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Effects of testosterone undecanoate on performance during multi-stressor military operations: A trial protocol for the Optimizing Performance for Soldiers II study

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ABSTRACT

Background: Previously, young males administered 200 mg/week of testosterone enanthate during 28 days of energy deficit (EDef) gained lean mass and lost less total mass than controls (Optimizing Performance for Soldiers I study, OPS I). Despite that benefit, physical performance deteriorated similarly in both groups. However, some experimental limitations may have precluded detection of performance benefits, as performance measures employed lacked military relevance, and the EDef employed did not elicit the magnitude of stress typically experienced by Soldiers conducting operations. Additionally, the testosterone administered required weekly injections, elicited supra-physiological concentrations, and marked suppression of endogenous testosterone upon cessation. Therefore, this follow-on study will address those limitations and examine testosterone's efficacy for preserving Solder performance during strenuous operations.

Methods: In OPS II, 32 males will participate in a randomized, placebo-controlled, double-blind trial. After baseline testing, participants will be administered either testosterone undecanoate (750 mg) or placebo before completing four consecutive, 5-day cycles simulating a multi-stressor, sustained military operation (SUSOPS). SUSOPS will consist of two low-stress days (1000 kcal/day exercise-induced EDef; 8 h/night sleep), followed by three high-stress days (3000 kcal/day and 4 h/night). A 23-day recovery period will follow SUSOPS. Military relevant physical performance is the primary outcome. Secondary outcomes include 4-comparment body composition, muscle and whole-body protein turnover, intramuscular mechanisms, biochemistries, and cognitive function/mood.

Conclusions: OPS II will determine if testosterone undecanoate safely enhances performance, while attenuating muscle and total mass loss, without impairing cognitive function, during and in recovery from SUSOPS.

Trial Registration: ClinicalTrials.gov Identifier: NCT04120363.

Abbreviations: BIA, bioelectrical impedance analysis; D_2O, deuterium; DXA, dual-energy x-ray absorptiometry; DSMB, data and safety monitoring board; ECW, extracellular water; EDef, energy deficit; EIEE, exercise-induced energy expenditure; FBR, fractional breakdown rate; FFM, fat-free mass; FSR, fractional synthetic rate; HR, heart rate; HRR, heart rate reserve; ICW, intracellular water; ID, identification; IRB, Institutional Review Board; MRE, Meal; Ready-to-Eat, OPS I; Optimizing Performance for Soldiers Trial I, OPS II; Optimizing Performance for Soldiers Trial II, PAR-Q+; Physical Activity Readiness Questionnaire+, PB; protein breakdown, PBRC; Pennington Biomedical Research Center, PLA; placebo experimental group, PS; protein synthesis, Q; whole-body nitrogen flux, RER; respiratory exchange ratio, RM; repetition maximum, RNA; ribonucleic acid, RPE; ratings of perceived exertion, SUSOPS; sustained, multi-stressor military operations; TBW, total body water; TDEE, total daily energy expenditure; TDEI, total daily energy intake; TEST, testosterone experimental group; VO_{2max}, maximal cardiorespiratory fitness; VO_{2peak}, peak oxygen uptake; WBGT, wet bulb globe temperature.

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1. Introduction

The collective stress of high physical activity, sleep deprivation, and energy deficit (EDef) (i.e., failing to match total daily dietary energy intake [TDEI] with total daily energy expenditure [TDEE]) during strenuous military training and operations can impair physical performance [1–4]. Performance decrements may be attributable, in part, to the suppressive effects of stress on the hypothalamic pituitary gonadal axis and endogenous testosterone synthesis [5,6]. Pharmacologic restoration of eugonadal testosterone concentrations may be an effective strategy to attenuate physiological decline and degraded performance during sustained, multi-stressor military operations (SUSOPS).

Recently, the Optimizing Performance for Soldiers (OPS) I study, a multi-institutional collaborative project, evaluated the physiological efficacy of supplemental testosterone during EDef [7–11]. OPS I was a 3-phase, randomized, double-blind, placebo-controlled trial involving 50 physically-active young males. Phase 1 was a 14-day, free-living, weight-maintaining diet phase. Phase 2 was a 28-day live-in, 55% exercise- and diet-induced EDef with (intervention group) or without (control group) exogenous testosterone (200 mg/week testosterone enanthate). Phase 3 was a 14-day recovery period [7]. The primary findings from OPS I were that participants receiving weekly intramuscular injections of testosterone gained lean mass and lost less total mass compared to controls, without an increased incidence of adverse events or negative cardiometabolic health biomarkers [8]. The change in total testosterone concentrations during the EDef was strongly correlated with changes in lean mass. Additionally, those given testosterone fully recovered their body mass and were 2.8 kg heavier at the end of the recovery period than controls, due entirely to gains in lean mass. However, despite differences in lean mass loss, lower-body muscular strength and endurance declined similarly between groups [8].

