Cover Page

PROTOCOL TITLE: The effects of varying essential amino acid intakes on resting and post-exercise skeletal muscle and whole-body protein kinetics during negative energy balance

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SECTION A: RESEARCH TEAM AND LOCATIONS

A1. RESEARCH TEAM

Study Role	Institution/Company and Contact Information
Sponsor	*Organization/Institution/Company:* Military Nutrition Division (MND), US Army Research Institute of Environmental Medicine (USARIEM) *Address:* 10 General Greene Ave, Bldg. 42 Natick, MA 01760 *Point of Contact:* N/A
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Research Monitor

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Ombudsmen

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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): Arny A Ferrando
Research Role: Associate Investigator
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach, and design. Assist PI with data analysis, particularly stable isotope assessments of skeletal muscle and whole-body protein kinetics. Dr. Ferrando will receive and analyze coded, de-identified blood and muscle samples for isotopic analysis. Dr. Ferrando will also be actively involved with the interpretation of the data and preparation of peer-reviewed manuscripts. He will observe test volunteers and the study process to ensure correct study implementation, but will not have access to personal identifiable information. ***A coded specimen transfer agreement is included with this submission.***

Name(s): Lee M Margolis
Research Role: Associate Investigator
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach, and design. Assist PI with data collection, management, and analysis and manuscript preparation. Prepare and administer test diets. Data collection will involve percutaneous skeletal muscle biopsies, phlebotomy, catheterization, monitoring submaximal exercise testing, and sample processing. In the event of Dr. Pasiakos' absence, Dr. Margolis can oversee, manage, and execute all study procedures, particularly the stable isotope infusion and muscle biopsy studies.

Name(s): Jess A Gwin
Research Role: Associate Investigator
Study Responsibilities: Assist PI with data collection, management, and analysis and manuscript preparation. Prepare and administer test diets. Data collection will involve percutaneous skeletal muscle biopsies, phlebotomy, catheterization, monitoring submaximal exercise testing, and sample processing. Dr. Gwin can execute the stable isotope infusion and muscle biopsy studies. She will also be actively involved with the interpretation of the data and preparation of peer-reviewed manuscripts and technical reports for publication.

Name(s): David C Church
Research Role: Associate Investigator
Study Responsibilities: Assist with data collection, biological sample processing, and biological sample analyses. David Church will not be involved in any credential requiring study processes.

Name(s): John W Carbone
Research Role: Consultant
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach, and design. Assist PI with data interpretation and manuscript preparation. Dr. Carbone will not receive coded data, nor be engaged in this collaborative research project. He will not interact or intervene with test volunteers, or have access to personal identifiable information.

Name(s): Adrienne Hatch
Research Role: Project Coordinator, Study Dietitian
Study Responsibilities: Supervise, manage, and coordinate study logistics and biological data collection. She will be involved with protocol development, menu development, volunteer briefing, preparation and administering study diets to volunteers, diet instruction, dietary assessment, and study implementation. She will actively participate in data collection to include phlebotomy, monitoring submaximal exercise testing, and interventions, and DEXA.

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

Name(s): Claire Whitney, Heather Fagnant, Jillian Allen
Research Role: Research Dietitians
Study Responsibilities: Menu development, preparation and administering study diets to volunteers, diet instruction, dietary assessment, and study implementation. Claire Whitney and Heather Fagnant will assist with DEXA. Claire Whitney will additionally assist with phlebotomy, monitoring submaximal exercise testing, and interventions.

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

Name(s): Christopher T Carrigan
Research Role: Research Physiologist
Study Responsibilities: Assist with data collection, phlebotomy, catheterization, DEXA, and biological sample processing.

Name(s): Marques Wilson
Research Role: Research Physiologist
Study Responsibilities: Assist with data collection and biological sample processing. Marques Wilson will also be involved in phlebotomy, catheterization, DEXA, and administration of maximal muscle exercise testing and intervention

Name(s): SPC Alaniz, SGT Cordell, SGT Patino, SPC Sarpong, SPC Sanchez, SSG Mason, SGT Rousayne, Patrick Radcliffe, Emily Howard, and Anthony Karis
Research Role: Research Assistants
Study Responsibilities: Assist with data collection, phlebotomy, and biological sample processing. SGT Cordell and SGT Patino may also assist with catheterization. Patrick Radcliffe and Emily Howard will not perform phlebotomy.

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 Guerriere, Katherine Mitchell, Karleigh Bradbury, Maria Canino, Caitlin Haven, Matthew Bartlett
Research Role: Ombudsman
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 Materiel Command. It is the Institute responsible for conducting basic and applied research to determine the effects of exposure to environmental extremes, occupational tasks, physical training, deployment, operational stress and nutritional factors on the health and performance of military personnel. The facility contains environmental chambers for controlling temperature and humidity, an environmentally controlled hypobaric chamber, a water immersion laboratory, as well as several dry and wet laboratories for animal and human experimentation. The dry laboratories are capable of a broad range of experiments, including biomechanical analysis, body composition, energy expenditure, 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.

UAMS, Little Rock, AR: The Center for Translation Research in Aging & Longevity (CTRAL), Donald W Reynolds Institute of Aging (RIOA), University of Arkansas for Medical Science (UAMS) has the basic laboratory facilities and equipment to analyze stable isotope kinetics, including 2 Agilent 5973 GC/MS, and 3 Agilent 5975 GC/MS, a Finnegan TSQ 7000 LC/MS/MS, and a Waters QTOF LC/MS. The CTRAL is led by the world's foremost expert in stable isotope assessments of human metabolism, Dr. Robert R Wolfe.

