PROTOCOL TITLE: Effects of severe negative energy balance on inflammation, iron absorption, nutritional status, skeletal muscle and whole-body metabolic homeostasis, cognitive, and physical performance during a simulated sustained operation (SUSOPS)

SECTION A: RESEARCH TEAM AND LOCATIONS

A1. RESEARCH TEAM

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A2. ROLES AND RESPONSIBILITIES

A2.1 Key Research Personnel

Name(s): Stefan M Pasiakos
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 the 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. Pasiakos will be involved in volunteer briefing, obtaining informed consent, data collection, performing muscle biopsies, catheterization, phlebotomy, exercise testing, and interventions.

Name(s): James P McClung, Stephen R Hennigar, Lee M Margolis, J Philip Karl, Harris R Lieberman, Nicholas D Barringer, and Tracey J Smith, Jessica A Gwin, Alyssa N Varanoske
Research Role: Associate Investigators
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach, and design. Assist PI with volunteer briefing, obtaining informed consent, exercise testing and interventions, oversight of daily volunteer monitoring, data collection, management, analysis, and manuscript preparation. Dr. Lee Margolis will also perform exercise testing, muscle biopsies, catheterization, and phlebotomy. Dr. Gwin will also perform exercise testing, muscle biopsies, catheterization and phlebotomy. MAJ Nicholas Barringer and Alyssa Varanoske will additionally assist with exercise testing, DEXA and phlebotomy. Drs. Margolis, Karl, Barringer, and Smith will assist with diet instruction and dietary assessment. Dr. Karl will also assist with SmartPill administration and analysis.

Name(s): Jennifer Rood
Research Role: Associate Investigator
Study Responsibilities: Receipt and analysis of coded data. Dr. Rood will not interact or intervene with subjects or their identifiable data or specimens.

Names (s): Svein Martini, Olav Vikmoen, Hilde Teien
Research Role: Associate Investigators
Study Responsibilities: Assist with study design, logistics, daily volunteer monitoring, meal preparation and administration of study diets to volunteers, data analysis, and report generation. Mr. Martini, Dr. Vikmoen, and Ms. Teien will only observe and assist with monitoring of credentialed USARIEM research procedures. They will not be preforming any credentialed procedure.

Name(s): Rasha Hammamieh, Graham Finlayson, John Caldwell, Oshin Vartanian
Research Role: Consultant
Study Responsibilities: Assist with study design, logistics, data analysis, and report preparation. These individuals will not interact or intervene with subjects or their identifiable data or specimens.

Name(s): Adrienne Hatch
Research Role: Project Coordinator
Study Responsibilities: Supervise, manage, and coordinate study logistics and biological data collection. She will be involved with protocol development, menu development, volunteer briefing, oversight, preparation and administering study diets to volunteers, diet instruction, dietary assessment, oversight of daily volunteer monitoring, and study implementation. She will actively participate in data collection to include phlebotomy, exercise testing, interventions, SmartPill administration, and DEXA. She will assist in management, analysis and interpretation of data, as well as preparation of manuscripts and technical reports for publication

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

Name(s): Claire Whitney, Heather Fagnant, Nicholes Armstrong, Jillian Allen
Research Role: Research Dietitians
Study Responsibilities: Menu development, preparation and administering study diets to volunteers, diet instruction, dietary assessment and study implementation, daily volunteer monitoring. Claire Whitney, Heather Fagnant, and Nicholes Armstrong will also assist with

DEXA. Claire Whitney will also actively participate in data collection to include phlebotomy, exercise testing, and interventions. Nicholes Armstrong will also assist with phlebotomy and catheterization. Ms. Fagnant will also assist with SmartPill administration and analysis.

Name(s): Nancy Murphy, Christopher Carrigan
Research Role: Biological Sample Coordinators
Study Responsibilities: Supervision, management, daily volunteer monitoring, and coordination of logistics, and biological data collection. They will be involved with protocol development and study implementation. Data collection responsibilities will involve sample processing, management, and oversight, and DEXA. Christopher Carrigan will also assist with phlebotomy and catheterization.

Name(s): Patrick Radcliffe, Emily Howard, Michelle Saillant
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Study Responsibilities: Assist with data collection, daily volunteer monitoring, meal preparation and administering study diets to volunteers, and biological sample processing. Mr. Radcliffe will also assist with SmartPill administration and analysis.

Name(s): Marques Wilson
Research Role: Research Assistant
Study Responsibilities: Assist with data collection, daily volunteer monitoring, logistics and biological sample processing. Marques Wilson will also be involved in phlebotomy, catheterization, DEXA, and exercise testing and intervention.

Name(s): SPC Chiusano, SGT Rousayne, SSG Mason, SPC Sarpong, SPC Sanchez, SPC Pounds, SPC Dare, SGT Hansen, SPC Galloway, SPC Bistolfi, SPC Figueroa, and Anthony Karis
Research Role: Research Assistants
Study Responsibilities: Assist with data collection, daily volunteer monitoring, meal preparation, administering study diets to volunteers, phlebotomy, and biological sample processing. SSG Mason will also assist with catheterization. Mr. Karis will also assist with SmartPill administration and analysis.

Name(s): Lauren Thompson, Philip Niro
Research Role: Research Assistants
Study Responsibilities: Facilitation and oversight of the mood, cognitive performance, and vigilance assessments; assist with daily volunteer monitoring.

Name(s): Susan McGraw, MAJ Jayne
Research Role: Research Assistant
Study Responsibilities: Assist with meal preparation, daily volunteer monitoring, and administering study diets to volunteers. Susan McGraw will additionally assist with DEXA.

Name(s): Karleigh Bradbury, Adam Luippold, Beau Yurkevicius
Research Role: Research Assistants
Study Responsibilities: Assist with meal preparation, daily volunteer monitoring, administering study diets to volunteers, exercise testing and intervention, and DEXA.

Name(s): MAJ Robin Cushing
Research Role: Research Monitor

Study Responsibilities: The research monitor shall 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.

Name(s): Katelyn I. Guerriere, Caitlin Haven, Matthew Bartlett
Research Role: Ombudsmen
Study Responsibilities: Observe group briefings for military volunteers not in the Human Research Volunteer program.

A3. RESEARCH LOCATIONS

USARIEM, Natick MA: USARIEM is a DoD research facility within the US Army Medical Research and Development 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, and 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. Staff computers are interfaced with a network server for easy, secure data handling and transfer. All testing (pre-study screening, baseline, and experimental testing) will take place at USARIEM.

SECTION B: RESEARCH METHODOLOGY

B1. ABSTRACT

Endurance exercise elicits skeletal muscle and systemic inflammation, reflected in large part by increases in circulating concentrations of the pleotropic cytokine, interleukin-6 (IL-6). Circulating IL-6 facilitates skeletal muscle tissue repair and serves as an energy sensor during prolonged endurance exercise by upregulating glycogenolysis to maintain blood glucose. The IL-6 response to endurance exercise is normally attenuated with adequate rest and recovery as skeletal muscle adapts with training. However, performing repeated bouts of prolonged and unaccustomed, muscle damaging (i.e., eccentric loading) endurance exercise may be detrimental to performance and limit the adaptive responses to exercise by diminishing the absorption of key nutrients (i.e., iron). Warfighters are commonly exposed to such exercise bouts during sustained training and combat operations (SUSOPS), the effects of which may be exacerbated by negative energy balance. We recently reported that IL-6 and hepcidin, a hepatic-derived protein that arises in response to inflammation and limits iron absorption, increased by approximately 245% and 33%, respectively in Norwegian Soldiers participating in a short-term (96 h) Arctic SUSOPS [1]. Circulating hepcidin concentrations post-SUSOPS were associated with total daily energy expenditure (r = 0.4), energy intake (r = -0.26), and energy balance (r = -0.43), suggesting that nutritional interventions designed to increase energy intake during SUSOPS may attenuate IL-6 and its untoward downstream effects by limiting the

magnitude of energy deficit. However, these data, which were derived from an uncontrolled military field study, are not indicative of causality and changes in iron absorption. Therefore, to define the putative role of energy balance on IL-6 and its downstream effects, we will conduct a controlled laboratory study that simulates the physiological stressors imposed during SUSOPS to determine if the IL-6 response is exacerbated by underfeeding. Fifteen male, weight-stable, active duty military personnel (aged 18 – 39 years) will be recruited to participate in a 22 d, longitudinal study. The study is comprised of four sequential phases: 1) a 72 h SUSOPS, 2) a 7 day recovery period (Recovery 1), 3) a second, 72 h SUSOPS, and 4) a final, 7 d recovery period (Recovery 2). During SUSOPS, subjects will be randomized to consume either sufficient food (combat rations) to maintain energy balance within ± 10% of estimated total daily energy expenditure (SUSOPS BAL) or will be provided only enough food to match 45% of total daily energy expenditure to elicit severe negative energy balance (SUSOPS NEG BAL). Testing procedures and primary outcome measures include dual energy x-ray absorptiometry (DEXA), maximal and sub-maximal aerobic exercise testing, load carriage exercise, controlled feeding, stable isotope assessments of energy expenditure, fractional iron absorption and whole-body protein turnover, percutaneous muscle biopsies to assess intramuscular IL-6, glycogen status, and regulators of muscle remodeling and substrate metabolism, intestinal permeability, gut microbiome composition and function, and blood sampling to assess circulating concentrations of IL-6 and other biomarkers of inflammation, muscle damage, nutritional, metabolic stress, and immune responses to SUSOPS BAL and NEG BAL. Physical performance will be assessed before and after each SUSOPS and during each recovery period using the vertical jump test. Self-reported mood, sustained attention, and cognitive performance will be assessed twice daily using the Profile of Mood States, the Psychomotor Vigilance Task, the Evaluation of Risk Questionnaire, the Match-to-Sample task, the Grammatical Reasoning test, and the N-Back task. Overall vigilance and nighttime sleep quality will be assessed via the wrist-worn USARIEM Vigilance Monitoring System, the Fatigue Science ReadiBand™ actigraph, Philips Respironics Actiwatch® Spectrum Plus, or equivalent device. This design will test the hypothesis that maintaining energy balance will attenuate the IL-6 response to SUSOPS, improve iron absorption, and attenuate the potential negative effects of excessive inflammation on whole-body metabolic homeostasis. Risks include those associated with venous catheterization, venipuncture, muscle biopsies, DEXA, exercise, and the discomfort of severe underfeeding.

B2. BACKGROUND AND SIGNIFICANCE

Physiology of the Inflammatory Response to Exercise

Prolonged strenuous exercise is characterized by a large increase in circulating concentrations of IL-6 (**Figure 1A**) followed by increases in cytokine inhibitors, such as IL-1 receptor antagonist (IL-1ra), and the anti-inflammatory cytokine IL-10 [2-4]. In fact, concentrations of IL-6 consistently increase more than any other cytokine during an exercise bout [5]. The immediate increase in circulating concentrations of IL-6 with exercise is mediated by the transcriptional upregulation and release of IL-6 by contracting skeletal muscle fibers [6, 7]. Although many cytokines are expressed in skeletal muscle (termed 'myokines') after prolonged exercise [8], IL-6 is the only myokine known to be released from skeletal muscle in appreciable concentrations. IL-6 synthesized and released by contracting skeletal muscle during prolonged exercise is thought to exert metabolic effects and is dependent on muscle glycogen availability [6, 7, 9] (**Figure 1B**). For example, reduction in intramuscular glycogen prior to exercise increases transcription of IL-6 mRNA in skeletal muscle and plasma IL-6 concentrations [6, 7], an effect which may be mediated through activation of AMP-activated protein kinase [10] and p38

mitogen activated protein kinase [6]. Moreover, infusion of humans with recombinant human IL-6 (rhIL-6) induces a dose-dependent rise in blood glucose concentrations [11] and increases in glucose disposal [12, 13], indicating that IL-6 may signal for hepatic glucose release to maintain blood glucose during exercise. In addition to increasing hepatic glycogenolysis and gluconeogenesis, rhIL-6 enhances lipolysis and fatty acid oxidation in adipocytes [14, 15], perhaps to provide free fatty acids and energy when glycogen stores are low.

Another source of IL-6 is immune cells. During recovery from exercise, microstructural damage as a result of microtrauma to contractile elements and connective tissue within muscle tissue signal immune cell infiltration and secretion of cytokines (tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-1ra, and IL-10) to heal the tissue. The rapid release of TNF-α and IL-1β stimulate the production of IL-6, which in turn, is thought to be the central mediator in activating the acute-phase response and the systemic release of hepatocyte-derived acute phase proteins [16]. These are likely delayed or secondary to the metabolic effects of IL-6 during exercise (i.e., during recovery), as peak circulating concentrations of muscle-derived IL-6 occur immediately following exercise and are acute, whereas IL-6 produced by immune cells occurs after this peak, is of lower magnitude, and may remain elevated for an extended period of time to repair the damaged tissue [5, 17, 18] (**Figure 1A**).

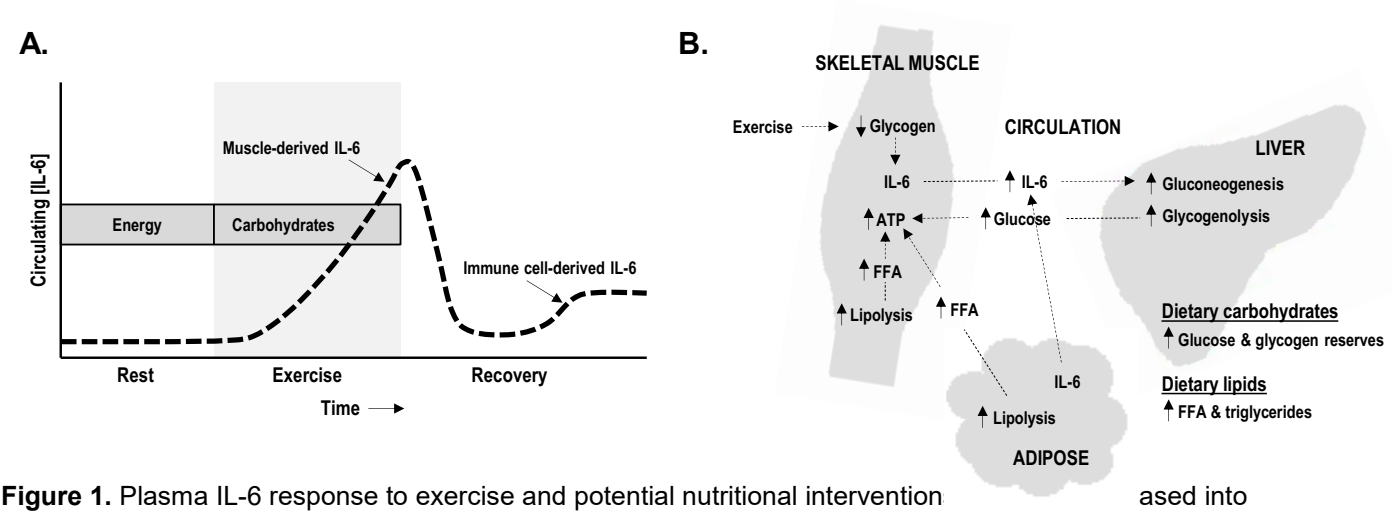


Figure 1. Plasma IL-6 response to exercise and potential nutritional intervention ased into circulation by contracting skeletal muscle (i.e., 'muscle-derived IL-6') during prolonged, strenuous exercise. This produces a large peak in circulating concentrations of IL-6 immediately after the completion of exercise followed by a rapid decline to baseline concentrations. If tissue injury occurs with exercise (e.g., strenuous eccentric exercise), immune cells infiltrate the muscle and release IL-6 to signal the acute-phase response and tissue repair (i.e., 'immune cell-derived IL-6'). This produces a rise in circulating concentrations of IL-6 that is lower in magnitude, but more sustained than concentrations of IL-6 in circulating following exercise. Sufficient calories to match the energy requirement prior to exercise and carbohydrate supplementation during prolonged, strenuous exercise may be an effective strategy to reduce increases in muscle-derived IL-6 immediately following exercise. (B) Glycogen stores are depleted with prolonged, strenuous exercise. Reductions in muscle glycogen signal for the transcriptional upregulation of IL-6. IL-6 is released from contracting muscle, resulting in a rise in circulating levels of IL-6. IL-6 signals for hepatic glycogenolysis and gluconeogenesis. Prolonged exercise also signals for the upregulation of IL-6 in adipose tissue and an increase in lipolysis. Circulating fatty acids and glucose and fatty acids released from lipolysis of intramuscular triglycerides are transported to muscle mitochondria for oxidation, resulting in increased ATP and energy for the exercising muscle. Dietary carbohydrate and lipid increase available glucose and free fatty acids and stores of glycogen and triglycerides, thereby sparing glycogen. ATP, adenosine triphosphate; FFA, free fatty acids; IL-6, interleukin-6 (figures adapted from Hennigar et al. [19]).

