Pennington Biomedical IRB FWA 00006218
Approved August 24, 2020

Protocol Title: A randomized, double-blind, placebo controlled trial of testosterone undecanoate for optimizing physical and cognitive performance during military operations (OPS II)

Running Title: OPS II

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Protocol Version Date: 3 August 2020

IRB Review History
Initial Approval V1.0 on May 10, 2019
Version 2.0 on August 21, 2019
Version 3.0 on August 23, 2019
Version 4.0 September 9, 2019
Version 5.0 December 23, 2019

Executive Summary

Warfighters frequently conduct training and combat operations under multi-stressor conditions that degrade muscle function [1]. The stressors contributing to functional decline during strenuous military operations include the integrated effects of high physical activity, sleep deprivation, and energy deficit. Energy deficits and sleep deprivation suppress skeletal muscle and whole-body anabolism and accelerate protein catabolism [2], in part by inhibiting endogenous testosterone synthesis [3, 4]. This results in a hypogonadal state and concomitant muscle atrophy, which contribute to declines in muscle function [5]. Pharmacologic restoration of eugonadal testosterone concentrations may be an effective strategy to attenuate functional decline by promoting anabolism and mitigating muscle mass losses typically observed during military operations. In the Optimizing Performance for Soldiers I (OPS I) study [6], healthy, non-obese men supplemented with 200 mg of testosterone enanthate per week during 28-d of exercise- and diet-induced energy deficit gained lean body mass (LBM) and lost less total body mass than controls, who experienced a 20% reduction in total testosterone concentrations. The beneficial effects of testosterone were sustained into recovery and occurred without concomitant increases in health-related risk. While OPS I demonstrated an anabolic benefit, several experimental limitations preclude definitive

recommendations to efficaciously adopt supplemental testosterone therapy as a Warfighter biomedical performance enhancement (BPE) strategy. The weekly intramuscular injections used in that study are not practical for Warfighters operating in austere environments. Further, the dose of the short-acting enanthate ester used produced testosterone concentrations exceeding the normal physiological range within days after administration, followed by a precipitous decline and prolonged hypogonadal state in recovery, which may not be well tolerated by Warfighters during combat and training operations. Third, the magnitude of stress imposed by physical activity and decreased energy intake did not adequately mimic the multi-faceted stress experienced during real-world military operations [4, 7, 8]. Finally, the muscle function tests used in OPS I lacked military relevancy and failed to demonstrate relevant performance improvements in participants receiving testosterone, despite LBM gains. ***The Optimizing Performance for Soldiers II (OPS II) study will address OPS I study limitations and test whether a standard, low dose of long-acting testosterone undecanoate (TU, 750 mg administered once) safely and steadily maintains normal testosterone concentrations, promotes anabolism, spares LBM, and enhances militarily-relevant measures of muscle function and performance without cognitive impairment during and in recovery from a highly-controlled, 20-d simulated, multi-stressor (i.e., high physical activity, sleep deprivation, and energy deficit) military operation.***

Objectives

1. Determine the effects of a single dose of TU on LBM, anabolism, muscle and cognitive function and performance during simulated operational stress.
2. Determine the effects of a single dose of TU on LBM, anabolism, muscle and cognitive function and performance recovery from simulated operational stress.

Hypotheses

1. TU will maintain testosterone within normal clinical range, spare LBM, stimulate anabolism, and sustain muscle function without cognitive impairment during simulated operational stress.
2. TU administration prior to 20-d of simulated operational stress will not cause hypogonadism during recovery, nor negatively affect LBM, muscle and cognitive functional recovery.

Background

Biomedical Performance Enhancement: Rationale & Results from OPS I

Modest energy deficits can reduce body mass and improve health of overweight and obese adults [9]. The composition of body mass lost during moderate energy deprivation (500-1000 kcal/d or 20-40% of total daily energy expenditure, TDEE) [9] is predominantly body fat mass (FM). However, when energy deficits are more severe (~1000-3500 kcal/d or ~50-100% of TDEE) than energy deficits imposed for healthy weight loss [10], substantial LBM loss is observed [8, 11]. Warfighters (e.g., light infantry, special operations forces) conducting strenuous training and combat operations commonly experience sustained periods of severe, unavoidable energy deficit, the effects of which resemble the pathophysiology of semi-starvation and lead to

significant LBM loss and degraded lower-body muscle function [8, 12, 13]. Further, some Warfighters experience these severe and unavoidable periods of energy deficit and associated loss of LBM repeatedly over their military career, raising concerns about the potential accumulated effects of those energy deficits on health-readiness. The US military has sponsored considerable research to develop nutritional countermeasures to mitigate LBM loss under these conditions; however, dietary interventions, including increased protein intake, have been, by themselves, ineffective in sparing LBM during severe energy deficit [14-16].

The extent to which LBM is lost in male Warfighters during strenuous, multi-stressor operations may, in part, be attributable to reductions in testosterone concentrations. Energy deficit inhibits endogenous testosterone synthesis by suppressing the hypothalamic-pituitary-gonadal (HPG) axis [3]. Reductions in testosterone are accompanied by muscle atrophy and decreased muscle strength [5]. The use of exogenous testosterone to maintain eugonadal concentrations during severe energy deficit may attenuate reductions in LBM and preserve muscle function. In 2015, Dr. Pasiakos of USARIEM and Dr. Rood of PBRC conducted the OPS I study, a multidisciplinary, multi-institutional collaborative project that tested the effects of supplementing men exposed to severe energy deficit with testosterone. OPS I was a three-phase, randomized, double-blind, placebo-controlled trial in 50 non-obese men (mean ± SD, 25 ± 5 y): 14-d run-in, free-living, eucaloric diet phase; 28-d live-in, 55% exercise- and diet-induced energy deficit (P2) with (200 mg testosterone enanthate per week, TEST) or without (placebo, PLA) exogenous testosterone phase; and 14-d recovery, free-living, ad libitum diet phase [6].

The primary findings from OPS I were that participants receiving weekly intramuscular injections of TEST gained LBM and lost less total body mass compared to those receiving placebo (**Figures 1A-B**). The change in total testosterone concentrations during the energy deficit was strongly correlated with changes in LBM ($r = .76$, $P < .001$). TEST fully recovered their body weight and were 2.8 kg heavier at the end of the 14-d recovery period than PLA, due entirely to LBM. TEST did not differ from PLA with respect to lower-body muscular strength and endurance decline, incidence of adverse events, or have any negative effect on cardio-metabolic health biomarkers during energy deficit or recovery (Pasiakos et al. unpublished).