Several experimental limitations in OPS I preclude definitive conclusions to suggest testosterone therapy for military personnel conducting strenuous SUOPS. First, the testosterone formulation required weekly injections, which are not practical for military operations conducted in austere locations. Further, the dose administered (200 mg of testosterone enanthate) produced testosterone concentrations exceeding the normal physiological range, followed by a precipitous decline upon cessation and prolonged hypogonadal state in recovery. Additionally, the inability to induce a hypogonadal state and lean mass loss in control participants suggests the magnitude and type of stress imposed by the EDef in OPS I was far less than the physiological stress typically endured during real-world training and combat operations (high physical activity, sleep deprivation, and EDef). Most importantly, the use of isometric and isokinetic dynamometry to measure performance were insufficiently sensitive to demonstrate militarily relevant benefits of testosterone on physical performance, despite gains in lean mass [7,8].

There are several alternatives to testosterone enanthate, including transdermal/nasal gels, patches, and buccal/bio-adhesive tablets. However, while these formulations are non-invasive and can be self-administered, they require daily applications (transdermal and nasal gels) and carry added risks, including skin-to-skin transfer (transdermal gels) and gum/nasal-related adverse events (buccal/bio-adhesive tablets, nasal gels) [12]. Long-acting formulations that require infrequent administration by clinicians may be a viable alternative to testosterone enanthate. A single intramuscular injection of testosterone undecanoate can maintain testosterone concentrations within the normal physiological range for 8–10 weeks [13] with considerably less clinical or logistical burden. Further, following cessation of testosterone undecanoate administration, testosterone concentrations decline gradually, which reduces the likelihood of becoming hypogonadal. This dosing regimen may be logistically feasible for military personnel vulnerable to muscle loss and performance decline while conducting strenuous operations as part of their annual training and deployment cycle. Thus, the OPS II study will address these limitations and test the hypothesis that a single dose of long-acting testosterone undecanoate (750 mg) will safely and steadily maintain normal testosterone concentrations and enhance military relevant measures of physical performance during, and in recovery from, a simulated, multi-stressor SUSOPS.

2. Materials and methods

2.1. Study design and setting

This 3-phase, interventional study will use a parallel, randomized, placebo-controlled, double-blind design to test whether testosterone undecanoate administration is effective at preventing the decline in physical and physiological outcomes typically experienced during multi-stressor SUSOPS. All participant testing will occur at a single site (Pennington Biomedical Research Center; PBRC) in Baton Rouge, LA. The Institutional Review Board (IRB) of PBRC (protocol 2019–017) and the US Army Human Research Protections Office (Fort Detrick, Fredericksburg, MD, USA) approved the study protocol and trial documents, including the consent form. The study protocol follows the recommendations of the Standard Protocol Items: Recommendations for Interventional Trials guidance for clinical trials [14,15]. The ClinicalTrials.gov identifier is NCT04120363.

2.2. Eligibility criteria and determination

Participants will be eligible for the trial if they meet the following criteria: male, aged 18–35 years old, healthy and physically active, meets age-specific U.S. Army body composition standards according to Army Regulation 600–9 [16], and has normal testosterone concentrations (300–1000 ng/dL). The eligibility criteria was designed to recruit volunteers with characteristics that most closely reflect the characteristics of actual Soldiers. Previous published papers from our laboratory, including the OPS I study (which provided the rationale for the current study), have used similar exclusion criteria; the descriptive characteristics of participants recruited for the OPS I study [8] were comparable to those reported in our previous published studies in Soldiers [17–19]. While recruiting actual Soldiers with previous military experience would increase the practical applicability of the current intervention, the time commitment and requirements of this study (e.g., a ~2-month leave period under highly-controlled laboratory conditions) would make recruiting active-duty Soldiers highly unlikely and unrealistic. Thus, the findings of this highly-controlled laboratory study in recreationally-active males will help provide preliminary information on whether a similar intervention should be evaluated in future field studies in Soldiers. A detailed list of inclusion and exclusion criteria is presented in Table 1.

