Protocol Title: Physiological and psychological effects of testosterone during severe energy deficit and recovery: a randomized, placebo-controlled trial

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IRB Review History

Executive Summary

Energy deficit during military operations is often ~50% of total daily energy requirements and can reach near complete starvation.[1,2] The physiological consequences of severe energy deficit include physical performance decrements, particularly energy deficit-induced hypogonadism and the loss of lean body mass, which cannot be overcome with macronutrient modification alone. [3-7] Prolonged energy deficit also impacts mood, attentiveness, and decision-making capabilities. The extent to which energy deficit-induced hypogonadism contributes to physiological and psychological declines during sustained energy deficit remains poorly understood. Therefore, the objective of this study is to determine whether maintaining a eugonadal state during severe, sustained energy deficit attenuates physiological decrements, particularly the loss of lean body mass, and maintains mental performance. To address these objectives and more (e.g., gut health, appetite regulation, physiological and psychological recovery), we will enroll up to 60 physically active men in a 3-phase, randomized, placebo-controlled study. After completing a 14-day (**free-living, phase 1**), energy-adequate, diet acclimation phase (protein, 1.6 $g \cdot kg^{-1} \cdot d^{-1}$; fat, 30% total energy intake; with remaining energy derived from carbohydrate), participants will be randomized to one of two experimental groups and undergo a 28-day (**live-in, phase 2**), 55% energy deficit phase: energy deficit alone (DEF) or energy deficit + exogenous testosterone (DEF+TEST). Recovery (**free-living, phase 3**) will be assessed after completing phase 2 to determine when body mass has been recovered within ± 2.5% of initial body mass (duration will vary, 42-day maximum for phase 3). Body composition, state-of-the-art stable isotope methodologies, proteomics, metabolomics, muscle biopsies, whole-room calorimetry, molecular biology, activity/sleep monitoring, personality and cognitive function

assessments, functional MRI (fMRI), biochemistries, and rigorously controlled diet and physical activity will be used to assess physiological and psychological responses to energy restriction and recovery feeding while in a hypogonadal versus eugonadal state. Results of the proposed experiments will provide a basis for future studies aimed at improving Warfighter resiliency to and recovery from negative energy balance.

Statement of Need
There is a critical need for effective and feasible interventions that sustain and optimize Warfighter health and performance during real-world training and combat operations. This study will delineate the contribution of testosterone declines from the physical and mental demands encountered by Warfighters during military training and combat operations on complex markers of physiological and psychological status, addressing a direct, consistently observed gap in knowledge.

Objective I: Effects of exogenous testosterone during energy deficit and recovery.

A. Determine the extent to which maintenance of a eugonadal state by exogenous testosterone administration attenuates the effects of severe, sustained energy deficit on body composition (body mass, lean body mass and fat mass), skeletal muscle (mass, strength/power/endurance, proteomics, intramuscular regulators of metabolism, protein synthesis and proteolysis), metabolism (energy expenditure, substrate oxidation and nitrogen balance) and physiological status (androgens, stress and metabolic hormones, inflammation, hepcidin, iron status, circulating and intramuscular substrates and blood lipids).
B. Determine the effects of exogenous testosterone administration during severe, sustained energy deficit on subsequent recovery of body composition, skeletal muscle, and metabolic and physiological status.
C. Identify metabolomic and genetic biomarkers associated with habitual dietary intake and severe, sustained energy deficit and their predictive association with body composition, skeletal muscle, metabolism, and physiological status.
D. Determine the extent to which exogenous testosterone administration modulates both metabolomic biomarkers and associations between the metabolome and diet, body composition, skeletal muscle, metabolism, and physiological status during severe, sustained energy deficit.

Hypotheses for Objective I:

A. Severe energy deficit will elicit a hypogonadal state and loss of total body mass, lean body mass, and fat mass; exogenous testosterone administration will attenuate the loss of lean body mass and skeletal muscle mass and enhance the loss of body fat. In addition, during severe energy deficit, protein (e.g., glycolytic, mitochondrion, muscle development, actin cytoskeleton, etc.) synthetic rates will be downregulated, whereas intramuscular proteolysis will be upregulated, resulting in a loss of skeletal muscle mass and diminished muscular strength, power, and endurance; exogenous testosterone administration will maintain or upregulate synthesis rates relative to pre-deficit levels, particularly protein synthetic rates of contractile proteins. Exogenous testosterone administration will preserve indices of muscular strength, power, and endurance during severe energy deficit. Energy expenditure will be downregulated by energy restriction, with an increased reliance on endogenous protein for energy metabolism;

exogenous testosterone administration will maintain measures of physiological status and energy expenditure and shift substrate oxidation to promote a greater reliance on body fat.

B. Severe, sustained energy deficit and associated hypogonadism will impact recovery, as hypogonadal individuals will gain a disproportionate amount of body fat relative to lean body mass upon refeeding, resulting in an incomplete recovery of skeletal muscle strength, power, and endurance. Exogenous testosterone administration during severe, sustained energy deficit will limit fat accretion and promote gains in lean body and skeletal muscle mass while eating ad libitum in recovery.
C. Genetic polymorphisms and specific circulating metabolites will be associated with dietary intake, body composition, skeletal muscle, metabolic, and physiological status responses to severe, sustained energy deficit and recovery.
D. Exogenous testosterone administration will elicit a metabolomic signature indicative of sustained muscle anabolism that is associated with a preservation of muscle mass, function, and physiological status.

Objective II: Effects of severe energy deficit and exogenous testosterone on personality, mood, and cognition, and their association with genetic markers of brain function, stress, and endocrine function.

A. Determine the effects of severe, sustained energy deficit and associated hypogonadism on mental fatigue and other aspects of mood, cognitive performance, brain function and sleep.
B. Identify metabolic biomarkers of fatigue and their association with cognitive performance and sleep during metabolic stress using global metabolomics.
C. Determine the extent to which the detrimental effects of sustained energy deficit on mood, cognitive performance, and sleep are attenuated by pharmacological testosterone treatment.
D. Determine the extent to which exogenous testosterone administration modulates associations between the metabolome, fatigue, and cognitive performance during sustained energy deficit.
E. Identify genetic markers of mood, personality, cognitive performance, and sleep that predict, in part, responses to energy deprivation and testosterone treatment.

Hypotheses:

A. Stress-induced hypogonadism (i.e., energy deficit induced by sustained/ increased physical activity and inadequate dietary intake) will increase fatigue and diminish mental performance.
B. Metabolic profiles and specific circulating metabolites will be associated with hypogonadism, fatigue, and measures of mental performance during sustained energy deficit.
C. Exogenous testosterone administration during sustained energy restriction will attenuate fatigue, prevent temporary alterations in personality characteristics, and protect against decrements in mental performance.
D. Exogenous testosterone administration during sustained, severe energy restriction will elicit a metabolomics signature indicative of enhanced central nervous system (CNS) function that is associated with lower fatigue and improved mental performance.

E. Polymorphisms in CNS, stress, and endocrine genes will be associated with energy deprivation and exogenous testosterone administration.

Objective III-A: Effects of energy deficit and exogenous testosterone on appetite regulation.

A. Determine the effect of testosterone maintenance on appetite and adaptive responses of appetite-mediating hormones during energy deficit and body mass recovery in non-obese adults.

Hypotheses:

A. During energy deficit, leptin concentrations will decrease while appetite and acyl ghrelin concentrations will increase. Following weight regain, hormone concentrations will not differ from pre-energy deficit concentrations.
B. Testosterone maintenance will augment the energy deficit-mediated decrease in leptin and increase in ghrelin concentrations but will not affect appetite.

Objective III-B: Effects of energy deficit and exogenous testosterone on the gut microbiome and intestinal permeability.

A. Determine the effects of energy deficit with and without testosterone treatment on gut microbiota composition, function, and activity.
B. Identify associations between gut microbiota composition and function, host energy/substrate metabolism, body mass change, and the composition of body mass loss and regain.

Hypotheses:

A. Energy deficit will have a detrimental impact on the gut as evidenced by reduced fecal short chain fatty acid (SCFA) concentrations, decreased gut bacteria diversity, increased relative abundance of pro-inflammatory and mucolytic bacteria, and increased intestinal permeability. Body mass regain will reverse these effects.
B. Testosterone treatment will mitigate the unfavorable effect of energy deficit on the gut microbiome and intestinal permeability.

Background

Objective I: Effects of exogenous testosterone during energy deficit and recovery.

Strenuous work and inadequate energy intake during military operations produce severe energy deficits, depleted body energy stores, muscle mass loss (primarily lean body mass), degraded performance, and increased injury risk.[8-12] The physiological consequences of military operations occur to varying degrees.[1] During sustained military training operations lasting 3.5-64 days, Warfighters have experienced body mass losses ranging from 3-16% of initial body mass, with lean body mass accounting for, on average, more than 50% of the total body mass lost. [3,13-21] Decrements in total body and lean body mass generally occur with concomitant reductions in circulating levels of anabolic hormones (e.g., testosterone).[5]

Recent attempts to attenuate lean body mass loss induced by military operations have largely focused on dietary protein manipulations given that higher-protein diets have consistently been shown to spare lean body mass and maintain muscle anabolic sensitivity during moderate energy deficit.[22-24] For example, physically active adults consuming 1.6 and 2.4 $g \cdot kg^{-1} \cdot d^{-1}$during a 21-day, 40% energy deficit lost more body fat, spared more muscle mass, and maintained muscle protein synthetic responses to feeding to a greater extent than those who consumed 0.8 $g \cdot kg^{-1} \cdot d^{-1}$.[24] There were also no differences between those consuming 2.4 $g \cdot kg^{-1} \cdot d^{-1}$and 1.6 $g \cdot kg^{-1} \cdot d^{-1}$, suggesting that during moderate, sustained energy deficit, there are no advantages of consuming protein beyond twice the RDA.[24,25] These findings, which were derived from a well-controlled clinical trial, were used to develop operationally relevant dietary protein recommendations.[26] However, consuming approximately 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ has generally been overwhelmed by the effects of severe energy deficit on protein retention during real-world military operations.[27]

We suspect that the dramatic reductions in testosterone that occur during severe energy deficit could diminish the efficacy of manipulating protein intake for sparing lean body mass during military operations. In healthy young males, the suppression of endogenous testosterone production has myriad adverse physiological consequences, including reduced lean body mass, increased adiposity, and decreased muscle strength.[28-31] Finkelstein et al. [29] recently demonstrated that decreased testosterone levels (from 530 to 350 $ng \cdot dL^{-1}$), achieved by goseralin acetate administration to reduce endogenous testosterone and estradiol production, result in increased adiposity, and further reductions to ≤ 200 $ng \cdot dL^{-1}$ are accompanied by skeletal muscle atrophy and decreased muscle strength. Importantly, testosterone decreases of this magnitude occur during military training and sustained operations, and are associated with concomitant decreases in lean body mass.[3-7] ***Although dietary macronutrient manipulations, to date, have proven unsuccessful at mitigating the endocrine response to severe negative energy balance,[32] pharmacologic interventions that restore anabolic hormone concentrations have been shown to promote nitrogen retention despite energy deficit.***[33-35] Whether preventing the decline in testosterone during conditions simulating severe, sustained operational stress enhances the protein-sparing effects of consuming a higher-protein diet has not been studied.

Although we suspect that maintaining testosterone production during severe, sustained energy deficit would attenuate the loss of lean body mass, the potential influence of testosterone maintenance on physiological and body composition in recovery from operational stress remains unclear. In general, refeeding following energy deficit is marked by the preferential accumulation of adipose tissue and not lean body mass.[36] This phenomenon of “rebound fatness” has been documented in Soldiers recovering from the sustained energy deficit experienced during US Army Ranger Training.[6] It is possible that the loss of body fat during energy deficit elicits a persistent suppression of metabolic rate during recovery, whereas the reductions in lean body mass promote hyperphagia in recovery from energy deficit.[37] Thus, we suspect that if testosterone levels are maintained during severe energy deficit, lean body mass will be spared, reducing subsequent hyperphagia and relative fat mass gain during refeeding and thereby setting the conditions for a more favorable recovery.

Results from the Functional Single Nucleotide Polymorphisms Associated with Human Muscle Size and Strength (FAMuSS) study suggest that genetic variants dictate individual skeletal muscle phenotypic and adaptive responses to stress (e.g., exercise training).[38] These findings suggest certain polymorphisms may predict skeletal muscle responses to underfeeding and testosterone treatment. More specifically, multiple genes involved in the regulation of muscle structure, growth, and inflammation were associated with sex-specific genetic skeletal muscle predisposition. Additional genes associated with body composition and skeletal muscle phenotype were highly predictive of whether an individual favorably responded to an exercise training stimulus. This study will therefore examine associations between genetic polymorphisms and body composition outcomes.

Objective II: Effects of severe energy deficit and exogenous testosterone on personality, mood, and cognition, and their association with genetic markers of brain function, stress, and endocrine function.

Testosterone appears to have a variety of effects on cognition including certain aspects of performance, mood, and sleep. Studies have reported linear[39-41] or nonlinear[42,43] relationships between serum testosterone levels and cognitive ability that suggest a U-shaped relationship between testosterone and performance.[44] Spatial abilities, verbal learning and memory, and nonverbal learning and memory all appear to be affected.[39-43] The literature also suggests a relationship between testosterone and socioemotional and economic behavior. High levels of endogenous testosterone have been associated with a greater tendency to pursue reward while ignoring potential threat,[45,46] and it has been shown that competitive success causes an increase in testosterone that encourages subsequent risk-taking behavior.[47] In men with low testosterone, supplementation exerts positive effects on mood and sexual behavior, reducing fatigue and depression and increasing self-esteem.[48-52] The literature also suggests that testosterone levels may impact behavioral characteristics such as the degree of masculine identification, social propensity, trust in others, and willingness to cooperate. However, relatively little work has been conducted to examine the impact of testosterone on brain function, cognition, and mood in healthy young men.

Beyond the apparent positive impact of testosterone on psychological status, testosterone administration may alter certain aspects of collaborative behavior by inducing individuals to increase the weight they assign to their own personal judgments relative to opinions of others.[53] Although testosterone does not appear to interfere with personal decision-making, its tendency to increase dominance and status-seeking behavior evidently promotes a degree of assertiveness. Zak et al.[54] found that subjects who had high levels of testosterone were less generous overall and more likely to punish ungenerous participants. Conversely, men with the lowest levels of testosterone were nearly 6 times more generous than their high-testosterone counterparts. These results are consistent with those from retrospective studies that have indicated high testosterone levels are associated with increased selfishness and a greater propensity to punish people who violate social norms.[55] They also are generally consistent with the idea that high testosterone is associated with dominance and aggression, as well as with a lack of trust and an increased responsiveness to perceived anger in others.[56,57]

Overall, published studies suggest testosterone has the potential to augment cognitive performance and mood and enhance assertiveness and risk-taking; however, much of the existing information is not definitive since it is based on correlational data, on subjects who may be anabolic steroid abusers, or on older individuals who are suffering from age-related hormonal changes. Testosterone treatment during a period of sustained energy deficit may reduce the adverse effects on mood associated with low testosterone levels. With regard to the sleep-disrupting (or possibly even sleep-improving) effects of either condition, additional research is essential since the available data also are confounded by the factors mentioned above.

The effect of testosterone supplementation on sleep quantity and quality has not been examined, but there may be a relationship between endogenous testosterone levels and sleep duration, as well as between endogenous testosterone and the length of wakefulness. An age-related decline in testosterone secretion has been observed, and it is thought this may be associated with age-related reductions in sleep quantity and quality.[58,59] With regard to testosterone supplementation, testosterone and anabolic steroids have been associated with reductions in sleep duration, increased insomnia, and sleep fragmentation, and several investigators have examined the relationship between testosterone and obstructive sleep apnea (OSA). While it has been concluded that OSA patients with low testosterone levels may experience sleep-related benefits from testosterone supplementation, little is known regarding the effects of testosterone on the sleep of normal young adults.[60]

Behaviors known to be associated with testosterone that will be assessed in this study, such as interpersonal trust, competition, and aggression, are known to be heritable and associated with polymorphisms in particular genes such as AVPR1a, OXTR, and HRT2A (genes coding respectively for arginine vasopressin, oxytocin, and the serotonin 2A receptor). At least 40% of the variability in testosterone-related behavioral functions may be explained by genetic factors.[61] In addition, it is well-established that a substantial proportion of individual variation in cognitive, emotional, and physical performance in response to stress is attributable to genetic differences. For example, 60% of the variance in the glucocorticoid response to stress may be due to genetic factors.[62] A variety of mechanisms may be responsible for differences in stress sensitivity, including the modulation of HPA axis sensitivity and variation in stress-induced neurotransmitter levels and receptor sensitivity.[63] For example, a single nucleotide polymorphism (SNP) within the GABRA6 gene (T1521C), a receptor for the inhibitory neurotransmitter GABA, is associated with blunted ACTH, cortisol, diastolic blood pressure, and mean blood pressure response to the Trier psychological stress test.[64] Similarly, repeat nucleotide polymorphisms in the gene encoding for tyrosine-hydroxylase (TCAT) are associated with differences in catecholamine secretion and cardiovascular reactivity to stress (Zhang et al., 2004).[65] Therefore, we will investigate polymorphisms associated with testosterone responsiveness, stress, and endocrine and brain function.