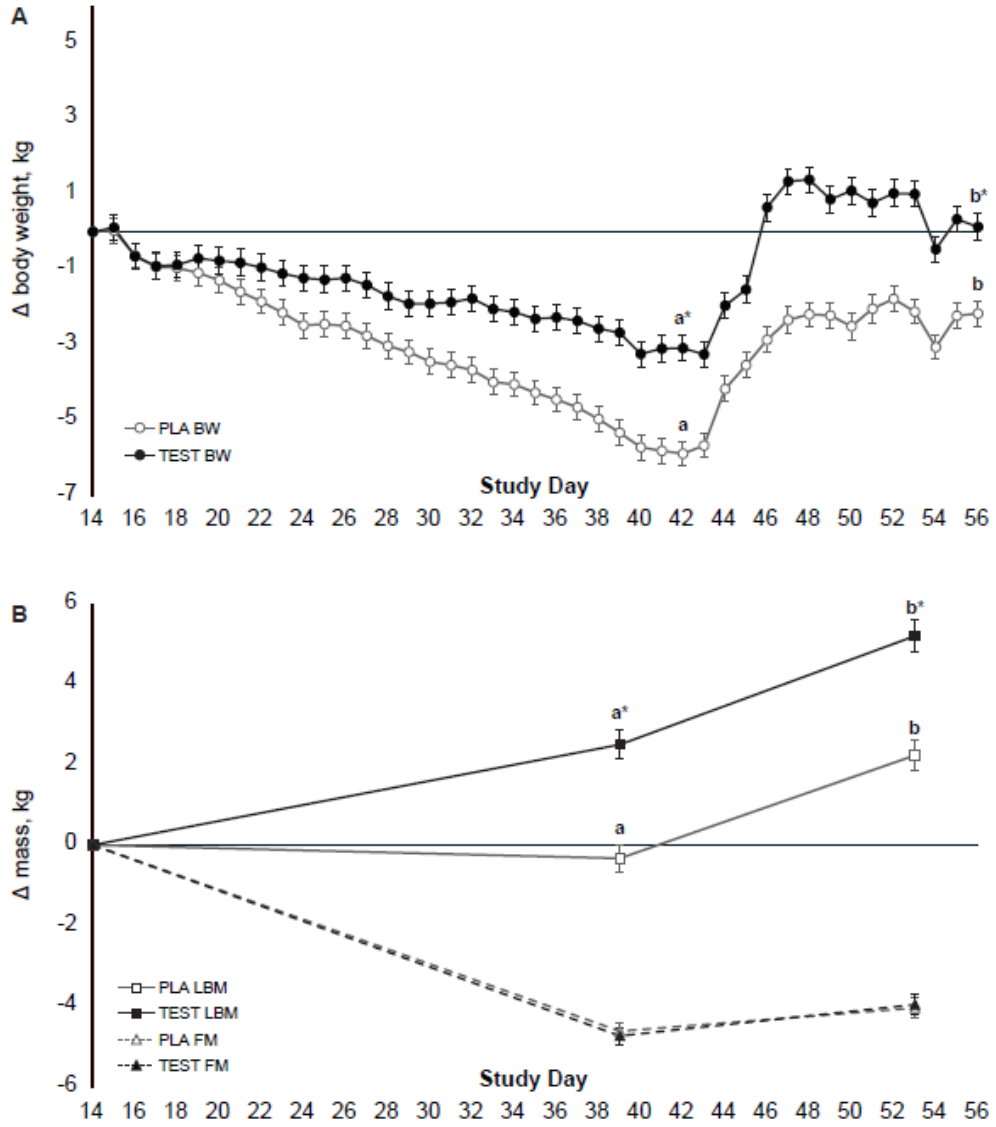


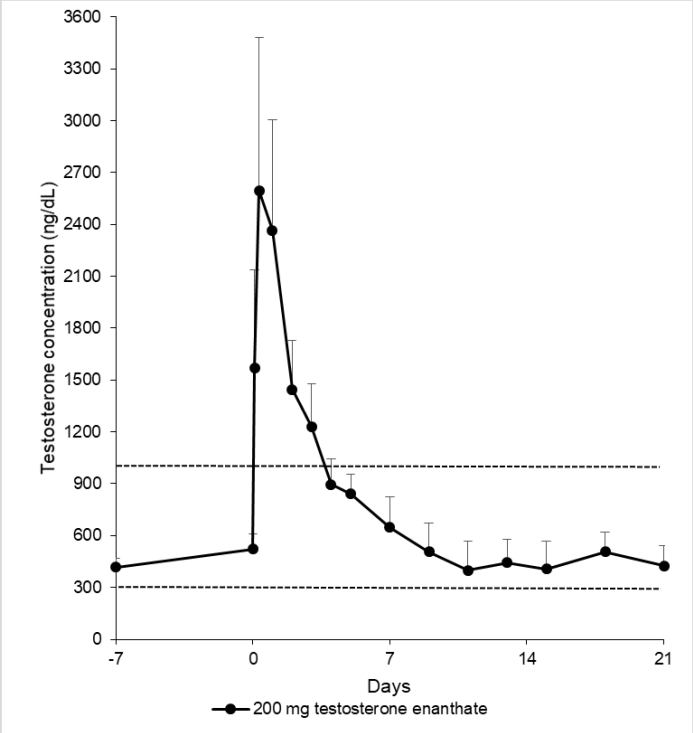
Figure 1. Change in body weight and composition during energy deficit and recovery. Least squares mean ± SE change in body weight (A) and composition (B, lean body mass, LBM; fat mass, FM) during phase 2 (P2, 55% energy deficit) and phase 3 (P3, recovery/weight regain) relative to the final body weight and composition measured during phase 1 (eucaloric diet) for TEST (n=24) and PLA (n=26). Data not sharing the same letter superscript within a treatment are different; and *indicates a between group difference (phase-by-treatment interaction). TEST, 55% energy deficit + 200 mg testosterone enanthate per week during P2; PLA, 55% energy deficit + 1 mL sesame seed oil placebo per week during P2 (Pasiakos et al. unpublished).

OPS I Lessons Learned & Justification for OPS II

The results from OPS I suggest that testosterone supplementation may be an effective and safe pharmacological BPE strategy to mitigate LBM losses experienced by Warfighters during sustained, multi-stressor training and combat operations. However, certain experimental limitations in OPS I currently preclude a definitive recommendation to adopt supplemental testosterone therapy as a Warfighter BPE strategy. The

testosterone dose, formulation, and administration schedule employed in OPS I is logistically impractical, and a more militarily feasible intervention is needed. The inability to induce a hypogonadal state and LBM loss in the participants receiving weekly placebo injections suggests the magnitude of stress imposed by the exercise- and diet-induced energy deficit in OPS I was far less than the stress Warfighters can endure during real-world training and combat operations, so our approach needs to be tested under more realistic conditions. Most importantly, the use of muscle performance measures that were insufficiently sensitive to demonstrate militarily relevant benefits of supplemental testosterone on muscle function, despite gains in LBM in OPS I, requires further investigation to conclusively demonstrate whether our proposed intervention can improve Warfighter performance. These limitations are clear gaps in knowledge that must be addressed before supplemental testosterone recommendations for Warfighters can be further considered.

Testosterone Formulations, Pharmacokinetics & Logistical Considerations



Testosterone is available in several therapeutic formulations with unique pharmacokinetic profiles, advantages, and disadvantages (**Table 1**; adapted from [17]). In OPS I, we administered 200 mg of testosterone enanthate per week by intramuscular (IM) injection. One 200 mg injection of testosterone enanthate can elicit a supraphysiological increase in testosterone concentrations that lasts ~7-d before declining back within the normal physiological range (**Figure 2**; adapted from [18]). The transient hypertestosteronemia and rapid decline in testosterone necessitates weekly (or biweekly) intramuscular injections, which if an injection is missed, can result in hypogonadal state until the next dose is delivered, or until endogenous testosterone production is recovered [17]. In OPS I, total and free testosterone concentrations were approximately 4% and 7% higher, respectively, than the upper-end of the normal physiological range at the end of the 28-d energy deficit. In recovery, concentrations declined to hypogonadal levels, 21-d after receiving the last dose of testosterone enanthate. Recovery of endogenous testosterone production was variable, ranging from 28-d to 11-mo. We attributed the mild hypertestosteronemia and delayed recovery of endogenous testosterone production to the administration of the short-acting enanthate ester and administering a greater dosage of testosterone enanthate (200 mg/wk) than typically recommended as the initial dose for clinical replacement therapy (75-100 mg/wk or 150-200 mg/2-wk [17]). Although there were no negative effects of short-term supplemental testosterone enanthate administration at

200 mg/wk in OPS I, providing weekly intramuscular injections of a supraphysiological dose during real-world training and combat operations is not likely to be practical or even feasible.

Table 1. Clinical pharmacology of testosterone (T) formulations approved in the United States and Europe (taken from [17]).

Formulation	Typical Starting Doses	Pharmacokinetic Profile	Advantages	Disadvantages
T enanthate or cypionate	150–200 mg IM every 2 wk or 75–100 mg/wk	After a single IM injection, serum T concentrations rise into the supraphysiological range, then decline gradually into the hypogonadal range by the end of the dosing interval	Relatively inexpensive, if self-administered; flexibility of dosing	Requires IM injection; peaks and valleys in serum T concentrations that may be associated with fluctuations in symptoms
T transdermal gels: 1%, 1.62%, or 2%	50–100 mg of 1% transdermal gel; 20.25–81 mg of 1.62% gel or 40–70 mg of 2%; transdermal gel applied to skin; check package insert for application site and instructions	With appropriate dose, restores serum T and E2 concentrations to the physiological male range; less fluctuation of T concentrations than T enanthate or cypionate	Provides flexibility of dosing, ease of application, good skin tolerability; less erythrocytosis than injectable T	Potential of transfer to a female partner or child by direct skin-to-skin contact; T concentrations may be variable from application to application; skin irritation in a small proportion of treated men; moderately high DHT concentrations (of unknown significance)
T Axillary Solution	60 mg of T solution applied in the axillae	Restores serum T and E2 concentrations to the physiological male range	Provides, good skin tolerability	Potential of transfer to a female partner or child by direct skin-to-skin contact; T concentrations may be variable from application to application; skin irritation in a small proportion of treated men; moderately high DHT concentrations (of unknown significance)
Transdermal T patch	One or two patches, designed to nominally deliver 2–4 mg of T during 24 h applied every day on nonpressure areas	Restores serum T, DHT, and E2 concentrations to the physiological male range	Ease of application	Serum T concentrations in some T-deficient men may be in the low-normal range; these men may need applications of two patches daily; skin irritation at the application site occurs frequently in many patients
Buccal, bioadhesive T tablets	30-mg controlled release, bioadhesive tablets twice daily	Restores serum T, DHT, and E2 concentrations to the physiological male range; absorbed from the buccal mucosa	Convenience and discreet	Gum-related adverse events in 16% of treated men
T pellets	Pellets containing 600–1200 mg T implanted SC; the number of pellets and the regimen may	Serum T peaks at 1 month and then is sustained in normal range for 3–6 mo, depending on formulation	Requires infrequent administration	Requires surgical incision for insertions; pellets may extrude spontaneously; rarely, local hematoma and infection may occur

	vary with formulation			
Injectable long-acting T undecanoate in oil	United States regimen: 750 mg IM, followed by 750 mg at 4 wk, and 750 mg every 10 wk	When administered at a dose of 750 mg IM, serum T concentrations are maintained in the normal range in most treated men	Requires infrequent administration	Requires IM injection of a large volume (3 or 4 mL); coughing episode reported immediately after injection in a small number of men
Nasal T gel	11 mg two or three times daily	Serum T concentrations are maintained in the normal range in most treated men	Rapid absorption and avoidance of first pass metabolism	Multiple daily intranasal dosing required; local nasal side effects, not appropriate for men with nasal disorders

There are several alternatives to testosterone enanthate, many of which do not require IM injections, including transdermal gels, patches, buccal, bio-adhesive tablets, and nasal gels (**Table 1**). However, while these formulations are non-invasive, and can be administered by the end-user, they require daily or multiple daily applications (transdermal and nasal gels), and carry added risks, including skin-to-skin transfer to others (transdermal gels) and gum- and nasal-related adverse events (buccal, bio-adhesive tablets, nasal gels). More importantly, allowing Warfighters to self-medicate with a Schedule III controlled substance (i.e., mild to moderate potential for abuse, physical and psychological dependence) while operating under extreme physiological and psychological stress raises health and ethical concerns. Long-acting testosterone formulations that require infrequent administration by licensed clinicians are much more viable options. Subcutaneous implantable testosterone pellets are an example of a long-acting testosterone formulation that maintains normal physiological testosterone concentrations for 3 to 6-mo (2-4 doses per year). However, the implantation requires a minor surgical procedure, which increases the risk of adverse events (e.g., infection, pellets may extrude from incision), and the overall logistical burden to the patient and Department of Defense.

