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

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Louisiana State University System
Pennington Biomedical Research Center
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14. ABSTRACT
The Collaborative Research to Optimize Warfighter Nutrition (CROWN) III project is a series of 5 projects that use 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 are collaborating 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 are 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 will provide 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

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1. INTRODUCTION:

The Collaborative Research to Optimize Warfighter Nutrition (CROWN) III project is a series of projects that use 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 and the Military Nutrition Division at the U.S. Army Research Institute of Environmental Medicine are collaborating on the design, execution and analysis of the research. The outcomes will 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 proposed 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 are conducting 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 are conducting 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, dates of completion, and current status.

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 2: Data Analysis (months 6-24)
Data analysis for this project began in the second quarter of year 1 and is continuing.

Analysis of questionnaires (including 3-day food records, cognitive tests and physical activity logs) is in progress.

Analysis of body composition data from DXA and brain imaging/function from fMRI has been completed. The primary outcome from DXA is lean body mass and fat mass and the distribution of these two compartments throughout the body. This year, a manuscript was accepted for presentation at an international conference next year and subsequent publication, and another manuscript was submitted for publication.

1. Pillai SR, Lieberman HR, Rood JC, Pasiakos SP, Murray K, Shankapal P, Carmichael OT. Constrained learning of task-related and spatially-coherent dictionaries from task fMRI data. Medical Image Computing and Computer-Assisted Intervention (MICCAI 2021) Workshop on Machine Learning in Clinical Neuroimaging – MLCN 2021. September 27, 2021. (Accepted)

2. 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. July 2021 (Submitted)

Exercise and strength testing data from VO2 max and Biodex testing has been compiled and is in progress of analysis. The three main variables are strength, total endurance and peak torque.

Major Task 3: Data Interpretation and Publishing of Results
Milestone #1: Co-author initial manuscript on findings from clinical study (months 6-12)
This milestone was met when the first manuscript was submitted for publication in year 2 and published in year 3.

An additional manuscript was published this year, and a copy of the published manuscript is included in the appendix.

1. 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.

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.

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 is being analyzed and published under this award as Major Task 1. The timeline for Milestone #2, Co-author initial manuscript on findings from clinical study, was months 1-12 of this award. This milestone was met in year 1.

An additional manuscript was published this year, and a copy of the published article is included in the appendix.

> 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.

This study examined the effects of consuming supplemental carbohydrate on aerobic exercise performance in recreationally active, healthy, young lowlanders at high altitude (HA), before and following 22 days of acclimatization while in a constant state of negative energy balance. 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 3: Data Interpretation and Publishing of Results

The projected timeline for this major task and Milestone #4, Co-author initial manuscript on findings from clinical study, was months 24-36 of the award. This was completed when the first two manuscripts for Project 3 were published this year. Copies of the manuscripts are included with this report.

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.

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 3: Data Interpretation and Publishing of Results

The projected timeline for this major task was months 36-48 of the award. The first two manuscripts were submitted for publication this year:

1. Kleinberger CA, Bukhari AS, Moylan EM, Kirkpatrick KM, Billington JL, Armstrong NJ, Cole RE, Deuster PA. Go for Green® Nutrition Program: Translating Evidence into Practice. J of Nutr Educ Behav. July 2021. (Resubmitted)

2. Bukhari AS, Champagne C, McGraw SM, Armstrong NJ, Moylan EM, Kleinberger C, Kirkpatrick K, Billington JL, Deuster PA, Cole RE. The Go For Green® Worksite Nutrition Intervention Improved Patrons' Food Choices. J of Nutr Educ Behav. July 2021. (Resubmitted)

One abstract for Project 4 was presented at an online conference this year, and three additional abstracts for Project 4 were accepted for presentation next year. A copy of the presented abstract is included in the appendix.

1. 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.

2. McGraw SM, Bukhari AS, Armstrong NJ, Champagne CM, 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. (Accepted)

3. 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. (Accepted)

4. 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. (Accepted)

A technical report also is planned. LTC Bukhari, COL Cole, the G4G team, and Dr. Champagne continue to discuss publications, abstracts and other deliverables from the G4G project.

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: Development of a valid and military-appropriate survey tool for the assessment of Soldier eating behaviors.

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

Major Task 3, Data Interpretation and Publishing of Results
Milestone #7: Co-author initial manuscript on findings from clinical study (months 36-48)
This milestone was met when the initial manuscript was published last year. Three additional manuscripts were published this year, and copies are included in the appendix:

1. 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.

2. 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.

3. 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.

An additional abstract using data from this study was accepted this year for presentation at a conference next year:

> 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. (Accepted)

This project has 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 beem 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?
Nothing to report

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?

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

Major Task 2: Data Analysis will continue.
Major Task 3: Data Interpretation and Publishing of Results will continue.

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

Major Task 3: Data Interpretation and Publishing of Results will continue.

Project 4: Go for Green (GFG) Effectiveness

Major Task 3: Data Interpretation and Publishing of Results will continue.

Project 5: Develop a military-specific eating behavior survey

Major Task 3: Data Interpretation and Publishing of Results will continue.

4. IMPACT:

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

Project 1
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
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
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
This project has 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 validated survey allows scientists to examine the role eating behavior plays in association with other variables such as body weight, body image, and physical fitness.

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
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report

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.

An additional manuscript for Project 1 was published this year. Also, a manuscript was accepted for presentation at an international conference next year and subsequent publication, and another was submitted for publication.

An additional manuscript for Project 2 and the first two manuscripts for Project 3 also were published in Year 4.

The first two manuscripts for Project 4 were submitted for publication this year.

Three additional manuscripts for Project 5 also were published this year.

Copies of the published manuscripts are included in the appendix.

1. 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.

2. 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.

3. 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.

4. 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.

5. 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.

6. 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.

7. 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.

8. Pillai SR, Lieberman HR, Rood JC, Pasiakos SP, Murray K, Shankapal P, Carmichael OT. Constrained learning of task-related and spatially-coherent dictionaries from task fMRI data. Medical Image Computing and Computer-Assisted Intervention (MICCAI 2021) Workshop on Machine Learning in Clinical Neuroimaging – MLCN 2021. September 27, 2021. (Accepted)

9. 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. July 2021 (Submitted)

10. Kleinberger CA, Bukhari AS, Moylan EM, Kirkpatrick KM, Billington JL, Armstrong NJ, Cole RE, Deuster PA. Go for Green® Nutrition Program: Translating Evidence into Practice. J of Nutr Educ Behav. July 2021. (Resubmitted)

11. Bukhari AS, Champagne C, McGraw SM, Armstrong NJ, Moylan EM, Kleinberger C, Kirkpatrick K, Billington JL, Deuster PA, Cole RE. The Go For Green® Worksite Nutrition Intervention Improved Patrons' Food Choices. J of Nutr Educ Behav. July 2021. (Resubmitted)

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers and presentations.

One abstract for Project 4 was presented at an online conference this year, and three additional abstracts for Project 4 were accepted for presentation next year. An additional abstract for Project 5 also was accepted this year for presentation at a conference next year.

A copy of the presented abstract is included in the appendix.

1. 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.

2. McGraw SM, Bukhari AS, Armstrong NJ, Champagne CM, 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. (Accepted)

3. 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. (Accepted)

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5. 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. (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 are being carried out in collaboration with scientists from the U.S. Army Research Institute of Environmental Medicine; however, funds from this award are covering personnel costs at Pennington Biomedical Research Center only. I have detailed the contributions of Pennington Biomedical Research Center employees during this year:

Name: Jennifer Rood
Project Role: Principal Investigator
ORCID ID: 0000-0001-5848-2987
Nearest Person Month Worked: 5.2
Contribution to Project: She oversees/manages/coordinates the award and acts as the point of contact for interactions with USARIEM scientists and oversee all regulatory processes. She also serves as PI for project 1.

Name: Catherine Champagne
Project Role: Co-investigator
ORCID ID: 0000-0001-6127-1072
Nearest Person Month Worked: 3.0
Contribution to Project: She serves as the investigator for project 4.

Name: Ray Allen
Project Role: Computer support
Nearest Person Month Worked: 2.5
Contribution to Project: He provides computer support for project 4.

Name: Bridget Conner
Project Role: medical technologist
Nearest Person Month Worked: 7.7
Contribution to Project: She provides analytical support in the clinical chemistry laboratory for projects 1, 2, and 3. She prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Valery Hymel
Project Role: research associate
Nearest Person Month Worked: 7.7

Contribution to Project: She provides analytical support in the Mass Spectrometry Laboratory. She prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Jessica Landry
Project Role: research specialist
Nearest Person Month Worked: 9.0
Contribution to Project: She provides analytical support in the clinical chemistry laboratory for project 3. She prepares specimen collection containers and processes specimens.

Name: Steve Lee
Project Role: medical technologist/lab manager
Nearest Person Month Worked: 7.7
Contribution to Project: He provides analytical support in the clinical chemistry laboratory for projects 1, 2, and 3. He prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Stacey Roussel
Project Role: medical technologist/lab manager
Nearest Person Month Worked: 7.7
Contribution to Project: She provides analytical support in the clinical chemistry laboratory for projects 1, 2, and 3. She prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Jonathan Savoie
Project Role: research associate
Nearest Person Month Worked: 7.7
Contribution to Project: He provides analytical support in the Mass Spectrometry Laboratory. He prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Tiffany Stewart
Project Role: Co-investigator
Nearest Person Month Worked: 1.8
Contribution to Project: She oversees project 5 and works on the development of a military specific eating behavior survey.

Name: Jamie Tuminello
Project Role: medical technologist
Nearest Person Month Worked: 12.0
Contribution to Project: She provides analytical support in the clinical chemistry laboratory for projects 1, 2, and 3. She prepares specimen collection containers, processes specimens, analyzes biological samples, and reports results.

Name: Dawn Turner
Project Role: research associate

Nearest Person Month Worked: 1.8
Contribution to Project: She provides visual estimation and food photography support for project 4.

