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

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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: - Determine the physiological and psychological effects of testosterone during severe energy deficit and recovery - Determine the substrate utilization, exercise performance, and skeletal muscle response to energy deficit and altitude acclimatization - Effects of energy balance and energy deficit on inflammatory response - Go for Green (GFG) Effectiveness - 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:

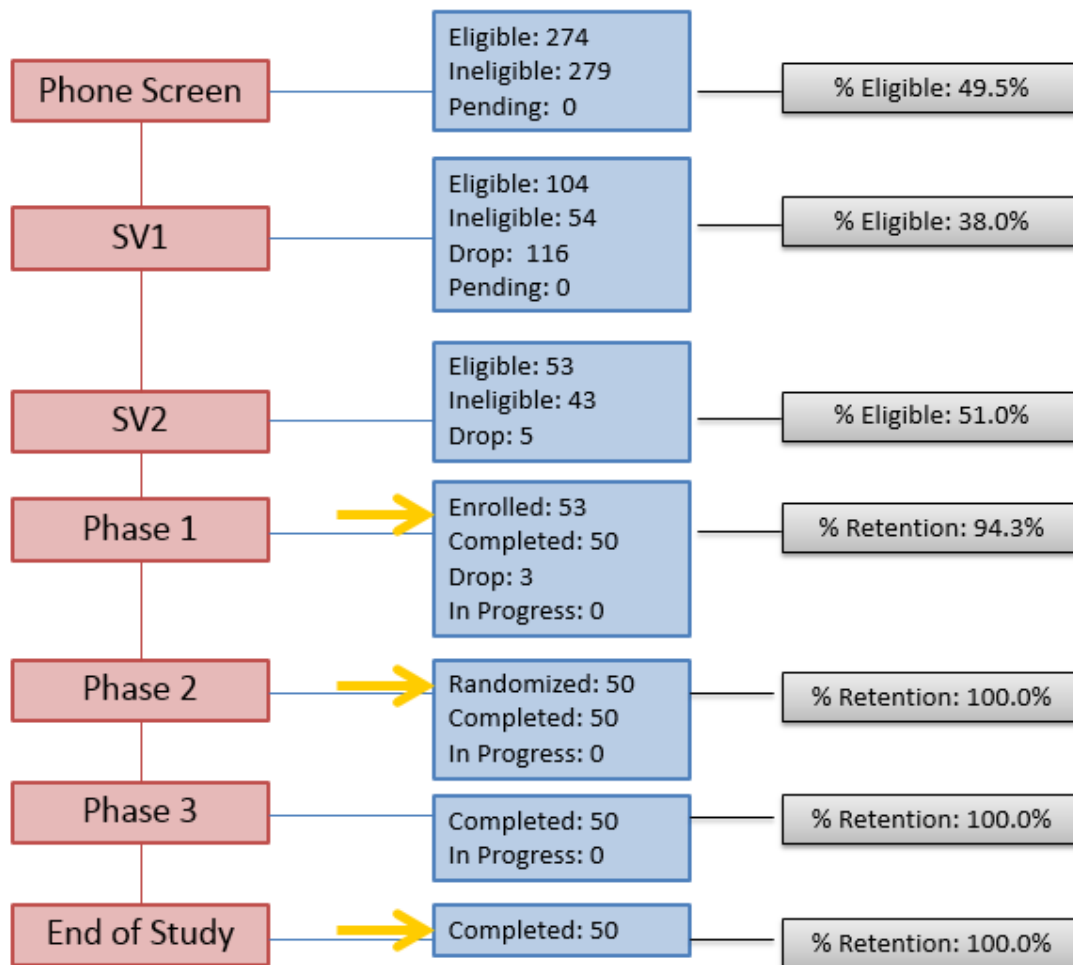
The major goals as defined in the statement of work involve 5 individual projects. Each of those projects is described below along with milestones, dates of completion, and current status.

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

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



I received continuing review approval for this project from the Pennington Biomedical Research Center Institutional Review Board (IRB) on September 20, 2017. All data was collected and I requested that the IRB status be changed from “active” to “data analysis only.” I received the approval on January 31, 2018. I notified HRPO of this status change to the project on February 22, 2018.

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

Data analysis for this project began in the second quarter and is continuing.

We have completed blood analysis on the following analytes: glucose, creatinine, potassium, uric acid, albumin, calcium, magnesium, CPK, ALT, alkaline phosphatase, iron, total

cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, complete blood count with differential, cortisol, insulin, total testosterone, free testosterone, FSH, LH, estradiol, SHBG, lipoprotein binding protein, DHEAs, ferritin, IGF-1, leptin, LpA, prolactin, prostate specific antigen, soluble transferrin receptor, hepcidin, active ghrelin and Interleukin 6. We also completed total nitrogen and creatinine on all of the urine samples collected during the study.

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

All of the DXA body composition data has been compiled and is being analyzed. In total, there are 132 body composition variables that are measured and/or calculated during the DXA test.

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 #2: Co-author initial manuscript on findings from clinical study (months 6-12)

The initial manuscript for this project is being prepared for submission.

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.

Major Task 1: Data Interpretation and Publishing of Results

Milestone #2: Co-author initial manuscript on findings from clinical study (months 1-12)

This milestone was achieved, as the first two articles from this project were published in the third quarter as detailed in the Products section. Three additional manuscripts were published in the fourth quarter, and a sixth is being prepared for submission. Copies of the five published articles are included with this report.

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

Specific Aim: Clinical study to assess the effects of energy balance and energy deficit on inflammatory response

Major Task 1: Conduct the clinical study, as determined during final protocol development, to determine effects of energy deficit on inflammation and nutritional status during short-term military training. (months 1-24)

During this year, the protocol was drafted, and the Statement of Work (SOW) was updated and submitted for approval in May 2018.

In brief, we will conduct a controlled laboratory study that simulates the physiological stressors imposed during sustained operations (SUSOPS) to determine if the IL-6 inflammatory response is exacerbated by underfeeding. Twenty-six male, non-obese (body mass index 19.9-29.9 kg/m²), active duty military personnel (aged 18-39 years) will be recruited to participate in a 32-day, longitudinal study. The study is comprised of four sequential phases: 1) a 96-hour SUSOPS, 2) a 7-day recovery period (Recovery 1), 3) a second, 96-hour SUSOPS, and 4) a 14-day recovery period (Recovery 2). During SUSOPS, subjects will be randomized to consume either sufficient food (combat rations) to maintain energy balance within $\pm 10\%$ of estimated total daily energy expenditure (SUSOPS BAL) or will be provided only enough food to match 45% of total daily energy expenditure to elicit severe negative energy balance (SUSOPS NEG BAL). Testing procedures and primary outcome measures include dual energy X-ray absorptiometry (DXA), maximal and sub-maximal aerobic exercise testing, load carriage exercise, controlled feeding, stable isotope assessments of energy expenditure, fractional iron absorption and whole-body protein turnover, percutaneous muscle biopsies to assess intramuscular IL-6, glycogen status, and regulators of muscle remodeling and substrate metabolism, intestinal permeability, gut microbiome composition and function, and blood sampling to assess circulating concentrations of IL-6 and other biomarkers of inflammation, muscle damage, nutritional, metabolic stress, and immune responses to SUSOPS BAL and NEG BAL. Physical performance will be assessed before and after each SUSOPS and during each recovery period using the vertical jump test. Self-reported mood, sustained attention, and cognitive performance will be assessed twice daily using the Profile of Mood States, the Psychomotor Vigilance Task, the Evaluation of Risk Questionnaire, the Match-to-Sample task, the Grammatical Reasoning test, the Visuomotor Integration task, and a 3D tracking task. Overall vigilance and nighttime sleep quality will be assessed via the wrist-worn USARIEM Vigilance Monitoring System. This design will test the hypothesis that maintaining energy balance will attenuate the IL-6 response to SUSOPS, improve iron absorption, and attenuate the potential negative effects of excessive inflammation on whole-body metabolic homeostasis. Risks include those associated with venous catheterization, venipuncture, muscle biopsies, DXA, exercise and the discomfort of severe underfeeding.

Milestone #3: Regulatory approval received (months 1-12)

At the time of the award, Project 3 was not completely detailed. The project was clearly defined in a second revision of the SOW that was submitted in May 2018 but was not accepted before the end of this reporting period. Milestone #3 was achieved in April 2018. The Headquarters, US Army Medical Research and Materiel Command Institutional Review Board (HQ USAMRMC IRB) approved the protocol on April 27, 2018. Pennington Biomedical Research Center's IRB determined Dr. Jennifer Rood's role in this project is "not human subjects research" as defined by 45 CFR Part 46 and does not require an additional IRB submission.

Project 4: Go for Green (GFG) Effectiveness

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

Major Task 1: Conduct the clinical study (months 1-24)

Milestone #5: Regulatory approval received (months 1-12)

Milestone #5 was met when regulatory approval was granted in the first quarter. Recruiting began in the first quarter. A total of 92 DFAC staff and 89 DFAC patrons were recruited for baseline data collection in year 1. All data collected was processed and sent to USARIEM. Nicholes Armstrong assumed leadership in place of LTC Renee Cole, who was transferred to San Antonio. Additional data collection was scheduled for year 2.

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

The draft survey was developed and IRB approval was obtained in the third quarter.

Major Task 2: Validation of new survey (months 24-36)

Initial pretests were conducted in soldier populations at Ft. Jackson, SC; Ft. Campbell, KY; Ft. Riley, KS; Joint Base San Antonio, TX; Ft. Carson, CO; Joint Base Lewis-McChord, WA; and Joint Base Elmendorf-Richardson, AK. Analysis and refinement of the survey began in the third quarter.

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 1: Conduct the clinical study. Biospecimen collection tubes and storage vials will be sent to field collection sites and samples will be collected.

Project 4: Go for Green (GFG) Effectiveness

Major Task 1: Conduct the clinical study: data collection will continue.

Project 5: Develop a military-specific eating behavior survey

Major Task 2: Validation of new Survey 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:

Fat free mass declines during periods of underfeeding for 21 days at high altitude despite increasing protein intake to twice the recommended dietary intake.

Appetite is suppressed during underfeeding at high altitude and this is associated with appetite-mediating hormones. Dietary protein did not impact appetite.

Underfeeding at high altitude inhibits skeletal muscle signaling pathways which leads to a loss of muscle.

Gene expression of the Fn14 gene may protect against the loss of muscle during underfeeding at high altitude.

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

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

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The contract was modified in November 2017 to incorporate the revised Statement of Work (SOW) dated 16 Nov 2017. This revision eliminated a milestone that was achieved under a separate award and updated the subject numbers and Gantt chart for Project 4, Go for Green (GFG) Effectiveness, to match the protocol approved by the Institutional Review Board and Human Research Protection Office. The increase in Project 4's maximum number of participants is not expected to affect the study's budget or timeline. Regulatory approval to increase the

maximum number of participants was sought based on a lower than anticipated completion rate in the first iteration. More participants were recruited in order to attain the budgeted sample size of participants completing the study.

At the time of the award, Project 3 was not completely detailed. The project was clearly defined in a second revision of the SOW that was submitted in May 2018 but was not approved before the end of this reporting period.

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

In the third quarter, Nicholes Armstrong assumed leadership of Project 4, Go for Green (GFG) Effectiveness, in place of LTC Renee Cole, who was transferred to San Antonio. Data collection was delayed due to the transfer, delays in implementing the protocol within the dining facility, and deployment schedules of soldiers. The data collection schedule for year 2 has been adjusted.

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.

Five manuscripts for Project 2 were published this year, and copies of the published articles are included. A sixth manuscript for Project 2 is being prepared for submission.

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

2. Karl JP, Cole RE, Berryman CE, Finlayson G, Radcliffe PN, Kominsky MT, Murphy NE, Carbone JW, Rood JC, Young AJ, and Pasiakos SM. Appetite suppression and altered food preferences coincide with changes in appetite-mediating hormones during energy deficit at high altitude, but are not affected by protein intake. *High Alt Med Biol.* 2018 Jun;19(2):156-169. doi: 10.1089/ham.2017.0155. Epub 2018 Feb 12. PMID: 29431471. PMCID: PMC6014054.
3. Margolis LM, Carbone JW, Berryman CE, Carrigan CT, Murphy NE, Ferrando AA, Young AJ, Pasiakos SM. Severe energy deficit at high altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. *FASEB J.* 2018 Jun 7:fj201800163RR. doi: 10.1096/fj.201800163RR. (Epub ahead of print) PMID: 29878853.
4. Pasiakos SM, Berryman CE, Carbone JW, Murphy NE, Carrigan CT, Bamman MM, Ferrando AA, Young AJ, Margolis LM. Muscle Fn14 gene expression is associated with fat-free mass retention during energy deficit at high altitude. *Physiol Rep.* 2018 Jul;6(14):e13801. doi: 10.14814/phy2.13801. PMID: 30009538. PMCID: PMC6046641.
5. Young AJ, Berryman CE, Kenefick RW, Derosier AN, Margolis LM, Wilson MA, Carrigan CT, Murphy NE, Carbone JW, Rood JC, Pasiakos SM. Altitude acclimatization alleviates the hypoxia-induced suppression of exogenous glucose oxidation during steady-state aerobic exercise. *Front Physiol.* 2018 Jul 9;9:830. doi: 10.3389/fphys.2018.00830. eCollection 2018. PMID: 30038576. PMCID: PMC6046468.
6. Karl J, Berryman C, Young A, Radcliffe P, Branck T, Pantoja-Feliciano I, Rood J, Pasiakos S. Associations between the gut microbiota and host responses to high altitude. *Am J Physiol Gastrointest Liver Physiol.* (in progress)

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 a national conference this year. Two others were accepted for presentation at meetings in year 2, and a fourth has been submitted but not yet accepted. Copies of the first three abstracts are included in the appendix.

1. Champagne CM, Allen HR, Cole RE, Armstrong NJ, Miketinas DC. Using the HEI-2010 vs HEI-2015 to determine measures of diet quality – where are the differences? American Society for Nutrition Annual Conference, June 2018, Boston.
2. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Diet Adequacy of Army Soldiers Supports the Need for a Planned Dining Facility Intervention. Society for Nutrition Education and Behavior Annual Conference, July 2018, Minneapolis.

3. Champagne CM, Allen HR, Cole RE, Armstrong N, McGraw SM, Moylan E, Miketinas DC. Baseline Dietary Intakes of Army Soldiers Prior to Go For Green® 2.0 Dining Facility Dietary Intervention. Military Health System Research Symposium, August 2018.
 4. Serrano EE, Armstrong NJ, Champagne CM, Hatch A, McGraw SM, Cole RE. A Nutrition Knowledge Comparison of Army Culinary Specialist and the Soldiers They Feed at a Fort Carson Dining Facility (DFAC). Society for Nutrition Education and Behavior. (submitted)
- **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: 8.4

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: 2.25

Contribution to Project: She serves as the investigator for project 4.

Name: Ray Allen

Project Role: Computer support

Nearest Person Month Worked: 3.09

Contribution to Project: He provides computer support for project 4.

Name: Bridget Conner

Project Role: Medical Technologist

Nearest Person Month Worked: 10.2

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: Melissa Harris

Project Role: project manager

ORCID ID: 0000-0001-9113-1695

Nearest Person Month Worked: 5.4

Contribution to Project: She serves as the project manager for project 1.

Name: Valery Hymel

Project Role: research associate

Nearest Person Month Worked: 10.2

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: Steve Lee

Project Role: medical technologist

Nearest Person Month Worked: 10.2

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

Nearest Person Month Worked: 10.2

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: 10.2

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

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.

Name: Dachuan Zhang

Project Role: biostatistician

Nearest Person Month Worked: 2.55

Contribution to Project: He provides data analysis for project 1.

Total Expenditures to Date: \$2,023,837.07

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?

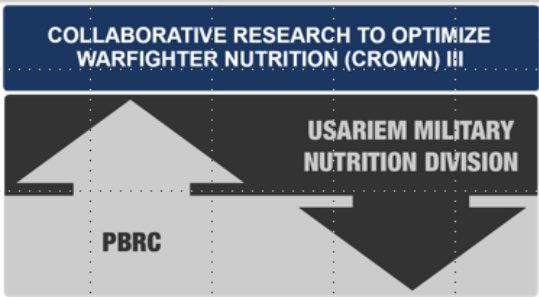
Organization Name: United States Army Research Institute of Environmental Medicine, Military Nutrition Division

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) Determine the interaction of gender and physiological strain on inflammation 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		Goals/Milestones:			
Activities	FY	17	18	19	20
Protocol Development/ Approval		[Bar]		[Bar]	
Data Analysis		[Bar]			
Publication of Results		[Bar]			
Estimated Budget (M)		\$2.165	\$1.5	\$1.5	\$1.5
Updated: (08/14/2018)					
FY17 – complete the clinical study for project 1, publish manuscript on methodology used in project 1, publish initial manuscript on findings from projects 1 and 2, draft protocol for project 3, obtain regulatory approval for project 4 FY18 – publish additional manuscripts from projects 1 and 2, conduct clinical study for project 3, conduct study for project 4 FY19 – development of a new/validated survey for project 5, publish additional findings from project 1-4 FY20 – publish initial manuscript for project 5, publish additional findings from projects 1-4 • Budget Expenditures to date: \$2,023,837					

9. APPENDICES: see following pages

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

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

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

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

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

For lowlanders sojourning at high altitude (HA >1500 m), maintaining energy balance can be challenging because of an increase in resting metabolic rate (RMR) (1, 2) and a decrease in appetite (3) caused by physiologic adaptations to hypoxia. The effects of hypoxia on energy balance are frequently compounded by limited access to food and high physical activity levels while at HA. Negative energy balance usually develops at HA and typically leads to

weight loss (4). Barnholt *et al.* (4) showed that individuals sustaining a 40% energy deficit (ED) for 21 d at HA lost more body weight than individuals adhering to the same controlled diet and physical activity at sea level (SL). Furthermore, some studies (5–8) have observed >50% of total body mass (TBM) loss as fat-free mass (FFM) during prolonged ED at HA, which is greater than the 25–35% typically observed at SL (9–11), suggesting protein turnover kinetics and substrate utilization during weight loss at HA may differ from that at SL.

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

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

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

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

MATERIALS AND METHODS

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

Participants

Recreationally active (programmed physical activity 2–4 d/wk) men ($n = 17$), aged 18–42 yr, who were not acclimatized to HA (i.e., born at altitudes <2100 m, currently residing at altitudes <1200 m, and who had not traveled to altitudes >1200 m for 5 d or more within 2 mo before beginning the study) were recruited. Participants were not taking any medications or were willing to refrain from all medication use for 4 wk before and throughout the entire study period, unless provided or approved by the study's medical oversight. Participants agreed to refrain from alcohol, smoking any nicotine product, using electronic cigarettes (vaping), chewing tobacco, caffeine, and dietary supplement use throughout the entire study period. Additional

exclusion criteria included metabolic diseases (e.g., diabetes and cardiovascular disease); injuries that would compromise exercise capability; gastrointestinal disorders; evidence of apnea or other sleeping disorders; prior HA pulmonary edema or HA cerebral edema diagnosis; allergies or intolerance to foods; vegetarian practices; history of complications with lidocaine; evidence of any physical, mental, or medical condition that would make participation more hazardous; present condition of alcoholism, anabolic steroid use, or other substance abuse; anemia; sickle cell anemia or trait; abnormal blood clotting; blood donation within 8 wk of beginning the study; any use of antibiotics, except topical antibiotics, within 3 mo of study participation; colonoscopy within 3 mo of study participation; use of laxatives, stool softeners, or antidiarrheal medications more than once a week.

Experimental design

This randomized, controlled trial included 2 phases conducted over 43 consecutive days. In phase 1 of the study (SL d 1–21, 21 d) participants were free-living and resided at SL (55 m, Natick, MA, USA). On d 21, participants were flown to Denver, CO, and transported by car to Colorado Springs, CO, where they stayed overnight under supervision in a local hotel. The following morning (HA d 1, ~3 AM), participants were transported by car to the USARIEM Maher Memorial Altitude Laboratory on the summit of Pikes Peak, CO (4300 m). Participants breathed supplemental oxygen (portable O₂ concentrators, FreeStyle 5; Air-Sep, Buffalo, NY, USA or EverGo, Respironics, Murrysville, PA, USA) after landing in Denver until they arrived at the summit to maintain normoxic oxygen saturation (means $\pm$ SD for SpO₂ measured hourly from arrival until 10 PM and every 30 min beginning at 3 AM was $97.0 \pm 0.9\%$, range 95–99%) in an effort to normalize exposure across volunteers and limit nonstudy hypoxic exposure. During phase 2 (HA d 1–22, 22 d), participants were randomized (computer generated, with 1:1 allocation for the 2 parallel groups) to a measured and controlled 40% ED diet (see Diet Intervention) and resided at the Maher Memorial Altitude Laboratory.

Assessment of energy intake and physical activity at SL

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

Diet intervention

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

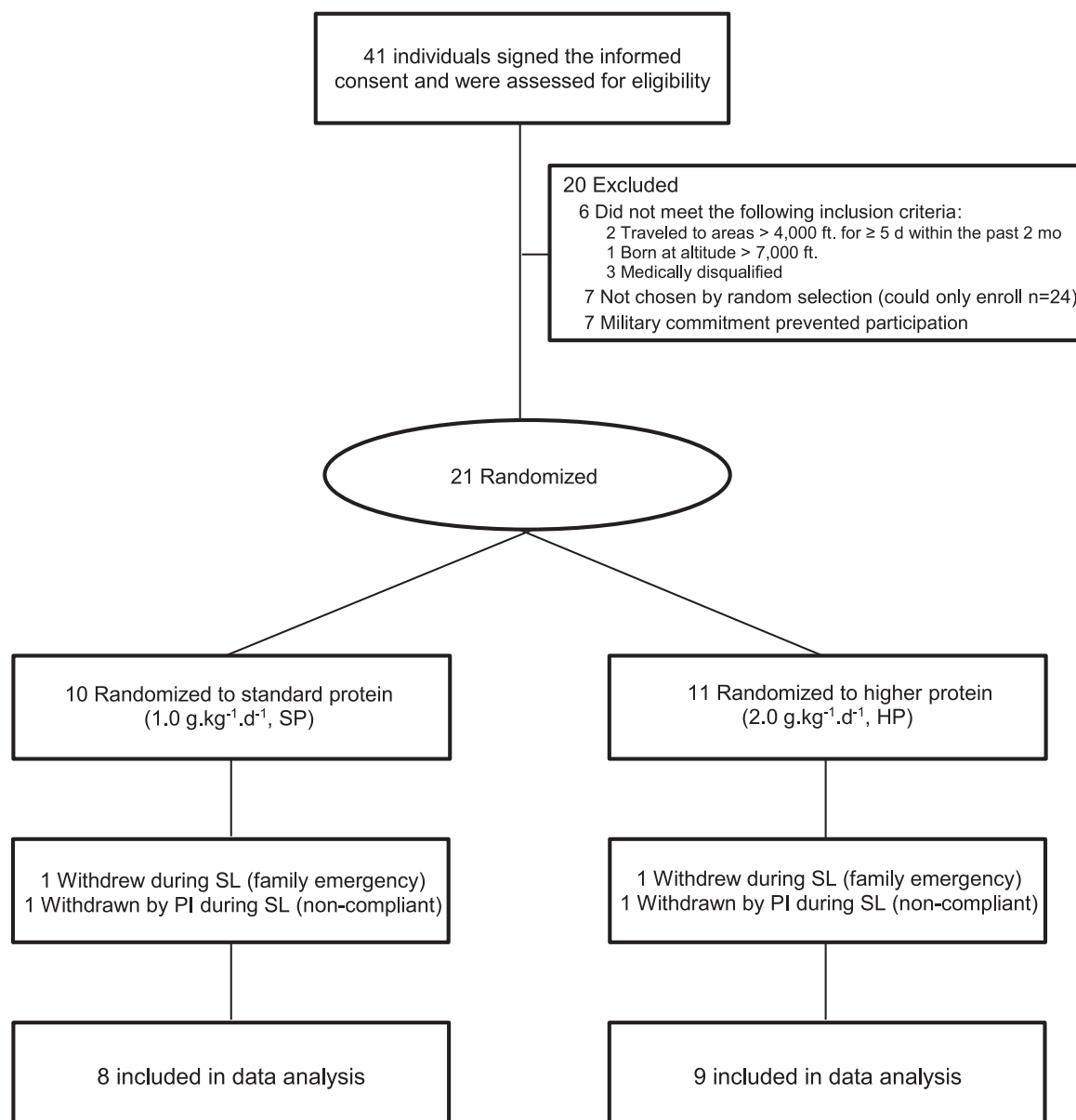


Figure 1. Volunteer selection chart.

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

During phase 2, energy intake was individualized for each participant. The controlled diet began on HA d 2 (HA d 2 to 22; 21 d diet intervention), after the stable isotope infusion protocol on HA d 1 (see Whole-Body Protein Turnover). To achieve the 40% ED, daily physical activity was increased by 10% (see Physical Activity Prescription) and daily energy intake was decreased by 30% relative to TDEE estimated at SL during energy balance. Registered dietitians developed individualized daily menus using the Combat Rations Database (U.S. Army Natick Soldier Research, Development, and Engineering Center and USARIEM) and Food Processor SQL (v.10.14; ESHA Research, Salem, OR, USA). Dietary carbohydrate provided 45% of total energy. Dietary protein provided either 1.0 ± 0.2 (SP) or 2.0 ± 0.2 (HP) g/kg per day. Fat provided the remainder of energy in the diets. The rationale for manipulating fat, instead of carbohydrate, to compensate for the 2 levels of protein intake was to avoid compromising a secondary study objective investigating carbohydrate

metabolism during exercise at SL and HA. Most of the foods in these diets were components of the military combat ration, Meal, Ready-to-Eat (MRE; menu 34; Ameriquel, Evansville, IN, USA). The remainder of energy came from non-MRE foods (*e.g.*, fruits, vegetables, olive oil, and ranch dressing) and whey protein beverages (*i.e.*, Isopure Zero Carb; Isopure, Hauppauge, NY, USA) to achieve individualized macronutrient requirements that could not be met using MRE rations alone. All foods and caloric beverages were weighed to the nearest 0.1 g by trained research staff. Meals and snacks were supervised, and participants were instructed to consume all provided foods and beverages. Any uneaten foods or beverages were weighed and documented. Noncaloric, noncaffeinated beverages (*e.g.*, water and carbonated drinks) were allowed *ad libitum*.

Physical activity prescription

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

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

Anthropometric measurements

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

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

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

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

Determination of peak oxygen uptake

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

Assessment of resting metabolic rate

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

on d 2 was also assigned to HA d 3). Thus, the 21 d at HA that coincided with the diet intervention (*i.e.*, HA d 2 to HA d 22) were assigned an RMR value to allow estimation of the cumulative contribution of RMR to the total ED.

