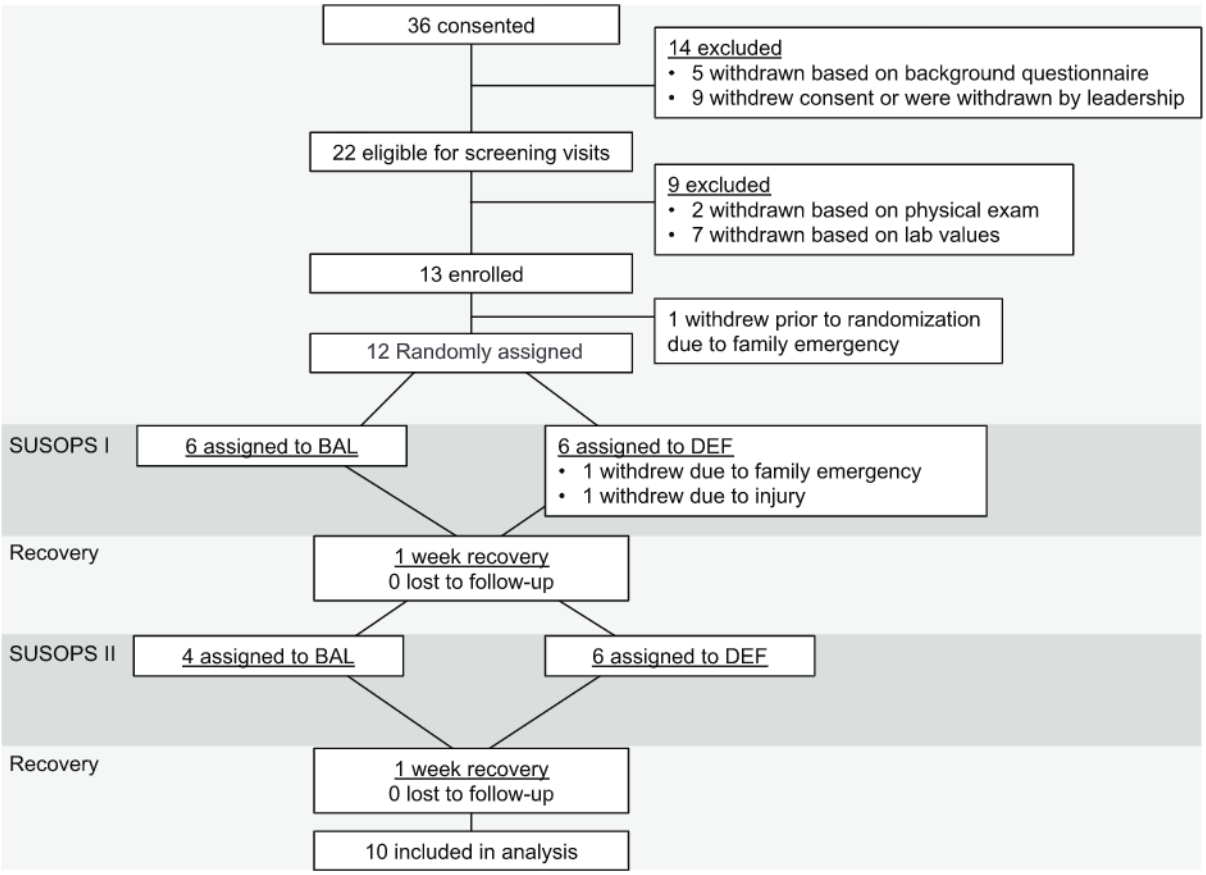


FIGURE 2 Participant flow chart. Thirty-six potential participants consented, 14 were excluded, 22 were eligible for screening visits, 9 were excluded following screening, 13 were enrolled, 1 discontinued participation prior to randomization, 12 were randomly assigned, and 10 completed the intervention. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations.

Shapiro-Wilk tests were used to determine normality of data. If normality was rejected ($P < 0.05$), log transformations were applied to normalize the data ($P > 0.05$). Differences in dietary intake for the controlled feeding days (rest, SUSOPS DEF, and SUSOPS BAL) were analyzed by 1-way ANOVA with Bonferroni correction for multiple comparisons. Paired Student *t*-tests were used to compare dietary intake during the recovery recall days and daily energy expenditure, thermic effect of food, activity-induced energy expenditure, energy balance, and energy deficit measured during SUSOPS DEF and SUSOPS BAL. General linear models with correlated errors were used to determine the main effects of the trial (i.e., DEF, BAL, and when relevant, rest; the trial included both SUSOPS and recovery when measurements were taken during recovery), time within trial (e.g., study day or minutes), and their interaction on biochemical measures and plasma iron isotope appearance. To test for carryover effects, a main effect of trial order (DEF first, BAL first) and an order-by-trial interaction were included in the model. No effects of trial order were observed, and these data are not shown. The residual maximum likelihood method was used to account for values that were missing completely at random on the dependent variable. If trial-by-time interactions were observed, a Bonferroni correction was applied for multiple comparisons. Statistical significance was set at $P < 0.05$. The P value was not adjusted for multiple endpoints, because the effectiveness of the multiple primary endpoints in the current study (IL-6, hepcidin, and dietary iron absorption) depended on the success of 2 or more primary endpoints (i.e., dietary iron absorption and hepcidin, dietary iron absorption and IL-6, or dietary iron absorption, hepcidin, and IL-6).

Results

Participants

Thirteen participants were enrolled, 12 were randomly assigned, and 10 completed the study (**Figure 2**). Baseline characteristics are shown in **Table 1**. Participants were young healthy males with sufficient iron status. With the exception of 1 participant who had a transferrin saturation of 14% (all other iron status indicators were in the normal range), none of the participants were iron deficient [ferritin < 20 ng/mL, sTfR > 32 nmol/L, and/or transferrin saturation < 20%, (21)] or anemic at baseline.

Dietary intake and physical activity

Dietary intake for the day preceding rest and during SUSOPS and recovery from SUSOPS are shown in **Table 2**. The

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TABLE 1 Baseline participant characteristics[1]

	$n = 10$
Race and ethnicity, *n* (%)	
Non-Hispanic black	1 (10)
Non-Hispanic white	7 (70)
Hispanic	0
Other	2 (20)
Age, y	22.4 ± 5.4
Body weight, kg	87.3 ± 10.9
BMI, kg/m^2	27.0 ± 3.5
VO_{2peak}, mL/kg/min	40.5 ± 4.6
Biochemical measures[2]	
IL-6, pg/mL	1.9 ± 0.6
CRP, mg/L	1.2 ± 0.9
Hemoglobin, g/dL	15.4 ± 0.9
Ferritin, ng/mL	77.0 ± 36.7
Transferrin saturation, %	45.3 ± 22.0
sTfR, nmol/L	19.8 ± 2.3
Hepcidin, ng/mL	7.2 ± 1.6
ERFE, ng/mL	0.2 ± 0.1

[1] Values are means ± SDs or *n* (%). BMI, body mass index; CRP, C-reactive protein; ERFE, erythroferrone; IL-6, interleukin-6; sTfR, soluble transferrin receptor; VO_{2peak}, peak oxygen uptake.

[2] All baseline biochemical measures were measured on day 2.

macronutrient distribution of the diets (i.e., the percentage of kcals from carbohydrate, protein, and fat) was similar during rest, SUSOPS DEF, and SUSOPS BAL; however, during rest and SUSOPS DEF participants consumed approximately 50% less energy (rest: −3005 ± 166, SUSOPS DEF: −2922 ± 227 kcal/d), carbohydrate (rest: −472 ± 28, SUSOPS DEF: −450 ± 31 g/d), protein (rest: −76 ± 8, SUSOPS DEF: −68 ± 11 g/d), and fat (rest: −108 ± 8, SUSOPS DEF: −99 ± 9 g/d) per day compared with SUSOPS BAL (*P*-adjusted < 0.0001 for all comparisons). Dietary iron intake was similar during rest and SUSOPS DEF, but both were less than during SUSOPS BAL (rest: −6.2 ± 2.0, SUSOPS DEF: −7.1 ± 1.8 mg/d; *P*-adjusted < 0.0001 for both). However, after accounting for supplemental iron given with meals during SUSOPS DEF, DEF and BAL consumed an equivalent amount of total iron during SUSOPS (0.7 ± 0.8 mg/d; *P*-adjusted = 1.00). Participants consumed similar diets during recovery from SUSOPS DEF and SUSOPS BAL.

Total time exercising (SUSOPS DEF: 283 ± 47, SUSOPS BAL: 283 ± 48 min/d; $P = 0.95$), mean exercise intensity (SUSOPS DEF: 7.5 ± 0.3, SUSOPS BAL: 7.6 ± 0.3 average metabolic equivalents; $P = 0.19$), and mean effort (SUSOPS DEF: 66 ± 9, SUSOPS BAL: 67 ± 9% of VO_{2peak}; $P = 0.27$) did not differ between SUSOPS. Total daily energy expenditure and activity-induced energy expenditure remained the same during SUSOPS DEF and SUSOPS BAL (Table 2). Combined with diet, participants were in a −2047 ± 920 kcal/d deficit (−43 ± 9% energy deficit) during SUSOPS DEF, which differed from SUSOPS BAL (18 ± 20% energy deficit, $P < 0.001$).

Metabolic markers

Mean responses of clinical biomarkers during SUSOPS and recovery are shown in **Table 3**. Participants lost weight during SUSOPS DEF (−1.9 ± 1.1 kg) and maintained weight during SUSOPS BAL (0.5 ± 0.7 kg, *P*-adjusted < 0.0001). Muscle glycogen and metabolic parameters were consistent with increased gluconeogenesis during SUSOPS DEF compared with SUSOPS BAL. There was a trial-by-time interaction for muscle glycogen (*P*-interaction = 0.03), free fatty acids (*P*-interaction < 0.0001), and glycerol (*P*-interaction < 0.0001). Muscle glycogen declined during SUSOPS DEF and was preserved during SUSOPS BAL (−188 ± 179 mmol/kg, *P*-adjusted < 0.01). Circulating concentrations of free fatty acids and glycerol increased by 142% and 147%, respectively, during SUSOPS DEF, but were unchanged during SUSOPS BAL (free fatty acids: 0.42 ± 0.19 mmol/L, *P*-adjusted < 0.0001; glycerol: 0.043 ± 0.037 mmol/L; *P*-adjusted < 0.0001). EPO increased during SUSOPS (6.5 ± 2.1 mIU/mL; *P*-time < 0.0001) with no differences between DEF and BAL (*P*-trial = 0.30).

Markers of muscle damage and inflammation

Time effects, but no trial or interaction effects, were observed for markers of muscle damage, such as myoglobin, CPK, and LDH (*P*-time < 0.0001 for all; Table 3). Post hoc comparisons for time revealed increased myoglobin, CPK, and LDH on day 3 of both SUSOPS trials compared with day 1 (*P*-adjusted < 0.0001). A trial-by-time interaction was observed for CRP (*P*-interaction < 0.01). CRP increased on day 3 of both SUSOPS trials compared with day 1. However, the increase in CRP was 59% greater during the DEF trial than during the BAL trial (2.6 ± 5.3 mg/L, *P*-adjusted < 0.0001). There was an effect of time for plasma IL-6 (*P*-time < 0.0001), but no trial (*P*-trial = 0.76) or interaction (*P*-interaction = 0.77) effects. Post-hoc comparisons for time demonstrated an increase in plasma IL-6 on day 2 of SUSOPS (11.1 ± 5.8 pg/mL) compared with day 1 of SUSOPS (2.6 ± 1.4 pg/mL; *P*-adjusted < 0.0001). A trial-by-time interaction was observed for plasma hepcidin (*P*-interaction = 0.001). Plasma hepcidin concentrations increased during SUSOPS DEF, but remained unchanged during SUSOPS BAL, such that hepcidin was 72% (7.8 ± 12.2 ng/mL, *P*-adjusted < 0.0001) greater on day 3 of SUSOPS and 59% (4.3 ± 10.4 ng/mL, *P*-adjusted < 0.01) greater on day 1 of recovery (day 4) during DEF compared with BAL.

Indicators of iron status

Trial-by-time interactions were observed for hemoglobin (*P*-interaction < 0.0001), ferritin (*P*-interaction = 0.01), serum iron (*P*-interaction < 0.0001), transferrin saturation (*P*-interaction < 0.0001), and sTfR (*P*-interaction = 0.05). There was a steady decline in hemoglobin during BAL beginning on day 2 of SUSOPS, reaching a 20% decrease on day 1 of recovery compared with day 1 of SUSOPS (−3.2 ± 0.7 g/dL, *P*-adjusted < 0.0001) before returning to pre-SUSOPS concentrations by day 3 of recovery (day 6) (Table 3). During DEF, there was an initial decline in hemoglobin on day 2 of SUSOPS, but hemoglobin rebounded to pre-SUSOPS levels on day 3, and then decreased again reaching a 15% reduction (−2.4 ± 0.8 g/dL, *P*-adjusted < 0.0001) on day 1 of recovery compared with day 1 of SUSOPS. Hemoglobin did not return to pre-SUSOPS levels until day 7 of recovery (day 10) following the DEF trial. Ferritin increased 33% on day 3 of SUSOPS DEF compared with day 1 (25.9 ± 12.7 ng/mL, *P*-adjusted = 0.02), but remained unchanged during SUSOPS BAL (−4.0 ± 8.9

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TABLE 2 Dietary intake and total daily energy balance at rest and during and after 72-h simulated sustained military operations[1]

		SUSOPS			Recovery		
	Rest	DEF	BAL	*P* value	DEF	BAL	*P* value
Absolute intake							
Energy, kcal/d	2382 ± 279[a]	2515 ± 171[a]	5437 ± 377[b]	<0.0001	2467 ± 811	2379 ± 1061	0.79
Carbohydrate, g/d	361 ± 44[a]	382 ± 26[a]	833 ± 51[b]	<0.0001	255 ± 98	245 ± 127	0.83
Protein, g/d	63 ± 6[a]	71 ± 3[a]	139 ± 12[b]	<0.0001	108 ± 36	106 ± 49	0.90
Fat, g/d	74 ± 10[a]	84 ± 7[a]	183 ± 15[b]	<0.0001	114 ± 37	110 ± 50	0.77
Dietary iron, mg/d	15.5 ± 2.9[a]	14.6 ± 2.3[a]	21.8 ± 1.3[b]	<0.0001	10.6 ± 5.7	11.4 ± 6.1	0.66
Total iron, mg/d[2]	15.5 ± 2.9[a]	22.5 ± 1.4[b]	21.8 ± 1.3[b]	<0.0001	—	—	—
Relative intake							
Energy, kcal/kg/d	26.7 ± 2.4[a]	29.1 ± 3.0[a]	62.0 ± 6.0[b]	<0.0001	28.3 ± 9.6	26.4 ± 10.3	0.61
Carbohydrate, g/kg/d	4.0 ± 0.4[a]	4.4 ± 0.5[a]	9.5 ± 1.0[b]	<0.0001	2.9 ± 1.2	2.7 ± 1.3	0.67
Protein, g/kg/d	0.7 ± 0.1[a]	0.8 ± 0.1[a]	1.6 ± 0.1[b]	<0.0001	1.2 ± 0.4	1.2 ± 0.5	0.77
Fat, g/kg/d	0.8 ± 0.1[a]	1.0 ± 0.1[a]	2.1 ± 0.2[b]	<0.0001	1.3 ± 0.4	1.2 ± 0.5	0.59
Energy intake, %							
Carbohydrate	61 ± 0.7[a]	59 ± 0.6[b]	60 ± 1.0[b]	<0.0001	40 ± 8.0	40 ± 7.8	0.91
Protein	11 ± 0.3[a]	11 ± 0.8[a]	10 ± 0.2[b]	<0.0001	18 ± 3	18 ± 3.0	0.75
Fat	28 ± 0.6[a]	30 ± 0.7[b]	30 ± 1.0[b]	<0.0001	41 ±5.8	43 ± 7.1	0.96
Daily energy expenditure, kcal/d[3]	—	4573 ± 989	4775 ± 940	—	—	—	0.69
Resting metabolic rate, kcal/d	1905 ± 241	—	—	—	—	—	—
Thermic effect of food, kcal/d	—	457 ± 99	478 ± 94	—	—	—	0.70
Activity-induced energy expenditure, kcal/d	—	2201 ± 802	2383 ± 737	—	—	—	0.69
Energy balance, kcal/d	—	–2047 ± 920	689 ± 852	—	—	—	<0.001
Energy deficit, %	—	–43 ± 9	18 ± 20	—	—	—	<0.001

[1] Values are means ± SDs; $n = 10$. Dietary intake for the day preceding rest (day 1), average intake across the SUSOPS period during DEF and BAL, and average intake from 3-d food records completed on days 6–8 and 16–18 during recovery from SUSOPS DEF or BAL. Differences in dietary intake for the controlled feeding days (rest, SUSOPS DEF, and SUSOPS BAL) were analyzed by 1-way ANOVA. Bonferroni corrections were used for post hoc comparisons. Different letters indicate significant difference. Paired Student *t*-tests were used to compare dietary intake during the recovery recall days and energy balance during SUSOPS DEF and SUSOPS BAL. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations

[2] Differences between dietary and total iron during SUSOPS are due to supplemental iron provided during SUSOPS DEF (i.e., dietary iron and total iron were the same during rest, SUSOPS BAL, and recovery).

[3] $n = 9$, 1 participant served as study control for doubly labeled water.

ng/mL, *P*-adjusted = 1.00); ferritin concentrations following SUSOPS DEF were 43% greater on day 3 (27.3 ± 33.2 ng/mL, *P*-adjusted < 0.0001) and 40% greater on day 1 of recovery (24.3 ± 36.0 ng/mL, *P*-adjusted < 0.0001) compared with BAL. There was an initial decline in serum iron (−43.3 ± 30.4 μg/dL, *P*-adjusted < 0.01) and transferrin saturation (−12.8 ± 10.2%, *P*-adjusted < 0.01) on day 2 of SUSOPS BAL compared with day 1, whereas concentrations were maintained during SUSOPS DEF (serum iron: −14.1 ± 30.4 μg/dL, *P*-adjusted = 1.00; transferrin saturation: −4.1 ± 9.6%, *P*-adjusted = 1.00). Serum iron and transferrin saturation declined during recovery from both SUSOPS DEF and SUSOPS BAL compared with day 1 of SUSOPS. sTfR declined on day 2 of SUSOPS BAL and continued to decline to day 1 of recovery compared with day 1 of SUSOPS (−0.37 ± 0.18 nmol/L, *P*-adjusted < 0.0001). sTfR was maintained during SUSOPS DEF, but then declined on day 1 of recovery compared with day 1 of SUSOPS (−0.26 ± 0.24 nmol/L, *P*-adjusted < 0.01) before returning to pre-SUSOPS levels. ERFE did not change during either SUSOPS trial or recovery.

Plasma iron isotope appearance

Trial, time, and interaction effects were observed for plasma iron isotope appearance ($P < 0.0001$ for all). Plasma iron isotope appearance peaked 120 min after ingesting the stable iron isotope following rest and mean appearance at 120 min was 74% lower following SUSOPS DEF and 49% lower following SUSOPS BAL compared with rest (**Figure 3**A). Participants absorbed 21.6 ± 8.7% of the 3.8-mg iron dose at rest, 12.9 ± 6.8% at the end of SUSOPS BAL, and 7.2 ± 3.7% at the end of SUSOPS DEF (*P*-trial < 0.0001, Figure 3B). There were trial, time, and interaction effects for hepcidin ($P < 0.0001$ for all). Hepcidin increased 360 min after ingestion of the stable iron isotope following rest (12.2 ± 8.2 ng/mL, *P*-adjusted < 0.0001) and following SUSOPS DEF (7.0 ± 7.9 ng/mL, *P*-adjusted < 0.0001) compared with 0 min, but did not increase following SUSOPS BAL (2.8 ± 4.2 ng/mL; *P*-adjusted = 0.71, Figure 3C). Trial and time effects ($P < 0.0001$ for both) were observed for serum iron, but no interaction (*P*-interaction = 0.17, Figure 3D). Mean serum iron concentrations were greater at the end of rest compared with SUSOPS DEF (38 ± 23 μg/dL; *P*-adjusted < 0.0001) and SUSOPS BAL (53 ± 31 μg/dL; *P*-adjusted < 0.0001) and at the end of SUSOPS DEF compared with SUSOPS BAL (14.6 ± 21 μg/dL; *P*-adjusted < 0.0001). Serum iron concentrations increased 360 min following ingestion of iron compared with 0 min (54 ± 27 μg/dL; *P*-adjusted < 0.0001). There was an effect of trial (*P*-trial < 0.0001), but no time (*P*-time = 0.27) or interaction effect (*P*-interaction = 1.00) for serum ferritin (Figure 3E). Serum ferritin was greater following SUSOPS DEF compared with rest (18 ± 30 ng/mL; *P*-adjusted < 0.0001) and SUSOPS BAL (23 ± 33 ng/mL; *P*-adjusted < 0.0001). Trial ($P < 0.0001$) and time ($P = 0.02$), but no interaction ($P = 0.15$) effects were found for ERFE.

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TABLE 3 Clinical biomarkers during and after 72-h simulated sustained military operations[1]

	DEF								BAL										
	SUSOPS, d			Recovery, d					SUSOPS, d			Recovery, d					*P* value		
	1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10	Trial	Time	TxT
Body weight, kg	88.3 ± 10.8	87.5 ± 10.6*	86.4 ± 10.8†*	87.3 ± 10.2*	88.2 ± 10.4	88.5 ± 10.7	88.6 ± 10.6	88.7 ± 10.7	88.1 ± 10.9	88.6 ± 10.8	88.6 ± 11.3	89.3 ± 10.3	88.7 ± 10.4	88.4 ± 10.3	88.5 ± 10.3	88.8 ± 10.4	<0.0001	<0.0001	<0.0001
Muscle glycogen, mmol/kg	495.1 ± 107.6	—	—	247.5 ± 75.1†*	—	—	—	—	498.4 ± 162.7	—	—	448.2 ± 174.7	—	—	—	—	0.02	0.002	0.032
Insulin, mIU/mL	10.1 ± 4.3	—	7.0 ± 3.4	—	11.2 ± 6.8	—	15.1 ± 12.2	15.7 ± 20.3	10.6 ± 5.4	—	7.1 ± 2.5	—	12.1 ± 8.4	—	12.9 ± 7.8	10.7 ± 6.4	0.62	0.02	0.62
Glucose, mg/dL	89.5 ± 8.9	—	84.1 ± 8.0	—	91.4 ± 11.8	—	93.1 ± 16.2	92.5 ± 12.3	92.2 ± 11.7	—	89.0 ± 9.1	—	93.0 ± 12.2	—	91.4 ± 11.9	89.6 ± 10.4	0.65	0.09	0.23
FFA, mmol/L	0.35 ± 0.08	—	0.86 ± 0.16†*	—	0.29 ± 0.10	—	0.20 ± 0.09	0.17 ± 0.13†	0.36 ± 0.15	—	0.44 ± 0.09	—	0.32 ± 0.15	—	0.22 ± 0.12	0.28 ± 0.19	0.03	<0.0001	<0.0001
Glycerol, mmol/L	0.032 ± 0.006	—	0.079 ± 0.040†*	—	0.036 ± 0.005	—	0.030 ± 0.000	0.036 ± 0.010	0.031 ± 0.003	—	0.036 ± 0.011	—	0.034 ± 0.011	—	0.038 ± 0.014	0.032 ± 0.004	<0.01	<0.0001	<0.0001
Testosterone, ng/dL	503.3 ± 102.0	—	439.5 ± 95.9	—	440.3 ± 118.2	—	518.1 ± 95.2	524.3 ± 116.3	497.5 ± 144.8	—	422.3 ± 107.1	—	465.8 ± 104.2	—	509.8 ± 122.2	521.2 ± 116.9	0.94	<0.0001	0.88
EPO, mIU/mL	7.28 ± 0.9	—	—	13.82 ± 1.7	—	—	—	—	6.79 ± 1.3	—	—	13.15 ± 2.5	—	—	—	—	0.30	<0.0001	0.85
Myoglobin, ng/mL	33.0 ± 8.9	—	47.2 ± 16.6	—	29.1 ± 13.0	—	26.6 ± 11.0	32.1 ± 16.5	30.1 ± 6.3	—	46.3 ± 20.9	—	30.6 ± 10.5	—	28.7 ± 10.5	30.4 ± 11.4	0.53	<0.0001	0.89
CPK, IU/L	126.9 ± 80.8	—	951.9 ± 1161.0	—	316.8 ± 251.7	—	157.7 ± 130.3	171.5 ± 195.6	135.2 ± 94.8	—	782.5 ± 666.1	—	200.4 ± 114.9	—	151.9 ± 120.2	149.5 ± 153.8	0.39	<0.0001	0.96
LDH, IU/L	121.2 ± 19.4	—	166.8 ± 47.2	—	147.1 ± 34.0	—	129.6 ± 25.3	127.9 ± 19.9	118.5 ± 14.0	—	157.7 ± 39.4	—	146.7 ± 24.5	—	136.6 ± 23.5	124.7 ± 22.6	0.52	<0.0001	0.44
CRP, mg/L	1.13 ± 0.9	3.38 ± 2.2*	7.83 ± 5.0†*	4.78 ± 3.5†	3.40 ± 2.2	1.94 ± 1.4	1.44 ± 1.0	1.27 ± 0.9	1.11 ± 0.7	2.18 ± 1.3	5.23 ± 3.4†	4.67 ± 3.6†	4.35 ± 3.3†	2.95 ± 2.0	2.10 ± 1.4	1.46 ± 1.1	0.06	<0.0001	<0.01
IL-6, pg/mL	2.85 ± 1.6	11.2 ± 6.5	3.6 ± 2.4	4.35 ± 4.1	5.15 ± 7.2	8.03 ± 7.8	1.57 ± 0.3	2.46 ± 2.2	2.29 ± 1.2	11.1 ± 5.3	3.70 ± 1.3	4.86 ± 6.4	5.70 ± 8.6	7.70 ± 6.8	5.96 ± 5.7	1.15 ± 1.4	0.76	<0.0001	0.77
Hepcidin, ng/mL	8.40 ± 2.2	11.91 ± 4.7	14.95 ± 10.1†*	13.73 ± 8.9†*	4.61 ± 1.8	3.95 ± 0.6	3.84 ± 0.8	5.52 ± 3.0	8.42 ± 2.6	11.08 ± 5.1	7.20 ± 3.4	9.47 ± 4.7	6.11 ± 3.7	4.79 ± 1.9	3.81 ± 1.3	4.80 ± 1.8	<0.0001	<0.0001	0.001
Hemoglobin, g/dL	15.7 ± 0.7	14.7 ± 1.1†	15.2 ± 1.0*	13.3 ± 1.0†	14.2 ± 0.8†	14.5 ± 0.9†	14.6 ± 1.4†*	15.3 ± 0.6*	16.1 ± 0.7	14.7 ± 0.8†	14.4 ± 0.8†	12.9 ± 1.0†	14.7 ± 0.9†	15.4 ± 0.9	15.2 ± 1.4	15.4 ± 0.7	0.10	<0.0001	<0.0001
Ferritin, ng/mL	77.3 ± 34.4	87.2 ± 35.1	103.2 ± 41.9†*	98.5 ± 38.0*	87.7 ± 27.9	73.6 ± 25.0	64.2 ± 20.7	65.0 ± 33.8	79.9 ± 33.7	77.0 ± 31.4	75.9 ± 31.6	74.1 ± 31.9	82.0 ± 33.6	79.8 ± 32.7	66.9 ± 29.7	57.5 ± 23.9	<0.0001	<0.0001	0.01
Serum iron, μg/dL	115.2 ± 35.8	101.1 ± 17.7*	125.5 ± 35.9*	128.5 ± 20.4	53.0 ± 11.4†	55.4 ± 10.5†	65.2 ± 24.3†	64.3 ± 21.6†*	101.7 ± 35.5	58.4 ± 15.8†	88.0 ± 14.8	130.9 ± 34.4	58.7 ± 16.5†	67.7 ± 25.0†	57.3 ± 13.7†	108.6 ± 38.1	0.11	<0.0001	<0.0001
Transferrin saturation, %	35.8 ± 11.5	31.7 ± 4.9*	40.6 ± 10.3*	46.0 ± 5.2	17.5 ± 3.4†	18.1 ± 4.2†	21.9 ± 10.0†	19.2 ± 7.1†*	31.9 ± 12.0	19.1 ± 5.4†	30.8 ± 6.9	47.2 ± 14.8†	19.0 ± 6.7	22.2 ± 10.0	18.6 ± 4.8†	32.6 ± 11.7	0.22	<0.0001	<0.0001
sTfR, nmol/L	20.7 ± 3.9	19.4 ± 4.0	18.9 ± 3.3	17.6 ± 2.5†	18.4 ± 3.9†*	19.8 ± 3.8	20.3 ± 3.8	24.0 ± 4.5†*	21.4 ± 3.7	19.0 ± 3.4†	18.1 ± 4.0†	17.0 ± 2.5†	19.7 ± 4.2	20.1 ± 3.6	20.7 ± 3.6	22.0 ± 3.4	0.29	<0.0001	0.05
ERFE, ng/mL	0.91 ± 1.5	0.90 ± 1.4	1.06 ± 1.4	1.10 ± 1.2	1.49 ± 1.3	1.04 ± 1.3	1.01 ± 1.5	0.90 ± 1.3	0.88 ± 1.5	0.98 ± 1.3	1.06 ± 1.4	1.06 ± 1.2	1.39 ± 1.5	1.04 ± 1.4	0.93 ± 1.4	1.05 ± 1.4	0.84	0.03	1.00

[1] Values are means ± SDs; $n = 10$. Effects of trial, time, and trial-by-time interactions were analyzed using linear mixed models. To test for carryover effects, a main effect of trial order and order-by-trial interaction was included in the model; no effects were observed, and these data are not shown. Bonferroni corrections were used for post hoc comparisons. †Different from day 1 SUSOPS within a trial ($P < 0.05$). *Trial difference within a timepoint ($P < 0.05$). BAL, energy balance; CPK, creatine phosphokinase; CRP, c-reactive protein; DEF, energy deficit; EPO, erythropoietin; ERFE, erythroferrone; FFA, free fatty acids; LDH, lactate dehydrogenase; sTfR, soluble transferrin receptor; SUSOPS, sustained military operations.

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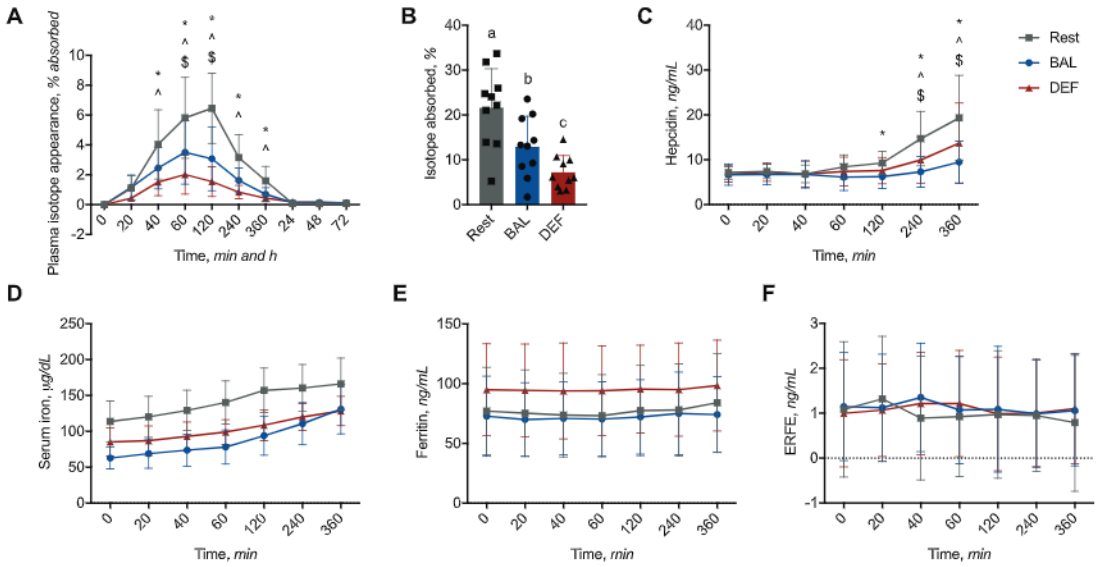


FIGURE 3 Time course of plasma iron isotope appearance, hepcidin, erythroferrone, and iron status indicators following consumption of a beverage containing an oral iron isotope. Time course of plasma iron isotope appearance (A). Data represent the percentage of isotope absorbed at a given time point of the total fraction that was absorbed, P-trial < 0.0001, P-time < 0.0001, P-interaction < 0.0001. Total amount of isotope absorbed (B). Time course of hepcidin (C), P-trial < 0.0001, P-time < 0.0001, P-interaction < 0.0001; serum iron (D), P-trial < 0.0001, P-time < 0.0001, P-interaction $= 0.17$; ferritin (E), P-trial < 0.0001, P-time $= 0.27$, P-interaction $= 1.00$; and ERFE (F), P-trial < 0.0001, P-time $= 0.02$, P-interaction $= 0.15$, after consuming an oral iron isotope following rest, SUSOPS BAL, and SUSOPS DEF. Values are means ± SD; $n = 10$. Effects of trial, time, and trial-by-time interactions were analyzed using linear mixed models. To test for carryover effects, a main effect of trial order and order-by-trial interaction was included in the model; no effects were observed, and these data are not shown. Bonferroni corrections were used for post hoc comparisons. *Rest different from BAL, $P < 0.05$; ^ rest different from DEF, $P < 0.05$; $BAL different from DEF, $P < 0.05$. Different letters indicate significant difference. BAL, energy balance; DEF, energy deficit; ERFE, erythroferrone.

Raw data for ERFE are shown but statistics were performed on log-transformed data. ERFE was lower following rest compared with SUSOPS DEF (−0.09 ± 0.63 ng/mL; P-adjusted < 0.0001) and SUSOPS BAL (−0.13 ± 0.56 ng/mL; P-adjusted < 0.0001).

Discussion

Those who frequently engage in strenuous physical activity are vulnerable to declines in iron status (5). Declines in iron status are associated with reduced physical performance and cognitive impairments (22), highlighting the importance of understanding the mechanisms by which iron status declines with physical activity. The major finding of this study was that 72 h of strenuous physical activity decreased dietary iron absorption compared with rest in nonanemic individuals with sufficient iron status. Moreover, findings indicate that energy deficit during strenuous physical activity increased hepcidin and diminished iron absorption compared with energy balance.

Few studies have directly examined the effects of physical activity on iron absorption using radioactive or stable iron isotopes. The first such study was conducted prior to the discovery of hepcidin and compared iron absorption in 8 male elite distance runners to 8 male blood donors with depleted iron stores (23). Using radiolabeled iron ($^{59}FeSO_4$) and measuring its incorporation into red blood cells, the authors found ∼50% (although statistically nonsignificant) reduction in iron absorption in runners compared with blood donors (runners 16 ± 13%, blood donors 30 ± 33%). A reduction in iron absorption is consistent with findings from the current study. However, recently, Moretti et al. conducted a cross-over intervention during which 10 iron-sufficient recreational male runners completed a 16-d control phase of no running followed by a training phase during which participants ran 8 km on alternate days for 22 d (24). On day 1 of the control phase and day 5 of the training phase participants were given a test beverage containing $^{57}FeSO_4$ to determine oral iron absorption and intravenous ^{58}Fe citrate to determine erythroid iron utilization. Whole blood was collected 16 days later to determine isotope incorporation into red blood cells. Despite an increase in IL-6, plasma hepcidin decreased and oral iron absorption increased with training compared with rest. The authors attribute these findings to the increased erythropoietic demand of exercise, which is consistent with previous studies that have demonstrated that erythropoiesis strongly suppresses hepcidin (25). In the current study, hepcidin increased with energy deficit during physical activity, but not energy balance, despite similar increases in IL-6 and EPO. The apparent discrepancy between findings from the current study and the study described above may be due to the length of the training program. Given the short duration of physical activity in the current study, it is unlikely that the observed increase in EPO produced a meaningful increase in erythropoiesis (26). Future studies should consider the interaction between erythropoiesis and gluconeogenic stimuli on the hepcidin response and its contribution to dietary iron absorption and iron status during short- and longer-term physical activity.

The increase in hepcidin in the current study is likely due to energy deficit, and not sleep deprivation, as a time-dependent increase in hepcidin has been observed in humans following

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an 18-, 42-, and 66-h fast, but not prolonged (50 h) sleep deprivation (27). The increase in hepcidin with energy deficit suggests that the PPARGC1A/CREBH pathway of hepcidin activation may contribute to the observed increase in hepcidin with physical activity. In this model, PPARGC1A and CREBH would stabilize CREBH binding and transactivate the hepcidin (*HAMP*) promoter in response to gluconeogenic stimuli during energy deficit (28, 13). CREBH, encoded by *CREB3L3*, is not only upregulated in response to gluconeogenic stimuli in energy-depleted conditions, but also by the unfolded protein response during endoplasmic reticulum stress and the acute-phase inflammatory response (28, 13). While most markers of inflammation and muscle damage increased with physical activity regardless of energy status, the slightly higher concentrations of CRP with energy deficit suggest a greater inflammatory response compared with energy balance. Likewise, the increase in ferritin with physical activity during energy deficit (and following ingestion of the oral iron isotope), but not energy balance, likely indicate a heightened acute phase response and not an improvement in iron stores, as changes in ferritin tend to reflect changes in inflammatory status and CRP. Thus, it is possible that the acute-phase response and gluconeogenic stimuli may cooperate to upregulate hepcidin during physical activity, particularly when energy stores are depleted. These findings do not necessarily rule out effects of other mediators, such as the mammalian target of rapamycin (29), on the hepcidin response to exercise and cellular iron metabolism.

Interestingly, although there was an increase in hepcidin with energy deficit during physical activity, hepcidin concentrations were not different when the oral iron isotope was ingested and remained the same 2 h postingestion. It is also noteworthy that the decrease in iron absorption during energy balance occurred despite no change in hepcidin with physical activity. This may be due, in part, to the timing of the blood draws and the transient nature of the IL-6 and hepcidin response. IL-6 and hepcidin both have short half-lives of several minutes (30, 31). Muscle-derived IL-6 peaks immediately following exercise and rapidly declines to baseline concentrations (17). Immune cells produce a more sustained increase in IL-6 to repair tissue damage, but the response is lower in magnitude than the initial peak in IL-6 from muscle. Alternatively, Zimmermann et al. reported that hepcidin is a modest predictor of dietary iron absorption, suggesting that other factors may influence iron absorption (19). Whether the decline in iron absorption during energy balance is dependent on hepcidin warrants further study.

Previous studies demonstrate that circulating concentrations of hepcidin are lowest in the early morning and increase throughout the day (27). Thus, it is likely that the increase in hepcidin following rest reflects diurnal variation (32, 33) and not an increase in response to ingestion of 3.8 mg iron. This would be consistent with studies demonstrating that hepcidin increases following ingestion of larger doses of iron (e.g., $>$ 40 mg iron) (6, 19, 34). The increased amplitude of the hepcidin response to diurnal variation following rest likely reflects that the participants had a relatively low iron requirement. In contrast, the suppressed amplitude of the hepcidin response following physical activity perhaps indicates an increased requirement for iron. Although the current study was not designed to produce robust changes in iron status, in general, iron status tended to decline with physical activity in both conditions. This is reflected by a decrease in transferrin saturation during recovery from physical activity and lower serum iron concentrations following ingestion of the oral iron isotope.

The strengths of this study include the cross-over design, the use of stable isotopes to determine dietary iron absorption, and the tightly controlled nature of all study conditions, including diet and exercise. This study has several limitations. First, the study included only 10 participants. Although the study employed a cross-over design, findings should be replicated with a larger sample size. Secondly, the numerous study outcomes increase the risk of making a type I error. Lastly, iron-sufficient male participants were chosen in the current study because low iron stores suppress the hepcidin response to physical activity, even in the presence of inflammation (35); however, future studies should include a more diverse study sample, including females and individuals with a range of iron stores.

To our knowledge, this is the first randomized controlled trial to demonstrate the relationship between physical activity–induced declines in iron absorption, the hepcidin response, and energy status. A major finding from the current study was that energy deficit during physical activity increased hepcidin concentrations and diminished iron absorption compared with energy balance, suggesting that interventions to maintain energy balance may be an effective strategy to prevent the decline in iron status with physical activity. These findings may be important for designing and implementing policies to prevent and treat iron deficiency in military personnel, endurance athletes, and potentially other populations that experience negative energy balance, such as in areas where malnutrition and infection are common.

Acknowledgments

We thank Andrew Young and Claire Berryman for reviewing early versions of the manuscript and the study team at the US Army Research Institute of Environmental Medicine, Natick, MA, USA (Jason Alaniz, Nicholes Armstrong, Nicholas Barringer, Joseph Bistolfi, Karleigh Bradbury, Kristine Chiusano, Lauren Dare, Heather Fagnant, Emily Howard, Julianna Jayne, Anthony Karis, Adam Luippold, Stephen Mason, Susan McGraw, Cornal Pounds, Patrick Radcliffe, Marcus Sanchez, Katakyie Sarpong, Tracey Smith, Alyssa Varanoske, Claire Whitney, and Beau Yurkevicius) for their technical support throughout the study.

The authors' responsibilities were as follows—SRH, JPM, JPK, LMM, SMP: conceived the study; all authors: conducted the study; SRH: wrote the manuscript with input from all authors; SRH: had primary responsibility for final content; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

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British Journal of Nutrition, page 1 of 14 doi:10.1017/S0007114521000131
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Effects of energy balance on appetite and physiological mediators of appetite during strenuous physical activity: secondary analysis of a randomised crossover trial

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(Submitted 20 September 2020 – Final revision received 4 December 2020 – Accepted 7 January 2021)

Abstract

Energy deficit is common during prolonged periods of strenuous physical activity and limited sleep, but the extent to which appetite suppression contributes is unclear. The aim of this randomised crossover study was to determine the effects of energy balance on appetite and physiological mediators of appetite during a 72-h period of high physical activity energy expenditure (about 9·6 MJ/d (2300 kcal/d)) and limited sleep designed to simulate military operations (SUSOPS). Ten men consumed an energy-balanced diet while sedentary for 1 d (REST) followed by energy-balanced (BAL) and energy-deficient (DEF) controlled diets during SUSOPS. Appetite ratings, gastric emptying time (GET) and appetite-mediating hormone concentrations were measured. Energy balance was positive during BAL (18 (SD 20) %) and negative during DEF (–43 (SD 9) %). Relative to REST, hunger, desire to eat and prospective consumption ratings were all higher during DEF (26 (SD 40) %, 56 (SD 71) %, 28 (SD 34) %, respectively) and lower during BAL (–55 (SD 25) %, −52 (SD 27) %, −54 (SD 21) %, respectively; $P_{condition} < 0{\cdot}05$). Fullness ratings did not differ from REST during DEF, but were 65 (SD 61) % higher during BAL ($P_{condition} < 0{\cdot}05$). Regression analyses predicted hunger and prospective consumption would be reduced and fullness increased if energy balance was maintained during SUSOPS, and energy deficits of ≥25 % would be required to elicit increases in appetite. Between-condition differences in GET and appetite-mediating hormones identified slowed gastric emptying, increased anorexigenic hormone concentrations and decreased fasting acylated ghrelin concentrations as potential mechanisms of appetite suppression. Findings suggest that physiological responses that suppress appetite may deter energy balance from being achieved during prolonged periods of strenuous activity and limited sleep.

Key words: **Eating behaviour: Military: Hunger: Satiety: Exercise: Sleep deprivation**

Energy deficit is common during prolonged periods of strenuous physical activity and limited sleep such as those experienced during military operations, disaster relief and ultra-endurance sporting events[1–5]. For example, military training exercises, known as sustained operations, and wildland firefighting are often characterised by multiple days of prolonged low-to-moderate intensity exercise and limited sleep (<4 h/night) resulting in total daily energy expenditures (TDEE) ranging from 16·7 to 29·3 MJ/d (4000 to 7000 kcal/d)[4,6–9]. In military studies, measured energy intakes rarely exceed 14·6 MJ/d (3500 kcal/d), 25–40 % of foods provided are often uneaten and energy deficits of about 40–60 % TDEE resulting in body mass losses of about 2–4 % are common[10–18]. Insufficient time, stress, environmental factors, food preferences and unwillingness or inability to carry enough food have all been cited as contributing factors[19]. Appetite suppression and the large volume of food required

Abbreviations: BAL, energy balance condition; DEF, energy deficit condition; GET, gastric emptying time; GLP, glucagon-like peptide; PAEE, physical activity energy expenditure; PP, pancreatic polypeptide; PYY, peptide-YY; REST, pre-SUSOPS sedentary condition; SUSOPS, simulated military operations; TDEE, total daily energy expenditure.

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to maintain energy balance may also play a role, but have received somewhat less attention[20,21]. Similarly, energy intakes during ultra-endurance sporting events appear to plateau at about 2·5 times BMR irrespective of TDEE resulting in energy deficits proportional to TDEE[1,2]. Thus, there appears to be an upper limit on energy intake during sustained periods of high physical activity which may impact the magnitude of energy deficit. Interest in identifying factors that influence this limit is driven by the fact that energy deficits, especially if prolonged, are catabolic and may compromise occupational and athletic performance[1,3,5,19,22].

Although the factors influencing energy intake during events requiring prolonged strenuous physical activity are multifactorial, physiological responses that suppress appetite likely contribute. For example, short-term (<14 d) increases in exercise generally do not elicit compensatory increases in energy intake sufficient to match the increase in physical activity energy expenditure (PAEE)[23,24]. That uncoupling of energy intake and expenditure has been shown to coincide with changes in 'episodic' (i.e. meal-to-meal[25]) mediators of appetite to include altered gastric emptying rate[26], increased circulating concentrations of the anorexigenic (i.e. appetite-suppressing) hormones peptide-YY (PYY), glucagon-like peptide (GLP)-1 and pancreatic polypeptide (PP), and decreased concentrations of the orexigenic (i.e. appetite-stimulating) hormone ghrelin[27]. However, studies reporting appetite suppression in response to increases in exercise have generally investigated exercise levels that elicit PAEE considerably less than those typical of military operations, wildland firefighting and ultra-endurance sporting events. Physically active individuals also appear to be more likely than those who are less active to adjust energy intake to match high energy expenditures[28]. Further, both body weight loss and sleep restriction stimulate appetite[29,30]. In some studies, increases in appetite with sleep restriction and weight loss have been observed in association with increases in ghrelin and decreases in PYY and GLP-1[31,32]. Many of the same studies also report reductions in insulin and leptin, both of which are considered 'adiposity' hormones or 'tonic' signals[25], that are secreted in proportion to body fat stores and act to defend body weight homoeostasis[33]. The collective effects of prolonged strenuous physical activity, weight loss and limited sleep on appetite and physiological mediators of appetite are therefore unclear. An improved understanding of those effects could help inform feeding strategies for mitigating energy deficits during events in which high energy expenditures are sustained over multiple days.

This study determined the effects of differences in energy balance on appetite, appetite-mediating hormones and gastric emptying during 72-h periods of prolonged low-to-moderate intensity physical activity and limited sleep. These measures were included as secondary outcomes in a randomised, crossover trial designed to determine the effects of energy balance on inflammation and Fe absorption during sustained military operations[34]. The study design included providing controlled energy-deficient and energy-balanced diets during otherwise identical 72-h testing periods. We hypothesised that appetite would be increased during energy deficit, but decline during energy balance, and those responses would correspond with changes in gastric emptying and appetite-mediating hormone concentrations that would collectively suggest a physiologically mediated appetite suppression under conditions of prolonged strenuous physical activity and limited sleep.

Methods

Study population

Generally healthy, recreationally active (2–4 d/week aerobic and/or resistance exercise), active duty male soldier volunteers, aged 18–39 years, with self-reported weight stability (±2·2 kg) for ≥2 months were recruited to participate in April and September 2019. Females were not recruited due to known sex differences in Fe metabolism and inflammation in response to exercise (primary study outcomes[34]). Additional exclusion criteria included unwillingness to eat the study diets, presence of any injuries or other conditions limiting exercise capability, cardiometabolic disease, gastrointestinal disease and anaemia. All participants were asked to abstain from alcohol, nicotine, caffeine and dietary supplement use throughout study participation and reported adhering to these instructions.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were reviewed and approved by the US Army Medical Research and Development Command Institutional Review Board (approval number: M-10731). Investigators adhered to the policies regarding the protection of human research participants as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. All participants provided written informed consent prior to participation. The trial was registered on www.clinicaltrials.gov as NCT03524690.

Study design

Full details of the study design are reported elsewhere[34]. Details relevant to the outcomes in this report are described below.

The trial used a randomised, crossover design consisting of a baseline period, and two 72-h periods of prolonged low-to-moderate intensity physical activity and limited sleep designed to simulate sustained military operations (SUSOPS). Each SUSOPS period was followed by a 7-d recovery period. Daily schedules were identical during both SUSOPS periods, sleep was restricted to 4 h/night (01.00–05.00 hours) and physical activity was prescribed to increase TDEE to a target of 20·9–25·1 MJ/d (5000–6000 kcal/d) in accord with recent observations during military field training exercises[10,11]. Daily physical activity during SUSOPS was divided into separate sessions that included walking with a weighted vest and backpack (mean weight: 33·5 (SD 0·2) kg) in the morning (64 (SD 6) min; 8·3 (SD 0·6) metabolic equivalents) and evening (122 (SD 7) min; 8·4 (SD 0·5) metabolic equivalents), a battery of military-relevant tasks in the late morning, and unweighted walking/jogging in the afternoon (94 (SD 46) min; 5·4 (SD 1·5) metabolic equivalents). The exact duration of activity was individualised to match the 20·9–25·1 MJ/d TDEE target. Energy expenditure predictions were calculated from measured RMR, the American College of

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British Journal of Nutrition

Sports Medicine's metabolic equations for steady-state exercise[35], and the compendium of metabolic equivalents for physical activities[36]. During SUSOPS, TDEE was measured using the doubly labelled water method as previously described[34].

The SUSOPS periods differed in the amount of food participants were provided. During one period, participants were provided enough food to maintain energy balance, defined as ±10 % of predicted TDEE during SUSOPS (BAL). During the other period, participants were kept in negative energy balance by providing only the amount of food estimated to meet 45 % of predicted TDEE during SUSOPS (DEF). Diets were designed, measured and provided by study dietitians, and the macronutrient distributions were matched between conditions (Table 1). Meal times were also matched between conditions, but participants could request snacks at any time. Participants could eat no more or less than the amount of food provided, and water was allowed *ad libitum*. Diets were composed mainly of the US Armed Services Meals, Ready-to-Eat ration, and also included other commercially available ready-to-eat products. Energy and macronutrient content was analysed using the Combat Rations Database, and Food Processor SQL (ESHA Research, version 10.14).

The order of DEF and BAL was randomised by the study coordinator using computer-generated randomisation. Participants resided in the laboratory throughout SUSOPS. During recovery periods, participants were free-living and ate *ad libitum*. Three days prior to the first SUSOPS period, volunteers spent 1 d (06.00–18.00 hours) in the laboratory for baseline appetite testing (REST) (see section Appetite and food preferences). Participants remained sedentary throughout the day and consumed a provided and measured diet containing an amount of energy predicted to maintain energy balance. This energy intake was calculated as the product of measured RMR and an activity factor of 1·3 to account for energy expended during activities of daily living. The macronutrient distributions of the diets provided during REST and both SUSOPS periods were matched (Table 1).

Table 1. Energy expenditure, energy balance and dietary intake over the 72-h sustained operations (SUSOPS) period
(Mean values and standard deviations)

			SUSOPS			
	REST		DEF		BAL	
	Mean	SD	Mean	SD	Mean	SD
Energy balance (MJ/d)†	–	–	–8·6	3·9	2·9*	3·6
Energy balance (%)†	–	–	–43	9	18*	20
TDEE (MJ/d)†	–	–	19·1	4·1	20·0	3·9
PAEE (MJ/d)†	–	–	9·2	3·4	10·0	3·1
Dietary intake‡						
Energy (MJ/d)	10·6	1·4	10·5	0·7	22·7§	1·6
Carbohydrate (g/d)	387	49	382	26	833§	51
Protein (g/d)	68	8	71	3	139§	12
Fat (g/d)	87	11	84	7	183§	15
Carbohydrate (%)	59	1	59	1	60	1
Protein (%)	11	0·5	11	0·8	10	0·2
Fat (%)	31	1	30	1	30	1

REST, pre-SUSOPS sedentary condition; DEF, energy deficit condition; BAL, energy balance condition; TDEE, total daily energy expenditure measured by doubly labelled water; PAEE, physical activity energy expenditure.
* Different from DEF, $P < 0{\cdot}001$.
† Energy expenditure measured during SUSOPS only. Between-condition comparisons analysed by paired-samples *t* test (n 9, one volunteer served as study control).
‡ Analysed by general linear model with correlated errors (n 10).
§ Different from REST and DEF, $P < 0{\cdot}001$.

Appetite and food preferences

During SUSOPS, self-reported appetite was measured daily before and after meals, and before and after the morning exercise session. During REST, measurements were collected before and after meals, and at the same times the morning exercise session was scheduled to begin and end during SUSOPS. Appetite measures relied on 10 cm pen and paper visual analogue scales which asked volunteers to rate their level of hunger, fullness, desire to eat and the amount of food that they thought they could eat (prospective consumption) at that moment[37]. All visual analogue scale responses were measured by one researcher. Random checks on those measurements were performed by a separate study team member, and all data were scrutinised for data entry errors prior to analysis.

Food preferences were measured by administering the Leeds Food Preference Questionnaire before and after lunch during REST and the final day of each SUSOPS period. The Leeds Food Preference Questionnaire is a computerised platform that uses pictures of individual food items selected from a validated database to measure three constructs encompassing different components of food preferences and hedonics: explicit liking (i.e. perceived hedonic impact of the food), explicit wanting (i.e. conscious desire to consume a food) and implicit wanting (i.e. subconscious motivation for a food)[38]. For this test, pictures of sixteen different foods were selected which varied in the dimensions of fat (high and low) and taste (sweet and savoury) (four pictures/category). Explicit liking was measured by a visual analogue scale that captured responses to the question 'how pleasant would you find the taste of this food right now.' Explicit wanting was measured by a visual analogue scale that captured responses to the question 'how much do you want to eat this food right now.' Implicit wanting was measured by showing participants two pictures and asking them to select the food they most wanted to eat at that moment. Both frequencies of selections within each food category and response time (i.e. relative preference) were recorded. The preference for different food types was computed for each construct by subtracting the mean scores (visual analogue scale scores or frequency of selection and reaction time) from a comparator group (low fat and savoury) relative to the matched reference group (high fat and sweet, respectively).

Gastric emptying time

Gastric emptying time (GET) was measured using the SmartPill™ wireless motility testing system (Covidien LLC). The system is FDA approved, recommended by American and European Neurogastroenterology and Motility Societies for evaluating suspected gastroparesis[39], and has shown strong correlations with gastric emptying scintigraphy[40] and manometry[41]. The system includes a SmartPill™ capsule that is ingested and transits the digestive tract transmitting pH (range 0·5–9·0, accuracy ± 0·5 units), temperature and pressure readings every 20–40 s to a data receiver worn by each participant.

British Journal of Nutrition

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For the test, a single SmartPill™ was ingested immediately following breakfast during REST and on the second morning of each SUSOPS period. After capsule ingestion, participants followed the same activity and meal schedule as on other SUSOPS days with no restrictions on snacking or water intake. As such, all participants participated in the morning exercise session within about 2 h and consumed another meal within about 5 h of ingesting the capsule. This procedure differed from standard clinical protocols used to assess gastroparesis wherein participants ingest the SmartPill™ with a standardised meal and then fast for 6 h[42]. However, we chose to deviate from standard protocol to assess physiological responses that may occur within the free living environments the study was designed to emulate.

After each capsule was passed, data from the receiver were downloaded and analysed using MotiliGI™ software version 3.1 (Given Imaging). Gastric emptying time was defined as the time from pill ingestion until an abrupt increase in pH of ≥3 units from a baseline gastric pH of <4 was observed[42], corresponding with transit from the acidic environment of the stomach to the more alkaline environment of the duodenum. As the SmartPill™ is not digestible, the pill is thought to exit the stomach after complete expulsion of the digestible components consumed during the initial test meal[41]. Therefore, the method is interpreted as an indirect measurement of gastric emptying, and GET can be prolonged if additional eating events occur before complete gastric emptying of the initial test meal. Normative values for GET measured by the SmartPill™ in healthy men according to standard protocol are 180–210 min with the 5th and 95th percentiles spanning 90–324 min[43,44].

Blood biochemistries

Circulating concentrations of insulin, leptin, acylated ghrelin, $PYY_{3–36}$, active GLP-1 and PP were measured in fasted blood samples collected during the first and final morning of each SUSOPS period (days 1 and 3), and on the second and fourth mornings of the 7-d recovery periods. Non-fasting blood samples of the same hormones, except leptin, were collected immediately after the morning exercise session (08.30–09.30 hours) on the first and final morning of each SUSOPS period (2·5–3 h after starting breakfast). All samples were collected by venepuncture, processed on site, and frozen as serum or plasma at −80°C until analysis.

For plasma acylated ghrelin measurements, blood was collected into chilled Monovettes containing EDTA-K_3 and 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (20 µl/ml whole blood). For plasma PYY measurements, blood was collected into chilled Monovettes containing EDTA-K_3 and protease inhibitor cocktail (40 µl/ml whole blood; complete, EDTA-free) and DPPIV-inhibitor (10 µl/ml whole blood). For plasma GLP-1 measurements, blood was collected into chilled Monovettes containing EDTA-K_3 and protease and DPPIV-inhibitor (10 µl/ml whole blood). Plasma aliquots for acylated ghrelin measurements were acidified with 50 µl 1 M HCl/ml plasma prior to freezing. Serum leptin was determined by RIA (EMD Millipore), serum insulin by automated immunoassay (Siemens Immulite 2000), serum PP by ELISA (EMD Millipore), plasma acylated ghrelin by RIA (EMD Millipore), plasma $PYY_{3–36}$ by RIA (EMD Millipore) and plasma active GLP-1 ($GLP\text{-}1_{7–36}$ amide and $GLP\text{-}1_{7–37}$) by ELISA (EMD Millipore). Intra-assay CV were 4·0 % for insulin, 5·0 % for leptin, 7·4 % for ghrelin, 8·7 % for PYY, 4·5 % for GLP-1 and 4·3 % for PP.

Anthropometrics and RMR

Height was measured in duplicate prior to the first SUSOPS period using a stadiometer. Nude, fasted body weights were measured each morning following first void during both SUSOPS and the recovery periods using a calibrated digital scale.

RMR was measured the week prior to the first SUSOPS period using indirect calorimetry (True Max 2400, ParvoMedics). Volunteers were instructed to fast for ≥8 h prior to testing and rested in a supine position for ≥30 min prior to starting the measurement. Twenty minutes of data were collected, and the final 10 min were averaged to determine RMR.

Statistical analysis

Sample size calculations used data from a laboratory study in which the same appetite-mediating hormones measured herein and appetite were assessed during severe short-term energy deficit[20,45]. Nine participants were estimated to be sufficient for detecting 1·4-fold between-condition differences in fasting hunger ratings and 1·7–3-fold between-condition differences in circulating concentrations of all appetite-mediating hormones except PP (for which n 14 would be needed) at $\alpha = 0{\cdot}05$ and power = 0·80. Sample size calculations for GET were based on studies reporting large effect sizes of physical activity[26] and fasting[46] on gastric emptying. It was estimated that eight participants would provide sufficient power to detect a large effect size (Cohen's $d = 1{\cdot}0$) for between-condition differences in GET at $\alpha = 0{\cdot}05$ and power = 0·80. Thirteen participants were enrolled to ensure adequate power for primary study outcomes while accounting for expected attrition.

Energy expenditure and energy balance data were compared between conditions using paired t tests. All other variables were analysed using general linear models with correlated errors and a compound symmetry covariance structure. All models included condition (DEF, BAL and REST when applicable), condition order (DEF then BAL or BAL then DEF) and their interaction as fixed factors. For outcomes measured more than once per condition (i.e. body weight, appetite ratings, food preferences, hormone concentrations), models included study day and, when relevant, time of day, as fixed factors in addition to the corresponding three-way (condition × day × time) and/or two-way (condition × day, condition × time) interactions. Models excluded the REST condition if study day was a factor in the model, and models for hormones that were measured post-exercise combined the time and day factors into a single factor (time point). Additionally, to examine appetite ratings on all 3 d of DEF and BAL relative to REST, separate models were used to test differences on each day of SUSOPS (i.e. REST was compared with DEF and BAL days 1, 2 and 3 in separate models). When statistically significant main effects or interactions were observed, *post hoc* testing was conducted using paired t tests and Fisher's least significant difference to identify differences between conditions or over time.

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In exploratory analyses, general linear models with correlated errors were used to examine associations between energy balance (independent variable) and percentage differences in appetite ratings from REST during DEF and BAL (dependent variable). These models provided a regression equation ($y = ax + b$) for each appetite rating where x = energy balance (% or MJ/d), y = percentage difference in appetite ratings from REST, and b = the y-intercept and corresponding 95 % CI. Each equation was then solved for $y = 0$ to find the x-intercept, and CI for the x-intercept were calculated using the Taylor expansion series. This analysis provided y-intercepts that represented the predicted difference in each appetite rating relative to REST at energy balance (i.e. energy deficit = 0), and x-intercepts that represented the predicted magnitude of energy deficit required to elicit increases in hunger, desire to eat and prospective consumption and decreases in fullness (see dashed lines in Fig. 2, for example).

All data were examined quantitatively and graphically prior to analysis, residual plots were evaluated to assess adherence to model assumptions (i.e. normal distribution and homogeneity of variance) and logarithmic or square root transformations were used when necessary to meet model assumptions. Cohen's f^2 was calculated as a measure of effect size for general linear models used in primary analyses, and Cohen's d was calculated as a measure of effect size for between-condition differences at single time points. Carryover effects were not detected for any outcome. Statistical analyses were completed using SPSS version 24.0 (IBM). Tests were two-sided and considered statistically significant at $P \leq 0{\cdot}05$. Results are presented as mean values and standard deviations or mean differences and 95 % CI in the text unless otherwise noted.

Results

Of the thirteen male volunteers enrolled, two volunteers withdrew for personal reasons and one withdrew due to injury. The ten volunteers (22 (SD 5) years, BMI: 27·0 (SD 3·5) kg/m^2) who completed the study were included in this analysis. PAEE and TDEE measured during SUSOPS did not differ between DEF and BAL, but were less than planned due to some participants not being able to complete the prescribed exercise on all SUSOPS days (Table 1). As a result, mean energy balance was positive during BAL (Δbody weight = 0·6 kg (95 % CI 0·2, 1·2)). However, as planned, energy intake during DEF was 46 % of that measured during BAL, resulting in energy deficit and body weight loss (–1·2 kg (95 % CI –1·7, –0·7)).

Appetite and food preferences

Between-condition differences in appetite ratings were evident within the first few hours of testing (online Supplementary Fig. S1), and mean ratings differed between BAL and DEF on each day of SUSOPS (Fig. 1(a),(c),(e) and (g)) and online Supplementary Table S1; Cohen's $f^2 = 0{\cdot}89$–$1{\cdot}19$). Aside from hunger ($P_{\text{condition} \times \text{day}} = 0{\cdot}02$), mean daily appetite ratings did not differ over time within BAL or DEF ($P_{\text{condition} \times \text{day}} > 0{\cdot}05$) though effect sizes for between-group differences were largest on the final day of SUSOPS (online Supplementary Table S1). When averaged across the three SUSOPS days, mean hunger, desire to eat and prospective consumption ratings were all higher during DEF relative to REST (mean percentage difference (95 % CI); hunger: 26 % (95 % CI –3, 54); desire to eat: 56 % (95 % CI 6, 107); prospective consumption: 28 % (95 % CI 4, 53)) and lower during BAL relative to REST (hunger: –55 % (95 % CI –73, –38); desire to eat: –52 % (95 % CI –71, –32); prospective consumption: –54 % (95 % CI –68, –39)) (Fig. 1(c)–(h)). In contrast, mean fullness ratings did not differ from REST during DEF (12 % (95 % CI –36, 61)), but were 65 % (95 % CI 21, 109) higher than REST during BAL (Fig. 1(a) and (b)).

When comparing changes in appetite ratings during BAL and DEF relative to REST, the mean decreases in hunger (–12 % (SD 31) % *v.* –55 % (SD 25) %, $P = 0{\cdot}003$) and prospective consumption (–17 % (SD 22) % *v.* –54 % (SD 21) %, $P = 0{\cdot}001$), but not desire to eat (–25 % (SD 30) % *v.* –52 % (SD 27) %, $P = 0{\cdot}09$), and the mean increase in fullness (10 % (SD 48) % *v.* 65 % (SD 61) %, $P = 0{\cdot}05$) were less when DEF was compared with REST relative when REST was compared with BAL, thereby indicating a larger effect of BAL on appetite ratings. When linear associations between percentage energy balance during SUSOPS and percentage differences in appetite ratings from REST were examined (Fig. 2 and Table 2), y-intercepts were negative for hunger and prospective consumption, but not desire to eat, and demonstrated a tendency towards being positive for fullness, which was statistically significant after removing one outlier (Table 2). Thus, the regression analyses predicted a mean suppression of hunger and prospective consumption, and an increase in fullness had energy balance been maintained during SUSOPS. Conversely, the energy balance during SUSOPS at which no change in appetite would be predicted (i.e. x-intercept) was negative for fullness, hunger and prospective consumption, representing a threshold of energy deficit beyond which appetite would be expected to start increasing during SUSOPS (Table 2). For example, hunger was not predicted to increase from REST until an energy deficit of 24 % or 5·4 MJ/d (1295 kcal/d) was exceeded (Table 2 and Fig. 2(b)).

Food preferences were neutral (i.e. CI for high *v.* low fat and sweet *v.* savoury crossed zero) and generally did not differ across study conditions (Fig. 3 and online Supplementary Table S2; Cohen's $f^2 = 0{\cdot}02$–$0{\cdot}06$ for sweet *v.* savoury and 0·09–0·10 for high *v.* low fat). Exceptions were a greater perceived liking of high-fat relative to low-fat foods ($P_{\text{condition}} = 0{\cdot}04$; $P_{\text{DEFvBAL}} = 0{\cdot}01$; Cohen's $d = 0{\cdot}42$), and a tendency towards a greater conscious desire for high-fat relative to low-fat foods ($P_{\text{condition}} = 0{\cdot}07$; $P_{\text{DEFvBAL}} = 0{\cdot}02$; Cohen's $d = 0{\cdot}49$) during DEF compared with BAL.

Gastric emptying time

GET did not differ from REST (median (interquartile range): 182 min (182)) during DEF (222 min (250); Cohen's $d = 0{\cdot}16$), but was increased during BAL (612 min (713)) relative to both REST ($P = 0{\cdot}01$; Cohen's $d = 0{\cdot}96$) and DEF ($P = 0{\cdot}05$; Cohen's $d = 1{\cdot}00$) ($P_{\text{condition}} = 0{\cdot}03$, Cohen's $f^2 = 0{\cdot}20$; Fig. 4(a)). GET exceeded 5 h, which was the approximate time elapsed between pill ingestion and the lunch meal, and a generally accepted upper limit for normal GET[43], for five participants during one

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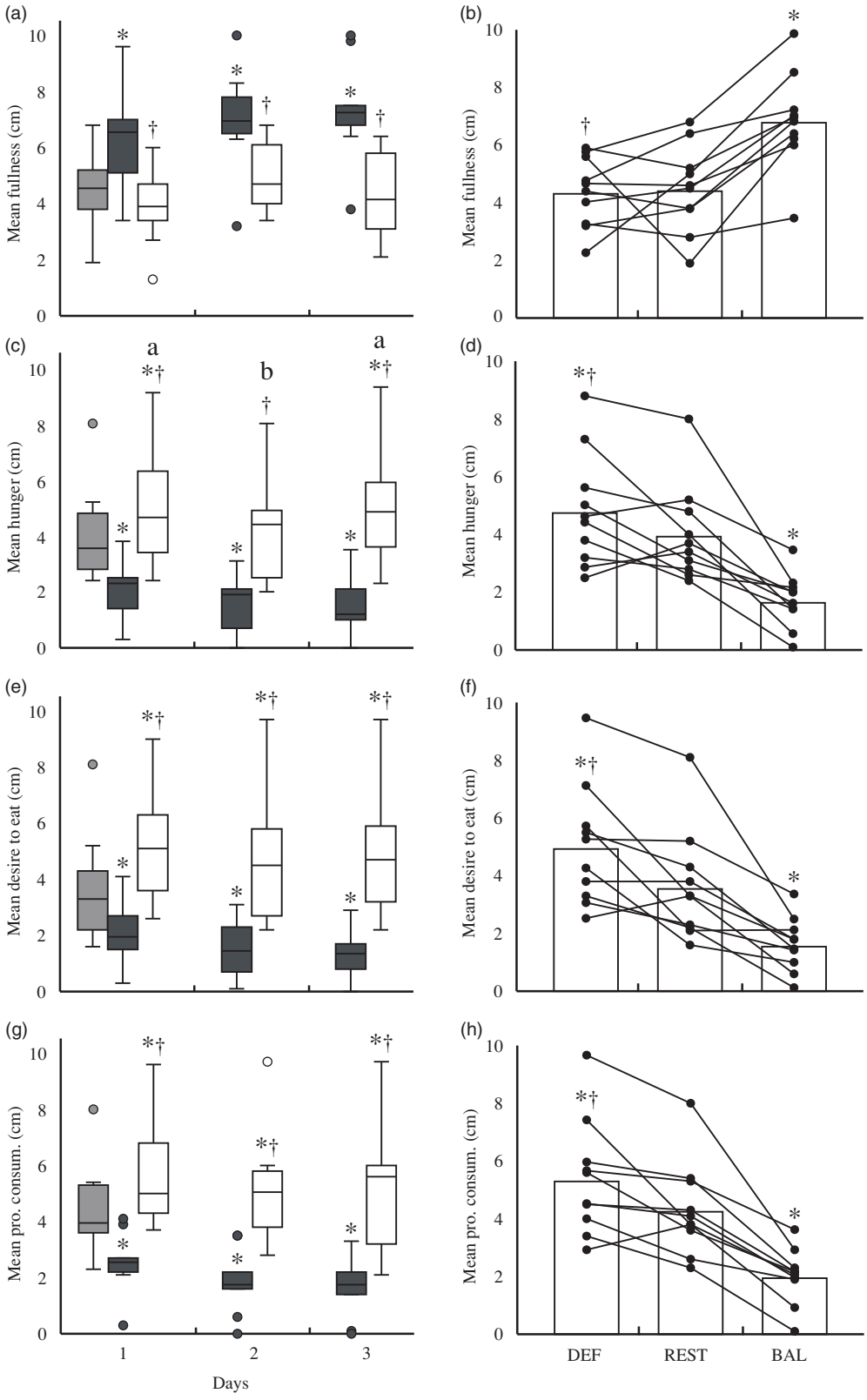


Fig. 1. Changes in mean appetite ratings during rest and during a 72-h simulated sustained military operation under conditions of energy balance (BAL) and energy deficit (DEF) (*n* 10). (a), (c), (e), (g) Boxes show median and interquartile range. Whiskers extend to 1·5 times the interquartile range or to minimum/maximum value if no values within that range. Differences between BAL and DEF analysed using general linear model with correlated errors, and condition, day, time and their interactions as fixed factors (model 1). Within a condition, boxes not sharing a letter are significantly different ($P < 0{\cdot}05$) ($P_{\text{condition x day}} < 0{\cdot}05$). Additionally, differences between BAL, DEF and REST analysed using separate general linear models with correlated errors for each study day, and with condition, time and their interaction included as fixed factors (model 2). Between-condition differences are denoted using symbols. (b), (d), (f), (h) Individual changes in mean appetite ratings. Bars represent the mean ratings over all 3 d of DEF and BAL, and over the full day of REST. Lines connect data collected from the same individual. Analysed using general linear model with correlated errors. (a)–(h) *,† Main effect of condition ($P < 0{\cdot}05$); * different from REST ($P < 0{\cdot}05$), † different from BAL ($P < 0{\cdot}05$). pro. consum., Prospective consumption; REST, baseline sedentary condition. ▒ REST; ■ BAL; □ DEF.

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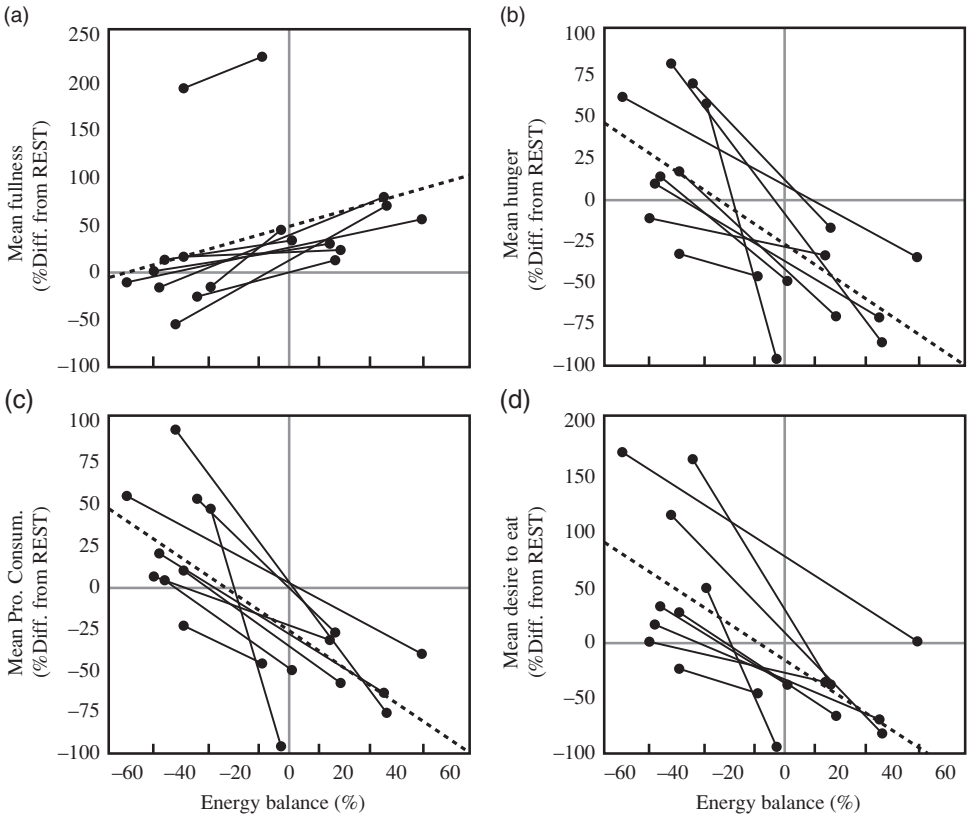


Fig. 2. Exploratory analysis of associations between mean changes in appetite ratings from REST (baseline sedentary condition) during a 72-h simulated sustained military operation relative to mean energy balance during the 72-h period (*n* 9). Solid lines connect data from the same individual. Dotted lines represent the best fit line calculated using general linear models with correlated errors (see Table 2). Diff., difference.

Table 2. Associations between energy balance during sustained operations (SUSOPS) and changes in appetite ratings*

	B	95 % CI	*P*	*Y*-intercept	95 % CI	*P*	*X*-intercept†	95 % CI
Energy balance (%)								
ΔFullness (%)‡	0·8	0·4, 1·2	0·001	48·6	−5·2, 102·4	0·07	−61 %	−117, 0
ΔHunger (%)	−1·1	−1·8, −0·4	0·004	−26·8	−49·0, −4·8	0·02	−24 %	−41, −7
ΔDesire to eat (%)	−1·6	−2·4, −0·8	0·001	−15·3	−55·1, 24·6	0·41	−10 %	−30, 11
ΔProspective consumption (%)	−1·1	−1·7, −0·5	0·003	−25·5	−45·2, −5·8	0·02	−23 %	−39, −8
Energy balance (MJ/d)								
ΔFullness (%)§	0·016	0·007, 0·026	0·004	49·2	−4·6, 103·0	0·07	−12·9 MJ/d	−25·2, 0·04
ΔHunger (%)	−0·021	−0·036, −0·006	0·01	−27·2	−48·4, −6·0	0·02	−5·4 MJ/d	−9·0, −1·7
ΔDesire to eat (%)	−0·033	−0·052, −0·015	0·003	−17·3	−53·7, 19·0	0·31	−2·2 MJ/d	−5·9, 1·6
ΔProspective consumption (%)	−0·020	−0·035, −0·006	0·01	−25·6	−44·4, −6·8	0·01	−5·4 MJ/d	−8·6, −1·9

* *n* 9. Associations determined using general linear model with correlated errors. Independent variable was energy balance (% or MJ/d). ΔDependent variables are percentage difference of mean ratings throughout energy deficit (DEF) and energy balance (BAL) conditions relative to mean ratings during sedentary condition (REST).
† CI for *x*-intercepts calculated using SD derived from Taylor expansion series.
‡ When one outlier removed: $\beta = 0{\cdot}8$ (0·4, 1·2), $P = 0{\cdot}003$; *y*-intercept = 25·1 (13·4, 36·8), $P = 0{\cdot}001$; *x*-intercept = −31 % (−48,−17).
§ When one outlier removed: $\beta = 0{\cdot}014$ (0·004, 0·024), $P = 0{\cdot}01$; *y*-intercept = 25·1 (11·9, 38·3), $P = 0{\cdot}002$; *x*-intercept = −7.5 MJ/d (−11.6, −3.2).

or more conditions (REST, *n* 1; DEF, *n* 2; BAL, *n* 5). In all of those cases, GET exceeded 10 h. Although excluding those participants from the analysis attenuated between-condition differences, GET during BAL (266 (SD 30) min) remained significantly higher than during REST (152 (SD 54) min, $P = 0{\cdot}01$; Cohen's $d = 2{\cdot}56$) and demonstrated a tendency to be higher relative to DEF (207 (SD 40) min, $P = 0{\cdot}06$; Cohen's $d = 1{\cdot}16$) ($P_{condition} = 0{\cdot}02$, Cohen's $f^2 = 0{\cdot}64$; Fig. 4(b)).

Appetite-mediating hormones

Condition-by-time point interactions were observed for all appetite-mediating hormones (Fig. 5 and online Supplementary Table S3). Fasting leptin concentrations decreased during both BAL and DEF, but to a greater extent during DEF, resulting in lower concentrations on the final day of SUSOPS during DEF relative to BAL ($P = 0{\cdot}002$; Cohen's $d = 0{\cdot}63$). Fasting acylated ghrelin concentrations also decreased during DEF and were lower on

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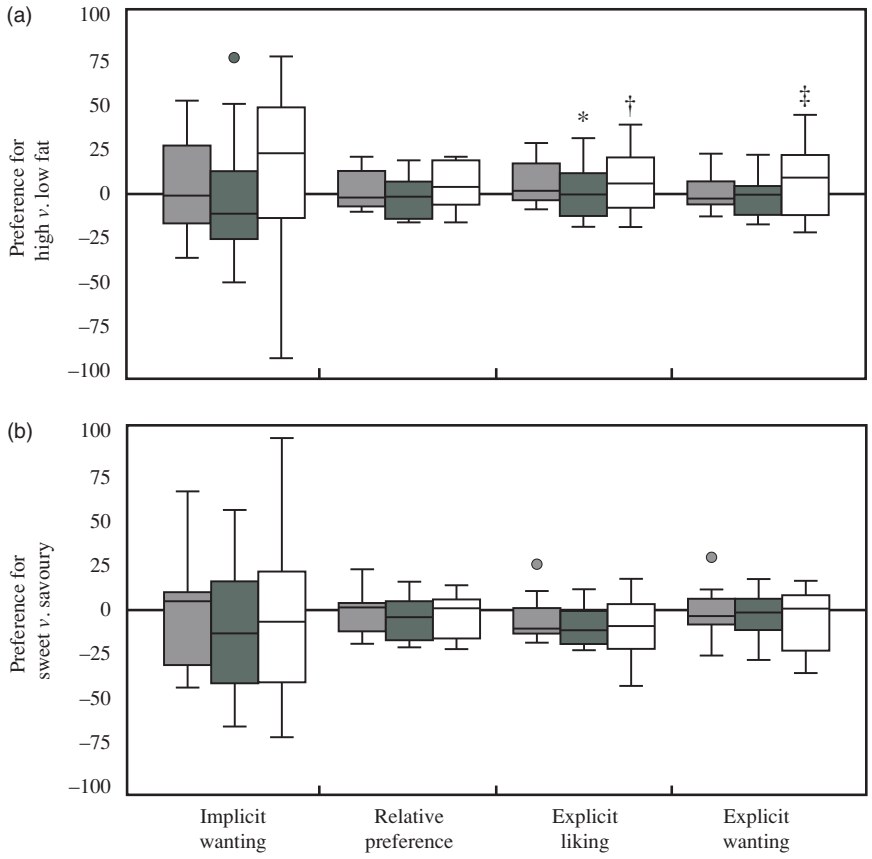


Fig. 3. Food preferences measured during rest and during a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF) (n 7). Preference for (a) high *v.* low fat and (b) sweet *v.* savoury foods measured by the Leeds Food Preference Questionnaire. Positive values indicate preference for high fat or sweet, negative values indicate preference for low fat or savoury. Boxes show median and interquartile range. Whiskers extend to 1·5 times the interquartile range or to minimum/maximum value if no values within that range. Each domain analysed separately using general linear model with correlated errors. For all models, data for n 3 were excluded for failure to adhere to test instructions. †,* Main effect of condition ($P = 0{\cdot}04$); † different from BAL ($P = 0{\cdot}01$), * difference from REST ($P = 0{\cdot}06$). ‡ Main effect of condition ($P = 0{\cdot}07$); different from BAL ($P = 0{\cdot}02$). REST, pre-SUSOPS sedentary condition. ▒ REST; ■ BAL; □ DEF.

the final day of SUSOPS during DEF relative to BAL ($P = 0{\cdot}03$; Cohen's $d = 0{\cdot}38$). In contrast, fasting insulin, PYY, GLP-1 and PP concentrations did not demonstrate any between-condition differences.

Mean concentrations of all hormones measured in the non-fasting state after exercise differed between DEF and BAL on both the first and final days of SUSOPS (Fig. 5 and online Supplementary Table S3). Acylated ghrelin concentrations were higher during DEF relative to BAL on both days (Cohen's $d = 1{\cdot}49$ (day 1), 1·25 (day 3); $P \leq 0{\cdot}001$). In contrast, PYY (Cohen's $d = 0{\cdot}90$ (day 1), 1·10 (day 3); $P \leq 0{\cdot}02$), GLP-1 (Cohen's $d = 1{\cdot}76$ (day 1), 0·76 (day 3); $P \leq 0{\cdot}01$), PP (Cohen's $d = 0{\cdot}80$ (day 1), 0·50 (day 3); $P \leq 0{\cdot}01$) and insulin (Cohen's $d = 1{\cdot}03$ (day 1), 1·90 (day 3); $P \leq 0{\cdot}001$) concentrations were all lower during DEF relative to BAL on both the first and final days of SUSOPS.

Discussion

Study results demonstrated that changes in appetite ratings during 72-h of prolonged low-to-moderate intensity physical activity and limited sleep simulating sustained military operations were not proportional to changes in energy balance. Associations between energy balance and appetite ratings predicted a mean appetite suppression at energy balance and provided preliminary evidence of a minimum energy deficit that may be expected independent of logistical and other factors that limit food intake during events characterised by prolonged periods of high physical activity and limited sleep. Between-condition differences in GET and circulating concentrations of appetite-mediating hormones identified slowed gastric emptying, increased concentrations of anorexigenic hormones and decreased acylated ghrelin concentrations as potential mechanisms for appetite suppression. Collectively, findings implicate a physiologically mediated attenuation of compensatory responses in appetite to increased PAEE as one factor contributing to the development of energy deficit during sustained military operations and possibly other events characterised by prolonged strenuous activity and limited sleep such as wildland firefighting and ultra-endurance sport.

The precise quantification of energy intake and expenditure along a spectrum of energy deficit to surplus within the same individuals is a strength of this study. The mean decrease in hunger and prospective consumption, and increase in fullness predicted to occur between the range of energy balance to about 25 % energy deficit (0 to about 5·4 MJ/d (1300 kcal/d) in this study; see negative y- and x-intercepts in Table 2) are consistent

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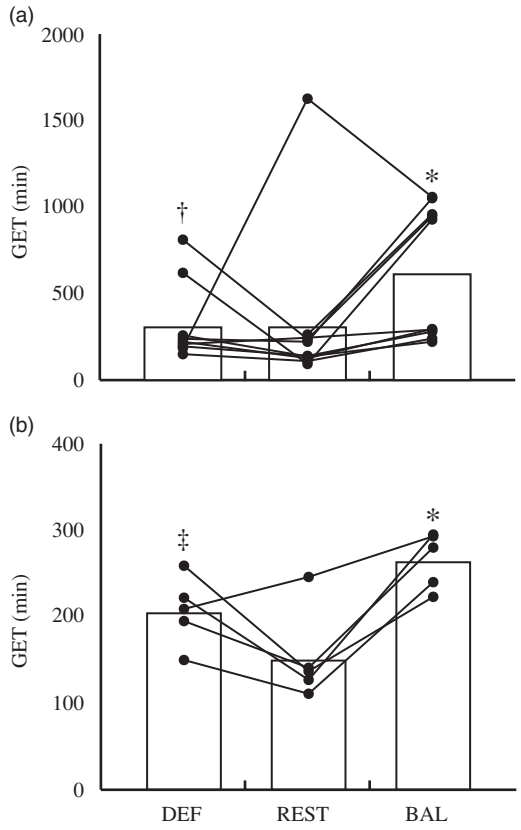


Fig. 4. Gastric emptying time (GET) measured during rest and during a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF). Bars represent mean GET for (a) all available data (n 10; square root-transformed for analysis), and (b) after excluding data for n 5 who demonstrated delayed gastric emptying during ≥1 conditions. Analysed using general linear model with correlated errors. *,†,‡ Main effect of condition ($P \leq 0{\cdot}03$). * Different from REST ($P \leq 0{\cdot}05$). † Different from BAL ($P < 0{\cdot}05$). ‡ Trend for difference from BAL ($P = 0{\cdot}06$).

with an exercise-induced appetite suppression which appears to supersede any stimulatory effects of sleep restriction and energy deficit on appetite. That result is consistent with laboratory *ad libitum* feeding studies which have reported that progressive increases in PAEE of up to 3·3–5·0 MJ/d (800–1200 kcal/d) over 7–14 d results in energy deficits of about 30 % (about 5·0 MJ/d (1200 kcal/d)) without significant increases in mean daily appetite ratings[47,48]. The findings also align with and extend those of studies which have concluded that energy intake does not increase to fully compensate for acute (<1 d) or short-term (2–14 d) increases in PAEE of lesser[23,24] or greater[2,5] magnitude than those experienced in this study.

Study findings extend those of previous studies conducted in military field training exercises. For example, increases in hunger (18 to about 40 %) that were proportionally less than the magnitude of energy deficit experienced were reported in two separate field training studies where TDEE was high (20·9–25·1 MJ/d (5000–6000 kcal/d)) and energy deficits were similar in magnitude to those imposed herein (40–55 % energy deficit over 4 d)[10,12]. Those studies and others have also reported that providing additional food or beverages to augment typical ration provisions (e.g. 3 rations/d) generally results in reduced consumption of the typical provisions[10,12,17,49,50]. Energy deficits are therefore attenuated, but not prevented when supplemental energy is provided. Collectively, these observations suggest that achieving energy balance while eating *ad libitum* during strenuous military operations is unlikely regardless of food availability because appetite does not increase sufficiently to match the high PAEE. Rather, when food availability is not limited, an energy deficit of 25 % may approximate a minimum deficit that can be expected independent of other factors limiting appetite and/or energy intake in those environments. Notably, recent findings suggest that an energy deficit of that magnitude (about 5·4 MJ/d (1300 kcal/d)) could be sustained for up to 2 week before reductions in lower-body physical performance would be expected[3]; however, decrements in mood and cognition may occur earlier[51,52].

The observed appetite suppression appeared to be physiologically mediated. In support, GET, which was increased during BAL, has been inversely associated with appetite at rest, and during and after exercise[26,53], likely due to effects on gastric distention and intestinal exposure to nutrients[54]. GET did not differ between REST and DEF, suggesting that the high physical activity may have attenuated any effects of energy deficit on slowing GET[53] and that the slowed GET during BAL was due, in part, to differences in diet volume. That conclusion would be consistent with results of one meta-analysis which reported that moderate-intensity exercise has no effect or slightly accelerates gastric emptying, while greater food volume is associated with slower gastric emptying during exercise[26]. In addition, the SmartPill™ used to measure GET generally passes from the stomach only after digestible solids are emptied[41]. That feature likely explains the above-average GET and prevalence of prolonged gastric retention of the SmartPill™ observed during BAL. Specifically, the increased diet volume and more frequent snacking during BAL relative to DEF and REST likely prevented the breakfast meal from completely emptying the stomach of some participants before the subsequent snack or lunch meal was consumed. Though the testing protocol used to measure GET did differ from clinical protocols using the SmartPill™, the approach is more relevant to the military environments the study was designed to emulate wherein individuals may eat meals or snacks prior to complete gastric emptying of the previous meal. Further, mean/median GET measured during DEF and REST, and during BAL after excluding outliers, was within the normal range for healthy males[43,44]. Taken together, these data suggest that the combination of higher volume meals and more frequent or higher volume snacks likely contributed to appetite suppression at energy balance, in part, by prolonging GET.