Name: Edie White
Project Role: administrative assistant
Nearest Person Month Worked: 6
Contribution to Project: She provides administrative support for the entire award.

Total Expenditures to Date: $6,156,045.47

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

QUAD CHART

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 will collaborate on 5 projects:

1) Determine the role of testosterone on physiological and psychological factors during energy deficit
2) Evaluate whether added dietary protein can sustain lean body mass when underfed and operating at high altitude
3) Assess the effects of energy balance and energy deficit during sustained operations on inflammation and cognitive and physical performance
4) Quantify the effectiveness of the military dining facility intervention Go For Green on Soldier food choices
5) Develop a valid and military appropriate survey tool for assessment of eating behavior



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
Protocol Development/ Approval				
Data Analysis				
Publication of Results				
Budget (M)	$2.165	$1.5	$1.5	$1.5

Updated: 8/13/2021

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 – Published additional findings from projects 1-5

Budget Expenditures to Date: $6,156,045.47

9. APPENDICES: see following pages

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

Emily E. Howard,[1,2,3] Lee M. Margolis,[1] Claire E. Berryman,[1,2,4] Harris R. Lieberman,[1] J. Philip Karl,[1] Andrew J. Young,[1,2] Monty A. Montano,[5,6,7] William J. Evans,[8,9] Nancy R. Rodriguez,[3] Neil M. Johannsen,[10] Kishore M. Gadde,[10] Melissa N. Harris,[10] Jennifer C. Rood,[10] and Stefan M. Pasiakos[1]

[1]*Military Nutrition Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts;* [2]*Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee;* [3]*University of Connecticut, Storrs, Connecticut;* [4]*Florida State University, Tallahassee, Florida;* [5]*MyoSyntax Corporation, Worcester, Massachusetts;* [6]*Harvard Medical School, Boston, Massachusetts;* [7]*Brigham and Women's Hospital, Boston, Massachusetts;* [8]*University of California at Berkeley, Berkeley, California;* [9]*Duke University, Durham, North Carolina; and* [10]*Louisiana State University's Pennington Biomedical Research Center, Baton Rouge, Louisiana*

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.

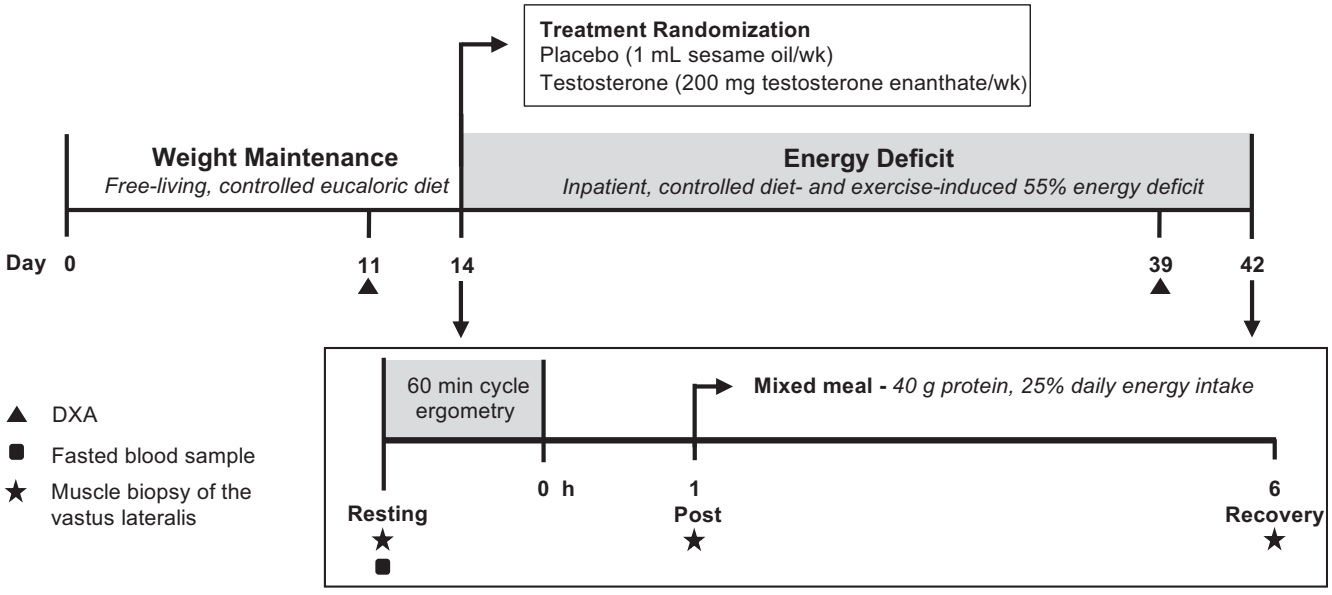


Fig. 1. Experimental design. The current analysis was part of a larger study assessing the effects of exogenous testosterone administration on changes in body composition after a 28-day exercise- and diet-induced energy deficit (ED) designed to be 45% of total energy needs (43). Biopsies were collected 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

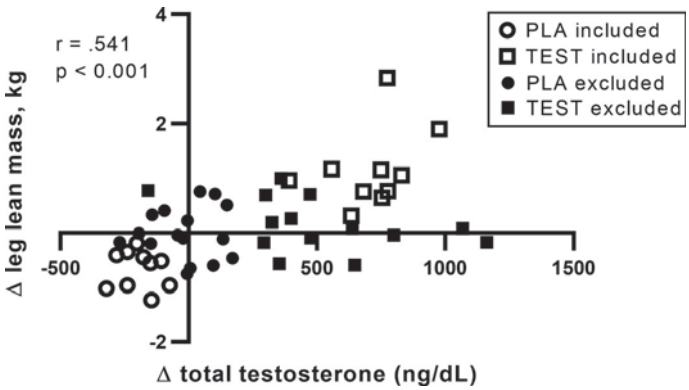


Fig. 2. Participant stratification according to leg lean mass and total testosterone. Changes in leg lean mass (kg) and total testosterone (ng/dL) were positively associated for all 50 participants. A subset of individuals exhibiting marked increases (TEST, n = 10) or decreases (PLA, n = 10) in both 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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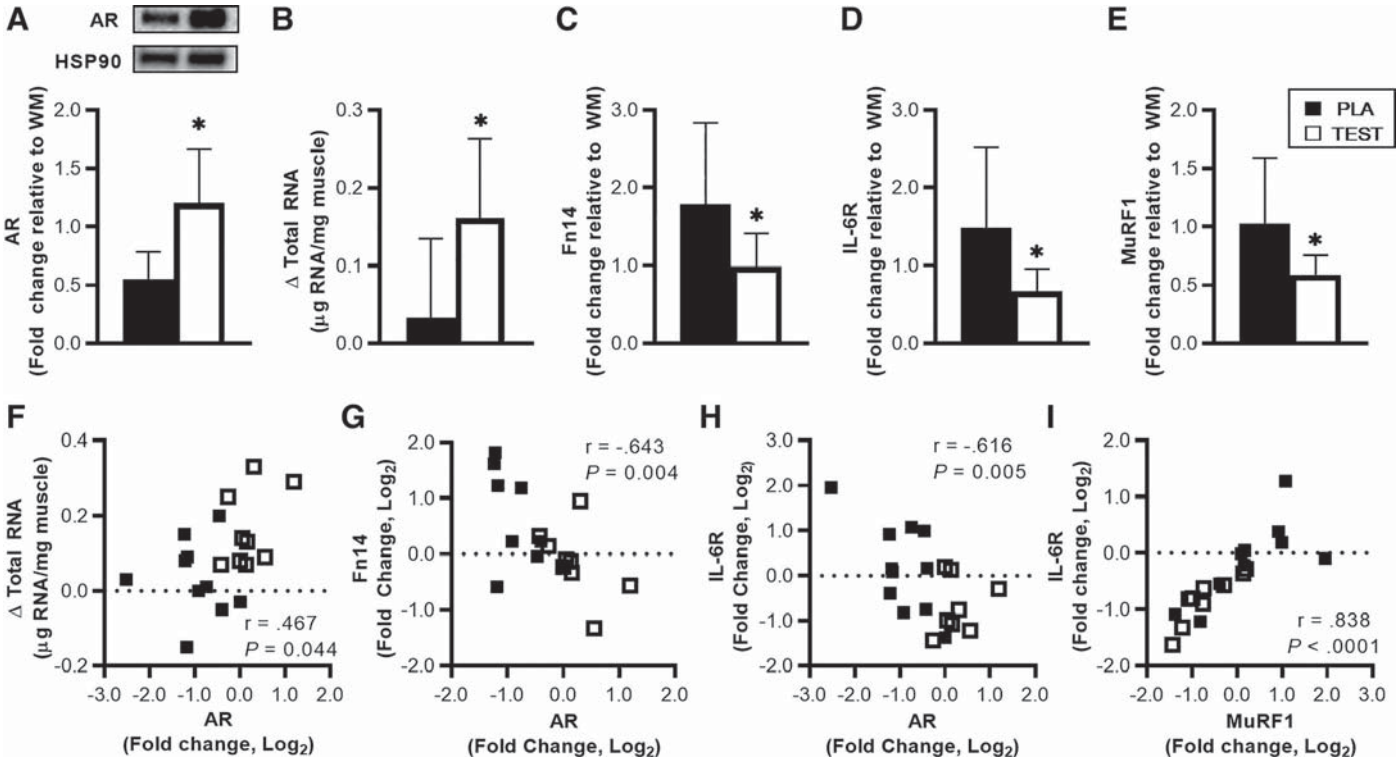