SECTION B: RESEARCH METHODOLOGY

B1. ABSTRACT

Short-term negative energy balance (i.e., energy deficit) downregulates muscle protein synthesis and upregulates whole-body proteolysis and amino acid (AA) oxidation, thereby increasing nitrogen excretion and exacerbating whole-body and skeletal muscle protein loss. Consumption of quality proteins high in essential amino acid (EAA, i.e., the active anabolic component of protein) content may attenuate protein loss during energy deficit by restoring whole-body and skeletal muscle anabolic potential to that observed in a eucaloric state. During energy balance, muscle protein synthesis appears to be maximally stimulated after consuming 15 g of EAA at rest and after conventional resistance-type exercise. In response to a short-term energy deficit that downregulated basal muscle protein synthesis by as much as 27%, consuming 15 g (~7.5 g EAA) and 30 g (~15 g EAA) of whey protein after a bout of resistance exercise restored muscle protein synthesis rates to resting, fasted rates observed in the eucaloric state in a dose dependent manner. The effect of EAA intakes above 15 g on resting and post-exercise muscle protein synthesis and the whole-body protein anabolic response during acute energy deficit has not been determined. To address this, we will assess resting and post-resistance exercise whole-body and skeletal muscle protein synthesis responses to across a spectrum of EAA intakes following a well-controlled, short-term (5-d) energy deficit (30% energy deficit). Using a randomized, double-blind, cross-over design, 20 resistance trained (≥ 2 d/wk for the past 6 mo) adults will undergo two, non-consecutive 5-d energy deficit periods, separated by a 14-d washout period. Resting and post-resistance exercise (single leg exercise model) whole-body protein turnover and skeletal muscle protein synthesis responses to two different doses of EAA (standard, 0.10 g/kg vs high, 0.30 g/kg) will be determined the morning after completing the 5-d energy deficit. This design will test the hypothesis that higher absolute doses of EAA are required to maintain resting and post-exercise anabolic responses during energy deficit. Risks include those associated with venous catheterization, muscle biopsies, DEXA, exercise, and the discomfort of moderate underfeeding.

B2. BACKGROUND AND SIGNIFICANCE

Periods of negative protein balance (i.e., breakdown > synthesis) are largely unavoidable during strenuous military operations, and is exacerbated by sub-optimal dietary protein and energy intake [1, 2]. Prolonged

negative protein balance and associated muscle loss resulting from reductions in muscle protein synthesis, and concomitant increases in whole-body proteolysis and amino acid oxidation, may compromise Warfighter performance and readiness [2, 3]. As such, studies that identify effective nutritional interventions that mitigate protein loss and sustain Warfighter health and performance during real-world operations are warranted.

Essential Amino Acids and the Regulation of Protein Metabolism

It is well-established that the EAA component of protein is responsible for the stimulation of muscle protein synthesis [4]. Net muscle protein accretion was assessed in adults ingesting either 40 g of free AA (18 g of EAA plus 22 g of non-essential amino acids (NEAA), 18 g of EAA alone [4], or 22 g of NEAA alone [5]. These seminal studies demonstrated that the EAA component of protein was entirely responsible for the net gain in muscle protein (**Figure 1**). Co-ingestion of NEAA with the EAA did not influence muscle protein accretion, nor did they stimulate a synthetic response when given alone.

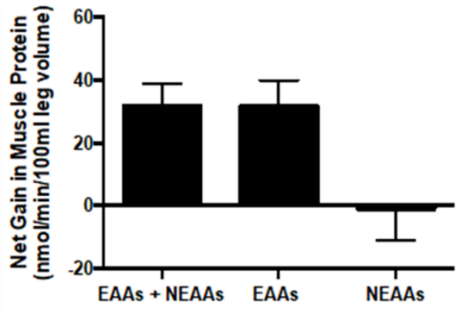


Figure 1. Net gain in muscle protein to ingestion of 40 g of free amino acids (18 g EAA + 22 g NEAA), 18 g EAA, or 7.56 g NEAAs alone.

There is a clear dose-response of EAA on muscle protein synthesis (**Figure 2)** [6-8]. Studies have shown that a dose as small as 3 g of EAA can stimulate muscle protein synthesis. The consumption of 7 g approximately doubled the 3 g response, while 15 g elicited a maximal response. Ingesting more than 15 g of EAA did not elicit a greater response. These findings are consistent with intact protein delivery of "matched" EAA content. Witard et al. [9], demonstrated that resting and post-resistance exercise muscle protein synthesis was maximally stimulated after consuming 20 g (~10-12 g EAA) of whey protein isolate, with no further increases in muscle protein synthesis after ingesting 40 g (~22 g EAA) of whey protein isolate. These findings have led to the widely accepted practice that 0.25-0.30 g of high-quality protein/kg per meal (20-30 g) maximally stimulates muscle protein synthesis in a eucaloric state, particularly when combined with the mechanical stress of resistance exercise. These findings form the basis for current recommendations issued by a number of organizations [10-12]. While these recommendations are scientifically sound, these studies were conducted during energy balance and focus solely on muscle protein synthesis.

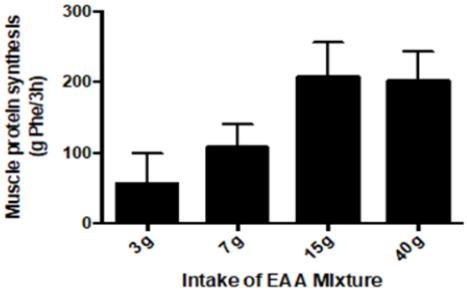


Figure 2. Dose response of muscle protein synthesis to ingestion of essential amino acids in human subjects.

Understanding Anabolism and its Quantification at the Whole-Body Protein Level

Anabolism is essentially the balance between protein synthesis and protein breakdown, which change in magnitude in response to various stimuli and stressors (e.g., EAA, exercise, hormones, stress, etc.). The application of stable isotope methodology allows for the measurement of these processes. In particular, quantifying labeled amino acid incorporation into intact proteins over time provides a measurement of fractional synthesis rate (FSR) of a given protein. In this regard, determining muscle FSR has become the most heavily relied upon method to evaluate the effects of various factors on muscle protein status. Conversely, assessing muscle fractional breakdown rate (FBR) is technically difficult, and the methods do not accurately account for a large bolus of EAA/protein intake over time [13-15]. As a result, muscle net protein balance is rarely measured, particularly in response to feeding. Instead, conclusions relevant to protein intake are based solely upon measurement of muscle FSR and the assumption that FSR is primarily responsible for changes in net protein balance in healthy young adults. As previously discussed, muscle FSR assessments alone have led to changes in sports nutrition recommendations regarding protein for muscle recovery, with no apparent consideration of net protein balance (i.e., anabolism). The potential contribution of protein breakdown (FBR) on net protein balance responses to increasing doses of EAA, particularly during negative energy balance, has never been studied.

While there is limited quality data on FBR, especially in response to feeding, the quantification of net protein balance at the whole-body level provides valuable additional insight on overall protein status, especially when combined with muscle FSR measures. In a recent study, postprandial muscle FSR and whole-body protein turnover were determined at rest and in response to resistance exercise to evaluate the overall body protein response to consuming mixed-meals with either 40 g or 70 g of high-quality protein [16]. As expected, muscle FSR was not different between protein intakes (**Figure 3**), indicating a similar, and probably maximal muscle protein synthetic response to each protein dose. However, whole-body net protein balance was significantly higher after consuming 70 g versus 40 g; an effect largely attributed to a dramatic decrease in whole-body protein breakdown (**Figure 4**).