Factors that affect the source and magnitude of IL-6 released from muscle and immune cells in response to exercise include mode and location of muscle mass, frequency, duration, intensity, and training status [reviewed in [5, 13, 17, 18]]. In general, eccentric and strenuous exercise involving multiple muscle groups for prolonged durations produces the greatest increase in IL-6 as a cytokine and myokine, respectively. Although skeletal muscle damage is not required for the release of IL-6 from muscle during and immediately following exercise [5], it is important to note that the release of muscle- and immune cell-derived IL-6 is not mutually exclusive. Higher concentrations of muscle-derived IL-6 during and immediately following exercise generally indicate greater muscle damage, leading to an increase in immune cell infiltration and the time required for tissue repair and regeneration.

Potential Detriments of Inflammation on Iron Absorption during Sustained Operations

The release of IL-6 during exercise has been described as a double-edged sword [20], as IL-6 has the potential for both beneficial and detrimental effects. Beneficial adaptations resulting from exercise occur when the stressor dose and the exercise bout are within a specific range and followed by a rest period [21], whereas detrimental effects may occur when the dose or exercise bout is excessive and not followed by periods of adequate rest and nutrition. For example, repeated bouts of exercise on the same day or over the course of two days elevates circulating concentrations of immune cells and induces a more pronounced increase in plasma IL-6 as compared to a single bout of exercise [22, 23]. Further, repeated bouts of exercise without adequate rest and nutrition may result in decreased performance, particularly if muscle glycogen concentrations are low prior to exercise (e.g., fasted state, inadequate recovery, etc.) or if the duration of exercise is too long to maintain glycogen stores.

Importantly, sustained elevations in IL-6 may result in a decline in nutrient absorption, particularly the nutritionally essential mineral iron. Circulating levels of iron increase immediately following exercise and decline with recovery [24]. Inflammation, specifically the release of IL-6, is known to contribute to the decline in iron (termed the ‘hypoferremia of inflammation’). For example, infusion of humans or animals with IL-6 reduces circulating concentrations of iron >50% [25, 26]. The mechanism for the hypoferremia of inflammation is well established and involves IL-6-induced increases in the hepatic, acute-phase protein hepcidin [26]. Hepcidin is thought to bind to Cys^{326} in the extracellular domain of the iron exporter ferroportin [27], resulting in the phosphorylation and subsequence ubiquitination of ferroportin [28]. The hepcidin-ferroportin complex is then endocytosed and degraded in the lysosome [29], thereby inhibiting iron efflux into circulation.

Human and rodent models suggest that IL-6 may contribute to the exercise-induced increase in hepcidin, as rises in hepcidin are subsequent to the post-exercise increase in muscle-derived IL-6 [19]. Work from our laboratory indicates declines in iron status during military training [30, 31], concomitant with increased inflammatory biomarkers, including IL-6 and hepcidin [32, 33]. As poor iron status is associated with a decrease in cognitive and physical performance [31] these data indicate that prolonged or repeated IL-6-mediated increases in hepcidin during training may be of concern if resulting in diminished iron status.

Nutritional Countermeasures to Inflammation: Role of Substrate Availability and Energy Balance

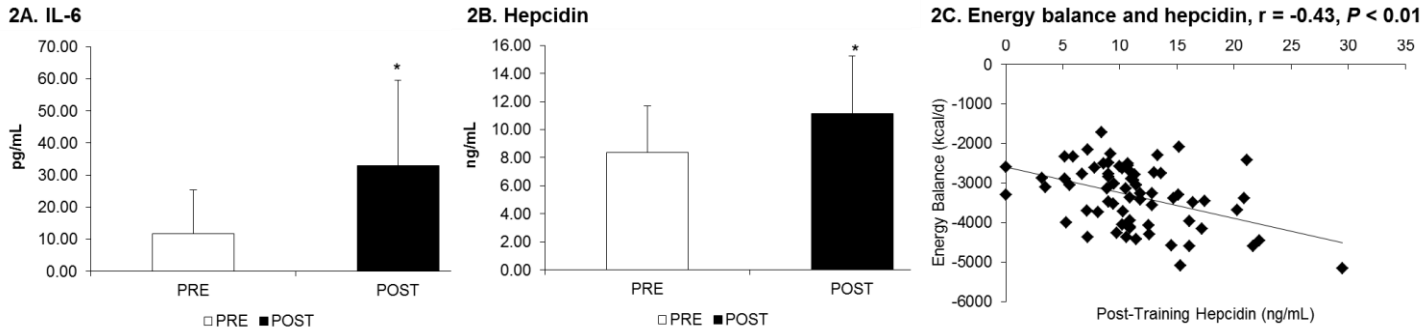
Sparing muscle glycogen and maximizing recovery is essential for optimal performance during exercise and in conferring the adaptive benefits of repeated exercise bouts with recovery (i.e.,

training). Because IL-6 is thought to respond to the energy available to the exercising muscle, various nutritional interventions have been studied with the goal of mitigating increases in circulating concentrations of IL-6 [19]. Collectively, these studies suggest that diets providing adequate energy and that are high in carbohydrate (>60%) prior to exercise may attenuate the depletion of glycogen stores and the resulting muscle-derived IL-6 response following exercise. This is consistent with the notion that IL-6 release from contracting skeletal muscle is related to pre-exercise glycogen availability [9] and that circulating concentrations of IL-6 post-exercise are negatively correlated with skeletal muscle glycogen concentrations [34] such that the duration and intensity of the exercise must be great enough to decrease skeletal muscle glycogen concentrations for diet to have an effect.

Sustained Operations, Energy Balance and Inflammation: Observations from Military Field Studies

Military personnel experience periods of negative energy balance resulting from increased energy expenditure, limited dietary intake, or combined effects of both during sustained combat and training operations (SUSOPS). Warfighters engaged in various SUSOPS (i.e., 1-7 d operations, separated by minimal rest (1-3 d), and repeated for ≥ a month) expend an average of 4600 kcal/d [35, 36]. In some cases, energy expenditure may exceed these levels; total energy expenditures for Marines engaged in mountain warfare training exceed 7000 kcal/d [37]. During military training and operations, energy supply and intake may be insufficient to maintain energy balance, thus resulting in extreme energy deficits [35, 38]. For example, mean energy expenditure during a 54-hr field training exercise was 6100 kcal/d for men, yet energy intake did not exceed 1500 kcal/d, resulting in a significant loss of total body mass [39]. Prolonged or repeat exposure to periods of negative energy balance can diminish physical performance, and may increase the risk of injury.

Military training and operations have been associated with inflammation and degraded nutritional status. For example, short-term (7 d) military training results in significant elevations in IL-6 and hepcidin [33]. Longer-term military training, such as basic combat training (7-10 wk), results in diminished iron status, which may be due to the physiologic response to repeated exposure to IL-6 [30-32]. A number of studies have investigated the effect of macronutrients, including carbohydrates, on the inflammatory and immune response to physical activity [40, 41], although these studies have not carefully controlled for energy intake. The relationship between energy balance and the inflammatory response, particularly the hepcidin response, is not well described. Previous studies from our laboratory show that IL-6 and hepcidin increased by approximately 245% (**Figure 2A**) and 33% (**Figure 2B**), respectively in Norwegian Soldiers participating in a short-term 96-h Arctic SUSOPS that produced high energy expenditures (~6000 kcal/d) and severe energy deficits (~50% total energy expenditure) [1]. Circulating hepcidin concentrations post-SUSOPS were associated with total daily energy expenditure (r = 0.4), energy intake (r = -0.26), and energy balance (r = -0.43, **Figure 2C**) [1]. Collectively, these



data indicate that interventions aiming at maintaining energy balance during periods of high energy expenditure should be considered in efforts to attenuate the inflammatory response associated with energy deprivation and military training. Although observational studies have uncovered statistical associations between energy balance and physiological outcomes, intervention studies have not directly tested the hypothesis that preventing energy deficit attenuates the inflammatory response, particularly the hepcidin response, to arduous physical activity.

Figure 2. Circulating IL-6 (A) and hepcidin (B) concentrations before (PRE) and after (POST) completing a 96 h Arctic SUSOPS, and the association between energy balance and post-SUSOPS hepcidin concentrations (C). *Different from PRE, $P < 0.05$ (data are mean ± SD). Figures adapted from Pasiakos et al. [1].

Secondary Objectives

Military Operational Stress, Intestinal Permeability, and Gut Microbiota Composition

Decreased gut barrier integrity has been reported following exposure to physical and psychological stressors such as those commonly experienced during military training [42-44]. The resulting increase in intestinal permeability can facilitate translocation of antigens from the gut into systemic circulation thereby inducing inflammation that can worsen gut barrier dysfunction [43]. A critical mediator of gut barrier integrity is the composition and activity of the bacteria residing in the human gut, the gut microbiota [45]. Our laboratory recently reported a positive association between changes in serum IL-6 concentrations and changes in intestinal permeability in Norwegian Soldiers participating in a 96-h Arctic SUSOPS characterized by severe energy deficit [44]. Changes in intestinal permeability and inflammation coincided with pronounced shifts in gut microbiota composition and activity, and changes in intestinal permeability were associated with both the composition of the microbiota before SUSOPS and changes in microbiota activity during SUSOPS [44]. Severe energy deficit alone has been shown to adversely affect gut barrier integrity, and gut microbiota composition and activity [46, 47]. The gap in knowledge is to what extent decrements in intestinal permeability and the gut microbiome can be mitigated by maintaining energy balance during SUSOPS. We will address this gap by measuring intestinal permeability and gut microbiome composition and function.

A primary finding from our Norwegian study was that of an increase in gut microbiota diversity which was associated with increases in intestinal permeability during SUSOPS. Greater diversity in the microbiota is generally considered a marker of a healthy and more resilient gut microbiota, and diversity has been reported to decrease in response to various stressors in animal models [48, 49]. It was recently shown that longer intestinal transit time is associated with increased gut microbiota diversity and changes in the relative abundance of multiple taxa [50], implicating transit time as an important confounder in studies assessing the gut microbiota, especially when studying effects of conditions known to impact intestinal transit. Exercise [51], underfeeding [52], and psychological and physical stress [53] have been independently shown to alter gastrointestinal motility. The effects of SUSOPS on gastrointestinal transit time, and its impact on gut microbiota composition, are undetermined.

Military Operational Stress, Appetite Regulation and Eating Behavior

Warfighters frequently endure substantial energy deficit during SUSOPS, even when enough food is provided to meet energy demands [54, 55]. This is counterintuitive because in most

settings, energy deficit elicits a strong counter-regulatory response which stimulates appetite to alleviate energy deficit and prevent weight loss. Various psychological, logistical, and environmental factors have all been cited as contributing to undereating during SUSOPS [54, 55], but whether appetite actually increases sufficiently to balance energy intake with elevated energy expenditure is unclear. The observation that endurance exercise transiently suppresses appetite, increases appetite-suppressing hormone concentrations (e.g., peptide-YY (PYY), glucagon-like peptide-1 (GLP-1), and pancreatic polypeptide (PP)), and depresses appetite-stimulating hormone concentrations (i.e., acylated ghrelin) suggests that the counter-regulatory response to energy deficit could be blunted during SUSOPS [56-58]. However, to our knowledge, this question has not been examined. Moreover, changes in gastrointestinal transit time have been associated with changes in appetite and appetite-mediating hormone secretion [52, 59] suggesting that exercise- and/or energy deficit-mediated decreases in transit time could contribute to appetite suppression during SUSOPS. An improved understanding of how appetite and biological factors regulating appetite (i.e., appetite-mediating hormones and gastrointestinal transit rate) respond during SUSOPS is needed to optimize feeding strategies within these environments, and to elucidate the contribution of these factors to eating behaviors (e.g., food choice) that promote weight regain following SUSOPS. As such, this study will measure appetite, food preferences, and biological factors mediating appetite and food choice before and after SUSOPS during both induced energy deficit and energy balance.

A Systems Biology Approach for Characterizing Military Operational Stress

Stress elicits systemic changes in gene and epigene expressions; behavior and performance; metabolism, and immune function. A comprehensive understanding of the spectrum of pathways involved in regulating the stress response to military-relevant stress, and how those pathways interact to influence health and behavior is needed to both identify biomarkers of stress responses in Warfighters, and to elucidate targets for precision therapeutics aiming to mitigate stress responses that may compromise Warfighter health and performance. For example, a systems biology approach interrogating the blood transcriptomic landscape was recently used to identify 1400 transcripts that were differentially expressed in Soldiers before and after Army Ranger Assessment and Selection [60], a notoriously grueling training program. The analysis identified transcripts involved in the immune response as the most impacted biological response to Ranger School, and elucidated pathways underlying impaired immune function in this population. This systems-biology approach will be expanded in this study by integrating genomic and epigenomic (saliva), metabolomic (blood, feces, saliva), microbial metagenomic (feces, saliva), and proteomic (saliva and blood) analyses. In addition to providing unique insight into the stress response in a military-relevant environment, this approach will enable studying interactions among the microbiome and host physiology, the "interactome", to elucidate how the interactome responds to SUSOPS and the role of negative energy balance in this response.

B3. MILITARY RELEVANCE

Warfighters are often exposed to physical and cognitive stress, which likely contribute to documented declines in iron status, physical performance, and cognitive function during military training [30, 31, 61]. Thus, it is critical to identify factors that contribute to the decline in iron status and effective interventions to sustain and optimize Warfighter health and performance. This highly-controlled study will delineate the effects of repeated bouts of strenuous activity endured during SUSOPS on the IL-6 and hepcidin response, iron absorption and status, whole-body and skeletal muscle homeostasis, physical and cognitive performance, and determine if

maintaining energy balance is an effective strategy to mitigate that response. Data obtained from this study may be used in the development of countermeasures to attenuate the detrimental physiological effects of negative energy balance during strenuous military operations. Data obtained from this study will also be used to identify novel potential biomarkers associated with the severity of stress responses to strenuous military operations.

B4. OBJECTIVES/SPECIFIC AIMS/RESEARCH QUESTIONS

Objectives

1. To determine the effects of a simulated 72-h SUSOPS on IL-6, hepcidin, and fractional iron absorption.

2. To determine whether energy balance and severe energy deficit modulate IL-6, hepcidin, and fractional iron absorption during a simulated 72-h SUSOPS.