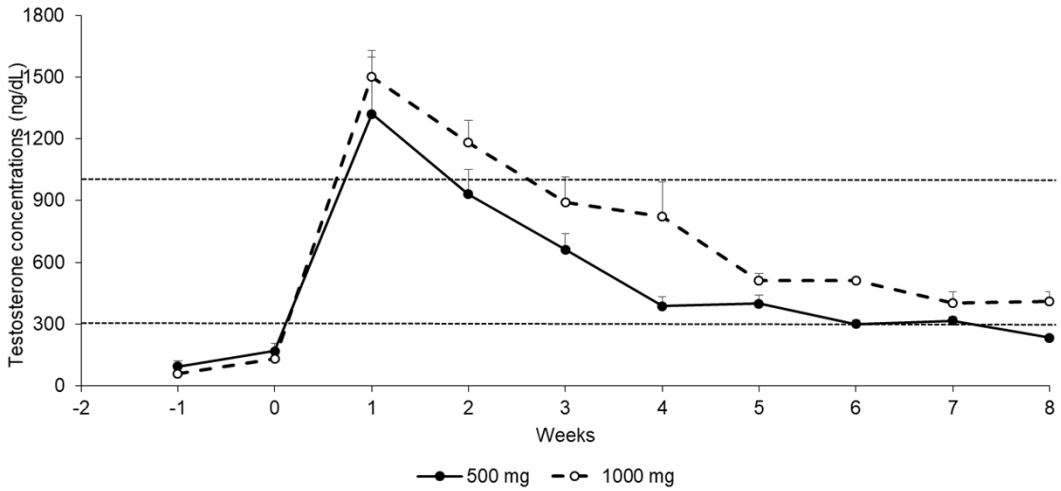


Figure 3. Serum testosterone response to 500 and 1000 mg intramuscular injection of testosterone undecanoate. Normal physiological range indicated between dotted lines (Zhang et al. 1998).

In contrast to the aforementioned approaches, intramuscular injection of testosterone undecanoate can maintain testosterone concentrations within the normal physiological

range for 8-10-wk (**Figure 3**; adapted from [19]) with considerably less clinical or logistical burden. The clinical testosterone undecanoate dosing regimen requires an initial 750 mg IM injection, followed by another 750 mg at wk 4, and 750 mg every 10-wk thereafter [17]. This dosing regimen may be logistically feasible for Warfighter populations (i.e., special operations forces, etc.) who are most vulnerable to muscle loss and functional decline while routinely conducting strenuous operations as part of their annual training and deployment cycle. Further, following cessation of testosterone undecanoate administration, testosterone declines gradually over time, which reduces likelihood of becoming hypogonadal between doses, or after ceasing treatment. ***Based on these factors, OPS II will test if testosterone undecanoate (TU, 750 mg administered once) safely and steadily maintains normal testosterone concentrations, promotes anabolism, spares LBM, and enhances militarily-relevant measures of muscle function without cognitive impairment during and in recovery from a 20-d simulated, multi-stressor military operation.***

Operational Stress, Testosterone Status, and Lean Mass Loss: Beyond Energy Deficit. In OPS I, we isolated the consequences of exercise- and diet-induced energy deficit from the additional stressors experienced during strenuous military operations. As a result, we were able to demonstrate that the overall metabolic stress and its associated consequences on testosterone and LBM during military operations is greater than can be accounted for by the energy deficit alone. More specifically, we observed a 20% reduction in total testosterone in OPS I study controls, which is comparable to declines observed in our previous controlled underfeeding study within a similar population [20], but much less than the decline in testosterone observed during strenuous military operations [4, 7, 8]. We employed an energy deficit (i.e., 55%) that was similar to energy deficits observed during real-world military operations, but total daily energy expenditure and the absolute energy deficit (~2000 kcal/d) were less than those observed in the field [8, 15, 16, 21], suggesting that a greater absolute energy deficit may have been necessary to induce the intended decline in total testosterone. In addition, we used discrete aerobic-type exercise bouts in OPS I to increase exercise-induced energy expenditure during the 28-d energy deficit, followed by periods of adequate rest. Energy deficits during real-world military operations often result from low-to-moderate increases in metabolic demand sustained for long periods. During real-world military operations, recovery is also minimal and Warfighters are often sleep deprived. Sleep restriction (5-h per night), alone, can suppress total testosterone by as much as 15% [4]. In a study that involved strenuous military training and produced a substantial energy deficit, continuous sleep (3-h), compared to non-continuous sleep, attenuated reductions in testosterone [22]. We did not restrict sleep in OPS I; therefore, the stressors applied (i.e., increased physical activity and decreased energy intake) may not have mimicked the multi-faceted stressors experienced during real-world military operations, which generally produce marked suppression of testosterone and catabolism of lean mass [11, 15, 16].

The preservation of LBM in OPS I study controls was also unexpected. We speculated that the combination of higher-protein feeding and the physical training stimulus in OPS I accounted for this finding. More specifically, energy deficiency suppresses muscle anabolism [23, 24], yet consuming a higher-protein diet (1.2-2.4 g protein/kg/d) while

underfed restores muscle anabolic rates to those observed during energy balance [24, 25]. The anabolic restorative effects of higher-protein diets during energy deficit are potentiated by training effects of repeated high-volume exercise [26], providing the conditions by which lean mass may be maintained [27].The addition of supplemental testosterone may have provided an anabolic stimulus that was additive to the stimulus provided by protein and exercise, explaining, in part, the gain in lean mass in the testosterone group. Studies showing that testosterone or testosterone analogue administration enhances protein synthetic efficiency support our speculation, meaning, for a given amount of intracellular essential amino acids, a greater proportion is routed towards protein synthesis than towards oxidative metabolism, thus conserving muscle protein mass [28, 29]. While plausible, Warfighters are unlikely to routinely meet operational dietary protein requirements (i.e., 1.5-2.0 g protein/kg/d) [30] while subsisting primarily on combat rations because rarely is a full daily allotment of combat rations provided [in the US, i.e., equal to three Meal, Ready-to-Eat (MRE), ~120 g of total protein (~1.5 g protein/kg/d for an average 80 kg male)] or consumed [31]. Thus, the synergistic effects of higher-protein feeding and high-volume exercise on lean mass retention likely does not occur during real-world military operations [15, 16]. Furthermore, whether testosterone modulates muscle and whole-body anabolic status during prolonged, severe energy deficit, regardless of amount of dietary protein consumed, has never been studied.

OPS II will address these limitations and gaps in knowledge by limiting dietary protein intake [15% of total energy provided (~0.8 g protein/kg/d based on an average 80 kg male and provision of ~two MREs per day)] during a 20-d simulated military operation comprised of very high total daily energy expenditures caused by sustained elevations in exercise-induced energy expenditure that, when combined with sleep deprivation, should cause severe absolute energy deficits, marked reductions in testosterone, and catabolism of lean mass. Muscle and whole-body anabolic status will also be assessed during OPS II by using stable isotope tracer techniques.

Warfighter Performance Enhancement

Lower-body muscle function declined similarly for both groups during the energy deficit in OPS I, despite no loss of LBM in either group. The decline in muscle function may have been due to neuromuscular fatigue [32] and reduced motivation over time [33, 34]. A similar inability to detect differences in muscle function with testosterone supplementation has also been demonstrated in young men receiving exogenous testosterone during 28-d of bed rest [35], in middle-aged, obese males receiving exogenous testosterone for 56-wk [36], and in older, overweight males receiving testosterone supplementation for 6-mo [37]. The discordance between the LBM change and muscle functional decline in OPS I for those receiving supplemental testosterone may be attributable, in large part, to the test metrics employed, as isokinetic and isometric dynamometry may not be sensitive to military-relevant performance improvement. ***OPS II will use a battery of muscle function and performance outcomes to test the possible performance enhancing effects of supplemental testosterone during strenuous training and combat operations. The test battery will include Warfighter occupational tasks commonly performed under stress,***

including load carriage, as well as reproducible and validated metrics of lower-body power, anaerobic and aerobic capacity.