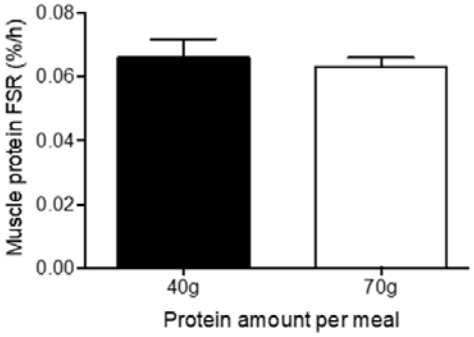


Figure 3. Muscle protein synthesis in response to a mixed-nutrient meal with 40 g or 70 g of quality protein intake in young men and women.

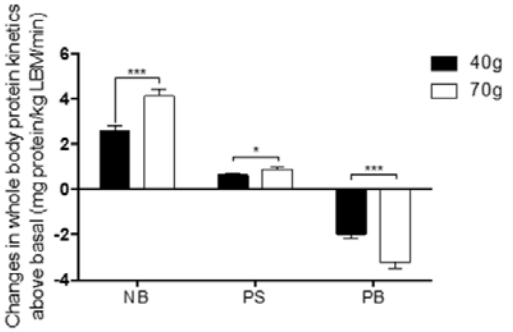


Figure 4. Whole-body protein kinetics in response to a mixed-nutrient meal with 40 g or 70 g of quality intake in young men and women[14]. NB - net protein balance; PS – protein synthesis; PB – protein breakdown. *p<0.05; ***p<0.0001.

These results point to a responsible metabolic mechanism that would ***not*** be evident if muscle protein synthesis was the only kinetic outcome studied. These results suggest that the abundance of circulating amino acids from exogenous sources reduces the reliance upon muscle protein breakdown to provide the amino acid precursors to sustain protein synthesis. Therefore, protein synthesis is maintained at a level commensurate with tissue intracellular concentrations. These data also indicate that relying upon only muscle FSR measures in response to protein intake may underestimate the total body anabolic response, which from a nutritional standpoint, reflects the total gain of body protein, and in more stressful circumstances (i.e., military operational stress), body protein health/status over time [17].

<u>Intracellular Amino Acid Availability and Whole-Body Protein Anabolism</u>

There appears to be a direct linear relationship between the rate of appearance of AA into the muscle intracellular pool (via inward AA transport and AA release from protein breakdown) and the rate of muscle protein synthesis [18, 19]. This finding indicates, in part, that increases in circulating AA concentrations are directly related to increases in protein synthesis. The controlling factor for synthesis appears to be the intracellular concentrations of AA, which remain relatively constant with modest increases in plasma AA [19]. Once the maximum rate of synthesis is reached, intracellular concentrations rise in relation to the continued increase in plasma AA [19]. However, the point at which intracellular AA increase does not necessarily coincide with the maximal *anabolic* response. Though protein synthesis may reach a maximum, net anabolism may continue if the rise in intracellular concentrations signal a reduction in the rate of protein breakdown [20]. Thus, even with a maximal synthetic response, further anabolism is achievable through a decrease in protein breakdown. Net anabolism is then dictated by the magnitude of decrease in protein breakdown. In this regard, the relationship between AA availability and net anabolism is linear, with no discernable plateau at higher levels of protein intake. This aspect of protein kinetics in response to EAA/protein intake is only evident when

both synthesis and breakdown are measured at the whole-body level. Preliminary work is consistent with this relationship, and demonstrates that whole-body net protein balance increases linearly with protein intake (**Figure 5**) [16]. This kinetic insight provides an integrated picture of whole-body protein status. Further, though not a direct measure of skeletal muscle protein turnover, changes in whole-body net protein balance are consistent with measures in muscle, due to the large contribution (by mass) of skeletal muscle to whole-body protein turnover. We suggest that assessing whole-body net protein balance may be of greater importance than measures in muscle that respond to the increasing stress of strenuous military operations, as it reflects the protein turnover status of many tissues that are less prominent in a non-stressed state. However, no studies have thoroughly examined the relationship between skeletal muscle and whole-body protein anabolism in response to various amounts of EAA during stress.

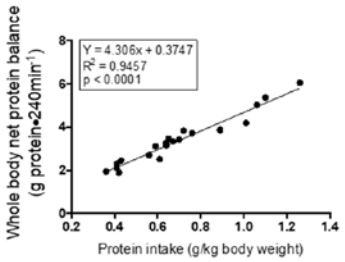


Figure 5. Relationship of protein intake in mixed meal and whole-body net protein balance.

Molecular Regulation of Skeletal Muscle Anabolism

Muscle protein synthesis rates (i.e., muscle FSR) are governed by the mechanistic target of rapamycin complex 1 (mTORC1) [21, 22]. The mTORC1 pathway can be influenced by exercise, energy balance, and EAA availability [23]. Resistance exercise and increased intracellular EAA concentrations can independently and synergistically activate mTORC1, resulting in a transient upregulation of anabolic signaling, mRNA translation initiation, elongation, and, ultimately, FSR [24-26]. We demonstrated blunted mTORC1 activation and downstream signaling following endurance exercise and recovery protein ingestion (25 g whey protein isolate) during a sustained energy deficit (unpublished data), yet whether consuming EAA in amounts greater than that present in 25 g of whey protein (~12.5g EAA) can overcome the apparent anabolic resistance during energy deficit has not been studied.

MicroRNA may be a potential mechanism governing adaptive mTORC1 signaling responses to anabolic stimulation [27]. Skeletal muscle-specific microRNA expression, myomiR; miR-1, miR-133a, miR-133b, miR-206, miR-208, miR-221, miR-222, miR-486, miR-499 [28], can be altered by acute anabolic stimulation [27, 29-31]. After resistance exercise, miR-1, miR-133a, miR133b, and miR-206 expression are downregulated [29, 32]. After endurance exercise, there is an upregulation or no change in expression of these miRNA [32-34]. However, when endurance exercise is combined with EAA ingestion, myomiR expression is downregulated or unchanged immediately post-exercise and during recovery. Divergent myomiR expression responses to various exercise modes, with or without EAA intake, may be sensitive to muscle protein synthesis rates, as miR-206 and miR-499 expression were recently shown to be inversely associated with muscle FSR during exercise [32]. MyomiR target and inhibit molecular markers within the mTORC1 pathway. As such, downregulation in their expression may enhance anabolic signaling. However, while these data suggest that myomiR expression is modulated in response to both exercise and EAA, this relationship has never been studied in the context of short-term energy deficit.