Estimation of substrate oxidation

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

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

$$\text{Glucose oxidation} = 4.55 \times \dot{V}CO_2 - 3.21 \times \dot{V}O_2 - 2.87 \times \text{urinary nitrogen} \quad (3)$$

$$\text{Fat oxidation} = 1.67 \times \dot{V}O_2 - 1.67 \times \dot{V}CO_2 - 1.92 \times \text{urinary nitrogen} \quad (4)$$

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

Metabolic and endocrine biomarkers

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

TABLE 1. *Prestudy characteristics*

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

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

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

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

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

Whole-body protein turnover

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

Blood sample processing for isotope enrichment

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

Whole-body protein turnover calculations

Whole-body PS and PB rates were calculated based on the determinations of the rate of appearance (*R_a*) into the plasma

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

$$\text{Total plasma } R_a = F/E \quad (5)$$

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

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

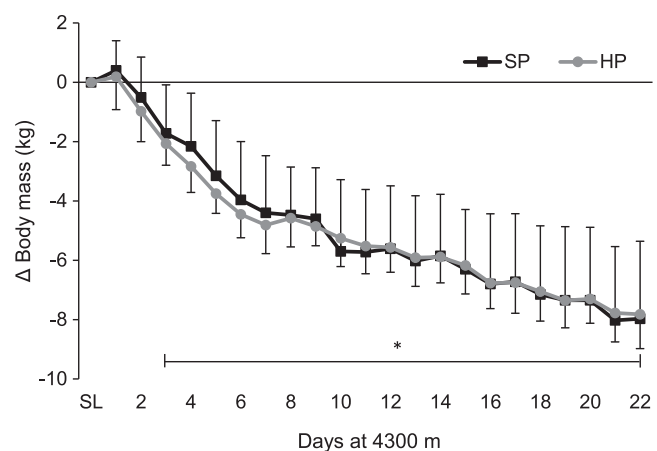


Figure 2. Mean change in body mass with consumption of an SP (*n* = 8) vs. an HP (*n* = 9) diet during ED at HA. Data were analyzed with linear mixed models with volunteer as the random effect and diet, time, and their interaction as fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni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

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

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

Linear mixed models were used to determine the main effects of diet: SP ($n = 8$) vs. HP ($n = 9$); time: SL d 20 vs. HA d 23, and their interaction. ^aNonaqueous FFM was estimated as 27% of total FFM (27). ^bAqueous FFM was estimated as 73% of total FFM (27).

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

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

$$NB = PS - PB \quad (10)$$

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

Statistical analysis

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

groups, an SD of 0.7 kg, and an α of 0.05, to provide more than 80% power to detect differences with 8 per group.

RESULTS

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

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

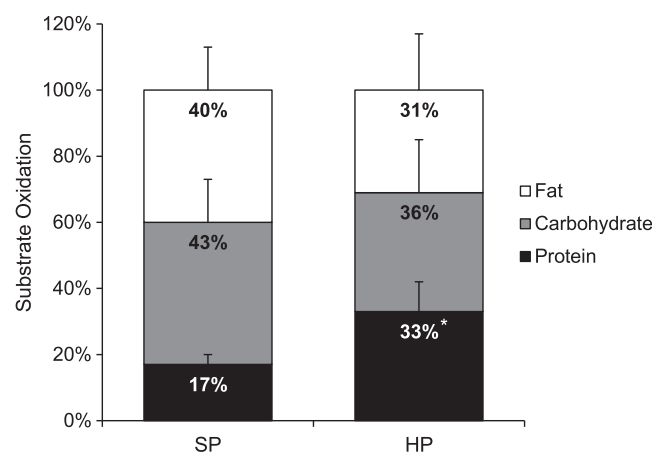


Figure 3. Substrate oxidation with consumption of an SP ($n = 8$) vs. HP ($n = 8$) diet during ED at HA. Independent Student's t tests were used to compare means between diet groups for each macronutrient. * $P < 0.05$.

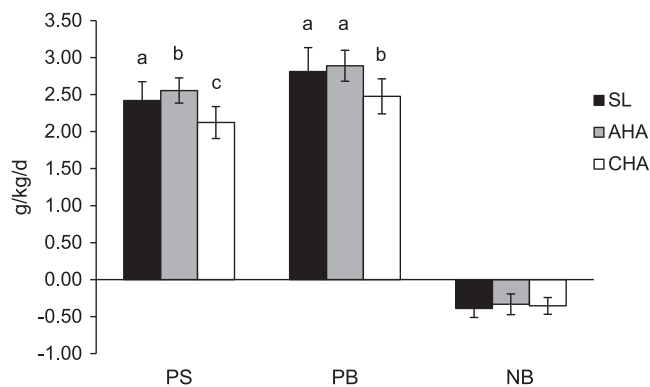


Figure 4. Postabsorptive whole-body protein turnover at SL, acute HA (AHA), and chronic HA (CHA). Linear mixed models, with volunteer treated as a random effect, were used to determine effects of time (fixed effect). Different lowercase letters within a variable indicate a significant difference. $P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

(1.00 ± 0.64 compared to 1.37 ± 0.55 g fat/kg/d) oxidation were not significantly different between HP and SP. Postabsorptive whole-body PS was increased at acute HA compared to SL and decreased at chronic HA compared to both SL and acute HA (main effect of time, $P < 0.01$; Fig. 4). PB was lower at chronic HA compared to both SL and acute HA (main effect of time, $P < 0.01$). NB did not differ by time ($P = 0.40$). To investigate diet-specific effects on whole-body protein turnover after chronic HA exposure,

PS, PB, and NB were examined, with adjustment for acute HA measures. HP and SP PS (2.08 ± 0.23 vs. 2.17 ± 0.21 g/kg/d; $P = 0.17$) and PB (2.52 ± 0.24 vs. 2.43 ± 0.24 g/kg/d; $P = 0.79$) were not significantly different; however, NB was more negative for the HP than the SP diet (-0.44 ± 0.08 vs. -0.26 ± 0.06 g/kg/d; $P < 0.01$).

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

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

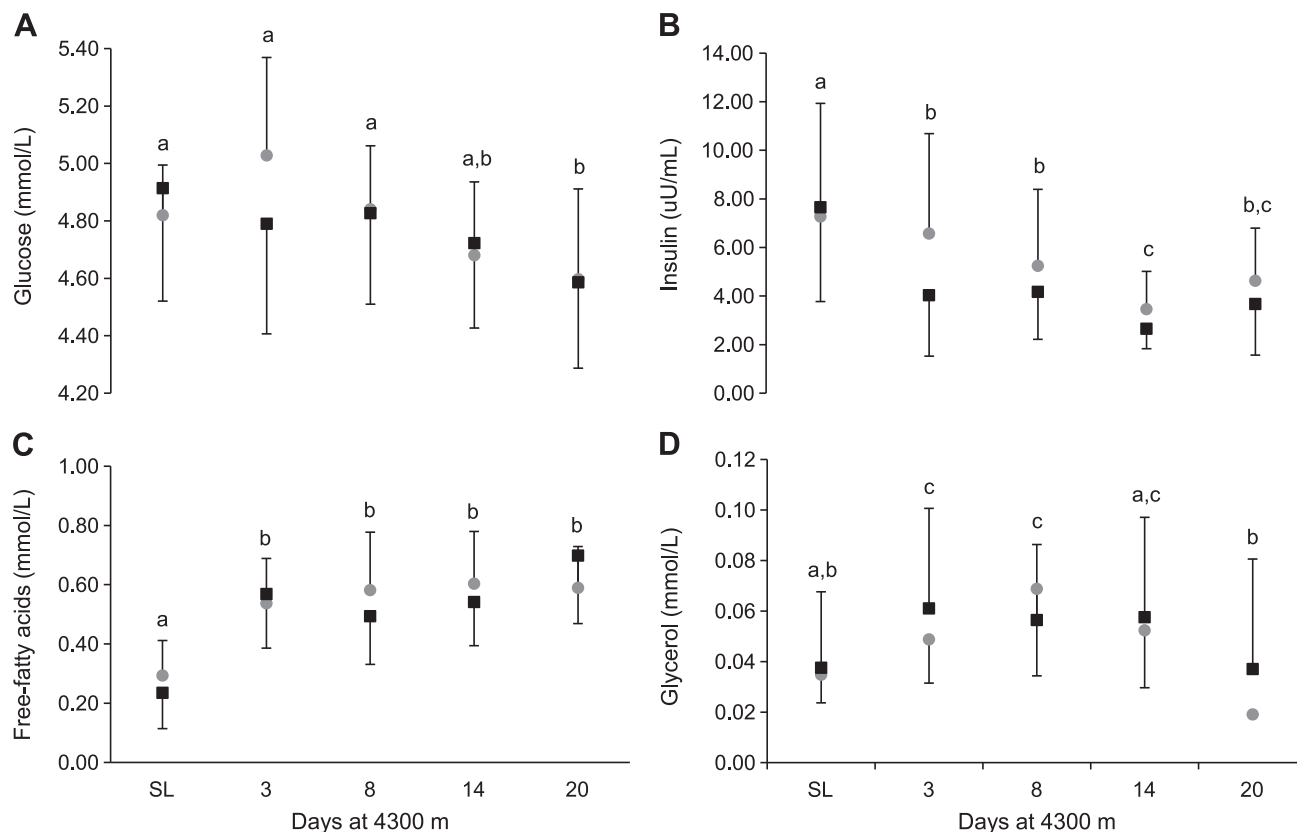


Figure 5. Longitudinal effects of a SP ($n = 8$) vs. HP ($n = 9$) diet on glucose (A), insulin (B), free-fatty acids (C), and glycerol (D) during energy deficit at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as fixed effects.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

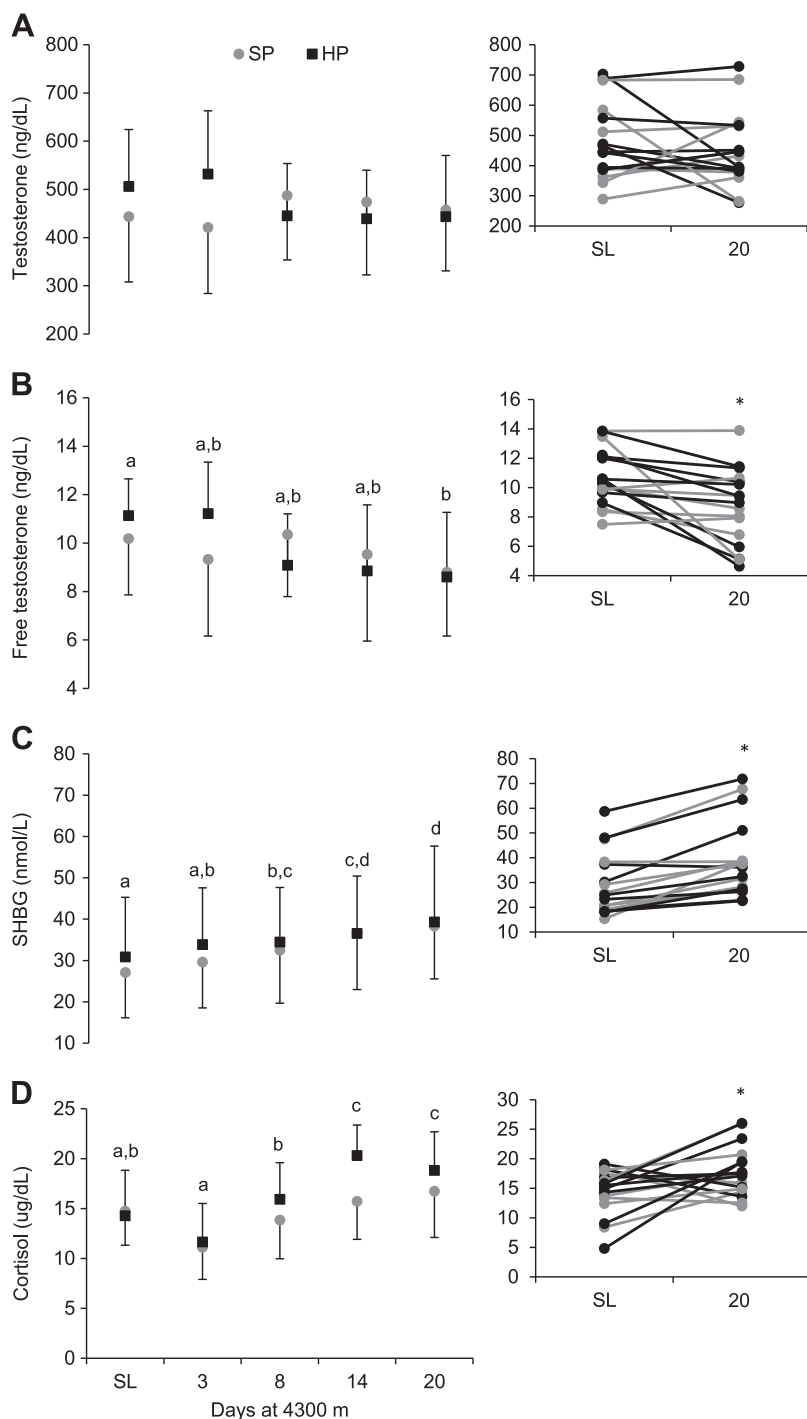


Figure 6. Longitudinal effects of an SP *vs.* HP protein diet on total testosterone (A), free testosterone (B), sex hormone-binding globulin (C), and cortisol (D) during ED at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as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

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

Participants who lost less FFM ($< 50\%$ of TBM loss; $n = 10$) had lower TBM and FM and higher aerobic fitness and total and free testosterone concentrations at SL than participants who lost more FFM ($> 50\%$ of TBM loss; $n = 7$; **Table 4**). In addition, at HA, participants who lost less

FFM had greater ED as measured by Δ ES, despite consuming significantly greater energy and carbohydrates per kilogram body weight than participants who lost predominantly FFM. Metabolic parameters and whole-body protein turnover measures at SL and substrate oxidation (absolute, relative, and percentage) at HA did not differ by FFM loss group (data not shown).

DISCUSSION

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

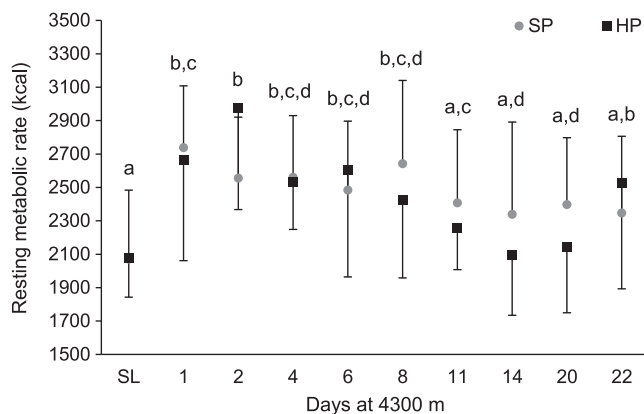


Figure 7. Longitudinal effects of an SP *vs.* HP diet on resting metabolic rate during energy deficit at HA. Data were analyzed using linear mixed models with volunteer as the random effect and diet, time, and their interaction as the fixed effects. *Post hoc* comparisons were adjusted with the Bonferroni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

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

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

Postabsorptive NB was more negative with an HP than with an SP diet at chronic HA, despite no significant differences in PS or PB. In another study, we demonstrated this effect at SL using ^{13}C -leucine whole-body protein turnover kinetics (41), showing that postabsorptive net

protein balance was more negative with increased protein intakes (0.8, 1.6, or 2.4 g protein/kg) because of a dose-dependent increase in whole-body protein oxidation. Similarly, in the current study, HP oxidized 75% more protein than SP. However, in the context of a 40% ED, HP diets have been shown to maintain an anabolic stimulus to feeding similar to that observed during weight maintenance, suggesting that although greater overall amounts of dietary protein are oxidized with increasing protein intakes, sufficient amino acid precursors remain to sustain anabolic sensitivity to protein-containing meals (12). In the current study, the additional protein provided to the HP group was almost completely oxidized (0.95 ± 0.32 g protein/kg/d more than in the SP group), precluding a sustained anabolic response to protein-containing meals and negating the FFM-sparing effects of the HP diet. The increase in whole-body PS at acute HA compared to SL is consistent with other studies showing increased PS in response to acute stress (17, 42, 43), which may be related to increased synthesis of acute phase proteins at HA (44). The observed suppression of both PS and PB at chronic HA is consistent with that in previous reports of whole-body protein kinetics during prolonged ED at SL (41, 45, 46), which indicates that protein and energy sparing are adaptive responses to sustained negative energy balance.

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

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

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

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

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

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

The prescribed ED and elevated RMR accounted for 74% of the total deficit. Studies have indicated a similar inability to account entirely for the apparent ED indicated by TBM loss at HA (3, 4, 50, 54). The thermic effect of food is ~10% of that measured for RMR, but may be greater when dietary protein intake is increased (55, 56). For example, those on the HP diet consumed ~65 g more protein than those on the SP diet, and if the thermic effect of metabolizing protein is 20–35% (compared to 5–15% for fat and carbohydrate), then the HP group may have expended 30–70 kcal/d more than the SP group. The unexplained deficit was numerically but not significantly greater in those consuming the HP diet, which is consistent with the greater energy cost associated with metabolizing protein, as compared to carbohydrate and fat (57, 58). The Pandolf

equation (23) has been shown to adequately predict EIEE at HA in young healthy males walking on a level or positive grade with oxygen uptake levels below 2.1 L/min (59), but a more recent study (60) evaluating energy expenditure during level walking in both males and females of a broader age, weight, and height range, suggests that the Pandolf equation (23) underestimates energy expenditure. Our findings are consistent with this underestimation, which may explain some of the unaccounted for energy expenditure. Finally, grade and terrain, 2 factors in the Pandolf equation (23, 61), were estimated from elevation maps and terrain coefficients (62), respectively, and errors in these estimations may also have contributed to an underestimation of EIEE.

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

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

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

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AUTHOR CONTRIBUTIONS

C. E. Berryman, A. J. Young, and S. M. Pasiakos designed the research; C. E. Berryman, A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, R. E. Cole, J. W. Carbone, I.-Y. Kim, A. A. Ferrando, and S. M. Pasiakos performed the research; C. E. Berryman and S. M. Pasiakos analyzed the data; C. E. Berryman, A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, J. W. Carbone, I.-Y. Kim, A. A. Ferrando, and S. M. Pasiakos interpreted the results; C. E. Berryman and S. M. Pasiakos prepared the figures; C. E.

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

REFERENCES

1. Butterfield, G. E., Gates, J., Fleming, S., Brooks, G. A., Sutton, J. R., and Reeves, J. T. (1992) Increased energy intake minimizes weight loss in men at high altitude. *J. Appl. Physiol.* (1985) **72**, 1741–1748
2. Grover, R. F. (1963) Basal oxygen uptake of man at high altitude. *J. Appl. Physiol.* **18**, 909–912
3. Butterfield, G. E. (1999) Nutrient requirements at high altitude. *Clin. Sports Med.* **18**, 607–621, viii
4. Barnholt, K. E., Hoffman, A. R., Rock, P. B., Muza, S. R., Fulco, C. S., Braun, B., Holloway, L., Mazzeo, R. S., Cymerman, A., and Friedlander, A. L. (2006) Endocrine responses to acute and chronic high-altitude exposure (4,300 meters): modulating effects of caloric restriction. *Am. J. Physiol. Endocrinol. Metab.* **290**, E1078–E1088
5. Rose, M. S., Houston, C. S., Fulco, C. S., Coates, G., Sutton, J. R., and Cymerman, A. (1988) Operation Everest. II: nutrition and body composition. *J. Appl. Physiol.* (1985) **65**, 2545–2551
6. Macdonald, J. H., Oliver, S. J., Hillyer, K., Sanders, S., Smith, Z., Williams, C., Yates, D., Ginnever, H., Scanlon, E., Roberts, E., Murphy, D., Lawley, J., and Chichester, E. (2009) Body composition at high altitude: a randomized placebo-controlled trial of dietary carbohydrate supplementation. *Am. J. Clin. Nutr.* **90**, 1193–1202
7. Wing-Gaia, S. L., Gershenoff, D. C., Drummond, M. J., and Askew, E. W. (2014) Effect of leucine supplementation on fat free mass with prolonged hypoxic exposure during a 13-day trek to Everest Base Camp: a double-blind randomized study. *Appl. Physiol. Nutr. Metab.* **39**, 318–323
8. Zaccagni, L., Barbieri, D., Cogo, A., and Gualdi-Russo, E. (2014) Anthropometric and body composition changes during expeditions at high altitude. *High Alt. Med. Biol.* **15**, 176–182
9. Chaston, T. B., Dixon, J. B., and O'Brien, P. E. (2007) Changes in fat-free mass during significant weight loss: a systematic review. *Int. J. Obes.* **31**, 743–750
10. Heymsfield, S. B., Gonzalez, M. C., Shen, W., Redman, L., and Thomas, D. (2014) Weight loss composition is one-fourth fat-free mass: a critical review and critique of this widely cited rule. *Obes. Rev.* **15**, 310–321
11. Weinheimer, E. M., Sands, L. P., and Campbell, W. W. (2010) A systematic review of the separate and combined effects of energy restriction and exercise on fat-free mass in middle-aged and older adults: implications for sarcopenic obesity. *Nutr. Rev.* **68**, 375–388
12. Pasiakos, S. M., Cao, J. J., Margolis, L. M., Sauter, E. R., Whigham, L. D., McClung, J. P., Rood, J. C., Carbone, J. W., Combs, G. F., Jr., and Young, A. J. (2013) Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial. *FASEB J.* **27**, 3837–3847
13. Longland, T. M., Oikawa, S. Y., Mitchell, C. J., Devries, M. C., and Phillips, S. M. (2016) Higher compared with lower dietary protein during an energy deficit combined with intense exercise promotes greater lean mass gain and fat mass loss: a randomized trial. *Am. J. Clin. Nutr.* **103**, 738–746
14. Mettler, S., Mitchell, N., and Tipton, K. D. (2010) Increased protein intake reduces lean body mass loss during weight loss in athletes. *Med. Sci. Sports Exerc.* **42**, 326–337
15. Picosky, M. A., Smith, T. J., Grediagin, A., Castaneda-Sceppa, C., Byerley, L., Glickman, E. L., and Young, A. J. (2008) Increased protein maintains nitrogen balance during exercise-induced energy deficit. *Med. Sci. Sports Exerc.* **40**, 505–512
16. Nindl, B. C., Barnes, B. R., Alemany, J. A., Frykman, P. N., Shippee, R. L., and Friedl, K. E. (2007) Physiological consequences of U.S. Army Ranger training. *Med. Sci. Sports Exerc.* **39**, 1380–1387
17. Berryman, C. E., Sepowitz, J. J., McClung, H. L., Lieberman, H. R., Farina, E. K., McClung, J. P., Ferrando, A. A., and Pasiakos, S. M. (2017) Supplementing an energy adequate, higher protein diet with protein does not enhance fat-free mass restoration after short-term severe negative energy balance. *J. Appl. Physiol.* (1985) **122**, 1485–1493