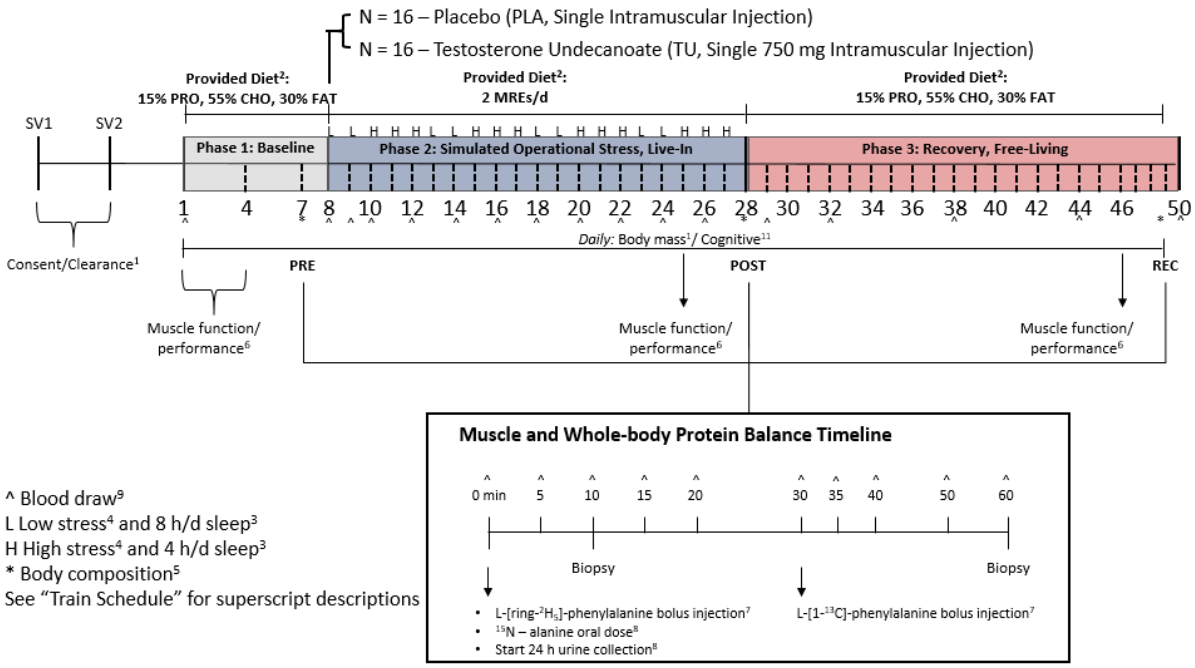
Military Relevance & Anticipated Deliverables

OPS II supports the Military Operational Medicine Research Program's Physiological Health and Performance Program Area under the BPE Work Unit. The intent of the BPE Work Unit is to execute basic and applied research to identify and develop biomedical strategies and solutions that safely and ethically enhance Warfighter physical, cognitive, sensory and behavioral performance in training and operational environments, enabling lethality and overmatch. The threat OPS II addresses is suboptimal performance and the capability gap we intend to close pertains to medical readiness, as Army units possess limited capacity to provide countermeasures to protect against health threats before and during deployment to sustain 90% medical fitness for duty rate for an operational environment. OPII will address the BPE near-term objective – to identify one effective androgen replacement therapy that enhances Warfighter physical and cognitive performance during simulated, multi-stressor military operations. Deliverables include evidence-based guidance for supplemental testosterone use for performance enhancement in Warfighters. Guidance will be transitioned to the United States Special Operations Command (USSOCOM), including the Army, Naval, Marine Corps Forces, Air Force, and Joint Special Operations Commands.

Experimental Design and Overview

The study will be a double-blind, randomized, placebo controlled trial in 32 physically-active men (18-35 y) exposed to 20 complete days (days 8-27) of simulated operational stress followed by 20 complete days of recovery (days 29-48, **Figure 4**). After completing baseline testing (Phase 1, P1), participants will be randomized to receive either a single intramuscular injection of testosterone undecanoate (TU; 750 mg, standard pharmaceutical dose [17]) or an iso-volumetric placebo (PLA, sesame oil solution) (day 8). The 20-d simulated operational stress (Phase 2, P2) will be highly controlled (live-in study on the inpatient unit at PBRC) and consist of 4 successive cycles of undulating stress, starting with 2 consecutive days of low stress followed by 3 consecutive days of high stress. Low and high stress days will result from low and high militarily-relevant exercise-induced energy expenditures, adequate and restricted sleep (8 h/d vs. 4/d), and diet restriction to produce energy deficits that range from approximately 960 kcal/d to 2975 kcal/d. After completing P2, participants will be released from PBRC, resume their habitual physical activity routines, and will be provided a controlled diet to consume, to assess physiological, endocrine, and cognitive recovery from sustained, severe operational stress (Phase 3, P3). Measures of muscle function will be the primary study endpoint. Secondary endpoints include body composition, whole-body and skeletal muscle anabolism, endocrine-, metabolic-, and safety-related biomarkers, cognitive function, and mood. This study design will allow us to test the hypothesis that a practical, single administration of TU elicits physiological enhancement during severe operational stress without psychological impairment.

Figure 4. Experimental design



Note: Corresponding Train Schedule is attached as Appendix A.

Primary Study Endpoint

Measures of muscle function will serve as the primary study endpoint. Body mass loss and adherence to the prescribed exercise, diet, and restricted sleep intervention will be used as a daily measures of compliance and conformance to the experimental design. Day 50 will signal the end of study (EOS) for all participants.

The study may be terminated if a participant withdraws, is unable to comply with the prescribed exercise, diet, and restricted sleep intervention, has complications with study procedures, particularly TU administration, muscle biopsies, and exercise. 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. OPS I recruited potential participants over a 2 year period to randomize and complete 50 participants. PBRC also has a Clinical Trials Recruitment Core with at least 2 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 completes 5000 phone screens per year from potential volunteers. Study recruitment will rely on the existing methods of the Clinical Trials Recruitment Core to advertise that were deemed successful in the recruitment of OPS I. Methods included but are not limited to local media (print, radio, TV), paid targeted digital campaigns, earned media, social media (Facebook, Twitter, etc.), community outreach events and presentations, and collaborative relationships with local universities, sport vendors, health food vendors, etc.

Inclusion and Exclusion Criteria
Inclusion Criteria

- Men aged 18-35 years
- Ability to understand verbal or written instructions/testing materials in English.
- Physically active (as determined by accelerometry and review of a physical activity log)
- 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 20 consecutive days
- 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

- Adults unable to consent
- Women
- Prisoners
- Sedentary or engages in insufficient quantities of physical activity per week (aerobic and/or resistance training as determined by accelerometry and review of a physical activity log)
- 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 Chronic Obstructive Pulmonary Disease or Obstructive Sleep Apnea
- Prostate-Specific Antigen (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.

Number of Subjects
Muscle function (lower-body muscular strength and power) is the primary study endpoint. The intended total energy deficit during the 20-d period of simulated operational stress [high total daily energy expenditures caused by low-to-moderate intensity physical work for extended periods of time (~≥10 h/d), restricted food intake and sleep] in the present study will be ~43,380 kcal (~2,169 kcal/d) which, based on our meta-regression of data generated from military field studies, should cause a ~9% reduction in total body mass and ~7% decline in lower-body muscle function (Murphy Sport Med). The magnitude of energy deficit and anticipated body mass loss exceeds the energy deficit and ~7% reduction in body mass that occurred during the first four weeks of US Army Ranger training that concomitantly lowered total testosterone concentrations by ~50% (pre-Ranger training, ~433 ng/mL; week four of Ranger training, ~202 ng/dL). We anticipate a similar decline in total testosterone and, based on the results from OPS I (Pasiakos et al. unpublished), expect body mass loss in TU to be ~50% less than PLA, such that lower-body muscular strength and power declines from baseline are attenuated by 50% in TU relative to PLA. The sample size necessary to determine the estimated differences between treatments is 15 per group (**Table 2**). To account for possible attrition (5% attrition in OPS I prior to randomization), 16 participants will be assigned to each group (32 total participants). To successfully enroll and complete testing on 32 participants, we request the ability to enroll up to 38 to randomize and complete 32. Enrollment will stop once 32 participants have completed the study.

Table 2. Power Analysis and Sample Size Justification

Hypothesized Effect (mean ± SD)	
Percent Δ lower-body strength and power following the 20-d intervention, %	
PLA	9.0 ± 6.75
TEST	4.5 ± 3.38
Effect Size	1.25
Alpha	0.05
Power	0.90
Sample Size	15 per group
5% study attrition	32 participants total

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 the first of two screening visits at 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**).
- A medical history, Physical Activity Readiness Questionnaire (PAR-Q) will be administered and CVD risk stratification assessed.
- Information regarding the participant's current medications 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 provided an accelerometer to wear. The accelerometer will be worn daily and only removed during water-required activities. Additionally, a physical activity log will be completed. An appointment will be scheduled approximately 1 week later for Screening Visit 2.

Screening Visit 2. SV2

- The participant's accelerometer & physical activity log will be reviewed for completeness and to determine activity levels.
- The participant will have an EKG and a complete physical exam where medical history will be reviewed by physician, NP, or PA.
- Weight, blood pressure, and pulse 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.
- Resting metabolic rate will be measured.
- The participant will be asked to wear the weighted pack and go on a practice ruck march.
- Eligibility criteria will be reviewed. All participants who maintain eligibility criteria will continue to wear an accelerometer, record their physical activity, 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 1 within 45 days of SV2.
- If more than 45 days lapse between screening measures and D1 scheduled date, participants may be asked to repeat measures to re-assess eligibility.