Secondary Objectives

1. To determine whether energy balance and severe energy deficit modulate intramuscular inflammation, transcriptional modifications (e.g., microRNA and factors involved with energy substrate metabolism), anabolic signaling, remodeling, and whole-body (e.g., protein balance, immune function, etc.) adaptive responses to a simulated 72-h SUSOPS.

2. To determine to what extent maintaining energy balance mitigates decrements in intestinal barrier integrity and in gut microbiome composition and function, and influences gastrointestinal transit time during a simulated 72-h SUSOPS.

3. To determine whether energy deficit increases appetite, and alters food preferences and biological mediators of appetite (i.e., appetite-mediating hormones and gastrointestinal transit time) during a simulated 72-h SUSOPS, and identify effects on food choice following SUSOPS.

4. To determine the effects of a simulated 72-h SUSOPS on physical performance, mood, cognitive performance, vigilance assessed continuously, and nightly sleep quality and determine whether energy deficiency exacerbates the effects of SUSOPS.

5. Characterize genomic and epigenomic stress markers in blood and saliva, particularly markers interacting with the microbiome from gut and saliva, to build networks that identify the “interactome” response associated with changes in energy homeostasis during SUSOPS.

Hypotheses

1. SUSOPS will increase circulating concentrations of IL-6 and hepcidin and decrease fractional iron absorption.

2. Energy balance will attenuate circulating concentrations of IL-6 and hepcidin and

improve fractional iron absorption compared to severe energy deficit.

Secondary Hypotheses

1. Energy balance will attenuate muscle glycogen depletion, intramuscular inflammation, minimize upregulation in microRNA transcription, facilitate muscle remodeling, and limit negative protein balance, and decrements in immune function.

2. Energy balance will attenuate increases in intestinal permeability, increases in gastrointestinal transit time, increases in markers of gut damage, and changes in gut microbiota composition and function during SUSOPS.

3. Energy deficit will increase appetite, change food preferences, increase circulating concentrations of appetite-suppressing hormones, and suppress circulating concentrations of appetite-stimulating hormones during SUSOPS, and alter food choices following SUSOPS.

4. SUSOPS will negatively impact physical performance, mood, cognition, and vigilance, and SUSOPS also will alter the sleep quality of participants. Negative energy balance will exacerbate the effects of SUSOPS on these parameters.

5. A systems biology approach integrating multi-omics data derived from fecal, blood, and saliva will elucidate genetic targets within host-microbiome relationship dynamics and biological pathways within the human host that are affected by military-relevant stress and are associated with physical and cognitive performance, and identify novel markers of physiological stress.

B5. RESEARCH PLAN

B5.1 Research Design

This randomized cross-over study will consist of four sequential phases (SUSOPS 1, Recovery 1, SUSOPS 2, and Recovery 2).

B5.2 Research Subjects/Population(s)

B5.2.1 Subject Population(s)

Healthy, recreationally-active adult men, who are active duty military, will be recruited to participate in this study. Male subjects were chosen because of known sex differences in iron status and absorption and differences in their response to exercise, including the IL-6 response to exercise [62, 63]. Pending the outcomes from this study, future, more targeted studies will be designed to address potential sex-based differences in physiological responses to SUSOPS.

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

This protocol requires 15 subjects complete testing to provide 80% power to detect significant differences in circulating hepcidin responses between SUSOPS 1 and SUSOPS 2. Up to 60 subjects will be enrolled to account for attrition and screening

failures in order to get complete data on 15 subjects. See section B5.8.1 for details on sample size calculations.

B5.2.3 Inclusion Criteria

- Men who are active duty military, aged 18 – 39 years
- Weight stable in the past 2 months (± 2.27 kg)
- Healthy without evidence of chronic illness, medication use, or musculoskeletal injury as determined by the USARIEM Office of Medical Support and Oversight (OMSO)
- Recreationally active (2-4 days per week aerobic and/or resistance exercise)
- Refrain from taking any pain-relievers (e.g., acetaminophen), nonsteroidal anti-inflammatory drugs (e.g., aspirin, Advil®, Aleve®, Naprosyn®), or any other aspirin-containing product for 10 days before starting and at least 5 days after completing the study
- Refrain from the use of alcohol and nicotine for the duration of the study
- Willing to refrain from alcohol, smoking any nicotine product (includes e-cigarettes), vaping, chewing tobacco, caffeine, and dietary supplement use, and from consumption of probiotic-containing foods (e.g., yogurt, cottage cheese, sauerkraut, etc.) and probiotic-containing supplements (e.g.,VSL#3, PRO-15, etc.) throughout the entire study period (vitamin/mineral supplements cannot be taken for at least 2 weeks before starting the study)
- Supervisor approval for non-HRV Active Duty Military working within the US Army Natick Soldier Systems Center
- Reports having a bowel movement at least as frequently as every-other-day

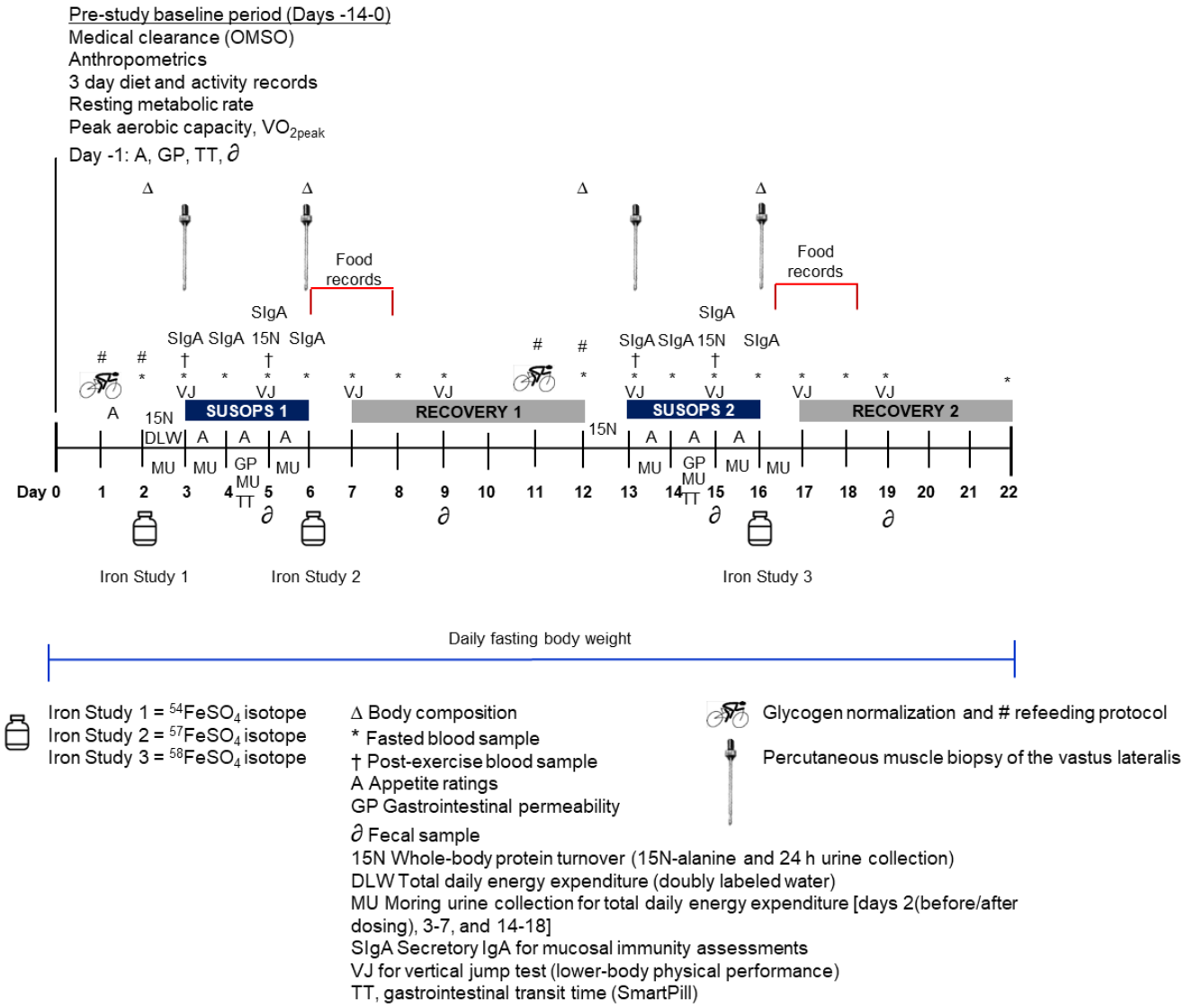
B5.2.4 Exclusion Criteria

- Musculoskeletal injuries that compromise exercise capability
- Metabolic or cardiovascular abnormalities (e.g., kidney disease, diabetes, cardiovascular disease, etc.)
- History of any disease or abnormality of the gastrointestinal tract including (but not limited to) diverticulosis, diverticulitis and inflammatory bowel disease, peptic ulcer disease, Crohn's disease, ulcerative colitis; or previous gastrointestinal surgery
- Anemic (plasma ferritin < 40 µg/L, hemoglobin < 13 g/dL) and Sickle Cell Anemia/Trait
- C-reactive protein (CRP) > 5 mg/dL
- Abnormal PT/PTT test or problems with blood clotting
- History of complications with lidocaine
- 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 or other substance abuse issues; use of anabolic steroids
- Blood donation within 4 months of beginning the study
- Oral antibiotic use within 3 months of participation
- Colonoscopy within 3 months of participation
- Use of laxatives, stool softeners, or anti-diarrheal medications more than once/month
- Currently using benzodiazepines, anti-depressants or anti-histamines
- Pacemaker or other implanted electronic medical device

- Are unwilling or unable to eat study diets and foods provided and/or follow exercise prescriptions

B5.3 Research Procedures

Figure 3. Experimental Design



Experimental Design

After completing pre-study baseline testing [e.g., medical clearance, anthropometrics, diet and activity records, resting metabolic rate (RMR), peak aerobic capacity (VO_{2peak})] and exercise and performance familiarization trials, volunteers will complete a glycogen normalization protocol and be provided a 48 h carbohydrate refeeding diet on days 1 and 2 to restore muscle glycogen content pre-SUSOPS (**Figure 3**). On day 2, following an ≥ 8 h fast (0000 h), volunteers will undergo the first of three fractional iron absorption studies (Iron Study 1), during which time volunteers will ingest a stable isotopically labeled iron ($^{54}FeSO_4$) drink followed by serial blood draws over the next 6 h. A fasting blood draw and percutaneous

muscle biopsy of the vastus lateralis will be collected on the morning of day 3. Volunteers will also complete approximately 3-4 mood/cognitive-performance familiarization sessions and 1 baseline session before the SUSOPS testing period. Immediately thereafter, volunteers will complete a 72-h SUSOPS, and will be provided either sufficient food (combat rations) to maintain energy balance within ± 10% of estimated total daily energy expenditure (SUSOPS BAL) or only enough food to match 45% of total daily energy expenditure to elicit severe negative energy balance (SUSOPS NEG BAL). Volunteers will reside at the Doriot Climatic Chambers during the SUSOPS beginning the evening of days 1 and 11 (volunteers will be provided standardized meals), and ending after final data collection on days 6 and 16. Throughout these periods, volunteers will wear the USARIEM Vigilance Monitoring System, the Fatigue Science ReadiBand™ actigraph, Philips Respironics Actiwatch® Spectrum Plus, or equivalent device on their non-dominant wrist. The activities performed will be comprised of load carriage and unloaded steady-state exercise and Warfighter operational tasks (e.g., 15 m timed casualty evacuation drag of a 123 kg dummy, move under fire, prepare fighting positions) to increase total daily energy expenditures. Physical performance will be assessed before and after each SUSOPS. Mood, cognitive performance and overall vigilance will also be assessed. Sleep will be restricted to four hours per night during SUSOPS to mimic recent field studies. On day 6, immediately following the final exercise bout and 8 h after dinner on day 5, a second muscle biopsy will be collected (alternate leg from biopsy one) and volunteers will undergo Iron Study 2 ($^{57}FeSO_4$). Days 7-12 will serve as a recovery period from SUSOPS 1 (Recovery 1). Fasted blood draws will be collected on days 7-9 during Recovery 1, and a second baseline mood/cognitive test session will occur during Recovery 1. Starting on day 11, volunteers will complete a second glycogen normalization and 48 h carbohydrate refeeding protocol. On day 13, following an overnight fast, a blood draw and a third muscle biopsy (alternate leg from biopsy two) will be collected. Immediately thereafter, volunteers will complete the second 72 h SUSOPS. All testing, planned activity, and sleep restrictions will be the same as described for SUSOPS I; however, volunteers will switch to either SUSOPS BAL or SUSOPS NEG BAL. Iron Study 3 ($^{58}FeSO_4$) and the fourth and final muscle biopsy (alternate leg from biopsy three) will occur on day 16 immediately following the final exercise bout and 8 h after dinner on day 15. Days 17-22will serve as Recovery 2 (7 d post-SUSOPS recovery blood draw will be collected on day 22 to match the 7 d recovery from SUSOPS 1) Diet records will be recorded on days 6-8, and 16-18.

SUSOPS BAL and SUSOPS NEG BAL

The SUSOPS will be comprised of a variety of Warfighter tasks, designed to elicit high energy expenditures, muscle damage and fatigue, sleep deprivation, and decrements in physical and cognitive performance as well as self-reported mood status. Most importantly, the SUSOPS will be designed to elicit a marked inflammatory response. Physical activity/exercise will be prescribed at levels to expend ~5000-6000 total kcal/d using the ACSM metabolic equations for steady-state exercise [64] and the compendium of metabolic equivalents for physical activities [65]. Total daily energy expenditure estimates will be consistent with our recent reports in Norwegian Soldiers and matched between SUSOPS BAL and NEG BAL [55, 66]. Combat rations (Meal Ready-to-Eat) will serve as the underlying diet during SUSOPS BAL and NEG BAL (refer to SUSOPS Diet Intervention section for details). Low-to-moderate intensity (30-65% VO_{2peak}) steady-state endurance-type exercise will be the primary exercise modality. Volunteers will perform three prolonged steady-state exercise bouts per day. All three exercise sessions will be conducted outdoors on Natick Soldier Systems Center grounds (NSSC fitness trail). Two of the three exercise bouts will be ~60-180-min load carriage exercise sessions, whereas the third will be unloaded. The total distance covered will be dictated by individual exercise prescriptions, and the load carried will be ~32 kg [comprised of the basic uniform (~5.3

kg), weapon and tactical equipment (~11.2 kg), and rucksack (~15 kg)]. The loads carried are consistent with infantry occupation standards. Volunteers will be permitted to consume water ad libitum throughout each exercise trial to maintain hydration. During the remainder of each day, volunteers will perform a number of Warfighter tasks [e.g., 15 m timed casualty evacuation drag of a 123 kg dummy, move under fire (15, 6.6 m rushes with weapon and full combat load (32 kg) for time, with 5 secs between rushes), prepare fighting positions (move 16, 18 kg sandbags 10 m while wearing full combat load minus a weapon (~26.5 kg)]. The Warfighter task measures will be performed daily to increase energy expenditure by simulating some operational tasks. Volunteers will perform at least two familiarization trials of each task to reduce injury risk during the pre-study baseline period. Mood, cognitive, and vigilance will also be assessed. Self-reported mood and cognitive performance will be assessed twice per SUSOPS day in addition to the sessions performed prior to SUSOPS 1 during the baseline testing phase of the protocol and the session performed during Recovery 1. Vigilance testing using the USARIEM Vigilance Monitoring System will only be active during specific intervals of wake time. Sleep will be restricted to 4 h per day to be consistent with previous USARIEM SUSOPS studies beginning the evening of days 3 and 13(i.e., volunteers will not be permitted to sleep until 0100 the morning of days 4 and 14) [67].

