PROTOCOL TITLE: The mechanistic effects of acute hypobaric hypoxia on exogenous carbohydrate utilization during steady-state aerobic exercise

SECTION A: RESEARCH TEAM AND LOCATIONS

A1. RESEARCH TEAM

Study Role	Institution/Company and Contact Information
Sponsor	*Organization/Institution/Company:* N/A
Principal Investigator	*Name, Rank, and Degree:* Lee M. Margolis, PhD, RD *Title:* ORISE Post Doctoral Fellow *Institution:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick MA 01760 *Phone Number:* 508-233-4591 *Email:* lee.m.margolis.ctr@mail.mil
Associate Investigator(s)	*Name, Rank, and Degree:* Stefan M. Pasiakos, PhD *Title:* Nutritional Physiologist *Institution/Company:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-6474 *Email:* stefan.m.pasiakos.civ@mail.mil *Name, Rank, and Degree:* Andrew J. Young, PhD *Title:* Nutritional Physiologist, ORISE Fellow *Institution/Company:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-5141 *Email:* Andrew.j.young.ctr@mail.mil *Name, Rank, and Degree:* Robert W. Kenefick, PhD *Title:* Physiologist *Institution/Company:* USARIEM / Thermal Mountain Medicine Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-6344 *Email:* robert.w.kenefick.civ@mail.mil *Name, Rank, and Degree:* Arny A. Ferrando, PhD *Title:* Professor *Institution/Company:* University of Arkansas for Medical Science (UAMS) *Address:* 4301 West Markham St, Slot 806 *Phone Number:* 501-526-5711 *Email:* AFerrando@uams.edu

Name, Rank, and Degree: Scott J. Montain, PhD
Title: Division Chief
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: 508-233-4564
Email: scott.j.montain.civ@mail.mil

Name, Rank, and Degree: Roy M. Salgado, PhD
Title: Physiologist
Institution/Company: USARIEM / Thermal Mountain Medicine Dvision
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: 508-233-5959
Email: roy.m.salgado.civ@mail.mil

Name, Rank, and Degree: Jessica A. Gwin, PhD
Title: ORISE Post Doctoral Fellow
Institution/Company: USARIEM / Military Nutrition Dvision
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: 508-233-6492
Email: jessica.a.gwin.ctr@mail.mil

Other Key Research Personnel (as applicable)

Name, Rank, and Degree: Marques Wilson, MS
Title: Project Coordinator
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: 508-233- 5681
Email: marques.a.wilson.civ@mail.mil

Other Individuals Supporting the Research (as applicable)

Name, Rank, and Degree: Nancy Murphy, MS
Title: Laboratory Manager
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4598
Email: nancy.e.murphy5.civ@mail.mil

Name, Rank, and Degree: Christopher Carrigan, BS
Title: Research Assistant
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508) 233-5101
Email: christopher.t.carrigan.civ@mail.mil

Name, Rank, and Degree: Claire Whitney, MS, RD
Title: Research Dietitian
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4875
Email: claire.c.whitney.civ@mail.mil

Name, Rank, and Degree: Adrienne Hatch, MS, RD
Title: Dietitian
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-5648
Email: adrienne.m.hatch.civ@mail.mil

Name, Rank, and Degree: Karleigh Bradbury, MS
Title: ORISE Research Fellow
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4977
Email: Karleigh.e.bradbury.ctr@mail.mil

Name, Rank, and Degree: Katherine M. Mitchell, MS
Title: Research Technician
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4177
Email: Katherine.m.mitchell15.civ@mail.mil

Name, Rank, and Degree: Beau R. Yurkevicius, BS
Title: ORISE Research Fellow
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-5950
Email: beau.r.yurkevicius.ctr@mail.mil

Name, Rank, and Degree: Pedro J. Claro, BS
Title: Chamber Crew Chief
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-5059
Email: pedro.j.claro.civ@mail.mil

Name, Rank, and Degree: SGT Ray Rivera
Title: Chamber Crew Chief
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4041
Email: raymond.riverakuilan.mil@mail.mil

Name, Rank, and Degree: SGT Brandon Cordell
Title: Altitude Chamber Operator
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-6985
Email: brandon.j.cordell.mil@mail.mil

Name, Rank, and Degree: SGT Alfonzo Patino, BS
Title: Altitude Chamber Operator
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-4715
Email: alfonzo.m.patino.mil@mail.mil

Name, Rank, and Degree: SGT Cassandra Rousayne
Title: Altitude Chamber Operator
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-5140
Email: cassandra.l.rousayne.mil@mail.mil

Name, Rank, and Degree: SPC Jason Alaniz
Title: Altitude Chamber Operator
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: N/A
Email: jason.alaniz3.mil@mail.mil

Name, Rank, and Degree: Patrick Radcliffe, BS
Title: ORISE Research Fellow
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: N/A
Email: patrick.n.radcliffe.ctr@mail.mil

Name, Rank, and Degree: Heather Fagnant, MS/MPH, RD
Title: ORISE Research Fellow
Institution/Company: USARIEM / Military Nutrition Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508) 233-5958
Email: heather.s.fagnant.ctr@mail.mil

Name, Rank, and Degree: Claudia Toussaint, MPH
Title: Chamber Crew Cheif
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508) 233-5956
Email: claudia.m.toussaint.ctr@mail.mil

Name, Rank, and Degree: LTC Frank Petrassi, PhD
Title: Chamber Crew Cheif
Institution/Company: USARIEM / Thermal Mountain Medicine Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508) 233-4861
Email: frank.petrassi.mil@mail.mil

Research Monitor

Name, Rank, and Degree: MAJ Robin Cushing, DPHS
Title: Physician Assistant
Institution/Company: USARIEM
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: 253-241-6526
Email: robin.e.cushing.mil@mail.mil

Ombudsman

Name, Rank, and Degree: Katelyn Guerriere, MS
Title: Ombudsman
Institution/Company: USARIEM / Military Performance Division
Address: 10 General Greene Ave, Bldg 42, Natick, MA 01760
Phone Number: (508)233-5619
Email: katelyn.i.guerriere.civ@mail.mil
Name, Rank, and Degree: Sarah Ross
Title: Ombudsman
Institution/Company: Human Research Volunteer Program
Address: 10 General Greene Ave, Bldg 2, Natick, MA 01760
Phone Number: (508)233-5619
Email: sarah.e.ross26.civ@mail.mil

A2. ROLES AND RESPONSIBILITIES

A2.1 Key Research Personnel

Name(s): Lee M Margolis
Research Role: Principal Investigator
Study Responsibilities: The Principal Investigator is responsible for the safe and scientifically sound conduct of the study. He will oversee all aspects of study, ensure safety and ethical treatment of volunteers; maintain required documentation for the study and obtain required approvals; and will have primary responsibility for data analysis, interpretation, and publication. Dr. Margolis will also be actively involved in the volunteer brief, obtaining consent, data collection, performing muscle biopsies, catheterization, phlebotomy, and exercise testing and interventions.

Name(s): Stefan M Pasiakos
Research Role: Associate Investigator
Study Responsibilities: Protocol concept development; formulation of protocol questions,

hypotheses, experimental approach and design. Assist PI with volunteer brief, obtain consent, perform muscle biopsies, catheterization, phlebotomy, exercise testing and interventions, data collection, management, and analysis and manuscript preparation.

Name(s): Andrew J Young, Robert W Kenefick, Scott J Montain, Roy M Salgado, and Jessica A Gwin
Research Role: Associate Investigators
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach and design. Assist PI with data collection, management, and analysis and manuscript preparation.

Name(s): Arny A Ferrando
Research Role: Associate Investigator
Study Responsibilities: Assist PI with the design, analysis, and interpretation of the stable isotope studies of glucose metabolism. Dr. Ferrando will receive and analyze coded, de-identified blood samples for isotopic analysis. He will not interact or intervene with study volunteers or have access to personal identifiable information. ***A coded specimen transfer agreement is included with this submission.***

Name(s): Marques Wilson
Research Role: Project Coordinator
Study Responsibilities: Supervise, manage, and coordinate study logistics and biological data collection. He will be involved with study implementation. He will actively participate in data collection to include catheterization, phlebotomy, exercise testing and interventions, and DEXA scans. He will assist in management, analysis and interpretation of data.

A2.2. Others Involved in the Research, as applicable

Name(s): Claire Whitney and Adrienne Hatch
Research Role: Study Dietitian
Study Responsibilities: Baseline diet assessments, study diet development, and prepare and administer test diets to volunteers. She will actively participate in data collection to include exercise testing and interventions, phlebotomy, and DEXA scans. She will assist in management, analysis and interpretation of data.

Name(s): Nancy Murphy
Research Role: Biological Sample Coordinator
Study Responsibilities: Supervision, management, and coordination of logistics and biological data collection and analysis. She will be involved with protocol development, study implementation. Data collection responsibilities will involve sample processing.

Name(s): Christopher Carrigan
Research Role: Research Assistant
Study Responsibilities: Assist with data collection and biological sample processing and analysis. Data collection responsibilities will involve with DEXA measurements, phlebotomy, and catheterization.

Name(s): Karleigh Bradbury, and Beau Yurkevicius
Research Role: Research Technician
Study Responsibilities: Assist with data collection to include catheterization, phlebotomy, exercise testing and interventions, DEXA scans and biological sample processing and analysis.

Name(s): Katherine Mitchell
Research Role: Research Technician

Study Responsibilities: Assist with data collection to include exercise testing and interventions and DEXA scans.

Name(s): Pedro Claro, SGT Ray Rivera, Claudia Toussaint, and LTC Frank Petrassi
Research Role: Altitude Chamber Crew Chief
Study Responsibilities: Will act as the Altitude Chamber Crew Chief, coordinate schedule for altitude chamber, and supervise altitude chamber operation.

Name(s): SGT Brandon Cordell, SGT Cassandra Rousayne, SGT Alfonso Patino, and SPC Jason Alaniz
Research Role: Altitude Chamber Operator
Study Responsibilities: Will act as the Altitude Chamber Operator.

Name(s): Patrick Radcliffe and Heather Fagnant
Research Role: Research Technician
Study Responsibilities: Assist with diet preparation and data collection.

Name(s): MAJ Robin Cushing
Research Role: Research Monitor
Study Responsibilities: The research monitor for this study is MAJ Robin Cushing. MAJ Robin Cushing is an appropriate subject matter expert not associated with the protocol. The research monitor shall, at a minimum, review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event. The PI and research monitor will discuss 'discontinuation criteria' for individual volunteers as the study progresses, based on their observations of the volunteer during testing or non-testing periods.

Name(s): Katelyn Guerriere and Sarah Ross
Research Role: Ombudsman
Study Responsibilities: Will act as the ombudsman for this study as necessary.

A3. RESEARCH LOCATIONS

USARIEM, Natick MA: The U.S. Army Research Institute of Environmental Medicine (USARIEM) is a DoD research facility within the U.S. Army Medical Research and Materiel Command. It is the Institute responsible for conducting basic and applied research to determine the effects of exposure to environmental extremes, occupational tasks, physical training, deployment, operational stress and nutritional factors on the health and performance of military personnel. The facility contains environmental chambers for controlling temperature and humidity, an environmentally controlled hypobaric chamber, a water immersion laboratory, as well as several dry and wet laboratories for animal and human experimentation. The dry laboratories are capable of a broad range of experiments, including biomechanical analysis, body composition, energy expenditure, muscle strength and endurance. The wet laboratories include general clinical chemistry analyzers, as well as equipment for ELISA, RIA, histology, and molecular biology assays. Each investigator at the facility has a personal computer with software for data management, analysis, presentation and report generation. Their computers are interfaced with a network server for easy, secure data handling and transfer. All testing procedures will take place at USARIEM.

University of Arkansas Medical Science, Center for Translational Research in Aging and Longevity, Little Rock, AR: The Center for Translation Research in Aging & Longevity (CTRAL), Donald W Reynolds Institute of Aging (RIOA), University of Arkansas for Medical Science (UAMS) has the basic laboratory facilities and equipment to analyze stable isotope kinetics, including 2 Agilent 5973 GC/MS, and 3 Agilent 5975 GC/MS, a Finnegan TSQ 7000 LC/MS/MS, and a Waters QTOF LC/MS. The

CTRAL is led by the world's foremost expert in stable isotope assessments of human metabolism, Dr. Robert R Wolfe.