2.3. Recruitment

This study will use a multi-stage screening process to recruit participants. Potential participants will undergo a web and/or telephone screen to determine eligibility based on inclusion/exclusion criteria. Individuals who meet the criteria will be invited to attend the first of two screening visits. During the first visit, the study consent form will be reviewed and signed, and height, body mass, blood pressure, and heart rate (HR) will be measured. Participants who do not meet the height/body mass criteria will have eligibility assessed by neck and waist circumference measurements to estimate percent body fat according to Army Regulation 600–9 [16]. Participants also will complete a Physical Activity Readiness Questionnaire (PAR-Q+) [20], a medical history questionnaire, and a cardiovascular disease risk assessment. Dietitians will meet with potential participants to discuss eating habits and dietary requirements for the study. Participants who maintain eligibility will be provided with an accelerometer (Actigraph wGT3X-BT, Pensacola, FL, USA) and will be scheduled for a second screening visit one week later. The accelerometer will be worn daily to assess physical activity, and participants will be required to fill out a daily physical activity log. After

Table 1
Study inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
• Males aged 18–35 years • Ability to understand verbal or written instructions/testing materials in English • Physically active (expends, on average, at least 300 kcal/day through structured aerobic and strength-training activities, 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 is $\geq$ 1–4 weeks •Wash-out period for caffeine and alcohol is $\geq$ 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 [16], which includes estimates of percent body fat based on height, body mass, and circumference measures (neck and waist) • Total testosterone concentration is within the normal physiological range for males (300–1000 ng/dL)	• Musculoskeletal injuries that compromise exercise capability • Diagnosed cardiometabolic disorders (i.e., hypertension, hyperlipidemia, kidney disease, diabetes, etc.) • Allergies or intolerance to foods or vegetarian practices • 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 • Females • 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 [16] • Previous history of kidney stones unless otherwise approved by the medical investigator • Systolic blood pressure $>$ 150 mmHg 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 $>$ 3 ng/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

review and approval of the accelerometer and physical activity log during the second visit, participants will undergo a physical exam, electrocardiogram, fasting blood and urine collection, and resting metabolic rate assessment. Blood will be analyzed for complete blood cell count, a Chem 26 panel, prostate specific antigen, and free and total testosterone concentrations. Urine will be analyzed for a urinalysis and drug screen. A psychological and behavioral barriers interview and Three Factor Eating Questionnaire will also be administered to exclude individuals with impediments to study completion and restrained eaters, respectively. Participants will then undergo a 1.5-mile mock march while carrying a loaded backpack to mimic the physical demands of the study. Participants who maintain eligibility criteria will continue to wear the accelerometer, record their physical activity, and complete a 3-day food record (2 weekdays, 1 weekend day) for one week prior to the start of the study.

2.4. Intervention

2.4.1. Study overview

Study participants will undergo a 3-phase, 50-day study, consisting of 7 days of baseline testing and diet acclimation (Phase 1, days 1–7), 20 days of simulated SUSOPS (Phase 2, days 8–27), and 23 days of recovery (Phase 3, days 28–50) (Fig. 1). On day 8, after completing baseline testing and diet acclimation (Phase 1), participants will be randomized to receive either a single intramuscular injection of testosterone undecanoate (TEST; 750 mg, standard pharmaceutical dose [12]) or an iso-volumetric placebo (PLA, sesame oil solution). The 20-day SUSOPS (Phase 2) will be highly controlled (live in the inpatient unit at PBRC), and consist of four consecutive cycles of undulating stress, starting with 2 days of 'low' stress, followed by 3 days of 'high' stress. After completing Phase 2, 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 SUSOPS (Phase 3).

2.4.2. Dietary intake determination

Participant physical activity patterns and exercise-induced energy expenditure (EIEE) will be assessed from accelerometer and PAR-Q+ data. Resting metabolic rate will be measured by indirect calorimetry using a Deltatrac II Metabolic Cart (Sensormedics, Yorba Linda, CA, USA) with a ventilated hood. The 3-day food records and resting metabolic rate measurements will be used to calculate individual TDEI.

Throughout all phases, participants will consume the same amount of total energy, and the macronutrient distribution will be fixed (approximately 15%, 55%, and 30% total energy from protein, carbohydrate, and fat, respectively). The macronutrient distribution of these diets is based on the composition of the Meal, Ready-to-Eat (MRE), a US combat ration and the primary food source for Phase 2. For Phases 1 and 3, dietitians will develop individualized menus consisting of breakfast, lunch, dinner, snacks, and energy-containing beverages. During these phases, breakfast meals will be consumed at PBRC under supervision and all other meals and energy-containing beverages will be provided for consumption offsite. Dietary compliance will be verified daily by assessing foods/beverages remaining in returned coolers and using questionnaires that allow participants to list any deviations from the diet. The energy content of the Phase 1 diet will be sufficient to maintain body mass within $\pm$2%. Participants will be weighed daily in each phase. TDEI will be adjusted incrementally ($\pm$200 kcal every 3 days) in Phase 1 as needed to achieve energy balance. Participants will be instructed to maintain pre-study activity levels and EIEE during Phases 1 and 3. Activity will be verified using a wrist-worn accelerometer and physical activity records.