Fig. 3. Resting androgen receptor (AR) protein content (*A*), muscle total RNA content (*B*), fibroblast growth factor-inducible 14 (Fn14, *C*), IL-6 receptor (IL-6R, *D*), and muscle ring-finger protein-1 (MuRF1, *E*) gene expression during energy deficit (ED) relative to weight maintenance (WM) and 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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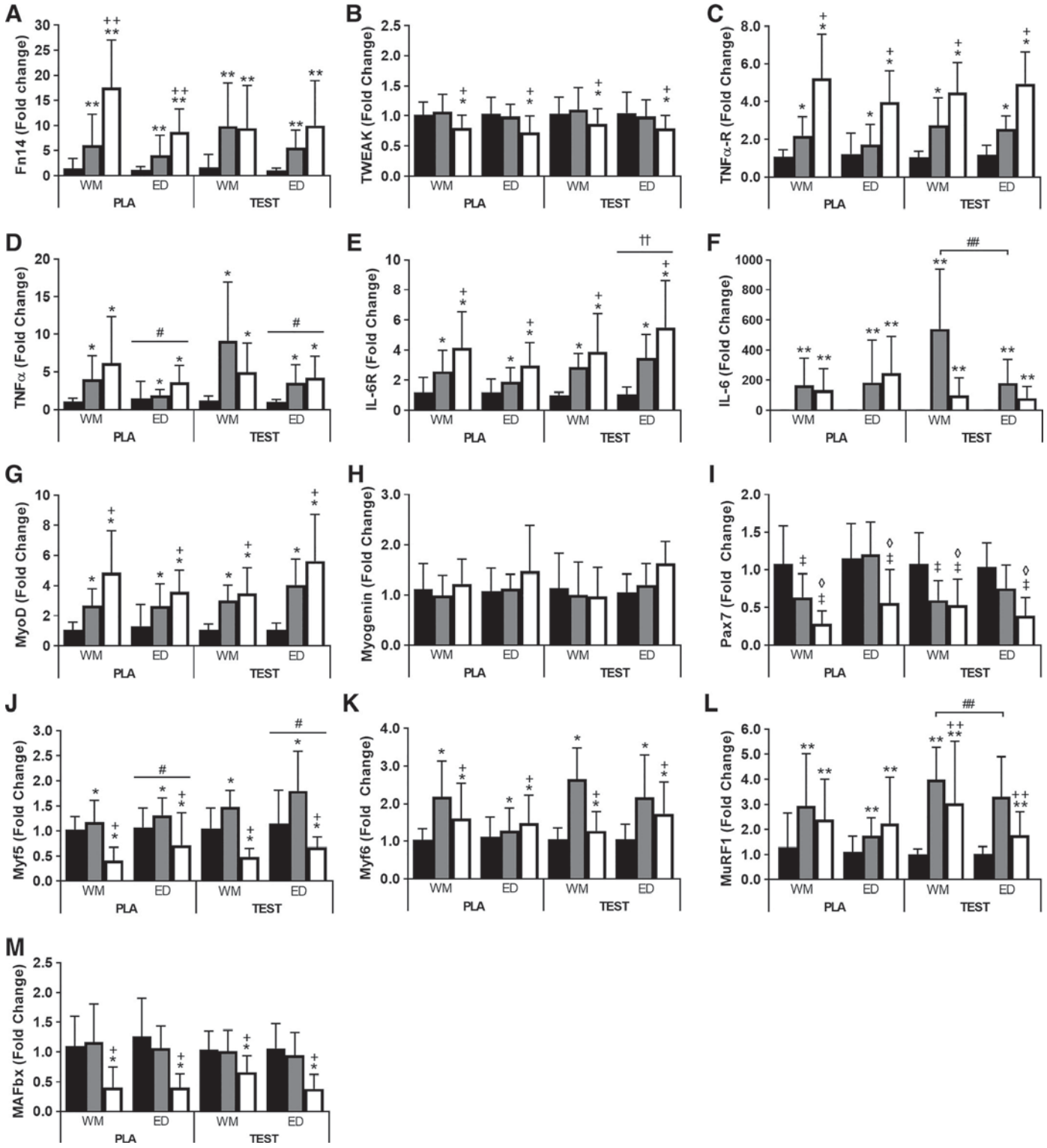


Fig. 4. Within [Resting (black bars), Post (gray bars), and Recovery (white bars)]- and between [weight maintenance (WM) and energy deficit (ED)]-study phase responses to exercise and a high-protein mixed meal for fibroblast growth factor-inducible 14 (Fn14, *A*), TNF-like weak inducer of apoptosis (TWEAK, *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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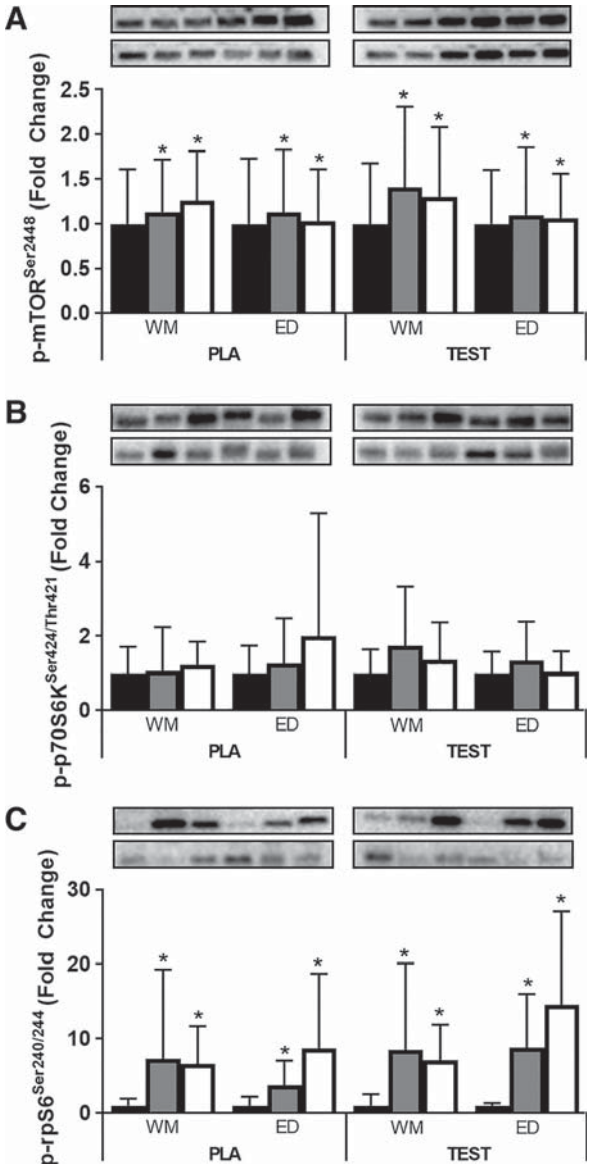


Fig. 5. Within [Resting (black bars), Post (gray bars), and Recovery (white bars)]- and between [weight maintenance (WM) and energy deficit (ED)]-study phase responses to exercise and a high-protein mixed meal for phosphorylated (p) mechanistic target of rapamycin (mTOR)^{Ser2448} (*A*), p-p70 ribosomal 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.

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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

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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).

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Data Availability: Data described in the manuscript and analytic code will be made available upon request.

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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

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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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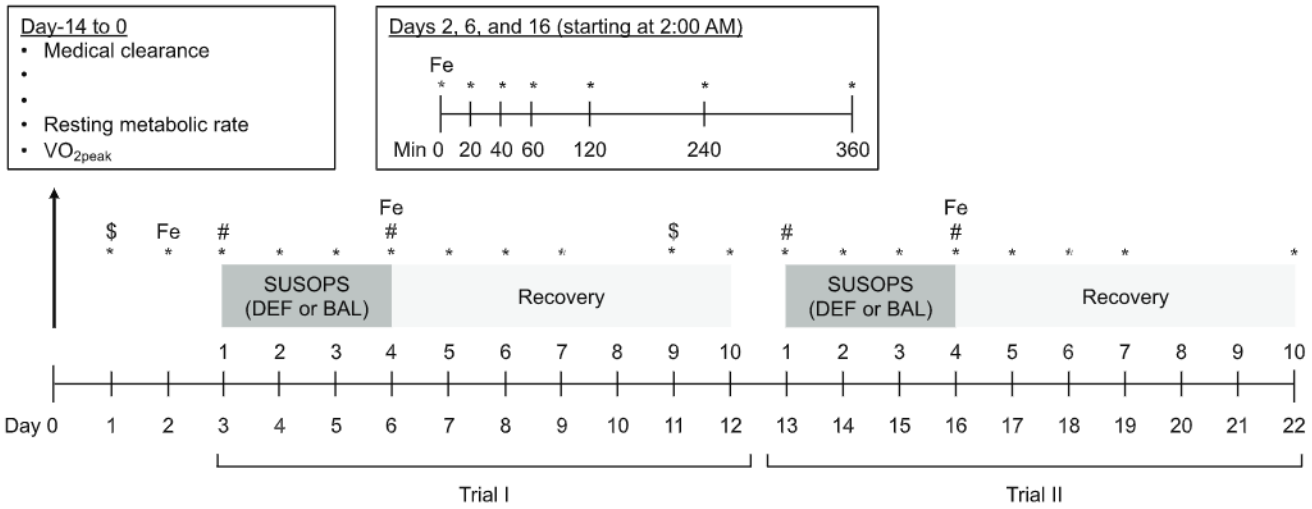