18. Henning, P. C., Park, B. S., and Kim, J. S. (2011) Physiological decrements during sustained military operational stress. *Mil. Med.* **176**, 991–997
19. Pasiakos, S. M., Austin, K. G., Lieberman, H. R., and Askew, E. W. (2013) Efficacy and safety of protein supplements for U.S. Armed Forces personnel: consensus statement. *J. Nutr.* **143**, 1811S–1814S
20. Margolis, L. M., Pasiakos, S. M., Karl, J. P., Rood, J. C., Cable, S. J., Williams, K. W., Young, A. J., and McClung, J. P. (2012) Differential effects of military training on fat-free mass and plasma amino acid adaptations in men and women. *Nutrients* **4**, 2035–2046
21. Ainsworth, B. E., Haskell, W. L., Herrmann, S. D., Meckes, N., Bassett, D. R., Jr., Tudor-Locke, C., Greer, J. L., Vezina, J., Whitt-Glover, M. C., and Leon, A. S. (2011) 2011 compendium of physical activities: a second update of codes and MET values. *Med. Sci. Sports Exerc.* **43**, 1575–1581
22. Roza, A. M., and Shizgal, H. M. (1984) The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. *Am. J. Clin. Nutr.* **40**, 168–182
23. Pandolf, K. B., Givoni, B., and Goldman, R. F. (1977) Predicting energy expenditure with loads while standing or walking very slowly. *J. Appl. Physiol.* **43**, 577–581
24. Pimental, N. A., and Pandolf, K. B. (1979) Energy expenditure while standing or walking slowly uphill or downhill with loads. *Ergonomics* **22**, 963–973
25. Heymsfield, S. B., Smith, R., Aulet, M., Bensen, B., Lichtman, S., Wang, J., and Pierson, R. N., Jr. (1990) Appendicular skeletal muscle mass: measurement by dual-photon absorptiometry. *Am. J. Clin. Nutr.* **52**, 214–218
26. Hoyt, R. W., Opstad, P. K., Haugen, A. H., DeLany, J. P., Cymerman, A., and Friedl, K. E. (2006) Negative energy balance in male and female rangers: effects of 7 d of sustained exercise and food deprivation. *Am. J. Clin. Nutr.* **83**, 1068–1075
27. Siri, W. E. (1956) The gross composition of the body. *Adv. Biol. Med. Phys.* **4**, 239–280
28. Weir, J. B. (1949) New methods for calculating metabolic rate with special reference to protein metabolism. *J. Physiol.* **109**, 1–9
29. Frayn, K. N. (1983) Calculation of substrate oxidation rates in vivo from gaseous exchange. *J. Appl. Physiol.* **55**, 628–634
30. Vermeulen, A., Verdonck, L., and Kaufman, J. M. (1999) A critical evaluation of simple methods for the estimation of free testosterone in serum. *J. Clin. Endocrinol. Metab.* **84**, 3666–3672
31. Paddon-Jones, D., Sheffield-Moore, M., Zhang, X. J., Volpi, E., Wolf, S. E., Aarsland, A., Ferrando, A. A., and Wolfe, R. R. (2004) Amino acid ingestion improves muscle protein synthesis in the young and elderly. *Am. J. Physiol. Endocrinol. Metab.* **286**, E321–E328
32. Volpi, E., Mittendorfer, B., Wolf, S. E., and Wolfe, R. R. (1999) Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *Am. J. Physiol.* **277**, E513–E520
33. Wolfe, R. R., and Chinkes, D. L. (2005) *Isotope Tracers in Metabolic Research*, Wiley, Hoboken, NJ, USA
34. Biolo, G., Fleming, R. Y., Maggi, S. P., and Wolfe, R. R. (1995) Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. *Am. J. Physiol.* **268**, E75–E84
35. Institute of Medicine. (2005) *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)*, The National Academies Press, Washington, DC
36. Calloway, D. H. (1975) Nitrogen balance of men with marginal intakes of protein and energy. *J. Nutr.* **105**, 914–923
37. Todd, K. S., Butterfield, G. E., and Calloway, D. H. (1984) Nitrogen balance in men with adequate and deficient energy intake at three levels of work. *J. Nutr.* **114**, 2107–2118
38. Wyckley, T. P., Moran, L. J., Clifton, P. M., Noakes, M., and Brinkworth, G. D. (2012) Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **96**, 1281–1298
39. Layman, D. K., Evans, E. M., Erickson, D., Seyler, J., Weber, J., Bagshaw, D., Griel, A., Psota, T., and Kris-Etherton, P. (2009) A moderate-protein diet produces sustained weight loss and long-term changes in body composition and blood lipids in obese adults. *J. Nutr.* **139**, 514–521
40. Margolis, L. M., Murphy, N. E., Martini, S., Gundersen, Y., Castellani, J. W., Karl, J. P., Carrigan, C. T., Teien, H. K., Madslien, E. H., Montain, S. J., and Pasiakos, S. M. (2016) Effects of supplemental energy on protein balance during 4-d Arctic military training. *Med. Sci. Sports Exerc.* **48**, 1604–1612
41. Pasiakos, S. M., Margolis, L. M., McClung, J. P., Cao, J. J., Whigham, L. D., Combs, G. F., and Young, A. J. (2014) Whole-body protein turnover response to short-term high-protein diets during weight loss: a randomized controlled trial. *Int. J. Obes.* **38**, 1015–1018
42. Margolis, L. M., Murphy, N. E., Martini, S., Spitz, M. G., Thrane, I., McGraw, S. M., Blatny, J. M., Castellani, J. W., Rood, J. C., Young, A. J., Montain, S. J., Gundersen, Y., and Pasiakos, S. M. (2014) Effects of winter military training on energy balance, whole-body protein balance, muscle damage, soreness, and physical performance. *Appl. Physiol. Nutr. Metab.* **39**, 1395–1401
43. Cayol, M., Tauveron, I., Rambourdin, F., Prugnaud, J., Gachon, P., Thieblot, P., Grizard, J., and Obled, C. (1995) Whole-body protein turnover and hepatic protein synthesis are increased by vaccination in man. *Clin. Sci.* **89**, 389–396
44. Boos, C. J., Woods, D. R., Varias, A., Biscocho, S., Heseltine, P., and Mellor, A. J. (2016) High altitude and acute mountain sickness and changes in circulating endothelin-1, interleukin-6, and interleukin-17a. *High Alt. Med. Biol.* **17**, 25–31
45. Hoffer, L. J., and Forse, R. A. (1990) Protein metabolic effects of a prolonged fast and hypocaloric refeeding. *Am. J. Physiol.* **258**, E832–E840
46. Stein, T. P., Rumpler, W. V., Leskiw, M. J., Schluter, M. D., Staples, R., and Bodwell, C. E. (1991) Effect of reduced dietary intake on energy expenditure, protein turnover, and glucose cycling in man. *Metabolism* **40**, 478–483
47. Henriksson, J. (1977) Training induced adaptation of skeletal muscle and metabolism during submaximal exercise. *J. Physiol.* **270**, 661–675
48. Horowitz, J. F., and Klein, S. (2000) Lipid metabolism during endurance exercise. *Am. J. Clin. Nutr.* **72** (2 Suppl), 558S–563S
49. Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K. E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R., and Storer, T. W. (2001) Testosterone dose-response relationships in healthy young men. *Am. J. Physiol. Endocrinol. Metab.* **281**, E1172–E1181
50. Mawson, J. T., Braun, B., Rock, P. B., Moore, L. G., Mazzeo, R., and Butterfield, G. E. (2000) Women at altitude: energy requirement at 4,300 m. *J. Appl. Physiol.* (1985) **88**, 272–281
51. Hannon, J. P., and Sudman, D. M. (1973) Basal metabolic and cardiovascular function of women during altitude acclimatization. *J. Appl. Physiol.* **34**, 471–477
52. Kellogg, R. H., Pace, N., Archibald, E. R., and Vaughan, B. E. (1957) Respiratory response to inspired CO₂ during acclimatization to an altitude of 12,470 feet. *J. Appl. Physiol.* **11**, 65–71
53. Stock, M. J., Norgan, N. G., Ferro-Luzzi, A., and Evans, E. (1978) Effect of altitude on dietary-induced thermogenesis at rest and during light exercise in man. *J. Appl. Physiol.* **45**, 345–349
54. Marriott, B. M., and Carlson, S. J., eds. (1996) Search of 500 kcal/day. In *Nutritional Needs In Cold and In High-Altitude Environments: Applications for Military Personnel in Field Operations*, Committee on Military Nutrition Research, Food and Nutrition Board, Institute of Medicine, Washington, DC
55. LeBlanc, J., Mercier, I., and Nadeau, A. (1993) Components of postprandial thermogenesis in relation to meal frequency in humans. *Can. J. Physiol. Pharmacol.* **71**, 879–883
56. LeBlanc, J., Diamond, P., and Nadeau, A. (1991) Thermogenic and hormonal responses to palatable protein and carbohydrate rich food. *Horm. Metab. Res.* **23**, 336–340
57. Feinman, R. D., and Fine, E. J. (2003) Thermodynamics and metabolic advantage of weight loss diets. *Metab. Syndr. Relat. Disord.* **1**, 209–219
58. Fine, E. J., and Feinman, R. D. (2004) Thermodynamics of weight loss diets. *Nutr. Metab. (Lond.)* **1**, 15
59. Cymerman, A., Pandolf, K. B., Young, A. J., and Maher, J. T. (1981) Energy expenditure during load carriage at high altitude. *J. Appl. Physiol.* **51**, 14–18
60. Ludlow, L. W., and Weyand, P. G. (2016) Energy expenditure during level human walking: seeking a simple and accurate predictive solution. *J. Appl. Physiol.* (1985) **120**, 481–494
61. Santee, W. R., Blanchard, L. A., Speckman, K. L., Gonzalez, J. A., and Wallace, R. F. (2003) *Load Carriage Model Development and Testing with Field Data*, Technical Note TN03-03, U.S. Army Research Institute of Environmental Medicine, Natick, MA, USA
62. Soule, R. G., and Goldman, R. F. (1972) Terrain coefficients for energy cost prediction. *J. Appl. Physiol.* **32**, 706–708
63. Krzywicki, H. J., Consolazio, C. F., Johnson, H. L., Nielsen, W. C., Jr., and Barnhart, R. A. (1971) Water metabolism in humans during acute high-altitude exposure (4,300 m). *J. Appl. Physiol.* **30**, 806–809

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

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Abstract

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

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

Introduction

LOWLANDERS SOJOURNING at high altitude (HA; 2500–5000 m) commonly experience unintentional weight loss (Westerterp and Kayser, 2006; Pasiakos et al., 2017). The

etiology is multifactorial, but reduced energy intake is a contributing factor (Hamad and Travis, 2006; Westerterp and Kayser, 2006), and coincides with a loss of appetite putatively characterized by a rapid reduction in hunger during meals and early satiation (Westerterp-Plantenga et al., 1999).

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

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

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

Materials and Methods

Participants

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

Study design

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

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

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

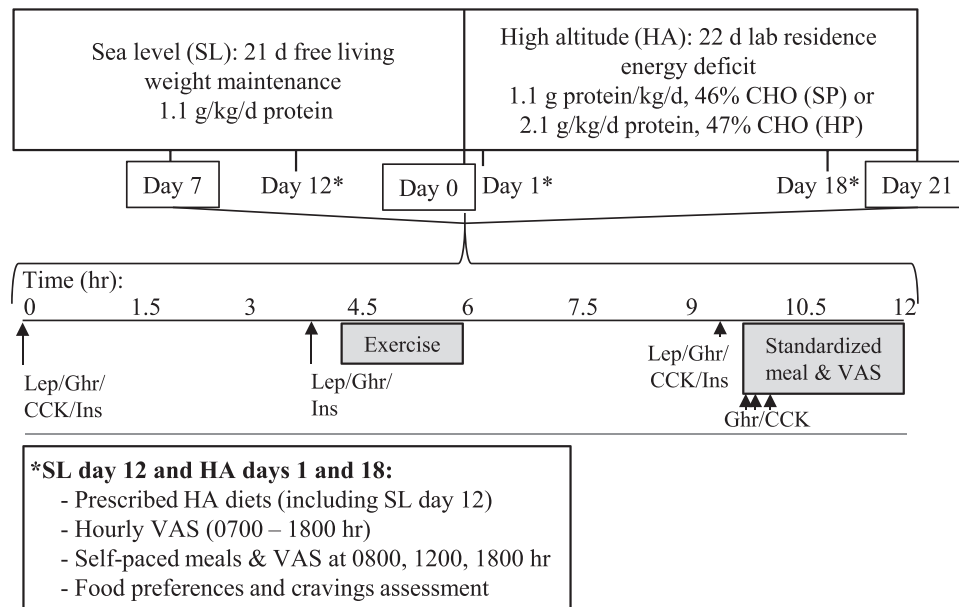


FIG. 1. Study design. Volunteers were followed for 21 days at SL, then for 22 days while living at HA (4300 m) and consuming standard-protein or higher protein hypocaloric diets. CCK, cholecystokinin; CHO, carbohydrate; Ghr, acylated ghrelin; Ins, insulin; Lep, leptin; VAS, visual analog scale measuring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

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

Study diets

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

During HA, all food and caloric beverages consumed were strictly controlled, weighed and measured, and provided by study staff. Meals and snacks were supervised, and participants were instructed to consume all provided food and beverages. Any uneaten foods were weighed and documented. Diets were individualized, followed a five-day rotating menu, and were designed to provide a standard (SP; 1.0 ± 0.2 g/kg/d) or higher (HP; 2.0 ± 0.2 g/kg/d) amount of protein, and 45% of total energy as carbohydrate each day. Fat intake was reduced in HP to accommodate the higher protein intake (Table 1). Most of the foods were entrées, sides, and snack items included in U.S. military Meals Ready-to-Eat rations. These items were supplemented with fresh fruits and vegetables, fruit snacks, olive oil, and ranch-flavored salad dressing. Protein intake was also manipulated using a whey-protein beverage (Isopure® Zero Carb; Isopure Co., Hauppauge, NY). Although the diets differed in appearance, participants were not told to which dietary group

they were assigned. Whether participants could correctly identify their group assignment was not assessed. Water and noncaffeinated calorie-free sodas were allowed *ad libitum*. Dietary intake was assessed using daily food records, the Combat Rations Database (U.S. Army Natick Soldier Research Development and Engineering Center and USAR-IEM), and Food Processor SQL v.10.14 (ESHA Research, Salem, OR).

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

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

TABLE 1. BASELINE PARTICIPANT CHARACTERISTICS AND ACTUAL DIETARY INTAKES OVER 22 DAYS AT HIGH ALTITUDE (4300 M)

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

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

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

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

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

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

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

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

Longitudinal changes in appetite and related hormones were examined on SL day 7 and HA days 0 and 21 by measuring circulating hormone concentrations throughout the day and by measuring appetite, CCK, and acylated ghrelin responses to a standardized meal. On these days participants did not consume their prescribed diets to conduct testing for

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

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

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

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

chilled Monovettes containing EDTA-K₃ and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (1 mg/mL whole blood) for acylated ghrelin determination or protease inhibitor cocktail (40 μ L/mL whole blood; cOmplete, EDTA-free) for CCK determination. Acylated ghrelin aliquots were combined with 50 μ L 1N HCl/mL plasma, and all samples were stored at -20°C to -80°C until analysis. Plasma acylated ghrelin was determined by RIA (EMD Millipore) and plasma CCK by ELISA (Cusabio, College Park, MD) according to manufacturer's instructions.

Visual analog scales

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

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

Statistical analyses

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

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

The effects of study day, diet, carbohydrate ingestion during exercise (145 g and 0 g carbohydrate), time, and their interactions on appetite ratings and hormone concentrations measured on SL day 7 and HA days 0 and 21 were tested using linear mixed models. All possible interactions except any between dietary protein group and carbohydrate supplement group were included in these models. Appetite ratings measured during and after the standardized meal test were analyzed using the metrics AUC, rate of change in appetite ratings during the meal, and rate of change in appetite

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

Results

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

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

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

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

TABLE 2. ACUTE MOUNTAIN SICKNESS, OXYGEN SATURATION, ACTUAL DIETARY INTAKES, AND MEAL ACCEPTABILITY MEASURED DURING APPETITE TESTING DAYS CONDUCTED AT SEA LEVEL AND WHILE LIVING AT HIGH ALTITUDE (4300 M)

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

Values are %n or mean ± SD.

¹Generalized linear mixed model with Bonferroni corrections; subject included as a random factor, and diet, day, and their interaction as fixed factors. Main effect of day ($p < 0.001$); incidence higher on HA days 1 and 18 relative to SL.

²Acute mountain sickness (AMS) was defined as a Lake Louise score > 0.7 on the shortened version of the Environmental Symptoms Questionnaire (Beidleman et al., 2007).

³Incidence of nausea defined as slight to extreme nausea reported on the Environmental Symptoms Questionnaire.

⁴⁻⁶Linear mixed model with Bonferroni corrections; subject included as a random factor, and diet, day, and their interaction as fixed factors. ⁴Diet-by-day interaction, $p < 0.05$. ^{a-c}Values within a dietary protein group sharing a superscript letter are not significantly different within that group. ⁵Main effect of group, $p < 0.001$. ⁶Main effect of day ($p < 0.001$); lower acceptability on HA days 1 and 18 relative to SL.

⁷Overall meal acceptability rated from 1 (dislike extremely) to 9 (like extremely). Values are the mean of ratings at breakfast, lunch, and dinner.

*Significantly different from the same day in the standard-protein group, $p < 0.05$.

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

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

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

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

Appetite responses to a standardized test meal. Appetite responses to the standardized meal test administered on SL day 7 and HA days 0 and 21 indicated changes in appetite responses over time that were independent of both dietary protein group and carbohydrate supplementation during exercise. Specifically, hunger AUC was lower on HA day 0, but not HA day 21, relative to SL, whereas fullness AUC was higher on HA day 21, but not HA day 0, relative to SL (main effect of day, $p \leq 0.03$; Fig. 4A, B). Overall appetite, pro-

spective consumption, and desire to eat AUC were lower on HA days 0 and 21 relative to SL (main effect of day, $p \leq 0.01$; Fig. 4C–E). These differences were attributable to a combination of differences in premeal appetite ratings (main effect of day, $p < 0.05$) and differences in the rate of change in appetite ratings during the postprandial period (main effect of day, $p \leq 0.01$) across study days (Fig. 4). No differences between days in the rate of change in any appetite rating measured during the meal (0–12 minutes) were observed (main effect of day, $p > 0.05$). The overall acceptability of the meal was rated as “moderately liked” (mean score = 7.1 ± 1.6), which did not change over time (main effect of day, $p = 0.10$) or differ between dietary protein groups (main effect of diet, $p = 1.00$).

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

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

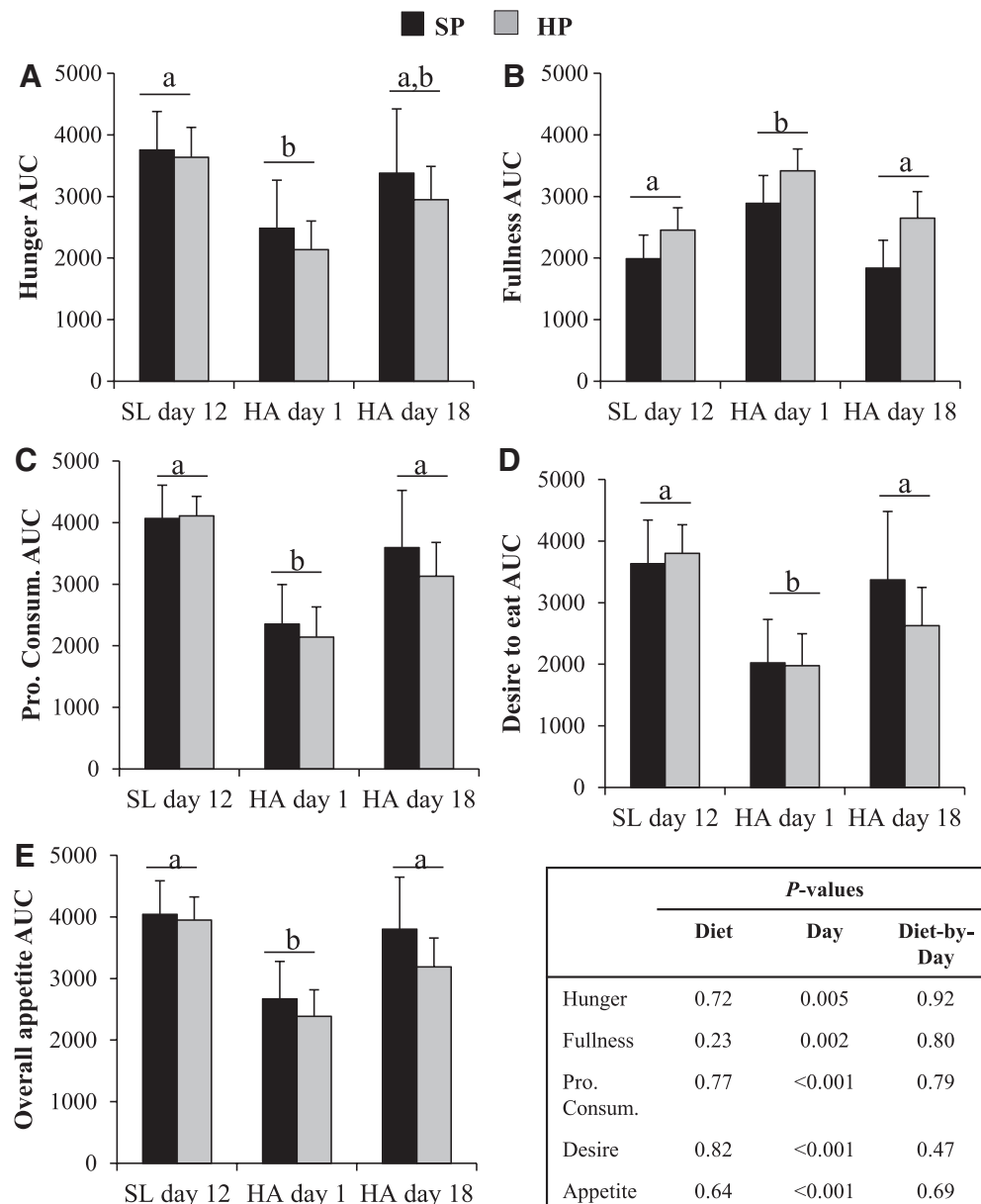


FIG. 2. Appetite at SL and HA (4300 m) while consuming hypocaloric standard-protein (SP, 1.1 g/kg/d, $n=8$) or higher protein (HP; 2.1 g/kg/d, $n=9$) diets. AUC for perceived hunger (A), fullness (B), prospective consumption (Pro. Consum.) (C), and desire to eat (D) measured at SL and after 1 and 18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

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

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

Discussion

The primary finding of this study was that dietary protein intake did not impact appetite during energy deficit at HA.

However, acute HA exposure was associated with appetite suppression concomitant to elevated concentrations of the anorexigenic hormones insulin, leptin, and CCK, depressed concentrations of the orexigenic hormone acylated ghrelin, and preferences for sweet and low-protein foods. Although anorexigenic hormone concentrations did not remain elevated following acclimatization and weight loss, appetite and acylated ghrelin concentrations remained depressed while food preferences shifted toward high fat foods.

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

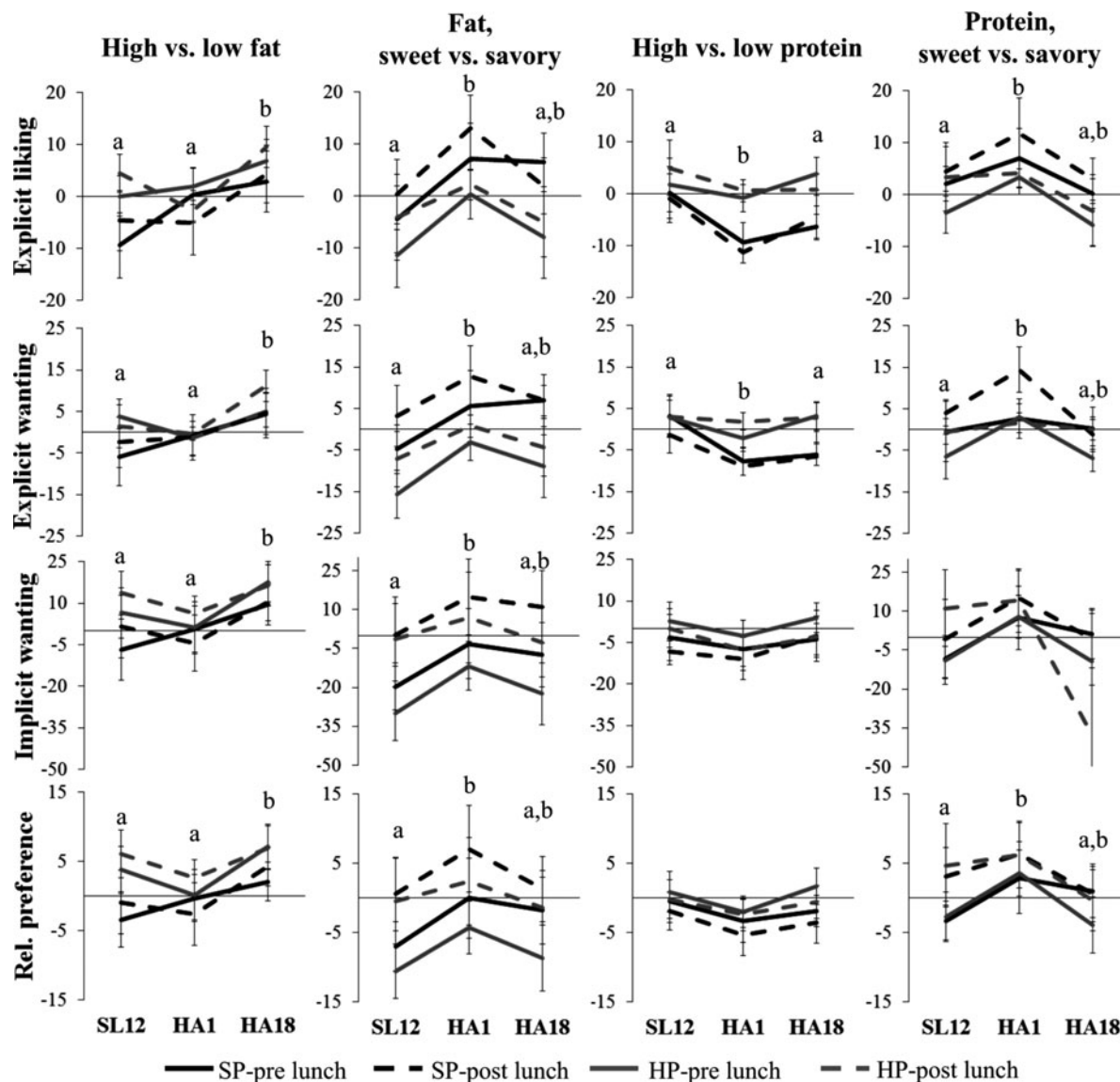


FIG. 3. Food preferences at SL and HA (4300 m) while consuming hypocaloric standard-protein (SP, 1.1 g/kg/d, $n=8$) or higher protein (HP; 2.1 g/kg/d, $n=9$) diets. Food preferences were measured before and after lunch on SL day 12 and after 1 and 18 days of living at HA using the Leeds Food Preference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

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

Additional factors may also have decreased the ability to detect significant effects of protein intake on appetite. First, between-group differences in protein intake ranged from 8 to 19 g/meal, which is less than the 30 g/meal difference others have suggested as a threshold for observing satiety-enhancing effects of higher protein meals (Paddon-Jones and Leidy, 2014). Second, the proportion of total energy intake

derived from liquids was higher in HP relative to SP as a result of the beverage used to increase protein intake, and liquids have been shown to elicit weaker satiety effects than solid foods (Leidy et al., 2011). Given these limitations, the hypothesis that higher protein diets could be counterproductive for maintaining fat-free mass at HA by exacerbating HA anorexia cannot be conclusively refuted and warrants further investigation.