During Phase 2, food will consist of approximately two MREs per day (menu 39; Ameriqual, Evansville, IN, USA) with the amount of energy and distribution of macronutrients the same as during Phase 1. Registered dietitians will develop individualized MRE-based menus. All meals will be eaten and monitored on the inpatient unit in Phase 2. Calorie-free seasonings, including hot sauce, will be allowed. A sample daily menu for Phases 1, 2, and 3 is provided in Table 2.

Water will be consumed *ad libitum* during all phases, but daily fluid intake will be recorded during Phase 2 to ensure proper hydration. Participants will also be allowed three energy-free approved beverages (i.e., energy-free sports drinks or sodas) per day during Phase 2, in addition to those provided as part of the diet. Hydration status will be tracked daily for safety via visual inspection of urine color. Participants will be weighed and wear a wrist-worn accelerometer daily throughout Phase 2.

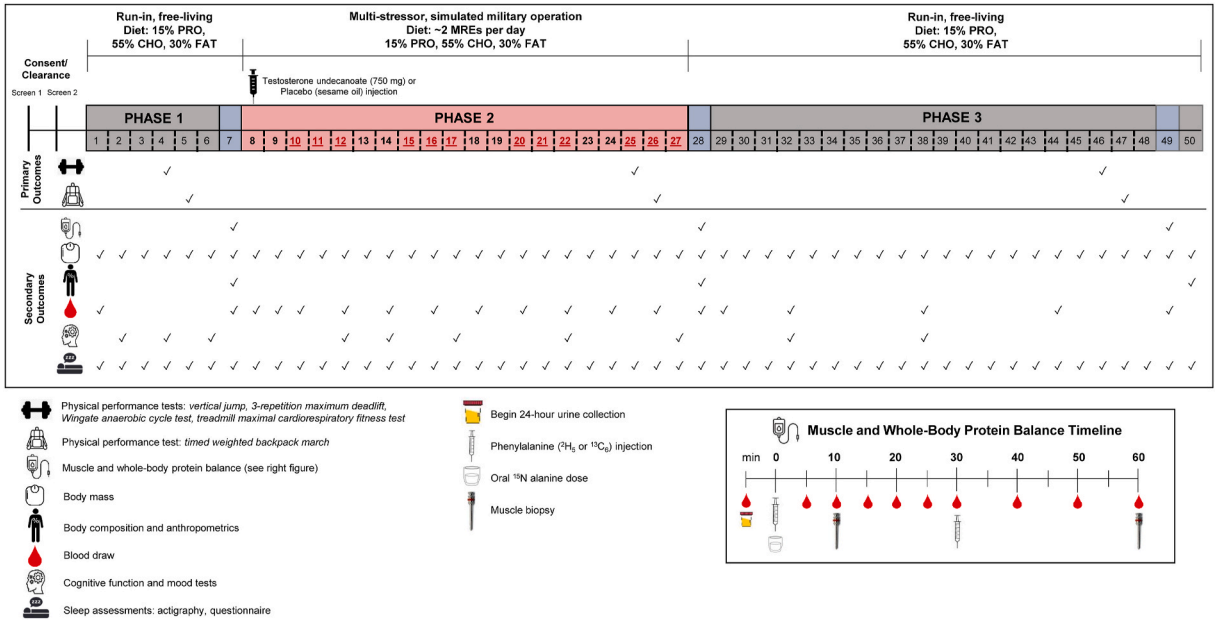

Fig. 1. Study design. Phases 1 (days 1–7) and 3 (days 28–50) are run-in and free-living with a standardized diet with energy derived from 15% protein (PRO), 55% carbohydrates (CHO), and 30% fat (FAT). Phase 2 (days 8–27) is a highly controlled, multi-stressor military operation (SUSOPS), consisting of four consecutive cycles of undulating stress, starting with 2 days of 'low' stress (1000 kcal/day exercise-induced energy deficit [EDef]; 8 h/night sleep; denoted by bolded number), followed by 3 days of 'high' stress (3000 kcal/day exercise-induced EDef, 4 h/night sleep; denoted by red bolded and underlined number). Participants will be randomized to receive either a single intramuscular injection of testosterone undecanoate (750 mg) or an iso-volumetric placebo (sesame oil solution) on day 8. Participants will consume the same total calories and macronutrient distribution in Phase 2, but food will be derived from the Meal, Ready-to-Eat, a US combat ration ([MRE] menu 39; Ameriqual, Evansville, IN, USA). Physical performance outcomes are the primary study outcomes, which will be measured in each phase of the study. Secondary outcomes include body composition, whole-body and skeletal muscle homeostasis, endocrine-, metabolic-, and safety-related biomarkers, and cognitive function/mood. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.4.3. SUSOPS