Metabolic Solutions, Nashua, NH: Metabolic Solutions is a state-of-the art stable isotope analytical laboratory that has the basic laboratory facilities and equipment to analyze stable isotope in breath and blood. Equipment onsite includes an Agilent 6110 LC-Tandem Mass Spectrometer (LC-MS/MS) Triple Quad with Agilent 1100 LC, Thermo Finnigan Delta XP Isotope Ratio Mass Spectrometer (IRMS) with GC Isolink for ^{13}C, ^{15}N and ^{2}H GC-IRMS and Conflow IV interface, and a Thermo Finnigan Delta V Advantage IRMS with trace GC-Combustion III unit for ^{13}C, ^{15}N and D GC-C-IRMS and Conflow IV interface and elemental Analyzer EA112HT ^{13}C, ^{15}N and D sample combustion unit. Metabolic Solutions has been an industry leader in developing new stable isotope tracer applications. Metabolic Solutions will analyze samples on a fee for service basis.

Pennington Biomedical Research Center (PBRC), Baton Rouge, LA: The laboratory is accredited by the College of American Pathologists (CAP) and participates in proficiency testing programs administered by the CAP. Biochemical analyses will be conducted at PBRC on a fee for service basis.

SECTION B: RESEARCH METHODOLOGY

B1. ABSTRACT

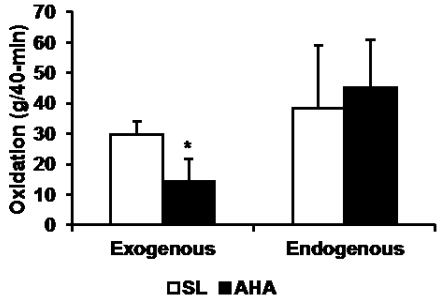
Service members deployed to high altitude (HA; hypobaric hypoxia) regions of the world experience aerobic performance decrements. Factors contributing to hypoxia-associated aerobic performance decrements, include an alteration in carbohydrate oxidation to metabolize energy to support the muscle workload. However, research has failed to consistently demonstrate an ergogenic advantage of consuming supplemental carbohydrate during strenuous work at HA. Moreover, there is evidence that oxidation of exogenous carbohydrate during exercise is blunted under acute hypobaric hypoxic conditions compared to normoxia (1, 2). The mechanisms by which hypoxia suppresses exogenous carbohydrate oxidation are not known. A better understanding how hypoxia modulates energy substrate metabolism during exercise is necessary for appropriate design and implementation of nutritional interventions aimed at maintaining physical performance at HA. The primary objective of this study is to confirm that acute HA exposure decreases exogenous carbohydrate oxidation during steady-state aerobic exercise compared to SL, and explore if the mechanism inhibiting plasma glucose uptake is insulin dependent or independent. This randomized crossover study will examine substrate metabolic responses to ingesting supplemental carbohydrate during steady-state aerobic-type exercise at sea level (SL) and following acute (~5 h) exposure to HA (4,300 m) conditions in 10 healthy, recreationally active males between the ages of 18-39 yrs. Following a 48-hr muscle glycogen normalization period, volunteers will complete 80-min of metabolically-matched, steady-state aerobic (same absolute workload corresponding to ~55 ± 5 % of VO_{2peak} at HA) exercise on a treadmill, and consume 145 g of glucose (1.8 $g \cdot min^{-1}$) at SL and HA. SL and HA trials will occur in the USARIEM hypobaric chamber and be separated by a minimum 7-d washout period. Glucose tracers will be used to assess glucose turnover, and contribution to exogenous and plasma glucose oxidation. Indirect calorimetry and urine collections will be used to determine total carbohydrate, fat, and protein oxidation during exercise at SL and HA. Serial blood draws will be collected during each trial to assess endocrine and circulating substrate responses to exercise, carbohydrate, and hypoxia. Muscle biopsies will be collected before and after steady-state exercise to examine muscle glucose transporter expression and translocation, glycogen status, and activity intramuscular enzymes in aerobic and anaerobic energy metabolism. The primary risks associated with this study include those associated with acute hypobaric hypoxia, exercise, blood draws, and muscle biopsies.

B2. BACKGROUND AND SIGNIFICANCE

Ingesting carbohydrate during aerobic exercise conducted at SL increases exogenous carbohydrate oxidation and limits endogenous carbohydrate utilization (3-6). Only two previous studies have investigated how hypoxia affects oxidation of exogenous carbohydrate ingested during exercise, and the observations reported from those studies differ (1, 2). When carbohydrate was ingested during exercise at 77% $\dot{V}O_{2max}$ in normoxia and hypoxia equivalent to 4,300 m, exogenous carbohydrate oxidation was the same (2). In contrast, when carbohydrate was ingested during lower intensity exercise (55% W_{max}, ~55-60% $\dot{V}O_{2max}$), exogenous carbohydrate oxidation was lower at high altitude (HA; 3,375 m) than normoxia (1). The reasons for the discrepant effects of hypoxia observed in these studies on exogenous carbohydrate oxidation during exercise are not clear, but might reflect differences in the exercise intensities employed.

Hypoxia reduces $\dot{V}O_{2max}$ (7, 8), so in the experiments described above (1, 2), exercise was performed at lower absolute intensity and metabolic rate in hypoxia to maintain relative exercise intensity (i.e. % $\dot{V}O_{2max}$) the same as normoxia. However, from a scientific (9, 10) and practical perspective (the metabolic rate during physical activities is the same at SL and HA, notwithstanding differences in relative intensity (i.e., % $\dot{V}O_{2max}$). We believe that differences in substrate metabolism during exercise at HA and SL should be investigated at the same absolute workload in both conditions. In one of the aforementioned studies (2), Péronnet et al. also compared exogenous carbohydrate oxidation during exercise performed at the same absolute intensity and metabolic rate (i.e., $\dot{V}O_2$ = ~ 2.2 $L \cdot min^{-1}$, 11.6 $kca \cdot min^{-1}$) in normoxia (54% $\dot{V}O_{2max}$) and hypoxia (78% $\dot{V}O_{2max}$) equivalent to 4,300 m. When subjects consumed 140 g of glucose during aerobic exercise that was metabolically matched exogenous glucose oxidation was numerically lower but not statistically different at HA compared to SL, while total and endogenous carbohydrate oxidation were significantly increased at HA compared to SL. Maximal exogenous carbohydrate oxidation rates are achieved between 51-64% of $\dot{V}O_{2max}$ (11). Therefore, in this previous study (2), 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. In a recent investigation, our laboratory sought to examine whether oxidation rates of exogenous glucose 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. Findings from this study (unpublished) indicated that exogenous glucose oxidation diminished by 46% from SL to HA when participants consumed 144 g carbohydrate while walking on a treadmill for 80-min at a fixed absolute workload ($\dot{V}O_2$ = ~1.7 $L \cdot min^{-1}$; 55% $\dot{V}O_{2max}$ at HA; **Figure 1**). While exogenous glucose oxidation was lowered in our investigations, endogenous carbohydrate oxidation was numerically but not statistically higher at HA compared to SL. These data suggested that the body's ability to oxidize exogenous glucose during exercise may be compromised during acute HA exposure.

Figure 1: Carbohydrate oxidation

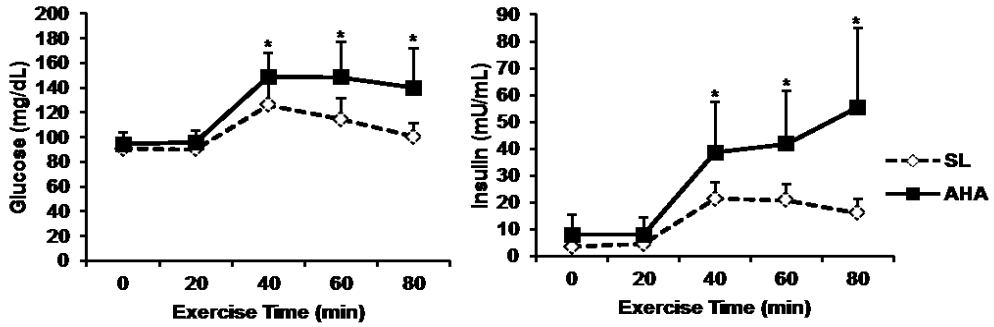


*Different than Sea Level; $P < 0.05$

Decreased insulin sensitivity is one potential mechanism for diminished ability to oxidize exogenous carbohydrate oxidation at HA (12-15). Concomitant with the suppression of exogenous carbohydrate oxidation in acute hypoxia, we also observed that blood glucose levels rose higher and remained elevated during exercise despite two to four fold higher insulin concentrations during HA exercise compared to SL (**Figure 2**). Under normal physiological conditions, elevated blood glucose levels stimulates a rise in circulating insulin to induce translocation of glucose transporter 4 (GLUT4), resulting in the uptake of glucose intake the cell. Several other investigations (12-15) have also observed this phenomenon of impaired insulin sensitivity upon acute exposure to HA in young healthy individuals. Therefore, it is possible that insulin resistance at HA may be a mechanism suppressing exogenous carbohydrate oxidation. However, little is known regarding alterations in intramuscular processes that

may regulate alterations in glucose uptake into the cell at HA compared to SL. Investigation is required on molecular pathways that regulate insulin dependent and independent translocation of GLUT4 to better understand the potential mechanism that may govern alterations in carbohydrate metabolism.

Figure 2: Glucose and insulin concentrations



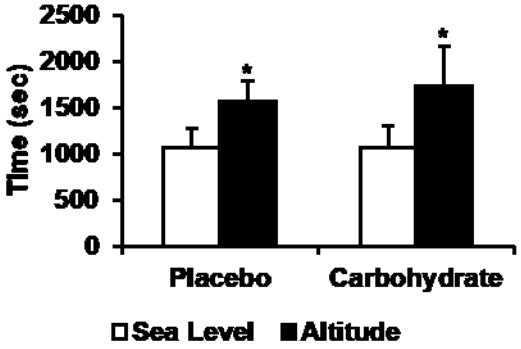
*Different than Sea Level; $P < 0.05$

B3. MILITARY RELEVANCE

Service members deployed to HA regions of the world experience aerobic performance decrements. Based on findings from our previous study, one contributing factor, at least during acute exposures to HA, is a decreased ability to oxidize exogenous glucose, with no difference in total and endogenous carbohydrate metabolism. During the initial exposure to HA there appears to be a diminished insulin sensitivity in response to carbohydrate consumption, resulting in a 46% reduction in exogenous glucose oxidation during exercise compared to SL.

Additionally, consumption of carbohydrate at HA did not minimize declines in physical performance compared to a matched non-nutritive placebo as determined by a 2-mile time trial (**Figure 3**). These data indicate that while dietary carbohydrate or carbohydrate supplementation may not support exercise performance during acute HA exposure. Though it was observed that exogenous glucose oxidations was lower during exercise at HA compared to SL, the mechanism leading to diminished exogenous glucose oxidation at HA remains unclear. The purpose of this study is to define the metabolic underpinnings for lower exogenous glucose oxidation to develop appropriate dietary recommendations and/or countermeasures to support acute HA military operations.

Figure 3: 2-mile time trial



*Different than Sea Level; $P < 0.05$

B4. OBJECTIVES/SPECIFIC AIMS/RESEARCH QUESTIONS

Objectives

Confirm results from our previous investigation that acute HA exposure decreased exogenous carbohydrate oxidation during steady-state aerobic exercise compared to SL, and explore if the mechanism inhibiting plasma glucose uptake is insulin dependent or independent.

Hypotheses

Exogenous carbohydrate oxidation will be lower at HA compared to SL during steady-state aerobic exercise at the same absolute workload, and potential regulation of diminished glucose uptake will be examined by insulin dependent or independent mechanisms.

B5. RESEARCH PLAN

B5.1 Research Design

This study will be a randomized crossover control trial.

B5.2 Research Subjects/Population(s)

B5.2.1 Subject Population(s)

Subject population will be representative of active duty male service members, being in good health and recreationally active. Only males are being studied so that results from the current study can be appropriately compared to our previous study (USARIEM protocol 16-02HC). It is apparent, that sex-based differences exist during low-to-moderate intensity endurance exercise under at SL. Several investigations (16-19) have reported that females oxidize proportionately more fat and less carbohydrate than males during aerobic exercise. Sex-based differences in substrate oxidation during exercise at SL may result in differences in response to change in substrate oxidation at HA between men and women. Based on findings from the current study, follow-up work may be conducted to assess sex differences in change in substrate oxidation during exercise at HA.