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

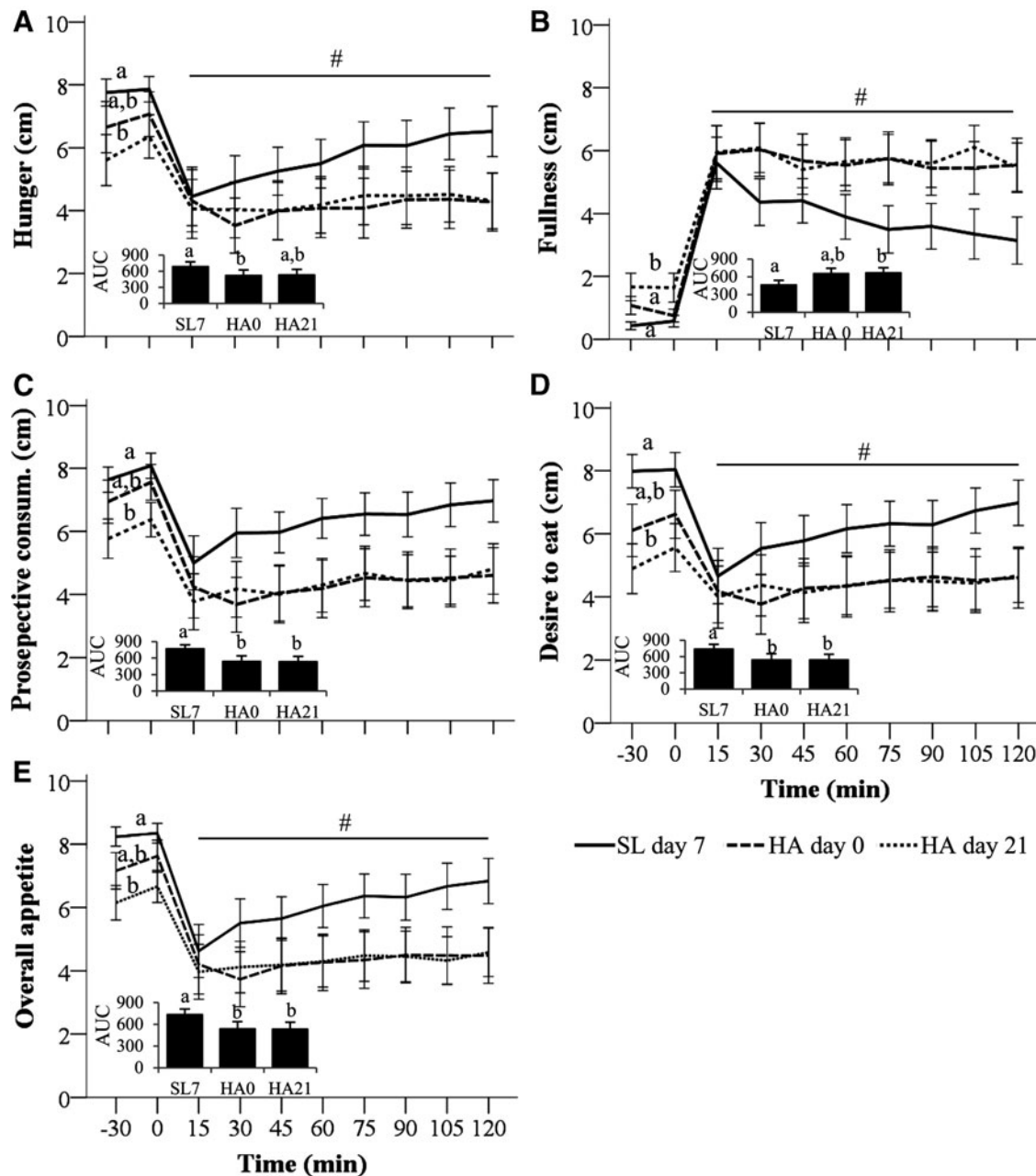


FIG. 4. Appetite response to a standardized meal at SL and HA (4300 m). Perceived hunger (**A**), fullness (**B**), prospective consumption (**C**), and desire to eat (**D**) measured during a standardized meal test at sea level (SL7), on the first day of HA exposure (HA0) and after 21 days of living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

this investigation (Millet et al., 2012). Furthermore, in the present study, we tightly controlled physical activity, climate, and diet, factors which may have confounded several previous field studies and contributed to inconsistencies in observations (Sierra-Johnson et al., 2008; Debevec, 2017). However, the absence of a SL control group is a limitation of the present study that prevents establishing causal effects of HA on study outcomes. Although this limitation is less likely

to impact study outcomes measured during acute HA, it does preclude definitively separating the impact of acclimatization to HA on study outcomes from factors such as weight loss, diet monotony, and living in a confined environment. Potential confounding due to weight loss is particularly important to consider because weight loss independently influences appetite, food preferences, and appetite-mediating hormones (Maclean et al., 2011; Sumithran and Proietto,

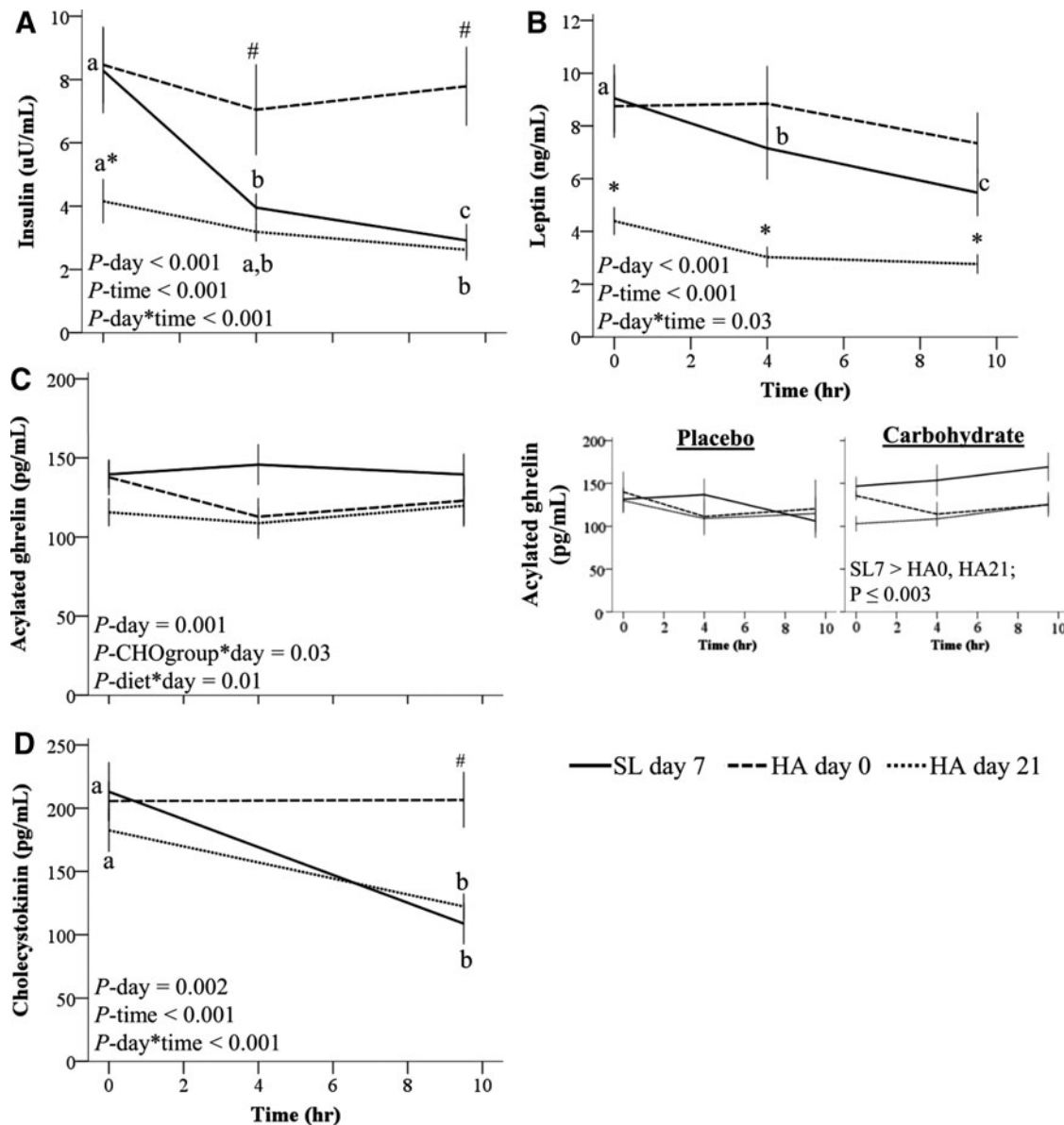


FIG. 5. Appetite-mediating hormones at HA (4300 m). **(A)** Insulin ($n=17$; $\log_{10}$ -transformed for analysis), **(B)** leptin ($n=16$), **(C)** acylated ghrelin ($n=17$), and **(D)** cholecystokinin ($n=17$) measured at SL, on the first day of HA exposure and after 21 days of living at HA. Volunteers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

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

Appetite suppression during acute HA exposure is a well-established response (Hamad and Travis, 2006; Westerterp and Kayser, 2006) and was supported in the present study (Figs. 2 and 4). While previous studies report that appetite remains suppressed at very high altitudes (>5000 m) for several weeks (Rose et al., 1988; Westerterp-Plantenga et al., 1999), others suggest that appetite suppression may be alleviated during chronic (>7 days) exposure to altitudes below

5000 m, at least under normobaric hypoxic conditions [3000–4000 m (Debevec et al., 2014, 2016; Mekjavic et al., 2016)]. In the present study, appetite was lower during the meal test on HA day 21 relative to SL and did not differ from SL on HA day 18 despite substantial weight loss. It is well established that weight loss stimulates compensatory mechanisms which act to stimulate appetite and restore body weight (Maclean et al., 2011; Sumithran and Proietto, 2013). Therefore, as we are not aware of any reports demonstrating weight regain in people sojourning at >4000 m for ≥ 3 weeks, these findings suggest that normal physiologic adaptations to weight loss

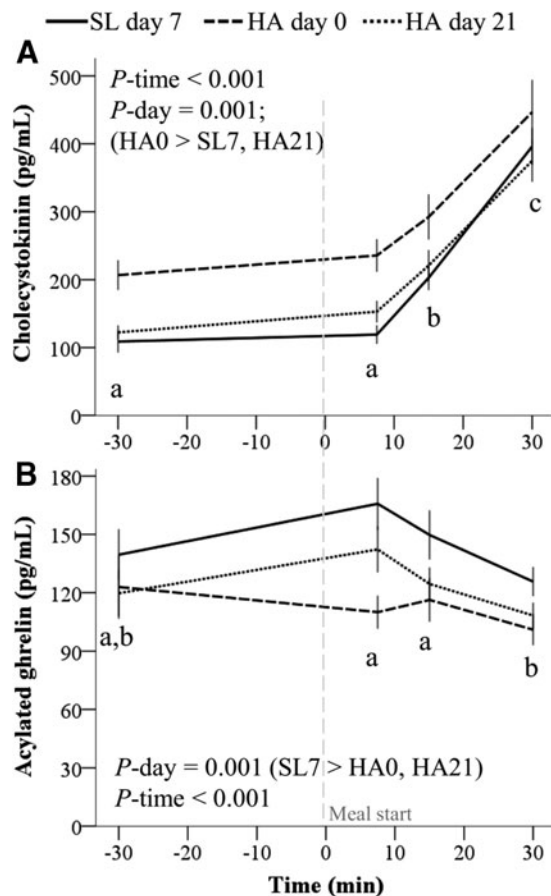


FIG. 6. Appetite-mediating hormone responses to a standardized meal at HA (4300 m). (A) Cholecystokinin ($n=17$) and (B) acylated ghrelin ($n=17$) responses to a fixed portion meal measured at SL, on the first day of HA exposure and after 21 days of living at HA. Volunteers consumed a hypocaloric standard-protein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

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

Our findings suggest, but do not prove, that the orexigenic hormone ghrelin may be one factor in the persistence of HA anorexia. Previous studies have reported that total ghrelin concentrations are transiently suppressed in response to hypoxia (Shukla et al., 2005; Riepl et al., 2012; Debevec et al., 2014, 2016; Mekjavic et al., 2016). However, the relevance of these findings to appetite regulation is unclear because the appetite-stimulating effects of ghrelin require posttranslational acylation (Lim et al., 2010), and acylated ghrelin comprises only a small variable fraction of total ghrelin concentrations (Liu et al., 2008). Previous studies measuring acylated ghrelin responses to HA have reported

suppressed concentrations during 5–7 hours of exposure to normobaric hypoxia simulating altitudes of 3500–4000 m (Wasse et al., 2012; Matu et al., 2017a, 2017b), but not < 3000 m (Morishima and Goto, 2016), and suppressed concentrations during a 12-day trek to 5140 m (Matu et al., 2017a, 2017b). Our findings are consistent with and extend those results by demonstrating that the suppression of acylated ghrelin concentrations upon acute HA exposure may be sustained for up to 21 days (Figs. 5C and 6B). Importantly, this observation is not likely confounded by weight loss as weight loss at SL is associated with increased acylated ghrelin concentrations (Martins et al., 2010).

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

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

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

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

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

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

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

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

Conclusion

Taken together, these findings suggest that maximizing the effectiveness of dietary strategies for mitigating weight loss at HA may require overriding physiologic signals that sup-

press appetite, accommodating food preferences, and adjusting diets to both the duration and elevation of the sojourn. These observations in combination with the absence of a SL control group highlight the need for additional research with appropriate controls to establish causal effects of prolonged HA exposure on appetite, appetite-mediating hormones, and food preferences. Contrary to our hypothesis, a higher protein diet did not exacerbate HA anorexia in the present study. However, study limitations preclude definitive conclusions, and the potential for the satiating properties of protein to reduce *ad libitum* food intake at HA and the possible incongruence of higher protein diets with food preferences at HA warrant further investigation.

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Disclaimers

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other official documentation. Citation of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such commercial items.

Author Disclosure Statement

No authors report a conflict of interest.

References

- Aeberli I, Erb A, Spliethoff K, Meier D, Gotze O, Fruhauf H, Fox M, Finlayson GS, Gassmann M, Berneis K, Maggiorini M, Langhans W, and Lutz TA. (2013). Disturbed eating at high altitude: Influence of food preferences, acute mountain sickness and satiation hormones. *Eur J Nutr* 52:625–635.
- Bailey DM, Ainslie PN, Jackson SK, Richardson RS, and Ghatei M. (2004). Evidence against redox regulation of energy homeostasis in humans at high altitude. *Clin Sci (Lond)* 107:589–600.
- Bailey DM, Davies B, Milledge JS, Richards M, Williams SR, Jordinson M, and Calam J. (2000). Elevated plasma cholecystokinin at high altitude: Metabolic implications for the anorexia of acute mountain sickness. *High Alt Med Biol* 1:9–23.
- Barnholt KE, Hoffman AR, Rock PB, Muza SR, Fulco CS, Braun B, Holloway L, Mazzeo RS, Cymerman A, and Friedlander AL. (2006). Endocrine responses to acute and chronic high-altitude exposure (4,300 meters): Modulating effects of caloric restriction. *Am J Physiol Endocrinol Metab* 290:E1078–E1088.

- Beidleman BA, Muza SR, Fulco CS, Rock PB, and Cymerman A. (2007). Validation of a shortened electronic version of the environmental symptoms questionnaire. *High Alt Med Biol* 8: 192–199.
- Benso A, Broglio F, Aimaretti G, Lucatello B, Lanfranco F, Ghigo E, and Grotto S. (2007). Endocrine and metabolic responses to extreme altitude and physical exercise in climbers. *Eur J Endocrinol* 157:733–740.
- Berryman CE, Young AJ, Karl JP, Kenefick RW, Margolis LM, Cole RE, Carbone JW, Lieberman HR, Kim I, Fernando A, and Pasiakos SM. (2017). Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: A randomized controlled trial. *FASEB J* [Epub ahead of print]; DOI: 10.1096/fj.201700915R.
- Berthoud HR. (2006). Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. *Obesity (Silver Spring)* 14 Suppl 5:197S–200S.
- Blundell J, de Graaf C, Hulshof T, Jebb S, Livingstone B, Lluch A, Mela D, Salah S, Schuring E, van der Knaap H, and Westerterp M. (2010). Appetite control: Methodological aspects of the evaluation of foods. *Obes Rev* 11:251–270.
- Boyer SJ, and Blume FD. (1984). Weight loss and changes in body composition at high altitude. *J Appl Physiol Respir Environ Exerc Physiol* 57:1580–1585.
- Dalton M, Finlayson G, Hill A, and Blundell J. (2015). Preliminary validation and principal components analysis of the control of eating questionnaire (CoEQ) for the experience of food craving. *Eur J Clin Nutr* 69:1313–1317.
- Debevec T. (2017). Hypoxia-related hormonal appetite modulation in humans during rest and exercise: Mini review. *Front Physiol* 8:366.
- Debevec T, Simpson EJ, Macdonald IA, Eiken O, and Mekjavic IB. (2014). Exercise training during normobaric hypoxic confinement does not alter hormonal appetite regulation. *PLoS One* 9:e98874.
- Debevec T, Simpson EJ, Mekjavic IB, Eiken O, and Macdonald IA. (2016). Effects of prolonged hypoxia and bed rest on appetite and appetite-related hormones. *Appetite* 107:28–37.
- Dhillon J, Craig BA, Leidy HJ, Amankwaah AF, Osei-Boadi Anguah K, Jacobs A, Jones BL, Jones JB, Keeler CL, Keller CE, McCrory MA, Rivera RL, Slobodnik M, Mattes RD, and Tucker RM. (2016). The effects of increased protein intake on fullness: A meta-analysis and its limitations. *J Acad Nutr Diet* 116:968–983.
- Dill DB, and Costill DL. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247–248.
- Ellis AC, Chandler-Laney P, Casazza K, Goree LL, McGwin G, and Gower BA. (2012). Circulating ghrelin and glp-1 are not affected by habitual diet. *Regul Pept* 176:1–5.
- Finlayson G, King N, and Blundell JE. (2007). Is it possible to dissociate ‘liking’ and for foods in humans? A novel experimental procedure. *Physiol Behav* 90:36–42.
- Griffioen-Roose S, Mars M, Siebelink E, Finlayson G, Tome D, and de Graaf C. (2012). Protein status elicits compensatory changes in food intake and food preferences. *Am J Clin Nutr* 95:32–38.
- Hamad N, and Travis SP. (2006). Weight loss at high altitude: Pathophysiology and practical implications. *Eur J Gastroenterol Hepatol* 18:5–10.
- Hoyt RW, Jones TE, Baker-Fulco CJ, Schoeller DA, Schoene RB, Schwartz RS, Askew EW, and Cymerman A. (1994). Doubly labeled water measurement of human energy expenditure during exercise at high altitude. *Am J Physiol* 266: R966–R971.
- Karl JP, Smith TJ, Wilson MA, Bukhari AS, Pasiakos SM, McClung HL, McClung JP, and Lieberman HR. (2016). Altered metabolic homeostasis is associated with appetite regulation during and following 48-h of severe energy deprivation in adults. *Metabolism* 65:416–427.
- Karl JP, Young AJ, and Montain SJ. (2011). Eating rate during a fixed-portion meal does not affect postprandial appetite and gut peptides or energy intake during a subsequent meal. *Physiol Behav* 102:524–531.
- Kayser B, Narici M, Milesi S, Grassi B, and Cerretelli P. (1993). Body composition and maximum alactic anaerobic performance during a one month stay at high altitude. *Int J Sports Med* 14:244–247.
- Leidy HJ, Bales-Voelker LI, and Harris CT. (2011). A protein-rich beverage consumed as a breakfast meal leads to weaker appetitive and dietary responses v. a protein-rich solid breakfast meal in adolescents. *Br J Nutr* 106:37–41.
- Leidy HJ, Clifton PM, Astrup A, Wycherley TP, Westerterp-Plantenga MS, Luscombe-Marsh ND, Woods SC, and Mattes RD. (2015). The role of protein in weight loss and maintenance. *Am J Clin Nutr* 101:1320S–1329S.
- Lim CT, Kola B, Korbonits M, and Grossman AB. (2010). Ghrelin’s role as a major regulator of appetite and its other functions in neuroendocrinology. *Prog Brain Res* 182:189–205.
- Liu J, Prudom CE, Nass R, Pezzoli SS, Oliveri MC, Johnson ML, Veldhuis P, Gordon DA, Howard AD, Witcher DR, Geysen HM, Gaylinn BD, and Thorner MO. (2008). Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *J Clin Endocrinol Metab* 93:1980–1987.
- Maclean PS, Bergouignan A, Cornier MA, and Jackman MR. (2011). Biology’s response to dieting: The impetus for weight regain. *Am J Physiol Regul Integr Comp Physiol* 301:R581–R600.
- Martens EA, Lemmens SG, and Westerterp-Plantenga MS. (2013). Protein leverage affects energy intake of high-protein diets in humans. *Am J Clin Nutr* 97:86–93.
- Martins C, Kulseng B, King NA, Holst JJ, and Blundell JE. (2010). The effects of exercise-induced weight loss on appetite-related peptides and motivation to eat. *J Clin Endocrinol Metab* 95:1609–1616.
- Matu J, Deighton K, Ispoglou T, and Duckworth L. (2017a). The effect of moderate versus severe simulated altitude on appetite, gut hormones, energy intake and substrate oxidation in men. *Appetite* 113:284–292.
- Matu J, O’Hara J, Hill N, Clarke S, Boos C, Newman C, Holdsworth D, Ispoglou T, Duckworth L, Woods D, Mellor A, and Deighton K. (2017b). Changes in appetite, energy intake, body composition, and circulating ghrelin constituents during an incremental trekking ascent to high altitude. *Eur J Appl Physiol* [Epub ahead of print]; DOI: 10.1007/s00421-017-3683-0.
- Mekjavic IB, Amon M, Kolegard R, Kounalakis SN, Simpson L, Eiken O, Keramidas ME, and Macdonald IA. (2016). The effect of normobaric hypoxic confinement on metabolism, gut hormones, and body composition. *Front Physiol* 7:202.
- Millet GP, Faiss R, and Pialoux V. (2012). Point: Hypobaric hypoxia induces different physiological responses from normobaric hypoxia. *J Appl Physiol* (1985) 112:1783–1784.
- Morishima T, and Goto K. (2016). Ghrelin, GLP-1, and leptin responses during exposure to moderate hypoxia. *Appl Physiol Nutr Metab* 41:375–381.
- Paddon-Jones D, and Leidy H. (2014). Dietary protein and muscle in older persons. *Curr Opin Clin Nutr Metab Care* 17: 5–11.

- Pasiakos SM, Austin KG, Lieberman HR, and Askew EW. (2013a). Efficacy and safety of protein supplements for U.S. Armed forces personnel: Consensus statement. *J Nutr* 143: 1811S–1814S.
- Pasiakos SM, Berryman CE, Carrigan CT, Young AJ, and Carbone JW. (2017). Muscle protein turnover and the molecular regulation of muscle mass during hypoxia. *Med Sci Sports Exerc* 49:1340–1350.
- Pasiakos SM, Cao JJ, Margolis LM, Sauter ER, Whigham LD, McClung JP, Rood JC, Carbone JW, Combs GF, Jr., and Young AJ. (2013b). Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: A randomized controlled trial. *FASEB J* 27:3837–3847.
- Pasiakos SM, Margolis LM, and Orr JS. (2015a). Optimized dietary strategies to protect skeletal muscle mass during periods of unavoidable energy deficit. *FASEB J* 29:1136–1142.
- Pasiakos SM, Sepowitz JJ, and Deuster PA. (2015b). U.S. military dietary protein recommendations: A simple but often confused topic. *J Spec Oper Med* 15:89–95.
- Premavalli KS, Wadikar DD, and Nanjappa C. (2009). Comparison of the acceptability ratings of appetizers under laboratory, base level and high altitude field conditions. *Appetite* 53:127–130.
- Raff H, Molthen R, Pan W, Kastin AJ, Ye J, Ozturk L, Xi L, Kukreja RC, Cabrera de Leon A, O'Donnell C, Vats P, Guerre-Millo M, Bigard X, and Simler N. (2008). Commentaries on viewpoint: Effect of altitude on leptin levels, does it go up or down? *J Appl Physiol* (1985) 105:1686–1690.
- Reynolds RD, Lickteig JA, Howard MP, and Deuster PA. (1998). Intakes of high fat and high carbohydrate foods by humans increased with exposure to increasing altitude during an expedition to mt. Everest. *J Nutr* 128:50–55.
- Riepl RL, Fischer R, Hautmann H, Hartmann G, Muller TD, Tschop M, Toepfer M, and Otto B. (2012). Influence of acute exposure to high altitude on basal and postprandial plasma levels of gastroenteropancreatic peptides. *PLoS One* 7:e44445.
- Rose MS, Houston CS, Fulco CS, Coates G, Sutton JR, and Cymerman A. (1988). Operation Everest. II: Nutrition and body composition. *J Appl Physiol* (1985) 65:2545–2551.
- Shukla V, Singh SN, Vats P, Singh VK, Singh SB, and Banerjee PK. (2005). Ghrelin and leptin levels of sojourners and acclimatized lowlanders at high altitude. *Nutr Neurosci* 8:161–165.
- Sierra-Johnson J, Romero-Corral A, Somers VK, and Johnson BD. (2008). Effect of altitude on leptin levels, does it go up or down? *J Appl Physiol* (1985) 105:1684–1685.
- Snyder EM, Carr RD, Deacon CF, and Johnson BD. (2008). Overnight hypoxic exposure and glucagon-like peptide-1 and leptin levels in humans. *Appl Physiol Nutr Metab* 33:929–935.
- Sumithran P, and Proietto J. (2013). The defence of body weight: A physiological basis for weight regain after weight loss. *Clin Sci (Lond)* 124:231–241.
- Thomas DT, Erdman KA, and Burke LM. (2016). American College of Sports Medicine joint position statement. Nutrition and athletic performance. *Med Sci Sports Exerc* 48:543–568.
- Tschop M, Strasburger CJ, Hartmann G, Biollaz J, and Bartsch P. (1998). Raised leptin concentrations at high altitude associated with loss of appetite. *Lancet* 352:1119–1120.
- Vats P, Singh VK, Singh SN, and Singh SB. (2007). High altitude induced anorexia: Effect of changes in leptin and oxidative stress levels. *Nutr Neurosci* 10:243–249.
- Veldhorst M, Smeets A, Soenen S, Hochstenbach-Waelen A, Hursel R, Diepvens K, Lejeune M, Luscombe-Marsh N, and Westerterp-Plantenga M. (2008). Protein-induced satiety: Effects and mechanisms of different proteins. *Physiol Behav* 94:300–307.
- Wasse LK, Sunderland C, King JA, Batterham RL, and Stensel DJ. (2012). Influence of rest and exercise at a simulated altitude of 4,000 m on appetite, energy intake, and plasma concentrations of acylated ghrelin and peptide YY. *J Appl Physiol* (1985) 112:552–559.
- Westerterp-Plantenga MS, Westerterp KR, Rubbens M, Verwegen CR, Richelet JP, and Gardette B. (1999). Appetite at “high altitude” [Operation Everest III (Comex-’97)]: A simulated ascent of Mount Everest. *J Appl Physiol* (1985) 87: 391–399.
- Westerterp KR, and Kayser B. (2006). Body mass regulation at altitude. *Eur J Gastroenterol Hepatol* 18:1–3.
- Westerterp KR, Kayser B, Wouters L, Le Trong JL, and Richelet JP. (1994). Energy balance at high altitude of 6,542 m. *J Appl Physiol* (1985) 77:862–866.
- White MA, and Grilo CM. (2005). Psychometric properties of the food craving inventory among obese patients with binge eating disorder. *Eat Behav* 6:239–245.
- Woods SC. (2009). The control of food intake: Behavioral versus molecular perspectives. *Cell Metab* 9:489–498.
- Woolcott OO, Ader M, and Bergman RN. (2015). Glucose homeostasis during short-term and prolonged exposure to high altitudes. *Endocr Rev* 36:149–173.
- Wycherley TP, Moran LJ, Clifton PM, Noakes M, and Brinkworth GD. (2012). Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: A meta-analysis of randomized controlled trials. *Am J Clin Nutr* 96:1281–1298.
- Zaccaria M, Ermolao A, Bonvicini P, Travain G, and Varnier M. (2004). Decreased serum leptin levels during prolonged high altitude exposure. *Eur J Appl Physiol* 92:249–253.