B3. MILITARY RELEVANCE

The use of a combat ration item designed to provide optimal protein quality and quantity to promote recovery from significant operational stress may increase combat effectiveness by sparing skeletal muscle and whole-body protein. Efforts to develop an optimized protein-containing food product would be consistent with recommendations from several internationally recognized organizations, and a recent consensus document developed specifically for military operations [10-12]. A consistent recommendation among these entities is the consumption of high-quality protein-containing products during periods of substantial metabolic stress, particularly during situations that elicit a negative energy and protein balance. This proposal will provide the initial evidence to support the production of a recovery-based food component for combat rations. It will define the metabolic responses of various EAA quantities. Future efforts will extend upon the present study's evidence and assess protein quality and the accompanying food matrix to support skeletal muscle and whole-body protein sustainment and/or recovery from military operational stresses. The proposed studies support the

joint effort between USARIEM and the US Army Combat Feeding Directorate, i.e., the Army Science and Technology Objective: Nutritionally Optimized Food Products for an Expeditionary Force (R.MRMC.2017.01).

B4. OBJECTIVES/SPECIFIC AIMS/RESEARCH QUESTIONS

Objectives

1. Characterize effects of essential amino acid (EAA) quantity on resting and post-resistance exercise muscle and whole-body protein kinetic responses during short-term energy deficit.

2. Determine the effects of essential amino acid (EAA) quantity on resting and post-resistance exercise skeletal muscle anabolic signaling during short-term energy deficit.

Hypotheses

1. Resting and post-resistance exercise muscle protein synthesis will be stimulated to a greater degree with higher (0.30 g/kg) versus lower (0.10 g/kg) EAA intake during acute, moderate energy deficit.

2. Resting and post-resistance exercise whole-body protein balance will be greater with higher (0.30 g/kg) versus lower (0.10 g/kg) EAA intake during acute, moderate energy deficit.

3. Resting and post-resistance exercise whole-body protein balance will be more positive with higher (0.30 g/kg) versus lower (0.10 g/kg) EAA intake during acute, moderate energy deficit due to a greater increase in whole-body protein synthesis.

4. Resting and post-resistance exercise skeletal muscle anabolic signaling will be stimulated to a greater degree with higher (0.30 g/kg) versus lower (0.10 g/kg) EAA intake during acute, moderate energy deficit.

B5. RESEARCH PLAN

B5.1 Research Design

This study will be a randomized, double-blind, cross-over controlled trial. Before the study begins, a research team member (Nancy Murphy) will generate the randomization scheme using a random number generator (http://randomization.com). Ms. Murphy will be responsible for preparing and coding the individualized EAA drinks for each trial and she will hold the randomization code until the study has been completed (i.e., data collected and analyzed). The study investigators will be blinded to this process. The code will be broken by Ms. Murphy after the primary outcomes (i.e., muscle protein synthesis and whole-body protein balance) have been statistically analyzed by the study investigators (i.e., Pasiakos, Ferrando, Margolis, Gwin, Church, and Carbone).

B5.2 Research Subjects/Population(s)

B5.2.1 Subject Population(s)

Twenty healthy, recreationally-active adult men and women representative of active duty military service members will be tested in this study. This study will recruit up to 70 individuals in order to complete testing on 20. These individuals may be recruited from the HRV population, civilians, and active duty military personnel (on and off the installation).

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

In previous studies of similar design, pre-study screening failure rate is approximately 25%. To account for screening failures and the possibility that actual study attrition rates may be greater, we estimate that 30 individuals will require screening in order to complete testing on the 20 volunteers necessary to reach statistical power in our primary outcomes. All screening will stop once complete data has been collected on 20 volunteers. Records and specimen collection are described in the Research Procedures and Data Collection sections. During briefings and consenting, potential participants will be informed that even though they may be eligible and want to participate, if we are able to obtain enough data from preceding subjects, they may not ultimately be tested.

B5.2.3 Inclusion Criteria

- Men and women aged 18 – 35 years
- Body mass index < 30.0 kg/m^2
- Healthy without evidence of chronic illness or musculoskeletal injury as determined by the USARIEM Office of Medical Support and Oversight (OMSO)
- Resistance exercise trained defined by self-report as performing ≥ 2 sessions/wk for previous 6 mo
- Refrain from taking any 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
- Willing to refrain from alcohol, smoking any nicotine product (includes e-cigarettes); vaping, chewing tobacco, caffeine, and dietary supplement use throughout the entire study period
- Supervisor approval for federal civilian employees and non-HRV active duty military personnel working within the US Army Natick Soldier Systems Center

B5.2.4 Exclusion Criteria

- Musculoskeletal injuries that compromise exercise capability as determined by the USARIEM Office of Medical Support and Oversight (OMSO)
- Metabolic or cardiovascular abnormalities, gastrointestinal disorders (e.g., kidney disease, diabetes, cardiovascular disease, etc.)
- Abnormal PT/PTT test or problems with blood clotting
- History of complications with lidocaine
- Present condition of alcoholism, anabolic steroids, or other substance abuse issues
- Blood donation within 8-wk of beginning the study
- Pregnancy (self-report or results of urine pregnancy test before body composition testing)
- Unwillingness or inability to consume study diets or foods provided

B5.3 Research Procedures

Experimental Design

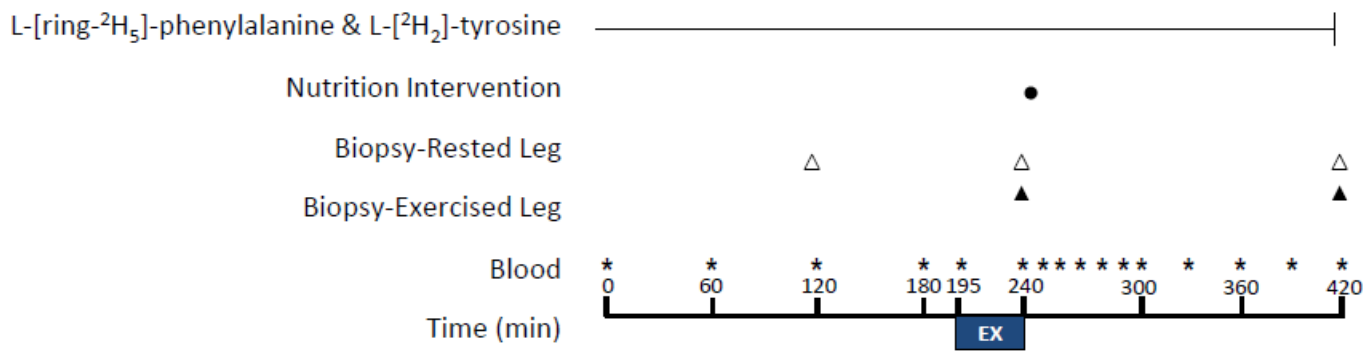
Twenty resistance-trained adults will be tested in this approximately 39-d randomized, double-blind cross-over study. Volunteers will participate in two, non-consecutive 5-d controlled energy deficit periods, separated by a 14-d washout period (i.e., sufficient time to restore nitrogen and metabolic homeostasis after moderate weight loss) [35-38]. Basal (fasting/resting), resting and post-resistance exercise (single leg exercise model) whole-body protein turnover and skeletal muscle protein synthesis responses to standard (0.10 g/kg) and high (0.30 g/kg) EAA doses will be determined the morning after completing each 5-d energy deficit. To limit potential confounding effects of pre-study diet, and to be consistent with our previous research [16, 39, 40], volunteers will be provided a 3-d run-in, eucaloric diet. After the 3-d eucaloric run-in diet, energy intake will be reduced by 30%. Physical activity will be completely restricted during the 3-d run-in diet and 5-d energy deficit to isolate the effects of the energy deficit on protein turnover and limit any residual effects of previous exercise on outcome measures [41].