FIGURE 1 Overview of study design. Participants were randomly assigned to complete the SUSOPS trials in DEF or BAL. *blood draw; #muscle biopsy; $start of glycogen normalization and refeeding; Fe consumption of a beverage containing a stable iron isotope. BAL, energy balance; DEF, energy deficit; SUSOPS, 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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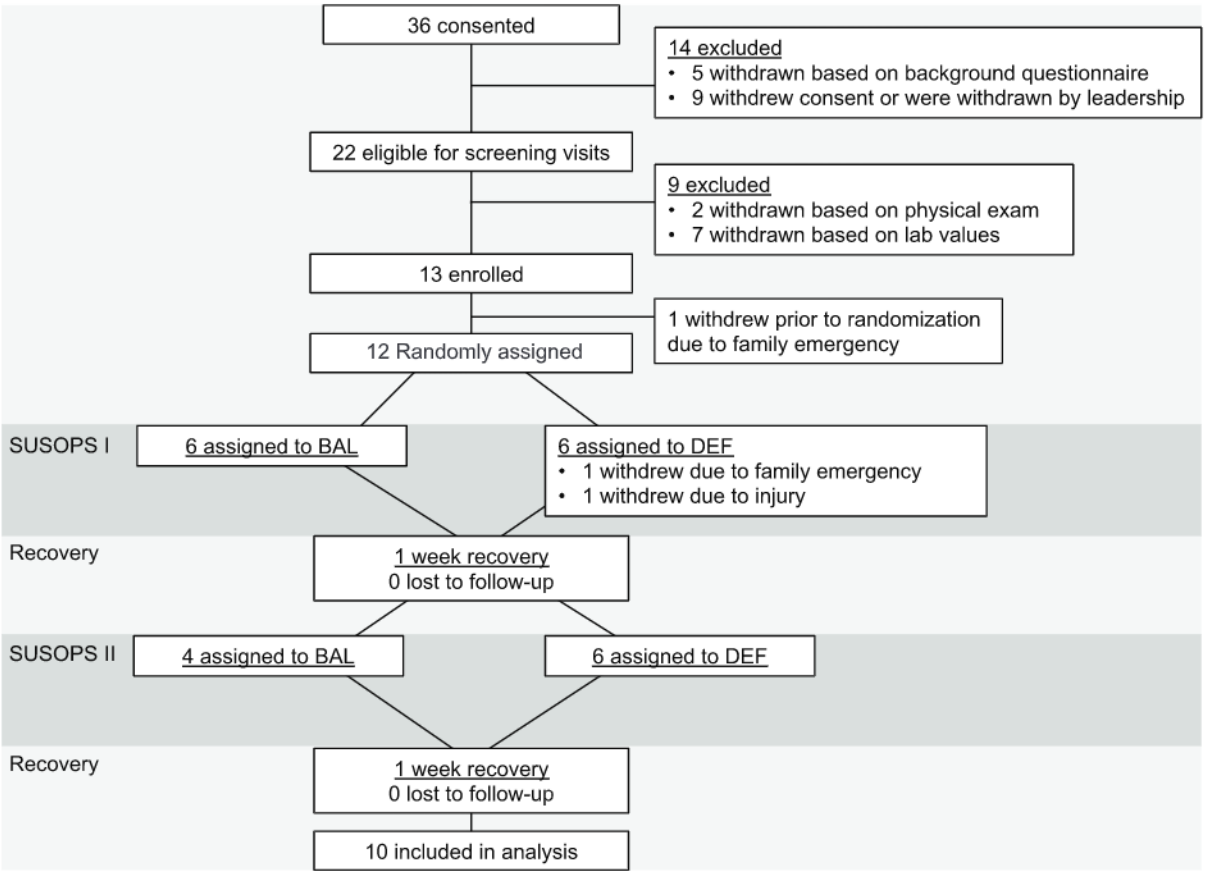


FIGURE 2 Participant flow chart. Thirty-six potential participants consented, 14 were excluded, 22 were eligible for screening visits, 9 were excluded following screening, 13 were enrolled, 1 discontinued participation prior to randomization, 12 were randomly assigned, and 10 completed the intervention. 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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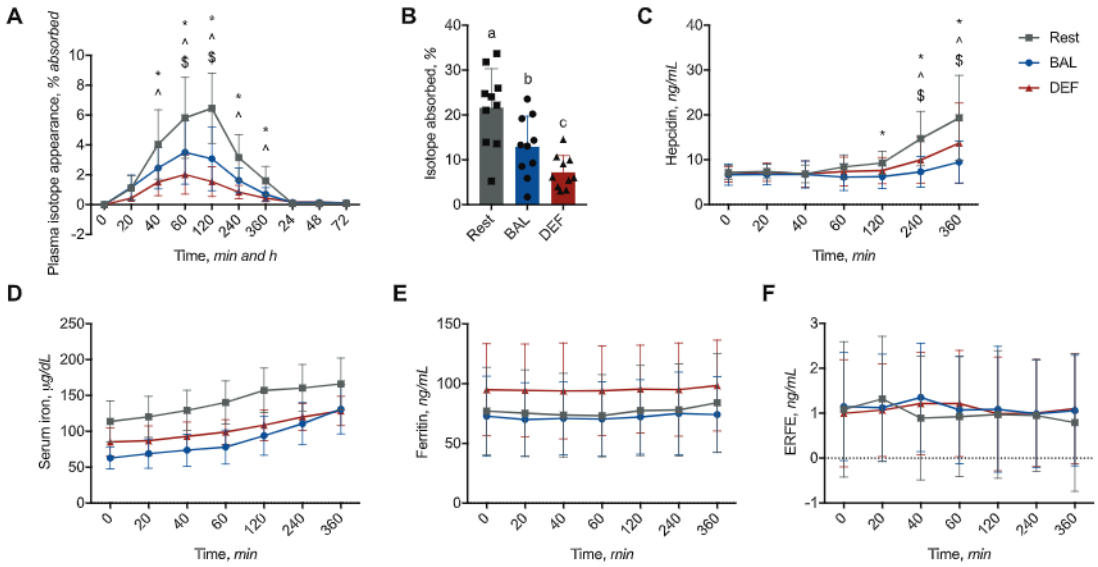


FIGURE 3 Time course of plasma iron isotope appearance, hepcidin, erythroferrone, and iron status indicators following consumption of a beverage containing an oral iron isotope. Time course of plasma iron isotope appearance (A). Data represent the percentage of isotope absorbed at a given time point 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.

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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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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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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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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.

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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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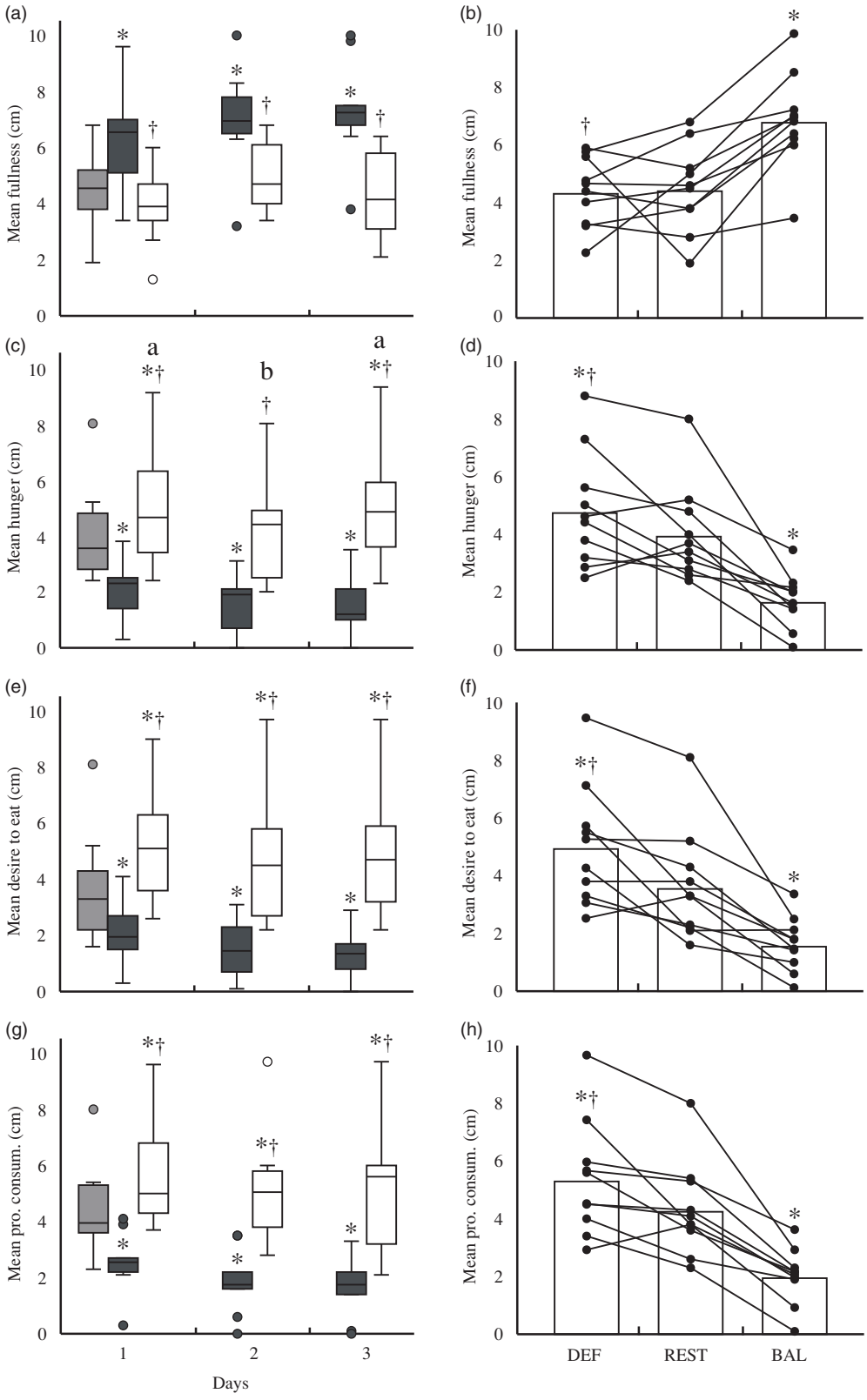