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

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

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

Lowlanders who sojourn at high altitude (HA; hypobaric hypoxia) often lose skeletal muscle mass (1–4). Dysregulated mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling and up-regulated ubiquitin proteasome-mediated proteolysis may regulate this muscle loss and modulate the extent to which muscle

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

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

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

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

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

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

MATERIALS AND METHODS

Participants

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

Study design

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

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

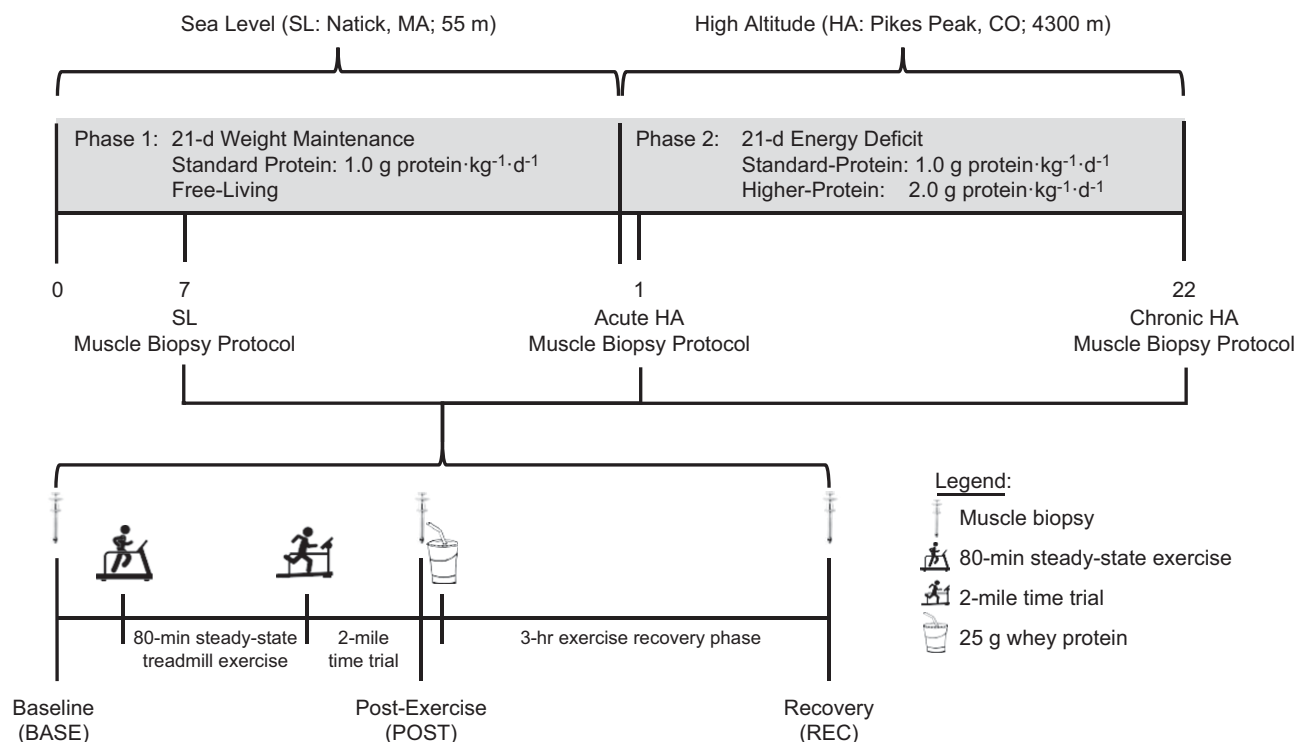


Figure 1. Study design. Energy deficit was designed to be 40% of total energy needs; however, on the basis of changes in fat mass and FFM, the actual deficit was closer to 70%. The current analysis was part of a larger study that assessed the impact of dietary protein intake on changes in body composition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

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

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

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

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

Aerobic exercise and muscle biopsy studies

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

treadmill time trial. Peripheral oxygen saturation (SpO₂; finger pulse oximetry, Model 9560; Nonin, Plymouth, MN, USA) was assessed throughout the exercise bout to document normoxic and hypoxic exposure. As expected, mean SpO₂ was different ($P < 0.05$) between all 3 study phases (SL, $96 \pm 1\%$; acute HA, $76 \pm 2\%$; chronic HA, $84 \pm 1\%$). In terms of intensity, participants averaged $\sim 85\%$ of SL $\dot{V}O_{2\text{peak}}$ and between ~ 75 and 85% of HA $\dot{V}O_{2\text{peak}}$ during 2-mile time trials. Immediately after the time trial, a postexercise (Post) muscle biopsy was obtained. Participants then consumed a bolus, 25 g of whey protein drink (Isopure Zero Carb) and rested in a chair for 3 h. After 3 h of recovery (Rec), a final muscle biopsy was obtained. Muscle biopsies were taken from 2 incisions on 1 leg per biopsy protocol day. BASE biopsies were taken from 1 incision, generally distal to the midpoint of the vastus lateralis. Post and REC biopsies were taken from the second incision that was proximal to the initial incision by ~ 7 cm. The angle of the biopsy needle was changed to limit excessive trauma and inflammation to any one area. Legs were alternated between SL and acute and chronic HA, and new incisions were made for each protocol day.

Muscle protein content

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

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

Intracellular signaling

Phosphorylation status and total protein expression of molecular markers in mTORC1-mediated anabolic signaling and ubiquitin proteasome-mediated proteolysis were determined from Western blot analysis. Homogenates used to estimate muscle protein content were centrifuged for 15 min at 10,000 g at 4°C. Supernatant (lysate) was collected and protein concentrations were analyzed (Thermo Fisher Scientific). Muscle lysates were solubilized in Laemmli buffer, and equal amounts of total protein (15 μg) were separated by SDS-PAGE using precast Tris-HCl gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred to PVDF membranes and exposed to commercially available primary Abs that were specific to Akt, p-Akt^{Ser473}, AMPK, p-AMPK^{Thr172}, TSC2, p-TSC2^{Thr1462}, mTOR, p-mTOR^{Ser2448}, p70 ribosomal protein S6 kinase (p70S6K), p-p70S6K^{Ser424/Thr421}, ribosomal protein S6 (rpS6), p-rpS6^{Ser235/236}, forkhead box O1 (FOXO1), p-FOXO1^{Thr24}, and ubiquitin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Secondary Ab (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology) and chemiluminescent reagent (Pierce, Rockford, IL, USA) were then

applied. Blots were quantified using ChemiDoc XRS (Bio-Rad) and Image Lab software (Bio-Rad). Heat-shock protein 90 (HSP90) was used to confirm that equal amounts of protein were loaded per well. Base phosphorylation was normalized to its total protein. Base total protein was normalized to HSP90. For time course analysis, phase-by-time, phosphorylation status was normalized to HSP90. Total protein ubiquitylation was expressed relative to free ubiquitin. To examine the differences in Base across study phases, Base acute HA and chronic HA were expressed relative to Base SL. To examine the effects of exercise and Rec protein feeding within and across study phases, Post and Rec data were expressed relative to Base within each study phase.

26S proteasome enzymatic activity

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

mRNA expression

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

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

Statistical analysis

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

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

RESULTS

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

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

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

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

TABLE 1. Muscle protein content

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

Values are means $\pm$ SEM. Muscle protein content = protein concentration (mg/ml) $\times$ homogenate vol (ml)/muscle wt (g) $\times$ leg FFM (g).

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

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

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

DISCUSSION

The primary observation of this investigation is that both acute HA exposure and chronic (21 d) HA exposure, coupled with a severe energy deficit, inhibited mTORC1 signaling at Post. Moreover, combined chronic HA exposure and energy deficit inhibited the anabolic sensitivity of muscle to consuming 25 g of whey protein. Reductions in mTORC1 signaling, despite lower SpO₂ at acute and chronic HA compared with SL, seem to be independent of purported hypoxia-mediated mTORC1 inhibition, as neither REDD1 mRNA expression, nor its coupling to 14-3-3 differed between study phases (SL, acute HA, and chronic HA). Markers of muscle protein breakdown were not indicative of elevated proteolysis at acute or chronic HA, which suggests a negligible effect of proteolysis in the modulation of skeletal muscle mass in response to chronic HA exposure and severe energy deficit in healthy individuals. These findings suggest that HA and severe energy deficit induces mTORC1-mediated anabolic resistance and may account, in part, for muscle loss during periods of sustained HA exposure.

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

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

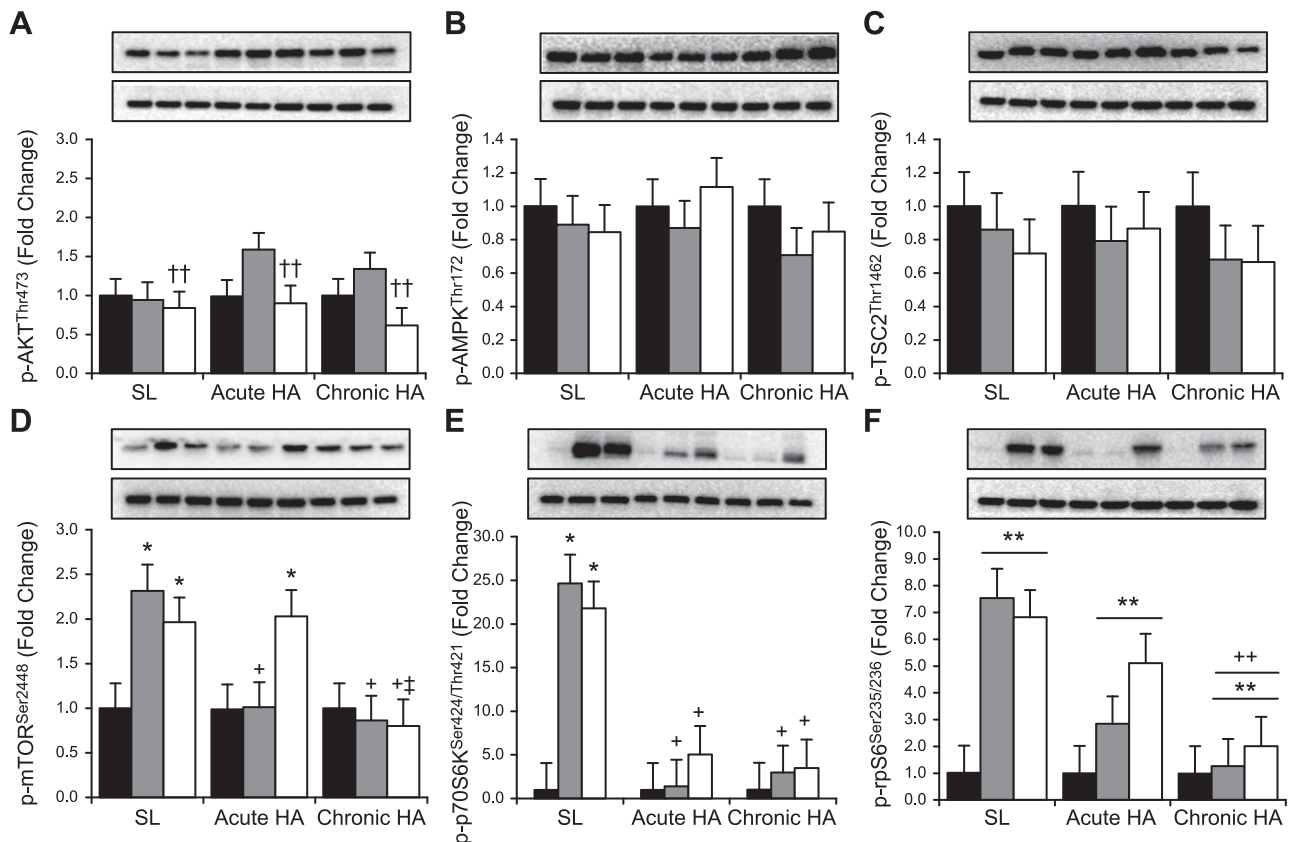


Figure 3. Values are presented as means $\pm$ SEM ($n = 8$). Within [Base (■), Post (▣), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and whey protein ingestion for p-AKT^{Thr473} (A), p-AMPK^{Thr172} (B), p-TSC2^{Thr1462} (C), p-mTOR^{Ser2448} (D), p-p70S6K^{Ser424/421}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

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

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

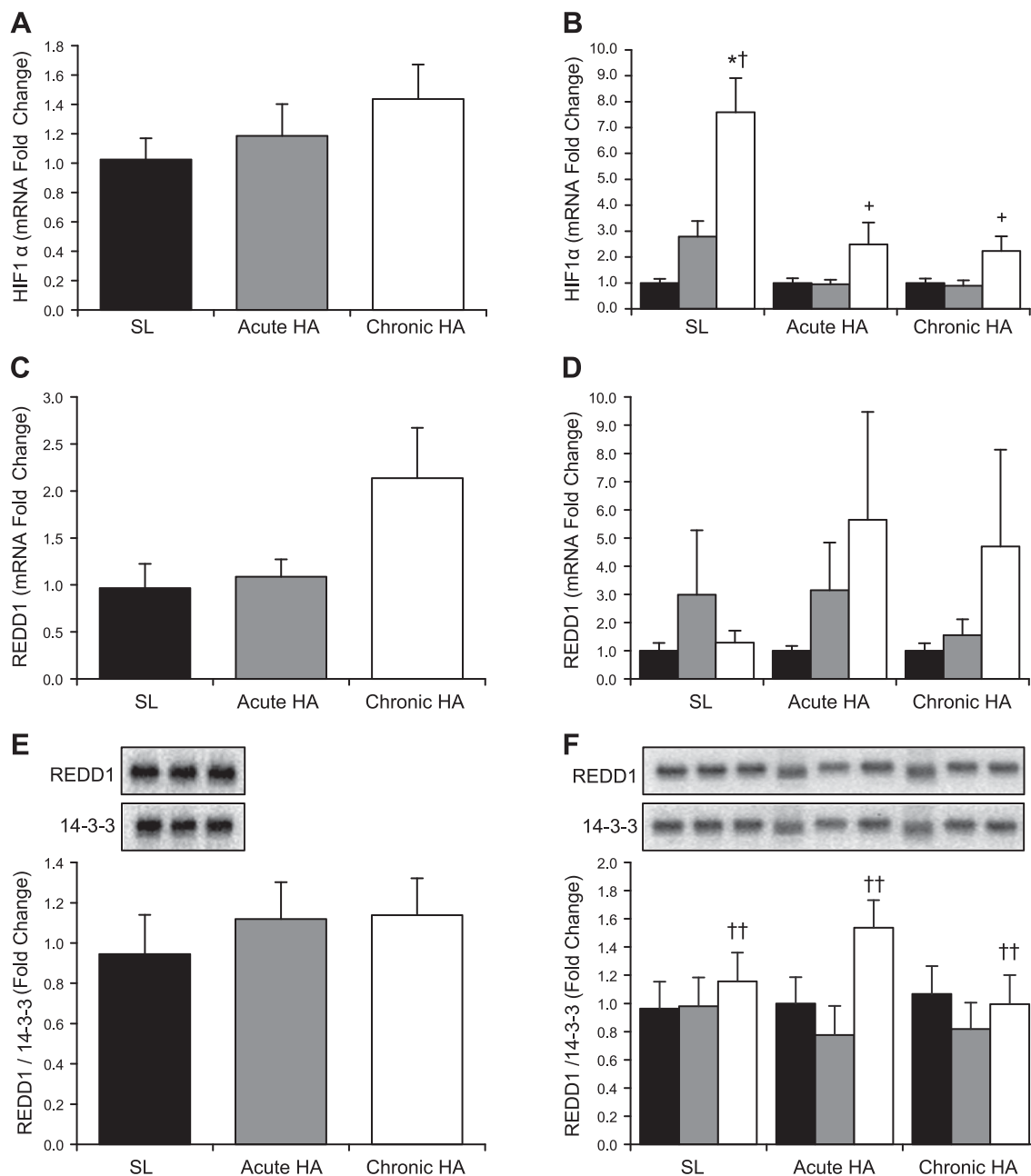


Figure 4. Values are presented as means $\pm$ SEM ($n = 8$). A, C, E) Base HIF-1 α (A) and REDD1 mRNA expression (C), and REDD1/14-3-3 coupling (E) between study phases. B, D, F) Within [Base (■), Post (■), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and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

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

proteolysis with acute HA exposure or after chronic HA exposure, in combination with severe energy deficit, suggests that muscle protein breakdown is not a major contributor to muscle loss during HA sojourns.

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

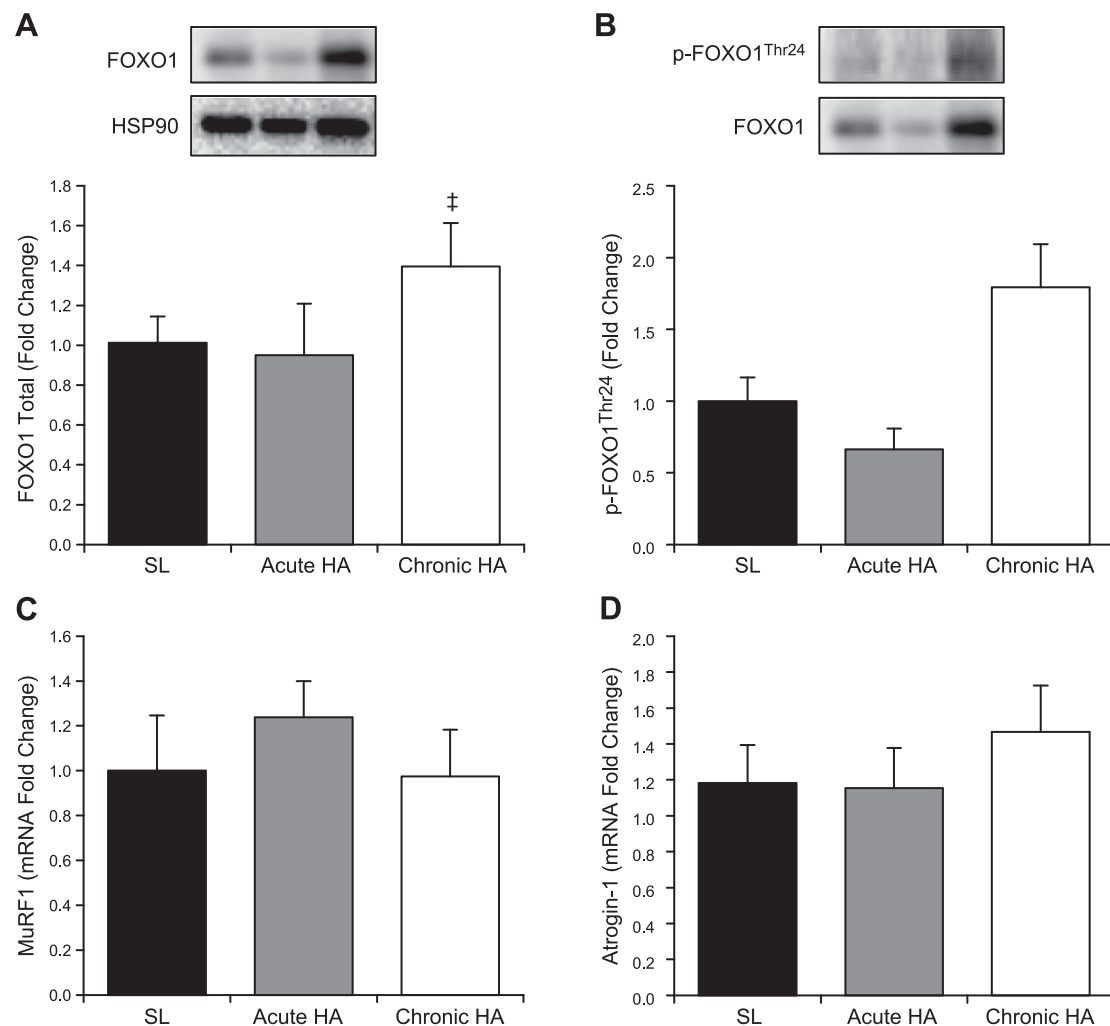


Figure 5. Values are presented as means $\pm$ SEM ($n = 8$). Base total FOXO1 (A) and p-FOXO1^{Thr24} (B), MuRF1 (C), and Atrogin-1 (D) mRNA expression at SL, acute HA, and chronic HA. [‡]Different from acute HA, time-by-phase interaction, $P < 0.05$.

energy deficit. However, there are several limitations. Despite observing robust changes in mTORC1-mediated anabolic signaling, we are unable to determine whether the signaling changes we observed manifested in tangible reductions in muscle protein synthesis. As such, we acknowledge that including stable isotope-derived estimates of muscle protein synthesis (and breakdown) would have strengthened our data and the interpretation of our findings; however, as a result of unexplainable analytical issues, those data are not available. Nevertheless, reductions in FFM (whole body and leg) and muscle protein content, which may be considered an indirect estimate of protein anabolism, suggest that muscle protein was lost in response to the 21 d of HA exposure and severe energy deficit. We also acknowledge that we are unable to determine the mechanism of inhibited mTORC1-mediated anabolic signaling in response to exercise at acute HA. Furthermore, the study design precluded us from determining whether the inhibition of mTORC1-mediated anabolic signaling at chronic HA was the result of prolonged exposure to HA, severe energy deficit, or a combination of both; however, our

study design was not intended to isolate the independent and synergistic effects of hypoxia and energy deficit on the molecular regulation of muscle mass. Our objective was to assess the environmental and physiologic stressors in concert, just as they would be experienced by military Service members (and other HA sojourners) performing strenuous work at HA. Observations made in the current study provide the scientific basis for future, more targeted, studies to delineate the role of hypoxia and underfeeding on the regulation of muscle mass.

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

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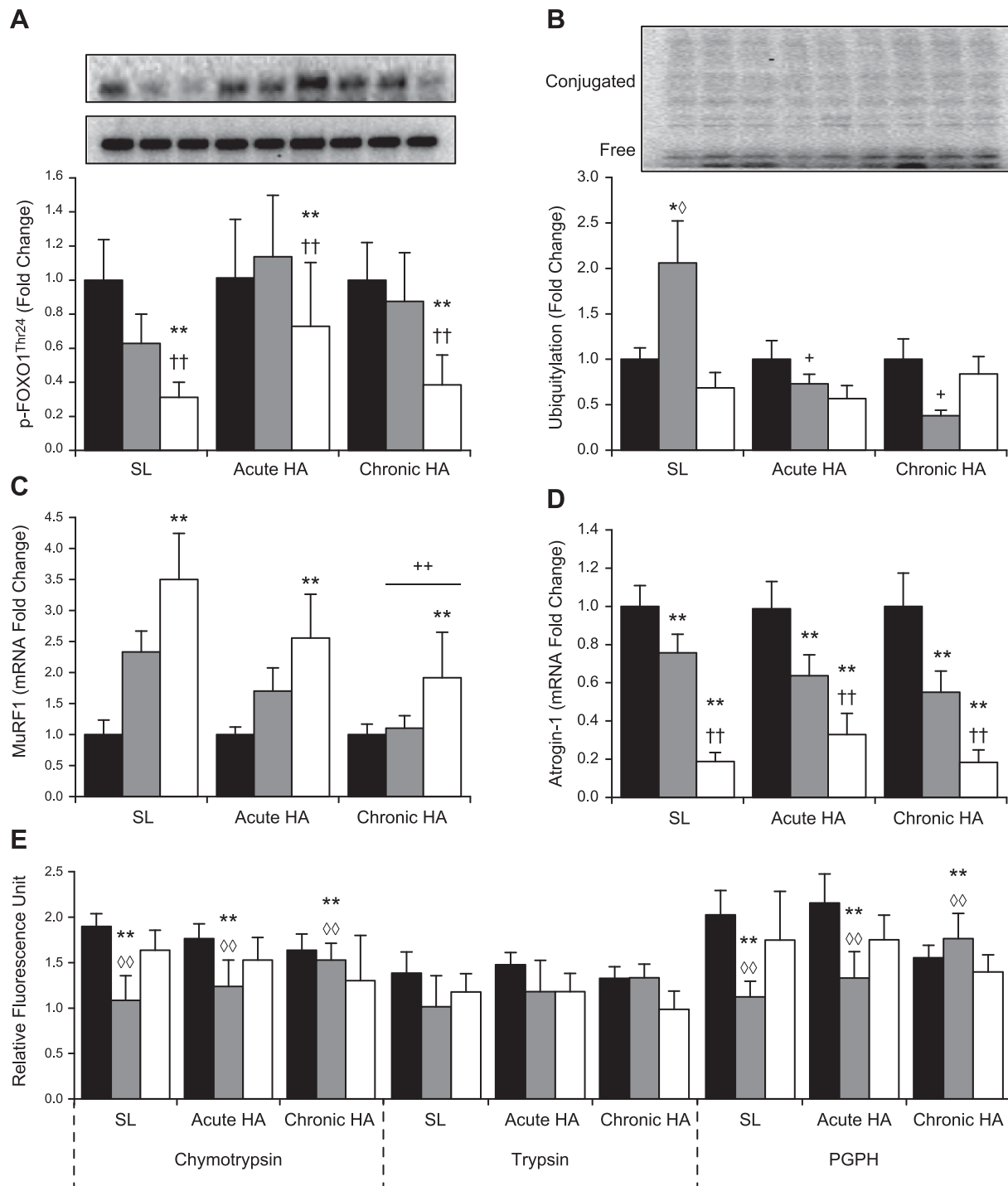


Figure 6. Values are presented as means $\pm$ SEM ($n = 8$). Within [Base (■), Post (▣), and Rec (□)] and between study phase (SL, acute HA, and chronic HA) responses to exercise and whey protein ingestion for p-FOXO1^{Thr24} (A), protein ubiquitylation (B), MuRF1 (C), and Atrogin-1 (D) mRNA expression,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

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AUTHOR CONTRIBUTIONS

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

REFERENCES

- Boutellier, U., Howald, H., di Prampero, P. E., Giezendanner, D., and Cerretelli, P. (1983) Human muscle adaptations to chronic hypoxia. *Prog. Clin. Biol. Res.* **136**, 273–285
- Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R., and Cerretelli, P. (1990) Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int. J. Sports Med.* **11** (Suppl 1), S3–S9
- MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P., and Houston, C. S. (1991) Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol. Scand.* **142**, 421–427
- Mizuno, M., Savard, G. K., Areskog, N. H., Lundby, C., and Saltin, B. (2008) Skeletal muscle adaptations to prolonged exposure to extreme altitude: a role of physical activity? *High Alt. Med. Biol.* **9**, 311–317
- Pasiakos, S. M., Berryman, C. E., Carrigan, C. T., Young, A. J., and Carbone, J. W. (2017) Muscle protein turnover and the molecular regulation of muscle mass during hypoxia. *Med. Sci. Sports Exerc.* **49**, 1340–1350
- Cam, H., Easton, J. B., High, A., and Houghton, P. J. (2010) mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1 α . *Mol. Cell* **40**, 509–520
- Hoppeler, H., and Vogt, M. (2001) Muscle tissue adaptations to hypoxia. *J. Exp. Biol.* **204**, 3133–3139
- DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D., and Ellisen, L. W. (2008) Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* **22**, 289–291
- Favier, F. B., Costes, F., Defour, A., Bonnefoy, R., Lefai, E., Baugé, S., Peinnequin, A., Benoit, H., and Freyssen, D. (2010) Downregulation of Akt/mammalian target of rapamycin pathway in skeletal muscle is associated with increased REDD1 expression in response to chronic hypoxia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R1659–R1666
- Chaudhary, P., Suryakumar, G., Prasad, R., Singh, S. N., Ali, S., and Ilavazhagan, G. (2012) Chronic hypobaric hypoxia mediated skeletal muscle atrophy: role of ubiquitin-proteasome pathway and calpains. *Mol. Cell. Biochem.* **364**, 101–113
- Rose, M. S., Houston, C. S., Fulco, C. S., Coates, G., Sutton, J. R., and Cymerman, A. (1988) Operation Everest. II: nutrition and body composition. *J. Appl. Physiol.* (1985) **65**, 2545–2551
- Butterfield, G. E., Gates, J., Fleming, S., Brooks, G. A., Sutton, J. R., and Reeves, J. T. (1992) Increased energy intake minimizes weight loss in men at high altitude. *J. Appl. Physiol.* (1985) **72**, 1741–1748
- Pasiakos, S. M., Vislocky, L. M., Carbone, J. W., Altieri, N., Konopelski, K., Freake, H. C., Anderson, J. M., Ferrando, A. A., Wolfe, R. R., and Rodriguez, N. R. (2010) Acute energy deprivation affects skeletal muscle protein synthesis and associated intracellular signaling proteins in physically active adults. *J. Nutr.* **140**, 745–751
- Murphy, C. H., Churchward-Venne, T. A., Mitchell, C. J., Kolar, N. M., Kassis, A., Karagounis, L. G., Burke, L. M., Hawley, J. A., and Phillips, S. M. (2015) Hypoenergetic diet-induced reductions in myofibrillar protein synthesis are restored with resistance training and balanced daily protein ingestion in older men. *Am. J. Physiol. Endocrinol. Metab.* **308**, E734–E743
- Hector, A. J., McGlory, C., Damas, F., Mazara, N., Baker, S. K., and Phillips, S. M. (2018) Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise. *FASEB J.* **32**, 265–275
- Areta, J. L., Burke, L. M., Camera, D. M., West, D. W., Crawshaw, S., Moore, D. R., Stellingwerff, T., Phillips, S. M., Hawley, J. A., and Coffey, V. G. (2014) Reduced resting skeletal muscle protein synthesis is rescued by resistance exercise and protein ingestion following short-term energy deficit. *Am. J. Physiol. Endocrinol. Metab.* **306**, E989–E997
- Hector, A. J., Marcotte, G. R., Churchward-Venne, T. A., Murphy, C. H., Breen, L., von Allmen, M., Baker, S. K., and Phillips, S. M. (2015) Whey protein supplementation preserves postprandial myofibrillar protein synthesis during short-term energy restriction in overweight and obese adults. *J. Nutr.* **145**, 246–252
- Pasiakos, S. M., Cao, J. J., Margolis, L. M., Sauter, E. R., Whigham, L. D., McClung, J. P., Rood, J. C., Carbone, J. W., Combs, G. F., Jr., and Young, A. J. (2013) Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial. *FASEB J.* **27**, 3837–3847
- Inoki, K., Zhu, T., and Guan, K. L. (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577–590
- Margolis, L. M., Rivas, D. A., Berrone, M., Ezzyat, Y., Young, A. J., McClung, J. P., Fielding, R. A., and Pasiakos, S. M. (2016) Prolonged calorie restriction downregulates skeletal muscle mTORC1 signaling independent of dietary protein intake and associated microRNA expression. *Front. Physiol.* **7**, 445
- Miller, B. F., Robinson, M. M., Bruss, M. D., Hellerstein, M., and Hamilton, K. L. (2012) A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction. *Aging Cell* **11**, 150–161
- Carbone, J. W., Pasiakos, S. M., Vislocky, L. M., Anderson, J. M., and Rodriguez, N. R. (2014) Effects of short-term energy deficit on muscle protein breakdown and intramuscular proteolysis in normal-weight young adults. *Appl. Physiol. Nutr. Metab.* **39**, 960–968
- Smiles, W. J., Areta, J. L., Coffey, V. G., Phillips, S. M., Moore, D. R., Stellingwerff, T., Burke, L. M., Hawley, J. A., and Camera, D. M. (2015) Modulation of autophagy signaling with resistance exercise and protein ingestion following short-term energy deficit. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **309**, R603–R612
- Carbone, J. W., Margolis, L. M., McClung, J. P., Cao, J. J., Murphy, N. E., Sauter, E. R., Combs, G. F., Jr., Young, A. J., and Pasiakos, S. M. (2013) Effects of energy deficit, dietary protein, and feeding on intracellular regulators of skeletal muscle proteolysis. *FASEB J.* **27**, 5104–5111
- Pasiakos, S. M., and Carbone, J. W. (2014) Assessment of skeletal muscle proteolysis and the regulatory response to nutrition and exercise. *IUBMB Life* **66**, 478–484