Warfighter Tasks

Move under Fire: During this task, volunteers will wear an approximately 32 kg fighting load [comprised of the basic uniform (~5.3 kg), weapon and tactical equipment (~11.2 kg), and rucksack (~15 kg)]. They will begin the task in the prone position. Upon command, volunteers will sprint approximately 6 m to a marker and assume the predetermined position for that marker (either the kneeling or prone position). They will remain in this position for approximately 5 seconds. Upon signal, volunteers will get up and sprint approximately 5 to 8 m to the next marker and assume the predetermined position for that marker. This will be repeated until they have covered a total of 100 m (15 rushes of ~6.6 m). After starting in the prone position, the next two positions will be kneeling, and then a prone position until the course is completed. Time to complete the task will be recorded. Each testing session will take approximately 1-2 minutes.

Casualty Evacuation Drag: Volunteers will drag a simulated casualty (approximately 123 kg) up to 15 m as fast as possible in 60 sec, while wearing an approximately 32 kg fighting load [comprised of the basic uniform (~5.3 kg), weapon and tactical equipment (~11.2 kg), and rucksack (~15 kg)]. If the volunteer is unable to pull the casualty the full 15 m in 60 sec, the distance the casualty was dragged will be measured to the nearest 0.25 m.

Prepare Fighting Positions (Sandbag Carry): While wearing a fighting load minus the weapon (approximately 26.5 kg), volunteers will lift and carry a total of 16 sandbags weighing 18 kg each, carry them 10 m, and place them on the floor in a 4 bag wide x 2 bag deep x 2 bag high formation as quickly as possible. The completion time will be recorded. The test is expected to take approximately 3-5 minutes per volunteer.

Diet and Total Daily Energy Expenditure Prescription during SUSOPS

Volunteers will complete a 3 d diet record and a 3 d activity log during baseline pre-study testing (days -14-0) and according to instructions provided by a registered dietitian to characterize pre-study diet and exercise habits (see “Diet Record” and “Activity Log”). To predict total daily energy expenditure (TDEE), volunteers’ resting metabolic rate (RMR) will be measured during the baseline pre-study testing period using standardized techniques and an indirect, open circuit respiratory system (True Max 2400, ParvoMedics, Sandy, Utah, USA). RMR will be multiplied

by a factor of 1.3 to estimate energy expenditures for activities of daily living. Together, these data will serve as the baseline total daily energy requirements for SUSOPS 1 and 2.

To increase TDEE to approximately 5000-6000 kcal/d, volunteers will be prescribed low-to-moderate intensity (30-65% of pre-determined peak oxygen uptake, VO_{2peak} and corresponding heart rate) endurance-type exercise using the ACSM metabolic equations for steady-state exercise [64] and the compendium of metabolic equivalents for physical activities (refer to descriptions of Determination of Peak Oxygen Consumption and SUSOPS BAL and SUSOPS NEG BAL below) [65].

For example, an 85 kg individual with a baseline total daily energy requirement (RMR of 2000 kcal/d * 1.3) of 2600 kcal/d and a VO_{2peak} of 45 mL/kg/min, would expend approximately 2468 kcal during two, 90-min load carriage exercise bouts combined [i.e., 85kg+32kg load = 117 kg * (~50% of VO_{2peak} is 6.7 METS *3.5 mL/kg/min)/1000) * (90 min of work * 5 kcal/L/min) = 1234 kcal/exercise bout], 600 kcal during a 60-min unloaded exercise session (calculations follow the same format), and approximately 100 kcal/d performing the Warfighter tasks, for an estimated TDEE of ~5768 kcal/d. TDEE prescriptions are individualized to each volunteer's requirements and will be held constant between SUSOPS 1 and 2. Exercise intensities, total duration exercised, and TDEE will likely differ between volunteers. Every attempt will be made to maintain TDEE between 5000-6000 kcal/d across all volunteers; however, some individuals may expend more and others less.

Registered Dietitians will develop individualized daily menus for SUSOPS 1 and 2 using Food Processor SQL (ESHA Research, Salem, OR, Version 10.14). The diets during each SUSOPS period will be derived primarily from components of the US military Meals Ready-to-Eat (MRE) rations to achieve appropriate macronutrient proportions. The macronutrient distribution of the MRE-based diets will not be manipulated. Thus, volunteers will be consuming a diet providing approximately 60% carbohydrate, 10-15% protein, and 25-30% fat. The macronutrient distribution, as a % of total energy, will remain constant during SUSOPS NEG BAL by uniformly reducing total energy intake across carbohydrate, protein, and fat. This feeding paradigm will most closely resemble field-feeding practices observed during recent USARIEM studies [55, 66]. The micronutrient content of the ration will not be altered. However, to limit the potential confounding effect of differing iron intakes across SUSOPS 1 and 2, volunteers will consume supplemental iron sulfate mixed in water (Ferrous sulfate drops, RxChoice, available OTC) during SUSOPS NEG BAL to match total iron consumed during SUSOPS BAL. This equates to only ~4-10 mg supplemental iron and will be consumed with one meal each day to mimic iron intake during SUSOPS BAL (iron content of SUSOPS BAL is approximately 20-25 mg/d). Water will be allowed ad libitum and total amount consumed will be recorded by study team members. During the 48 hours prior to each SUSOPS period (beginning on days 1 and 11, volunteers will be fed meals derived of commercially available foods which will meet calculated energy requirements to maintain their body weight.

Volunteers will receive instructions from study dietitians on how to consume an ad libitum diet with the same macronutrient distribution during the two recovery periods (see "Choosing Your Foods During Recovery" document). Food records will be maintained by study volunteers on days 6-8 and 16-18. Volunteers will meet with study dietitians to review these records. These records will be used to determine how energy deficit during SUSOPS impacts food selection during recovery. Physical activity will be restricted during both recovery periods.

Determination of Total Daily Energy Expenditure

Doubly labeled water (DLW) will be used to determine actual TDEE during SUSOPS 1 and SUSOPS 2 and verify the accuracy of estimated TDEE. DLW will be administered during the first iron study on day 2 (~0000 h after an 8 h fast). Immediately before drinking the DLW, volunteers will provide a urine sample to determine the natural abundance of ^{18}O and ^{2}H. They will not eat or drink anything for 4 h (~0400 h) after consuming a total of 120 g DLW containing 10% $H_2{}^{18}O$ (~0.285 g $H_2{}^{18}O$·kg total body water [TBW]$^{-1}$) and 99% $^{2}H_2O$ (~0.15 g $^{2}H_2O$·kg TBW^{-1}; Sigma-Aldrich, St. Louis, MO or similar company). The DLW dose container will be rinsed with local tap or bottled water, which the volunteer will drink. Urine samples will then be collected approximately 4 h and 6 h after the DLW dosing for initial TBW determinations to be made. Volunteers will be free to eat and drink after these urine samples.

Two volunteers will be chosen at random to consume only locally available drinking water to control for natural changes in ^{2}H and ^{18}O abundance (local water will be analyzed to determine isotopic enrichments). Rate of disappearance of ^{18}O and ^{2}H for volunteers dosed with DLW will be corrected for mean changes in background enrichments based on controls. Morning urine samples will be collected daily during each SUSOPS period to determine elimination rates over time. TBW will be calculated by determining the regression line for the elimination of ^{2}H and ^{18}O and extrapolated to a maximum enrichment. All urine samples will be collected and stored in 5ml tubes and shipped to the Pennington Biomedical Research Center (PBRC) for analysis.

Enrichments of ^{2}H and ^{18}O will be measured using isotope ratio mass spectroscopy (Finnigan Mat 252, Thermo Fisher Scientific, Waltham, MA or similar model). The ^{2}H and ^{18}O isotope elimination rates (k_H and k_O) will be calculated by linear regression using the isotopic disappearance rates during each training phase.

$$rCO_2 = (N/2.078)(1.01k_O - 1.04k_H) - 0.0246rH_2O_f$$

where N is TBW; k_O and k_H are ^{18}O and ^{2}H isotope disappearance rates respectively, and rH_2O_f is the rate of fractionated evaporated water loss and is estimated to be 1.05 N * (1.01 k_O – 1.04 k_H). Total daily energy expenditure will then be calculated using the energy equivalent of CO_2 for a respiratory quotient of 0.86 [68].

Anthropometric Data

Anthropometrics and body composition will be performed using standardized techniques and equipment to characterize study volunteers, and evaluate responses to SUSOPS 1 and 2. Height will be measured in duplicate to the nearest 0.1 cm using a stadiometer at baseline. Body weight will be measured, nude (scale will be placed in a locked bathroom) and after an overnight fast (≥ 8 h), using a calibrated digital scale to the nearest 0.1 kg at baseline (days -14-0) and then daily throughout the 22 d study to ensure weight maintenance and/or weight loss.

Body composition will be determined on days 2, 6, 12, and 16 using a four-compartment model derived from dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI) and bio-electrical impedance (InBody 720, BIOSPACE, Korea) [69]. The DEXA technique allows for the non-invasive assessment of soft tissue composition by region with a precision of 1-3%. The volunteer will lay face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet, and will be asked to remain motionless for the 8-10 min scan. The InBody measure takes less than a minute to complete, will be performed immediately before or after the DEXA, with volunteers wearing the same clothing described for DEXA. These data will be used to calculate body water, protein (i.e., lean mass), mineral (i.e., bone), and fat mass.

Determination of Peak Oxygen Uptake

Peak oxygen uptake (VO_{2peak}) will be determined using an indirect, open circuit respiratory system (True Max 2400, ParvoMedics, Sandy, Utah, USA) on a cycle ergometer. The value will be used to better estimate exercise-induced energy expenditure (EIEE) during SUSOPS and to determine the workloads necessary for the glycogen normalization protocol (see below). Volunteers will be clothed in appropriate athletic attire and perform this assessment in a temperature and humidity controlled room. The volunteer will be allowed to warm-up by pedaling at 70 W for 5 min. 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. Every minute, workload intensity will be progressively increased by 30 W until the volunteer is fatigued or unable to maintain a pedaling rate that either maintains or increases O_2 consumption. Heart rate will be monitored using a heart-rate monitor (Polar Electro Inc, Oulu, Finland) and recorded during the last 30 sec of each workload. 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 the testing equipment fails.

Glycogen Normalization Protocol

To normalize muscle glycogen content and its potential influence on inflammation leading into both SUSOPS periods [days 1 and 11 following an overnight fast (≥ 10 h)], participants will perform a glycogen normalization protocol followed by a 2-d refeeding protocol. The glycogen normalization protocol will be completed on a cycle ergometer. The intensity will be based on VO_{2peak}. Volunteers will begin with a 5 min warm-up at 50% VO_{2peak} before beginning the protocol. After a warm-up period, the cycle ergometer protocol is comprised of repeated periods of 2 min of work at 80 ± 5% $\dot{V}O_{2peak}$ followed by 2 min of recovery at 50 ± 5% VO_{2peak}. The protocol will last approximately 50-min (12 work:rest cycles). To ensure familiarity with the testing procedures, volunteers will perform one practice session during the baseline pre-study period. Volunteers will be permitted to consume water *ad libitum* during the protocol.

After completing the glycogen depletion protocol, volunteers will be fed a controlled diet prescribed to maintain energy balance and provide at least 6.0 g carbohydrate $\cdot kg^{-1} \cdot d^{-1}$ (~55-60% of total energy consumed) to ensure adequate glycogen repletion and homogeneous glycogen levels within and across volunteers between SUSOPS phases (BAL and NEG BAL). 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.

Determination of Fractional Iron Absorption

Volunteers will undergo three acute fractional iron absorption studies (Iron Studies 1, 2, and 3). The studies will occur on days 2, 6, and 16 in the morning (~0000 h) after an 8 h fast. The iron studies on days 6 and 16 will occur ~3 h after completing the last exercise bout of the SUSOPS to correspond to peak hepcidin responses to exercise. An indwelling 18 gauge intravenous catheter will be placed in the antecubital fossa (or distally) and a baseline blood sample will be drawn before consuming the iron isotope (0 min). Volunteers will then consume a 300 mL drink containing 3.8 mg iron (representative of dietary iron in an iron-rich meal) as isotopically labeled $^{54}FeSO_4$, $^{57}FeSO_4$, or $^{58}FeSO_4$ (Trace Sciences International). Venous blood samples will be collected at 20, 40, 60, 120, 240, and 360 min-post iron loading [70] to assess the effects of a transient exercise-induced increase in IL-6 (and hepcidin) on the appearance of absorbed iron in blood.

Percutaneous Muscle Biopsy of the Vastus Lateralis

Percutaneous muscle biopsies will be obtained 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 [71, 72]. The biopsy procedures will be performed after a ≥ 8 h fast immediately before starting and after completing each SUSOPS period (days 3, 6, 13, and 16). The muscle biopsies collected on days 6 and 16 will occur within 30 min of completing the final exercise bout before starting iron tracer studies 3 h post-exercise (refer to Determination of Fractional Iron Absorption). The muscle samples obtained will be analyzed for muscle glycogen content, intramuscular markers of inflammation, proteolytic, energy-sensing, and anabolic cell signaling. Volunteers will undergo four total biopsies. Each biopsy will require a new incision and the biopsied leg will alternate between days.

Whole-Body Protein Utilization

Whole-body protein turnover (^{15}N-alanine) will be assessed before (days 2 and 12) and on the last day of each SUSOPS period (days 5 and 15), as previously reported in several studies from this laboratory [55, 66, 73, 74]. Total nitrogen, ammonia, and urea (^{15}N-nitrogen, ^{15}N- ammonia, and ^{15}N-urea) enrichments will be used to measure whole-body protein turnover [75]. After providing a urine sample to determine background ^{15}N enrichments, volunteers will consume a single dose of ^{15}N-alanine (300 mg·d^{-1}, Cambridge Isotope Laboratories, Andover, MA) and collect their urine for the next 24 h. Total nitrogen intake will be determined from ration dietary analysis.

Urine samples will be frozen and shipped to Metabolic Solutions Inc. (Nashua, NH) for isotopic analysis using a fee-for-service contract. The ^{15}N enrichment of urinary ammonia and N (ratio of tracer to tracee, t:t) will be determined using isotope ratio mass spectroscopy. The t:t ratio for the cumulative sample will be corrected for the background ^{15}N-ammonia enrichment. Nitrogen intake (*I*) during the 24 h period will be determined by ration analysis. Nitrogen flux (*Q*), protein synthesis (PS), protein breakdown (PB), and net protein balance (NET) will be calculated according to Stein et al.[76].

Determining Lower-Body Performance

Physical performance (i.e., lower-body power) will be assessed before and after each SUSOPS and during each recovery period on days 3, 5, 7, 9, 13, 15, 17, 19using the vertical jump test (Vertec™ device). Volunteers will place their feet at shoulder width with their knees slightly bent while standing on a flat, clean surface. On command, volunteers will perform an arm swing and a countermovement to a self-selected depth and jump with maximal effort. Volunteers will tap the fins of the Vertec™ at peak jump height. Volunteers will receive three attempts separated by one minute of recovery. Vertical displacement (jump height) will be calculated as the difference between maximal jump height and reach height and peak power will be calculated using the following equation [77].