Fig. 1. Changes in mean appetite ratings during rest and during a 72-h simulated sustained military operation under conditions of energy balance (BAL) and energy deficit (DEF) (*n* 10). (a), (c), (e), (g) Boxes show median and interquartile range. Whiskers extend to 1·5 times the interquartile range 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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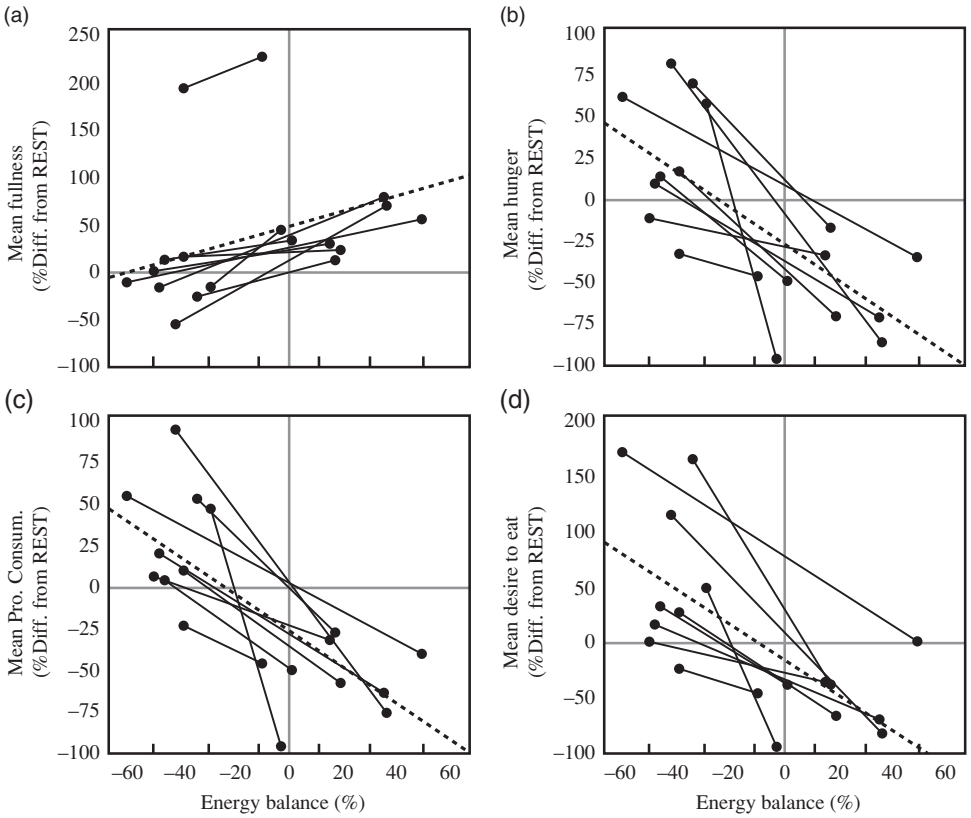


Fig. 2. Exploratory analysis of associations between mean changes in appetite ratings from REST (baseline sedentary condition) during a 72-h simulated sustained military operation relative to mean energy balance during the 72-h period (*n* 9). Solid lines connect data from the same individual. Dotted 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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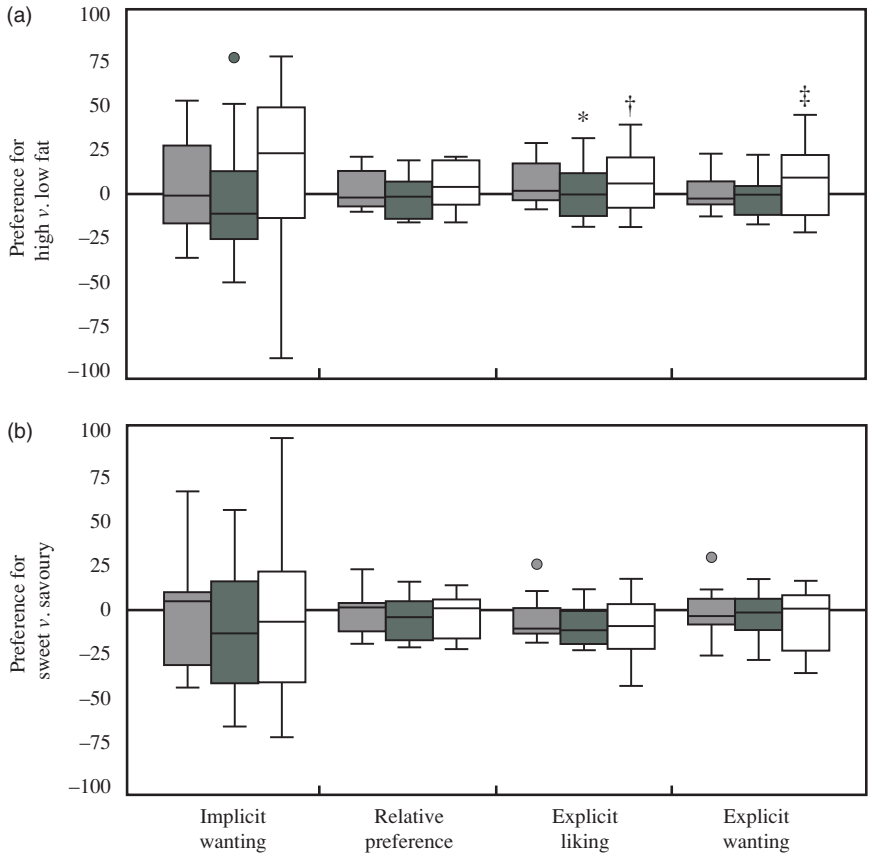


Fig. 3. Food preferences measured during rest and during a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF) (n 7). Preference for (a) high *v.* low fat and (b) sweet *v.* savoury foods measured by the Leeds Food Preference Questionnaire. 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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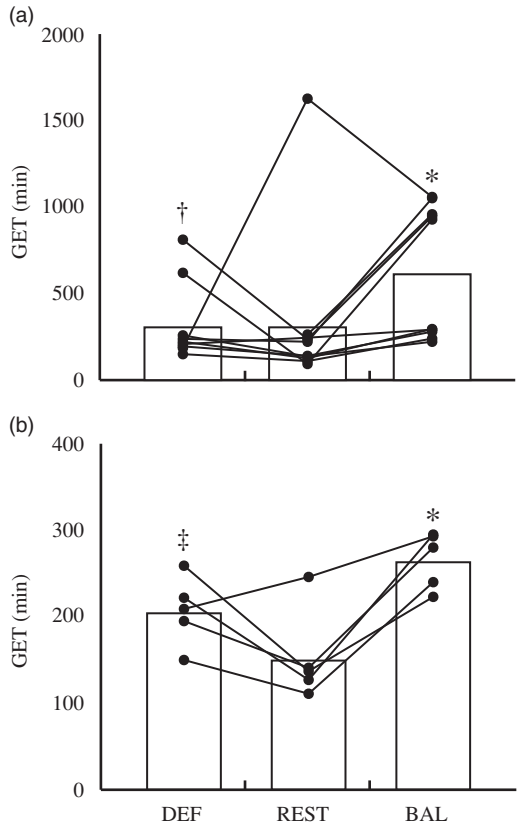


Fig. 4. Gastric emptying time (GET) measured during rest and during a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF). Bars represent mean GET for (a) all available data (n 10; square root-transformed for analysis), and (b) after 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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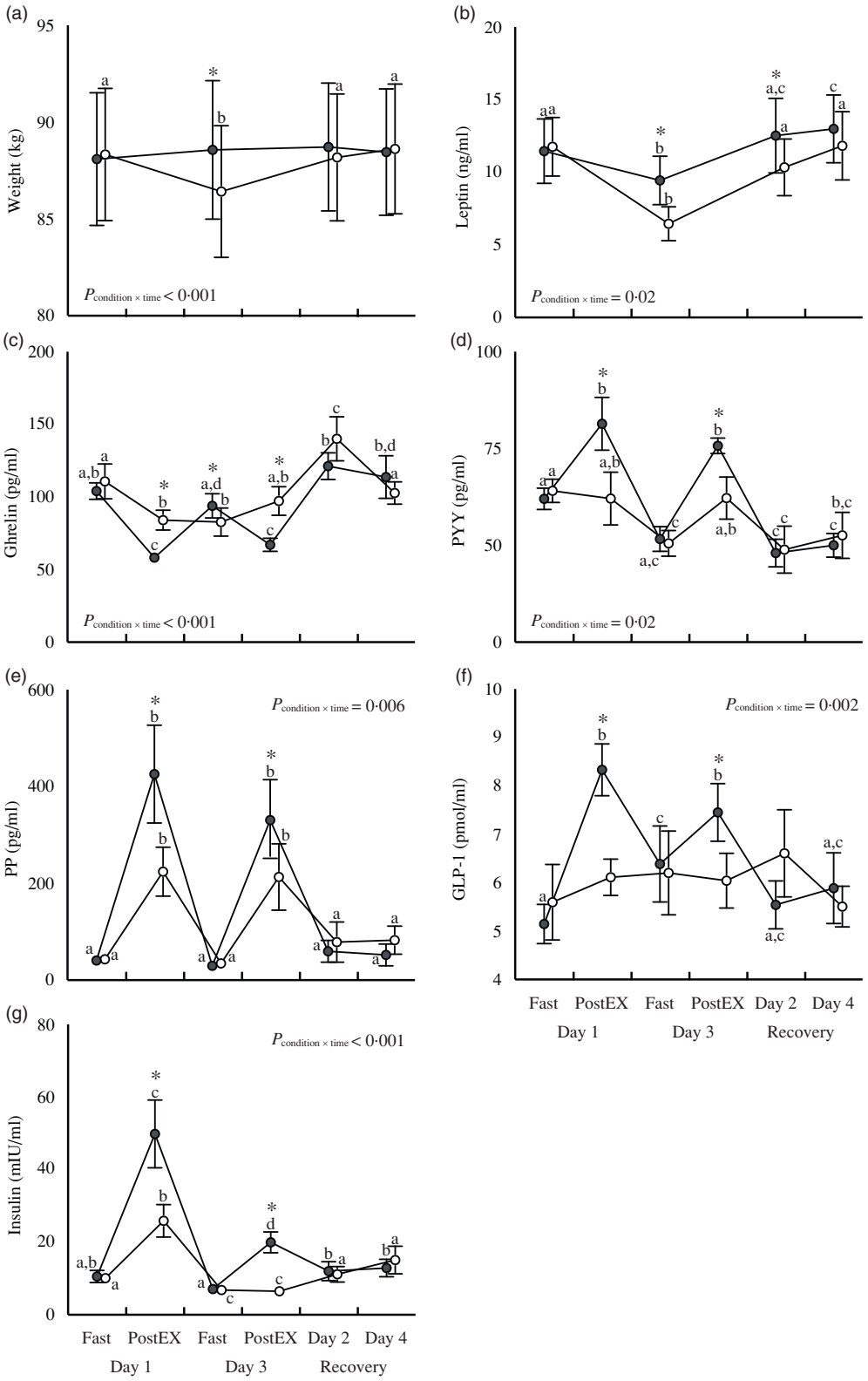


Fig. 5. Body weight (a) and appetite-mediating hormone concentrations (b)–(g) measured during (days 1 and 3) and after (Recovery (Rec.) days 2 and 4) a 72-h simulated sustained military operation (SUSOPS) under conditions of energy balance (BAL) and energy deficit (DEF) (*n* 10). Values are mean values 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.