B5.2.2 Number of Subjects, Records, and/or Specimens

We estimate that 30 individuals will require screening in order to complete testing on the 10 volunteers necessary to reach statistical power. All screening will stop once complete data has been collected on 10 volunteers. Records and specimen collection are described in the Research Procedures and Data Collection sections. During briefings and the consenting process potential volunteers will be informed that even though they may be eligible and want to participate, if we are able to obtain enough data from preceding subjects, they may not ultimately be tested.

B5.2.3 Inclusion Criteria

- Men aged 18 – 39 years
- Born at altitudes less than 2,100 m (~7,000 feet; Examples include Santa Fe, New Mexico; Laramie, Wyoming; Etc.)
- Physically active based on assessment of physical activity history (2-4 days per week aerobic and/or resistance exercise)
- Have supervisor approval (permanent party military and civilians)
- Willing to refrain from alcohol, smokeless nicotine products and dietary supplement use during study periods
- Refrain from taking any nonsteroidal anti-inflammatory drugs (NSAIDs; e.g. aspirin, Advil®, Aleve®, Naprosyn®, or any aspirin-containing product) for 10 days before and at least 5 days AFTER each muscle biopsy. (*Tylenol® or acetaminophen is ok to use if needed for discomfort)

B5.2.4 Exclusion Criteria

- Born at altitudes greater than 2,100 m (~7,000 feet; Examples include Santa Fe, New Mexico; Laramie, Wyoming; Etc.)

- Living in areas that are more than 1,200 m (~4,000 feet), or have traveled to areas that are more than 1,200 m for five days or more within the last 2 months (Examples include Ft. Huachuca, Arizona; Lima, Peru; Feldberg, Germany, Etc.)
- Musculoskeletal injuries that compromise exercise capability
- Metabolic or cardiovascular abnormalities (determined by resting ECG), gastrointestinal disorders (e.g., kidney disease, diabetes, cardiovascular disease, etc.)
- Medication that affects macronutrient metabolism (i.e., diabetes medications, statins, corticosteroids, etc) and/or the ability to participate in strenuous exercise
- Evidence of apnea or other sleeping disorders
- Prior diagnosis of high altitude pulmonary edema (HAPE) or high altitude cerebral edema (HACE)
- Presence of asthma or respiratory tract infections (< 1 month prior)
- Allergies or intolerance to foods (including but not limited to lactose intolerance/milk allergy), vegetarian practices, or medications (including, but not limited to, lidocaine) to be utilized in the study
- Smoking or vaping
- History of complications with lidocaine
- Taking medications that interfere with oxygen delivery and transport (Includes sedatives, sleeping aids, tranquilizers and/or any medication that depresses ventilation, diuretics, alpha and beta blockers)
- Evidence of any physical, mental, and/or medical conditions that would make the proposed studies relatively more hazardous as determined by OMSO
- Present condition of alcoholism, anabolic steroids, or other substance abuse issues
- Anemia (HCT <38% and HBG <12.5 g/dL) and Sickle Cell Anemia/Trait
- Abnormal PT/PTT test or problems with blood clotting
- Blood donation within 8 weeks of beginning the study

B5.3 Research Procedures

Study Design

This randomized crossover study will examine substrate metabolic responses to ingesting supplemental carbohydrate during steady-state aerobic-type exercise at sea level (SL) and following acute (~5 h) exposure to HA (4,300 m) conditions in 10 healthy, recreationally active adults between the ages of 18-39 yrs (**Table 1**). Following a 48-hr muscle glycogen normalization period, volunteers will complete 80-min of metabolically-matched, steady-state aerobic exercise on a treadmill, and consume 145 g of glucose (1.8 $g \cdot min^{-1}$) at SL and HA. Treadmill exercise will be performed at the same absolute workload, with speed and grade being the same at SL and HA to induce the same absolute workload between phases. SL and HA trials will occur in the USARIEM hypobaric chamber and will be separated by a minimum 7-d washout period between each protocol day. 6-6-[$^{2}H_{2}$] glucose will be used as a tracer to assess glucose turnover. Indirect calorimetry, breath sampling for $^{13}C/^{12}C$ expired in CO_2, and urine collections will be used to determine carbohydrate, fat, and protein oxidation during exercise at SL and HA. Serial blood draws will be collected during each trial to assess endocrine and circulating substrate responses to exercise, carbohydrate, and hypoxia. Muscle biopsies will be collected before and after steady-state exercise to examine intramuscular glucose transport expression and translocation, glycogen status, and activity enzyme intermediates in aerobic and anaerobic energy metabolism.

Study Day	SV	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Medical Screening	X															
Body Composition		X														
Height		X														
Weight		X						X							X	
Fitness Assessment		X	X													
Blood Sampling								X							X	
Exercise Practice Sessions				X	X											
Muscle Biopsies								X							X	
Carbohydrate Depletion Exercise						X							X			
Steady-State Exercise								X							X	
Study Diet						X	X						X	X		
Carbohydrate Tracer Study								X							X	
Post Biopsy Follow-Up									X							X

Table 1. Study Timeline

Note that this is a potential timeline, specific study days and length of time to complete the study may be dependent on volunteer's availability, so total time commitment may be longer.

Research Procedures

Anthropometric Data

Anthropometrics, performed using standardized techniques and equipment, will be used to determine volunteer eligibility and characterize study volunteers. Height will be measured to the nearest 0.1 cm using a stadiometer at screening. Body mass will be measured after an overnight fast (≥ 8 hr), using a calibrated digital scale to the nearest 0.1 kg at screening. Body mass will be measured at baseline and the morning of protocol days.

Body composition will be determined using dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI). The DEXA technique allows for the non-invasive assessment of soft tissue composition by region with a precision of 1-3% (20). The volunteer will lay face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet. Volunteers will be asked to remain motionless for the 8-10 min scan. These data will be used to calculate total body mass, fat-free mass, and fat mass. Calibration to external standards will be performed before actual data collection. The operator remains in the room with the volunteer during the scan. Measurements of body composition will be used for participant characterization and to assess if fat mass and fat-free mass are confounding variables for substrate oxidation at SL and HA.

Determination of Peak Oxygen Uptake

Volunteers will complete two separate maximal aerobic exercise sessions to determine peak oxygen uptake ($\dot{V}O_{2peak}$) on a treadmill. Two treadmill $\dot{V}O_{2peak}$ assessments will be conducted because HA exposure of 4,300 m produces a decrease in $\dot{V}O_{2peak}$ of ~27% compared to SL (21). As such, volunteers will complete one treadmill $\dot{V}O_{2peak}$ at SL and one at HA so $\dot{V}O_{2peak}$ is known under both conditions. $\dot{V}O_{2peak}$ will be determined using an indirect, open circuit respirator system (True Max 2400, Parvomedics, Sandy, Utah, USA). Volunteers will be instructed to fast overnight (≥ 8 hr) before testing. Volunteers will be clothed in appropriate athletic attire and perform this assessment at standard ambient indoor temperature (20-22°C) and humidity conditions (30-80%). Volunteers will be given adequate time to become familiar with the testing procedures and allowed a 3-min self-paced warm-up on the treadmill. At the start of testing, the volunteer will put on a nose clip and a mouthpiece connected to a 2-way respiratory valve, which is attached to a head piece to hold it in place. The volunteers will

begin by running for 4 min at a pace predetermined as comfortable at a 0% grade. At 4-min, the grade will be increased to 4% followed by an additional 2% every 2 min thereafter until volitional exhaustion.

Heart rate will be monitored using a heart-rate monitor (Polar Electro Inc, Oulu, Finland) the last 30 seconds of each workload during all testing. The test will be stopped immediately if the subject reports angina-like symptoms, exertional syncope, shows signs of poor perfusion (i.e., light-headedness, confusion, ataxia, pallor, cyanosis, nausea, or cold and clammy skin), or if testing equipment fails.

Steady-State Treadmill Exercise Prescription

Results from $\dot{V}O_{2peak}$ assessment will be used to prescribe steady-state treadmill exercise for protocol days. At SL and HA volunteers will complete 80-min of steady-state exercise on a treadmill at 55 ± 5% of their VO_{2peak} determined at HA, performing the same absolute work under both study conditions. The speed and grade of the treadmill will be determined using the ACSM metabolic equation for walking based on desired $\dot{V}O_2$ (22). Prior to the protocol day, participants will perform a practice session of the treadmill exercise to confirm the prescribed speed and grade are appropriate to induce target $\dot{V}O_2$. The speed and grade determined during the practice session will be used at SL and HA to induce the same absolute $\dot{V}O_2$ between phases.

Glycogen Normalization Period

A glycogen depletion protocol will be completed on a cycle ergometer, with intensity based on results from VO_{2peak} assessment. After the warm-up period volunteers will complete 2-min of high-intensity cycling (work period) at 90% VO_{2peak}, followed by 2-min recovery period where volunteers will cycle at 50% VO_{2peak}. This work-to-recovery ratio will be maintained until volunteers are no longer able to complete 2-min of cycling at 90% VO_{2peak}, determined as the inability to maintain a cycling cadence of 60 rpm for 15-sec. Cycling intensity will then be lowered to 80% VO_{2peak}. When the volunteer is unable to complete 2-min of cycling at 80% VO_{2peak}, cycling intensity will be lowered to 70% VO_{2peak}. Once the volunteer can no longer complete 2-min of cycling at 70% VO_{2peak}, cycling intensity will be lowered to 60% VO_{2peak}. The glycogen depletion protocol will be terminated once the volunteer is unable to complete 2-min of cycling at 60% VO_{2peak}. For each drop in the intensity of the work period, the cycling intensity during the recovery period will be maintained at 50% VO_{2peak} for 2-min. This exercise protocol will maximally deplete muscle glycogen stores (23). Prior to the protocol day, volunteers will perform a practice session to ensure they are familiar with the glycogen depletion protocol procedures. Following familiarization volunteers will complete a glycogen depletion protocols 48-hrs before testing.

After completing the glycogen depletion protocol, volunteers will be fed a controlled diet providing at least 6.0 g carbohydrate $\cdot kg^{-1} \cdot d^{-1}$ (~55-65% of total energy consumed) to ensure adequate glycogen repletion and homogeneous glycogen levels within and across volunteers between phases (SL and HA). All food and beverages (except water) will be prepared and provided by study dietitians and consist largely of military combat ration and supplemental food items. Since ^{13}C will be measured from breath on protocol days, volunteers will be instructed to avoid foods high in natural abundance of ^{13}C, such as corn and cane sugar. Diets provided during the two day normalization phase will not contain these products.