Peak Power (Watts) = [60.7 x jump height (cm)] + [45.3 x body mass (kg)] - 2055

Volunteers will receive detailed instruction, demonstration by study staff, and complete two familiarization trials during the pre-study period. All experimental conditions will be held constant between testing days.

Eating behavior, appetite and food preferences and cravings

Volunteers will rate self-perceived appetite using visual analog scales (see “VAS” document). The VAS will be administered before and after meals, and before and after morning exercise on

study days -1 (may be adjusted ± 2d if needed), 3-5, and 13-15, (note on day -1 the VAS will be administered at the same time of day as the morning exercise sessions on days 3-5, and 13-15). Visual analog scales will be administered using paper and pencil, or via electronic means (e.g., tablet or laptop). During study day -1, participants will remain in the lab under staff supervision from breakfast through dinner (~0600-~1900). All meals will be provided in sufficient quantity to meet weight maintenance energy requirements, and participants will remain sedentary throughout the day. Appetite-mediating hormones (acyl ghrelin, leptin, GLP-1, PYY, PP, and insulin) and metabolic markers putatively involved in appetite regulation (glucose, FFA, BHB) will be measured following a ≥8 hr fast on the mornings of study days 3, 5, 7, 9, 13, 15, 17, and 19 and following the morning ruck march on days 3, 5, 13 and 15.

Food preferences will be measured using the Leeds Food Preference Questionnaire (LFPQ) which will be administered before and after lunch on study days -1 (may be adjusted ± 2d if needed), 5, and 15. The LFPQ is a computerized platform that measures different components of food preference and hedonics, and is administered on a computer [78]. For the tests, 32 pictures of individual food items that vary in 3 dimensions; protein (low and high), taste (sweet and savory), and/or fat (low and high) will be selected from a validated database of common foods. At least four pictures from each food type will be selected. To measure explicit liking (i.e., perceived hedonic impact of the food), the volunteer will be shown each picture in random order and asked to respond to the question “how pleasant would you find the taste of this food right now” using a visual analog scale. To measure explicit wanting (i.e, conscious desire to consume a food), the volunteer will be shown each picture in random order and asked to respond to the question “how much do you want to eat this food right now”, using a visual analog scale. Mean ratings for each food category will be computed to determine explicit liking and explicit wanting for each food type. Implicit wanting (i.e., automatic subconscious attraction to a food) will be measured using forced choice methodology. Pairs of food images from separate food categories will be presented in randomized pairs on the computer screen. Volunteers will be asked to as quickly as possible select the food that they most want to eat at that moment. Both frequencies of selections within each food category and response time will be recorded. A familiarization session will be conducted before study day -1. The LFPQ will be will be purchased or used with permission and takes ~15min to complete

Gut microbiome composition and activity

Volunteers will collect 5 separate fecal samples to determine the effects of SUSOPS with and without energy deficit on gut microbiome composition and activity. A single fecal sample will be collected during baseline (days -1-0 (may be adjusted ± 2d if needed)), and study days 5-6, 9-10, 15-16, and 19-20. At each time point participants will be given a 48-h window to collect a usable sample. A usable sample is defined as being >15g wet weight, and having been delivered to study staff as soon as possible and within 12 hr of defecation while being kept cold but not frozen from the time of collection to delivery. If a participant does not provide a usable sample within the timeframe noted above, the collection period will be extended until a usable sample is produced. To collect fecal samples, all participants will be given pre-labeled containers with covers and a plastic device to hold the container in the toilet. Participants will defecate into the collection container which will then be given to study staff. During free-living phases of the study participants will be given plastic sealable bags, a cooler or insulated bag, and ice packs to store and transport the samples (see “Fecal Collection Instructions”). Gut microbiota composition, function, and activity will be measured at the US Army Center For Environmental Health Research (USACEHR), Ft Detrick using next generation shotgun-sequencing and non-targeted metabolomics. Additional aliquots will be archived by USARIEM at -80°C and may be analyzed for other gut related outcomes at a later date.

Gastrointestinal permeability

A differential sugar absorption test will be used to provide a functional assessment of gastrointestinal permeability [79]. For this test participants will consume 2 g sucralose and 2 g erythritol dissolved in 180 mL water in the morning during study days -1 (may be adjusted ± 2d if needed), 4, and 14. Sucralose and erythritol are sugar substitutes commonly used in a variety of food products. Consumption of the solution will be conducted under staff supervision. Participants will then collect urine produced over the subsequent 24 hr. Urine aliquots will be taken after 5 hr and 24 hr and frozen immediately. Urine sucralose and erythritol concentrations will be analyzed by PBRC, and will provide a measure of small intestinal, large intestinal and whole-gut permeability [79]. Coolers or insulated bags with ice packs will be used to keep urine cool and for transport of samples outside of the lab (see "Fecal Collection Instructions").

Gastrointestinal transit time

Gastrointestinal transit time will be measured on days -1, 4 and 14 using the SmartPill (Medtronic, Minneapolis, MN). The SmartPill is an ingestible FDA-approved wireless motility capsule similar in size to a multi-vitamin pill that transits the gastrointestinal tract while transmitting data to a receiver kept near the body [http://www.medtronic.com/covidien/products/motility-testing/smartpill-motility-testing-system#]. The device continuously measures gastrointestinal pH, core temperature, gastrointestinal pressure, and gastrointestinal transit time for up to 5 d, and can isolate transit through the stomach, small bowel, and colon [80]. One sterile SmartPill will be ingested immediately after breakfast on days -1, 4, and 14 under staff supervision. The pill is easy to swallow and volunteers will be given ample instruction. Elimination of the pill from the gastrointestinal tract will be confirmed by a temperature decrease when the capsule passes from the body into toilet water. If a pill stops transmitting before being eliminated from the body, a new pill may be administered and fecal sample collections will be used to visually search for and confirm exit of the pill that is no longer transmitting. We expect most volunteers will pass the capsule within 24-48 hr based on data from Rao et al. [81] who reported median [IQR] whole gut transit time of 30 hr [22-46 hr] in 81 healthy adults. Volunteers will wear a bracelet indicating the pill is inside of them until elimination from the body is confirmed by study staff. Transit time will be used in interpretation of the gut microbiota, gastrointestinal permeability, and appetite results as changes in transit time could impact all of these measures. A subset of volunteers will be recruited randomly to participate in this procedure using a random number generator. If any volunteer who is randomly selected reports difficulty swallowing large pills or any other contraindication (see below), they will be replaced with a randomly selected alternate.

Contraindications:

- History of gastric bezoar
- Swallowing disorders; severe dysphagia to food or pills
- Suspected or known strictures, fistulas, or physiological/mechanical GI obstruction
- Implanted or portable electro-mechanical medical devices
- Using proton pump inhibitors
- Cannot stop use of the following medications within 48hr of procedure: histamine blockers, GI motility-altering medications, antiemetics & 5HT3 antagonists, macrolides, anticholinergics, 5HT4 partial agonists, antacids

Gastrointestinal health log

Frequency of bowel movements and subjective ratings of gastrointestinal symptoms (e.g., flatulence, constipation, loose stool) will be assessed during study days -1 (may be adjusted ± 2d if needed), 3, 6, 13, and 16, using modified versions of the Irritable Bowel Syndrome-Symptom

Severity Score Questionnaire [82] and the Gastrointestinal Quality of Life Index [83] (see "Gastrointestinal Health Log"). Both questionnaires are publicly available and take <5min to complete.

Assessment of mucosal immunity

Secretory IgA will be assessed from saliva samples at baseline and each morning of SUSOPS to evaluate mucosal immunity. Saliva samples will be collected using the non-invasive commercially-available Salimetrics polyester Oral Swab (SOS) technology (Salimetrics, CA, USA). Subjects will be asked to place one SOS under their tongue for 3 minutes before returning it to the provided vial. Participants will then place a second SOS under their tongue until the swab is saturated with saliva. This sampling technique will enable us to obtain the required amount of saliva to determine sIgA concentration and the salivary flow rate at each time point. Salivary SIgA is a well-documented biomarker of mucosal innate immunity, used to predict upper respiratory tract infections and to study the influence of stress and dehydration on immune function [84-86]. The collected saliva will be stored at -80°C until analysis. Saliva osmolality will be determined using a freezing point depression osmometer, and samples will be analyzed simultaneously for sIgA concentration using a commercially-available ELISA.

Self-Reported Mood, Cognitive Performance, and Vigilance Assessments

Several mood and cognitive performance assessment sessions will be conducted during the pre-SUSOPS period of testing so that volunteers become familiar with the test battery and their performance is stable. The last of these sessions will serve as the baseline data for statistical analyses. During the simulated SUSOPS periods, a morning and evening mood/cognitive test session will be conducted on each day. Ambulatory vigilance will be assessed via the wrist-worn USARIEM Vigilance Monitoring System. The Fatigue Science ReadiBand™ actigraph, Philips Respironics Actiwatch® Spectrum Plus, or an equivalent device will record sleep data at night and daytime motor activity. (see "POMS" and "EVAR" documents).

The Profile of Mood States (POMS): The Profile of Mood States (POMS) Questionnaire [87] is a 65-item inventory of self-reported mood states that is sensitive to a wide variety of nutritional manipulations including undernutrition[88] and environmental factors including hypoxia [89], sleep loss, and sub-clinical doses of various drugs [90-92]. Participants rate each of 65 mood-related adjectives on a five-point scale, in response to the question, "How are you feeling right now?" The adjectives factor into six mood sub-scales (tension/anxiety, depression/dejection, anger/hostility, vigor/activity, fatigue/inertia, and confusion/bewilderment. The POMS will be used to assess the overall mood states of the participants in the present study. The POMS Questionnaire takes less than 5 minutes and will be administered via computer software. This questionnaire will be purchased and used with permission.

Evaluation of Risks Scale (EVAR): The Evaluation of Risks (EVAR) Questionnaire measures willingness to take risks through participant responses to 24 items which they mark on a visual analog scale [93]. Each end of the line is anchored by descriptors such as "not at all" and "very much." Respondents simply mark the point on the line that best describes their current feeling state. The scale yields five factors, including Self-control, Danger-seeking, Energy, Impulsiveness, and Invincibility, as well as a Total Risk-Taking Propensity score, which is derived by summing all 24 items. These items are internally consistent, yielding a coefficient a of 0.78 [94]. The scale has been shown to differentiate individuals who routinely engage in risky behavior, and it also correlates with measures of sensation-seeking and other risk-related traits [93, 94]. The EVAR takes less than 5 minutes to complete. This questionnaire is publicly available.

The Psychomotor Vigilance Test (PVT): The Psychomotor Vigilance Test (PVT) measures simple visual reaction time which is particularly sensitive to the vigilance decrements associated with sleep restriction or disruption [95]. A series of stimuli are presented at random intervals on a screen and the subject must respond as rapidly as possible when a stimulus appears. The subject hits either the left or right arrow keys to respond to the stimulus. Parameters recorded include reaction time, false alarms, and number of lapses (long duration responses). PVT performance lapses refer to the times when a subject fails to respond to the task in a timely manner (i.e., > 500 msec). The test requires subjects to sustain attention and respond to a randomly appearing stimulus on a computer screen by pressing a button. The PVT takes 10 minutes to complete and will be administered via computer software. This test was developed at USARIEM and does not require any permission for use.

Matching to Sample: The Matching to Sample Test assesses short-term spatial memory (working memory) and pattern recognition skills [90, 91]. The participant responds by pressing the down arrow key when the word "READY" appears on the screen. The participant is then presented with an 8 X 8 matrix of a red and green checkerboard on a color screen. The matrix is on the screen for 4 seconds. Afterwards, the sample is removed and followed by a variable delay interval during which the screen is blank (except for the word delay at the bottom of the screen). After the delay, two matrices are presented on the screen: the original sample matrix and a second matrix that differs slightly in that the color sequence of two of the squares will be reversed. The participant selects the comparison matrix by responding on the left or right arrow key that matches the original sample matrix. A response (left or right arrow key) must be made within 15 seconds; otherwise a time-out error will be recorded. Correct responses will also be recorded, as will response time to choose a matrix. The task lasts approximately 5 minutes and will be administered via computer software. After the delay, two matrices are presented: the sample matrix and a second matrix that differs slightly in that the color sequence of two of the squares is reversed. The participant selects the comparison matrix by responding on the left or right arrow key that matches the original sample matrix. A response must be made within 15 seconds; otherwise a time-out error will be recorded. Correct responses will also be recorded, as will response time to choose a matrix. The task lasts approximately 5 minutes and will be administered via computer. This test was developed at USARIEM and does not require any permission for use.

Grammatical Reasoning: The Grammatical Reasoning Test assesses language-based logical reasoning and has been used to assess the effects of various treatments on cognitive function [96]. It has been adapted from the Baddeley Grammatical Reasoning Test. On each trial, the letters AB or BA follows a statement. The participant must decide whether or not each statement correctly describes the order of the two letters. The "T" key on the keyboard is pressed for correct (statement is true) and the "F" key is pressed for incorrect (statement is false). Statements can be positive/negative or active/passive, and a given letter may precede/follow the other letter. A session lasts for 32 trials and is made up of the above combination of statements. The time to complete this test is approximately 5 minutes and it will be administered via computer software. This test was developed at USARIEM and does not require any permission for use.

The N-back Task: The N-back Task measures working memory [97]. It requires on-line monitoring, updating, and manipulation of remembered information and allows for the parametric assessment of different working memory loads. Participants will be shown a series of letters one at a time in the center of a computer screen and will be required to mentally take note of those depicted letters. They will then respond by pressing the spacebar if the letter presented is the same as the previous letter (1-back condition), 2 previous letters back (2-back condition), or 3 previous letters back (3-back condition). Dependent measures include response time and accuracy. This task takes

approximately 15 min to complete and will be administered via computer software. This version of the task was developed at USARIEM and does not require any permission for use.

The Balloon Analogue Risk Task: The Balloon Analogue Risk Task (BART) is a computerized test designed to measure willingness to take risks versus "play it safe" and requires the participant to fill a simulated balloon with air [98]. Points are given for keeping the balloon as full as possible. The more expanded the balloon gets, the more points are earned. However, all points are lost if the balloon is over-inflated and pops. The object of this task is to earn as many points as possible by keeping the balloon inflated without popping. Additionally, there is a risk-learning component to this task as some balloon colors pop with less inflation and others with more, while a third category is unpredictable. Standard administration of this task allows 30 trials. The BART takes approximately 10 min to complete and will be administered via computer software. This test is publicly available and does not require permission for use.

Ambulatory Vigilance and Sleep Evaluation: The USARIEM developed wrist-worn Vigilance Monitoring System (produced in collaboration with PCD, Inc., Ft. Walton Beach, FL) will be used to continuously measure a variety of key behavioral and environmental factors while volunteers are engaged in daily activities. The monitors are lightweight devices slightly larger than a wristwatch and are worn on the non-dominant wrist. Each monitor contains a microprocessor, non-volatile memory, and several other sensors. The monitors measure ambient temperature, sound intensity, and environmental light levels as well as vigilance, rest, and activity. The monitors will be programmed such that at random intervals, averaging approximately 10 times an hour, an audible tone sequence, a light stimulus and/or a vibratory stimulus, similar to the vibration of a pager or cell phone, will be emitted. Vigilance during Baseline, SUSOPS and recovery will be assessed during specific standardized times. The volunteer will be required to push a small button on the monitor in response to the stimuli. Vigilance and response time will be assessed by monitoring correct responses and the latency to respond to the tone. These measures provide estimates of different aspects of vigilance, the ability to detect stimuli and the ability to respond as rapidly as possible. Measurements of sleep and activity will be collected using the Fatigue Science ReadiBand™ actigraph, Philips Respironics Actiwatch® Spectrum Plus, or equivalent device. Volunteers will wear the monitors during the baseline and simulated SUSOPS portion of the research.