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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.)

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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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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.

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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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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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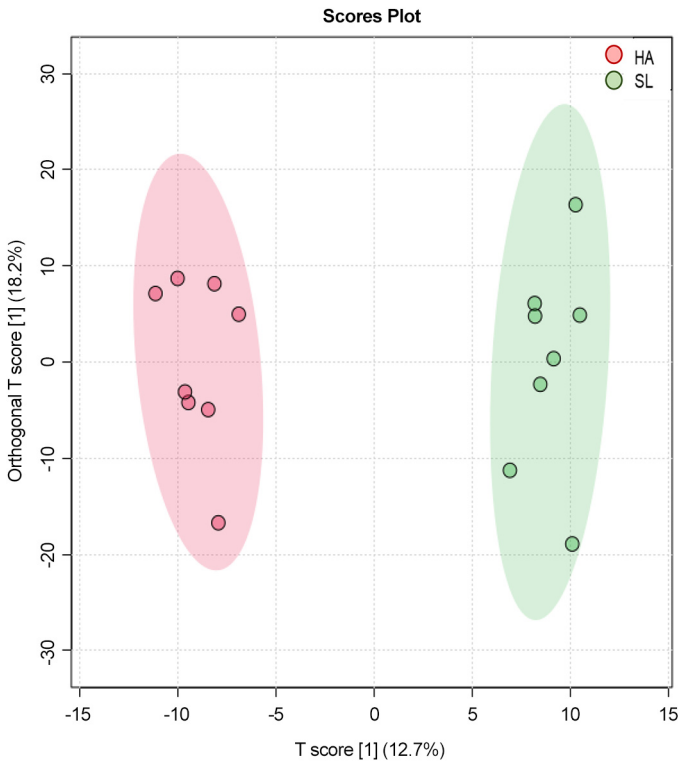


Figure 1. Orthogonal projections to latent structures discriminant analysis score plot for all metabolite features samples based on subject (n = 8) and condition [sea level (SL) and high altitude (HA)]. Circles represent individuals participants under each experimental condition.

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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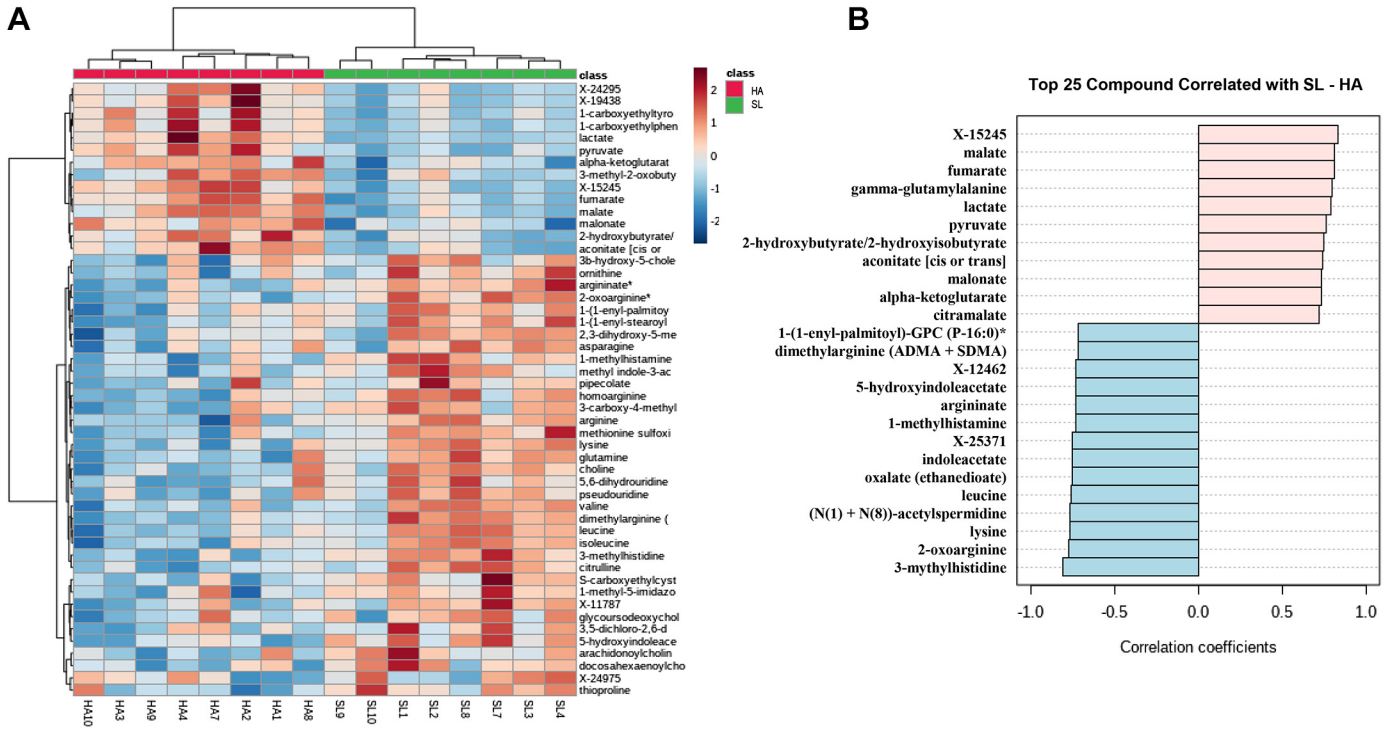


Figure 2. *A*: heatmap of hierarchical cluster analysis of the 50 metabolites with the lowest *Q* values between changes in sea level (ΔSL) and high altitude (ΔHA). *B*: pathfinder analysis of the 25 metabolites most highly associated with differences between ΔSL and ΔHA.

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

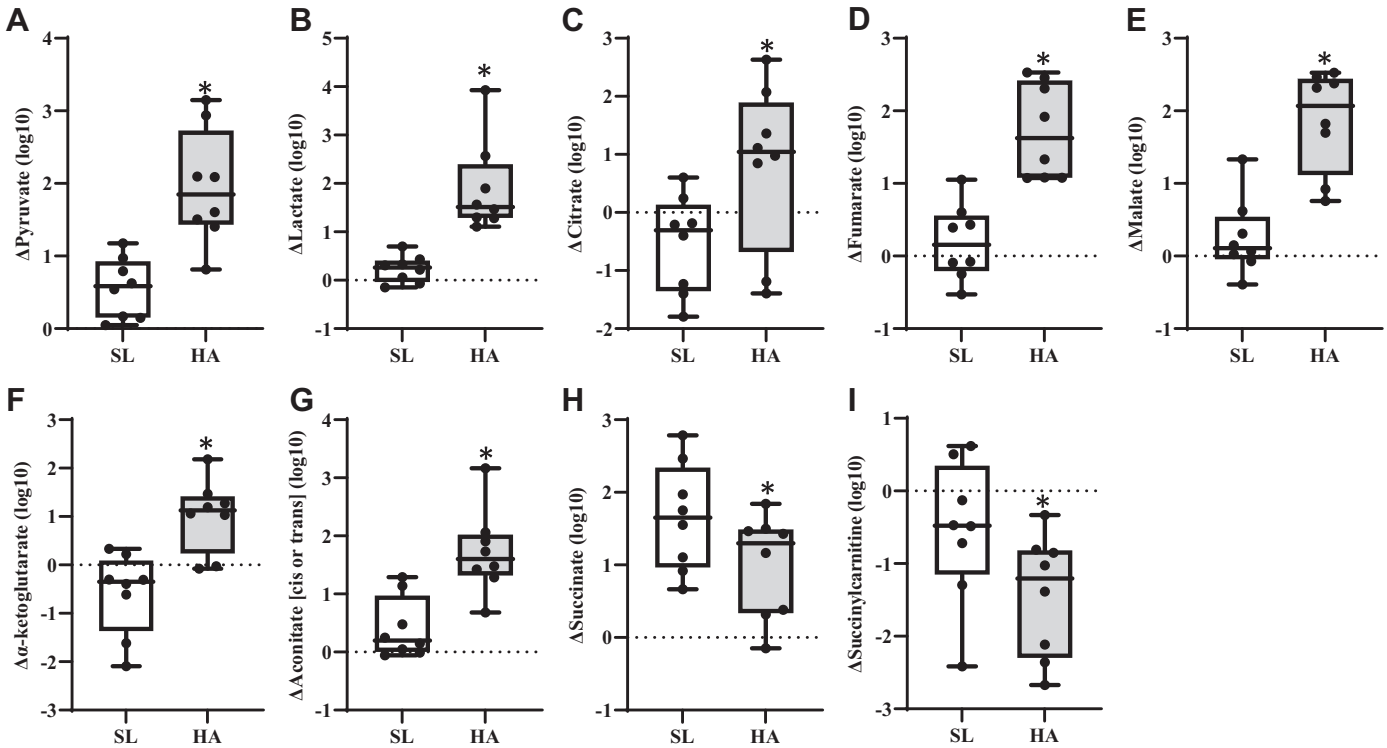


Figure 3. Box plots of difference from resting, fasted conditions at sea level (SL) in glycolysis (*A* and *B*), and tricarboxylic acid (TCA) metabolites (*C*–*I*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental Table S1.