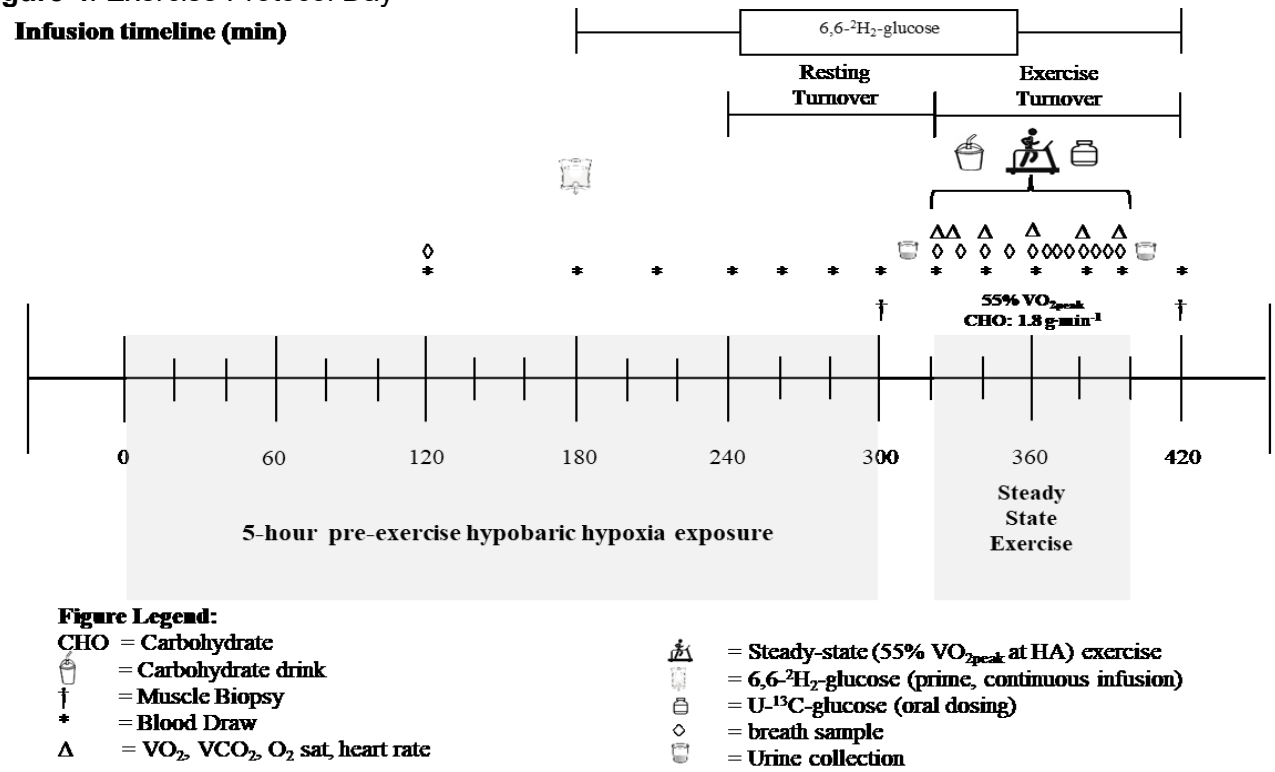
Altitude Chamber

To simulate ascension to high altitude volunteers will perform one arm of this crossover study in a hypobaric (altitude) chamber under temperate (20°C, 30-50% rh; 12-16°C°C Wet Globe Bulb Temperature (WGBT)) ambient conditions. The chamber will reduce barometric pressure, and thus ambient oxygen pressure, to a value similar to that at the summit of Pike Peak, CO (4,300 m). Volunteers will be exposed to hypobaric/hypoxic conditions for 5-hrs before beginning steady-state exercise to mimic our past investigations. Total time in the chamber on the protocol day will be ~7-8-hrs. All activities within the altitude chamber will be monitored by trained study staff. The chamber is on a regular maintenance schedule and chamber performance is documented each test day.

Exercise Protocol Day

Two catheters will be placed into the lower arm (one in each arm). One arm will be used for infusion of 6,6-[$^2H_2$] glucose tracer and the other will be used for blood sampling under resting and exercise conditions. Following an initial blood and breath sample collection to determine background enrichments, a primed, continuous infusion of 6,6-[$^2H_2$] glucose will begin (prime, 82.2 μmol·kg^{-1}; continuous rate, 0.78 μmol·kg^{-1}·min^{-1}, **Figure 4**) (24, 25). Volunteers will complete 80-min of steady-state exercise on a treadmill at 55 ± 5% of their VO_{2peak} determined at HA, performing the same absolute work under both study conditions. $\dot{V}O_2$, $\dot{V}CO_2$, HR, and SaO_2 will be measured at approximately 320, 330, 340, 360, 380, and 395 min. During exercise volunteers will consume a CHO beverage at 1.8 g·min^{-1} to examine the impact of altitude exposure on exogenous carbohydrate oxidation efficiency. Drinks will contain 145 g glucose, with a total volume of 1450 mL. The drink will be consumed in four boluses throughout the 80-min of steady-state exercise (320, 340, 360, and 380-min). The first bolus will be 550 mL, with the remaining three being 300 mL. The CHO beverage consumed during exercise will be enriched with 200 mg U-^{13}C-glucose (Cambridge Isotope Laboratory, Andover, MA, USA) to increase the isotopic enrichment well above natural levels and optimize the measurement of exogenous carbohydrate oxidation. Before exercise, during the HA phase, volunteers will be exposed to hypobaric hypoxic conditions for 5-hrs to mimic our previous investigation. Muscle samples will be obtained using percutaneous muscle biopsy, taken from the vastus lateralis using a 5-mm Bergstrom needle with manual suction while the volunteer is under local anesthesia (1% lidocaine) according to the approved USARIEM SOP (26, 27). Muscle samples will be collected before (300 min) and immediately (420 min) after 80-min of steady state exercise during both the SL and HA protocol study days. The two muscle biopsy collections will occur from one incision each protocol day. Following a minimum 7-d washout period, volunteers will return to the laboratory to complete the second arm of the investigation. A total of four muscle biopsies will be conducted per volunteer, two per protocol day, with one incision made on each leg.

Figure 4: Exercise Protocol Day



B5.4 Data Collection

Blood Sampling

Blood samples will be collected using intravenous access by a USARIEM credentialed phlebotomist on throughout protocol days under SL and HA conditions (**Figure 4**). Baseline blood sampling will occur after a 10-hr overnight fast. A total of 26 blood draws will be completed during this study taking 438 mL of blood sampled from each volunteer over the two protocol days (**Table 2**). Blood samples will be used for isotope analysis, assessment of nutrient status, hormone response, and circulating microRNA (**Table 3**).

Table 2. Total Volume of Blood Sampled for Protocol Days

Tube	Number of Tubes	Total Volume (mL)
7.5 mL Red Top Tube	8	60
4 mL Red Top Tube	8	32
4 mL Green Top Tube	16	64
1.2 mL Green Top Tube	16	20
6 mL Purple Top Tube	18	108
4 mL Purple Top Tube	28	112
2.6 mL Gray Top Tube	16	42
Total	**110**	**438**

Table 3. Blood Analytes

	Time Point (min)												
Analyte	**120**	**180**	**210**	**240**	**260**	**280**	**300**	**320**	**340**	**360**	**380**	**400**	**420**
Glucose[1]	x						x	x	x	x	x	x	x
Insulin[2]	x						x	x	x	x	x	x	x
Free fatty acids[2]	x						x	x	x	x	x	x	x
Lactate[2]	x						x	x	x	x	x	x	x
Glycerol[2]	x						x	x	x	x	x	x	x
Norepinephrine[2]	x						x	x	x	x	x	x	x
Epinephrine[2]	x						x	x	x	x	x	x	x
Hemoglobin[1]	x						x			x			x
Hematocrit[1]	x						x			x			x
^{13}C-glucose[3]								x	x	x	x	x	
6,6-[$^{2}H_2$] glucose[4]		x	x	x	x	x	x	x	x	x	x	x	x
c-miRNA[*1]	x						x			x			x
Exosomal miRNA[1]	x						x			x			x
PBMC[+]	x						x						x
Archive	x	x	x	x	x	x	x	x	x	x	x	x	x

[*] Analytes: c-miRNA, circulating microRNA, [+]Peripheral blood mononuclear cell

Samples will be analyzed at [1]USARIEM, [2]Pennington Biomedical Research Center (Baton Rouge, LA) [3]Metabolic Solutions (Nashua, NH), and [4]The Center for Translational Research in Aging and Longevity (Little Rock, AR). All blood samples will be separated into plasma and serum through centrifugation,

aliquoted, and stored at –80˚C until analysis or shipment. Study samples will be collected and aliquoted at USARIEM and specific samples will be shipped on dry ice to Pennington Biomedical Research Center, Metabolic Solutions and the Center for Translational Research in Aging and Longevity. Blood sent out to other laboratories for analysis will not have any remaining samples after analysis is completed. USARIEM will retain all archive samples.

Blood Sample Processing for Enrichment

Blood samples for 6,6-[$^2H_2$] glucose analysis will be collected at 180, 210, 240, 260, 280, 300, 320, 340, 360, 380, 400, and 420-min to calculate plasma glucose turnover at rest and in response to exercise and carbohydrate intake under SL and HA conditions. Serial blood sampling for analysis will be collected in EDTA-coated tubes for plasma collection. The tracer/tracee ratio (6,6-[2H2] glucose/glucose) will be measured on the pentaacetate derivative by gas-chromatography-mass spectrometry (GCMS; Agilent Technologies, Santa Clara, CA) (24).

Blood samples for ^{13}C-glucose analysis will be collected at 320, 340, 360, 380, and 400-min to calculate the rate of plasma glucose oxidation. $^{13}C/^{12}C$ in plasma glucose will be measured using isotope-ratio mass spectroscopy (Metabolic Solutions, Inc., Nashua, NH).

Breath Sample Processing for Isotopic Enrichment

The natural enrichment of the glucose ingested and the $^{13}C/^{12}C$ in expired gas samples will be analyzed using isotope-ratio mass spectroscopy (Metabolic Solutions, Inc., Nashua, NH). Breath samples will be collected at approximately 320, 330, 340, 350, 360, 365, 370, 375, 380, 385, 390, and 395-min to determine exogenous carbohydrate oxidation using single-patient breath collection bags (Quin-Tron Instrument Company, Milwaukee, WI, USA). A baseline breath sample will also be collected prior to correct for background of naturally occurring ^{13}C. All breath samples will be transferred to 10 mL evacuated tubes.

Plasma Glucose Turnover Calculations

For calculation of plasma glucose turnover the Steele equation with modifications for non-steady state will be used (28). Enrichment (E) will be expressed as mole percent excess (MPE); calculated as (TTR)/(1 + TTR), where TTR is the tracer to tracee ratio. Appropriate corrections for skewed abundance distribution and overlapping spectra will be made for the TTR of the glucose tracers, 6,6-[$^2H_2$] glucose and U-^{13}C-glucose (28). From these calculations, total glucose Ra will be comprised of rates of appearance of exogenous (i.e., ingested) glucose and of endogenous (i.e., hepatic glucose production and negligible renal glucose production or splanchnic glucose) glucose (28):

$$\text{Total glucose } R_a \text{ (Total } R_a) = (F - ((pV \times ((C_2 + C_1)) / 2) \times ((E_2 - E_1) / (t_2 - t_1)))) / ((E_2 + E_1) / 2)$$

$$\text{Glucose } R_d = \text{Total } R_a - (pV (C_2 - C_1) / (t_2 - t_1))$$

$$\text{Exogenous glucose } R_a \text{ (Exo } R_a) = ((\text{Total Ra} + F) \times ((G2 + GE1) / 2) + ((pV \times ((C2 + C1) / 2)) \times ((G2 - G1) / (t2 - t1)))$$

$$\text{Endogenous glucose } R_a = \text{Total } R_a - \text{Exo } R_a$$

$$\text{Metabolic Clearance Rate (MCR)} = \text{Glucose } R_d / ((C_2 + C_1) / 2)$$

Where F represents the infusion rate of 6,6-[$^2H_2$] glucose; pV is the effective volume of distribution for glucose, C_1 and C_2 are plasma glucose concentrations at t_1 and t_2, respectively, E_1 and E_2 are plasma enrichments of 6,6-[$^2H_2$] glucose at t_1 and t_2, respectively, and E_d and E_p are tracer enrichments of U-^{13}C-glucose from the test drink and plasma, respectively.

Assessment of Urinary Nitrogen Excretion

Volunteers will void urine before starting steady state exercise, with a subsequent urine sample immediately after (400-min) the conclusion steady-state exercise to measure nitrogen excretion during exercise. Urine volume will be measured and aliquots stored at -20ºC until analyzed. Total nitrogen content of the urine will be determined using pyrochemiluminescence (Antek 9000, Houston, TX).

Calculations of Carbohydrate, Fat, and Protein Oxidation

Carbohydrate, fat, and protein oxidation rates will be calculated from V̇O2 (L/min), V̇CO2 (L/min), and urinary nitrogen excretion rate (g/min) during the 80-min exercise bout as described by Jeukendrup and Wallis (29):

Fat oxidation (g/min) = 1.695 x $\dot{V}O_2$ – 1.701 x $\dot{V}CO_2$ – 1.77 x urinary nitrogen
Carbohydrate oxidation (g glucose/min) = 4.210 x $\dot{V}CO_2$ – 2.962 x $\dot{V}O_2$ – 2.37 x urinary nitrogen
Protein oxidation (g/min) = 6.25 x urine nitrogen excreted (g)/ urine collection time (min)

Calculations of Exogenous and Endogenous Glucose Oxidation

Exogenous and plasma glucose oxidation will be calculated as (1):

Exogenous glucose (g/min) = $\dot{V}CO_2$ [(Rexp - Rref)/(Rexo - Rref)]/k
Plasma glucose (g/min) = $\dot{V}CO_2$ [(Rexp - Rref)/(Rglu - Rref)]/k

where $\dot{V}CO_2$ is in L/min, Rexp is the observed isotopic composition of expired CO_2, Rref is the isotopic composition of expired CO_2 at rest before ingestion of the first dose of ^{13}C-glucose, Rexo is the isotopic composition of the exogenous glucose ingested, Rglu is the isotopic composition of plasma glucose, and k (0.747 L/g) is the volume of CO_2 provided by the complete oxidation of glucose. Total endogenous glucose oxidation can be calculated by subtracting exogenous glucose oxidation from total CHO oxidation. Endogenous glucose oxidation derived from muscle and liver can be determined by subtracting plasma glucose oxidation from total carbohydrate oxidation (muscle), and subtracting exogenous carbohydrate oxidation (liver) from plasma glucose oxidation (1). The first 40 min of steady-state exercise will allow for equilibration between the $^{13}C/^{12}C$ in expired CO_2 and the $^{13}C/^{12}C$ in CO_2 produced in tissues (30). Thus, endogenous glucose oxidation will only be calculated from samples obtained in the last 40 min of steady-state exercise (40 to 80 min).