Differences in appetite-mediating hormone concentrations may also have contributed to appetite suppression at energy balance. Relative to both fasting concentrations and those measured during DEF, non-fasting post-exercise insulin, PYY, GLP-1 and PP concentrations were elevated during BAL, whereas non-fasting post-exercise acylated ghrelin concentrations were reduced. Those differences were likely attributable to greater food intake during the breakfast meal (consumed 2·5–3 h prior to the post-exercise blood draw) during BAL, as exercise was matched between conditions. Effects of PP, PYY and GLP-1 include promoting satiety and slowing gastric emptying, whereas ghrelin

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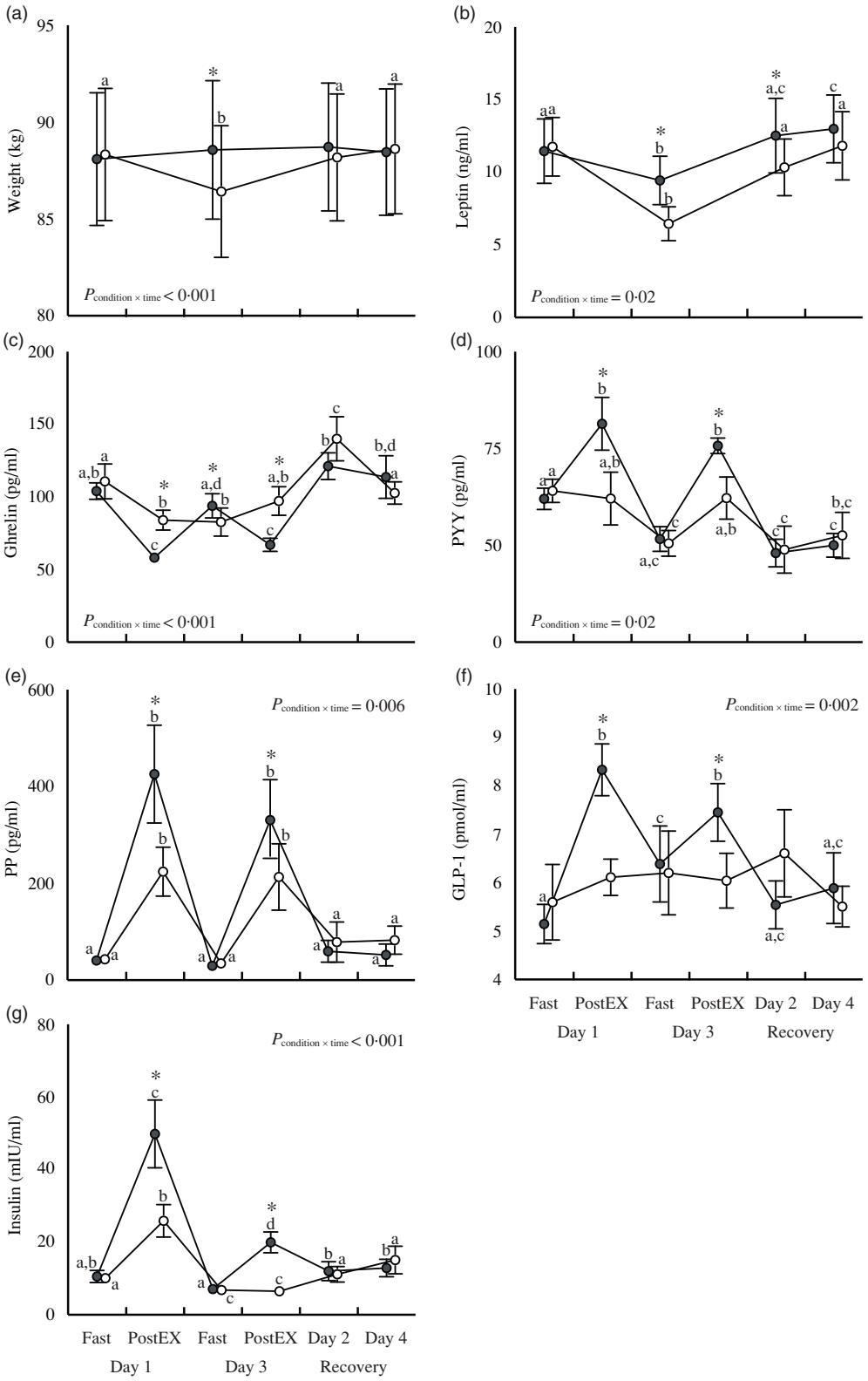


Fig. 5. Body weight (a) and appetite-mediating hormone concentrations (b)–(g) measured during (days 1 and 3) and after (Recovery (Rec.) days 2 and 4) a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF) (*n* 10). Values are mean values and standard errors. Analysed by general linear models with correlated errors. [a,b,c,d] Within a condition, time points not sharing a superscript letter are significantly different ($P < 0{\cdot}05$). * Difference between BAL and DEF ($P < 0{\cdot}05$). Fast, fasting measurement; PostEx, non-fasting measurement collected immediately after morning exercise bout. ■ BAL; □ DEF.

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stimulates hunger and accelerates emptying[55–57]. Thus, a prolonged postprandial elevation in circulating anorexigenic hormone concentrations and suppression of acylated ghrelin concentrations would collectively be expected to slow gastric emptying, increase satiety and suppress hunger.

Between-group differences in fasting appetite-mediating hormone responses to SUSOPS were notably different from between-group differences in non-fasting measurements. Only fasting acylated ghrelin and leptin differed, both decreasing to a greater extent during DEF than BAL. While it seems counterintuitive that the orexigenic hormone ghrelin would decrease during energy restriction, similar responses have been observed during severe, short-term energy deficit in other studies[20,45,58,59], and animal studies suggest that energy deprivation increases neuronal ghrelin sensitivity[60,61]. The larger decrease in leptin during DEF may have also contributed to changes in ghrelin sensitivity and appetite. Circulating leptin concentrations are known to decrease immediately and substantially during energy deficit[62]. This response alters central nervous system sensitivity to episodic signals mediating appetite during and between meals[33] and drives the common tendency to regain lost body mass[63], as was observed in this study. Thus, the relative appetite suppression observed at energy balance and rapid weight regain following energy deficit, when considered within the context of the between-condition differences in appetite-mediating hormones and GET, are consistent with the concept that biological responses to perturbations in energy balance effectively match energy intake and expenditure, but over timescales longer than meal-to-meal or day-to-day[64,65]. These findings suggest that responses of episodic and tonic signals mediating appetite and body weight homoeostasis are likely to deter voluntary increases in energy intake to match energy expenditure during short-term periods of high PAEE, but will stimulate weight regain once energy expenditure returns to usual levels, and highlight the importance of allowing adequate rest and recovery to restore body mass losses.

Mitigating rather than preventing energy deficit may therefore be a more realistic goal during periods of prolonged strenuous activity and limited sleep. One means of achieving that goal could include exploiting hedonic factors that influence the preference and desire for particular foods. Hedonic factors are recognised as important determinants of energy intake which can override episodic and tonic mediators of energy balance homoeostasis and promote overconsumption[66], and food acceptability is a known factor influencing energy intake in military environments[67] and ultra-endurance sport[5]. For those reasons, it is notable that desire to eat changed in proportion to energy balance and volunteers demonstrated a greater preference, albeit small, for higher fat foods during SUSOPS when in energy deficit. Fat is less satiating per unit energy than carbohydrate or protein[68] and is energy dense. Higher energy density meals and diets consistently result in higher energy intakes, usually without affecting perceived appetite[69], and despite postprandial appetite-mediating hormone responses that track energy intake[70]. Higher-fat diets (50–60 % *v.* 30–35 % of energy) and meals have also been shown to mitigate exercise-induced energy deficits[71–74] and contribute to increased energy intake in military field training environments[75]. Increasing fat content and energy density of foods (e.g. military rations) consumed during sustained periods of prolonged low-to-moderate intensity physical activity may therefore warrant consideration in future research as possible strategies for attenuating energy deficit. However, those studies will need to consider whether increasing dietary fat content affects the ability to consume recommended intakes of carbohydrate and protein, and any impacts on physical and cognitive performance[5,10,76].

The results of this study should be interpreted within the context of several limitations. First, although the study was adequately powered to detect between-condition differences for all of the secondary study outcomes reported herein except PP, the sample size was small and multiple outcomes were assessed, which increases risk of type 1 error. As such, findings warrant cautious interpretation and require replication in larger cohorts. Additionally, the study was not designed to control for all factors that may influence appetite, GET or appetite-mediating hormone concentrations. However, any effects of those factors should have been mitigated by the standardised time periods between eating and blood measurements, and by measuring appetite at multiple time points throughout each day. A second limitation was the mean positive energy balance during BAL. The correlations between energy deficit and appetite ratings which were used to address that limitation do strongly suggest that appetite would have been suppressed had energy balance been maintained during BAL. However, it is possible that the relationship between energy balance and appetite is not linear as the analysis assumed. Third, appetite ratings and food preferences do not necessarily predict eating behaviour[37]. Allowing *ad libitum* food intake would have increased the external validity of the study, but would also have precluded assessing physiological responses across the spectrum of negative to positive energy balance. Fourth, intra-individual variability in GET measured by the SmartPill™ may be high, as suggested in one study wherein the coefficient of variability for measurements made 2–4 week apart ranged from 20 to 40 %[44]. However, those values were skewed by a small sample size and 10 % of participants whose GET measured >10 h during one measurement. When those outliers were removed, intra-individual differences in repeated measurements of GET averaged <1 h. Notably, that value is similar to or less than the mean between-condition differences observed after excluding five participants with GET > 10 h from the analysis in the present study (Fig. 4(b)). Finally, study results should not be generalised to women as others have reported sex differences in appetite and related physiological responses to increased exercise[77].

Reasons for undereating during events requiring prolonged strenuous physical activity and limited sleep such as sustained military operations and ultra-endurance sporting competitions are multi-factorial and often include logistical constraints and limited access to food[19]. Study findings suggest that energy balance is unlikely to be achieved by *ad libitum* eating in these environments, even if food availability is not constrained, due in part to a physiologically mediated suppression of appetite. The resulting energy deficit, if sustained, would be expected to degrade physical performance, cognition and immunity, and prolong recovery[3,21,22,51]. Study findings therefore provide a physiological basis for testing feeding strategies known to

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promote overconsumption during events requiring multiple days of prolonged low-to-moderate intensity physical activity such as sustained military operations. Such strategies may include timing meals or snacks to allow for gastric emptying, and providing rapidly digestible, higher-fat, energy-dense foods and beverages.

Acknowledgements

We thank the volunteers who participated in the study. We also thank Dr. Jennifer Rood and the Clinical Chemistry Laboratory team at the Pennington Biomedical Research Center for their assistance with the hormone analyses, Philip Niro for assistance with the figures and Dr. Andrew Young for critical review of the manuscript. Finally, we thank MAJ Julianna Jayne, LTC Nicholas Barringer, Dr. Tracey Smith, Dr. Emily Howard, Dr. Alyssa Varanoske, Svein Martini, Hilde Teien, Nancy Murphy, Chris Carrigan, Anthony Karis, Marques Wilson, Nicholes Armstrong, Susan McGraw, Claire Whitney, Beau Yurkevicius, Karleigh Bradbury, Adam Luippold, SGT Marcus Sanchez, SPC Kristine Chiusano, SPC Cornal Pounds, SSG Stephen Mason, SPC Jason Alaniz, SGT Katakyie Sarpong, SGT Joseph Bistolfi, and SPC Lauren Dare for their expert technical support throughout the study.

Funded by the US Army Medical Research and Development Command, Military Operational Medicine Research Program. The funder had no role in the study design, collection, analysis and interpretation of data, writing of the report or decision to publish.

J. P. K., A. H. M., G. F., J. A. G., L. M. M., S. R. H., J. P. M. and S. M. P. designed the study; A. H. M and J. T. A. coordinated data collection; J. P. K., A. H. M., J. A. T., H. S. F., P. N. R., J .A. G., L. M. M., S. R. H., J. P. M. and S. M. P. participated in data collection; J. P. K., A. H. M., J. T. A., H. S. F., G. F. and P. N. R. managed the data; J. P. K. analysed the data and wrote the manuscript; all authors critically reviewed the manuscript and approved the final version.

The authors declare that there are no conflicts of interest.

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Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0007114521000131

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Research Article

Eating Behaviors Are Associated With Physical Fitness and Body Composition Among US Army Soldiers

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ABSTRACT

Objective: Examine associations between soldiers' eating behaviors, compliance with body composition and fitness standards, and physical performance.

Design: Cross-sectional study.

Setting: Eight Army installations.

Participants: US Army Soldiers (n = 1,591; 84% male).

Main Outcome Measures: Characteristics, eating behaviors, compliance with body composition and physical fitness standards, and fitness level were assessed via questionnaire.

Analysis: Bivariate and multivariable logistic regression.

Results: Eating mostly at a dining facility was associated with lower odds of body composition failure (odds ratio [OR], 0.44; 95% confidence interval [CI], 0.26−0.73); whereas, eating at a fast rate (OR, 1.51; 95% CI, 1.05−2.17) or often/always ignoring satiety cues (OR, 2.12; 95% CI, 1.06−4.27) was associated with higher odds of body composition failure. Eating mostly fast-food/convenience meals (OR, 1.75; 95% CI, 1.19−2.59) and eating at a fast rate (OR, 1.42; 95% CI, 1.04−1.93) was associated with higher odds of physical fitness failure. Skipping breakfast was associated with lower odds of high physical performance (OR, 0.41; 95% CI, 0.23−0.74); whereas, nutrition education was associated with higher odds of high physical performance (OR, 1.02; 95% CI, 1.01−1.04).

Conclusions and Implications: As eating behaviors are modifiable, findings suggest opportunities for improving the specificity of Army health promotion and education programs.

Key Words: military, eating behaviors, eating rate, body composition, performance (*J Nutr Educ Behav.* 2021;53:480−488.)

Accepted January 12, 2021. Published online March 3, 2021.

INTRODUCTION

Specific eating behaviors such as eating rate, listening to satiety cues, and distracted eating are associated with body mass index (BMI).[1−4] In addition, eating behaviors that promote proper timing and nutrient intake in relation to physical training demands facilitate training adaptations that optimize physical fitness.[5] Thus, in addition to what one eats, behaviors dictating how and where one eats impacts health and physical performance.[6] Eating behaviors are therefore highly relevant to US Army soldiers, individuals who must be prepared to perform at a high level without notice, and for whom meeting body composition and physical fitness standards is a condition for military entry and retention.[1,2]

For more than a decade, the US Army has actively engaged in public health campaigns aiming to improve the eating and nutrition-related behaviors of soldiers.[7,8] New programs currently being piloted, such as the *Holistic Health and Fitness Program* and the *Healthy Army Communities Performance Dining Card Initiative*,[9,10] emphasize the importance of healthy eating behaviors to promote high occupational performance and readiness (the ability of soldiers to be ready to perform all aspects of military duty).[6,11] The effectiveness of these campaigns and programs is dependent on multiple factors, including modifying intractable eating behaviors, which may be shaped by early military training experiences and reinforced by the Army environment.[12,13] For example, in initial military training, recruits are integrated into the military in a highly controlled food environment that limits access to less nutritious foods. However, in that highly controlled environment, food access is restricted to

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Conflict of Interest Disclosure: The authors have not stated any conflicts of interest.

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Published by Elsevier Inc. on behalf of Society for Nutrition Education and Behavior.
https://doi.org/10.1016/j.jneb.2021.01.013

designated meal times, outside food is not allowed,[14] and trainees must eat quickly without regard to internal hunger and satiety cues.[15,16] These and other experiences throughout a soldiers' military career may foster the development of undesirable eating behaviors that have previously been associated with high BMI and poor diet quality in both military and civilian populations.

Other factors relevant to soldiers' eating behaviors are mandatory assessments of compliance with body composition and physical fitness standards. A BMI-based screening assessment occurs twice yearly for all soldiers. Any soldier who exceeds the BMI standard for their sex and age undergoes a circumference-based body fat assessment called the tape test.[17] If soldiers exceed the maximum allowable body fat percentage, they are considered to have failed body composition standards and are then required to complete nutrition education, are reweighed and tape tested every 30 days, are mandated to increase physical activity, and cannot be promoted until they reach an acceptable body fat percent.[17] Soldiers who fail to reach or maintain body composition standards can be involuntarily discharged from service.[17] Soldiers must also pass a physical fitness test twice yearly that assesses cardiovascular endurance and strength.[18,19] Soldiers who fail physical fitness standards are penalized similarly to those who fail body composition standards. A soldier's performance and ability to meet body composition and physical fitness standards are likely influenced by nutrition-related behaviors such as how, what, and where one eats.

Relationships between eating behaviors and weight and performance outcomes remain an understudied area of research on military performance and readiness. Previous studies have investigated diet quality,[20] intuitive eating,[21] motivations for eating behaviors,[22] and mediators of eating behaviors[23] in military populations as strategies for informing health promotion and education programs. In addition, the adoption of undesirable eating behaviors has been demonstrated in recruits,[12] but the specific eating behaviors that may contribute to failing body composition and physical fitness standards of fully trained soldiers have not been thoroughly examined.[24] An improved understanding of the specific eating behaviors associated with soldiers' ability to meet these standards is needed to inform interventions aimed at promoting soldier health and readiness.[25]

This cross-sectional study aimed to (1) examine associations between eating behaviors, soldier characteristics, and failing body composition and physical fitness standards; and (2) identify eating behaviors and characteristics of soldiers that discriminate those who score low from those who score high on the physical fitness test. The authors hypothesized that eating behaviors, including the location where a soldier most frequently eats, their typical eating rate, and whether they ignored satiety cues, would be associated with body composition and physical fitness standard failure, and that there would be a difference between the eating behaviors and characteristics of low- and high-performing soldiers.

METHODS

Between 2016 and 2019, 1,789 soldiers from geographically and operationally diverse Army installations at Fort Jackson, SC, Joint Base Lewis–McChord, WA, Fort Riley, KS, Fort Sam Houston, TX, Fort Carson, CO, Joint Base Elmendorf–Richardson, AK, Natick Soldier System Center, MA, and Fort Campbell, KY were briefed in person and asked to complete a 100 item questionnaire to assess eating behaviors, habits, history of compliance with body composition and physical fitness standards, and physical fitness assessment scores, in addition to sociodemographic information (age, race/ethnicity, level of education, and marital status). Collection of race/ethnicity followed Office of Management and Budget guidelines,[26] but because of small numbers of volunteers in some categories, responses were collapsed to 4 categories. Soldiers completed the questionnaire in a conference room type setting with researchers standing by to answer questions. The data for this study were gathered during a study to validate a tool for understanding the eating behaviors of soldiers (R.E.C., unpublished data, 2020). The questionnaire was pretested in a focus group setting and with a group of experienced soldiers and nutrition experts before use in this sample and took, on average, 40 minutes to complete. Volunteers were given an optional break to avoid survey fatigue. A total of 1,591 soldiers, 89% of whom ranged in age from 18 to 58 years (mean, 23.7; SD, 5.2) completed surveys after they were briefed, were assured of the confidentiality of their responses, and gave written informed consent. A *post hoc* power analysis using G*Power (Heinrich Heine University Düsseldorf, www.gpower.hhu.de, 2020) determined the sample size to be ample for regression models with 15 predictors to detect an odds ratio of 1.2 with 80% probability at a significance level of 0.05. The study was approved by the US Army Medical Research and Development Command Institutional Review Board, investigators complied with human research policies outlined in Army Regulation 70–25, and the study was conducted in adherence with the requirements of 32 CFR Part 219.

Dependent Variables

Body composition and physical fitness standards failure, physical fitness test scores, and fitness level. Army body composition assessment involves measuring the circumference of the neck, waist, and hip for women and the neck and waist for men.[17] Body composition standards failure was determined by a yes response to the question, Have you ever failed the body fat tape test?, with possible responses being yes, no, or never taken, or tested. Those who had never taken the tape test are those who either never exceeded screening BMI or who had not been in the Army long enough to be screened. Physical fitness test failure was determined by a yes response to the question, Have you failed a record Physical Fitness/Readiness test since initial military training?, with possible responses being yes, no, or never taken, or tested. The Army Physical Fitness Test consists of 3 timed events: push-ups, sit-ups, and a 2-mile run. Those who achieve ≥90 points on each

event (≥270 out of a possible 300 points) on the Army Physical Fitness Test receive the US Army Physical Fitness Excellence Badge.[19] Low performers were designated as those who had failed physical fitness standards 1 or more times and whose most recent US Army Physical Fitness Test score was ≤269 points, and high performers as those who never failed physical fitness standards and whose most recent US Army Physical Fitness Test score was ≥270 points.

Independent Variables

Eating behaviors and soldier characteristics. Where soldiers ate most of their meals was determined by the question, In the past 30 days, where did most of your meals and snacks come from?, with options that allowed the volunteer to indicate where and how often they had typically consumed breakfast, lunch, dinner, and snacks. Options included home/barracks, field rations (eg, meals-ready-to-eat, military dining facility, fast-food/vending/convenience store locations), or full-service restaurants, or if they typically skipped these meals or snacks. How often a volunteer had eaten while distracted was determined by the question, I ate my meals while being distracted (eg, using phone/computer, watching TV, driving, working). Responses were captured by a 5-point Likert-type scale that ranged from never to always. The eating rate was determined by combining 2 questions that asked volunteers how fast they think they typically eat, with responses ranging from very slow/extremely slow to very fast/extremely fast. The level of hunger before meals was determined by the prompt, Over the past 30 days, on average, I got so hungry that my stomach felt like a bottomless pit, and I was hungry between meals. Responses ranged from never to always and were combined to create a variable that reflected the level of hunger before meals. How often soldiers followed ignored satiety cues was determined by the prompts, Over the past 30 days on average, I continued to eat after feeling full, I cleaned my plate regardless of feeling full, I ate even though I wasn't hungry, I ate so much that after eating, I felt sick to my stomach, and I relied on feelings of fullness to tell me when to stop eating. Responses ranged from never to always and were combined to create a variable that reflected how often soldiers reported ignoring satiety cues. The prompt I relied on feelings of fullness to tell me when to stop eating was reverse coded, so the directionality of all responses would agree. Nutrition education was determined by the prompt, How have you received nutrition education, guidance, or instruction during your military career with options for selected various types of formal (eg, a class taught by a dietitian) or informal nutrition education (eg, researched nutrition on own). Body mass index was calculated from self-reported height and weight, which has been found to be highly correlated ($r = 0.88$) to measured BMI in a previous study in soldiers.[27] Army Physical Fitness Test score was self-reported. The International Physical Activity Questionnaire was used to gather general physical activity information.[28] Time spent doing physical activity was calculated by combining total minutes of self-reported moderate and vigorous physical activity and reported as minutes per day.

Statistical Analysis

Data were analyzed using Stata (version 15.1, StataCorp, College Station, TX, 2017). Two hundred (13%) soldiers had missing data, with the most common variables missing data being: physical activity (6%), nutrition education (4%), where most meals were eaten and eating rate (2%), and distracted eating (1%). Less than 1% of soldiers had missing responses for body composition and physical fitness failure, hunger level, satiety cues, or sociodemographic variables. No other variables used in the analysis had missing data. Investigating patterns among missing variables revealed no differences in the distribution between missing and nonmissing variables such that missing data can be described as missing completely at random. There were 289 soldiers who were new to the Army and had not yet taken the Army Physical Fitness Test. The characteristics and eating behaviors of low and high performers were examined using ANOVA and chi-square statistics with *post hoc* testing for between-group differences. Multivariate adjusted logistic regression models were implemented to examine associations between soldiers' characteristics, eating behaviors, failing body composition or physical fitness standards, and Army Physical Fitness Test performance. Models were adjusted for sociodemographic variables, including sex, age, rank (enlisted vs officer), training status (trainee vs fully trained), marital status, education level, and race/ethnicity. Initial military training soldiers who had yet to be assessed for body composition failure or had not taken the Army Physical Fitness Test were excluded from models. Regression diagnostics to include screening for multicollinearity among variables was performed. The receiver operating characteristic curve was calculated to assess model discrimination.

RESULTS

Volunteers were majority male (84%), of a racial or ethnic minority group (53%), single (63%), had at least some college education (52%), and a mean age of 24.1 years (Table 1). These demographics are generally representative of the Army population.[29] There were significant differences in the eating behaviors and characteristics of soldiers on the basis of performance level. Referring to significant results, high-performing soldiers ate more frequently at home, ate less from fast-food/vending locations, and skipped breakfast less often compared with low-performing soldiers. High-performing soldiers also reported eating while distracted less frequently than low-performing soldiers, were more likely to have received formal nutrition education, and had less obesity.

In adjusted logistic regression models of body composition or physical fitness failure, compared with those who reported eating most meals at home, eating mostly in the field or meals-ready-to-eat field rations raised the odds of body composition failure by 2.34 times (95%

Table 1. Eating Behaviors and Characteristics of Soldiers, Stratified by Performance Level

Sociodemographic, Characteristics or Eating Behavior Variables, Mean (SD) or %	All Soldiers n = 1,591	Low Performers n = 190[a]	High Performers n = 328[a]	Difference Between High and Low Performers (*P*)
Most meals eaten[b]				
Home/barracks	35.9	31.2	40.9	0.03
Field/meals, ready-to-eat	4.8	5.4	3.7	0.38
Dining facility	32.6	26.3	27.0	0.89
Fast-food/vending/convenience	17.7	27.4	18.1	0.01
Full-service restaurant	9.0	9.7	10.3	0.85
Skipped breakfast	10.5	17.4	7.0	< 0.001
Ate while distracted				
Never/rarely	32.4	18.4	25.6	0.06
Sometimes	31.7	33.7	37.8	0.35
Often/always	35.9	47.9	36.6	0.01
Eating rate				
Slow	5.6	5.8	3.7	0.26
Normal	49.3	51.9	52.6	0.87
Fast	45.1	42.3	43.7	0.76
Extreme hunger before meals				
Never	25.3	18.4	25.0	0.09
Sometimes	45.5	51.6	48.5	0.49
Often	29.3	30.0	26.5	0.40
Ignored satiety cues				
Never/rarely	8.0	6.8	9.2	0.34
Sometimes	21.9	20.0	24.0	0.29
Often/always	70.1	73.2	66.8	0.13
Formal nutrition education	28.7	23.9	36.7	0.01
BMI, kg/m^2				
Underweight/normal: ≤ 24.9	44.7	31.1	49.7	< 0.001
Overweight: 25.0–29.9	42.6	44.2	45.7	0.32
Obese: ≥ 30.0	12.7	24.7	4.6	< 0.001
Physical fitness test score	256.4 (31.7)	226.0 (28.9)	287.7 (9.9)	< 0.001
Physical activity, min/d[c]	108.6 (80.3)	100.8 (65.9)	96.9 (63.08)	0.51
Sex				
Male	83.5	87.4	87.8	0.88
Female	16.5	12.6	12.2	0.88
Age, y	24.1 (5.2)	24.3 (5.2)	24.3 (5.4)	0.64
Training status				
Trainee	19.4	0.5	0.6	0.90
Rank categories				
Enlisted	93.3	97.9	39.6	< 0.001
Officer	6.7	2.1	60.4	< 0.001
Marital status				
Single	62.7	60.0	60.7	0.94
Married	37.3	40.0	39.3	0.94
Education level				
High school/GED	48.3	55.3	40.6	< 0.001
Some college	38.6	40.0	33.9	0.17
Degree	13.1	4.7	25.5	< 0.001
Race/ethnicity				
Non-Hispanic White	47.4	47.1	48.5	0.76
Non-Hispanic Black	18.5	19.6	16.5	0.37
Non-Hispanic other	12.7	10.1	12.2	0.46
Hispanic	21.4	23.3	22.8	0.91

BMI indicates body mass index.

[a]Bivariate analysis between high and low performers does not include soldiers who had not taken the fitness test or those who are neither low nor high performers; [b]most meals eaten was defined as the location where >50% of meals were eaten in the previous 30 days; [c]exercise time is a combination of moderate and vigorous exercise totals.

Note: Low performers were those who had failed fitness standards 1 or more times and whose most recent Army Physical Fitness Test score was ≤269 points; high performers were those who never failed fitness standards and whose most recent Army Physical Fitness Test score was ≥270 points. Significance set at $P < 0.05$.

confidence interval [CI], 1.21−4.50) and physical fitness failure by 1.91 times (95% CI, 1.02−3.60) (Table 2). Eating most meals at a dining facility or a full-service restaurant lowered the odds of body composition and physical fitness failure by 2.27 times (odds ratio, 0.44; 95% CI, 0.26−0.73) and 3.33 times (odds ratio, 0.30; 95% CI, 0.13−0.66), respectively, and eating most meals from fast-food or vending locations raised odds of physical fitness failure by 1.75 times (95% CI, 1.19−2.59). Often/always ignoring satiety cues raised the odds of body composition failure by 2.12 times (95% CI, 1.06 −4.27), and eating at a fast rate raised the odds of failing body composition and physical fitness standards by 1.51 times (95% CI, 1.05−2.17) and 1.42 times (95% CI, 1.04−1.93), respectively. Those who skipped breakfast had 2.43 lower odds of reporting high physical fitness performance (0.41; 95% CI, 0.23−0.74), but having formal nutrition raised the odds of reporting a high physical fitness performance by 1.02 times (95% CI, 1.01−1.04).

Table 2. Results of Logistic Regression Models of Failing Body Composition Standards (n = 1,160), Failing Physical Fitness Standards (n = 1,223), or High Physical Fitness Test Performance (n = 964)

Sociodemographic Characteristics or Eating Behavior Variables	Body Composition Standards Failure	Physical Fitness Test Failure	High Fitness Test Performance
Most meals eaten			
Field/meals, ready-to-eat	2.34 (1.21−4.50)	1.91 (1.02−3.60)	0.88 (0.40−1.97)
Dining facility	0.44 (0.26−0.73)	1.31 (0.86−1.98)	1.04 (0.69−1.57)
Fast-food/vending/convenience	0.89 (0.57−1.39)	1.75 (1.19−2.59)	0.87 (0.56−1.32)
Full-service restaurant	0.30 (0.13−0.66)	1.34 (0.78−2.30)	0.83 (0.48−1.45)
Skipped breakfast	1.44 (0.86−2.40)	1.47 (0.95−2.27)	0.41 (0.23−0.74)
Ate while distracted			
Sometimes	0.68 (0.43−1.07)	1.30 (0.87−1.92)	1.09 (0.73−1.61)
Often/always	0.93 (0.59−1.46)	1.47 (0.99−2.19)	0.80 (0.53−1.19)
Eating rate			
Slow	0.53 (0.23−1.21)	1.20 (0.62−2.31)	0.85 (0.46−1.59)
Fast	1.51 (1.05−2.17)	1.42 (1.04−1.93)	1.01 (0.73−1.40)
Extremely hungry before meals			
Sometimes	0.95 (0.63−1.43)	1.29 (0.89−1.86)	1.16 (0.81−1.60)
Often	0.64 (0.39−1.04)	1.24 (0.82−1.87)	1.05 (0.68−1.06)
Ignored satiety cues			
Sometimes	1.27 (0.59−2.74)	0.76 (0.43−1.35)	0.58 (0.31−1.06)
Often/always	2.12 (1.06−4.27)	0.79 (0.47−1.33)	0.61 (0.35−1.05)
Formal nutrition education	1.02 (0.99−1.04)	1.00 (0.99−1.02)	1.02 (1.01−1.04)
Physical activity, mins/d	1.00 (0.99−1.01)	1.00 (0.99−1.01)	1.07 (0.99−1.08)
Control variables			
Female	1.97 (1.23−3.13)	1.30 (0.84−2.02)	0.63 (0.38−1.03)
Age, y	0.22 (0.06−0.73)	1.04 (1.00−1.07)	0.98 (0.94−1.01)
Officer	0.81 (0.72−0.92)	0.28 (0.08−0.95)	4.27 (1.87−9.74)
Not a trainee	1.37 (0.69−2.74)	1.49 (0.63−3.50)	4.91 (1.05−9.83)
Married	1.02 (0.70−1.50)	1.22 (0.87−1.71)	1.13 (0.79−1.62)
Some college	0.81 (0.55−1.18)	0.84 (0.61−1.17)	1.32 (0.93−1.88)
Degree	0.62 (0.29−1.34)	0.29 (0.13−0.65)	1.76 (0.86−3.59)
Non-Hispanic Black	1.43 (0.88−2.33)	1.09 (0.72−1.65)	1.34 (0.86−2.10)
Non-Hispanic other	0.93 (0.52−1.65)	0.82 (0.51−1.32)	0.97 (0.61−1.61)
Hispanic	1.53 (1.00−2.35)	1.07 (0.74−1.54)	1.32 (0.90−1.95)
Constant	0.81 (0.66−0.98)	0.03 (0.01−0.15)	0.17 (0.02−1.30)

Note: Values are odds ratio (95% confidence interval). Model sample size is as follows: body composition failure (failing the body fat tape test) excluded 285 soldiers who had never been tested for body composition and 146 with missing data; fitness failure (failing a physical fitness test since initial military training) excluded 289 who had never taken the physical fitness test and 79 with missing data; high Army Physical Fitness Test performance (those who never failed fitness standards and whose most recent fitness test score was ≥270 points) excluded 289 soldiers who had never taken the Army Physical Fitness Test, 195 who did not know their score, and 143 who had missing data. The area under the receiver operating characteristic curve statistic for body composition, fitness test failure, and high fitness test performance was 0.78, 0.71, and 0.69, respectively. The referent group was eating at home/barracks, never skipping breakfast, never eating while distracted, normal eating rate, never extremely hungry, never ignoring satiety cues, men, enlisted soldiers, recruits, single, high school education, non-Hispanic White. The level of significance was 95% confidence intervals corresponding to $P < 0.05$.

DISCUSSION

This study aimed to identify eating behaviors of soldiers associated with the ability to meet Army body composition and physical fitness standards and that discriminate soldiers with high physical fitness from those with lower physical fitness. No individual eating behavior was associated with all of those outcomes. However, the location where most meals were consumed, eating rate, ignoring internal satiety cues, and skipping breakfast were associated with 1 or more outcomes. These behaviors may therefore be targets for behavioral interventions aiming to improve soldier body composition and physical fitness.

No statistically significant difference was found between the amount of time spent doing physical activity reported by low- and high-performing soldiers, but the results of this study suggest that other behaviors may play a role in physical fitness. In particular, eating behaviors may contribute, as high performing relative to low-performing soldiers reported engaging more frequently in positive nutrition behaviors such as eating fewer fast-food/convenience meals, not eating while distracted, eating breakfast, not allowing themselves to get extremely hungry before meals, and not ignoring satiety cues.

Notably, formal nutrition education was associated with slightly higher odds of high physical fitness test performance and was reported by a greater percentage of high-performing soldiers than low-performing soldiers. This finding could indicate that Army programs aiming to provide more contact with registered dietitians may be an effective strategy for educating soldiers on performance nutrition concepts.[9] For example, the Joint Position Statement on Nutrition and Athletic Performance by the American College of Sports Medicine and the Academy of Nutrition and Dietetics emphasizes the role of meal timing for performance and body composition.[5] Among athletes in civilian settings, nutrition educations programs have been effective in improving nutrition benchmarks.[30] Similar to athletes, soldiers must always be physically prepared to perform and may receive a dual benefit from nutrition education that underscores the importance of meal timing for performance.

The location where most meals were consumed was associated with both body composition and physical fitness standards failure. Compared with soldiers who mostly ate at home, soldiers who ate most meals at a dining facility, and those eating primarily at full-service restaurants demonstrated lowered odds of failing body composition standards. Although dining facilities have been criticized for serving unappealing and unhealthy foods,[31] when compared with fast-food/convenience options, dining facilities provide a better variety of fresh fruits and vegetables as well as lower-fat options and reasonable portion sizes.[32] Dining facilities are also an effective venue for delivering nutrition education and interventions. In support, several studies have demonstrated reduced fat[32] and energy intakes,[33] and improved diet quality among service members following the implementation of dining facility-based nutrition interventions.[34] These studies showed point-of-sale nutrition labeling, increased variety, and availability of fruits and vegetables, limited fried foods, and increased lean protein options to be effective strategies to improve dietary intake among soldiers.[32–34] As 74% of soldiers who are located on an Army installation consume at least 1 meal per day at a dining facility,[33] these findings reinforce the importance of these facilities as a source of high quality, nutritious meal offerings that may help sustain military readiness. Ultimately, where a soldier chooses to eat is influenced by his or her irregular schedule and long work hours, combined with a food environment on most military installations that features fast and convenience type foods.[31] These findings support the notion that the overall military food environment should include less fast-food/convenience type food offerings or should perhaps explore options to increase the nutrition of those food offerings while promoting military dining facilities as a source of healthy nutrition. This concept is being explored in initiatives that aim to increase the availability of healthy options on posts while providing quality nutrition within dining facilities.[35,36]

In this sample, 45% of soldiers reported a fast eating rate, which is higher than the 25% to 31% prevalence reported in civilian populations,[37–39] and notable because eating at a fast relative to moderate rate was associated with 51% and 42% higher odds of failing body composition and physical fitness standards, respectively. These findings are consistent with studies in both military and civilian populations that have linked eating fast to higher BMI.[4,12] Findings are also consistent with previous studies suggesting that Army training may facilitate the development of eating behaviors that contribute to undesirable weight and performance outcomes among soldiers.[12,13,16] For example, to keep recruits on strict training schedules, soldiers in the initial military training environment must consume large portions of food quickly without regard to internal hunger or satiety cues.[12,40] This experience may foster the development of undesirable behaviors that are retained throughout adulthood[13] and are not conducive to healthy weight management and optimal performance.[3]

Military training and work environments that encourage fast eating may also reduce the ability of individuals to recognize satiety cues.[41] For example, reliance on satiety cues to stop eating when full has been shown to significantly decline during initial military training, as eating rate significantly increased during the same period.[12] In this study, the authors found that soldiers who often or always ignore satiety cues have more than 2 times the odds of failing body composition standards, compared with soldiers who report never ignoring satiety cues. Although not directly examined in this study, these findings are consistent with those in civilian populations, demonstrating that the combination of fast eating and ignoring internal satiety cues may additively influence undesirable body weight and body composition outcomes.[42] Conversely, studies have shown that intuitive eating or increasing reliance on physiologic

hunger and satiety cues[43] are effective weight management strategies in a variety of populations.[21,43] Thus, the results for the present study identify eating rate and attention to internal satiety cues as potential targets for behavioral modification to promote compliance with military body composition and physical fitness standards.

When soldiers train in austere environments, field rations, typically meals-ready-to-eat, are supplied with limited or no supplementation with fresh fruits and vegetables. This study found that compared with soldiers who ate most meals at home, consuming primarily meals-ready-to-eat was associated with increased odds of failing body composition and physical fitness standards. Meals-ready-to-eat are designed for the specific purpose of short-term provision of adequate energy and nutrients to all service members operating in environments in which high energy expenditures are common.[44,45] As such, although meals-ready-to-eat are of high nutritional quality,[46] they are also energy-dense and are distributed to each soldier regardless of individual energy needs. Energy density is positively associated with overeating and higher BMI in civilian populations[47]; therefore, the present findings suggest using meals-ready-to-eat for as short a period as possible may help avoid undesirable effects.

The strengths of this study were the large, diverse sample that included soldiers from multiple locations across the US. A limitation of this study is the cross-sectional design that does not demonstrate causality in the relationship between eating behaviors and study outcomes. Furthermore, all data were gathered using questionnaires and, therefore, may be subject to self-reporting bias, such as from reluctance to report body composition failure. It is also possible that misclassification bias could have occurred if soldiers did not correctly recall their physical fitness test scores. However, a previous study on the validity of self-report physical fitness test data among soldiers found a high correlation ($r = 0.78–0.98$) between self-reported and measured scores on the same physical fitness test used in this study, which suggests that soldiers can recall their physical fitness test scores accurately.[27] In addition, this study was conducted in a sample of young military personnel, limiting the generalizability of findings to the general public, although findings may have generalizability to collegiate athletes or similar populations.

IMPLICATIONS FOR RESEARCH AND PRACTICE

The findings of this study provide new information relevant to health practitioners and leaders who are in a position to influence the nutrition behaviors of military personnel and athletes and are relevant to informing initiatives and programs that aim to improve the eating behaviors and nutrition environment for soldiers.[9,10] Specifically, this study identified eating location, eating rate, and attention to internal satiety cues as potentially modifiable eating behaviors that are associated with outcomes of critical importance to the health and readiness of soldiers. Notably, key aspects of Army eating environment behaviors associated with body composition and physical fitness test failure are modifiable. These aspects include the amount of time allowed for eating during initial military training and the availability of fast-food operations on military installations, among others. Because of the difficulty in reversing behaviors once established,[13] and in both losing weight and sustaining weight loss,[48] lifestyle and behavioral modifications that consistently and persistently promote behaviors that deter weight gain are highly desirable from a public health perspective in both the general and specialized populations, such as the military. Thus, these findings identify opportunities to improve the specificity of Army health promotion programs as well as the Army eating environments to promote the development of eating behaviors that support health, performance, and readiness.

ACKNOWLEDGMENTS

This study was funded by the Army Medical Research and Development Command. The soldiers who participated in this research are gratefully acknowledged, and the research staff and soldiers who assisted with study implementation and data collection. Dr Andrew Young is also acknowledged for editorial assistance.

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Am J Physiol Regul Integr Comp Physiol 321: R1–R11, 2021.
First published May 5, 2021; doi:10.1152/ajpregu.00076.2021

RESEARCH ARTICLE

Don't Deny Your Inner Environmental Physiologist: Investigating Physiology with Environmental Stimuli

Metabolomic profiles are reflective of hypoxia-induced insulin resistance during exercise in healthy young adult males

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Abstract

Hypoxia-induced insulin resistance appears to suppress exogenous glucose oxidation during metabolically matched aerobic exercise during acute (<8 h) high-altitude (HA) exposure. However, a better understanding of this metabolic dysregulation is needed to identify interventions to mitigate these effects. The objective of this study was to determine if differences in metabolomic profiles during exercise at sea level (SL) and HA are reflective of hypoxia-induced insulin resistance. Native lowlanders (n = 8 males) consumed 145 g (1.8 g/min) of glucose while performing 80-min of metabolically matched treadmill exercise at SL (757 mmHg) and HA (460 mmHg) after 5-h exposure. Exogenous glucose oxidation and glucose turnover were determined using indirect calorimetry and dual tracer technique ([^{13}C]glucose and [6,6-$^{2}H_2$]glucose). Metabolite profiles were analyzed in serum as change (Δ), calculated by subtracting postprandial/exercised state SL (ΔSL) and HA (ΔHA) from fasted, rested conditions at SL. Compared with SL, exogenous glucose oxidation, glucose rate of disappearance, and glucose metabolic clearance rate (MCR) were lower ($P < 0.05$) during exercise at HA. One hundred and eighteen metabolites differed between ΔSL and ΔHA ($P < 0.05$, $Q < 0.10$). Differences in metabolites indicated increased glycolysis, tricarboxylic acid cycle, amino acid catabolism, oxidative stress, and fatty acid storage, and decreased fatty acid mobilization for ΔHA. Branched-chain amino acids and oxidative stress metabolites, Δ3-methyl-2-oxobutyrate ($r = -0.738$) and Δγ-glutamylalanine ($r = -0.810$), were inversely associated ($P < 0.05$) with Δexogenous glucose oxidation. Δ3-Hydroxyisobutyrate ($r = -0.762$) and Δ2-hydroxybutyrate/2-hydroxyisobutyrate ($r = -0.738$) were inversely associated ($P < 0.05$) with glucose MCR. Coupling global metabolomics and glucose kinetic data suggest that the underlying cause for diminished exogenous glucose oxidative capacity during aerobic exercise is acute hypoxia-mediated peripheral insulin resistance.

branched-chain amino acids; fatty acids; glycogenolysis; high altitude; substrate oxidation

INTRODUCTION

Acute high-altitude (HA) exposure (<8 h) suppresses exogenous glucose oxidation during steady-state aerobic exercise compared with metabolically matched exercise at sea level (SL) (1–4). In two separate studies (1, 4), our laboratory observed that consuming exogenous glucose during metabolically matched, steady-state aerobic exercise was associated with higher concentrations of circulating glucose and insulin, and lower rates of exogenous glucose oxidation during acute HA exposure compared with SL. Furthermore, glucose rate of disappearance (R_d) and metabolic clearance rate (MCR), kinetic measures indicative of glucose uptake and utilization (5, 6), were both lower at HA compared with SL (1). Collectively, these metabolic dysregulations suggest that acute hypoxia elicits peripheral insulin resistance in healthy exercising adults (7). Although our glucose kinetics and circulating glucose and insulin responses suggest impaired insulin sensitivity as a potential mechanism contributing to the reduction in exogenous glucose oxidation, a better understanding of this metabolic dysregulation is required to identify interventions to mitigate these effects of acute HA exposure.

Nontargeted metabolomics analysis is a methodological approach that may provide greater insight into metabolic alterations that manifest when exogenous glucose oxidation

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Submitted 15 March 2021 / Revised 3 May 2021 / Accepted 4 May 2021

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is impaired at HA. This approach provides a comprehensive and sensitive analysis, allowing for the simultaneous measurement of hundreds to thousands of metabolites which can capture changes in whole body metabolism (8, 9). Metabolite profiling may provide greater insight into hypoxia-induced insulin resistance as a mechanism resulting in changes in metabolic pathways associated with impaired substrate oxidation, mobilization, and storage. Recent investigations have used metabolite profiling to gain a greater understanding into metabolic alterations with altitude acclimatization of ≥14 days (10, 11). This approach has also been effectively used to characterize metabolic dysregulation between healthy individuals and those with insulin resistance or type 2 diabetes (12–14). In addition, individual metabolites of branched-chain amino acid (BCAA) metabolism and oxidative stress have been identified as markers of these disease states (12–14). Identifying similar patterns in healthy adults during aerobic exercise at HA would strengthen evidence of hypoxia-induced insulin resistance and aid in identifying potential therapeutic targets.

The objective of this study was to assess the effects of acute HA exposure on changes in global metabolomic profiles during aerobic exercise while consuming carbohydrate. We hypothesized that metabolomic profiles would differ between SL and HA, and that these differences would be reflective of hypoxia-induced insulin resistance. In addition, metabolites that differ between SL and HA will be associated with reductions in exogenous glucose oxidation rate, glucose R_d, and MCR at HA compared with SL.

METHODS AND MATERIALS

Participants

Participants in this study were a part of a larger randomized crossover study that examined the impact of acute HA (hyperbaric hypoxia) on exogenous glucose oxidation and glucose turnover during metabolically matched, steady-state aerobic exercise compared with SL (1). Eight healthy, recreationally active men (age: 23 ± 2 yr) completed the study. Individuals were excluded from participation if they had any metabolic, cardiovascular, or gastrointestinal disorders, prior diagnosis of high-altitude pulmonary edema or high-altitude cerebral edema, evidence of apnea or other sleeping disorders, presence of asthma or respiratory tract infection (<1 mo of data collection), taking medications that interferes with oxygen delivery, anemia (HCT < 38% and Hgb < 12.5 g/dL), sickle cell anemia/trait, born at altitudes >2,100 m, living at altitudes >1,200 m, were smokers, refused to abstain from alcohol, smokeless tobacco, and dietary supplement use during the study, had musculoskeletal injury that compromised ability to exercise, or donated blood with 8 wk of beginning the study. All data collection took place at the United States Army Research Institute of Environmental Medicine (USARIEM, Natick, MA), during November and December 2018. This study was approved by the Institutional Review Board at the US Army Medical Research and Development Command (MRDC, Fort Detrick, MD; www.clinicaltrials.gov; NCT03851744).

Height (Seritex, Inc., Carlstadt, NJ), body mass (WB-110A, Tanita, Tokyo, Japan), and body composition (dual energy X-ray absorptiometry, DPX-IQ, GE Lunar Corporation, Madison, WI), were used to characterize the participants (body mass: 83 ± 9 kg, height: 178 ± 7 cm, body mass index: 26 ± 3 kg/m^2, fat mass: 21 ± 5 kg, fat-free mass: 63 ± 5 kg). Peak oxygen uptake ($\dot{V}O_{2peak}$) was assessed during a progressive intensity, treadmill (Trackmaster TMX425C, Newton, KA) running exercise test using an indirect, open circuit respiratory system (True Max 2400, Parvo Medics, Sandy, UT) to prescribe exercise intensities. Participants completed assessments of $\dot{V}O_{2peak}$ under SL (4.3 ± 0.2 L/min) and HA (2.9 ± 0.2 L/min) conditions.

Study Design

As previously described (1), to normalize the effects of diet and exercise on endogenous carbohydrate stores before the experiments, 48 h before testing, participants completed a glycogen depletion protocol by cycling (Lode, BV, The Netherlands) at various intensities until failure (15), and were then fed a controlled diet (5.9 ± 0.2 g/kg/day carbohydrate, 1.2 ± 0.1 g/kg/day protein, and 1.0 ± 0.1 g/kg/day fat) before each study arm. At the conclusion of the normalization phase, participants reported to the hypobaric chamber after a 10-h overnight fast to complete the trial under SL (757 mmHg) and HA (460 mmHg) conditions. To match our previous work (16), participants rested quietly while exposed to SL or HA conditions for 5 h, and then they completed 80 min of metabolically matched, steady-state exercise on a treadmill, while consuming 145 g of glucose (1.8 g/min). Glucose turnover was assessed using 6-6-[$^2H_2$]glucose tracer methodologies. Indirect calorimetry and breath sampling for $^{13}C/^{12}C$ expired in CO_2 were used to determine carbohydrate and fat oxidation during exercise at SL and HA. Blood samples for metabolomics analysis were collected 20 min before exercise under resting, fasted conditions at SL, and after 40 min of aerobic exercise at both SL and HA conditions. After a minimum 7-day washout period, participants returned to the laboratory to complete the second arm of the investigation. Treatment (SL vs. HA) order was randomized using a random numbers generator to avoid order bias.

Steady-State Treadmill Exercise

After 48 h of controlled feeding and exercise, participants reported to the hypobaric chamber after a 10-h overnight fast to complete the trial under SL (757 ± 10 mmHg) or HA (459 ± 2 mmHg) conditions. To match our previous work (4), participants sat quietly while exposed to SL or HA conditions for 5 h. They then completed 80 min of steady-state exercise on a treadmill, while consuming 145 g of glucose (1.8 g/min) enriched with 200 mg [^{13}C]glucose (Cambridge Isotope Laboratory, Andover, MA). Treadmill speed (3.7 ± 0.3 mph) and grade (2% ± 0%) were matched between conditions to match the absolute exercise intensity between SL ($\dot{V}O_2$: 1.66 ± 0.14 L/min, 329 ± 28 kcal) and HA ($\dot{V}O_2$: 1.59 ± 0.10 L/min, 320 ± 19 kcal). Glucose turnover was assessed using a primed (82.2 μmol/kg), continuous (0.78 μmol/kg/min) infusion of 6-6-[$^2H_2$]glucose, provided 2 h before and throughout exercise. Participants ingested 58 g carbohydrate immediately before exercise, followed by consumption of 29 g carbohydrate at 20, 40, and 60 min during exercise. The carbohydrate drink was prepared by the Combat Feeding Directorate (Natick,

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MA), containing corn-derived dextrose (CERELOSE, Ingredion, Westchester, IL). Nutrient content was confirmed using gas chromatography (Covance Laboratories, Inc., Madison, WI). Indirect calorimetry and breath sampling for $^{13}C/^{12}C$ expired in CO_2 were used to determine carbohydrate and fat oxidation during exercise at SL and HA.

Exogenous Glucose Oxidation and Turnover

As previously reported (1), exogenous glucose oxidation during metabolically matched, steady-state aerobic exercise was calculated using equations by Peronnet et al. (17) under SL and HA conditions. The Steele equation (18) with modifications for nonsteady state was used to calculate glucose R_d and MCR under SL and HA conditions. The differences in exogenous glucose oxidation, R_d, and MCR between SL and HA conditions were used in the current analysis to determine associations of these primary outcome measures of the parent study to metabolite concentrations during exercise.

Metabolomics

Serum samples for metabolomics analysis were collected by antecubital intravenous draw at 20 min before exercise under resting, fasted at SL, and after 40 min of aerobic exercise while consuming carbohydrate at SL and HA conditions. Samples were centrifuged at 3,000 rpm at 4°C for 10 min. Serum was then stored at −80°C until analysis. Samples were analyzed using four separate methods: two separate reverse phase (RP)/ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), a RP/UPLC-MS/MS method with negative ion mode ESI, and a hydrophilic interaction (HILIC)/UPLCMS/MS method with negative ion mode ESI (Metabolon Inc., Morrisville, NC). Technical replicates, blanks, internal standards, and several recovery standards were analyzed with experimental samples for quality control. Raw data were extracted, peaks identified, and quality control processed using proprietary hardware and software. The relative quantitation values are based on integrated peak areas (area under the curve). All samples were analyzed on an equivalency basis based on volume.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a references library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon (Metabolon, Inc.) (19, 20). The level of identification for the majority of the compounds detected meets the highest standard of metabolite identification according to the Metabolomics Standards Initiative (21). Several types of controls were analyzed in concert with the experimental samples. A pooled matrix sample was generated by taking a small volume of each experimental sample to serve as a technical replicate throughout the dataset. Extracted water samples served as process blanks. A cocktail of quality control standards that would interfere with the measurement of endogenous compounds was spiked into every analyzed sample to allow instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample before injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., noninstrument standards) present in 100% of the pooled matrix samples.

Statistical Analysis

Sample size calculations were based on primary study outcome of exogenous glucose oxidation, which has been previously reported (1). Analyses were completed using R v4.0.3, SPSS v26 (IBM Analytics; Armonk, NY), ArrayStudio (Omicsoft, Corp.; Cary, NC), and MetaboAnalyst v.5.0 (22). Before analysis of metabolomics data, any missing values were imputed using the minimum observed peak area for each compound. Peak area for each metabolite was then normalized to set the mean equal to 0, and log10 transformed to meet model assumptions. Metabolites were analyzed as change (Δ), calculated by subtracting peak area during postprandial/exercised state SL (ΔSL) and HA (ΔHA) from peak area during fasted, rested conditions at SL.

Orthogonal projections to latent structures discriminant analysis, hierarchical clustering of Euclidean distances, and pattern hunter analysis were conducted using MetaboAnalyst v.5.0 (22) to assess the effect of condition (ΔSL vs. ΔHA) on changes in global metabolite profiles. Paired t test was also used to determine differences between ΔSL and ΔHA. Statistically significant differences were then associated with between-condition differences (Δ) in exogenous glucose oxidation, glucose R_d, and MCR using Spearman's correlation coefficient. To account for multiple comparisons, the Benjamini–Hochberg method was used to estimate false discovery rate (Q value). Statistical significance was set at $P < 0.05$ and $Q < 0.10$.

RESULTS

As previously reported (1), exogenous glucose oxidation was 0.09 ± 0.09 g/min lower ($P < 0.05$), Glucose R_d was 1.66 ± 1.69 mg/kg/min lower, and MCR was 3.11 ± 3.00 mg/kg/min lower ($P < 0.05$) during metabolically matched, steady-state exercise at HA compared with SL.

Metabolomics analysis measured 1,162 metabolites. Of those, 952 could be identified. Orthogonal projections to latent structures discriminant analysis demonstrated a clear separation in metabolite profiles by condition (Fig. 1). Hierarchical clustering and pattern search analysis identified multiple metabolites that clustered together and were most strongly associated with differences between conditions (Fig. 2, *A* and *B*). In total, 188 metabolites demonstrated significant ($P < 0.05$, $Q < 0.10$) differences between ΔSL and ΔHA (Supplemental Table S1; see https://doi.org/10.6084/m9.figshare.14597352). Of those significant metabolites, 8% were within glycolysis and tricarboxylic acid (TCA) cycle pathways, 39% were within amino acid metabolism pathways, 2% were oxidative stress pathways, and 15% were within fatty acid metabolism pathways.

Within the glycolysis pathway, increases in lactate and pyruvate were higher in ΔHA compared with ΔSL (Fig. 3, *A* and *B*). Increases in the TCA cycle metabolites malate, fumarate,

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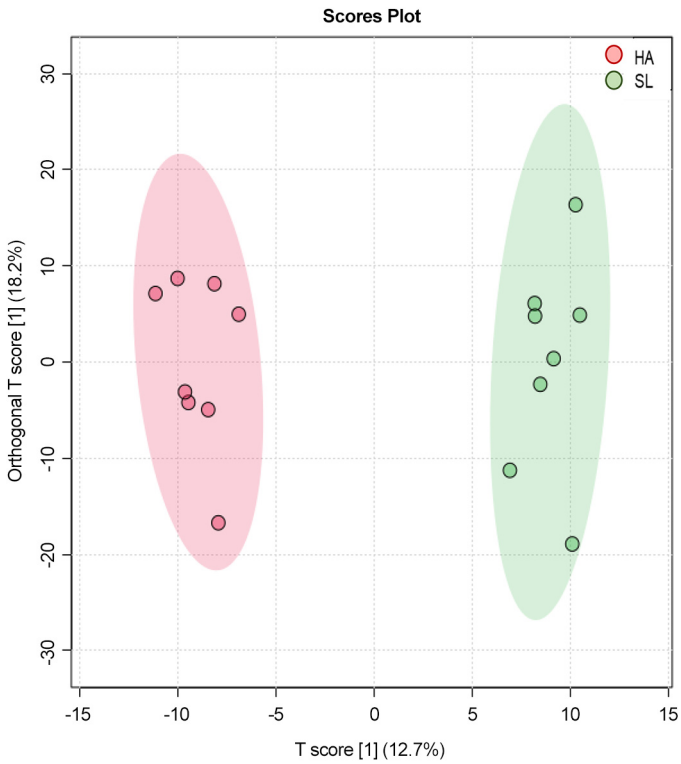


Figure 1. Orthogonal projections to latent structures discriminant analysis score plot for all metabolite features samples based on subject (n = 8) and condition [sea level (SL) and high altitude (HA)]. Circles represent individuals participants under each experimental condition.

citrate, aconitate (*cis* or *trans*), and α-ketoglutarate were also higher ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL (Fig. 3, *C–G*). Conversely, increases in succinate were lower ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL, and larger decreases ($P < 0.05$, $Q < 0.10$) in succinylcarnitine were observed for ΔHA compared with ΔSL (Fig. 3, *H* and *I*).

Decreases in branched-chain amino acids (BCAA), leucine, isoleucine, and valine, were greater ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL (Fig. 4, *A–C*). Downstream BCAA metabolites, 3-hydroxyisobutyrate, 3-methyl-2-oxobutyrate, and 4-methyl-2-oxopentanoate, were higher ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL (Fig. 4, *D–F*). ΔValine was positively associated with Δexogenous glucose oxidation ($P < 0.05$, $r = 0.786$) and Δglucose R_d ($P < 0.05$, $r = 0.786$; Fig. 4, *G* and *H*). Δ3-Hydroxyisobutyrate and Δ3-methyl-2-oxobutyrate were inversely associated with ΔMCR ($P < 0.05$, $r = -0.762$) and Δexogenous glucose oxidation ($P < 0.05$, $r = -0.738$), respectively (Fig. 4, *I* and *J*). Within other pathways of amino acid metabolism, larger reductions in multiple histidine and urea cycle-related metabolites were observed for ΔHA compared with ΔSL (Table 1).

Changes in γ-glutamylalanine and 2-hydroxybutyrate/2-hydroxyisobutyrate, both markers of oxidative stress, were higher ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL (Fig. 5, *A* and *B*). Δ2-Hydroxybutyrate/2-hydroxyisobutyrate was inversely associated with ΔMCR ($P < 0.05$, $r = -0.738$; Fig. 5*C*). Δγ-Glutamylalanine was inversely associated with Δexogenous glucose oxidation ($P < 0.05$, $r = -0.810$) and Δglucose R_d ($P < 0.05$, $r = -0.881$; Fig. 5, *D* and *E*).

Changes in several fatty acid-related metabolites were different ($P < 0.05$, $Q < 0.10$) between ΔHA compared with ΔSL (Table 2). Decreases in multiple carnitine and choline metabolites were greater ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL. In contrast, increase in malonate, a metabolite in the fatty acid synthesis pathway, was higher ($P < 0.05$, $Q < 0.10$) for ΔHA compared with ΔSL.

DISCUSSION

The main finding of this study was that acute HA exposure altered circulating metabolomics profiles during metabolically matched, steady-state aerobic exercise compared with SL conditions. Significant differences were observed in metabolites within sub-pathways of glycolysis, TCA cycle, BCAA metabolism, oxidative stress, and fatty acid metabolism. Changes in several of these metabolites were associated with decreases in exogenous glucose oxidation, glucose R_d, and MCR at HA, suggesting that changes in activity within these metabolic pathways under acute HA conditions may mediate hypoxia-induced insulin resistance.

The primary outcome from the parent study (1) was that exogenous glucose oxidation was reduced during aerobic exercise under acute HA conditions compared with SL. Accompanying lower rates of exogenous glucose oxidation were increased circulating glucose and insulin concentrations and reductions in glucose R_d and MCR (1). These shifts in glucose kinetics are characteristic of insulin resistance (7) and suggest that reductions in peripheral glucose uptake may be a primary factor contributing to lower rates of exogenous glucose oxidation at HA. We thus hypothesized that the observed dysregulations in glucose metabolism were the result of hypoxia-induced insulin resistance. Using nontargeted metabolomics analysis in this secondary investigation demonstrated greater increases in metabolites indicative of oxidative stress, γ-glutamylalanine, and 2-hydroxybutyrate/2-hydroxyisobutyrate, during exercise at HA relative to SL, which were associated with larger declines in exogenous glucose oxidation, MCR, and glucose R_d. Increased oxidative stress is common during unacclimitized high-altitude exposure, potentially due to decreased oxygen pressure resulting in elevated free radical production and reductions in plasma antioxidant capacity (23, 24). Higher concentrations of these metabolites have also been observed in individuals with type 2 diabetes and insulin resistance (25, 26).

Increased oxidative stress in the current study may be associated with hypoxia-induced lipid accumulation under HA compared with SL conditions (27, 28). Lower concentrations of choline and acyl choline metabolites, carnitine, and higher concentrations of malonate under HA conditions indicate decreased fatty acid mobilization and fatty acid transport into the mitochondria, and increased fatty acid synthesis (29, 30). Low choline availability impairs fatty acid β-oxidation and results in mitochondrial dysfunction increasing oxidative stress (28, 31). Low carnitine and high acyl carnitine concentrations also increase oxidative stress and have been linked to insulin resistant populations (32, 33). Accumulation of acyl carnitines with hypoxia exposure has been reported in human (34) and rodent models (35), with the latter also reporting concurrent reductions in fat oxidation. Declines in the rate fat oxidation with HA exposure

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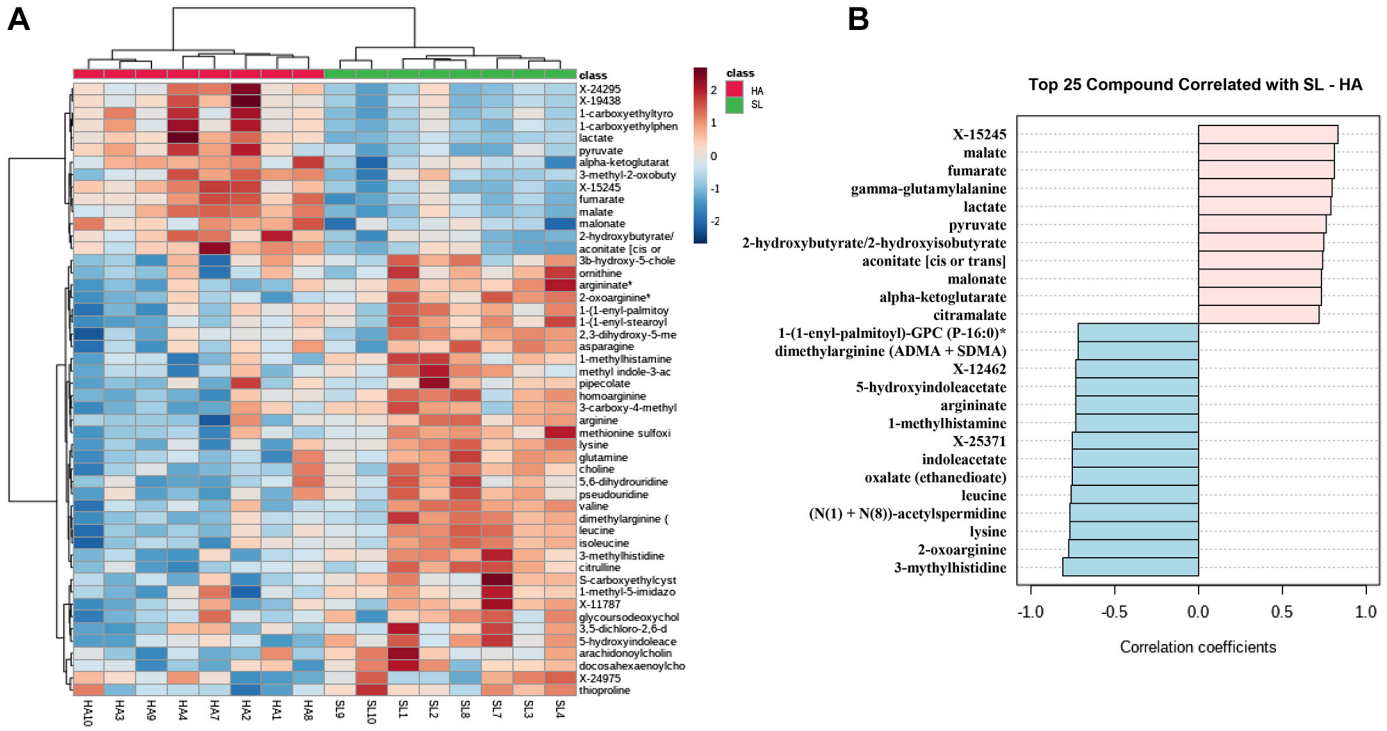


Figure 2. *A*: heatmap of hierarchical cluster analysis of the 50 metabolites with the lowest *Q* values between changes in sea level (ΔSL) and high altitude (ΔHA). *B*: pathfinder analysis of the 25 metabolites most highly associated with differences between ΔSL and ΔHA.

were reported in our parent study (1) and may have been the result of hypoxia-induced reductions in carnitine, which is essential for transporting long-chain fatty acids into the mitochondria for β-oxidation. Interestingly, supplementation of L-carnitine during hypoxia exposure functions as an antioxidant, reduced oxidative stress, and improved physical performance in rats (36, 37). Whether L-carnitine supplementation with acute HA exposure in

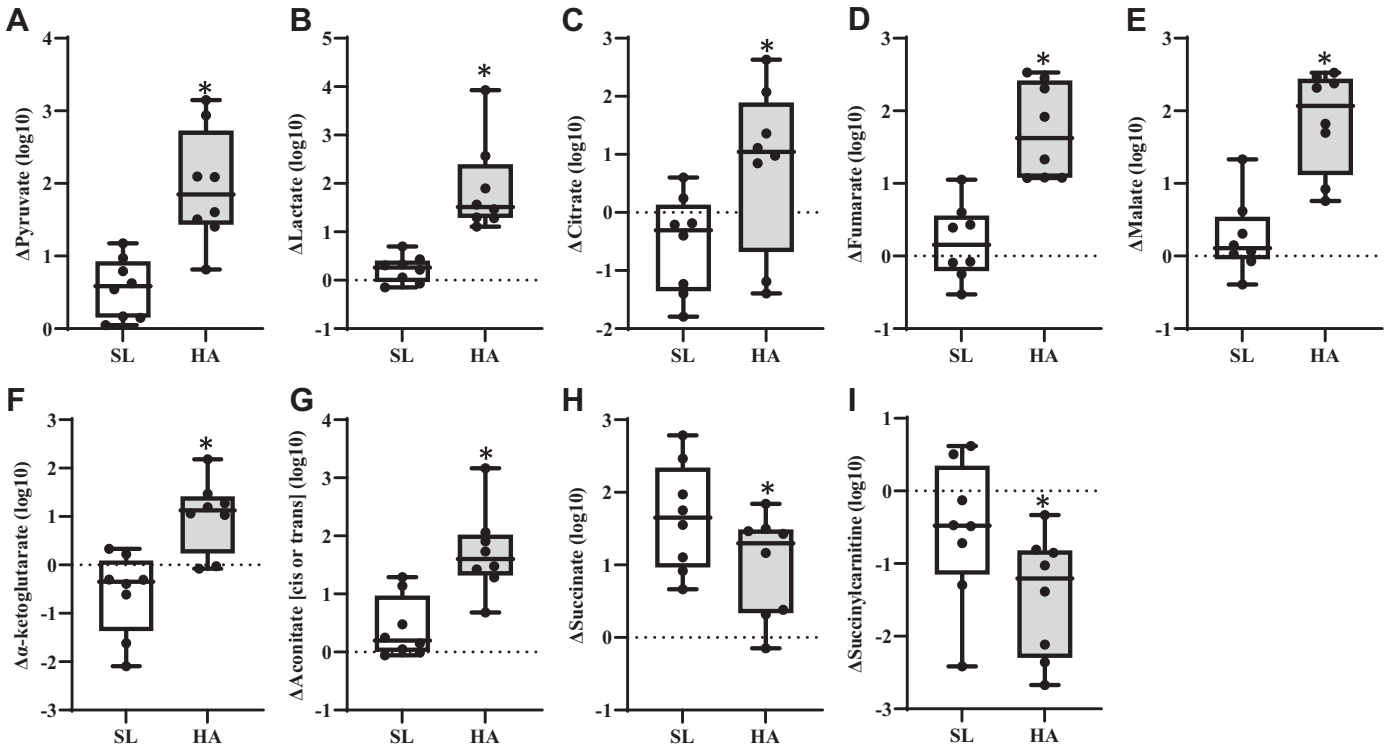


Figure 3. Box plots of difference from resting, fasted conditions at sea level (SL) in glycolysis (*A* and *B*), and tricarboxylic acid (TCA) metabolites (*C*–*I*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental Table S1.

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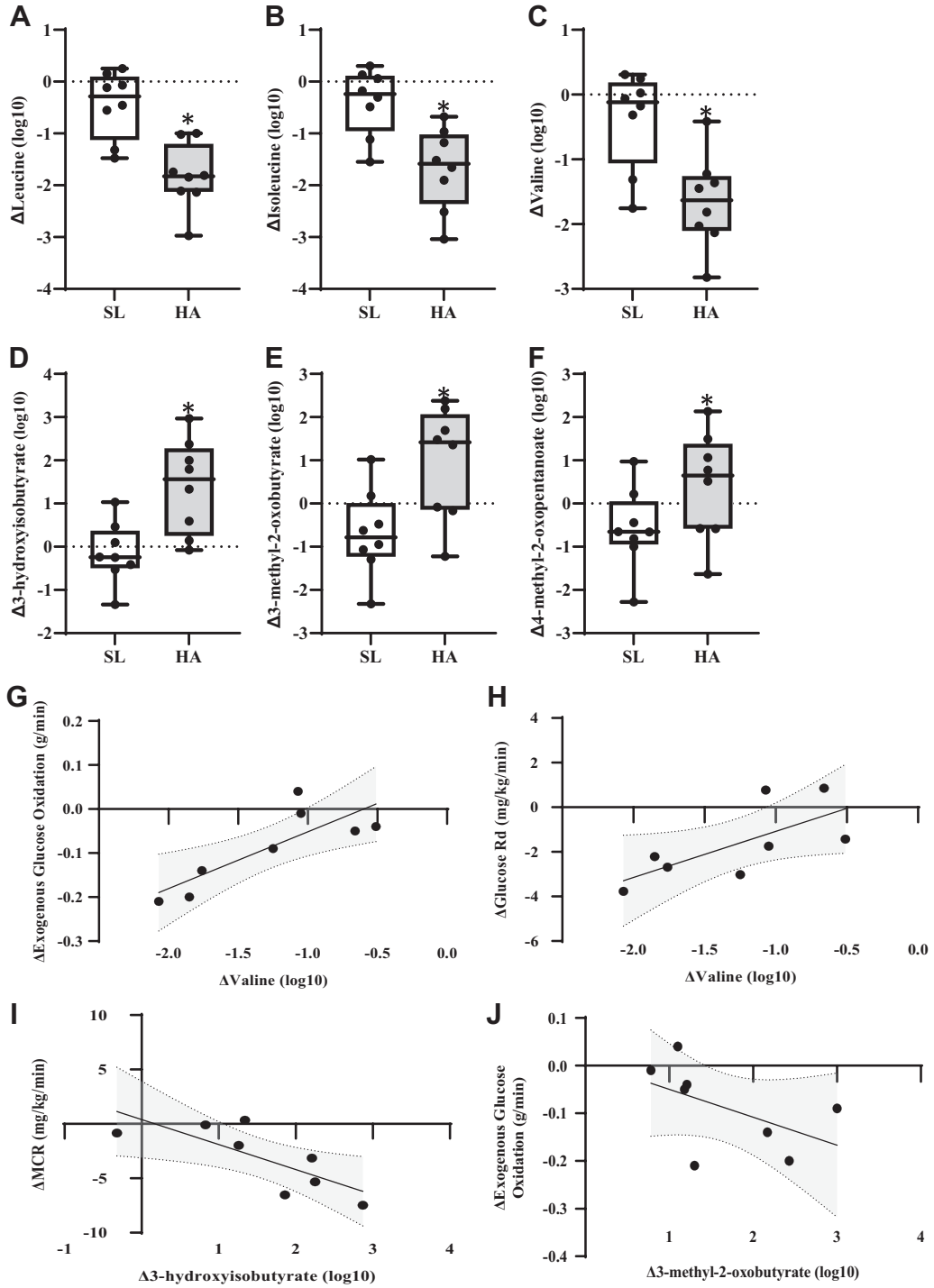


Figure 4. Box plots of difference from resting, fasted conditions at sea level (SL) in branched-chain amino acids (BCAA) metabolites (*A*–*F*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental Table S1. Significant associations in delta (HA-SL) BCAA metabolites to delta exogenous glucose oxidation, metabolic clearance rate (MCR), and glucose R_d (*G*–*J*).

humans can reduce oxidative stress to mitigate hypoxia-induced insulin resistance, and improve exercise performance is unclear.

Several BCAA metabolites that had a greater increase for ΔHA compared with ΔSL have also been previously associated with type 2 diabetes and insulin resistance (12). Higher concentrations of the BCAA metabolites 3-hydroxyisobutyrate, 3-methyl-2-oxobutyrate, and 4-methyl-2-oxopentanoate are associated with insulin resistance and type 2 diabetes in both human and mouse models (13, 38–40). In agreement with previous animal and human diseased state studies, our results demonstrate that greater increases in 3-hydroxyisobutyrate and 3-methyl-2-oxobutyrate were associated with lower MCR, an indicator of glucose uptake

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Table 1. *Histidine and urea cycle metabolites*

Subpathway	Metabolite	ΔSea Level	ΔHigh Altitude	*P* Value	*Q* Value
Histidine metabolism	1-Methyl-5-imidazoleacetate	0.30 (−0.20, 0.79)	−0.76 (−1.55, 0.03)	0.001965	0.034
	1-Methyl-5-imidazolelactate	0.23 (−0.27, 0.73)	−1.00 (−1.96, −0.04)	0.004776	0.048741
	1-Methylhistamine	0.62 (0.09, 1.16)	−0.68 (−1.23, −0.13)	0.000161	0.01762
	3-Methylhistidine	−0.52 (−0.95, −0.09)	−1.81 (−2.22, −1.40)	2.40 *E*−05	0.010267
	Histidine	−0.56 (−0.81, −0.31)	−1.56 (−2.36, −0.76)	0.009504	0.07169
	N-acetylhistidine	0.13 (−0.35, 0.61)	−0.74 (−1.34, −0.15)	0.004244	0.044842
Urea cycle metabolism	2-Oxoarginine	0.28 (−0.22, 0.77)	−0.97 (−1.41, −0.54)	0.001858	0.03393
	Argininate	0.07 (−0.43, 0.56)	−1.16 (−1.70, −0.63)	0.000257	0.01762
	Arginine	−0.72 (−1.16, −0.29)	−1.87 (−2.55, −1.19)	0.001006	0.025854
	Citrulline	−0.90 (−1.50, −0.29)	−2.06 (−2.47, −1.64)	0.000376	0.01762
	Dimethylarginine (ADMA + SDMA)	−0.18 (−0.98, 0.62)	−1.75 (−2.27, −1.23)	0.001237	0.026432
	Homoarginine	−0.41 (−1.10, 0.28)	−1.79 (−2.42, −1.16)	0.000442	0.01762
	N-acetylarginine	−0.36 (−1.23, 0.52)	−1.20 (−1.93, −0.48)	0.006469	0.056118
	Ornithine	−0.43 (−1.31, 0.44)	−1.47 (−2.22, −0.72)	0.001856	0.03393

Values mean (95% confidence interval) log10, presented as the delta during postprandial/exercise under sea level and high-altitude conditions minus fasted, rested sea level conditions. Main effect of condition for all metabolites; $P < 0.05$ and $Q < 0.10$.

into peripheral tissue, and exogenous glucose oxidation, respectively.

Hypoxia-mediated changes in BCAA metabolites may, in part, be due to increased glycogenolysis and reductions in endogenous glucose stores during acute HA exposure. Our laboratory (1) and others (41–43) have shown endogenous glucose oxidation is higher and fat oxidation is lower during metabolically matched aerobic exercise under acute HA compared with SL. Increased glycogenolysis was reflected in the current study with higher increases in glycolysis metabolites pyruvate and lactate at HA. Higher pyruvate with HA exposure may explain the larger increase in TCA cycle metabolites during HA. However, concomitant increases in lactate production under the hypoxic conditions suggest increased anaerobic glycolysis. Higher pyruvate and lactate at HA may be due to increased hypoxia inducible factor 1α (HIF-1α) upregulating PDK1 via deactivation of PDH, preventing the conversion of pyruvate to acetyl-CoA (44). Increases in TCA cycle metabolites under HA conditions may instead reflect alternate carbon flow for oxidation (45). It is unlikely that increases in TCA metabolites are derived from fatty acids, as both fat oxidation and related fatty acid

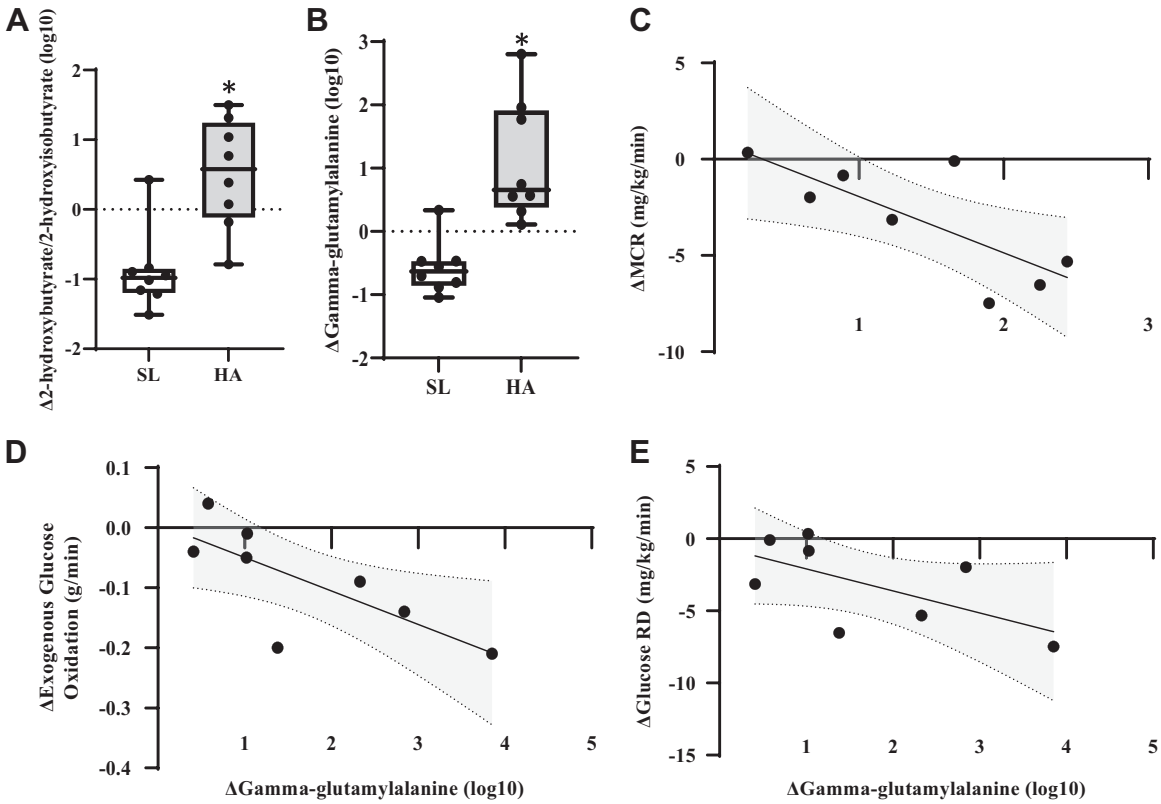


Figure 5. Box plots of difference from resting, fasted conditions at sea level (SL) in oxidative stress metabolites (*A* and *B*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental Table S1. Significant associations in delta (HA-SL) oxidative stress metabolites to delta exogenous glucose oxidation, metabolic clearance rate (MCR), and glucose R_d (*C*–*E*).

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Table 2. *Fatty acid metabolites*

Subpathway	Metabolite	ΔSea Level	ΔHigh Altitude	*P* Value	*Q* Value
Carnitine Metabolism	Carnitine	−0.19 (−0.74, 0.35)	−1.06 (−1.89, 0.23)	0.007039	0.058157
	Deoxycarnitine	−0.16 (−1.01, 0.69)	−0.76 (−1.55, 0.03)	0.006051	0.053835
Fatty acid metabolism (acyl carnitine, hydroxy)	(*S*)-3-Hydroxybutyrylcarnitine	0.34 (−0.17, 0.84)	1.45 (0.54, 2.37)	0.016166	0.096671
Fatty acid metabolism (acyl choline)	Arachidonoylcholine	−1.21 (−1.67, −0.74)	−1.97 (−2.43, −1.52)	0.000187	0.01762
	Docosahexaenoylcholine	−1.07 (−1.56, −0.57)	−1.85 (−2.28, −1.41)	0.000287	0.01762
	Eicosapentaenoylcholine	−1.09 (−1.69, −0.50)	−1.65 (−2.37, −0.92)	0.002779	0.036451
Fatty acid synthesis	Malonate	−0.91 (−1.81, −0.01)	1.01 (0.35, 1.67)	4.47 *E*−05	0.010267
Fatty acid, dicarboxylate	3-Carboxy-4-methyl-5-pentyl-2-furanpropionate (3-CMPFP)	−0.16 (−0.74, 0.43)	−1.46 (−2.22, −0.70)	0.002219	0.035351
	Octadecanedioate (C18)	−0.28 (−1.00, 0.44)	0.51 (−0.24, 1.27)	0.010877	0.076246
Phospholipid metabolism	Choline	−0.22 (−0.96, 0.51)	−1.63 (−2.49, −0.77)	0.002523	0.035729

Values mean (95% confidence interval) log10, presented as the delta during postprandial/exercise under sea level and high-altitude conditions minus fasted, rested sea level conditions. Main effect of condition for all metabolites; $P < 0.05$ and $Q < 0.10$.

metabolites were reduced at HA. Greater decreases in leucine, isoleucine, and valine, with increases of downstream BCAA metabolites at HA may indicate increased reliance on BCAA carbon skeletons for energy production via the TCA cycle (46). Similar alterations in glycolysis, TCA, and BCAA metabolite profiles have been reported during ascent to Everest base camp (47) and following 4 days of exposure to hypobaric hypoxia at 5,300 m (34). Low energy or glycogen availability may increase the reliance of BCAA for substrate to be used for energy production and maintenance of glucose homeostasis (48, 49). Leucine and isoleucine can be broken down and converted to acetyl-CoA to enter the TCA cycle for energy production (46, 50). Acetyl-CoA then enters the TCA through its conversion to citrate, which aligns with higher concentrations of citrate at HA compared with SL in the current study.

Despite increases in the majority of TCA metabolites, succinate decreased to a greater extent during aerobic exercise at HA compared with SL. Lower succinate may be reflective of changes in BCAA metabolites. Unlike leucine and isoleucine, valine enters the TCA cycle through conversion to succinyl-CoA, which in turn is converted to succinate (46). Increase in 3-hydroxyisobutyrate, a valine metabolite, during exercise at HA may suggest impaired conversion of this metabolite to enter the TCA cycle to be used for energy. The subsequent higher increase in fumurate within the TCA cycle at HA may have resulted from the conversion of urea cycle metabolites for energy use (51). This increased reliance on urea and other amino acid metabolites for oxidative purposes may be a marker of negative net protein balance during acute hypoxia exposure. Our laboratory (52) has previously reported that reductions in plasma urea and histidine metabolites occur concurrently with negative net protein balance following 4 days of ∼55% activity-induced energy deficit. Reductions in concentrations of these metabolites have also been reported with decreased dietary protein intake (53), which results in negative protein balance. Taken together, these findings suggest that increased reliance on amino acids for oxidative purposes may, in part, help explain declines in mechanistic target of rapamycin complex 1 anabolic signaling (54), negative net protein balance (55, 56), and reductions in muscle mass (55–57) that occur in unacclimatized lowlanders sojourning at HA.

Our current study provides novel insight into the changes of metabolomics profiles while consuming carbohydrate during aerobic exercise under acute HA exposure. However, several limitations should be acknowledged. A sample size of eight for the parent study was generated based on anticipated differences of −14 ± 8 g/40 min exercise in exogenous glucose oxidation at HA compared with SL (1). This sample size was appropriate to address the primary outcome of differences in substrate oxidation and glucose turnover between SL and HA conditions; however, it is relatively small for metabolomics analysis. Though relatively small, other studies of similar sample size, $n = 14$ (7 men, 7 women) and $n = 10$ (6 men and 4 women) have effectively used metabolomics analysis to characterized metabolic alterations after 16 (10), 3, and 14 days (11) of hypoxia exposure. In addition, in the current study, the alterations in metabolomic profiles largely align with the results from the substrate oxidation in the parent study (1). Agreement between indirect calorimetry/isotope and metabolomic datasets, along with larger differences in effects between study conditions, enhances the physiological relevance and likely validity of the results. Furthermore, to account for the small sample size we assessed our data as deltas during exercise under acute high altitude and sea level conditions from our control resting/fasted sample collected under sea level conditions. This approach not only allowed us to isolate the effects of the exercise response between the two conditions, but also limited the number of data points being compared which enhance our statistical power. Similarly, the use of a crossover study designed enhanced the statistical power of our work by allowing each participant to act as their own control. It should also be noted that the results of our study should be taken in the context of our current study design. Our study exposed male participants to conditions equal to an altitude 4,300 m for ∼8 h. Only males were included in this investigation so outcome measures from the parent study could be appropriately compared with our previous work, which only had male participants. Sex-based differences in substrate oxidation during exercise at SL may result in differences in response to change in substrate oxidation at HA between men and women. Variant results may be observed using female participants, different severities of altitude, and more prolonged exposures.

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It is important to state that while changes in metabolomics profiles during HA are reflective of changes in substrate oxidation and mirror profiles of insulin resistant populations, causality cannot be determined. The information obtained from this analysis can be used to identify potential interventions for future investigation to overcome changes in metabolite profiles to improve glucose tolerance to support physical performance at HA. Based on the present study findings, short-term use of insulin sensitizing drugs, such as metformin or pioglitazone, may be appropriate with unacclimatized HA exposure to efficiently metabolize dietary carbohydrate. Alternatively, potential nutrition interventions such as antioxidant supplement L-carnitine or choline supplementation to reduce oxidative stress and enhance fat oxidation may improve metabolic dysregulation by increasing reliance on fatty acids for fuel during exercise, minimizing oxidative stress associated with insulin resistance.

In conclusion, results from this study show differences in circulating metabolite profiles during exercise under acute HA compared with SL conditions, indicating increased glycolysis and TCA cycle activity, amino acid breakdown, oxidative stress, and fatty acid storage, and decreased fatty acid mobilization. Increased concentrations and inverse associations of metabolites within BCAA and oxidative stress pathways with exogenous glucose oxidation, glucose R_d, and MCR suggest that changes in metabolite profiles under acute HA conditions may be reflective of hypoxia-induced insulin resistance. These data provide new insight into the potential underlying alterations in metabolic pathways that govern metabolic dysregulation in substrate oxidation under acute HA exposure.

SUPPLEMENTAL DATA

Supplemental Table S1: doi.org/10.6084/m9.figshare.14597352.

ACKNOWLEDGMENTS

The authors thank the volunteers for participating in this research experiment. The authors acknowledge our medical oversight team for support of this study. The authors sincerely thank Dr. David Chuch, Dr. Robert Wolfe, Dr. Jess Gwin, Claire Whitney, Christopher Carrigan, Nancy Murphy, Patrick Radcliffe, Adrienne Hatch-McChesney, and Heather Fagnant from the US Army Research Institute of Environmental Medicine, and Danielle Anderson from the Combat Feeding Directorate for contributions to the project.

GRANTS

This material is based on the work supported by DHP JPC-5/MOMRP and through fellowship appointments to the US Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education (to J. L. Coleman) through an interagency agreement between the US Department of Energy and the US Army Medical Research and Development Command.

DISCLAIMERS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.M.M., A.A.F., A.J.Y., and S.M.P. conceived and designed research; L.M.M., M.A.W., A.J.Y., and S.M.P. performed experiments; L.M.M., J.P.K., and J.L.C. analyzed data; L.M.M., J.P.K., A.J.Y., and S.M.P. interpreted results of experiments; L.M.M. and J.L.C. prepared figures; L.M.M. drafted manuscript; L.M.M., J.P.K., M.A.W., J.L.C., A.A.F., A.J.Y., and S.M.P. edited and revised manuscript; L.M.M., J.P.K., M.A.W., J.L.C., A.A.F., A.J.Y., and S.M.P. approved final version of manuscript.

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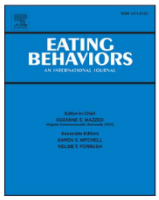
Eating Behaviors 42 (2021) 101532



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Exceeding body composition standards is associated with a more negative body image and increased weight cycling in active duty U.S. soldiers

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ARTICLE INFO

Keywords:
Body image
Weight cycling
Body composition
Military

ABSTRACT

Negative body image is more often identified in individuals with overweight or obesity. More than 65% of U.S. soldiers have a body mass index (BMI) that classifies them with overweight or obesity. Additionally, all soldiers must meet body composition and physical fitness standards which may increase the risk of negative body image. This cross-sectional study aimed to identify associations between compliance with body composition standards, body image, and weight cycling through surveying 969 active-duty soldiers (86% male, 24.0 ± 5.5 years of age, BMI 26.0 ± 3.6 kg/m^2). Compliance with body composition standards was measured by whether a soldier had ever taken and failed the circumference-based body fat assessment. Weight cycling was self-reported as ≥3 weight fluctuations of ≥5% of body weight during their military career. Multivariate linear and logistic regression models were used to examine the relationship between compliance with body composition standards, body image, weight cycling, and sociodemographic characteristics while controlling for BMI. Failing the circumference-based body fat assessment was associated with increased concern with conforming to military image. Negative body image was associated with higher odds of weight cycling. Further examination is warranted to understand the effects of body composition standards on soldiers' body image and weight cycling.