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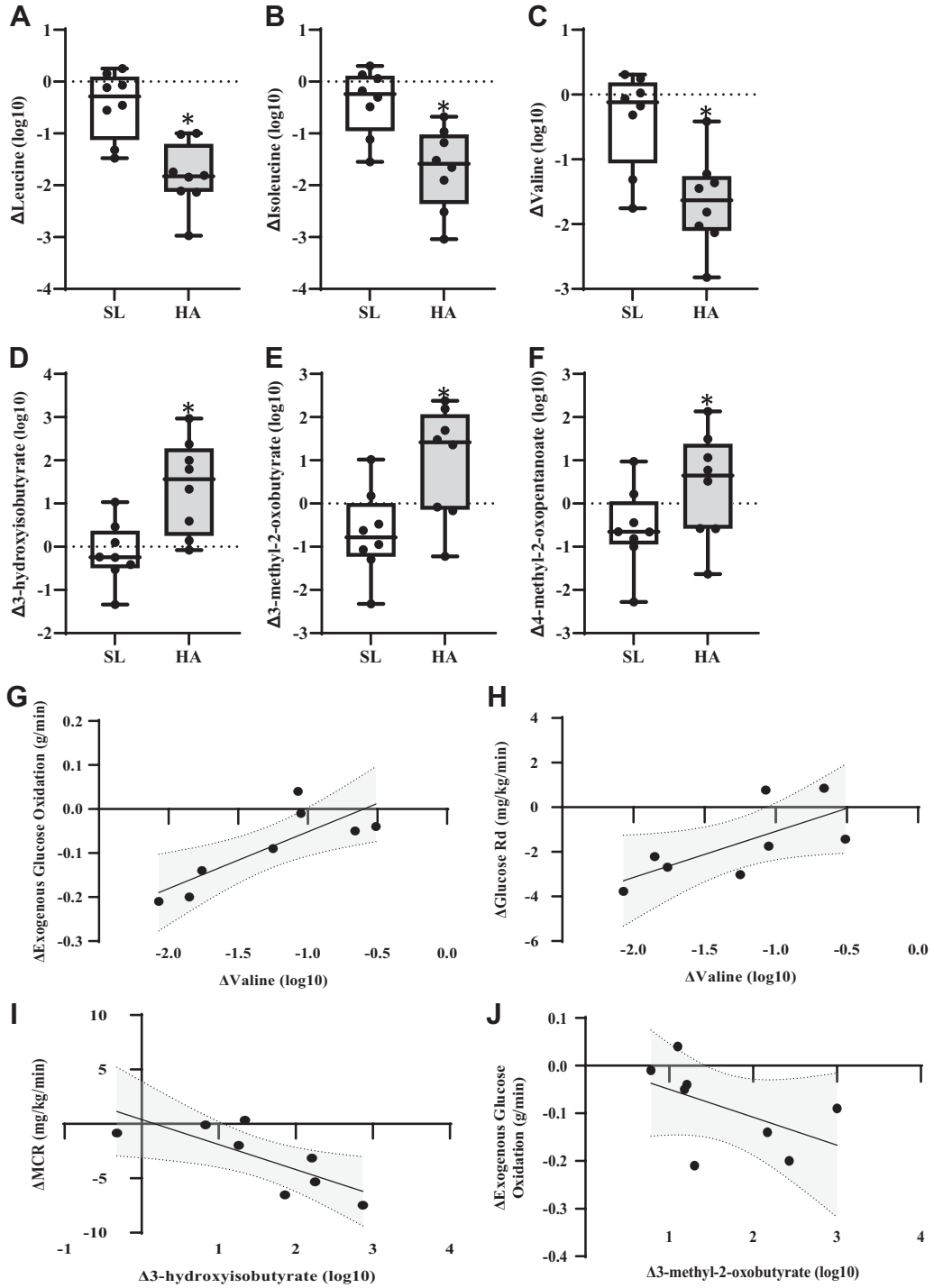


Figure 4. Box plots of difference from resting, fasted conditions at sea level (SL) in branched-chain amino acids (BCAA) metabolites (*A*–*F*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental 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

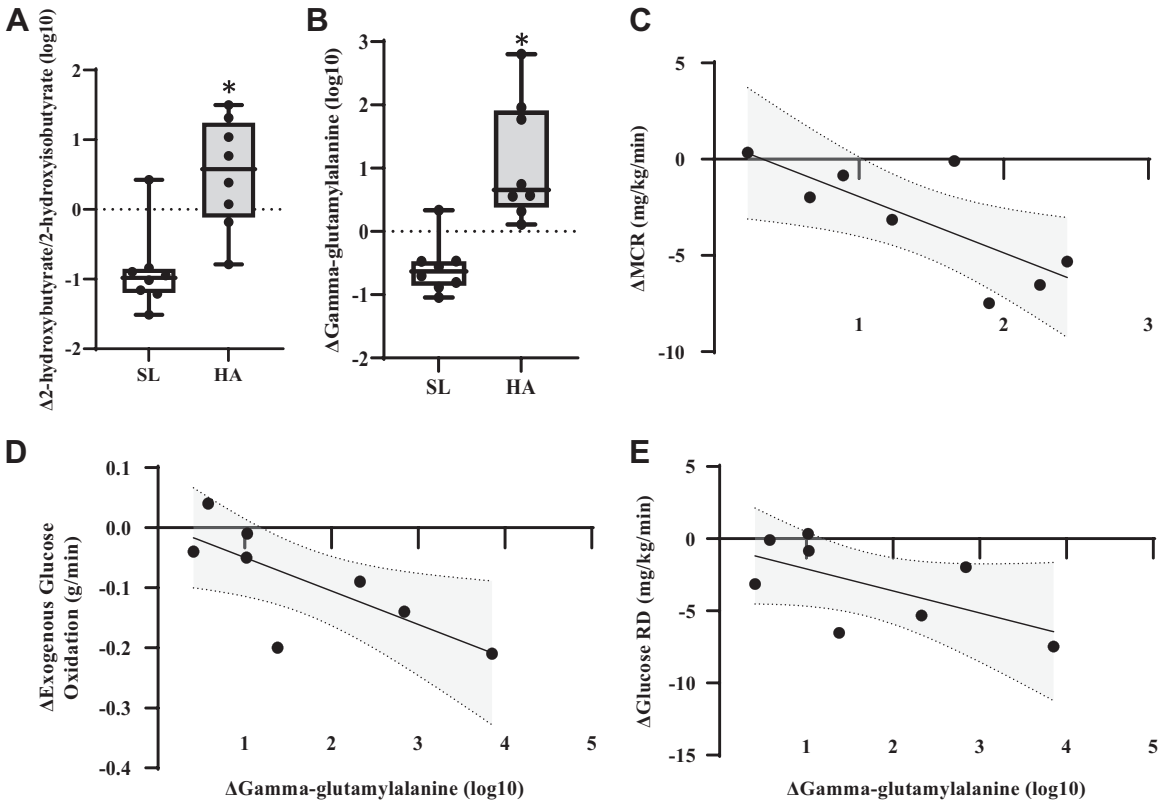


Figure 5. Box plots of difference from resting, fasted conditions at sea level (SL) in oxidative stress metabolites (*A* and *B*) during exercise at SL and high altitude (HA). *Significantly different from SL, $P < 0.05$, $Q < 0.10$. Exact *P* and *Q* values are reported in Supplemental Table S1. Significant 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.

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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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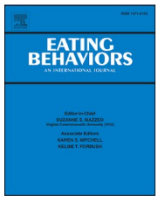
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Exceeding body composition standards is associated with a more negative body image and increased weight cycling in active duty U.S. soldiers

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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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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 [illegible] their current physical appearance and [illegible]apabilities 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

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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-[illegible] relative to not undergoing the [illegible]ment 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.

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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 [illegible]age, 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

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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 who perceive weight stigma are more inclined to [redacted] 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.

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of the data, writing the manuscript, or the decision to submit the paper for publication.

Declaration of competing interest

None.

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Disclaimer

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Questionnaire Development Research Methods

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;000:1−13.)

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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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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,

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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 [illegible] 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;