B5.4 Data Collection

Determination of Fractional Iron Absorption and Circulating Biomarkers of Inflammation and Muscle Damage and Nutritional, Metabolic, and Androgen Status

Fasted (≥ 8 h) blood draws, and post-exercise blood draws will be collected as outlined below to assess fractional iron absorption and relevant biomarkers of inflammation, muscle damage, appetite, and nutritional and metabolic stress responses to SUSOPS (**Tables 1**). A total of 42 blood draws (≤ ~530 mL) will be collected during the entire study.

Table 1. Blood sample collection per analyte[1]

		SUSOPS 1				Recovery 1				SUSOPS 2				Recovery 2			
Days	2	3	4	5	6	7	8	9	12	13	14	15	16	17	18	19	22
Tracer/tracee[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
Hgb/Hct	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Erythroferrone[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
c-myomiRNA		x		x	x					x		x	x				
Exo miRNA		x		x	x					x		x	x				

Ferritin[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
sTfR	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Hepcidin[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
Iron[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
TIBC	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
IL-6[2]	7	x	x	x	7	x	x	x	x	x	x	x	7	x	x	x	x
hs-CRP	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Erythropoietin		x			x				x			x				
Cortisol		x		x		x		x	x	x		x		x		x	x
CK		x		x		x		x	x	x		x		x		x	x
LDH		x		x		x		x	x	x		x		x		x	x
Myoglobin		x		x		x		x	x	x		x		x		x	x
T-test		x		x		x		x	x	x		x		x		x	x
SHBG		x		x		x		x	x	x		x		x		x	x
LH		x		x		x		x	x	x		x		x		x	x
DHEA-S		x		x		x		x	x	x		x		x		x	x
Insulin[3]		2		2		x		x	x	2		2		x		x	x
FFA[3]		2		2		x		x	x	2		2		x		x	x
Glycerol[3]		2		2		x		x	x	2		2		x		x	x
Glucose[3]		2		2		x		x	x	2		2		x		x	x
β-HB[3]		2		2		x		x	x	2		2		x		x	x
Osmolality	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x
Leptin[3]		x		x		x		x		x		x		x		x	
Acyl ghrelin[3]		2		2		x		x		2		2		x		x	
PYY[3]		2		2		x		x		2		2		x		x	
GLP-1[3]		2		2		x		x		2		2		x		x	
PP[3]		2		2		x		x		2		2		x		x	
I-FABP[3]		2		2						2		2					
Zonulin[3]		2		2				x		2		2				x	
LBP[3]		2		2				x		2		2				x	
S100B[3]		2		2				x		2		2				x	
Ex vivo PBMC	x				x								x				
Serum archive[4]	8	2	x	2	8	x	x	x	x	2	x	2	8	x		x	x
EDTA archive[4]	x	2	x	2	x	x	x	x	x	2	x	2	x	x		x	x
LiHep archive[4]	x	2	x	2	x	x	x	x	x	2	x	2	x	x		x	x
mRNA-seq	x				x			x					x			x	
Metabolomics	x				x			x					x			x	

[1]Analyte abbreviations: Hbg/Hct, hemoglobin/hematocrit; c-myoMIR, circulating muscle-specific microRNA; sTfR, soluble transferrin receptor; T-saturation, transferrin saturation; TIBC, total iron binding capacity; IL-6, interleukin-6; hs-CRP, high-sensitivity C-reactive protein; CK, creatine kinase; LDH, lactate dehydrogenase; T-test; total testosterone; SHBG, sex-hormone binding globulin; LH, luteinizing hormo33.5.ne; DHEA-S, dehydroepiandrosterone-sulfate; FFA, free-fatty acids; β-HB, beta-hydroxybutyrate; GLP-1, glucagon-like peptide-1; PYY, peptide-YY: PP, pancreatic polypeptide; I-FABP, intestinal fatty acid binding protein; LBP, lipopolysaccharide binding protein, PBMC, peripheral blood mononuclear cells. [2]Blood will be sampled seven times during the fractional iron absorption studies through an indwelling venous catheter for the notated analytes (0, 20, 40, 60, 120, 240, and 360 min) for a total of 21 blood draws (seven per iron study). A final blood draw will be taken on each of the iron study mornings (1 per iron study morning; 3 total) through the indwelling venous catheter that is separate from the seven draws for the iron studies in order to analyze the remaining markers (marked with an x on days 2, 6, and 16) at the same time of day as those measured on the other study mornings when blood draws

occur after an overnight fast (venipuncture). [3]A second blood sample will be collected post-exercise for the notated analytes on days 3, 5, 13, and 15. [4]Serum, EDTA, LiHep archived blood samples are derived from the blood draws at each specific time point, and stored in the event certain assays require re-analysis, or for future analysis if any sample remains. Based on this explanation, the total number of study blood draws is 42 (iron study days, 8 x 3 = 24; 2 on days 3, 5, 13, and 15, for 8 total draws; and 1 on every other indicated blood draw day, 10 total). Serum and plasma will be archived from each blood draw. The total volume of blood collected per participant is ≤ ~530 mL.

Saliva analysis

Saliva collection is a non-invasive technique with easy achievability that may facilitate sample collection in austere environments by study volunteers with minimal instruction. The technique may therefore provide an efficient alternative to using blood and/or fecal collection to monitor physiological status in Warfighters engaged in SUSOPS or during deployment. However, the validity of saliva collection as a deployable solution for monitoring physiologic status requires validation. As such, saliva and blood samples will be collected in tandem. Blood will be subjected to mRNA-seq, targeted micro-RNA (see *mRNA, myomiR, and c-myomiR Expression* below) and metabolomics analyses. Saliva will be subjected to microRNA profiling, ion abundance measurement and metabolomics analyses. Results will be compared between sample types, and in relation to the gut microbiome, and cognitive and physical performance outcomes. In addition to validating the approach, analyses will be undertaken to explore associations between the salivary microbiome and stress responses.

Saliva samples will be collected on study days 2, 6, 9, 16 and 19 at the same time blood samples will be collected for mRNA-seq, microRNA-seq and metabolomics assays, respectively. During saliva collection, a Salivette tube with opaque white cap (SARSTEDT, Inc.) will be opened; the swab will be removed and placed in the mouth. After 45 seconds (gentle chewing possible, but not required), the saliva-soaked swab will be returned to the Salivette® which will be closed with the plug for storage and transport. The expected yield is 0.8-1.4 mL saliva. A second round of sample collection will occur 30 minutes later following the same procedure. The tubes will be frozen at -80^{o}C and shipped to USACEHR. Upon receiving the tubes, the tubes will be centrifuged at room temperature at 1000g for 2 minutes. The first tube will be used for microRNA sequencing and microbiome shot gun sequencing. The second tube will be used for metabolomics and targeted proteomics (inflammatory panel of 65 protein markers will be tested using the Luminex platform).

Determination of Fractional Iron Absorption

Stable isotope concentrations in whole blood will be determined by inductively coupled plasma mass spectrometry (ICP-MS) by the Energy and Environmental Sustainability Laboratory (EESL) at Pennsylvania State University (PSU) using a fee-for-service contract.

The amount of absorbed iron in circulation will be calculated using isotope dilution as described previously [70, 99]. Briefly, the amount of absorbed iron circulating in blood will be calculated based on the amount of stable isotope administered, the amount of stable isotope detected in the blood, hemoglobin concentration, and blood volume, which will be estimated based on volunteer height and weight [100]. Isotope dilution will also be used to calculate fractional iron incorporation into red blood cells after administration of the stable isotope and assuming 80% incorporation [70].

An example calculation using ^{54}Fe tracer and endogenous concentrations of ^{56}Fe is included below:

$$^{54}Fe_{inc} = \frac{\left(\frac{^{54}Fe}{^{56}Fe_{enr}} - \frac{^{54}Fe}{^{56}Fe_{base}}\right) Fe_{circ} \cdot NA_{54}}{\frac{^{54}Fe}{^{56}Fe_{base}}}$$

$^{54}Fe_{inc}$ = quantity of ^{54}Fe incorporated into red blood cells
$^{54}Fe/^{56}Fe_{enr}$ = enriched isotope ratio
$^{54}Fe/^{56}Fe_{base}$ = baseline isotope ratio
Fe_{circ} = total circulating iron
NA_{54} = natural abundance of ^{54}Fe

Muscle Glycogen

Approximately 20 mg of muscle from each muscle biopsy 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. Muscle glycogen will be in solution at this point. Glycogen will be quantified by a fluorometric assay (Sigma-Aldrich, St. Louis, MO, USA or equivalent). Muscle glycogen will be quantified to determine the contribution of endogenous carbohydrate availability on inflammation in response to SUSUPS BAL and NEG BAL.

mRNA, myomiR, and c-myomiR Expression

Total RNA will be isolated from approximately 25 mg of muscle using a mirVana™ miRNA isolation kit (Invitrogen, Carlsbad, CA, USA) or equivalent. Quantity and quality of RNA will be assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA will be synthesized into cDNA for analysis of mRNA (iScript™ Advanced cDNA Synthesis Kit; Bio-Rad or equivalent) and a TaqMan® microRNA RT kit (Applied Biosystems, Foster City, CA, USA) or equivalent. Individual primers will be used to determine the mRNA expression of known muscle inflammatory, anabolic, proteolytic, myogenic, and metabolic markers susceptible to stress, to include but not limited to, TNF-α, TNF-αR, IL-6, IL-6R, TWEAK, TWEAK-R, REDD1, Atrogin, MuRF-1, MyoD, Myogenin, Myogenenin, Pax7, PGC-1α, SIRT1, p53, ACC, and AMPK. microRNA analysis will be conducted using individual Taqman® probes (Applied Biosystems) or equivalent, assessing microRNA that may be associated with inflammation and metabolism. This microRNA targets will include, but not be limited to, miR-1, miR-23a/b, miR-26, miR-29, miR-34a, miR-103, miR-107, miR-133a/b, miR-146, miR-206, miR-208a, miR-486, and miR-499a.

Following identification of skeletal muscle microRNA that had a significant change, Taqman® probes (Applied Biosystems) or equivalent will be used to assess the expression of these microRNA in serum to determine their potential use as noninvasive markers of altered metabolism in response to elevated inflammation during and following SUSOPS. Circulating miRNA will be extracted from 200 µL serum using miRNeasy Serum/Plasma kit, which allows for extraction and purification of small (< 200 nucleotides) cell-free RNA (Qiagen, Valencia, CA, USA or equivalent). To avoid introduction of potentially contaminating material, prior to RNA extraction serum samples will be centrifuged for 10 min at 4°C to remove cellular debris. Supernatant will be removed and transferred to a new tube without disturbing the pellet. Due to

the small amount of RNA in the serum, 3.5 µL of a Spike-In Control (C. elegans miR-39; Qiagen or equivalent) will be added to all samples prior to extraction of RNA to determine the yield of template recovered. After extraction 3 µl of serum RNA will reverse transcribed using the TaqMan® microRNA RT kit (Applied Biosystems) or equivalent with miRNA-specific stem-loop RT primers pooled in 1X-Tris-EDTA (TE) buffer for a final dilution of 0.05X. 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) or equivalent for miRNA of interest at 0.05X concentration in 1X TE buffer. All serum miRNA will be normalized to the geometric mean of external (Spike-In Control C. elegans miR-39) and internal controls to allow for both technical and inter-individual normalization [101]. Geometric mean of controls will be used to correct for possible outlying values and abundance differences between the different controls [102].

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 or similar model). A StepOnePlus™ real-time PCR system (Applied Biosystems) or similar model will be used to perform all mRNA and miRNA analysis. Fold changes will be calculated using the ΔΔcycle threshold ($\Delta\Delta C_T$) method as described below in statistical analysis section.

Bioinformatics Analysis

microRNA with significant changes in their expression will be uploaded to DNA Intelligent Analysis (DIANA)-miRPath 3.0 (Alexander Fleming Biological Sciences Research Center [BSRC], Athens, Greece; http://diana.cslab.ece.ntua.gr) to determine potential molecular pathways that these microRNA have previously been reported to regulate. Relevant Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways will be identified using experimentally verified targets from TarBase 7.0 (Alexander Fleming BSRC). Based on findings from this analysis, any additional gene and protein expression of relevant targets will be assessed.

Western Blotting

Approximately 30 mg of muscle will be homogenized in ice-cold buffer (1:10 w/v) containing 50 mM Tris–HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benz-amidine, 1 mM PMSF, 10 µg mL−1 trypsin inhibitor and 2 µg mL−1 aprotinin. Homogenate will be centrifuged for 15 min at 10,000 × g at 4°C. Protein concentration of supernatant (lysate) will be determined using 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA or equivalent). Phosphorylation status and total protein expression of intramuscular inflammation and markers of muscle carbohydrate metabolism will be determined by Western blot. 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 difluoride (PVDF) membranes and incubated with commercially available primary antibodies of intracellular markers involved in inflammation, anabolism, proteolysis, and substrate metabolism [e.g., IL-6, TNFα, NF-kB, IKKα/β, Akt, mTORC1, p70SK1, AMPKα, p38 MAPK, PGC-1α , SIRT1, CaMK, p53, and ACC (Cell Signaling Technology, Danvers, MA, USA)] 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 or equivalent). Blots will be quantified using the ChemiDoc XRS from Bio-Rad and Image Lab software (Bio-Rad) or similar model. To confirm equal protein loading per well a normalizing protein such as HSP90 or GAPDH will be assessed.

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. Subject ID numbers 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 enrolled 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 or the project coordinator's office, or kept in a computer file with password-protected access restricted to the principal investigator and the project coordinator. Social security numbers and banking information will be collected to process volunteer payments. The master list link, social security numbers, and banking information will be deleted immediately after the study has been completed and payment has been confirmed. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. Study biological samples will be processed on site at USARIEM and stored in Military Nutrition laboratory freezers (rooms 322, 304) using the subject identification number until analyzed on site or until sample aliquots are shipped to other laboratories for analysis. Aliquots will be made of all study samples and stored on site in Military Nutrition laboratory freezers indefinitely for reanalysis if necessary. Aliquots of coded biological samples will be shipped overnight on dry ice to Dr. Hammamieh at USACEHER, Dr. Rood at PBRC, Metabolic Solutions Inc., and to the Energy and Environmental Sustainability Laboratory (EESL PSU) at Pennsylvania State University for analyses. Once samples have been analyzed by these respective laboratories, there will be no remaining sample for storage. Coded data will be transmitted between the abovementioned laboratories and USARIEM, as well as between USARIEM and the University of Leeds via encrypted email, a secure file transfer site, or using an approved removable media. Coded specimen/data transfer agreements have been obtained for PRB and University of Leeds. Further, USARIEM has existing collaborative agreements in place with the Norwegian Defence Research Establishment (CRADA W81XWH-12-0270) and the University of Leeds (W81XWH-16-0498). An institutional collaborative agreement is currently in review between USARIEM and USACEHR.

Only personnel assigned to the research study by the principal investigator will have access to the data. Only the principal investigator and project coordinator will have access to personal identifiable data. No outside laboratory will have access to identifiable 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 ten years after the study has been completed.

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

Any use of the samples outside of the broad scope of this protocol will be submitted as a protocol amendment or a new protocol. Once the protocol is closed, samples will be retained for further analyses that may or may not align with the hypotheses set forth in the protocol. Samples will be retained for future analyses once the protocol is closed.