Objective III-A. Effects of energy deficit and exogenous testosterone on appetite regulation.

The appetite-mediating hormones, including anorexigenic hormones peptide-tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1) and the orexigenic hormone ghrelin, are thought to contribute to the drive to eat and hunger after weight loss and may underlie the common tendency for weight regain.[66] However, supporting evidence is predominantly derived from studies conducted in obese populations. Given the high prevalence of dieting for weight loss and body weight cycling in non-obese populations, including Warfighters, and the increased risk of obesity that accompanies these behaviors,[67] improving current understanding of adaptive responses of appetite-mediating hormones to weight loss and weight regain in non-obese populations is imperative.

Of particular interest is the hormone ghrelin. Ghrelin is the only known orexigenic hormone to be secreted from the gastrointestinal tract. Current evidence indicates that ghrelin concentrations increase during energy deficit, but the evidence is limited to mainly studies that have measured only total (inactive + active) ghrelin concentrations. However, ghrelin circulates in both an inactive form (des-acyl ghrelin) and an active, appetite-stimulating form (acyl ghrelin).[68] Evidence from our group (O'Connor et al., unpublished data) and others[69] suggests that in non-obese adults, des-acyl and acyl ghrelin concentrations may respond differently from one another to acute energy deficit. These studies have employed near total energy restriction, which may prevent the conversion of des-acyl ghrelin to acyl ghrelin. The extent to which differences in des-acyl and acyl ghrelin responses are influenced by the magnitude of energy deficit and whether differential responses are extant over prolonged energy deficit have not been characterized in non-obese adults. This study will characterize adaptive responses of appetite mediating hormones in non-obese adults during energy deficit and determine whether these responses persist after body weight is restored to pre-intervention levels. Both des-acyl and acyl ghrelin will be measured to provide novel insight into the regulation of ghrelin activity in response to weight loss and regain in non-obese adults.

Hypothalamic-pituitary-gonadal axis function is one potential, but understudied, factor in adaptive responses of appetite-mediating hormones to energy deficit. Estrogens have been shown to modulate appetite-mediating hormone secretion and CNS sensitivity to gastroenteropancreatic hormones in both murine models and human studies.[70] Comparatively little is known about the effects of androgens on appetite-mediating hormones and eating behavior. Testosterone and ghrelin concentrations are positively associated in healthy men,[71] and testosterone therapy for hypogonadism has been accompanied by increases in ghrelin[72] and decreases in leptin concentrations.[73] This study will expand the current evidence base by determining to what extent testosterone maintenance alters appetite-mediating hormone concentrations and appetite during energy restriction and refeeding. Moreover, it will provide novel information regarding the role of testosterone on acyl ghrelin responses to energy availability and subsequent effects on appetite.

Objective III-B. Effects of energy deficit and exogenous testosterone on the gut microbiome and intestinal permeability.

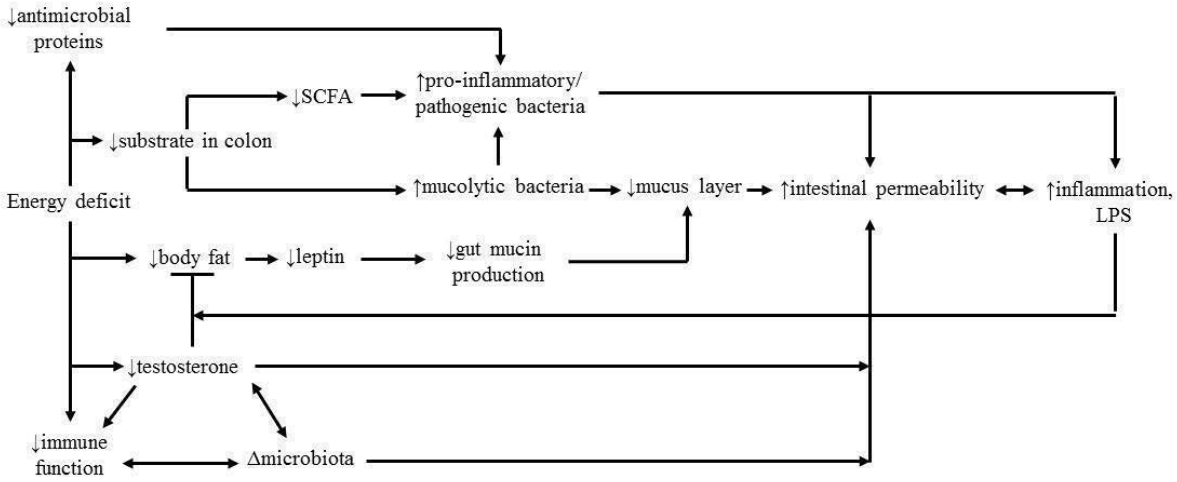
The lining of the gastrointestinal tract is both a physical and immunological barrier, acting to protect the host by deterring translocation of potentially harmful bacteria,

toxins, and antigens into systemic circulation.[74,75] The immunological barrier consists of several specialized cell types that secrete antimicrobial compounds and participate in antigen-sensing and immune response activation.[76] The physical barrier includes both a mucus layer and a layer of epithelial cells connected by protein complexes known as tight junctions. Tight junctions are particularly important to the integrity of the physical barrier, acting to regulate permeability through the opening and closing of paracellular channels through which contents of the intestinal lumen can pass.[75] Disruption or dysfunction of the gastrointestinal barrier can increase intestinal permeability, causing translocation of bacteria and their pro-inflammatory components (e.g., lipopolysaccharide [LPS], also known as endotoxin) into systemic circulation.[74] The resulting low-grade systemic inflammation increases susceptibility to acute and chronic disease,[77] and may compromise nutrient status (e.g., iron status),[78] adversely impact cognitive and physical performance,[74] and exacerbate gastrointestinal barrier dysfunction.[75]

Evidence suggests that changes in gut microbiota due to energy deficit may alter intestinal permeability (**Figure 1**). Specifically, gut bacteria capable of degrading host-derived glycans within the mucus layer outcompete other bacteria populations as energy restriction decreases the availability of undigested dietary substrates.[79] Sulfates released during degradation of mucins within the mucus layer facilitate the growth of pro-inflammatory, sulfate-reducing bacteria,[80] which can lead to decreased bacterial fermentation and short-chain fatty acid (SCFA) production. The resulting increase in colonic pH is conducive to the growth of pathogenic bacteria that suppress antimicrobial protein secretion and immune function. This combination of factors can degrade the mucus barrier, increase intestinal permeability, and subsequently affect inflammation and metabolic dysfunction. Additionally, reduced leptin secretion may alter intestinal permeability and gut microbiota composition during energy deficit as leptin stimulates gut mucin production,[81] and reduced leptin concentrations during energy deficit have been associated with changes in gut microbiota composition in mice.[82]

Emerging evidence suggests testosterone may mediate intestinal permeability. Specifically, sex hormones, including testosterone, increase expression of tight junction proteins in multiple tissues.[83,84] Testosterone is also thought to regulate immunity, with recent evidence indicating that testosterone may have a bi-directional relationship with the gut microbiome.[85] These findings suggest that testosterone maintenance could attenuate energy deficit-mediated decrements in gut microbiota composition and intestinal permeability. To our knowledge, this hypothesis has not been empirically examined. This study will address this knowledge gap by determining the effects of energy deficit and testosterone maintenance during energy deficit on the gut microbiome, intestinal permeability, and inflammation.

Figure 1. Proposed mechanisms of energy deficit-mediated effects on the gut microbiome and intestinal permeability.



Experimental Design and Overview

Physically active men (n=60) will be recruited for a 3-phase, randomized, placebo-controlled study to assess physiological and psychological responses to testosterone administration, at a dosage designed to maintain eugonadal status, during severe and sustained energy restriction. **Free-living, phase 1** will begin immediately after baseline measures are complete. Participants will be prescribed and provided individualized, 14-day eucaloric lead-in diets with 1.6 g protein·kg^{-1}·d^{-1}, 30% of total daily energy requirements from fat, and the remaining energy from carbohydrate. The 14-day lead-in diet will ensure sufficient time to acclimate to the prescribed study diet. After completing phase 1, participants will be randomly assigned to one of two, highly controlled (**live-in, phase 2**), 28-day treatment groups: energy deficit (DEF, 55% of total daily energy expenditure, TDEE) or energy deficit + testosterone maintenance (DEF+TEST). Protein intake (1.6 g protein·kg^{-1}·d^{-1}, based on day 14 total body mass) will remain constant throughout the 28-day intervention, fat will contribute 30% of total energy, and the remaining energy will be derived from carbohydrate. Exercise-induced energy expenditure (EIEE) will be increased 50% above TDEE for all participants. Energy intake will be 45% of the elevated TDEE, resulting in a 55% energy deficit (**see Table 1 for an example**).

Table 1. Example total daily energy expenditure and dietary energy and macronutrient content of the 28-d experimental diets.

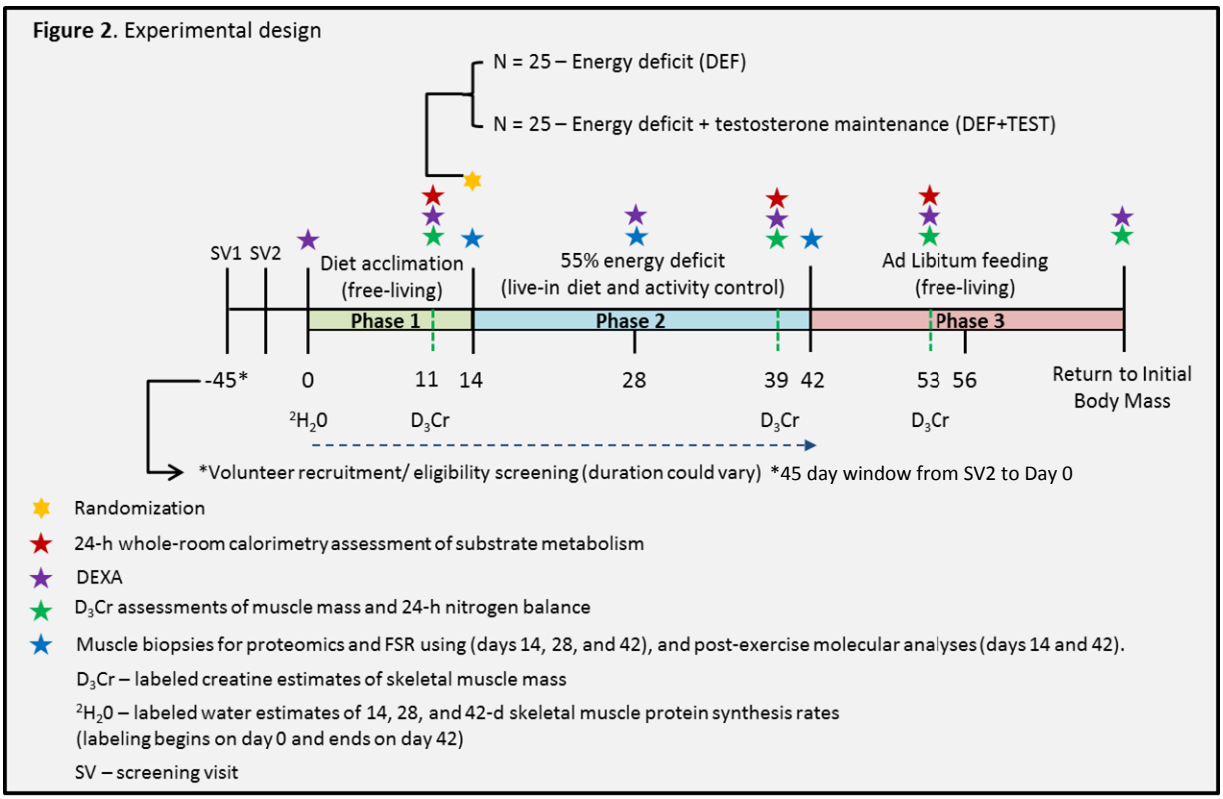
Days 0-14		**Days 15-42**	
BMR+TEF	2250	TDEE days 0-14	2500
EIEE	250	50% ↑ EIEE	1250
TDEE	2500	TDEE for all groups	3750

Energy intake, deficit, diet composition, and estimated loss of body mass during days 15-42

	DEF, DEF+TEST
TDEE	3750
Energy intake, kcal (% of TDEE)	1687 (45)
Energy deficit, kcal (% of TDEE)	2063 (55)
Protein, g (g/kg, kcal)	128 (1.6, 512)
Fat, g (% total energy, kcal)	56 (30, 506)
Carbohydrate, g (g/kg, kcal)	168 (2.1, 670)
Body mass loss, kg (% of initial body mass)	7.5 (9.4)

[1]Representative data based on a 2500 kcal TDEE for an 80 kg male for days 0-14.

Participants will be released from the metabolic center the day after completing phase 2. Participants will be allowed to return to their habitual diet and physical activity patterns to assess total body mass and, more importantly, skeletal muscle mass recovery from the intervention (**free-living, phase 3**). Recovery will be assessed after completing phase 2 to determine when body mass has been recovered within ± 2.5% of initial body mass (end of study, EOS). The duration of phase 3 will vary by participant, depending on each individual's rate of body mass regain (42-day maximum for phase 3). The experimental design is presented in **Figure 2**.



Details regarding study procedures can be found in the *Study Parameters & Procedure Descriptions* section below. See **Appendix A** Train Schedule for timing of study-specific measures.

Enrollment will begin during the spring of 2016 and we anticipate enrollment to continue for at least one year (summer 2017).

We expect that all data will be collected by September 2017. Complete data analysis, including the preparation of the final report and peer-reviewed manuscripts, will be completed in the winter of 2018.

Rationale for Duration and Severity of Energy Deficit

The duration and severity of energy deficit proposed in the current study was based on prior studies showing decrements in testosterone concentrations during energy deficit (unpublished data, Pasiakos 2015).[3,5,17,18,32] Special operations field training has been shown to decrease testosterone levels by 50-85% and total body mass by 6-16% in response to short (7 days) and longer-term (56 days) energy deficits of ≥1,000 kcal/d (unpublished, Pasiakos 2015).[3,17] A tightly controlled clinical trial that reduced energy intake by ~750 kcal/d (40%) for 21 days produced smaller reductions in testosterone (16%) and total body mass (4%).[32] Study duration, physical activity quantity and intensity, energy deficit, and dietary composition are critical factors to consider when comparing results from field and clinical studies. Therefore, in the current study, we have proposed a 28-day energy deficit that increases energy expenditure (+50%) and decreases energy intake (-55%) to levels comparable to the extreme energy deficits observed in military field studies. An energy deficit of this magnitude should produce

reductions in total body mass, lean body mass, and testosterone large enough for the intervention to be effective and create a biologically relevant and statistically significant difference between treatment groups.

Primary Study Endpoint

Total body mass will serve as the primary study endpoint and be used as a daily measure of accuracy and compliance with the diet and exercise intervention. Recovery of total body mass to pre-deficit levels (± 2.5%) or 42 days in phase 3 without recovery of body mass to pre-deficit levels will signal EOS. No other endpoint will signal EOS for any particular participant.

However, the study may be terminated if a participant withdraws, is unable to complete the prescribed exercise, has complications after undergoing the biopsy procedure, or if there is proof of noncompliance. Participants are required to complete all study measures at the given time points. In the event a procedure cannot be completed or is refused, participant continuation in the trial will be determined by the PI and MI. Noncompliance to the intervention will be addressed on a case by case basis.