The 30% energy deficit from diet alone is consistent with our previous energy deficit studies [42] and work demonstrating that a 10-d, 20% diet-induced energy deficit (i.e., ~500 kcal/d) downregulates resting skeletal muscle protein synthesis by as much as 19% [43]. Others have shown that 5-d of moderate energy deficit (reducing energy availability from 50 kcal/kg fat-free mass to 30 kcal/kg fat-free mass) is long enough to downregulate resting skeletal muscle protein synthesis by 27% [35]. In that study, ingesting 15 g and 30 g of whey protein isolate after a bout of resistance exercise restored muscle protein synthesis rates to resting, fasting rates observed during energy balance in a dose dependent manner. This proposal will extend these findings and determine if the observed muscle protein synthetic plateau after conventional resistance exercise [9] during energy balance persists during moderate energy deficit when EAA are ingested relative to body mass (0.10 g/kg versus 0.30 g/kg). Relative EAA intakes were chosen to minimize inter-subject variability and to provide a spectrum of responses to varying absolute EAA doses in order to delineate the breakpoint, and practical dose, at which skeletal muscle and whole-body anabolic responses are optimal [44]. This study will be the first to assess whole-body protein kinetics in response to consuming different doses of EAA and their associations with muscle protein synthesis during short-term energy deficit.

The stable isotope infusion protocol will occur in the morning following an overnight fast (**Figure 6**). Two intravenous catheters will be placed; one for the infusion of stable isotopes, and the other heated for serial "arterialized" blood sampling. As previously described [45], a baseline blood sample will be drawn (0-min) prior to primed continuous infusions of L-[ring-$^2H_5$]-phenylalanine and L-[3,3-$^2H_2$]-tyrosine. To appropriately reach isotopic equilibrium of L-[ring-$^2H_4$] tyrosine enrichment derived from L-[ring-$^2H_5$]-phenylalanine tracer, a priming dose of L-[ring-$^2H_4$]-tyrosine also will be injected at 0-min [45]. All isotopes will be purchased from Cambridge Isotope Laboratories (CIL, Andover, MA) and solutions will be constituted by a licensed pharmacy and certified sterile and pyrogen free before administration (pharmacy to be determined).

At 120-min, a muscle biopsy of the vastus lateralis will be taken on the non-exercised leg only to assess basal (fasting/resting) muscle protein kinetics. After the muscle biopsy, volunteers will remain rested until they perform a heavy-load, high volume, single leg-resistance exercise bout at 195-min that consists of 8 sets X 10 repetitions on the leg-press and leg-extension machines [9]. Both exercises will be performed at 80% of a pre-determined one repetition maximum (1RM) with a 2-min rest interval between sets. Volunteers will have ~45-min to complete the exercise bout. In the event the volunteer is unable to complete the prescribed workload; the workload will be reduced by 4.5 kg as previously described [9]. This will be repeated until the participant can complete the prescribed work. Approximately 5-min after completing the exercise bout (240-min), a muscle biopsy will be taken from the vastus lateralis of the exercised and non-exercised (rested) legs (the exercised and non-exercised leg will be the same between trials). Immediately after the biopsies, volunteers will consume the appropriate dose (i.e., an artificially flavored beverage) of free-form EAA in 5-10-min [Reginator™; EAA content per 10 g of the mixture: 0.15 g histidine, 3.66 g leucine, 0.94 g isoleucine, 1.65 g lysine, 0.3 g methionine, 0.63 g phenylalanine, 0.89 g threonine, 0.1 g valine, 0.006 g tryptophan, and 0.81 g arginine (conditionally essential under stress)]. The phenylalanine content of the beverage will be artificially

Figure 6. Infusion Protocol



enriched with L-[ring-$^{2}H_{5}$]-phenylalanine to 4% to limit potential disturbances in isotopic steady-state. Volunteers will remain rested during serial blood sampling over a 3-h period following ingestion. Final biopsies will be taken from each leg at the end of the 3-h period. There will be a total of 2 incisions per EAA trial, one on the rested leg and one on the exercised leg. A total of 5 muscle biopsies will be collected from each volunteer per EAA trial; 2 through 1 incision on the exercised leg and 3 through 1 incision on the rested leg (2 new incisions will be made on the subsequent EAA trial. The incisions on EAA trial 1 will NOT be used for trial 2). Blood samples will be collected at regular intervals to assess isotopic enrichments for kinetic calculations, insulin, blood urea nitrogen, and amino acid concentrations (**Figure 6**).

3-d Run-In and 5-d Energy Deficit

Volunteers will complete a baseline, pre-study 3-d diet record (**Appendix A**) and a 3-d activity log (**Appendix B**) according to instructions provided by study team dietitians. The data will be analyzed using Food Processor SQL™ (Salem, OR Version 10.0) and collected to predict volunteers' total daily energy requirements for the 3-d eucaloric run-in and 5-d energy deficit diets. The volunteers will receive standardized meals derived primarily from military combat rations (Meals Ready-to-Eat, MRE) for each diet phase to replicate the source of nutrition during short-term military operations. The energy content and macronutrient distribution of the MRE-based, 3-d run-in diet will be sufficient to maintain body weight and provide between 50-65% carbohydrate and 1.6 ± 0.2 g protein·kg^{-1}·d^{-1}, amounts habitually consumed by Warfighters in past USARIEM studies [39, 42]. Total energy content of the 5-d energy deficient diet will be reduced by 30%, primarily by reducing the total amounts of carbohydrate and fat consumed, while keeping dietary protein constant at 1.6 ± 0.2 g protein·kg^{-1}·d^{-1}. All meals will be prepared and provided to volunteers by study team dietitians during the 3-d run-in diet. During the 5-d energy deficit, all meals will be prepared and provided to the volunteers.. Volunteers will be completely restricted from all non-study physical activity during the 3-d run-in and 5-d energy deficit diets to minimize complications and the potential for carryover effects on study parameters. If necessary, military volunteers will be provided study-specific profiles to restrict physical activity. This approach has been applied successfully in past USARIEM studies [39].