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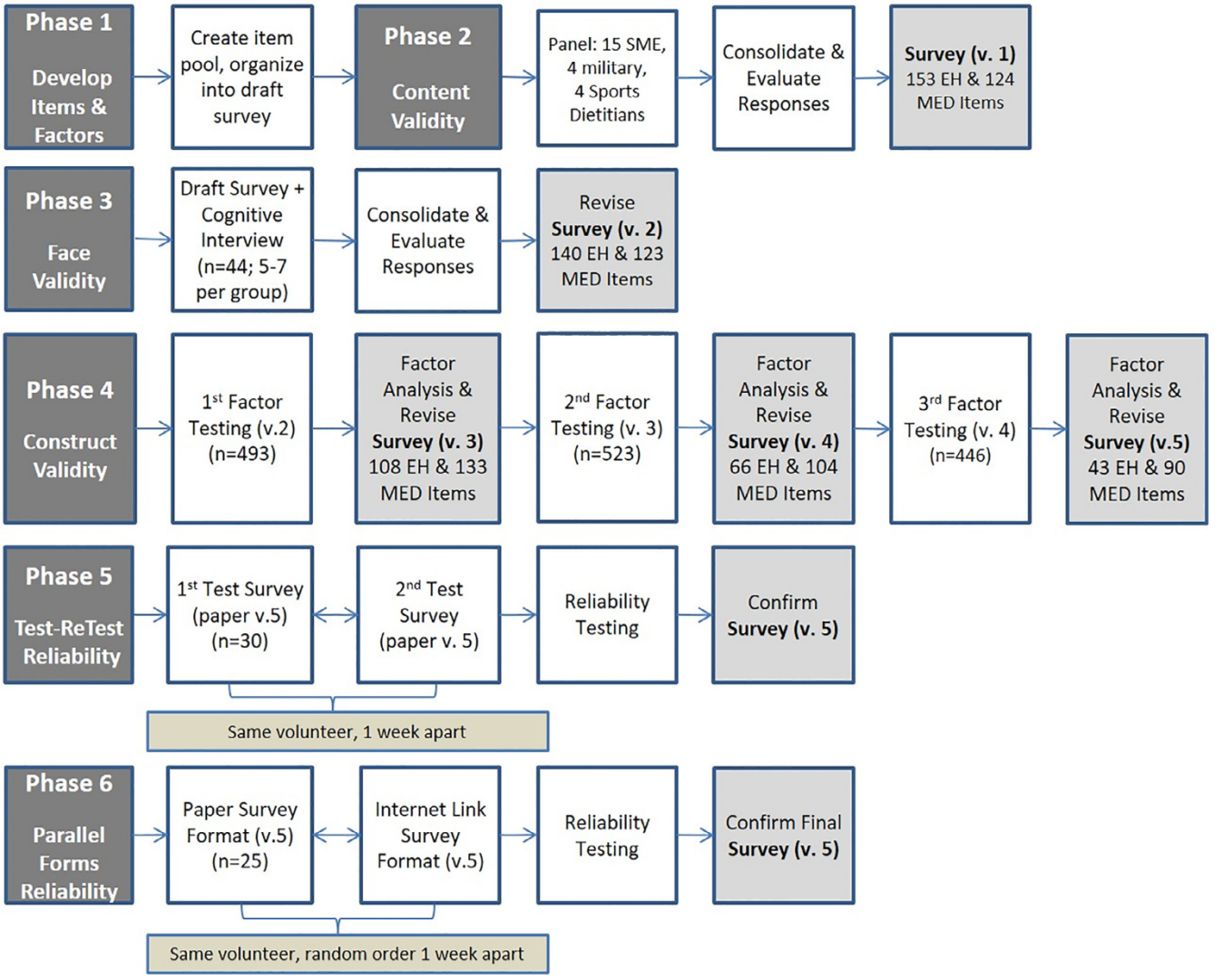


Figure 2. Diagram depicting the development and validation process of the Military Eating Behavior Survey. EH indicates eating habits; MED, mediators of eating behavior; SME, subject matter experts; V, version. Note: The number of items retained in each survey version is denoted as EH or MED in the light 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

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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 con- t 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 ≥0.35 on a factor and if the item loaded ≥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 ≥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]

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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 re- 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

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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.

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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 [illegible]tive 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.

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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[d]	0.683[d]
Satiety	0.71	0.711[d]	0.845[e]
Food craving	0.75	0.792[e]	0.704[d]
Meal pattern	0.76	0.673[d]	0.933[e]
Restraint	0.76	0.920[e]	0.915[e]
Dietary rigidity	0.73	0.892[e]	0.820[e]
Emotional eating	0.84	0.613[d]	0.844[e]
Fast eating rate	0.72	0.844[e]	0.921[e]
Slow eating rate	0.65	0.856[e]	0.742[d]
Environmental triggers	0.78	0.699[d]	0.898[e]
Situational eating	0.63	0.607[f]	0.829[e]
General health supplement	0.52	NV	0.785[e]
Performance supplement	0.82	0.914[e]	0.849[e]
Food choice (HES-7)	0.52	0.887[e]	0.941[e]
Mediators of Eating Behavior			
Body composition strategy	0.87	0.815[e]	0.734[d]
Perceived stress	0.87	0.879[e]	0.889[e]
Food access	0.68	0.717[d]	0.808[e]
Sleep habits	0.55	0.775[e]	0.895[e]
Military fitness	0.68	0.879[e]	0.783[e]
Physical activity quantity	0.67	0.675[d]	0.838[e]
Military body image scale	0.60	0.874[e]	0.871[e]
Concern with military image	0.54	0.793[e]	0.821[e]
Satisfaction with body shape	0.88	0.904[e]	0.776[e]
Agreement with ideal military physique	0.63	0.742[e]	0.894[e]
Nutrition knowledge	0.72	0.915[e]	0.865[e]

HES indicates Healthy Eating Score; ICC, intraclass correlation coefficient; NV, no variance.
[a]n = 1,462; [b]n = 30; [c]n = 25; [d]$P < 0.01$; [e]$P < 0.001$; [f]$P < 0.05$.
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 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

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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)[b]	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)[c]	18.5 (3.3)	16.8 (4.3)[b]	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)[c]	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)[c]	11.9 (2.5)	9.8 (3.1)[d]	10.7 (3.0)	10.0 (3.2)[c]	10.7 (3.0)
Emotional eating	0–12	2.7 (2.7)[d]	3.6 (2.2)	2.7 (2.8)	3.0 (2.1)	2.5 (2.5)	3.0 (2.9)	2.6 (2.7)[b]	2.9 (2.7)
Fast eating rate	0–12	6.4 (2.5)[c]	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)[b]	2.9 (1.7)	2.4 (1.6)	2.5 (1.8)	2.7 (1.7)[d]	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)[b]	3.5 (1.4)	3.9 (1.4)[b]	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)[c]	0.6 (0.7)	0.3 (0.6)[c]	0.7 (0.7)	0.3 (0.6)	0.4 (0.7)	0.3 (0.6)[c]	0.5 (0.8)
Performance	0–4	1.0 (1.4)[c]	0.3 (0.5)	0.9 (1.3)[d]	1.5 (1.6)	0.8 (1.3)[b]	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)[d]	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)[b]	1.3 (1.6)	1.0 (1.5)[d]	0.5 (1.1)	0.4 (0.9)[c]	1.4 (1.7)	0.8 (1.4)[b]	1.1 (1.6)
Military perceived stress	0–40	14.6 (6.6)	14.8 (5.4)	14.8 (6.6)[b]	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)[c]	9.7 (1.7)	8.9 (2.1)[b]	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)[c]	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)[b]	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)[b]	12.5 (3.9)	12.6 (4.2)[c]	14.2 (4.0)	13.4 (4.2)	13.5 (4.1)
AMP	3–15	8.6 (2.3)[b]	7.7 (2.8)	8.5 (2.4)	8.2 (2.0)	7.7 (2.1)[c]	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)[d]	5.3 (1.5)	4.8 (1.7)	4.6 (1.6)	4.5 (1.7)[b]	4.9 (1.6)
Military fitness test	0–100	86.9 (10.0)	88.5 (9.9)	85.9 (10.0)[c]	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)[c]	68.5 (45.4)	102.1 (82.9)[d]	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)[c]	50.6 (8.6)	41.0 (8.9)[b]	42.8 (9.5)	40.0 (7.8)[c]	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); [b]$P < 0.05$; [c]$P < 0.001$; [d]$P < 0.01$.
Note: Independent sample *t* test comparison for body mass index and age groups and Mann-Whitney U comparison for sex and rank group.

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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.

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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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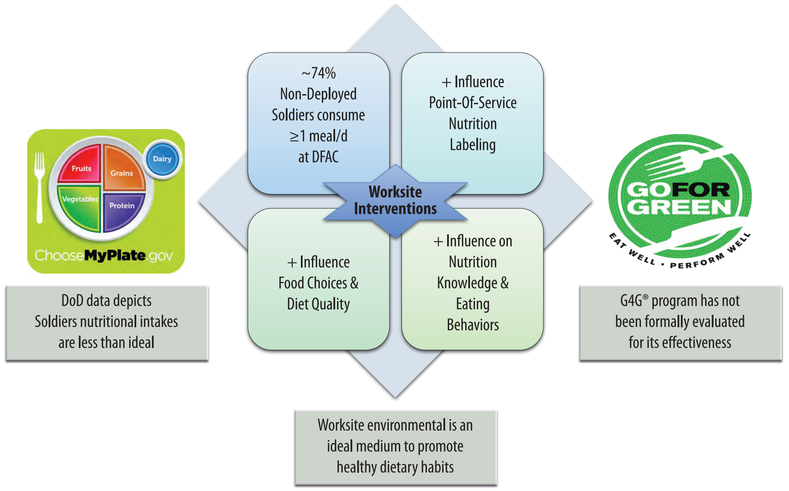
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(32% pre; 57% post; $p<0.001$), vegetarian options were available (25% pre; 38% post; $p=0.023$), and they used nutrition labels to choose performance foods (38% pre; 54% post; $p=0.039$).

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

	GO FOR GREEN			
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	

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 $p<0.05$).

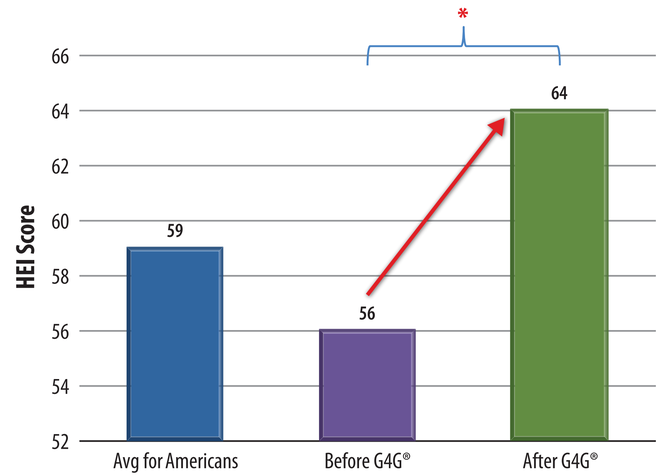
FOOD PHOTOGRAPHY



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; $p=0.039$).

** p <0.05 pre- post intervention*



HEI, Healthy Eating Index
*$p<0.05$ pre- post intervention
https://www.fns.usda.gov/hei-scores-americans

Figure 1. HEI-2015 Overall Score Change

Table 2. Diet Quality – HEI-2015 Score

HEI-2015 Domains & Total Score (points)	Control DFAC Patron Intake				
	Max Points Available	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*

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.