Muscle Glycogen

Approximately 20 mg of muscle will be dehydrated in a freeze dryer. Samples will then be ground to powder and visible connective tissue will be removed. Powdered muscle will be placed in 500 µl 2 N HCl. Samples will then be placed in an incubator for 120 min at 100˚C. Following incubation samples will be neutralized with 1500 µl 0.67 N NaOH and glycogen will be quantified by a fluorometric assay (Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence staining, microscopy, and image analysis

To determine GLUT4 translocation to the cellular membrane in response to exercise and carbohydrate intake immediately after sample collection by muscle biopsy, excess blood and any visible collagen and/or fat will be removed and muscle will be embedded in Tissue Tek Optimum Cutting Temperature (OCT) compound (VWR International, Leicester, UK) and frozen in liquid nitrogen. Frozen muscle biopsy samples will be cryosectioned to a thickness of 5 µm onto uncoated glass microscope slides (VWR International). Sections will be fixed in 75% acetone and 25% ethanol solution for 5 min, then washed 3 times for 5 min in phosphate-buffered saline (PBS). A previously validated (31, 32) GLUT4 and dystrophin primary antibody (Abcam, Cambridge, UK) will be applied to muscle sections at a 1:200 and 1:400, respectively, dilution in 5% normal goat serum (Invitrogen, Carlsbad, CA, USA) for 2-hr at room temperature. Dystrophin staining will allow for visualization of the plasma membrane to determine GLUT4 location. Following primary antibody incubation, sections will be washed 3 times for 5 min in PBS and then incubated in 1:200 dilutions of secondary antibodies AlexaFluor 488-conjugated goat anti-rabbit IgG (Invitrogen; GLUT4) and AlexaFluor 594-conjugated goat anti-mouse IgG2b (Invitrogen; Dystrophin) for 30 min at room temperature. Sections will then be washed 3 times for 5 min in PBS and glass coverslips will be mounted with 20 µL mowiol mounting medium [6 g glycerol (Sigma-Aldrich), 2.4 g mowiol 4–88 (Sigma-Aldrich) and 0.026 g 1,4-Diazabicyclo[2.2.2]octane (DABCO) (Sigma-Aldrich) dissolved in 18 mL 0.2 M Tris-buffer (pH 8.5) (Sigma-Aldrich)]. Images will be captured using a Nikon TI-U inverted microscope equipped with a Retiga 2000R camera and NIS-Elements

image analysis software (Nikon, Tokyo, Japan) to determine colocalization of GLUT4 and dystrophin. Quantitation of GLUT4 in the plasma membrane (dystrophin-stained region) will be performed using Imaging software.

mRNA and microRNA Expression

To understand potential molecular regulation of changes in substrate metabolism during exercise at SL and HA this study will examine alterations in expression profiles of mRNA and microRNA that are associated with substrate metabolism. Total RNA will be isolated from approximately 25 mg of muscle using a mirVana™ miRNA isolation kit (Invitrogen, Carlsbad, CA, USA). Quantity and quality of RNA will be assessed using a Nanodrop ND-1000spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA will be synthesized into cDNA for analysis of mRNA (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA, USA) and a TaqMan® microRNA RT kit (Applied Biosystems). Individual primers will be used to determine the mRNA expression of known intracellular targets regulating substrate metabolism, through glycolysis, TCA cycle, fatty acid oxidation, and glucose uptake. Individual primers will be used to determine microRNA expression of microRNA that have been reported to impact metabolism within the muscle.

Circulating miRNA will be extracted from serum using previously published methodologies (33). Due to the small amount of RNA in the serum, 3.5 µL of a Spike-In Control (C. elegans miR-39; Qiagen) A pre-amplification step will be performed after reverse transcription to increase cDNA template using a primer pool of 20 X Taqman® Small RNA Assays (Applied Biosystems) for miRNA of interest at 0.05X concentration in 1X TE buffer. All serum miRNA will be normalized to the geometric of external (Spike-In Control C. elegans miR-39) and internal controls to allow for both technical and inter-individual normalization (34). Geometric mean of controls will be used to correct for possible outlying values and abundance differences between the different controls (35).

All reverse transcription for mRNA and miRNA, and pre-amplification of serum miRNA will be conducted in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). A StepOnePlus™ real-time PCR system (Applied Biosystems) will be used to perform all mRNA and miRNA analysis. Fold changes will be calculated using the $\Delta\Delta$ cycle threshold ($\Delta\Delta C_T$) method as described below in statistical analysis section.

Western Blotting

To understand potential molecular regulation of changes in substrate metabolism during exercise at SL and HA this study will examine alterations in phosphorylation and total protein content of signaling proteins associated with substrate metabolism. Following previously published methods (36), ~30 mg of muscle will be homogenized in ice-cold buffer to extract proteins for Western blotting. Protein concentration of supernatant (lysate) will be determined using 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Phosphorylation status and total protein expression of molecular markers associated with regulating substrate metabolism, through glycolysis, TCA cycle, fatty acid oxidation, and glucose uptake. Muscle lysates will be solubilized in Laemmli buffer, with equal amounts of total protein (15 µg) separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad). Proteins will be transferred to polyvinylidene fluoride (PVDF) membranes and exposed to commercially available primary antibodies at 4°C overnight. Labeling will be performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent will be applied (Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots will be quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). To confirm equal protein loading per well a normalizing protein such as GAPDH or HSP90 will be assessed.

Enzyme Activity

Colorimetric enzyme activity assays will be conducted to assess the impact of altitude exposure on substrate energy metabolism, to include but not limited to pyruvate dehydrogenase (PDH) and citrate synthase.

Pyruvate dehydrogenase (PDH) activity will be measured from approximately 10 mg of muscle homogenized in 100 µl of ice-cold PDH Assay Buffer (Sigma-Aldrich). Samples will be loaded into a 96 well clear plate with PDH developer (Sigma-Aldrich) and incubated at 37°C for 3 min. Initial PDH activity will be read at 450 nm. Samples will continue to be incubated at 37°C and read every 5 min until the highest standard value (12.5 nmol) is met. A final absorbance measurement will be taken at 450 nm. Change in absorbance will be calculated from initial and final read.

Homogenate from muscle samples prepared for Western blotting will be used to assess citrate synthase activity, by combining 10 µl of diluted (1:10; 0.1 M Tris HCl pH 8.1) sample to 150 µl of reaction master mix (1 mL DNTB, 3 mg Acetyl CoA, and 8 mL 0.1 M Tris HCl pH 8.1). The reaction will be initiated when 10 µl of 10 mM oxaloacetate will be added to each well (37). Samples will be read at 412 nm. Data will be normalized to protein content of muscle homogenate.

Enzyme activity will be determined using a colorimetric assay analyzed on an SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Any use of the data or samples outside of this defined research plan will be submitted as a protocol amendment or a new protocol.

B5.5 Managing Data and/or Human Biological Specimens for this Research

All data and medical information obtained will be considered privileged and held in confidence. Study volunteers will be assigned unique subject identification (ID) numbers that will not contain any personal identifiers such as name, social security number, address, date of birth, zip code, etc. This study subject ID number will be used on all data collection instruments, to include questionnaires, data collection forms, computer records, etc. A number will be assigned as each volunteer is medically cleared for participation. A master list linking the volunteers' names and ID numbers will be kept in a separate locked file in the principal investigator's office, or kept in a computer file with password-protected access restricted to the principal investigator. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. Study samples will be processed on site at USARIEM. All samples will be stored using the subject identification number. De-identified blood and urine will be shipped on dry ice to PBRC for analysis. De-identified samples for isotopic analysis will be shipped on dry ice to UAMS (blood) and Metabolic Solutions (blood and breath). All samples will be shipped via FedEx and stores in these laboratories until analyzed. Once analyzed, there will be no remaining sample for storage.

Only personnel assigned to the research study by the principal investigator will have access to the data. Hard copy data records will be stored for a minimum of three years from the time the study is completed. Electronic data records will be maintained for a period of at least five years after the study has been completed.

B5.6 Managing Data and/or Human Biological Specimens for <u>Future</u> Research

The master list linking subjects with their data will be destroyed when the study is closed. De-identified study samples will be stored in -80ºC freezer at USARIEM in room 322 or 304 for potential future use. All samples will be de-identified and maintained indefinitely. Only personnel assigned to the research study by the principal investigator will have access to samples. The de-identified data and samples will remain under the control of the PI and may be shared with outside collaborators for future research. Any use of the samples outside of this defined protocol will be submitted as a protocol amendment or a

new protocol.

B5.7 Devices, Drugs, Dietary Supplements, Nutritional Supplements, And Biologics

B5.7.1 Devices

5.7.1.1 FDA-approved device being used in this research according to the approved labeling. DEXA, DPX-IQ, Lunar Corporation, True Max 2400, Parvomedics, Sandy, Utah, USA, Polar Electro Inc, Oulu, Finland

5.7.1.2 FDA-approved device being used in this research in a manner other than its approved labeling
N/A

B5.7.2 Drugs
N/A

B5.7.2.1 FDA-approved and used in accordance with the approved labeling
N/A

B5.7.2.2 FDA-approved and used in a manner not in accordance with its approved labeling
N/A

B5.7.2.3 Any drug not approved by the FDA
N/A

B5.8 Statistical Analysis

B5.8.1 Sample Size Estimation

Statistical power and sample size were determined from the effect size of exogenous carbohydrate oxidation (-14 ± 8 g/40-min exercise) that we previously observed at HA compared to SL. Using this information the sample size necessary to determine statistical significance between intervention arms is 7, with an alpha of 0.05 and 80% power. To account for greater variance in the response to altitude between volunteers and increased study power to 90%, 10 volunteers will be enrolled to complete this investigation. Based on our previous studies we request to consent 30 individuals to complete 10.

B5.8.2 Data analysis

Statistical analyses will be conducted using either SPSS (IBM Corp. Armonk, NY), SAS 9.3 (SAS Institute Inc., Carey, NC), or equivalent. Common descriptive statistics will be used to describe volunteer characteristics. Shapiro-Wilk tests will be used to determine normality of data. Paired t-test will be performed to determine main effects of phase (SL versus HA) for glucose turnover and glucose oxidation. Mixed model repeated measures ANOVA will be performed to determine main effects of time (baseline versus post-exercise), phase (SL versus HA) and time-by-phase interactions for glucose and insulin concentrations, GLUT4 translocation, glycogen, mRNA and microRNA, Western blots and enzyme activity. If interactions are significant, appropriate post-hoc correction will be used to examine these relationships. Correlation coefficients and multiple regression analysis will be used to evaluate relationships between study outcome measures. The alpha level will be adjusted for multiple comparisons, with the level for statistical significance set at $P < 0.05$.

SECTION C: HUMAN RESEARCH PROTECTIONS

C1. RECRUITMENT AND CONSENT

C1.1 Identification and Selection of Subjects

Potential Volunteers will be recruited from the U.S. Army Natick Soldier Systems Center (NSSC) pool of Human Research Volunteers (HRVs). Additional volunteers will be recruited from civilian population and active duty military stationed at NSSC, as well as civilians from the local surrounding area using flyers and electronic posting, such as newsletters and social media. In addition, other military organizations may be recruited through coordination with NSSC Soldier and Squad Optimization & Integration Team (SSOI-T). The SSOI-T will schedule the consent briefing for the military research volunteers outside the NSSC and Ms. Katelyn Guerriere will serve as the ombudsman for non-HRV Soldiers.