1. Introduction

Body image is a multifactorial construct related to a person's perceptions and attitudes towards their body, and is linked to a person's level of body satisfaction (Tylka & Wood-Barcalow, 2015a). Conceptually, body image can be divided into two subcategories: positive and negative body image (Tylka & Wood-Barcalow, 2015a, 2015b). Positive body image is correlated with less instances of depression and fewer unhealthy dieting behaviors (Gillen, 2015); while negative body image is associated with greater body shame and body dissatisfaction (Denny et al., 2013; Gast et al., 2012; Tylka & Wood-Barcalow, 2015b). In addition, individuals with negative body image are more likely to internalize negative concepts about themselves and be preoccupied with how they look (Tylka & Wood-Barcalow, 2015b). Promoting positive body image may therefore lead to better overall mental and physical health outcomes (Jones, 2004; Tiggemann, 2003; Tylka & Wood-Barcalow, 2015a).

In civilian populations, negative body image is more often identified in individuals with overweight and obesity (Gillen, 2015). Similar to U.S. civilian populations, 67% of U.S. soldiers have a BMI that classifies them as having overweight or obesity, indicating that the prevalence of negative body image in soldiers may be high (Health of the Force, 2019). Additionally, all soldiers must meet body composition and physical fitness standards during their time-in-service (Army Regulation 600-9, 2019; Department of Defense Directive, 2004). Soldiers undergo a BMI-based weight assessment every six months and if sex- and age-

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https://doi.org/10.1016/j.eatbeh.2021.101532
Received 22 April 2020; Received in revised form 13 May 2021; Accepted 19 May 2021
Available online 24 May 2021
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specific BMI cut-offs are exceeded, soldiers must undergo further assessment using body circumference measurements to assess body fat percentage (Army Regulation 600-9, 2019). Twice yearly, they must also complete a standardized physical fitness test. Failure to meet either the body circumference measurement or physical fitness standard requires additional weight and physical fitness monitoring and can slow or prevent career progression. Whether the scrutiny created by these standards negatively impacts body image and leads to stigma surrounding overweight and physical fitness abilities is not clear.

Attempting to adhere to the "ideal" military image and to adhere to the body composition standards could lead to unhealthy weight loss techniques among soldiers, especially those who have greater difficulty meeting standards (Piche et al., 2014). Unhealthy weight-loss techniques noted among soldiers with overweight and obesity include the use of diet pills (49%), skipping meals/fasting (45%), and laxatives (22%) (Piche et al., 2014). These and other unhealthy weight loss techniques have also been associated with negative body image and stress in meeting body composition standards among soldiers (Cole et al., 2018). Extreme measures to induce weight loss can result in weight cycling, a repetitive cycle of weight loss and weight regain (Mackie et al., 2017). Evidence suggests that weight cycling could be predictive of future weight gain (Kroke et al., 2002), which could increase difficulty in complying with body composition standards, exacerbate negative body image, and contribute to a cycle of unhealthy weight loss techniques to counteract weight regain.

Physical fitness and body composition requirements in military personnel may affect body image (Clark et al., 2017; Cole et al., 2018). However, the relationships between body composition standard compliance, body image, and weight cycling have not been thoroughly studied. This cross-sectional study aimed to examine associations between body composition standards, body image, and weight cycling. It was hypothesized that failing body composition standards would be associated with more negative body image and higher odds of weight cycling.

2. Methods

2.1. Study population

This study assessed 969 (835 males and 134 females) active duty Army soldiers surveyed between May 2017 and February 2018 at five U.S. military installations. Data used for this study was part of a larger study which developed and validated a survey for assessing eating behaviors and mediators of eating behaviors specifically in military personnel (manuscript in press). Questions for the survey were developed based on validated surveys used in civilian populations. The surveys used obtained information related to body image, compliance with body composition standards, weight cycling, and sociodemographic information (weight, height, age, time-in-service, gender, rank, marital status, education level, and race/ethnicity). Inclusion criteria were active duty U.S. Army soldiers, at least 18 years of age, and who had completed initial entry military training. The U.S. Army Medical Research and Development Command Institutional Review Board approved the study. All participants provided written informed consent prior to participation.

2.2. Body image

The construct of body image was measured by the recently developed and validated Military Body Image Scale (manuscript in press). The Military Body Image Scale is comprised of three subscales with additive scores: Concern with Conforming to Military Image, Agreeance with the Ideal Military Physique, and Satisfaction with Body Shape. The Concern with Conforming to Military Image subscale included four questions focused on soldiers' perception of their current physical appearance and its impact on their professional capabilities within the military. The Agreeance with the Ideal Military Physique subscale included three questions centered on perceived physique characteristics related to muscularity, thinness, and fatness. The Satisfaction with Body Shape subscale included five questions about desire to change specific areas of the body. All items were scored on a 5-point Likert-type Scale ranging from 1 = definitely disagree to 5 = definitely agree. Scores ranged from 12 to 60 points with a higher score reflective of a more negative body image and a lower score reflective of a more positive body image.

2.3. Weight cycling

Weight cycling was assessed by a question stating, "During your military career, how many times has your weight gone up AND down by more than 5% of your body weight (excluding pregnancy or illness)?". Researchers used the value of 5% as a meaningful weight change that may have clinical significance (Jortberg et al., 2015) and significance to meeting Army body composition standards since even a five pound weight change can be the difference between a soldier being in or out of compliance (Army Regulation 600-9, 2019). The survey provided examples of 5% weight change for 120–300 lbs to provide clarification to soldiers answering this question (ex: 120 lb. = 6 lbs). Response options ranged from "never" with continuous number options up to the final response of "10 or more times". Responses were dichotomized by identification of weight cycling or not; responses with $\geq$3 reports of weight fluctuations of >5% were defined as weight cycling based on previous research using this cut-off (Jayne et al., 2019; Field et al., 1999).

2.4. Body composition standards, and body mass index

Compliance with body composition standards is assessed twice yearly with a height/weight screening. Those that exceed the maximum allowable weight for their height undergo a circumference-based body fat assessment often referred to as the tape test. The maximum allowable weight for height is at or above 25.0 kg/m^2 based on gender and age cutoffs given that soldiers may have more muscle mass than civilian populations (U.S. Army Public Health Center, n.d.). For soldiers under the age of 21, BMI cutoffs are 25.0 kg/m^2 for females and 25.9 kg/m^2 for males. For soldiers between the age of 21–27, BMI cutoffs are 25.3 kg/m^2 for females and 26.5 kg/m^2 for males. For soldiers between the age of 28–39, BMI cutoffs are 25.6 kg/m^2 for females and 27.2 kg/m^2 for males. For soldiers 40 and older, BMI cutoffs are 26.0 kg/m^2 for females and 27.5 kg/m^2 for males. Males are measured at two circumference sites: neck and waist. Females are measured at three circumference sites: neck, waist, and hip. In this study, compliance with body composition standards was assessed using one question "Have you ever failed the body fat tape test?" answered with either a "yes," "no," or "never taken or tested." "Yes" indicates a soldier underwent the circumference-based body fat assessment and failed the tape test. "No" indicates a soldier underwent the circumference-based body fat assessment but passed the assessment. "Never taken or tested" indicates a soldier passed the weight for height assessment and did not have to undergo further assessment in the form of the tape test. Dummy variables were used to code this variable for linear regression analysis using never taken or tested as the reference category. Body weight and height were self-reported and used to calculate body mass index (BMI). BMI was analyzed as a continuous variable.

2.5. Race/ethnicity, education level, marital status, time in service, and rank

Race/ethnicity was assessed by two questions asking about ethnic background and racial background and was split into four groups: Non-Hispanic White, Non-Hispanic Black, Non-Hispanic Other, and Hispanic. Education level was assessed by one question asking for the highest level of education completed by the soldier and was grouped into three

groups: high school/GED, some college/associates degree, and bachelor's degree or higher. Marital status was assessed by one question and was dichotomized into two groups: single, widowed, divorced, separated and married or living with partner. The self-reported "time-in-service" or length of time a soldier had been in the Army was analyzed continuously. Soldier's rank was self-reported.

2.6. Data analysis

Analyses were conducted using SPSS Statistics v.24 (IBM Corp.; Armonk, NY). Statistical significance was set at $p < 0.05$. Cronbach's alpha was analyzed to assess scale reliability for the Military Body Image Scale, Concern with Conforming to Military Image subscale, Agreeance with the Ideal Military Physique Subscale, and Satisfaction with Body Shape subscale.

The Military Body Image Scale contains three distinct constructs that help to differentiate which aspects of military body image were most associated with failing the circumference-based body fat assessment. Thus, two sets of analyses were conducted to determine whether certain subscales may contribute more or less strongly to the association between body image and weight cycling. To determine the association between failing body composition standards and body image, separate multiple linear regression models were conducted with the Military Body Image scale, Concern with Conforming to Military Image subscale, Agreeance with the Ideal Military Physique subscale, and Satisfaction with Body Shape subscale as the dependent variables, respectively. Each model included adherence to body composition standards as the independent variable and six covariates (time-in-service, BMI, gender, race/ethnicity, education level, marital status, and rank). To assess the association between body image and weight cycling, a multivariate logistic regression was conducted with weight cycling as the dependent variable. The model included one independent variable (Military Body Image scale) and six covariates (time-in-service, BMI, gender, race/ethnicity, education level, marital status, and rank). To determine the association between the three body image subscales (Concern with Conforming to the Ideal Military Image subscale, Agreeance with the Ideal Military Physique subscale, and Satisfaction with Body Shape subscale) and weight cycling, a multivariate logistic regression was conducted with weight cycling as the dependent variable. During survey development, a portion of the participants were newly enlisted soldiers only three to 12 days into their initial military training. They had no "military career" opportunities for weight fluctuation at that time. Therefore, only 439 Soldiers who completed their initial military training were included in this analysis resulting in a smaller sample subset for analyses that included weight cycling. For all regression models, assumptions were tested by regression diagnostic techniques and were met. For linear regression models, variance explained was calculated to assess model fit and for logistic regression models, the area under the receiver operator characteristics (ROC) curve was used to assess model prediction and discrimination.

3. Results

A majority of the population was male, junior enlisted soldiers (76.4%) with an average BMI of 26.0 ± 3.6 kg/m^2, and mean age of 24.0 ± 5.5 years (Table 1). A total of 85.4% of soldiers underwent body composition testing at some point in their military career, with 15.5% reporting having failed standards. Weight cycling was reported in 34.5% of the sample. Of the participants with a healthy BMI (18.5–24.9 kg/m^2), 31.0% reported having weight cycled. For this sample (n = 969), Concern with Conforming to Military Image, Agreeance with the Ideal Military Physique, Satisfaction with Body Shape, and Military Body Image showed good internal consistency (Table 2).

In the multiple linear regression models, failing the circumference-based body fat assessment relative to not undergoing the circumference-based body fat assessment was positively associated with Concern with Conforming to Military Image, but was not associated with the Military Body Image scale score, the Satisfaction with Body Shape subscale score or Agreeance with the Ideal Military Physique subscale score. Additionally, undergoing the circumference-based body fat assessment and passing relative to not undergoing the circumference-based body fat assessment was positively associated with the Satisfaction with Body Shape subscale score. BMI was positively associated with the Military Body Image score, the Satisfaction with Body Shape subscale score, and the Agreeance with the Ideal Military Physique subscale score (Table 3).

In the multivariate logistic regression model, each one unit increase in the Military Body Image scale score was associated with a 1.07 higher odds of weight cycling (Table 4).

In the multivariate logistic regression model, for each one unit

Table 1
Characteristics of soldiers (N = 969).

Sociodemographic variables	Mean ± SD or %
Failed body composition standards	
Yes	15.5
No	69.9
Never taken or tested	14.3
Weight cycling	
Yes	34.5
No	65.5
Age, years	24.0 ± 5.5
Body mass index, kg/m^2	26.0 ± 3.6
Underweight	0.6
Normal weight	55.1
Overweight	43.5
Obese	0.7
Time-in-service, years	3.5 ± 4.2
Gender	
Male	86.0
Female	14.0
Rank categories	
Enlisted	90.9
Officer	9.1
Marital status	
Single, widowed, divorced, separated	59.8
Married or living with Partner	40.2
Education level	
High school/GED	50.8
Some college/associates degree	35.4
Bachelor's degree or higher	13.7
Race/ethnicity	
Non-Hispanic White	49.0
Non-Hispanic Black	17.0
Non-Hispanic Other	12.0
Hispanic	22.0

Note: Weight cycling was defined as responses with ≥3 reports of weight fluctuations of 5% during a soldier's military career. GED = General Education Degree; SD=standard deviation; Non-Hispanic Other = Native American/Alaskan Native, Asian, Native Hawaiian/Pacific Islander, and other; Hispanic = Hispanic or Latino.

Table 2
Descriptive statistics for Military Body Image Scale scores in soldiers (N = 969).

Variable	Range	Mean	SD	Sk	Ku	α
Military Body Image Scale	12–60	35.49	8.18	−0.22	−0.24	0.88
Concern Conforming to Military Image	4–20	11.93	3.05	−0.49	0.34	0.83
Agreeance with the Ideal Military Physique	3–15	8.42	2.28	0.16	−0.11	0.63
Satisfaction with Body Shape	5–25	15.18	5.45	−0.30	−0.67	0.71

Note: Items were rated on a 5-point Likert scale ranging from 1 (definitely disagree) to 5 (definitely agree). All body image scales were coded using a 1 to 5 scale. SD=standard deviation; Sk = skewness; Ku = kurtosis; α = Cronbach alpha coefficient.

Table 3
Results of Multiple linear regression analyses for Military Body Image Scale (N = 944), concern Conforming to Military Image Scale (N = 960), Agreeance with the Ideal Military Physique Subscale (N = 958), and Satisfaction with Body Shape Scale (N = 952) by failing the circumference-based body fat assessment in soldiers.

	Military Body Image Scale		Concern Conforming to Military Image		Agreeance with the Ideal Military Physique		Satisfaction with Body Shape	
	$\beta \pm SE$	p	$\beta \pm SE$	p	$\beta \pm SE$	p	$\beta \pm SE$	p
Predictor								
Failed body fat assessment								
Yes	1.35 ± 2.02	0.50	1.12 ± 0.41	0.01*	0.18 ± 0.38	0.63	11.45 ± 6.19	0.06
No	−2.32 ± 1.68	0.17	0.03 ± 0.28	0.91	−0.18 ± 0.28	0.51	9.94 ± 4.98	0.04*
Covariates								
Body mass index	0.50 ± 0.11	0.02*	0.04 ± 0.04	0.27	0.23 ± 0.05	<0.001*	0.51 ± 0.19	0.01*
Time-in-service	0.98 ± 0.52	0.06*	–	–	0.16 ± 0.14	0.26	0.03 ± 0.14	0.85
Male	1.52 ± 1.02	0.14	1.05 ± 0.39	0.01*	0.81 ± 0.44	0.06*	−1.11 ± 0.97	0.25
Officer/warrant officer	−2.00 ± 2.05	0.33	–	–	–	–	–	–
Some college	−1.09 ± 1.26	0.39	−1.71 ± 0.44	<0.001*	−0.29 ± 0.43	0.51	−0.97 ± 1.13	0.39
Bachelor's degree or higher	2.24 ± 2.96	0.45	1.10 ± 0.93	0.24	−0.11 ± 0.60	0.85	−2.73 ± 1.35	0.04*
Married	−11.04 ± 4.21	0.01*	−2.48 ± 1.49	0.10	0.67 ± 0.50	0.18	−6.70 ± 2.69	0.01*
Non-Hispanic White	−5.03 ± 1.89	0.01*	−0.26 ± 0.33	0.43	−1.42 ± 1.33	0.29	−0.52 ± 0.60	0.39
Non-Hispanic Black	−5.69 ± 2.40	0.02*	−1.22 ± 0.43	<0.001*	−1.34 ± 1.57	0.39	−1.74 ± 0.75	0.02*
Non-Hispanic Other	0.26 ± 2.56	0.92	−0.54 ± 0.50	0.29	0.25 ± 1.67	0.88	−0.28 ± 0.84	0.74

Note: Variance explained: 12.0%, 6.8%, 18%, and 7.7% for Military Body Image, Concern Conforming to Military Image, Agreeance with the Ideal Military Physique, and Satisfaction with Body Shape, respectively. Stepwise analyses conducted; no information indicates covariate was not included in stepwise linear regression analysis. Referent group is never taken or tested for failed body fat assessment, enlisted, Hispanic, female, some high school education, and single. Column β refers to standardized regression coefficients. Level of significance: $p < 0.05$.

* Significant p values.

Table 4
Results of logistic regression model of weight cycling by military body image (N = 438).

Predictors and control variables	OR [95% CI]	p
Military Body Image Scale	1.07 [1.03, 1.10]	<0.001*
Body mass index	1.11 [1.04, 1.18]	<0.001*
Time in service	1.19 [1.06, 1.33]	<0.001*
Gender		
Female	1.28 [0.61, 2.69]	0.52
Male	1.00	–
Rank categories		
Officer/warrant officer	0.82 [0.24, 2.85]	0.76
Enlisted	1.00	–
Marital status		
Married or living with partner	0.76 [0.47, 1.22]	0.25
Single, widowed, divorced, separated	1.00	–
Education level		
Some college/associates degree	0.96 [0.59, 1.57]	0.86
Bachelor's degree or higher	1.54 [0.50, 4.70]	0.45
Some high school/GED	1.00	–
Race/ethnicity		
Non-Hispanic Black	1.46 [0.79, 2.71]	0.23
Non-Hispanic Other	1.14 [0.54, 2.40]	0.73
Hispanic	1.29 [0.76, 2.21]	0.35
Non-Hispanic White	1.00	–

Note: Area under the operating characteristic (ROC) curve statistic for the model was 0.66. Non-Hispanic Other = Native American/Alaskan Native, Asian, Native Hawaiian/Pacific Islander, and other; Hispanic = Hispanic or Latino. Model excluded 7 soldiers with missing data. Level of significance was 95% confidence intervals corresponding to $p < 0.05$.

Table 5
Results of logistic regression model of weight cycling by Military Body Image subscales (N = 439).

Predictors and control variables	OR [95% CI]	p
Agreeance with the Ideal Military Physique	1.14 [1.02, 1.27]	0.02*
Concern Conforming to Military Image	1.02 [0.95, 1.10]	0.61
Satisfaction with Body Shape	1.06 [1.01, 1.12]	0.02*
Body mass index	1.10 [1.03, 1.18]	0.01*
Time in service	1.20 [1.07, 1.34]	<0.001*
Gender		
Female	1.33 [0.63, 2.82]	0.45
Male	1.00	–
Rank categories		
Officer/warrant officer	0.85 [0.24, 2.96]	0.80
Enlisted	1.00	–
Marital status		
Married or living with partner	0.75 [0.47, 1.21]	0.24
Single, widowed, divorced, separated	1.00	–
Education level		
Some college/associates degree	0.93 [0.57, 1.53]	0.47
Bachelor's degree or higher	1.52 [0.50, 4.65]	0.40
Some high school/GED	1.00	–
Race/ethnicity		
Non-Hispanic Black	1.51 [0.81, 2.84]	0.20
Non-Hispanic Other	1.11 [0.53, 2.34]	0.79
Hispanic	1.28 [0.74, 2.19]	0.34
Non-Hispanic White	1.00	–

Note: Area under the operating characteristic (ROC) curve statistic for the model was 0.63. Non-Hispanic Other = Native American/Alaskan Native, Asian, Native Hawaiian/Pacific Islander, and other; Hispanic = Hispanic or Latino. Model excluded 7 soldiers with missing data. Level of significance was the 95% confidence intervals (CI) corresponding to $p < 0.05$.

increase in the Agreeance with the Ideal Military Physique subscale score and the Satisfaction with Body Shape subscale score, the odds of weight cycling are expected to increase by a factor of 1.14 and 1.06, respectively (Table 5). The Concern Conforming to Military Image subscale score and weight cycling did not have a statistically significant association.

4. Discussion

This study aimed to identify associations between adherence to body composition standards, body image, and weight cycling among U.S. Army soldiers. While several studies have reported that having to adhere to body composition standards in athletics and professional occupations is associated with a more negative body image, there has not been a study assessing the relationship between these constructs in military populations (DeFeciani, 2016; Fortes et al., 2013; Khodaee et al., 2015; Kong & Harris, 2014). In addition, to the best of our knowledge, there has not been a study assessing the relationship between these constructs along with weight cycling. The main findings of this study demonstrate that failing the circumference-based body fat assessment is associated with the Concern with Conforming to Military Image subscale of the

Military Body Image scale. Additionally, the Military Body Image scale, the Satisfaction with Body Shape subscale, and the Agreeance with the Ideal Military Physique were associated with weight cycling.

Failing the circumference-based body fat assessment, was associated with higher concern with conforming to military image. This may suggest that soldiers who do not meet the body composition standards have greater concern with conforming to the military image. Additionally, BMI was associated with the Military Body Image score, the Satisfaction with Body Shape subscale score, and the Agreeance with the Ideal Military Physique subscale score. This could suggest that soldiers with a higher BMI have a more negative body image and lower agreeance with the ideal military image versus soldiers with a lower BMI. These results are consistent with literature comparing athletes in sports with body composition standards (DeFeciani, 2016; Fortes et al., 2013; Khodaee et al., 2015). For example, Kong & Harris reported that female athletes involved in sports where leanness is emphasized, such as gymnasts and lightweight rowers, report higher body dissatisfaction and exhibit greater disordered eating symptomatology (Kong & Harris, 2014). Additionally, individuals with greater body dissatisfaction and unhealthy eating behaviors are more likely to normalize these thoughts and behaviors (Tantleff-Dunn et al., 2011). Trying to conform to an ideal physique can lead to athletic-ideal internalization, an aspect of negative body image that involves the idea that one must be physically fit for acceptance (Bell et al., 2016). Findings of this study suggest a similar phenomenon may occur among soldiers though the study design precludes demonstrating a causal relationship.

Results of this study also demonstrated that a more negative body image was associated with higher odds of weight cycling, a result which was driven by lower agreeance with military physique and decreased satisfaction with body shape. This observation could suggest that weight stability (i.e., less weight cycling) positively impacts these aspects of body image, and/or that negative body image and increased frequency of weight cycling are interrelated. Athletes in sports with body composition standards have decreased self-esteem and higher frequency of weight cycling (Franchini et al., 2012). For these athletes, rapid weight loss has been shown to have negative psychological and physiological effects including increased fatigue, depression, decreased concentration, and a decrease in both aerobic and anaerobic performance (Franchini et al., 2012). Military personnel are often considered similar to athletes who participate in sports with body composition requirements, due to high physical demands, the routine assessment of body composition, and requirements for physical fitness. It is not known whether weight cycling among soldiers has similar effects on mental health outcomes. However, it has been found that greater amounts of weight lost over a shorter period of time have been found to be more predictive of weight regain (Turicchi et al., 2019). As such, weight cycling could ultimately make it more difficult for soldiers to adhere to the body composition standards, contributing to a cycle of difficulty in meeting body composition standards and negative body image. Conversely, soldiers with a more positive body image may be less likely to experience weight cycling which may ultimately support adherence to body composition standards and performance requirements.

The observed relationships between adherence to body composition standards, body image and weight cycling were independent of BMI, and 31% of soldiers with a healthy BMI reported weight cycling. This suggests that pressure to adhere to body composition standards may negatively impact soldiers' body image and weight stability regardless of whether or not they have a BMI that would put them at risk for failing the standard. This finding could also suggest that many soldiers who are meeting the body composition standards do so with a great deal of effort that may result in more frequent bouts of weight cycling. Both possibilities are concerning given that negative body image and weight stigma are associated with unhealthy eating behaviors, body shame, and body dissatisfaction (Schaefer et al., 2017; Shank et al., 2019). Further, individuals with a greater perceived weight stigma are more inclined to use compensatory behaviors including unhealthy eating behaviors to lose weight (Araiza & Wellman, 2017). Often the use of unhealthy weight loss techniques is seen in individuals who weight cycle and can lead to future weight gain (Khodaee et al., 2015; Saarni et al., 2006). Research has indicated that unhealthy weight loss techniques are more likely to be used by soldiers with a negative body image (Cole et al., 2018). Although, weight loss methods used among soldiers who had negative body image and weight cycling were not assessed in this study, and causality cannot be determined, findings may suggest that body image promotion strategies should not be restricted to only soldiers who have high BMI.

Strengths of this study include the use of a survey created using language and questions tailored specifically for military populations, and the inclusion of a relatively large sample of soldiers from multiple military installations with diverse occupations. However, several limitations should be noted. In particular, the study relied on self-report measures, and causal relationships between body image, body composition compliance, and weight cycling could not be determined. Given that 86% of soldiers were male and over 90% were enlisted, these results may not be generalizable to women and/or officers in the Army. Another limitation of this study was that volunteers could have incorrectly chose "no" instead of "never taken or tested" when asked whether they had ever failed the tape test, which would result in misclassification. Weight cycling definitions vary across research studies making it difficult to compare results to previous research studies (Kroke et al., 2002; Field et al., 1999; Delahanty et al., 2014). Some varying definitions for weight cycling include unintentional weight gain and intentional weight loss of more than 5 kg during the last 2 years, intentional weight loss of $\geq$9 kg $\geq$3 times over 5 years, and weight gain and loss of $\geq$2.25 kg since last weight change (Kroke et al., 2002; Field et al., 1999; Delahanty et al., 2014). Ultimately, longitudinal studies are needed to definitively elucidate whether military standards influence soldiers' perception regarding body image and the effect on weight management behaviors and health outcomes. Additionally, future studies should examine weight loss techniques associated with weight cycling to determine how soldiers use weight cycling and weight loss techniques in the context of meeting body composition standards.

5. Conclusion

Study findings demonstrate an association between failing U.S. Army body composition standards, body image, and weight cycling among U.S. Army soldiers. Both negative body image and weight cycling have been associated with greater weight gain during an individual's lifetime, unhealthy weight loss practices, and undesirable health outcomes that may collectively degrade soldier health and readiness. As such, the extent to which health promotion efforts focused on promoting a positive body image and reducing weight cycling could improve soldier health and readiness warrant further investigation.

CRediT authorship contribution statement

Maria J. Stukenborg: conceptualization, methodology, formal analysis, investigation, writing – original draft. **Bethany A. Deschamps:** conceptualization, writing – review & editing, supervision. **Julianna M. Jayne:** conceptualization, formal analysis, investigation, writing – review & editing, supervision. **J. Philip Karl:** investigation, writing – review & editing. **Susan M. McGraw:** investigation, data curation, writing – review & editing. **Adam J. DiChiara:** investigation, writing – review & editing. **Renee E. Cole:** conceptualization, investigation, writing – review & editing, supervision.

Role funding sources

The work was supported by the US Army Medical Research and Development Command Fort Detrick, MD (Grant #18640). The MRDC did not influence the study design, collection, analysis or interpretation

of the data, writing the manuscript, or the decision to submit the paper for publication.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank the soldiers who volunteered to participate in this study. Additionally, we would like to thank Zachary Colburn for his assistance with the statistical analyses

Disclaimer

The views and information presented are those of the author and do not represent the official position of the U.S. Army Medical Department Center and School Health Readiness Center of Excellence, the U.S. Army Training Doctrine Command, or the Departments of the Army/Navy/Air Force, Department of Defense, or U.S. Government. The investigators have adhered to the policies for protection of human subjects as prescribed in 45 CFR 46

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Development and Validation of the Military Eating Behavior Survey

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ABSTRACT

Objective: To describe the Military Eating Behavior Survey (MEBS), developed, and validated for use in military populations.

Design: Questionnaire development using a 6-phase approach that included item generation, subject matter expert review, cognitive interviewing, factor analysis, test-retest reliability testing, and parallel forms testing.

Setting: US Army soldiers were surveyed at 8 military bases from 2016 to 2019 (n = 1,561).

Main Outcome Measure: Content, face, and construct validity and reliability of the MEBS.

Analysis: Item variability, internal consistency, and exploratory factor analysis using principal coordinates analysis, orthogonal varimax rotation, and scree test (correlation coefficient and Cronbach alpha), as well as consistency and agreement (intraclass correlation coefficient) of test-retest reliability and parallel forms reliability.

Results: Over 6 phases of testing, a comprehensive tool to examine military eating habits and mediators of eating behavior was developed. Questionnaire length was reduced from 277 items to 133 items (43 eating habits; 90 mediating behaviors). Factor analysis identified 14 eating habit scales (hunger, satiety, food craving, meal pattern, restraint, diet rigidity, emotional eating, fast/slow eating rate, environmental triggers, situational eating, supplement use, and food choice) and 8 mediating factor scales (body composition strategy, perceived stress, food access, sleep habits, military fitness, physical activity, military body image, and nutrition knowledge).

Conclusions and Implications: The MEBS provides a new approach for assessing eating behavior in military personnel and may be used to inform and evaluate health promotion interventions related to weight management, performance optimization, and military readiness and resiliency.

Key Words: military personnel, survey methods, questionnaire design, eating behavior (*J Nutr Educ Behav.* 2021;53:798–810.)

Accepted April 29, 2021. Published online June 30, 2021.

INTRODUCTION

Eating habits can be defined as modifiable behaviors and attitudes related to eating patterns,[1,2] eating rate,[3,4] food appeal,[5,6] and food choice,[6,7] to include the influence of appetite,[8,9] satiation,[6,9] and food rules[10,11] on dietary intake. Mediators of eating behavior can be defined as factors that influence eating behavior, such as physical activity,[7,12] sleep habits,[13] body satisfaction,[11,14] perceived stress,[15,16] tobacco and alcohol use,[17,18] nutrition knowledge,[19,20] and the eating environment.[21–23] There is a well-documented relationship between eating habits and mediators of eating behaviors and physical and mental performance, weight status, and overall health.[24–27]

The US military requires its personnel to maintain physical and mental fitness, readiness, and the Department of Defense develops and encourages participation in health and resilience promotion programs aimed at fostering nutrition- and health-related behaviors.[28,29] For example, Department of Defense-wide public health campaigns have stressed the importance of eating habits such as increasing fruit and vegetable intake and whole-grain consumption.[30,31] Despite these

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Conflict of Interest Disclosure: The authors have not stated any conflicts of interest.

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https://doi.org/10.1016/j.jneb.2021.04.467

efforts, US military service members (SMs) do not meet recommended dietary guidelines established for the US population at large and/or more active subpopulations (such as athletes).[24,32] In addition, more than half (52%) of SMs are overweight (body mass index [BMI] 25.0–29.9 kg/m^2), and 17% are obese (BMI ≥ 30 kg/m^2),[33] leaving them at risk for weight-related injuries and comorbidities.[34] Entry and retention in military service are contingent on meeting service-specific body composition standards.[35] Poor eating behaviors may jeopardize SMs' ability to meet those standards, increase the probability of military separation, and increase the risk of chronic disease. Thus, there is interest in understanding the eating habits of SMs, how those behaviors evolve, and the factors shaping that evolution.

The eating habits of SMs and the factors mediating those behaviors may be influenced by unique characteristics of military environments. Military SMs often experiences long training days or night operations that are physically demanding, disrupt sleep patterns, and sometimes restrict access to foods and the time allowed for meal consumption. Furthermore, SMs are subject to mandatory body composition testing and appearance standards, noncompliance with which can result in their discharge from the service.[35] Those unique environmental mediating factors can shape eating habits by altering perceptions of hunger/satiety signals[6,7,9,26]; increasing impulsivity, craving, and preoccupation of food[36–38]; and increasing desire for high-energy/low nutrient-dense foods.[6,39] By achieving a better understanding of the relationships between unique military environments, eating habits, dietary intake, and related health outcomes, new insights regarding the etiology of unhealthy or disordered eating patterns, unintentional weight gain, altered body satisfaction, and weight cycling in SMs which may subsequently contribute to separation from the service, chronic disease, and injury in this population can be gained (Figure 1).[16,22,36,37,40–42]

Eating habits and the mediating factors influencing eating behaviors are commonly assessed by quantitative survey methods. This enables longitudinal monitoring of eating habits and mediators of behavior in large cohorts to assess the effectiveness of interventions for modifying behaviors and associations with health and diet-related outcomes. Although numerous validated eating behavior surveys exist, most focus on only 1 specific aspect of behavior, knowledge, or attitude; do not examine an individual in a comprehensive, holistic manner; or have unnecessary or irrelevant content for a military population. Futhermore, to the authors' knowledge, no survey exists that adequately accounts for the unique aspects of military culture and environments that may mediate eating habits in SM. To address this gap, a Military Eating Behavior Survey (MEBS) was developed and validated.

METHODS

Research Design

The development and validation of the MEBS followed best practices in survey development,[43–53] and involved 6 phases: (1) item generation and development of the draft MEBS, (2) determination of content validity by an expert panel, (3) determination of face validity by cognitive interview of the target population in groups sessions, (4) determination of construct validity (ie, factor development) by exploratory factor analysis, (5) conducting test-retest evaluations to confirm internal consistency and reliability, and (6) performing parallel forms testing to assess the agreement of an electronic survey version (Figure 2). The investigative team consisted of a combination of military and civilian professionals that were registered dietitians, statisticians, behavioral health and consumer science professionals, and survey research experts. The team was involved in discussions and decisions at each stage of the survey development process. All study procedures were approved by the Institutional Review Board of the US Army Medical Research and Development Command, and all volunteers provided written informed voluntary consent before study participation. In all phases of the study, soldiers were eligible if they were aged ≥18 y, spoke English, and had completed initial military entry training with the exception of the construct validity phase, in which a subset of soldiers (n = 309) were purposefully enrolled during initial military training. Initial military training is when soldiers are first exposed to the military environment, values/beliefs, and requirements. The inclusion of initial trainees at this stage of the survey development was intentional to capture a sample of individuals undergoing this indoctrination process. The inclusion of initial trainees earlier in survey development would not have added value because of their lack of military experience.

Phase 1: Item Generation and Factor Development

Content for the survey was characterized conceptually by 2 main constructs: eating habits and mediators of eating behavior. Eating habits were defined as encompassing food-related behavior, habits, and attitudes related to eating patterns, eating rate, food appeal, food choice, and includes the influence of appetite, satiation, and food rules on dietary intake. Mediators of eating behavior were defined as factors previously documented in literature influencing eating behavior, habits, and attitudes. These mediators included stress, emotions, social and environmental triggers, physical activity, body image, nutrition knowledge, sleep habits, tobacco use, and weight management efforts.

Existing validated questionnaires for measuring constructs of interest were identified by performing a literature search with PubMed and the Defense Technical Information Center (Supplementary Table 1). Thirty-two existing validated questionnaires were identified as potential contributors to these 2 constructs (Supplementary Table 1). The research team identified relevant items (ie,



Figure 1. Military-specific eating behavior and mediators of behavior concept.

questions, statements, and descriptive responses) from the validated surveys and crafted new items specific to the military environment and culture. A pool of 271 items was identified for initial consideration and organized into 2 parts (139 eating habit items and 132 mediating factor items). The verbiage of some items was revised to employ military-specific language (eg, cafeteria was replaced with dining facility/galley), converted to a consistent past-tense format, grouped by time (eg, past 7 days for meal timing and physical activity, past 30 days for lifestyle behaviors, and 6 months for weight management efforts), and some categorical items were converted to 5-point Likert-type scale response options using a consistent format for scaling throughout the MEBS. Minor adjustments were made to the original validated perceived stress items: unable to was converted to (1) able to cope or (2) able to control, which required reverse coding; and 5-point Likert response options were modified for consistency with other item response options (never, rarely, sometimes, often, and always).

Phase 2: Content Validity

Content validity of the 2-part survey was completed with a 19-member expert review panel. The expert panel consisted of 15 subject matter experts from the government and academia with knowledge or experience in eating habits, weight management, behavioral theory, military culture, nutrition and performance, and survey development. The panel also included 4 enlisted soldiers who provided their perspective on initial military training and the military eating environment. A separate 4-member military officer panel of certified sports dietitians provided feedback specific to items assessing nutrition knowledge of general and performance nutrition topics. Expert panel members were asked to assess the nonvalidated items to determine if: (1) the item was succinct and clearly written; (2) the item response options adequately captured the information (exclusive and/or exhaustive); (3) the item added value to the proposed factors; (4) the item should be retained; (5) if so, rewording suggestions to improve clarity;

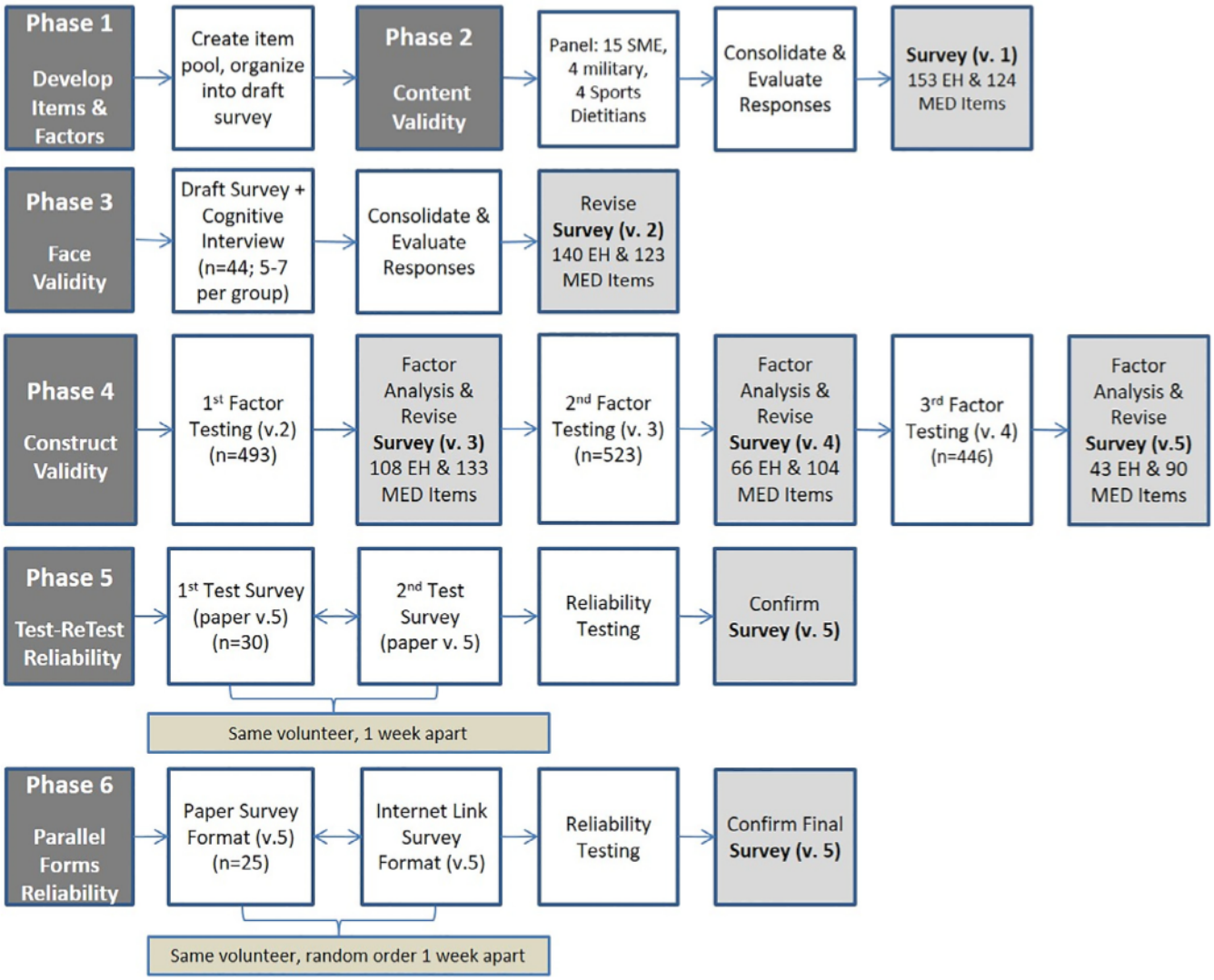


Figure 2. Diagram depicting the development and validation process of the Military Eating Behavior Survey. EH indicates eating habits; MED, mediators of eating behavior; SME, subject matter experts; V, version. Note: The number of items retained in each survey version is denoted as EH or MED in the light gray boxes.

and (6) new item additions to fill a gap area. The panelist's feedback was consolidated into 1 document and reviewed by the research team. Study staff used the expert panel results to direct subsequent modifications and format survey version 1 (V1) for testing.

Phase 3: Face Validity

Face validity was tested using cognitive interviewing techniques[44] to determine if the draft survey items were interpreted by the target population as intended by the research team. Ethnographic qualitative research with a purposive sampling strategy was conducted through group cognitive interview sessions to understand the thought process of participants as they answered survey items.[54] Group cognitive interview sessions were designed to discuss the understandability of language, appropriateness, applicability, format, and interpretation of survey V1 items and identify gap areas not previously identified that could contribute to further factor and/or construct definition.

A recruitment goal of 1:10 officer-to-enlisted and female-to-male ratios were sought to represent current US Army demographics during face and construct validity phases.[55] There is no consensus on the exact sample size required for qualitative cognitive interview methodology; however, the literature supports a sample size between 20 and 60 participants divided into groups of ≤10 as sufficient to reach response saturation in which no new themes arise.[54,56] Forty-four participants were recruited, provided informed consent in advance, and participated in scheduled cognitive interview sessions. Sessions were conducted by study team members experienced in qualitative methodology following rehearsal sessions to ensure consistency among the 3 interview facilitators.

Group interviewer procedures recommend limiting the group size <12 participants[44]; however, groups

of 4–7 military participants were chosen to foster ample discussion in a welcoming environment while accommodating the size of the interview room. Each cognitive interview session was conducted over a 4-hour time period that included at least two 15-minutes breaks. Participants were given 1 hour to complete the MEBS survey V1, were asked to mark confusing or questionable items, and annotate any specific thoughts on individual items. After consolidating all of the survey comments, the facilitator interviewed the participants as a group, using a probing method in which survey items were read aloud, and participants were asked to verbalize their thoughts as they decided how to respond. The facilitator focused initially on items marked by participants to ensure they were addressed within the allotted time. Cognitive interview sessions were also audio recorded with participant approval. Study staff listened to recorded interviews to ensure all concerns, suggestions, and gap areas were captured and consolidated for review.

The verbal responses in the cognitive interview recordings were consolidated into 1 document and reviewed by the research staff. The assessment included determining if each item was interpreted and comprehended as intended or presented cultural or level of education concerns. Research staff agreed on suggestions for clarification, changes to survey formatting and instructions, removal and revisions of items, and the creation of new questions related to survey gap areas. Follow-up cognitive interview sessions with the same participants were conducted on newly revised items to verify proper interpretation and comprehension. Study staff used the interview results to direct subsequent modifications to the survey version 2 (V2). The original section title (ie, Eating Behaviors) was changed to eating habits to prevent respondent confusion with mediators of eating behavior.

Phase 4: Construct Validity

Construct validity was assessed by pretesting the survey to ensure convergent and discriminant validity of the proposed factors from version 2 (V2) to version 4 (V4) (Figure 2). Testing determined item variance, internal consistency, and reliability to develop the structure of the survey through exploratory factor analysis, guided item removal, and shaped the final draft before Phase 5 (test-retest validity).[57,58] The sample size for exploratory factor analysis was based on the ratio of volunteers to items. O'Rourke and Hatcher[59] recommend that a ratio of at least 10:1 be used to ensure well-powered results. The largest number of items for any proposed factor was 21, requiring a sample size of 210 per testing iteration for a homogenous sample; however, the enrollment goal was 400 participants per testing iteration for adequate power given the sample diversity (male/female, enlisted/officer, and training/regular duty assignment) and the potential for missing data (estimated at 20%).

Participants in Phase 4 testing were enrolled in or had completed initial military entry training. They were recruited from the following 7 military installations over 3 iterations: Fort Jackson, SC (initial entry trainees only) and Joint Base Lewis-McChord, WA (V2 survey testing: January-March 2017); Joint Base San Antonio, TX, Fort Riley, KS, and Fort Carson, CO (V3 testing: May-August 2017); and Fort Campbell, KY and Joint Base Elmendorf-Richardson, AK (version 4 [V4] testing: February 2018). Half of the participants completed the eating habits survey first, whereas the other half completed the mediators of eating behavior survey first, with a scheduled break to reduce bias associated with survey fatigue. Study staff was available to answer questions and review surveys for missing data.

Both eating habits and mediating factors were revised, if needed, on the basis of the variance explained by each factor after testing each survey version. Analyses were conducted using SAS (version 9.4, SAS Institute Inc, 2013) and included assessing item variability, internal consistency reliability, and exploratory factor analysis. Item variability was examined to determine if items provide variability within the responses and were completed using descriptive analysis of response frequencies.[60] Exploratory factor analysis is a data reduction technique to identify the relationship between (or internal structure) for a set of variables.[57] To define the factor structure of the survey and reduce extraneous items, the data were subject to a principal coordinates analysis (PCA) and orthogonal variance maximizing (varimax) rotation of the original variable space.[59] For PCA analysis, the factors were based on an analysis of the total variance in the original data. The initial analysis examined the correlation matrix set at a perfect correlation of 1, implying that 100% of the variance is common or shared between the variables. The varimax rotation was used to re-orient and refine the factor loading obtained from PCA. The goal was to maximize the variability of the factor while minimizing the variance around the factor. Items were retained if they have a factor loading of $\geq$0.35 on a factor and if the item loaded $\geq$0.3 on only 1 factor. A Scree test was performed to determine the number of factors to be retained.[57,59] Each pretest iteration resulted in repeat PCA and varimax rotation analysis to provide the best fit of survey items to define the factors and/or subscales. Sample diversity (sex, assignment, and rank) was accounted for during analysis.

Internal consistency was examined using Cronbach alpha, which measured how well each item agreed with other items within each proposed factor. This enabled creation of a scale (an additive set of items that describe the eating habit or mediator factor represented as a point score), maximized variability of the scale, and minimized the variance around the scale.[61] Cronbach alpha was computed for each version of the survey to further reduce the number of items. Items were retained if they had an absolute alpha value factor loading of $\geq$0.5.[57,60] A Cronbach alpha value of 0.7–0.8 was defined as acceptable, whereas a value of >0.8 was defined as preferred.[62] Analysis was conducted both iteratively and by combining all of the data into the final analysis. Assumptions about missingness of data were investigated using a statistical test derived from Little.[63]

Following each testing iteration, descriptive data were analyzed with SPSS software (version 25, IBM Corp, Armonk, NY, 2017), and factor analysis results were discussed by the research team. Removal and revisions to existing items were discussed and agreed on by the research team. The survey was reformatted after testing each survey version, and the revised survey was tested at the subsequent iterations (V2–V4), resulting in V5 for test-retest reliability testing. The readability of the final version (ie, V5) identified a Flesch readability ease at 58.3 and a Flesch-Kincaid grade level of 7.9.

Phase 5: Test-Retest Reliability

The purpose of the survey test-retest was to assess the consistency of participant responses on the MEBS V5 when administered to the same participant on 2 separate occasions. Thirty-two soldiers were recruited from the Natick Soldier System Center in Natick, MA, from August to December 2018, and 30 participants completed the MEBS (V5) and demographic surveys on 2 separate occasions, approximately 1 week apart. One week separation is considered sufficient time to reduce the risk of survey response recall while short enough time to mitigate a major life event influencing responses.[64–66]

To examine test-retest reliability, consistency, and agreement, the intraclass correlation coefficient (ICC) was examined, comparing participant data at both time points using SPSS software. A 2-way mixed-effect model with absolute agreement defined reliability. The ICC estimate was defined as moderate (ICC, 0.50–074), good (ICC, 0.75–90), or excellent (ICC, >0.90) with 95% confidence.[67] The research team discussed analysis results and confirmed the survey was ready for the final parallel forms testing (V5).

Phase 6: Parallel Forms Reliability

Similar to the test-retest analysis, reliability was assessed between 2 formats: a paper survey and an electronic survey accessed via an internet link. Thirty soldiers were recruited from Joint Base Lewis-McChord, WA, in April 2019. Twenty-five soldiers completed the test-retested MEBS (V5) and demographic surveys on 2 occasions separated by 1 week and assigned in a random format order (paper-online vs online-paper). To examine parallel forms reliability, consistency, and agreement, the ICC was examined using the same parameters described previously.[67]

Scale Examination Between Common Military Groups

Data collected from MEBS (V5), consolidated from Phases 4–6, were stratified by sex, military rank, BMI weight status, and age groupings to examine differences in established scales by commonly assessed groups within the military population. Scale score results were reported as mean ± SD. Independent sample t test or Mann-Whitney U analyses were conducted on the basis of meeting parametric assumptions. Alpha was set at 0.05 with 80% power.

RESULTS

A summary of survey activities and participant demographics during Phases 2–6 are presented in Table 1, with specific noteworthy results from the survey development process reported below.

> The Military Eating Behavior Survey comprises 43 eating habits and 90 mediating behavior items, 22 validated scales, and takes 30–60 minutes to complete.

Phase 3: Face Validity

Forty-four Army soldiers participated in >19 cognitive interviewing sessions. In addition to the revisions noted in Table 1, a unique addition was made to the 40-item nutrition knowledge assessment. The majority of participants (73%) acknowledged guessing on most of the nutrition knowledge assessment items. As the intent of the knowledge assessment is to inform future nutrition interventions, the research team agreed that beyond true or false responses per item, a column should be included asking about confidence in his/her answer (yes/no). In addition, the researchers identified that the body image/satisfaction items did not accurately define the construct from a military perspective. Thus, 16 new body image items were created to replace the initial body image/satisfaction items. A cognitive interview session was included to verify the new items. The items were tested as a stand-alone subset with 6 participants who tested the original questions, as well as with 4 new participants in the complete survey format. The new body image questions were adequately interpreted as intended and deemed more appropriate by participants.

Phase 4: Construct Validity

Three survey versions were tested using a total of 1,462 soldiers (Table 1). Participants were predominately male (83%), enlisted (94%), unmarried (64%), White (63%), with postsecondary education (51%). Factor analysis began with V2 of the survey with 140 items in eating habits and 123 items in mediators of eating behavior. Figure 2 depicts how the survey evolved to a V5 containing 43-items in Part 1: Eating Habits and 90-items in Part 2: Mediators of Eating Behavior (Supplementary Tables 2 and 3 include factor loading results). The estimated time for participants to complete the survey decreased from 1.5–2 hours in V1 to 35–60 minutes in V5 (3–5 minutes demographic survey, 15–30 minutes for Part 1, and 15–30 minutes for Part 2).

Exploratory factor analysis indicated 14 eating habit scales and 8 mediating scales (Table 2 and Supplementary Tables 2 and 3). Each confirmed scale was scored by adding the response coding for each item in that factor to yield a point range. For example, the hunger scale comprised 2 items scored on a 5-point Likert scale (0, never; 1, rarely; 2, sometimes; 3, often; and 4, always). The

Table 1. Summary of Military Eating Behavior Survey Revisions During the Content, Face, and Construct Validity and Reliability Phases

Phase Information	Content Validity (Phase 2)	Face Validity (Phase 3)	Construct Validity (Phase 4)	Test-Retest Reliability (Phase 5)	Parallel Forms Reliability (Phase 6)
Study Activity	SME review	19 Cognitive interviews	3 Survey test iterations	Survey test wk 1 and retest wk 2	2 Survey formats tested wk 1 and 2
Participants	No participants 15 SMEs, 4 enlisted soldiers, and 4 sports dietitians officers	Recruited and completed: 44 41 M/3 F 40 E/4 O	Recruited: 1,605 Completed: 1,462 1,218 M/244 F 1,364 E / 93 O/5 U Age: 23.5 ± 5.2 y	Recruited: 32 Completed: 30 28 M/3 F 39 E/OE Age: 22.7 ± 4.8 y	Recruited: 32 Completed: 25 21 M/4 F 20 E/5 O Age: 28.1 ± 6.0 y
Locations/sessions	SMEs from academia and military in a variety of locations	NSSC	493 (FJS, JBLM) 523 (FCC, FRK, JBSA) 446 (JBER, FCK)	NSSC	JBLM
Summary	74 survey items impacted[a]; 66 revised for clarity, grammar, or to reduce the burden; 13 item response options altered; 7 items deleted; 8 items added	184 survey items impacted; 73 revised for clarity, grammar, or to reduce burden[c]; 81 item response options altered[d]; 8 items deleted; 22 items added[e]	247 survey items impacted; 42 word choice revised[b]; 46 item response options altered[b]; 1 food security item added; 3 items moved from EH to MED; 155 items deleted[f]	No changes made to survey; Scales were confirmed; Survey includes a 23-item nutrition knowledge assessment and military demographic questions	No changes made to survey
Revised Survey Version	V1	V2	V3–V5	V5	V5
Part 1: EH items	153	140	43	43	43
Part 2: MED items	124	123	90	90	90

E indicates enlisted; EH, eating habits items; F, female; FCC, Fort Carson, CO; FCK, Fort Campbell, KY; FJS, Fort Jackson, SC; FRK, Fort Riley, KS; JBLM, Joint Base Lewis-McChord; M, male; MED, mediators of eating behavior items; NSSC; Natick Soldier Systems Center, MA; O, Officer; SME, subject matter experts; U, Unknown rank (not reported).

[a]Twenty items had more than 1 type of revision made; thus, summed no. of items revised does not equal total items impacted; [b]Revised to reflect military language for Army Reserve Officer Training Corps, Air Force, Navy, Marine, and Coast Guard, revised body image items, and improved instructions; [c]Converted 12 items from choose best option statements to single statements within the 5-point Likert scale item section; converted Perceived Stress Scale items no. 2 and 6 to able to (from unable to); [d]Included the N/A (not applicable) response option on 51 items and confident (Y/N) column with the true/false responses on the 40-item nutrition knowledge assessment; converted Perceived Stress Scale 5-point Likert Scale to the following: never, rarely, sometimes, often, and always, for consistency with other survey items; [e]Included 16 items to assess military body image/satisfaction; [f]Deleted items by each testing iteration: 46 items from the first iteration, 71 items from the second iteration, and 37 items from the third iteration.

hunger scale score ranges from 0 to 8 points. The higher the score, the greater the hunger. The scores for all scales are identified in Table 3, with additional details about the item composition of each factor scale and scoring interpretation provided in Supplementary Tables 2 and 3).

Eight eating habit items and 29 mediating behavior items were not identified for factor scales but were retained in the MEBS. These items do not contribute to a scale score but yield adequate item variance, internal consistency, and reliability, as they provide relevant descriptive information to understand the population (eg, meal location, work schedule, eating style and reason, weight management efforts and reasons, and use of nutrition assistance programs).

> The Military Eating Behavior Survey can be administered on paper or through an online platform, increasing population reach.

The 40-item nutrition knowledge assessment was reduced to 23-items on the basis of item variance and initial factor analysis results; however, not all items factor together because of distinct knowledge concepts. Items focused on general health and nutrition performance most relevant to military operations: macronutrients (8 items), micronutrients (7 items), hydration (3 items), and energy sources (5 items). Current scoring mechanisms for knowledge assessments only account for correct vs incorrect responses and cannot differentiate between correct responses that result from actual knowledge and those that result from guessing. Current scoring mechanisms also cannot identify incorrect responses in which participants are confident in their inexperience. Determining whether participants are guessing (whether correctly or not) and their confidence in the responses may help discriminate erroneous from factual knowledge and might be useful for tailoring nutrition education and combating misinformation. To address this, the investigative team adopted a novel scoring mechanism that accounts for participant confidence (guessing by participants). Scoring was assigned on a 4-point scale for each item: 0, incorrect and confident; 1, incorrect and not confident (guessing); 2, correct and not confident (guessing); 3, correct and confident. The 23-items totaled for a score ranging from 0 to 69 points. A higher score represents accurate knowledge, whereas a lower score indicates a lack of knowledge. Collectively using the new scoring mechanism yielded an acceptable Cronbach alpha, excellent test-retest internal consistency, and good parallel forms internal consistency (Table 3). Results indicated that data were completely missing at random.

Phase 5: Test-Retest Validity

Thirty participants completed both surveys (Table 1). Participants were predominately male (93%), enlisted (100%), unmarried (83%), White (79%), with postsecondary education (45%). Of the 22 scales, 1 had no test-retest variability, 8 met moderate internal consistency (ICC, 0.50−0.74), 10 met good internal consistency (ICC, 0.75−0.90) and 3 met excellent internal consistency (ICC, >0.90) (Table 2). The 3 subscales comprising the Military Body Image Scale met either good or excellent internal consistency (Table 2). No changes were made to V5 because of all scales assessing various aspects of eating habits that are relevant to SMs.

Phase 6: Parallel Forms Validity

Twenty-five participants completed the paper and internet survey formats (Table 1). Participants were predominately male (84%), enlisted (80%), married (64%), White (68%), and with postsecondary education (80%). Of the 22 scales, 4 met moderate internal consistency (ICC, 0.50−0.74), 14 met good internal consistency (ICC, 0.75−0.90), and 4 met excellent internal consistency (ICC, >0.90), demonstrating the interchangeability of paper and online survey formats (Table 2). The 3 subscales comprising the Military Body Image Scale met either good or excellent internal consistency (Table 2). Mean ± SD scale score results for MEBS (V5) consolidated data (n = 501) are reported by sex, military rank (enlisted vs officer), weight status (<25 vs ≥25 kg/m^2), and age group (18−22 y vs 23−42 y) (Table 3). Significant differences were identified between commonly assessed military groups. Males exhibited higher scores than females in satiety, fast eating rate, situational eating, performance supplements, agreement with the military's ideal physique, and physical activity. In contrast, females scored higher than males in emotional eating, slow eating rate, general health supplements, body composition strategies, and food access. Enlisted soldiers scored higher than officers in perceived stress, satisfaction with body shape, and physical activity; however, officers scored higher than enlisted soldiers in meal pattern, diet rigidity, situational eating, general and performance supplements, food choice, sleep habits, military fitness, and nutrition knowledge. Participants who had a BMI in the normal range (18.5−24.9 kg/m^2) scored higher than those with a BMI in the overweight range (25.0−29.9 kg/m^2) in meal patterns and slow eating rate. In contrast, overweight participants scored higher than normal-weight participants in restraint, dietary rigidity, performance supplements, body composition strategies, the military body image total score and subscale scores, and nutrition knowledge. Soldiers aged 23 y and older scored higher than younger soldiers (18-22 y) in dietary rigidity, emotional eating, general health supplements, body composition strategies, sleep, and nutrition knowledge.

> The Military Eating Behavior Survey provides a new comprehensive approach for assessing eating behaviors in a military population.

Table 2. Validity Statistics by Factor for the Military Eating Behavior Survey

Construct and Scale	Cronbach α[a]	Test-Retest ICC[b]	Parallel Forms ICC[c]
Eating Habits			
Hunger	0.67	0.649**	0.683**
Satiety	0.71	0.711**	0.845***
Food craving	0.75	0.792***	0.704**
Meal pattern	0.76	0.673**	0.933***
Restraint	0.76	0.920***	0.915***
Dietary rigidity	0.73	0.892***	0.820***
Emotional eating	0.84	0.613**	0.844***
Fast eating rate	0.72	0.844***	0.921***
Slow eating rate	0.65	0.856***	0.742**
Environmental triggers	0.78	0.699**	0.898***
Situational eating	0.63	0.607*	0.829***
General health supplement	0.52	NV	0.785***
Performance supplement	0.82	0.914***	0.849***
Food choice (HES-7)	0.52	0.887***	0.941***
Mediators of Eating Behavior			
Body composition strategy	0.87	0.815***	0.734**
Perceived stress	0.87	0.879***	0.889***
Food access	0.68	0.717**	0.808***
Sleep habits	0.55	0.775***	0.895***
Military fitness	0.68	0.879***	0.783***
Physical activity quantity	0.67	0.675**	0.838***
Military body image scale	0.60	0.874***	0.871***
Concern with military image	0.54	0.793***	0.821***
Satisfaction with body shape	0.88	0.904***	0.776***
Agreement with ideal military physique	0.63	0.742***	0.894***
Nutrition knowledge	0.72	0.915***	0.865***

HES indicates Healthy Eating Score; ICC, intraclass correlation coefficient; NV, no variance.
[a]n = 1,462; [b]n = 30; [c]n = 25.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Note: Cronbach alpha (factor loading using exploratory factor analysis; 0.7–0.8 was defined as acceptable, whereas > 0.80 was defined as preferred; 2-way mixed-effects reliability model with absolute agreement at ICC ≥ 0.70 with 95% confidence); NV were all no use responses.

IMPLICATIONS FOR RESEARCH AND PRACTICE

The validated MEBS provides a new comprehensive tool to characterize the relationship between eating habits and mediators of eating behavior in military personnel. Many valid nutrition-related behaviors, attitudes, and knowledge survey tools were considered during the development of the MEBS. Most have a narrow focus, unable to provide a holistic assessment in the context of the unique aspects of military culture and environment. In addition, content and face validity testing indicated that not all items from survey tools previously available were relevant to and understandable by military populations, and not all the items from those previous survey tools used the same response format. Hence, the MEBS was created to consolidate questionnaires assessing different eating habits constructs and reduce redundancy while revising questions/concepts adapted from other validated surveys using military language to increase comprehension and standardize response options. Those revisions subsequently required a systematic, phased approach using best practice methods to create,[43,49,52] operationally define,[44,49,53,54,57,59,60,66] and validate[45–51,65,67] the eating habits and mediators of behavior constructs identified by the MEBS. The result is a comprehensive, standardized, holistic, and valid tool to examine eating habits and mediators of eating behavior in US military personnel.

Military personnel live and work in a unique culture that includes physically, emotionally, and cognitively demanding activities that often result in restricted food access, elevated stress, and sleep deprivation, all of which may influence eating habits. Developing undesirable eating habits could be an unintended consequence of that culture that ultimately may make maintaining body composition standards more difficult and increase injury and chronic disease risk during and after military service. As such, the MEBS will enable a better understanding of the eating habits of SMs and the factors mediating those behaviors, which is required for developing empirically based, effective interventions for improving military health, weight management, performance optimization, and military readiness and resiliency. In addition, the MEBS provides an approach to assess the effectiveness of nutritional strategies

Table 3. Mean and SD of Military Eating Behavior Survey Scale Results (n = 501) by Sex, Military Rank, Body Mass Index Status, and Age

		Mean Point Score (SD) by Demographic Category Grouping							
Scale	**Score Range[a]**	**Male (n = 453)**	**Female (n = 48)**	**Enlisted (n = 444)**	**Officer (n = 56)**	**< 25 kg/m^2 (n = 234)**	**≥ 25 kg/m^2 (n = 267)**	**18–22 y (n = 278)**	**23–42 y (n = 233)**
Hunger	0–8	2.9 (1.7)	2.5 (1.5)	2.8 (1.7)	3.1 (1.5)	2.8 (1.7)	2.8 (1.6)	2.7 (1.7)	3.0 (1.6)
Satiety	0–20	5.9 (3.5)*	4.8 (2.7)	5.8 (3.6)	5.8 (2.8)	5.6 (3.3)	5.9 (3.6)	5.6 (3.5)	5.9 (3.4)
Food craving	0–8	3.1 (1.9)	3.3 (1.6)	3.1 (1.9)	3.2 (1.7)	3.1 (1.9)	3.1 (1.9)	3.1 (2.0)	3.1 (1.8)
Meal pattern	0–21	16.4 (4.4)	16.1 (4.3)	16.1 (4.5)***	18.5 (3.3)	16.8 (4.3)*	16.1 (4.5)	16.1 (4.6)	16.8 (4.2)
Restraint	0–16	5.9 (3.6)	6.7 (3.1)	5.9 (3.6)	6.5 (3.2)	4.5 (3.4)***	7.2 (3.3)	5.5 (3.6)	6.6 (3.5)
Dietary rigidity	0–16	10.2 (3.1)	10.8 (2.6)	10.1 (3.2)***	11.9 (2.5)	9.8 (3.1)**	10.7 (3.0)	10.0 (3.2)***	10.7 (3.0)
Emotional eating	0–12	2.7 (2.7)**	3.6 (2.2)	2.7 (2.8)	3.0 (2.1)	2.5 (2.5)	3.0 (2.9)	2.6 (2.7)*	2.9 (2.7)
Fast eating rate	0–12	6.4 (2.5)***	4.9 (2.0)	6.2 (2.5)	6.3 (2.9)	6.0 (2.5)	6.4 (2.5)	6.2 (2.4)	6.2 (2.7)
Slow eating rate	0–8	2.4 (1.7)*	2.9 (1.7)	2.4 (1.6)	2.5 (1.8)	2.7 (1.7)**	2.2 (1.6)	2.5 (1.6)	2.4 (1.7)
Environmental triggers	0–12	4.9 (2.5)	5.3 (2.5)	4.9 (2.5)	5.1 (2.5)	5.2 (2.5)	4.8 (2.5)	5.1 (2.5)	4.8 (2.5)
Situational eating	0–12	4.0 (1.4)*	3.5 (1.4)	3.9 (1.4)*	4.3 (1.2)	3.9 (1.3)	3.9 (1.5)	3.9 (1.4)	4.0 (1.4)
Supplement use									
General health	0–3	0.3 (0.7)***	0.6 (0.7)	0.3 (0.6)***	0.7 (0.7)	0.3 (0.6)	0.4 (0.7)	0.3 (0.6)***	0.5 (0.8)
Performance	0–4	1.0 (1.4)***	0.3 (0.5)	0.9 (1.3)**	1.5 (1.6)	0.8 (1.3)*	1.1 (1.4)	0.9 (1.4)	1.0 (1.4)
Food choice (HES-7)	0–35	18.0 (5.5)	19.5 (4.8)	17.9 (5.5)**	20.0 (4.6)	18.1 (5.5)	18.2 (5.4)	17.7 (5.6)	18.6 (5.2)
Body composition strategy	0–6	0.9 (1.5)*	1.3 (1.6)	1.0 (1.5)**	0.5 (1.1)	0.4 (0.9)***	1.4 (1.7)	0.8 (1.4)*	1.1 (1.6)
Military perceived stress	0–40	14.6 (6.6)	14.8 (5.4)	14.8 (6.6)*	13.0 (5.0)	14.2 (6.1)	15.1 (6.8)	14.9 (6.3)	14.3 (6.7)
Food access	0–12	8.9 (2.1)***	9.7 (1.7)	8.9 (2.1)*	9.6 (1.9)	9.0 (2.1)	9.0 (2.1)	8.9 (2.1)	9.2 (2.1)
Military body image scale		34.1 (7.2)	32.7 (7.8)	34.1 (7.4)	33.0 (5.9)	32.0 (7.0)***	35.7 (7.1)	33.6 (7.5)	34.4 (7.0)
CMI	4–20	12.0 (3.0)	11.7 (3.4)	11.9 (3.1)	12.3 (2.6)	11.7 (3.0)*	12.3 (3.0)	11.8 (3.2)	12.3 (2.8)
SBS	5–25	13.5 (4.2)	13.3 (3.8)	13.6 (4.2)*	12.5 (3.9)	12.6 (4.2)***	14.2 (4.0)	13.4 (4.2)	13.5 (4.1)
AMP	3–15	8.6 (2.3)*	7.7 (2.8)	8.5 (2.4)	8.2 (2.0)	7.7 (2.1)***	9.2 (2.3)	8.4 (2.3)	8.6 (2.4)
Sleep habits	0–8	4.7 (1.7)	4.9 (1.7)	4.6 (1.7)**	5.3 (1.5)	4.8 (1.7)	4.6 (1.6)	4.5 (1.7)*	4.9 (1.6)
Military fitness test	0–100	86.9 (10.0)	88.5 (9.9)	85.9 (10.0)***	95.6 (5.0)	87.8 (9.0)	86.3 (10.8)	86.4 (10.0)	87.7 (10.0)
Physical activity level, min/d		102.6 (82.5)***	68.5 (45.4)	102.1 (82.9)**	75.6 (46.6)	92.9 (70.0)	104.9 (88.3)	97.5 (66.0)	101.5 (95.3)
Nutrition knowledge	0–69	41.8 (9.2)	44.1 (9.7)	40.9 (8.8)***	50.6 (8.6)	41.0 (8.9)*	42.8 (9.5)	40.0 (7.8)***	44.4 (10.3)

AMP indicates agreement with ideal military physique; CMI, concern conforming to military image; HES, Healthy Eating Score; SBS, satisfaction with body shape.

[a]Scales comprise a varying number of items with an additive point score on the basis of item response options (Supplementary Tables 2 and 3 define items and score interpretation).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Note: Independent sample *t* test comparison for body mass index and age groups and Mann-Whitney U comparison for sex and rank group.

aimed at modifying diet-related behaviors and associated health outcomes.

Several limitations are worth noting. The heterogeneous sample of male enlisted soldiers enrolled in Phase 4 was representative of current Army sex and rank proportions; however, future assessment with a larger sample size of officers and females, and greater demographic diversity, is warranted to increase generalizability. Scales that comprised <3 items should be interpreted with caution. The MEBS contained 2 highly correlated items for the hunger, food craving, slow eating rate, sleep, and physical activity scales. In addition, 7 of the created scales did not have a desired Cronbach alpha >0.7, warranting future reassessment with a robust representative sample.

In summary, this newly developed and validated survey enables examination of eating habits and mediating behavior factors and demonstrated sensitivity to identify significant differences in sex, rank, weight status, and age, all commonly assessed groups within the military population. The survey imposes a relatively low subject burden and can be administered to large cohorts in person or a web-based format. As such, the MEBS will enable future large military cohort studies to include a validated and standard method for assessing longitudinal eating habits change over the course of military careers, and the factors that mediate those changes to include sex, rank structure, military occupations, and environmental exposures.

ACKNOWLEDGMENTS

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the US government. This study was funded by the Army Medical Research and Development Command. The soldiers who participated in this research are gratefully acknowledged, and the research staff, soldiers, and dietetic interns who assisted with study implementation and data collection. Dr Andrew Young is also acknowledged for editorial assistance.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jneb.2021.04.467.

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NeuroImage 243 (2021) 118496

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/neuroimage

Effects of testosterone administration on fMRI responses to executive function, aggressive behavior, and emotion processing tasks during severe exercise- and diet-induced energy deficit ☆,☆☆

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ARTICLE INFO

Keywords:
Energy deficit
Testosterone
fMRI
Cognition

ABSTRACT

Background: Clinical administration of testosterone is widely used due to a variety of claimed physical and cognitive benefits. Testosterone administration is associated with enhanced brain and cognitive function, as well as mood, in energy-balanced males, although such relationships are controversial. However, the effects of testosterone administration on the brains of energy-deficient males, whose testosterone concentrations are likely to be well below normal, have not been investigated.

Methods: This study collected functional magnetic resonance imaging (fMRI) data from 50 non-obese young men before (PRE) and shortly after (POST) 28 days of severe exercise-and-diet-induced energy deficit during which testosterone (200 mg testosterone enanthate per week in sesame oil, TEST) or placebo (sesame seed oil only, PLA) were administered. Scans were also collected after a post-energy-deficit weight regain period (REC). Participants completed five fMRI tasks that assessed aspects of: 1) executive function (Attention Network Task or ANT; Multi-Source Interference Task or MSIT; AXE Continuous Processing Task or AXCPT); 2) aggressive behavior (Provoked Aggression Task or AGG); and 3) latent emotion processing (Emotional Face Processing or EMO).

Results: Changes over time in task-related fMRI activation in *a priori* defined task-critical brain regions during performance of 2 out of 5 tasks were significantly different between TEST and PLA, with TEST showing greater levels of activation during ANT in the right anterior cingulate gyrus at POST and during MSIT in several brain regions at REC. Changes over time in objective task performance were not statistically significant; testosterone-treated volunteers had greater self-reported anger during AGG at POST.

Conclusions: Testosterone administration can alter some aspects of brain function during severe energy deficit and increase levels of anger.

1. Introduction

Androgen replacement therapy is widely practiced in the United States, with over 5 million testosterone prescriptions written in 2017 (Kane, 2019). Some professional societies have recommended testosterone administration to improve sexual function among men with pathological hypogonadism, (Dimopoulou et al., 2016; Yeap et al., 2016) and among post-menopausal women (The role of testosterone therapy in postmenopausal women: position statement of The North American Menopause Society, 2005; Davis et al., 2019). In addition,

☆ **Clinical Trials Registry**: NCT02734238 (www.clinicaltrials.gov).
☆☆ **Data Availability:** Data for this study is available at **Clinical Trials Registry**: NCT02734238 (www.clinicaltrials.gov).

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https://doi.org/10.1016/j.neuroimage.2021.118496.
Received 23 February 2021; Received in revised form 10 August 2021; Accepted 19 August 2021
Available online 20 August 2021.
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many men consume dietary supplements that claim to increase testosterone concentrations due to supposed benefits for mood, sexual function, well-being, muscle mass and bone density even though these supplements have not been proven to increase testosterone (Petering and Brooks, 2017). Because prescription testosterone and dietary supplements that purportedly increase testosterone are widely used, examining the effects of testosterone on brain function, behavior, and mechanisms underlying any effects of them is of considerable importance.

Among the claimed effects of testosterone in men is modification of cognitive functioning and mood. Greater endogenous testosterone may be associated with greater objective measures of aggression among adult men (Archer, 1991). Associations between testosterone and depressive symptoms have been inconsistent overall (Amiaz and Seidman, 2008) (Seidman and Weiser, 2013; Ponce et al., 2018), although specific sub-populations may purportedly benefit (Vartolomei et al., 2020; Johnson et al., 2013; Zarrouf et al., 2009; Amanatkar et al., 2014) such as those on relatively high dose testosterone regimens (Walther et al., 2019). Although data on young men is sparse, associations between endogenous testosterone and cognitive functioning among older men is mixed (Boss et al., 2014; Holland et al., 2011; J.T. Hua et al., 2016; Beauchet, 2006; Lv et al., 2016) as are randomized controlled trials of testosterone administration effects on cognition (Beauchet, 2006; J.T. Hua et al., 2016; Huo et al., 2016). Nearly all reviews of this literature note substantial methodological variability across studies as a reason for inconsistent results, including differences in baseline testosterone concentration, demographic factors such as age, treatment formulation, dose regimen, and cognition and mood assessment methods used. Due to the mixed state of the literature, there is broad agreement that additional clinical trials are required to determine the supposed cognitive and mood effects of testosterone.

Contributing to the lack of clarity about testosterone effects on cognitive functioning and mood are mechanistic studies attempting to clarify the brain functional underpinnings of such effects. These studies have not produced conclusive results due to methodological heterogeneity and lack of replication across studies. Correlations between endogenous testosterone concentrations and functional magnetic resonance images (fMRI) signals collected during tasks related to emotional stimulus processing have been reported in healthy populations (Volman et al., 2011; Spielberg et al., 2015; Tyborowska et al., 2016; Denson et al., 2013; Ackermann et al., 2012; Stanton et al., 2009; Derntl et al., 2009; Kuo et al., 2012; Manuck et al., 2010), and acute administration of exogenous testosterone appears to provoke such fMRI changes (S.M. Goetz et al., 2014; Bos et al., 2010; Van Wingen et al., 2009; van Wingen et al., 2010; Bos et al., 2016; Hermans et al., 2008; Bos et al., 2013; van Honk et al., 2005; Radke et al., 2015; Bos et al., 2012). Most of these studies implicate the amygdala as a brain region whose activity during emotion processing tasks is associated with either endogenous levels or administration of acute exogenous testosterone. This finding aligns with a large literature implicating the amygdala in emotional stimulus processing, especially when threat or aggressive behavior are assessed. However, these studies do not agree about the degree to which other brain regions previously shown to be involved in various aspects of emotion processing (including the orbitofrontal, ventromedial prefrontal, and dorsolateral prefrontal cortex, as well as insula, posterior cingulate, and precuneus) are similarly associated with testosterone levels.

Two interventional studies with higher-order cognitive tasks (Sommer et al., 2008; Pintzka et al., 2016) suggested associations between administered testosterone and brain function in regions believed to contribute substantially to the task (anterior temporal structures including the entorhinal cortex and hippocampus for the spatial learning and navigation task of (Pintzka et al., 2016); Brodmann areas 39 and 40 for the mental rotation task of (Sommer et al., 2008)) but the very small number of such studies limits our ability to make conclusive statements. Similarly, only a small number of studies of brain function and testosterone have been completed in other domains, including risk and reward processing (de Macks et al., 2011; Hermans et al., 2010; Kopsida et al., 2016), processing of sexual stimuli (Lee et al., 2015), and resting state fMRI signals. (Peters et al., 2015). Like the cognitive literature, these studies are methodologically diverse, using a variety of testosterone formulations, including sublingual (Bos et al., 2010, 2016, 2013, 2012; Hermans et al., 2008, 2010; van Honk et al., 2005; Radke et al., 2015; Pintzka et al., 2016), transdermal (Goetz et al., 2014; Kopsida et al., 2016), and intranasal (Van Wingen et al., 2009, 2010); time between testosterone administration and fMRI scans of 30 min (Van Wingen et al., 2009, 2010) to 4 h (Bos et al., 2010, 2016, 2013, 2012; Hermans et al., 2008, 2010; van Honk et al., 2005; Radke et al., 2015; Pintzka et al., 2016); and populations that included males (Goetz et al., 2014), females (Bos et al., 2010, 2016, 2013, 2012; Van Wingen et al., 2009, 2010; Hermans et al., 2008, 2010; Radke et al., 2015; van Honk et al., 2005; Pintzka et al., 2016), or both (Kopsida et al., 2016). Despite this variability, the broad pattern of the evidence suggests that testosterone administration may modify the functioning of brain circuitry underlying important aspects of daily functioning.

In addition to methodological heterogeneity, there are additional limitations to the existing literature on the brain functional effects of testosterone. For example, to our knowledge all but one (Davis et al., 2014) of the interventional fMRI studies administered a single dose of testosterone on a single day. Thus, it is unclear the extent to which reported brain effects of testosterone are due to transient changes in neurochemistry as opposed to shifts in steady-state functioning that might emerge over the course of days or weeks of administration. Furthermore, the endogenous concentration of testosterone in neuroimaging study participants was often not documented and likely varied substantially between participants since there are large differences among eugonadal men. This difference in endogenous concentration may have led to differential effects of administration. Finally, to our knowledge, all fMRI studies to date have been conducted in individuals who are nominally energy balanced. Prolonged energy deficit—an excess of energy expenditure relative to energy intake extending over the course of days to weeks – occurs in a variety of settings, including athletic training (Loucks, 2004), intentional weight loss (Hall, 2008), aging (Hays and Roberts, 2006), military training (Margolis et al., 2013), congenital diseases (Kramerova et al., 2009), psychiatric disorders (Pinkston et al., 2001), and cancer treatment (Laviano et al., 2005). Because severe energy deficits are associated with substantial declines in circulating testosterone (Henning et al., 2014), this paradigm provides a unique model for assessing the effects of testosterone in otherwise healthy, young individuals who could potentially benefit from it.

The purpose of this study was to assess brain functional changes associated with chronic testosterone administration in healthy young men with experimentally-induced testosterone deficits exposed to a 28-day period of energy deficit followed by a 14-day weight regain period. In a double-blind, placebo-controlled, randomized controlled trial, participants were tested with a battery of five fMRI tasks spanning emotional and higher-order cognitive domains before, during, and after administration with sustained-release testosterone and after weight regain. We compared changes in fMRI signals and associated behavior over the course of prolonged energy deficit between groups receiving testosterone or placebo.

2. Methods and materials

2.1. Participants

Participants were enrolled in a randomized, double-blind, placebo-controlled trial designed to assess the effects of testosterone replacement on muscle mass and physical performance among healthy young men exposed to prolonged energy deficit. All participants were recruited from the Baton Rouge, LA community. Procedures regarding recruitment, randomization, and follow-up of this study have been described previously, as has a CONSORT diagram (Pasiakos et al., 2017, 2019).

Briefly, participants were healthy (no cardiometabolic or other disorders), physically active (≥ 2 days per week of aerobic and/or resistance exercise) men aged 18–39 years, with total testosterone concentrations within the normal physiological range (10.4–34.7 nmol/L). Participants met age-specific US Army body composition standards (Pasiakos et al., 2013). Participants with prostate-specific antigen (PSA) concentrations > 3 μg/L, hematocrit > 50%, a positive urine drug screen, or reporting anabolic steroid, human growth hormone, or nutritional testosterone precursor-like prescription drug or dietary supplement use within the past 6 months were excluded. All volunteers were examined prior to enrollment in the protocol to insure they did not have a mental disorder. This study was approved by the Pennington Biomedical Research Center (PBRC) Institutional Review Board and the Human Research Protection Office of the US Army Medical Research and Materiel Command. Participants provided written informed consent. Participants were studied between April 12, 2016 and September 15, 2017. This study is registered at www.clinicaltrials.gov as NCT02734238.

2.2. Study design

The study design has been described in detail elsewhere (Pasiakos et al., 2017). Briefly, this 3-phase, randomized, double-blind, placebo-controlled trial was conducted at PBRC to assess the effects of exogenous testosterone administration on body composition changes during prolonged energy deficit. The testosterone dose was chosen in an attempt to restore plasma testosterone to near-normal concentration during a prolonged energy deficit, which is known to substantially lower testosterone concentration (Pasiakos et al., 2017). Phase 1 (P1) was a 14-day (days 1–14), free-living, eucaloric diet period during which participants maintained their habitual physical activity levels. Phase 2 (P2: days 15–42) was a 28-day inpatient stay with a highly controlled energy deficit providing food intake equal to 45% of total daily energy expenditure. During P2, exercise energy expenditure was increased by 50% over P1 levels, and energy intake reduced to 45% of the individual's estimated energy expenditure. During P2, all meals were provided and consumed under supervision, and sleep was not restricted. After completing P2, participants were released from the inpatient unit and instructed to return to their pre-study, ad libitum, habitual diet and physical activity routines; body mass and composition were assessed during this recovery period (phase 3, P3; days 43–56). Over the course of the study mood state, including aggression and anxiety, were assessed on a regular basis using standardized questionnaires. Energy expenditure was measured via a 24-hour stay in a metabolic chamber, with one such 24-hour stay occurring in each of the PRE, POST, and REC phases of the study.

2.3. Testosterone/Placebo dosing

At the start of P2 (day 15), a software-generated plan (SAS Institute, Cary, NC, USA, version 9.4) was used to randomize volunteers to the testosterone or placebo condition. The testosterone group (N = 24) received 4 intramuscular injections of testosterone enanthate (200 mg of testosterone/week) in a sesame oil carrier which slows release of testosterone compared to testosterone enanthate administered intramuscularly in a non-lipid carrier. The placebo group (N = 26) received 4 doses of just sesame oil (1 mL sesame oil/week). Testosterone or placebo was administered at 0700 on days 15, 21, 28, and 35. The dose was selected to maintain near normal testosterone plasma concentrations despite the imposed severe energy deficit which was expected to lower endogenous testosterone concentrations of placebo-treated volunteers (Friedl et al., 2000). Fasted blood samples collected on days 0, 14, 42, and 56 between 6:00 and 09:00 AM were analyzed to assess total testosterone, and free testosterone was determined by calculation (Vermeulen et al., 1999). Study volunteers and study staff were blinded to group assignment until study completion and initial data analyses were conducted.

2.4. fMRI

Each fMRI task described below was collected at three time points per person: once during P1 (PRE, on day 5, 9, or 12); once during P2 (POST, on day 36, 37, or 38); and once during P3 (REC, on day 55, 56, or 57).

2.5. MRI acquisition

We acquired functional magnetic resonance imaging (fMRI) scans on a 3T MRI scanner (General Electric, 750 W Discovery, 32ch quadrature head coil) using a blood oxygen level dependent echo-planar imaging (BOLD-EPI) pulse sequence. Participants wore a respiratory monitoring belt and pulse oxygenation sensor during scanning to allow for post hoc correction of cardiac and respiratory influences on fMRI signals. Key acquisition parameters were: voxel size: $3.4 \times 3.4 \times 3.5$ mm^3, TR: 3 s, number of slices: 52, flip angle: 90°, and TE: 30 ms. Structural images acquired for data analysis were obtained using a T1-weighted magnetization-prepared gradient echo pulse sequence with the following parameters: TR: 8.7 ms, TE: 3.8 ms, FA: 8°, number of slices: 176, and voxel size: $1 \times 1 \times 1$ mm^3.

2.6. fMRI data analysis

Functional MRI data was analyzed using MATLAB R2016a and the Statistical Parametric Mapping 12 (SPM12) toolbox. Preprocessing steps included realignment to correct for head motion, co-registration to the T1-weighted structural scan, slice timing correction, smoothing using a 6 mm full width at half maximum Gaussian kernel, and warping to the Montreal Neurological Institute (MNI) template. Cardiac and respiratory components of each time series were removed using the RETROICOR algorithm (Gary et al., 2000). Time points representing volumes with excessive head motion (defined as greater than 1.5° of rotation or 1.5 mm of translation from the previous time point) and activation spike artifact (defined as global mean brain activation greater than 2.3 standard deviations above the mean across all time points) were removed from analysis. The data for each subject was entered into a first-level voxel-wise analysis using the general linear model. Each trial was modeled as a boxcar function convolved with the canonical hemodynamic response function that began at the onset of the stimulus presentation. First level beta maps, performed at the single subject level, quantified differences in BOLD signal within an individual between pairs of conditions; all such condition pairs, corresponding to our contrasts of interest, are listed in the description of tasks below. To encourage reproducibility of fMRI findings, current best practices for fMRI analysis emphasize the importance of testing effects in brain regions hypothesized *a priori* to be affected (Poldrack et al., 2017, 2008; Kriegeskorte et al., 2009; Nichols et al., 2016). Such an approach avoids the identification of spurious fMRI effects in unexpected brain regions– a common pitfall of classical voxel-level analysis followed by application of statistically-complex multiple comparison correction methods. Therefore, BOLD signal differences were averaged within 5mm-diameter regions of interest (ROI) that were identified before the beginning of this project as task-relevant in previously-published reports on each of the tasks, as listed below and in Supplemental Table 3.

2.7. fMRI tasks

On each day of scanning, participants practiced the tasks to be performed on a laptop computer outside the MRI scanner. Inside the scanner, headphones were placed over the ears to reduce MRI scanner noise. Detailed descriptions of the parameter settings and design choices for each task are provided in the Supplemental material. We chose to provide three executive function tasks (the Multiple Source Interference Task, AXE continuous performance task, and Attention Network Task) due to prior reports of associations between endogenous testosterone

levels or testosterone supplementation and performance on executive function tasks (Holland et al., 2011; Nguyen et al., 2017; Martin et al., 2007, 2008; Lašaitė et al., 2017), as well as fMRI signals during execution of executive tasks (Alarcón et al., 2014). In addition, we chose a task probing provoked aggression due to prior findings that greater endogenous testosterone may be associated with more aggressive behavior (Archer, 1991). Finally, we chose an implicit emotion processing task due to prior reports that suggested that acutely-administered testosterone provokes changes in fMRI responses to these tasks (Goetz et al., 2014; Bos et al., 2010, 2016, 2013, 2012; Van Wingen et al., 2009, 2010; Hermans et al., 2008; van Honk et al., 2005; Radke et al., 2015).

- **Multiple Source Interference Task (MSIT, 10 min):** In the multi-source interference task (MSIT), each trial presents a string of three characters including at least one occurrence of a digit between 1 and 3. The participant is required to use a 3-button response box to identify which of the three numbers appears only once in the string. In difficult trials of the task, there are two sources of interference: the presence of two other distracting numbers in the string, and the positioning of the probe number discordant with the position of the corresponding button on the response box. In easy trials, the two other characters in the string are the number "0", and the probe number is positioned concordantly with button assignment. Each administration of the task required the participant to complete 4 blocks of trials, with each block containing an equal number of easy and difficult trials. Specifically, each block began with 70 s of difficult trials; if the participant competed a certain number of difficult trials in that amount of time, the remainder of the block consisted of that same number of easy trials. In total, participants completed an average of 142 (standard deviation: 11.97) difficult trials and 142 (sd: 11.97) easy trials per administration of the task. The task accuracy rate (i.e., the percentage of trials in which the correct button was pressed), mean reaction times (i.e., times between presentation of the character string and button press) for easy and difficult trials, and an interference score calculated as the difference in mean easy and difficult reaction times, were analyzed. Similarly, differences in BOLD signals between difficult and easy trials were calculated at a set of ROIs identified in a prior study as repeatably providing signal differences between these two conditions (Bush and Shin, 2006).
- **Provoked aggression (AGG, 45 min):** Participants were fitted with an MR-safe device designed to apply an uncomfortable electrical stimulus to an application site at the tip of the ring finger of the non-dominant hand (STM100C, BioPac Systems, Inc). The participant then engaged in a simple reaction time game against a fictitious opponent, following a previously published design (Lotze et al., 2007). Briefly, the participant was introduced to a fictitious human opponent at the beginning of the game. In each trial of the game, the participant was instructed to click a button as fast as possible when a stimulus appeared on the screen; after clicking, the participant was told whether or not he clicked before the opponent did and therefore won the trial. On a win, the participant was instructed to set the intensity level of a painful stimulus applied to the opponent, on a scale between 1 and 5, with higher settings providing a greater number of electric shocks. The participant then viewed a video showing the opponent's reaction to the painful stimulus. On a loss, the participant observed the opponent setting the intensity level of the painful stimulus to be applied to the participant and received that number of electric shocks. The participant was never told that there was no real human opponent competing in the game, nor that trial outcomes and intensities of opponent-applied shocks were predetermined. Trials of the game were organized into four blocks, with each successive block featuring a greater number of shocks per trial. Each block featured 9 trials in which the participant won, and 11 trials in which the participant lost. After each block, the participant provided self-reported ratings of anger, compassion, and sympathy for the opponent, on a 1–5 scale. Within each of the four task blocks, the mean level of participant-applied shock, as well as the mean reaction time, was analyzed. Sympathy, compassion, and anger ratings were analyzed. Similar to prior work (Lotze et al., 2007), the BOLD contrasts of primary interest were between trials with high levels vs. low levels of participant-applied punishment, and between trials with high levels vs. low levels of opponent-applied punishment. These BOLD signal contrasts were evaluated within ROIs from a prior study (Lotze et al., 2007).
- **AX continuous performance task (AXCPT, 15 min):** Each trial of the AX continuous performance task (Carter et al., 1998) presents a single cue letter on a screen, followed by a single probe letter. The task requires individuals to click one button when the cue letter is A and the probe letter is X; and click a different button on every other combination of cue and probe letter. Task accuracy rate (i.e., the percentage of trials in which the correct button was clicked) and reaction times for A vs. non-A cues and X vs. non-X probes (i.e., the amount of time that elapsed between presentation of the cue or probe and the button press) were analyzed. The main BOLD contrast of interest was between AXE trials and all other trials. This BOLD contrast was evaluated at a set of ROIs identified as task-involved in prior studies (Lesh et al., 2013; Lopez-Garcia et al., 2016). The total number of trials that had an A cue with X probe, A cue with non-X probe, non-A cue with X probe, and non-A cue with non-X probe, were 104, 16, 16, and 8.
- **Attention Network Task (ANT, 20 min):** In each trial of the Attention Network Task (ANT), a line of arrows is presented in one of two locations on the screen. Participants clicked a left-hand or right-hand button depending on whether the center arrow pointed to the left or right. The participant was required to suppress distracting flanker arrows on either side of the center arrow, and had to process a spatial cue that appeared prior to the arrow line, either in the same location as the eventual arrow line, in both possible locations of the eventual arrow line, or not at all. The alerting score was the difference in mean reaction time (i.e., the amount of time between presentation of the line of arrows and the button click) between trials with and without a spatial cue. The orienting score was the difference in reaction time between trials with the spatial cue in the same vs. different location as the arrow line. The executive control score was the difference in mean reaction time between trials with congruent and incongruent flankers. The total number of trials with and without a spatial cue, with a spatial cue in the same location as the arrow line vs both possible locations of the arrow line vs no cue at all, and with congruent vs. incongruent flankers was 101, 51, 45, 56, 51, 77, and 75. Accuracy rate across trials was also analyzed (i.e., the percentage of trials in which the correct button was pressed). BOLD signal contrasts corresponding to the alerting, orienting, and executive control scores were assessed at ROI locations reported previously (Fan et al., 2005).
- **Emotional reactivity (EMO, 8 min):** Each trial of this implicit emotional face processing task presented a probe image of a human face in the center of the screen that must be matched to a gallery face on the left or right side of the screen. In each trial, the emotional valences of probe and gallery faces were all the same, and only one gallery face had the same identity as the probe face. In control trials, the probe and gallery images showed black oval shapes on a white background rather than human faces. The number of trials showing shapes, as well as neutral, angry, fearful, and surprised faces, were 30, 6, 6, 6, and 6. Accuracy rate (i.e., the percentage of trials in which the selected gallery image showed the same individual as the probe image) and reaction times (i.e., the amount of time between presentation of the probe image and the button press for selecting a gallery image) for each probe image emotion (e.g., angry, fearful) were analyzed. Differences in BOLD signals between trials with emotionally valent probe faces (angry, fearful, surprised) and emotionally neutral probe faces were analyzed. These BOLD contrasts were assessed at ROIs previously identified as providing differing

Table 1
Characteristics of participants randomized to testosterone or placebo.