Recruitment Methods

The PBRC staff has an extensive history of successfully recruiting and conducting large funded trials on exercise and dietary restriction interventions. Study recruitment will rely on previous methods, which have proven successful at enrolling volunteers who are consistent with the demographics of the region. PBRC also has a Clinical Trials Recruitment Core with 6 full-time staff dedicated solely to recruiting for clinical trials. In addition to phone calls, participants can screen via the PBRC website (https://www.pbrc.edu/clinical-trials/) and study-specific web pages are designed by the Recruiting Core. Both methods, phone and web, are funneled into a computerized participant-tracking system that allows for eligibility checks and real-time reporting. Investigators are able to track the recruitment status of their studies in real time using the PBRC intranet.

The metro Baton Rouge, LA, area has a population of 411,000. The Clinical Trials Recruitment Core receives 3,200 calls per month from potential volunteers. Study recruitment will rely on the existing methods of the Clinical Trials Recruitment Core to advertise via local media (print, radio, TV), paid targeted digital campaigns, earned media, social media (Facebook, Twitter, etc.), and collaborative relationships with local universities, sport vendors, health food vendors, etc. Targeted populations for recruitment will include PBRC employees, healthy adults (ages 18 and older), and non-military personnel.

Inclusion and Exclusion Criteria

Inclusion Criteria

- Men aged 18-39 years
- Physically active (at least 2 days per week aerobic and/or resistance exercise)

- Not taking any prescription medications and/or willing to refrain from all medication use prior to and throughout the entire study period, unless provided/approved by the study physician
- Willing to refrain from alcohol, smoking, e-cigarettes or use of any nicotine product, caffeine, and dietary supplement use throughout the entire study period.
    - At the discretion of the study physician, wash-out period for medications, supplements, and over the counter medications (OTCs) is ≥ 1-4 weeks
    - Wash-out period for caffeine and alcohol is ≥ 7 days
- Willing to live on the PBRC inpatient unit for 28 consecutive days
- Willing to have a urine drug screening
- Meets age-specific US Army body composition standards according to Army Regulation 600-9, which includes estimates of percent body fat based on height, weight, and circumference measures (neck and waist)
- Total testosterone concentration is within the normal physiological range (300-1,000 ng/dL).

Exclusion Criteria

- Musculoskeletal injuries that compromise exercise capability
- Diagnosed cardiometabolic disorders (i.e., hypertension, hyperlipidemia, kidney disease, diabetes, etc.)
- Allergies or intolerance to foods, vegetarian practices, or history of complications with lidocaine
- Anabolic steroid, human growth hormone, or nutritional testosterone precursor-like supplement use within the past 6 months
- Will not refrain from smoking (any nicotine product), alcohol, caffeine, or any other dietary supplement during the study
- Any use of antibiotics, except topical antibiotics, within 3 months of study participation
- Colonoscopy within 3 months of study participation
- Chronic use of laxatives, stool softeners, antacids, or anti-diarrheal medications (≥ once a week)
- History of gastrointestinal disease (e.g., celiac, irritable bowel syndrome, colitis, Crohn's disease)
- Restrained eater (the Three-Factor Eating Questionnaire) as assessed by the study's psychological and behavioral assessment staff
- Adults unable to consent
- Women
- Prisoners
- Metal implants, claustrophobia, head size incompatible with MRI equipment, etc.
- Sedentary or engages in <2 days of physical activity per week (aerobic and/or resistance training)
- Exceeds age-specific US Army body composition standards according to Army Regulation 600-9
- Previous history of kidney stones unless otherwise approved by the medical investigator
- Systolic blood pressure > 150 or diastolic blood pressure > 95 mmHg
- Previous history of breast or prostate cancer

- Previous history of COPD or Obstructive Sleep Apnea (OSA)
- Findings of lab results of PSA > 3ng/ml, Hematocrit > 50%, or positive urine drug screening
- Based on the investigative team's clinical judgment, a subject may not be appropriate for participation in the study.

Enrolling individuals based on US Army body composition standards (**Appendix B)** will ensure a population representative of the US military is studied.

Power analysis

Relevant data (means ± standard deviations) demonstrating the effects of moderate-to-severe energy deficit on lean body mass were used to determine statistical power and sample size. The percentage total body mass loss (2.7 kg) attributed to lean body mass during a 21-d, higher-protein (1.6 $g \cdot kg^{-1} \cdot d^{-1}$), moderate energy deficit was approximately 30% (0.8 kg).[24] However, the proportion of total body mass loss (5.8 kg) attributed to lean body mass in response to a short-term (~7-d), military training-induced, severe energy deficit was approximately 55% (3.1 kg) (unpublished data). Based on these results, and given the proposed study will induce a 55% energy deficit for 28 days in men consuming a higher-protein diet (1.6 $g \cdot kg^{-1} \cdot d^{-1}$), lean body mass will likely account for 40% of the total body mass loss. Maintaining testosterone within normal physiological ranges is expected to attenuate the loss of lean body mass by 25%, such that those assigned to the testosterone group will lose proportionally 30%, the same percentage of lean body mass as demonstrated in our previous study. Based on these estimates, the sample size necessary to determine the estimated differences between treatments is 22 per group. However, based on previous variability in lean body mass loss in response to moderate-to-severe energy deficit, 25 participants per group is a more conservative estimate to allow for detection of treatment effects in the current study. To account for possible attrition (20%), 30 participants will be assigned to each group (60 total participants). To successfully enroll 60 participants, we request the ability to consent up to 240 participants, given only a third of those briefed and consented in past projects, with more lenient study-specific eligibility criteria, were enrolled. ***At least 50 participants will complete the intervention and enrollment will stop once 50 participants have completed the study.***

Table 2. Power Analysis and Sample Size Justification

Hypothesized Effect (mean ± SD)	
Δ Lean body mass following the 28-d intervention, kg	
DEF	3.0 ± 0.75
DEF+TEST	2.25 ± 0.75
Effect Size	1.0
Alpha	0.05
Power	0.90
Sample Size	22 per group
Expected variability	25 per group
20% study attrition	60 participants total
75% eligibility screen failure	240 total consented individuals
Enrollment will stop once 50 participants (25 per group) complete the intervention	

Study Timeline

Individuals who respond to recruitment materials and indicate interest in study participation will be provided with study-specific information. If still interested, potential participants will be asked a series of demographic and health-related questions to assess eligibility via phone/web-based methods (approximately 15 min). Individuals who meet the initial inclusion/exclusion criteria via **self-reported** responses will be invited to attend two screening visits at Pennington Biomedical Research Center (PBRC). Details of each study visit are described below. See Train Schedule in **Appendix A**.

Screening Visit 1. SV1

- The study consent form will be reviewed with the participant to ensure all questions and concerns are clarified before the participant signs the consent form and before any procedures are conducted. Those who are still interested in participating will be asked to sign the consent form.
- Height, weight, blood pressure, and pulse will be measured. Participants who do not meet the height/weight criteria will have eligibility assessed by neck and waist circumference measurements to estimate percent body fat according to Army Regulation 600-9 (**Appendix B**). Circumference measurements will not be repeated during the study.
- A Physical Activity Readiness Questionnaire (PAR-Q) will be administered and CVD risk stratification assessed.
- Information regarding the participant's current medications and medical history will be recorded.
- A study dietitian will meet with the participant to discuss current eating habits and dietary requirements for each phase of the study.
- Eligibility criteria will be reviewed. All participants who maintain eligibility criteria will be scheduled for an appointment approximately 1 week later for Screening Visit 2.

Screening Visit 2. SV2

- The participant will have an EKG and a complete physical exam.
- Weight, blood pressure, pulse, and head size will be measured.
- Fasting blood will be drawn for CBC, Chem 26, PSA, testosterone, and study archives.
- Urine will be collected for a urinalysis and urine drug screening.
- A barriers interview will be conducted (**Appendix C**).
- A Three Factor Eating Questionnaire (TFEQ), or equivalent measure, will be administered to exclude restrained eaters.
- Information regarding any changes in medications or any adverse events will be recorded.
- Eligibility criteria will be reviewed. All participants who maintain eligibility criteria will be provided an accelerometer to wear, will complete a 3 day physical activity log, and will complete a 3-day food record (2 weekdays, 1 weekend day) one week prior to the start of phase 1. An appointment will be scheduled for Day 0 within 45 days of SV2.

- If more than 45 days lapse between screening measures and D0 scheduled date, participants may be asked to repeat measures to re-assess eligibility.

Phase 1: Diet Acclimation (free-living). Day 0–Day 14

- During the diet acclimation phase, participants will return to Pennington Biomedical once a day to eat and receive additional meals for the day. Participants will be weighed daily and caloric adjustments made if needed. Participants will be asked to record their activity daily throughout the phase. Additionally, 2 accelerometers (wrist- and waist-worn) will be applied at Day 0 and worn through day 14.
- Blood draws will occur on days 0, 7, and 14.
- Cognitive training will be performed on days 0, 2, 4, 6, 8, and 10 for approximately 1.5-2 hours each day. Cognitive testing will be performed on days 5 and 13 over a 2-hour period each day. The timing of training sessions is not critical.
- A DXA will be done on days 0 and 11.
- A 1-hour fMRI will be done approximately on days 5, 9, and 12.
- A food intake test with VAS and gut hormone assessments will be done on day 7.
- Participants will consume 150 mL/d $^{2}H_2O$ (i.e., heavy water) from day 0 to 7 and 100 mL/d $^{2}H_2O$ from day 8 to 14.
- Participants will be asked to provide a saliva sample on days 3, 7, and 11 to assess body water enrichment.
- Participants will ingest D_3-creatine (D_3Cr) (60 mg capsule) on day 11 for estimates of muscle mass at the end of phase 1 (urine collections on days 11, 13, 14).
- On day 11, participants will stay in the metabolic chamber and have urine nitrogen and urine creatinine collected for 24 hours.
- Participants will consume 2 g sucralose dissolved in 180 mL water on the morning of study day 11. A 24-hour urine sample collected with the nitrogen and creatinine testing will be used to analyze the sugar substitute ingestion.
- Participants will be asked to provide a stool sample on day 11 (+72 hours) for gut microbiome analysis.
- Information regarding adverse events will be collected on days 0, 7, and 14.
- A biodex familiarization test will be performed on day 0 and the actual biodex test will be performed on day 13.
- A VO_{2max} treadmill test will be performed on day 0. The test will be used to prescribe exercise intensities.
- Eating Inventories (TFEQ, FCI, FCI trait) will be completed on day 14.
- A bike familiarization ride will be performed within the first week of Phase I. The familiarization ride will be used to establish appropriate settings and exercise workloads for the experimental testing procedures.
- Muscle biopsies will be collected pre, 60 min and 360 min post a 60 min cycle ergometry exercise session on day 14.
- Time commitment during phase 1 will be about 34 hours per day on days 0-6, 8-10 and 12-13. On day 7, the time commitment will be about 4 hours. On days 11 (whole-room calorimetry day) and 14 (end of phase 1, check into PBRC metabolic ward after testing), participants will be at the facility for 24 hours.

- Participants will be randomly assigned to 1 of 2 experimental groups on day 14.

Randomization

Immediately following phase 1 testing on day 14, participants will be randomized to one of two experimental groups, either a 28-d (live-in), 55% energy deficit phase (DEF) or a 28-d (live-in), 55% energy deficit phase with exogenous testosterone administration (DEF+TEST). A randomization scheme will be determined using a block design (n=60) and age stratification (< 29 years or ≥ 29 years). Randomization will be done by a biostatistician with no direct study affiliation. Prior to the start of the study, the randomization schedule will be given to the pharmacist. The PBRC clinical research pharmacist will have no direct contact with participants. Treatment administration will be performed by a physician assistant, nurse practitioner, or nurse who will not be aware of treatment assignments. Participants and all study personnel will be blinded to treatment group. The code will be kept as a locked electronic file on a secure server by the pharmacist until study completion or there is a need to break the code for safety of the participant.

Phase 2: 55% Energy Deficit (live-in diet & activity control). Day 15–Day 42

- During the energy deficit phase, participants will live on Pennington Biomedical's inpatient unit in a 24-hour-a-day controlled setting. Once a day, participants will have their vitals (BP, pulse) measured. All meals will be eaten on the unit and monitored. Daily, participants will be weighed. Additionally, a wrist-worn accelerometer will be used throughout phase 2.
- Throughout phase 2, participants will complete supervised exercise sessions 1-4 times per day. Every 2 weeks, participants will undergo submax met carts for each mode of exercise utilized.
- Participants will consume 100 mL/d $^{2}H_{2}O$ (i.e., heavy water) from day 15 to 42.
- Participants will be asked to provide a saliva sample on days 15, 19, 23, 27, 31, 35, and 39 to assess body water enrichment.
- Blood draws will occur on days 28 and 42.
- Cognitive testing will be performed on days 15, 20, 22, 27, 29, 34, 36, 40 and 41 over a 1.5-2 hour period each day.
- A DXA scan will be done on days 28 and 39.
- A 1 hour fMRI will be done on days 36, 37, and 38.
- An injection (placebo or testosterone) will be administered on days 15, 21, 28, and 35.
- Eating Inventories (TFEQ, FCI, FCI trait) will be completed on day 42.
- Muscle biopsies will be collected at rest on day 28 and pre, 60 min and 360 min post a 60 min cycle ergometry exercise session on day 42. ***Participants will undergo 7 total muscle biopsy procedures during the entire study period (3 on one leg and 4 on the opposite leg, 3 total incisions).***
- Participants will ingest D_3-creatine (D_3Cr) (60 mg capsule) on day 39 for estimates of muscle mass at the end of phase 2 (urine collections on days 39, 41, 42).
- On day 39, participants will stay in the metabolic chamber and have urine nitrogen and urine creatinine collected for 24 hours.

- Participants will consume 2 g sucralose dissolved in 180 mL water on the morning of study day 39. A 24-hour urine sample collected for nitrogen and creatinine testing will be used to analyze the sugar substitute ingestion.
- Exercise testing for muscular strength (biodex) will be performed on day 41.
- Participants will be asked to provide a stool sample on days 25 and 39 (+72 hours) for gut microbiome analysis.
- Information regarding adverse events will be collected weekly on days 21, 28, 35, and 42.
- Participants will reside in the metabolic ward at PBRC during phase 2, thus the time commitment for this phase is 24 hours per day.
- Upon completion of each week of Phase 2, participants will receive an incentive item to aid in retention and adherence to the study protocol. Incentive items include a towel, water bottle, t-shirt, and gym bag.

Phase 3: Ab Libitum Feeding (free-living). Day 43–Return to Body Mass

- For days 43 (participants will be released after completing the final test on day 43) to EOS, participants will return to PBRC at least weekly to weigh-in.
- While participants will report to PBRC approximately once a week to weigh-in, each participant will be provided a scale to take home for daily weight measurements during this time frame. All measures will be semi-nude (t-shirt, shorts, socks) and performed after an overnight fast. Prior to distribution of the scales, they will be verified via PBRC's scale calibration verification SOP with a 50 lb. standard weight. At each weekly check, the participant will be asked to return the scale for reverification to ensure accurate weight measurements.
- Participants will complete a 3-day food record (2 weekdays, 1 weekend day) one week into phase 3 (days 50 to 56).
- Accelerometers (wrist and waist worn) will be worn on days 43 through EOS.
- A food intake test with VAS and gut hormones will be done on day 43.
- Blood draws will occur on day 56.
- Cognitive testing will be performed on day 54
- A DXA will be done on day 53.
- A 1 hour fMRI will be done on days 55, 56, and 57.
- Exercise testing for muscular strength (biodex) will be performed on day 55.
- On day 53, participants will stay in the metabolic chamber and have urine nitrogen and urine creatinine collected for 24 hours.
- Participants will consume D_3-creatine (D_3Cr) (60 mg capsule) on day 53 for estimates of muscle mass (urine collected on days 53, 55, 56).
- Time commitment during phase 3 will be about 2-3 hours per day on days 54-57. On day 43, the time commitment will be about 4 hours. On day 53 (whole-room calorimetry day), participants will be at the facility for 24 hours. On the once a week weigh-in, participants will be at the facility for approximately 30 minutes.

EOS: End of Study. EOS – EOS + 3 days

- During EOS, participants will return to Pennington Biomedical 4 consecutive days (EOS, EOS + 1, EOS +2, EOS + 3). During each visit, the participants will be weighed.