C1.2 Recruitment Process

Superiors of Service members (e.g., unit officers, senior NCOs, and equivalent civilians) / supervisors of DoD civilians (e.g., military and civilian supervisors or anyone in the supervisory structure) will not be present at any recruitment sessions or during the consent process in which members of units under their command / personnel under their supervision are afforded the opportunity to participate as human subjects of research.

For Soldiers in the NSSC pool of military volunteers, the Principal Investigator will furnish a copy of the consent form to the Human Research Volunteer Program Coordinator or designee. The Coordinator will schedule the consent briefing for the military human research volunteer platoon and will serve as ombudsman during the briefing. Civilians and permanent party military will be recruited for the study using flyers posted or electronically distributed or through informational briefs. Principal Investigator or Project Coordinator will receive and respond to inquiries submitted from these recruitment materials, and will schedule informed consent briefings for these potential volunteers. Katelyn Guerriere will be the ombudsman for group briefings of non-HRV Soldiers, the HRV coordinator will be the ombudsman for HRV briefings and all civilian briefings will be done one-on-one.

C1.3 Eligibility

Volunteers will be medically cleared by the Office of Medical Support and Oversight (OMSO) before participation in accordance with USARIEM procedures outlined for screening volunteers for research involving exercise, muscle biopsies, and altitude exposure either at USARIEM or at the potential volunteer's duty station. Potential military and civilian volunteers will undergo the same clearances used at USARIEM for research involving maximal aerobic exercise testing and high altitude exposure. In addition, volunteers will be screened for problems with blood clotting, including prothrombin time (PT)/ partial thromboplastin time (PTT), which is a specific criterion for research involving muscle biopsies. Health problems identified during the screening process will be documented and a copy provided to the volunteer. The volunteer will be encouraged to make an appointment with their primary care provider for a full evaluation of the problem. Volunteers with evidence of any physical, mental, and/or medical conditions that would make the proposed studies relatively more hazardous will be excluded. Any personal health information collected during this screening process will be destroyed at the time of study withdrawal or at the completion of the study.

All volunteers must be willing to consume only food and beverages provided by study staff during the

48-hr normalization period and on protocol days. Additionally, volunteers must be willing to refrain from any additional exercise during this period.

C1.4 Consent Process

Informed consent documents will be provided to each prospective volunteer in writing, as well as in an oral presentation by the principal investigator or his designee. The purpose of this study, procedures involved, risks, and expectations of volunteers will be explained. The principal investigator or designee will answer all group and private questions. Interested volunteers will sign the informed consent form prior to undergoing initial screening for which they will have blood drawn for study-specific clearance. If they meet all the medical selection criteria after completing the screening health assessment they will be scheduled to begin data collection. A copy of the informed consent will be provided to the volunteer with the original kept for study documentation. No study procedures will occur prior to the volunteer giving informed consent. Volunteers who have already consented will be informed of any new information or changes to the protocol that may affect their willingness and ability to continue participation in the study using an approved consent addendum.

C1.4.1 Research involving subjects with cognitive impairment or who lack capacity to provide informed consent: N/A

C1.4.2 Research involving non-English speaking subjects: N/A

C1.4.3 Research involving a waiver of the requirement to obtain informed consent OR alteration of the elements of informed consent: N/A

C1.4.4 Research involving a waiver of the requirement for investigator to obtain a signed consent form: N/A

C1.4.5 Waivers of assent or parental permission when the research involves children: N/A

C1.4.6 Research involving data collection for the USAMRMC Volunteer Registry Database

It is the policy of USAMRMC that data sheets are to be completed on all volunteers participating in greater than minimal risk research for entry into the U.S. Army Medical Research and Materiel Command Volunteer Registry Database. The information to be entered into this confidential database includes name, address, social security number, study name, and dates. The intent of the database is twofold: first, to readily answer questions concerning an individual's participation in research sponsored by the USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at the USAMRMC for a minimum of 75 years.

C2. COMPENSATION FOR PARTICIPATION

Military and civilian personnel will receive $25 for each successful blood draw. There are 26 blood draws during the two protocol days (13 per protocol day). Volunteers completing all 26 draws will receive $650. If a volunteer does not complete the entire study, they will be compensated for the number of successful blood draws they did complete. Volunteers will not be eligible for any other form of compensation during this study.

Military personnel will have paperwork submitted to petition for hazardous duty pay for performing any testing under hypobaric hypoxic conditions.

C3. WITHDRAWAL FROM RESEARCH PARTICIPATION

Volunteers will be allowed to withdraw at any time without penalty or loss of benefits to which they would otherwise be entitled. If a participant wishes to withdraw mid-study while the chamber is mimicking hypoxic conditions they will enter a separate portion of the chamber that will allow them to descend back to SL conditions. Once descent is completed the participant will be allowed to leave the chamber. An investigator may stop an individual's participation in the study if the participant is unwilling or unable to complete study procedures. An investigator or the medical monitor may also withdraw a participant if the individual becomes ill or injured or it would not be in the participant's best interest to continue.

C4. PRIVACY FOR SUBJECTS

To protect the participant's privacy, any of their research-related records will be labeled or "coded" with an assigned research participant number that will not include their name or social security number. Dr. Lee M. Margolis will keep the link between participant number and the participant's research records in a locked cabinet. Any documents that will require the participant's name, such as the consent form, will be kept in a locked cabinet separate from any research documents that contain the participant's ID number. The principal investigator is the only person who will be able to match the research participant number with any of their personal identifying information.

When the results of the research are published or discussed in conferences, no information will be included that would reveal the volunteers identity to others. If photographs, videos, or audio-tape recordings of you will be used for educational purposes, your identity will be protected or disguised. All identifiable or recognizable information (e.g., names and faces) will be covered in any photographs unless volunteers agree to sign a photo release form.

C5. CONFIDENTIALITY PROCEDURES FOR RESEARCH RECORDS, DATA, HUMAN BIOLOGICAL SPECIMENS

All data and medical information obtained will be considered privileged and held in confidence. Study volunteers will be assigned unique subject identification (ID) numbers that will not contain any personal identifiers such as name, social security number, address, date of birth, zip code, etc. This study subject ID number will be used on all data collection instruments, to include questionnaires, data collection forms, computer records, etc. A number will be assigned as each volunteer is medically cleared for participation. A master list linking the volunteers' names and ID numbers will be kept in a separate locked file in the principal investigator's office, or kept in a computer file with password-protected access restricted to the principal investigator and project manager. The master list linking subjects with their data will be destroyed when the study is closed. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. Study samples will be processed on site at USARIEM. All samples will be stored using the subject identification number. Samples will be shipped for analysis to Pennington Biomedical Research Center, Metabolic Solutions, and The Center for Translational Research in Aging and Longevity. Remaining samples will be stored at USARIEM in a -80ºC freezer in room 322 or 304. The volunteers name or other identifiable information will not be included on any data, data collection sheets, specimens, or other research records. De-identified samples will be maintained indefinitely.

Only personnel assigned to the research study by the principal investigator will have access to the data. Hard copy data records will be stored for a minimum of three years from the time the study is completed. Electronic data records will be maintained for a period of at least five years after the study has been completed.

C6. RISKS OF HARM, MEASURES TO REDUCE THE RISKS OF HARM, AND BENEFITS OF PARTICIPATION

C6.1 Risks of Harm

Research Procedure Name: DEXA Scan
Research Procedure Description: Volunteer will lay face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet. Volunteers will be asked to remain motionless for the 8-10 min scan.
Research-related Risks: The DEXA scan is an X-ray and is considered to be a low risk procedure. The radiation dose of the whole-body DEXA scan is 0.1 mrem. This dose is equivalent to approximately 1/250 of normal annual background radiation, 1/9 of the radiation received in a transatlantic flight, or 1/30 of the radiation received in a chest X-ray.
Measures to Minimize Risks of Harm: (Precautions, safeguards): A quality assurance check will be completed on the DEXA each day prior to its use; the software will not allow the use of the DEXA densitometer if the quality assurance check fails.

Research Procedure Name: Indwelling Catheters
Research Procedure Description: A needle will be used to guide a catheter into the antecubital vein of the volunteer. The catheter will be attached to saline to keep the line patent for multiple blood draws.
Research-related Risks: The risks of blood sampling are small and usually limited to local bruising or swelling. Also sometimes volunteers feel faint or may faint. If the volunteer has had problems with fainting during blood draws in the past, they may be more prone to them during future procedures. If the catheter, the tube that is left in the arm after the needle is removed, becomes clogged at any time during the protocol, we will have to replace this to continue blood sampling and therefore the study. This will require another needle to be inserted into your arm. In addition, the catheter can cause irritation, bruising, swelling, infection, or an allergic reaction.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Trained technicians will use sterile techniques to place the catheter; however, in spite of being careful there is a chance that the site may become infected. Volunteers should not donate blood for eight weeks before or after this study.

Research Procedure Name: Venipuncture
Research Procedure Description: A needle will be used for single blood draws of the antecubital vein.
Research-related Risks: Venipuncture is a routine clinical procedure the medical community commonly uses to obtain blood samples. The immediate complications may be slight pain during the entry of the needle into the skin, possible dizziness, and syncope. Dizziness or syncope constitutes no long-term harm, and immediate relief is achieved by having the subject put their head down between their knees or lie down. Additionally, a hematoma may result from the venipuncture, but this is more unsightly than risk producing. Late complications might include thrombosis of the vein due to trauma or infection. These complications are extremely rare.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Participant monitoring, aseptic technique, including sterile disposable blood collection apparatus and adherence to standard medical precautions reduce risk. Trained technicians will perform all venipuncture.

Research Procedure Name: Lidocaine Injection
Research Procedure Description: Roughly 8-10 mL of Lidocaine will be injected using a needle at the site of the incision, superficially (i.e., skin) and within the vastus lateralis.
Research-related Risks: Slight pain at the site of injection might occur. Although rare, anaphylactic reactions may also occur following administration of lidocaine. Unlikely, but possible side effects could include: dizziness, confusion, shakiness, visual changes, nausea, and unusually slow heartbeat.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Volunteers will be instructed to Notify study coordinator or PI immediately if an allergic (i.e., swelling, itching, rash, hives, difficulty swallowing, or difficulty breathing) reaction occurs. In the case of severe reaction, lidocaine use will be discontinued.

Research Procedure Name: Muscle Biopsy
Research Procedure Description: A small incision will be made in the skin and fascia of the vastus lateralis. A 5-mm Bergstrom biopsy needle will pass through these incisions with manual suction while the volunteer is under local anesthesia (1% lidocaine) to collect muscle samples.
Research-related Risks: Percutaneous needle muscle biopsies have been established as a non-routine, but safe research procedure. Similar to blood draws, there is a risk that volunteers will feel faint or may faint. If the participant has had problems with fainting during blood draws or muscle biopsies in the past, they may be more prone to them during future procedures. There is some risk of post-biopsy infection, which can be minimized by employing correct sterile procedures and carefully instructing volunteers on care of the wound. Moderate stiffness, hematoma and swelling around the biopsy site may occur following the procedure, but this usually resolves itself within several days. Some minimal scarring will accompany healing of the incision and formation of a hypertrophic scar or keloid is possible. Although this is a rare event in fair-skinned persons, the incidence of hypertrophic scarring or keloid formation associated with healing of a primarily closed skin biopsy site (i.e., one which was closed with sutures immediately afterward) is 5-10% in dark-skinned persons. Complications of bleeding can be reduced by applying direct pressure to the wound following the biopsy. If symptoms should occur, they usually do not interfere with normal walking or heavier exercise. Volunteers with evidence of bleeding diatheses or with local skin infection or irritation, or recent anticoagulant medication (including aspirin), will not be used.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Volunteers will be instructed about precautions against hematoma and infection. They will be given a handout outlining instructions for proper care of the incision site (**Appendix A**). Dr. Margolis or Dr. Pasiakos will perform the procedure with sterile technique according the USARIEM Muscle Biopsy SOP and record notes for each biopsy (**Appendix B**). The medical staff at USARIEM will follow-up with volunteers within 3 days post-biopsy to monitor for any sign of infection, bleeding, or hematoma. To minimize the likelihood of hypertrophic scarring or keloid formation, biopsy wounds will be closed as promptly as feasible.