	Overall $N = 50$	Testosterone $N = 24$	Placebo $N = 26$
Age (years)	24.80 ± 4.95	24.92 ± 5.00	24.69 ± 5.00
Race and Ethnicity (N,%)			
Non-Hispanic Black	12 (24.00%)	5 (20.83%)	7 (26.92%)
Non-Hispanic White	29 (58.00%)	14 (58.33%)	15 (57.69%)
Hispanic	3 (6.00%)	3 (12.5%)	0 (0%)
Other	6 (12.00%)	2 (8.33%)	4 (15.38%)
VO2_peak	3.51 ± 0.68	3.60 ± 0.78	3.43 ± 0.59
Total testosterone at baseline	461.10 ± 117.93	494.67 ± 130.23	430.12 ± 97.87
Free testosterone at baseline	9.31 ± 2.84	9.72 ± 2.78	8.93 ± 2.90

BOLD signals under differing probe image valences (Goetz et al., 2014).

The tasks were collected on the following study days and times:

- MSIT: days 5, 36, and 55; start time approximately 10:00 AM
- AGG: days 12, 38, and 57; start time approximately 9:00 AM
- AXCPT: days 9, 37, and 56; start time approximately 9:15 AM
- ANT: days 9, 37, and 56; start time approximately 9:40 AM
- EMO: days 12, 38, and 57; start time approximately 8:45 AM

2.8. Statistical analysis

SPM8/12 analysis description to get the fMRI summary measures. Analyses were carried out using R version 3.3.3. For each behavioral and fMRI outcome variable, two-way repeated measures ANOVA models tested the main effects of treatment group (TEST vs. PLA) and time point (PRE vs. POST vs. REC) on the outcome, as well as the interaction between treatment group and time point. For models with a significant interaction term, post-hoc pairwise F tests were used to assess treatment group differences at each time point. Data are reported as mean ± SEM with a significance threshold of $p \leq 0.05$. Effect size for all significant effects is reported in terms of generalized eta squared (GES) (Bakeman, 2005; Olejnik and Algina, 2003).

3. Results

3.1. Participants

Baseline characteristics of participants, including demographics, fitness, and circulating testosterone, are shown in Table 1. Participants were in their mid-twenties on average, represented predominantly white and African American communities, levels of physical fitness typical of physically active young adult males, and concentrations of circulating testosterone within the normal range. Overall, extent of data exclusion was typical of that of prior studies. There were no substantive differences between TEST and PLA in terms of data exclusions at any stage of the study. There were no significant differences between the placebo and testosterone-treated volunteers and no indication that testosterone treatment had adverse effects.

3.2. Brief summary of energy deficit and circulating testosterone

Changes in energy balance and circulating testosterone during the lead-in, energy deficit, and weight regain periods have been described elsewhere (Pasiakos et al., 2019) and are summarized in Table 2. Briefly, participants were weight stable during the 14-day lead-in period, and at the end of that period there were no significant differences between TEST and PLA in circulating testosterone or total daily energy expenditure, after controlling for age and baseline values. A 52.35 +/- 4.23 percent deficit in total daily energy expenditure was achieved during energy deficit, an amount that did not significantly differ between groups.

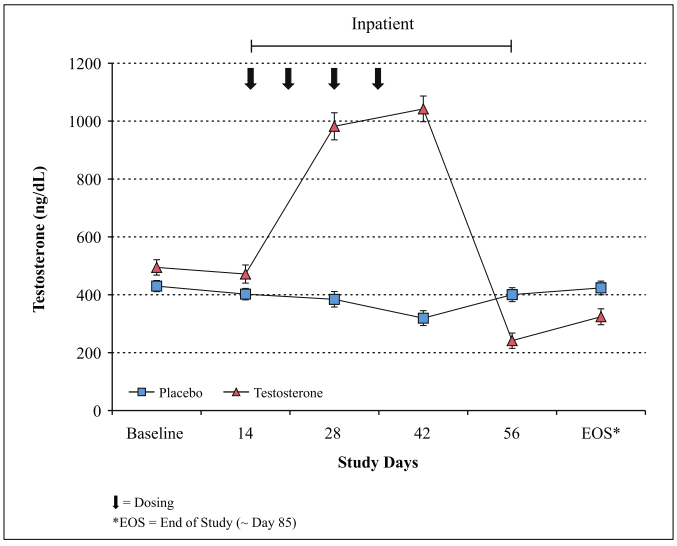


Fig. 1. Time course of circulating total testosterone in testosterone and placebo groups.

Total daily energy expenditure in both groups recovered almost to baseline levels at REC. Energy deficit was associated with weight loss of 2.95% in TEST and 6.96% in PLA on average. After weight recovery, body weight recovered to approximately 1.77% above baseline levels in TEST but was still approximately 2.67% below baseline levels in PLA on average. The TEST group attained supraphysiological concentrations of circulating total and free testosterone during energy deficit, and these values dipped substantially below baseline concentrations at REC as typically occurs when chronic treatment with testosterone is halted (see Fig. 1 and Table 2). Total and free testosterone concentrations in PLA decreased significantly during energy deficit and recovered to near baseline concentrations during weight recovery.

3.3. Overall fMRI data quality

The percentages of scans, averaged over all tasks, that were censored from analysis due to poor task performance, inadequate levels of task engagement, and excessive head motion are shown in Supplemental Table 1. The percentage of scans censored due to each cause were relatively low, in most cases below 10%. There was no clear pattern of TEST or PLA accounting for the larger share of censored scans. The percent of scans censored due to inadequate task engagement and excessive head motion increased steadily between baseline, energy deficit, and weight regain measurements; the percent of scans censored due to poor task performance increased between baseline and energy deficit measurements, then were somewhat lower at the weight regain measurement.

Table 2
Effects of intervention on body weight, circulating testosterone, and energy balance among participants randomized to testosterone or placebo.

	Overall $N = 50$	Testosterone $N = 24$	Placebo $N = 26$
Percent change in total testosterone during energy deficit, relative to baseline	55.92 ± 104.03	134.98 ± 94.37	−17.06 ± 38.49
Percent change in total testosterone after weight regain, relative to baseline	−21.20 ± 35.14	−48.23 ± 18.69	1.69 ± 29.06
Percent change in free testosterone during energy deficit, relative to baseline	59.53 ± 147.41	172.91 ± 144.44	−40.76 ± 32.80
Percent change in free testosterone after weight regain, relative to baseline	−26.57 ± 34.14	−51.06 ± 20.36	−5.86 ± 29.47
Percent change in total daily energy expenditure during energy deficit, relative to baseline	62.85 ± 21.89	63.70 ± 21.63	62.06 ± 22.52
Percent change in total daily energy expenditure during weight regain, relative to baseline	3.78 ± 6.92	5.90 ± 6.20	1.83 ± 7.10
Percent change in body weight during energy deficit, relative to baseline	−5.04 ± 2.89	−2.95 ± 2.55	−6.96 ± 1.52
Percent change in body weight during weight regain, relative to baseline	−0.54 ± 4.12	1.77 ± 3.89	−2.67 ± 3.10
Percent energy deficit during energy deficit phase, relative to baseline	52.35 ± 4.23	52.04 ± 4.82	52.64 ± 3.68

3.4. Behavioral and imaging data from fMRI tasks

Results from each fMRI task are as follows. Detailed statistical models referenced below with significant effects are in Supplemental Table 4.

- **MSIT**
  - **Behavioral:** There were no significant group-by-time interactions for any of the behavioral variables analyzed from MSIT (see Supplemental Table 2). Reaction times on the MSIT became significantly faster between PRE, POST, and REC time points (congruent trials: p = .0033, numDF=2, denDF= 70, F = 6.2190, GES=0.15; incongruent trials: p < .0001, numDF=2, denDF=70, F = 50.5681, GES=0.59).
  - **fMRI:** In the MSIT, the activation level of deactivation-related ROIs showed a significant group-by-time interaction (p=.02, numDF=2, denDF=69, F = 4.126731, GES=0.11), with post hoc analysis suggesting PLA had greater de-activation of these ROIs at REC only (p=.007, Fig. 3).
- **AGG**
  - **Behavioral:** The self-reported anger rating provided after the first block of AGG showed a significant group-by-time interaction (p=.033, numDF=2, denDF=74, F = 3.5705, GES=0.09) with post-hoc analysis demonstrating anger ratings were significantly higher in TEST than in PLA at the POST time point (p=.002, Fig. 2). Average applied punishment levels significantly increased (p=.0010, numDF=2, denDF=74, F = 7.62173, GES=0.1) and self-reported compassion (p=.0038, numDF=2, denDF=74, F = 6.01918, GES=0.14) and sympathy (p=.0003, numDF=2, denDF=74, F = 9.17545, GES=0.20) ratings after the first block correspondingly decreased over time in AGG.
  - **fMRI:** There were no significant group-by-time interactions for any fMRI variables derived from AGG.
- **AXCPT**
  - **Behavioral:** There were no significant group-by-time interactions for any of the behavioral variables analyzed from AXCPT (see Supplemental Table 2). Reaction times on the AXCPT became significantly faster between PRE, POST, and REC time points(p=.0072, numDF=2, denDF=54, F = 5.4061, GES=0.17). AXCPT task accuracy was significantly lower at REC compared to PRE and POST (p=.0231, numDF=2, denDF=54, F = 4.043, GES=0.13).
  - **fMRI:** There were no significant group-by-time interactions for any fMRI variables derived from AXCPT.
- **ANT**
  - **Behavioral**: There were no significant group-by-time interactions for any of the behavioral variables analyzed from ANT (see Supplemental Table 2). Reaction times on the ANT became significantly faster between PRE, POST, and REC time points (reaction times on no-cue trials: p=.0061, numDF=2, denDF=76, F = 5.4570, GES=0.13).
  - **fMRI:** In the ANT, the executive control contrast in the right anterior cingulate gyrus showed a significant group-by-time interaction (p=.048, numDF=2, denDF=76, F = 3.159, GES=0.08), with post hoc tests suggesting that PLA had significantly greater contrast values than TEST at POST only (p=.014,Fig. 4). The main effect of time was significant in ANT for the mean fMRI contrast across all executive control ROIs (p=.05, numDF=2, denDF=76, F = 3.108, GES=0.08), with post hoc analysis suggesting reduced contrast levels at REC compared to PRE and POST.
- **EMO**
  - **Behavioral**: There were no significant group-by-time interactions for any of the behavioral variables analyzed from EMO (see Supplemental Table 2).
  - **fMRI:** In EMO, only one fMRI variable (right basolateral amygdala or BLA activation to surprised faces) showed a trend toward a group-by-time interaction (p=.0656, numDF=2, denDF=74, F = 2.827, GES=0.07), although this interaction was difficult to interpret due to poor matching of values between groups at baseline. Two fMRI variables (periaqueductal gray or PAG activation to angry faces and right BLA activation to fearful faces) showed a pattern of increasing activation over time across groups (p=.012 and p=.049; numDF=2 and numDF=2;denDF=74 and denDF=74;F = 4.69 and F = 3.15; GES= 0.11 and GES= 0.08).

4. Discussion

This randomized controlled trial of 28-day sustained-release testosterone administration among energy-deficient young men had three main findings. First, those receiving testosterone experienced changes in fMRI-assessed brain function over the treatment period during two of five cognitive tasks administered, compared to those receiving placebo. Second, those who were treated with testosterone exhibited greater self-reported levels of anger during a provoked aggression task shortly after testosterone was discontinued and testosterone concentrations were still elevated, compared to those who received placebo. Third, overall patterns of change over time in brain functioning and task performance in this cohort of energy-deficient individuals were largely congruent with those observed previously in energy-balanced individuals. Overall, these findings suggests that 28 days of sustained-release testosterone treatment has significant behavioral and brain functional effects on energy-deficient young men. However, some effects of testosterone observed in other studies were not observed, possibly due to differing participant characteristics, different energy status, and especially different dose, time course, and formulation of testosterone treatment.

To our knowledge, testosterone trials to date have not collected fMRI data during execution of the ANT or MSIT, and we found group differences in brain activation during these tasks in brain regions that are consistent with the literature. In particular, the anterior cingulate cortex has been shown in a large body of literature to be critically involved in adjudicating between conflicting sources of information during decision making, as in the executive control component of the ANT

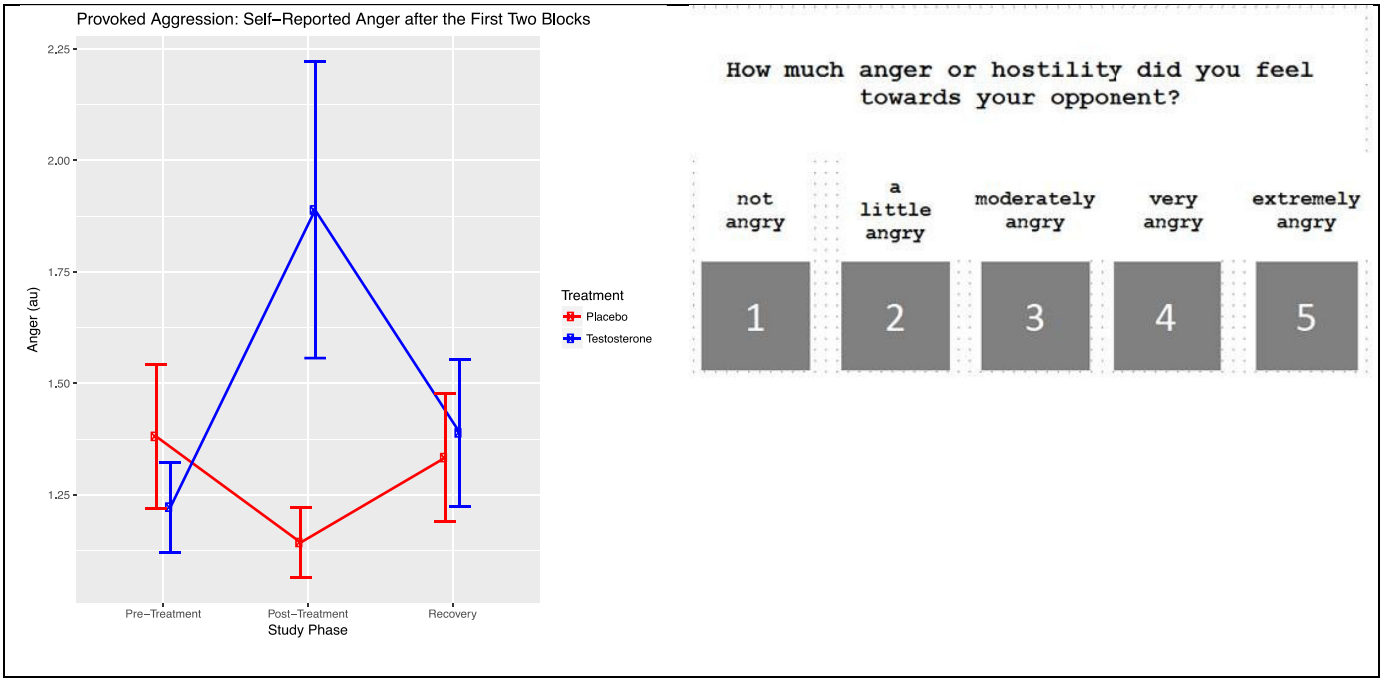


Fig. 2. **Left**: Self-reported anger ratings after the first two blocks of AGG, in both study groups and all three study phases. **Right**: The prompt for self-reported anger ratings presented to the participant in the scanner.

(Van Veen and Carter, 2002; Botvinick et al., 2004), and some evidence from other populations and task domains suggests activation of this region may be modifiable (Yoshimura et al., 2014; Copland et al., 2009; Russo et al., 2017). Similarly, the MSIT deactivation brain regions (the amygdala, posterior insula, parahippocampal gyrus, superior frontal gyrus, lentiform nucleus, middle temporal gyrus, angular gyrus, and posterior and anterior cingulate gyrus) we evaluated were identified in one prior study (Sheu et al., 2012), but similar sets of regions have been shown to deactivate during MSIT execution in studies by at least two other laboratories (Bush et al., 2003; Robertson et al., 2014). This agreement with prior data lends confidence in the validity of these findings.

Using best practices designed to enhance repeatability and validity of fMRI findings (Poldrack et al., 2008; Kriegeskorte et al., 2009; Nichols et al., 2016), we only evaluated group differences in fMRI signals at a small number of brain regions that were identified as task-related in reference papers prior to data collection. By design, the brain regions at which we found group differences in fMRI signals are regions defined in these reference papers, but in a large literature of unrelated papers these same regions have been implicated as central to performance of these tasks and have proven to be modifiable by interventions. In contrast, most other fMRI papers in this area took a voxel-based approach to analysis, which current research suggests may have led to a higher probability of false positive detections (Poldrack et al., 2008; Kriegeskorte et al., 2009; Nichols et al., 2016).

Each of the identified differences observed in this study between testosterone and placebo groups were specific to a single time point in the intervention process. This was to be expected due to the dramatic differences in circulating testosterone across session and treatment. Specifically, fMRI activation during the ANT task, and self-reported anger ratings after executing the provoked aggression task, differed between groups at the post-intervention assessment only. In addition fMRI activation during the MSIT task differed between groups only after the post-intervention recovery period. We speculate that the time specificity of these differences may be due to the dynamic time course of changes in circulating testosterone over the study which we documented by regular assessment of testosterone levels. Circulating testosterone among those receiving it rose to roughly double the baseline concentration during the administration period, then quickly fell to concentrations below baseline after administration ceased, and did not fully recover to baseline concentrations at the post-recovery time point (See Fig. 1). The post-intervention measurements took place when circulating concentrations were still elevated, and the post-recovery measurements occurred when the testosterone group still had significantly lower circulating concentrations than the placebo group. We suggest the differing testosterone dynamics between these two time points could have resulted in differing effects on behavior and brain function, although further study of precise relationships between circulating testosterone concentration and brain outcomes are needed in specific contexts of elevation to supraphysiological concentrations, dynamic changes over time, and depletion below physiological concentrations.

Several of the behavioral findings in this study are consistent with previous reports. In particular, that level of punishment applied to the opponent steadily increases in response to increases in the level of punishment applied by the simulated opponent in our provoked aggression task, and self-reported levels of compassion and sympathy for the opponent go down over time, as would be expected in a behavioral paradigm of this type (Anderson et al., 2008; Chermack et al., 1997). In addition, the simple reaction times required by AGG slowed over the course of the intervention, in line with studies suggesting similar slowing in physically exhausted individuals (Lorenzo et al., 1995). In contrast, choice response times on the higher-order cognitive tasks (ANT, MSIT, AXCPT) became faster with repeated administrations of the task with no apparent decrement in task accuracy, suggestive of practice effects that have been reported previously for these tasks (Bush et al., 2003; Ishigami and Klein, 2010; Fan et al., 2002; Strauss et al., 2014). This consistency with the literature indicates our behavioral data were collected in a valid fashion.

Although these aspects of the findings are plausible, there are nonetheless aspects of the fMRI findings that are not directly reflected in earlier testosterone studies. In particular, prior reports sugeted relationships between endogenous testosterone levels (Volman et al., 2011; Spielberg et al., 2015; Tyborowska et al., 2016; Denson et al., 2013; Ackermann et al., 2012; Stanton et al., 2009; Derntl et al., 2009;

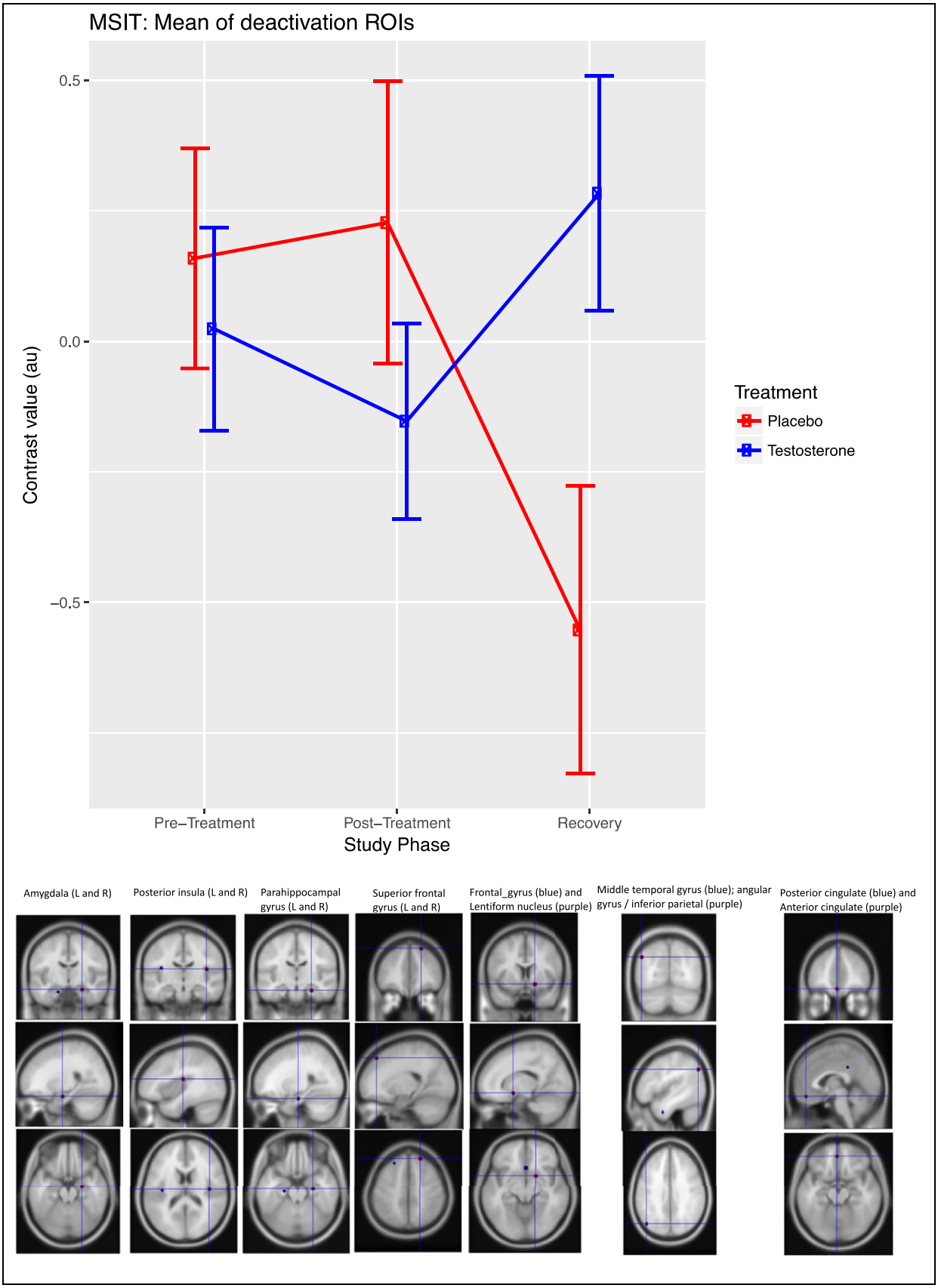


Fig. 3. Top: Mean activation level across all deactivation ROIs during execution of the MSIT. Bottom: 3D locations of all Multi-Source Interference Task (MSIT) de-activation regions of interest (ROIs), shown in MNI space. ROI locations are taken from (Bush and Shin, 2006).

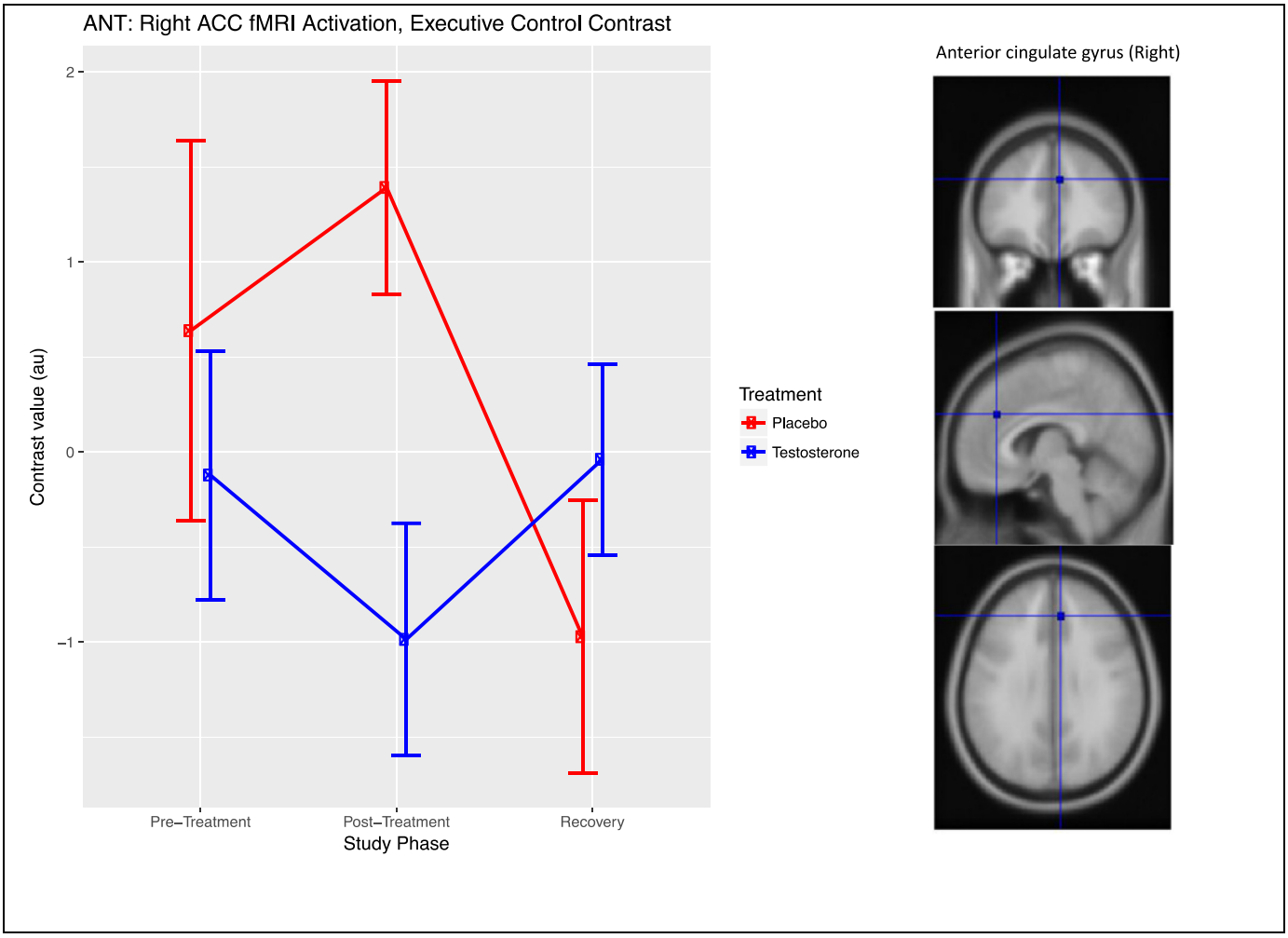


Fig. 4. Left: Executive control related fMRI activation in the right anterior cingulate gyrus during the ANT task. Right: 3D location of the anterior cingulate gyrus region of interest (ROI), shown in MNI space. The ROI location was taken from (Fan et al., 2005).

Kuo et al., 2012; Manuck et al., 2010) or exogenous testosterone administration (Goetz et al., 2014; Bos et al., 2010, 2016, 2013, 2012; Van Wingen et al., 2009, 2010; Hermans et al., 2008; van Honk et al., 2005; Radke et al., 2015) on one hand, and brain function during processing of emotional stimuli on the other, while we observed no such relationships. Similarly there has been at least one prior report of differences in brain function during tasks related to aggression between individuals who did, vs. did not, receive a single dose of exogenous testosterone (Wagels et al., 2019), while we observed no such differences. There are several potential reasons for these differences from prior studies. First, many aspects of the testosterone regimen in this study differed from that of prior fMRI studies: unlike all but one fMRI study we are aware of to date, (Davis et al., 2014) we used a chronic sustained-release treatment that increased circulating concentrations over the course of 28 days rather than a single day or only a few days of treatment. Unlike most fMRI studies to date, testosterone was administered intramuscularly rather than transdermally (Goetz et al., 2014) (Kopsida et al., 2016), sublingually (Bos et al., 2010; Bos et al., 2016; Bos et al., 2013; Bos et al., 2012) (Hermans et al., 2008; Hermans et al., 2010) (van Honk et al., 2005; Radke et al., 2015) (Pintzka et al., 2016), or intranasally (Van Wingen et al., 2009; van Wingen et al., 2010). Second, the physically active young male population studied here differed from that of most prior fMRI studies, many of whom enrolled women (Bos et al., 2010; Van Wingen et al., 2009; van Wingen et al., 2010; Bos et al., 2016; Hermans et al., 2008; Bos et al., 2013; Radke et al., 2015; Bos et al., 2012; Hermans et al., 2010) (van Honk et al., 2005; Pintzka et al., 2016), mixed samples of men and women (Kopsida et al., 2016), and middle aged adults (Van Wingen et al., 2009; Davis et al., 2014). Third, unlike most prior fMRI studies, testosterone administration occurred in the context of severe energy deficit, during which circulating testosterone concentrations drop dramatically (Henning et al., 2014). This means that our group comparisons were between a testosterone-depleted control group and a group with supraphysiological circulating concentrations; most prior studies compared a eugonadal control group to a group with elevated circulating concentrations. The only other fMRI study to our knowledge that compared a testosterone-depleted control group to a supplemented group was an acute study in which a single dose of testosterone or placebo was preceded by a dose of gonadotropin releasing hormone which transiently suppressed lutenizing hormone release and thus reduced circulating testosterone (Goetz et al., 2014). Our results differed from those of this prior study for several possible reasons, including our administration of chronic (not acute) testosterone and our use of energy deficit (not a drug) to suppress circulating testosterone. Fourth, given evidence of severe energy deficit effects on cognitive function (Lieberman et al., 2006), the severe energy deficit imposed on all participants in this study may have had its own independent effects on brain function that were pronounced and obfuscated any other effects testosterone administration may have had. Fifth, while most prior studies tested for group differences in fMRI signals at each voxel, our study followed current best practices (Poldrack et al., 2008; Kriegeskorte et al.,

2009; Nichols et al., 2016) by only testing for such differences at brain locations previously reported to be task-related.

This approach reduced our sensitivity to detect any and all group differences, including those that are difficult to replicate. Finally, our tasks showing group differences in fMRI measures were the same tasks that have well established test-retest repeatability, while the repeatability of our other tasks is less well understood. Specifically, the high repeatability of MSIT fMRI data is well known (Bush and Shin, 2006; Sheu et al., 2012; Bush et al., 2003), and in our own hands ANT fMRI summary measures collected from healthy young adults have high repeatability (*e.g.*, Pearson correlation of 0.75 for the orienting contrast, 0.67 for the executive control contrast; data not shown). Conversely, the repeatability of data from emotional face fMRI tasks, which underlie a large portion of the testosterone-related fMRI literature, has recently been called into question (Nord et al., 2017). Future work should investigate each of these factors in turn to build a more comprehensive understanding of factors that influence testosterone effects on fMRI measurements.

A limitation of the study was a lack of an energy-balanced control arm, which would dissociate effects of energy deficit and testosterone administration on the cognitive and brain variables assessed. Another limitation of the study is that we repeatedly assessed identical versions of each fMRI task at each study phase, rather than assessing different variants of the task with similar measurement properties at different phases, as has been shown to be advantageous for other fMRI tasks (King et al., 2018). As a result, for statements about overall changes in performance or fMRI signals over time, we cannot rule out the possibility that practice effects influenced the magnitudes of those changes. Key strengths of this study include its randomized, double-blind, placebo-controlled design; rigorous measurement of, and control over, energy deficit and testosterone concentrations; its multi-domain fMRI evaluation of brain functioning; and *a priori* specified fMRI analyses to reduce the risk of difficult to reproduce results.

In conclusion, this study found that in a group of healthy young men subjected to 28 days of severe energy deficit, testosterone administration was associated with differences in behavior and brain functioning at the end of the administration period, as well as differences in brain functioning at a post-recovery time point. These results point to the need for additional research on the neurocognitive effects of chronic testosterone administration in diverse realistic settings.

5. Disclaimer

The views expressed in this paper are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or U.S. Government. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Funding for this research was provided by the U.S. Army Medical Research and Development Command (USAMRDC) and the Defense Medical Research Program.

Credit authorship contribution statement

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Acknowledgments

Funding for this project was provided by Collaborative Research to Optimize Warfighter Nutrition projects I and II, Joint Program Committee-5, funded by the US Department of Defense. Additional support provided by the Pennington Biomedical Research Foundation. Authors have no conflicts of interest to report.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2021.118496.

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British Journal of Nutrition, page 1 of 8 doi:10.1017/S000711452100338X
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Weight management behaviours mediate the relationship between weight cycling, BMI and diet quality among US Army Soldiers

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(Submitted 25 March 2021 – Final revision received 22 June 2021 – Accepted 29 July 2021)

Abstract

Weight cycling is prevalent in sports/professions with body composition standards, and has been associated with weight management behaviours that may contribute to suboptimal diet quality and weight gain. US Army Soldiers may be at increased risk of weight cycling relative to civilians due to mandated body composition standards. However, the relationship between weight cycling, weight management behaviours, BMI and diet quality among Soldiers is unknown. In this cross-sectional study, 575 Soldiers (89 % enlisted, 90 % male, 23 ± 4 years) at Army installations at Joint Base Elmendorf-Richardson, AK, Joint-Base Lewis McChord, WA, and Fort Campbell, KY completed questionnaires on food frequency, health-related behaviours and history of weight cycling (≥ 3 weight fluctuations ≥ 5 % body weight). Weight cycling was reported by 33 % of Soldiers. Those who reported weight cycling reported higher BMI (27 ± 4 *v.* 25 ± 3 kg/m^2, $P < 0{\cdot}001$) and higher prevalence of engaging in weight management behaviours prior to body weight screening but did not report lower dietary quality (Healthy Eating Index-2015 (HEI) scores 59 ± 10 *v* 59 ± 11, $P = 0{\cdot}46$) relative to those who did not report weight cycling. Results of mediation analyses suggested that weight cycling may affect BMI both directly ($c' = 1{\cdot}19$, 95 % CI: 0·62, 1·75) and indirectly ($ab = 0{\cdot}45$, 95 % CI: 0·19, 0·75), and HEI scores indirectly ($ab = 0{\cdot}69$, 95 % CI: 0·20, 1·35) through the adoption of weight management behaviours. Weight cycling is common in Soldiers and is associated with higher BMI and higher prevalence of engaging in weight management behaviours that mediate associations between weight cycling, BMI and diet quality.

Key words: **Weight cycling: Eating behaviours: Body composition: Diet quality: Military**

Weight cycling is characterised by 'repeated cycles of weight loss and regain' and is common among individuals who participate in sports or work in professions where a certain body weight or image must be maintained[1,2]. To lose weight quickly, individuals in these sports/professions often adopt behaviours such as restricting food intake, skipping meals or using extreme methods such as dehydration, sweat suits, saunas, diet pills, laxatives and vomiting[3,4]. While these behaviours can facilitate rapid weight loss, they may also compromise sport-specific performance[4] and contribute to metabolic adaptations that promote increased adiposity and weight regain[5] in excess of the amount of weight originally lost[6]. This may lead to multiple bouts of weight cycling that, over the long-term, increase risk of chronic diseases such as obesity, hypertension and type 2 diabetes[1,6–10].

US Army Soldiers may be at risk of adopting behaviours that promote weight cycling due to Army-specific body weight and composition standards and a military culture that stresses a 'soldierly' appearance (i.e. neat and fit professional image)[11,12]. All Soldiers are assessed bi-annually for adherence to these standards using a BMI-based screening. Those who exceed their maximum screening weight undergo a circumference-based body fat assessment to confirm compliance with mandatory body composition standards[12]. Soldiers who exceed these standards are enrolled in an Army-specific weight management programme that involves weight management counselling, monthly monitoring of weight and body fat, and includes punitive measures such as restriction from training opportunities or promotion until the Soldier becomes compliant with standards[13]. The incentive to comply is high as Soldiers who are unable to successfully meet the standards or have reoccurrences of failing standards can be involuntarily discharged from service[13].

Beyond body weight and composition standards, military culture emphasises notions that excessive body fat reflects lack

Abbreviations: HEI, Healthy Eating Index;; MEBS, Military Eating Behavior Survey.

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British Journal of Nutrition

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of personal discipline, distracts from a soldierly appearance and indicates poor health[10,14,15]. This emphasis is thought to contribute to body dissatisfaction and stigmatisation, which, in combination with body composition standards, may foster the adoption of eating behaviours such as over-restriction and binging[10,12,16]. These behaviors, in turn, have been associated with excess weight gain, stress, decreased occupational performance, and increased risk of injury and mental health disorders[17]. Indeed, US active duty military and veterans are at increased risk for weight cycling compared with civilians[10,12,16], and mean BMI of Soldiers has increased over the past 10 years with BMI trajectories suggestive of weight cycling apparent in the population[18]. Soldiers who show BMI fluctuations indicative of weight cycling have a higher incidence of failing body composition standards and are more likely to show a trajectory of weight gain *v* weight stability[18]. Thus, military standards and culture may promote weight cycling.

Nutrition is a key element in overall health, optimal body composition, physical and cognitive performance, psychological resilience, and recovery for military personnel[19–21]. Soldiers with higher diet quality are more likely to have normal body composition, Army Physical Fitness Test (APFT) scores in the top quartile and higher emotional fitness scores[19]. High-quality diets are also thought to support successful long-term maintenance of a healthy body weight and composition[22,23]. Whether or not weight cycling is associated with suboptimal diet quality in Soldiers has not been examined. However, if weight cycling in Soldiers is associated with poor diet quality, then improving the diets of these individuals may be one strategy to break the futile cycle. It has been proposed that dieting behaviours, especially behaviours such as periods of over-restriction that lead to episodic uncontrolled eating, are one explanation for the occurrence of weight regain after weight loss[24]. Since rapid weight loss may be achieved through weight management behaviours characterised by poor nutritional intake, it is important to consider how weight cycling, weight management behaviours, BMI and diet quality may be related.

Although the prevalence of overweight and obesity is lower among service members compared with civilian populations, rates among US military personnel demonstrate a continuing rise over the last two decades[18,25] and current estimates indicate that 17 % of USmilitary personnel have a BMI that classifies them as having obesity[25]. Recent findings have demonstrated that Soldiers of all ranks report experiencing several barriers within the Army eating environment that restrict behaviour change and the ability to make healthy choices[26]. Common barriers include lack of access to nutritious options, excess exposure to fast food, limited time to eat meals or prepare healthy foods, time constraints that promote fast eating, and increased exposure to stress/trauma compared with the civilian population[26,27]. Thus, overweight and obesity are increasingly common in US military personnel, and identification of contributing factors is needed to inform development of prevention strategies.

This cross-sectional study aimed to examine associations between weight cycling, weight management behaviours, BMI and diet quality in US Army Soldiers. We hypothesised that Soldiers who reported weight cycling would also report a higher prevalence of adopting weight management behaviours prior to body weight screenings and have a higher BMI and poorer diet quality than those who did not report weight cycling. Additionally, we hypothesised that being more likely to adopt weight management behaviours prior to body weight screening would mediate associations between weight cycling and BMI and weight cycling and diet quality, respectively.

Methods

Study design and population

From 2016 to 2017, 1789 Soldiers from eight Army installations were surveyed in person during the development and validation of the Military Eating Behavior Survey (MEBS)[28]. The MEBS contains military-relevant phrasing of questions assessing eating behaviours, mediators of eating behaviours, food and supplement intake, nutrition knowledge, history of weight management practices, current and past body weight, and demographics. Groups of Soldiers were selected by their command to be briefed in-person prior to completing the MEBS. All active-duty Soldiers who were ≥ 18 years of age, spoke English and had completed initial military training were eligible to participate. A subset of Soldiers (*n* 309) were intentionally enrolled during initial military training in order to capture individuals first being exposed to the military environment, culture, values and body composition requirements[28]. No additional inclusion/exclusion criteria were applied.

The MEBS development study was approved by the Institutional Review Board of the US Army Medical Research and Development Command, Fort Detrick, Maryland. Investigators adhered to the policies regarding the protection of human subjects as prescribed in Army Regulation 70-25 and the research was conducted in adherence with the provisions of 32 CFR Part 219. All Soldiers were assured of the confidentiality of their responses and provided written, voluntary informed consent.

The sample for the cross-sectional analyses reported herein is from 575 Soldiers (aged 18 to 42 years (mean ± SD 22·6 ± 3·4 year)) who completed the final version of the MEBS at Army installations at Joint Base Elmendorf-Richardson, AK (*n* 192), Joint-Base Lewis McChord, WA (*n* 98), Natick Soldier System Center, MA (*n* 31) and Fort Campbell, KY (*n* 254). In total, 607 Soldiers at these installations were briefed for potential participation, 577 of them provided informed consent and 575 completed the MEBS. From this same group of volunteers, a subsample of 446 Soldiers at Joint Base Elmendorf-Richardson, AK, and Fort Campbell, KY, were asked to complete a FFQ[27], of which 441 Soldiers agreed. A *post hoc* power analysis using G × Power[29] determined the sample size was sufficient for regression models with up to 10 predictors to detect an R^2 as small as 0·02 or an odds ratio of 1·2 with 80 % probability at a significance level of 0·05.

Dépendent variables

BMI and Healthy Eating Index score. BMI was calculated from self-reported height and weight. Height was captured by asking for 'Your height in feet and inches (without footwear)', and

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weight was captured by asking for 'Your current weight (without clothing in pounds)'. Biologically implausible values and outliers based on Army regulations for minimum and maximum height and weights[11] were excluded from the analysis (n 2). Self-reported height and weight have been shown to have a high correlation ($r = 0{\cdot}78$–$0{\cdot}88$) to measured values among Soldiers[30].

The Healthy Eating Index (HEI) is a validated measure of diet quality used to evaluate nutritional adequacy of the foods consumed based on food components included in the Dietary Guidelines for Americans (DGA)[31]. The HEI has been used to assess diet quality in both the general population and military populations[20,21,31]. HEI-2015 includes thirteen components that are scored based on their alignment with the 2015–2020 DGA recommendations. These component scores are based on adequacy of intake of total fruits, whole fruits, total vegetables, greens and beans, whole grains, dairy, total protein foods, seafood and plant proteins, and fatty acids. Because these food groups are encouraged, higher scores of these components reflect more desirable dietary intake. Components that are scored based on moderate consumption include refined grains, Na, added sugars and saturated fats. Higher scores for these components reflect lower intake due to the DGA recommendation to limit consumption. Each component can be scored independently and can also be evaluated together to assess overall diet quality, which is reflected by the total HEI score. The total HEI scoring system ranges from 0 to 100, with higher scores reflecting more optimal diet quality[31,32]. To facilitate interpretation, HEI-2015 scores are assigned letter grades, with scores between 90 and 100 interpreted as a grade of A, 80 to 89 a B, 70 to 79 a C, 60 to 69 a D and 0 to 59 an F[32]. HEI-2015 total scores were calculated from the validated Block 2014 FFQ[33] which assesses frequency of food and beverage intake over the past 6 months. Paper copies of the FFQ were administered along with photographs for portion size estimations. The study team, led by a Registered Dietitian, provided instruction and was available to answer questions. Questionnaires were analysed for macro- and micronutrient intakes, and HEI total and component scores by Nutrition Quest (Berkeley, CA, USA) using the US Department of Agriculture Food and Nutrient Database for Studies version 1.0.

Independent variables

Self-Reported weight cycling. Similarly to others, weight cycling was defined as ≥ 3 events of weight fluctuations[18,32], as determined by the question 'During your military career, how many times has your weight gone up AND down by more than 5 % of your body weight (excluding pregnancy or illness)?', to create a dichotomous variable for self-reported weight cycling (yes or no). To improve ability to identify 5 % changes in body weight, examples were provided for eight body weights ranging between 120 and 300 pounds. Socio-demographic variables were used to determine Soldier characteristics and used as control variables in analyses. Specifically, sex, race/ethnicity, education level and marital status were used as categorical control variables, and years in service as a continuous control variable, to reduce confounding. Additionally, tobacco use was dichotomised and controlled for in analyses since tobacco may be used as a weight management technique.

Mediator

Weight management behaviours. The measures of weight management behaviours used in this study are derived from the validated MEBS that provides a comprehensive, standardised, holistic and valid tool to examine eating habits mediators of eating behaviour in US military personnel[28]. Weight management behaviours were captured by the question 'Did you use any of the following methods leading up to your last Height & Weight/Tape test?' with options including 'Diet: changed what I ate, cut calories, skipped meals, eliminated a food group, or drank more water', 'Added new physical activity, increased time or intensity of usual exercise', 'Used muscle building, fat burning, or weight loss supplements', 'Neck exercises or wore a waist trainer to improve body fat measurements', 'Took diuretics, took laxatives (including Mg), or made myself vomit', 'Sat in a sauna/steam room, sat in Epsom salt bath, covered my body with Preparation H® and Saran Wrap, or stopped drinking water', and 'Other' which had an option to write in a response.

Statistical analysis

Data were analysed using Stata, v.15.1 (StataCorp) or SPSS, v.26 (IMB Statistics). Data were complete for all but three Soldiers (0·01 %) who had a missing response on race/ethnicity and one Soldier (0·002 %) who had a missing response on years of service. The data were screened for violations of assumptions prior to analysis. BMI and HEI score skewness and kurtosis were 0·35 and 2·94 and −0·11 and 2·97, respectively, but to aid interpretation, both were used without transformation[18]. No other violations were noted. The characteristics of Soldiers by frequency of self-reported weight cycling were examined using χ^2 and ANOVA statistics with *post hoc* tests to show between group differences. Additionally, we examined the characteristics of Soldiers based on those who did or did not complete the FFQ for between group differences.

For this cross-sectional study, multiple linear regression using the Stata *sem* command with full-information maximum likelihood estimation using the *mlvm* option was implemented to examine associations between weight cycling (dichotomous variable), weight management behaviours (categorical variable), and BMI or HEI scores (continuous variables). This estimation method assumes missing data among independent variables is missing at random, which allows the use of data from all participants who do not have missing data on the dependent variable. Root mean square error of approximation was used to assess goodness of fit.

Based on Hayes[34], simple associations between BMI, HEI score, weight cycling and weight management methods are not required as a pre-condition for pursuing a mediation analysis. Therefore, regardless of the presence or absence of simper associations, simple mediation models as described by Hayes[34] were implemented using the *process* command in SPSS to test the relationship between weight cycling and BMI, also known as the direct effect, and examine if weight management behaviours acts as a mediator between weight cycling and BMI, also known as the indirect effect. Using the same method, an additional model was implemented to test the same mediator relationship with the outcome of HEI score. The *process* command assumes complete

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British Journal of Nutrition

data and excludes participants that have missing data on any of the variables included in the models. For mediation models, weight management behaviours were consolidated to create a categorical variable because the *process* command does not allow binary mediators. To ensure the normality of the sampling distribution and to assess the indirect effect of weight cycling on BMI and HEI score, through weight management behaviours, bootstrap CI were generated based on 10 000 bootstrap samples. Weight management behaviours were considered to be a mediator if the bootstrap 95 % CI for the indirect effect was above 0. All statistical models included several control variables which were selected a priori. Control variables included years of military service, sex, race/ethnicity, highest level of education achieved, military rank and marital status. Additionally, tobacco use was included as a control in all models since tobacco may be used for weight management purposes.

Results

Overall, 33 % of Soldiers reported weight fluctuations that met the criteria for weight cycling. BMI was higher (+1·8 kg/m^2, $P < 0{\cdot}001$), but HEI score did not differ between Soldiers who reported weight cycling relative to those who did not ($P = 0{\cdot}46$; Table 1). Soldiers who reported weight cycling were also more likely to engage in one or more weight management behaviours prior to body weight screening and had more years of service in the Army relative to those who did not report weight cycling (Table 1). For differences based on FFQ completion, no differences were observed on key variables of interest such as weight cycling or weight management methods; however, there were differences in age, years in service, education and marital status which were controlled for in statistical models and are likely due to heterogeneity at Army posts where the volunteers were recruited (online Supplementary Table 1).

In regression models, weight cycling was associated with a 1·09 point higher BMI which equated to 5·45 pounds ($P < 0{\cdot}001$), using dieting with a 1·67 points higher BMI or 8·35 pounds ($P =< 0{\cdot}001$), exercise with a 1·0 point higher BMI or 5·0 pounds ($P = 0{\cdot}007$), and dehydration methods with a 1·46 point higher BMI or 7·3 pounds ($P = 0{\cdot}003$), respectively (Table 2). Use of muscle building, fat burning or weight loss supplements prior to body weight screening was associated with

Table 1. Characteristics of soldiers, stratified by self-reported weight cycling (Percentages; mean values and standard deviations)

	Self-reported weight cycling									
	All (*n* 575)			Yes (*n* 190)			No (*n* 385)			
Socio-demographic variables, mean (SD) or %	%	Mean	SD	%	Mean	SD	%	Mean	SD	*P*
BMI (kg/m^2)		25·7	3·6		26·9	3·8		25·1	3·3	**< 0·001**
Underweight/normal: ≤ 24·9	44·9			30·5			52·0			**< 0·001**
Overweight: 25·0–29·9	43·8			51·6			40·0			**0·008**
Obese: ≥ 30·0	11·3			17·9			8·0			**< 0·001**
HEI-2015 total score		59·0	10·5		59·1	10·3		58·7	10·8	0·46
Weight management methods (%)										
Dieted	26·1			36·3			21·0			**< 0·001**
Increased exercise	30·3			38·4			26·2			**0·004**
Supplement use	13·0			17·9			10·6			**0·02**
Neck exercises/waist trainer	7·7			12·2			5·5			**0·01**
Diuretics/laxatives/vomiting	4·7			8·9			2·6			**0·001**
Dehydration (sauna/steam room)	11·3			18·4			7·8			**< 0·001**
Other methods	0·2			0·0			0·3			1·00
Sex										0·87
Male	89·6			90·0			89·4			
Female	10·4			10·0			10·6			
Age, years		23·4	4·4		23	4·3		23	4·5	0·14
Years of service in the army		2·3	2·8		3·0	3·3		2·0	2·5	**0·0001**
Race/ethnicity										
Non-Hispanic White	51·2			47·1			53·3			0·18
Non-Hispanic Black	14·7			17·5			13·3			0·21
Non-Hispanic Other	11·4			24·9			21·7			0·40
Hispanic	22·7			10·6			11·7			0·78
Education level										
High school/GED	53·0			49·5			54·8			0·25
Some college	32·0			34·2			30·9			0·45
Degree	15·0			16·3			14·3			0·54
Marital status										0·59
Single	61·0			59·5			61·8			
Married/living with a partner	30·0			40·5			38·2			
Use tobacco products	29·4			29·0			29·6			0·87
Rank categories										0·89
Enlisted	88·8			89·4			88·5			
Officer	11·2			10·6			11·5			

GED, General Educational Development Test; HEI, Healthy Eating Index.
Numbers in bold indicate differences significant at the $P < 0{\cdot}05$ level.

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Table 2. Results of regression models of BMI or Healthy Eating Index (HEI) score by weight cycling and weight management methods (Coefficient values and standard errors)

	BMI (kg/m^2) (*n* 575)			HEI score (*n* 441)*		
Predictor/mediator	*β*	SE	*P*	*β*	SE	*P*
Weight cycling	1·09	0·28	< **0·001**	–0·35	1·05	0·74
Dieted	1·67	0·40	< **0·001**	0·72	1·60	0·65
Increased exercise	1·00	0·39	**0·01**	1·19	1·53	0·44
Supplement use	–0·84	0·45	0·06	1·99	1·76	0·26
Neck exercises/waist trainer	1·05	0·58	0·07	–1·82	2·29	0·43
Diuretics/laxatives/vomiting	0·94	0·68	0·17	–2·21	2·66	0·41
Dehydration (sauna/steam room)	1·46	0·49	**0·003**	3·76	1·84	**0·04**
Other methods	1·21	3·11	0·70	8·69	10·47	0·41
Control variables						
Women	–2·70	0·44	< **0·001**	0·92	1·73	0·60
Years of service	0·08	0·05	0·08	0·23	0·24	0·13
Non-Hispanic Black	–0·31	0·38	0·42	0·76	1·43	0·28
Non-Hispanic Other	0·22	0·42	0·60	–2·43	1·59	0·18
Hispanic	0·24	0·33	0·47	1·36	1·25	0·28
Some college	0·54	0·29	0·07	1·52	1·14	0·18
Degree	0·80	0·39	**0·04**	3·92	1·44	**0·01**
Married	0·57	0·28	**0·04**	–0·07	1·08	0·95
Used tobacco products	–0·01	0·29	0·98	–2·40	1·10	**0·03**
Constant	21·52	6·25	0·001	39·81	20·97	0·06

β, unstandardized regression coefficient. Weight cycling refers to ≥ 3 weight fluctuations of ±5 % of body weight. Referent group was no weight cycling, men, non-Hispanic White, high school education, single, no tobacco. Numbers in bold indicate level of significance at $P < 0·05$.
* Model included the 441 Soldiers who had HEI scores. Root mean square error of approximation for both models was 0·01.

lower BMI of 0·84 points or 4·2 pounds ($P = 0·02$). Using dehydration methods prior to body weight screening was associated with 3·76 higher HEI score ($P = 0·04$), which is of low practical significance.

The simple mediation models showed that weight cycling indirectly influenced both outcomes of BMI and HEI scores through its effect on weight management behaviours (Fig. 1). Thus, weight management behaviours mediated the relationship between weight cycling and BMI and weight cycling and HEI scores (Fig. 1). For the BMI model, both the direct and indirect effects were statistically significant demonstrating that weight cycling may affect BMI directly and through weight management behaviours as a mediator. In the model of HEI scores, there was no evidence that weight cycling influenced HEI scores independently of its effect on weight management behaviours showing that its effect on HEI scores was mediational only.

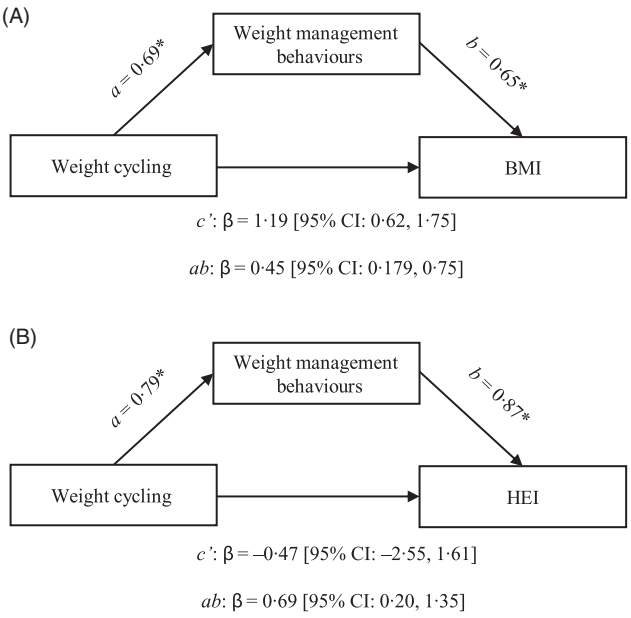


Figure 1. Mediation effect of weight management behaviors on the association between self-reported weight cycling and (A) BMI (*n* 571), and (B) Healthy Eating Index (HEI; *n* 441) score among Soldiers. Models adjusted for sex, years of service, race/ethnicity, education level, marital status and tobacco use. 95 % CI were generated from 10,000 bootstrap samples. Weight management behaviours were considered to be a mediator if the bootstrap 95% CI for the indirect effect was above zero. *ab*, indirect effect; *c*, direct effect. *$P \leq 0.001$.

Discussion

This study examined the relationships between self-reported weight cycling, weight management behaviours prior to body weight screening, BMI and diet quality among US Army Soldiers. Findings indicate that weight cycling is common among US Army Soldiers and is associated with higher BMI and greater likelihood of adopting weight management behaviours prior to body weight screening. When examined further, adopting any weight management behaviour prior to body weight screening mediated associations between weight cycling and BMI and weight cycling and HEI scores. To our knowledge, these findings are the first to link weight management behaviours to weight cycling, BMI and diet quality in US Army Soldiers and suggest that weight management behaviours could be potentially modifiable intervention targets for Soldiers who weight cycle.

The 33 % prevalence of weight cycling observed in this study suggests that weight cycling is common in US Army Soldiers. In comparison, reports on weight cycling in civilian populations indicate a prevalence of 18 %–34 % in men and 20 %–55 % in women[35–39]. Prevalence of weight cycling in athletes is unclear, but previous studies estimated that around 50 % of athletes

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participating in combat sports (e.g. boxing, wrestling, mixed martial arts, judo, jiu-jitsu, karate, taekwondo), use rapid weight loss methods prior to competition[40,41]. Though studies differ in their definitions of weight cycling, the predominantly male population in the present study suggests that weight cycling in Soldiers may be as prevalent as that observed in civilian populations.

Soldiers who reported weight cycling were more likely to report higher BMI and adopting various weight management behaviours prior to body weight screening. Though causality cannot be demonstrated with these data, the association between weight cycling and BMI is consistent with robust data from civilian populations linking weight cycling with weight gain and higher BMI[2,6–10,42]. Notably, the observed association between weight cycling and BMI was independent of years of service in the Army. This may suggest that factors other than exposure to the Army environment, such as physiologic adaptations to weight loss that promote weight regain[4–6], may contribute to the relationship. Aspects of Army culture related to body composition standards may exacerbate the weight cycling relationship identified due to the stigma of being enrolled in a weight loss programme and potential impact on career progression. Previous studies have also demonstrated that military service members cyclically engage in weight management behaviours to comply with biannual physical fitness and body weight/composition screenings[10,12,43–45]. Those behaviours include purging or using diet pills, body wraps and saunas, and have been associated with body weight dissatisfaction and poor mental health outcomes[10,12,14,46]. Similarly, athletes participating in combat sports report behaviours such as increasing physical activity, using saunas and using plastic clothing to achieve rapid weight loss prior to competition[40]. Taken together, these findings suggest that pressure to meet the Army's body composition standards may be one factor which promotes the adoption of weight management behaviours in some Soldiers. Those behaviours, in turn, may contribute to higher BMI which would increase difficulty in complying with body weight and composition standards, thereby perpetuating cycles of losing and regaining weight.

Whether high BMI or an initial weight loss initiated weight cycling in the study population cannot be determined. However, regardless of the initiating factor, the cycle is likely to persist without interventions that prevent or modify the weight management behaviours mediating the association between weight cycling and BMI. In regression models, weight cycling was associated with a 1·09 point higher BMI which equated to 5·45 pounds ($P < 0·001$). Given that a Soldier's career can hinge on relatively small differences in body weight, these differences in BMI are occupationally meaningful. Potential interventions might include holistic initiatives that promote sustainable weight loss and healthy weight maintenance. For example, exposure to an Army eating environment, which is often characterised by limited and high-cost nutrient dense items, abundance of fast foods or hyper palatable foods, time constraints, and stress[16,27,46,47] early in a Soldier's career may foster the development of undesirable eating behaviours, such as fast eating and eating irrespective of hunger and fullness cues[16,47]. This exposure may inadvertently set Soldiers on a trajectory by which they must adopt weight management behaviours to compensate for these habits. In support, military personnel from various ranks have acknowledged that environmental barriers not only hinder behaviour changes but make application of healthy nutrition behaviours seem unrealistic[26]. The current military eating environment and social norms also do not acknowledge the multitude of factors that impact eating behaviours beyond the individual, such as environment and psychosocial influences[26,48]. When environmental and psychosocial factors are included within Army health promotion programmes, greater change in behaviours has resulted[48]. Creating a culture of health, removing barriers, moving beyond nutrition education alone and taking a more holistic approach to influencing behaviour change in Soldiers may therefore increase the effectiveness and sustainability of weight management interventions and warrant consideration in future research as aiming to prevent unwanted weight gain and weight cycling.

Results of the present study suggest that interventions aimed at improving diet quality also warrant consideration. Contrary to our hypothesis, weight cyclers did not report worse diet quality than those who did not weight cycle. This could reflect the lack of variability and relatively low HEI scores within the study population, and that dieters often eat healthier foods during periods of weight loss[3]. Notably, HEI scores for both those who did and did not report weight cycling received an 'F' for how closely dietary intakes conformed to the 2015 Dietary Guidelines for Americans, with totals scores being 59·1(10·3) and 58·7(10·8), respectively. Poor diet quality among Soldiers is consistent with recent studies that have reported Soldier eating patterns do not provide optimal energy availability, adequate macronutrient distribution or sufficient micronutrients when compared with sports nutrition guidelines or Military Dietary Reference Intakes[21,26,49]. Soldiers with more optimal diet quality are more likely to have more desirable body composition, APFT scores and emotional fitness scores[19]. Thus, diet quality, body weight and composition, mental health and occupational performance appear to be closely linked among US Army Soldiers. Notably, mediation analysis suggested that weight cyclers who adopted weight management behaviours had higher diet quality. However, that population also reported higher BMI. Taken together, those findings reinforce the need for holistic interventions aimed at promoting not just optimal diet quality, but also sustainable eating habits that facilitate long-term healthy body weight maintenance.

Strengths of this study include the use of a survey tailored to military populations, and the inclusion of Soldiers with diverse occupations and from multiple military installations located in different areas of the USA. However, despite this demographic and geographic diversity, the study population was slightly overrepresented by lower-ranking Soldiers in comparison to the full Active Duty population[50]. For example, officers comprise about 19 % of the full Active Duty population, but only 11 % of the study population. Thus, while findings are likely generalisable to enlisted Soldiers, the generalisability to officers requires further investigation. Several additional study limitations should also be noted. Foremost, the cross-sectional design precludes determining cause and effect. Another limitation was that the *process* command used in the mediation analysis, while widely

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used and accepted for estimating direct and indirect effects in mediation models[51], did not allow us to distinguish which specific weight management behaviour(s) acted as a mediator between study outcomes. Additionally, although both the MEBS and FFQ are validated tools, responses could have been influenced by social desirability bias, recall bias or individual biases. Height and weight were also self-reported which may introduce misreporting or recall bias. However, previous studies indicate that Soldiers are accurately able to report height and weight[30]. Finally, there is no standard definition of weight cycling which complicated comparisons of the prevalence of weight cycling in this cohort with that reported in other populations.

Conclusion

This study demonstrated that self-reported weight cycling is associated with higher BMI, and higher prevalence of adopting weight management behaviours prior to body composition screening in active duty Soldiers. This study is the first to suggest that weight management behaviours mediate the relationship between weight cycling and BMI and weight cycling and HEI scores among US Army Soldiers. Though cause and effect cannot be established, these findings extend the evidence base suggesting a relationship between Army body weight and composition standards, though well intentioned, and the adoption of weight management behaviours by some Soldiers that, rather than promoting health, could ultimately compromise health and occupational performance. As discussed above, aspects of Army culture related to body composition standards may exacerbate the weight cycling relationship identified due to the stigma of being enrolled in a weight loss programme and potential impact on career progression. Utilising the Army's arsenal of resources of medical and specialty providers to proactively educate Soldiers on the undesirable long-term consequences of weight cycling and the importance of good diet quality in an environment that models healthy behaviours warrants investigation as one strategy to healthy weight management and ensuring a nutritionally fit force.

Acknowledgements

The Soldiers who participated in this research are gratefully acknowledged as well as the research staff and soldiers who assisted with study implementation and data collection. Dr Andrew Young is also acknowledged for editorial assistance.

This study was funded by the Army Medical Research and Development Command.

Authors R. C. and J. K. designed the study and wrote the protocol with assistance from S. M., K. O. and A. D. J. J., J. A. and J. K. developed the research questions for this manuscript with input from R. C. J. J., J. K., S. M., K. O., A. D., and R. C. collected the data. J. J. conducted the statistical analysis with input from J. A. and J. K. J. A. wrote the first draft of the manuscript. All authors contributed to subsequent manuscript drafts and have approved the final version.

The authors declare no conflicts of interest

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S000711452100338X

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Healthy Eating Score–7 as a Measure of Diet Quality in a Military Population

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ABSTRACT

Objective: Identify potential revisions to the Healthy Eating Score (HES–5) that improve associations with the Healthy Eating Index (HEI) –2015 total and component scores.

Methods: Pearson *r* correlations were determined from soldiers' (n = 433) survey data, including the HES, proposed additional questions, and the Block Food Frequency Questionnaire.

Results: Adding sugar-sweetened beverages and energy drink questions (HES–7) strengthened the HES and HEI–2015 correlation (HES–5; $r = 0.42$, n = 433, $r^2 = 0.18$, $P < 0.001$) (HES–7; $r = 0.52$, $r^2 = 0.27$, $P < 0.001$). The HES components and Block Food Frequency Questionnaire consumption correlations were as follows: quantity of fruit ($r = 0.37$, $r^2 = 0.14$, $P < 0.001$), vegetables ($r = 0.41$, $r^2 = 0.17$, $P < 0.001$), whole grains ($r = 0.35$, $r^2 = 0.12$ $P < 0.001$), dairy ($r = 0.34$, $r^2 = 0.12$, $P < 0.001$), fish ($r = 0.31$, $r^2 = 0.10$, $P < 0.001$), and energy drink ($r = 0.59$, $r^2 = 0.35$, $P < 0.001$).

Conclusions and Implications: HES–7 had the strongest correlation with HEI–2015. Future studies can explore if including consumption quantity in the HES improves its representation of diet quality.

Key Words: Healthy Eating Score–5, Healthy Eating Index–2015, diet assessment, Block Food Frequency Questionnaire, military (*J Nutr Educ Behav.* 2022;54:455–464.)

Accepted September 29, 2021. Published online January 6, 2022.

INTRODUCTION

In 2015, > 325,000 or 69.4% of US soldiers[1] were reported to be overweight or obese,[2] and in 2019, 17% of military service members were reported to be obese.[1] Overweight and obesity can be detrimental to soldiers' readiness or ability to execute all duty-required tasks, putting them at greater risk of musculoskeletal injuries[3] and chronic diseases.[3,4] Force readiness is a focus of the US Army[5] and emphasizes all aspects of soldiers' health, including nutritional fitness.[6] Nutritional fitness is a concept that encompasses providing and consuming foods in the quantity and quality needed to perform optimally and prevent disease and/or injury.[7] The US Joint Chiefs' Total Force Fitness framework identifies nutritional fitness as a cornerstone of warfighter health with multiple nutrition-focused programs implemented across all military branches.[8]

A method of assessing nutritional fitness is diet quality.[7] Diet quality describes the wholesomeness and nutrient content of foods and whether the diet meets an individual's nutritional requirements.[9] Better diet quality is related to reduced obesity[10–12] and improved physical fitness,[13] weight maintenance,[14,15] and weight loss.[14,15] The diets of military personnel are generally low in fruits, vegetables, and whole grains and high in refined grains and saturated fats,[16,17] and military environments may promote these diet patterns.[18] These diet patterns may impact training, injury risk, and increase the risk of chronic diseases that may reduce performance. In addition, high diet quality is associated with mental resilience in military personnel[19] and better physical performance in elite soldiers.[20]

The validated Healthy Eating Index (HEI) is the gold standard for measuring diet quality in the US.[21] The HEI–2015 assesses diet quality in American populations by examining the consumption of 13 food groups and nutrient components (Table 1).[22] The HEI is updated every 5 years to reflect changes to the Dietary Guidelines for Americans and captures how closely an individual's diet pattern aligns with the most current recommendations.[23] Many tools are used to capture diet consumption, but some may not be suitable for studies involving data collected from a large number of subjects for various reasons.[24] Twenty-four–hour diet recalls are time-consuming and require multiple days of gathering data.[24] Dietary histories gather information

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Conflict of Interest Disclosure: The authors have not stated any conflicts of interest.

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Published by Elsevier Inc. on behalf of Society for Nutrition Education and Behavior.
https://doi.org/10.1016/j.jneb.2021.09.019

Table 1. Nomenclature for Survey Instruments Used to Collect Data (n = 433)

Instrument	Abbreviation	Description of Data Gathered	Assesses	Current Use
Healthy Eating Score–5	HES-5[21,23]	Fruit (fresh, frozen, canned or dried, or 100% juices), vegetables (fresh, frozen, canned, cooked or raw: dark green vegetables [broccoli, spinach, most greens] orange vegetables [carrots, sweet potatoes, winter squash, pumpkin], legumes [dry beans, chick peas, tofu], starch vegetables [corn, white potatoes, green peas], and other [tomatoes, cabbage, celery, cucumbers, lettuce, onions, peppers, green beans, cauliflower, mushrooms, summer squash, etc]), whole grains (rye, whole-wheat, or heavily seeded bread; brown or wild rice; whole-wheat pasta or crackers; oatmeal; corn tacos), dairy (regular/whole fat milk; low- or reduced-fat milk (2%, 1%, 0.5%, or skim), yogurt, cottage cheese, low-fat cheese, frozen low-fat yogurt, soy milk, or other calcium-fortified foods (orange juice, soy/rice milk, breakfast cereals, etc), fish (tuna, salmon, or other nonfried fish)	Diet quality	Military
Healthy Eating Score–6_1	HES–6_1[21,23]	All HES–5 foods and SSBs (Coke, Sprite, flavored soda, Mountain Dew, sweet tea, lemonade, Frappuccino)	Diet quality	Not in use
Healthy Eating Score–6_2	HES–6_2[21,23]	All HES–5 foods and energy drinks (Monster, Red Bull, Rip-It, NOS, 5-Hour)	Diet quality	Not in use
Healthy Eating Score–7	HES–7[21,23]	All HES–5 foods and SSBs (Coke, Sprite, flavored soda, Mountain Dew, sweet tea, lemonade, Frappuccino) and energy drinks (Monster, Red Bull, Rip-It, NOS, 5-Hour)	Diet quality	Not in use
Block Food Frequency Questionnaire	Block FFQ[25]	Eggs and dairy foods; cereals, grains, and breads; vegetables; fruits; beans, tofu, and meat substitutes; soup, mixed dishes, and noodles; meat and chicken; fish, seafood; nuts, seeds, snacks; sweets and desserts; spreads, sauces, other foods; and beverages	Frequency and quantity of foods consumed	Nonmilitary and military
Healthy Eating Index–2015	HEI–2015[22,23]	Total vegetables, greens and beans, total fruit, whole fruit, whole grains, dairy, total protein, seafood and plant proteins, fatty acids, refined grains, sodium, added sugars, saturated fats[22] Two adequacy categories: maximum of 5-point score components (total fruit, whole fruit, total vegetables, greens and beans, total protein foods, and seafood, plant proteins), maximum of 10-point score components (whole grains, dairy, fatty acids). One moderation component: a maximum of 10-points (refined grains, sodium, added sugars, and saturated fats)	Diet quality	Nonmilitary and military

Note: Healthy Eating Score-6_1 indicates HES-5 plus sugar-sweetened beverages; Healthy Eating Score 6_2 indicates HES-5 plus energy drinks.

over a longer period but are costly and time-consuming because they involve a trained surveyor guiding participants through a survey.[24] Food frequency questionnaires (FFQ) are comprehensive surveys usually self-administered, capturing 6–12 months' worth of food intake data.[24] Although appropriate for data collection from large populations, FFQs are still time-consuming for participants and expensive to evaluate.[24] The Block FFQ is a type of FFQ that can be used to estimate HEI–2015 scores.[25] The Block FFQ collects food intake data for the HEI computation but carries the limitations of taking 60 minutes to complete and is costly to score and analyze.[26] Because of length and respondent burden, the Block FFQ has a 24% nonresponse rate in some studies and is not practical to administer in certain environments in which access to volunteer populations is limited.[26] A validated, shorter survey to assess diet quality may improve response rates by reducing participants' time burden and assessment time and costs.[27]

In 2012, the Healthy Eating Score –5 (HES–5) was designed to assess diet quality in a large cross-sectional military population.[10] The HES–5, scored on a scale of 0–25, is a survey developed to be a proxy for the HEI –2005 and consists of 5 questions assessing consumption frequency, but not quantity, of fruit, vegetables, whole grains, dairy, and fish over 30 days.[10] Results from the HES–5 found that service members who scored in the highest quartile of the HES–5 slept better, less frequently reported binge drinking, consumed less soda, and met the national recommendations for physical activity compared with service members in the lowest quartile.[10] Although the HES–5 has been successfully implemented as a field expedient tool to assess diet quality on the basis of HEI –2005 guidelines, it is not reflective of the current HEI–2015 version and evidence surrounding its correlation with health and performance outcomes in military populations is limited.[23,27]

Including new HES questions to measure other food and nutrient components measured in the HEI –2015 should improve the ability of

the HES to measure diet quality. The proposed questions to add to the HES−5 were selected from the Military Eating Behaviors Survey (MEBS) that is designed to assess eating behaviors and factors influencing eating behaviors among military personnel.[28] There were 5 questions from the MEBS that could be added to the HES−5 that captured alcohol consumption, dining out frequency, breakfast consumption, sugar-sweetened beverage (SSB) consumption, and energy drink (ED) consumption.[28] Alcohol consumption and meal timing (breakfast consumption and dining out frequency) are not concepts captured in the HEI−2015.[21] Therefore, those 3 questions were not added to the HES−5. However, the HEI−2015 captures added sugar as its own component,[23] and thus adding SSBs and energy drinks as new components of the HES−5, creating the HES−7, may strengthen the correlation to the HEI−2015.[21,23]

An improved ability to quickly and accurately assess diet quality within large populations, to include military cohorts, would provide researchers, policymakers, and health professionals greater insight into diet quality within populations and associations between diet quality and health outcomes. The purpose of this study was to determine the strength of associations between the HES−5 and HEI−2015 and to determine if that association could be improved by adding 2 questions (SSB and ED consumption) to the HES−5 to create the HES−7. A secondary aim was to determine the association between HES−5, SSB, and ED component scores that only assess the frequency and not the quantity of consumption and total intake of foods measured by FFQ.

METHODS

Research Design, Population, and Recruitment Procedures

This was a cross-sectional study that used data collected during the development and validation of a questionnaire designed to assess the eating behaviors of military personnel.[28] The Institutional Review Board of the US Army Medical Research and Development approved the study. Soldiers were recruited from Joint Base Elmendorf-Richardson, AK, and Fort Campbell, KY, in February 2018. All soldiers were orally briefed on the study before providing consent to participate. Inclusion criteria were active-duty US Army soldiers aged ≥ 18 years, with English as a primary language, and completed initial military training (eg, basic combat training). There were no exclusion criteria. A total of 467 soldiers were briefed, 448 soldiers provided voluntary written informed consent; however, 15 participants departed the auditorium without completing the survey or notifying the research staff, such that 433 soldiers completed the questionnaires described below. The research staff reviewed all completed questionnaires before participant departure to minimize errors and missing data.

Survey Instruments

The validated MEBS[28] and the Block FFQ were administered. The MEBS is a paper survey comprised 3 questionnaires: demographics (25-items), eating habits (43-items), and mediators of eating behavior (90-items). The descriptive demographics data from the questionnaire used for this analysis included sex, age, military rank, education level, marital status, and race/ethnicity (Table 2). Male and female were the only 2 options participants could select to identify their sex. Questions on the ethnic and racial backgrounds were asked in 2 separate questions. The first question, "What is your ethnic background?" had 2 options, Hispanic/Latino and not Hispanic/Latino. The second question, "What is your racial background? (choose all that apply)," had 6 options: White/Caucasian, Black/African American, Native American/Alaskan Native, Asian, Native Hawaiian/Pacific Islander, and other (open response). The only information included in this analysis from the eating habits questionnaire was the HES−5 and two questions related to ED and SSB intake (noted below). Weight and height

Table 2. Summary of Participants' Demographic Characteristics (n = 433)

Characteristic	Range	Mean ± SD or n (%)
Sex		
Male		392 (90.6)
Female		41 (9.4)
Age, y	18−42	23.0 ± 3.4
Rank categories		
Enlisted		381 (88.6)
Officer		52 (12.4)
Education level		
High school/GED		242 (56.3)
Some college		125 (28.5)
Degree or higher		66 (15.3)
Marital Status		
Single		277 (64.3)
Married		156 (36.7)
Race/ethnicity		
Non-Hispanic White		218 (49.1)
Non-Hispanic Black		72 (16.2)
Non-Hispanic Other		50 (11.4)
Hispanic		103 (23.3)
BMI, kg/m^2	16.3−39.1	25.7 ± 3.8
HES−5 Score	7−18	11.0 ± 4.0
HES−7 Score	7−33	20.0 ± 5.0
HEI−2015 Score	28−86	59.0 ± 10.0

BMI indicates body mass index; GED, General Education Development; HEI, Healthy Eating Index; HES, Healthy Eating Score.

information from the MEBS was used to calculate body mass index (BMI).

Healthy Eating Score and adaptations. The HES−5 is a validated questionnaire designed to quickly examine how well soldiers met the Dietary Guidelines for Americans.[10] The foundational question asked: "Over the last 30 days, how often did you eat/drink the following foods/beverages?" Immediately following the question were headings that represented the 5 original HES−5 food components (fruit, vegetables, whole grains, dairy, fish) and the 2 new beverage components (SSB and ED) (Table 1). These components were chosen because they capture added sugar consumption that is part of the HEI−2015 but not part of the HES −5.[21,23] The scoring mechanism is detailed in Table 3 with points ranging from 0−5 points. The highest scores represent meeting the dietary guidelines. The scoring mechanism for fish differed slightly as the guidelines reflect weekly intake, not daily; thus, the scores were collapsed to 0, 3, and 5 points (Table 3). A limitation of the HES−5 is that no serving quantity was included. The researchers included the HES−5 as intended and did not modify the instrument.

The SSB and ED items were reversed scored and added to the HES −5 in all 3 variations for analysis (Table 4). The maximum HES−5, HES -6_1, HES-6_2, and HES−7 scores were 25, 30, 30, 35, respectively (Table 4). The HEI−2015 total and component scores were calculated using the standardized scoring method.[23]

Block FFQ. Administered via paper, the Block FFQ asked participants to report the consumption quantity and frequency of > 100 food and beverage items over the last 6 months (Table 1).[25] Pictures of 4 different portions of food, including one-fourth, one-half, 1, and 2 cups, were provided in the Block FFQ to assist the participant in estimating how much was eaten.[25] Other portion sizes were asked and included the following questions: How many eggs a day? How many tablespoons? How many slices? How many pieces? How many buns? etc.[25] The Block FFQ asked how often, on average, a type

Table 3. Healthy Eating Score−5 (HEI−5), Proposed Additional Healthy Eating Score (HES) Components and Scoring Rubrics

		Frequency of Consumption and Scores					
Rubric	**Components**	**≥ 3 times/d**	**2 times/d**	**1 time/d**	**3−6 times/wk**	**1−2 times/wk**	**Rarely or Never**
HEI−5 Components and Scoring Rubric[a]	HES−5						
	Fruit[b]	5	4	3	2	1	0
	Vegetables[c]	5	4	3	2	1	0
	Whole Grains[d]	5	4	3	2	1	0
	Dairy[e]	5	4	3	2	1	0
	Fish[f]	5	5	5	5	3	0
Proposed Additional HES Components and Scoring Rubric[g]	New HES						
	Sugar-sweetened beverages[h]	0	1	2	3	4	5
	Energy drinks[i]	0	1	2	3	4	5

[a]Table adapted from Purvis et al[10]; [b]Fresh, frozen, canned or dried, or 100% juices; [c]Fresh, frozen, canned, cooked, or raw: dark green vegetables (broccoli, spinach, most greens), orange vegetables (carrots, sweet potatoes, winter squash, pumpkin), legumes (dry beans, chickpeas, tofu), starchy vegetables (corn, white potatoes, green peas), and other (tomatoes, cabbage, celery, cucumbers, lettuce, onions, peppers, green beans, cauliflower, mushrooms, summer squash, etc); [d]Rye, whole-wheat, or heavily seeded bread; brown or wild rice; whole-wheat pasta or crackers; oatmeal; corn tacos.; [e]Regular/whole fat milk; low- or reduced-fat milk (2%, 1%, 0.5%, or skim), yogurt, cottage cheese, low-fat cheese, frozen low-fat yogurt, soy milk, or other calcium-fortified foods (orange juice, soy/rice milk, breakfast cereals, etc); [f]Tuna, salmon, or other nonfried fish; [g]Table adapted from a validated questionnaire assessing soldiers' eating behaviors[28]; [h]Examples: Coke, Sprite, flavored soda, Mountain Dew, sweet tea, lemonade, Frappuccino; [i]Examples: Monster, Red Bull, Rip-It, NOS, 5-Hour.

Table 4. Results of the Correlation Analysis between Variations of the Healthy Eating Score (HES) and the Healthy Eating Index (HEI)−2015 (n = 433)

	New Item Options					
HES Combination	**Sugar-Sweetened Beverages**	**Energy Drinks**	***r***	***r*²**	***P***	**Maximum Score**
HES−5			0.42	0.18	0.001	25
HES−6_1	✓		0.50	0.24	0.001	30
HES−6_2		✓	0.47	0.22	0.001	30
HES−7	✓	✓	0.52	0.27	0.001	35

Note: Checked blocks indicate which variables were included in each variation.

of food was eaten.[25] The 9 options for how often included: never, a few times per 6 months, once per month, 2−3 times per month, once per week, 2 times per week, 3−4 times per week, 5−6 times per week, and every day.[25] The Block FFQ was used to determine HEI−2015 scores.[25]

Health Eating Index−2015. The HEI −2015 is a validated diet quality index that captures how closely an individual's diet pattern meets the 2015−2020 Dietary Guidelines for Americans recommendations.[22,23] The HEI−2015 used a density method (per 1,000 kcal) to standardize the scoring across energy needs, allowing an evaluation of diet quality regardless of daily energy needs.[22,23] The HEI−2015 scores ranged from 0 to 100, and higher scores indicated better diet quality.[25] The HEI−2015 had 2 main components: adequacy and moderation. Adequacy components are categories individuals should include more of in their diet; moderation components are categories individuals should limit in their diets.

Block FFQ quantity and frequency components. The total quantity and frequency of consumption for individual FFQ food items within food groups assessed by the HES−5 and SSB and ED were summed to assess the total frequency (ie, servings/d) and quantity (ie, g/d) of consumed foods within each food group (Table 5 footnote legend).

Data Analysis

All analyses, including Pearson *r* coefficient correlation, coefficients of determination, and descriptive statistics, were performed using SPSS software (version 25.0, IBM Corp, Armonk, NY, 2017) with statistical significance set at $P < 0.05$. All tests were 2-sided. Descriptive statistics were used to assess the population and reported as either mean ± SD or percent. Pearson *r* coefficient correlation and corresponding coefficients of determination were then used to compare scores and assess variability explained, respectively, for the different HES versions with HEI−2015 scores, average frequency, and quantity of consumption of foods within individual food groups. To test for potential differences between HES versions and HEI−2015 scores, the correlations were transformed into *z*-scores using Fisher *r*-to-*z* transformation. The r^2 squared values were calculated to estimate the variation in HEI−2015 scores explained by the HES version. Fisher exact test was used to determine if demographic bias existed in the survey population. Shapiro-Wilk normality test was used on all surveys to determine the normality of data. After Lin's Concordance Correlation was performed, Bland-Altman plots were generated to assess the agreement of the HES and HEI scores to 1.96 SDs using a 95% confidence interval. Because of the suspected impact of differently scaled data, this analysis was performed again after the scores had been normalized and the scores of total energy (kcal) outliers were assessed. Distribution of the total energy (kcal) outliers was observed, and a 5% to 95% winsorization was conducted. Winsorization did not alter the distribution, and energy outliers were not significantly connected to HEI or HES scores; thus, the outliers were not removed. Fisher's exact test, Shapiro-Wilk normality test,[29] Lin's Concordance Correlation,[30] and Bland-Altman plots were generated using R (version 4.0.5, R Core Team, Vienna, Austria, 2018).

RESULTS

The study population was predominantly single, young, White, enlisted men, representing the broader Army population[31] except for the study population being generally younger (Table 2).

Healthy Eating Score Variations and HEI−2015 Pearson Correlation Coefficient

There was a statistically significant correlation between the score of HES −7 and the HEI−2015 total score. Furthermore, data were both normalized and monotonic. Correlation coefficients between the HEI−2015 and HES versions are shown in Table 4. The HES−5 was weakly correlated with HEI−2015. Correlations increased with the addition of SSB and ED questions, with the HES−7 (SSB and ED) having the strongest correlation with the HEI−2015. Relative to the HES−5, the HES−6_1, HES −6_2, and HES−7 captured an additional 4% to 9% of the total variation in HEI−2015 scores (Table 4).

FFQ Frequency, FFQ Quantity, and HEI−2015 Components and HES Component Pearson Correlation Coefficient

Table 5 and Figure details FFQ frequency components, FFQ quantity

Table 5. Healthy Eating Score (HES), Healthy Eating Index–2015 (HEI–2015), and Block Food Frequency (FFQ) Components (n = 433)

HES Components	*r*	*x'*	r^2	*P*
FFQ frequency components				
Fruit[a]	0.39	0.41	0.15	0.001
Vegetables[b]	0.55	0.62	0.30	0.001
Dairy[c]	0.50	0.55	0.25	0.001
Whole Grains[d]	0.24	0.24	0.05	0.001
Fish[e]	0.51	0.56	0.26	0.001
Energy drinks[f,g]	0.79	1.07	0.62	0.001
Sugar-sweetened beverages[h,i]	0.57	0.65	0.32	0.001
FFQ quantity components				
Fruit[a]	0.37	0.39	0.14	0.001
Vegetables[b]	0.41	0.44	0.17	0.001
Dairy[c]	0.34	0.35	0.12	0.001
Whole Grains[d]	0.35	0.37	0.12	0.001
Fish[e]	0.31	0.32	0.10	0.001
Energy drinks[f,g]	0.59	0.68	0.35	0.001
Sugar-sweetened beverages[h,i]	0.39	0.41	0.15	0.001
HEI–2015 components				
Fruit[a]	0.42	0.45	0.18	0.001
Vegetables[b]	0.48	0.52	0.23	0.001
Dairy[c]	0.37	0.39	0.14	0.001
Whole Grains[d]	0.30	0.31	0.09	0.001
Fish[e]	0.29	0.30	0.08	0.001
Energy drinks[f,g]	0.24	0.24	0.06	0.001
Sugar-sweetened beverages[h,i]	0.37	0.39	0.14	0.001

[a]FFQ fruit frequency component includes melons, berries, bananas, apples, oranges, peaches, other fresh fruit, dried fruit, and canned fruit. FFQ fruit quantity component includes grams consumed per d of all fruits in the frequency component; [b]FFQ vegetables frequency component includes broccoli, carrots, corn, green beans, cooked greens, cabbage, green salad, raw tomatoes, sweet potatoes, potatoes, other vegetables, beans, and tomato juice. FFQ vegetable quantity component includes gram consumed per d of all vegetables included in the frequency component, Brussel sprouts, pickles, and sauerkraut; [c]FFQ dairy frequency component includes milk, yogurt, cottage cheese, cream cheese, sliced cheese, and cocoa. FFQ dairy quantity component includes grams consumed per d of milk, yogurt, cheese, and milk substitutes; [d]FFQ whole-grain frequency component includes whole-grain cereals and crackers, grits, brown rice, popcorn, and corn chips/bread. FFQ whole-grain quantity component includes grams consumed per d of whole-grain cereals, noodles, and crackers; grits; brown rice; cornbread/tortillas; and whole and multigrain bread; [e]FFQ fish frequency component includes tuna, salmon, fried or breaded fish, and other fish. FFQ fish quantity component includes grams consumed per d of high omega 3 fish, fried fish, fish sticks, and other fish; [f]FFQ energy drink frequency component energy drinks or shots. FFQ energy drink quantity component includes grams consumed per d of total energy drinks, low sugar energy drinks, and sugary energy drinks; [g]These variables are not in the original HES–5. They have suggested additions; [h]FFQ sugar-sweetened beverage frequency component includes sugary beverages. FFQ sugar-sweetened beverage quantity component includes grams consumed per d of sugary beverages.

components, and HEI–2015 components with the corresponding HES component. The HES ED component had the highest correlation with the corresponding FFQ frequency components followed by SSB, vegetables, fish, dairy, and fruit. Whole grains had the lowest correlation. The correlations between HES components and FFQ quantity components were generally weaker (except for the whole-grain component) than the correlations between the HES components and FFQ frequency components. Similarly, the HES ED component had the highest correlation with the corresponding FFQ quantity component, followed by vegetables, SSB, fruit, and dairy. Fish had the lowest correlation. All *r* values had a corresponding significance of $P < 0.001$. The *r* values, r^2 values, *z*-scores, and *P* values between the HES, HEI–2015, and Block FFQ components are shown in Table 5. Although all correlations are statistically significant, the correlations were generally low.