- Participants will be asked to complete a 3-d food record (2 weekdays, 1 weekend day) prior to the EOS visit. When the participant has reached approximately + 3% of initial body weight, he will be asked to begin the 3 day food record.
- Accelerometers (wrist and waist worn) will continue to be worn through EOS + 3.
- Cognitive testing, a DXA, and muscular strength (biodex) will be measured at EOS.
- A blood draw and 24 hour urine creatine and urine nitrogen will be collected at EOS.
- Participants will consume D_3-creatine (D_3Cr) (60 mg capsule) on EOS for estimates of muscle mass at the end of phase 3 (urine collected at EOS, EOS + 2, EOS + 3).
- Participants will consume 2 g sucralose dissolved in 180 mL water on the morning of EOS. A 24-hour urine sample collected for nitrogen and creatinine testing will be used to analyze the sugar substitute ingestion.
- Participants will be asked to provide a stool sample on EOS (+72 hours) for gut microbiome analysis.
- Eating Inventories (TFEQ, FCI, FCI trait) will be completed on EOS.
- A food intake test with VAS and gut hormones will be done on EOS+1.
- Information regarding any changes in medications or any adverse events will be recorded.
- Time commitment during EOS will be about 4-5 hours at EOS, 4 hours at EOS+1, and approximately 1 hour at EOS+2 and EOS+3.

PRN: As Needed Follow-up Visit

- After day 42, if the participant's testosterone levels have not returned to normal levels, a blood draw will occur every 90 days until levels have returned to within normal range (testosterone concentration ≥ 300 ng/dL).

Note: If timeline deviations are unavoidable (e.g. equipment issues, participant illness, unforeseen delays, etc.) schedule alterations, that will not affect study outcomes, may be necessary and are at the discretion of study staff.

Study Parameters & Procedure Descriptions

Objective I: Methodology

Determination of habitual dietary intake and physical activity levels

Eligible participants will complete a 3-day food diary according to instructions provided by the research team prior to the start of phase 1 (**Appendix D**). Habitual physical activity will be determined from accelerometer data obtained during the pre-study period. Physical activity patterns will be maintained at pre-study levels during phase 1. Physical activity will be verified using an accelerometer and physical activity logs during screening, phases 1 and 3; physical activity will be highly controlled and monitored during phase 2.

Determination of body composition

Height will be measured using a stadiometer. Weight will be measured (semi-nude and performed after an overnight fast) at PBRC using a calibrated digital scale at SV1 and

SV2 and daily during phase 1 and phase 2. After completing phase 2, participants will report to PBRC approximately once a week to weigh in and each participant will be provided a scale to take home for daily weight measurements (semi-nude and performed after an overnight fast).

Body composition will be determined using DXA (GE iDXA) on days 0, 11, 28, 39, 53 and EOS. Volunteers will undergo 6 DXA scans. DXA allows the non-invasive assessment of soft tissue composition by region with a precision of 1-3%.[86] Volunteers lay in the supine position on the densitometer table in shorts, t-shirts, and socks. They will be asked to remain motionless for the 5-10 min scan. These data will be used to calculate lean body mass, fat mass, bone mineral content, bone mineral density, and total body tissue mass. Calibration to external standards will be performed prior to actual data collection.

Estimates of skeletal muscle mass will be determined using the creatine (methyl-d_3) dilution method. The creatine dilution method uses basic principles of muscle creatine and creatinine biology to estimate muscle mass based on the irreversible conversion of creatine to creatinine and subsequent excretion in the urine. Participants will provide a fasting urine sample (to correct for background enrichments; **all urine samples will be second morning void before eating**) prior to ingesting a single, 60 mg dose (capsule) of D_3Cr on days 11, 39, 53, and EOS. Fasting urine samples will be collected 48 and 72 hours after each creatine pill ingestion to assess longitudinal changes in muscle mass. Research staff will provide labeled tubes for each urine collection. De-identified urine samples will be processed and frozen for future analysis by liquid chromatography–mass spectrometry (LCMS).

Determination of aerobic capacity

Aerobic capacity (i.e., peak oxygen uptake, VO_{2peak}) will be measured using an indirect open circuit respiratory system on a treadmill (day 0) during phase 1. Aerobic capacity will be used as a reference point to determine the appropriate exercise workloads necessary to meet the energy requirements for phase 2. In brief, participants will be clothed in appropriate athletic attire and perform this assessment at standard ambient indoor temperature (20-22°C) and humidity conditions (30-80%). Participants will be instructed to refrain from the consumption of food and caffeine for a minimum of 3 hours, following an overnight fast before testing, or following a light snack. Following instruction, participants will be given adequate time to become familiar with the testing procedures and allowed a 5-min self-paced warm-up on the treadmill. At the initiation of testing, the participant will put on a nose clip and a mouthpiece connected to a two way respiratory valve, which is attached to a head piece to hold it in place. Prior to beginning the running protocol, participants will walk for 5 minutes at predetermined comfortable speed and a 0% grade. The participants will then run for 4 minutes at a pace predetermined as comfortable at a 0% grade. At 4-min, the grade will be increased to 4%, followed by an additional 2% every two min thereafter until volitional exhaustion. Heart rate and ratings of perceived exertion (RPE) will be recorded during each stage. Although the testing endpoint will be volitional exhaustion, 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 there is a failure of testing equipment.

Testosterone and placebo administration

Participants will receive either 200 mg testosterone enanthate or placebo (sesame oil) by intramuscular injection at the conclusion of all phase 1 testing (morning of day 15) and then weekly on days 21, 28, and 35. Doses and syringes will be prepared by the PBRC clinical research pharmacy and injected by PBRC medical staff. Based on previous dose-response studies, 200 mg of testosterone enanthate was chosen as an effective dose to maintain testosterone within normal physiological ranges while minimizing risk of secondary health effects.[28,87,88]

Endocrine, hematological and physiological biomarkers

Fasted blood samples will be collected biweekly on days 0, 14, 28, 42, 56 and EOS. Blood samples will be analyzed for total testosterone, free testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormone binding globulin (SHBG), insulin-like growth factor-1 (IGF-1), estradiol, insulin, cortisol and a complete blood count with differential (CBC w/diff). In addition, blood samples will be analyzed for amino acids, lipopolysaccharide-binding protein (LBP), and a chem 15 panel on days 0, 14, 42, 56 and EOS, Prostate-specific antigen (PSA) will be assessed on days 14 and 42. Blood samples collected on days 14, 28, and 42 will be collected before the first muscle biopsy procedure. Additional blood will be collected and archived to allow for future analyses. Blood measure collection time points are shown in **Appendix E**. In addition, testosterone measurements will be taken every 90 days (starting from day 42) to assure full recovery (testosterone concentration ≥ 300 ng/dL) once the study has been completed.

Experimental physical activity

Physical activity will be controlled and supervised for accuracy starting on day 15 (i.e., start of phase 2). Varied low, moderate, and high intensity (40-85 % of predetermined VO_{2peak}) endurance-type exercise will be performed during the 28-d live-in phase to increase participants' EIEE 50% above the 14-d acclimation period TDEE. Intensity is defined as <40% of HRR for low intensity, ≥ 40% and < 60% of HRR for moderate intensity, and ≥ 60% and < 90% of HRR for high intensity. Energy expenditure will be achieved by performing at least one, but no more than four, exercise sessions per day, using a variety of endurance-type modalities (outdoor walking, treadmill [walk, run, and load carriage w/weighted vest equal to 20-35% body mass], cycle ergometer, and elliptical). **Exercise intensity will be verified biweekly and adjusted accordingly using an open circuit indirect calorimeter.**

Exercise intensity and exercise modalities will be programmed to limit the risk of developing an overuse or acute injury by alternating exercise sessions between low intensity weight-bearing modes and moderate to high intensity non-weight bearing exercise. Light calisthenics will be incorporated into the exercise regimen approximately every 3-4 days to decrease the monotony of the prescribed aerobic exercise and better simulate field operations (**Appendix F**). Standardized caloric expenditures will be determined for calisthenics exercises and integrated into individual exercise prescriptions to meet target expenditure. Calisthenics will not be done within 48 hours of testing (i.e. muscle biopsy, Biodex). Exercise will be performed daily, unless the day has been designated as a light exercise (days 21, 28, 35) or testing (days 14 and 42)

day. Light exercise days will not be void of all programed physical activity but the total increase in EIEE will be reduced by half so that participants only have to perform half the amount of exercise they perform on all other days (i.e., exercise will be walking at a low intensity). **Energy intake will be adjusted to account for the reduced level of exercise to maintain the 55% energy deficit** (e.g., for an individual with 3750 kcal/d TDEE during phase 2, 55% ED, EIEE is 1250 kcal/d and energy intake 1687 kcal/d; on light exercise days EIEE should be 625 kcal and energy intake 1062 kcal). Exercise, with the exception of the exercise prescribed for particular experimental measures (i.e., muscle biopsy studies), will not be performed on testing days 14 and 42. **Energy intake will be adjusted to account for the reduced level of exercise on test days that occur during the acclimation and 28-d intervention period to maintain body mass and the 55% energy deficit, respectively.**

Experimental diet

Energy intake will be individualized for study participants using the Mifflin St Jeor Equation with an activity factor of 1.3-1.6.[89] Diets will provide 1.6 ± 0.2 g protein·kg^{-1}·d^{-1} distributed equally across meals, 30% of total daily energy requirements from fat, with the remaining energy derived from carbohydrate.[24,26]

Registered dietitians will develop individualized 6-d menus (consisting of breakfast, lunch, dinner, snacks, and beverages) (see **Appendix G** for example menu). Meals will be prepared in advance by PBRC kitchen staff to ensure the composition of the diets is accurate. The energy content of the phase 1 diet will be sufficient to maintain body mass within ±2%. Intake will be adjusted incrementally (± 200 kcal) to achieve energy balance.

Participants will consume breakfast at the PBRC metabolic kitchen during phase 1. Participants will be weighed (see *Determination of body composition*) by research staff before breakfast is provided. Lunch, dinner, snacks, and remaining beverages (water is allowed ad libitum and not accounted for on the planned menus) will be packed and consumed offsite. Dietary compliance will be verified daily by assessing foods/beverages remaining in returned coolers and using pack-out questionnaires that allow participants to list any deviations from the provided diet (**Appendix H**). Participants will stay onsite on day 14 after testing has been completed, undergo randomization, and begin the 28-d experimental phase (phase 2) the next morning.

Energy intake will be 45% of TDEE after accounting for the 50% increase in EIEE, resulting in a 55% total daily energy deficit.

The micronutrient content of the acclimation diet will be consistent with current recommendations. Micronutrient intake during the 28-d, 55% energy deficit will likely be diminished. **To maintain operational relevance and to explore the impact of sustained, severe energy deficit on micronutrient-related markers of nutritional status, intake will not be augmented with supplementation.**

Participants will resume their self-selected, ad libitum diet during the recovery phase (phase 3). Participants will complete 3-day food records (1 weekend day, 2 weekday

days) within the week prior to day 56 and prior to EOS to be turned in to PBRC staff for monitoring and assessment.

Deuterium labeling, muscle biopsies and determination of muscle protein synthesis of individual proteins throughout the proteome

Deuterium (^2H_2O) labeling, muscle biopsies, and proteomic analyses will be used to determine protein synthesis rates for individual muscle proteins in response to the intervention. Participants will consume ^2H_2O (70%; Cambridge Isotope Laboratories, Andover MA, USA) beginning on day 0 and ending on day 42. Days 0-7 will serve as an isotopic priming phase, where participants will consume 3, 50mL doses (150 mL total) each day to achieve a target enrichment of 1-2%. During the priming phase, the first dose of each day will be consumed before breakfast and supervised by study staff to ensure compliance. The additional 2 doses will be packed out for participants to consume with subsequent meals. Participants will consume 2, 50mL doses (100 mL total) each day for the remainder of the study (days 8-42) to maintain isotopic enrichment. For days 8 through 14, participants will continue to receive one supervised dose with breakfast and the other dose at home with the dinner meal. During phase 2 (days 15-42), doses will be supervised with breakfast and dinner meals. **Body water enrichment will be determined from blood collected during the muscle biopsy days 14, 28, and 42 and saliva samples collected on days 3, 7, 11, 15, 19, 23, 27, 31, 35 and 39 (Appendix I)**.

Muscle biopsy samples of the vastus lateralis will be collected while the participants are under local anesthesia (1% lidocaine) using a 5-mm Bergstrom needle with manual suction on days 14, 28, and 42 after an overnight fast. Participants will undergo three muscle biopsy procedures (pre-exercise, 60-min and 360-min post-exercise) on days 14 (either right or left leg) and 42 (contralateral leg from day 14). The leg selected will be done via random assignment with considerations for age and treatment arm. Each of the three specimens collected on days 14 and 42 will be taken from the same incision, with the needle inserted at different angles to separate sample sites by ~5 cm. One muscle biopsy will be performed on day 28 (mid-point of the intervention). That procedure will be performed on the same leg sampled on day 14, with the incision ~7 cm apart from the incision made on day 14. Up to 250 mg (multiple passes are likely required to obtain adequate sample) of tissue will be collected with each procedure. Visible blood and connective tissue will be removed from the specimens, and samples will be frozen in liquid nitrogen before being transferred on dry ice to a -80°C freezer for storage and, ultimately, analysis. Participants will be fasted for pre-exercise and 60-min post-exercise biopsies; participants will be provided a standardized meal (25% of energy requirements) after the 60-min post-exercise biopsy.

A segment of the first (pre-exercise) muscle biopsy sample taken on days 14 and 42 and the sample collected on day 28 will be used for muscle proteomics (***detailed methods provided below***) and molecular analyses of muscle glycogen, anabolic intracellular signaling, mitochondrial biogenesis, amino acid transporter expression, androgen receptor expression, and ubiquitin-mediated proteolysis using qRT-PCR, SDS-PAGE, Western Blot, and enzymatic activity assays as previously described.[90-92] The same molecular analyses will be performed on both post-exercise specimens collected 60-min and 360-min from exercise initiation (cycle ergometry for 60-min at 2.6-

3.0 L/min) on days 14 and 42. Prior to Day 14, a cycle ergometry familiarization ride will be performed within the first week of Phase I. The familiarization ride will be used to establish appropriate settings (seat height) and exercise workloads for the experimental testing procedures. To establish experimental exercise workloads, the familiarization ride will include intermittent indirect calorimetry assessments of oxygen kinetics. The familiarization trial will be no longer than the 60 min experimental trial and stopped once the appropriate workload has been established. An absolute intensity was chosen to match the metabolic cost and total work performed and to limit the confounding effects of weight loss on relative exercise intensity. The workload will approximate 65-75% VO2peak based on an anticipated average VO2peak of 4L/min (Pasiakos PLoS One 2015, Pasiakos FASEB 2013, Pasiakos AJCN 2011).

De-identified muscle, plasma and saliva samples for proteomic and body water assessments will be frozen for future analysis.

Determination of Proteome Dynamics using Liquid chromatography-tandem mass spectrometry (LCMSMS)

Muscle samples will be thawed and homogenized for 75-sec in PBS containing 1 mM PMSF and 5 mM EDTA using a Mini-BeadBeater 8 (BioSpec, Bartlesville, OK) placed on ice for 1 min. This procedure is repeated twice and the resulting homogenate will be diluted to 10% (w/v) in PBS containing 1 mM PMSF. Protein from prepared homogenates are uniformly reduced by incubation in 10 mM DTT and SDS-PAGE sample loading buffer for 5 min at 95°C. The reduced samples are then alkylated by incubating in 15 mM iodoacetamide for 1-h at room temperature. Proteins are then fractionated by SDS-PAGE (BioRad). Using in-gel molecular weight markers, each sample will be divided into 10 molecular weight regions and subjected to overnight trypsin digestion at 37°C (Trypsin Gold, Promega, Madison, WI). Alternatively, muscle samples may be fractionated by sequential extraction of proteins into buffers containing 0.08%SDS, or 4M guanidine to yield 2 soluble fractions and 1 insoluble fraction followed by overnight in-solution digestion with trypsin at 37°C. The peptides from the resulting samples will then be extracted from the gel, dried, reconstituted in 5% acetonitrile/5% formic acid for analysis by LCMSMS.