During the 14-d washout period, volunteers will be instructed to consume an ad libitum diet. Routine physical activity (exercise) can be resumed during the wash-out phase at levels consistent with amounts reported in pre-study 3-d activity records. Diet records (24-h) and activity logs (24-h) will be completed every third day and then reviewed (the following day) during the 14-d washout period to assess dietary intake and physical activity. Volunteers will meet with study dietitians to review these records.

Anthropometric Data

Anthropometrics, performed using standardized techniques and equipment, will be used to characterize study volunteers, and evaluate responses to each 5-d energy deficit. 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 and then daily during each 3-d run-in and 5-d energy deficit period to ensure weight maintenance and/or weight loss. Body mass will be measured every third day during the 14-d washout period (measurements will correspond with the days diet records and activity logs are reviewed described above).

Body composition will be determined at baseline and after the 14-d washout by using a dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI). The DEXA technique allows for the non-invasive assessment of soft tissue composition by region with a precision of 1-3%. 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. These data will be used to calculate total body mass, fat-free mass, and fat mass. Calibration to external standards will be performed before actual data collection. The operator remains in the room with the volunteer during the scan.

B5.4 Data Collection

Blood Sampling and Total Blood Volume Collected

Serial blood draws collected during the stable isotope infusion studies (see **Figure 6**) will be analyzed for isotopic enrichments, blood urea nitrogen, insulin, and AA concentrations. There are 16 blood draws per infusion study. 9.5mL of blood will be drawn at each of the 16 draws, for a total of 152 mL per infusion study and 304 mL over the course of the study. Sufficient blood will be drawn and archived to allow for additional analyses of related metabolic biomarkers sensitive to EAA, resistance exercise, and energy deficit.

Stable Isotope Analysis and Calculations of Whole-body and Muscle Protein Kinetics

All stable isotope analyses on de-identified, coded blood and muscle samples will be conducted by Dr. Ferrando at UAMS (data sharing agreement attached is provided with this protocol). No samples will be stored at UAMS for future analyses because the entire sample provided will be processed for the assays below.

Blood Sample Processing for Isotopic Enrichment
Blood samples will be precipitated with 125 µL of 10% sulfosalicylic acid (SSA) and centrifuged. The internal standard technique will be used to determine blood EAA concentrations. Free amino acids (AA) will be extracted from 300 µL supernatant fluid by cation exchange chromatography and dried under Speed Vac (Savant Instruments, Farmingdale, NY or similar model). Enrichments of phenylalanine and tyrosine will be measured on the tert-butyldimethylsilyl derivative with the use of gas chromatography-mass spectrometry (models 7890A/5975; Agilent Technologies, Santa Clara, CA or similar model) [46, 47]. Ions of mass-to-charge ratio of 234, 235, and 239 for phenylalanine, of 302 and 308 for leucine, and of 466, 467, 468, and 470 for tyrosine will be monitored with electron impact ionization and selective ion monitoring.

Muscle Sample Processing for Isotopic Enrichment
Muscle biopsy tissue samples taken from the vastus lateralis muscle will be cleaned of visible fat, blotted of excess blood, then frozen in liquid nitrogen, and stored in a −80°C freezer until later analysis. Upon thawing, muscle tissues will be weighed, and tissue proteins precipitated with 0.5 mL of 4% SSA. The tissues will then be homogenized and centrifuged for collection of supernatant. The procedure will be repeated two more times, and tissue intracellular free AAs extracted from the pooled supernatant via the same cation exchange chromatography stated in plasma analyses and then dried under the Speed Vac. The remaining muscle pellet will be washed, dried, and hydrolyzed in 0.5 mL of 6 N HCl at 105°C for 24-h. Enrichments from muscle free and bound tracers will be determined as in plasma analyses.

Whole-body Protein Turnover and Muscle Fractional Synthesis Calculations
Calculations of whole-body protein synthesis (PS) and protein breakdown (PB) rates will be performed based on the determinations of the rate of appearance (Ra) into the plasma of phenylalanine and tyrosine and the fractional Ra of endogenous tyrosine converted from phenylalanine [48]. Plasma enrichments of phenylalanine and tyrosine tracers will be curve-fitted with a cubic spline method using Graphpad Prism 5 (Graphpad Software, La Jolla, CA or equivalent). Whole body protein turnover will be calculated by dividing kinetic values of a single essential amino acid (i.e., phenylalanine), which cannot be synthesized in the body, by its fractional contribution to protein. For the calculations for whole-body PB rate, contribution from exogenous EAA and tracers infused will be subtracted from total Ra. Appropriate skew correction will be made for ^{13}C-labeled tracers [48].

Calculation of resting and post-exercise muscle FSR will be determined as the incorporation of the phenylalanine tracer from biopsy one (180 min) to biopsy two (420 min) using the precursor-product model [49].

The following equations will be used for calculations of whole-body and muscle protein kinetics:

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

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

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

$$PS = [(R_{a|Phe} – \text{Phe hydroxylation}) \text{ X } 25]$$

$$PB = [(R_{a|Phe} – F_{Phe}) \text{ X } 25 – PRO_{MEAL}]$$

$$NB = PS – PB$$

$$FSR\ (\%) = [(E_{p2}\text{-}E_{p1})/(E_m \text{ X } t)] \text{ X } 60 \text{ X } 100$$

where enrichment (E) is expressed as tracer-to-tracee ratio (TTR) for calculation of PB or mole percent excess for calculation of muscle FSR, calculated as TTR/(TTR + 1). E is enrichment of respective tracers at plateau. F is respective tracer infusion rate into a venous side: F_{Phe} for phenylalanine tracer. $E_{Tyr\ M+4}$ and $E_{Phe\ M+5}$ are plasma enrichments of tyrosine tracers at M+4 and M+5 relative to M+0, respectively. Correction factor of 25 is for conversion of value for phenylalanine to protein based on the assumption that contribution of phenylalanine to protein is 4% (100/4 = 25) [50]. PRO_{MEAL} is the amount of exogenous EAA (g) that appeared in the circulation, which was calculated as total amount of EAA provided (if any), based on the assumption that 85% of the EAA ingested was absorbed [51]. Phenylalanine hydroxylation is the R_a of tyrosine derived from phenylalanine through hydroxylation. E_{P1} and E_{P2} are the enrichments of bound L-[*ring*-$^2H_5$]-phenylalanine in the 180 min and 420 min biopsies, respectively. E_m is the calculated mean value of the enrichments of [*ring*-$^2H_5$]-phenylalanine in the intracellular or plasma pool. *t* is the time in minutes elapsed between muscle biopsies. Factors 60 and 100 were used to express muscle FSR in percent per hour.