HES–7 and HEI–2015 Lin's Correlation Coefficient

Lin's correlation coefficient for HES –7 and HEI–2015 was 0.03. Although the HES–7 and HEI–2015 scores had a very weak linear correlation, this effect was exaggerated by the difference in scale 1–35 and 1 –100, respectively. When normalized, a concordance of 0.40 was shown (concordance correlation coefficient, 0.40; 95% confidence interval, 0.34–0.45). A concordance

of 0.40 is on the upper end of the weak concordance range and the lower end of the moderate concordance range.

DISCUSSION

This study examined associations between current HES−5 questions (which assess consumption frequency but not consumption quantity) and diet quality (measured by the HEI −2015), and whether adding 2 items assessing beverage intakes to the HES improved the association. To our knowledge, only 1 other published study has assessed different variations of the HES−5 and diet quality.[32] Shams-White et al[32] added frequency of breakfast, postexercise snack, and SSB consumption to the HES−5, and determined that adding postexercise snack and SSB consumption improved correlation with the HEI−2015 (increased from $r = 0.41$ to $r = 0.53$). However, breakfast consumption and postexercise snack consumption measure eating behaviors rather than intake of individual foods and are therefore not direct measures of diet quality. Although Purvis et al[10] discussed the creation of the HES−5 and compared the top quartiles of HES−5 scores with other military fitness scores (emotional, social, physical, etc), the HES−5's ability to capture diet quality was not assessed in Purvis et al.[10] The current study indicates adding SSB and ED to the HES−5 to create the HES−7 (HES −5 plus SSB and ED) improved the correlation between the HES and HEI −2015. This can be explained because the HES−7 captures added sugar consumption, whereas the HES −5 does not.[21,33] Therefore, these findings confirm and extend those of Shams-White et al[32] by demonstrating that adding both SSB and ED consumption to the HES improves its correlation with the HEI−2015. However, because HES−7 only includes diet quality components and the Shams-White et al[32] HES version does not, the HES−7 more accurately assesses diet quality than the Shams-White et al[32] HES version.

If the HES−7 is to be used to measure diet quality, caution must be applied because its correlation with the HEI−2015 is moderate. One factor contributing to the moderate correlation is the difference between the time frames captured in the Block FFQ (last 6 months) and HES (last 30 days). Another contributing factor is that the HES−7 lacks the food and nutrient components of the HEI −2015,[21] including saturated fats, sodium, refined grains, and fatty acids.[21,23] However, many of these may be difficult to measure in the HES format as they are not discrete food groups. In addition, the HES−7 captures fruit, vegetables, and protein (fish) in a single category for each food group, whereas the HEI −2015 captures those food components in 2 separate categories for each food group (vegetables-total vegetables/greens and beans, fruit-total fruit/whole, protein-total protein foods/seafood, and plant proteins).[10,21,23] Furthermore, it must be acknowledged that SSBs are not the only source of added sugars in an individual's diet. In addition, it would be preferable to use 1 question to capture all SSBs instead of 2 separate questions for SSBs and EDs and to clarify that the ED question excludes sugar-free versions. However, as previously stated, questions were taken from the MEBS; therefore, the opportunity to adjust or add questions was not available in this study. Including more of the HEI−2015 food and nutrient components in the HES−7 would likely improve its correlation with the HEI−2015. Therefore, the HES−7 may be useful for identifying some areas of concern and shaping targeted nutrition interventions, but it is not a strong tool in its current form.

The second focus of this study was to examine the strength of correlations between HES−7 component scores and frequency and consumption of intake within those food groups. When HES questions were compared with Block FFQ consumption frequency and quantity (ie, the combination of frequency and serving size) components, nearly all frequency correlation coefficients (except whole grains) were higher than quantity correlation coefficients. These results suggest only collecting frequency underestimates total quantity, potentially because individuals consume more than a single serving at 1 time. Interestingly, ED strongly correlated with the consumption quantity and frequency components of the Block FFQ. This may be because, in the HES, the ED question provides examples of items sold in preportioned quantity sizes, aligning frequency and quantity more closely than other HES questions.

The whole-grain intake had one of the lowest correlations between HES, HEI−2015 scores, and Block FFQ consumption quantity data. This may be because the HES question does not provide much detail on examples of whole grains. Identifying whole-grain foods may also be more challenging for a participant than identifying an ED or SSB. In addition, whole-grain serving sizes may be difficult for individuals to conceptualize. It is possible that FFQ frequency and quantity components are better correlated with HES questions, which provide examples that are consumed in preportioned quantities close to serving sizes used by the Block FFQ. However, evidence suggests participants may not respond accurately to questions about quantity on the Block FFQ.[34] Although respondent recall bias is a limiting factor for all surveys,[35] it is still the best method of gathering valuable data from large populations.[36]

This 90.6% male sample consisting of only soldiers, although representative of the demographics in the US Army,[1] limits the generalizability of the results in civilian populations. A weakness of the study includes the respondent time burden as the Block FFQ and the validated questionnaire assessing soldiers' eating behaviors took between 45 to 90 minutes each to complete, potentially resulting in survey fatigue.

IMPLICATIONS FOR RESEARCH AND PRACTICE

In both military and general populations, creating interventions to improve nutritional status is vital. Understanding the diet quality of the military may shape the future direction of nutrition strategies that support military service members. On the basis of our findings, further

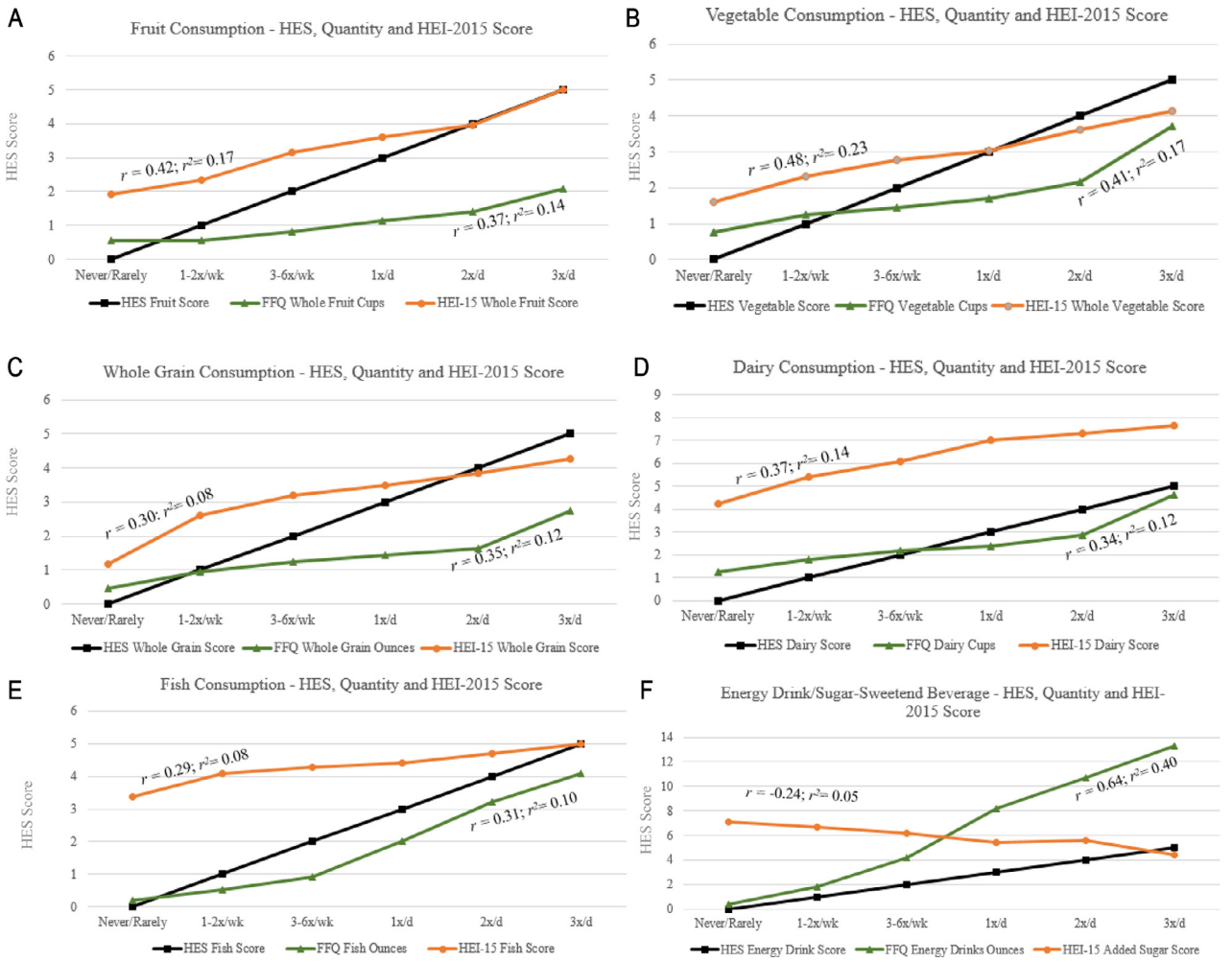


Figure. Correlations of Healthy Eating Score component scores, Healthy Eating Index–2015 component scores, and Block Food Frequency Questionnaire consumption quantity (n = 433). Note: The line representing quantity shows information captured by the HEI-2015 component but not captured by the related HES component. The *r* values above the orange lines are the correlations between the HEI-15 score and HES score for each food item. The *r* values below the green lines are the correlations between the food frequency questionnaire quantity component and the HES score for each food item. For all correlations $P < 0.001$. (A) Correlations between HES fruit question, Block FFQ fruit quantity, and HEI-15 fruit score. (B) Correlations between HES vegetable question, Block FFQ vegetable quantity, and HEI-15 vegetable score. (C) Correlations between HES whole grain question, Block FFQ whole grain quantity, and HEI-15 whole grain score. (D) Correlations between HES dairy question, Block FFQ dairy quantity, and HEI-15 dairy score. (E) Correlations between HES fish question, Block FFQ fish quantity, and HEI-15 fish score. (F) Correlations between HES energy drink question, Block FFQ energy drink quantity, and HEI-15 added sugar score.

research is warranted before the HES–7 can be considered an accurate measure of diet quality. Specifically, future research is needed to strengthen the HES by creating and validating questions to capture more of the food and nutrient components of the HEI–2015 that are not currently represented in the HES–7. When considering questions to add, investigators may consider using commonly consumed foods rather than nutrient categories (eg, SSBs vs added sugars, or red meat and whole fat dairy vs saturated fat) to reduce respondent burden. In particular, questions capturing total proteins and green vegetables and beans may be considered as additions to the HES. These findings also support revising current questions or adding additional questions to capture consumption quantity information included in the HEI–2015. Future research may include a more cohort, specifically women and other military branches, to determine if these other populations impact the HES–7's ability to represent diet quality. If these future additions improve the correlation between the HES and the HEI–2015, the HES may then serve as a useful, cost- and time-effective survey tool for measuring overall nutritional fitness in large military populations.

ACKNOWLEDGMENTS

The work was supported by the US Army Medical Research and Development Command Fort Detrick, MD.

The Medical Research and Development Command did not influence the study design, collection, analysis, or interpretation of the data, writing the manuscript, or the decision to submit the paper for publication. The authors would like to thank Mr Joshua Sakai, MS, for his assistance with commuting and analyzing the statistics.

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Go for Green® Nutrition Program: Translating Evidence Into Practice

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ABSTRACT

Introduction:

Go for Green® (G4G) is an evidence-based, multi-component nutrition program for military dining facilities (DFAC) to improve nutritional fitness among Service Members. The program evolved from supporting "fueling" during initial Army training into a robust intervention across all U.S. Military branches. The current G4G program consists of eight program requirements to optimize the nutrition environment, including traffic light labeling, nutritious menus, choice architecture, food promotion, marketing, and staff training. The evolution of the G4G program, development of standardized program requirements, and lessons learned are described.

Materials and Methods:

The latest scientific evidence, best practices in health promotion and nutrition education, results and data from G4G implementation in the military community support the current version of G4G. Feedback and observations from program developers, military branch foodservice headquarters, installation leadership, and local G4G DFAC teams provided insight into implementation challenges, successes, facilitators, and barriers.

Results:

The G4G program has evolved and expanded from its initial inception over 10 years ago to its current version. Research studies, nutrition science, and feedback from military community stakeholders have informed programmatic changes and improvements.

Conclusions:

G4G 2.0 is a robust, innovative, multi-component, performance nutrition program with clear program element requirements. Value was added to elevate the G4G program by setting program requirements, expanding program components, and establishing a centralized resource hub. Performance nutrition initiatives in local military DFAC for dining facilities, such as G4G 2.0, has great potential to impact the health and well-being of Service Members.

INTRODUCTION

Optimal nutrition contributes to mission readiness of warfighters (Service Member) by sustaining physical and cognitive performance during military operational stress. To that end, in 2007, the Consortium for Health and Military Performance at the Uniformed Services University of the Health Sciences hosted a workshop entitled "Warfighter Nutrition: Current Opportunities and Advanced Technologies".[1] The goal of the workshop was to identify research and technology gaps in nutrition areas impacting the health, well-being, and performance of Service Member (SMs). Two research objectives identified were as follows: (1) evolve military dining facilities (referred to as DFAC) into twenty-first century warfighter nutrition centers and (2) identify effective

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Information pertaining to this manuscript have been presented:

- Oral presentation: *Go for Green®: A Performance Nutrition Initiative.* Understanding and Overcoming the Challenge of Obesity and Overweight in the Armed Forces: A Workshop, National Academy of Science, May 7, 2018.
- A platform presentations on "*Go for Green® program Updates"* was provided at the Department of Defense Nutrition Subcommittee key stakeholders in March and September 2021.

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doi:https://doi.org/10.1093/milmed/usab503

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"cafeteria" interventions to promote healthy eating in DFAC by military consumers.

To address those objectives, military DFAC began testing nutrition programs based on the U.S. Dietary Guidelines for Americans with special consideration for the military environment and its mission.[2] The mission of nutrition interventions and education, much like in athletics, is to optimize mental and physical performance. The chosen intervention, a traffic light style labeling system, was founded on social cognitive theory.[3–8] It is intended to standardize labeling programs across the Department of Defense (DoD) to simplify SMs' food choice decisions and prompt more nutritious options to support performance optimization in addition to military weight control and health promotion programs. The U.S. Army 2010 Soldier Fueling Initiative was developed for use in Initial Military Training to establish a "fueling" standard for Soldiers.[9] The Soldier Fueling Initiative development group recognized that without appropriate fueling and performance-based dietary menu standards, Soldiers would be unable to maintain their health or sustain core mission competencies. They identified a number of aspects of DFAC feeding that could be modified to enhance nutrition quality and promote healthier eating, including menus, recipes, preparation methods, and portion sizes. A process was developed for DFAC patrons to identify and discriminate between nutritious and less nutritious options. The most nutritious items were coded "Green" and the least nutritious as "Red." This intervention was the initial Go for Green® (G4G) program, which is based upon other food labeling programs.[7,8,10–13] The traffic light color-coding system employed in G4G has been shown to be more effective at shifting attention toward nutritious items and away from less healthy items compared to other numerical labeling (e.g., calories) and other methods for increasing healthy food choices.[11,14,15] This initial version also included a marketing campaign emphasizing connections among nutrition quality, well-being, health, and performance.

An evaluation of G4G by the Army Public Health Center confirmed that Soldiers reported using the color labels to guide their food choices.[9] Nearly 2/3 (66.0%) reported using the labels at least once a day.[9,16] Soldiers also stated that the performance nutrition education provided to them generally helped them make performance enhancing food choices.[5] Another important finding from the evaluation was that unit and command level leadership were key influencers of food choice and eating behaviors of Soldiers in the training environment, which indicates that leaders at every echelon should be educated on performance nutrition and good eating habits.[9,17]

Since 2008, the G4G program has evolved based on nutrition science, changes in nutrition guidance, data from conducted research, and feedback from key stakeholders (Table I). The G4G version 2.0 (G4G 2.0) is a rebrand of the original G4G with a new and trademarked logo, which may not be used by commercial entities in advertising or product packaging labels. The G4G menu guidelines are based on the U.S. Dietary Guidelines for Americans, Military Dietary Reference Intakes, and DoD nutrition and foodservice standards/regulations.[2,18,19] Because the G4G nutrition program is intended to support mental and physical performance and improve the nutrition environment on military installations, program requirements (PRs) were established to verify dining facilities using the G4G logo met program goals. The objective of this report is to provide an overview of the evolution of the G4G program using an evidence-based approach to utilize military dining avenues as a platform to optimize warfighter nutrition fitness, performance and military readiness. This report does not represent human subject's research but instead provides the methodological context to understand the G4G 2.0 program. However, the U.S. Army Research Institute of Environmental Medicine and U.S. Army Medical Research and Materiel Command Institutional Review Boards granted approval to the G4G 2.0 evaluation research.

TABLE I. Evolution of the Go for Green® Program

Program requirement (PR)	Original G4G (2008)	G4G 1.5 (2015)	G4G 2.0 (current)
PR 1: Standardized Training for Management	–	√	√
PR 2: Assign Traffic Light Color Codes Based Only on Approved Coding Algorithm	√	√	√
• Coding by only designated professionals	–	√	√
PR 3: Menu Targets: Minimum Green-coded Items	–	√	√
PR 4: Standardized Food Cards	√	√	√
PR 5: Food Placement Strategies	–	√	√
PR 6: Promotion of Green-coded Items	–	–	√
PR 7: Marketing and Education			
• Printed material	√	√	√
• Social media, other media, and press	–	–	√
• Nutrition education for diners	–	–	√
PR 8: Standardized Training for all Staff			
• Initial	–	√	√
• Ongoing	–	–	√

Abbreviations: PR = Program Requirement; G4G = Go for Green®.

GO FOR GREEN PROGRAM REQUIREMENTS METHODOLOGY

Eight PRs were established to achieve the program goals of improving the food environment where SMs live and work and

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increasing the variety of and access to more nutritious options to optimize performance, readiness, and health. Additionally, standardized PRs help maintain the integrity and accuracy of G4G 2.0 across hundreds of facilities in the military. By achieving these requirements, programs build trust across the intended SM patrons and ensure the same minimum standards are achieved, regardless of service branch or location. New science, best practices in health promotion, G4G pilot tests, and feedback from key stakeholders guided the inclusion and necessity of eight PRs. The "Program Fidelity Assessment" outlines the requirements for displaying G4G 2.0 program logo and materials. This tool can be used during planning to outline major action steps necessary for program implementation and upon implementation to evaluate fidelity of each PR. Currently, G4G 2.0 is intended for government-funded military DFAC, galleys, and medical treatment facilities, with expected expansion to other food service entities (e.g., non-government commercial cafes and restaurants and ready-to-use-foods) located on installations. Description of each PR follows.

PR 1: Standardized Training for Management

Standardized training is a key aspect of successful implementation of G4G 2.0. Online training was determined as the most accessible and feasible way to disseminate the G4G program across multiple branches of the military and installations with DFAC throughout the world. Training for local DFAC G4G team leaders to plan and execute G4G 2.0 involves completing online modules on PRs and staff training on the Human Performance Resources by Consortium for Health and Military Performance website (https://www.hprc-online.org/nutrition/go-green/training/classes). Best practices support a local G4G team of at least two to four members depending on the size of the facility and the structure of the military branch's foodservice operations, including where aspects such as menu creation and recipe coding are centralized. Installation or headquarters nutrition assets, such as registered dietitians, health promotion educators, or diet technicians along with DFAC managers and assistant managers, staff shift supervisors, and installation leadership are the intended audiences for the different G4G trainings. Training modules include (1) G4G 2.0 Coder; (2) G4G 2.0 Staff Trainer; (3) G4G 2.0 Program Requirements; and (4) G4G 2.0 Executive Summary (high-level overview of G4G 2.0 for leadership). All modules allow opportunity for course evaluation whereas the G4G 2.0 Coder training has two learning assessments (coding recipes, coding ready-to-use items) in which a score of $\geq$75% is required for course completion. Additionally, tips and guidance are provided to those leading G4G 2.0 implementation, including sample timelines, planning team templates, position descriptions, and sample briefs to leadership.

PR 2: Assign Traffic Light Color Codes Based on Approved Coding Algorithm

The G4G 2.0 Coding Algorithm includes the algorithm, criteria and calculator, and was developed by a joint service team of dietitians to ensure replicable code assignment quality control maintenance. The evidence-based nutritional criteria on which the algorithm is based were also determined by a joint service team of dietitians and align with the U.S. Dietary Guidelines for Americans, Military Dietary Reference Intakes, and DoD menu regulations.[2,18,19] The algorithm is expected to be reviewed and updated every five years or as needed, such as when dietary recommendations or criteria change (Fig. 1).

The algorithm assigns points to food items based on nutritional information including trans fatty acids content, total calories, percent calories from fat, fiber content, sugar content, degree of processing, additives, and preparation method. If an item receives 0–4 points, it is coded Red, 5–8 points is coded Yellow, and 9–13 points is coded Green. The algorithm also assigns a sodium code based on sodium content per serving in each food category. Beverage codes are not assigned through the coding algorithm, but instead are precoded based on added sugar content, presence of artificial ingredients, saturated fat content, and amount of naturally occurring healthful nutrients, such as vitamins and minerals (details available: https://www.hprc-online.org/nutrition/go-green/getting-started-implementation/prep-your-facility/program-requirements-1). The Armed Forces Recipe Service verified the coding with more than 2,000 recipes to ensure the calculator consistently assigned the same color code. To improve reliability across users, the coding algorithm was deployed in a protected manner. First, the algorithm is available in two user-friendly formats, locked spreadsheet and web-based calculator. Second, "coder" certification training was created for nutrition or food product subject matter experts to preserve the integrity of the code assignment in the decentralized DoD menu system. Third, a team of expert coders is available upon request (g4gteam@usuhs.edu) for hard-to-code products and to help food programs who choose to monitor for coding discrepancies.

PR 3: Menu Targets: Minimum Green-Coded Items

Each DFAC must offer a sufficient number of Green-coded food options to use the G4G 2.0 trademarked logo; the target for Green-coded food items offered is modest. Specifically, a minimum of 30% Green-coded items must be offered at every meal, within every station and within each food category (i.e., entrees, starchy sides, non-starchy sides or vegetables, dressings, beverages, and desserts). Additionally, some maximums for Red-coded food items were set; for example, no more than three Red-coded items on a breakfast bar.

Several new DoD food service features assist DFAC in achieving these menu targets. Most notably, the Armed

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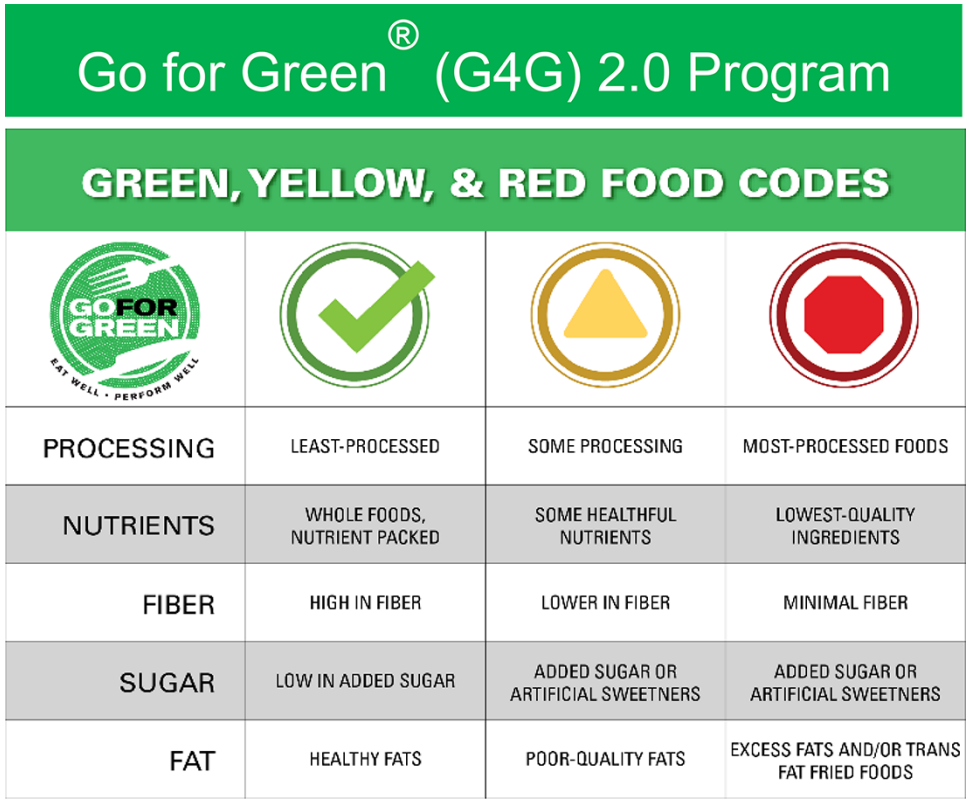


FIGURE 1. G4G 2.0 standardized traffic light labeling criteria and logos. Check mark = Green, caution sign = Yellow, stop sign = Red.

Forces Recipe Service has increased the availability of Green-coded recipes from 377 (20%) to 819 (37%) across 2017–2020. Recipes now incorporate more nutritious food items (e.g., lentils, beans, Greek yogurt, vegetables), healthier cooking methods (e.g., roasted vegetables), and enhanced seasonings to increase flavor in lieu of excess salt, sugar, and less healthy fats (e.g., saturated) compared to earlier recipes. Many plant-based and vegetarian options are now available for menus. Furthermore, sample menus that adhere to G4G 2.0 menu targets are easily accessible (https://www.hprc-online.org/nutrition/go-green/g4g-getting-started/menus-and-guidelines).

PR 4: Standardized Food Cards

The G4G 2.0 program food labels utilize a standardized template with two distinct identifiers: Green, Yellow, or Red color codes for nutritional quality and a salt shaker shown as Low, Medium, or High code to designate sodium content. The separation of sodium content from the overall color code was deemed important for SMs because their sodium needs vary based on general health, environmental conditions, physical demands, and assigned operational duties.[14] The color and sodium codes offer separate, but complementary, labels to inform and guide the diners to make healthy choices. Information is displayed to diners by using one of three options at the point of selection: food and beverage cards, coding signs, or digital menu boards. Individual DFAC are responsible for populating standardized G4G templates with their menu offerings. Across military DFAC, digital menu boards are rare; thus, a combination of the analog food cards and coding signs are used in most facilities.

PR 5: Food Placement Strategies

Numerous studies have shown that choice architecture, also known as strategic placement of foods, encourages the selection of healthy foods.[20–22] Choice architecture was woven into the G4G 2.0 concept to encourage diners to select Green-coded foods and beverages. The DFAC is often limited in its ability to move certain food service equipment and structures, such as salad bars. Therefore, more feasible changes were encouraged, such as making plates available at the salad bar for entrée salads, displaying infused water in clear visually appealing cannisters, placing fruit in attractive bowls, and modifying the serving spaces at existing bars to display Red-coded items in physically smaller containers.

For each menu, a planogram or "visual menu" is used to depict where and how food service staff could optimally place foods at each station. The G4G program instructs that Green-coded items are placed first for diners to view easily at each station, followed by Yellow, and then Red. Additional strategies to encourage selection of Green-coded items include (1) placing Red-coded items out of sight for

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Salami (R)	Bologna (R)	Bacon (R)	Cheese (Y)	Onions (G)	Banana peppers (G)	Olives (G)	Coleslaw (Y)	Mayo (R)	Caesar dressing (R)
Lean deli turkey (Y)	Lean ham (Y)	Lean roast Beef (Y)	Cheese (Y)	Tomatoes (G)	Bell peppers (G)	Pickles (G)	Tabbouleh (G)	Pesto (Y)* *Depending on recipe	Mustard (Y)
Roasted/ grilled Portobello or Eggplant (G)	Tuna salad (G)	Roasted chicken slices (G)	Cheese (Y)	Spinach, lettuce, or mixed greens (G)	Cucumbers (G)	Avacado (G)	Legumes (G)	Hummus (G)	Oil and vinegars (G)
→ Diner flow of traffic →									

- Place high-volume, Green-coded options in the largest pans your bar accommodates.
- Place Red-coded items in the smallest pans your bar accommodates, and pair them with your smallest serving utensils.
- Serve Green-coded fats/oils, such as olive oil and avocado, in the smallest pans with the smallest serving utensils to encourage diners to use the designated portions.
- Keep whole-grain bread options in sight, use as the default option, and promote them with the "Featured Sandwich."
- Keep white bread options out of sight, for example, under the serving line so that diners have to ask for them.

FIGURE 2. The G4G 2.0 program provides detailed food placement diagrams for various food stations for easy access to Green-coded food options (example for Sandwich Bar). G = Green-coded item, Y = Yellow-coded item, R = Red-coded item.

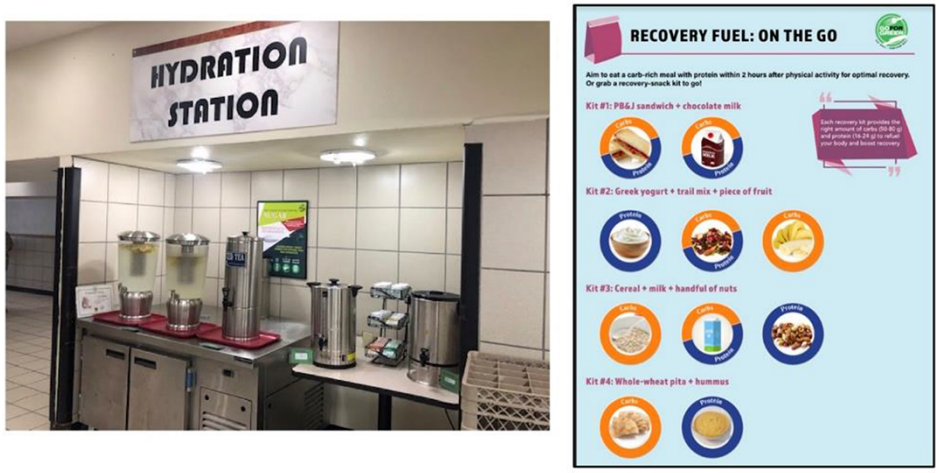


FIGURE 3. Examples of New Serving Stations—Hydration Station and Recovery Station to increase variety and availability of Green-coded food and beverages.

request-only access; (2) offering a Green-coded vegetable at all stations including the short order/grill; and (3) increasing the number of healthy toppings and dressings available (Fig. 2).

Choice architecture also introduces new concepts for food and beverage stations, which were created to help the DFAC keep pace with food trends and support optimal nutrition. Examples of the new stations include hydration, recovery, deli, and grab and go. Hydration stations provide healthy alternatives to the standard soft drink and juice fountains and instead feature infused water, very lightly sweetened iced teas, and cold-brew coffee. The recovery station includes posters to educate diners and provides grab and go options for optimal refueling and recovery after physical activity, particularly relevant to SMs with limited time in their duty day (Fig. 3). Grab and go kits are designed to provide an evidenced-based amount of carbohydrate and protein shown to optimize recovery.[23–25] Additionally, "build-your-own" stations can

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be designed to personalize and create nutritious meals with flexibility.

PR 6: Promotion of Green-Coded Foods

Promotional signs provide important value as visual signaling to assist diners in their decision-making process, and encourage selection of Green-coded items. Signs placed at the front of the serving line or station allow diners to immediately see the most nutritious option being offered. Three types of promotional signs are used in G4G 2.0. "Featured meal" signs showcase a "Green" meal highlighting the most nutritious entrée option with complementary sides for diners while "featured item" signs promote selection of individual Green-coded items. For example, grilled chicken was a Green featured item on the short order menu, whereas a fruit and granola parfait was a Green featured item at the dessert bar. In addition to featured meals and featured items, "build-your-own" signs for self-serve stations suggest combinations of foods to help diners use new or unique ingredients in their bowls, salads, sandwiches, etc. For example, build-your-own breakfast parfaits offered a variety of nutritious food items to encourage selection of nutrient-dense choices. The featured meal, featured item, and build-your-own meal signs may become habitual tools for the diner that encourages quick nutrient-dense food choice decisions. Templates were created to reduce staff burden and provide flexibility along with marketing strategies (social media).

PR 7: Marketing and Education

A marketing and education campaign complements the other PRs by encouraging and promoting high-performance eating as part of the overall G4G 2.0 program. Aims of the campaign are to educate diners on the G4G 2.0 labeling system, generate interest in nutrition and G4G 2.0, and motivate increased selection of Green-coded foods and beverages. The cornerstone of the G4G 2.0 marketing and educational campaign is eye-catching posters grouped into message sets that are rotated every three to four months. Military DFAC operate predominantly in analog versus digital, but digital versions of posters are also available for those with electronic display platforms. Digital and social media marketing is also recommended to optimize exposure. Press releases and article templates, talking points, graphics, and guidance to create a marketing plan are available in a comprehensive toolkit on the G4G website to assist DFACs in marketing their menu, facility, and G4G 2.0 program.

Facilities are encouraged to post G4G updates and messages on social media accounts at least twice a week. Currently, many pre-crafted social media posts are available on the G4G 2.0 website to promote the G4G 2.0 program features. The G4G 2.0 Facebook page provides access to additional content available for re-posting by facilities.

Education for SMs on nutrition, operational readiness, and optimal performance will coincide with G4G 2.0 program implementation. Ideally, a registered dietitian or other qualified health promotion specialist educates SMs new to the G4G 2.0 program. Additionally, a year-long nutrition education curriculum intended for distribution via social media was created by G4G 2.0 subject matter experts. The curriculum offers a unique approach to provide nutrition education in a succinct, user-friendly manner by utilizing a platform (social media) that most SMs use regularly. Additionally, it is intended to extend the reach of registered dietitians in the military, since they are not available at every military installation.

PR 8: Standardized Training for All Staff

After completing the online G4G 2.0 staff trainer course, the certified staff trainer conducts in-person training for all DFAC supervisors and foodservice staff by using six standardized modules. The modules categorize the G4G 2.0 program requirements into high impact daily tasks, such as following recipes, food placement, and food cards. All DFAC staff are required to complete in-person G4G 2.0 training. Shift leaders and supervisors must complete two additional modules on quality control for program maintenance and marketing. Refresher training is required at regular intervals, as is new staff training.

LESSONS LEARNED

Over 10 years since its inception, G4G has evolved and expanded based on the latest evidence from nutrition science, best practices in health promotion and nutrition education, and from implementation in the military community. Lessons learned from implementing this performance nutrition program in multiple military DFAC have provided valuable insights from various key stakeholders for future military or civilian programs and their impact on diner behavior. Lessons learned were gleaned from program developers, military branch foodservice headquarters, installation leadership, and local G4G DFAC teams through in-person discussions, phone and email communication about firsthand experiences, and work group meetings. Program data collected using the program fidelity assessment tool also provided critical input. Additionally, earlier research using focus groups with foodservice management and staff provided insights from users' perspectives on barriers and areas to enhance similar DFAC nutrition programs.[26]

Value in Setting Program Requirements

Establishing requirements is essential for program integrity and quality control. The PRs established in 2017 were instrumental in setting benchmarks to implement and sustain the G4G 2.0 program. These PRs also facilitated the development of the "Program Fidelity Assessment" tool by the G4G Program Office in collaboration with the Army Public Health Center. The intent of the tool is to assess program compliance across various DFAC and capture areas of success and barriers to overcome. These findings resulted in iterative

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enhancements to the program framework, resource development, and trainings. At this time, the fidelity tool is used by G4G 2.0 research sites at specified time points including baseline ("pre-G4G 2.0 program implementation") and G4G 2.0 grand opening ("post-G4G 2.0 implementation"). Non-research sites should conduct an annual assessment.

Value in Expanding Program Components

Throughout the revision of G4G, additional program components were introduced to emphasize training, knowledge, and implementation strategies. The newly introduced components not only added a new dimension to the program, but they promoted a synergistic effect between multiple components. The emphasis of the G4G 2.0 on marketing enabled not only promotion of the program itself but also the dining facility and menu options. The significance of educating SMs on performance nutrition along with G4G was also identified as an important program component. Lastly, training requirements for foodservice staff and management were expanded to include standardized training modules, refresher sessions, and interactive hands-on modules to improve staff knowledge of the program, communication, and engagement.

Value in Establishing Centralized Resources Hub

The demand to embrace G4G 2.0 resulted in the establishment of a centralized website with standardized resources to enable easy scale-up across military DFACs. The G4G website (https://www.hprc-online.org/nutrition/go-green) serves as a hub to host toolkits, worksheets, and templates to enable customization and use at military installations worldwide. This enables DFAC managers to focus on daily program implementation and quality control tasks instead of program material creation, especially related to time-intensive activities such as social media marketing and menu revisions. This website, maintained by the Consortium for Health and Military Performance, continues to evolve with feedback from program users.

IMPLICATIONS FOR RESEARCH AND PRACTICE

The G4G 2.0 program is a robust, innovative, multicomponent, performance nutrition program with clear PRs and goals. Full implementation with all of the PRs described above is necessary for optimal success. The G4G 2.0 has great potential to impact the health and well-being of SMs across the DoD. Although initially designed for DFAC within a recruit training program, G4G has expanded throughout all military branches in a joint effort with significant momentum. Best practices support that implementation of any new DFAC nutrition intervention includes evaluation to demonstrate program effectiveness in terms of meal satisfaction and improved diet quality. Additionally, periodic reassessment of PRs and clear guidance is essential for an effective nutrition intervention.

FUNDING

This research was funded by the Joint Program Committee-5 and the US Army Research and Material Command Military Operational Research Program (No. 18630).

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

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The Journal of Clinical Endocrinology & Metabolism, 2022, **XX**, 1–10
https://doi.org/10.1210/clinem/dgac295
Advance access publication 9 May 2022
Clinical Research Article

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Effects of Testosterone on Mixed-Muscle Protein Synthesis and Proteome Dynamics During Energy Deficit

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Abstract

Context: Effects of testosterone on integrated muscle protein metabolism and muscle mass during energy deficit are undetermined.

Objective: The objective was to determine the effects of testosterone on mixed-muscle protein synthesis (MPS), proteome-wide fractional synthesis rates (FSR), and skeletal muscle mass during energy deficit.

Design: This was a randomized, double-blind, placebo-controlled trial.

Setting: The study was conducted at Pennington Biomedical Research Center.

Participants: Fifty healthy men.

Intervention: The study consisted of 14 days of weight maintenance, followed by a 28-day 55% energy deficit with 200 mg testosterone enanthate (TEST, n = 24) or placebo (PLA, n = 26) weekly, and up to 42 days of ad libitum recovery feeding.

Main Outcome Measures: Mixed-MPS and proteome-wide FSR before (Pre), during (Mid), and after (Post) the energy deficit were determined using heavy water (days 1-42) and muscle biopsies. Muscle mass was determined using the D_3-creatine dilution method.

Results: Mixed-MPS was lower than Pre at Mid and Post ($P < 0.0005$), with no difference between TEST and PLA. The proportion of individual proteins with numerically higher FSR in TEST than PLA was significant by 2-tailed binomial test at Post (52/67; $P < 0.05$), but not Mid (32/67; $P > 0.05$). Muscle mass was unchanged during energy deficit but was greater in TEST than PLA during recovery ($P < 0.05$).

Conclusions: The high proportion of individual proteins with greater FSR in TEST than PLA at Post suggests exogenous testosterone exerted a delayed but broad stimulatory effect on synthesis rates across the muscle proteome during energy deficit, resulting in muscle mass accretion during subsequent recovery.

Key Words: negative energy balance, dynamic proteomics, fractional synthesis rates, heavy water, translational capacity, anabolism

Abbreviations: ^2H_2O, heavy water; CV, coefficient of variation; D_3Cr, D_3-creatine; DXA, dual-energy X-ray absorptiometry; EOS, end of study; FSR, fractional synthesis rate; GC-MS, gas chromatography-mass spectrometry; LBM, lean body mass; LC-MS, liquid chromatography-mass spectrometry; MPB, muscle protein breakdown; MPS, muscle protein synthesis; PBRC, Pennington Biomedical Research Center; PLA, placebo; TEST, testosterone enanthate

We recently reported that supplemental testosterone increases lean body mass (LBM) in young men during a military-relevant energy deficit (1). However, the mechanisms underlying this effect have not been fully elucidated. Data from our laboratory suggest that the anabolic stimulus derived from testosterone administration during energy deficit may result from an increase in translational capacity and decrease in proteolysis at rest (2). These static molecular measures are consistent with controlled laboratory studies using short-term IV tracer infusions and arterial-venous balance methods, which show that exogenous testosterone modulates muscle protein turnover (3). Administration of testosterone or oxandrolone, a synthetic testosterone analog, over short (5 days) and longer (3 weeks to 6 months) durations increases acute, postabsorptive muscle protein synthesis (MPS) in young and old men (4-8) and women (9, 10). Although these studies are informative, they only measured MPS at isolated time points over the course of a few hours

Received: 14 February 2022. **Editorial Decision:** 3 May 2022. **Corrected and Typeset:** 1 June 2022
Published by Oxford University Press on behalf of the Endocrine Society 2022. This work is written by (a) US Government employee(s) and is in the public domain in the US.

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under highly controlled conditions. As such, this work may not fully account for the integrated effects of metabolic state (ie, fed vs fasting), daily activity, energy balance, and diurnal changes in hormone levels on MPS over several weeks under free-living conditions. Whether the changes in gene expression and protein abundance observed in participants from the current study (2), and the reported effects of testosterone on acute measures of MPS (4-10), result in adaptations over time remains uncertain.

Participants in the current study were administered heavy water (^2H_2O) to measure the time-integrated effects of exogenous testosterone on MPS. Heavy water administration for extended durations combined with gas chromatography (GC)-mass spectrometry (MS) and liquid chromatography (LC)-MS analyses allows for determination of integrated MPS and fractional synthesis rate (FSR) of a large number of individual muscle proteins of different ontologies (11, 12). This metabolic labeling approach provides unique insights into muscle adaptations to interventions over time (days to weeks). For example, during administration of a selective androgen receptor modulator, an increase in FSR of multiple muscle proteins over 10 days in rats was associated with gains in muscle mass at day 28 (13). These findings suggest that the synthetic rates of individual muscle proteins across the proteome were predictive of subsequent muscle hypertrophy (13). In the current study, ^2H_2O administration was used to measure skeletal muscle protein dynamics across the proteome in response to testosterone administration during energy deficit, including changes in individual myofibrillar, mitochondrial, and sarcoplasmic proteins. D_3-creatine (D_3Cr) was also administered at points throughout the study to examine potential relationships between changes in proteome dynamics and changes in muscle mass. While we have previously reported the effect of testosterone supplementation on LBM (1), the D_3Cr dilution method more specifically assesses skeletal muscle mass by measuring creatine pool size, as 98% of body creatine is found in skeletal muscle (14).

Short-term energy deficit reduces acute measures of mixed-MPS (15, 16). Whether exogenous testosterone mitigates those effects or influences proteome-wide FSR is not known. The purpose of this study was to examine mixed-MPS and muscle proteome dynamics during energy balance and in response to 28 days of exercise- and diet-induced energy deficit in healthy, nonobese men administered supplemental testosterone or a placebo weekly. D_3Cr muscle mass was also assessed after weight maintenance and energy deficit phases, and with ad libitum recovery feeding. We hypothesized that supplemental testosterone would attenuate the expected decline in mixed-MPS during energy deficit, and increase FSR of individual muscle proteins, resulting in greater D_3Cr muscle mass compared with the placebo.

Methods

This study reports data from the Optimizing Performance for Soldiers trial, which examined the effects of supplemental testosterone during 28 days of a severe exercise- and diet-induced energy deficit (17). The primary outcomes of body composition, endocrine status, and muscle function were reported previously (1), and the effects of testosterone on appetite (18), hematological (19), and intramuscular molecular (2) responses to severe energy deficit have also been published elsewhere. This study was approved by the institutional review board at the Pennington Biomedical Research Center (PBRC, Baton Rouge, LA) and by the Human Research Protection Office of the US Army Medical Research and Development Command (Ft. Detrick, Fredericksburg, MD). All volunteers provided written informed consent before participation.

Participants

Participants were healthy, physically active (≥ 2 days/wk aerobic and/or resistance exercise) men aged 18 to 39 years, with total testosterone concentrations within the normal physiological range (10.4-34.7 nmol/L). Additional inclusion/exclusion criteria and details of the recruitment process are published elsewhere (17).

Experimental Design

Full details of the Optimizing Performance for Soldiers trial have been published previously (1, 17). In brief, this randomized, double-blind, placebo-controlled trial consisted of a screening period followed by 14 days of weight maintenance, 28 days of a highly controlled exercise- and diet-induced energy deficit, and a free-living recovery period in which participants returned to their prestudy, ad libitum diet and physical activity routines until body mass was restored within ± 2.5% of initial body mass. Figure 1 provides an overview of the experimental design including timing of ^2H_2O and D_3Cr administration, muscle biopsies, and muscle mass and LBM assessments. Mixed-MPS and proteome dynamics were measured at the end of weight maintenance (Pre), and at the middle (Mid) and end (Post) of the energy deficit. D_3Cr muscle mass and LBM were assessed before and after the energy deficit (Pre and Post), after 11 days of ad libitum recovery feeding (Recovery), and once initial body mass was restored or study day 84, whichever occurred first (end of study [EOS]).

Habitual dietary intake and physical activity patterns were assessed during screening using accelerometry and 3-day diet and physical activity records (17). Participants maintained habitual levels of physical activity during weight maintenance (days 1-14) while also consuming a weight-maintaining diet providing 1.6 g protein/kg/d, 30% energy intake from fat, and remaining calories from carbohydrate. Body weight was measured daily after an overnight fast and morning void using a calibrated digital scale (GSE Inc. model 450; GSE Scale Systems, Novi, MI). Caloric adjustments were made if necessary to maintain body weight within ± 2%.

Participants were admitted to an inpatient unit at PBRC after the weight maintenance phase to begin a 28-day exercise- and diet-induced energy deficit (days 15-42). Participants were randomly assigned at the beginning of this phase to receive intramuscular injections of either testosterone enanthate (200 mg/wk, TEST) or a placebo (1 mL sesame oil/wk, PLA) on days 14, 21, 28, and 35. Participants performed ~3.5 sessions of low-, moderate-, and high-intensity aerobic-type exercise daily (ie, treadmill and/or outdoor walking and/or running, elliptical, stationary bike, walking with a weighted backpack containing ~30% body weight) to increase exercise-induced energy expenditure and elevate total daily energy expenditure by 50% over weight maintenance values (1). These discrete bouts of steady-state aerobic-type exercise were prescribed to reflect the aerobic-type physical work typically performed during strenuous military training and operations. Light calisthenics were also incorporated every 3 to 4 days to better simulate field operations but were not performed within 48 hours of muscle biopsies. Exercise prescriptions were verified

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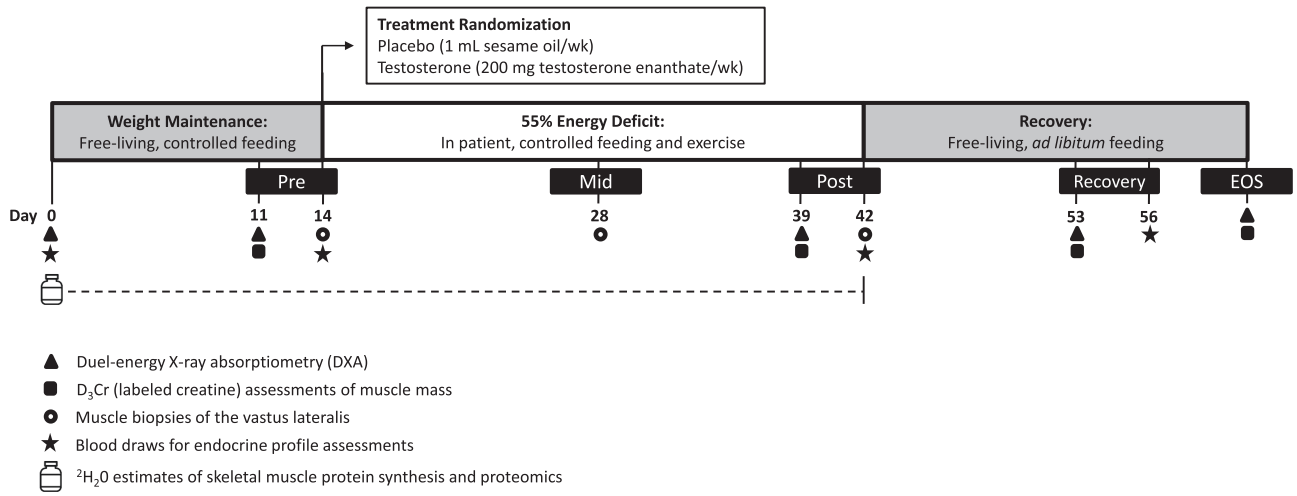


Figure 1. Experimental design. The study consisted of 14 days of weight maintenance, followed by a 28-day 55% energy deficit with 200 mg testosterone enanthate (TEST, n = 24) or placebo (PLA, n = 26) weekly, and up to 42 days of ad libitum recovery feeding. EOS measures occurred an average of 10 ± 9 days after the Recovery time point.

biweekly using open circuit indirect calorimetry (ParvoMedics TruOne 2400, East Sandy, UT) and adjusted when needed to maintain the prescribed exercise-induced energy expenditure (1). Dietary energy intake was restricted to 45% of the elevated total daily energy expenditure to elicit a 55% energy deficit. The macronutrient distribution of the energy deficit diet was consistent with the weight maintenance phase and was not different between groups. Participants consumed a self-selected, ad libitum diet after the energy deficit during a free-living recovery period (day 43 to EOS).

Metabolic Labeling With Heavy Water

Participants consumed multiple aliquots of 70% ^2H_2O (Cambridge Isotope Laboratories, Andover, MA) daily during weight maintenance and energy deficit phases to label newly synthesized muscle proteins with ^2H_2O and assess mixed-MPS (by GC-MS) and muscle proteome-wide protein dynamics (LC-MS). Participants ingested three 50-mL doses of 70% ^2H_2O (150 mL/d) on days 0 to 7 to increase body water enrichment to 1% to 2%. The first dose of each day was consumed before breakfast under supervision of study staff, and the additional 2 doses were packed for participants to consume with subsequent meals at home. Thereafter (days 8-42), participants consumed two 50-mL doses daily (100 mL/d) to maintain body water ^2H enrichment. Participants continued to receive 1 supervised dose with breakfast and consumed the additional dose at home with dinner on days 8 through 14. ^2H_2O consumption was supervised by study staff at breakfast and dinner meals during the energy deficit phase (days 15-42).

Tissue Sampling

Biopsies of the vastus lateralis were collected under rested, fasted conditions at Pre (day 14), Mid (day 28), and Post (day 42) to assess mixed-MPS and muscle proteome dynamics. Fasted blood samples were collected on days 0, 14, 42, 56, and EOS to assess endocrine profiles and body water ^2H enrichment. Saliva was also collected on days 3, 7, 11, 15, 19, 23, 27, 31, and 35 to measure body water enrichment. Participants were instructed not to eat or drink for 30 minutes before saliva collection. Body water ^2H enrichment was determined from blood and saliva using methods described previously (20). All samples were stored at -80°C until analysis.

Mixed MPS

Mixed-MPS was measured as the incorporation of ^2H into protein-bound alanine isolated from hydrolysis of muscle proteins using previously described methods (21). Briefly, ~20 mg of muscle tissue was hydrolyzed and resulting hydrolysates were ion exchanged, dried under vacuum, and suspended in 1 mL of 50% acetonitrile and 50 mM K_2HPO_4, pH 11. The mixture was then incubated at 100°C following the addition of pentafluorobenzyl bromide (20 µL; Pierce Scientific, Rockford, IL), and derivatives were extracted into ethyl acetate. The top organic layer was subsequently removed and dried by addition of solid Na_2SO_4 followed by vacuum centrifugation. The derivatized amino acids were analyzed by GC-MS on a DB-225 gas chromatograph column with negative chemical ionization, and selected ion monitoring of mass-to-charge ratios of 448, 449, and 450, representing M0, M1, and M2 mass isotopomers of derivatized alanine. The newly synthesized fraction of muscle proteins (f) was determined for each sample as the ratio of the measured excess fractional M + 1 enrichment (EM1) to the asymptotic or maximal (equilibrium) value of EM1 ($EM1_{max}$) using the following equation:

$$f = EM1_{sample}/EM1_{max}$$

with values obtained from mass isotopomer distribution analysis calculations using the heavy water exposure during the labeling period as described previously (22). Mixed-MPS was calculated from the changes in enrichment of ^2H-labeled alanine at Pre, Mid, and Post. The analytical coefficient of variation (CV) for mixed-MPS was 3.6%.

Muscle Proteome Dynamics

Muscle samples were processed as previously described to examine muscle proteome-wide protein dynamics (11, 23). Briefly, muscle biopsy tissue was suspended in 0.08% SDS at a 10:1 volume:weight ratio and vortexed to extract cellular proteins. The resulting supernatant was removed and incubated overnight with ethanol (5:1 ethanol:extraction buffer) at -20°C, and centrifuged (1600*g* for 45 minutes) to

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precipitate soluble proteins. Pelleted proteins were then rinsed with 90% ethanol, allowed to air dry, and resuspended in 8 M urea before trypsin digestion. Up to 80 µg of the soluble protein sample was denatured using Protease-Max surfactant (0.1%; Promega, Madison, WI) and 4 M urea in 25 nM ammonium bicarbonate (pH 8). Proteins were reduced with TCEP (5 mM) and incubated with iodoacetamide (10 mM) to chemically modify reduced cysteines. Proteins were then digested with trypsin (Promega) at 37°C overnight using a 1:25 trypsin:protein mass ratio. The following day, formic acid was added to a total concentration of 5%, and samples were centrifuged at 14,000*g* for 30 minutes. The supernatant was transferred to a fresh tube, desalted, dried via vacuum centrifugation, and resuspended in 0.1% formic acid/3% acetonitrile before LC-MS analysis.

Trypsin-digested peptides were analyzed on a 6550 quadruple time-of-flight mass spectrometer with a 1260 Chip Cube nano ESI source (Agilent Technologies, Santa Clara, CA). Each sample was injected twice per analysis, once for protein/peptide identification in data-dependent MS/MS mode and once for peptide isotope analysis in MS mode. Acquired MS/MS fragmentation spectra were extracted and analyzed using Spectrum Mill Proteomics Workbench software (Agilent Technologies, RRID:SCR_022171, https://scicrunch.org/resources/data/record/nlx_144509-1/SCR_022171/resolver?q=022171&l=022171&i=rrid:scr_022171) and the Uniprot/Swissprot database (August 2010). Search results were validated with a global false discovery rate of 1%. A filtered list of peptides was collapsed into a nonredundant peptide formula database containing peptide elemental composition, mass, and retention time. This was used to extract mass isotope abundances (M0-M3) of each peptide from MS-only acquisition files with Mass Hunter Qualitative Analysis software (Agilent Technologies, RRID:SCR_019081, https://scicrunch.org/resources/data/record/nlx_144509-1/SCR_019081/resolver?q=SCR_019081&l=SCR_019081&i=rrid:scr_019081). An in-house software program was used to calculate peptide elemental composition and curve fit parameters for predicting peptide isotope enrichment based on precursor body water enrichment and the number of amino acid C-H positions per peptide actively incorporating hydrogen and deuterium from body water (23, 24). The analytical CV for muscle proteome dynamics was 8.7%.

Subsequent data handling was performed using python-based scripts, with input of MIDA precursor enrichment for each subject, to yield FSR data at the protein level. FSR data were filtered to only include protein measurements with ≥ 2 peptide isotope measurements per protein and that were measured in at least 10 participants per group. Additional details of the FSR calculations and data filtering criteria were as described in detail previously (23, 24). FSR of individual proteins (%/day) was calculated as the fraction of newly synthesized protein present at Pre, Mid, and Post, and the proportion of individual proteins with greater FSR in TEST compared with PLA was also determined. This approach has been implemented previously by our group to examine changes in proteome dynamics (11, 25).

Body Composition and Endocrine Profile

Body composition and endocrine profile data at Pre, Post, and Recovery were previously reported as primary outcomes of the parent study (1) and are included in this manuscript along with EOS values to provide context as to the overall effects of the intervention. Body composition (total body mass, lean body mass, and fat mass) was measured via dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, Madison, WI, USA) at the start of the study (day 0) and toward the end of each study phase on days 11, 39, 53, and EOS. DXA scans were conducted after an overnight fast and morning void using participant positioning standards described previously (1). LBM was calculated as total body mass minus fat mass and bone mineral content.

Endocrine profiles were analyzed as described previously (1, 2). In brief, total testosterone (Siemens cat. no. L2KTW2, RRID:AB 2756391, http://antibodyregistry.org/AB_2756391) and SHBG (Siemens cat. no. L2KSH2, RRID:AB_2819251, http://antibodyregistry.org/AB_2819251) were analyzed using an Immulite 2000 system (Siemens, Llanberis, UK). Intra- and interassay CV for Immulite assays were 9.8% and 12% for total testosterone, and 2.6% and 4.2% for SHBG, respectively. All assays were run with standards and appropriate quality control material by PBRC, which is accredited by the College of American Pathologists. PBRC also runs external proficiency samples and results are compared with other laboratories across the country. Free testosterone was determined by calculation (26). Blood was sampled between 6:00 am and 9:00 am to limit the potential confounding effects of diurnal variation of total testosterone in young men (27).

D_3-Creatine Dosing

Participants ingested a single 60-mg dose of D_3Cr toward the end of each study phase on days 11, 39, 53, and EOS to assess longitudinal changes in muscle mass using the D_3Cr dilution method. The D_3Cr dilution method uses basic principles of muscle creatine and creatinine biology to estimate muscle mass based on the irreversible conversion of creatine to creatinine and subsequent excretion in the urine (28). Urine samples were collected before dosing to correct for background enrichments. Fasting urine samples were collected 48 and 72 hours after each dose and analyzed for D_3-creatinine, unlabeled creatinine, and creatine using LC-MS (AB SCIEX QTRAP 6500 plus system, RRID:SCR_021831, https://scicrunch.org/resources/data/record/nlx_144509-1/SCR_021831/resolver?q=SCR_021831&l=SCR_021831&i=rrid:scr_021831). The 48- and 72-hour time points were averaged for determination of muscle mass. The urine creatine/creatinine ratio was used to correct for any spillage of the D_3Cr dose and to calculate muscle mass as previously described (29). The analytical CV for the measure was 2.9%.

Statistical Analyses

The effect of testosterone on mixed-MPS was determined using a 2-way repeated measures ANOVA that examined the effects of treatment (TEST or PLA) and phase (Pre, Mid, Post), with Tukey multiple comparison post hoc tests (GraphPad Prism, La Jolla, CA). Cross-sectional comparisons of proteome dynamics between TEST and PLA were determined at each time point using *t* tests with Benjamini-Hochberg corrections for multiple comparisons (GraphPad Prism). Two-tailed binomial tests were used to examine whether the proportion of individual myofibrillar, mitochondrial, and sarcoplasmic proteins with higher FSR in TEST than PLA at each time was statistically significant (GraphPad Prism). The effects of treatment (TEST or PLA), phase (Pre, Post, Recovery, EOS), and their interaction on body composition and endocrine profiles

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were examined using linear mixed models adjusted for age and prestudy values (day 0), with Bonferroni corrections for post hoc comparisons (SPSS v26.0, IBM, Armonk, NY). The effects of treatment, phase, and their interaction on muscle mass were examined using mixed-effects model, with post hoc Bonferroni multiple comparison tests (GraphPad Prism). All data are presented as mean ± SD unless otherwise noted. The α level of significance for all statistical tests was two-tailed and set at $P < 0.05$.

Results

Body water ^{2}H enrichment increased over the study period to approximately 1.0% of total body water (Figure 2). Mixed-MPS was 40% and 33% lower at Mid and Post compared to Pre, respectively (Figure 3, $P < 0.0005$), and was not different between TEST and PLA at any time point.

The FSR of individual myofibrillar, mitochondrial, and sarcoplasmic proteins at Pre (Figure 4A), Mid (Figure 4B), and Post (Figure 4C) were not different between TEST and PLA at any time point. However, the proportion of proteins with numerically higher synthesis rates in TEST compared with PLA at Post was significant by a 2-tailed binomial test (52/67; $P < 0.05$, Figure 5). These 52 proteins were 22 ± 13% greater on average in TEST compared with PLA. The proportion of individual proteins at Pre (44/75) and Mid (32/67) with numerically greater FSR in TEST compared with PLA were not significant using binomial tests.

DXA-derived body composition and endocrine biomarker values have been reported previously (1) and are included here with EOS values for descriptive purposes (Table 1). EOS measures occurred an average of 10 ± 9 days after the Recovery time point. In brief, lean mass increased in TEST and was maintained in PLA during the energy deficit and increased in both groups during the recovery phase (Table 1, $P < 0.0001$). Total body mass decreased during the energy deficit and increased during recovery in both groups ($P < 0.0001$), with PLA losing more total body mass. Total testosterone and free testosterone increased in TEST and remained unchanged in PLA during the energy deficit, and decreased in TEST during recovery such that they were lower than PLA ($P < 0.0001$). D_3Cr muscle mass was greater across all phases in TEST compared with PLA (main effect of treatment; $P < 0.05$). Although there was no phase-by-treatment ($P > 0.05$), post hoc pairwise comparisons revealed that muscle mass was greater in TEST compared with PLA at Recovery and EOS ($P < 0.05$; Figure 6).

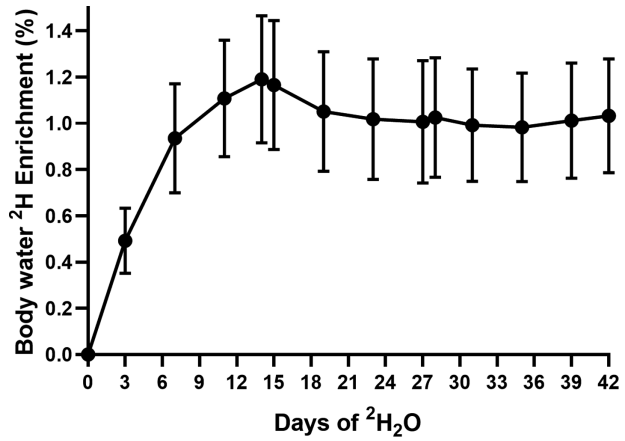


Figure 2. Body water ^{2}H enrichment measured from blood and saliva samples during the 42-day labeling period. Values are mean ± SD.

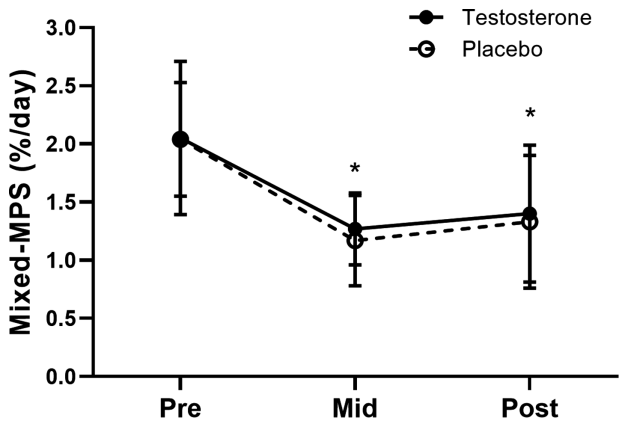


Figure 3. Mixed-muscle protein synthesis (MPS; %/day) before (Pre), during (Mid), and after (Post) a 28-day energy deficit in participants receiving 200 mg testosterone enanthate per week (TEST) or placebo (PLA). Differences between groups were examined using a 2-way ANOVA (time × treatment). Values are mean ± SD. *Different from Pre ($P < 0.05$).

Discussion

The current study explored the effects of a 28-day, severe exercise- and diet-induced energy deficit on mixed-MPS, muscle proteome dynamics, and muscle mass in nonobese men provided either TEST (200 mg testosterone enanthate) or PLA weekly. The primary findings show that severe energy deficit markedly suppressed mixed-MPS at the middle and end of the energy deficit, but, contrary to our hypothesis, this suppression was not affected by testosterone supplementation. In contrast, proteome dynamic analyses revealed a stimulatory effect of supplemental testosterone. Although FSR of individual muscle proteins were not statistically different between TEST and PLA at any time point, the proportion of proteins with numerically higher FSR in TEST compared with PLA was significant at Post using a binomial test. This was followed by a delayed accretion of muscle mass in TEST during ad libitum recovery feeding. These findings reveal a novel time-dependent and proteome-wide stimulatory effect of exogenous testosterone on protein synthesis during energy deficit that is only apparent with longer administration.

The suppressive effect of energy deficit on mixed-MPS in the current study is consistent with previous work (15, 16, 30-32). We previously showed that 10 days of a 20% energy deficit in healthy young men and women reduced postabsorptive mixed-MPS by ~19% (15), and 21 days of a 40% energy deficit in participants consuming the recommended dietary allowance for protein (0.8 g/kg/day) attenuated the muscle protein synthetic response to a protein-containing mixed-meal (16). Work from Hector et al (31) showed that changes in acute mixed-MPS during energy deficit follow a similar pattern to integrated myofibrillar MPS, which was attenuated after 10 days of a 40% energy deficit in young men consuming either lower (1.2 g/kg/day) or higher (2.4 g/kg/day) amounts of protein. The current study confirms the suppressive effect of energy deficit on integrated MPS, which occurred with energy deficit of greater magnitude (55%) and longer duration (14 and 28 days).

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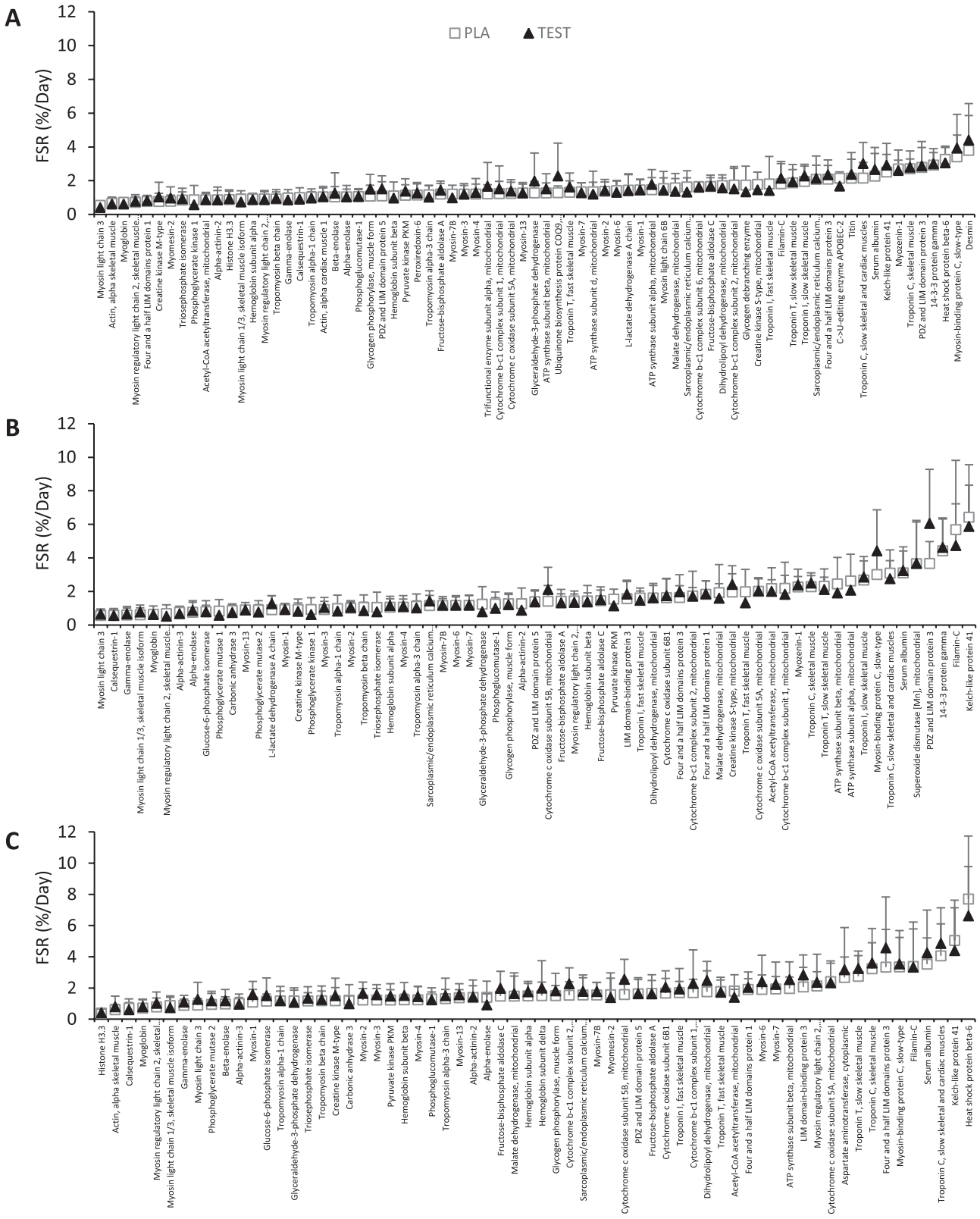


Figure 4. Pre (A), Mid (B), and Post (C) fractional synthesis rates (%/day) of individual myofibrillar, mitochondrial, and sarcoplasmic proteins in participants receiving 200 mg testosterone enanthate (TEST) or placebo (PLA) weekly during the energy deficit. Cross-sectional comparisons between TEST and PLA were determined using *t* tests with Benjamini-Hochberg corrections for multiple comparisons. Values are mean ± SD. No differences between groups ($P > 0.05$).

Testosterone administration did not change the mixed-MPS response to energy deficit. These findings contrast previous work during energy balance conditions showing short (5 days) and long-term (6 months) testosterone administration increases acute measures of postabsorptive MPS (4-6, 8). Although these discrepancies may be attributed to energy

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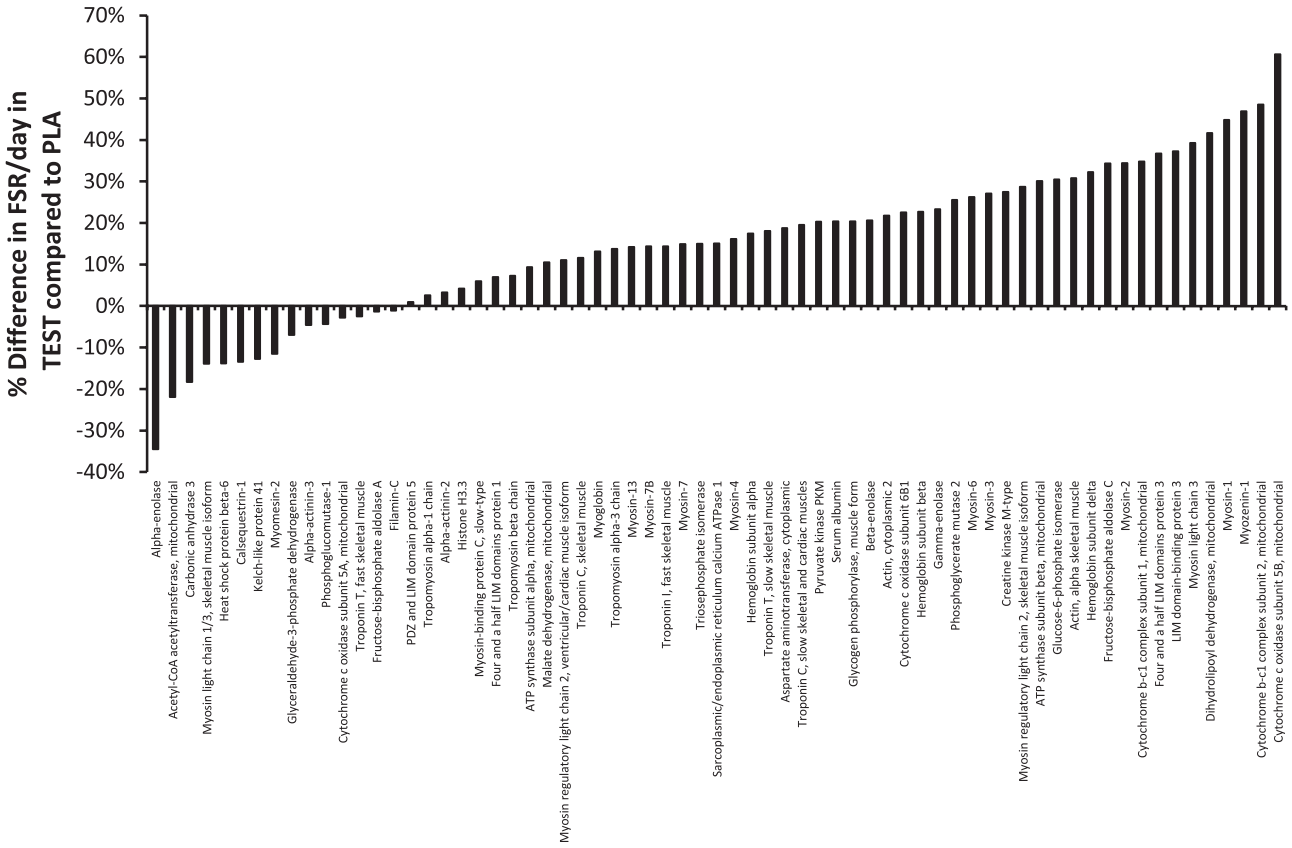


Figure 5. Magnitude difference (%) in FSR for individual myofibrillar, mitochondrial, and sarcoplasmic proteins in TEST (200 mg testosterone enanthate/week) compared with PLA (placebo) at Post. Positive values represent greater synthesis rates for proteins in TEST compared with PLA. The proportion of individual proteins with greater synthesis rates in TEST compared with PLA was significant using a 2-tailed binomial test ($P < 0.05$).

status (ie, energy balance or energy deficit), they may also result from methodological differences in measuring MPS. Studies examining the effect of testosterone administration on MPS have generally used primed constant infusion of stable isotope-labeled amino acids to measure the synthetic response over the course of a few hours, rather than integrated MPS over the course of many days (^2H_2O technique). Acute increases in postabsorptive mixed-MPS observed during controlled laboratory settings may or may not translate to net changes over days or weeks. Integrated MPS is an average of proteins that turnover slowly and rapidly, making comparisons between these methods difficult.

FSR of individual proteins did not differ significantly between groups at any time point. However, analyzing the proportion of individual proteins with greater FSR in TEST compared with PLA revealed a stimulatory effect of supplemental testosterone during the second half of the energy deficit. The proportion of myofibrillar, mitochondrial, and sarcoplasmic proteins with numerically greater FSR in TEST compared with PLA (52/67) was significant using a binomial test at Post, but not at Mid (32/67). These findings suggest that testosterone administration during energy deficit provides a modest but relatively broad and time-dependent stimulatory effect across the muscle proteome. This mechanism is consistent with controlled laboratory studies showing testosterone supplementation stimulates protein synthesis by promoting a greater use of intracellular amino acids to improve synthetic efficiency (4). This is also consistent with studies examining acute vs chronic effects of supplemental testosterone on MPS. Although stimuli such as exercise, amino acids, and insulin have strong acute effects on amino acid kinetics and MPS (33), short-term elevations in blood testosterone have minimal influence (34). Church et al (34) showed that testosterone infusion (0.25 mg/min) did not affect direct or model-derived measures of MPS over a 5-hour period despite significant increases in circulating testosterone. These findings suggest that chronic testosterone administration affects MPS through cellular adaptations that manifest over time, rather than transient anabolic signaling events.

The idea that longer term cellular adaptations contributed to a broad stimulation of FSR across the proteome in TEST compared with PLA is consistent with reported changes in muscle translational capacity following testosterone administration. Muscle total RNA content and total RNA content per "cellular unit" (RNA:DNA ratio) have been used as indirect measures of ribosomal capacity for protein synthesis (35-37) because ribosomal RNA comprises the majority of cellular RNA (> 85%) (38). Gharahdaghi et al (39) reported greater muscle total RNA content and RNA:DNA ratios at week 6 but not week 3 of a resistance exercise training program in older men supplemented with testosterone compared with placebo. These findings suggest that chronic testosterone supplementation may lead to delayed increases in translational capacity. We previously reported an increase in muscle total RNA content in a subset of TEST participants from the current analysis (2), suggesting changes in translational capacity may underlie the proteome-wide and time-dependent stimulus derived from testosterone supplementation during energy deficit.

D_3Cr muscle mass did not change during the energy deficit contrary to the increase in DXA-derived LBM reported

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Table 1. DXA-derived body composition and endocrine profiles

	Testosterone (n = 24)				Placebo (n = 26)				*P*		
	Pre	Post	Recovery	EOS	Pre	Post	Recovery	EOS	Phase	Treat	Phase × Treat
Total mass (kg)	78.1 ± 0.4^{a}	75.8 ± 0.4^{b,*}	79.3 ± 0.4^{c,*}	78.9 ± 0.4^{a,c,*}	78.3 ± 0.3^{a}	73.3 ± 0.3^{b}	76.4 ± 0.3^{c}	76.9 ± 0.3^{c}	<0.0001	<0.0001	<0.0001
Lean mass (kg)	57.9 ± 0.3^{a}	60.4 ± 0.3^{b,*}	63.1 ± 0.3^{c,*}	61.7 ± 0.3^{d,*}	58.3 ± 0.3^{a}	58.0 ± 0.3^{a}	60.5 ± 0.3^{b}	59.9 ± 0.3^{b}	<0.0001	<0.0001	<0.0001
Fat mass (kg)[1]	16.8 ± 0.2	12.0 ± 0.2	12.8 ± 0.2	13.8 ± 0.2	16.8 ± 0.2	12.2 ± 0.2	12.8 ± 0.2	13.9 ± 0.2	<0.0001	0.78	0.96
TT (nmol/L)	15.6 ± 0.9^{a}	34.4 ± 0.9^{b,*}	7.8 ± 0.9^{c,*}	10.5 ± 0.9^{c,*}	14.6 ± 0.9^{a,b}	11.8 ± 0.9^{a}	14.6 ± 0.9^{a,b}	15.4 ± 0.9^{b}	<0.0001	<0.0000	<0.0001
FT (nmol/L)	0.33 ± 0.02^{a}	0.84 ± 0.02^{b,*}	0.16 ± 0.02^{c,*}	0.22 ± 0.02^{c,*}	0.31 ± 0.02^{a}	0.18 ± 0.02^{b}	0.29 ± 0.02^{a}	0.31 ± 0.02^{a}	<0.0001	<0.0001	<0.0001
SHBG (nmol/L)	31.6 ± 1.6^{a,b}	36.5 ± 1.6^{a,*}	30.7 ± 1.6^{b,*}	31.2 ± 1.6^{b}	31.0 ± 1.5^{a}	52.3 ± 1.5^{b}	35.6 ± 1.5^{a}	33.8 ± 1.5^{a}	<0.0001	<0.0001	<0.0001

Values are least squares mean ± standard error. Data were analyzed using linear mixed models, adjusted for age and prestudy (day 0) values. Bonferroni corrections were used for post hoc comparisons. Data not sharing the same superscripted letter within a treatment are different by phase.
DXA, dual-energy X-ray absorptiometry; FT, free testosterone; TT, total testosterone.
*Indicates a between-group difference at a particular phase (phase-by-treatment interaction).
[1]Indicates all phases are different (phase main effect). $P < 0.05$ considered statistically significant.

Figure 6. Muscle mass as determined by the D_3Cr dilution method in TEST and PLA at Pre, Post, Recovery, and EOS. Values are mean ± SD. Although there was no phase-by-treatment interaction, post hoc comparisons are noted in the figure as #different from PLA. †Main effect of treatment ($P < 0.05$).

previously for TEST participants over the same period (1). The divergent values of LBM and muscle mass in the current study are in line with previous work showing DXA-derived LBM is not an accurate surrogate measure of muscle mass (40). Increased LBM in TEST likely resulted from changes in fluid content rather than contractile tissue, as testosterone supplementation has been shown to increase extracellular water content in hypogonadal men (41). We also report a main effect of treatment such that muscle mass was greater across all phases in TEST compared with PLA. Although there was no phase-by-treatment interaction, post hoc pairwise comparisons revealed that the main effect of treatment was likely driven by greater muscle mass in TEST compared with PLA at Recovery and EOS. A lack of change in muscle mass during the energy deficit may be attributed to the body's use of dietary protein to support energy production rather than MPS, which can occur with more severe energy deficits (42). In contrast, the proteome-wide stimulus derived from testosterone resulted in muscle accretion once energy and dietary essential amino acids were readily available during recovery. This increase in muscle mass occurred despite a decrease in circulating total testosterone, which likely resulted from limited endogenous testosterone production once supplementation was stopped (43). These findings suggest that muscle mass increased as a result of testosterone-mediated cellular adaptations during the energy deficit phase that persisted during early recovery.

The current study did not examine mixed-MPS or FSR of individual muscle proteins during recovery, limiting our ability to confirm whether changes in these measures contributed to the greater muscle mass reported in TEST compared with PLA during this phase. Additionally, the current study did not examine the potential contribution of muscle protein breakdown (MPB) to changes in muscle mass. Studies reporting the effect of testosterone on isotopic measures of MPB have yielded conflicting results. Although MPB was unaltered 5 days after a single testosterone injection (4) and with 5 days of oxandrolone treatment (8), Ferrando et al (44, 45) reported that fasting net protein balance (NB = MPS – MPB) was less negative after 6 months of testosterone administration in healthy older men because of a decrease in fasting protein

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breakdown. Testosterone-mediated changes in markers of MPB have also been observed in cell and animal models (46, 47), and work from our laboratory reported a decrease in markers of proteolysis at rest in a subset of TEST participants from the current analysis (2). Whether alterations in MPB contribute to changes in muscle mass with testosterone supplementation remains an important question for future work.

In conclusion, these data demonstrate that energy deficit has a strong suppressive effect on mixed-MPS, which was markedly lower at the middle and end of the 28-day energy deficit. Mixed-MPS did not differ between TEST and PLA; however, testosterone supplementation did result in a high proportion of proteins across the muscle proteome with numerically greater synthesis rates than placebo during the second half of the energy deficit. Muscle mass measured by D_3Cr dilution did not change during the energy deficit phase but was greater in TEST than PLA with ad libitum recovery feeding. The divergent values of LBM and muscle mass are consistent with DXA-based lean mass not being an accurate surrogate measure of muscle mass. These findings collectively reveal a stimulatory effect of supplemental testosterone on synthesis rates across the muscle proteome during prolonged energy deficit that results in muscle mass accretion with subsequent periods of positive energy balance.

Acknowledgments

The authors thank the participants who consented and participated in this research experiment. We also thank our other co-investigators and the study team at Pennington Biomedical Research Center for their significant contributions to study management and conduct, data collection, and analysis.

Funding

The research reported in this publication was supported by the Collaborative Research to Optimize Warfighter Nutrition II and III projects and the Joint Program Committee-5, funded by the US Department of Defense, and appointments to the US Army Research Institute of Environmental Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Army Medical Research and Development Command. The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Author Contributions

Conception and design of the study: S.M.P., M.S., C.E.B., L.M.M., H.R.R., J.P.K., A.J.Y., M.A.M., W.J.F., M.K.H., and J.C.R. Data collection and biochemical analyses: M.S., M.D.M., A.B., E.N., H.N., M.N.H., and J.C.R. Analysis and interpretation of the data: E.E.H., S.M.P., M.S., L.M.M., W.J.F., and M.K.H. Manuscript drafting: E.E.H., S.M.P., M.S., C.E.B., L.M.M., H.R.R., J.P.K., A.J.Y., M.A.M., W.J.F., M.K.H., and J.C.R. All authors critically evaluated the manuscript and approved it for submission. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Disclosures

M.D.M., A.B., E.N., H.M., W.J.E., M.K.H., and J.C.R. reported that their institutions received funding from the US Department of Defense for work associated with this publication. All remaining authors declare no competing interests. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the US Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

Clinical Trial Information

ClinicalTrials.gov identifier no. NCT02734238 (registered April 12, 2016).

Data Availability

Original data generated or analyzed during this study are included in this published article or in the data repositories listed in the references section.

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Constrained Learning of Task-related and Spatially-Coherent Dictionaries from Task fMRI Data

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Abstract. Dictionary learning and sparse coding techniques overcome limitations of traditional voxel-level analyses of task-based functional magnetic resonance imaging (fMRI) data by identifying broader temporal and spatial patterns of brain activity. However, prior applications of these methods to task-related fMRI data are not simultaneously optimized to find temporal patterns of activity that change in concert with changes in task conditions and spatial patterns that leverage existing neuroscience knowledge. In this study we present a new sparse dictionary learning method that uses prior knowledge of the temporal pattern of task conditions and the locations of brain regions hypothesized to be involved in the task to decompose fMRI data into temporal patterns of signals that loosely differ between task conditions and sparse spatial patterns that are at least partially similar to known functional network hubs. An efficient on-line optimization framework identifies the temporal and spatial patterns. The method identifies spatial and temporal patterns programmed into synthetic task fMRI data. The proposed method also identifies spatial locations known *a priori* to be activated by the Attention Network Task (ANT) more completely than competing methods when applied to real fMRI data from 20 healthy young individuals aged 18 to 39 years. Simultaneously leveraging the known temporal structure of the task and biasing solutions towards hypothesized network hubs increases the usefulness of sparse dictionary learning methods applied to task fMRI data.

Keywords. Sparse Dictionary Learning, fMRI, constrained learning.

1 Introduction

A current focus of human neuroscience research is the identification and study of functional brain networks (FBN) that work in a coordinated fashion to execute cognitive tasks [1]. FBNs consist of distributed brain regions ("hubs") each of which has a characteristic temporal pattern of activity. Due to the central role of FBNs in brain development, aging, and diseases [2], many techniques have emerged that use sparse dictionary learning methods to automatically identify the locations of hubs and temporal patterns, based on fMRI data [3, 4]. This application of sparse dictionary learning

methods to FBN identification has been supported by biological findings suggesting that sparse coding is implemented in the brain [5].

To date, most sparse dictionary learning methods for FBN estimation have been applied to resting-state fMRI data and, therefore, do not attempt to identify hubs that are task-engaged (*i.e.*, hubs with temporal patterns of activity that are correlated with changes in task conditions). While resting-state analysis is important, identifying task-engaged FBNs remains one of the central goals of cognitive neuroscience. Supervised dictionary learning is an exception to this rule [6, 7], but this method strictly constrains its temporal patterns to exactly match the temporal patterns of task conditions. It is therefore susceptible to errors when there is even a minor mismatch between the timing of real brain activity relative to task condition timing. In addition, supervised dictionary learning does not leverage prior knowledge about the hypothesized spatial locations of task-related activity. Assisted dictionary learning [8] avoids the problem of strictly adhering to temporal constraints and does not leverage prior knowledge of spatial locations of activity.

This paper presents a method for sparse dictionary learning of FBN spatial and temporal patterns from task fMRI data (Figure 1) that uses known task condition time courses and hypothesized FBN hub locations to identify FBN hubs that are spatially coherent and task-engaged. We apply the method to synthetic task fMRI data where FBN spatial and temporal properties are known *a priori* and to real task fMRI data where expected FBN hub locations are known from existing research. We compare the performance of the method with that of existing dictionary learning methods designed for fMRI data.

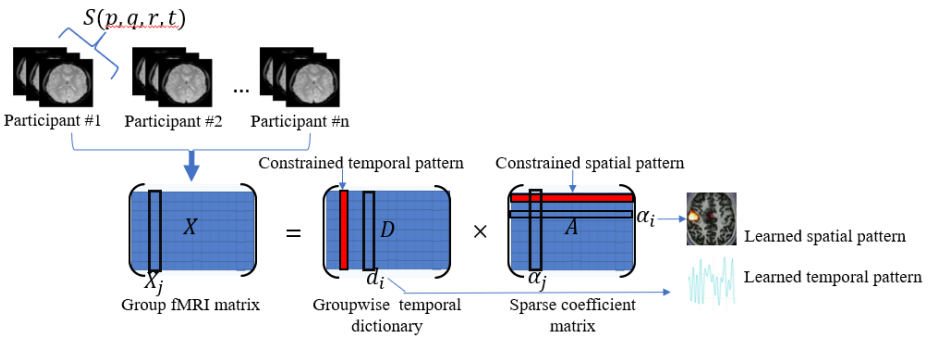


Fig. 1. Dictionary Learning of task fMRI data

This paper presents a baseline sparse dictionary learning method as a starting point (Section 2). Enhancements that allow the method to leverage known task-related time courses are discussed in Section 2.1. We discuss how to make the method leverage hypothesized spatial hub locations and spatial coherence in Section 2.2. Optimization within this new framework is described in Section 2.3. Section 3 covers experiments on synthetic and real fMRI data.

2 Constrained Online Dictionary Learning

2.1 Dictionary learning of fMRI data

Let $S[p, q, r, t]$ denote the fMRI scan of a single participant. Here, $p, q,$ and r are spatial coordinates, and t denotes time. The S from individual j is vectorized into a column vector $X_j \in \mathbb{R}^T$, and all n of these are concatenated to form the group data matrix X (Figure 1). The aim of sparse dictionary learning is to represent every X as a linear combination of a small number of temporal patterns $\{d_i\}_{i=1}^K$ in a dictionary $D \in \mathbb{R}^{T \times K}$, *i.e.*, $X_j = D\alpha_j$ where $\alpha_j \in \mathbb{R}^K$ represents a spatial pattern within which the temporal patterns are expressed. A is the matrix formed by stacking the column vectors α_j. Typically, $T < K$ and A encouraged to be sparse, *i.e.*, contain a large number of zeroes. We use training signals $\{X_j\}_{j=1}^n$ to learn D and A [9]:

$$\arg\min_{D,\mathrm{A}} \sum_{j=1}^{n} \left(\left\| X_j - D\alpha_\mathrm{j} \right\|_2^2 + \lambda \left\| \alpha_j \right\|_1^1 \right), \left\| d_j \right\|_2 < 1 \; \forall \, \mathrm{j} \tag{1}$$

The first term in the sum encourages low reconstruction error while the second encourages sparsity of α_j. λ controls the tradeoff between these competing terms.