Phase 1: Out-Patient Baseline Testing. Days 1–7

- During the P1, participants will return to PBRC once a day to eat and receive additional meals for the day. Participants will be weighed daily and energy adjustments made if needed. Participants will be asked to record their food and activity daily throughout P1. Additionally, participants will wear a wrist and waist-worn accelerometer throughout P1. Time commitment for P1 is ~1-5-h per day.
- Blood sample by venipuncture will occur on day 1.
- Practice on the cognitive tests will be performed on days 2, 4, and 6 for approximately 1.5-2-h. The timing (day of testing or time of day) of training sessions is not critical.
- Sleep will be monitored daily using a wrist-worn monitor and by completion of the Modified Pittsburg Sleep Quality Index (PSQI) each day.
- Body composition (dual energy X-ray absorptiometry, DXA; bioelectrical impedance, BIA; circumference measurements) will be measured on day 7.
- To measure total body water, a urine sample will be collected prior to having the consuming deuterium water on day 7. A 4 hour post urine sample will be collected after dosing.
- Skeletal muscle protein balance will be measured on day 7 (PRE on **Figure 4**), including 2, bolus intravenous injections of stable isotopes ($^{2}H_{5}$-phenylalanine, 1-^{13}C-phenylalanine), 2 percutaneous muscle biopsies of the vastus lateralis, and 10 intravenous blood draws.
- Whole-body protein balance will be measured on day 7 (PRE) using an oral dose of ^{15}N-alanine and 24-h urine sample. Participants will have a blood draw to measure BUN and to complete the 24 hour urine sample on day 8.
- Muscle function and performance will be assessed on day 4 and 5, including the vertical jump test, deadlift, Wingate, treadmill VO2peak, and time trial.

Familiarization will occur on day 1. *May be administered on alternative visit days (+/- 2 days) as scheduling and availability permits.

Randomization
Immediately following P1 testing on day 7, participants will be randomized to receive either a single IM injection of testosterone undecanoate (TU; 750 mg, standard pharmaceutical dose [17]) or an iso-volumetric placebo (PLA, sesame oil solution) on the morning of day 8 after checking into the in-patient unit at PBRC and before initiating the 20-d of simulated operational stress. A randomization scheme will be determined using a block design (n=32) 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: Simulated Operational Stress. Days 8-28

- During the simulated operational stress phase, participants will live on the in-patient unit at PRBC in a 24-h per day controlled setting. Once a day, participants will have their vitals (BP, pulse) measured. All meals (~2 rations per day) will be eaten on the unit and monitored. Participants will be weighed daily and wear a wrist-worn accelerometer throughout P2. Since participants will reside on the in-patient unit during P2, the time commitment for this phase is 24-h per day.
- An IM injection (placebo or testosterone) will be administered on day 8, after a blood sample is collected.
- Throughout phase 2, participants will be highly physically active, completing supervised exercise sessions to elicit low (days 8, 9, 13, 14, 18, 19, 23, and 24) and high stress (days 10, 11, 12, 15, 16, 17, 20, 21, 22, 25, 26, and 27) energy expenditure and deficit prescriptions.
- Participants will be allowed 8-h of sleep on low (approx. 2200-0600-h; days 8, 9, 13, 14, 18, 19, 23, and 24) and 4-h of sleep on high stress (approx. 0100-0500-h; days 10, 11, 12, 15, 16, 17, 20, 21, 22, 25, 26, and 27) days. Sleep outside these defined periods will not be allowed. Sleep will be monitored daily using wrist-worn monitors.
- Blood sample by venipuncture will occur on days 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, and 26.
- Cognitive testing will be performed on days 12, 14, 17, 22, and 27 over a 1.5-2-h period each day.
- Sleep will be monitored daily using a wrist-worn monitor and by completion of the Modified Pittsburg Sleep Quality Index (PSQI) each day.
- Muscle soreness will be measured each day using a Visual Analog Scale (VAS).
- Body composition (DXA, BIA, and circumferences) will be measured on day 28.

- To measure total body water, a urine sample will be collected prior to having the consuming deuterium water on day 28. A 4 hour post urine sample will be collected after dosing.
- Skeletal muscle protein balance will be measured on day 28 (POST on **Figure 4**), including 2, bolus intravenous injections of stable isotopes ($^{2}H_5$-phenylalanine, 1-^{13}C-phenylalanine), 2 percutaneous muscle biopsies of the vastus lateralis, and 10 intravenous blood draws.
- Whole-body protein balance will be measured on day 28 (POST) using an oral dose of ^{15}N-alanine and 24-h urine sample. Participants will have a blood draw to measure BUN and to complete the 24 hour urine sample on day 29.
- Muscle function and performance will be assessed on day 25 and 26, including the vertical jump test, deadlift, Wingate, treadmill VO2peak, and time trial. Familiarization will occur on day 23. *May be administered on alternative visit days (+/- 2 days) as scheduling and availability permits

Phase 3: Recovery. Days 29-50

- For days 29-50 (i.e., day 50 constitutes the EOS; participants will be released from the in-patient unit after completing all tests on day 28, POST) participants will return to PBRC once a day to eat and receive additional meals for the day. Participants will be weighed daily and wear a wrist and waist-worn accelerometer throughout P3. The energy provided will be the same as that provided during P1. Participants will be asked to record their food and activity daily throughout P3. Time commitment during P3 is about 1-5-h per day.
- Blood sample by venipuncture will occur on days 29, 32, 38, 44, and 50.
- Cognitive testing will be performed on days 32 and 38 over a 1.5-2-h period each day.
- Sleep will be monitored daily using a wrist-worn monitor and by completion of the Modified Pittsburg Sleep Quality Index (PSQI) each day.
- Body composition (DXA, BIA, and circumferences) will be measured on day 49.
- To measure total body water, a urine sample will be collected prior to having the consuming deuterium water on day 49. A 4 hour post urine sample will be collected after dosing.
- Skeletal muscle protein balance will be measured on day 49 (recovery, REC on **Figure 4**), including 2, bolus intravenous injections of stable isotopes ($^{2}H_5$-phenylalanine, 1-^{13}C-phenylalanine), 2 percutaneous muscle biopsies of the vastus lateralis, and 10 intravenous blood draws. *Participants will undergo 6 total muscle biopsy procedures during the entire study period (4 on one leg and 2 on the opposite leg, 3 total incisions).*
- Whole-body protein balance will be measured on day 49 (POST) using an oral dose of ^{15}N-alanine and 24-h urine sample.
- Muscle function and performance will be assessed on day 46 and 47, including the vertical jump test, deadlift, Wingate, treadmill VO2peak, and time trial. Familiarization will occur on day 44. *May be administered on alternative visit days (+/- 2 days) as scheduling and availability permits
- Participants will return on day 50 to have a blood draw to measure BUN and to return the 24 hour urine sample.

PRN: As Needed Follow-up Visit

- After day 50, if the participant's testosterone concentrations are not within normal reference range (300-1000 ng/dL), a blood sample by venipuncture will occur every 90 days until levels have returned to within normal reference range.

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

Total daily energy requirements and P1 and P3 dietary intake

During the screening visits, participants will meet with registered dietitians and complete a 3-day food and activity records to determine habitual dietary intake, physical activity patterns, and exercise-induced energy expenditure. Pre-study activity levels and exercise-induced energy expenditures will be maintained during P1 and P3. Activity will be verified during P1 and P3 using accelerometry and activity records. Physical activity will be highly controlled and monitored during P2.

The 3-d records will also be used by registered dietitians to calculate daily energy needs for P1 and P3. Resting metabolic rate will be measured by indirect calorimetry using a Deltatrac II Metabolic Cart (Sensormedics, Yorba Linda, CA) with a ventilated hood prior to the start of P1. During P1 (baseline, pre-operational stress), participants will be provided a controlled, weight-maintaining diet designed to match the macronutrient distribution of the diet during P2 (15%, 55%, and 30% total energy from protein, carbohydrate, and fat, respectively). During P3 (recovery, post-operational stress), participants will be provided the same controlled diet provided in P1 to assess weight regain and physiological recovery. The macronutrient distribution of these diets is based on a combination of observations made during field operations [15, 16, 38, 39], the composition of Meal, Ready-to-Eat (MREs, primary food source for P2), and the current Military Dietary Reference Intakes (as stated in Army Regulation 40-25). Registered dietitians will develop individualized menus consisting of breakfast, lunch, dinner, snacks, and energy-containing beverages for P1 and P3. Breakfast meals will be consumed at PBRC under supervision and all other meals and energy-containing beverages will be provided for consumption offsite. P1 and P3 diet compliance will be verified by research staff.

Note: The micronutrient content of the P1 and P3 will be consistent with current recommendations. Micronutrient intake during P2 may be lower than P1 and P3. 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.

20-d Simulated Operational Stress

The 20-d simulated operational stress will be highly controlled (live-in study on the inpatient unit at PBRC) and consist of 4 successive cycles of undulating stress, starting with 2 consecutive days of low stress followed by 3 consecutive days of high stress

(refer to **Figure 4**). Low and high stress days will result from low and high militarily-relevant exercise-induced energy expenditures, adequate and restricted sleep (8 h/d vs. 4/d), and diet restriction (~2 combat rations per day throughout the 20-d intervention) to produce energy deficits that range from approximately 960 kcal/d to 2975 kcal/d. This level of deficit was selected based on a recent meta-analysis of field studies conducted in military environments that identified this level as the upper end of mean energy deficits observed during various military training exercises, and will produce a total energy deficit over 20-d (~43,380 kcal) that is expected to induce decrements in body mass, LBM, testosterone concentrations, and muscle function [13].