mRNA and microRNA 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-1000spectrophotometer (Nanodrop, Wilmington, DE, USA) or equivalent. Equal amounts of total RNA will be synthesized into cDNA for analysis of mRNA (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA, USA or equivalent) and a TaqMan® microRNA RT kit (Applied Biosystems) or equivalent. Individual primers will be used to determine the mRNA expression of known intracellular targets regulating protein metabolism, to include but not limited to atrogin, MurF-1. LARS, LAT1, SNAT2, REDD1, and IGF1. Individual primers will be used to determine microRNA expression of microRNA that have been reported to impact skeletal muscle anabolism, to include but not limited to miR-1, miR-133a/b, miR-206, miR-208, miR-486, and miR-499

To determine if circulating microRNA reflect changes within skeletal muscle to exercise and dietary protein intake total circulating microRNA will be extracted from 200 µL serum using miRNeasy Serum/Plasma kit or equivalent, which allows for extraction and purification of small (< 200 nt) cell-free RNA (Qiagen, Valencia, CA, USA). Additionally, to assess if changes in circulating microRNA differ between total and exosomal circulating microRNA, exosomes will be extracted from 1000 µL serum using a miRCURY™ Exosome Isolation Kit-Serum and Plasma (Exiqon, Woburn, MA) or equivalents. 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) 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 of external (Spike-In Control C. elegans miR-39) and internal controls to allow for both technical and inter-individual normalization. Geometric mean of controls will be used to correct for possible outlying values and abundance differences between the different controls [52].

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 ΔΔ cycle threshold ($\Delta\Delta C_T$) method as described below in statistical analysis section.

Western Blotting

Approximately 30 mg of muscle will be homogenized in ice-cold buffer (1:10 wt/vol) 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 molecular markers associated with anabolism will be determined using Western blotting techniques. Muscle lysates will be solubilized in Laemmli buffer, with equal amounts of total protein (15 μg) separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad). Proteins will be transferred to polyvinylidene fluoride (PVDF) membranes and exposed to commercially available primary antibodies of intracellular markers involved with skeletal muscle anabolism and proteolysis to include but not limited to Akt, p-Akt^{Ser473}, mTOR, p- mTOR^{Thr2448}, p70S6K, p-p70S6K^{Thr424}, rpS6, and rpS6$^{Ser235/236}$ (Cell Signaling Technology, Danvers, MA, USA or equivalent) at 4°C overnight. Labeling will be performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent will be applied (Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots will be quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad) or similar models. To confirm equal protein loading per well a normalizing protein such as GAPDH or HSP90 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. This study subject ID number will be used on all data collection instruments, to include questionnaires, data collection forms, computer records, etc. (**Appendices A - D**). 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 samples will be processed on site at USARIEM and stored in Military Nutrition laboratory freezers (room 322, 304) usingthe subject identification number indefinitely for renalysis if necessary. Coded study samples (blood and muscle) for isotopic and amino acid analysis will be shipped on dry ice to Dr. Arny Ferrando at UAMS via FedEx and stored in his laboratory until analyzed. Once analyzed, there will be no remaining sample for storage. .

Only personnel assigned to the research study by the principal investigator will have access to the data. 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.

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

Any use of the samples outside of this defined protocol will be submitted as a protocol amendment or a new protocol. Samples will be retained for further analyses related to metabolic biomarkers sensitive to EAA, resistance exercise, and energy deficit once the protocol is closed. Participants will be made aware of this during the informed consent process.

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

B5.7.1 Devices

5.7.1.1 FDA-approved device being used in this research according to the approved labeling

DEXA, DPX-IQ, Lunar Corporation, Madison, WI

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

Means (SD) from Macnaughton et al. [53] and Witard et al. [9] demonstrating the effects of graded protein dose on resting and post-resistance exercise muscle FSR were used to determine statistical power and sample size. Using those data, we anticipate observing a mean (SD) difference in muscle FSR of ~0.015 %/h (0.02 %/h) for both the rested and exercised leg between EAA doses. The sample size necessary to determine differences between EAA doses (assuming an alpha of 0.05, mean difference of 0.015 %/h, standard deviation of 0.02 %/h, and 80% power) is 16. However, 20 volunteers will be studied to account for the potential for greater variability than those reported by others and to increase our statistical power to 90% in order to detect differences between the 0.10 g/kg and 0.3 g/kg EAA doses. This estimate also provides greater than 95% power to detect differences in whole-body protein balance between EAA doses [16]. We request the ability to increase our enrollment to 40 volunteers to account for screening failures and study attrition. Enrollment and testing will cease once data has been collected on 20 volunteers.

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. An ANOVA will be performed to determine the effects of EAA dose (0.10 g/kg vs. 0.3 g/kg) on muscle FSR and whole-body protein turnover. Correlation coefficients and multiple regression analysis will be used to evaluate relationships between study outcome measures. The alpha level will be adjusted for multiple comparisons, with the level for statistical significance set at $P < 0.05$.

SECTION C: HUMAN RESEARCH PROTECTIONS

C1. RECRUITMENT AND CONSENT

C1.1 Identification and Selection of Subjects

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 (**Appendix C**). If still eligible, volunteers will make an appointment for a medical screening. If an individual fails the medical screening for whatever reason or does not meet eligibility criteria, their screening data will be destroyed.

The medical clearance will take place at USARIEM (Natick, MA) by OMSO staff. Volunteers recruited through SSIT may undergo 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).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 to pre-study, baseline testing.

C1.2 Recruitment Process

Volunteers will be recruited from the federally and non-federally employed civilian population, the Natick Human Research Volunteer (HRV) Pool, NSSC active duty military personnel, 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 Human Research Subjects 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 (either Katelyn Guerriere, Katherine Mitchell, Karleigh Bradbury, Maria Canino, Matthew Bartlett, or Caitlin Haven) will be present during the briefing.