2.2 Incorporating task characteristics

Task fMRI is characterized by the imposition of differing experimental conditions on the participant at differing moments in time. Let $\delta(t)$ represent a time course of task conditions numerically, i.e., $\delta(t) = 0$ (*vs.* 1) at time *t* when the first (vs. second) experimental condition is imposed [1]. Given several δ, $\{\delta_1, \delta_2, \ldots \delta_m\}$, we encourage the discovery of temporal patterns that change in concert with changes in experimental conditions by constraining *D* to the set of matrices *C*, each with members that contain columns similar to the δ:

$$C \triangleq \left\{ D \in \mathbb{R}^{T \times K} \middle| \begin{array}{c} \| \, d_j - \delta_j \, \|^2 \leq c_\delta, \; j = 1, \ldots, m \\ d_j^T d_j \leq 1, \; j = m+1, \ldots, K \end{array} \right\} \tag{2}$$

Constraining D to the set C requires D to include elements that at least loosely follow the temporal pattern of experimental conditions. Adding this constraint requires us to change the dictionary update step of our optimization algorithm [9] to a two-step process of modifying the constrained and unconstrained temporal patterns separately; otherwise, optimization proceeds as it did without the constraints.

[1] Note that the case of two experimental conditions is shown for ease of exposition, but this formulation trivially extends to larger numbers of experimental conditions via multiple δ.

2.3 Constraining spatial patterns

Although λ empirically determines the minimum number of voxels that the spatial patterns of FBNs occupy [10], it does not actively encourage biological plausibility of the spatial patterns. To encourage such plausibility, we constrain a subset of the spatial patterns to be similar to those identified as task-engaged in previous fMRI studies. Specifically, for each vectorized spatial pattern α_i, which is a row in A, we generate P_i, a binary version of α_i that has a value of 1 wherever α_i is non-zero. We encourage any *a priori* spatial pattern M to have high spatial overlap with P_i by requiring the Dice coefficient R between P_i and M to be high:

$$R(P_i, M) = \frac{|\mathrm{P_i} \cap M|}{|M|} \tag{3}$$

The orthogonal matching pursuit algorithm in the sparse coding step of optimization [9] described in the next section is constrained to incorporate a new Dice-related constraint $\mathfrak{D}_\mathrm{C}$ on m designated rows of A. The columns of A are also constrained, as in prior work [10], to be sparse, *i.e.*, to include no more than L nonzero pixels.

$$A \in \mathbb{R}^{K \times n} \; s.t \; \left\|\alpha_j\right\|_0^0 \leq L \, , R(P_i, M) \geq \mathfrak{D}_C, \;\; i = 1, \ldots, m \tag{4}$$

2.4 Optimization

Because equation (1) is non-convex with respect to D and A jointly, an optimization approach that alternates between D and A is typically utilized, following the Majorization-Minimization (MM) principle [11] [12]. An alternating optimization calculates the sparse code a_j for X_j and updates the temporal patterns in D for a fixed number of iterations over the entire training data [13]. Our work accelerates this traditional approach via an online algorithm inspired by prior work [9]. The representation error $X_j - D\alpha_\mathrm{j}$ in (1) is minimized by considering one X_j at a time and updating the temporal patterns based on stochastic block-coordinate descent following prior suggestions that stochastic gradient algorithms provide the fastest solutions among competing approaches for these types of problems [14] while requiring lower speed and less memory than traditional batch optimizers. In addition to its speed advantages, this is a second order optimization method that does not require explicit tuning of a learning rate. This optimizer was implemented on top of the base online optimization code within the SPAMS toolbox [15].

3 Application and results

3.1 Synthetic fMRI data generation using SimTB

We used SimTB [16] to generate synthetic task-related fMRI data sets. We generated 15 spatiotemporal patterns, each consisting of a single spatial pattern defined on a 100

x 100 voxel image slice and a temporal pattern of 300 time points (TR = 2s). One of the temporal patterns corresponded to a time course of changing task conditions convolved with a canonical hemodynamic response function (δ) as depicted in Fig.2, upper right; the other 14 temporal patterns and their corresponding spatial patterns, representing various physiological sources of fMRI signal variability, were taken from a previous publication [8]. We generated 20 synthetic datasets by linearly combining the 15 patterns using randomly generated amplitude scalings of the temporal patterns (with scalings drawn uniformly from [0.5, 1]). Translational head motion in the x and y directions was then drawn uniformly from the range of 0.02 to 0.5 times one voxel size and added to each synthetic scan. Finally, Gaussian amplitude noise at multiple levels (SNR of 10, 20 and 50 dB) was added to create the final synthetic dataset.

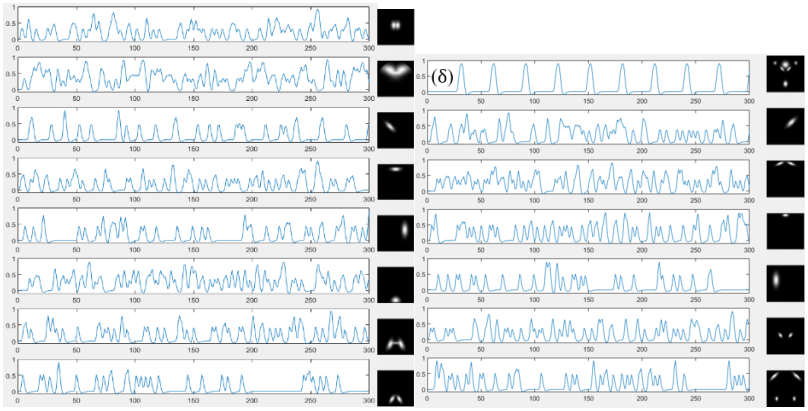


Fig. 2. Spatial patterns and corresponding temporal patterns programmed into the synthetic dataset

3.2 Evaluation of sparse dictionary learning algorithms

The ground-truth spatial patterns were vectorized into the rows of matrix A_g, and the ground-truth temporal patterns were vectorized into the columns of matrix D_g. We evaluated the proposed algorithm and three competing algorithms in terms of similarity between estimated A and D matrices and these ground-truth matrices. The time course δ was provided as a constraint to the optimizer. The spatial pattern M provided as a constraint to the optimizer is a binarized version of the spatial pattern for one source. For each run of each algorithm, D and A were initialized by randomly drawing entries from a normal distribution (zero mean and variance one) and normalizing columns to unit length. We completed differing runs with differing levels of sparsity L (see equation 4), which control the maximum number of nonzero elements in columns of A. Sparsity levels 2, 3, and 4 correspond to 20%, 30% and 40% of the column being nonzero. Within each run, each paired step of optimization consisting of sparse coding and dictionary learning was executed 180 times. We completed 30 runs at each sparsity level for each tested algorithm, with each run applied to a different set of generated

synthetic data. Each of the 15 learned temporal patterns in D were correlated with ground-truth time courses in D_g via Pearson correlation. A ground-truth temporal pattern was considered "recovered" if there existed an estimated temporal pattern in D whose Pearson correlation to it was greater than 0.9. Every recovered temporal pattern had a corresponding spatial pattern that had at least 50 percent overlap with the ground-truth spatial pattern. The average number of recovered temporal patterns across all runs at a given sparsity level were then tabulated. We evaluated learning rates γ between 0.001 and 0.25 for K-singular value decomposition (K-SVD) [11] and K-SVD-sparse (K-SVDs); the ones that gave the best results (γ =0.18) in terms of maximal correlation between estimated temporal pattern and task condition temporal pattern (δ) was selected. The temporal pattern level sparsity parameter for K-SVDs was set to 10.

3.3 Synthetic data results

The mean percentages of recovered temporal patterns at all sparsity and noise levels are shown in Table 1.

Table 1. Average percentage of recovered atoms for the Constrained Dictionary Learning (CDL) compared to other algorithms KSVD [11], KSVDs, and Online Dictionary Learnning (ODL)

	Sparsity Level (L)	CDL	KSVD (γ = 0.18)	KSVDs (Atom sparsity level 10)	ODL
SNR 10 dB	2	**87.3**	72.8	61.20	86.47
	3	87.80	71.20	55.73	**88.69**
	4	**68.60**	28.53	38.93	65.16
SNR 20 dB	2	**90.67**	81.40	81.70	88.17
	3	91.67	82.50	75.67	**92.50**
	4	**93.60**	81.79	72.53	89.20
SNR 50 dB	2	90.67	**90.80**	88.57	89.52
	3	**91.67**	88.69	87.12	90.11
	4	**93.60**	91.55	90.54	87.42

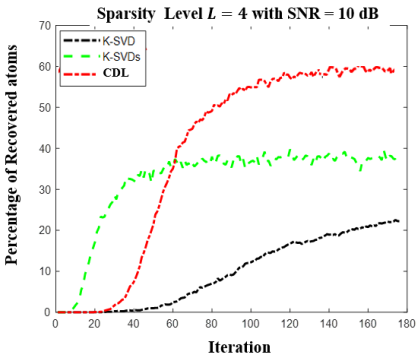


Fig. 3. Average percentage of atoms recovered after each iteration.

The proposed algorithm, Constrained Dictionary Learning (CDL), provides higher percentages of recovered temporal patterns than the three competitor algorithms (Table 1). This was especially true at lower SNR levels and at lower sparsity levels. The recovery rates of ODL were similar to the proposed algorithm at higher sparsity levels. Figure 3 depicts the average percentage of recovered atoms as a function of iteration number (sparsity s = 4, SNR = 10 dB). The figure suggests that K-SVDs was allowed to run to convergence but still arrived at a poorer solution than the proposed method. K-SVD had a much lower recovery rate possibly because it needs many more iterations to converge[2].

3.4 Real Task fMRI data

The real fMRI data set was acquired as part of a clinical trial among healthy young men [17] ages 18 to 39. Functional magnetic resonance imaging (fMRI) scans were acquired on a 3T MRI scanner (General Electric, 750W Discovery, 32-channel quadrature head coil) using a blood oxygen level dependent echo-planar imaging (BOLD-EPI) pulse sequence. The fMRI scans collected during execution of the Attention Network Task [18] were analyzed from 50 participants, with congruent and incongruent trial types providing the two experimental conditions represented as 0 and 1 in the temporal constraint δ after convolution with a canonical hemodynamic response function. Spatial patterns of regions known to have different activity between congruent and incongruent trials, based on published reports (*i.e*., the anterior cingulate cortex or ACC; and lateral prefrontal cortex or LPC)[19], were obtained from Automated Anatomical Atlas 3 (AAL3)[20]. These spatial patterns were used as the spatial constraints M in equation (3). A classical fMRI preprocessing pipeline of head motion correction, co-registration to the structural scan, slice timing correction, spatial smoothing using a 6 *mm* full width at half maximum Gaussian kernel, and warping to the Montreal Neurological Institute (MNI) template was applied using Statistical Parametric Mapping 12 (SPM12) with MATLAB R2020b. Participants wore a pulse oxygenation sensor during scanning, and cardiac / respiratory influences on fMRI signals were removed based on its data [21]. Data from the 20 participants with highest task performance accuracy was selected for analysis.

Dictionary Learning: Similar to the process carried out for the synthetic data, the data matrix was formed by the vectorized preprocessed images. The data matrix $X \in \mathbb{R}^{T \times n}$, was then down sampled by a factor of 8 along the column direction. A dictionary of size $T \times 40$ was initialized using random vectors (columns) taken from X. The same algorithms tested on synthetic data were applied to real data with sparsity level s set to 3. During optimization we constrained one time course δ (corresponding to congruent and incongruent trials) and one spatial pattern M (corresponding to executive control regions) and examined our algorithm's ability to learn spatial patterns corresponding to other FBNs known to be involved in task execution (the alerting and orienting

[2] The convergence rate of ODL is not included in the figure since the code did not provide this information.

networks) as well as temporal patterns corresponding to other relevant task conditions (*i.e.*, task trials with differing alerting and orienting requirements). As for synthetic data, the Pearson correlation between estimated and ground-truth temporal patterns were calculated, and the maximum correlation for each ground-truth pattern is reported as an indicator of recovery. Similarly, the Dice coefficient between estimated and ground-truth spatial patterns is reported as an indicator of spatial pattern recovery.

Table 2. Results for real fMRI data

	(A) Highest correlation coefficient between estimated temporal pattern and time course of task conditions				(B) Highest dice coefficient between recovered spatial pattern and known task-evoked networks			
Contrast	CDL	KSVD	KSVDs	ODL	CDL	KSVD	KSVDs	ODL
Executive control	**0.96**	0.53	0.61	0.68	**0.81**	0.59	0.52	0.45
Alerting	**0.83**	0.58	0.70	0.52	**0.65**	0.61	0.42	0.58
Orienting	**0.89**	0.47	0.39	0.48	**0.69**	0.51	0.32	0.51

Recovery of spatial and temporal patterns from the real fMRI data is shown in Table 2. As expected, our CDL method recovers the executive control spatial and temporal patterns because these were provided as constraints to the optimizer. Additionally, CDL does the best job of all algorithms at recovering the alerting and orienting related spatial and temporal patterns even though information about these spatial and temporal patterns were not provided to the optimizer. Interestingly, although the algorithm was encouraged to find a spatial pattern matching the spatial pattern of an executive control network, the spatial pattern providing the best such match was not perfectly correlated with the ground-truth spatial pattern (Pearson's r=.81), suggesting that requiring spatial patterns to exactly match ground-truth [6] may be too rigid to allow accurate data representation.

4 Conclusion

Using task information to partially constrain dictionary learning allowed this method to identify task related networks in real and synthetic fMRI data. Future work should assess how additional constraints help or hurt performance and how the method can be applied to more novel tasks with involved networks that are less certain. Standard online optimizer enhancements should also be explored such as 1) sampling the training data intelligently, 2) using Minibatch extension, and 3) purging the dictionary of unused atoms.

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Acknowledgment: Funding for this project was provided by Collaborative Research to Optimize Warfighter Nutrition projects I and II, and the Defense Health Program, Joint Program Committee-5, Military Operational Medicine Research Program. Additional support provided by the Pennington Biomedical Research Foundation. Authors have no conflicts of interest to report.

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U.S. Army Research Institute of Environmental Medicine

Natick, Massachusetts

TECHNICAL REPORT NO. T22-11
DATE 21 April 2022

EFFECTIVENESS OF GO FOR GREEN® NUTRITION PROGRAM TO IMPROVE WARFIGHTER MEAL QUALITY AND NUTRITION KNOWLEDGE

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USARIEM TECHNICAL REPORT T22-11

Effectiveness of Go for Green® Nutrition Program to Improve Warfighter Meal Quality and Nutrition Knowledge

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Report Documentation Page

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
20 April 2022	Technical Report	2017-2022

4. TITLE AND SUBTITLE
Effectiveness of Go for Green® Nutrition Program to Improve Warfighter Meal Quality and Nutrition Knowledge

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
LTC Asma S. Bukhari, Catherine M. Champagne, Nicholes J. Armstrong, Susan M. McGraw, H. Raymond Allen, Katie M. Kirkpatrick, Elizabeth M. Moylan, Carolyn A. Kleinberger, Jennifer L. Billington, Patricia A. Deuster, COL Renee E. Cole

5d. PROJECT NUMBER
USARIEM protocol: 17-09-HC

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Research Institute of Environmental Medicine, Natick, MA, 10 General Greene Ave, Natick, MA 07160-5007 and Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808-4124

8. PERFORMING ORGANIZATION REPORT NUMBER
T22-11

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command, Fort Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Military dining facilities (DFACs) are important avenues to fuel Service Members (SM) for optimal performance. Nutrition-based interventions can both provide and educate SM on high-performance fueling options. Go for Green® 2.0 (G4G 2.0) is a multi-component intervention in DFACs to optimize access, availability, and knowledge of nutritious food choices. The study determined the fidelity of G4G 2.0 program implementation, along with impact on DFAC diner meal quality, diner satisfaction, and plate cost. A time series, multi-site, non-controlled intervention was conducted with data collected from consenting participants at pre- and post-G4G 2.0 implementation using food photography and surveys. The G4G 2.0 program has eight requirements, including a traffic light color labeling system guiding food selection, menus offering tasty, nutrient-dense (Green-coded) foods, placement of Green-coded foods for maximum visibility, DFAC staff training, and marketing campaign. Participants freely selected food choices. Digital food photography captured diners' food selections and analyzed nutrient composition and Healthy Eating Index (HEI) scores to measure meal quality. Surveys gathered demographic, lifestyle, nutrition knowledge and diner satisfaction data. Data on food cost and program fidelity were also collected. Data were analyzed using descriptive, independent t-test, Mann-Whitney U, chi-square analyses and 2-step cluster analysis (α=0.05, 80% power). The study was approved by the Institutional Review Board. Participants were active-duty Soldiers (n=100) who provided food intake data from at least one breakfast, lunch, and dinner meal at the intervention DFAC (92% male, median age 22.0 years, 63 pre; 37 post-intervention). Meal quality (pre/post HEI scores, Median ± IQR) improved from 55.0 ± 12.8 to 64.1 ± 12.04, p<0.001. There were significant improvements in selection of whole grains, seafood and plant proteins, fewer refined grains, and improved trends for other food groups. More diners agreed that main dishes were nutritious/performance-based (32% pre; 57% post; p<0.01) and used color-coded labels to choose performance foods (38% pre; 54% post; p=0.04). The intervention resulted in an 11% increase in plate cost. The overall nutrition knowledge scores of diners and foodservice staff was below 60% post program implementation, nevertheless, a greater proportion of correct responses to nutrition knowledge questions was observed for those participants exposed to the G4G program and received at least one hour of nutrition education. Program fidelity assessment indicated successful implementation in six of eight program requirements. Results indicate that the G4G 2.0 program is feasible, efficacious, and leads to improved meal quality and satisfaction of diners. Additional strategies are needed to further enhance nutrition knowledge of both patrons and diners and find ways to improve program marketing. Future research should continue evaluating facilitators and barriers to availability, access, cost, and consumption of performance-based food options, and collect outcomes related to health and performance. Improving access to nutritious food choices that SMs enjoy supports fueling needs to support optimal performance and military readiness.

15. SUBJECT TERMS Go for Green®; Meal Quality; Food Photography; Nutrition Knowledge

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	unclassified	unclassified	Unclassified	109	LTC Asma Bukhari

TABLE OF CONTENTS

Section	Page
List of Tables ……..	vi
List of Figures ……	vii
Background …….....	ix
Acknowledgments …….....	x
Executive Summary ……..	1
Introduction …….	3
Study Objectives / Specific Aims / Research Questions …….....	7
Research Design and Methods ……..	8
Go for Green® Program Intervention……..	11
Digital Photography and Meal Quality …….	13
Paper Surveys ……	15
Plate Cost ……	18
Statistical Analysis ……	18
Results…… …….	19
Discussion ……..	24
Conclusions and Implications …….....	30
Recommendations ……	31
References …….	33

Section Page

Appendix A: G4G 2.0 Menu Standards and Guidelines …….. 58

Appendix B: Demographic & Lifestyle Survey …….. 72

Appendix C: Dining Facility Satisfaction Survey ……..... 77

Appendix D: Nutrition Knowledge Survey ……. 78

Appendix E: G4G Awareness for Diner ……. 80

Appendix F: G4G Awareness for Staff …….. 86

Appendix G: Demographic & Lifestyle for Staff ……. 92

Appendix H: Satiety Labeled Intensity Magnitude (SLIM) Scale

Appendix H1: Pre-SLIM …….. …….. 94

Appendix H2: Post-SLIM ……. 95

Appendix I: G4G Program Fidelity Checklist ……. 96

LIST OF TABLES

Table Page

1. Evolution of the G4G 2.0 Program ……. 37

2. Demographic and lifestyle characteristics of active-duty military personnel consuming meals at two large military dining facilities pre and post-G4G 2.0 program implementation (n=100)……. 38

3. G4G 2.0 program requirements with benchmarks and results from two sites 39

4. Perceived changes in components of meal satisfaction and point-of-service nutrition labeling reported by participants consuming meals at two large military dining facilities pre- and post-G4G 2.0 program implementation (n=100) …….. 40

5. Demographic and lifestyle characteristics of military dining facility diners who completed nutrition knowledge surveys pre- and post-G4G program implementation …… 41

6. Nutrition knowledge subgroup from the 18-item nutrition knowledge questionnaire …….. 43

7. Demographic characteristics of foodservice staff from two large military dining facilities based on level of exposure to the performance nutrition programs (n=184) …… 44

LIST OF FIGURES

Figure Page

1. G4G 2.0 standardized traffic light labeling criteria and logos …… 45

2. G4G 2.0 study implementation and timeline …… 46

3. Digital food photography station example …… 47

4. Healthy Eating Index-2015 component food group and total median scores among participants consuming meals at two large military dining facilities pre- and post-G4G 2.0 program implementation ……. 48

5. Examples of meals with high and low Healthy Eating Index 2015 scores 49

6. Overall meal and per meal changes in Green, Yellow, and Red food items of participants consuming meals at two large military dining facilities before and after G4G 2.0 program implementation (n=100)… 50

7. Nutrition knowledge levels reported by military dining facility diners (n=269) …….. 51

8. Extent of military/civilian nutrition education received by military dining facility diners (n=269) …… 52

9. Hours of nutrition education received by military dining facility diners (n=269) …… 53

10. Responses to 18 nutrition knowledge questions by military dining facility diners (n=269) …….. 54

11. Level of food service staff training during G4G 2.0 program implementation …….. 55

12. Food service staff knowledge improvement with exposure to G4G 2.0 program (n=184) …….. 56

13. Responses to 18 nutrition knowledge questions by food service staff … 57

BACKGROUND

The impact of nutritional fitness is increasingly recognized across the Department of Defense as a critical component of overall Service Member (SM) mission readiness. For example, nutritional readiness is one of the pillars of the Army's Holistic Health and Fitness (H2F) System, which is designed to address Soldier performance (ref: FM7-22). Along with H2F, there is a renewed interest and desire at all echelons to optimize the nutrition environment of our SMs. Nutritional fitness impacts mental and physical performance, military readiness, and recovery following injury. Military dining facilities (DFACs) provide an opportunity to offer and educate warfighters on how to identify and use performance-based fuel. The current Go for Green® (G4G 2.0) nutrition program was developed as an intervention to inform and motivate SMs to make more performance-focused food and beverage choices. Demonstrating that the revised G4G 2.0 is effective in improving Soldier dietary intake, and cost effective and durable, will help ensure the program receives necessary support and resources for DoD wide implementation.

ACKNOWLEDGMENTS

The authors thank the following team members for their critical support in conducting this study. Staff from US Army Research Institute of Environmental Medicine to include Marques A. Wilson, MS; Anthony J. Karis, BS; and several other research assistants. Support from Pennington Biomedical Research Center with food photography: Christopher Gelpi, BS; Dawn Turner, BS; and several other research assistants. Support from consultations: Kathryn DeWitte, BS and Shawn Saco, MS, CNS, LDN from CHAMP; food technologists from the Armed Forces Recipe Service; experts from the Joint Culinary Center of Excellence. Support from study sites: MAJ Brigette Grimes, MS, RD, CSSD (Division Dietitian, 4th Infantry Division); SGT Hermes Matos (68M); SGT Brittany Basye (68M); CPT Sara Crews, RD; CPT Jonathan Allen, RD along with food advisors, DFAC management, DFAC staff, and diners. We also deeply appreciate Dr. Andrew Young's editorial support.

Obtained written permission from all persons named in the Acknowledgment.

Keywords: Healthy Eating Index, Food Photography, Go for Green, Diner Satisfaction.

Funding: US Army Medical Research & Development Command, Military Operational Medicine Research.

Conflict of Interest: The authors have no financial interests or relationships to disclose.

EXECUTIVE SUMMARY

Military dining facilities (DFACs) are important avenues to fuel Service Members (SM) for optimal performance, and at the same time educating them on nutrient-dense ("high performance") menu options. The Go for Green® 2.0 program (G4G 2.0) is a multi-component intervention in DFACs designed to optimize access, availability, and knowledge of high-performance food choices. The G4G 2.0 evaluation study aimed to determine the fidelity of G4G 2.0 program implementation, along with the program's impact on DFAC meal quality, diner satisfaction, nutrition knowledge and plate cost. This project employed a time series, multi-site (Fort Hood and Fort Carson), non-controlled intervention with data collected from consenting participants at pre- and post-G4G 2.0 program implementation using food photography and surveys. The study was approved by the U.S. Army Research Institute of Environmental Medicine and U.S. Army Medical Research and Materiel Command Institutional Review Boards. The G4G 2.0 program has eight requirements, including a traffic light, color-coded labeling system, menus that offer tasty and nutrient-dense (Green-coded) foods, strategic placement of Green-coded foods to maximize visibility, DFAC staff training, and a robust marketing campaign. Participants freely selected meals from food choices served at the DFAC. Digital food photography captured diners' food selections and analyzed nutrient composition and Healthy Eating Index (HEI) scores to measure meal quality. Surveys gathered demographic, lifestyle, diner satisfaction data and nutrition knowledge. Data on food cost and G4G 2.0 program fidelity were also collected. Data were analyzed using descriptive, independent t-test, Mann-Whitney U, chi-square analyses and 2-step cluster analysis (α=0.05, 80% power). Participants were active-duty Soldiers (n=100) with food intake data from at least one breakfast, lunch, and dinner meal at the intervention DFAC (92% male, median age 22.0 years, 63 pre; 37 post-intervention). HEI scores improved significantly from pre: 55.0 ± 12.8 to post: 64.1 ± 12.04, p<0.001 (Median ± IQR). Significant improvements were noted in selection of whole grains, seafood and plant proteins, fewer refined grains, and improved trends for other food groups. More diners agreed that main dishes

were nutritious/performance-based (32% pre; 57% post; $p<0.01$) and reported using color-coded labels to choose performance foods (38% pre; 54% post; $p=0.04$). The intervention resulted in an 11% increase in plate cost. The overall nutrition knowledge scores of diners was below 60% post program implementation. Nevertheless, a greater proportion of correct responses to nutrition knowledge questions was observed for those participants exposed to the G4G 2.0 program and who received at least one hour of nutrition education. Program fidelity assessment indicated successful implementation of six of the eight program requirements. The G4G 2.0 program is feasible, efficacious, and improved meal quality and satisfaction of diners. Implementing G4G 2.0 per the program requirements provides a greater amount and variety of strategically placed Green-coded (high-performance) menu items to create a more support nutrition environment. Also, G4G 2.0 program has a potential to improve performance nutrition knowledge, which could translate to overall improved nutrition-related behaviors both within and outside DFAC. Future research should identify key facilitators and barriers to providing and choosing performance-based food options, as well as consider outcomes related to health and performance, rather than just diner satisfaction. Improving opportunities for SM to fuel with nutritious and tasty food choices along with strategic nutrition education messaging are crucial for optimal nutritional fitness and military readiness.

INTRODUCTION

Reports indicate that 63.5% of Department of Defense (DoD) Service Members (SMs) are overweight and 14.4% are obese per body mass index guidelines.[1] Overweight and obesity are associated with poor physical fitness and may adversely affect mission readiness. Moreover, data from across the DoD indicate that SM intake of nutrient-dense foods is less than ideal, which may contribute to overweight and obesity. [2-4]

In a recent study, food choices of SMs in a military dining facility (DFAC) were rated as poor as evidenced by a meal quality score of 49 of 100 points on the Healthy Eating Index (HEI). [2] Of note, the average reported HEI-2015 score for Americans is 59 points.[5] Similar to the civilian setting, gaps in access and availability of nutrient-dense foods exist in the military nutrition environment, which negatively influence SM eating behaviors.[6,7] Providing adequate nutritional fueling is critical for mental and physical performance optimization as poor food choices and suboptimal meal quality may negatively impact mission readiness.

Targeted nutrition interventions in DFACs are key opportunities to improve SM knowledge and behavior.[8] Several DFAC interventions have been shown to be effective, especially when the taste and quality of menu items were improved.[9] One such intervention involving menu enhancements (increased fruits, vegetables, and whole grains; reduced fat and sugar; included at least one lean meat or vegetarian entree), staff education, marketing posters, and use of traffic light food labels at ten Army DFACs resulted in reduced intakes of energy, total fat, saturated fat, and refined grains and improved customer satisfaction.[8] Similarly, a nutrition program at two U.S. Air Force basic military trainees DFACs demonstrated that diners reduced daily fat intake from 35% to 19% of total calories, whereas those dining on

the standard fare DFAC increased their fat intake.[10]

Traffic light labels identifying menu items as Green, Yellow, and Red, when placed at the point of selection, have been suggested as a population-wide strategy to encourage better food choices.[11] This strategy is based on social cognitive theory, which focuses on the inter-relationship of diner knowledge regarding nutritious food choice, personal responsibility to make better choices to support optimal health and performance, and the influence of the social and physical environment when deciding what foods to choose.[12-14] Compared to other labeling options, traffic light labeling was shown to be more effective in educating consumers to select healthier food options.[15-18]

Interventions using food labels have had mixed results, ranging from improvement to no change in eating behaviors.[9,19,20] However, it is unclear whether awareness and/or perceived healthfulness actually translate into healthier food choices.[17] Two systematic reviews of food labeling have highlighted the 1) the inability of menu labeling with calories alone to promote healthier choices, 2) a need for more contextual or interpretive nutrition information on restaurant menus to assist consumers in selecting fewer calories, and 3) a need for well-designed studies on menu labeling in various settings.[21,22] Thus, a nutrition DFAC intervention that includes traffic light labels with choice architecture is warranted to determine effectiveness and ultimately to encourage better food choices and improve mission readiness of SM.

In 2008, Go for Green® (G4G 1.0 or original) was implemented to standardize labeling in Army DFACs and educate Soldiers to identify and choose nutritious, “high-performance” options. Initially there were three program components: 1) traffic light color labels, 2) standardized food cards, and 3) printed marketing posters (Table 1). This was based upon research suggesting

that traffic light color labels displayed on food cards influenced diners to choose more nutritious food options.[23] The program was then expanded to the other military service branches with the potential to impact SMs worldwide.

The original G4G 1.0 program underwent a major revision by incorporating feedback from DoD dietitians, foodservice operators, and other key stakeholders along with advancements in nutrition and health promotion research. Revisions addressed outdated coding criteria, lack of a standardized coding approach, lack of standardized staff training, outdated marketing and education strategies, and menus with inadequate amounts of Green-coded items.[24,25] Additionally, a larger emphasis on the connection between optimal fueling and SM performance was needed to prioritize resources and buy-in by leaders and diners for DFAC interventions, such as G4G. Across 2015, the G4G program was pilot tested by the G4G Program Office at the Consortium for Health and Military Performance (CHAMP) at the Uniformed Services University of the Health Sciences and the Army Public Health Center in conjunction with the Performance Triad pilot at several Army installations.

Based on the available data and feedback, the G4G program was substantially revised and rebranded to G4G version 2.0 (G4G 2.0) and managed by the G4G Program Office at CHAMP.[26] One of the most significant revisions was development of an algorithm to standardize assigning foods or beverages as Green, Yellow, or Red code (Figure 1). This assigned code aligns with the U.S. Dietary Guidelines for Americans, Military Dietary Reference Intakes, DoD nutrition and foodservice standards and regulations, and the latest nutrition science literature. Specific emphasis was placed on “nutritional quality” to capture the degree of processing, total sugar, quality (type) of fat, and fiber instead of only calories, percent total fat, and sodium. Additionally, sodium was designated as a separate, but complementary code (Low, Moderate, and High) based on the variable sodium needs of SMs. This program revision also supported

the Armed Forces Recipe Service's initiative to update their catalog of military recipes to increase whole grains, add more vegetables, utilize leaner meats, optimize flavors through more seasonings (less salt), and employ healthier preparation techniques (e.g., roasting vegetables, baked vs fried).

The second significant G4G 2.0 program modification was the establishment of program requirements (PRs). This included five newly added PRs to increase the availability, access, and promotion of Green-coded menu items. The G4G 2.0 PRs are: 1) standardized management training, 2) assign traffic light codes based on approved coding algorithm by trained coders, 3) menu with minimum number of Green-coded items targets, 4) standardized food cards, 5) food placement strategies, 6) promotion of Green-coded items, 7) marketing (printed materials, social media, press) and education, and 8) standardized staff training. Program resources, templates, menus and recipes, news, and support are available on the public-facing website, hosted by CHAMP: https://www.hprc-online.org/nutrition/go-green. Key programmatic elements are highlighted in Appendix A.

An opportunity arose to implement the newly revised G4G 2.0 program at Army DFACs. CHAMP requested assistance from USARIEM to assess the effectiveness of G4G 2.0. Both PubMed and Defense Technical Information Center (DTIC) searches were conducted (14 November 2016) using the keywords: "dining facility" (or "dining hall"); "military forces" (or "military"); "Health Eating Index 2010" (or "HEI 2010"); "menu planning"; "healthy eating"; and "performance". These searches indicated that this protocol would not be a duplication of previous research efforts. The purpose of this study was to test the feasibility and efficacy of improving ad libitum nutritional intake, nutrition knowledge, and diner satisfaction of Wolf DFAC, Fort Carson and Freeman Café, Fort Hood Soldiers following implementation of the revised G4G 2.0 program.

Study Objectives / Specific Aims / Research Questions

Primary Objectives:

1. Determine the effectiveness of the revised G4G 2.0 program to improve meal quality, satisfaction and nutrition knowledge of DFAC diners.
2. Determine the G4G 2.0 program feasibility through changes in average plate cost during G4G 2.0 implementation.

Secondary Objectives:

1. Assess whether the G4G 2.0 program intervention promotes changes in diners' self-reported lifestyle behaviors and attitudes towards the impact of nutrition on health and wellness factors.
2. Determine if the G4G 2.0 program intervention is associated with changes in subjective rating of appetite/satiety before and after eating in the DFAC.
3. Capture DFAC staff perspectives on barriers, challenges, and experiences related to G4G 2.0 program planning, implementation, and sustainment.

Hypotheses:

1. Implementation of the G4G 2.0 program will result in improved meal quality, meal satisfaction and nutrition knowledge of diners.
2. Average plate cost for G4G 2.0 will be feasible and comparable to a standard garrison DFAC.
3. The G4G 2.0 program intervention will promote changes in Soldiers' self-reported lifestyle behaviors and attitudes with potential future impact of nutrition on health and wellness factors.
4. The G4G 2.0 program intervention will be associated with enhanced diners subjective rating of appetite/ and satiety.

5. Understanding the DFAC staff perspectives on barriers, challenges, and experiences related to G4G 2.0 program planning, implementation, and maintenance will assist with future mitigation strategies and useful to improve program fidelity.

RESEARCH DESIGN AND METHODS

Research Design

This was a time series, multi-site, non-controlled intervention study evaluating the implementation and effectiveness of the G4G 2.0 program between May 2017 and September 2019 at two U.S. Army DFACs (in Colorado and Texas). Data were collected pre- and post-G4G 2.0 program implementation. At each time point, uniquely enrolled participants were assessed for meal quality of, and satisfaction with, three meals (minimum of one breakfast, one lunch, and one dinner). The study was approved by the U.S. Army Research Institute of Environmental Medicine and U.S. Army Medical Research and Materiel Command Institutional Review Boards.

Participant Population

Participants included diners at the Wolf DFAC (primarily Soldiers from the 4th Sustainment Brigade (4SB), 4th Engineers (4EN), 759 Military Police (MP), Division Artillery (DIVARTY), and Headquarters and Headquarters Battalion (HHBN) of the 4th Infantry Division, Fort Carson, CO) and diners at the Freeman Café DFAC (primarily Soldiers from the 1st Cavalry (1CAV), 4SB, 13th Sustainment Command (Expeditionary), DIVARTY, and HHBN 1CAV, of the III Corps, Fort Hood, TX). The plan was to recruit a total of 720 DFAC Soldiers and 250 DFAC staff: 180 Soldiers over four iterations; 50 DFAC staff over five iterations.

Inclusion and Exclusion Criteria

Participants were included if on active duty, adults (18 years and older), consuming meals for at least one month at either Wolf DFAC or Freeman Café. Participants needed to be willing to consume three meals each day for two testing days at the intervention DFAC (minimum of one breakfast, one lunch, and one dinner to attain average daily intake). If willing and available to participate, they could volunteer for subsequent data collection. No exclusion criteria were set. Participants were not monetarily compensated for study participation; however, they were offered the privilege of moving to the front of the DFAC line on study days to allow maximum time for meal consumption and tray photography.

Sample Size Estimations

SPSS SamplePower 3.0.1[41] was used to estimate sample size based upon the anticipated change in HEI-score as the primary outcome by using an independent t-test analysis (different participants per group); power was set at 80% and alpha 0.05 (two-tailed analysis). A mean change in HEI-2015 was expected at 5-6 points pre- to post-implementation with a 10-point variance. This estimate was based on a Kirkpatrick et al.[27] report noting that HEI standard deviations of approximately 11-12 points for adults would equate to a moderate effect size of 0.5. To detect that magnitude of HEI change required a minimum of 17 participants per time point at each location. Based upon results from USARIEM protocol #15-04-HC[2] (same methodology), a 30-50% increase in sample size would be needed to account for attrition and participant non-compliance (not attending three of six meals. Therefore, a sample size of n=180 per iteration for a total n=540 over three iterations was considered sufficient.

Sample size estimate for change in nutrition knowledge before and after implementation

assessment required 16 per group (pre vs. post) considering a 10-point difference in knowledge scores, a 10-point standard deviation, at 80% power and an alpha set at 0.05 (two-tailed analysis). Thus, the sample size (n=180) required for the HEI-score dietary quality assessment was sufficient to complete the nutrition knowledge assessment as well.

Stratified, purposeful sampling was conducted to obtain feedback from Wolf DFAC and Freeman Café staff in both supervisory and non-supervisory roles. Published studies using focus groups for health-related outcomes range in sample size of 10-60 participants.[28-31] Research supports a minimum of 15 participants to represent each group (in this case supervisory and non-supervisory staff role).[32] USARIEM protocol #15-04-HC enrolled 7-10 DFAC supervisors and 26-33 non-supervisory DFAC staff at each data collection iteration and reached thematic saturation (no new themes identified during final focus group of each iteration). The researchers therefore requested up to 50 DFAC staff members (combination of supervisory/non-supervisory) from Wolf DFAC and Freeman Café to once again increase likelihood of thematic saturation (maximum sample size of n=50 at focus groups held at pre, mid, and post-G4G 2.0 implementation at Wolf DFAC; pre- and post-G4G 2.0 implementation at Freeman Café; total of n=250 for the study).

A total sample of n=790 was requested to account for both the DFAC diner assessment (n=540) and DFAC staff member focus groups (n=250).

Research Methods

The G4G Program Office at CHAMP served as consultants for G4G 2.0 program implementation along with local DFAC teams and USARIEM along with Pennington Biomedical Research Center (PBRC) staff were responsible for G4G 2.0 program evaluation. The USARIEM research team planned to travel to Fort Carson for three

iterations and Fort Hood at two iterations as noted in Figure 2: pre at 0-month (T-0), mid-launch (T-1), and post-G4G 2.0 intervention (T-2). Noted below are the participant populations (DFAC diners or staff), number of participants, measures and timing of data collection, and the intent for each iteration.

Participants

The plan was to recruit up to 180 DFAC diners at pre- and post-iterations for each site, consent, ask to complete four surveys (lifestyle behaviors, nutrition knowledge, G4G awareness and customer satisfaction), and participate in digital food photography nutrient intake assessments. In addition, we also planned to recruit and consent up to 50 DFAC staff members for five iterations (three at Fort Carson and two at Fort Hood) to complete three surveys (nutrition knowledge and G4G awareness).

Time Point Description

T-0 – Pre-G4G 2.0 Intervention (baseline): This iteration examined DFAC diner and staff outcome measures (noted above) prior to G4G program implementation.

T-1 – Mid (Fort Carson only): This iteration captured staff experiences, challenges, and suggestions to improve G4G 2.0 implementation thus far and to provide leadership with additional insights from the DFAC staff perspective.

T-2 - Post-G4G 2.0 intervention: This iteration examined DFAC diner and staff outcome measures after full G4G 2.0 program implementation.

Go for Green® (G4G) 2.0 Program Intervention

The G4G Program Office at CHAMP worked with Wolf DFAC, Fort Carson and Freeman Café, Fort Hood DFAC staff and supervisors to execute G4G 2.0 planning, implementation, and

sustainment. During the study, the G4G Program Office at CHAMP conducted in-person management training (PR1), assigned color and sodium codes to a 21-day menu with 12 specialty bar options (PR2) to meet G4G 2.0 menu targets (PR3), and printed materials and other marketing strategies (PR7). The team conducted site visits for hands-on assistance, staff training, and course correction and was engaged with the local DFAC team, Registered Dietitian and nutrition assets, and leadership via email and teleconferences.

Research in the emerging field of nutrition implementation science highlights the need to maximize program quality and impact to ensure interventions are effective, achievable, and efficient in practice. To do so, evaluation tools are necessary to provide objective assessment of program compliance to established standards or benchmarks. In the 10-year history of the G4G program, a standardized, comprehensive evaluation instrument with benchmarks has not been used. The G4G Program Office at CHAMP, in collaboration with the Army Public Health Center, developed the first version of the G4G 2.0 PFA tool. The PFA tool evaluates the degree to which DFACs have implemented the eight established PRs.

The validity and reliability of this tool was established during the current G4G 2.0 evaluation effort. During this time, G4G Program Office at CHAMP team tested and refined the PFA tool. First, the team identified actionable tasks to evaluate whether the facility successfully implemented each PR. Examples of PR tasks include: percentage of Green-coded menu items by review of the facility's menu, the number of choice architecture strategies to promote Green-coded options, use of various marketing strategies to market the DFAC and menu, and percentage of DFAC staff trained. Multiple onsite visits by the G4G Program Office at CHAMP team led to iterative revisions that refined the tool for a more accurate assessment of adherence to the desired benchmarks. In particular, the review of serving a nutritious menu (PR 3),

program and facility marketing (PR 7), and DFAC staff training (PR 8) were expanded to better capture tasks critical to implementation efforts.

Lastly, a scoring system was developed to objectively assess PR fidelity with a set benchmark of 75-100%. Consideration was given to establishing a realistic benchmark but allowing room to surpass minimum standards to motivate staff and DFACs to achieve excellence. A 75% benchmark was set for most PRs: menu targets, standardized food cards, food placement strategies, promotion of Green-coded items, and marketing and education. Benchmarks for DFAC facility management training and assigning traffic light codes for menu items were set at 100% because they are essential to program integrity and success. Despite its criticality to program and staff engagement, staff training was set at 80% due to high staff turnover inherent to operating DFACs. Overall, the PFA consisted of 15 scored sections covering the eight PRs for an objective assessment of program compliance. The G4G 2.0 program was considered fully implemented if the DFAC met benchmark standards for all eight PRs. This project evaluated G4G 2.0 program implementation at two Army DFACs to assess the impact of this performance nutrition intervention.

Digital Photography and Diet Quality

Digital photography, a field expedient food photography method that accurately captures food selected and consumed by diners, was used to quantify food selection and intake of study participants (Figure 3). Researchers added the DFAC's recipes to the USDA Food and Nutrient Database for Dietary Studies (FNDDS).[33] Total nutrient intake was defined as the difference between estimations of food amounts in pre- and post-meal photographs reviewed by trained visual estimators. Final nutrient intake estimates were averaged and analyzed using the FNDDS system. Specific participant nutrient intake information was generated. Studies with adults have demonstrated this methodology to be highly reliable and valid.[34,35]

The dietary intake measures included, but were not limited to total calories, fatty acids, carbohydrate, protein, fiber, vitamins, and minerals. The Food Pattern Equivalents Database generated food serving information necessary for assessing food pattern components to calculate the HEI.[36,37] The HEI-2015 is a measure of diet quality as relative adherence to the 2015-2020 Dietary Guidelines for Americans (DGA).[38] The HEI-2015 score is comprised of 13 food components with a maximum of 5-10 points per component with 100 points as the highest potential score. Adequacy of food component scores increase as nutrient intake aligns with federal recommendations, while the moderation component scores increase with lower intake of nutrients to limit (refined grains, saturated fats, added sugars, and sodium).

HEI-2015 can be used to describe adherence to the DGA: overall scores of 90 to 100, or component scores that are 90% to 100% of maximum score: A; overall scores of 80 to 89, or component scores that are 80% to 89% of maximum score: B; overall scores of 70 to 79, or component scores that are 70% to 79% of maximum score: C; overall scores of 60 to 69, or component scores that are 60% to 69% of maximum score: D; and overall scores of 0 to 59, or component scores that are 0% to 59% of maximum score: F. The U.S. National HEI score average over the past ten years has ranged from 48-57 points.[39,40] Previously, Basiotis et al.[41,42] described diets according to the following: HEI scores > 80 indicate a “good” diet, scores ranging from 51 to 80 reflect a diet that “needs improvement,” and HEI scores < 51 imply a “poor” diet. Adequacy of food component scores increase as nutrient intake aligns with federal recommendations, while the moderation component scores increase with lower intake of nutrients to limit (refined grains, saturated fats, added sugars, and sodium). The HEI-2015 total and component scores of participants were assessed as a daily average of three meals (breakfast, lunch, and dinner; examples shown and reported as median ± interquartile score range (IQR).

Paper-Based Surveys

1) Demographics & Lifestyle Survey (Appendix B)

Participant demographic data and lifestyle information was collected in-person via paper survey. Demographic data included: age, height and weight, ethnic and racial background, highest education level, and military rank. Twenty-eight lifestyle questions were included relating food choice to meal timing and location, lifestyle habits and perceptions regarding physical activity, sleep, energy level, and performance. The survey was successfully used for Protocol #15-04-HC and enables future comparison between DFAC studies.[2]

2) Dining Facility Satisfaction (Appendix C)

The diner satisfaction survey examined sensory qualities of food provided and consumed in the DFAC (taste, texture, temperature, and appearance), availability, thoughts on quality and health impact, and usefulness of labels to promote selection of high-performance foods. The survey consisted of 17 items on a 7-point Likert Scale ranging from “Strongly Agree" to “Strongly Disagree”. The survey was successfully used for Protocol #15-04-HC and enables future comparison between DFAC studies.[2]

3) Nutrition Knowledge (Appendix D)

Data collected examined a combination of general and performance-based nutrition knowledge (NK) of diners and DFAC staff. This diner NK survey was created as a subscale to a Military-Specific Eating Behavior Survey (Protocol #16-12-HC)[43] and tailored for this protocol using 40 true/false questions. Of the survey, 18 items were validated as part of the Military-Specific Eating Behavior Survey and consisted of general and performance nutrition questions. The NK questionnaire consisted of true/false questions on performance nutrition - macronutrients, micronutrients, energy, dietary supplements, and hydration and confidence in the response

(yes/no). Nutrition knowledge was compared between diners who were and were not exposed to G4G 2.0 program and those with and without any prior nutrition education. The survey served two purposes: 1) identify the key gaps in nutrition knowledge; and 2) assess if changes in nutrition knowledge impact food choice (used in conjunction with food photography) regardless of the G4G 2.0 program. The DFAC staff at two DFACs completed questionnaires pre- and/or post-G4G 2.0 program implementation on NK and the G4G 2.0 program information. A demographic survey was added after the first study location pre-data collection with a resultant smaller sample size.

4) G4G Awareness for Diner (Appendix E) and Staff (Appendix F)

Specific surveys were created in conjunction with the G4G 2.0 Program Office at CHAMP consultants. The diner survey examined the effectiveness of the G4G 2.0 marketing campaign as well as beliefs and attitudes towards the usefulness of G4G 2.0. The diner survey consisted of 29 items that were a combination of multiple-choice, 5-point rating scales and yes/no questions. The staff survey examined the effectiveness of the G4G 2.0 staff training as well as beliefs and attitudes towards the new program and barriers to G4G 2.0 implementation. This staff survey consisted of 32 items that are a combination of problem solving, multiple-choice, 5-point rating scales and open answer questions. These documents will be incorporated as future program evaluation tools.

5) G4G Staff Demographics (Appendix G)

Participant demographic data information was captured by self-report and included: self-reported sex, age, height, weight, rank, ethnicity, racial background, education, Military Occupational Skill (MOS), and years in the military.

Satiety Labeled Intensity Magnitude (SLIM) Scale (Appendix H)

Research suggests the level of hunger influences the types of food selected.[44-46] The investigators were interested in understanding the relationship between hunger levels pre- and post-meal and the impact on food choice quality and quantity. Results from the USARIEM protocol #15-04-HC explained 54% of the variance in magnitude of satiation (SLIM score change) was due to the initial degree of hunger, protein and carbohydrate intake, whole grain intake, meal length and having enough time to eat.[2]

Hunger and satiety were assessed pre- and post-meal consumption at the time of tray photography. Each participant was asked by a research staff member to view the SLIM scale and identify the level of hunger or satiety that was the closest representation to their current state. The SLIM scale is an 11-item diagram drawn on a 10-cm line with descriptive labels ranging in self-perceived hunger/fullness ranking from greatest imaginable hunger to greatest imaginable fullness. Scoring ranges from -100 points (greatest level of hunger) to +100 points (greatest level of fullness). The change in SLIM scores is calculated as the difference between the pre- and post-SLIM scores (total of 200 points). The SLIM scale was shown to be a sensitive, reliable, and easy-to-use scale for measuring perceived satiety.[47] Perceived satiety was compared to HEI-2015 scores and specific nutrient intake.

Pre-meal SLIM scale also included questions on previous mealtime and snack choice because prior snacks may impact food choices and rate of eating (Appendix H 1). Post-meal SLIM scale asked two questions related to the rate of eating (Appendix H 2). Three additional questions (dependent on time availability) were asked at post-meal photography: (1) “How satisfied are you with your meal selection today?”, (2) “If you could recommend one change in the DFAC, what would it be?”, and (3) If a significant amount of plate waste was noted, “What is the reason for leftover food on your plate?” Responses to these

questions were digitally recorded along with the meal tray. This provided additional insight into diner satisfaction immediately following meal consumption. The SLIM data are analyzed separately and will not be presented in this report.

Plate Cost

Military DFACs receive funds allocated to feed each diner per day (called a "basic daily food allowance") from which to plan and execute meals.[48] Concerns exist regarding potential increased cost of performance-focused menu modifications due to the addition of fresh produce, seafood, and whole grains, as well as conversion to more scratch-made vs ready-to-serve options. Average plate cost of the G4G 2.0 compliant menu used was determined from the total cost of food prepared divided by the number of diners fed.

Focus groups (FG) conducted with DFAC staff and FG data are analyzed separately and will not be presented in this Technical Report.

Statistical Analysis

SPSS version 26[49] was used for statistical analyses. Demographic descriptive data between baseline and post-test were examined using Mann Whitney U or Chi-square analyses as location and time points varied in sample size and enrolled different participants at each time point. A two-step cluster analysis examined HEI scores (pre-intervention and change pre-to-post), by location (Fort Carson vs. Fort Hood), sex, education, and rank variables, to determine cluster effects of non-normal distribution and unequal sample sizes at the two locations. Demographic descriptive data are reported as median and IQR or frequency and percent based on the scale of measurement. Mann-Whitney U analysis compared total HEI-2015 and HEI

component scores between pre- and post-G4G 2.0 implementation stratified by meal and location with data reported as median and IQR. Differences in Green-, Yellow-, or Red-coded foods consumed at each meal were compared by Chi-square analysis. Results are combined between two study locations due to attrition and inadequate numbers of subjects to assess differences by installation. The fidelity of G4G 2.0 implementation was measured by adherence to the G4G 2.0 program requirements benchmarks. The total cost of food prepared divided by the number of diners fed determined the average plate cost of the G4G 2.0 menu used in this study. Nutrition knowledge assessment consisted of analysis on the subjects' percentage of correct responses and the NK responses were re-categorized into four response outcomes: confident and correct, confident but incorrect, unconfident and correct, or unconfident and incorrect. Recategorized values were summed for a total NK score ranging from 1-54 points. The NK data were analyzed for descriptive statistics and are reported as mean ± standard deviation (SD) or frequency (%). Wilcoxin rank sum test was used to compare total NK score and each NK question between staff with or without G4G training. Response percentages were reported as either confidently correct or confidently incorrect.

RESULTS

Subject Demographics

Of the 282 total enrolled participants, 100 participants consumed at least one breakfast, lunch, and dinner meal (n=63 pre- and n=37 post-G4G 2.0 implementation) to meet criteria for data analysis inclusion. The majority (92%) of participants were male and young (median±IQR: 22±4.0 years). Demographic data depicted that the majority were junior ranking personnel, had a healthy Body Mass Index, were physically active, and had physical readiness ratings of "good" to "best shape" (Table 2). The only significant difference was a greater proportion of senior

enlisted personnel participated during post-G4G 2.0 implementation compared to pre-G4G 2.0 (14% vs. 2%; p=0.02).

G4G 2.0 Program Fidelity

The implementation process resulted in the development and refinement of content and benchmarks to objectively assess program fidelity by using the first G4G Program Fidelity Assessment (PFA) tool (Appendix I). Assessment of G4G 2.0 program fidelity identified the extent of adherence to eight program requirements (Table 3.). After a phased implementation, Wolf DFAC successfully met the benchmarks for 8 of 15 PFA scored sections. The PFA was conducted at the Freeman Café G4G 2.0 grand opening and again three months later. Although the time between the PFAs was short, the initial (grand opening) PFA followed a period of close support and guidance from the G4G Program Office at CHAMP. Per the initial PFA, the DFAC successfully met the benchmarks for 12 of 15 scored sections. The second PFA indicated the DFAC met benchmarks in 10 of 15 scored sections.

Overall, the DFACs were successful in meeting the following PR benchmarks after G4G 2.0 implementation: standardized management training, assignment of traffic light codes, menu targets with a minimum of Green-coded items, standardized food cards, food placement strategies, and staff training. Specifically, the updated G4G 2.0 DFAC menus increased availability of high-performance choices. Prior to G4G 2.0, the DFAC menu was 5% Green (breakfast) and 43% Green (lunch/dinner) compared to post-G4G 2.0 implementation, which transitioned to 41% Green (breakfast) and 61% Green (lunch/dinner). Adherence was below benchmarks for requirements related to marketing of the overall G4G 2.0 program and certain strategies to promote Green-coded items. Overall, G4G 2.0 was implemented such that the evaluation of program effectiveness is valid and relevant. Extent of program adherence at the two study sites are highlighted in Table 3.

Meal Quality

Improved menus translated into higher availability of high-performance foods and more opportunity for diners to select them. This critical component of the G4G 2.0 intervention resulted in a significant improvement of overall meal quality with HEI-2015 scores increasing from 55.0±12.8 at baseline to 64.1±12.0 post-G4G 2.0 implementation (p<0.001). Although sample size at each location was not large enough to warrant comparative analysis between locations, cluster analysis identified that Fort Carson was most predictive of the change in HEI score (1.00; 100% Fort Carson contributing to pre-HEI score at 55.5 vs post-HEI score at 64.1 points) followed by pre-post intervention (0.82; 70% of Fort Carson had a pre-HEI at 58.5 points). Figure 4 illustrates the median changes in 13 HEI component scores from baseline to post-G4G 2.0 implementation. Whole grains (+3.0 points, p<0.001) and seafood and plant proteins (+3.5 points, p<0.01) increased significantly whereas intake of refined grains decreased (1.49 points, p<0.001). Some examples of meals with high and low HEI-15 scores are shown in Figure 5. After G4G 2.0 implementation, significantly more Green-coded (+8%, p<0.05) and fewer Red-coded (-7%, p<0.05) options were selected overall (Figure 6). More Green options were selected for lunch (+8%, p<0.05) and dinner (+13%, p<0.05) and Red item selections decreased (-10%, p<0.05). At breakfast, a significant decrease in Red-coded items was noted (-5%, p<0.05), although no change in choice of Green-coded items was observed (27% Green-coded items selected).

Diner Satisfaction

Significantly more diners agreed that the main dishes served were healthy and performance-based and appreciated the availability of vegetarian food choices post-G4G 2.0 implementation (Table 4). Diners reported that food labeling enabled performance-based choices. No significant

differences were noted for other survey components pre- to post-G4G 2.0.

Plate Cost

The average plate cost for the G4G 2.0 menu was $13.11 per day. This value was based on two weeks of consolidated data when the G4G Program Office team at CHAMP was confident that the planned menu reflected the food costs actually served. This is approximately 11% higher than the established basic daily food allowance of $11.78 per day.[48]

Nutrition Knowledge of Diners

Nutrition knowledge (NK) survey was completed by 269 diners at the two DFACs pre- and post-G4G 2.0 program implementation (Table 5). Nutrition knowledge was compared between diners who were and were not exposed to G4G 2.0 program (n=166) and on prior nutrition education, with the majority (n=201, 75%) having no prior nutrition education. The nutrition knowledge subgroup from the 18-item questionnaire is shown in Table 6. The demographic and lifestyle variables (BMI, sleep and physical activity) were similar between diners with or without G4G 2.0 exposure or nutrition education.

The overall NK scores were similar between G4G 2.0 program exposure groups: 59.8±16% not exposed vs. 59.9±16% exposed (p=0.75). The questions were further categorized into four topics: macronutrients, vitamins and minerals, energy, and hydration. Of the four topics, only macronutrients yielded a significant difference in responses between the groups: 11±3 for G4G 2.0-exposed vs. 10±0 for not exposed (score - range 0-18 points; p=0.043). A greater proportion of G4G-exposed diners were correct and confident regarding the difference in nutrient source between whole, 2%, and 1% milk (22.3% vs. 15.5%, p=0.046). When grouped by nutrition education, a greater proportion of diners with at least one hour of nutrition education were

confident and correct in their responses for the following two NK questions compared to diners without nutrition education: “At least half of the food on their plate should be fruits and vegetable” (53.7% vs. 67.6%; *p*=0.029) and “Dark green vegetables, eggs and fortified cereal are sources of dietary iron sources” (42.8% vs. 66.2%; *p*=0.001) (Figures 7-9).

When examining NK (Figure 10) of the overall sample of diners (n=269), results were concerning for the proportion of confidently incorrect for the questions related to performance nutrition basics. The questions included:: “A recovery drink or snack should always be consumed after exercise” (65.4%); “Protein is an important source of energy for physical activity” (46.5%); “Most military personnel require four times more protein than civilians” (32.7%); “Sports beverage are a preferred beverage when exercising at moderate intensity” (25.7%); “Dietary fats are unimportant for a balanced diet” (20.4%); “Meat is a source of fiber” (24.5%); and “Vitamins and minerals are a source of energy (25.3%).” Only 17% of diners self-reported being extremely/very knowledgeable about their overall nutrition knowledge. The top five sources of nutrition information diners identified as “helpful” were nutrition websites (41%), nutrition health professionals (39%), gym and fitness personnel (38%), fellow Soldiers (33%), and social media (31%).

Nutrition Knowledge of Foodservice Staff

Foodservice staff (n=184) at the two DFACs completed questionnaires pre- and/or post-G4G 2.0 program implementation on NK and G4G program information. Study participants were primarily active-duty military (73%), non-supervisory (73%; 72% of which were junior enlisted), male (60%), and aged (mean ± SD) 25.7±8 year (Table 7). Only 25% of staff completed 50% (4 of 8 modules) of the G4G 2.0 training and 2.7% completed 100% (8 of 8 modules) of the training (Figure 11). The percent of NK questions correctly answered was 55.0±18% with a mean score of 29.2 ± 9 points (NK scores ranged 0-54); however, the percent correct was significantly

higher among trained G4G 2.0 staff compared to those without G4G 2.0 training (58.0±19% vs. 52.9±17%, p=0.025). A greater proportion of G4G 2.0-trained staff were confident and correct in their responses compared to those without training for questions on (1) protein requirements for military (38% vs. 21%, p=0.013) and (2) identifying good sources of iron (62% vs. 41%, p=0.006) (Figure 12).

Although there were no other significant differences between NK responses for G4G-trained and untrained staff, over 25% of staff were confidently incorrect on the ten NK areas (Figure 13): need for a recovery beverage or snack post exercise (72.8%), protein as a source of energy (64.7%), dietary supplement purity and safety (57%), nutrient source between whole, 2%, and 1% milk (42.4%), protein needs of military personnel (41.8%), recommendation for sports drinks after moderate intensity exercise (39.7%), vitamins and mineral as sources of calories (34.8%), meat as a source of fiber (34.2%), meeting dietary vitamin and mineral needs (33.7%) and good food sources of calcium (27.7%). Regardless of the level of G4G 2.0 training or NK, over 85% of staff acknowledged the importance of correct food labeling to maintain diner trust in the G4G 2.0 program.

DISCUSSION

This study evaluated the revised G4G 2.0 program as an intervention to improve Soldier meal quality and diner satisfaction at two Army DFACs. Our results indicate that diner meal quality can be enhanced without compromising satisfaction. Importantly, offering and supporting a high-quality nutrition program implementation is critical to optimizing performance-based foods in DFACs. Although the two DFACs met the benchmarks for most of the PRs, it is possible that if a greater proportion of PRs benchmark were met, meal quality may have improved to an even greater extent. This will require further research.

Although this study is the first G4G 2.0 program evaluation, our findings are consistent with other military DFAC interventions that employed similar elements. In a cross-sectional study, Crombie et al. observed lower intake of energy, fat, discretionary fat, and refined grains along with improved satisfaction in diners at 6 and 12 months after the menu changes, traffic light labels, and presence of educational materials compared to control DFACs.[8] Similarly, a performance nutrition DFAC intervention evaluation by Cole et al. reported an 11-point improvement in HEI-2010 after four months, which was sustained at 12 months compared to the control DFAC.[2] In a military eating environment multiple factors influence food choices and decision-making processes. Ghoniem et al. reported that food availability must be considered in combination with intrinsic motivational factors, such as desire for healthful food and previous learning experiences, as these factors interact to promote choice.[50] The G4G 2.0 program incorporated additional interventional strategies to address these complexities, but it is uncertain as to whether these strategies are sufficient.

Program Fidelity Assessment

The PFA is an objective tool to determine the success of DFACs in implementing the G4G 2.0 program. Evaluating G4G 2.0 implementation at both DFACs using the PFA revealed compliance strengths as well areas for improvement. Scores ranged from 0-100% highlighting the wide range of barriers and facilitators to program implementation success. Of note, the G4G Program Office at CHAMP team provided staff and management training (PR 1, PR 8), food and beverage coding (PR 2), a menu with at least 30% Green-coded items (PR 3), and printed marketing materials (PR 7), all of which facilitated 100% compliance on these PR subsections at both DFACs.

Areas where DFACs did not meet the benchmarks were menu targets and recipe fidelity (PR 3), promotion of Green-coded items (PR 6), and marketing and education (PR 7). A detailed review of the G4G 2.0 implementation data revealed specific challenges with program implementation:

current DFAC infrastructure (physical layout of food stations), limited Green-coded food placement, lack of Green-coded items, and limited promotion of such foods. The foodservice information management system prevented efficient loading and updating of standardized recipes, and frequent staff turnover challenged training efforts. The SMEs used results to focus resource creation, training, and support.

Evaluation of nutrition intervention implementations, such as G4G 2.0, can inform successes, challenges, and areas for improvement. Knowing the degree of program fidelity is critical for overall program execution, improvement, and potential impact on the intended audience of SMs eating in DFACs.

Meal Quality

The G4G 2.0 program facilitated an increase in diner selection and consumption of Green-coded foods with a decrease in consumption of Red-coded foods. The breakfast menu was particularly challenging to update given the nature of traditional breakfast food items with the lowest percentage of Green-coded items (41% vs 61% for lunch and dinner). Increasing nutrient-dense ingredients in DFAC recipes (whole grains, vegetables, beans, fish) translated to increased diner access to more nutritious Green-coded foods. Improvement in meal HEI score was primarily attributed to significant increases in participant selection of Green-coded whole grains, seafood, and plant protein along with a reduced selection of Red-coded refined grains. These results are consistent with those found in the civilian setting. Chen et al. also transformed food choices in a buffet-style cafeteria, successfully increasing diner selection of Green and Yellow-coded foods, with a resultant decrease of Red-coded foods.[23]

Diner Satisfaction

Similar to past military DFAC interventions, the G4G 2.0 program was well-received [8] [2] with diner satisfaction related to the availability of nutritious and performance-based main dishes,

availability of vegetarian options, and the presence of color-coded labels to help select performance-based foods. Maintaining diner satisfaction is crucial to DFAC success as preserving “diner headcount” (number of diners served per meal) can be a leadership concern with “healthy menu” initiatives. Balancing the desire to provide performance-focused menus built from nutrient-dense foods while serving familiar “comfort” foods is challenging yet essential to the success of any military nutrition intervention.

Plate Cost

Each DFAC receives funds allocated to feed each diner per day (“basic daily food allowance”) from which they plan and execute meals.[48] A performance-focused menu raises concerns that incorporating fresh produce, seafood, and whole grains will likely exceed food costs. Similar food cost anecdotal remarks from leaders were observed when planning a menu conversion from ready-to-serve options to scratch-made recipes. Modest increases in the average plate cost of the G4G 2.0 menu may influence the perception of program feasibility by higher-level decision-makers, despite improved meal quality and favorable diner satisfaction results. In the current study, food cost increases appeared attributable to large variations in daily projected diner headcount, which disrupted accurate forecasting and production. A transient increase in plate cost was expected with the initial G4G 2.0 menu implementation due to significant menu changes. Future research is needed to assess the plate cost changes with improvements in meal forecasting and reduction ins food waste reduced.

Nutrition Knowledge

<u>Diners’ Nutrition Knowledge</u>

This study highlights the widespread suboptimal nutrition knowledge (<60% correct responses) among warfighters on general and performance nutrition despite G4G program exposure and previous nutrition education. The G4G 2.0 program is designed to translate nutritional guidance

into easily identifiable high-performance menu options; however, it does not target education on the NK deficit areas. The reported sources for NK may contribute to the high proportion of confidently incorrect responses. Poor NK could be one of the many factors hindering improvement in overall meal selection.

DFAC Staff Nutrition Knowledge

Regardless of G4G 2.0 training completion or type of G4G training sessions attended, the DFAC staff scored poorly on the NK test. Foodservice staff can be a force multiplier when implementing a nutrition intervention as their efforts may determine the quality of food service outcome. Foodservice staff with a poor understanding of nutrition concepts will likely be challenged to implement a nutritious menu comprised of nutrient rich recipes using healthy food preparation practices. Innovative interactive strategies are needed to assess, educate, and empower foodservice staff at fast-paced military DFAC operations.

Strengths and Limitations

The greatest strength of this study was a closely engaged G4G Program Office team at CHAMP, who were involved with study site program implementation, including designing the study menu (led by collaborators at the Armed Forces Recipe Service), printing marketing materials, assembling color-coded food cards, and leading in-person training to DFAC staff and management. The implementation team was not involved with survey or meal quality data collection. Other strengths included program components that emphasized user-friendly traffic light labels, educational materials, and food placement for quick and easy selection of nutritious offerings. Additionally, digital food photography served the dual purpose of participant burden reduction with data collection and provision of accurate insight into diner food consumption. Given the fixed price per diner, there was no ability to track sales of specific items offered or to

assess food selection changes. Digital photography of food consumed is a much stronger reflection of changes in actual food intake than sales data (citation from food photography section).

Despite the strengths, several limitations were identified. In relation to the study design, a control DFAC was not available as a reference for G4G 2.0 implementation comparison. The current study gathered data at only two time points (pre- and post-G4G 2.0 implementation) over a shorter period compared to other military DFAC studies.[2,8] Use of different participants at pre- and post-G4G 2.0 implementation precluded the ability to assess within and between subject changes over time. Participant attrition in the current study was likely due to factors out of the participants' control. Modifiable personal preferences to attain meals elsewhere may influence dining location despite the voluntary commitment to the study. In addition, although SM may routinely eat at the study DFAC, changes in training missions, time and travel constraints, and unexpected additional duties may divert them away from dining at the study DFAC. These factors are anecdotally observed in military studies, are understandable given mission priority, but nonetheless challenging to researchers.

Regarding program fidelity, uncontrollable factors such as frequent turnover of DFAC management resulted in challenges with adequate training and marketing, and inaccurate preparation of menu items. Lessons learned from the current intervention will inform future studies seeking to improve diner access to tasty, appealing high-performance food choices. There is a need for on-going, well-designed research to evaluate nutrition program implementation and its impact as well as to better understand diners' food-related behaviors. Additional, increasing the leadership's awareness of optimal nutrition and how they can support soldier fueling, may reduce program barriers impeding full program implementation.

CONCLUSIONS AND IMPLICATIONS

G4G 2.0 is a comprehensive DFAC nutrition intervention aimed to offer tasty high-performance (Green-coded) menu items to optimize nutritional fueling. Despite system-wide related barriers, the revised G4G 2.0 program's multifaceted approach yielded positive results in meal quality and diner satisfaction at two fast-paced DFACs. Through this study, a much-needed mechanism to assess program fidelity was developed and iteratively refined.

G4G 2.0 successfully improved meal quality by nine points through significant improvements in selection of whole grains, seafood and plant proteins, fewer refined grains, and improved trends for other food groups. Menu revisions built on updated recipes (more whole grains, vegetables, and seasonings beyond salt) increased the availability of Green-coded foods and reduced the number of Red-coded foods successfully translating to significantly increased diner selection of Green-coded and fewer Red-coded food choices.

The improvement in meal quality occurred without detrimental impact on diner satisfaction and only a modest increase in plate cost. Some aspects of satisfaction improved which infers high acceptability of performance-focused, nutrient-dense menu items. Challenges observed in program implementation are not unique to the military settings. Overcoming these challenges could further enhance program implementation by positively impacting availability, access, and knowledge of performance-boosting foods.

Nutrition knowledge of DFAC foodservice staff and diners may impact the quality of foods served and actual meals consumed. Innovative interactive strategies are needed to educate and empower foodservice staff at fast-paced military DFAC operations. Enhanced NK can enable DFAC staff as stewards of nutrition programs, helping to translate program requirements into actual foods served. Pre-crafted G4G messaging through social media and the G4G website may increase SM awareness about performance-focused food choices. In addition, standardized nutrition education for SM at fitness centers, health clinics, and by nutrition

professionals embedded within the units, can provide the foundational knowledge needed to make better food choices.

RECOMMENDATIONS

Future research should continue to evaluate facilitators and barriers to availability, access, cost, and consumption of performance-based food options, and outcomes related to health and performance. Several recommendations are proposed:

1. Invest in nutrition programs implemented within military dining facilities as they provide crucial opportunities for pairing high-performance fueling options with education on nutritional fitness and mission readiness.
2. Develop standardized education materials (class, infographics, messaging, etc.) that increases the nutrition knowledge of DFAC diners as well as the DFAC foodservice staff. Arming SMs and leaders with credible nutrition information can influence optimal nutrition-related decisions even in an environment with limited choices. Innovative interactive strategies are needed to assess, educate, and empower DFAC staff at fast-paced operations.
3. Further develop a G4G 2.0 program communication plan to increase awareness that the G4G program is an evidenced-based best practice program. Upon completion of this study, the G4G Program Office at CHAMP prioritized communication of program resources and support. They launched a quarterly G4G Newsletter, including a spotlighted success story, targeted at G4G program operators and advocates. The G4G Team developed a focused social media schedule on the G4G Facebook page to target resources, news, success stories, and program updates for program operators. The G4G Program Office at CHAMP posted material created for the study site DFACs (templates, marketing materials, toolkits) on the public-facing website for DoD G4G

program operators and advocates (https://www.hprc-online.org/nutrition/go-green). Efforts are underway to close the gaps in resources and further develop the G4G website.

4. Conduct a new G4G study protocol (IRB approved by USUHS) in which several sites focus on 2-3 targeted G4G PR areas found deficient from this study. Program components such as menu enhancements, staff training, marketing, social media promotion are self-identified areas of interest by local facilities. The study should target key areas such as increasing menu and serving options (plant-forward, station style feeding operations) and non-traditional dining venues (snack bars, fueling stations, dining in afloat facilities).
5. Support G4G 2.0 required 5-year cycle program updates.

Evidence from this research study, the current targeted research studies, along with input from nutrition and foodservice experts across the DoD will contribute to programmatic changes. Future program implementation could involve more customized options for PRs, as this research showed not all PRs need to be implemented completely for diner impact. High-quality nutrition interventions in DFACs have the potential to impact SM nutritional fitness and mission readiness by improving the availability, access, and knowledge of performance-focused choices.

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Table 1. Evolution of the Go for Green® Program

Program Requirement (PR)	Original G4G (2008)	G4G 1.5 (2015)	G4G 2.0 (Current)
PR 1: Standardized Training for Management	—	√	√
PR 3: Assign Traffic Light Color Codes Based only on Approved Coding Algorithm	√	√	√
• Coding by only designated professionals	—	√	√
PR3: Menu Targets: Minimum Green-Coded Items	—	√	√
PR 4: Standardized Food Cards	√	√	√
PR 5: Food Placement Strategies	—	√	√
PR 6: Promotion of Green-coded Items	—	—	√
PR 7: Marketing and Education			
• Printed materials	√	√	√
• Social media, other media & press	—	—	√
• Nutrition education for diners	—	—	√
PR 8: Standardized Training for all Staff			
• Initial	—	√	√
• Ongoing	—	—	√

PR = Program Requirement; G4G = Go for Green.

Table 2. Demographic and lifestyle characteristics of active-duty military personnel consuming meals at two large military dining facilities pre- and post-G4G 2.0 program implementation (n=100)

Variables[a]	Pre G4G[b] (n=63)	Post G4G (n=37)	*P*-value
Age, y (median±IQR[c])	22.0±4	22.0±4.5	0.64
Sex			0.98
Male, n (%)	58 (92.1)	34 (91.9)	
Female, n (%)	5 (7.9)	3 (8.1)	
Race, n (%)			0.08
White	39 (61.9)	29 (78.4)	
Black / African American	14 (22.2)	5 (13.5)	
Native American/Alaskan Native/Hawaiian	2 (3.2)	1 (2.7)	
Asian	4 (6.3)	2 (5.4)	
Other	4 (6.3)	0 (0)	
Ethnicity, n (%)			0.47
Hispanic	10 (15.9)	8 (21.6)	
Not Hispanic	53 (84.1)	29 (78.4)	
Education, n (%)			0.80
Some High School/High School	38 (60.3)	23 (62.2)	
Post High School/Associates Degree	22 (34.9)	13 (35.1)	
Bachelors/Graduate Degree	3 (4.8)	1 (2.7)	
Military Rank, n (%)[d]			**0.02**
E1-E4 (Junior Enlisted)	62 (98.4)	32 (86.5)	
E5-E7 (Senior Enlisted)	1 (1.6)	5 (13.5)	
BMI, kg/m^2 (median±IQR)[e]	25.4±5.5	25.4±4.4	0.66
Physical Readiness Rating, n (%)			0.74
Good-Best Shape	48 (76.2)	27 (73)	
Neither Good Nor Bad	13 (20.6)	9 (24.3)	
Not Good- Worst Shape	2 (3.2)	1 (2.7)	
Daily Vigorous activity, hours (median±IQR)	1.5±1.0	1.0±1.0	0.63
Daily Moderate activity (hours) (median±IQR)	1.0±1.3	1.0±1.0	0.38
Daily Walking (hours) (median±IQR)	1.0±2.5	1.5±3.5	0.56
Total Sitting/week (hours) (median±IQR)	4.0±4.0	5.0±3.3	0.62
Army Physical Fitness Test Score (median±IQR)	249±64	244±55	0.89
Sleep hours (median±IQR)	6.5±2.0	6.0±1.5	0.44

[a] Self-reported data; [b]G4G =Go for Green® intervention; [c] IQR= interquartile range

[d] Rank, E=Enlisted. E1-E4 = Private, Private First Class, and Specialist; E5-E7 = Sergeant, Staff Sergeant, Sergeant First Class.

[e] BMI = Body Mass Index, estimated from self-reported height and body weight.

Table 3: G4G 2.0 Program Requirements with Benchmarks & Results from Two Sites

PR # and Description	Benchmark %	Site 1 %	Site 2A %	Site 2B %
PR1: Standardized Training for Management	100	100	100	100
PR2: Assign Traffic Light Color Codes	100	100	100	100
PR3: Menu Targets	75	66	99	81
• PR3.1: Minimum Green-coded items	100	100	100	100
• PR3.2: Planned vs. Served Menu	75	98	96	61
• PR3.3: Recipe Fidelity	75	0	100	NA[a]
PR4: Standardized Food Cards	75	89	97	91
PR5: Food Placement Strategies	75	75	93	97
PR6: Promotion of Green-coded Items	75	67	50	44
PR7: Marketing and Education	75	37	45	42
• PR7.1: Print Materials	75	100	100	100
• PR7.2: Social Media	75	0	0	0
• PR7.3: G4G Grand Opening	75	70	80	80
• PR7.4: Post-Grand Opening	75	14	NA[b]	29
• PR7.5: Nutrition Education	75	0	0	0
PR8: Standardized Training for all Staff	80	84	66	66
• PR8.1: Led by Certified Staff Trainer	100	100	0	0
• PR8.2: Standardized Slide Deck Training	80	68	99	99
• PR8.3: Hands-on Training	80	NA[c]	100	100

PR: Program Requirement

Site 1: Wolf DFAC

Site 2: Freeman Café; 2A= Grand opening; 2B= After 3 months of G4G implementation

Green highlighted= Compliance to PR (areas to sustain)

Red highlighted = Noncompliance to PR (areas to improve upon)

Table 4. Perceived changes in components of meal satisfaction and point-of-service nutrition labeling reported by participants consuming meals at two large military dining facilities pre and post-G4G program implementation (n=100)

17-Item Patron Satisfaction Survey[a]	Pre G4G[b] (n=63) Agreements		Post G4G (n=37) Agreements		Pre-Post *P*-value
	n	%	n	%	
1. Appearance of the food is pleasing	36	58	18	49	0.49
2. Flavor and taste of the food is good	33	52	16	43	0.67
3. Choices available are adequate	27	44	18	49	0.33
4. Availability of healthy foods is adequate	33	52	25	68	0.07
5. Availability of performance foods is adequate	31	49	17	46	0.79
6. Portion sizes are appropriate	29	47	22	60	0.14
7. Availability of fresh fruit is adequate	45	71	23	62	0.51
8. Salad bar offers a variety of fresh vegetables	32	51	22	60	0.38
9. Main dishes served: healthy & performance-based	20	32	21	57	**<0.01**
10. The side dishes are served without added fat	27	43	16	43	0.43
11. Healthy/performance-based dessert are available	15	24	11	30	0.07
12. Temperature of hot and cold foods is just right	32	51	19	51	0.71
13. Vegetarian food choices are available	16	25	14	38	**0.02**
14. DFAC[c] nutrition labels easy to use	30	48	23	62	0.19
15. Nutrition labels enables performance-based choices	30	48	24	65	0.09
16. Use DFAC nutrition labels to choose healthy foods	26	41	20	54	0.05
17. Use nutrition labels to choose performance foods	24	38	20	54	**0.04**

[a] Self-reported data; [b]G4G = Go for Green® intervention; [c] DFAC = Dining Facility

Table 5. Demographic and lifestyle characteristics of DFAC patrons who completed nutrition knowledge survey pre- and post-Go for Green® 2.0 program implementation

Variables[a]	Patron (n=269) n(%)
Age, y (mean ± SD[c])	22.41±3.3
Sex	
Male, n (%)	216 (80.3)
Female, n (%)	53(19.7)
Race, n (%)	
White	161(60.1)
Black / African American	71 (26.5)
Native American/Alaskan Native/Hawaiian	7 (2.6)
Asian	14 (5.2)
Native Hawaiian/Pacific Islander	1 (0.4)
Other	14 (5.2)
Ethnicity, n (%)	
Hispanic	73 (27.1)
Not Hispanic	196 (72.9)
Education, n (%)	
Some High School/High School	164 (61.0)
Post High School/Associates Degree	95 (35.3)
Bachelors/Graduate Degree	10 (3.7)
Years of Service	2.4 ± 2.8
Military Rank, n (%)[d]	
E1-E4 (Junior Enlisted)	257 (95.5)
E5-E9 (Senior Enlisted)	12 (4.5)
Warrant Officer	-

BMI (Adjusted), kg/m^2 (mean ± SD)[e]	25.3±3.1
Physical Readiness Rating, n (%)	
Good-Best Shape	180 (66.9)
Neither Good Nor Bad	79 (29.4)
Not Good- Worst Shape	8 (2.9)
Daily Vigorous physical activity, hours (mean ± SD)	1.5±1.0
Daily Moderate physical activity (hours) (mean ± SD)	1.4±1.4
Army Physical Fitness Test Score	243±43
Sleep hours (average)	6.3±1.5

[a] Self-reported data

[b] G4G = Go for Green intervention

[c] SD = standard deviation

[d] Rank, E = Enlisted; E1-E4 = Private, Private First Class, and Specialist; E5-E7 = Sergeant, Staff Sergeant, Sergeant First Class

[e] BMI = Body Mass Index, estimated from self-reported height and body weight

Table 6: Nutrition Knowledge Subgroups from the 18-Item Nutrition Knowledge Questionnaire

Macronutrients	
	• Most plants, fish, nuts and seeds are sources of unsaturated fats. • Whole milk is a better source of protein than 2% or skim milk . • Dietary fat is not considered an important part of a balanced diet. • At least half of the food on your plate should be fruits and vegetables. • Most military personnel require about four times more protein than civilians. • Meat is a good source of fiber**.**
Vitamins & Minerals	
	• Dietary supplements are regulated by the government for purity (cleanliness) and safety before sale. • Good sources of calcium include bread, steak, and corn. • Vitamins and minerals are sources of calories. • Iron is found in dark green vegetables, eggs, and fortified cereal. • As long as enough calories are consumed, vitamin and mineral needs of military personnel are met.
Energy	
	• As long as I am physically active and not overweight, I can eat whatever I want and be healthy. • A recovery beverage or snack should always be consumed after exercise. • Protein is the most important source of energy (calories) for physical activity. • Carbohydrates are the main fuel for mental performance. • Body fat is an important source of energy at rest and during long-duration exercise.
Hydration	
	• Replacing lost body weight from an exercise session with fluid is important. • Sports drinks are always the preferred beverage when exercising at moderate intensity.

Table 7. Demographic characteristics of food service staff from two large military dining facilities based on level of exposure to the performance nutrition programs (n=184)

Variables[a]	Staff[b] (n=184) n(%)
Age, y (mean ± SD[c])	25.67±8
Supervisor	38 (27)
Non-supervisor	102 (72)
Sex	
Male, n (%)	109 (60)
Female, n (%)	73 (40)
Education, n (%) (n=135)	
Some High School/High School	70(52)
Post High School/Associates Degree	57 (42)
Bachelors/Graduate Degree	8 (6)
Military Rank, n (%)[d] (n=134)	
Officer/Warrant Officer	2 (2)
Enlisted	132 (99)
E1-E4 (Junior Enlisted)	97 (72)
E5-E9 (Senior Enlisted)	35 (26)
Time Working at Dining Facility	
Less than 1 year	84 (46)
1 year but less than 3 years	78 (42)
3 or more years	22 (12)
Attended staff training session on G4G in last 6 months	77(42)

[a] Self-reported data; [b] Civilian and active duty military personnel; [c] SD = standard deviation
[d] Rank, E = Enlisted; E1-E4 = Private, Private First Class, and Specialist; E5-E9 = Sergeant, Staff Sergeant, Sergeant First Class, Master Sergeant, Sergeant Major

Figure 1. G4G 2.0 standardized traffic light labeling criteria and logos



Symbols: Checkmark = Green, caution sign = Yellow, stop sign = Red

Figure 2. G4G Study Implementation and Timeline
Note: T-1 occurred at only 1 site

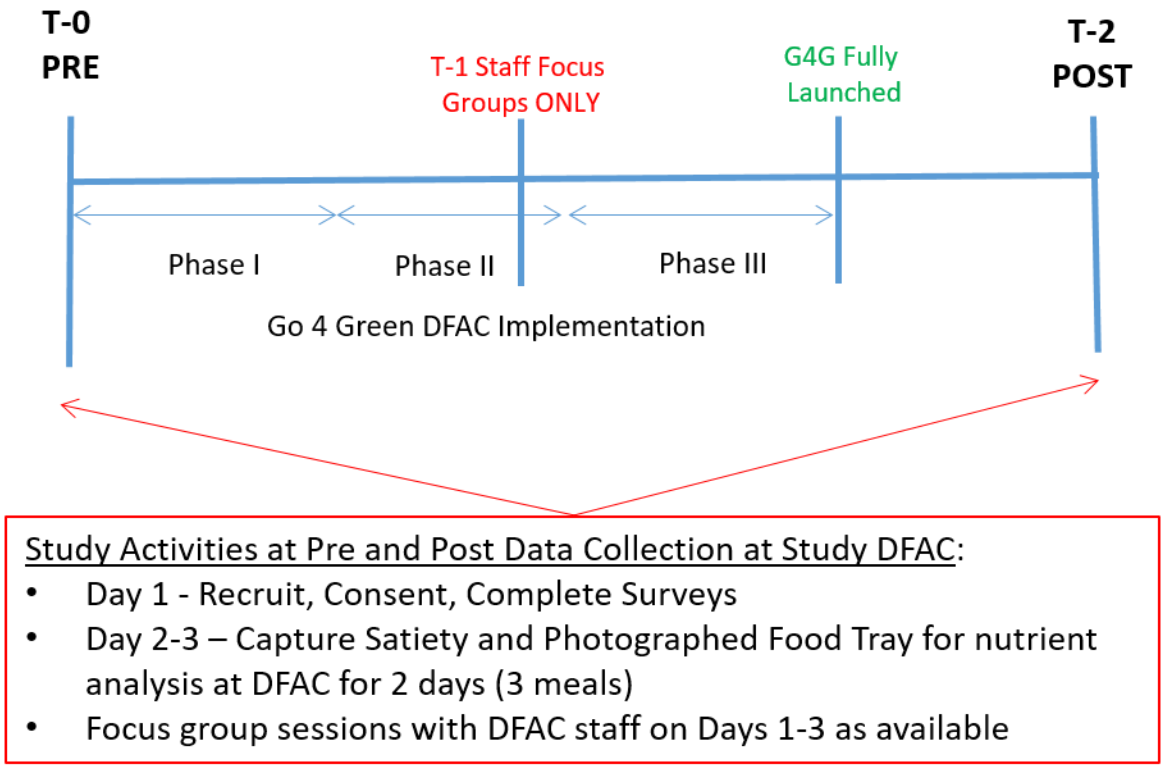


Figure 3. Digital Food Photography Station Example



Figure 4. Healthy Eating Index (HEI-2015) component food group and total median scores among participants consuming meals at two large military dining facilities pre- and post-Go for Green® (G4G) program implementation (n=100)

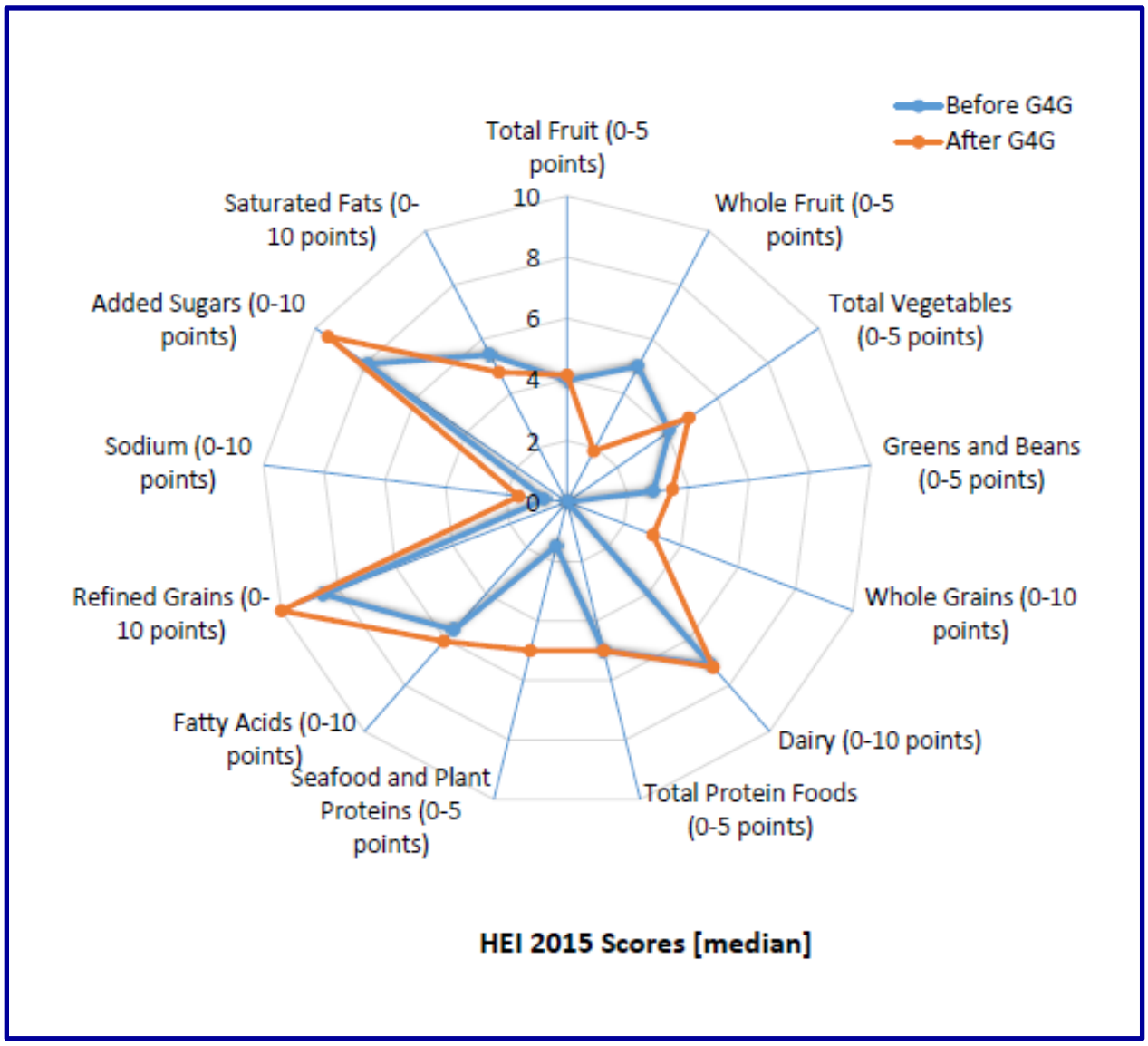
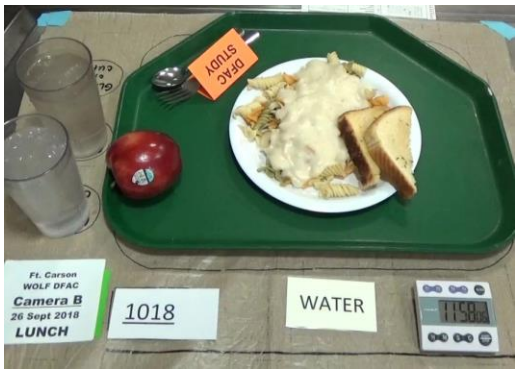


Figure 5. Examples of meals with high and low HEI-2015 scores

Meal	High HEI	Low HEI
Breakfast	 HEI =86	 HEI =25
Lunch	 HEI =88	 HEI =33
Dinner	 HEI =84	 HEI =37

Note: HEI scores calculated based on amount consumed vs. selected.