For physical activity, varied low-, moderate-, and intermittent high-intensity endurance and muscle loading-type exercise will be performed to reproduce conditions normally observed during strenuous, sustained military operations [1], and increase participants' exercise-induced energy expenditure to approximately 960 kcal/d to 2975 kcal/d above their P1 total daily energy expenditure, and to generate an energy expenditure of approximately 3600 kcal/d to 5625 kcal/d (based on an estimated mean P1 total daily energy expenditure of 2650 kcal/d; exact values will differ based on actual P1 energy expenditures). The increase in total daily energy expenditures from physical activity will be achieved by performing multiple exercise sessions daily lasting 2-3-h per session, using a variety of endurance and muscle loading modalities that mimic movements and activities common during field training and combat operations. Steady-state load carriage endurance-type exercise will be the primary exercise modality (~30-40% of total body mass carried). However, activities will also include walking and hiking without a loaded pack, jogging and field-based military activities. These activities will be completed both in the laboratory using exercise equipment and in a modified military training grounds on the PBRC campus. The military activities will include Army Physical Readiness-type training, simulated body evacuations, an obstacle course and others. The 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 while still maintaining the prescribed low and high stress-induced deficits.

The same total energy provided during P1 (and P3) will also be provided during P2. However, the primary food source during P2 will be US combat rations, specifically the Meal, Ready-to-Eat (MRE, on average participants will receive the equivalent of ~2 MREs, ~2600 kcal/d). Registered dietitians will develop individualized MRE-based menus. The macronutrient distribution of the P2 diets will be consistent with P1 and P3 (15%, 55%, and 30% total energy from protein, carbohydrate, and fat, respectively).

Anthropometrics and body composition

Height will be measured using a stadiometer. Semi-nude body weight will be measured after an overnight fast and morning void using a calibrated digital scale (GSE Inc. Model 450, GSE Scale Systems, Novi, MI) during each screening visit and daily throughout P1, P2, and P3. Body composition (bone, fat, lean, and body water) and circumferences will be measured after an overnight fast and morning void on days 7 (PRE), 28 (POST), and 49 (REC) using a 4-compartment model derived from DXA (Hologic DXA, Discovery A, Hologic, Marlborough, MA) and BIA (3D Fit3D system, 3D Size Stream

system, Impedimed SFB7, InBody S10, Jawon Cozy 930; or similar) [40]. Multi-compartment methods (2, 3, and 4-compartment) will be used to calculate total body fat, FFM, protein and hydration (TBW/FFM, ECW/ICW).

Total body water (TBW) will also be measured by deuterium dilution on days 7 (PRE), 28 (POST), and 49 (REC). Participants will provide a pre-dose urine sample and will then be orally dosed with deuterium water (99.9% D2O) at 0.15 g/kg body weight. The water will be given orally and then the dose cup rinsed with 50 mL of unlabeled (tap) water. A second urine sample will be collected 4 hours post dose. The enrichment of deuterium in the urine at 4 hours will be used to determine TBW.

Muscle Function and Performance

A battery of muscle function and performance tests will be conducted on days 4, 5, 25, 26, 46, and 47 to assess lower-body strength, power, anaerobic and aerobic capacity during P1, P2, and P3 (**Table 3**). Participants will be familiarized to the test battery on days 1, 23, and 44. The order and timing of tests will be standardized (i.e., strength/power → anaerobic capacity → aerobic capacity, as listed in **Table 3**). Ratings of perceived exertion (RPE) will be recorded during each battery. The RPE scale will be presented on a video monitor and participants will be asked to give a verbal response that will be recorded by a research team member (< 30 sec to complete).

Table 3. Muscle Function and Performance Test Battery

Test	Purpose
Vertical Jump Test [41]	Measure lower-body peak power
Deadlift (component of the Army Combat Fitness Test)[1]	Operational test used in the Army to assess upper- and lower-body muscular strength, anaerobic and cardiorespiratory endurance, and lower-body and upper-body explosive power
Wingate Test	Measures anaerobic capacity
Treadmill VO_{2peak}	Measures peak aerobic capacity. Will be used as an outcome and as a reference point to determine the appropriate exercise workloads for the exercise bouts before starting P2
Time Trial[2]	Militarily-relevant aerobic performance outcome

[1]OPS II will employ a modified execution of the deadlift from the Army Combat Fitness Test. [2]Load carriage is an essential aerobic-based Warfighter task and according to Army Techniques Publication (ATP) 3-21.18, Soldiers are expected to carry a standard fighting load of 68.9 lbs. and move at a rate of 4 km per hour in an ideal situation. As such, OPS II will test time to complete a 4 km (2.5 miles) ruck march (i.e., load carriage) with participants wearing a 31.3 kg (68.9 lbs.) pack.

Cognitive Function, Personality, Mood, and Sleep

A battery of cognitive performance tests and questionnaires will be administered to assess cognition, personality, and mood during P1, P2, and P3 (**Table 4**). Sleep will be monitored throughout the study. During P2, participants will be allowed 8-h of sleep between 2200-0600-h for low stress days, whereas, participants will be allowed only 4-h of sleep between 0100-0500-h on high stress days. Sleep outside these defined periods will not be permitted during P2.

Table 4. Cognitive Function, Personality, Mood, and Sleep Test Battery

Test	Description
Cognitive tests	
Balloon Analogue Risk Task (BART) [43]	Measure of willingness to take risks versus "play it safe" using a simulated balloon task. The more expanded the balloon gets, the more points are earned. All points are lost if the balloon is over-inflated and pops. There is also a risk-learning component to this task.
Scanning Visual Vigilance Task [44]	This test of vigilance is sensitive to a wide variety of environmental conditions, nutritional factors, sleep loss, and very low doses of hypnotic drugs and stimulants. It was designed to simulate various critical military activities that require maintenance of vigilance such as sentry duty and sonar watch.
Psychomotor Vigilance [45]	Measure of simple visual reaction time which is particularly sensitive to the vigilance decrements associated with sleep restriction or disruption
Match to Sample [46]	Assesses short-term spatial memory (working memory) and pattern recognition skills
N-back Task [47]	Measures working memory and requires on-line monitoring, updating, and manipulation of remembered information and allows for the parametric assessment of different working memory loads.
Provoked Aggression [48]	This task is referred to as the Electrical Stimulation Task in the informed consent form. This task assesses participant's propensity toward retaliatory increases in applied pain level. Two electrodes will be attached to the skin and to an electrical stimulator (STMISO, Biopac Systems, Inc.). The stimulator applies a brief pulse of electrical current to provide an uncomfortable stimulus to the participant. The electrodes are placed at two standard locations on any limb, following a manufacturer protocol, to prevent electrical current from traveling to the rest of the body beyond the limb. A gel is rubbed onto the skin at the electrode site to enhance conductance. In addition, the device conforms to the IEC 601-2-10 international regulatory standard that prescribes limits on the voltage, pulse energy, and pulse width that can safely be applied by the device to human subjects. The levels of stimulation applied to the participant are tested by study staff before they are applied to any research participant. During the task, the participant engages in a simple game with a digital opponent; after each round, the winner is allowed to apply a stimulus to the opponent, and is instructed to set the intensity of the applied pain 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. The task assesses the degree to which a study intervention heightens the propensity toward retaliatory aggression against a provoking adversary, across varying levels of provocation. The provoked aggression task involves partial participant deception, and the procedure requires

	incomplete disclosure on the informed consent form. The reason to incorporate deception is that the outcome of primary interest is the participant's intent to engage in aggressive acts toward a human being that is provoking him. Proxy measures, including self-reported feelings of aggression and biomarkers of arousal, are not informative enough to accurately depict this intent. Because the participant must be given the opportunity to engage in actions that appear to be aggressive, we have no realistic option besides creating the illusion that the participant is engaging in a retaliatory act, when in fact no actual discomfort is inflicted on any other human being. Thus, deception is unavoidable to collect the required data. At the end of their involvement in the study, participants will **not** be debriefed about the partial deception present in the provoked aggression task. The key reason for non-disclosure is that completing participants who learn about the deception will be able to alert new study recruits about the deception, thus invalidating any future data collected from them. Because Baton Rouge is not a large city, we expect that there will be many opportunities for study participants to have social interactions outside of PBRC, and therefore there will be many opportunities for completing participants to alert new recruits to the deception.
	*Will only be administered on D6, D27, D32, and D38 of cognitive battery (+/- 2 days)
Psychology/Personality	
Buss-Perry Aggression Questionnaire [49, 50]	29-item questionnaire measuring levels of Physical Aggression, Verbal Aggression, Anger, and Hostility and has shown utility for examining the impact of testosterone on aggressive impulses.
Profile of Mood States [46, 51-54]	65-item inventory of self-reported mood states that is sensitive to a wide variety of stressors including undernutrition and sleep loss
Evaluation of Risks Scale [55]	Assesses willingness to take risks through participant responses to 24 items on a visual analog scale
Modified Pittsburgh Sleep Quality Index (PSQI) [56]	Self-rated questionnaire which assesses sleep quality and disturbances on a nightly basis for each night of the protocol. Generates scores on subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, and sleep disturbances.
Sleep Monitoring/Actigraphy	Assesses spontaneous motor activity, circadian rest/activity cycles, and sleep using a watch-sized, wrist-worn device

<u>PRE, POST, and REC Muscle and Whole-Body Anabolism</u>

Skeletal muscle and whole-body anabolic status (protein synthesis, protein breakdown, and net protein balance) will be measured on days 7 (PRE), 28 (POST), and 49 (REC) by using the minimally invasive pulse bolus tracer injection technique [57] and the end-

product method [58]. Following an overnight fast (no less than 8-h), intravenous catheters will be placed into a forearm vein on each arm, one for blood sampling and one for bolus stable isotope administration. A blood sample will be drawn to establish background amino acid enrichment before the tracer injections. Immediately thereafter, participants will provide a urine sample to establish background isotopic enrichments for whole-body measures. At the start of the 60-min tracer study (0 min; **Figure 4**), a bolus injection of $^{2}H_5$ phenylalanine (35 μmol/kg) will be administered [59]. At the same time, participants will consume a single oral dose of ^{15}N alanine (333 μmol/kg) and begin collecting their urine for the next 24-h. A bolus injection of $^{13}C_6$ phenylalanine (35 μmol/kg) will be administered 30-min after the first bolus was administered.