26. Pasiakos, S. M., Margolis, L. M., and Orr, J. S. (2015) Optimized dietary strategies to protect skeletal muscle mass during periods of unavoidable energy deficit. *FASEB J.* **29**, 1136–1142
27. Hoyt, R. W., Jones, T. E., Baker-Fulco, C. J., Schoeller, D. A., Schoene, R. B., Schwartz, R. S., Askew, E. W., and Cymerman, A. (1994) Doubly labeled water measurement of human energy expenditure during exercise at high altitude. *Am. J. Physiol.* **266**, R966–R971
28. Hoyt, R. W., Jones, T. E., Stein, T. P., McAninch, G. W., Lieberman, H. R., Askew, E. W., and Cymerman, A. (1991) Doubly labeled water measurement of human energy expenditure during strenuous exercise. *J. Appl. Physiol.* (1985) **71**, 16–22
29. Berryman, C. E., Young, A. J., Karl, J. P., Kenefick, R. W., Margolis, L. M., Cole, R. E., Carbone, J. W., Lieberman, H. R., Kim, I. Y., Ferrando, A. A., and Pasiakos, S. M. (2018) Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. *FASEB J.* **32**, 894–905
30. Margolis, L. M., Rood, J., Champagne, C., Young, A. J., and Castellani, J. W. (2013) Energy balance and body composition during US Army special forces training. *Appl. Physiol. Nutr. Metab.* **38**, 396–400
31. Pandolf, K. B., Givoni, B., and Goldman, R. F. (1977) Predicting energy expenditure with loads while standing or walking very slowly. *J. Appl. Physiol.* **43**, 577–581
32. Pimential, N. A., and Pandolf, K. B. (1979) Energy expenditure while standing or walking slowly uphill or downhill with loads. *Ergonomics* **22**, 963–973
33. Hoyt, R. W., Opstad, P. K., Haugen, A. H., DeLany, J. P., Cymerman, A., and Friedl, K. E. (2006) Negative energy balance in male and female rangers: effects of 7 d of sustained exercise and food deprivation. *Am. J. Clin. Nutr.* **83**, 1068–1075
34. Siri, W. E. (1956) The gross composition of the body. *Adv. Biol. Med. Phys.* **4**, 239–280
35. Barnholt, K. E., Hoffman, A. R., Rock, P. B., Muza, S. R., Fulco, C. S., Braun, B., Holloway, L., Mazzeo, R. S., Cymerman, A., and Friedlander, A. L. (2006) Endocrine responses to acute and chronic high-altitude exposure (4,300 meters): modulating effects of caloric restriction. *Am. J. Physiol. Endocrinol. Metab.* **290**, E1078–E1088
36. Mawson, J. T., Braun, B., Rock, P. B., Moore, L. G., Mazzeo, R., and Butterfield, G. E. (2000) Women at altitude: energy requirement at 4,300 m. *J. Appl. Physiol.* (1985) **88**, 272–281
37. Margolis, L. M., Murphy, N. E., Martini, S., Gundersen, Y., Castellani, J. W., Karl, J. P., Carrigan, C. T., Teien, H. K., Madslie, E. H., Montain, S. J., and Pasiakos, S. M. (2016) Effects of supplemental energy on protein balance during 4-d arctic military training. *Med. Sci. Sports Exerc.* **48**, 1604–1612
38. Margolis, L. M., Murphy, N. E., Martini, S., Spitz, M. G., Thrane, I., McGraw, S. M., Blatny, J. M., Castellani, J. W., Rood, J. C., Young, A. J., Montain, S. J., Gundersen, Y., and Pasiakos, S. M. (2014) Effects of winter military training on energy balance, whole-body protein balance, muscle damage, soreness, and physical performance. *Appl. Physiol. Nutr. Metab.* **39**, 1395–1401
39. Margolis, L. M., McClung, H. L., Murphy, N. E., Carrigan, C. T., and Pasiakos, S. M. (2017) Skeletal muscle myomiR are differentially expressed by endurance exercise mode and combined essential amino acid and carbohydrate supplementation. *Front. Physiol.* **8**, 182
40. Pasiakos, S. M., McClung, H. L., Margolis, L. M., Murphy, N. E., Lin, G. G., Hydren, J. R., and Young, A. J. (2015) Human muscle protein synthetic responses during weight-bearing and non-weight-bearing exercise: a comparative study of exercise modes and recovery nutrition. *PLoS One* **10**, e0140863
41. Pasiakos, S. M., McClung, H. L., McClung, J. P., Margolis, L. M., Andersen, N. E., Cloutier, G. J., Pikosky, M. A., Rood, J. C., Fielding, R. A., and Young, A. J. (2011) Leucine-enriched essential amino acid supplementation during moderate steady state exercise enhances postexercise muscle protein synthesis. *Am. J. Clin. Nutr.* **94**, 809–818
42. Pasiakos, S. M., McClung, H. L., McClung, J. P., Urso, M. L., Pikosky, M. A., Cloutier, G. J., Fielding, R. A., and Young, A. J. (2010) Molecular responses to moderate endurance exercise in skeletal muscle. *Int. J. Sport Nutr. Exerc. Metab.* **20**, 282–290
43. Thomson, D. M., and Gordon, S. E. (2005) Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation. *J. Appl. Physiol.* (1985) **98**, 557–564
44. Thomson, D. M., and Gordon, S. E. (2006) Impaired overload-induced muscle growth is associated with diminished translational signalling in aged rat fast-twitch skeletal muscle. *J. Physiol.* **574**, 291–305
45. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45
46. Gorissen, S. H., Horstman, A. M., Franssen, R., Kouw, I. W., Wall, B. T., Burd, N. A., de Groot, L. C., and van Loon, L. J. (2017) Habituation to low or high protein intake does not modulate basal or postprandial muscle protein synthesis rates: a randomized trial. *Am. J. Clin. Nutr.* **105**, 332–342
47. Etheridge, T., Atherton, P. J., Wilkinson, D., Selby, A., Rankin, D., Webborn, N., Smith, K., and Watt, P. W. (2011) Effects of hypoxia on muscle protein synthesis and anabolic signaling at rest and in response to acute resistance exercise. *Am. J. Physiol. Endocrinol. Metab.* **301**, E697–E702
48. Masschelein, E., Van Thienen, R., D'Hulst, G., Hespel, P., Thomis, M., and Deldicque, L. (2014) Acute environmental hypoxia induces LC3 lipidation in a genotype-dependent manner. *FASEB J.* **28**, 1022–1034
49. D'Hulst, G., Jamart, C., Van Thienen, R., Hespel, P., Francaux, M., and Deldicque, L. (2013) Effect of acute environmental hypoxia on protein metabolism in human skeletal muscle. *Acta Physiol. (Oxf.)* **208**, 251–264
50. D'Hulst, G., Ferri, A., Naslain, D., Bertrand, L., Horman, S., Francaux, M., Bishop, D. J., and Deldicque, L. (2016) Fifteen days of 3,200 m simulated hypoxia marginally regulates markers for protein synthesis and degradation in human skeletal muscle. *Hypoxia (Auckl.)* **4**, 1–14
51. Viganò, A., Ripamonti, M., De Palma, S., Capitanio, D., Vasso, M., Wait, R., Lundby, C., Cerretelli, P., and Gelfi, C. (2008) Proteins modulation in human skeletal muscle in the early phase of adaptation to hypobaric hypoxia. *Proteomics* **8**, 4668–4679
52. Miller, B. F., Robinson, M. M., Reuland, D. J., Drake, J. C., Peeler F. F. III, Bruss, M. D., Hellerstein, M. K., and Hamilton, K. L. (2013) Calorie restriction does not increase short-term or long-term protein synthesis. *J. Gerontol. A Biol. Sci. Med. Sci.* **68**, 530–538

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ORIGINAL RESEARCH

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

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Abstract

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

Introduction

Hypoxia exposure during high-altitude (HA) sojourns elicits a persistent systemic inflammatory response (Mazzeo et al. 1985; Hartmann et al. 2000; Lundby and Steensberg 2004). This response represents a homeostatic

adaptation to hypoxia that may promote acute-phase protein synthesis, angiogenesis, and erythropoiesis (Mazzeo et al. 1985; Klausen et al. 1997; Mazzeo 2005). Sojourners at HA are also subject to additional pro-inflammatory stressors, such as high physical activity levels (Hoppeler et al. 1990; Hoyt et al. 1994) and energy deficits

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

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

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

and energy deficit, and the magnitude of this response would be directly associated with the magnitude of FFM loss experienced by individuals exposed to the stress of hypoxic exposure and energy deficit.

Methods

Participants

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

Experimental design

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

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

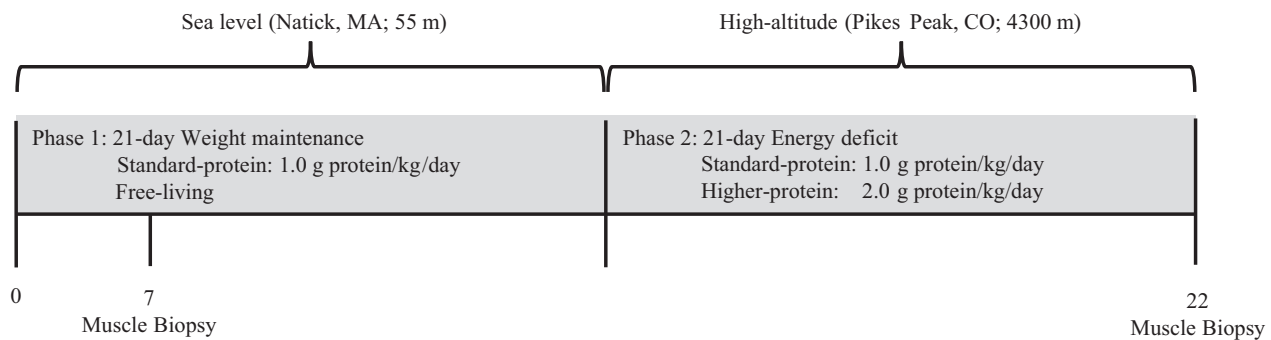


Figure 1. Study design. Energy deficit was designed to be 40% of total energy needs; however, on the basis of changes in body energy stores, the actual deficit was closer to 70% (-1862 ± 525 kcal/day). The current analysis was part of a larger study that assessed the impact of dietary protein intake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

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

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

Body mass was measured daily at HA and body composition (total body mass, TBM; FFM; and fat mass, FM) was determined at baseline, before ascent to HA (SL day 20), and immediately after descent from HA ($\sim$ 0630–0800 on

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

Muscle inflammatory and myogenic mRNA

Muscle interleukin 6 (IL-6), IL-6 receptor (IL-6R), TNF α , TNF α -receptor (TNF α -R), TWEAK, Fn14, paired box protein-7 (PAX7), MYOD, MYOGENIN, MuRF1, and ATROGIN-1 gene expression were determined using primers from Bio-Rad Laboratories (Hercules, CA, USA). TRIzol (ThermoFisher, Waltham, MA, USA) was used to isolate total RNA from 20 mg of muscle followed by quantity and quality assessments using a Nanodrop ND-2000 spectrophotometer (ThermoFisher). cDNA was then synthesized from 500 ng of total RNA using iScriptTM Advanced cDNA Synthesis Kits from Bio-Rad Laboratories and a T100TM Thermal Cycler (Bio-Rad). cDNA amplifications were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Samples were run in 20 μ L reactions in duplicate using iTaqTM Universal SYBR[®] Green Supermix (Bio-Rad). mRNA data were normalized to the geometric mean of β -actin, GAPDH, and B2M mRNA, and fold changes (HA vs. SL) were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). The MURF1 and ATROGIN-1 gene expression responses to the HA and severe energy deficit have been reported previously (Margolis et al. 2018), but are reported briefly in this paper to explore the potential

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

NF- κ B phosphorylation and total protein expression

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

26S proteasome enzymatic activity

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

Statistical analysis

This study was a secondary objective of a larger randomized controlled trial powered to detect differences in FFM between standard- and higher-protein diets following HA exposure and concomitant energy deficit (Berryman et al. 2018). As reported, dietary protein intake had no effect on FFM in response to the combined physiological and environmental stress (Berryman et al. 2018). Therefore, we did not expect dietary protein group to affect skeletal muscle inflammation, regeneration, and proteasome enzymatic activities after 21 days of exposure to HA and energy deficit. After testing for normality (Shapiro-Wilk), and log₂

transformation of skewed data (all mRNA endpoints), we examined the effects of dietary protein on study outcomes at HA relative to SL using repeated measures ANOVA with phase (SL and HA) and dietary protein group (standard- and higher-protein) as within- and between-subjects factors, respectively. As anticipated, there were no effects of dietary protein group on any study outcome.

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

Results

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

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

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

($r = -0.513$), and Fn14 ($r = -0.715$) gene expression were associated ($P < 0.05$) with less FFM loss. Based on these results, participants were dichotomized into HIGH and LOW according to the median Fn14 gene expression at HA, respectively (Fig. 4A). HIGH lost less FFM and more FM ($28 \pm 28\%$ and $72 \pm 28\%$), as a proportion of

TBM loss, than LOW ($58 \pm 26\%$ and $42 \pm 26\%$; Fig. 4B, $P < 0.05$). MYOGENIN gene expression after HA exposure and severe energy deficit was also greater for HIGH versus LOW (Fig. 4C, $P < 0.05$). MYOGENIN gene expression changes in response to HA and severe energy deficit were associated ($r = 0.530$, $P < 0.05$) with the

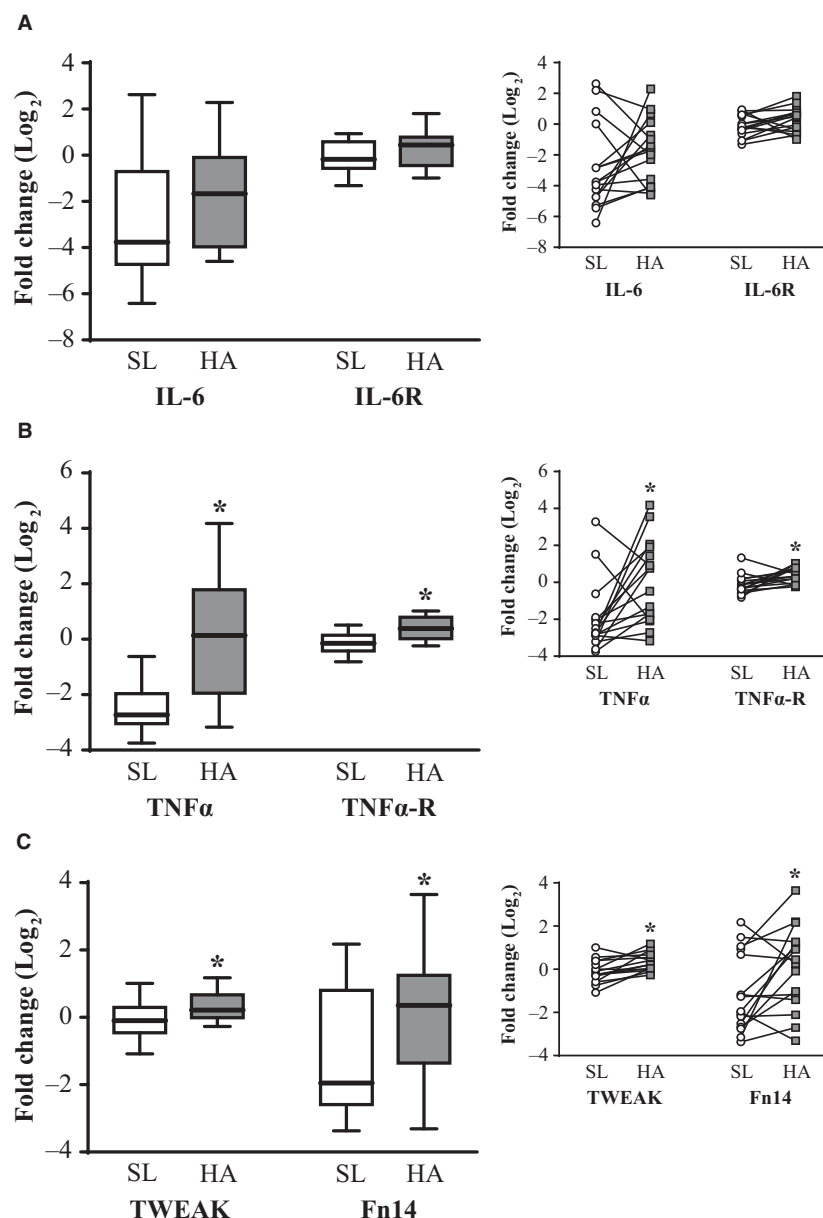


Figure 2. Skeletal muscle IL-6 and IL-6R (A), TNF α and TNF α -R (B), and TWEAK and Fn14 (C) gene expression in male ($n = 16$) lowlanders exposed to 21 days of energy deficit at high altitude (4300 m). Data were normalized to the geometric mean of β -actin, GAPDH, and B2M gene expression, and fold changes were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001). HA data were expressed as fold change relative to SL to examine differences using paired t -tests after data were log₂ transformed. Values are presented as mean $\pm$ SD and individually per participant. *HA different than SL, $P < 0.05$. IL-6, interleukin 6; IL-6R, interleukin 6 receptor; TNF α , tumor necrosis factor alpha; TNF α -R, tumor necrosis factor alpha-receptor; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; Fn14, fibroblast growth factor-inducible 14; SL, day 7 at sea level; and HA, day 22 at high altitude.

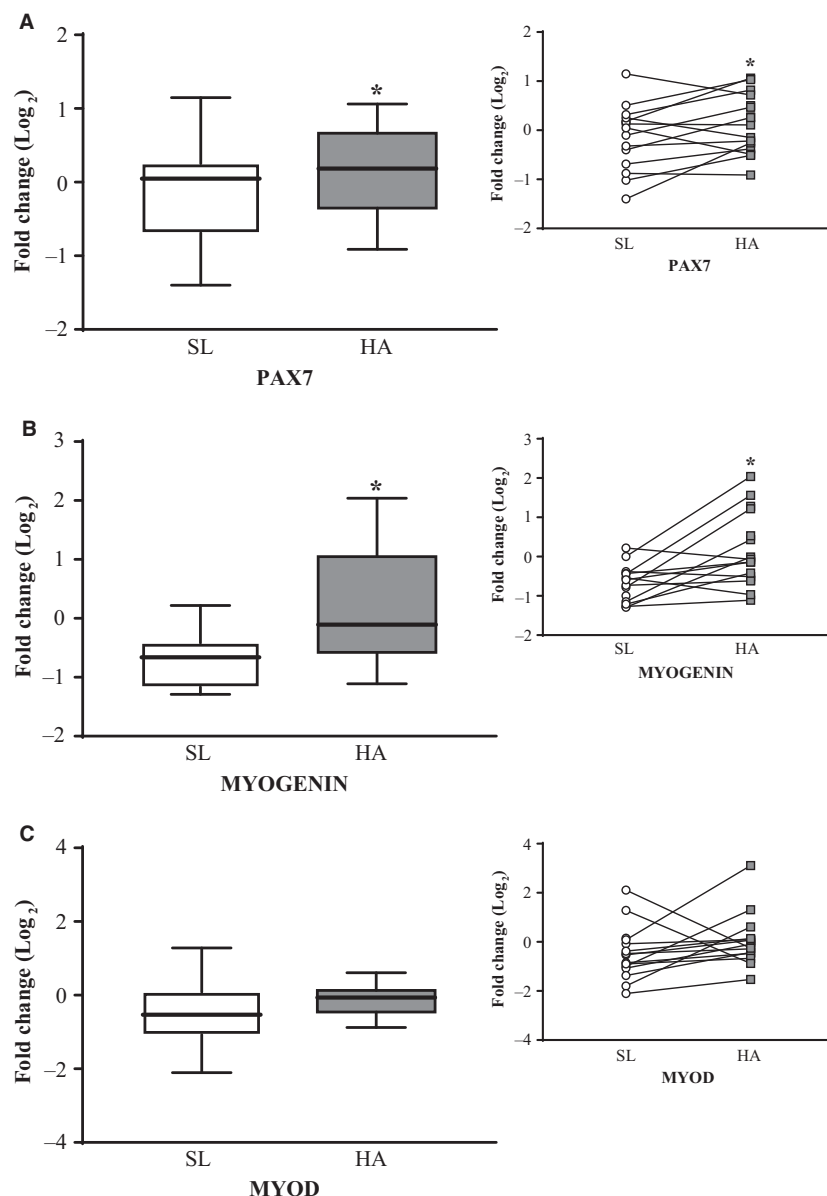


Figure 3. Skeletal muscle PAX7 (A), MYOGENIN (B), and MYOD (C) gene expression in male ($n = 16$) lowlanders exposed to 21 days of energy deficit at high altitude (4300 m). Data were normalized to the geometric mean of β -actin, GAPDH, and B2M gene expression, and fold changes were calculated using the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

change in Fn14. There were no significant differences in MuRF1 or ATROGIN-1 gene expression, or chymotrypsin-like, trypsin-like, and PGPH-like enzymatic activities between HIGH and LOW. No significant differences were observed in EIEE between the two groups (HIGH: 686 ± 156 kcal day⁻¹, LOW: 655 ± 196 kcal day⁻¹, $P > 0.05$) or in the estimated energy deficit (HIGH: -201 ± 228 kcal day⁻¹, LOW: -1702 ± 693 kcal day⁻¹, $P > 0.05$).

Discussion

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

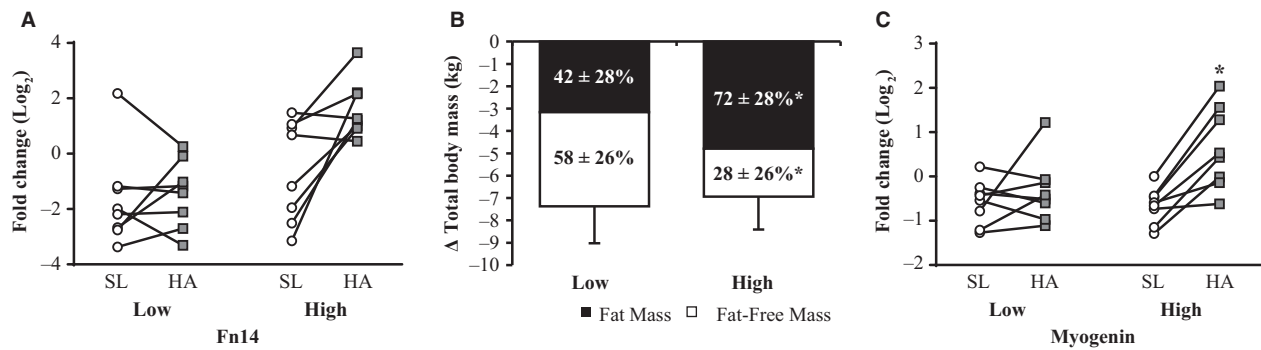


Figure 4. Fn14 (A), FM and FM loss as a proportion (%) of total body mass loss (B) and MYOGENIN gene expression (C) in male ($n = 16$) lowlanders dichotomized based on median Fn14 gene expression at HA relative to SL into LOW and HIGH after being exposed to 21 days of energy deficit at high altitude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

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

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

Muscle inflammation and its potential downstream effects on proteolytic activity in healthy individuals exposed to chronic HA and concomitant underfeeding, to our knowledge, has not been explored. We observed modest increases in muscle TNF α , TNF α -R, TWEAK, and Fn14

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

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

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

Based on a previous study by Bamman et al. (2015), we used regression analysis and subsequent dichotomization to explore whether the interindividual variability in muscle inflammation dictated the magnitude of FFM loss in response to energy deficit at HA. In the Bamman study (Bamman et al. 2015), patients undergoing elective total hip arthroplasty were dichotomized based on perioperative Fn14 gene expression in the gluteal muscle of the surgical limb, and deemed either susceptible (high Fn14 expression relative to controls) or not susceptible (low Fn14 gene expression relative to controls) to excessive muscle inflammation. Patients with high Fn14 gene expression had lower muscle protein synthesis rates and higher NF- κ B activity and MURF1 gene expression compared to patients with lower Fn14 gene expression (Bamman et al. 2015). Bamman et al. (2015) concluded that elevated Fn14 gene expression may be a prognostic indicator of poor recovery from surgery. Accordingly, we expected that the muscle inflammatory response to 21 days of energy deficit at HA would be detrimental, such that those with an elevated muscle inflammatory response would lose more FFM than participants exhibiting minimal changes in muscle inflammation. After regression analysis confirmed Fn14 was the strongest predictor of FFM loss, participant dichotomization into HIGH and LOW Fn14 demonstrated that those classified as HIGH lost significantly less FFM and more FM than

those classified as LOW. We suspect that the apparent beneficial effect of increased muscle inflammation, particularly Fn14, in the current study was due to the upregulation in myogenic regulatory factor gene expression. This is highlighted by the finding that MYOGENIN gene expression in response to chronic HA and concomitant energy deficit was greater in HIGH than LOW. These results, although in contrast to our original hypothesis, are in accordance with studies that show muscle inflammation in response to routine exercise is not overtly catabolic and aids in the repair, remodeling, and maintenance of healthy muscle tissue (Raue et al. 1985a,b; Warren et al. 2002; Karalaki et al. 2009).