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.

Civilian volunteers and other active duty personnel will be recruited by “word of mouth”, posted flyers (“off-site recruitment flyer” to be used for any flyers posted outside of NSSC, “recruitment flyer” to be used for postings within NSSC), or electronic distribution of the “off-site” flyer to include a text-only, approved version of the flyer. Recruiting materials will be distributed around NSSC, surrounding community, and on bulletin boards at local universities. 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). Recruiting may also be conducted through information meetings presented to college classes, clubs, sports teams, or other organizations. 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 (**Appendix C**). 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 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). Potential military and civilian volunteers will undergo the same clearances. 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, as determined by OMSO, 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 females will be given a urine pregnancy test during the initial screening and the morning of or at least 24 hours prior to each DEXA scan. The test will be read by a female member of the study team and the results will be shared with the volunteer. If the pregnancy test is positive, the volunteer will be excluded from further participation in this study.

All volunteers must be willing to consume only food and beverages provided by study staff during the run-in and energy deficit 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. No study procedures will occur prior to any volunteer giving their informed consent. 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. 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. An ombudsman will not be required for any individual briefings or for civilian group 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 USAMRMC Volunteer Registry Database
It is the policy of USAMRMC that data sheets are to be completed on all volunteers participating in this research for entry into the U.S. Army Medical Research and Materiel Command Volunteer Registry Database. The information to be entered into this confidential database includes name, address, social security number, study name, and dates. The intent of the database is twofold: first, to readily answer questions concerning an individual's participation in research sponsored by the USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at the USAMRMC for a minimum of 75 years.

C2. COMPENSATION FOR PARTICIPATION

Volunteers who participate in the study will receive $33.75 per blood draw (32 total draws), for a total of $1,080 for completing the study.

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. 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 him/herself, all further data collection will discontinue. Participants will be compensated for any blood draws they completed up until that point, and they will be asked to return any remaining food items that were provided, in addition to any wrappers and diet/activity 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 coordinator 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 coordinator's office, or kept in a computer file with password-protected access restricted to the principal investigator and project coordinator. 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 and stored on site at USARIEM in Military Nutrition laboratories (rooms 304, 322). Study samples will shipped to Dr. Arny Ferrando's laboratory at UAMS will be processed in their entirety for analyses (i.e., no additional samples will be stored at UAMS). All samples coded with a 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.

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: Venous Catheterization
Research Procedure Description: A needle will be used to guide a catheter into a superficial vein. 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 8 weeks before or after this study.

Research Procedure Name: Stable Isotope Administration
Research Procedure Description: Volunteers will be infused intravenously with stable isotopes of amino acids on two occasions.
Research-related Risks: The primary risks associated with tracer studies are those related to venous catheterization. The catheter can cause irritation, bruising, or infection. There are no known risks or reported side effects associated with administration of 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 infusions proposed have no appreciable effect on the total abundance of the isotopes present in the body. The risks associated with the infusion include volume overload, infection, and allergic reaction to the infused substance. There have been no occurrences of volume overload, no occurrences of infection or allergic reaction attributable to iodine used prior to venipuncture in any of the 200 infusion protocols that the principal investigator has been involved with over the last 8 years. To minimize the likelihood of these events occurring, infusion rate will be closely monitored and maintained at less than 50 ml/hr throughout the entire 7-h infusion period.
Measures to Minimize Risks of Harm: All staff who directly participate in the stable isotope studies will be properly trained how to safely monitor (i.e., infusion pumps and IV lines) isotope infusion studies from Dr. Pasiakos, who has extensive experience with stable isotopes. In addition, infusates will be prepared sterile, pyrogen-free, and in the proper dosages by a licensed pharmacist and administered by Dr. Pasiakos, Dr. Margolis, and Dr. Gwin.

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%) [54, 55]. Panic episode, bleeding, and edema have also been reported (0.21%, 0.42%, and 0.84%, respectively) [54]. 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 (**Appendix D**). Muscle biopsies will be performed using sterile procedures by Dr. Pasiakos, Dr. Margolis, or Dr. Gwin, 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, OMSO will be notified immediately and lidocaine use will be discontinued. Dr. Pasiakos, Dr. Margolis, and Dr. Gwin 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: Resistance Exercise
Research Procedure Description: Resistance exercise includes maximal leg press and leg extension testing, and prescribed resistance exercise bouts during the infusion studies.
Research-related Risks: Resistance exercise is generally considered safe and the primary risks associated with resistance exercise include local muscle discomfort/soreness, ranging in intensity from mild to severe that can persist for 1-7 d. Other risks include muscle strains and hyper-extension and/or flexion of the joint.
Measures to Minimize Risks of Harm: To reduce these risks, volunteers will be familiarized with the exercise tests and protocol well in advance of the criterion measure. Volunteers will be closely monitored by experienced and qualified research team members, and asked to report any pain or discomfort resulting from exercise, followed up if necessary by medical examination and postponement or curtailment of further testing.

Research Procedure Name: Diet Interventions
Research Procedure Description: Volunteers will be instructed to follow a eucaloric 3-d run-in diet to maintain body mass, followed by a 5-d period of moderate energy deficit (20%). Combat rations will be the primary food source in addition to some commercially available pre-packaged foods.
Research-related Risks: The foods and MRE components used in this study pose no known risks to volunteers. 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 diet 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: Volunteers 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. All females will be required to have a pregnancy test the day before, or the day of testing. Pregnant females will not be scanned, nor allowed to continue the study.

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 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)?
- Did you perform any exercise or physical activities outside of study activities 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 MRMC IRB as needed.

C8. REPORTABLE EVENTS

C8.1 Expected adverse events

An adverse event is defined as any untoward or unfavorable medical occurrence in a human research 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, if one was appointed for the study.

Expected adverse events which are not serious are reported to the IRB at the time of continuing review of the protocol. These events include bruising, infection, swelling and slight pain from the IV placement; slight pain from the lidocaine injection; pain, soreness, infection, bruising from the muscle biopsy; feeling faint with IV placement, blood draw or biopsy; fatigue and muscle soreness from study exercises; 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

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 USAMRMC Institutional Review Board 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 or definitely related to participation will be promptly forwarded to the ORQC and HQ USAMRMC IRB.

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

AA, amino acids; EAA, essential amino acids; MRE, Meal Ready-to-Eat; NEAA, non-essential amino acids; FSR, fractional synthesis rate; FBR, fractional breakdown rate; PS, protein synthesis; PB, protein breakdown; net, net protein balance; DEXA, dual energy x-ray absorptiometry

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