The isotopic distributions of peptides will be measured using an Agilent 6520QToF with Chip Nano source (Agilent, Santa Clara CA). Each sample will be injected two times per analysis. Mobile phase for the LC is 3% v/v acetonitrile, 0.1% formic acid, in 18MΩ water (Buffer A) and 95% acetonitrile, 0.1% formic acid in18MΩ water (Buffer B). During the first injection, data dependent MSMS fragmentation spectra will be collected with the instrument set to collect 4 MS scans per second with up to 6 MSMS spectra from each scan. MSMS fragmentation data will be analyzed using the Agilent software package Spectrum Mill (B0.3) and protein identifications will be based on the Uniprot/Swissprot database (08/2010). The kinetic information in the isotopomer patterns will be extracted from the MS scan data using the Mass Hunter software package (B0.4) from Agilent. The peptide list with calculated neutral mass, elemental formula and retention time will be used to filter the observed isotope clusters. A visual basic application will be used to calculate peptide elemental composition from lists of peptide sequences and calculate isotopomer patterns over a range of precursor body $^{2}H_2O$ enrichments (p), for the

number (n) of C-H positions actively incorporating ^{2}H from body water. Fractional synthesis rates of proteins are calculated by deconvoluting the mass isotopomer pattern of newly-synthesized species as compared to unlabeled species, as described previously.[93]

Determination of fiber type (fast/slow) and cross-sectional area (CSA)
Muscle biopsy material from days 14 and 42 (pre-exercise samples) will be utilized for comparative histology, as follows: A) The proportion of muscle fiber types (type II/fast-twitch/strength and type I/slow-twitch/endurance) will be measured at day 14 (baseline) to establish subject-specific fiber type composition and again at day 42 for both treatment groups. B) CSA for each fiber type, for each subject, will be measured to identify muscle wasting based on fiber type. C) Expression changes based on cellular distribution of Smad2/3, Tak1, PGC1a, S6K will be measured to link wasting with specific cell signaling on a per fiber basis.

De-identified muscle samples for muscle phenotyping will be shipped frozen to MyoSyntax for analysis.

Determination of anabolic responsiveness/resistance
Muscle precursor cells (MPCs) from muscle biopsy segments obtained on days 14 and 42, pre and 360-min post exercise, will be used to measure cell-specific response *in vitro* to anabolic agents (*e.g.,* testosterone, DHT, Leucine) to determine whether DEF reduces anabolic responsiveness and increases anabolic resistance, and whether this can be prevented with TEST administration in the DEF+TEST group, and whether exercise in these groups can influence anabolic responsiveness/ resistance in the context of energy deficit. Participant-specific expression of PGC1a and S6K will be measured in response to anabolic exposure *in vitro* to capture cell-population based distributions of anabolic response for each subject.

De-identified muscle samples for MPCs isolation will be shipped frozen to MyoSyntax for analysis.

Determination of inflammatory profiles in blood derived cells (PBMCs) and muscle tissue derived leukocytes.
Blood and muscle derived monocyte/macrophage (e.g., M1, M2) and T cell subsets (e.g., Tregs, CD8+ T cells) will be measured to determine whether energy deficit influences inflammation in peripheral blood, in correlation with infiltration of inflammatory cell subsets and regulatory T cells into muscle, to skew muscle maintenance towards wasting. Whether catabolic/ inflammatory factors (soluble and cellular) are elevated in the DEF arm, and attenuated in the TEST+DEF arm will be determined. Cell subsets in blood and muscle will be compared with serum inflammatory factors measured as part of other sub-aims in this study proposal.

De-identified muscle and plasma samples for leukocyte and PBMC inflammatory profile analysis will be shipped frozen to MyoSyntax for analysis.

Determination of muscular strength and power

To assess the severe energy deficit, with or without testosterone treatment, muscular strength/power will be measured with isometric and isokinetic knee extension tests on days 13, 41, 55, and at EOS. A familiarization session will take place on day 0. Testing will take place prior to all daily exercise bouts. Isometric quadriceps strength, maximal power and muscular endurance will be quantified using an isokinetic dynamometer (Biodex Medical Systems, Shirley, NY). Isometric muscle strength will be measured at 75° knee flexion. This procedure will be performed three times, each separated by a 30 sec rest period. Maximal muscle strength (maximal voluntary contraction, MVC) will be determined from six maximal knee extensions at a fixed speed of 60° per second, while muscular endurance capability will be quantified during 20 repeated maximal knee extensions with movement speed fixed at 180° per second. Unilateral testing will be done on the dominant leg. The data collection form for muscular strength and power can be found in **Appendix J**.

Determination of energy expenditure, substrate oxidation and nitrogen balance.
Energy expenditure, substrate oxidation, and nitrogen balance will be measured for a 24-h period in a metabolic chamber on days 11, 39, and 53 (see **Figure 2**). The metabolic chamber stays will begin before breakfast, after completing the DXA scan, (~0800) and end before receiving breakfast the following morning (~0800).[94] Participants will receive the same meals, snacks, and beverages to control for potential differences between menu days. The timing of those meals will be similar to other days of the study, with the exception of the biopsy study days. While in the metabolic chamber, participants will perform the same amount of daily exercise typically performed during acclimation, 28-d deficit and recovery phase. For uniformity, all exercise will be performed on a cycle ergometer. Lights will be turned off at 2230, and the participants will be awakened at 0630. All urine will be collected for measurement of urinary nitrogen and creatinine excretion rates to determine nitrogen balance. Energy expenditure will be calculated by indirect calorimetry corrected for urinary nitrogen excretion and respiratory quotient.[95] Energy expenditure and substrate oxidation will be calculated for the 24-h period and also partitioned between rest, exercise and sleep. Substrate oxidation (carbohydrate, fat, and protein) will be calculated using standard equations.[96]

Objective I: Statistics

All data analyses will be based on the intention-to-treat principle using SPSS statistical software, unless otherwise noted. Data will be examined quantitatively and graphically for outliers and other artifacts that might have an undue impact on the analyses. Logarithmic or similar transformations will be applied when necessary to insure the validity of statistical procedures. All tests will be two-sided and considered statistically significant at $p < 0.05$.

Physiological outcomes
Mixed-model repeated measures ANOVA will be used to test the effects of testosterone maintenance on changes in body composition, skeletal muscle function, protein dynamics, metabolism, and biomarkers of physiological status. Mixed-models will include subject as a random factor, study day and group as fixed factors, and the day-

by-group interaction. Akaike's information criterion will be used to determine appropriate covariance structures. When a statistically significant time-by-group interaction is detected ($P < 0.05$), all possible within- and between-group comparisons will be completed, and the familywise error rate adjusted using the Bonferroni correction.

Objective II: Methodology

A comprehensive battery of questionnaires, evaluations, cognitive performance tests, personality assessment and the actigraphic assessment of daily spontaneous motor activity, sleep and circadian rhythms will be used to evaluate the effects of energy deficit and exogenous testosterone administration. Validated questionnaires will be used to examine fatigue and other aspects of mood such as depression, aggressive thoughts and perceived hunger. Risk-taking propensity and dominance will be evaluated using standardized objective procedures, and beliefs about other's feelings and intentions will be determined via a validated projective technique. Vigilance, memory and reaction time will be assessed with a standardized cognitive test battery that has established sensitivity to the impact of a wide array of real-world stressors typical of military operations.[97,98] Questionnaires, evaluations, cognitive performance tests, and personality assessments are described below and can be found in **Appendix K**.

Questionnaires/Evaluations

The **Minnesota Multiphasic Personality Inventory[99]** is one of the oldest and most widely used psychological assessment tools.[100]. Although it was originally designed to establish psychological diagnoses, the MMPI provides a broader picture of a person's basic personality characteristics. Generally, results remain stable over time; but intensive interventions have been shown to produce alterations over the course of relatively short time spans.[101] To date the MMPI-2 has not been used to study the combined impact of energy restriction and testosterone supplementation in healthy young men; however, statistically significant changes in a wide array of MMPI–2 scale scores (to include Depression and Masculinity-Femininity) were found over the course of 3 months after initiating testosterone treatment to transgender men.[102] In the present study, the MMPI-2 will be administered on days 5 and 40. The MMPI-2 takes approximately 1-2 hours to complete, depending on reading level (it is designed to require a sixth-grade reading level), and will be administered via computer software.

The **Buss–Perry Aggression Questionnaire**[103] is a 29 item questionnaire in which participants rank certain statements along a 5 point continuum from "extremely uncharacteristic of me" to "extremely characteristic of me." Examples of items include: "Once in a while I can't control the urge to strike another person", "Given enough provocation, I may hit another person", and "If somebody hits me, I hit back." The scores are normalized on a scale of 0 to 1, with 1 being the highest level of aggression. Results are shown in terms of scores on 4 scales: Physical Aggression, Verbal Aggression, Anger, and Hostility. This test has shown utility for examining the impact of testosterone on aggressive impulses,[104] and it will be administered to participants in the present study at several time points. The Buss-Perry Aggression Questionnaire takes approximately 2-3 minutes and will be administered via computer software or paper and pencil.

The **Profile of Mood States** (POMS) Questionnaire[105] is a 65-item inventory of subjective mood states that is sensitive to a wide variety of nutritional manipulations, environmental factors, sleep loss and sub-clinical doses of various drugs.[106-110] 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, depression, anger, vigor, fatigue, and confusion). 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 or paper-and-pencil.

The **Satiety Labeled Intensity Magnitude** (SLIM) questionnaire is a brief questionnaire designed to assess subjective feelings of hunger and fullness. The SLIM has been shown to be a sensitive, reliable, and easy-to-use scale for measuring perceived hunger and fullness.[111] Briefly, the scale is a vertical, 100 mm, bidirectional hunger/fullness scale anchored by the terms "greatest imaginable fullness" and "greatest imaginable hunger." The participant is directed to mark the scale anywhere along the axis corresponding to their level of hunger or fullness "right now." A rating anywhere above the midpoint of the scale indicates that some degree of fullness is perceived. This scale will provide valuable information on the perceived impact of the testosterone and feeding interventions in the present study. The SLIM questionnaire takes 1 minute to complete and will be administered via paper-and-pencil or computer.

Objective Risk Assessment Tests

The **Balloon Analogue Risk Task** (BART) is a computerized test designed to measure willingness to take risks versus "play it safe." It requires the participant to fill a simulated balloon with air.[112] Points are given for maintaining the flow of air and keeping the volume of 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 minutes to complete and will be administered via computer software.

Objective Cooperation/Dominance Test

The **Ultimatum Game** is a test of negotiation and cooperation among two people. It has long been of interest in behavioral research due to the fact that results often conflict with what would be logically expected based on rational economic theory alone. In this game, two players are offered a chance to win a sum of money, and all they must do is simply divide it. The proposer suggests how to split the sum, and the responder either accepts or rejects the deal. If the deal is rejected, neither player gets anything. The rational solution, suggested by game theory, is for the proposer to offer the smallest possible share and for the responder to accept it; however, the most frequent outcome is a fair share. In fact, low proposals are often rejected by responders, and it is thought that this is due to the fact that people wish to manage their reputations (i.e., show that they will not accept being treated unfairly). Since testosterone modulates the manner in

which individuals respond aggressively to challenges, and low offers on the Ultimatum Game can be viewed as expressions of a dominance challenge, the Ultimatum Game has been used to explore aggressiveness/cooperation associated with testosterone levels. In one study,[113] it was found that men who rejected low offers had significantly higher testosterone levels than those who accepted. The Ultimatum Game takes less than 5 minutes to complete and will be administered via computer software.

Beliefs, Intentions, & Desires-of-Others Assessment

The **Reading the Mind in the Eyes Test** (RTMITE)[114] was designed to examine the ability to attribute or infer beliefs, intentions and desires of others. The test has been largely used in research addressing social cognition deficits in persons with autism, Asperger's syndrome, and schizophrenia;[115] however, it would appear useful for exploring the impact of testosterone and energy deficit in normal subjects since both of these interventions have been observed to affect mood state, and since testosterone levels have been associated with aggression, selfishness, and suspicion about the intentions of others. In the present study, participants will first be presented with an oral description of the test followed by presentation of a single image of a person's eyes and their immediate eye region, along with four descriptive words. Then they will be asked to choose the word that best describes the emotional or mental state of the person in the image. This procedure will be carried out for the 1 practice picture and then the 36 test pictures. A score of 1 will be given for each correctly chosen target word and a score of 0 will be given if a foil word is chosen. The total score thus will range from 0 to 36. The test will take approximately 10 minutes to complete and will be administered via computer software.

Cognitive/Vigilance Tests

The **Scanning Visual Vigilance Task** is a test sensitive to a wide variety of environmental conditions, nutritional factors, sleep loss, and very low doses of hypnotic drugs and stimulants.[116] The subject must continuously scan a laptop or desktop computer screen to detect the occurrence of infrequent, difficult to detect stimuli. It was designed to simulate various critical military activities that require maintenance of vigilance such as sentry duty, standing radar and sonar watch and communications monitoring. The participant must detect a faint stimulus that appears randomly on a computer screen for two seconds. Typically the stimulus will occur on average once a minute. Upon detection of the stimulus the participant will press the space bar on the keyboard as rapidly as possible. The computer will record whether or not a stimulus is detected and the response time (in milliseconds) for the detections. Responses made before or after stimulus occurrence will be recorded as false alarms. This task takes 10 minutes to complete and will be administered via computer software.

The **Psychomotor Vigilance Test** (PVT) is a test of simple visual reaction time.[117] 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.

The **Matching to Sample** is a test assessing short-term spatial memory (working memory) and pattern recognition skills.[107,109] 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.

The **Grammatical Reasoning** test assesses language-based logical reasoning and has been used to assess the effects of various treatments on cognitive function.[118] 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.

The **Borg Rating of Perceived Exertion Scale** (RPE) will be administered during exercise sessions. The RPE scale assesses self-reported perceived physical exertion. It is administered during each exercise test session immediately before and immediately after the cognitive assessment period. The scale will be presented on a video monitor and participants give a verbal response that will be recorded by an experimenter (< 1 minute).

The **N-back** task measures working memory. 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 asked to monitor the identity or location of a series of verbal (letters) stimuli and to indicate when the currently presented stimulus is the same as the one presented “n” trials back (e.g. 0, 1, 2, or 3). For example, participants will be presented with letters one at a time in the center of the monitor and asked to determine if the letter presented is the same as a predetermined letter (0-back condition), the previous letter (1-back condition), 2 previous letters back (2-back condition) and so on. Dependent measures include response time and accuracy. This task takes approximately 15 minutes to complete and will be administered via computer software.

Sleep assessments

Actigraphic measures of sleep will be collected throughout the study using wrist-worn monitors. Actigraphy offers an easy-to-implement alternative to "gold-standard" polysomnography for measuring sleep without the need for electroencephalographic, electromyographic and electrooculographic monitoring. Actigraphy is a non-invasive method to objectively assess spontaneous motor activity, circadian rest/activity cycles and sleep using a watch-sized, wrist-worn device that uses a sensitive digital accelerometer to track the frequency of movements on a minute-by-minute basis. The information is processed through various algorithms to establish sleep/wake and sleep quality measures. The Standards of Practice Committee of the American Academy of Sleep Medicine (2007) has concluded that actigraphy provides an accurate estimate of sleep patterns in healthy people.[119]

In the present investigation, actigraphic data will be collected with the Fatigue Science ReadiBand™ actigraph or equivalent device. The wrist-worn ReadiBand™ has been validated in comparison to polysomnography (as have any alternative devices which would warrant consideration as "equivalent"), and the results have shown concordance of 90% or greater in terms of sleep-scoring accuracy. The device contains a 3-D accelerometer sampled at 16 Hz, a storage chip, and a 1.5 V battery, and it is waterproof and shock resistant. It will be worn on the dominant wrist continuously, 24-hours per day, during the acclimation (days 0 to 14), intervention (days 14 to 42), and recovery (days 42 to EOS) phases of the protocol. At the end of each week of data collection, data will be downloaded, scores for indications of sleep quantity, sleep quality, and sleep/wake timing will be calculated, and the results will be archived for subsequent analysis. Daily spontaneous motor activity will also be assessed.