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

SmartPill, Polar Electro heartrate monitor, True Max 2400 ParvoMedics, Fatigue Science ReadiBand™ actigraph, Philips Respironics Actiwatch® Spectrum Plus, InBody 720, and DEXA will be used in research according to approved labeling.

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

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

Power analyses were performed using www.biomath.info/power/prt.htm and indicate that 11 participants will provide adequate power (alpha = 0.05, power = 0.8) to detect significant changes in circulating concentrations of IL-6 and hepcidin in response to stress and differences in energy balance. To account for potential attrition (~30% attrition in 16-02-HC), 4 additional subjects are requested for a total of 15 subjects. Data used in calculating samples size estimates (i.e., means and standard deviations) are derived from previous studies that found significant increases in IL-6 and/or hepcidin in 1) male athletes (n=12) after a 7-day training block [103], 2) male athletes (n=9) after two 75 min endurance exercise bouts separated by 3 h of rest [22], 3) male athletes (n=8) after two high-intensity endurance exercise bouts over two days [23], and 4) male athletes (n=11) completing a 1.5 h treadmill run [104]. The outcome measures, means, standard deviations (SD), n, and alpha and power levels are included below:

Study	Outcome	Mean difference	SD of difference	Alpha	Power	n/group
[103]	Hepcidin	4.7	4.9	0.05	0.8	11
	IL-6	2.7	1.3	0.05	0.8	5
[22]	IL-6	4.8	1.8	0.05	0.8	6
[23]	IL-6	3.2	2.0	0.05	0.8	6

[104]	Hepcidin	7	7.4	0.05	0.8	11
	IL-6	9	6.6	0.05	0.8	7

We have previously shown that an increase of intestinal permeability of 55-60% is associated with inflammation in military-relevant environments (USARIEM protocol 14-33HC [44]). Using means (pre-SUSOPS sucralose excretion = 2.0%), standard deviations (SD = 1.1), and correlations for repeated measurements of sucralose excretion (r = 0.5) from 14-33HC, we estimate that 14 subjects will allow detection of a main effect of study phase (BL vs. SUSOPS-NEG BAL vs. SUSOPS-BAL) if the effect size of SUSOPS-BAL vs. SUSOPS-NEG BAL on intestinal permeability is medium (~20% decrease) at power = 0.80 and alpha = 0.017 (https://glimmpse.samplesizeshop.org/). Of note, larger effect sizes have been reported in studies investigating the effects of nutritional interventions on intestinal permeability during exercise [105]. Additionally, we feel that it is likely that the SDs measured in this laboratory study will be slightly lower than those measured in our previous study (14-33HC) which was conducted in a field environment. A 25% decrease in the SD would allow us to detect a main effect of phase with 11 subjects at power = 0.80 and alpha = 0.017. As such we expect an n of 11 to 15 will provide adequate power to detect a main effect of phase on intestinal permeability.

Power calculations for appetite-mediating hormones outcomes were conducted using data obtained during USARIEM protocol H10-09 [73, 106]. In that study an energy deficit (3700 kcal/d) slightly greater than that which will be imposed in this study resulted in a 2.5-fold decrease in fasted acyl ghrelin, a 2.2 fold decrease in leptin, a 1.7-fold decrease in PYY, and a 3-fold increase in PP concentrations concomitant to an ~11-fold increase in appetite and overeating during recovery from energy deficit. Based on these results we expect to observe the hormone responses in the table below where fasting concentrations will be approximately maintained during BAL, and the maximal difference between BAL and NEG BAL will be observed on SUSOPS day 3 followed by a full recovery of hormone concentrations by recovery day 3. For all but PP, 12 subjects will provide ≥80% power at alpha = 0.01 to detect a phase-by-time interaction.

	BAL				**NEG BAL**						
	S-d1	**S-d3**	**R-d1**	**R-d3**	**S-d1**	**S-d3**	**R-d1**	**R-d3**	**SD**	**r**	**n**
Ghrelin	185	185	185	185	185	75	135	185	90	P,0.65; T, 0.60	11
Leptin	9	9	9	9	9	4.1	6.5	9	5	P,0.60; T, 0.75	11
PYY	170	170	170	170	170	100	135	170	170	P,0.85; T, 0.85	12
PP	65	65	65	65	65	195	130	65	100	P,0.30; T, 0.30	19

Samples sizes (n) calculated using https://glimmpse.samplesizeshop.org/. r, correlation between phases (P) and over time within each phase (T); R, recovery; S, SUSOPS; SD, standard deviation. Values based on those measured during USARIEM protocol H10-09 [109]].

Using data obtained using the SmartPill in a study of 73 healthy adults we estimate that mean gastrointestinal transit time at baseline will be 29.5 hr (SD = 11.0 hr) [107]. Horner et al. [108] reported a large effect size of 1.0 in a meta-analysis of high-quality studies that measured changes in orocecal transit time in response to exercise. A similar effect size was reported by Corvilain et al. [109] who measured gastric emptying in healthy

adults before and after a 4-d fast. Using these data and a conservative correlation between repeated measurements of r = 0.50 (Diaz-Tartera et al. [107] reported r = 0.99), we estimate that n = 8 participants will provide >80% power to detect a main effect of condition at alpha = 0.05. This sample size will also provide >80% power to detect a main effect should the effect size be medium (effect size = 0.5) and the correlation between repeated measurements (r = 0.90) more similar to that reported by Diaz-Tartera et al. [107]. We will enroll at least n = 9 in this procedure to ensure complete data is collected on 8 participants. Due to equipment availability, n = 9 is also the maximum number of participants we can measure simultaneously. However, should multiple iterations be needed to complete data collection, we will enroll up to n = 9 participants during each iteration. Although n = 8 is sufficient to detect a main effect of condition, enrolling more than n = 9 will provide greater power to detect associations between changes in gastrointestinal transit time and changes in gut microbiota composition, intestinal permeability, and appetite-related outcomes.

Next Generation Sequencing (maximum 200 million pair-end reads) of blood mRNA samples from 15 participants will yield 60% statistical power to detect a true difference in expression of at least 1.5 fold with group coefficient variation 0.4, and a conservative false discovery rate of 0.05 using moderated *t*-tests and with transcriptome coverage of 9 over reference human genome with sequence length of 150 base pairs. Note, a more liberal false discovery rate (e.g., 0.20) may be used to reduce type 2 error rates and increase power. For microbiome analysis, shotgun sequencing (maximum 50 million pair end reads) of 15 participants will achieve ≥69% power to detect a true difference in relative abundance of at least 1.5 fold with group coefficient variation 1.3, and false discovery rate of 0.05. Again, a more liberal false discovery rate (e.g., 0.20) may be used to reduce type 2 error rates and increase power. Power calculations were conducted using pairwise distances and PERMANOVA power with respect to effect size. However, power will be enhanced by correlating the blood mRNA data with other omics readouts derived from saliva (microRNA-seq, metabolomics and microbiome) and blood (targeted microRNA and metabolomics), and by temporal sample collection. As such, we expect that 15 participants will provide adequate power to detect differences in blood mRNA expression, and gut microbiome community and genome composition between study phases, and correlations between these parameters and other –omics readouts.

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, and transformations will be applied as appropriate to ensure model assumptions are met. Correlation and multiple regression will be used to evaluate relationships between study outcomes.

Primary Objectives

Linear mixed models will be used to determine main effects of phase (SUSOPS BAL and SUSOPS NEG BAL), time within phase, and their interaction on inflammation, iron absorption, mood/cognitive, and measures of muscle, nutritional, and metabolic homeostasis. If phase by time interactions are observed, a Bonferroni correction will be applied for multiple comparisons. Statistical significance will be set at $P < 0.05$. To test for carryover effects a main effect of group (SUSOPS BAL first, SUSOPS NEG BAL first)

and a group-by-phase interaction will be included in initial models. These terms will be removed if they do not significantly contribute to the overall fit of the model. When significant phase-by-time interactions are observed post hoc comparisons will be made using paired t-tests with Bonferroni corrections.

Secondary Objectives

Linear mixed models will be used to examine changes in intestinal permeability and gastrointestinal transit time. Models will include subject as a random factor, and study phase as a fixed factor. To test for carryover effects a main effect of group (SUSOPS BAL first, SUSOPS NEG BAL first) and a group-by-phase interaction will be included in initial models. These terms will be removed if they do not significantly contribute to the overall fit of the model. When a significant main effect is observed post hoc comparisons will be made using paired t-tests with Bonferroni corrections.

Fecal metabolomics data will be visualized using hierarchical average-linkage clustering and principal components analysis. Bacterial taxonomic data will be visualized using hierarchical average-linkage clustering and principal coordinates analysis of beta (i.e., between samples) diversity scores (e.g., Bray-Curtis, and weighted and unweighted UniFrac). Alpha (i.e., within-sample) diversity will be calculated for taxonomic data using Shannon, Simpson and Chao1 indices. Linear mixed models will be used to examine changes in biomarkers of gut health, fecal metabolite concentrations, and alpha diversity over time. Models will include subject as a random factor, and study phase, time within phase, and their interaction as fixed factors. To test for carryover effects a main effect of group (SUSOPS BAL first, SUSOPS NEG BAL first) and a group-by-phase interaction will be included in initial models. These terms will be removed if they do not significantly contribute to the overall fit of the model. Models for bacterial taxa and gene abundance will be analyzed using the R statistical software package “DESeq2” or equivalent. The Benjamini-Hochberg correction will be used to control the false discovery rate in microbiome-specific and metabolite models. Data analysis will be completed using SPSS, XLSTAT, R, Qiime, or similar software as needed.

Linear mixed models will be used to examine changes in appetite-mediating hormone concentrations, appetite ratings, food preferences, food choice during recovery (i.e., diet macronutrient proportion and energy intake). Models will include subject as a random factor, and study phase, time within phase, and their interaction as fixed factors. Separate analysis will be conducted for fasted and post-exercise appetite-mediating hormones. To test for carryover effects a main effect of group (SUSOPS BAL first, SUSOPS NEG BAL first) and a group-by-phase interaction will be included in initial models. These terms will be removed if they do not significantly contribute to the overall fit of the model. When significant phase-by-time interactions are observed post hoc comparisons will be made using paired t-tests with Bonferroni corrections.

The mRNA seq strand-specific libraries will be assayed using the Illumina HiSeq platform. Paired end reads of the strand-specific RNA sequences (RNA-Seq) will be generated using NextSQ from one flow cell with eight lanes, producing at least 30 million reads of 100 bases per sample. Based calling and expression analysis will be conducted using the Illumina-provided tool CASAVA. miRNA (20-40 bp) will be size selected from total RNA and will be processed by Illumina NextSQ. Image analysis and base calling will be performed using the newest available version of the Illumina pipeline. Preprocessing of raw base calls and sample de-multiplexing will be performed using the

standard open source tool CASAVA. The microRNA read count matrix will be generated by a series of tool kits of latest version including CutAdapt, short read aligner Bowtie and PICARD. Metabolomics and proteomics reads will be processed by the software offered by the vendors namely Waters Corporation and BioRad, respectively.

Using a systems approach, we will integrate multiple levels of biological information. We have published the analysis pipeline and implemented it successfully in the past. Our pipeline uses industry standards and our established SOPs. We will start by analyzing and processing data at the analyte level for each data type such as mRNA expression, miRNA expression, microbiome, proteomics, and metabolomics. We will first examine the confounding factors or batch effects caused by non-biological factors, such as the technical error, sample processing date etc., using principal component analysis and ANOVA. If necessary, corrections will be made using the COMBAT algorithm. All datasets will be visually inspected using PCA, heatmaps, and boxplots before and after any required normalization, correction, or filtering, to assure quality control.

Using R package the significantly altered analytes will be mined by t-test p-values (<0.05) and fold-change 1.5. The R package BETR in conjunction with standard and lagged correlation analysis will be used to find biological signatures which vary systematically with the phenotypic data. Data dimension reduction, specifically latent factor analysis for phenotypic (clinical indices of disease onset) and PCA/PCoA for omics data will be used to uncover underlying mechanisms and dominating trends. Venn diagrams will be used to identify the biological signatures, including the molecules and enriched networks/pathways, which will emerge common/unique to different treatment groups and common/unique to control vs. treated specimens. To assess gene ontology and pathway enrichments, and analyze regulatory networks and common pathways we will use the Hypergeometric Test (false discovery rate, $q < 0.05$) of Bingo 2.44 and ClueGO (Cytoscape 3.0.1 plugins; http://www.cytoscape.org/), Fisher's Exact Test of Ingenuity Pathway Analysis (IPA, Ingenuity, Inc., Redwood, CA), and Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea/index.jsp) software sets. Gene-metabolite condition 'interactomes' will be constructed and visualized using Gephi.0.8.2 beta (www.gephi.org). Using a suite of platforms such as ClueGo, Bingo, IPA, David and IMPaLA, we will deliver a panel of networks informed by multiple levels of molecular evidence that are unique to treatment groups. From these analyses we will produce a suite of networks informed by multiple levels of molecular evidence that are unique to treatment groups.

We will employ our in-house developed tool, Core Module Biomarker Identification with Network Exploration (COMBINER), to look for those novel functional groupings of molecules, which are statistically perturbed across time course. In addition, Feature Assisted Clustering for Time-series (FACT), a pipeline for exploratory analysis and visualization of longitudinal data, will identify differential expressed molecules, pathways/GO terms, separate clusters of similar patterns, and compare pathway dynamics. Finally, we will use commonly accepted criteria of Receiver Operator Curve (ROC), using Area Under Curve (AUC), sensitivity, and specificity to evaluate the performance of individual molecules.

SECTION C: HUMAN RESEARCH PROTECTIONS

C1. RECRUITMENT AND CONSENT

C1.1 Identification and Selection of Subjects

Interested volunteers who have been briefed on study procedures will be provided the opportunity to consent to participate. After consent, and before medical clearance, study eligibility will be determined based on volunteer responses to questions pertaining to self-reported study inclusion and exclusion criteria (see "Background Questionnaire"). If still eligible, volunteers will make an appointment for a medical screening. If an individual fails the screening, their screening and demographic data will be destroyed. An additional demographic and nutritional questionnaire will be administered during the pre-study baseline period to those participants who are enrolled in the study (see "Additional Demographic and Nutritional Survey").

The medical clearance will take place at USARIEM (Natick, MA) by OMSO staff. Volunteers recruited through SSIT may undergo OMSO medical clearance at their home duty station prior to arrival at USARIEM (clearances will be coordinated between the PI, OMSO, and the unit's Brigade Surgeon). The clearance will include a blood draw to assess health status and inclusion/exclusion criteria. The medical screening visit will take approximately 1 hr. If any medical screening tests show a possible medical concern, the volunteer will be notified. Those who receive study clearance and meet the inclusion/exclusion criteria will continue on pre-study, baseline testing.

C1.2 Recruitment Process

Volunteers will be recruited from the Natick Human Research Volunteer (HRV) Pool, and the active duty population located at other military organizations, to include coordination with NSSC Soldier/Squad Interface Team (SSIT).

For HRVs, the Principal Investigator will provide a copy of the informed consent document to the HRV 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. The HRV Coordinator may also organize consent briefings for Soldiers at their Advanced Individual Training unit. The Coordinator will serve as an ombudsman for the offsite consent briefings. In addition, other military organizations may be recruited through coordination with NSSC SSIT. The NSSC SSIT Coordinator will schedule the consent briefing for the military research volunteers and an ombudsman will be present during briefing. Ms. Katelyn Guerriere will serve as the ombudsman for the current study.