Research Procedure Name: Altitude Exposure
Research Procedure Description: Participants will exercise at 4300 m simulated altitude.
Research-related Risks: Hypoxemia, lightheadedness, AMS, HAPE, HACE, ear pain/discomfort, and peripheral/facial edema.
Measures to Minimize Risks of Harm: (Precautions, safeguards):

- During hypobaric exposures, volunteers will always be accompanied by one or more trained staff that will monitor signs and symptoms of research-related risks. To additionally minimize risk, volunteers will be familiarized with safety procedures and equipment in the hypobaric chamber.
- *Hypoxemia*- Exposure to hypobaric hypoxia introduces the hazard of hypoxemia. Depending upon the degree and duration of hypobaric exposure, hypoxemia may be well tolerated without consequences, or may present a full spectrum of clinical entities ranging from mild discomfort to potentially lethal conditions. Participants will be monitored by study staff and participants will be allowed to terminate testing early if they desire.
- *Lightheadedness*- Exercising at altitude can cause an increased risk of lightheadedness which may occur just after stopping heavy exercise. Lightheadedness increases the risk that the volunteer could fall. This risk (rare) will be minimized by having at least one staff member in close proximity to the volunteer (as is done for all exercise tests regardless of location) so that he/she will not fall off. If a volunteer becomes lightheaded and feels faint, he/she will be assisted off the treadmill and instructed to lay down with feet up until fully recovered. Volunteers will be assisted on and off exercise and other experimental apparatus as needed.
- *AMS*- AMS is a self-limited syndrome that is common when unacclimatized lowlanders are exposed to higher altitudes. Symptoms of AMS include headache, nausea, anorexia, lethargy, dizziness, tiredness, weakness, insomnia, and sometimes vomiting. The prevalence and severity of AMS increase directly in proportion to ascent rate and elevation. With rapid ascent, and no beneficial treatment (e.g., breathing supplemental O_2) symptoms commonly appear

within four to six hours. Symptoms of AMS will reach their severity within 18 to 24 hrs of altitude exposure, and be the most severe after awakening. With rapid ascent to the altitudes of 3000 m and 4000 m for a 24-hr period, more than 30-90% of the untreated volunteers will likely experience AMS, with symptoms ranging from mild (10 to 40%), to moderate (20 to 40%) to severe (10 to 20%). Due to the duration (~7-8 hrs) of the altitude exposure in the current study, AMS may occur but should not reach its most severe symptoms.

- *HAPE/ HACE*- The incidence of clinical HAPE in unacclimatized sea-level residents being exposed to high altitude for at least a few days appears to be less than 1%. As a clinical condition, the possibility of HACE occurring when sea-level residents are exposed to high altitude appears to be even lower than the incidence of HAPE. If either or both are recognized early and treated by descent, they are completely reversible. With regard to the proposed study, the risk of any volunteer developing either HAPE or HACE is considered remote and is not expected. If a volunteer shows developing symptoms of HAPE or HACE they will be removed from the simulated high altitude environment.
- *Ear Pain/Discomfort*- Changes in ambient gas pressure during induction of or return from hypobaric conditions can cause untoward effects due to gas trapped in the body. At the relatively slow rates of pressure change during normal operation (simulated ascent and/or descent) of the hypobaric chamber, some individuals may experience discomfort in their ears, paranasal sinuses, teeth, or abdomen. In healthy individuals, pressure in the middle ear and sinuses can be equalized with ambient atmospheric pressure by swallowing, yawning, or tensing the muscles of the throat, procedures that contract the pharyngeal muscles and open the Eustachian tubes, thereby ventilating the middle ear and sinus. Another more effective means of ventilating the middle ear and sinuses is forced expiration against a closed nose and mouth. Adjunctive therapy may also include the use of short-acting antihistamines or decongestants to reduce swollen Eustachian tubes and sinus openings due to inflammation or infection. During recompression (descent), moderately severe ear pain may be experienced by volunteers who cannot maintain Eustachian tube patency. If ear or sinus pain occurs, recompression will be stopped and the affected volunteer will be decompressed until the pain resolves; recompression will then be restarted at a slower rate. Damage to eardrums is likely only under the unusual situation where deliberately rapid recompression is necessitated by dire emergency.
- *Peripheral/Facial Edema*- Peripheral and facial edema occurs in some individuals when initially exposed to high altitude. It is characterized by pronounced edema of the face and upper extremities, decreased urine output and weight gain. Although uncomfortable, it is a benign condition. The peripheral and facial edema resolves with descent.

Research Procedure Name: Exercise
Research Procedure Description: Exercise testing will occur on a cycle ergometer or treadmill. Exercise will be at various levels of intensity based on exercise protocol.
Research-related Risks: Exercise is generally considered safe and beneficial for individuals without cardiovascular disease. The U.S. prevalence of fatal events is approximately 1:100,000 to 1:300,000 in competitive high school athletes and increases to 1:15,000 to 1:50,000 in athletes over the age of 35. Current civilian and military guidelines state that individuals less than 40 years of age who have no symptoms of or known presence of heart disease or major coronary risk factors have a low risk for cardiac complications during vigorous exercise. All volunteers in this study fall into this low risk category. Local muscle discomfort and fatigue may occur in active muscles during and shortly after exercise. Muscle soreness, ranging in intensity from mild to severe, may persist for 1 to 7 days.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Studies have confirmed the safety of maximal exercise testing, particularly among apparently healthy persons without significant cardiovascular risk factors. As a precaution, there will be at least one spotter during all exercise sessions, and heart rate will be monitored in real time during testing. In addition, exercise monitors and test administrators will be CPR-certified.

Research Procedure Name: 6,6-[$^2H_2$] glucose infusions
Research Procedure Description: 6,6-[$^2H_2$] glucose will be infused using a indwelling catheter and ^{13}C-glucose will be consumed in a carbohydrate beverage.
Research-related Risks: The primary risks associated with tracer studies are those related to venous catheterization. The catheter can cause irritation, bruising, or infection. There are no known risks or reported side effects associated with administration of 6,6-[$^2H_2$] glucose infusions to humans during clinical or experimental studies. The risks associated with the infusion include volume overload, infection, and allergic reaction to the infused substance. There have been no occurrences of volume overload, no occurrences of infection or allergic reaction attributable to iodine used prior to venipuncture in any of the 200 infusion protocols that the investigators have been involved. To minimize the likelihood of these events occurring, infusion rate will be closely monitored and maintained at less than 30 ml/hr throughout the entire infusion protocol day.
Measures to Minimize Risks of Harm: All staff who directly participate in the infusion studies will be properly trained how to safely monitor (i.e., infusion pumps and IV lines) infusion studies from Drs. Margolis and Pasiakos, who have extensive experience with infusion studies. In addition, infusates will be prepared sterile, pyrogen-free, and in the proper dosages by a licensed pharmacist.

Research Procedure Name: ^{13}C-glucose ingestion
Research Procedure Description: ^{13}C-glucose will be consumed in a carbohydrate beverage.
Research-related Risks: There are no known risks of consuming stable isotope of ^{13}C-glucose. ^{13}C is naturally occurring in the diet (~1%). It is a nonradioactive isotope. A trace amount will be used to establish carbohydrate oxidation (200 mg in 145 g of glucose, < 0.1%), which is a safe increase in ^{13}C.
Measures to Minimize Risks of Harm: Pyrogen-free isotope will be purchased from reputable supplier.

C6.2 Incidental or Unexpected Findings

Health problems identified during the screening process will be documented and a copy provided to the volunteer. The volunteer will be encouraged to make an appointment with their primary care provider for a full evaluation of the problem. Volunteers with evidence of any physical, mental, and/or medical conditions that would make the proposed studies relatively more hazardous will be excluded.

C6.3 Potential Benefits

There is no health or other benefits related to participation in this study. Information obtained from this research may benefit other individuals in the future.

C7. DATA AND SAFETY MONITORING

C7.1 Monitoring

The PI will, with the assistance of Associate Investigators, continuously evaluate recruitment, the informed consent process, adverse events, and protocol adherence and deviations in order to identify unanticipated problems or risks to the volunteers associated with the research. The PI will ensure that the number of volunteers recruited for this study complies with the protocol. Every morning, volunteers will be asked the following questions to evaluate their readiness to test.

- How have you been feeling well since the last test in our laboratory (below average, average, above average)?
- Do you have any pain or symptoms to report that may affect our testing today (e.g., sinus congestion, fatigue, muscle soreness, fever, tooth pain)?
- Have you reported all food and beverages consumed in the last 24 h that were not provided to you by study staff?
- What time did you fall asleep last night and awake this morning?

- Did you perform any exercise or physical activities outside of study activities in the last 24 h?

The PI or project manager will monitor data monthly during the data collection portion of the protocol. A log will be created listing any missing data.

C7.2 Research Monitor (as applicable)

The research monitor for this study is MAJ Robin Cushing. This individual is an appropriate subject matter expert not associated with the protocol. The research monitor shall, at a minimum, review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event. The PI and research monitor will discuss "discontinuation criteria" for individual volunteers as the study progresses, based on their observations of the volunteer during testing or non-testing periods. Other responsibilities may be assigned by the MRMC IRB as needed.

C8. REPORTABLE EVENTS

C8.1 Expected adverse events

An adverse event is defined as any untoward or unfavorable medical occurrence in a human research participant, including any abnormal sign (e.g., abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the individual's participation in the research, whether or not considered related to the individual's participation in the research.

Expected adverse events are some level of AMS under HA conditions, potential fainting during blood draws and muscle biopsies, local bruising and soreness from blood draws and muscle biopsies, and muscle soreness or pain from exercise. Moderate stiffness, hematoma and swelling may occur around the muscle biopsy site.

All medical events that the USARIEM Office of Medical Support and Oversight (OMSO) evaluates will be reported to the ORQC. The PI will report all adverse events to the Research Monitor, if one was appointed for the study.

Expected adverse events which are not serious are reported to the IRB at the time of continuing review of the protocol.

C8.2 Unexpected adverse events and unanticipated problems

A serious adverse event is any adverse event temporally associated with the subject's participation in research that is fatal, life-threatening, permanently disabling, requires inpatient hospitalization, or results in congenital anomalies/birth defect, overdose or cancer, or based on appropriate medical judgment, may jeopardize the participant, or may require medical or surgical intervention to prevent one of the above outcomes.

All medical events will be reported to USARIEM's Office of Medical Support and Oversight (OMSO). OMSO staff will retain a copy of the report in the subject's OMSO medical file as a means of tracking and analyzing trends in medical events. The PI will report all adverse events to the Research Monitor, if one was appointed for the study.

All unanticipated problems involving risk to subjects or others, serious adverse events that are unexpected and determined to be at least possibly or definitely related to study participation, will be promptly reported within one working day by phone (508-233-6306/4811) or email (usarmy.natick.medcom-usariem.mbx.usariem-rqc-protocol@mail.mil) to the USARIEM ORQC and the

Commander. These events will also be reported to the HQ USAMRMC IRB within one working day by phone (301-619-6240), or by e-mail (usarmy.detrick.medcom-usamrmc.other.irb-office@mail.mil)

Adverse events assessed by the PI as not serious and serious adverse events that are deemed to be unrelated to participation in the study will be reported to the IRB at the time of continuing review of the protocol.

The research monitor is required to review all unanticipated problems involving risk to volunteers or others, serious adverse events and all volunteer deaths associated with the protocol and provide an unbiased written report of the event. At a minimum, the research monitor should comment on the outcomes of the event or problem, and in the case of a serious adverse event or death, comment on the relationship to participation in the study. The research monitor should also indicate whether he or she concurs with the details of the report provided by the study investigator. Reports for events determined by either the investigator or research monitor to be: possibly related, unexpected, and serious or suggest that the research places subjects or others at increased risk of harm during participation, will be promptly forwarded to the ORQC and HQ USAMRMC IRB.

In the event of a medical emergency at facilities on the Natick Soldier Center, the local Emergency Medical Services (EMS) will be contacted immediately by dialing 5911. The installation security personnel will direct the ambulance to the proper location on the installation. While awaiting their arrival, Basic Life Support will be rendered by study personnel or on-site medical coverage. EMS response time to USARIEM is approximately 5 minutes. Transport time to definitive care is approximately 8 minutes.