Questionnaires, MMPI-2, RTMITE Test, Risk-Assessment, and Cognitive/Vigilance Tests

Training: All volunteers will undergo training and familiarization with all behavioral tests (with the exception of the MMPI-2, on which there is no "training curve"). Introductory training and familiarization on the subjective scales, RTMITE test, the risk-assessment task and Ultimatum Game, and the cognitive/vigilance assessments will occur at 6 different points during the 14-day lead-in phase of the study. The entire battery of tests (questionnaires, RTMITE, and computerized cognitive and risk tests) will be administered upon arrival at the test facility on Day 0. Subsequently, on days 2, 4, 6, 8, and 10, following the packing of take-home meals and snacks, there will be an additional test-training session (one per day) which will include all of the previously-mentioned tests with the exception of select questionnaires (i.e., those on which there is no "training curve").

Baseline Testing: On Day 13, volunteers will complete a final lead-in, pre-intervention test battery which will consist of the entire suite of tests to include the questionnaires. Data from this test session which will serve as the baseline data (i.e., before the energy deficit/testosterone phase). Providing subjects with 6 pre-baseline test-training sessions along with 1 baseline questionnaire-familiarization session should be more than sufficient to minimize the influence of training effects on all of these instruments,

and therefore should ensure valid baseline results from the Day-13, pre-energy-deficit/testosterone-intervention phase of the protocol.

28-d Energy deficit testing: Once the live-in, intervention portion of the protocol begins, volunteers will be asked to complete all of the subjective questionnaires (excluding the MMPI—see below), and risk/cognitive tests at 8 different points prior to the time at which they will return to free living. These cognitive test sessions will occur on Days 15, 20, 22, 27, 29, 34, 36, and 41. Note that 3 pairs of these sessions (D20 and D22, D27 and D29, and D34 and D36) are positioned so that they immediately surround the second, third, and fourth testosterone (or placebo) administrations on days 21, 28, and 35 in order to facilitate the examination of acute pre/post dose effects. Session on D15 occurs on the same day as the first injection. A determination regarding which sets of sessions will most appropriately describe the overall cumulative impact of the testosterone/calorie-deprivation intervention will depend on whether or not a preliminary analysis indicates the presence of significant differences between the 3 pairs of pre/post testosterone-administration sessions. Regardless of the outcome of this analysis, the session on Day 41 which is 1 day prior to the end of the entire intervention cycle (and 6 days after the final testosterone dose administration) will provide a valid end-point assessment of any cumulative calorie-deprivation/testosterone effects. With regard to the MMPI-2, it will be administered only on a single pre-intervention day (Day 5) and a single near-end-of-intervention day (Day 40) to explore the impact of caloric restriction and testosterone supplementation on several of the more stable aspects of personality.

Recovery/Ad-libitum feeding testing: During the final portion of the protocol, volunteers will be asked to complete two final questionnaire/test assessment sessions. The first will be on Day 54, and the second will occur at EOS.

Functional MRI assessments

Participants will perform a total of six tasks during acquisition of functional MRI (fMRI) data within the MRI machine. Each task will be performed during phases 1, 2, and 3. Tasks 1 and 2 will be performed on days 5, 36, and 55; tasks 3 and 4 will be performed on days 9, 37, and 56; and tasks 5 and 6 will be performed on days 12, 38, and 57. The duration of each task is expected to be about 20 minutes. With 40 minutes of task performance, 5-10 minutes of structural MRI scanning performed to facilitate fMRI data analysis, and 5-10 minutes transitioning into and out of the MRI scanner, each MRI session is expected to take approximately 1 hour. For timing purposes, task coupling (ex: 1 and 2 varied to 1 and 3) may be adjusted to maintain approximately 1 hour of MRI total time. Additionally, scans in phase 1 may be completed on different train schedule days (Day 1 through Day 13) in the phase to aid in scheduling.

General MRI methodology: At the time of recruitment, each participant will first fill out a detailed screening form indicating all possible contraindications to MR scanning. Study personnel will review the form and clarify any uncertainties with the participant. On the day of scanning, participants will be asked to sit in front of a laptop computer outside the MRI scanner and perform practice versions of the tasks they are scheduled to perform in the scanner that day. Then, the MR technician will re-screen the participant for MRI contraindications out of caution. The MR technician

will then insure that all metallic items are removed from the exterior of the body of the participant, and that clothing that might cause image artifacts are removed. Participants wearing simple cotton clothing are expected to be able to remain clothed although those with suspected metallic materials in upper body clothing may be required to change into a hospital gown. Headphones are placed over the ears to remove MRI scanner noise and deliver audio stimuli during the task. The MR technician will then place the participant on the MR table, supplying cushioning under the neck and legs as needed to insure comfort. A blanket will be provided to participants that feel cold on the MR table. A belt-like respiratory monitor will be affixed around the waist and a pulse oximeter will be clipped onto a finger or toe for physiological monitoring. The MR coil will then be placed over the head, button response boxes are placed in the hands, and the participant is then inserted into the MRI tube by the MR technician. Inside the MRI tube, the participant listens to audio stimuli, views images and words projected onto a screen, and initiates task responses by clicking buttons with their fingers.

Task 1: Risk taking propensity: The life-cash version of The Gambling Task assesses the propensity for high-risk, high-reward decisions, with differing reward domains (life and cash respectively) [120]. In repeated trials of a simple game, participants are asked to click buttons to choose between a high-risk, high-reward outcome; and a low-risk, low-reward outcome. The task will determine whether the testosterone intervention increases the tendency to make risky decisions in the context of prolonged energy deficit.

Task 2: Provoked aggression: In this task, the participant will be fitted with an MR-safe device designed to apply a electrical stimulus to an application site on the arm (STM100C, BioPac Systems, Inc). The player engages in a simple game with a digital opponent; after each round of the game, the winner of the round is allowed to apply the stimulus to the opponent, and is instructed to set the intensity of the applied pain (modulated by the number of electrical stimuli applied to the arm) to a value of their choosing. The participant is not told that there is no real human opponent, and both the outcomes of the trials and the intensities of the applied electrical stimulus are actually preordained. These task parameters are set in such a way as to test specific hypotheses about the participant's propensity toward retaliatory increases in applied pain level. The task gives a sense of the degree to which prolonged energy deficit and the testosterone intervention heighten the propensity toward retaliatory aggression against a provoking adversary, across varying levels of provocation [121].

Task 3: Multiple Source Interference Task (MSIT): In the multi-source interference task (MSIT), each trial shows the participant a string of three numbers including digits 1 to 3, and requires the participant to use a 3-button response box to identify which of the three numbers appears only once in the string. There are two sources of interference: the two other distracting numbers in the string, and positioning of the probe number that is discordant with the position of the corresponding button on the response box [122]. Besides providing an indication of inhibitory control function, this may be the most robustly validated fMRI task in existence, with extensive data showing similar patterns of activation across individuals and across MRI scanners.

Task 4: Working memory (AX CPT): The AX continuous performance task[123] requires individuals to click a response button when they observe an X that has been followed by an A in a stream of characters displayed on the screen. Performance on the task reflects the ability to maintain a shifting buffer of recently seen characters. The CNTRCS consortium selected this task as the test of working memory that is most well characterized and ready for translation into clinical trials in neuropsychiatric disorders. The AXCPT also has a robust and growing fMRI literature.

Task 5: Attention Network Task (ANT): The Attention Network Task (ANT) probes multiple aspects of attentional and inhibitory control. Participants are presented with a line of arrows, and is required to click a lefthand or righthand button depending on whether the center arrow points to the left or right respectively. The participant is required to suppress multiple distracting spatial cues to provide this correct response. It is very well characterized from a large number of behavioral studies and has several fMRI studies as well. It provides an assessment of spatial attention and how it is modified by the presence of distracting information.

Task 6: Emotional reactivity: In this task, participants are presented with a series of trials in which a probe image of a human face shown in the center of the screen must be identified to a face shown either on the left or right side of the screen. The facial expression shown in the probe image has differing emotional valence (*i.e.,* angry or neutral). Emotional face matching provides an assessment of brain reactivity to emotional stimuli even when the task does not require overt emotion processing [124]. These tasks have consistently shown greater brain activity due to emotionally valent image content in normal individuals and enhancement of this effect in individuals who underwent acute testosterone supplementation. The task will assess whether the effects of testosterone on covert emotional processing is enhanced by prolonged energy deficit.

Objective II: Statistics

Individualized scale scores from each of the questionnaires, the MMPI-2, and the RTMITE, the scores from the Ultimatum Game, the number of points earned on the Balloon Task, and dependent measures (such as accuracy scores and reaction times) on the vigilance, memory, and reasoning tasks will be analyzed with a series of mixed-factorial ANOVAs. Significant main effects and interactions will be pursued with Analysis of Simple Effects followed by appropriate linear contrasts and pairwise comparisons.

On all assessments there will be 2 levels of the grouping factor: *Intervention* (Energy Deficit-Androgen Maintenance and Energy Deficit+Androgen Maintenance). For preliminary examination of immediate pre/post-dose effects (on tests other than MMPI), the primary analysis will include the single grouping factor of *Intervention* with two within-subjects factors: *Dose-iteration* (Dose2, Dose3, Dose4) and *Pre-post* (Pre-dose, Post-dose). For examination of cumulative treatment effects (on tests other than MMPI), the primary analyses will include the *Intervention* grouping factor (Energy

Deficit-Androgen Maintenance and Energy Deficit+Androgen Maintenance) and a single within-subjects factor labeled *Session*, minimally with 7 levels (Baseline, Intervention1, Intervention2, Intervention3, Intervention4, Recovery1, and Recovery2). The specific sessions chosen for this "cumulative-treatment-effect" analysis will depend on whether or not acute testosterone dose effects are found in the previously-described separate examination of immediate pre/post dose effects (described above). If acute post-dose effects appear to have occurred, the *Sessions* will consist of: Baseline-Day13, Live-In-Day21, Live-In-Day27, Live-In-Day34, Live-In-Day41, AdLib-Day54, and AdLib-Day83. In this case, note that all of the Live-In sessions are 6 days removed from (i.e., following) the testosterone dose administrations. If the preliminary analysis indicates no differences between the immediate pre/post dose administrations, an analysis may be performed on all of the data-collection sessions, or possibly only on the Live-In sessions that immediately follow each of the testosterone dose administrations (i.e., Baseline-Day13, Live-In-Day15, Live-In-Day22, Live-In-Day29, Live-In-Day36, AdLib-Day54, and AdLib-Day83. In this latter case, note that all of the Live-In sessions occur on the very next day after testosterone has been injected. With regard to the MMPI-2, since it is only to be administered twice, there will be 2 levels of the *Intervention* grouping factor and 2 levels of the *Session* within-subjects factor (Baseline, and End-Of-Intervention Day 40).

ReadiBand activity/sleep-monitoring data (sleep duration, sleep latency, and sleep efficiency) will be analyzed via a mixed factorial ANOVA for *Intervention* (Energy Deficit-Androgen Maintenance and Energy Deficit+Androgen Maintenance) by *Period* (Baseline, Live-In1, Live-In2, Live-In3, Live-In4, Live-In5, Ad-lib). In addition, average sleep data during the acclimation (free-living) phase will be more generally compared to average sleep data during the intervention and ad-libitum (free-living) phases with a 2x3 ANOVA. Significant interactions from both sets of analyses will be followed up with Analysis of Simple effects and appropriate post-hoc comparisons. Depending on the pattern of observed effects, a more fine-grained analysis (which would potentially include every night of ReadiBand data) may be conducted as well.

Polymorphism assays for genes related to behavior will be conducted using nucleotides extracted from whole blood collected at the initial or a subsequent blood sample (~ 1 mL). A series of polymorphisms associated with differences in the stress response, testosterone-related behavioral traits such as cooperation, completion and aggression, cognitive and endocrine function as well as skeletal muscle adaptive responses to stress will be assessed and may include (but not be limited to): OXTR, AVPR1a (social and emotional regulation) CRHR1 and FKBP5 (HPA axis reactivity); COMT, 5-HTTLPR, DRD2, GABRA6, TCAT (neurotransmitters); and BDNF (synaptic plasticity).[62-64] All polymorphism assays analyses will be performed on a fee-for-service basis at the Lincoln Laboratory at MIT; all samples will be coded using participant number.

fMRI data will be managed locally by Pennington Biomedical's Imaging Core. Across the 6 tasks, fMRI data will be analyzed in conjunction with investigators/consultants at USARIEM. Data will be analyzed for *Intervention* (Energy Deficit-Androgen Maintenance and Energy Deficit+Androgen Maintenance) by *Period* (Baseline, Live-In1, Live-In2, Live-In3, Live-In4, Live-In5, Ad-lib) effects.

Objective III A-B: Methodology

Determination of appetite and endocrine mediators of appetite
Assessment of appetite and endocrine mediators of appetite will be completed after 1 week of diet acclimation (day 7, baseline), on day 43 (post-energy deficit), and upon return to initial body mass (EOS+1). Participants will receive a fixed-portion breakfast meal (0 min) and an ad libitum lunch meal (180 min). Subjectively rated appetite and blood samples will be collected before and periodically following each meal (**Table 3 and Table 4**).

Table 3. Baseline (day 7) and recovery (EOS+1) appetite experiment timeline.

Time (min)	-15	0	30	60	120	180	185	Post-meal
Meal		x					x	
VAS	x		x	x	x	x		X
Bloods	x		x	x	x	x		

Table 4. Post-energy deficit appetite experiments (day 43).

Time (min)	-15	0	30	60	120	180	185	215	245	305	365
Meal		x					x				
VAS	x		x	x	x	x		X	x	x	x
Bloods	x		x	x	x	x		X	x	x	x

VAS, visual analog scale.

A fixed-portion test meal will be served as a breakfast meal on the morning of study days 7, 43 and at EOS+1. This meal will have mixed macronutrient content and be prepared according to a standard procedure. For each participant the energy content of the meal will be equivalent to 20% of the TDEE prescribed for that individual on study days 0-14. Water in the amount of 240 g will be provided during the meal. Participants will be instructed to consume all of the water before completing the meal and will not be permitted additional water during the meal.

A second meal, the lunch meal, will be consumed *ad libitum* 180 min following provision of the breakfast meal. The lunch meal will have a mixed macronutrient content, consist of a single item (e.g., lasagna), and be prepared according to a standard procedure by research staff. A portion calorically equivalent to at least 75% of the TDEE prescribed for that individual on study days 0-14 will be served to ensure that food intake is not limited by the amount served. Participants will be instructed to eat until comfortably full, and be permitted to eat as much or as little as desired. There will be no restrictions on meal duration. The amount of uneaten food will be weighed and the energy content of the portion consumed calculated. Water in the amount of 240 g will be provided. Participants will be instructed to consume all of the water before completing the meal and will not be permitted additional water during the meal. (**Diets will be adjusted on day 7 to account for the appetite assessment**)

In the intervals between meals participants will be required to drink 360 mL water, and will not be permitted access to any additional food or beverage.

At each blood draw, self-reported fullness, hunger, prospective consumption, and desire to eat will be measured using 100-mm visual analog scales (**Appendix K**).[125] The visual analog scales will be administered by paper and pencil or may be administered on a computer.

Biomarkers of appetite regulation.
An indwelling intravenous catheter into the antecubital vein or forearm of the participant's arm will be placed 30 min prior to the breakfast meal. A fasted blood sample will be taken 15 min before beginning the meal. Blood will be sampled according to the timelines in **Tables 3 and 4**. At each time point 14.5 mL blood will be collected for a total of 275.5 mL over the full study to measure endocrine mediators of appetite. If at any point during the testing period the catheter becomes clogged or is no longer patent it will be removed and replaced. Serum will be analyzed for leptin and insulin (measured at -15 min only),serum for glucose,and plasma for acyl ghrelin and des-acyl ghrelin at PBRC using commercially available assays. Additional samples will be collected at each timepont and archived for future tests. All samples will be collected using appropriate preservatives to minimize degradation. Blood measure collection time points are shown in **Appendix E**.

Determination of gut microbiome composition and activity, and intestinal permeability
A single fecal sample will be collected at 4 time points (end of run-in diet [days 11-14], mid-point [days 25-28] and end of intervention [days 39-42], and upon return to initial body weight [EOS-EOS+3]) during the study to assess gut microbiota composition and function. At each time point participants will be given a ≤72-h window to collect a usable sample. A usable sample is defined as being > 25g wet weight, and having been delivered to study staff within 12-h 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 and ice packs to store and transport the samples from home (**Appendix L**).