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

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Conflict of Interest

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

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

References

- Bamman, M. M., A. A. Ferrando, R. P. Evans, M. J. Stec, N. A. Kelly, J. M. Gruenewald, et al. 2015. Muscle inflammation susceptibility: a prognostic index of recovery potential after hip arthroplasty? *Am. J. Physiol. Endocrinol. Metab.* 308: E670–E679.
- Berryman, C. E., A. J. Young, J. P. Karl, R. W. Kenefick, L. M. Margolis, R. E. Cole, et al. 2018. Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. *FASEB J.* 32:894–905.
- Burd, N. A., and M. De Lisio. 2017. Skeletal muscle remodeling: interconnections between stem cells and protein turnover. *Exerc. Sport Sci. Rev.* 45:187–191.
- Butterfield, G. E., J. Gates, S. Fleming, G. A. Brooks, J. R. Sutton, and J. T. Reeves. 1985. Increased energy intake minimizes weight loss in men at high altitude. *J. Appl. Physiol.* 72:1992.
- Cai, D., J. D. Frantz, N. E. Jr Tawa, P. A. Melendez, B. C. Oh, H. G. Lidov, et al. 2004. IKK β /NF- κ B activation causes severe muscle wasting in mice. *Cell* 119:285–298.
- Carbone, J. W., L. M. Margolis, J. P. McClung, J. J. Cao, N. E. Murphy, E. R. Sauter, et al. 2013. Effects of energy deficit, dietary protein, and feeding on intracellular regulators of skeletal muscle proteolysis. *FASEB J.* 27:5104–5111.
- Chen, S. E., B. Jin, and Y. P. Li. 2007. TNF- α regulates myogenesis and muscle regeneration by activating p38 MAPK. *Am. J. Physiol. Cell Physiol.* 292:C1660–C1671.
- Dogra, C., H. Changotra, S. Mohan, and A. Kumar. 2006. Tumor necrosis factor-like weak inducer of apoptosis inhibits skeletal myogenesis through sustained activation of nuclear factor- κ B and degradation of MyoD protein. *J. Biol. Chem.* 281:10327–10336.
- Dogra, C., H. Changotra, N. Wedhas, X. Qin, J. E. Wergedal, and A. Kumar. 2007a. TNF-related weak inducer of apoptosis (TWEAK) is a potent skeletal muscle-wasting cytokine. *FASEB J.* 21:1857–1869.
- Dogra, C., S. L. Hall, N. Wedhas, T. A. Linkhart, and A. Kumar. 2007b. Fibroblast growth factor inducible 14 (Fn14) is required for the expression of myogenic regulatory factors and differentiation of myoblasts into myotubes. Evidence for TWEAK-independent functions of Fn14 during myogenesis. *J. Biol. Chem.* 282:15000–15010.
- Girgenrath, M., S. Weng, C. A. Kostek, B. Browning, M. Wang, S. A. Brown, et al. 2006. TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. *EMBO J.* 25:5826–5839.
- Hagobian, T. A., K. A. Jacobs, A. W. Subudhi, J. A. Fattor, P. B. Rock, S. R. Muza, et al. 2006. Cytokine responses at high altitude: effects of exercise and antioxidants at 4300 m. *Med. Sci. Sports Exerc.* 38:276–285.
- Hartmann, G., M. Tschop, R. Fischer, C. Bidlingmaier, R. Riepl, K. Tschop, et al. 2000. High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. *Cytokine* 12:246–252.
- Hennigar, S. R., J. P. McClung, and S. M. Pasiakos. 2017. Nutritional interventions and the IL-6 response to exercise. *FASEB J.* 31:3719–3728.
- Hoppeler, H., E. Kleinert, C. Schlegel, H. Claassen, H. Howald, S. R. Kayar, et al. 1990. Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int. J. Sports Med.* 11(Suppl 1):S3–S9.
- Hoyt, R. W., T. E. Jones, C. J. Baker-Fulco, D. A. Schoeller, R. B. Schoene, R. S. Schwartz, et al. 1994. Doubly labeled water measurement of human energy expenditure during exercise at high altitude. *Am. J. Physiol.* 266:R966–R971.
- Hoyt, R. W., P. K. Opstad, A. H. Haugen, J. P. DeLany, A. Cymerman, and K. E. Friedl. 2006. Negative energy balance in male and female rangers: effects of 7 d of sustained exercise and food deprivation. *Am. J. Clin. Nutr.* 83: 1068–1075.
- Karalaki, M., S. Fili, A. Philippou, and M. Koutsilieris. 2009. Muscle regeneration: cellular and molecular events. *In Vivo* 23:779–796.
- Klausen, T., N. V. Olsen, T. D. Poulsen, J. P. Richalet, and B. K. Pedersen. 1997. Hypoxemia increases serum interleukin-6 in humans. *Eur. J. Appl. Physiol. Occup. Physiol.* 76: 480–482.
- Kumar, A., S. Bhatnagar, and P. K. Paul. 2012. TWEAK and TRAF6 regulate skeletal muscle atrophy. *Curr. Opin. Clin. Nutr. Metab. Care* 15:233–239.
- Lundby, C., and A. Steensberg. 2004. Interleukin-6 response to exercise during acute and chronic hypoxia. *Eur. J. Appl. Physiol.* 91:88–93.
- Margolis, L. M., D. A. Rivas, M. Berrone, Y. Ezzyat, A. J. Young, J. P. McClung, et al. 2016. Prolonged calorie restriction downregulates skeletal muscle mTORC1 signaling independent of dietary protein intake and associated microRNA expression. *Front. Physiol.* 7:445.
- Margolis, L. M., J. W. Carbone, C. E. Berryman, C. T. Carrigan, N. E. Murphy, A. A. Ferrando, et al. 2018. Severe energy deficit at high-altitude inhibits skeletal muscle mTORC1-mediated anabolic signaling without increased ubiquitin proteasome activity. *FASEB J.* fj-201800163RR. <https://doi.org/10.1096/fj.201800163RR>. [Epub ahead of print].
- Mazzeo, R. S. 2005. Altitude, exercise and immune function. *Exerc. Immunol. Rev.* 11:6–16.
- Mazzeo, R. S., D. Donovan, M. Fleshner, G. E. Butterfield, S. Zamudio, E. E. Wolfel, et al. 1985. Interleukin-6 response to exercise and high-altitude exposure: influence of alpha-adrenergic blockade. *J. Appl. Physiol.* 91:2001.

- McClung, J. P., S. Martini, N. E. Murphy, S. J. Montain, L. M. Margolis, I. Thrane, et al. 2013. Effects of a 7-day military training exercise on inflammatory biomarkers, serum hepcidin, and iron status. *Nutr. J.* 12:141.
- Merritt, E. K., M. J. Stec, A. Thalacker-Mercer, S. T. Windham, J. M. Cross, D. P. Shelley, et al. 1985. Heightened muscle inflammation susceptibility may impair regenerative capacity in aging humans. *J. Appl. Physiol.* 115:2013.
- Merritt, E. K., J. M. Cross, and M. M. Bamman. 2012. Inflammatory and protein metabolism signaling responses in human skeletal muscle after burn injury. *J. Burn Care Res.* 33:291–297.
- Merritt, E. K., A. Thalacker-Mercer, J. M. Cross, S. T. Windham, S. J. Thomas, and M. M. Bamman. 2013. Increased expression of atrogenes and TWEAK family members after severe burn injury in nonburned human skeletal muscle. *J. Burn Care Res.* 34:e297–e304.
- Mittal, A., S. Bhatnagar, A. Kumar, E. Lach-Trifilieff, S. Wauters, H. Li, et al. 2010. The TWEAK-Fn14 system is a critical regulator of denervation-induced skeletal muscle atrophy in mice. *J. Cell Biol.* 188:833–849.
- Pandolf, K. B., B. Givoni, and R. F. Goldman. 1977. Predicting energy expenditure with loads while standing or walking very slowly. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* 43:577–581.
- Pasiakos, S. M., J. J. Cao, L. M. Margolis, E. R. Sauter, L. D. Whigham, J. P. McClung, et al. 2013. Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial. *FASEB J.* 27:3837–3847.
- Pasiakos, S. M., L. M. Margolis, N. E. Murphy, H. L. McClung, S. Martini, Y. Gundersen, et al. 2016. Effects of exercise mode, energy, and macronutrient interventions on inflammation during military training. *Physiol. Rep.* 4:1.
- Pasiakos, S. M., C. E. Berryman, C. T. Carrigan, A. J. Young, and J. W. Carbone. 2017. Muscle protein turnover and the molecular regulation of muscle mass during hypoxia. *Med. Sci. Sports Exerc.* 49:1340–1350.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
- Pimental, N. A., and K. B. Pandolf. 1979. Energy expenditure while standing or walking slowly uphill or downhill with loads. *Ergonomics* 22:963–973.
- Raue, U., B. Jemiolo, Y. Yang, and S. Trappe. 1985a. TWEAK-Fn14 pathway activation after exercise in human skeletal muscle: insights from two exercise modes and a time course investigation. *J. Appl. Physiol.* 118:2015.
- Raue, U., T. A. Trappe, S. T. Estrem, H. R. Qian, L. M. Helvering, R. C. Smith, et al. 1985b. Transcriptome signature of resistance exercise adaptations: mixed muscle and fiber type specific profiles in young and old adults. *J. Appl. Physiol.* 112:2012.
- Rose, M. S., C. S. Houston, C. S. Fulco, G. Coates, J. R. Sutton, and A. Cymerman. 1985. Operation everest. II: nutrition and body composition. *J. Appl. Physiol.* 65:1988.
- Roza, A. M., and H. M. Shizgal. 1984. The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. *Am. J. Clin. Nutr.* 40:168–182.
- Tedesco, F. S., A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J. Clin. Invest* 120:11–19.
- Warren, G. L., T. Hulderman, N. Jensen, M. McKinstry, M. Mishra, M. I. Luster, et al. 2002. Physiological role of tumor necrosis factor alpha in traumatic muscle injury. *FASEB J.* 16:1630–1632.
- Yarar-Fisher, C., C. S. Bickel, N. A. Kelly, M. J. Stec, S. T. Windham, A. B. McLain, et al. 2016. Heightened TWEAK-NF-kappaB signaling and inflammation-associated fibrosis in paralyzed muscles of men with chronic spinal cord injury. *Am. J. Physiol. Endocrinol. Metab.* 310:E754–E761.



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

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

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

INTRODUCTION

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

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

Sojourners at HA experience both anorexia and increased total daily energy expenditure (TDEE) (Westerterp et al., 1994;

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

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

MATERIALS AND METHODS

Participants

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

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

Experimental Design and Diet

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

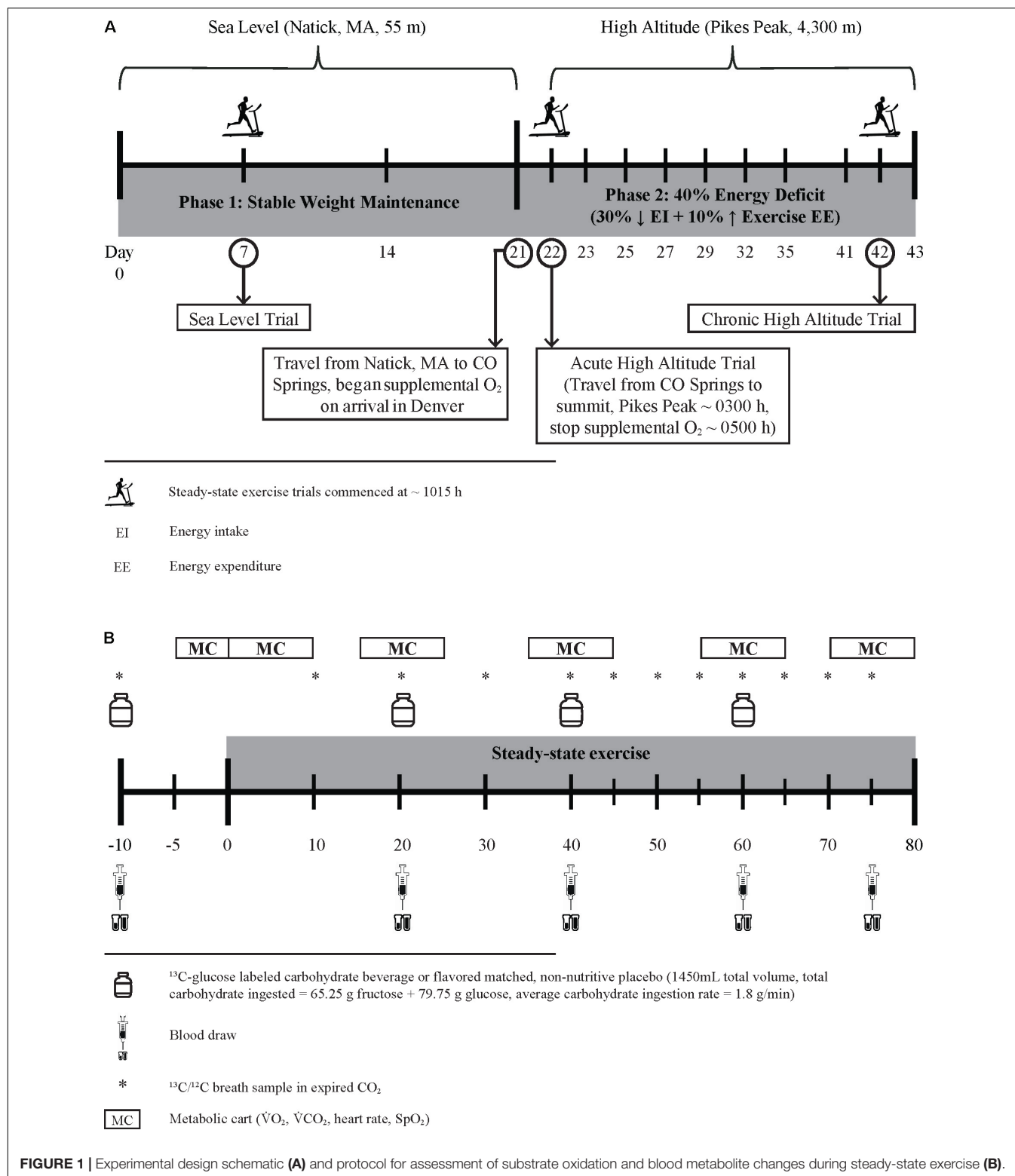
On day 21, participants were flown to Denver, CO (1,630 m) and transported by research staff to Colorado Springs, CO (1,840 m), where they stayed overnight, under supervision, in a local hotel before being transported ($\sim 03:00$ h) to the USARIEM Maher Memorial Altitude Laboratory on the summit of Pikes Peak, CO (4,300 m, day 22). Participants breathed supplemental oxygen (portable O₂ concentrators, FreeStyle 5, AirSep, Buffalo, NY, United States or EverGo, Respironics, Murrysville, PA, United States) after landing in Denver until they arrived at the summit about 13-h later, so as to maintain normoxic oxygen saturation (mean $\pm$ SD for peripheral oxygen saturation [finger pulse oximetry, Nonin, Model 9560, Plymouth, MN, United States] measured hourly from arrival until 22:00 h and every 30 min beginning at 03:00 h was $97.0 \pm 0.9\%$, range 95–99). This allowed the initiation of hypoxic exposure before experimental testing began to be standardized among all of the participants (~ 315 min). Participants did not breathe supplemental oxygen until they arrived in Denver, and thus

experienced a mild, 4-h hypoxic exposure equivalent to $\sim 1,900$ m (Hampson et al., 2013) during the 4-h flight from Boston. Starting the morning following the AHA experiment, participants were randomized and consumed either the standard or higher protein [amount consistent with recommendations for strenuous military operations, (Pasiakos et al., 2013a)] diet for the next 21 days (HA days 2–22). The carbohydrate content of the diets was fixed at 45% of total energy consumed so as to avoid confounding effects of variations in daily carbohydrate intake on substrate oxidation during exercise during the CHA trial. The remainder of the energy consumed was derived from fat. For HA, Registered Dietitians created diets based on individualized energy requirements to maintain body mass at SL, consisting primarily of components of the military combat ration, Meal, Ready-to-Eat (MRE; menu 34; AmeriQual, Evansville, IN, United States). The remainder of the energy was derived from perishable foods and whey protein drinks (Isopure® Zero Carb), as previously described (Berryman et al., 2018). All foods and caloric drinks were weighed to the nearest 0.1 g, and preparation and consumption of all meals and snacks were supervised by trained research staff. Non-caloric, non-caffeinated drinks were allowed *ad libitum*.

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

Peak Oxygen Uptake

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



Steady-State Exercise Protocol

Substrate oxidation during steady-state exercise (treadmill walking) was assessed following the same protocol (**Figure 1B**) at SL, AHA, and CHA. Participants completed all three

of the steady-state exercise tests following a 15-h fast, and all three exercise tests were performed at the same absolute $\dot{V}O_2$. The absolute $\dot{V}O_2$ (i.e., L/min) for the exercise experiments was selected to correspond to ~55% of each individual's absolute

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

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

from respiratory exchange measurements as described by Jeukendrup and Wallis (2005):

Energy expenditure rate

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

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

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

Measurement of Substrate Oxidation During Steady-State Exercise

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

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

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

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

Calculation of protein oxidation assumed that nitrogen lost as sweat urea was negligible. The natural enrichment of the fructose-glucose ingested and the $^{13}\text{C}/^{12}\text{C}$ in expired gas samples were analyzed using isotope-ratio mass spectroscopy (Metabolic Solutions, Inc., Nashua, NH, United States). The isotopic enrichments were expressed in ‰ difference by comparison with VPDB (see **Figure 2** for expired $\delta^{13}\text{CO}_2$ versus VPDB during each endurance exercise substrate utilization protocol) and used to calculate exogenous glucose oxidation rate as follows:

$$\text{Exogenous glucose oxidation (g/min)} = \dot{V}\text{CO}_2 [(R_{\text{exp}} - R_{\text{ref}})/(R_{\text{exo}} - R_{\text{ref}})]/k,$$

where $\dot{V}\text{CO}_2$ is in L/min, R_{exp} is the observed isotopic composition of expired CO_2 , R_{ref} is the isotopic composition of expired CO_2 at rest before ingestion of the first dose of ^{13}C -glucose, R_{exo} is the isotopic composition of the exogenous glucose ingested. The volume of CO_2 provided by the complete oxidation of glucose is 0.747 L/g (k) (Trimmer et al., 2001; Ruzzin et al., 2003). Subtracting exogenous glucose oxidation from total carbohydrate oxidation yielded the amount of “other” carbohydrates oxidized. For PLA, the “other” carbohydrate oxidized was entirely comprised of endogenous carbohydrate (i.e., glucose provided by muscle and liver glycogen stores) oxidation. For CHO, the “other” carbohydrate oxidized was comprised of endogenous carbohydrate oxidation and exogenous fructose oxidation, the latter of which was not captured by

the tracer techniques employed in the CHO group. These calculations assume that, in response to exercise, ^{13}C labeled glucose is not irreversibly lost in pools of tricarboxylic acid cycle intermediates (Ruzzin et al., 2003) and/or bicarbonate (Trimmer et al., 2001) allowing for near complete $^{13}\text{CO}_2$ recovery in expired gases. Due to the slow equilibration of the $^{13}\text{C}/^{12}\text{C}$ in expired CO_2 (i.e., mouth) with $^{13}\text{C}/^{12}\text{C}$ in tissue derived CO_2 (Pallikarakis et al., 1991), and to account for transient effects of hyperventilation (Jones and Jurkowski, 1979), only data and samples from the final 40 min of the steady-state exercise test were used to calculate expenditure and substrate oxidation (rates, g/min, and total amounts in 40 min, g).

Blood and Urine Analyses

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

Statistical Analyses

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

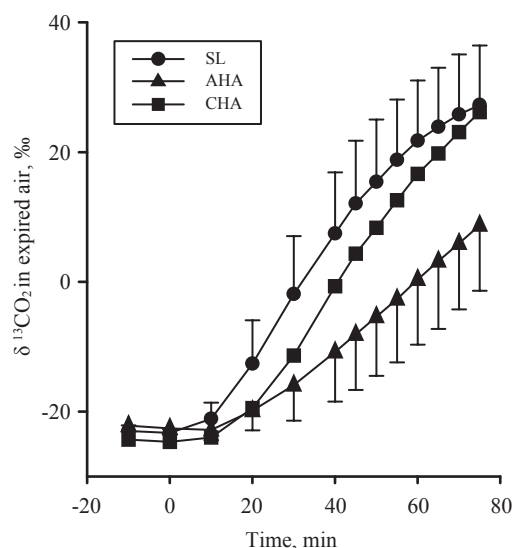


FIGURE 2 | The expired $\delta^{13}\text{CO}_2$ versus VPDB in participants provided 1.8 g/min carbohydrate (CHO, $n = 8$, 65.25 g fructose + 79.75 g glucose, closed symbols, during 80-min steady-state exercise (treadmill walking) at sea level (SL) and at 4,300 m altitude, within 5–7 h after arrival (acute high-altitude, AHA) and again on the 22nd day of continuous residence at high-altitude (chronic high-altitude, CHO).

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

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

RESULTS

Participant Characteristics

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

TABLE 1 | Detailed listing of physical characteristics of participants.

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

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

TABLE 2 | Detailed dietary intake for participants.

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

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

same for both groups, averaging 690 ± 178 kcal/d for the CHO and 695 ± 185 kcal/d for PLA.

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

Respiratory Gas Exchange During Steady-State Exercise

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

Substrate Oxidation During Steady-State Exercise

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

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

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

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

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

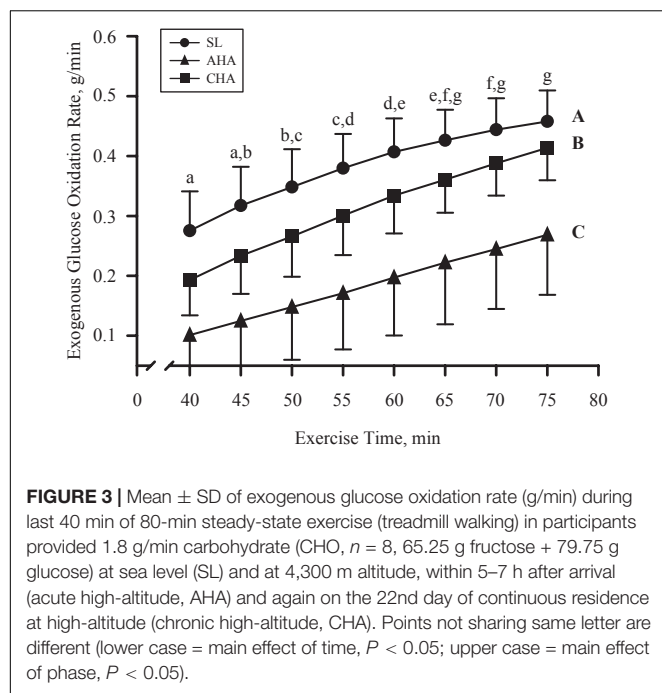
	SL		AHA		CHA	
	CHO	PLA	CHO	PLA	CHO	PLA
Carbohydrate (g) glucose						
Total* [†]	68.3 ± 19.8	45.9 ± 21.4	59.7 ± 18.9	42.9 ± 15.7	45.5 ± 13.3	28.2 ± 17.9
Exogenous glucose [†]	15.3 ± 2.2	—	7.4 ± 3.7	—	12.4 ± 2.3	—
Other [†]	53.0 ± 20.1	45.9 ± 21.4	52.3 ± 16.9	42.9 ± 15.7	33.0 ± 12.6	28.2 ± 17.9
Fat* [†]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

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

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

The contribution of protein oxidation to total energy production during exercise did not differ between groups or

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

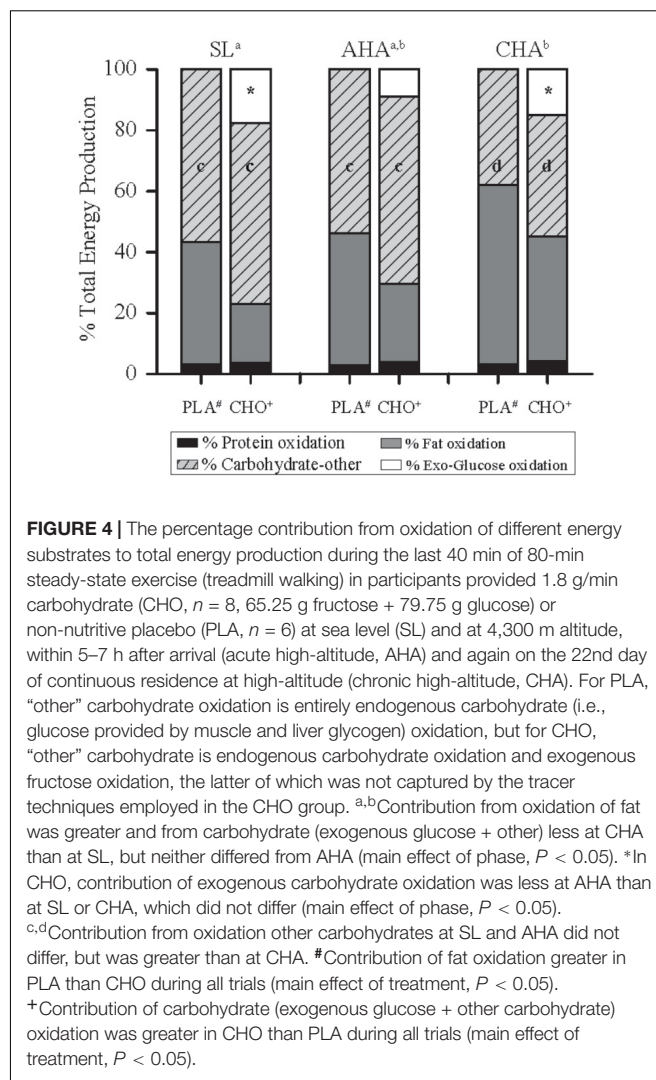


contributed more to total energy production at CHA than SL ($P < 0.05$).