Figure 6. Overall meal and per meal changes in Green, Yellow, and Red food items of participants consuming meals at two large military dining facilities pre- and post-Go for Green® 2.0 program implementation (n=100)



Figure 7. Nutrition knowledge levels (%) reported by military dining facility diners (n=269)

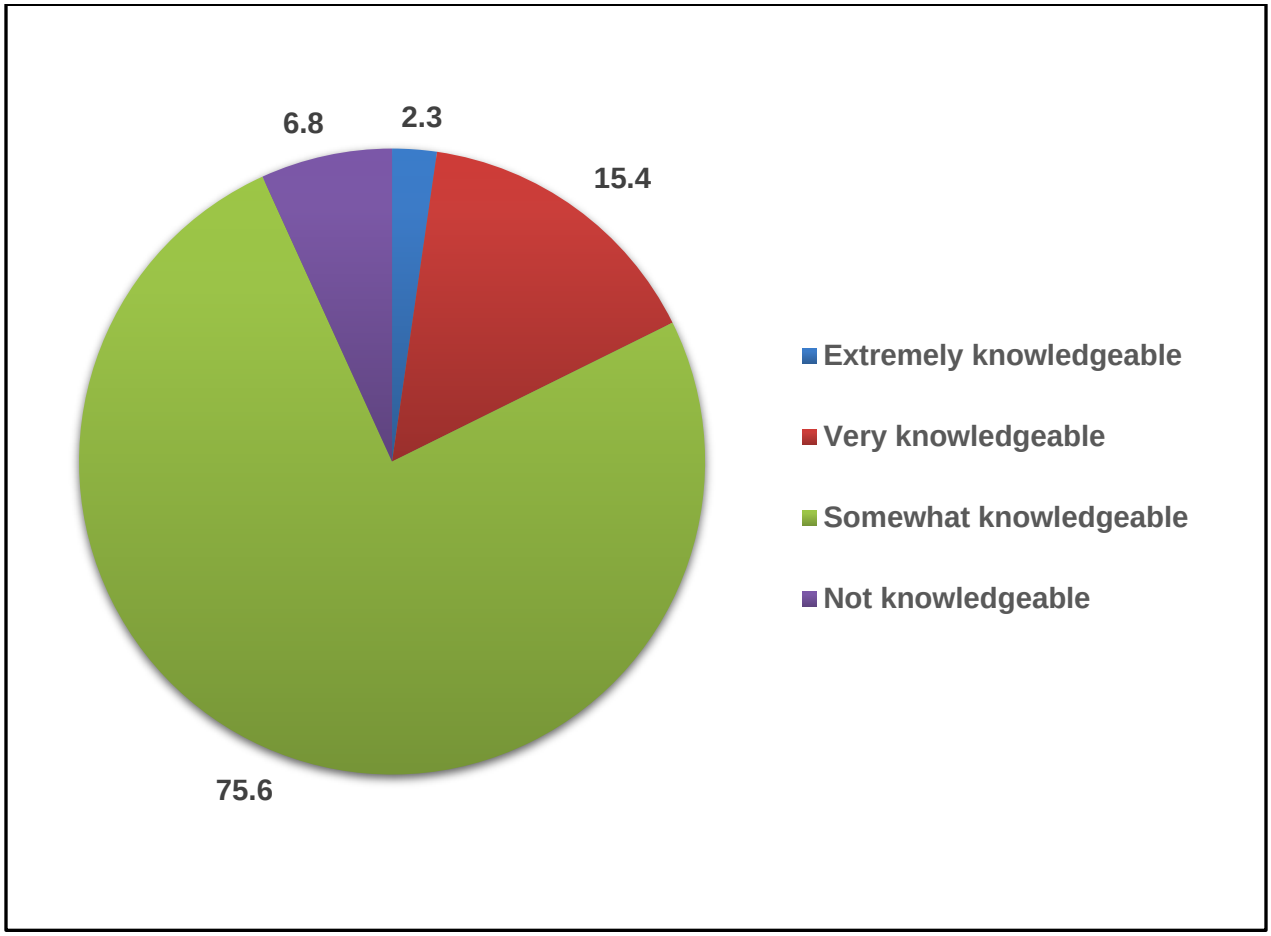


Figure 8. Extent of military/civilian nutrition education received by military dining facility diners (n=269)

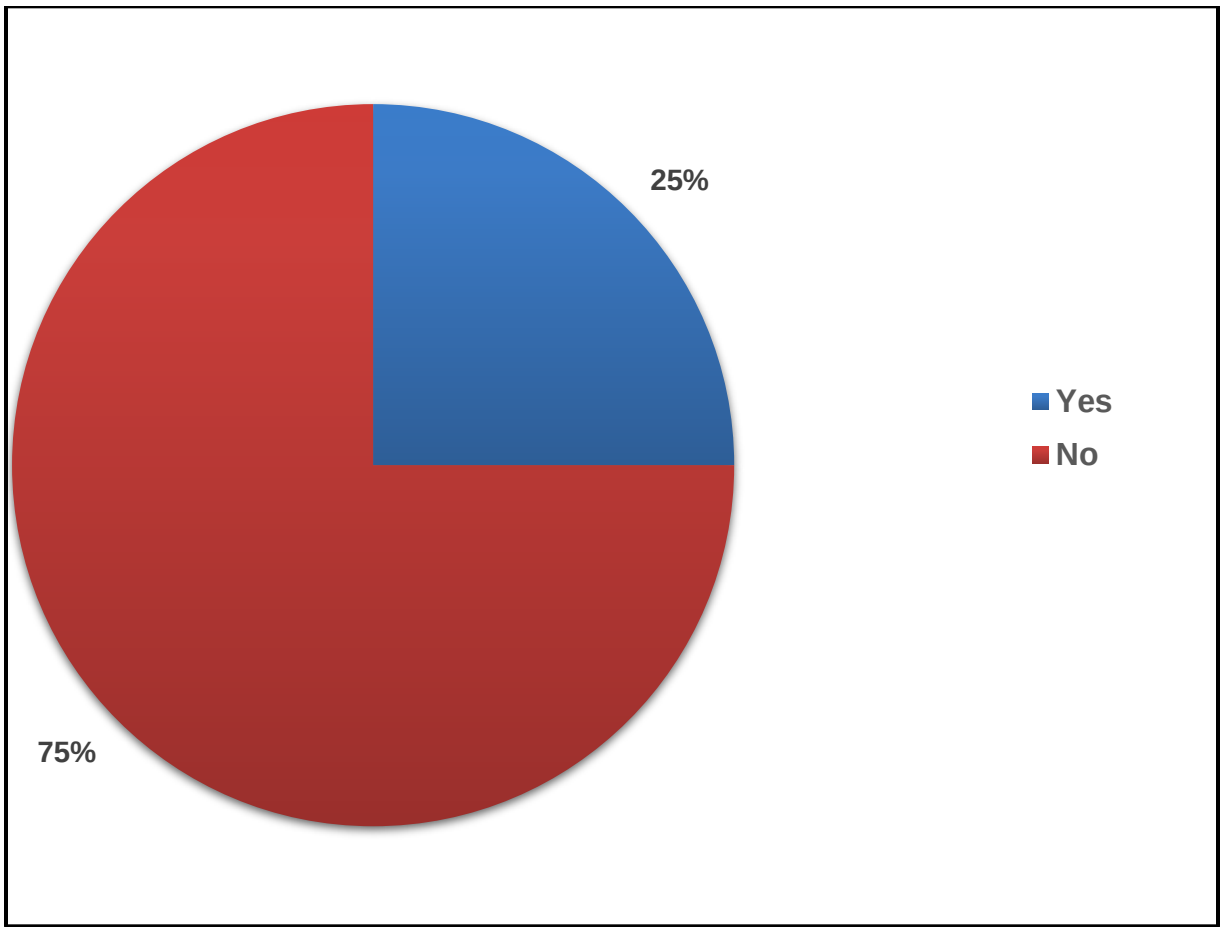


Figure 9. Hours of nutrition education received by military dining facility diners (n=269)

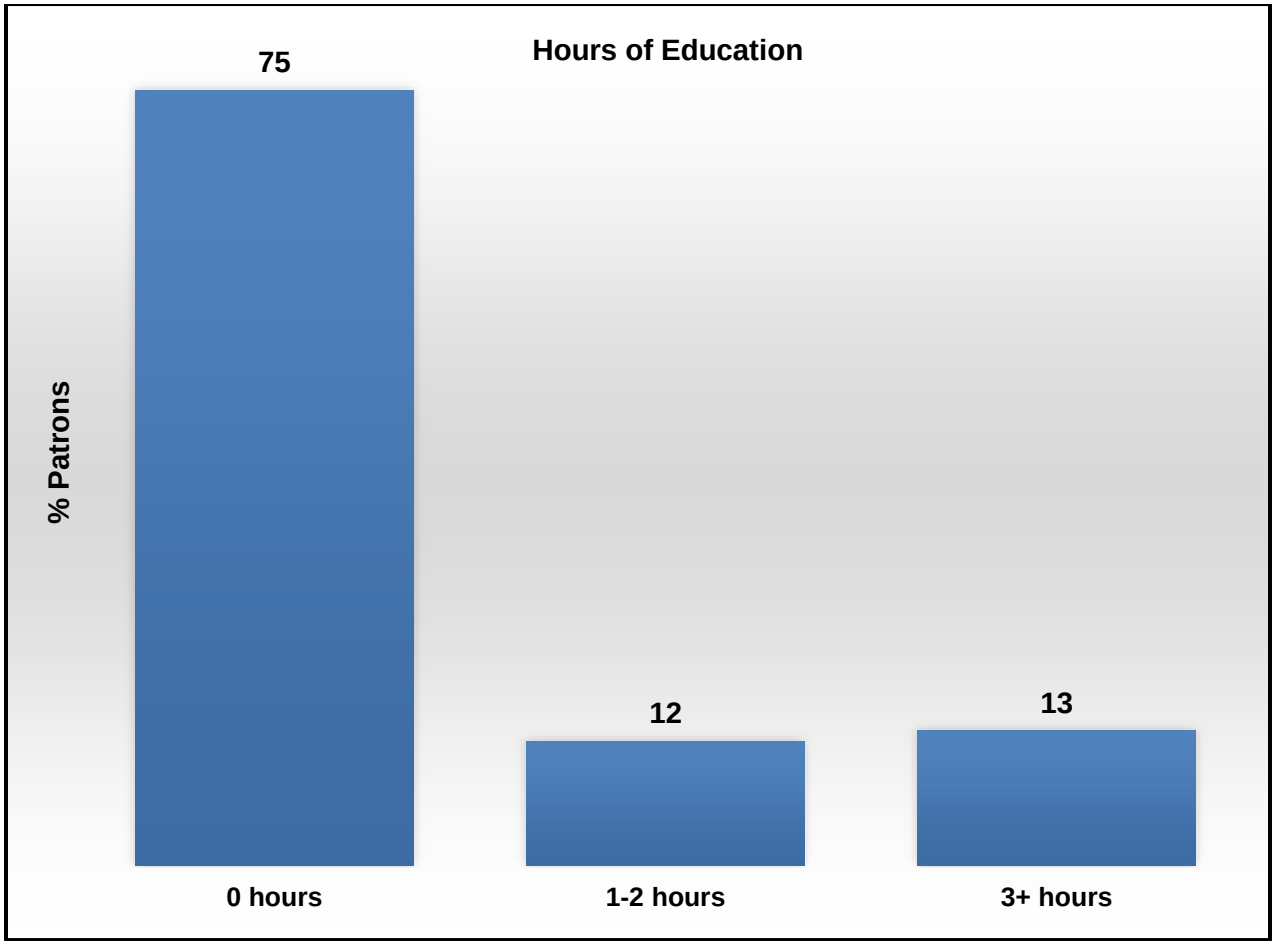


Figure 10. Military dining facility diners responses to 18 nutrition knowledge questions (n=269)

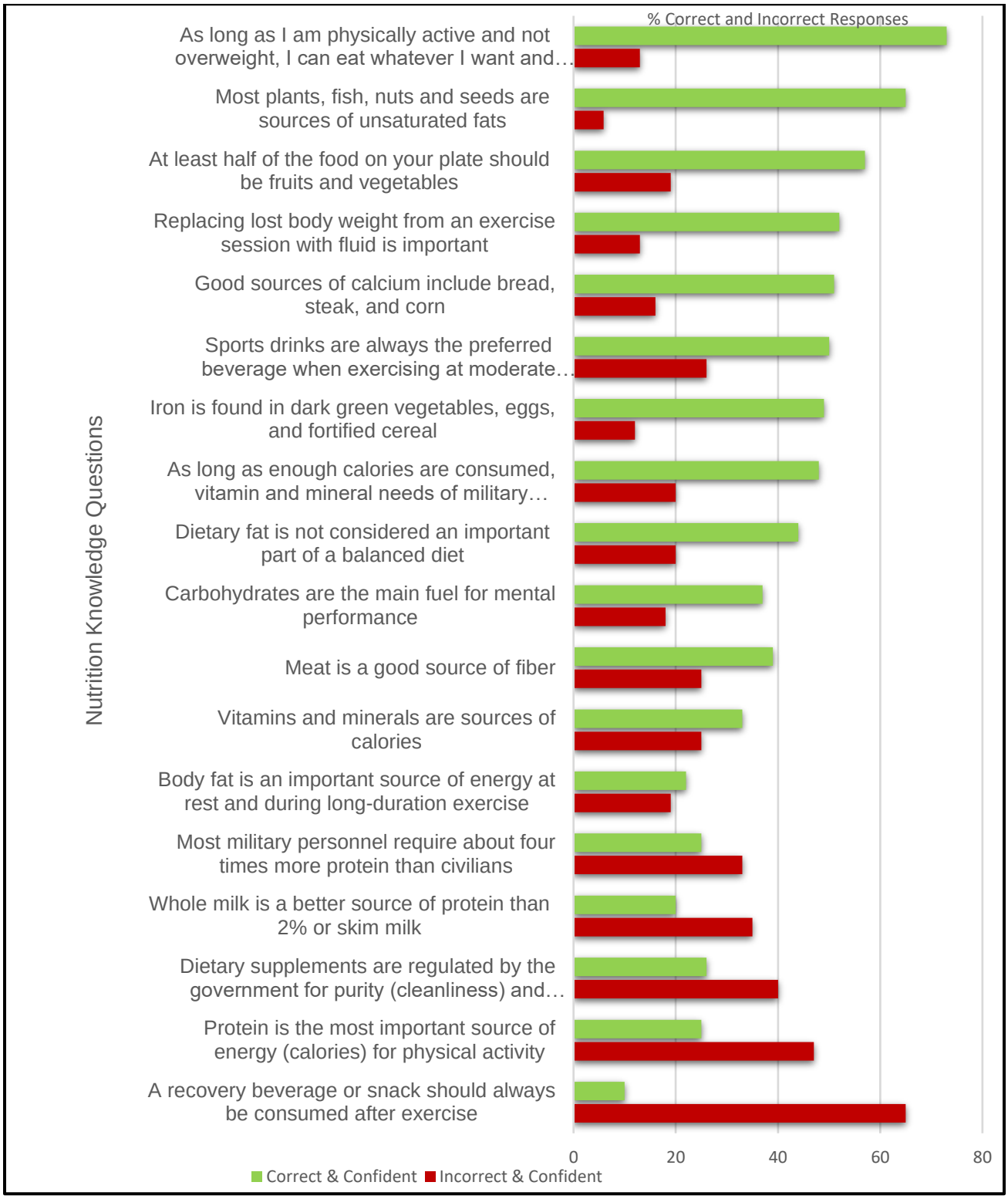
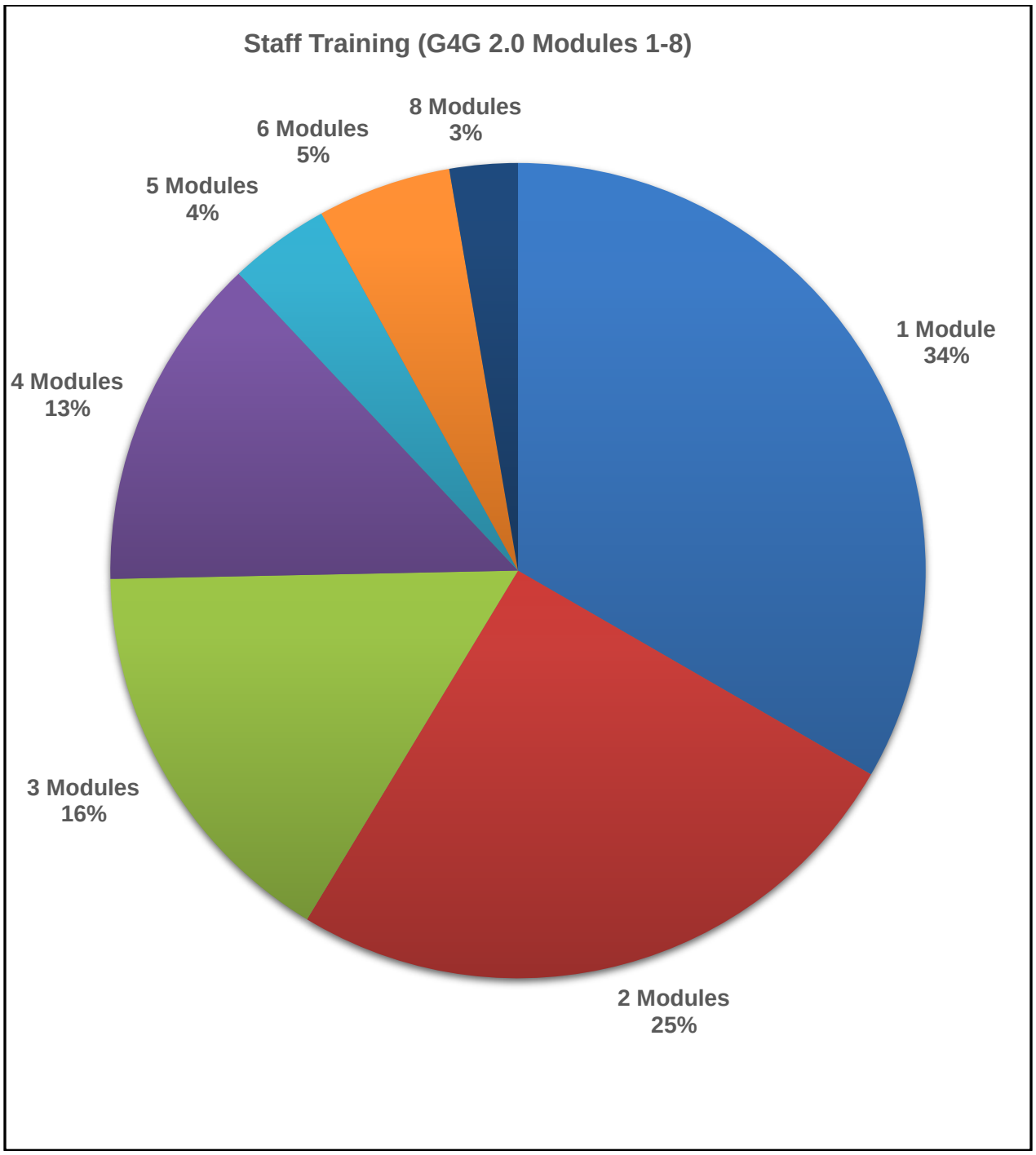
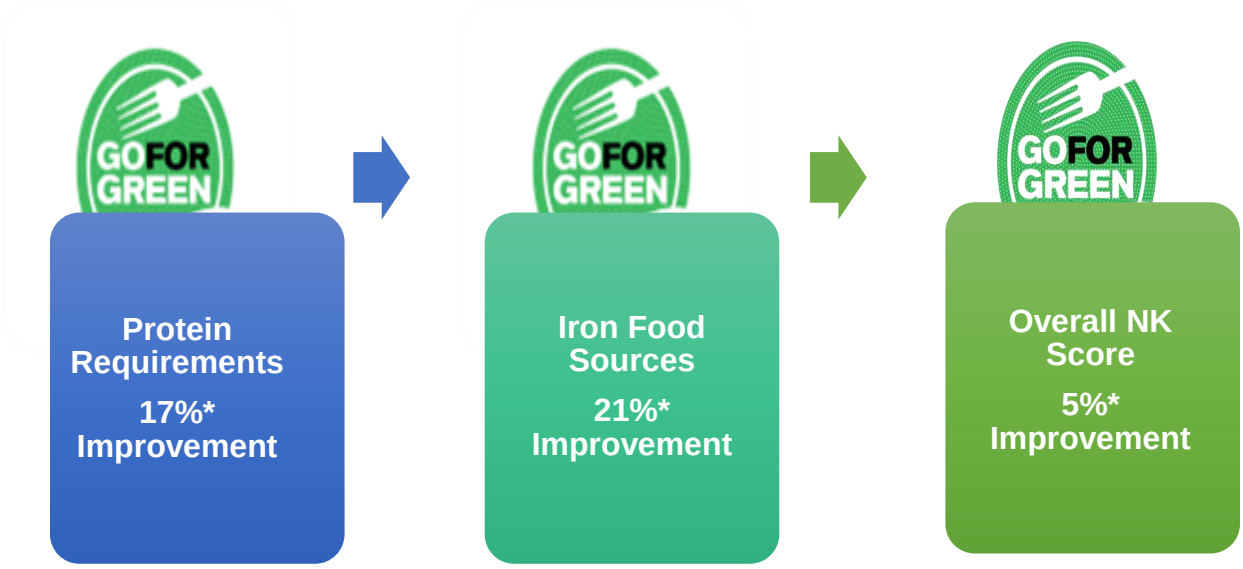


Figure 11. Level of food service staff training during G4G 2.0 program implementation



Foodservice staff exposed to G4G, only 25% completed 50% (4-8 modules) of the G4G training and 2.7% completed 100% (1-8 modules) of the training.

Figure 12. Food service nutrition staff knowledge improvement with exposure to G4G 2.0 program (n=184)

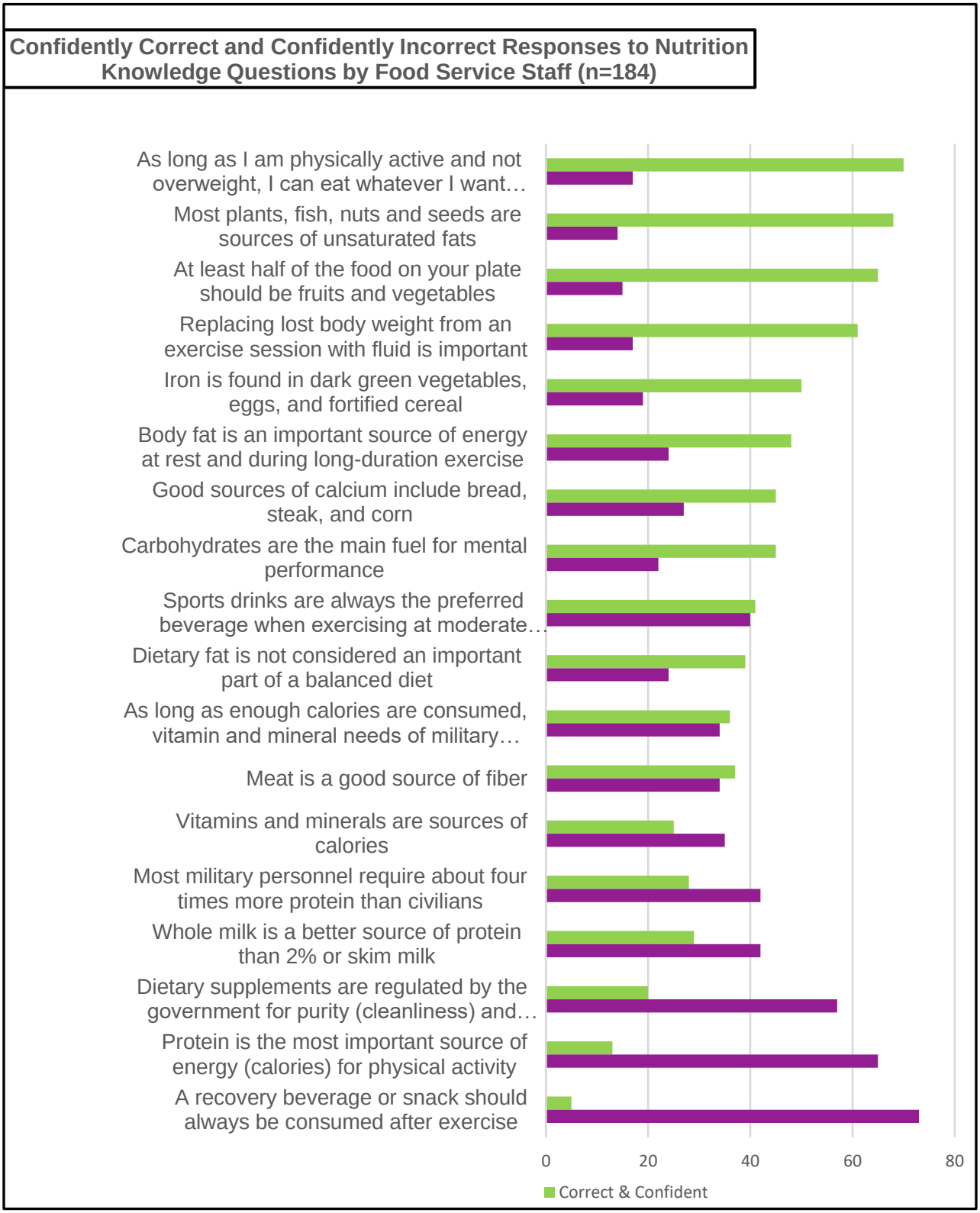


NK=Nutrition Knowledge

*P <0.05

Improvement in Food Service Staff Nutrition Knowledge Scores with any Exposure of G4G 2.0 Program (n=184)

Figure 13. Food service staff response to 18 nutrition knowledge questions



Appendix A: Go for Green® 2.0 Program Requirements

Note: The information is from below listed website and used here with permission of collaborators.
For full details on G4G program: https://www.hprc-online.org/sites/default/files/document/G4G_GR_Revise%2520Program%2520Requirement%2520links_011018.pdf

The Go for Green® (G4G) 2.0 food and recipe coding criteria are based on established guidelines, such as the Dietary Guidelines for Americans 2010[1] and subsequently 2015–2020 Dietary Guidelines for Americans[2], Military Dietary Reference Intakes (MDRIs)[3], joint regulation Nutrition and Menu Standards for Human Performance Optimization (AR 40–25/ OPNAVINST 10110.1/ MCO 10110.49/AFI 44–141)[4], Department of Defense Food Service Program (DoDI 1338.10)[5], and Joint Subsistence Policy Board DoD Menu Standards (DoDM 1338.10)[6]. G4G coding criteria also consider the specific and unique nutrition needs of the military community, where certain nutrient requirements are affected by extreme physical activity and environments[3].

Through its criteria for Green-coded foods and beverages, G4G promotes a balanced nutrient-dense eating pattern of fresh fruits and vegetables, whole grains, lean protein, low-fat dairy products, and healthful fats, which mirrors the focus of the 2015–2020 Dietary Guidelines for Americans. This dietary pattern optimizes intake of naturally occurring electrolytes (such as potassium, calcium, and magnesium), antioxidants, phytochemicals, vitamins, minerals, and dietary fiber. When Service Members select appropriately across all food groups, they eat a balance of macronutrients (carbohydrates, protein, and fats) and micronutrients to optimize performance, readiness, and health.

The G4G Criteria consider the complexity of the overall nutritional value of a food, beverage, or recipe and summarize the results in one of three easy-to-read color codes: Green, Yellow, or Red. This stoplight-labeling system makes it easier for Service Members to identify the best fuel for their performance and health. Research supports using stoplight-labeling systems because consumers find the information effective and easy to understand[7]. There already is a strong association between colors (Green, Yellow, and Red) and meaning (Green means "go")[8], which minimizes the amount of education Service Members need on how to use G4G.

G4G promotes eating more Green-coded foods—to at least half of all food choices—when feasible. Yellow- and Red-coded menu items still will be offered daily in military dining facilities. These items provide variety and fit into an overall nutritious eating pattern when consumed in moderation. Red-coded "comfort" foods and beverages also can help boost morale at times.

Revision and Approval Cycle

The science and research in the field of nutrition is constantly evolving. Therefore, G4G coding criteria will be reviewed, updated, and reapproved for major changes every five years to include the latest nutrition information and recommendations. In addition, G4G Criteria review and reapproval will occur when significant changes are made to the U.S. Food and Drug Administration (FDA) Nutrition Facts panel or when substantial policy changes are made requiring alignment. The next scheduled criteria review will occur in 2020 in order to allow time for adoption of the revised G4G 2.0 initiative as determined by each Service. In addition, this time point will align with the publication of the 2020–2025 Dietary Guidelines for Americans.

Alignment with G4G Coding Algorithm

The G4G Coding Algorithm is the practical application of the G4G Criteria, which are evidence-based but not readily operationalized. These two documents enable G4G coding, or the assignment of color codes to menu items. To maintain standardization, only a Certified G4G Coder can assign color and sodium codes to menu or ready-to-use items.

Evidence Basis for Criteria by Component

G4G 2.0 color-code assignment of foods and beverages is based on the following criteria: saturated fats, fiber, sugar, processing, and total fat. No one criterion (fat, sugar, fiber, etc.) determines the color code for an item;

instead, coding is assigned based on a combination of all criteria. Similarly, no single ingredient determines the color code of a recipe. A few exceptions—such as the presence of MSG, use of trans fats, or deep-fry cooking method—result in an automatic Red code. The G4G Criteria aim to identify the overall nutritional quality of foods and beverages. Items coded Green must provide nutritional value, not just lack undesirable nutrients. The following table is a snapshot of the criteria and is not meant as a coding tool.

GREEN, YELLOW, & RED FOOD CODES			
GO FOR GREEN · EAT WELL · PERFORM WELL			
PROCESSING	LEAST-PROCESSED	SOME PROCESSING	MOST-PROCESSED FOODS
NUTRIENTS	WHOLE FOODS, NUTRIENT PACKED	SOME HEALTHFUL NUTRIENTS	LOWEST-QUALITY INGREDIENTS
FIBER	HIGH IN FIBER	LOWER IN FIBER	MINIMAL FIBER
SUGAR	LOW IN ADDED SUGAR	ADDED SUGAR OR ARTIFICIAL SWEETNERS	ADDED SUGAR OR ARTIFICIAL SWEETNERS
FAT	HEALTHY FATS	POOR-QUALITY FATS	EXCESS FATS AND/OR TRANS FAT FRIED FOODS

Saturated Fats

The 2015–2020 Dietary Guidelines for Americans emphasize limiting saturated fats to less than 10% of daily calories[2]. Encouraging the consumption of natural oils from plants (for example, canola, olive, and safflower), nuts, seeds, seafood, olives, and avocados promotes monounsaturated and polyunsaturated fatty acids over saturated and trans fat. G4G Criteria reflect this nutritional goal.

Trans Fats

Given the evidence-based link between trans fats and cardiovascular risk, the 2015–2020 Dietary Guidelines for Americans recommends limiting trans-fat consumption to a minimum[2]. Despite a decrease in use, artificial trans fats from partially hydrogenated oils are still found in some margarines and packaged foods. Naturally occurring trans fats are found in small amounts in dairy and meat and do not need to be entirely avoided.

G4G Criteria support limiting trans-fat consumption by coding all packaged foods, ready-to-eat items, and recipes containing ingredients with artificial trans fats as Red. The absence of trans fats will be determined by the ingredients list for products showing zero grams of trans fats on the Nutrition Facts panel. In addition, all deep-fried foods, including those that are pre-deep fried and heated in an oven, such as French fries, are automatically coded Red.

Fiber

The Institute of Medicine recommends women 50 and younger consume 25 grams of fiber and men 50 and younger consume 38 grams of fiber per day[9]. Food-labeling guidelines indicate that "good" sources of fiber must contain ≥2.5 grams (or 10% of the recommended daily value of 25 grams) and "high-fiber" sources must contain ≥5 grams (or 20% of the recommended daily value of 25 grams)[10]. These calculations are based on recommendations for women. To account for the recommended 38 grams per day for men, "good" sources of fiber would contain ≥3.8 grams and "high-fiber" sources would contain ≥7.6 grams. G4G Criteria use an adaptation of the official fiber recommendations and FDA food-labeling guidelines. The criteria for "good" sources of fiber at 3–6 grams and "high-fiber" sources at >6 grams align with the Institute of Medicine's recommendations for men and women.

FDA has proposed a new dietary fiber definition that includes "added (isolated or synthetic) non-digestible carbohydrates (≥3 monomeric units) that FDA has determined to have a physiological benefit."[11] G4G Criteria will be updated based on further guidance from FDA regarding added non-digestible carbohydrates, such as inulin, found in packaged foods and not yet proven to be beneficial.

Sugar

The 2015–2020 Dietary Guidelines for Americans recommends a daily maximum consumption of 10% of calories from added sugar[2]. Current information about added sugar is either limited or unavailable for most foods and beverages; therefore, the following approach uses total sugar (grams) to approximate the recommendation based on added sugars. To obtain sugar recommendations, G4G nutrition experts applied the 10% maximum recommendation to a 2,800-calorie diet, on which the Basic Daily Food Allowance (BDFA) is based. The recommendation of 70 grams of added sugar as the daily maximum was doubled to 150 grams to account for naturally occurring sugars. To translate this recommendation into G4G Criteria, G4G nutrition experts conducted a detailed review of food categories where the presence of sugar would be expected (for example, beverages, desserts, yogurts, and sauces) and identified low, moderate, and high levels of total sugar per category.

When the new Nutrition Facts panel includes "Added Sugars" on packaged products beginning in 2018[12], the G4G evaluation criteria will be revised: Products and recipes will be evaluated based on "added sugars" instead of "total sugars."

Non-nutritive Sweeteners

Non-nutritive sweeteners are a popular replacement for the calories from added sugar found in beverages, yogurts, and desserts. However, the research is inconclusive about their effectiveness for long-term weight management[2]. Given non-nutritive sweeteners have not been found to be healthful or provide any nutritional value, beverages (that is, "diet" drinks) and foods containing non-nutritive sweeteners do not meet the G4G Criteria to qualify as Green-coded choices. These will code Yellow, at most.

Total Fat

Nutrition recommendations have shifted focus from percentage of calories from total fat to the types of fat. "Calories from fat" was removed from the updated Nutrition Facts panel to reflect new scientific information that shows the type of fat is more important to health than the amount of fat[12]. The benefits of the Mediterranean Diet—which is high in unsaturated fats from olive oil, nuts, and seeds—are well documented. Research has shown the benefits of the Mediterranean-style eating pattern on cardiovascular risk factors, which experts attribute to the combination of high-unsaturated-fat and low-saturated-fat content[13].

G4G Criteria reflect the updated research and recommendations on total fat by focusing on the types of fat. This allows for highly nutritious foods such as nuts, seeds, avocadoes, and healthful oils—which would be coded Red based on their percentages of calories from fat—to be coded Green instead.

Processing

Recommendations in the 2015–2020 Dietary Guidelines for Americans move away from an emphasis on individual nutrients and foods and instead focus on a healthy-eating pattern by encouraging the consumption of nutrient-dense foods and beverages across and within all food groups[2]. Recipes made with minimally processed or mostly whole-food ingredients such as fruits, vegetables, whole grains, seafood, lean meats and poultry, eggs, legumes, nuts, seeds, and oils contribute to a healthy eating pattern. Processing ingredients—such as with refined grains, meats/poultry, and processed packaged foods—might remove healthy nutrients, such as key vitamins or fiber, and add undesirable components such as saturated or trans fats.

G4G Criteria mirror the 2015–2020 Dietary Guidelines for Americans by coding minimally processed foods Green to encourage their intake. Moderately processed packaged foods and ingredients tend to be coded Yellow, while highly processed packaged foods and ingredients code Red.

Monosodium Glutamate

Monosodium glutamate (MSG) occurs naturally in many foods and is also a popular food additive due to its flavor enhancing properties. According to DoD Manual 1338.10 section 10b, "Products containing monosodium glutamate as an ingredient must be avoided."[6]

Caffeine

Research has shown that caffeine use is associated with increased alertness and enhanced physical performance[14]. Moderate caffeine use is generally safe. However, there is a wide range of individual response to caffeine (that is, caffeine sensitivity) affected by factors including genetics, stimulant or drug use, stress, and relevant health conditions[14]. Given these findings, G4G Criteria do not incorporate caffeine level into coding for food and beverages, but encourage Service Members to be mindful of their caffeine consumption through G4G educational material and resources such as Operation Supplement Safety. Unsweetened teas and coffees fall into the Green category. As beverages combine caffeine along with added sugar or high saturated-fat ingredients, they tend to code as Yellow or Red.

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G4G Coding Calculator

Color Code

Nutrition Component	Value	Scoring
Preparation	Is the cooking method deep-frying?	☐ Yes * Items are automatically marked Red if this answer is "yes"
Trans Fat	Does the product contain "partially hydrogenated" fat/oil (trans fat)?	☐ Yes * Items are automatically marked Red if this answer is "yes"
Additives	Does the product contain added monosodium glutamate (MSG)?	☐ Yes * Items are automatically marked Red if this answer is "yes"
Total Calories	Enter total number of calories This is used to determine the percentage of total and saturated fat only.	cal
% of calories from saturated fat	How many grams of SATURATED fat? or What % of calories are from SATURATED fat?	g or %
% of calories from total fat	How many grams of TOTAL fat? or What % of calories are from TOTAL fat?	g or %
Fiber	Grams of fiber per serving	g
Sugar	Grams of total sugar per serving	g
Processing	How processed are the ingredients in the product? Mostly:	○ Whole foods ○ Lightly processed ○ Moderate to highly processed

Results

Green
9-13 Points

Yellow
5-8 Points

Red
<5 Points

Current Score: 0

Sodium Code

Type of Food	Select type of food from list Note: beverages and fruits will not be labled for sodium	-- Choose One --
Mg of Sodium	Enter total mg of sodium per serving	mg

Results

select type of food



High:

Moderate:

Low:

Final Score:

Go for Green® Coding Algorithm

Color Code			
Nutrition Component	**Value**	**Possible Points**	**Points**
Preparation	Is the cooking method of the product deep-frying?	Stop here: Automatically Red	
Trans Fat	Does the product contain "partially hydrogenated" fat/oil (trans fat)?	Stop here: Automatically Red	
Additives	Does the product contain added monosodium glutamate (MSG)?	Stop here: Automatically Red	
% of calories from saturated fat	Calculate % of calories from SATURATED fat or use nutrient analysis	≤ 10% sat fat = 2 11–15% sat fat = 1 ≥ 16% sat fat = 0	
% of calories from total fat	Calculate % of calories from TOTAL fat * Total fat may be higher than 30% and still score 2 points for select items if the saturated fat is ≤ 10%. Items that may qualify for this exception include salmon and salad dressings or condiments made with heart-healthy fats.	≤ 30% total fat* = 2 31–49% total fat = 1 ≥ 50% total fat = 0	
Fiber	Grams of fiber per serving	> 6 grams = 3 4–6 grams = 2 2–3.9 grams = 1 < 2 grams = 0	
Sugar	Grams of total sugar per serving	< 12 grams of sugar = 3 12–18 grams of sugar = 1 > 18 grams of sugar = 0	

Color Code			
Nutrition Component	**Value**	**Possible Points**	**Points**
Processing ► Processing that minimally affects nutritional value = canning, dehydrating, freezing, etc. What is added during processing may be unhealthy, but the processes themselves are not harmful ► Processing that affects nutritional value = processes/ingredients where nutrients have been stripped away and/or artificial flavors and/or colors have been added; curing	How processed are the ingredients in the product? Mostly: ► Whole-food ingredients: fresh or frozen vegetables, fresh fruits, nuts, seeds, whole grains, unprocessed meat, poultry, and seafood, canned tomatoes and beans, plain dairy products ► Some processed ingredients: canned plain vegetables, canned fruits in juice or water, dehydrated fruits or vegetables, canned soups, meat or poultry injected with solution, uncured deli meats ► Moderately to highly processed and/or refined ingredients: refined (white) grains (for example, white bread, rice, and pasta), canned fruits or vegetables with added saturated fat and/or low to moderate amounts of added sugar, nitrates/nitrites, artificial flavors, non-nutritive sweeteners: Acesulfame potassium ("Sunett" or "Sweet One"), Aspartame ("Equal" or "NutraSweet"), Neotame, Saccharin ("Sweet'N Low"), Sucralose ("Splenda"), Truvia, Stevia	Mostly whole-food ingredients = 3 Lightly processed, but still of moderate nutritional value = 1 Moderately to highly processed and/or refined ingredients = 0	
		Total Points	
Scoring (total points)		**Green = 9–13** **Yellow = 5–8** **Red = < 5**	

Sodium Code
Reference Sodium Criteria table to identify appropriate Low-, Moderate-, or High-sodium code.
Category Name:
Sodium Code:

Sodium Table‡

Sodium is part of the Go For Green® initiative. Sodium levels of foods are labeled by the Low, Moderate, or High salt-shaker symbols shown below.

The sodium code works together with the Green, Yellow, and Red code to help diners choose foods that are acceptable for them. Sodium needs vary from person to person, depending on activity level and health concerns.

Sodium Content	 LOW	 MODERATE	 HIGH
Full-plate Meal*	< 800 mg	800–1500 mg	>1500 mg
Entrée** Protein, vegetables, starch	< 650 mg	650–1100 mg	>1100 mg
Entrée** Protein and vegetables	< 450 mg	450–800 mg	> 800 mg
Entrée** Protein and starch (carb)	< 600 mg	600–950 mg	> 950 mg
Protein only	< 350 mg	350–800 mg	> 800 mg
Grains & Other Starches	< 300 mg	300–700 mg	> 700 mg
Vegetables	< 150 mg	150–400 mg	> 400 mg
Soup	< 450 mg	450–600 mg	> 600 mg
Dairy	< 150 mg	150–300 mg	> 300 mg
Condiments, Gravies	< 200 mg	200–300 mg	> 300 mg
Dessert	< 300 mg	300–600 mg	> 600 mg
Beverages	Will not be labeled for sodium		
Fruits	Will not be labeled for sodium		

‡ Sodium values will be revised at three-year intervals, as more reduced-sodium products and recipes become available.

** Full-plate meal includes fruit and dairy.*

*** Entrees exclude fruit and dairy.*

Go for Green® Coding Algorithm: Beverage Table

The Go for Green® Coding Algorithm (web-based or handwritten) shouldn't be used to code beverages. Instead, use the following coding table. Beverages are coded based on added sugar content, artificial ingredients, saturated fat content, and healthful nutrients such as vitamins and minerals. Provide G4G educational materials to Service Members to help them make informed choices too.

Code	Beverages
	► Water (plain or carbonated) ► Naturally flavored water, including fruit/vegetable/herb-infused (no artificial sweeteners) ► Herbal tea ► Unsweetened iced or hot tea ► Unsweetened iced or hot coffee ► 100% vegetable juice ► Milk, unsweetened (skim, 1%) ► Milk alternatives: soy, almond, rice, unsweetened or plain with added calcium and vitamin D
	► Sports drinks ► 100% fruit juice ► Lightly sweetened iced or hot tea ► Coffee with small amounts of sugar, cream, or milk ► Artificially sweetened beverages (diet or light sodas, tea, juices, and many flavored waters) ► Milk, unsweetened (2%) ► Flavored milk (skim, 1%, 2%) (vanilla, chocolate, etc.) Flavored milk alternatives: coconut, soy, almond, and rice ► Hot chocolate made with water or milk (skim, 1%, 2%)
	► Energy drinks ► Sweet tea ► Coffee with large amounts of whole milk or cream and sugars or syrups ► Sweetened beverages of any kind (sodas, fruit punches, and juice drinks) ► Milk, plain or flavored (whole) ► Hot chocolate made with whole milk, cream, or half-and-half

NOTE: Use the point-based approach (pages 28 and 29 of this document).

G4G Menu Coding Goals

These Menu Coding Goals are intended to define—for dining facilities and galleys—what will qualify as menus compliant with Go for Green® 2.0. The overall goal is to shift menu design away from an overabundance of Red-coded items towards more Green-coded items. In general, there should always be a Green-coded option for each meal part (entrée, starchy side, non-starchy side, etc.) at each station (Main line, Short Order, specialty bar, etc.) for each meal served daily.

The goals are designed around an average-size facility. The size of your foodservice operation—particularly small or exceptionally large—might impact how these goals are applied. For example, in very small facilities where only one entrée is offered on the Main line and one on the Short Order at lunch, the goal might be that only one of those can be a Red-coded entrée and the other should be a Green-coded entrée. For an exceptionally large facility where there might be four or more entrées on the Main line, at least one must be a Green-coded entrée and no more than one Red-coded entrée on each line. This leaves options for additional Green- and/or Yellow-coded entrées. The goals are presented both by meal and by overall menu for two different ways to assess your menus.

Guideline Cards are available for various types of specialty bars and other areas within the dining facility, including Breakfast, Dessert Bar, and Beverage Bar. Refer to these **Guideline Cards** for specific guidance on how to give your dining facility a performance-boosting makeover!

Go for Green® Menu Coding Goals: Breakfast

Menu Coding Goals for Breakfast are presented separately from those for Lunch and Dinner. As more Green- and Yellow-coded breakfast recipes become available, more Green- and Yellow-coded items should be offered. Over time, Menu Coding Goals (percentage of Green-coded items) will be the same for Breakfast as for Lunch and Dinner.

Dining Facility/Galley Serving Line Component	Green-coded Items in Overall Menu	Green-coded Items per Meal	Red-coded Items per Meal
Main/Hotline Entrées	At least 30%	At least 1	—
Main/Hotline Starchy Sides	At least 30%	At least 1 (every other day)	—
Main/Hotline Non-Starchy Sides	At least 30%	At least 1	—
Grill/Short-Order Entrées: Omelet Station Breakfast Entrees (where applicable)	At least 30%	At least 1 (omelet or entrée) At least 4 omelet toppings/fillings	—
Grill/Short-Order Sides	At least 30%	At least 1	—
Breakfast/Fitness Bar	At least 30%	At least 6	No more than 3
Cereals, Cold	At least 30%	At least 2	No more than 2
Cereals, Hot	At least 30%	At least 1	–
Beverages	At least 30%	At least 1	—
Smoothies (if offered)	At least 30%	—	—

Go for Green® Menu Coding Goals: Lunch and Dinner

Separate Menu Coding Goals are included for Breakfast vs. Lunch and Dinner. At present, an insufficient number of Green-coded recipes are available for Breakfast. As more Green- and Yellow-coded breakfast recipes become available, more Green- and Yellow-coded items should be offered. Over time, Menu Coding Goals (percentage of Green-coded items) will be the same for Breakfast as for Lunch and Dinner.

Dining Facility/Galley Serving Line Component	Green-coded Items in Overall Menu	Green-coded Items per Meal	Red-coded Items per Meal
Main/Hotline Entrées	At least 30%	At least 1	No more than 1
Main/Hotline Starchy Sides	At least 30%	At least 1	No more than 1
Main/Hotline Non-Starchy Sides	At least 30%	At least 1	No more than 1
Grill/Short-Order Entrées	At least 30%	At least 1	No more than 50%
Grill/Short-Order Sides	At least 30%	At least 1	No more than 2
Sandwich Line (Cold) (featured)	At least 30%	At least 2	—
Salad Bar (excluding dressings)	At least 50%	Unlimited	No more than 3–5 toppings on the bar
Legumes	At least 30%	Unlimited	—
Desserts	At least 30%	Unlimited	—
Beverages	At least 30%	—	—
Dressings	At least 30%	At least 3	No more than 3
Chips/Portable Snacks/ Sides	At least 30%	Unlimited	—
Specialty Bars (Pizza, Potato, Taco, Chicken, etc.)	At least 30%	At least one entrée and one side	—

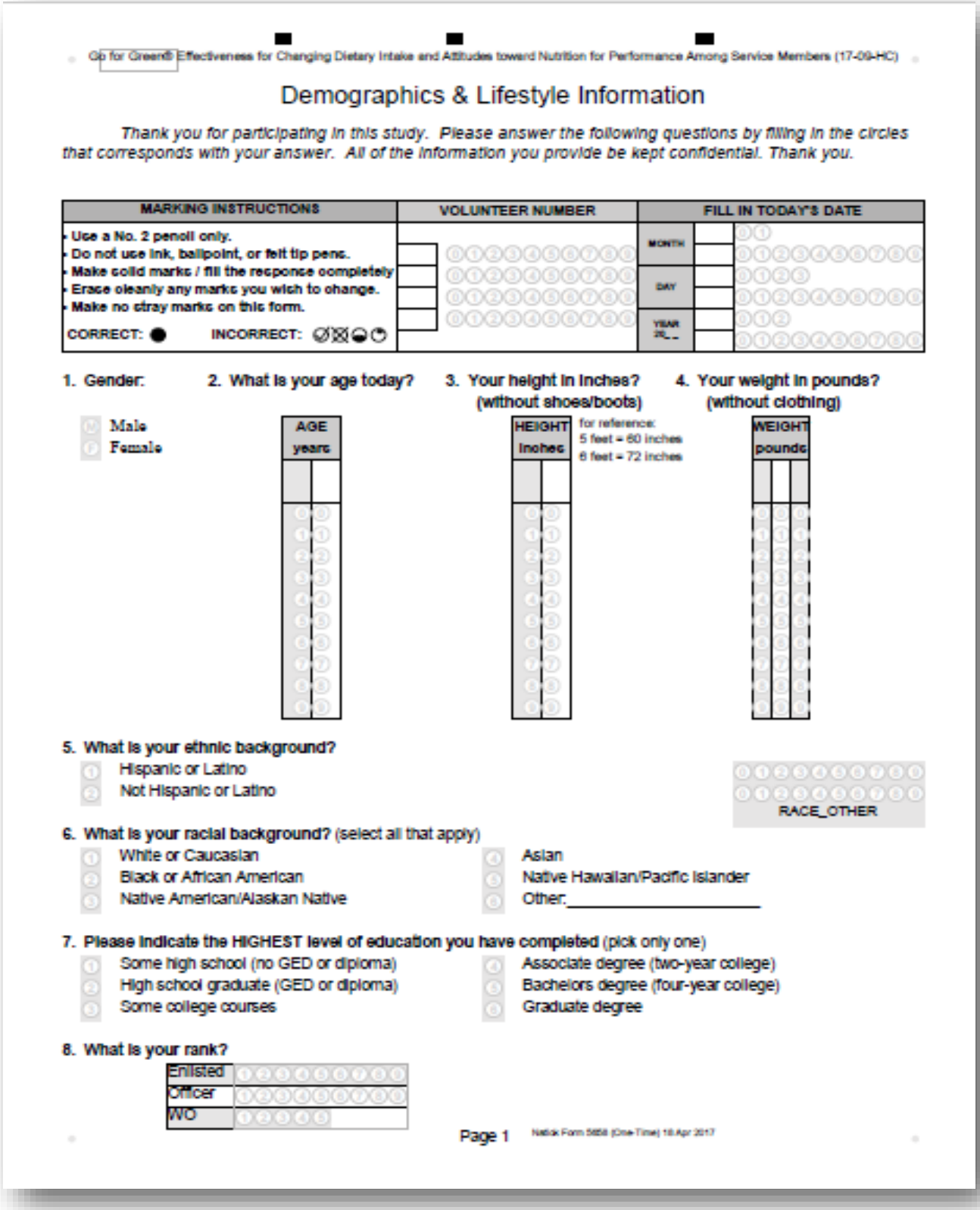
G4G Food-placement Goals

For each serving area, implement at least 3 food-placement goals.

Bar/Station	Food-placement Goals
Hotline/Main Line	Place Green-coded items first, followed by Yellow-coded ones, and then Red-coded items
	Offer 2 non-starchy vegetables
	Offer whole-grain versions of starches and place first in line
	Examples: brown rice, whole-wheat pasta
	Offer Red-coded sauces on the side, at the end of the line
Short Order/Grill	Place Green-coded items first, followed by Yellow-coded ones, and then Red-coded items
	Make whole-grain bread, wraps, and pita the default
	Place white bread, rolls, wraps, and pita out of sight
	Offer 4 vegetables as burger or sandwich toppings
	Offer only one fried starch (French fries or onion rings)
	Offer grilled chicken
	Offer veggie burgers
	Offer baked fries instead of deep-fried ones
Deli/Sandwich Bar	Place Green-coded items first, followed by Yellow-coded ones, and then Red-coded items
	Make whole-grain bread, wraps, and pita the default
	Place white bread, rolls, wraps, and pita out of sight, such as under the counter
	If offering pre-made sandwiches, place Green-coded options at or above eye level
	Offer Green-coded spreads such as hummus or guacamole; place before Red-coded options such as mayonnaise or creamy dressings
	Offer at least 6 vegetables as sandwich toppings
	Place Red-coded toppings in smaller containers at the end of the line
	Offer meatless options such as Portobello mushrooms or marinated tofu

Bar/Station	Food-placement Goals
Salad Bar	Offer at least 10 Green-coded vegetables
	Offer whole-grain starchy salads (for example, those made with brown rice or quinoa)
	Offer Green-coded dressings such as olive oil and homemade vinaigrettes; place before Red-coded options
	Offer Green-coded fats such as nuts, seeds, or avocados; place before Red-coded options
	Place Red-coded toppings in smaller containers at the end of the line
	Make whole-grain bread, wraps, and pita the default
	Place white bread and pita out of sight, such as under the bar
Specialty Bar	Place Green-coded items first, followed by Yellow-coded ones, and then Red-coded items
	Offer at least 4 vegetables
	Offer meatless options such as Portobello mushrooms, beans, or marinated tofu
	Place Red-coded toppings in smaller containers at the end of the line
	Offer Green-coded fats such as nuts, seeds, or avocadoes; place before Red-coded options
	Offer whole-grain versions of starches and place first in line *Examples:* brown rice, whole-wheat pasta
Dessert Bar	Offer fruit, whole or cut-up
	Place Dessert Bar out of the main traffic flow
	Decrease the physical space of the Dessert Bar
	Offer fruit-and-yogurt parfaits
	Offer more Yellow-coded options than Red-coded ones
Beverages	Offer infused or "spa" water
	Serve water in multiple places
	Assign water its own full fountain tab on the drink machine
	Offer unsweetened tea, and place in line before sweet tea
	Add lemon slices to water or unsweetened tea
	Move Red-coded beverages to the end of the station
	Place low-fat and plain milks before flavored (chocolate) milk
	Offer 100% juice rather than juice drinks or fruit punches

Appendix B: Demographic & Lifestyle Survey



Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09-HC)

Demographics & Lifestyle Information

Thank you for participating in this study. Please answer the following questions by filling in the circles that corresponds with your answer. All of the information you provide be kept confidential. Thank you.

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ∅ ⊠ ◒ ◔		MONTH DAY YEAR 20__

1. Gender:
   - (M) Male
   - (F) Female

2. What is your age today?
   AGE years

3. Your height in inches? (without shoes/boots)
   HEIGHT inches
   for reference:
   5 feet = 60 inches
   6 feet = 72 inches

4. Your weight in pounds? (without clothing)
   WEIGHT pounds

5. What is your ethnic background?
   - (1) Hispanic or Latino
   - (2) Not Hispanic or Latino

   RACE_OTHER

6. What is your racial background? (select all that apply)
   - (1) White or Caucasian
   - (2) Black or African American
   - (3) Native American/Alaskan Native
   - (4) Asian
   - (5) Native Hawaiian/Pacific Islander
   - (6) Other:_____

7. Please indicate the HIGHEST level of education you have completed (pick only one)
   - (1) Some high school (no GED or diploma)
   - (2) High school graduate (GED or diploma)
   - (3) Some college courses
   - (4) Associate degree (two-year college)
   - (5) Bachelors degree (four-year college)
   - (6) Graduate degree

8. What is your rank?
   - Enlisted
   - Officer
   - WO

Page 1 Natick Form 5858 (One-Time) 18 Apr 2017

9. What is your primary MOS? Description:_____

10. How long have you been in the Armed Services?

Active Duty	1 Less than one year	OR	If a year or more, please fill in the number of years (start with leading zero's when needed).	

11. During the past 7 days, which meals and snacks did you eat on a regular basis (at least 5 times per week)?
Please fill in one response for each line:

Yes	No	
Y	N	A. Early morning snack
Y	N	B. Breakfast
Y	N	C. Morning snack
Y	N	D. Lunch
Y	N	E. Afternoon snack
Y	N	F. Dinner
Y	N	G. Evening snack

12. During the past 7 days, where did *MOST* of your meals and snacks come from? Select ONLY ONE choice for each meal and snack.

	Do not eat a meal/snack at this time	Home / Barracks (current residence)	Military dining facility	Restaurants / Dine in	Restaurants / Take-out	Convenient stores / PX / Shoppette	Other _____
Early Morning Snack	0	1	2	3	4	5	6
Breakfast	0	1	2	3	4	5	6
Morning Snack	0	1	2	3	4	5	6
Lunch	0	1	2	3	4	5	6
Afternoon Snack	0	1	2	3	4	5	6
Dinner	0	1	2	3	4	5	6
Evening Snack	0	1	2	3	4	5	6

Q12_oth

For questions 13-16, during the last 7 days, think about only those physical activities that you did continuously for at least 10 minutes at a time. On how many days did you do each type of activity and on the average, for how long?

13. VIGOROUS physical activity

(makes you breathe much harder than usual with heavy sweating; e.g. lifting weights, aerobics, or fast running / bicycling)?

Number of days per week ○○○○○○○ X

TIME per day	
Hr.	Min.
0 0	0 0
1 1	1 1
2 2	2 2
3 3	3 3
4 4	4 4
5 5	5 5
6 6	
7 7	
8 8	
9 9	

14. MODERATE physical activity

(makes you breathe somewhat harder than usual; e.g. jogging, carrying light loads, or bicycling at a regular pace)?

Number of days per week ○○○○○○○ X

TIME per day	
Hr.	Min.
0 0	0 0
1 1	1 1
2 2	2 2
3 3	3 3
4 4	4 4
5 5	5 5
6 6	
7 7	
8 8	
9 9	

15. WALK for at least 10 minutes at a time?

This includes walking at work and at home, walking to travel from place to place, and other walking that you did solely for recreation, sport, exercise or leisure.

Number of days per week ○○○○○○○ X

TIME per day	
Hr.	Min.
0 0	0 0
1 1	1 1
2 2	2 2
3 3	3 3
4 4	4 4
5 5	5 5
6 6	
7 7	
8 8	
9 9	

16. How much time in total did you usually spend SITTING on a week day?

This includes while at work or home, while doing course work and during leisure time, sitting at a desk, visiting friends, reading, traveling in a vehicle, and sitting or lying down to watch television.

TIME per day	
Hr.	Min.
0 0	0 0
1 1	1 1
2 2	2 2
3 3	3 3
4 4	4 4
5 5	5 5
6 6	
7 7	
8 8	
9 9	

Use the table below to indicate how many hours during a typical day (24 hrs), during the week and then again during the weekends, you spend engaged in electronic activities.

17. DURING WEEKDAYS							
WATCHING TV		VIDEO GAMES		USING COMPUTER FOR WORK OR SCHOOL		USING COMPUTER FOR LEISURE	
0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9	

18. DURING WEEKEND							
WATCHING TV		VIDEO GAMES		USING COMPUTER FOR WORK OR SCHOOL		USING COMPUTER FOR LEISURE	
0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9		0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9	

19. How would you rate your physical readiness for military training or combat at this time?

1. Best physical shape in my life
2. Good physical shape
3. Neither good nor bad physical shape
4. Bad physical shape
5. Worst physical shape in my life

APFT

20. APFT score

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9

21. Number of pushups

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9

22. Number of sit ups

0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9

23. 2 MILE RUN TIME

minutes		/ seconds	
		/	
0	0		0
1	1		1
2	2		2
3	3		3
4	4		4
5	5		5
6	6		6
7	7		7
8	8		8
9	9		9

24. Alternate APFT event:

1. Bike
2. Walk
3. Swim
4. Other_____

26. DATE OF LAST APFT		
MONTH		0 1 0 1 2 3 4 5 6 7 8 9
DAY		0 1 2 3 0 1 2 3 4 5 6 7 8 9
YEAR 20__		0 1 2 0 1 2 3 4 5 6 7 8 9

SLEEP

26. During the last 7 days, how would you rate your sleepiness during the day?

1. Feeling active, vital, alert, or wide awake
2. Functioning at high level, but not at peak; able to concentrate
3. Awake but relaxed; responsive but not fully alert
4. Somewhat foggy; let down
5. Foggy; losing interest in remaining awake; slowed down
6. Sleepy, woozy, fighting sleep; prefer to lie down
7. No longer fighting sleep, sleep onset soon; having dream-like thoughts

27. During the last 7 days, on average how many hours of sleep did you get in a 24-hour period? *(to nearest ½ hr)*

Hours per day	
Hr.	Min.
00	00
01	01
02	02
03	03
04	04
05	05
06	06
07	07
08	08
09	09
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20
21	21
22	22
23	23
24	24

PERFORMANCE

28. During the last 7 days, did your food choices in the dining facility have an effect on:

	NEVER	SOMETIMES	MOST OF THE TIME	ALWAYS
Feeling energized throughout the day?	1	2	3	4
Improving your mood during the day?	1	2	3	4
Feeling satisfied for several hours after meals (not over hungry nor over full)?	1	2	3	4
Improving your mental performance (e.g. ability to think clearly, focus, learn, and ability to recall information during the day)?	1	2	3	4
Improving your level of physical performance (e.g. during workout or military training)?	1	2	3	4
Improving your ability to sustain physical performance longer?	1	2	3	4
Feeling good about yourself?	1	2	3	4
Improving your recovery after a vigorous physical activity OR workout? *(recovery refers to how quickly your muscles and cardiovascular systems rebound after a workout or physical activity)*	1	2	3	4
Improving your recovery after a moderate physical activity OR workout? *(recovery refers to how quickly your your muscles and cardiovascular systems rebound after a workout or physical activity)*	1	2	3	4
Reducing injury?	1	2	3	4
Improving your sleep quality?	1	2	3	4
Improving your response to emotional or psychological stress?	1	2	3	4

Appendix C: Dining Facility Satisfaction Survey

Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09-HC)

Dining Facility Satisfaction Survey

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊗ ◒ ◔		MONTH, DAY, YEAR 20__

Thank you for participating in this study. Please answer the following questions by filling in the circles that reflect your experience with the food service. All of the information you provide be kept confidential. Thank you.

Complete by marking the boxes that reflect your experience with our Food service.

		STRONGLY AGREE	MODERATELY AGREE	AGREE	UNDECIDED	DISAGREE	MODERATELY DISAGREE	STRONGLY DISAGREE
1.	Appearance of the food is pleasing	1	2	3	4	5	6	7
2.	Flavor and taste of the food is good	1	2	3	4	5	6	7
3.	Choices available are adequate	1	2	3	4	5	6	7
4.	Availability of healthy foods is adequate	1	2	3	4	5	6	7
5.	Availability of performance foods is adequate	1	2	3	4	5	6	7
6.	Portion sizes are appropriate	1	2	3	4	5	6	7
7.	Availability of fresh fruit is adequate	1	2	3	4	5	6	7
8.	The salad bar offers a variety of fresh vegetables	1	2	3	4	5	6	7
9.	The main dishes served are healthy and performance-based	1	2	3	4	5	6	7
10.	The side dishes are served without added fat (ex: butter)	1	2	3	4	5	6	7
11.	Healthy and performance-based dessert choices are available	1	2	3	4	5	6	7
12.	Temperature of food (ex: hot food is hot) is just right	1	2	3	4	5	6	7
13.	Vegetarian food choices are available	1	2	3	4	5	6	7
14.	I find the DFAC nutrition labels easy to use	1	2	3	4	5	6	7
15.	Nutrition labels provide knowledge to make performance based choices	1	2	3	4	5	6	7
16.	I use the DFAC nutrition labels to chose healthy foods	1	2	3	4	5	6	7
17.	I use the DFAC nutrition labels to chose performance foods	1	2	3	4	5	6	7

18. How long have you been eating at this dining facility *(i.e., years/months OR weeks)*? *Please use leading zeros when needed. If you are not reporting in a row, please fill in a zero.*

YEARS	
MONTH	
-OR-	
WEEKS	

Natick Form 5667 (One-Time) 18 Apr 2017

Appendix D: Nutrition Knowledge Survey

Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09-HC)

Nutrition Knowledge

Thank you for participating in this study. Please answer the following questions by filling in the circles that corresponds with your answer. All of the information you provide will be kept confidential. Thank you.

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊠ ◒ ◔		MONTH DAY YEAR 20__

This section is intended to assess your general knowledge about nutrition:

First tell us if each statement is TRUE or FALSE, then tell us if you are confident with your answer (YES), or not confident in your answer (NO).

This section is intended to assess your general knowledge about nutrition:
First tell us if each statement is TRUE or FALSE, then tell us if you are confident with your answer (YES), or not confident in your answer (NO).

		TRUE	FALSE	Are you confident with your answer? YES	NO
1.	Dietary supplements are regulated by the government for purity *(cleanliness)* and safety before sale.	T	F	Y	N
2.	Feeling fatigued midway through a workout may be a sign of insufficient carbohydrate calories in your diet.	T	F	Y	N
3.	Protein helps to build and repair muscle.	T	F	Y	N
4.	Replacing lost body weight from an exercise session with fluid is important.	T	F	Y	N
5.	Protein, carbohydrate, and fat all provide the same amount of calories per gram.	T	F	Y	N
6.	Vitamin D helps your body better absorb calcium for bone health.	T	F	Y	N
7.	Common foods that are low in saturated fat include cheese, pizza, and fried chicken breast.	T	F	Y	N
8.	Weight loss occurs from eating fewer calories, burning more calories, or both.	T	F	Y	N
9.	Thirst sensation during or after exercise is an indicator that your body is already dehydrated.	T	F	Y	N
10.	As long as I am physically active and not overweight, I can eat whatever I want and be healthy.	T	F	Y	N
11.	Good sources of calcium include bread, steak, and corn.	T	F	Y	N
12.	Most plants, fish, nuts and seeds are sources of healthy unsaturated fats.	T	F	Y	N
13.	Iron deficiency can cause mental and physical fatigue.	T	F	Y	N
14.	Military personnel should avoid carbohydrates in their diet.	T	F	Y	N
15.	Whole milk is a better source of protein than 2% or skim milk.	T	F	Y	N
16.	A recovery beverage or snack should always be consumed after exercise.	T	F	Y	N
17.	Military personnel who are vegetarian need to consume a daily protein supplement because they cannot get enough protein from their diet.	T	F	Y	N
18.	Vitamins and minerals are sources of calories.	T	F	Y	N
19.	A fluid deficit can lead to an electrolyte imbalance that is not easily overcome by simple rehydration.	T	F	Y	N

Natick Form 9654 (One-Time) 18 Apr 2017

First tell us if each statement is TRUE or FALSE, then tell us if you are confident with your answer (YES), or not confident in your answer (NO).

		TRUE	FALSE	Are you confident with your answer? YES	NO
20.	Dietary fat is not considered an important part of a balanced diet.	○	○	○	○
21.	Protein is the most important source of energy *(calories)* for physical activity.	○	○	○	○
22.	Men need more iron than women.	○	○	○	○
23.	At least half of the food on your plate should be fruits and vegetables.	○	○	○	○
24.	Most military personnel require about four times more protein than civilians.	○	○	○	○
25.	Meat is a good source of fiber.	○	○	○	○
26.	Vitamin E is important for turning the food you eat into energy.	○	○	○	○
27.	Carbohydrates are the main fuel for mental performance.	○	○	○	○
28.	As long as enough calories are consumed, vitamin and mineral needs of military personnel are met.	○	○	○	○
29.	Fiber is important for regular bowel movements.	○	○	○	○
30.	Sports drinks are always the preferred beverage when exercising at moderate intensity.	○	○	○	○
31.	Common carbohydrate-rich foods are pasta, potato, cereal and bread.	○	○	○	○
32.	Iron is found in dark green vegetables, eggs, and fortified cereal.	○	○	○	○
33.	Carbohydrates *(stored as muscle glycogen)* are the most important energy source for high-intensity activity.	○	○	○	○
34.	To lose weight, recommendations say that skipping meals is best.	○	○	○	○
35.	Good sources of protein include yogurt, black beans and nuts.	○	○	○	○
36.	A healthy person should be able to get all of the vitamins and minerals the body needs from a balance of food choices.	○	○	○	○
37.	Fat is an important source of energy at rest and during long-duration exercise.	○	○	○	○
38.	Eating a poor-quality diet may increase risk for injury or illness in military personnel.	○	○	○	○
39.	Vitamin C is sometimes called the sunshine vitamin because the sun helps your body make it.	○	○	○	○
40.	Consuming more than 2 grams of protein per kg of body weight per day will build muscle.	○	○	○	○

Thank you for taking the time to fill out this survey. Please check over the questions to be sure nothing was missed.

Appendix E: G4G Awareness for Diner

Diners Survey - Patrons

Thank you for participating in this study. Please answer the following questions by filling in the circles that corresponds with your answer. All of the information you provide will be kept confidential. Thank you.

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊠ ◒ ◔		MONTH DAY YEAR 20__

Instructions: For all questions and/or statements presented in this survey, please select only one (1) response unless the question and/or statement indicate otherwise.

1. **Do you believe the foods you eat will impact your physical performance?**
   - Yes
   - No

2. **Do you believe the foods you eat will impact your mental performance?**
   - Yes
   - No

3. **What source do you use the most in helping to understand nutrition?** *(Choose all that apply)*
   - Social media *(Facebook, Twitter, Instagram, etc.)*
   - Health professionals such as Nurses, Physicians or Dietitians
   - Fellow Soldiers
   - Command leadership
   - Family members
   - Blogs
   - Health and fitness magazines
   - Gym/fitness personnel
   - Specialty foods or supplement stores *(GNC, Whole Foods, etc.)*
   - Posted information *(posters, table tents, brochures)*
   - Websites
   - Podcasts
   - Other, please specify_____

4. **Have you received any military or civilian education on nutrition, not including any Go for Green trainings?**
   - Yes
   - No (skip to question 5)

   If you selected *YES*, how many hours of nutrition training have you received?
   - 1-2 hours
   - 3-5 hours
   - 6-8 hours
   - 9 hours or more

Natick Form 3952 (One-Time) 18 Apr 2017

5. **How would describe your overall nutritional knowledge?**

① Extremely knowledgeable
② Very knowledgeable
③ Somewhat knowledgeable
④ Not knowledgeable

6. **Which of the logos are you familiar with?**





① ② Both ③ Neither ④

7. **If you recognized this logo** 

which of the following statements do you most associate with the logo *(Choose all that apply)?*

Ⓐ Performance focused foods
Ⓑ New menu items
Ⓒ New promotions such as posters, brochures and social media
Ⓓ Food labels
Ⓔ Education
Ⓕ All of the above
Ⓖ None of the above
Ⓗ I do not recognize this logo

8. **From which of the following did you learn about Go for Green®?** *(Choose all that apply)*

YES	Source
①	Print Media *(newspaper, press release, command newsletter, etc.)*
②	Social Media *(Facebook, Twitter, etc.)*
③	Posters
④	Table tents in the dining facility
⑤	Email
⑥	Unit Leadership
⑦	Word of Mouth *(friends, peers, dining facility staff, etc)*
⑧	Other, please specify: _____

9. **Do you use Go for Green® to help make food choices in the dining facility?**

① Yes
② No

10. To what extent do you agree or disagree with the following statements about the Go for Green® initiative.

	STRONGLY DISAGREE	SOMEWHAT DISAGREE	NEITHER AGREE NOR DISAGREE	SOMEWHAT AGREE	STRONGLY AGREE
I find Go for Green® useful	1	2	3	4	5
Go for Green® motivates me to pay attention to what I eat/drink	1	2	3	4	5
I find Go for Green® simple to use	1	2	3	4	5
Go for Green® provides accurate information about food and nutrition	1	2	3	4	5

11. **Do you believe that you have received enough information about the Go for Green® approach to nutrition to help make decisions about what to eat or purchase outside of the dining facility?**
    1. yes
    2. no (skip to question 12)

"If you selected *YES*, where else do you use the Go for Green® guidelines to help make food choices?
- A. Commissary or local grocery store
- B. Restaurants and local eateries
- C. Cooking at home
- D. Other, please specify_____

12. **Go for Green® initiative encourages Soldiers to eat *Green-Coded* items.**
    1. Yes
    2. No

13. **Which of the following is most true about a *Green-coded* food or beverage?** *(Pick one response)*
    1. High in fiber
    2. Low in fiber
    3. Contains artificial sweeteners
    4. Contains trans fat

14. **Using the Go for Green® guidelines which of the following foods or beverages would most likely be *Green-coded*?** *(Pick one response)*
    1. Chocolate milk
    2. Broccoli with cheese sauce
    3. 100% fruit juice
    4. Frozen vegetables

15. **A *Green code* on a food or beverage means which of the following within Go for Green®?** *(Pick one response)*
    1. It must be a green colored food such as dark green leafy vegetables
    2. It must be low in calories
    3. It is the best choice for performance and should be eaten often
    4. All of the above

16. **Which of the following is most true about a *Yellow-coded* food or beverage?** *(Pick one response)*
    1. Nutrient packed whole food
    2. Automatically higher in sodium than Green-coded food or beverage
    3. May contain artificial sweeteners
    4. Mostly processed food, often with multiple unhealthy ingredients

17. **Which of the following is most true about a *Yellow-coded* food or beverage?** *(Pick one response)*
- Low-performance food that should be eaten rarely
- Moderate-performance food that should be eaten occasionally
- High-performance food that should be eaten often
- None of the above

18. **Using the Go for Green® guidelines what type of milk would be considered *Yellow-coded*?** *(Pick one response)*
- Skim, fat-free, or 1% milk
- Chocolate milk (made with skim, fat-free or 1% milk)
- Whole milk
- Half and half

19. **Which of the following is most true about a *Red-coded* food or beverage?** *(Pick one response)*
- Low-performance food that should be eaten rarely
- Moderate-performance food that should be eaten occasionally
- High-performance food that should be eaten often
- None of the above

20. **Which of the following is most true about a *Red-coded* food or beverage?** *(Pick one response)*
- Naturally packed with nutrients
- High in fiber
- Low in sodium
- High in added sugar

21. **Using the Go for Green® guidelines which of the following fruits would most likely be *Red-coded*?** *(Pick one response)*
- Fresh peach
- Sweetened applesauce
- Canned pears in light syrup
- Frozen strawberries without added sugar

22. **How often do you use the Go for Green® Food Card to help you decide what foods you will eat?**
- Never
- Rarely
- Sometimes
- Often
- Always

23. **How often do you use the Go for Green® Beverage Cards to help you decide what beverages you will drink?**
- Never
- Rarely
- Sometimes
- Often
- Always

24. **How helpful/informative are the following Go for Green® materials? Circle a rating of 0-5 with 0 =not helpful/informative at all to 5 = most helpful/informative for <u>each</u> material listed.**

	Have NOT seen	Rate between 0-5: 0 (not helpful/informative) to 5 (most helpful/informative)					
Go for Green® materials	(a)	0	1	2	3	4	5
Food and Beverage Cards in DFAC	(a)	0	1	2	3	4	5
Pamphlets/brochures	(a)	0	1	2	3	4	5
Social media (Facebook, Twitter, etc) post from DFAC	(a)	0	1	2	3	4	5
Social media (Facebook, Twitter, etc) post from Command	(a)	0	1	2	3	4	5
Posters in DFAC	(a)	0	1	2	3	4	5
Table tents DFAC	(a)	0	1	2	3	4	5

25. **From which sources would you <u>*PREFER*</u> to learn about G4G and about Performance Nutrition?**
(Choose all that apply)

(A) Print Media *(newspaper, press release, command newsletter, etc.)*
(B) Social Media *(Facebook, Twitter, etc.)*
(C) Posters
(D) Table tents in the dining facility
(E) Email
(F) Brief/class by Unit Leadership
(G) Informal discussion with Unit Leadership
(H) Brief/class with Performance-focused Dietitian
(I) Word of Mouth *(friends, peers, dining facility staff, etc)*
(J) Other, please specify: _____

26. **Please select how much you agree or disagree with each statement by placing a mark in the corresponding box.**

	STRONGLY DISAGREE	SOMEWHAT DISAGREE	NEITHER AGREE NOR DISAGREE	SOMEWHAT AGREE	STRONGLY AGREE
I'm just not that interested in the information on Go for Green® Food Cards.	1	2	3	4	5
The difference in meaning between the colors on the Go for Green® Food Cards is hard to understand.	1	2	3	4	5
It takes too much time to read the Go for Green® Food Cards.	1	2	3	4	5
I prefer getting nutrition information from other sources besides Go for Green® Food Cards.	1	2	3	4	5
I find the Go for Green® Food Cards easy to use.	1	2	3	4	5
I use the Go for Green® Food Cards to choose healthy foods.	1	2	3	4	5
I use the Go for Green® Food Cards to choose performance food.	1	2	3	4	5
I do not use the Go for Green® Food Cards since I already know which foods to eat.	1	2	3	4	5
I find it easier to choose healthy performance optimizing foods since the revised/rebranded Go for Green® came to the dining facility.	1	2	3	4	5
The name of the food on Food Card does not match the food being served, making it difficult to use.	1	2	3	4	5
I do not trust that the information on the Go for Green® Food Cards is accurate.	1	2	3	4	5
Colored Cards (e.g. Green, Yellow, Red) are more useful than caloric labeling.	1	2	3	4	5
I find the Go for Green® Food Cards useful, when making decisions about what to eat.	1	2	3	4	5
I am learning more about nutrition/fueling for performance through the Go for Green® Food Cards.	1	2	3	4	5

26. Continued: Please select how much you agree or disagree with each statement by placing a mark in the corresponding box.

	STRONGLY DISAGREE	SOMEWHAT DISAGREE	NEITHER AGREE NOR DISAGREE	SOMEWHAT AGREE	STRONGLY AGREE
I am learning more about nutrition/fueling for performance through the Go for Green® posters and table tents.	1	2	3	4	5
I am learning more about nutrition/fueling for performance through the Go for Green® Facebook and Twitter messages.	1	2	3	4	5
I choose more performance based foods since Go for Green® arrived.	1	2	3	4	5
I am NOT learning about nutrition from Go 4 Green®	1	2	3	4	5

27. Have you shared any of the information you learned from the Go for Green® program with others?

- Y Yes
- N No (skip to question 28)

*If you selected *YES*, with whom have you shared the information with?

- A Family members
- B Friends
- C Peers
- D Subordinates
- E Superiors
- F Other, please specify:_____

28. Do you intend to share any of the information you learned from Go for Green® with others?

- Y Yes
- N No (skip to question 29)

*If you selected *YES*, with whom do you intend to share the information with?

- A Family members
- B Friends
- C Peers
- D Subordinates
- E Superiors
- F Other, please specify:_____

29. What are the other ways you would recommend improving the Go for Green® initiative?

_____

_____

_____

Appendix F: G4G Awareness for Staff

Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09-HC)

Staff Survey

Thank you for participating in this study. Please answer the following questions by filling in the circles that corresponds with your answer. All of the information you provide will be kept confidential. Thank you.

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘⊠◒◔		MONTH, DAY, YEAR

Instructions: For all questions and/or statements presented in this survey, please select only **one (1)** response unless the question and/or statement indicate otherwise.

Participant Demographics

1. **How long have you worked at your current dining facility?**
   - Less than 1 month
   - 1 month to 3 months
   - 4 months to 7 months
   - 8 months but less than 1 year
   - 1 year but less than 3 years
   - 3 or more years

2. **What is your affiliation with this installation?**
   - Civilian Employee
   - Contractor
   - Military Service Member
   - Other, please specify: _____

3. **If you selected <u>Civilian Employee</u> in Question 2, what is your GS grade or equivalent?** (*Please bubble your selection*) If you did not select Civilian Employee in Question 2, please bubble "N/A".

   - OR -

4. **If you selected <u>Military Service Member</u> in Question 2, what is your rank?** (*Please bubble your selection*) If you did not select Service Member in Question 2, please bubble "N/A".

Enlisted	
Officer	
WO	

- OR -

Natick Form 5656 (One-Time) 18 Apr 2017

5. **What is your current job title at this dining facility?**
   1. Food Program Advisor
   2. Dining Facility Manager
   3. NCOIC
   4. Shift leader/ supervisor
   5. 92G *(Non-Supervisor)*
   6. Administrative
   7. Other *(please specify)*:_____

6. **In the last 6 months, did you attend any staff training sessions on G4G** *(or key G4G concepts not limited to food placement and maintaining food cards)*?
   1. Yes*
   2. No *(skip to question 7)*
   3. Unsure/Cannot remember

*If you chose ***YES***, how many G4G staff training modules *(15-30 minutes each)* have you participated in?

1 2 3 4 5 6 7 8 9

Participant Knowledge of G4G program

Select a one response for your level of agreement with the following statements:

	STRONGLY DISAGREE	DISAGREE	NEITHER AGREE NOR DISAGREE	AGREE	STRONGLY AGREE
7. **I fully understand my role and duties for the G4G program.**	1	2	3	4	5
8. **I understand that labeling a food item correctly** ***(e.g., Eggs are not labeled as pancakes)*** **is important to maintaining diner trust in the program.**	1	2	3	4	5

9. **When a food item is given the** ***wrong Food Card*** ***(e.g., Baked Chicken Food Card placed on Corn)*****, the** ***best*** **way to address it is to:**
   1. Replace the card myself
   2. Notify another staff member to replace the card
   3. Notify the NCOIC or DFAC manager
   4. No need to do anything; it's an equivalent product.
   5. If you can't find the correct card, address at the next meal

10. **When a food item is labeled with the** ***wrong color code*** ***(e.g., French fries are coded Green)*****, the** ***best*** **way to address is to:**
    1. Replace the card myself
    2. Notify another staff member to replace the card
    3. Notify the NCOIC or DFAC manager
    4. No need to do anything; it's an equivalent product.
    5. If you can't find the correct card, address at the next meal

11. **You notice there is a new recipe** ***(or an existing recipe has an ingredient change)*****, how can you** ***best*** **get the recipe re-evaluated for a possible new food card code?** *(Pick only one answer)*
    1. I can choose the codes myself
    2. Notify the admin team who can assign the codes
    3. Notify the NCOIC or DFAC manager who can assign the codes
    4. Notify the NCOIC or DFAC manager who can obtain the codes from the Dietitian
    5. No need to do anything; it's an equivalent product

12. **Which option is incorrect?**
   1. Green = High Performance Food
   2. Red = Low Performance Food
   3. Yellow = Low Performance Food
   4. All of the above

13. **Which of the following is a key part of the revised G4G program?** *(Pick only one answer)*
   1. Food placement with easy access to Red-coded foods and beverages
   2. Food labeling with a revised two-part coding: Green, Yellow, or Red for nutritional quality and Low, Moderate, or High for sodium content
   3. Coding that highlights only low-calorie options
   4. Any staff member can determine the coding for Green, Yellow, or Red food items if he/she thinks the color code is wrong
   5. Food and beverages with a Red color code means it is a high-performance fuel

14. **A food item's color code is Green. Which of these actions could result in a change of the item's actual color code to Yellow or Red?**
   1. Changing the cooking technique or preparation method for a food item *(e.g., fry vs. bake)*
   2. Adding or replacing an ingredient that is not in the recipe *(e.g., butter instead of oil)*
   3. Using a different type or cut of meat than the recipe states
   4. Adding more low-fat cheese or sour cream than is in the recipe
   5. All of the above can change the color code of a food item

15. **Which of the following can be found on G4G food and beverage cards?** *(Select all that apply)*
   - A. Sodium code *(Low, Moderate, or High)*
   - B. Calorie count
   - C. Color code *(Green, Yellow, or Red)*
   - D. Food or beverage name
   - E. Grams of sugar

16. **In the spaces provided, match the form of a cooked white potato with its correct color code.**

	GREEN	YELLOW	RED
Deep-fried French Fries	G	Y	R
Baked with skin	G	Y	R
Baked French Fries	G	Y	R

17. **Can Green-coded foods be high in sodium?**
   - Y. Yes
   - N. No
   - U. Unsure

Awareness of G4G implementation at dining facility

Food placement describes the different ways that food choices could be presented to encourage selection of specific menu offerings and promote healthy eating (e.g., placing Green-coded items first on the hot line, putting fruit in highly visible locations, etc.)

18. Does your dining facility use *food placement* to encourage healthy choices?

- Yes *
- No
- Unsure

*If *Yes*, please describe some of the food placement efforts at your dining facility. Please list the change and the serving station name. Example: "Fruit was placed next to dessert options."

_____

_____

_____

_____

19. Match the color code to the order each item should appear on the hotline:

	GREEN	YELLOW	RED
1st on Serving Line			
2nd on Serving Line			
Last on Serving Line			

20. Are original G4G promotional materials currently on display within your dining facility?

- Yes
- No
- Unsure

21. Which of the revised G4G promotional materials are found within your dining facility?
(Choose all that apply)

- Posters
- Table tents or napkin dispensers
- Brochures
- Other (please specify):_____
- Revised G4G promotional sources are not displayed

22. What are the biggest changes your dining facility has made to promote Green-coded foods? Please describe:

_____

_____

_____

Attitudes/Beliefs about the revised G4G's implementation

Answer the following questions on your thoughts and feelings of Go for Green® program and your dining facility environment

	NEVER	RARELY (~20% OF TIME)	SOMETIMES (~50% OF TIME)	OFTEN (~80% OF TIME)	ALWAYS	N/A - NOT INVOLVED WITH FOOD PREP
23. **How often does the Food Card NOT match the food item *(e.g., pancake label for bacon)*?**	0	1	2	3	4	
24. **How often do you see the kitchen staff make changes to recipes *(e.g., add butter or oil)* during food preparation?**	0	1	2	3	4	
25. **How often does the color code seem wrong for the food item *(e.g., steamed vegetables coded red)*?**	0	1	2	3	4	
26. **How often do you make changes to recipes *(e.g., add butter or oil)* during food preparation without relabeling?**	0	1	2	3	4	5

27. **To what extent do you agree or disagree with the following statements:**	STRONGLY DISAGREE	DISAGREE	NEITHER AGREE NOR DISAGREE	AGREE	STRONGLY AGREE
a. The G4G program encouraged our dining facility to add more healthy food options into our menu.	1	2	3	4	5
b. The G4G program makes it easier to find the performance nutrition/healthy options in our facility.	1	2	3	4	5
c. The G4G program is a valued program in our dining facility.	1	2	3	4	5
d. The G4G program is easy to apply at our dining facility/galley.	1	2	3	4	5
e. The G4G program's healthy items are popular with our diners.	1	2	3	4	5
f. The G4G program's implementation is time consuming to dining facility staff.	1	2	3	4	5
g. It is a hassle to find or display G4G Food Cards.	1	2	3	4	5
h. Adding more healthy items on the menu is a priority at our dining facility/galley.	1	2	3	4	5

28. **Please choose any of the following challenges you experienced while implementing the G4G program.** *(Choose all that apply)*

- (A) It was difficult to follow the recipes exactly as printed on the recipe cards.
- (B) It was difficult to place the correct G4G food card when food items change.
- (C) It was difficult to maintain G4G Food Cards *(e.g., clean and orderly)*.
- (D) Diners were confused by G4G Food Cards.
- (E) Food service staff could not answer diner's questions about the G4G program.
- (F) Implementing the G4G program was time consuming.
- (G) Sometimes we did not have the right ingredients to implement the new menus.
- (H) Other, please list _____
- (I) None, there were no challenges with implementation.

29. **Please explain in more detail some of the biggest challenges you had when implementing G4G program.**

30. **What recommendation(s) would you make to improve the G4G program within this dining facility/galley?**

31. **What resources would help the staff at this dining facility/galley to improve G4G implementation?**

32. **How can the program become more useful for diners?**

Appendix G: Demographic & Lifestyle for Staff

Demographics & Lifestyle Information

Thank you for participating in this study. Please answer the following questions by filling in the circles that corresponds with your answer. All of the information you provide be kept confidential. Thank you.

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊠ ◒ ◔		MONTH, DAY, YEAR 20__

1. Gender:
   - ○ Male
   - ○ Female

2. What is your age today?

AGE years

3. Your height in inches? (without shoes/boots)

HEIGHT inches

for reference:
5 feet = 60 inches
6 feet = 72 inches

4. Your weight in pounds? (without clothing)

WEIGHT pounds

5. What is your ethnic background?
   - ○ 1 Hispanic or Latino
   - ○ 2 Not Hispanic or Latino

RACE_OTHER

6. What is your racial background? (select all that apply)
   - ○ 1 White or Caucasian
   - ○ 2 Black or African American
   - ○ 3 Native American/Alaskan Native
   - ○ 4 Asian
   - ○ 5 Native Hawaiian/Pacific Islander
   - ○ 6 Other:_____

7. Please indicate the HIGHEST level of education you have completed (pick only one)
   - ○ 1 Some high school (no GED or diploma)
   - ○ 2 High school graduate (GED or diploma)
   - ○ 3 Some college courses
   - ○ 4 Associate degree (two-year college)
   - ○ 5 Bachelors degree (four-year college)
   - ○ 6 Graduate degree

8. What is your rank?

Enlisted	
Officer	
WO	

9. What is your primary MOS? Description: _____

10. How long have you been in the Armed Services?

Active Duty

1 Less than one year **OR** If a year or more, please fill in the number of years (start with leading zero's when needed).

Appendix H: Satiety Labeled Intensity Magnitude (SLIM) Scale
Appendix H1: Pre-SLIM

Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09-HC)

Pre-SLIM scale

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊠ ◒ ◔		MONTH DAY YEAR 20__

TIME (24 HR FORMAT)	PRE-MEAL ONLY: WHEN DID YOU LAST EAT SOMETHING	PRE-MEAL ONLY: WHAT WAS YOUR LAST MEAL	IF A SNACK, WHAT DID YOU EAT?
Station (A B C D)	1 Early morning snack* 2 Breakfast 3 Morning snack* 4 Lunch 5 Afternoon snack* 6 Dinner 7 Evening snack*	A B C	

Please indicate your level of hunger or fullness right now by placing a hash mark (/) on the scale below.