Samples will be processed as soon as possible and within 12-h of defecation. Aliquots for 16s rRNA gene sequencing, transcriptomics, metabolomics, short-chain fatty acids (SCFA) analysis and archiving will be frozen at -80°C. DNA and RNA will be extracted from samples using commercially available kits. Microbiota composition will be assessed by sequencing amplicons targeting the V4 region of the bacterial 16S rRNA gene using appropriate primers and unique barcodes for each sample.[126] Transcriptomics will be completed using RNA-seq. Sequencing will be completed on the Illumina MiSeq or a similar platform, [127] and completed at the Broad Institute (Cambridge, MA) or by a similar entity on a fee-for-service basis. As gut microbiota composition analysis is a rapidly evolving field, the selection of primers, the 16S rRNA

gene variable region to sequence, and the sequencing platform may be modified to be consistent with the literature and technology available at the time of analysis. Quality control of reads and taxonomic assignment (phyla to operational taxonomic unit-level) will be completed using established software and databases.[128] Metabolomics will be completed by Metabolon (Durham, North Carolina) or by a similar entity on a fee-for-service basis. SCFA will be measured as markers of bacterial fermentation at the US Army Natick Soldier Research Development and Engineering Center (NSRDEC) or elsewhere on a fee-for-service basis using gas chromatography and a flame-ionization detector [129] or an equivalent method.

Urine collection and assessment of gastrointestinal permeability

Differential sugar absorption tests will be used to provide a functional assessment of gastrointestinal permeability. [130] For this test participants will consume 2 g sucralose dissolved in 180 mL water on the morning of study days 11, 39, and upon return to initial body mass (EOS). Sucralose is a sugar substitute commonly used as a sweetener in a variety of food products. Consumption of the solution will be conducted under staff supervision. Participants will then collect a urine sample 24-h after sugar substitute ingestion. Aliquots will be taken and frozen immediately. The sucralose concentration will be analyzed by PBRC. The sucralose ratio in the 24hr urine collection will provide measures of whole gut permeability. [130] Prior to testing days, participants will undergo a 2-day washout period of sucralose- containing beverages and foods.

Determination of eating attitudes and behaviors

Several questionnaires will be administered with participants in a fasted state at SV2 and on study days 14, 42 and EOS to determine the effects of testosterone maintenance during energy deficit on eating behaviors and food cravings. We will also explore associations between these outcomes and gut microbiota composition. The Three Factor Eating Questionnaire (TFEQ) will be used to measure hunger, dietary restraint and disinhibition.[131] Food cravings will be assessed using the Food Cravings Questionnaire-trait (FCQ-trait) and the Food Cravings Inventory 2 (FCI-2) which measures the frequency of cravings for specific types of foods. The TFEQ and FCQ-trait are publicly available. The FCI will be purchased from PBRC or used with permission. The TFEQ takes about 10 minutes, the FCQ-trait 5 minutes, and the FCI-2 5 minutes to complete and will be administered via the REDCap system.

Objective III A-B: Statistics

Data will be examined quantitatively and graphically for outliers and other artifacts that might have an undue impact on the analyses. Logarithmic or similar transformations will be applied when necessary to insure the validity of statistical procedures. All tests will be two-sided and considered statistically significant at $P < 0.05$. Data analysis will be completed using SPSS statistical software unless otherwise noted.

Physiological outcomes

Mixed-model repeated measures ANOVA will be used to test the effects of testosterone maintenance on changes in appetite, postprandial endocrine responses, intestinal permeability, and SCFA concentrations over time. Postprandial appetite ratings and responses of endocrine mediators of appetite will be summarized using area under the

curve and peak/nadir concentration prior to analysis. Mixed-models will include subject as a random factor, study day and group as fixed factors, and the day-by-group interaction. Akaike's information criterion will be used to determine appropriate covariance structures. When a statistically significant time-by-group interaction is detected ($P < 0.05$), all possible within- and between-group comparisons will be completed, and the familywise error rate adjusted using the Bonferroni correction.

Psychological outcomes

Questionnaires will be scored according to questionnaire-specific procedures. Scores will then be analyzed by mixed-model repeated measures ANOVA. Mixed-models will include subject as a random factor, test session or study day and group as fixed factors, and the session-by-group or day-by-group interaction. Akaike's information criterion will be used to determine appropriate covariance structures. When a statistically significant time-by-group interaction is detected ($P < 0.05$), all possible within- and between-group comparisons will be completed, and the familywise error rate adjusted using the Bonferroni correction.

Gut microbiota composition and metabolomics

Metabolomics data will be visualized using hierarchical average-linkage clustering and principal components analysis. 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. Mixed-model repeated measures ANOVA will be used to test the effects of testosterone maintenance on changes in metabolite concentrations, alpha diversity, and the relative abundance of individual taxa over time. Models will include study day and group as fixed factors, and their interaction. The Benjamini-Hochberg correction will be used to control the false discovery rate for main effects of time, group and the time-by-group interactions resulting from taxa-specific models. When a statistically significant time-by-group interaction is detected ($Q < 0.05$), post hoc within- and between-group comparisons will be completed and the familywise error rate adjusted using the Bonferroni correction. Linear discriminant analysis will be used to identify between-group differences in metabolite concentrations and relative abundances of taxa at each time point. Data analysis will be completed using SPSS, XLSTAT, R, Qiime or similar software as needed.

Relationships between gut microbiota composition, metabolites, and physiological and psychological outcomes

Changes in gut microbiota composition, metabolites, and physiological and psychological outcomes during DEF and ad libitum feeding will be examined separately by Pearson's correlation or Spearman's rank correlation as appropriate. False discovery rate will be controlled using the Benjamini-Hochberg correction. Procrustes analysis will be used to examine the relationship between gut microbiota composition and metabolite profiles. Redundancy analysis will be used to examine the amount of variance in physiological and psychological outcomes explained by gut microbiota composition and metabolite profiles. Data analysis will be completed using XLSTAT, R or similar software as needed.

Data and Specimen Management

Study participants will be assigned unique subject identification (ID) numbers. Study subject ID numbers will be used on all data collection instruments, to include questionnaires, data collection forms, biological specimen tubes, and computer records. A master list linking the participants' names and ID numbers will be kept in a password-protected computer file with access restricted to the PI and study navigator. Biological samples that are moved off-site (including to USARIEM) for analysis will not contain any personally identifiable information and will be labeled with only the unique subject ID numbers. Staff at these sites will not have access to the master list at any time.

Data collection forms will be kept under lock and key, or password-protected if computerized, and under the control of the PI, associate PIs, and project coordinator. Only personnel assigned to the research study by the PI will have access to the data. Hard-copy data records will be stored for a minimum of 3 years and a maximum of 5 years from the time the study is completed and then destroyed.
The PBRC has a fully integrated, campuswide, automated data management system. All data are entered into a Central Database using existing methodology that has been fully validated and undergoes continuous quality assurance by the PBRC Research Computing Core and NORC. Most data are automatically uploaded from the instruments that measure the endpoint. All self-report inventories and questionnaires will be completed in REDCap via surveys. Participants will be asked to complete the survey via laptop, computer, or tablet. Data will be exported from REDCap for analysis. Exercise testing data will be downloaded from the Parvo Medics' TrueOne® 2400 cart directly following each test and reviewed for integrity. All data are backed up daily, and the Research Computing Core at the PBRC oversees all data management.

Blood, muscle, and urine samples will be stored frozen at PBRC until analysis can be completed. Specific muscle, saliva, and blood samples for proteomic and body water assessments will be frozen for future analysis. Specific muscle and blood samples will be shipped to MyoSyntax for further analysis. Fecal samples will be initially processed at PBRC and stored frozen until analysis. Fecal samples or bacterial DNA extracted from fecal samples will be frozen and shipped to NSRDEC and the Broad Institute (Cambridge, MA) for analysis. Packaging and shipping of biological samples will be overseen by the PI, associate PIs, or study navigator, and will be completed in accordance with International Air Transport Association regulations to ensure that viable biological samples reach their intended destination.

Any blood, muscle, urine, or fecal samples remaining after analysis will be stored indefinitely to assess biomarkers associated with the study outcomes. This includes bacterial RNA analysis in collected fecal samples, metabolomics analysis in archived fecal/plasma/serum samples, and any biomarkers of nutritional status not currently identified in the protocol. Any use of the samples outside of this defined protocol will be submitted as a protocol amendment or a new protocol.

Standardization of Procedures and Quality Control

The research team has extensive experience using the procedures and methods required to conduct this study. Standard operating procedures in place throughout the units at Pennington Biomedical will be utilized for repeatable, valid data collection and

quality.

Data Analyses

See specific statistic sections listed under each objective.

Provisions to Monitor the Data to Ensure the Safety of Subjects

The PI and/or IRB of record will appoint a Research Monitor or monitors to ensure that voluntary participation is clearly and adequately stressed during the recruitment process. The monitor may be a member of the DSMB. The appointed DSMB member to serve as the Research Monitor is Dr. Timothy Church. This appointee will also ensure that the information provided about the research is clear, adequate and accurate. He can be called upon to interview human subjects, consult with others outside of the study or with the investigators. He will have the responsibility to promptly report his observations and finding to the IRB or other designated official. The research monitor has the authority to stop the research protocol in progress, remove an individual subject from the protocol and take whatever steps are necessary to protect the safety and well-being of human subject until the IRB can assess the monitor's report.

Data and Safety Monitoring Board

This study will use a data and safety monitoring board (DSMB). The DSMB will receive quarterly reports via email. One or more meetings each year may be conducted in person or via conference call if deemed appropriate by the DSMB chair. Prior to the start of recruitment the DSMB will give formal approval of the study protocol and informed consent.

- Size and Composition: The DSMB will consist of 4 members both internal and external to the Pennington Biomedical Research Center. The planned composition is as follows: Biostatistician (1), Exercise Physiologist (1), Clinician (1), Layperson (1)
- Major Responsibilities of Members:
  -Sign and abide by a statement of confidentiality
  -Disclose any actual or potential conflicts of interest
  -Oversee safety of participants to include review of adverse events
  -Review reports of related studies as appropriate
  -Review major proposed modifications
  -Monitor recruitment and adherence
- Reports: Following each meeting, the DSMB will provide written documentation regarding findings for the study as a whole and any relevant recommendations related to continuing, changing, or terminating the study. All DSMB recommendations will be submitted to the Principal Investigator and/or his designee, with a copy provided to the Pennington Biomedical Research Center IRB. Annually, the DMSB chair will provide a written summary report approving that the study can continue.
- Qualifications and Responsibilities of the Safety Officer: The Safety Officer for this trial will be familiar with the adverse event definitions and reporting requirements for the study. The Safety Officer will review reports sent by the study coordinator

as they occur and will determine whether there is any corrective action or stopping rule violation. The safety officer will send written documentation of the decision to the PI.

Adverse Events

Serious adverse events are defined to include:

- Death
- A life-threatening event
- Severe illness including worsening of a pre-existing condition, injury or accidents
- An inpatient hospitalization, surgical procedure, or a treatment to prevent a SAE
- A permanent disability or incapacity
- A clinically significant abnormal laboratory or diagnostic test result
- Any other event that, in opinion of the principal investigator or study physician, might have resulted in a serious adverse event if medical intervention had not been initiated

For this trial's purposes, an **adverse event or experience** is defined as any health-related unfavorable or unintended medical occurrence that happens after randomization. Examples of Adverse Events include but are not limited to the following:

- A clinically significant laboratory or clinical test result at follow up assessments
- An event that results in 3 consecutive missed exercise sessions
- An event that requires a visit to a physician because it alters participant's ability to do physical activity
- An event that occurs as a result of a study procedure which is not listed in the Risks section of the consent

Adverse events will be reported to the study PI, study coordinator, Chair of IRB, Chair of the study DSMB, and Safety Officer throughout the trial as necessary. Adverse event data will be collected from Baseline (Day 0) until the final closeout visit. Adverse Events classified as serious will be reported from the date of consent through the final closeout visit. Adverse Event Data will be analyzed quarterly, but serious or life-threatening adverse events require immediate reporting and follow-up. We anticipate most adverse events will be mild and the participant will be able to resume intervention activities within a day or two of reporting the event.

In the event an adverse event occurs on campus results in a serious or life threatening situation, the investigator or other project staff present will begin emergency measures, as appropriate, and call 911.

- For minor physical injury, the individual will be encouraged to see a health care practitioner of his or her choice.
- If the study participant experiences psychological or emotional distress, the project staff will cease research activities and attempt to calm and reassure the participant. The participant will be directed to an appropriate health care practitioner for further assessment and treatment as needed.

- The investigator and/or project staff will record detailed narrative notes describing the adverse event they witnessed or that was reported by participant. The Medical Safety Officer will complete the form Notification of an Adverse Event.

Adverse Event reporting will follow the requirements of the IRB of the Pennington Biomedical Research Center. Serious adverse events that are unexpected and related to the study will be reported within 48 hours. Other adverse events that are not serious but are unexpected and are associated with the study procedures will be reported within 10 days.

Safety Measures During Physical Activity

Exercise interventions are conducted on-site and all sessions are conducted and supervised by **trained PBRC exercise interventionists,** who monitor potential adverse experiences and symptoms. During the physical activity sessions a defibrillator and on-site trained staff are available to deal with medical emergencies. Also, institutional and community EMS services are activated if needed. Participants will be taught the importance and proper method of **warming-up** prior to and **cooling-down** following structured activity sessions. Heart rate will be monitored throughout the intervention sessions. If at any point during a physical activity session, participants develop chest pain, shortness of breath, or dizziness, they are instructed to rest and to contact their physicians if these symptoms persist or recur with further physical activity.

Procedures to minimize discomfort include **warm-up and cool-down activities** that include stretching, light walking or cycling. Participants are also **supervised and instructed** on correct physical activity techniques.

If for any reason the participant reports an injury, chest pain, shortness of breath, or dizziness, they are referred to their doctor, or the study clinician calls the doctor or other health care provider. In addition, specific criteria for **suspending or stopping physical activity** are developed to adjust the program for **intercurrent illness**.

Stopping Rules

There is more than minimal risk for participating in this trial. Nevertheless, in addition to monitoring recruitment and compliance to the intervention, we also will monitor the rates of injury in our participants. The safety officers, in conjunction with the study investigators, will alert the IRB and DSMB if a larger than reasonably expected injury rate occurs in the treatment groups. Other issues that are related to the stopping rules include:

- New information – It is unlikely that new information will become available during this study that would result in discontinuing the trial.
- Limits of assumption – It is possible that the value of data analysis will be limited by differences between the intervention groups at baseline or because of study dropouts or missing data. Baseline differences will be analyzed annually and effects on the power to detect differences in the outcome measures will be evaluated and discussed with the PI, safety officer, and the USARIEM Project Officer. Although an excessive number

of dropouts could occur, this has not been our past experience. In similar studies completed by USARIEM, the dropout rate was approximately 10%. If the dropout rate for the proposed study exceeds 20%, the safety officer will initiate a meeting with the PI to discuss strategies to increase retention. If the dropout rate exceeds 50%, the safety officer will meet with the study investigators to determine whether or not the study should continue.

- Limit of rules – We acknowledge that circumstances, other than what are listed, may justify stopping the study.

Withdrawal of Subjects

We will attempt to retain program participants once randomized at a retention rate of 80% for study completion through Day 42 and close-out visit. It is our desire to analyze results on all participants who were included into the program (e.g. completed SV1 visit). In accordance with the declaration of Helsinki/Tokyo/Venice/Hong Kong, participants have the right to withdraw from the program at any time for any reason. The investigator also has the right to withdraw participants from the program treatments in the event of inter-current illness, adverse experience, treatment failure, protocol violation, or other reasons. Should a participant decide to withdraw from treatment, all efforts will be made to complete and report follow-up observations as thoroughly as possible.

Risks to Subjects

This study does not involve major risk to screeners and trial participants. Efforts to minimize the potential risks of the assessment methods and outcome variables include frequent monitoring by the investigators to assure that no participant suffers any adverse effects from participating in the research. The study procedures include:

- $^{2}H_{2}O$ (i.e., heavy water).The extra neutron in the heavy water is not radioactive and has no risk. Children and pregnant women have been given this “special” water.
- Body weight. There is no risk to participants who record their body weight.
- Blood Pressure Testing. Participants may experience temporary discomfort during blood pressure recordings due to the pressure of the cuff on their arm.
- Venipuncture (blood draw). There is the possibility of pain and bruising at the vein on the participant’s arm where the needle is inserted. Aseptic (sterile) technique and trained personnel minimize these risks.
- Venous catheterization. There is a possibility of pain, bruising, or infection at the site of the needle insertion for the IV line. Trained personnel minimize this risk.
- Dual Energy X-ray Absorptiometry (DXA). The amount of radiation used for this procedure is very small. The radiation dose for this scan is equivalent to the radiation an individual is naturally exposed to in the environment in less than one day. The completion of 6 scans over the course of the trial is 500 times below the limit for radiation exposure per year. Per Pennington SOP, the quantity of scans completed in the trial is within the 10 DXA scans per year limit.