Changes in Blood Metabolites During Steady-State Exercise

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

Serum free fatty acid concentrations (**Figures 5E,F**) before exercise and beverage consumption did not differ between groups or phases. However, during exercise, free fatty acid concentrations progressively increased ($P < 0.05$) in PLA, but progressively decreased ($P < 0.05$) in CHO. By 40 min of exercise and thereafter, free fatty acid concentrations were higher ($P < 0.05$) in PLA than CHO. In both CHO and PLA, free fatty acid concentrations were higher at AHA than



SL, ($P < 0.05$) and higher at CHA than AHA ($P < 0.05$). Serum glycerol concentrations (**Figures 5G,H**) before exercise and beverage consumption were on average higher (main effect of treatment, $P < 0.05$) in CHO than PLA. Glycerol concentrations in PLA rose progressively through 60 min of exercise, and remained plateaued thereafter, but glycerol concentrations in CHO remained unchanged throughout exercise. In both groups, glycerol concentrations during exercise were highest at CHA, intermediate at AHA, and lowest at SL ($P < 0.05$).

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

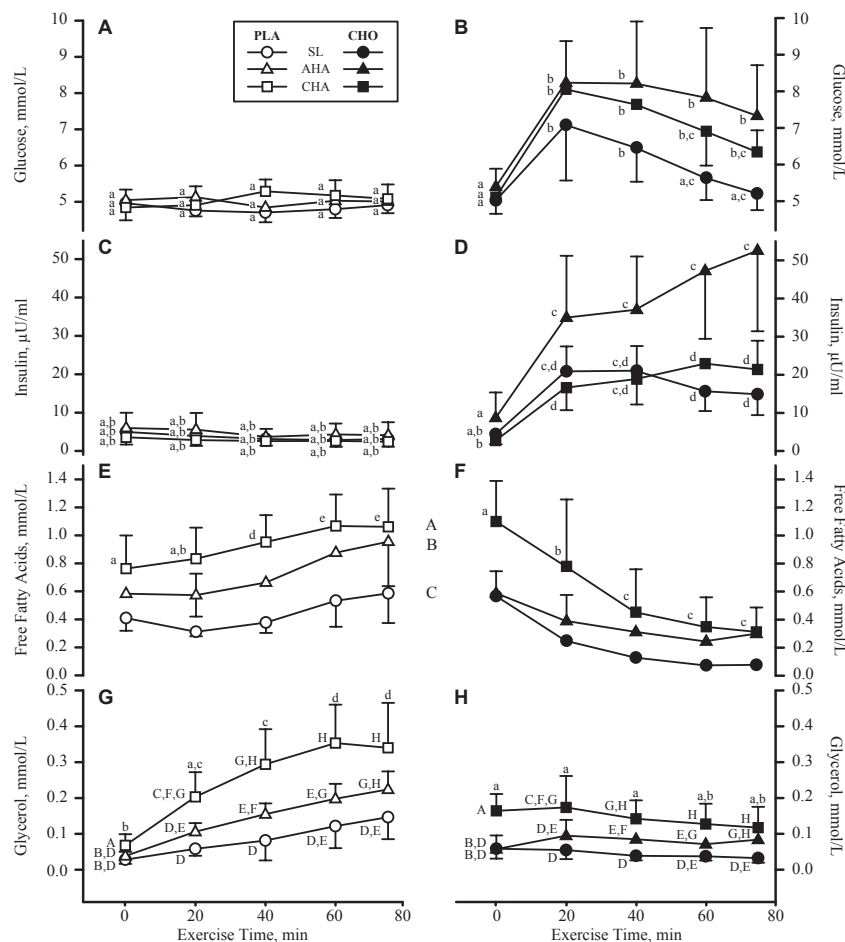


FIGURE 5 | Mean + SD of serum concentrations of glucose (mmol/L, **A,B**), insulin (μ U/mL, **C,D**), free fatty acids (mmol/L, **E,F**) and glycerol (mmol/L, **G,H**) concentrations in participants provided non-nutritive placebo (PLA, $n = 6$, open symbols, left column of panels) or 1.8 g/min carbohydrate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

DISCUSSION

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

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

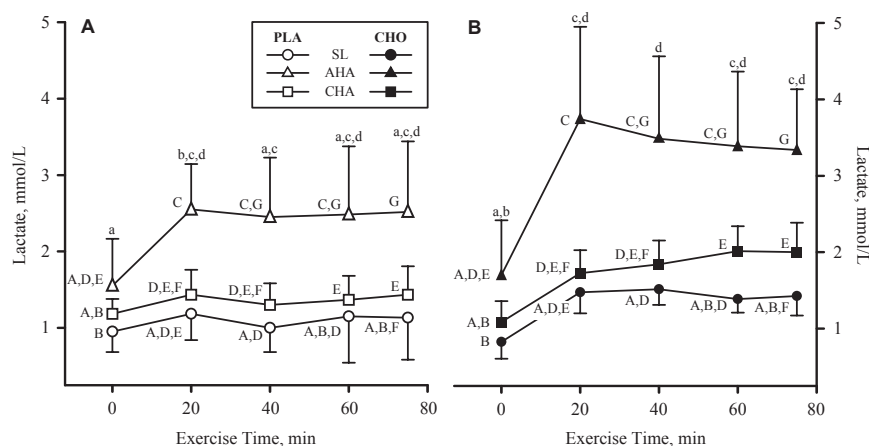


FIGURE 6 | Mean + SD of plasma lactate concentrations in participants provided non-nutritive placebo (PLA, $n = 6$, open symbols, **(A)** or 1.8 g/min carbohydrate (CHO, $n = 8$, 65.25 g fructose + 79.75 g glucose, closed symbols, **(B)**) during 80-min steady-state exercise (treadmill walking) at sea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

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

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

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

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

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

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

In summary, our study demonstrated that exogenous glucose oxidation during exercise is suppressed by acute hypoxia, but after 21 days of altitude acclimatization and energy deficit, that suppression is alleviated. Whether altitude acclimatization alleviates hypoxic suppression of exogenous glucose oxidation in fully fed sojourners maintaining stable body weight remains to be demonstrated. From a practical perspective, our observations suggest that traditional recommendations for

consuming carbohydrate during exercise may not have the same effects on substrate oxidation during exercise on the first few days of a HA sojourn as during exercise at SL, but following a period of HA acclimatization, metabolic effects of consuming exogenous carbohydrate during exercise may normalize.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

- Ainsworth, B. E., Haskell, W. L., Herrmann, S. D., Meckes, N., D. R. Jr., Tudor-Locke, C., et al. (2011). 2011 compendium of physical activities: a second update of codes and MET values. *Med. Sci. Sports Exerc.* 43, 1575–1581. doi: 10.1249/MSS.0b013e31821eece12
- American College of Sports Medicine (2014). *ACSM's Guidelines for Exercise Testing and Prescription*, 9th Edn. Baltimore, MD: Wolters Kluwer.
- Berryman, C. E., Young, A. J., Karl, J. P., Kenefick, R. W., Margolis, L. M., Cole, R. E., et al. (2018). Severe negative energy balance during 21 d at high altitude decreases fat-free mass regardless of dietary protein intake: a randomized controlled trial. *FASEB J.* 32, 894–905. doi: 10.1096/fj.201700915R
- Braun, B., Butterfield, G. E., Dominick, S. B., Zamudio, S., McCullough, R. G., Rock, P. B., et al. (1998). Women at altitude: changes in carbohydrate metabolism at 4,300-m elevation and across the menstrual cycle. *J. Appl. Physiol.* 85, 1966–1973. doi: 10.1152/jappl.1998.85.5.1966
- Braun, B., Rock, P. B., Zamudio, S., Wolfel, G. E., Mazzeo, R. S., Muza, S. R., et al. (2001). Women at altitude: short-term exposure to hypoxia and/or alpha(1)-adrenergic blockade reduces insulin sensitivity. *J. Appl. Physiol.* 91, 623–631. doi: 10.1152/jappl.2001.91.2.623
- Brooks, G. A., Butterfield, G. E., Wolfe, R. R., Groves, B. M., Mazzeo, R. S., Sutton, J. R., et al. (1991). Increased dependence on blood glucose after acclimatization to 4300 m. *J. Appl. Physiol.* 70, 919–927. doi: 10.1152/jappl.1991.70.2.919
- Butterfield, G. E. (1999). Nutrient requirements at high altitude. *Clin. Sports Med.* 18, 607–621, viii. doi: 10.1016/S0278-5919(05)70171-X
- Butterfield, G. E., Gates, J., Fleming, S., Brooks, G. A., Sutton, J. R., and Reeves, J. T. (1992). Increased energy intake minimizes weight loss in men at high altitude. *J. Appl. Physiol.* 72, 1741–1748. doi: 10.1152/jappl.1992.72.5.1741
- Coyle, E. F., Jeukendrup, A. E., Wagenmakers, A. J. M., and Saris, W. H. (1997). Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. *Am. J. Physiol.* 273(2 Pt 1), E268–E275. doi: 10.1152/ajpendo.1997.273.2.E268
- Gautier, J. F., Pirnay, F., Jandrain, B., Lacroix, M., Mosora, F., Scheen, A. J., et al. (1993). Endogenous substrate oxidation during exercise and variations in breath ^{13}C Co 2 /12Co 2 . *J. Appl. Physiol.* 74, 133–138. doi: 10.1152/jappl.1993.74.1.133
- Hampson, N. B., Kregenow, D. A., Mahoney, A. M., Kirtland, S. H., Horan, K. L., Holm, J. R., et al. (2013). Altitude exposures during commercial flight: a reappraisal. *Aviat. Space Environ. Med.* 84, 27–31. doi: 10.3357/ASEM.3438.2013

- Hoyt, R. W., Jones, T. E., Baker-Fulco, C. J., Schoeller, D. A., Schoene, R. B., Schwartz, R. S., et al. (1994). Doubly labeled water measurement of human energy expenditure during exercise at high altitude. *Am. J. Physiol.* 266(3 Pt 2), R966–R971. doi: 10.1152/ajpregu.1994.266.3.R966
- Jentjens, R. L. P. G., Moseley, L., Waring, R. H., Harding, L. K., and Jeukendrup, A. E. (2004). Oxidation of combined ingestion of glucose and fructose during exercise. *J. Appl. Physiol.* 96, 1277–1284. doi: 10.1152/japplphysiol.00974.2003
- Jeukendrup, A. (2014). A step towards personalized sports nutrition: carbohydrate intake during exercise. *Sports Med.* 44(Suppl. 1), S25–S33. doi: 10.1007/s40279-014-0148-z
- Jeukendrup, A., Brouns, F., Wagenmakers, A. J., and Saris, W. H. (1997). Carbohydrate-electrolyte feedings improve 1 h time trial cycling performance. *Int. J. Sports Med.* 18, 125–129. doi: 10.1055/s-2007-972607
- Jeukendrup, A. E. (2004). Carbohydrate intake during exercise and performance. *Nutrition* 20, 669–677. doi: 10.1016/j.nut.2004.04.017
- Jeukendrup, A. E. (2008). Carbohydrate feeding during exercise. *Eur. J. Sport Sci.* 8, 77–86. doi: 10.1080/17461390801918971
- Jeukendrup, A. E., Moseley, L., Mainwaring, G. I., Samuels, S., Perry, S., and Mann, C. H. (2006). Exogenous carbohydrate oxidation during ultraendurance exercise. *J. Appl. Physiol.* 100, 1134–1141. doi: 10.1152/japplphysiol.00981.2004
- Jeukendrup, A. E., and Wallis, G. A. (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int. J. Sports Med.* 26, S28–S37. doi: 10.1055/s-2004-830512
- Jones, N. L., and Jurkowski, J. E. (1979). Body carbon-dioxide storage capacity in exercise. *J. Appl. Physiol.* 46, 811–815. doi: 10.1152/jappl.1979.46.4.811
- Larsen, J. J., Hansen, J. M., Olsen, N. V., Galbo, H., and Dela, F. (1997). The effect of altitude hypoxia on glucose homeostasis in men. *J. Physiol.* 504, 241–249. doi: 10.1111/j.1469-7793.1997.241bf.x
- Lundby, C., and Van Hall, G. (2002). Substrate utilization in sea level residents during exercise in acute hypoxia and after 4 weeks of acclimatization to 4100 m. *Acta Physiol. Scand.* 176, 195–201. doi: 10.1046/j.1365-201X.2002.01030.x
- Margolis, L. M., Murphy, N. E., Martini, S., Spitz, M. G., Thrane, I., McGraw, S. M., et al. (2014). Effects of winter military training on energy balance, whole-body protein balance, muscle damage, soreness, and physical performance. *Appl. Physiol. Nutr. Metab.* 39, 1395–1401. doi: 10.1139/apnm-2014-0212
- Margolis, L. M., Pasiakos, S. M., Karl, J. P., Rood, J. C., Cable, S. J., Williams, K. W., et al. (2012). Differential effects of military training on fat-free mass and plasma amino acid adaptations in men and women. *Nutrients* 4, 2035–2046. doi: 10.3390/nu4122035
- O'Hara, J. P., Woods, D. R., Mellor, A., Boos, C., Gallagher, L., Tsakirides, C., et al. (2017). A comparison of substrate oxidation during prolonged exercise in men at terrestrial altitude and normobaric normoxia following the coingestion of 13C glucose and 13C fructose. *Physiol. Rep.* 5:e13101. doi: 10.14814/phy2.13101
- Oltmanns, K. M., Gehring, H., Rudolf, S., Schultes, B., Rook, S., Schweiger, U., et al. (2004). Hypoxia causes glucose intolerance in humans. *Am. J. Respir. Crit. Care Med.* 169, 1231–1237. doi: 10.1164/rccm.200308-1200OC
- Pallikarakis, N., Sphiris, N., and Lefebvre, P. (1991). Influence of the bicarbonate pool and on the occurrence of Co-13(2) in exhaled air. *Eur. J. Appl. Physiol. Occup. Physiol.* 63, 179–183. doi: 10.1007/Bf00233844
- Pandolf, K. B., Givoni, B., and Goldman, R. F. (1977). Predicting energy expenditure with loads while standing or walking very slowly. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* 43, 577–581. doi: 10.1152/jappl.1977.43.4.577
- Pasiakos, S. M., Austin, K. G., Lieberman, H. R., and Askew, E. W. (2013a). Efficacy and safety of protein supplements for U.S. Armed Forces personnel: consensus statement. *J. Nutr.* 143, 1811S–1814S. doi: 10.3945/jn.113.176859
- Pasiakos, S. M., Cao, J. J., Margolis, L. M., Sauter, E. R., Whigham, L. D., McClung, J. P., et al. (2013b). Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial. *FASEB J.* 27, 3837–3847. doi: 10.1096/fj.13-230227
- Pasiakos, S. M., Margolis, L. M., McClung, J. P., Cao, J. J., Whigham, L. D., Combs, G. F., et al. (2014). Whole-body protein turnover response to short-term high-protein diets during weight loss: a randomized controlled trial. *Int. J. Obes.* 38, 1015–1018. doi: 10.1038/ijo.2013.197
- Peltonen, G. L., Scalzo, R. L., Schweder, M. M., Larson, D. G., Luckasen, G. J., Irwin, D., et al. (2012). Sympathetic inhibition attenuates hypoxia induced insulin resistance in healthy adult humans. *J. Physiol.* 590, 2801–2809. doi: 10.1113/jphysiol.2011.227090
- Peronnet, F., Massicotte, D., Folch, N., Melin, B., Koulmann, N., Jimenez, C., et al. (2006). Substrate utilization during prolonged exercise with ingestion of (13)C-glucose in acute hypobaric hypoxia (4,300 m). *Eur. J. Appl. Physiol.* 97, 527–534. doi: 10.1007/s00421-006-0164-2
- Pirnay, F., Crielaard, J. M., Pallikarakis, N., Lacroix, M., Mosora, F., Krzentowski, G., et al. (1982). Fate of exogenous glucose during exercise of different intensities in humans. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* 53, 1620–1624.
- Roberts, A. C., Reeves, J. T., Butterfield, G. E., Mazzeo, R. S., Sutton, J. R., Wolfel, E. E., et al. (1996). Altitude and beta-blockade augment glucose utilization during submaximal exercise. *J. Appl. Physiol.* 80, 605–615. doi: 10.1152/jappl.1996.80.2.605
- Rose, M. S., Houston, C. S., Fulco, C. S., Coates, G., Sutton, J. R., and Cymerman, A. (1988). Operation Everest. II: nutrition and body composition. *J. Appl. Physiol.* 65, 2545–2551. doi: 10.1152/jappl.1988.65.6.2545
- Rowlands, D. S., Thorburn, M. S., Thorp, R. M., Broadbent, S., and Shi, X. (2008). Effect of graded fructose coingestion with maltodextrin on exogenous 14C-fructose and 13C-glucose oxidation efficiency and high-intensity cycling performance. *J. Appl. Physiol.* 104, 1709–1719. doi: 10.1152/japplphysiol.00878.2007
- Roza, A. M., and Shizgal, H. M. (1984). The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. *Am. J. Clin. Nutr.* 40, 168–182. doi: 10.1093/ajcn/40.1.168
- Ruzzin, J., Peronnet, F., Tremblay, J., Massicotte, D., and Lavoie, C. (2003). Breath [¹³CO₂] recovery from an oral glucose load during exercise: comparison between [U-¹³C] and [1,2-¹³C]glucose. *J. Appl. Physiol.* 95, 477–482. doi: 10.1152/japplphysiol.00095.2003
- Santee, W. R., Blanchard, L. A., Speckman, K. L., Gonzalez, J. A., and Wallace, R. F. (2003). *Load Carriage Model Development and Testing with Field Data*. Natick, MA: US Army Research Institute of Environmental Medicine.
- Tharion, W. J., Lieberman, H. R., Montain, S. J., Young, A. J., Baker-Fulco, C. J., Delany, J. P., et al. (2005). Energy requirements of military personnel. *Appetite* 44, 47–65. doi: 10.1016/j.appet.2003.11.010
- Trimmer, J. K., Casazza, G. A., Horning, M. A., and Brooks, G. A. (2001). Recovery of ¹³CO₂ during rest and exercise after [1-¹³C]acetate, [2-¹³C]acetate, and NaH¹³CO₃ infusions. *Am. J. Physiol. Endocrinol. Metab.* 281, E683–E692. doi: 10.1152/ajpendo.2001.281.4.E683
- Westerterp, K. R., Kayser, B., Wouters, L., Le Trong, J. L., and Richalet, J. P. (1994). Energy balance at high altitude of 6,542 m. *J. Appl. Physiol.* 77, 862–866. doi: 10.1152/jappl.1994.77.2.862
- Young, A. J., Cymerman, A., and Burse, R. L. (1985). The influence of cardiorespiratory fitness on the decrement in maximal aerobic power at high altitude. *Eur. J. Appl. Physiol.* 54, 12–15. doi: 10.1007/BF00426291
- Young, A. J., and Reeves, J. T. (1998). "Human adaptation to high terrestrial altitude," in *Textbook of Military Medicine, Part III: Disease and the Environment, Volume: Medical Aspects of Deployment to Harsh Environments*, eds R. E. Burr and K. B. Pandolf (Washington, DC: Department of the Army), 644–688.

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Using the HEI-2010 vs HEI-2015 to determine measures of diet quality – where are the differences?

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Objective: The Healthy Eating Index (HEI)-2015 is a measure of diet quality that reflects the 2015-2020 Dietary Guidelines for Americans. The current version differs from HEI-2010 by separating the empty calories (moderation) component into saturated fats and added sugars. Legumes are now also represented in four components: total vegetables, greens and beans, seafood and plant proteins, and total protein foods. Diet quality studies completed with a military population have used HEI-2010. It has yet to be determined if these past HEI-2010 diet quality studies are translatable to the new HEI-2015. The objective of this study was to compare the 2010 vs 2015 versions of the HEI to determine whether these different calculation methods result in equivalent scores.

Methods: A subset of 143 Soldiers (with complete dietary data from breakfast, lunch and dinner) were selected from 2 dining facility studies. Digital photography methodology was used to capture meal trays before and after food consumption. Foods consumed were estimated by two trained dietitians and nutrient content calculated using the FNDDS 2013-2014. Dietary data were evaluated using both HEI calculation methodologies and analyzed using SAS® version 9.4 software.

Results: Mean HEI total scores differed significantly ($p < 0.001$) between the 2015 and 2010 calculation methods (68.90 ± 11.5 vs. 69.76 ± 10.4 , respectively). Component scores differed significantly for total vegetables ($p = 0.005$), greens and beans ($p = 0.013$), seafood and plant proteins ($p < 0.001$), and total protein ($p < 0.001$). Due to the change in the moderation category, the score in HEI-2015 vs HEI-2010 was lower (32.31 ± 4.2 vs 34.10 ± 5.2 ; $p < 0.001$).

Conclusions: These results identified significant differences between HEI-2010 & 2015 versions for total scores, moderation composite scores, and legume-containing component scores. Direct translation between methodologies may not be possible and researchers should use caution when comparing results between HEI analysis versions. Although statistically different scores were obtained, clinical meaningfulness of the data should also be considered. Results may not be generalizable to the civilian population and should be confirmed with a larger sample size.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government

Baseline Diet Adequacy of Army Soldiers Supports the Need for a Planned Dining Facility Intervention

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Background: Recent studies depict sub-optimal diet quality scores of Soldiers at a Fort Bragg dining facility (DFAC). Poor diet quality may contribute to the increasing military overweight rates and associated comorbidities. It is unclear if similar diet quality scores will be attained at other military installations.

Objective: The objective of this study was to determine Soldiers' current dietary intake and diet quality at a Colorado military dining facility (DFAC).

Study Design, Settings, Participants: In 2017, Pennington Biomedical Research Center partnered with US Army researchers to assess dietary intakes of Soldiers at a designated Fort Carson, CO DFAC. A total of 89 Soldiers were recruited and asked to consume three meals in the DFAC over a 2-day period. Soldiers' dining trays were captured using digital photography methodology before and after food consumption. Foods consumed were estimated by two trained dietitians.

Outcome Measures and Analysis: Foods consumed were analyzed for nutrient content using the FNDDS 2013-2014. The Healthy Eating Index-2015 (100 points maximum score), a measure of diet quality that reflects the most recent version of the 2015-2020 Dietary Guidelines for Americans, was calculated for soldiers who had complete dietary data. Data were analyzed using SAS® version 9.4 software.

Results Summary: Forty-one soldiers provided complete dietary data for at least one day (all 3 meals). Mean HEI-2015 total score was 63.1 ± 9.0 and the lowest component score was whole grain consumption (0.25 ± 0.7 points). Total protein reached 94.5% of the maximum score; while total fruits, vegetables and dairy reached 64.7%, 80.6%, and 67.4% of the target, respectively.

Conclusions and Implications: Low diet quality scores support the need for a DFAC nutrition intervention aimed to improve food offerings and Soldiers' healthy eating scores at the Fort Carson DFAC. These results echo the need for interventions to increase diet quality in military dining facilities. Soldiers tasked with defending our nation need to be in optimal nutritional status.

Disclosure: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government

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Baseline Dietary Intakes of Army Soldiers Prior to Go For Green® 2.0 Dining Facility Dietary Intervention

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Background: Recent studies depict sub-optimal diet quality of Soldiers at a Fort Bragg dining facility (DFAC) using the Healthy Eating Index (HEI). The HEI-2015 (100 points maximum score) reflects the most recent version of the 2015-2020 Dietary Guidelines for Americans, while Military Dietary Reference Intake (MDRI) recommendations account for specific military operational needs and should also be considered. Poor diet quality may contribute to the increasing military overweight rates and associated comorbidities and may be linked to poor job performance related to less than optimal dietary intakes. This has prompted further efforts to implement interventions in DFACs focusing on healthier options for soldiers and increasing diet quality. The Go For Green® version 2.0 (G4G 2.0) initiative, which specifically incorporates MDRI requirements, is undergoing revisions and being implemented at a number of DFACs in the U.S. The G4G 2.0 implementation team is working with a designated DFAC in Fort Carson, CO to launch the revised program.

Objective: The objective of this study was to determine Soldiers' current dietary intake and diet quality at baseline at a Fort Carson, CO, DFAC.

Study Design, Settings, Participants: In 2017, Pennington Biomedical Research Center partnered with US Army researchers to assess dietary intakes of Soldiers at a designated Fort Carson, CO DFAC. A total of 89 Soldiers were recruited and asked to consume three meals in the DFAC over a 2-day period. Soldiers' dining trays were captured using digital photography methodology before and after food consumption. Foods consumed were estimated by two trained dietitians.

Outcome Measures and Analysis: Foods consumed were analyzed for nutrient content using the Food and Nutrient Database for Dietary Studies (FNDDS) 2013-2014. HEI-2015 scores are based upon 1000 calorie intake and were calculated for soldiers who had complete dietary data. HEI-2015 components were revised and now consist of: fruit, total vegetable, greens and beans, whole grains, refined grains, dairy, total protein foods, seafood & plant proteins, fatty acid ratio, sodium, added sugars, and saturated fats. A good/healthy quality diet is defined as a HEI-2015 total score of ≥81 points, while 50-80 points needs improvement and <50 points is considered a poor quality diet. Data were analyzed using SAS® version 9.4 software.

Results Summary: Thirty-eight male soldiers provided complete dietary data for at least one day (all 3 meals). Comparisons of the average nutrient intakes were made between recommendations for men

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specified in the Military Dietary Reference Intakes (MDRI). Average caloric intake was 2867 ± 617 kcal/day, falling short of the Military Dietary Reference Intake (MDRI) general value of 3250 kcal/day. Protein intakes averaged 119.8 ± 23.7 g/day or 16.6% of energy, more than adequately meeting the MDRI for males of 91 g/day (range of 65-154 g/day) and 10-15% of protein from total energy intake. Dietary fat intake averaged 37.6% of energy; although there is no MRDI for dietary fat intake beyond the recommendation to not exceed 30% of energy intake. Dietary cholesterol intake averaged 626 ± 187 mg/day, more than double the MDRI recommendation for less than 300 mg/day. Dietary fiber averaged 20.4 ± 5.2 g/day falling short of the Dietary Guidelines for Americans recommendation (14 g per 1000 kcal) as there are no dietary fiber recommendations in the MDRI. Mean HEI-2015 total score was 62.9 ± 9.0 points (range 42.7 to 81.8) and the lowest component score was whole grain consumption (0.27 ± 0.7 points). Total protein reached 94.4% of the maximum score; while total fruits, vegetables and dairy reached 63.4%, 80.6%, and 68.5% of the target, respectively.

Conclusions and Implications: These data suggest that although certain target nutrients were met or exceeded, the overall average consumption falls short of good/healthy diet quality classification. Low diet quality scores support the need for a DFAC nutrition intervention aimed to improve food offerings and Soldiers' healthy eating scores at the Fort Carson DFAC. Emphasis should be placed on specific components of the diet contributing to a low HEI diet quality score as well as meeting the MDRI and then re-evaluated to assess the effectiveness of the nutrition intervention. This is the first military study using HEI-2015 scores to determine Soldier diet quality while past studies have used previous version of the HEI. Thus researchers and healthcare providers should use caution when comparing and interpreting HEI scores from previous studies as the component scores have changed. Direct translation between methodologies may not be possible and should be further investigated.

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