Venous blood samples will be obtained in 5-min intervals for the first 30-min of the tracer study and at 10-min intervals for the remaining 10-min (10 total blood samples over 60-min). Muscle biopsies of the vastus lateralis will be obtained at the 10-min and 60-min point of the tracer study. Muscle biopsies will be snap frozen in liquid nitrogen and stored at -80ºC. Blood samples will be centrifuged and serum will be stored at -80ºC. Muscle, blood, and urine samples will be analyzed for isotope enrichments using gas and liquid chromatography mass spectrometry [57].

Mixed-muscle fractional synthesis rate (FSR) will be calculated using the formula:

$$\mathrm{FSR} = \frac{[\mathrm{E_B}(t_2) - \mathrm{E_B}(t_1)]}{\int_1^2 E_M(t)\mathrm{d}t}$$

where $E_B(t)$ is the enrichment of bound phenylalanine enrichment at time *t* and $E_M(t)$ is the enrichment of free phenylalanine at time *t* [57].

Mixed-muscle fractional breakdown rate (FBR) will be calculated using the formula:

$$FBR = \frac{[\mathrm{E_M}(t_2) - \mathrm{E_M}(t_1)] \cdot \int_2^3 [\mathrm{E_A}(t) - \mathrm{E_M}(t)\mathrm{d}t) - [\mathrm{E_M}(t_3) - \mathrm{E_M}(t_2)] \cdot [\int_1^2 \mathrm{E_A}(t) - \mathrm{E_M}(t)\,\mathrm{d}t]}{\int_2^3 \mathrm{E_M}(t)\mathrm{d}t \cdot \int_1^2 \mathrm{E_A}(t)\mathrm{d}t - \int_1^2 \mathrm{E_M}(t)\mathrm{d}t \cdot \int_2^3 \mathrm{E_A}(t)\mathrm{d}t} \cdot (\mathrm{Q_M}/\mathrm{T_M})$$

where $E_A(t)$ and $E_M(t)$ are the arterialized and muscle free phenylalanine enrichments at time *t* and Q_M/T_M is the ratio of free to bound phenylalanine in muscle [57].

Net muscle protein balance (M-NET) will be calculated as the difference between FSR and FBR.

Whole-body nitrogen flux (Q, g N/24 h) will be determined using urinary urea enrichment according to Fern et al. [60]. Whole-body protein synthesis (PS) and breakdown (PB) will be calculated according to Stein et al. [61].

$$Q = PS + N_{EX} \text{ and } Q = PB + N_{IN}$$
$$PB = Q - N_{IN} \text{ and } PS = Q - N_{EX}$$

where N_{EX} is urinary urea nitrogen excretion and N_{IN} represents nitrogen intake during the 24-h urine collection period.

Net whole-body protein balance (WB-NET) will be calculated as the difference between whole-body PS and PB.

Note: Muscle biopsies will be collected while the participants are under local anesthesia (1% lidocaine) using a 5-mm Bergstrom needle with manual suction [62]. Two specimens will be taken on each tracer study day (PRE, POST, and REC) from the same incision (3 total incision and 6 total biopsies if all tracer studies are completed). Up to 250 mg of muscle tissue (multiple passes may be required to obtain adequate sample) will be collected during each biopsy procedure.

A segment of muscle collected at each time point will used to explore the potential mechanisms by which testosterone regulates muscle anabolism and the metabolic response to the simulated operational stress. In brief, testosterone targets several molecular pathways regulating muscle mass [63]. Rodent models providing exogenous testosterone or using castration models to diminish endogenous testosterone have identified alterations in androgen receptor activation of the Akt-mTORC1 signaling pathway [64-66], satellite cell proliferation and differentiation [67-69], SMAD/FOXO activation of proteolytic atrogene expression [70], and autophagy [70]. Additionally, exogenous testosterone may increase mitochondrial biogenesis and quality by upregulating PGC-1α [71], which may indirectly support muscle mass by facilitating greater utilization of fatty acids as an energy substrate. OPS II will provide a unique opportunity to explore these pathways in healthy individuals exposed to severe metabolic stress.

To assess these pathways we will employ global gene array analysis using Illumina next-generation sequencing (Illumina Inc., San Diego, CA, USA). Total RNA will be isolated from muscle using the Trizol/ethonal precipitation. Quantity and quality of RNA will be assessed using a Nanodrop ND-1000spectrophotometer (Nanodrop, Wilmington, DE, USA). 500 ng total RNA will be used to construct sequencing libraries. Samples will be amplified using index-tagged primers to facilitate multiplexing. Image analysis and base calling will be performed using the Illumina pipeline. Genes will be defined as differentially expressed when >±1.5-fold compared with baseline values and a value of $P < 0.05$.

Following RNA-Seq, identified target pathways will be further assessed using Western blot. Briefly, muscle will be homogenized in ice-cold buffer (1:10 wt/vol) and centrifuged for 15 min at 10,000 × g at 4°C. Protein concentration of supernatant (lysate) will be determined. Muscle lysates will be solubilized in Laemmli buffer, with equal amounts of total protein (15 µg) separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad). Proteins will be transferred to polyvinylidene fluoride (PVDF) membranes and exposed to commercially available primary antibodies at 4°C overnight. Labeling will be performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent will be applied

(Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots will be quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). To confirm equal protein loading per well a normalizing protein will be assessed.

Finally, microRNA regulating identified pathways by RNA-Seq will also be assessed as a potential mechanism contributing to testosterone and energy deficit induced alterations in muscle mass. Equal amounts of total RNA will be synthesized into cDNA for analysis using a TaqMan® microRNA RT kit (Applied Biosystems). Individual probes or microarrays will be used to assess changes in microRNA expression.

Statistics

All analyses will be considered 2-tailed, with $\alpha = 0.05$ considered statistically significant. All primary analyses will be based on the intention-to-treat principle using SAS (SAS Institute version 9.4, Cary, NC, USA), unless otherwise noted. Between group comparisons for all baseline, pre-study variables and P1, P2, and P3 dietary intake, total daily energy expenditure, exercise-induced energy expenditure, total sleep, % and absolute energy deficit will be assessed using two sample Student's t-tests. Primary analysis of muscle function and performance, body composition, muscle and whole-body anabolism, endocrine, metabolic, and safety biomarkers will be performed using a mixed effect linear model. Treatment (TU and PLA), phase (P1, P2, and P3), phase-by-treatment interaction, age, and pre-study values (only for body composition and clinical parameters) will be considered fixed effects covariates in the model. The random effect will include an unstructured covariance matrix to account for the correlation within-participants over time. Least squares means from the model will be used to estimate interaction effects. Familywise error rate will be adjusted using the Bonferroni correction when appropriate.

Data and Specimen Banking

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 onsite PI (Dr. Rood of PBRC) and study navigator (Ms. Harris). 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, including the USARIEM PI (Dr. Pasiakos) 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 onsite PI (Dr. Rood of PBRC) and study navigator (Ms. Harris). Only personnel assigned to the research study by Dr. Rood will have access to the data. Hard-copy data records will be stored for a minimum of 5 years and a maximum of 10 years from the time the study is completed and then destroyed.

The PBRC has a fully integrated, campus-wide, 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, blood, and urine samples will be shipped to the University of Arkansas for Medical Sciences (Dr. Ferrando) for isotopic analyses of muscle and whole-body anabolism. Specific muscle samples will also be shipped to USARIEM (Dr. Pasiakos) for molecular analyses of endocrine-medicated anabolic signaling. Packaging and shipping of biological samples will be overseen by the onsite PI (Dr. Rood) or study navigator (Ms. Harris), 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, or urine samples remaining after analysis will be stored indefinitely to assess biomarkers associated with the study outcomes, which includes any biomarkers of endocrine, metabolic, or safety 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 detailed **Statistics** section described above.

Power analysis.
See in **Number of Subjects** section listed under inclusion and exclusion section for detailed statistical power analyses.

Data and Safety Monitoring Board
This study will use a data and safety monitoring board (DSMB) and Safety Officer. 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. Detailed descriptions of the DSMB and Safety Officer are provided below:

- Size and Composition of DSMB: The DSMB will consist of 4 members both internal and external to PBRC. The planned composition is as follows: Biostatistician (1), Exercise Physiologist (1), Clinician (1), and Layperson (1).
- Major Responsibilities of DSMB 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 onsite PI (Dr. Rood), with a copy provided to the PBRC Institutional Review Board (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 navigator 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 onsite PI (Dr. Rood).