GREATEST IMAGINABLE FULLNESS
EXTREMELY FULL
VERY FULL
MODERATELY FULL
SLIGHTLY FULL
NEITHER HUNGRY NOR FULL
SLIGHTLY HUNGRY
MODERATELY HUNGRY
VERY HUNGRY
EXTREMELY HUNGRY
GREATEST IMAGINABLE HUNGER

DO NOT WRITE IN THIS BOX

slim code	snack code
	snack-1 snack-3 snack-5 snack-7

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Appendix H: Satiety Labeled Intensity Magnitude (SLIM) Scale
Appendix H2: Post-SLIM

Go for Green® Effectiveness for Changing Dietary Intake and Attitudes toward Nutrition for Performance Among Service Members (17-09HC)

Post-SLIM scale

MARKING INSTRUCTIONS	VOLUNTEER NUMBER	FILL IN TODAY'S DATE
• Use a No. 2 pencil only. • Do not use ink, ballpoint, or felt tip pens. • Make solid marks / fill the response completely • Erase cleanly any marks you wish to change. • Make no stray marks on this form. CORRECT: ● INCORRECT: ⊘ ⊠ ◒ ◔		MONTH DAY YEAR 20__

TIME (24 HR FORMAT)	POST-MEAL ONLY
Station (A) (B) (C) (D)	1. Was today's meal: (1) Shorter than usual (2) Typical (3) Longer than usual 2. Did you have enough time to eat what you want? (Y) Yes (N) No

Please indicate your response for the next questions by placing a hash mark (/) on the scale below.

3. How would you describe the speed you ate?

AS FAST AS POSSIBLE

DO NOT WRITE IN THIS BOX

AS SLOW AS POSSIBLE

4. Please indicate your level of hunger or fullness right now.

GREATEST IMAGINABLE FULLNESS
EXTREMELY FULL
VERY FULL
MODERATELY FULL
SLIGHTLY FULL
NEITHER HUNGRY NOR FULL
SLIGHTLY HUNGRY
MODERATELY HUNGRY
VERY HUNGRY
EXTREMELY HUNGRY
GREATEST IMAGINABLE HUNGER

DO NOT WRITE IN THIS BOX

Natick Form 5655 (One-Time) 18 Apr 2017

Appendix I: G4G Program Fidelity Checklist

Note: Reference with permission from G4G consultants.

G4G Management Checklist – Daily

Print for use during your daily maintenance walk-throughs.

Date: _____

Meal period (choose one): _____

Completed by: _____

Prepare menu items from approved recipes, and label them with the correct color and sodium codes.

Yes	No	Ensure Menu Coding Goals are met
		Is at least one Green-coded menu item offered on each line (Hotline, Deli Bar, Salad Bar, etc.) in each category as stated in the Menu Coding Goals?

Place Food Cards in the appropriate locations, and ensure they're clean and serviceable.

Yes	No	Check appearance of Food Cards or G4G Coding Signs
		Does the name on the Food Card match the food item prepared and served to diners?
		Does the color code make sense for the identified food?
		Does each menu item have an individual Food Card near or in front of it with color and (except beverages and fruit) sodium codes? Or is the G4G 8.5" × 11" Coding Sign displayed near the appropriate bar/serving station?
		Are Food Cards arranged in a way that is uncluttered and easy to read? Or is the G4G 8.5" × 11" Coding Sign clearly displayed and easy to read?
		Are Food Cards and holders clean and neat?
		If a menu item is switched out during service (for example, if one item ran out and was replaced with a different item), was the Food Card for the original item removed and replaced with a card that matched the new item?

Each serving area (Hotline, Deli Bar, Salad Bar, etc.) must have at least 3 food-placement strategies in place to market high-performance foods and beverages. See list in Appendix H: G4G Food-placement Goals.

Yes	No	Ensure placement of healthy food items
		For each serving line (Hotline, Deli Bar, Salad Bar, etc.), are Green-coded items displayed first on the line, followed by Yellow-coded ones, and then Red-coded items?
		Is water placed prominently at the Beverage Bar? For example, is there a tab on the soda fountain for water, or is infused water offered?
Serving Area:		Evaluate one serving area in detail for food-placement goals.
Goal 1:		
Goal 2:		
Goal 3:		

Food Promotion

Yes	No	Ensure promotion of healthy food choices
		Phases 1 and 2: Is there at least one Green-coded "Featured Meal" offered and displayed with a sample plate, photograph, or sign? **Phase 3 and Maintenance:** Is there at least one Green-coded "Featured Meal" offered and displayed with a sample plate, photograph, or sign at 3 stations?
Names of stations:		Which station(s) offered "Featured Meals" today?

G4G Management Checklist – Monthly

Print and complete monthly.

Take corrective action for any item that is not in compliance with your checklist. Identify any item(s) assessed as "not meeting" standards. These should include a record (including supervisor's name) of the reported issue(s), temporary solutions taken to address the matter(s), and recommendations for long-term solutions that will help prevent future recurrences.

Date: _____

Completed by: _____

Menu Items: Breakfast

Yes	No	Menu-item changes
		Have any new menu items been added?
		Have any recipe ingredients changed?
		If "yes," has this menu item been sent to the Certified G4G Coder for coding?
		If "yes," is a temporary white Food Card being used until coding is completed?
		Is coding completed and a new Green, Yellow, or Red Food Card being used?
		Are the Food Cards and white sticky labels or G4G Coding Signs in good condition (that is, no tears, holes, stains, etc.)?

Menu Items: Lunch

Yes	No	Menu-item changes
		Have any new menu items been added?
		Have any recipe ingredients changed?
		If "yes," has this menu item been sent to the Certified G4G Coder for coding?
		If "yes," is a temporary white Food Card being used until coding is completed?
		Is coding completed and a new Green, Yellow, or Red Food Card being used?
		Are the Food Cards and white sticky labels or G4G Coding Signs in good condition (that is, no tears, holes, stains, etc.)?

Menu Items: Dinner

Yes	No	Menu-item changes
		Have any new menu items been added?
		Have any recipe ingredients changed?
		If "yes," has this menu item been sent to the Certified G4G Coder for coding?
		If "yes," is a temporary white Food Card being used until coding is completed?
		Is coding completed and a new Green, Yellow, or Red Food Card being used?
		Are the Food Cards and white sticky labels or G4G Coding signs in good condition (that is, no tears, holes, stains, etc.)?

Marketing and Education

Yes	No	Marketing and educational materials
		Are posters displayed at eye level?
		Are permanent posters (Traffic Light, Food Cards, and Sodium) displayed at entrances and serving areas?
Date		Key-message posters should be rotated every 4 months. When were these last changed?
		Are G4G brochures neatly displayed in a highly visible location?
		Are table tents and table signs displayed on each table in the dining room?
		Are all marketing and educational materials in good condition (that is, no tears, holes, fading, stains, etc.)?
Yes	**No**	**Marketing and educational strategies**
		Has G4G been marketed outside your dining facility? Examples include articles in the installation newspaper or website, features in the health promotion or wellness department newsletter, and email announcements.
		Has G4G been marketed on social media? For example, have any G4G messages or graphics been posted on dining facility-level and command-level Facebook or Twitter accounts?
		Were any performance nutrition and/or G4G education talks offered to Service Members outside the dining facility this month? Refer to the nutrition asset on the local G4G Planning Team for verification.

Staff Training

Yes	No	Staff training should be up-to-date and verified
		Is 80% of current staff trained on G4G? Review staff training records.
		Have any new staff members arrived in the past month?
		If "yes," have they been oriented to G4G (target: within one month)?
		Was a G4G in-service, refresher training, or program update session provided to staff this month?
Module/Topic		**If "yes," which module or training topic?**
		Have staff members been trained on how to prepare and display any new menu items or ingredients?
Menu items		**If "yes," which items?**

Using the HEI-2010 vs HEI-2015 to determine measures of diet quality – where are the differences?

Champagne CM[1], Allen HR[1], Cole RE[2], Armstrong NJ[2], Miketinas DC[1]
[1]Pennington Biomedical Research Center, Baton Rouge, LA
[2]US Army Research Institute of Environmental Medicine, Natick, MA

Objective: The Healthy Eating Index (HEI)-2015 is a measure of diet quality that reflects the 2015-2020 Dietary Guidelines for Americans. The current version differs from HEI-2010 by separating the empty calories (moderation) component into saturated fats and added sugars. Legumes are now also represented in four components: total vegetables, greens and beans, seafood and plant proteins, and total protein foods. Diet quality studies completed with a military population have used HEI-2010. It has yet to be determined if these past HEI-2010 diet quality studies are translatable to the new HEI-2015. The objective of this study was to compare the 2010 vs 2015 versions of the HEI to determine whether these different calculation methods result in equivalent scores.
Methods: A subset of 143 Soldiers (with complete dietary data from breakfast, lunch and dinner) were selected from 2 dining facility studies. Digital photography methodology was used to capture meal trays before and after food consumption. Foods consumed were estimated by two trained dietitians and nutrient content calculated using the FNDDS 2013-2014. Dietary data were evaluated using both HEI calculation methodologies and analyzed using SAS® version 9.4 software.
Results: Mean HEI total scores differed significantly ($p<0.001$) between the 2015 and 2010 calculation methods (68.90 ± 11.5 vs. 69.76 ± 10.4, respectively). Component scores differed significantly for total vegetables ($p=0.005$), greens and beans ($p=0.013$), seafood and plant proteins ($p<0.001$), and total protein ($p<0.001$). Due to the change in the moderation category, the score in HEI-2015 vs HEI-2010 was lower (32.31 ± 4.2 vs 34.10 ± 5.2; $p<0.001$).
Conclusions: These results identified significant differences between HEI-2010 & 2015 versions for total scores, moderation composite scores, and legume-containing component scores. Direct translation between methodologies may not be possible and researchers should use caution when comparing results between HEI analysis versions. Although statistically different scores were obtained, clinical meaningfulness of the data should also be considered. Results may not be generalizable to the civilian population and should be confirmed with a larger sample size.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government

Soldiers Who Have Failed U.S. Army Body Composition Standards Report a Faster Eating Rate than Soldiers Who Have Not

Kristie L. O'Connor, J. Philip Karl, Susan M. McGraw, Adam J. DiChiara, Renee E. Cole

Eating at a fast rate is associated with overweight and obesity. Anecdotal reports, but little empirical evidence, suggest that Soldiers adapt to a faster eating rate during initial military training (IMT).

Objectives: To examine associations between self-reported eating rate (ER), years of military service, BMI, and history of passing U.S. Army body composition standards.

Methods: Questionnaires were administered to U.S Army recruits at the beginning of IMT (n=144) or 2 wk into IMT (n=162), and to Soldiers with ≤2 yr (n=296) or >2 yr (n=414) of military service. Mean ER over the past 30 d was assessed using both a categorical response question and 100 mm visual analog scale. Height, weight, and whether a Soldier had ever failed an Army body composition assessment was self-reported. Associations between ER and study outcomes, and whether these associations differed by time in service was assessed by ANCOVA and logistic regression.

Results: ER was 26% faster in recruits who were 2 wk into IMT relative to beginning recruits, 33% faster than Soldiers with ≤2 yr service, and 31% faster than those with >2 yr service ($P < 0.001$). Of those who were 2 wk into IMT, 73% reported eating at a fast/very fast rate relative to 49%, 38% and 40% of those in the beginning IMT, ≤2 yr, and >2yr groups ($P < 0.001$), respectively. There were no associations between ER and BMI. However, among active duty Soldiers (n = 700), the odds of having failed a body composition test were 2.8 times [95% CI: 1.1-7.2] higher in those who reported eating fast/very fast relative to those who reported eating slow/very slow ($P = 0.03$), and ER was 10% higher [95% CI: 3-16%] ($P < 0.001$).

Conclusion: Findings suggest an association between eating at a faster rate and being in IMT, and that eating at a fast rate is associated with a higher odds of having failed a body composition assessment. The cross-sectional design precludes determining whether the habit of eating fast develops during IMT, is sustained following IMT or whether perception of ER adjusts to a faster rate following IMT. Nonetheless, these data support the need for longitudinal studies assessing the impact of IMT on ER and the effect on body composition.

Disclaimer: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. Funded by Medical Research and Materiel Command.

Baseline Diet Adequacy of Army Soldiers Supports the Need for a Planned Dining Facility Intervention

Champagne CM[1], Allen HR[1], Cole RE[2], Armstrong N[2], McGraw SM[2], Moylan E[3], Miketinas DC[1]
[1]Pennington Biomedical Research Center, Baton Rouge, LA
[2]US Army Research Institute of Environmental Medicine, Natick, MA
[3]Uniformed Services University, Bethesda, MD

Background: Recent studies depict sub-optimal diet quality scores of Soldiers at a Fort Bragg dining facility (DFAC). Poor diet quality may contribute to the increasing military overweight rates and associated comorbidities. It is unclear if similar diet quality scores will be attained at other military installations.
Objective: The objective of this study was to determine Soldiers' current dietary intake and diet quality at a Colorado military dining facility (DFAC).
Study Design, Settings, Participants: In 2017, Pennington Biomedical Research Center partnered with US Army researchers to assess dietary intakes of Soldiers at a designated Fort Carson, CO DFAC. A total of 89 Soldiers were recruited and asked to consume three meals in the DFAC over a 2-day period. Soldiers' dining trays were captured using digital photography methodology before and after food consumption. Foods consumed were estimated by two trained dietitians.
Outcome Measures and Analysis: Foods consumed were analyzed for nutrient content using the FNDDS 2013-2014. The Healthy Eating Index-2015 (100 points maximum score), a measure of diet quality that reflects the most recent version of the 2015-2020 Dietary Guidelines for Americans, was calculated for soldiers who had complete dietary data. Data were analyzed using SAS® version 9.4 software.
Results Summary: Forty-one soldiers provided complete dietary data for at least one day (all 3 meals). Mean HEI-2015 total score was 63.1 $\pm$ 9.0 and the lowest component score was whole grain consumption (0.25 $\pm$ 0.7 points). Total protein reached 94.5% of the maximum score; while total fruits, vegetables and dairy reached 64.7%, 80.6%, and 67.4% of the target, respectively.
Conclusions and Implications: Low diet quality scores support the need for a DFAC nutrition intervention aimed to improve food offerings and Soldiers' healthy eating scores at the Fort Carson DFAC. These results echo the need for interventions to increase diet quality in military dining facilities. Soldiers tasked with defending our nation need to be in optimal nutritional status.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government

A Nutrition Knowledge Comparison of Army Culinary Staff and the Soldiers They Feed

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Background (Background, Rationale, Prior Research, and/or Theory): Warfighter diet quality has a direct impact on health and performance. Military dining facilities (DFAC) are a common source of dietary intake and DFAC culinary staff often provides nutrition information to service members during meal selection. However, it is uncertain if staff members possess a higher nutrition knowledge (NK) than their patrons.

Objective: Evaluate and compare the NK of DFAC staff members and their patrons.

Study Design, Setting, Participants, Intervention: A 19-question true/false military NK survey was administered to 48 staff and 125 patrons at Fort Carson, CO. A confidence score was also included to stratify NK scores. Possible question outcomes were confident and correct (CC), confident but incorrect (CI), unconfident and correct, or unconfident and incorrect. Data analysis was restricted to CC and CI responses. Volunteers were predominately male (71%), junior enlisted Soldiers (90%) between the ages of 18 and 34 years.

Outcome Measures and Analysis: Descriptive data are reported as mean ± standard deviation (SD) or frequency (n / %). Independent t-test and chi-square analyses were conducted comparing patron and staff total NK score, %CC, and %CI scores.

Results: CC scores (mean ± SD) were similar between culinary staff (40 ± 16%) and patrons (41 ± 21%), and CI scores were higher for staff (31 ± 16%) compared to patrons (25 ± 16%; P = .024). More staff responded CC that at least half of the plate should be fruits and vegetables (90% vs. 70%; P = .021) but staff had lower CC scores on questions related to dietary fat (50% vs. 77%; P = .008) and protein as an energy source (13% vs. 38%; P = .005). Patrons who had ≥3 hours of nutrition education had higher NK scores (53% CC) compared to only 1–2 hours (39% CC; P = .044) and no education (39% CC; P = .015).

Conclusions and Implications: DFAC culinary staff are not more knowledgeable about nutrition than patrons and may unknowingly provide inaccurate information. Future initiatives should consider a minimum three hours of nutrition education for DFAC staff to promote dissemination of accurate dietary guidance.

Funding: U.S. Army Research Institute of Environmental Medicine.

Baseline Dietary Intakes of Army Soldiers Prior to Go For Green® 2.0 Dining Facility Dietary Intervention

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Background: Recent studies depict sub-optimal diet quality of Soldiers at a Fort Bragg dining facility (DFAC) using the Healthy Eating Index (HEI). The HEI-2015 (100 points maximum score) reflects the most recent version of the 2015-2020 Dietary Guidelines for Americans, while Military Dietary Reference Intake (MDRI) recommendations account for specific military operational needs and should also be considered. Poor diet quality may contribute to the increasing military overweight rates and associated comorbidities and may be linked to poor job performance related to less than optimal dietary intakes. This has prompted further efforts to implement interventions in DFACs focusing on healthier options for soldiers and increasing diet quality. The Go For Green® version 2.0 (G4G 2.0) initiative, which specifically incorporates MDRI requirements, is undergoing revisions and being implemented at a number of DFACs in the U.S. The G4G 2.0 implementation team is working with a designated DFAC in Fort Carson, CO to launch the revised program.

Objective: The objective of this study was to determine Soldiers' current dietary intake and diet quality at baseline at a Fort Carson, CO, DFAC.

Study Design, Settings, Participants: In 2017, Pennington Biomedical Research Center partnered with US Army researchers to assess dietary intakes of Soldiers at a designated Fort Carson, CO DFAC. A total of 89 Soldiers were recruited and asked to consume three meals in the DFAC over a 2-day period. Soldiers' dining trays were captured using digital photography methodology before and after food consumption. Foods consumed were estimated by two trained dietitians.

Outcome Measures and Analysis: Foods consumed were analyzed for nutrient content using the Food and Nutrient Database for Dietary Studies (FNDDS) 2013-2014. HEI-2015 scores are based upon 1000 calorie intake and were calculated for soldiers who had complete dietary data. HEI-2015 components were revised and now consist of: fruit, total vegetable, greens and beans, whole grains, refined grains, dairy, total protein foods, seafood & plant proteins, fatty acid ratio, sodium, added sugars, and saturated fats. A good/healthy quality diet is defined as a HEI-2015 total score of ≥81 points, while 50-80 points needs improvement and <50 points is considered a poor quality diet. Data were analyzed using SAS® version 9.4 software.

Results Summary: Thirty-eight male soldiers provided complete dietary data for at least one day (all 3 meals). Comparisons of the average nutrient intakes were made between recommendations for men

specified in the Military Dietary Reference Intakes (MDRI). Average caloric intake was 2867 ± 617 kcal/day, falling short of the Military Dietary Reference Intake (MDRI) general value of 3250 kcal/day. Protein intakes averaged 119.8 ± 23.7 g/day or 16.6% of energy, more than adequately meeting the MDRI for males of 91 g/day (range of 65-154 g/day) and 10-15% of protein from total energy intake. Dietary fat intake averaged 37.6% of energy; although there is no MRDI for dietary fat intake beyond the recommendation to not exceed 30% of energy intake. Dietary cholesterol intake averaged 626 ± 187 mg/day, more than double the MDRI recommendation for less than 300 mg/day. Dietary fiber averaged 20.4 ± 5.2 g/day falling short of the Dietary Guidelines for Americans recommendation (14 g per 1000 kcal) as there are no dietary fiber recommendations in the MDRI. Mean HEI-2015 total score was 62.9 ± 9.0 points (range 42.7 to 81.8) and the lowest component score was whole grain consumption (0.27 ± 0.7 points). Total protein reached 94.4% of the maximum score; while total fruits, vegetables and dairy reached 63.4%, 80.6%, and 68.5% of the target, respectively.

Conclusions and Implications: These data suggest that although certain target nutrients were met or exceeded, the overall average consumption falls short of good/healthy diet quality classification. Low diet quality scores support the need for a DFAC nutrition intervention aimed to improve food offerings and Soldiers' healthy eating scores at the Fort Carson DFAC. Emphasis should be placed on specific components of the diet contributing to a low HEI diet quality score as well as meeting the MDRI and then re-evaluated to assess the effectiveness of the nutrition intervention. This is the first military study using HEI-2015 scores to determine Soldier diet quality while past studies have used previous version of the HEI. Thus researchers and healthcare providers should use caution when comparing and interpreting HEI scores from previous studies as the component scores have changed. Direct translation between methodologies may not be possible and should be further investigated.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. Funded by Medical Research and Materiel Command.

A Nutrition Knowledge Comparison of Army Culinary Staff and the Soldiers They Feed

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Background: Warfighter diet quality has a direct impact on health and operational performance. Military dining facilities (DFAC) are a common source of dietary intake and DFAC culinary staff often provides nutrition information to service members during meal selection. However, it is uncertain if staff members possess a higher nutrition knowledge (NK) than their patrons. More so, data captured from previous studies examining nutrition knowledge are limited to correct versus incorrect scores, which may not provide the clearest insight in knowledge gaps.

Methods: Two questionnaires were administered to 48 staff and 125 patrons at Fort Carson, CO that collected data on NK, sources of nutrition information, and demographic information. Nineteen true/false questions were used to assess NK. A dichotomous measure of confidence was included to stratify NK scores. Possible question outcomes were confident and correct, confident but incorrect, unconfident and correct, or unconfident and incorrect. Data analysis was restricted to confident correct and incorrect responses. Data were analyzed for descriptive statistics using IBM Statistical Package for Social Sciences (SPSS) v24. Descriptive data are reported as mean ± standard deviation (SD) or frequency (n / %). Independent t-test and chi-square analyses were conducted comparing patron and staff total NK score, % confidently correct, and % confidently incorrect scores.

Results: Volunteers were predominately male (71%), junior enlisted Soldiers (90%) with less than 2 years of military service (50%) between the ages of 18 and 34 years. Confidently correct scores (mean±SD) were similar between culinary staff (40±16%) and patrons (41±21%), and confidently incorrect scores were higher for staff (31±16%) compared to patrons (25±16%; p=0.024).

When looking at individual questions, more staff responded confidently correct that at least half of the plate should be fruits and vegetables (90% vs. 70%; p=0.021) but staff had lower confidently correct scores on questions related to dietary fat (50% vs. 77%; p=0.008) and protein as an energy source (13% vs 38%; p=0.005). Staff and patrons were confidently incorrect in questions related to whole milk vs. 2% or skim milk (76% patron vs 61% staff), the need for post-exercise recovery beverage or snack (87% patron vs. 97% staff), and the protein supplementation with vegetarian lifestyle (65% patrons vs. and 69% staff).

In comparison between demographic groups, patrons who had ≥3 hours of nutrition education had higher confidently correct NK scores (53%) compared to only 1-2 hours (39%; p=0.044) and no education (39%; p=0.015). Secondary findings demonstrate no difference related to factors such as rank, Army Physical Fitness Test scores, age, or sex within the patron population. Patrons who had some college or a college degree (n=52) had higher confidently correct NK scores (48%) then Patrons with only a high school degree (37%; p=0.008).

Staff and patrons provided sources of information in attaining NK. The top sources of information were websites (29%), gym personnel (25%), health professionals (24%), fellow Soldiers (24%), and social media (22%). Volunteers who listed only health professionals as their source of information (n=42) scored higher (46%) than volunteers who listed other or multiple sources (38%; p=0.038).

Conclusions: This is the first study that asked volunteers about their response confidence to determine if the lack of nutrition knowledge is based upon misconception of truth versus guessing. While confidently incorrect answers could be identified in an open response format, the analytical burden is high compared to a dichotomous measure of confidence. With this additional insight, military nutrition education programs can accurately capture true gaps in knowledge. For instance in this study, DFAC culinary staff were not more knowledgeable about nutrition compared to patrons; however, low confidently correct and high confidently incorrect scores amongst staff supports the possibility of offering inaccurate information to the patron's they serve. It is unclear if the source of information is contributing to the knowledge misconception as 25% of the volunteers reported attaining information from unreliable sources. Future initiatives should consider a minimum 3 hours of nutrition education for DFAC staff and warfighters, and target the most common sources of information, to promote dissemination of accurate dietary guidance.

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Soldier Body Satisfaction is Associated with Undesirable Weight Management Behaviors and Stress in Meeting Body Composition Standards.

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Background: In civilian populations, poor body image amongst individuals in weight-class sports or professions where body dimensions are considered important is associated with undesirable eating and weight management behaviors. Military personnel have pressure to meet performance, appearance, and body composition standards biannually to maintain career progression. This purpose of this study was to compare body image among Soldiers to civilian norms, and examine associations between personal body image, perceived stress / confidence in meeting body composition standards, and self-reported weight management practices. Body image was assessed by the Multi-Dimensional Body-Self Relation Questionnaire (MBSRQ). The MBSRQ is a validated tool commonly used to assess body image in civilian populations, but has not been used in a military population.

Methods: In this cross-sectional study, 435 U.S. Army Soldiers (72% M; 22±4 yr, BMI range: 17-39 kg/m^2) were administered the MBSRQ and additional questionnaires that assessed height and weight, eating behaviors, and weight management practices. Five MBSRQ subscales were examined (scale 0-5 points for each): 1) Appearance Evaluation (AE; happiness with physical appearance), 2) Appearance Orientation (AO; importance of appearance and grooming), 3) Overweight Preoccupation Scale (OPS; fat anxiety, weight vigilance, dietary restraint), 4) Body Area Satisfaction Scale (BASS; happiness with body area size), and 5) Self-Classified Weight Class (SCW; perception of one's weight). AE and BASS data were dichotomized into normal vs. abnormal (<3.1 pts for AE and BASS) body image groups based upon previous studies in which the MBSRQ was evaluated in individuals with body dysmorphia. Misclassification of body weight was defined as a discrepancy between actual and perceived BMI categories (i.e., underweight, normal weight, overweight). Descriptive and comparative statistics were performed using SPSS v.24 with alpha = 0.05.

Results: Mean MBSRQ subscale scores of the Soldiers (mean±SD) were similar to civilian norms (18-29 y): 3.6±0.7 AE (normative scores: 3.6 pts), 3.5±0.8 BASS (normative scores are 3.4 pts female; 3.6 pts male), and 3.1±0.7 SCW (normative scores are 3.0-3.6 pts). However, 24% of Soldiers reported abnormal AE scores (mean±SD: 2.6±0.4 pts), 30% reported abnormal BASS scores (2.6±0.5 pts), 40% misclassified their BMI, and 46% of normal BMI Soldiers fell within the abnormal body image category. BMI was higher in the abnormal vs normal AE group (26.0±3.8 vs 24.9±3.5kg/m^2; p=0.01), but did not differ between BASS groups (normal 25.0±3.4 vs. abnormal 25.6±3.9 kg/m^2). Of the 40% that misclassified their BMI (83% male; 17% female), 79% of men self-classified into a lower-than-calculated BMI category, whereas 63% females self-classified into a higher-than-calculated BMI category (p<0.001). Relative to those with normal AE and BASS scores, those with abnormal body image scores more frequently reported

often/always skipping meals (20% abnormal vs 6% normal), taking laxatives (7% vs 2%), sitting in a sauna/steam room (11% vs 5%), doing neck exercises to improve body fat measurement (12% vs 8%), and using Preparation H and/or wrapping body in plastic (9% vs. 4%) as a method to meet body composition standards (all $p<0.05$). In addition, relative to those with normal AE and BASS scores, a greater proportion of those in the abnormal AE and BASS groups reported feeling very stressed about meeting the body composition standards (20% vs. 5%), not being confident that they could pass their next body composition assessment (30% vs. 9%), and were intentionally trying to lose weight (62% vs. 40%)(all $p<0.05$).

Conclusions:
While mean body image scores in this military population were similar to civilian norms, nearly one third of the Soldiers possessed a poor body image, half of whom were classified as normal weight. Soldiers with a poor body image score were more likely to engage in undesirable weight management behaviors, and were more stressed, and less confident in meeting the Army body composition standards. Previous research suggests that problematic behaviors related to body image may lead to detrimental health outcomes (i.e. increased injuries, chronic dehydration, eating pathologies). Whether improving body image could reduce undesirable weight management behaviors in Soldiers, and the relationship between poor body image with health and injury risk in Soldiers, warrants further examination.

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Funded by Medical Research and Materiel Command.

Eating Behaviors are Associated with Body Composition and Physical Fitness Standards Failure among US Army Soldiers

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Abstract

Objective: US Army Soldiers must comply with body composition (BC) and physical fitness (PF) standards as a condition for military entry and retention. Whether eating behaviors are associated with meeting these standards is unknown. This cross-sectional study aimed to determine the specific eating behaviors associated with failing BC and PF standards, and to characterize the eating behaviors and other habits of Soldiers who are high performers as defined by high PF scores and consistently meeting BC standards.

Methods: Soldiers (N=1,460, 93% enlisted, 83% male, 23.5 ± 5.2 years of age with 3.5 ± 4.0 years of service) completed a questionnaire to assess their eating behaviors and history of compliance with BC and PF standards. Multivariate adjusted logistic regression models using maximum likelihood estimation were used to examine relationships between Soldiers' eating behaviors and failing standards. Bivariate analysis was used to test differences between high (HP) and low performers (LP).

Results: Eating mostly (>50% of meals in last 7 days) at a dining facility lowered the odds of BC failure by 2.63 times (OR: 0.38, 95% CI: 0.22, 0.64); whereas, eating mostly Meals, Ready-to-Eat (MRE), eating at a fast rate, and ignoring fullness cues raised the odds by 2.23 times (95% CI: 1.15, 4.35), 2.65 times (95% CI: 1.18, 5.94) and 1.90 times (95% CI: 1.16, 3.11), respectively. Eating mostly at restaurants, being distracted while eating, and extreme hunger prior to meals, raised the odds of PF failure by 1.70 times (95% CI: 1.17, 2.48), 1.68 times (95% CI: 1.13, 2.51), and 1.55 times (95% CI: 1.08, 2.24), respectively. HP were significantly less likely than LP to eat mostly at restaurants (25% HP, 39% LP) and were significantly more likely than LP to report longer work hours (33% HP, 28% LP) ($p<0.05$).

Conclusions: Findings suggest that specific eating behaviors of Soldiers are associated with failing BC and PF standards. As eating behaviors may be modifiable, these findings could identify opportunities for improving the specificity of Army health promotion and education programs aimed at increasing force readiness and performance.

Funding Source: US Army Medical Research and Materiel Command

Disclaimer: Authors' views do not reflect official DoD or Army policy.

Testosterone Administration During Energy Deficit Suppresses Hepcidin Concentrations and Increases Iron Utilization in Healthy Males (OR15-08-19)

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Objectives: Military personnel are exposed to periods of energy deficit and stress, which is typically accompanied by reductions in testosterone concentrations and declines in iron status. Exogenous testosterone administration may increase erythropoiesis and iron availability.

The objective of this study was to determine the effects of exogenous testosterone on iron homeostasis in healthy young males during energy deficit.

Methods: Fifty males (24.8 ± 5.0 y, 78.6 ± 12.2 kg) completed this randomized, double-blind, placebo-controlled trial. Participants were provided a eucaloric diet during a 2-week free-living phase (day 1–14). Following the free-living phase, participants completed a 4-week live-in phase (day 15–42). During the live-in phase participants were randomized to 200 mg testosterone enanthate/week ($n = 24$) or 1 mL sesame oil/week (placebo, $n = 26$) and were in an energy- and exercise-induced energy deficit equal to 53 ± 4%. Blood was collected on days 14 and 42 to determine concentrations of testosterone, hemoglobin, hematocrit, ferritin, hepcidin, and erythroferrone. Data are reported as change scores (mean ± SD).

Results: Total testosterone concentrations increased with testosterone and declined with placebo (569 ± 271 nmol/L and −83 ± 142 nmol/L, $P < 0.0001$). Testosterone attenuated the decline in hemoglobin (−0.04 ± 0.7 g/L and −0.6 ± 0.8 g/dL, $P < 0.01$) and hematocrit (0.6 ± 2.2% and −1.3 ± 2.1%, $P < 0.01$) that occurred with placebo. Hepcidin (−3.7 ± 5.0 ng/mL and 2.1 ± 5.0 ng/mL, $P < 0.001$) and ferritin (−32.5 ± 38.6 ng/mL and 15.4 ± 41.2 ng/mL, $P < 0.0001$) were reduced with testosterone as compared to placebo. Erythroferrone (−0.5 ± 2.1 ng/mL and 0.5 ± 2.0 ng/mL, $P = 0.11$) did not differ when comparing testosterone to placebo.

Conclusions: Testosterone administration during energy deficit suppressed hepcidin concentrations and increased iron utilization in healthy young males. The reduction in hepcidin with testosterone administration appears to occur through an erythroferrone-independent mechanism.

Funding Sources: The views expressed are those of the authors and do not reflect the official policy of the Army, DoD, or the U.S. Government. Supported by the Collaborative Research to Optimize Warfighter Nutrition II and III projects and the Defense Health Program Joint Program Committee-5.

Military Dining Facility Staff Perspectives on the Implementation Barriers and Facilitators of Go for Green 2.0, a Department of Defense Nutrition Initiative

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Background: In an effort to optimize performance, readiness, and health of military service members, the Department of Defense (DoD) created a performance nutrition initiative, Go for Green (G4G). Challenges led to program revision and development of G4G 2.0, however successful implementation has yet to be accomplished.

Objective: The purpose of this study was to improve the G4G 2.0 program framework by informing and developing best practices through explication of dining facility (DFAC) staff perspectives.

Study Design, Settings, Participants: Focus groups were conducted in two phases (May 2017 to February 2018) with supervisors and staff of Wolf DFAC, at Fort Carson, Colorado prior to G4G 2.0 implementation. Participants were recruited by purposive sampling (n = 89). Focus groups were audio recorded to capture all verbal responses, transcribed verbatim, and lasted an average of 31 minutes (range 24 to 58 minutes).

Measurable Outcome/Analysis: Focus group transcripts of DFAC staff perspectives were reviewed via qualiP175 (continued) Continued on page S113 S112 Poster Abstracts Journal of Nutrition Education and Behavior Volume 51, Number 7S, 2019 tative content analysis using an exploratory, inductive approach to elucidate emergent themes from the data.

Results: DFAC staff perspectives of barriers to G4G implementation included lack of, or ineffective training, inconsistent preparation of food items, fidelity issues, staff perceptions of patron preferences, lack of patron familiarity to new G4G food items and lack of buy-in. Implementation facilitators included training, G4G team site visits, and patron exposure to G4G menu items. Staff suggestions for G4G implementation and program improvement included training on preparation methods, having a flexible implementation timeline, up front provision of all G4G materials, for the G4G team to conduct a needs assessment, and G4G and nutrition education and awareness outside the DFAC.

Conclusion: Results demonstrated that barriers to G4G 2.0 implementation outweighed facilitators, and supported the idea that there may be more viable, less time and work-intensive methods to promote healthier food selection by patrons. Incorporating methods to improve patron selection of healthful items in DFACs could play an instrumental role in improving Service Member health, thereby enhancing readiness to meet mission requirements.

Funding: United States Army Research Institute of Environmental Medicine.

Using the Healthy Eating Score-5 as a measure of diet quality in an Active Duty Military population

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Abstract

Background: Having an accurate and time-efficient method of assessing diet quality is important to understand the nutritional health of a population, and to guide health promotion and dietary interventions. The five-question Healthy Eating Score-5 (HES-5) was developed to quickly assess diet quality in a military population in place of the 110-question Block food frequencies questionnaire (FFQ) and Healthy Eating Index (HEI) developed in 2005. Although the HEI was updated in 2015, the association with the HES-5 has not been re-assessed. This study aimed to determine the correlation between the HES-5 and the HEI-2015, and if that correlation could be strengthened by adding questions to the HES-5.

Methods: Soldiers (N = 446, 91% male, 89% enlisted, 23 ± 3 years old) completed the HES-5, five additional diet-related questions, and the validated Block FFQ. HEI-2015 scores were calculated from responses to the Block FFQ. The HES-5 asked respondents about their frequency of vegetable, fruit, whole grain, dairy, and fish consumption. Each category was scored on a 5-point scale with higher scores indicating more frequent consumption. The five additional questions assessed alcohol (ALC), energy drink (ED), sugar-sweetened beverage (SSB), and breakfast consumption (BC), and dining out (DO) frequency. ALC, ED, SSB, and DO were reverse scored. Various combinations of these questions were then added to the HES-

5. Normality was assessed, and Pearson's correlation was used to determine which combination of HES-5 and additional questions best correlated to the HEI-2015.

Results: Mean ± SD HEI-2015 scores were 59 ± 10 out of 100 points matching the average HEI-2015 score in the American population and reflecting poor diet quality. HES-5 scores averaged 11 ± 4 out of 25 points. The means of the highest and lowest quartile of HES-5 scores were 16 ± 2 and 6 ± 2 respectfully. Only 0.2% of the sample received a score of 25 which indicates meeting the dietary guidelines. There was as moderate, positive correlation between the original HES-5 and HEI-2015 (r = 0.41, N = 433, p < 0.01). Of the various HES question combinations assessed, this correlation increased the most with the addition of the SSB, DO and ED questions (HES-8; r = 0.54, N = 433, p < 0.01; mean ± SD 22 ± 5 out of 40 points).

Conclusion: Results indicate that, in our population, the correlation between the HES-5 and HEI-2015 was similar to previously reported correlations between the HES-5 and HEI-2015 (r = 0.41, N = 333, p < 0.0001). However, adding three questions related to SSB, ED, and DO strengthened the correlation between the HES-5 and the HEI-2015. The HES-8 therefore provides a better assessment of diet quality than the currently used HES-5. In future versions of the HES, rephrasing HES questions to target consumption amount instead of frequency may improve correlation with the HEI-2015 which is based on amount consumed. Research to assess the validity of the HES-8 using more precise dietary assessment methods is also needed.

Disclaimer: The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of the Army/Navy/Air Force, Department of Defense, or U.S. Government.

Funding Source: US Army Medical Research and Materiel Command

Emotional Eating Behavior Mediates the Association between Perceived Stress and Body Weight Status

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Abstract

Background: Overweight and obesity among Soldiers is a major concern for the US Army because it must maintain a healthy and fit fighting force. Research in civilian populations demonstrates overweight and obesity to be associated with eating in response to external cues, such as perceived stress, but this association has not been examined in Soldiers who face unique stressors and eating environments. This cross-sectional study aimed to examine the relationship between failing body composition (BC) standards, body mass index (BMI), perceived stress (PS), and emotional eating (EE) among Soldiers, and whether the effect of EE on study outcomes differed by sex. **Methods:** Soldiers (N=1,460, 83% male, 93% enlisted, 23.5 ± 5.2 years of age with 3.5 ± 4.0 years of service) from seven Army posts completed validated surveys that obtained information on eating behaviors or mediators of eating behaviors including, PS, EE, adherence with military BC standards, and sociodemographic information. Hierarchical logistic and linear multiple regression analysis was implemented to test EE as a mediator between PS and BMI, and between PS and BC failure. Sobel-Goodman test of mediation was used to determine the percentage of the relationship between study outcomes and PS explained by EE. **Results:** Mean ± SD PS score were 17.5 ± 4.7 and 17.7 ± 4.4 for men and women, respectively (p=0.46), indicating moderate PS. Sex differences in Soldiers reporting EE behaviors sometimes or often were significantly different at 29% and 44% of men and women, respectively (p<0.001). Higher PS was associated with more frequent self-reported EE behaviors (p<0.001), higher BMI (p<0.001), and higher odds of BC failure (95% CI: 1.02, 1.09). EE was a complete mediator in the relationship between BMI and PS and a partial mediator in the relationship between BC failure and PS. The effect of EE on BMI and BC failure was statistically significant with approximately 30% (p=0.01) and 35% (p<0.001) of the total effect being mediated, respectively.

Conclusions: EE behaviors fully explain the association between PS and BMI. PS was also associated with BC failure, with the odds of BC failure being higher among Soldiers who exhibited EE behaviors compared to those who did not. These findings suggest EE behaviors may be potential intervention targets within multidisciplinary behavioral health programs aiming to prevent and manage overweight and obesity among Soldiers.

The association between military weight status and body image in a sample of active duty Soldiers

Maria J. Stukenborg, Bethany A. Deschamps, Susan M. McGraw, Julianna M. Jayne, J. Philip Karl, Renee E. Cole

Background: Negative body image has been correlated with depression, unhealthy dieting behaviors, and elevated body mass index (BMI). Studies indicate that athletes in sports with body composition requirements often report increased body dissatisfaction. Soldiers are likewise required to meet body composition and physical fitness standards during their time in service. However, the impact of military weight status on body image among Soldiers has not been thoroughly studied. This study aimed to identify the association between military weight status, as measured by compliance with body composition standards, and time in service with body image among Soldiers.

Methods: Surveys were administered to active-duty Soldiers (N=969, 86% male, 91% enlisted, 23.5 ± 5.2 years of age, 3.5 ± 4.2 years of service) to collect data on military weight status, time in service, body image, and demographics. Twelve body image questions were rated on a 5-point Likert-type scale wherein higher scores represented a more unfavorable body image. In statistical models, body image questions were grouped together (12 items; α=0.88) and Soldiers were dichotomized into 2 groups based on the mean of the scores: less-favorable (≥ 35.5 points) and favorable (< 35.5 points body) image groups. Military weight status was determined by whether a Soldier had ever failed a body fat tape test during their military career. Multivariate logistic regression models were used to examine the relationship between military weight status, time in service, and body image with the covariates of sex, education, marital status, current duty station, race/ethnicity, and rank.

Results: The mean ± SD total body image score was 35.5 ± 8.2 and was normally distributed. Soldiers who reported having failed the body fat tape test (N=148, 25.5 ± 5.6 years of age, BMI of 29.5 ± 3.1) were 5.65 times ($p < 0.001$; 95% CI: 3.30, 9.70) more likely to have a less-favorable body image than those who had never undergone the body fat tape test (N = 138, 23.5 ± 5.3 years of age, BMI of 23.5 ± 2.4). Soldiers who had been taped but never failed the test (N = 659, 23.8 ± 5.5 years of age, BMI of 25.7 ± 3.3) were 1.51 times ($p = 0.034$; 95% CI: 1.03, 2.22) more likely to have a less-favorable body image than those who had never undergone the body fat tape test. There was not a significant relationship between time in service and body image.

Conclusion: Findings support an association between undergoing the body fat tape test and a less-favorable body image among Soldiers. Since negative body image in civilian populations has been linked to increased depression, undesirable eating behaviors, and a higher BMI, further studies examining the effects of body composition standards on Soldiers' body image are warranted.

Current Word count: 448

Disclaimer: The views expressed in this abstract are those of the author(s) and do not reflect the official policy of the Department of the Army/Navy/Air Force, Department of Defense, or U.S. Government.

Funding Source: US Army Medical Research and Materiel Command

NOTE: MHSRS Requirements:
Title restricted to 255 characters
Abstract restricted to 500 words

Does Go For Green 2.0 Joint Service Performance-Nutrition Initiative Improve Dietary Intake of Soldiers?

Champagne CM[1], Miketinas DC[2], Allen HR[1], Cole RE[3,4], McGraw SM[3], Moylan E[5], Armstrong N[3]
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Background: Poor diet quality may contribute to increased military overweight rates and associated comorbidities. Go For Green® version 2.0 (G4G 2.0) is a project to improve soldiers' food environment to optimize performance, readiness, and health. Objective - to determine if soldiers' diet quality at baseline improved after implementation of G4G 2.0 at a Colorado military dining facility (DFAC).
Methods: Soldiers recruited at baseline (n=89) and post G4G 2.0 implementation (n=45) were asked to consume three meals in the DFAC over a 2-day period per iteration. Soldiers consuming only one meal were excluded. Soldiers' before/after dining trays were captured using digital photography. Foods consumed, estimated by two trained dietitians, were analyzed for nutrient content (FNDDS 2015-2016). Healthy Eating Index-2015 (HEI-2015; 100 points maximum score), diet quality measure reflecting the 2015-2020 Dietary Guidelines for Americans, was calculated for soldiers consuming at least 2 different meals. Data analysis used SAS® version 9.4 software.
Results: At baseline, HEI-2015 total score was 62.9 $\pm$ 9.0 pts (n=75). Lowest component score was whole grain (0.45 $\pm$ 1.5 pts); total protein was 94.0% of maximum score, and total fruits, vegetables and dairy were 69.6%, 77.7%, and 63.3% of target, respectively. After G4G 2.0 implementation, mean HEI-2015 total score was 71.2 ± 10.9 pts (n=40, $p < 0.001$). Whole grain consumption increased (3.9 ± 2.9, $p<0.001$), protein was 97.5% of maximum score, and total fruits, vegetables and dairy were 68.1%, 79.1%, and 72.0% of target, respectively.
Conclusions: Soldiers tasked with defending our nation should be in optimal nutritional status. Low baseline diet quality scores supported need for a DFAC nutrition intervention aimed to improve food offerings and soldier nutrient intake. These results suggest that G4G intervention can help to increase diet quality in military dining facilities.

Disclosure: Views expressed do not reflect Dept. of Army/Defense or U.S. Government official policy.

126 May 27 10:15 AM - 10:30 AM

Testosterone Supplementation Upregulates Myogenesis And Attenuates Proteolytic Gene Expression During Severe Energy Deficit

Emily E. Howard[1], Lee M. Margolis[1], Claire E. Berryman[2], Harris R. Lieberman[1], J. Philip Karl[1], Andrew J. Young, FACSM[1], Monty A. Montano[3], William J. Evans[4], Nancy R. Rodriguez, FACSM[5], Neil M. Johannsen[6], Kishore M. Gadde[6], Melissa N. Harris[6], Jennifer C. Rood[6], Stefan M. Pasiakos, FACSM[6].
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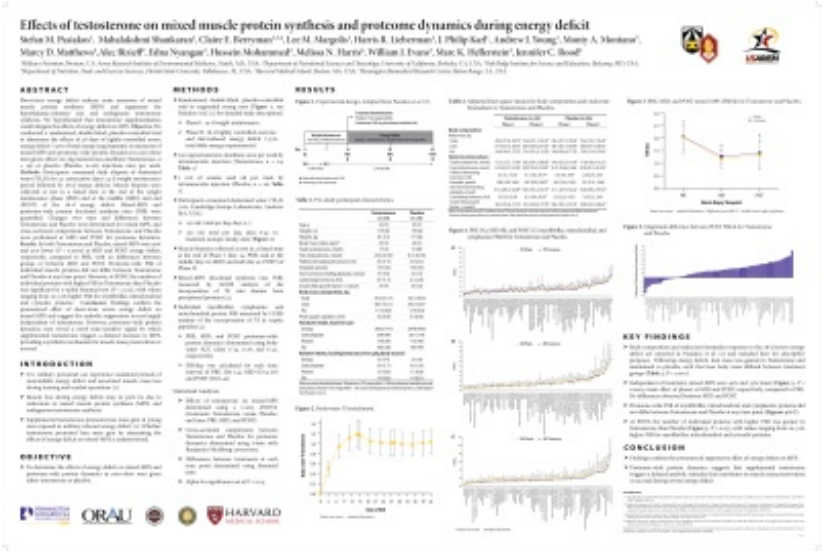
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BACKGROUND: Testosterone supplementation promotes whole-body lean mass accretion during severe energy deficit in non-obese, young men. The intramuscular mechanisms mediating this effect remain undefined.
PURPOSE: Determine the effect of supplemental testosterone on androgen receptor (AR), anabolic, proteolytic, inflammatory, and myogenic pathways during energy deficit.
METHODS: Fifty men (mean ± SD; 25 ± 5 y, 25 ± 3 kg/m^2) completed a 14-d weight maintenance (WM) phase, followed by a 28-d, 55% diet and exercise-induced energy deficit (ED) with 200 mg testosterone enanthate/week (TEST, n = 24) or placebo (PLA, n = 26). Muscle biopsies (vastus lateralis) from a subset (n = 10 per group) of participants exhibiting the greatest increase or decrease in leg lean mass (DEXA) and total testosterone were assayed to assess phosphorylation status, total protein and gene expression using Western blotting and RT-qPCR. Biopsies were collected at the end of WM and ED at baseline, and at 1 and 6 h after exercise (1 h cycle ergometry matched between phases for power and total work), with a mixed-meal (40 g protein) consumed following the first post-exercise biopsy.
RESULTS: Change (ED – WM) in leg lean mass (1.2 ± 0.7 vs. -0.7 ± 0.4 kg) and total testosterone concentrations (712 ± 159 vs. -193 ± 78 ng/dL) differed between TEST and PLA (P < 0.05). Relative to WM, baseline AR total protein was 118 % higher for TEST than PLA (P < 0.05) and was associated with changes in leg lean mass and fibroblast growth factor-inducible 14 (Fn14) gene expression (r = .540 and -.563, P < 0.05). Baseline Fn14 gene expression relative to WM was 52 % lower for TEST than PLA (P < 0.05) and tended to associate with changes in leg lean mass (r = -.455, P = 0.058). After exercise (6 h), muscle atrophy F-box and muscle ring finger-1 gene expression was lower (61 and 44 %) and MyoD expression was 122 % higher for TEST than PLA (P < 0.05), relative to WM. Mechanistic target of rapamycin-mediated anabolic signaling was not different between groups.
CONCLUSIONS: The hypertrophic effect of testosterone on lean mass during severe energy deficit may be due, in part, to reduced ubiquitin-mediated proteolysis and increased myogenesis after exercise and feeding, subsequent to modulation of upstream AR and Fn14 signaling. Supported by USAMRDC; authors' views not official U.S. Army or DoD policy.

(P10-042-20) Effects of Testosterone on Mixed Muscle Protein Synthesis and Proteome Dynamics During Energy Deficit

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Topical Area: Energy and Macronutrient Metabolism


Effects of testosterone on mixed muscle protein synthesis and proteome dynamics during energy deficit

Objectives: Short-term energy deficit reduces acute measures of mixed muscle protein synthesis (MPS) and suppresses the hypothalamic-pituitary axis and endogenous testosterone synthesis. We hypothesized that testosterone supplementation could mitigate the effects of energy deficit on MPS. We conducted a randomized, double-blind, placebo-controlled trial to determine the effects of 28 days of tightly-controlled severe energy deficit (deficit 55% of total energy requirements) on measures of mixed-MPS and proteome-wide protein dynamics in non-obese men either given 200 mg testosterone enanthate (Testosterone, n = 24) or placebo (Placebo, n=26) injections per week.

Methods: Participants received daily aliquots of deuterated water (^2H_2O) for 42 consecutive days (14-d weight maintenance period followed by 28-d energy deficit). Muscle biopsies were collected at rest in a fasted state at the end of the weight maintenance phase (PRE) and at the middle (MID) and end (POST) of the 28-d energy deficit. Mixed-MPS and proteome-wide protein fractional synthesis rates (FSR) were quantified. Changes over time and differences between Testosterone and Placebo were determined for mixed-MPS, and cross-sectional comparisons between Testosterone and Placebo were performed at MID and POST for proteome dynamics.

Results: In both Testosterone and Placebo, mixed-MPS were 40% and 33% lower ($P <$ 0.0005) at MID and POST energy deficit, respectively, compared to PRE, with no differences between groups or between MID and POST. Proteome-wide FSR of individual muscle proteins did not differ between Testosterone and Placebo at any time point.

However, at POST, the number of individual proteins with higher FSR in Testosterone than Placebo was significant by 2-tailed binomial test ($P < 0.05$), with values ranging from 20-32% higher FSR for myofibrillar, mitochondrial and cytosolic proteins.

Conclusions: Findings confirm the pronounced effect of short-term severe energy deficit on mixed-MPS and suggest the anabolic suppression occurs largely independent of testosterone. However, proteome-wide protein dynamics may reveal a novel time sensitive signal by which supplemental testosterone triggers a delayed increase in MPS, providing a synthetic mechanism for muscle mass preservation or accrual.

Funding Sources: Supported by DHP JPC-5/MOMRP; authors' views not official U.S. Army or DoD policy

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(P10-007-20) Longitudinal Comparison of Lean Body Mass by Dual Energy X-Ray Absorptiometry and Muscle Mass by Creatine (methyl-d3) Dilution in Response to a 28-d Severe Energy Deficit

eventscribe.com/2020/ASN/fsPopup.asp

Topical Area: Energy and Macronutrient Metabolism

Objectives: We reported that healthy males supplemented with testosterone gained lean body mass (LBM) during 28-d of energy deficit and 14 d of ad libitium feeding when measured by dual energy x-ray absorptiometry (DXA), but with no increase in muscle strength. We were unable to determine whether LBM gains were due to muscle mass accrual since DXA does not deliniate muscle from visceral organs and body water.
Objectives: To assess the effects of testosterone supplementation on muscle mass as measured by creatine (*methyl*-d_3) dilution, and determine the relationship between muscle mass and DXA-measured LBM in response to short-term energy deficit.

Methods: Secondary analysis of a 3-phase, randomised, double-blind, placebo controlled trial in healthy males: 14-d free-living, eucaloric phase (P1); 28-d live-in, 55% energy deficit phase with (200 mg testosterone enanthate/wk, TEST, n = 24) or without (PLA, n = 26) testosterone (P2); and 14-d free-living, ad libitum feeding phase (P3). Muscle mass was measured by creatine dilution and LBM by DXA at the end of each phase.

Results: We previously reported increased LBM in TEST (mean change from P1 ± SEM; 2.5 ± 0.4, $P < 0.01$), but not PLA (-0.3 ± 0.3 kg, $P > 0.05$), following P2. Both TEST (5.2 ± 0.4 kg) and PLA (2.2 ± 0.4 kg) gained LBM in P3 ($P < 0.01$). There was a treatment-by-phase trend for change in muscle mass (P-interaction = 0.054), but muscle mass data were highly variable and no post-hoc comparisons met statistical significance (PLA, P2: -0.3 ± 1.6; TEST, P2: -0.7 ± 1.7; PLA, P3: -0.3 ± 1.6; TEST, P3: 3.8 ± 1.7 kg; $P > 0.05$). Cross-sectional measures of muscle mass were correlated ($P < 0.001$) with total and appendicular LBM at P1 ($r = 0.63$ and 0.67), P2 ($r = 0.71$ and 0.72), and P3 ($r = 0.55$ and 0.48), respectively. However, changes in muscle mass were not associated with changes in total or appendicular LBM at P2 or P3 ($P > 0.05$).

Conclusions: Testosterone supplementation increased LBM in response to short-term energy deficit and recovery feeding, but had no significant effect on muscle mass or muscle function. The discordance between changes in LBM and muscle mass underscore the inherent limitations of LBM as a surrogate measure for skeletal muscle mass, particularly in response to short-term intervention studies.

Funding Sources: DHP JPC-5/MOMRP; authors' views not official U.S. Army or DoD policy.

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Nutrition interventions in military dining facilities can enhance diet quality and meal satisfaction

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ABSTRACT

Objective: Determine the effectiveness of the Go for Green® (G4G) nutrition program to improve diet quality and meal satisfaction of US Army soldiers. G4G® incorporates strategic food placement, new recipes, and color-coded nutrition labels.

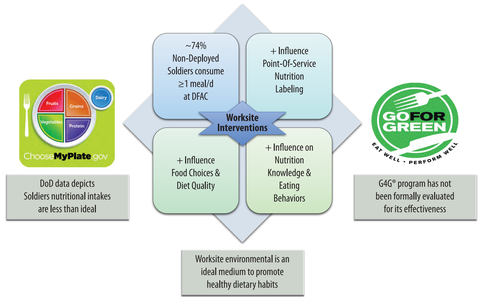
Methods: Of 179 study participants who consumed at least one meal, 100 (92% M, 22.6±3.2 years, 63 pre; 37 post - G4G® program) consumed 3 daily meals at a military dining facility in a time-series study. Questionnaires and food photography estimations provided data on demographics, diet quality (Healthy Eating Index – HEI 2015) and meal satisfaction. Independent t-test, Mann Whitney U, or chi-square were conducted using SPSS version 26 to assess changes pre/post (alpha set at $p<0.05$).

Results: Diet quality (pre/post HEI scores, Mean ± SD) improved from 55.7 ± 9.5 to 64.1 ± 8.8, $p<0.001$; attributed predominantly to 3 of 13 HEI constructs (3.6 pts whole grains, 1.1 pts seafood/plant proteins and 1.9 pts refined grains, all $p<0.05$). When any combination of 3 meals were assessed, fatty acids increased by 1.2 pts ($p=0.038$). More patrons agreed that main dishes were healthy/performance-based (32% pre; 57% post; $p<0.001$), vegetarian options were available (25% pre; 38% post; $p=0.023$), and they used nutrition labels to choose performance foods (38% pre; 54% post; $p=0.039$).

Conclusions: G4G® program successfully improved access to healthy/performance-based foods and improved the patron's satisfaction and meal quality

Learning Objective: Describe a strategy to improve and evaluate meal quality and satisfaction in a cafeteria-style environment.

BACKGROUND



INTERVENTION: GO FOR GREEN® 2.0

Go for Green 2.0 (G4G®) is a multifaceted Department of Defense dining facility program, designed to improve the nutritional fitness of service members and increasing access to high-performance foods.

What you eat matters; Go for Green® (G4G) 2.0 promotes fueling to optimize performance, readiness and health.

✓ Menu Coding Goals	✓ Food Placement
✓ Promotion of Green-coded Items	✓ Standard Food Cards
✓ Printed Materials	✓ Marketing Plan
✓ Standardized Staff Training	✓ Program Requirements

GREEN, YELLOW, & RED FOOD CODES

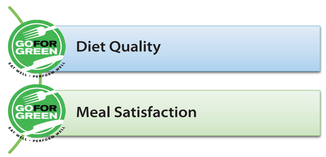
PROCESSING	LEAST-PROCESSED	SOME PROCESSING	MOST-PROCESSED FOODS
NUTRIENTS	WHOLE FOODS, NUTRIENT PACKED	SOME HEALTHFUL NUTRIENTS	LOWEST-QUALITY INGREDIENTS
FIBER	HIGH IN FIBER	LOWER IN FIBER	MINIMAL FIBER
SUGAR	LOW IN ADDED SUGAR	ADDED SUGAR OR ARTIFICIAL SWEETNERS	ADDED SUGAR OR ARTIFICIAL SWEETNERS
FAT	HEALTHY FATS	POOR-QUALITY FATS	EXCESS FATS AND/OR TRANS FAT FRIED FOODS





OBJECTIVES AND STUDY DESIGN

Determine the effectiveness of the G4G® 2.0 program to increase/improve garrison dining facility (DFAC) patrons



- **Design:** Time series. New participants recruited pre and post G4G®.
- **Locations:** Wolf DFAC at Ft. Carson and Freeman Café at Ft. Hood.
- **Subjects:** Military patrons consuming meals at study location DFACs.

METHODS

- Of 179 study participants who consumed at least one meal, 100 (92% M, 22.6±3.2 years, 63 pre; 37 post - G4G® program) consumed 3 daily meals at a military dining facility in a time-series study.
- Questionnaires and food photography estimations provided data on demographics, diet quality (Healthy Eating Index – HEI 2015) and meal satisfaction.
- Independent t-test, Mann Whitney U, or chi-square were conducted using SPSS version 26 to assess changes pre/post (alpha set at $p<0.05$).

FOOD PHOTOGRAPHY



RESULTS

- Diet quality (pre/post HEI scores, Mean ± SD) improved from 55.7 ± 9.5 to 64.1 ± 8.8, $p<0.001$
- Improvements in to 3 of 13 HEI constructs (3.6 pts whole grains, 1.1 pts seafood/plant proteins and 1.9 pts refined grains, all $p<0.05$).
- When any combination of 3 meals were assessed, fatty acids increased by 1.2 pts ($p=0.038$).
- More patrons agreed on following improvements:
  - Main dishes were healthy/performance-based (32% pre; 57% post; $p<0.001$)
  - Vegetarian options were available (25% pre; 38% post; $p=0.023$)
  - They used nutrition labels to choose performance foods (38% pre; 54% post; $p=0.039$).

RESULTS

Table 1. Demographic Characteristics

Variables	Pre (n=63)	Post (n=37)	P-value
Age (years)	22.7±3.2	22.5±3.2	0.643
Gender (% Male)	92%	92%	0.976
Education			0.802
Some HS/HS	60%	62%	
Post HS to AAS	35%	35%	
BS or Grad	5%	3%	
Rank			0.016*
E1-E4	95%	87%	
E5-E7	2%	14%	
BMI	25.3±2.9	25.0±3.0	0.41
Physical Readiness Rating			0.74
Good-Best Shape	76%	73%	
Neither Good Nor Bad	21%	24%	
Not Good- Worst Shape	3%	3%	

*$p<0.05$ pre- post intervention

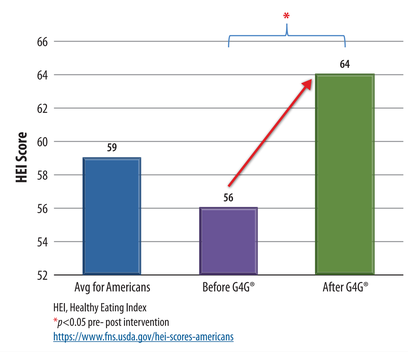


Figure 1. HEI-2015 Overall Score Change

Table 2. Diet Quality – HEI-2015 Score

HEI-2015 Domains & Total Score (points)	Max Points Available	Control DFAC Patron Intake Pre (n=63) Mean (SD)	Post (n=37) Mean (SD)	Change	P-value
Total Fruit	5	3.4 (1.8)	3.4 (1.9)	No change	0.88
Whole Fruit	5	3.8 (1.9)	2.7 (2.2)	Decreased	0.06
Total Vegetables	5	3.8 (1.2)	4.3 (1.0)	Improved	0.10
Greens and Beans	5	2.8 (1.8)	3.1 (1.9)	Improved	0.28
Whole Grains*	10	0.3 (0.9)	3.9 (2.9)	Improved	0.00*
Dairy	10	6.8 (2.9)	6.7 (2.9)	No change	0.76
Total Protein Foods	5	4.8 (0.4)	4.9 (0.1)	Improved	0.09
Seafood and Plant Proteins*	5	2.0 (2.1)	3.2 (2.1)	Improved	0.01*
Fatty Acids	10	5.8 (2.5)	6.8 (2.3)	Improved	0.09
Refined Grains*	10	7.6 (2.6)	9.5 (1.6)	Improved	0.00*
Sodium	10	2.1 (2.4)	2.2 (2.4)	Improved	0.85
Added Sugars	10	7.2 (2.9)	8.0 (2.6)	Improved	0.19
Saturated Fats	10	5.3 (2.8)	5.3 (2.7)	No change	0.68
HEI Total Score	100	55.68 (9.5)	64.1 (8.8)	Improved	<0.001*

Significant change, $p<0.05$

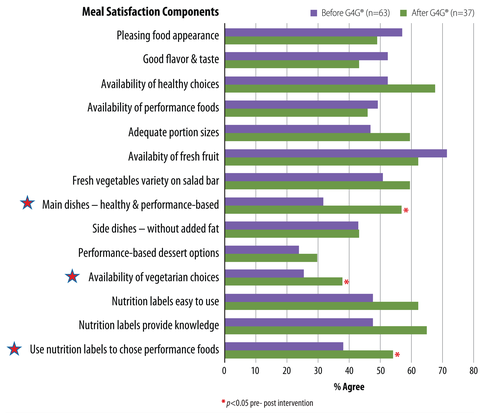
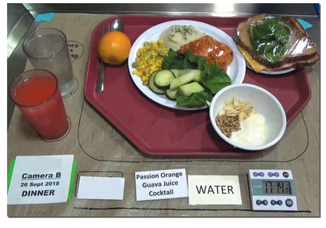


Figure 2. Meal Satisfaction

KEY FINDINGS

- G4G® was successful in improving the meal quality and either maintained or improved meal satisfaction
- G4G® was successful in enabling patrons to select performance-based food choices.
- Several barriers were identified; strategies could be developed to mitigate challenges.



CONCLUSIONS

- G4G® program successfully improved access to healthy/performance-based foods and improved the patron's satisfaction and meal quality.

IMPLICATIONS

- Military dining facilities are important avenues to improve diet quality and nutrition-related behaviors to optimize warfighters fueling requirements.

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FUNDING SOURCE:
US Army Medical Research & Development Command, Military Operational Medicine Research.

DISCLAIMER:
The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government.

Nutrition interventions in military dining facilities can enhance diet quality and meal satisfaction

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Objective: Determine the effectiveness of the Go for Green® (G4G) nutrition program to improve diet quality and meal satisfaction of US Army soldiers. G4G® incorporates strategic food placement, new recipes, and color-coded nutrition labels.

Methods: Of 179 study participants who consumed at least one meal, 100 (92% M, 22.6±3.2 years, 63 pre; 37 post - G4G program) consumed 3 daily meals at a military dining facility in a time-series study. Questionnaires and food photography estimations provided data on demographics, diet quality (Healthy Eating Index – HEI 2015) and meal satisfaction. Independent t-test, Mann Whitney U, or chi-square were conducted using SPSS version 26 to assess changes pre/post (alpha set at $p<0.05$).

Results: Diet quality (pre/post HEI scores, Mean ± SD) improved from 55.7 ± 9.5 to 64.1 ± 8.8, $p <0.001$; attributed predominantly to 3 of 13 HEI constructs (3.6 pts whole grains, 1.1 pts seafood/plant proteins and 1.9 pts refined grains, all $p<0.05$). When any combination of 3 meals were assessed, fatty acids increased by 1.2 pts ($p=0.038$). More patrons agreed that main dishes were healthy/performance-based (32% pre; 57% post; $p<0.001$), vegetarian options were available (25% pre; 38% post; $p=0.023$), and they used nutrition labels to choose performance foods (38% pre; 54% post; $p=0.039$).

Conclusions: G4G program successfully improved access to healthy/performance-based foods and improved the patron's satisfaction and meal quality.

Implications: Military dining facilities are important avenues to improve diet quality and nutrition-related behaviors to optimize warfighters fueling requirements.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government.

Funding: US Army Medical Research & Development Command, Military Operational Medicine Research.

Leaning Objective: Describe a strategy to improve and evaluate meal quality and satisfaction in a cafeteria-style environment.

2021 MHSRS Abstract: Diners Nutrition Knowledge

Title: Nutrition knowledge deficit identified among military dining facility patrons

Asma S. Bukhari, PhD, RDN[1], Nicholes J. Armstrong MS, RDN[1]Catherine M. Champagne, PhD[2], Susan M McGraw, BS[1], Katie M. Kirkpatrick, MS, RDN[3,4], Jennifer L. Billington, MS, RDN[5], Patricia A. Deuster, PhD, MPH[3], Renee E. Cole, PhD, RDN[6]

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The authors have no financial interests or relationships to disclose.

Funding: US Army Medical Research & Development Command, Military Operational Medicine Research.

Learning Objectives

1. Discuss baseline nutrition knowledge of warfighters using military dining facilities.
2. Describe change in diners nutrition knowledge with exposure to a dining facility nutrition programs such as Go for Green® (G4G) program.
3. Elucidate top five sources of nutrition information utilized by warfighters using military dining facilities.

ABSTRACT

Background: Nutrition programs, such as Go for Green® (G4G) used in military dining facilities (DFAC), are designed to enable warfighters to easily select nutritious food choices. The

G4G program promotes a performance-based menu through strategic food placement and user-friendly food labeling at point of service. Nutrition knowledge of military DFAC diners may impact food choices and requires further investigation.

Objective: This analysis aimed to assess the nutrition knowledge of warfighters consuming meals at military DFAC as part of a larger effort to evaluate the G4G program.

Methods: Nutrition knowledge (NK) of diners (n=269) was assessed at two military DFAC pre- and post- G4G 2.0 program implementation. The 18-item NK assessment tool is a scale from a validated survey used to assess military eating behaviors (Military-Specific Eating Behavior Survey) and consists of general and performance nutrition questions. Self-reported demographics, health, and lifestyle data were collected. NK scores were compared between diners with (n=103) and without (n=166) G4G program 2.0 exposure and with (n=201) and without (n=68) at least one hour of nutrition education. Data were analyzed for descriptive statistics by using IBM Statistical Package for Social Sciences (SPSS) v26 and reported as mean ± standard deviation (SD) or frequency (%). The Wilcoxon signed rank sum test compared NK total score and specific NK question responses (four response outcomes: confident and correct, confident but incorrect, unconfident and correct, or unconfident and incorrect) with or without G4G 2.0 program exposure and nutrition education.

Results: DFAC diners were 100% enlisted, (mean ± SD) 22.4±2.2 years, 80% male with 2.4±2.8 year of service. There were no significant differences between baseline variables related to demographics, body mass index, sleep and physical activity among diners with or without G4G 2.0 program exposure or nutrition education. The overall NK scores were similar between G4G program 2.0 exposure groups: 59.8±16% not exposed vs. 59.9±16% exposed (p=0.75). Of the 18 NK questions, only one yielded significant difference is response proportion between the G4G exposure groups. A greater proportion of G4G exposed diners were correct and confident regarding the difference in nutrient source between whole, 2%, and 1% milk (22.3% vs. 15.5%, p=0.046). When grouped by nutrition education, a greater proportion of diners with at least one hour of nutrition education were confident and correct in their responses for two NK questions compared to diners without nutrition education: at least half of the food on their plate should be fruits and vegetable (53.7% vs. 67.6%; p=0.029) and dark green vegetables, eggs and fortified cereal are sources of dietary iron sources (42.8% vs. 66.2%; p=0.001). When NK of the overall

sample of diners (n=269) was examined by question, concerning results were identified for the propotion of confidentally incorrect in the following seven areas: a recovery drink or snack should always be consumed after exercise (65.4%), protein is an important source of energy for physical activity (46.5%), most military personnel require four times more protein than civilians (32.7%), sports beverage are a preferred beverage when exercising at moderate intensity (25.7%), dietary fats is unimportant for a balanced diet (20.4%), meat is a source of fiber (24.5%) and vitamins and minerals as a source of energy (25.3%). Only 18% of diners self-reported being extremely/very knowledgeable about their overall nutrition knowledge. The top five sources of nutrition information identified as 'helpful' by diners were nutrition websites (40.5%), nutrition health professionals (38.7%), gym and fitness personnel (37.9%), fellow soldiers (33.1%), and social media (31.2%).

Conclusions: This study highlights the widespread knowledge deficits (<60% correct) among warfighters on general and performance nutrition. The NK scores were similiar in those with or without G4G 2.0 program exposure or with or without nutrition education. Knowlegdge deficits were primarly attritubted to eight NK areas. The G4G 2.0 program is designed to translate nutritional guidelines into high-performance menu options and promote selection of more nutritious food choices; however, but does not educate on these eight NK deficit areas. The top sources for NK reported by diners may be contributing to the high proportion of confidentally incorrect responses. Poor NK could be one of the many factors hindering improvement in overall meal quality as noted during the G4G 2.0 program evaluation.

Implications: Strategies to enhance basic and performance NK of warfighters is needed. Diners eating in military DFAC could benefit from strategically placed nutrition messaging in close proximity to performance-based menu choices. The G4G 2.0 program has precrafted social media messages on the G4G website for education about the G4G program and performance nutrition basics. These can be utilized to increase reach and promote selection of nutritious food choices. Arming diners with credible nutrition information can influence nutrition-related decisions regardless of the eating environment.

Go for Green® program evaluation finds nutrition knowledge gaps among foodservice staff

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The authors have no financial interests or relationships to disclose.

Funding: US Army Medical Research & Development Command, Military Operational Medicine Research.

Learning Objectives

1. Discuss the role of foodservice staff in optimizing warfighters fueling efforts in military dining facilities.
2. Describe the use of a novel approach to assess nutrition knowledge including incorporating confidence along with correct and incorrect responses to highlight nutrition knowledge gaps.
3. Discuss the foodservice staff nutrition knowledge gaps and level of nutrition program associated training.

ABSTRACT

Background: Research on effectiveness of nutrition programs in military dining facilities (DFAC) such as Go for Green® (G4G) and others have shown significant improvement in warfighter food choices. However, compliance to such programs implementation has been less than optimal. Foodservice staff are essential stakeholders in such endeavors to maximize

program implementation and fidelity. The nutrition knowledge (NK) of foodservice staff has not been previously investigated.

Objective: The purpose of this study was to examine the nutrition knowledge of foodservice staff during G4G program implementation at two DFAC.

Methods: Foodservice staff (n=184) at two DFAC completed two questionnaires either pre or post G4G program implementation on NK and G4G program information. A demographic survey was added after the first study location pre data collection with a resultant smaller sample size (n=134). The NK questionnaire consisted of true/false questions on performance nutrition - macronutrients, micronutrients, energy, dietary supplements, and hydration and if confident in the response (yes/no). In addition to percent correct responses, the NK responses were recatorgized into four response outcomes: confident and correct, confident but incorrect, unconfident and correct, or unconfident and incorrect. Recategorized values were summed for a total NK score ranging from 1-54 points. Data were analyzed for descriptive statistics by using IBM Statistical Package for Social Sciences (SPSS) v26. Descriptive data are reported as mean ± standard deviation (SD) or frequency (%). Wilcoxin rank sum test was used to compare total NK score and each NK question between staff with or without G4G training. Response precentages were reported as either confidently correct or confidentaly incorrect.

Results: Study participants were primary military (73%), non-supervisory (73%; 53% of which were junior enlisted), male (60%), aged (mean ± SD) 25.7±8 year, with 2.4±2.8 years in service. Only 25% of staff completed 50% of the G4G training and 2.3% completed 100% of the training. More staff agreed they understood their role and duties for the G4G program after G4G training compared to those responding as no/unsure to G4G training (84% vs. 54%). Overall percent of NK questions correctely anwered was 55.0±1.3% with a mean score of 29.2±0.6 points; however, NK percent correct were significantly higher among G4G-trained staff compared to those without G4G training (58.0±1.7% vs. 52.9±2.1%, p=0.025). A greater proportion of G4G-trained staff were confident and correct in their responses compared to those without training regarding protein requirements for military (38% vs. 21%, p=0.013) and identifying good sources of iron (62% vs. 41%, p=0.006). Although there were no other significant difference between NK responses for G4G-trained and untrained staff, over 25% of staff were confidently incorrect on ten NK areas: need for a recovery beverage or snack post exercise (72.8%), protein

as a source of energy (64.7%), dietary supplement purity and safety (57%), nutrient source between whole, 2%, and 1% milk (42.4%), protein needs of military personnel (41.8%), recommendation for sports drinks after moderate intensity exercise (39.7%), vitamins and mineral as sources of calories (34.8%), meat as a source of fiber (34.2%), meeting dietary vitamin and mineral needs (33.7%) and good food sources of calcium (27.7%). Regardless of the level of training or NK, over 85% of staff acknowledged the importance of correct food labeling to maintain diner trust in the program.

Conclusions: Poor NK scores were noted for those with or without the provided G4G training sessions. This could be attributed to poor attendance to full G4G training, as well as the focus of training on program implementation vs. improving nutrition knowledge. Foodservice staff are force multipliers during nutrition interventions implementation and poor nutrition knowledge could limit engagement during various phases of program implementation and program maintenance. Innovative interactive strategies are needed to assess, educate, and empower foodservice staff at fast-paced military DFAC operations.

Implications: Enhanced nutrition knowledge can enable staff to operate as better stewards of the DFAC nutrition programs as they translate program requirements to actual foods served.Enhancing nutrition knowledge of military DFAC staff can likely benefit themselves and those they feed.

Nutrition interventions in military dining facilities can enhance diet quality and meal satisfaction

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Objective: Determine the effectiveness of the Go for Green® (G4G) nutrition program to improve diet quality and meal satisfaction of US Army soldiers. G4G incorporates strategic food placement, new recipes, and color-coded nutrition labels.

Methods: A time series study enrolled 179 participants (92% M, 22.6±3.2 years) who consumed at least one meal of which 100 (55.9%) consumed a breakfast, lunch and dinner meal at a military dining facility before or after the G4G program implementation (63 pre- and 37 post-test). Demographic and meal satisfaction data was collected via survey. Food photography provided data on total diet quality (Healthy Eating Index [HEI-2015]) and 13 component scores by meal and meal type. Independent t-test, Mann Whitney U, or chi-square were conducted using SPSS version 26 to assess changes pre/post (alpha set at $p<0.05$ with 80% power).

Results: Diet quality (pre/post HEI scores, Mean ± SD) improved from 55.7 ± 9.5 to 64.1 ± 8.8, $p <0.001$; attributed predominantly to 3 of 13 HEI constructs (3.6 pts whole grains, 1.1 pts seafood/plant proteins and 1.9 pts refined grains, all $p<0.05$). HEI changes by meal: Breakfast Pre (n=160) 48.1±12.3 to Post (n=73) 57.5±11.8 points ($P<0.001$); Lunch: Pre (n=127) 48.9±10.1 to Post (n=78) 55.2±11.7 points ($P<0.001$); and Dinner: Pre (n=102) 47.2±9.3 to Post (n=67) 52.2±10.6 pts ($P=0.008$). Patrons who consumed at least three meals in any combination during the study period (for example breakfast with either two lunches or dinners) dietary fatty acids increased by 1.2 pts ($p=0.038$). More patrons agreed that main dishes were healthy / performance-based (32% pre; 57% post; $p<0.001$), vegetarian options were available (25% pre; 38% post; $p=0.023$), and they used nutrition labels to choose performance foods (38% pre; 54% post; $p=0.039$).

Conclusions: G4G program successfully improved access to healthy/performance-based foods and improved the patron's satisfaction and meal quality. G4G program enabled patrons to select performance-based food choices. Strategies to overcome program barriers can further improve patron's diet quality.

Implications: Military dining facilities are important avenues to improve diet quality and nutrition-related behaviors to optimize warfighters fueling requirements.

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The authors have no financial interests or relationships to disclose.

Funding: US Army Medical Research & Development Command, Military Operational Medicine Research.

Leaning Objective:
1. Describe a strategy to improve meal quality and satisfaction in a cafeteria-style environment.
2. Leverage technology to capture food consumed by warfighters at fast-paced dining venues.
3. Discuss the use of Healthy Eating Index (HEI) as a tool to measure and score warfighters nutrient intake and identify areas for future improvements.

Weight Management Behaviors Mediate the Relationship between Weight Cycling, Body Mass Index, and Diet Quality among US Army Soldiers

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Abstract

Objective: Weight cycling is prevalent in sports/professions with body composition standards, and has been associated with weight management behaviors that may contribute to suboptimal diet quality and weight gain. US Army Soldiers may be at increased risk of weight cycling relative to civilians due to mandated body composition standards. However, the relationship between weight cycling, weight management behaviors, body mass index (BMI), and diet quality among Soldiers is unknown.

Methods: In this cross-sectional study, 575 Soldiers (89% enlisted, 90% male, 23±4yr) from Army installations at Joint Base Elmendorf-Richardson, AK, Joint-Base Lewis McChord, WA, and Fort Campbell, KY completed questionnaires on dietary intake, health-related behaviors, and history of weight cycling (≥3 weight fluctuations ≥5% body weight). Mediation models tested weight management behaviors as a mediator of associations between weight cycling and BMI and weight cycling and diet quality, respectively.

Results: Weight cycling was reported by 33% of Soldiers. Those who reported weight cycling reported higher BMI (27±4 vs. 25±3 kg/m^2, p<0.001) and higher prevalence of engaging in weight management behaviors prior to body weight screening, but did not report lower dietary quality (Healthy Eating Index-2015 (HEI) scores 59±10 vs 59±11, p=0.46) relative to those who did not report weight cycling. Results of mediation analyses suggested that weight cycling may affect BMI both directly (c'= 1.19, 95% CI: 0.62, 1.75) and indirectly (ab=0.45, 95% CI: 0.19, 0.75), and HEI scores indirectly (ab=0.69, 95% CI: 0.20, 1.35) through the adoption of weight management behaviors.

Conclusions: Weight cycling is common in Soldiers and is associated with higher BMI and higher prevalence of engaging in weight management behaviors that mediate associations between weight cycling, BMI and diet quality.

Funding Source: US Army Medical Research and Development Command

Disclaimer: Authors' views do not reflect official DoD or Army policy.

Learning Objectives:

1. Define why weight cycling is a concern among Soldiers.
2. Describe weight loss strategies Soldiers use to meet body composition standards.
3. Discuss behavioral differences between Soldiers who weight cycle and those who do not and how this information may be used to create a military culture of health to educate Soldiers and improve readiness.

Educational Session Proposal Form
2021 Food & Nutrition Conference & Expo™ – New Orleans, LA
(Call Site Opens September 1 - Closes November 18, 2020)

Session Details

Proposed Session Title* *(Please keep to 10 words or less. Containing no abbreviations.)* *150 characters maximum*

Eating Behavior within the Military Environment and Culture: Development, Results, and Utilization of the Military Eating Behavior Survey

Session Format* - How will this session be presented?

X	Lecture		Demo		Debate
	Panel Discussion		Interview		Other

If "Other" is chosen, use the space provided to include a brief description of the format style.

Session Duration*

X	60 minutes
	90 minutes

Session Track*- Select the most appropriate track for your session.

	Art and Experience of Food
	Behavioral and Mental Health
	Business and Communications
	Career Development and Advancement
	Community and Population Health
	Food Systems Development and Sustainability
	Health Equity, Access and Opportunity
	Interprofessional Collaboration
	Medical Nutrition Therapy
	Nutrition in Academia
	Nutrition through the Lifecycle
	Professional Standards and Advocacy
X	Sports, Fitness and Performance
	Technology Impacting Practice

Session CPE Level*: *Identify the CPE Level. The review process favors advanced-level (Level 2 or 3) submissions.*

	Level 1 (basic, requires no prior knowledge/experience)
X	Level 2 (intermediate, requires some prior knowledge/experience)
	Level 3 (advanced, require an expert level of knowledge/expertise)

Session Description*

Explain the session content and rationale for presentation to conference attendees. This statement may be used in supportive promotional materials for the session and should be reflective of the previously stated objectives. *1,000 characters maximum*

A gap exists regarding the ability to measure eating behaviors and mediators of eating behaviors among Warfighters that accounts for the unique military environments, culture, and requirements. To fill this gap, the Military Eating Behavior Survey (MEBS) was developed. The session will educate nutrition professionals on the survey development process to include the constructs the survey measures, phase of development, and the validation process. Speakers in the session will also share results from data gathered by the MEBS which has now been used to

examined eating behaviors associated with higher BMI and failing military body composition and physical fitness standards; eating behaviors of high and low performers; emotional eating among service members; body image; relationship between stress, emotional eating, and BMI; weight cycling among Warfighters; and eating behaviors associated with weight cycling. This information can be used by military and civilian health professionals to guide public health initiatives, patient interactions, and patient education materials.

References: if submitting a proposal based on emerging research, please include up to three links to references.

(1) Jayne, J.M., Ayala, R., Karl, J.P., Deschamps, B.A., McGraw. S.M., O'Connor, K., DiChiara, A.J, Cole, R.E. Body Weight Status, Perceived Stress, and Emotional Eating among US Army Soldiers: A Mediator Model. *Eating Behaviors* (2020). DOI: https://doi.org/10.1016/j.eatbeh.2020.101367; PMID: 32018191
(2) Cole, R.E., Jayne, J.M., O'Connor, K., McGraw. S.M., Beyl, R., DiChiara, A.J., Karl, J.P., Development and Validation of the Military Eating Behavior Survey. Submitted to *Journal of Nutrition Education and Behavior*, October 20. (In review)
(3) Stukenborg, M.J., Deschamps, B.A., Jayne, J.M., Karl, J.P., McGraw, S.M., DiChiara, A.J., Cole, R.E. The Association Between Military Weight Status and Body Image in a Sample of Active Duty US Soldiers. Submitted to *Eating Behaviors*, April 2020. (In review)

Learning Objectives

Provide three learning objectives that are clear, measurable, and achievable. (*All objectives are to complete the following statement: "After presentation, attendees will be able to…") 30 words maximum*

(1) Upon completion, participants will be able to explain the rationale and methods used to develop the Military Eating Behavior Survey and the knowledge gap it addresses.
(2) Upon completion, participants will be able to identify key nutrition behaviors related to performance and health outcomes that have been identified by the Military Eating Behavior Survey, with application to civilian populations.
(3) Upon completion, participants will be able to describe how findings from the Military Eating Behavior Survey can be used to guide public health initiatives, patient interactions, and patient education materials.

Additional Session Details

Is this proposal being submitted as a DPG/MIG spotlight session? * *(All submissions must be approved by the DPG/MIG Chair)*

- [X] No
- [ ] Yes

If yes, please provide name of the group chair that approved this spotlight submission:

* *indicates a required field*

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Is this session proposal being submitted on behalf of or in conjunction with a specific professional association, corporation or organization? *

- [X] No
- [ ] Yes

If yes, please provide name of professional association, corporation or organization:

If your proposal is declined, do you wish to be considered for a webinar? * This question will be omitted from the review process.

- [ ] No
- [X] Yes

Performance Indicators*: Please include three performance indicators for your session. For more detail on performance indicators, you may review the Commission on Dietetic Registration's Essential Practice Competencies guide.

(1) 6.2.2 Demonstrates knowledge of research instruments and tools (e.g., surveys, interview tools) to support research initiatives.
(2) 4.1.2 Interprets and integrates evidence-based research and literature in decision making.
(3) 6.2.5 Applies research/evidence-based findings to improve practice, service delivery, and health and nutrition of customers. Submitted 8.1

Session Participants

Program Planner*

The program planner is administratively responsible for the session. All fields are required.

First Name	Julianna
Last Name	Jayne
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Moderator*

Due to the responsibility of managing the question and answer technology, the moderator cannot also be a session speaker. All fields are required.

First Name	J. Phillip
Last Name	Karl
Credentials	PhD, RDN
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Have you presented at FNCE® in the last two years?

- [x] No
- [ ] Yes

If yes, when?

Speaker #1*

This Speaker will be the first Speaker in the session. All fields on this form are required.

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Have you presented at FNCE® in the last two years?

- [X] No
- [ ] Yes

If yes, when?

Speaker #1 – CV*

This will be uploaded as an attachment in the web portal. This must be a 1 MB .PDF/.doc/.docx format

Speaker #1- Bio*

Please keep succinct. This will be published to the website and mobile app if the proposal is accepted for presentation at FNCE®.

Colonel Renee Cole is an Army Registered Dietitian with over 14 years of experience in nutrition, education and research. She is the Program Director for the U.S. Military-Baylor University Graduate Program in Nutrition at Joint Base San Antonio-Fort Sam Houston, TX. She has authored / co-authored numerous publications in nutrition focused on eating behaviors, weight management, human performance optimization, and disease mitigation/treatment.

Optional: Professional Social Media Networks and Past Speaking Videos

Speakers can include professional social media profiles and/or short video clips of a previous speaking engagement.

* *indicates a required field*

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2021 Food & Nutrition Conference & Expo™ – New Orleans, LA
(Call Site Opens September 1 - Closes November 18, 2020)

Speaker #2*
This Speaker will be the second Speaker in the session. All fields on this form are required.

First Name	Julianna
Last Name	Jayne
Credentials	PhD, RDN, CHES
Academy Member	974393
Employer	US Army Research Institute of Environmental Medicine
Position/Title	Principal Investigator
Home Address	10 General Greene Ave., Natick, MA 01760
Email Address	julianna.m.jayne.mil@mail.mil
Phone Number	508-206-2314

Have you presented at FNCE® in the last two years? *

[ ] No
[x] Yes

If yes, when?

10/18/2020 – Poster presentation

Speaker #2 – CV*
This will be uploaded as an attachment in the web portal. This must be a 1 MB .PDF/.doc/.docx format

Speaker #2- Bio*
Please keep succinct. This will be published to the website and mobile app if the proposal is accepted for presentation at FNCE®.

Major Julianna Jayne is a Principal Investigator and the Military Deputy Chief of the Military Nutrition Division at the United States Army Research Institute of Environmental Medicine (USARIEM) in Natick, MA. She has been a registered dietitian for 12 years and has been in a research role since 2018. Her research focuses on the eating behaviors of Soldiers and factors that influence body weight status, nutrition-related health outcomes, and combat readiness. MAJ Jayne has multiple ongoing research studies and publications on these topics.

Optional: Professional Social Media Networks and Past Speaking Videos
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Speaker Outlines*

Briefly describe each speaker's presentation: key points, teaching methods, parallels to the session's learning objectives, and any unique audiovisual or equipment requirements. Highlight new and/or significant contributions, and/or cutting-edge information to the profession. A separate outline for each speaker, with individualized talking points, is required.

Speaker #1 – Presentation Outline*

1. Description of the gap area that necessitated the need to develop the Military Eating Behavior Survey (MEBS) **(LO1)**
    a. Similarities and differences between military and civilian populations
    b. Unique aspects of the military and how this may affect Warfighter's behaviors
2. Definition of eating behaviors and mediators of eating behaviors **(LO1)**
    a. How these contribute to undesirable health outcomes
    b. How the military environment may influences eating behaviors
    c. Conceptualization of the survey and how it would be used
3. The process of the development of the MEBS **(LO1)**
    a. Constructs within the survey
        i. How the MEBS captures military specific constructs
        ii. How these constructs compare to those seen in civilian populations
    b. Development phases
    c. Validation process
        i. Sample size, methods, and testing locations
        ii. Description of statistical methods
    d. Unique aspects of the MEBS

Speaker #2 – Presentation Outline*

1. Overview of methods related to analyzing data gathered from the Military Eating Behavior Survey (MEBS)
2. Results and findings from studies that used the MEBS **(LO2)**
    a. Study: Eating Behaviors are Associated with Physical Fitness and Body Composition among US Army Soldiers
        i. Eating behaviors associated with higher BMI
        ii. Eating behaviors associated with failing body composition and physical fitness standards
        iii. Eating behaviors of high and low performers on physical fitness tests
    b. Study: Body Weight Status, Perceived Stress, and Emotional Eating among US Army Soldiers: A Mediator Model
        i. Emotional eating among service members
        ii. Relationship between stress, emotional eating, and BMI
    c. Study: Weight Cycling is associated with Higher Body Mass Index and Undesirable Weight Loss Behaviors in US Army Soldiers
        i. Weight cycling among Warfighters
        ii. Eating behaviors among weight cyclers
        iii. Weight management methods of weight cyclers
    d. Study: The association between body composition standards and body image in a sample of active duty U.S. Soldiers
        i. Body image among Warfighters
        ii. Relationship between body image and weight cycling
3. How findings are generalizable to practitioners working with military and civilian populations (e.g. student athletes; college students) **(LO3)**

** indicates a required field*

a. Potential applications in military context
b. How findings can be used to guide public health initiatives, patient interactions, and patient education materials

Speaker #3 – Presentation Outline* (if applicable)