Superiors of Service members (e.g., unit officers, senior NCOs, and equivalent civilians) shall not be present at any recruitment sessions or during the consent process in which members of units under their command are afforded the opportunity to participate as human subjects of research.

Other active duty personnel may also be recruited by "word of mouth" and posted flyers Recruiting materials will be distributed around NSSC. The text-based flyer will be posted on various USARIEM social media sites and used in distribution media requiring a text

format (e.g., electronic newsletters). Approvals from the requisite parties will be obtained prior to any recruitment activities.

C1.3 Eligibility

All potential volunteers will complete the background questionnaire pertaining to the study inclusion and exclusion criteria. Volunteers must then be medically cleared by OMSO for participation in accordance with USARIEM procedures outlined for screening volunteers for research involving exercise. Volunteers recruited through SSIT may undergo OMSO medical clearance at their home duty station prior to arrival at USARIEM (clearances will be coordinated between the PI, OMSO, and the units Brigade Surgeon). Volunteers will be screened for anemia and 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 SUSOPS phases of the protocol, and they must be willing to adhere to exercise and physical activity prescriptions and restrictions. If the results of all screening tools reveal the volunteer fits the screening criteria, they will be eligible to volunteer for the study.

C1.4 Consent Process

Prior to providing informed consent, discussions with potential volunteers (such as over the telephone) will not involve the collection of any personally identifiable information besides their name, email, and telephone number. The principal investigator, an associate investigator or the project coordinator will brief potential volunteers about the nature, purpose, procedures involved, risks, expectations and requirements for participation in the study. Study briefings will be scheduled to occur in-person, and will not occur over the phone. Prospective volunteers will be familiarized with the study procedures and informed verbally and in writing of their rights to withdraw from any part of the study without penalty or prejudice. The principal investigator or designee will answer all group and private questions. Potential volunteers will have at least one hour after they are briefed, with the ombudsman remaining present, to read and review the Informed Consent document and decide whether they wish to consent to participate. No study procedures will occur prior to any volunteer giving their informed consent. An ombudsman will not be required for any individual briefings. A copy of the informed consent document will be provided to the volunteer with the original kept for study documentation. If they meet all the medical selection and eligibility criteria after completing the screening health assessment and consenting to participate, they will begin preliminary testing. 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 USAMRDC Volunteer Registry Database

It is the policy of USAMRDC that data sheets are to be completed on all volunteers participating in this research for entry into the U.S. Army Medical Research and Development 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 USAMRDC; and second, to ensure that the USAMRDC 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 USAMRDC for a minimum of 75 years.

C2. COMPENSATION FOR PARTICIPATION

Volunteers will receive a total of $1050 for completing the study. This is based on an amount of $25 per blood draw (42 total draws).

Note: *Volunteers who receive more than $600 in a calendar year will have this income reported to the Internal Revenue Service.*

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. An investigator may stop an individual's participation in the study if the volunteer is unwilling or unable to complete study procedures or follow study diets/exercise prescriptions. An investigator may also withdraw a volunteer if the individual becomes ill or injured or it would not be in the volunteer's best interest to continue. If the participant is withdrawn by the investigator or decides to voluntarily withdraw himself, all further data collection will discontinue, but the data that was collected up to the point of withdrawal may

still be used for analysis. Participants will be compensated for any blood draws they completed up until that point, and they will be asked to return any study food and/or wrappers, study supplies that were provided, in addition to any study logs that they had completed up to the point of withdrawal.

C4. PRIVACY FOR SUBJECTS

To protect the volunteer's privacy, all of their research-related records will be labeled or "coded" with an assigned research volunteer number that will not include their name or any other form of identifiable information. The principal investigator or project coordinator will keep the link between volunteer number and the volunteer's research records in a locked cabinet. Any documents that will require the volunteer's name, such as the consent form, will be kept in a locked cabinet separate from any research documents that contain the volunteer's ID number. The principal investigator and project manager are the only people who will be able to match the research volunteer 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 volunteer's identity to others. If photographs, videos, or audio-tape recordings of volunteers are used for educational purposes, volunteer 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. If volunteers do not sign a photo release form, any photographs taken of them will be destroyed.

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 or project manager's office, or kept in a computer file with password-protected access restricted to the principal investigator and project manager. 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, and off-site at PBRC, USACEHR, and at the fee-for-service labs. All samples will be stored using the subject identification number. The volunteers name or other identifiable information will not be included on any data, data collection sheets, specimens, or other research records. Coded data from the LFPQ will be provided to the University of Leeds for analysis using encrypted email or a secure file transfer website (https://safe.amrdec.army.mil/SAFE/ Welcome.aspx). Coded study data may be shared with the USACEHR for integration with –omics data sets. Any shared data will be transferred using encrypted email, a secure file transfer website (https://safe.amrdec.army.mil/SAFE/Welcome.aspx) or the SysBioCube platform (https://sysbiocube-abcc.ncifcrf.gov/). No personally identifiable information will be shared with PBRC, the University of Leeds, or USACEHR.

Only personnel assigned to the research study by the principal investigator will have access to the data. Only the principal investigator and project coordinator will have access to personal

identifiable 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 ten 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: Venipuncture
Research Procedure Description: A needle is used to draw blood from a superficial vein.
Research-related Risks: Venipuncture is a routine clinical procedure the medical community commonly uses to obtain blood samples. The risks of venipuncture are small and usually limited to local bruising or swelling. Sometimes volunteers feel faint or may faint during or right after venipuncture. If the volunteer has had problems with fainting during blood draws in the past, they may be more prone to them during future procedures. Dizziness or faintness constitutes no long-term harm, and immediate relief is achieved by having the subject put their head down between their knees or lie down. In addition, 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: Volunteer 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: Venous Catheterization
Research Procedure Description: A needle will be used to guide a catheter into a superficial vein in the antecubital fossa (or distally). The catheter will either be attached to saline, or flushed periodically with saline, to keep the line patent for serial blood draws.
Research-related Risks: The risks of venous catheterization are small and usually limited to local bruising or swelling. Sometimes volunteers feel faint or may faint during or right after the catheter is placed. If the volunteer has had problems with fainting during blood draws in the past, they may be more prone to them during future procedures. Dizziness or faintness constitutes no long-term harm, and immediate relief is achieved by having the subject put their head down between their knees or lie down. If the catheter becomes clogged at any time during the protocol, it will be replaced to continue blood sampling and therefore the study. This will require another needle to be inserted.
Measures to Minimize Risks of Harm: Trained technicians will use aseptic techniques to place the catheter; however, in spite of being careful there is a chance that the site may become infected. Volunteers should not give blood for 4 months before and 2 months after the study.

Research Procedure Name: Oral Stable Isotope Administration
Research Procedure Description: Volunteers will consume a drink containing stable isotopically labeled iron or amino acids on multiple occasions.
Research-related Risks: There are no known risks or reported side effects associated with oral administration of stable isotopes to humans during clinical or experimental

studies. This is because there is relatively little mass difference between the isotopic tracers and the more prevalent natural isotopes, and the body's naturally occurring pool of stable isotopes is high enough that types of experimental protocols proposed have no appreciable effect on the total abundance of the isotopes present in the body.
Measures to Minimize Risks of Harm: All staff who directly participates in the stable isotope studies will be properly trained to prepare and administer from Dr. Pasiakos, who has extensive experience with stable isotopes.

Research Procedure Name: Percutaneous Skeletal 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 applied to collect muscle samples, while the volunteer is under local anesthesia (1% lidocaine).
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 right after a muscle biopsy. If the volunteer has had problems with fainting during blood draws or muscle biopsies in the past, they may be more prone to them during future procedures. The most common risks associated with muscle biopsies are pain (~1.27%), erythema (~1.27%), and ecchymosis (1.27%).[110, 111] Panic episode, bleeding, and edema have also been reported (0.21%, 0.42%, and 0.84%, respectively).[110] Denervation, numbness, and atrophy may occur but have not been verified in the literature. 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.
Measures to Minimize Risks of Harm: 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 diathesis should be excluded during medical clearance; those with local skin infection or irritation or recent use of anticoagulant medication not identified during initial medical screening (including aspirin) will be withdrawn by the PI in consultation with OMSO. 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 (see "Biopsy Care Instructions"). Muscle biopsies will be performed using sterile procedures by Dr. Stefan Pasiakos or Dr. Lee Margolis, who will abide by USARIEM's Percutaneous Skeletal Muscle Biopsy SOP (OMSO-approved USARIEM SOP for Invasive Procedures, Chapter 10) of 11 July 2017 in all regards. The PI and OMSO will follow-up with volunteers within 3 d post-biopsy to monitor for any sign of infection, bleeding, or hematoma.

Research Procedure Name: Lidocaine Injection
Research Procedure Description: Approximately 8-10 mL of 1% lidocaine will be injected using a 25 g 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: Volunteers will be instructed to notify a study investigator or the project coordinator 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 and OMSO will be notified immediately. Dr. Pasiakos or Dr. Margolis will be the only ones administering the lidocaine, and medical staff will be onsite. The PI and study staff will closely monitor the volunteers throughout the procedure.

Research Procedure Name: Exercise
Research Procedure Description: Exercise includes peak aerobic capacity and glycogen normalization studies on a cycle ergometer, outdoor load carriage exercise, steady-state elliptical and cycle ergometer exercise sessions, exercise associated with the Warfighter tasks.
Research-related Risks: Exercise is generally considered safe and beneficial for individuals without cardiovascular disease. The US 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. Exercise often carries a risk of injury, including dehydration, acute musculoskeletal strains and sprains, overuse injuries, and accidental injuries caused by the test apparatus. The risk of musculoskeletal injury from bouts of endurance exercise is minimal. Muscle soreness, ranging in intensity from mild to severe, may persist for 1 to 7 days. Additional risks associated with exercise include foot blisters, skin chafing, muscle cramps, stress fracture, trauma due to falling, and hypotension following the completion of the exercise bout. The frequency of many of these risks increases with the duration of the exercise bout.
Measures to Minimize Risks of Harm: 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/monitor during all exercise sessions, and heart rate will be monitored in real time during testing. Exercise monitors and test administrators will be CPR-certified. Additional safeguards taken to minimize risk during exercise include: (a) qualified personnel will administer the maximal exercise tests, (b) the volunteer will be asked to report any pain or discomfort resulting from exercise, followed up if necessary by medical examination and postponement or curtailment of further testing, (c) volunteers will be required to wear correctly sized footwear, and (d) volunteers will have access to water (ad libitum) to remain hydrated.

Research Procedure Name: Energy Balance and Negative Energy Balance Diet Interventions
Research Procedure Description: Volunteers will be fed adequate energy to maintain body mass during SUSOPS BAL and only 45% of total energy expenditure during SUSOPS NEG BAL to elicit negative energy balance (where dietary intake is < energy expenditure). Combat rations will be the primary food source (some perishable whole-foods).
Research-related Risks: The foods and MRE components used in this study pose no known risks to volunteers. All of the meals that volunteers will be fed during both 96 hour

SUSOPS periods consist solely of MRE components. Sudden changes to the diet can cause gas, cramping, bloating, constipation, or other abdominal discomfort in some individuals. The main discomfort associated with a low energy diets is hunger. Volunteers will be shown copies of study menus and food lists at the initial study recruitment brief. This will be used to determine if prospective volunteers have an allergy, intolerance, or personal preference to foods listed.
Measures to Minimize Risks of Harm: All efforts will be made to accommodate the volunteers with regard to dietary preferences while keeping the major constituents of the diets consistent with study design and between volunteers. Those who have an allergy or intolerance to a menu component, which cannot be accommodated, will not be enrolled in the study.

Research Procedure Name: Dual energy X-ray absorptiometry (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: 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.

There are no risks associated with bio-electrical impedance body composition measures

There are no risks associated with saliva sampling measures

Research Procedure Name: Multi-sugar absorption test for intestinal permeability measurement
Research Procedure Description: Participants will consume 2 g sucralose and 2 g erythritol dissolved in 180 mL water. All urine produced over the subsequent 24hr will be collected.
Research Related Risks: Sucralose and erythritol are commonly consumed sugar substitutes which may cause gas, cramping, diarrhea or bloating in some individuals.
Measures to Minimize Risks of Harm: Any participant reporting gastrointestinal distress following the test will be given the option of not participating in the test at the next opportunity.

Research Procedure Name: SmartPill
Research Procedure Description: The SmartPill is an ingestible FDA-approved wireless motility capsule that transits the gastrointestinal tract while continuously measuring pH, temperature, pressure, and gastrointestinal transit time. A single pill will be ingested by volunteers and allowed to pass normally through the gastrointestinal tract.
Research Related Risks: Choking, aspiration, retention gastrointestinal tract.
Measures to Minimize Risks of Harm: Potential participants with contraindications for use will not be allowed to participate in the SmartPill procedure. If they are randomly

selected for SmartPill testing, an alternate will be chosen to participate instead. Pills will be ingested under staff supervision.

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 direct health or other benefits related to participation in this study.

C7. DATA AND SAFETY MONITORING

C7.1 Monitoring

The PI will, with the assistance of Associate Investigators and project coordinator, 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. The PI will submit a monthly summary of all adverse events to the Research Monitor to determine whether the number of adverse events is excessive for the risks outlined in the research protocol. The PI and onsite physician or PA will discuss “discontinuation criteria” for individual volunteers as the study progresses, based on their observations of the volunteer during testing or non-testing periods. 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, gastrointestinal pain, etc.)?
- 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?
- What type of exercise or physical activities have you performed in the last 24 h?

C7.2 Research Monitor

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. Other responsibilities may be assigned by the USA MRDC 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 volunteer, 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.

A Serious Adverse Event is defined as 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 volunteer, or may require medical or surgical intervention to prevent one of the above outcomes.

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.

Expected adverse events which are not serious are reported to the IRB at the time of continuing review of the protocol. These events include bruising, infection, swelling and slight pain from the IV placement; slight pain from the lidocaine injection; pain, soreness, infection, and bruising from the muscle biopsy; feeling faint with IV placement, blood draw or biopsy; fatigue and muscle soreness from study exercises and SUSOPS activities; hunger, bloating, gas, cramping, constipation from the dietary invention; fatigue and headaches during the negative energy balance portion of the dietary intervention.

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, and 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@mail.mil) to the USARIEM ORQC and the Commander. These events will also be reported to the HQ

USAMRDC 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 USAMRDC IRB.

In the event of a medical emergency at facilities on the Natick Soldier Systems Center, the local installation emergency management will be contacted immediately by dialing x5911. 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

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SECTION E: ABBREVIATIONS AND ACRONYMS

ACSM, American College of Sports Medicine; BART, balloon analogue risk test; BAL, energy balance; EVAR, evaluation of risks scale; DEXA, dual energy x-ray absorptiometry; DLW, doubly labeled water; GLP-1, glucagon-like peptide-1; IL-6, interleukin 6; LFPQ, Leeds Food Preference Questionnaire; NEG BAL, MRE, Meals Ready-to-Eat; negative energy balance; PP, pancreatic polypeptide; POMS, profile of mood states; PVT, psychomotor vigilance test; PYY, peptide-YY; RMR, resting metabolic rate; SIgA, Secretory IgA; SUSOPS, sustained operations; TBW, total body water; TDEE, total daily energy expenditure; TNF-α, tumor necrosis factor alpha; VAS, visual analogue scale; VO_{2peak}, peak oxygen consumption; refer to table 1 for appropriate definitions for analyte abbreviations.

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

Click in the appropriate box See the "Guide for Investigators" for definitions and further information.

☒ 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