- Functional magnetic resonance imaging (fMRI). There are no known biological risks associated with magnetic resonance scanning. It has been used routinely for over 20 years. It produces side effects in very few situations. Those situations include:

    - **Metal**: Because the magnetic resonance machine uses a magnetic field, it can move any metallic objects that are inside the body. *This disruption of metal inside the body is extremely dangerous and may even be life threatening.* If the participant thinks he/she may have a cardiac stent, metallic implant, metallic piercings, shrapnel, or any other metallic material in the body, it is of utmost importance that the participant alert the study coordinator or MR technician. If the participant has metallic materials in the body that cannot be removed, we will exclude the participant from this study.
    - **Electronics**: Magnetic resonance imaging involves the use of radio frequency energy that can disrupt the functioning of electronic devices. If the participant possesses a pacemaker or any other electronic medical device inside the body, the participant will be excluded.
    - **Tattoos and cosmetics**: Some tattoos and cosmetics contain metallic materials that can heat up during scanning, especially if they are located on the part of the body being scanned. If the metallic material heats up enough, the participant may feel an uncomfortable burning sensation, and a skin burn may develop. In some cases, the amount of metallic material in the area being scanned is so excessive that the scan cannot proceed without risk of a burn developing. In other cases, a cold compress placed over the metallic material can be used to prevent burning.
    - **Confinement**: During the MR scan, the participant will be lying down on a table inside of a metal tube. The metal tube is a confined place. This might produce a feeling of claustrophobia, which can be distressing. A participant who has experienced claustrophobia in the past might become too distressed to complete the scan. In this case, the scan will be halted.
    - **Noise:** The MRI machine creates a loud, rhythmic noise that sounds like grinding or churning. This can be distressing to those who are sensitive to loud noises. The participant will be provided with earplugs to reduce the noise. But, if the participant finds the machine noises distressing, the MR technician can halt the scan.
    - **Peripheral nerve stimulation:** During the MR scan, the magnetic field around the body goes through rapid changes. These changes are all within safety limits set by the Food and Drug Administration. But, some people experience twitching in the nerves of their arms or legs as a result of these magnetic field changes. This twitching is generally not painful, and it stops at the end of the MR scan. But the feeling of inadvertent muscle twitching may make individuals feel disoriented or uncomfortable. Any participant who experiences this and wishes to stop the scan as a result will be allowed to do so.
    - **Electrical stimulus:** The BioPac device providing painful electrical stimulus has been safely applied in human subjects under a variety of conditions. Although the experimental design requires that the stimulus be painful, the electrical stimulation has no physical effects that last

beyond the duration of the task. There is a risk that the application of a painful stimulus may be psychologically distressing to the participant. In this event, the task will be terminated.

- **Mental tasks performed during fMRI:** There are no anticipated risks from performing these tasks.

- Self-report Questionnaires. There are no anticipated risks from completing self-report questionnaires. If signs of minor stress or fatigue are apparent, participants will be given time to take a break from completing the questionnaires. It is estimated that the questionnaires will take from 90 to 120 minutes to complete. The questions contained in some of the questionnaires may make people feel uncomfortable since they ask about topics such as how they feel about their body size. Responses to the questions will be coded to protect confidentiality, and participants may choose to not answer questions.
- Accelerometry. There is no risk associated with measuring activity with accelerometers. Accelerometers fit comfortably on the participant's arm and at the waist and can easily be removed should they become uncomfortable.
- Archive of Biological (Blood) Sample. The primary risk to participants regarding blood to be banked for future research is the risk of loss of confidentiality and/or privacy. Most banks need to maintain a link between the identities of donors and coded specimens to be able to collect valuable clinical follow-up information about the donor. To insure participants' privacy and confidentiality, their samples will be labeled with a unique series of letters and numbers. Pennington Biomedical will store these samples with unique identifiers and a minimum number of personal identifiers to meet laboratory standards. Storage and disposal of tissue will be conducted in a manner conforming to the appropriate care and handling of biological specimens as outlined through the Institutional Biohazard Committee Guidelines.
- Muscle Biopsy. Mild to severe pain, soreness, bruising, and a small scar are common risks. A hematoma (collection of blood in the tissue)) may occur. There is a slight risk that a superficial nerve may be cut; the nerve may heal, or it may result in a permanent loss of sensation in the skin at the biopsy site. Although infrequent, there is risk of infection at the biopsy site, which may need treatment with antibiotics.
- Metabolic Chamber. A participant may experience some level of claustrophobia or discomfort from staying in the chamber and being continuously monitored by a camera. However, participants will not be locked in and will be able to open the door in case of an emergency. The camera has been installed for participants' safety and no one is allowed access to the monitor except chamber personnel.
- 24 Hour Urine Collection. There are no known risks of collecting urine into a container.
- Fecal Collection. There are no known risks of collecting a fecal sample.
- Consumption of D_3-creatine (D_3Cr) (60 mg capsule). This is a stable isotope, and thus is not radioactive. Consuming this isotope at levels described in this study is considered safe.
- Testosterone. Potential side effects of testosterone treatment include acne, oiliness of skin, increased growth of body hair, breast tenderness, a reversible increase in hemoglobin, sleep apnea, leg edema and weight gain.
- EKG. There are minimal risks associated with this test. There is a small possibility there may be some redness or irritation while cleaning the skin prior to applying the

electrodes or if a participant happens to be allergic to the adhesive on the electrodes.

- Exercise testing. There is minimal risk of injury or a cardiovascular event during exercise testing. We believe the risk of an event during exercise testing is minimized with a pretest review of the medical history, physical examination by a physician or mid-level health care professional, use of a highly trained staff, and well-defined emergency procedures. Participants may experience temporary discomfort during blood pressure recordings due to the pressure of the blood pressure cuff on the arm. All tests are conducted in the presence of an exercise physiologist with extensive experience in conducting maximal exercise tests as well as a Research Associate specializing in cardiology. All laboratory staff are trained in basic CPR and/or ACLS (advanced cardiac life support). In the event of a life threatening emergency, the subject would be treated with ACLS by a staff M.D. and research nurses and subsequently be transported to the nearest acute care medical-surgical facility via Emergency Medical Services which is a parish wide paramedic response unit. The closest facility is approximately 0.25 miles away.
- Exercise interventions. The proposed exercise interventions are unlikely to cause major problems. We have conducted numerous exercise training studies and have never had a serious adverse event. There is the possibility of adverse events ranging from minor musculoskeletal problems to, in very rare cases, cardiovascular events. Exercise bouts will include a variety of modes (walking, cycling, elliptical, running, and pack walking) and intensities (low to high) at the discretion of the trainer to ensure compliance to the intervention as well as minimizing injury. Occasionally study participants experience minor orthopedic problems, but most are self-correcting with rest and standard first aid. These orthopedic injuries will be minimized by gradually progressing participants to their prescribed dose at the start of the study and alternating exercise sessions between cycle ergometers and treadmills as necessary. Exercise supervisors are trained in first aid and basic CPR. Each staff member is trained in either advanced or basic life support, and an automated external defibrillator and fully-stocked crash cart are kept on site. Although some study participants will be at moderately elevated risk for CVD, they will receive a thorough health screen including a physical examination by a study physician or mid-level health care professional and a maximal exercise test. According to the available data on adverse events resulting from the types of exercise proposed here, risk should be low in this study. Fatal events during exercise are extremely rare.
- Energy Deficit. There is a small risk of hypoglycemia when starting a diet lower in calories than participants are accustomed to and increasing caloric expenditure via exercise. Regular blood work and monitoring (including vitals) will be completed throughout the phases for safety checks. Prior to inclusion in the trial, all participants are screened to exclude those with medical or mental disorders that are unsuitable for the trial. Additionally, a barriers interview will be completed during screening to further explain study demands, assess barriers to participation, and screen for psychological and behavioral contraindications to the trial. The clinical site physician will review the medical procedures and psychological health of each participant and attest to suitability for inclusion to into the trial. Once enrolled in the trial, study staff with psychological and behavioral expertise will make daily rounds on the unit to assess mental stability and health of the participants. Any participant

displaying undue distress or other negative psychological symptoms as a result of the trial procedures, the PI and MI will assess if continuation in the trial is warranted. An on-call physician is also available after hours and on weekends.

Potential Benefits to Subjects

No direct benefits to the participants are expected.

Sharing of Results with Subjects

Participants will be provided a summary results sheet at the completion of their participation in the study. The summary results will include body composition andphysical fitness tests, and available lab work results.

The MR scan data collected for the study is not designed to be used by a doctor to evaluate physical health the way a scan done in a hospital or clinic is. That means that any abnormalities in the body that are relevant to personal health will not necessarily be noticed by study personnel. However, MR scanning may result in discovery of an unexpected incidental finding as described above. Therefore, there is a small chance that this study will provide benefit by revealing problems with personal health that would not have been discovered without MR scanning.

Setting

Clinical Facility

A detailed description of the clinical research facilities at Pennington Biomedical can be found in **Appendix M**.

Exercise Facility

The PBRC site has a 2,300 square foot Exercise Training Facility, which is under the management of the Preventive Medicine Department. Dr. Church is the Medical Director of this core. The facility offers state-of the-art equipment, professional intervention technicians, and optional training data capturing capabilities. The cardiovascular fitness training room contains treadmills, stationary bikes, and ellipticals. Multiple televisions hang from the ceilings in each room and a variety of magazines are available for use by the participants. Locker rooms are equipped with lockers, showers, and towels. Calibrated scales are available to measure body weight and work stations with computers are available that provide a private area to meet with participants. A dedicated parking lot with handicap parking is located immediately outside of the facility. The fitness center is supported by a trained staff composed of full-time technicians, exercise interventionists, post-doctoral researchers and internship students working on exercise-related degrees. Each is trained in either advanced or basic life support, and an automated external defibrillator.

Compensation

We will provide $6,000 per individual as an incentive for participation in the study. We think this amount is appropriate given the amount of time and procedure burden that the participants will spend both during the free living phase and live-in diet phase.

Participants will receive $500 after the completion of screening and free-living diet acclimation (randomization at day 14). They will also receive $3500 for completion of the live-in diet and activity phase (day 15 through day 42). If the participant does not complete the entire live-in diet phase, the compensation will be prorated based on the number of days completed. Lastly, participants will receive $2000 for completion of the free-living phase (close out visit). Total compensation will be up to $6000.

Confidentiality

All participants are assured of their confidentiality both verbally and in the informed consent form. The clinical facilities are strictly limited to the staff of the research institution and to research participants. This is accomplished by a variety of stringent security measures. All medical records are stored in locked areas. Access to these areas is limited to the clinical support staff, director of the clinical facilities, and the PIs. Participants' medical records are filed according to ID numbers. All forms on the chart display the ID number. Electronic data storage is similarly restricted with only the PIs and authorized persons having access to databases containing confidential clinical records, i.e. those containing name OR other identifying information.

Data, including body weight, body composition, exercise testing, etc. will be collected from participants. Data are confidentially collected from study participants and are only used for research purposes. All records are kept in locked file cabinets, and participant data can be identified only by number. Data are used only in aggregate, and no identifying characteristics of individuals are published or presented.

The MRI screening form contains potentially sensitive information about the health condition of the participant. These forms are considered study records and are kept in Medical Records or in a locked cabinet in the MRI Suite. Each participant is assigned a numerical identifier that is assigned to all MRI files collected as part of the study. All PHI is removed from these files when the numerical identifier is assigned to them. The mapping from subject name to numerical identifier is maintained by the Research Computing Group.

Data Sharing

The participant is asked to allow their study data to be stored and used for research at a later time. Participants that refuse to have study data kept for future research will be excluded from the study. The study data will be stored indefinitely. The data may be given to other investigators for future research as well. The future research may take place at Pennington Biomedical and may involve Pennington Biomedical Researchers in this study. The future research may not take place at Pennington Biomedical Research Center and may not be reviewed by Pennington Biomedical Research Center's Institutional Review Board. For privacy and confidentiality, study data will be labeled with a unique series of letters and numbers. Pennington Biomedical will store study data with this unique identifier. The research done with study data may help to develop new products in the future, or may be used to establish a diagnostic test that could be patented or licensed. The participant will not receive any financial compensation for any patents, inventions or licenses developed from this research.

Incidental Findings

We will follow the recommendations of a Presidential panel in handling incidental findings on MRI [132]. First, we indicate to the participant whether or not there is a well-established set of incidental findings for the scan they are undertaking, and if so what those incidental findings are. For scans without a well established set of incidental findings, we will provide a list of incidental findings that we feel may be possible. We will then describe the difference between clinically actionable incidental findings and non-clinically-actionable incidental findings. We will then ask the participant to decide whether they want to be informed of non-clinically-actionable incidental findings. Participants will be informed that informing them of clinically-actionable incidental findings is required for participation in the study. In the event that study personnel identify MR abnormalities, they will consult with a radiologist who will determine the clinical relevance of the abnormalities. Participant identity will not be shared with the radiologist in this event. If the radiologist determines that an MR abnormality is relevant to personal health, the radiologist will then determine whether the finding is clinically actionable. In the event that the finding is clinically actionable, or if the participant consented to be informed of non-clinically actionable incidental findings, study personnel will provide the information to the study Medical Investigator so that he or she can discuss the relevance of the finding with the participant. In the event of an incidental finding that is to be released to the participant, the imaging findings flow from the study staff, to a radiologist, to the Medical Investigator who explains the findings with the participant.

Compensation for Research-Related Injury

In the unlikely event a participant becomes injured as a result of participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By participating in this study, participants are not waiving any rights they have against PBRC/USARIEM for injury resulting from negligence of PBRC/USARIEM or its investigators.

Roles and Responsibilities

Principal Investigator (PBRC)

The Principal Investigator (PI) will assist in all aspects of the study including scheduling, briefing potential volunteers, being responsible for record keeping, quality assurance issues, maintenance of confidentiality, and notification to the DSMB in the event of an adverse incident. Procedures will be performed only by privileged personnel or by personnel who are under direct supervision of the privileged personnel.

Jennifer C. Rood, Ph.D.: Dr. Rood will be the PI of record and assume responsibility for the safe and scientifically sound conduct of the study. She will oversee all aspects of the study, assist with data collection, ensure safety and ethical treatment of participants, maintain required documentation for the study, obtain required approvals, have primary responsibility for data analysis and assist with data interpretation and publication.

Co-Investigators (USARIEM)

USARIEM co-investigators will be non-engaged, as they will not obtain: information about participants of the research through intervention or interaction with them, identifiable private information about the participants of the research, or the informed consent of human subjects involved in the research. All data files and biological samples (muscle, blood, urine, fecal samples) sent from PBRC to USARIEM will be de-identified. USARIEM co-investigators will assist with concept development, formulation of protocol objectives, hypotheses, experimental approach and design. Co-investigators will maintain active communication with the PI and study staff.

Stefan M. Pasiakos, Ph.D. and Claire E. Berryman, Ph.D.: Drs. Pasiakos and Berryman will assist with concept development, formulation of protocol objectives, hypotheses, experimental approach and design. They will have primary responsibility for data interpretation and preparation of manuscripts and technical reports for publications. Drs. Pasiakos and Berryman will share primary responsibility for all communication with PBRC and routinely visit PBRC to ensure study progress and proper execution of the project.

APPENDICES

Appendix A: Train schedule
Appendix B: AR 600-9 (Army Body Comp Program)
Appendix C: Semi-structured barriers interview
Appendix D: 3-d food diary and instructions
Appendix E: Blood measures
Appendix F: Example PT circuit
Appendix G: 6-d cycle menu example
Appendix H: Daily food diary
Appendix I: Saliva collection instructions and log
Appendix J: Biodex performance data form
Appendix K: Questionnaires, evaluations, cognitive performance tests, and personality assessments
Appendix L: Fecal collection instructions
Appendix M: Detailed Description of the Clinical Research Facilities at Pennington Biomedical Research Center

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