C8.3 Adverse device effects: N/A

C8.4 FDA-regulated research under IND and IDE: N/A

SECTION D: REFERENCES

1. O'Hara JP, Woods DR, Mellor A, Boos C, Gallagher L, Tsakirides C, Arjomandkhah NC, Holdsworth DA, Cooke CB, Morrison DJ, et al. 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 2017;5(1). doi: 10.14814/phy2.13101.
2. Peronnet F, Massicotte D, Folch N, Melin B, Koulmann N, Jimenez C, Bourdon L, Launay JC, Savourey G. Substrate utilization during prolonged exercise with ingestion of (13)C-glucose in acute hypobaric hypoxia (4,300 m). Eur J Appl Physiol 2006;97(5):527-34. doi: 10.1007/s00421-006-0164-2 [doi].
3. Jeukendrup A, Brouns F, Wagenmakers AJ, Saris WH. Carbohydrate-electrolyte feedings improve 1 h time trial cycling performance. Int J Sports Med 1997;18(2):125-9. doi: 10.1055/s-2007-972607.
4. Jeukendrup AE. Carbohydrate intake during exercise and performance. Nutrition 2004;20(7-8):669-77. doi: 10.1016/j.nut.2004.04.017 [doi];S0899900704001169 [pii].
5. Jeukendrup AE. Carbohydrate feeding during exercise. Eur J Sport Sci 2008;8(2):77-86. doi: 10.1080/17461390801918971.
6. Trommelen J, Fuchs CJ, Beelen M, Lenaerts K, Jeukendrup AE, Cermak NM, van Loon LJ. Fructose and Sucrose Intake Increase Exogenous Carbohydrate Oxidation during Exercise. Nutrients 2017;9(2). doi: 10.3390/nu9020167.
7. Young AJ, Cymerman A, Burse RL. The influence of cardiorespiratory fitness on the decrement in maximal aerobic power at high altitude. Eur J Appl Physiol 1985;54:12-5.
8. Young AJ, Reeves JT. Human adaptation to high terrestrial altitude. Edtion ed. In: Burr RE, Pandolf KB, eds. Textbook of Military Medicine, Part III: Disease and the Environment, Volume: Medical Aspects of Deployment to Harsh Environments. Washington, D.C.: Office of the Surgeon General, Department of the Army, and Borden Institute, 1998:644-88.

9. Roberts AC, Reeves JT, Butterfield GE, Mazzeo RS, Sutton JR, Wolfel EE, Brooks GA. Altitude and beta-blockade augment glucose utilization during submaximal exercise. J Appl Physiol (1985) 1996;80(2):605-15.
10. Brooks GA, Butterfield GE, Wolfe RR, Groves BM, Mazzeo RS, Sutton JR, Wolfel EE, Reeves JT. Increased dependence on blood glucose after acclimatization to 4300 m. J Appl Physiol 1991;70:919-27.
11. Pirnay F, Crielaard JM, Pallikarakis N, Lacroix M, Mosora F, Krzentowski G, Luyckx AS, Lefebvre PJ. Fate of exogenous glucose during exercise of different intensities in humans. J Appl Physiol Respir Environ Exerc Physiol 1982;53(6):1620-4.
12. Larsen JJ, Hansen JM, Olsen NV, Galbo H, Dela F. The effect of altitude hypoxia on glucose homeostasis in men. J Physiol-London 1997;504(1):241-9. doi: DOI 10.1111/j.1469-7793.1997.241bf.x.
13. Braun B, Rock PB, Zamudio S, Wolfel GE, Mazzeo RS, Muza SR, Fulco CS, Moore LG, Butterfield GE. Women at altitude: short-term exposure to hypoxia and/or alpha(1)-adrenergic blockade reduces insulin sensitivity. Journal of Applied Physiology 2001;91(2):623-31.
14. Oltmanns KM, Gehring H, Rudolf S, Schultes B, Rook S, Schweiger U, Born J, Fehm HL, Peters A. Hypoxia causes glucose intolerance in humans. Am J Resp Crit Care 2004;169(11):1231-7. doi: 10.1164/rccm.200308-1200OC.
15. Peltonen GL, Scalzo RL, Schweder MM, Larson DG, Luckasen GJ, Irwin D, Hamilton KL, Schroeder T, Bell C. Sympathetic inhibition attenuates hypoxia induced insulin resistance in healthy adult humans. J Physiol-London 2012;590(11):2801-9. doi: 10.1113/jphysiol.2011.227090.
16. Carter SL, Rennie C, Tarnopolsky MA. Substrate utilization during endurance exercise in men and women after endurance training. Am J Physiol Endocrinol Metab 2001;280(6):E898-907.
17. Devries MC, Hamadeh MJ, Phillips SM, Tarnopolsky MA. Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. Am J Physiol Regul Integr Comp Physiol 2006;291(4):R1120-8. doi: 10.1152/ajpregu.00700.2005.
18. Horton TJ, Pagliassotti MJ, Hobbs K, Hill JO. Fuel metabolism in men and women during and after long-duration exercise. J Appl Physiol (1985) 1998;85(5):1823-32.
19. Phillips SM, Atkinson SA, Tarnopolsky MA, MacDougall JD. Gender differences in leucine kinetics and nitrogen balance in endurance athletes. J Appl Physiol (1985) 1993;75(5):2134-41.
20. Lohman TG, Harris M, Teixeira PJ, Weiss L. Assessing body composition and changes in body composition. Another look at dual-energy X-ray absorptiometry. Ann N Y Acad Sci 2000;904:45-54.
21. Young AJ, Cymerman A, Burse RL. The influence of cardiorespiratory fitness on the decrement in maximal aerobic power at high altitude. Eur J Appl Physiol Occup Physiol 1985;54(1):12-5.
22. ACMS' Guidelines for Exercise Testing and Prescription: Seventh Edition. 2006.
23. McInerney P, Lessard SJ, Burke LM, Coffey VG, Lo Giudice SL, Southgate RJ, Hawley JA. Failure to repeatedly supercompensate muscle glycogen stores in highly trained men. Med Sci Sports Exerc 2005;37(3):404-11.
24. Kim IY, Schutzler SE, Azhar G, Wolfe RR, Ferrando AA, Coker RH. Short term elevation in dietary protein intake does not worsen insulin resistance or lipids in older adults with metabolic syndrome: a randomized-controlled trial. BMC Nutr 2017;3. doi: 10.1186/s40795-017-0152-4.
25. Kim IY, Williams RH, Schutzler SE, Lasley CJ, Bodenner DL, Wolfe RR, Coker RH. Acute lysine supplementation does not improve hepatic or peripheral insulin sensitivity in older, overweight individuals. Nutr Metab (Lond) 2014;11(1):49. doi: 10.1186/1743-7075-11-49.
26. Evans WJ, Phinney SD, Young VR. Suction applied to a muscle biopsy maximizes sample size. Med Sci Sports Exerc 1982;14(1):101-2.
27. Pasiakos SM, McClung HL, McClung JP, Urso ML, Pikosky MA, Cloutier GJ, Fielding RA, Young AJ. Molecular responses to moderate endurance exercise in skeletal muscle. Int J Sport Nutr Exerc Metab 2010;20(4):282-90.
28. Wolfe RR, Chinkes DL. Isotope tracers in metabolic research: principles and practice of kinetic analysis: John Wiley & Sons, 2005.
29. Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. Int J Sports Med 2005;26 Suppl 1:S28-37. doi: 10.1055/s-2004-830512.
30. Pallikarakis N, Sphiris N, Lefebvre P. Influence of the bicarbonate pool and on the occurrence of 13CO2 in exhaled air. Eur J Appl Physiol Occup Physiol 1991;63(3-4):179-83.
31. Bradley H, Shaw CS, Bendtsen C, Worthington PL, Wilson OJ, Strauss JA, Wallis GA, Turner AM, Wagenmakers AJ. Visualization and quantitation of GLUT4 translocation in human skeletal muscle following glucose ingestion and exercise. Physiol Rep 2015;3(5). doi: 10.14814/phy2.12375.
32. Bradley H, Shaw CS, Worthington PL, Shepherd SO, Cocks M, Wagenmakers AJ. Quantitative immunofluorescence microscopy of subcellular GLUT4 distribution in human skeletal muscle: effects of endurance and sprint interval training. Physiol Rep 2014;2(7). doi: 10.14814/phy2.12085.

33. Margolis LML, S.J.; Ezzyat, Y.; Fielding, R.A.; Rivas, D.A. Circulating microRNA are predictive of aging and acute adaptive response to resistance exercise in men. J Gerontol A Biol Sci Med Sci 2016;EPub Ahead of Print.
34. Marabita F, de Candia P, Torri A, Tegner J, Abrignani S, Rossi RL. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. Brief Bioinform 2016;17(2):204-12. doi: 10.1093/bib/bbv056.
35. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7):RESEARCH0034.
36. Margolis LM, Rivas DA, Berrone M, Ezzyat Y, Young AJ, McClung JP, Fielding RA, Pasiakos SM. Prolonged Calorie Restriction Downregulates Skeletal Muscle mTORC1 Signaling Independent of Dietary Protein Intake and Associated microRNA Expression. Front Physiol 2016;7:445. doi: 10.3389/fphys.2016.00445.
37. Lessard SJ, Rivas DA, Alves-Wagner AB, Hirshman MF, Gallagher IJ, Constantin-Teodosiu D, Atkins R, Greenhaff PL, Qi NR, Gustafsson T, et al. Resistance to aerobic exercise training causes metabolic dysfunction and reveals novel exercise-regulated signaling networks. Diabetes 2013;62(8):2717-27. doi: 10.2337/db13-0062.

SECTION E: ABBREVIATIONS AND ACRONYMS

HA; high altitude, SL; sea level, ATP; adenosine triphosphate, GLUT4; glucose transporter 4, PDHC; pyruvate dehydrogenase complex, HIF-1α; hypoxic-inducible factor 1α, PDK; pyruvate dehydrogenase kinase, TCA; tricarboxylic acid cycle, MORE; Modular Operational Ration Enhancement, CHO; carbohydrate, DEXA; dual energy x-ray absorptiometry, HR; heart rate, SaO_2; oxygen saturation, miRNA; microRNA, TTR; tracer to tracee ratio, MPE; mole percent excess, PBR; phosphate-buffered saline, OCT; Optimum Cutting Temperature, PVDF; polyvinylidene fluoride

SECTION F: DoD PRIVACY RULE AND PROTECTED HEALTH INFORMATION (HIPAA)

☒ NA – institution is not a covered entity

☐ NA – will not use or disclose protected health information

☐ HIPAA authorization will be obtained

☐ An application for waiver/alteration of HIPAA authorization will be submitted

Appendices

Appendix A: Muscle Biopsy Care

The mechanistic effects of acute hypobaric hypoxia on exogenous carbohydrate utilization during steady-state aerobic exercise

PRINCIPAL INVESTIGATOR:
Lee M. Margolis, Ph.D.

Muscle Biopsy Care
Information for the Participant

You have just had a percutaneous muscle biopsy. The muscle biopsy site is closed with a steri-strip or butterfly bandaid. This is covered with 4 x 4 gauze pad and then an elastic bandage.

As the anesthetic wears off, you may feel a dull ache at the biopsy site for 1-3 days. Many people have no pain at all. Instructions for the care of the biopsy site are as follows:

1. You may perform all your normal activities immediately after the biopsy.
2. Leave the ACE bandage in place for 2 hours after the biopsy. After this time, remove everything except the steri-strip or butterfly bandaid.
3. Do not get the incision wet for 24 hours after the biopsy.
4. Do not take any aspirin or aspirin-containing products for 5 days after the biopsy unless MD approves. Also avoid 'aspirin-like' drugs such as Motrin. Indocin, etc.
5. After 2 days, no special precautions are required and the site should be almost completely healed.
6. If any of the signs below occur, contact:
   - Fever
   - Bleeding from biopsy site
   - Inflammation at biopsy site (warmth, redness, tenderness, pus formation, clear discharge, opening of incision, lump under incision, or rash around incision). If you are not sure if it is healing normally, alert study staff
   - Persistent numbness in the leg
   - Pain at the biopsy site more than one week after the biopsy

Lee M. Margolis, PhD, RD
U.S. Army Research Institute of Environmental Medicine
Building 42, Room 203
10 General Greene Ave
Natick, MA 01760
Office Phone: 508-233-4591
Cell Phone: 203-522-0624

Appendix B: Muscle Biopsy Notes

Participant ID: _____

Trial 1 / 2

Date:	
Biopsy One	
Leg: L or R	
Time:	
Lidocaine	
Size:	
Notes:	
Biopsy Two	
Leg: L or R	
Time:	
Lidocaine	
Size:	
Notes:	