Adverse Events
Serious adverse events (SAE) 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 OPS II 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 exercise.
- An event that occurs as a result of a study procedure which is not listed in the Risks section of the informed consent.

Adverse events will be reported to the onsite PI (Dr. Rood), project manager (Ms. Harris), Chair of the PBRC IRB, Chair of the study DSMB, and Safety Officer throughout the trial as necessary. Adverse event data will be collected from Baseline (day 1) 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 that 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 Safety Officer will complete the form Notification of an Adverse Event.

Adverse Event reporting will follow the requirements of the PBRC IRB. Serious adverse events that are unexpected and related to the study will be reported within 48-h. Other adverse events that are not serious but are unexpected and are associated with the study procedures will be reported within 10-d.

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 Officer, in conjunction with the study investigators, will alert the IRB and DSMB if a larger than reasonably expected injury rate occurs in either of 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 PIs (Drs. Rood and Pasiakos), Safety Officer, and the USARIEM Project Officer. Although an excessive number of dropouts could occur, this has not been our past experience, particularly with OPS I. In OPS I, only 3 participants withdrew and did so prior to randomization. There were no dropouts once the study began – i.e., retention was 100%. However, this study will expose participants to greater overall stress and we expect that dropout rates could reach 20%. If that occurs, the Safety Officer will initiate a meeting with the PIs (Drs. Rood and Pasiakos) to discuss strategies to increase retention. If the dropout rate exceeds 50%, the safety officer will meet with the PIs 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 50 (REC). 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 intercurrent illness, adverse experience, treatment failure, protocol violation, or other reasons. Should a participant decide to withdraw, 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 study. The study procedures include:

- 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) and Bio-electrical Impedance (BIA). The amount of radiation used for DXA 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 3 scans over the course of OPS II is 500 times below the limit for radiation exposure per year. The OPS II protocol will follow Pennington SOP 1121 related to radiation dose limits. There are no risks associated with BIA.
- Archive of Biological (Blood, Muscle, and Urine) Samples. The primary risk to participants regarding archiving biological samples 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 subject ID. PBRC, USARIEM, UAMS, and other study collaborators will store participant samples with unique IDs and a minimum number of personal identifiers to meet laboratory standards. Storage and disposal of samples 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.
- 24-h Urine Collection. There are no known risks of collecting urine into a container.
- 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, pain at the injection site, leg edema, weight gain, aggressiveness, oil embolism, and emotional lability. Emotional lability is when an individual experiences rapid or exaggerated changes in mood.
- 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.
- 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, 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. 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 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 energy than participants are accustomed to and increasing energy 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 onsite PI (Dr. Rood) and MI (Dr. Greenway) will assess if continuation in the trial is warranted. An on-call physician is also available after hours and on weekends.

- Stable isotope tracer studies. The amino acid isotope administered intravenously and orally are stable, and thus are not radioactive. The isotopes used in this study are considered safe.
- Accelerometry. There are no risks associated with measuring activity and sleep with accelerometers. Accelerometers fit comfortably on the participant's arm and at the waist and can be easily removed should they become unconformable.
- Sleep deprivation. The possible risks of sleep deprivation include fatigue, irritability, bad mood, and slowing of mental performance.
- Electrical Stimulation. The purpose of the electrical stimulator is to provide an aversive stimulus to the participant. The experience of pain caused by the stimulation could be mentally distressing. We will minimize this risk by carefully explaining the electrical stimulus to the participant. To insure that the level of discomfort experienced during the test is not excessive, we will expose the participant to incrementally increasing levels of electrical stimulation to identify the level of stimulation required to induce moderate but not extreme discomfort. Similar electrical stimulation procedures are commonly used in the human research literature with no reports of injury. [72-75] Some subjects may experience irritation of the skin from the conductance gel used in the sensors. This clears up quickly once the sensors and the gel are removed.
- Muscle function and performance testing. The possible risks of the muscle function and performance testing include soreness, joint injuries, muscle strains, blisters, lower-back and shoulder pain. Risks will be mitigated by allowing familiarization and ensuring proper technique with constant verbal feedback by trained exercise physiologists.
- Total Body Water: The extra neutron in the water is not radioactive and has no risk.

Potential Benefits to Subjects
No direct benefits to the participants are expected.

Vulnerable Populations
This research does not include vulnerable populations.

Sharing of Results with Participants
Participants will be provided a summary results sheet at the completion of their participation in the study. The summary results will include body composition and muscle function/performance tests, and available lab work results.

Resource Availability and Setting
Clinical Facility, Equipment and Personnel

A detailed description of the clinical research facilities at PBRC can be found in the PBRC Clinical Resources Facility description that is maintained by the clinic administrator of the CTU. This document is updated and approved annually.

Exercise Facility & Campus
Pennington Biomedical is a 234 acre campus that is dedicated to research and education in nutrition and preventive medicine. The PBRC site has a 2,300 square foot

Exercise Training Facility, which is under the management of the Interventional Resources Department. Melissa Harris is the Director of this core. The exercise facility includes a pool, tennis court, and lighted jogging path around a 17 acre lake. Additionally, the campus has a 5 acre plot on the south side where a modified military training grounds will be established. The exercise facility offers state-of the-art equipment, professional intervention technicians, and optional training data capturing capabilities. The aerobic training room contains treadmills, stationary bikes, and elliptical machines. The resistance training room contains free weights and 15 different resistance-training machines to work a variety of muscle groups and movements. 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 exercise facility 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 exercise interventions, basic life support and an automated external defibrillator and may have ACSM or ACE personal trainer certifications. Exercise related supplies and equipment will be provided by the study. These include but are not limited to: water bottles, towels, first aid and injury prevention supplies, musculoskeletal support supplies, rain gear, tennis shoes, and other exercise supplies and equipment to implement the military activities of the intervention protocol.

Prior Approvals
Before initiation of the study protocol, radiation safety will be assessed. The research proposed herein is funded by the US Army Medical Research and Materiel Command and, therefore, will require approval from the US Army Human Protections research Office (HRPO) subsequent to the PBRC IRB approval. However, the study will not require a full IRB review by the US Army (see the signed Institutional Agreement for Intuitional Review Board Review (IAIR)).

Compensation
We will provide $7,500 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 study phases. Participants will receive $500 after the completion of screening and P1. They will also receive $5000 for completion of P2. If the participant does not complete the entire P2 live-in phase, the compensation will be prorated based on the number of days completed. Lastly, participants will receive remaining $2000 for completion of P3. Total compensation will be up to $7500.

Provisions to Protect the Privacy Interests of Subjects
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

onsite PI (Dr. Rood). 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 onsite PI (Dr. Rood) 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.

Volunteer Registry Database

It is the policy of the US Army Medical Research and Development Command that data sheets (60-R forms) are to be completed on all enrolled volunteers participating in research for entry into this Command's Volunteer Registry Database. The information entered into this confidential database includes your name, address, Social Security Number, study name, and dates. The intent of the database is two-fold: first, to readily answer questions concerning an individual's participation on research sponsored or funded by the USAMRDC; and secondly, to ensure that the USAMRDC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information with the stored at the USAMRCC for a minimum of 75 years.

60-R Form Completion: Enrolled participants will complete Part A and Part B during Phase 1 of participation. Part C will be completed at the end of study participation. Completed forms will be submitted to USAMRDC by study staff.

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.

Economic Burden to Participants

Participants in this study will not incur any personal costs.

Consent Process

Refer to **Study Timeline** for description of the informed consent process.

Drugs or Devices

Study drug will be stored, dispensed and handled through the clinical trials unit pharmacy and pharmacist according to all pharmacy SOPs and the study protocol. Compliance and dispensing records will be kept in the pharmacy and individual charts. Nursing staff on the inpatient unit will administer the study drug per protocol.

Roles and Responsibilities

Onsite Principal Investigator (PBRC)

The PBRC, onsite 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 event. 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 PBRC onsite 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, and share (with Dr. Pasiakos) primary responsibility for data analysis and assist with data interpretation and publication. Dr. Rood will maintain institutional coordination between USARIEM, PBRC, and UAMS investigators and the PBRC IRB and US Army Medical Research and Materiel Command HRPO.

Offsite Principal Investigator (USARIEM)

Stefan M. Pasiakos, Ph.D.: Dr. Pasiakos will be the USARIEM offsite PI of record and share responsibility for the safe and scientifically sound conduct of the study. He 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, and share (with Dr. Rood) primary responsibility for data analysis and assist with data interpretation and publication. Dr. Pasiakos will maintain institutional coordination between USARIEM, PBRC, and UAMS investigators and the PBRC IRB and US Army Medical Research and Materiel Command HRPO.

Sub-Investigators

Drs. Varanoske and Margolis (USARIEM), and Dr. Ferrando (UAMS) will be engaged in this research trial. They will assist Drs. Rood and Pasiakos with protocol concept development, execution, data collection (i.e., Varanoske, Margolis, and Ferrando: tracer studies, muscle function and performance assessments, operational stress intervention), analysis, interpretation, and publication.

Consultant

Dr. Lieberman (USARIEM) will consult on the cognitive testing concept development, execution, and data collection.

Appendices

A. Train schedule
B. AR 600-9 (Army Body Composition Program)
C. Semi-structured Barriers interview

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