The 20-day SUSOPS (Phase 2) will consist of four consecutive cycles of undulating stress, starting with 2 days of 'low' stress followed by 3 days of 'high' stress. Low and high stress days will entail low and high militarily relevant EIEE, adequate and restricted sleep (8 h/day vs. 4 h/day). Since TDEI remains the same as during Phase 1, the increase in EIEE will produce EDef of approximately 1000 kcal/day and 3000 kcal/day, respectively (Table 3). This level of EDef was selected based on a recent meta-regression of field studies conducted in military environments, indicating that a total EDef of ~43,380 kcal over 20 days of will result in meaningful reductions in body mass and physical performance [2].

Varied low-, moderate-, and intermittent high-intensity endurance and muscle loading-type exercise will be performed to simulate physical activity typically observed during strenuous, SUSOPS [21], and increase participants' EIEE to approximately 1000 kcal/day and 3000 kcal/day above their Phase 1 EIEE. This will generate an energy expenditure of approximately 3700 kcal/day on low days and 5700 kcal/day on high days (based on an estimated mean Phase 1 TDEE of 2700 kcal/day; exact values will differ based on actual Phase 1 TDEE). The increase in TDEE from physical activity will be achieved by performing multiple exercise sessions daily, each session lasting 1–5 h, using a variety of endurance and muscle loading modalities that mimic movements and activities common during military operations. Steady-state load carriage (i.e., marching with a weighted backpack) endurance-type exercise will be the primary exercise modality (~30% of total body mass carried) and will account for approximately 50% of daily EIEE prescription. Other activities will include walking and hiking without a weighted backpack, jogging, running, cycling, elliptical, field-based operational activities, calisthenics, stretching, and yoga which will completed in the laboratory using exercise equipment and in modified military training grounds on the PBRC campus. The operational activities will include Army Physical Readiness-type training such as load carries, ladder runs, jump roping, battle ropes, tire flips/pulls, sled drags/pulls, sandpit digging, and others. EIEE will be determined for each participant and activity using the Compendium of Physical Activities [22] or the participant's body mass and exercise duration/distance/intensity according to published equations [23,24]. Individualized participant spreadsheets embedded with formulas to meet target energy expenditures per will be created to accurately document EIEE. The exercise intensity and 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 maintaining the prescribed low and high EDef. HR (Zephyr Bioharness™, Zephyr Technology Corporation, Annapolis, MD, USA) will be tracked and recorded throughout all exercise sessions to evaluate exercise intensity (percent HR reserve [HRR]) based on the resting HR from the screening visit and the maximum HR obtained via the maximal cardiorespiratory fitness test in Phase 1. While there is not a specified HR range for exercises, most exercises will likely elicit HR within 40–90% of HRR.

Outdoor exercise sessions will be reserved for earlier and later sessions in the day (weather permitting) to avoid extreme weather conditions. The wet bulb globe temperature (WBGT) index, which combines ambient temperature, sunlight, humidity, and wind speed, will be used to ensure participant safety (Extech HT30 Heat Stress WBGT Meter, Flir Systems Inc., Nashua, NH, USA). Participants will not be allowed to exercise outdoors if WBGT readings are above 32.2 °C (89.9 °F). Staff will restrict outdoor exercise time based on the WBGT during that session and will suggest adequate fluid consumption for corresponding WGBT ranges. If weather does not permit scheduled outdoor activities, most exercises can be completed indoors.

Table 2
Sample daily menu items and macronutrient breakdown in each phase of the study.

Phases 1 and 3						Phase 2					
Food Item	Mass (g)	PRO (g)	CHO (g)	FAT (g)	Kcal	MRE Food Item	Mass (g)	PRO (g)	CHO (g)	FAT (g)	Kcal
Breakfast						**Breakfast**					
Corn grits, Quaker, white, instant, dry	93	6.6	73.0	0.9	332	Snack bread, multigrain	34.4	3.3	21.3	1.3	111
Butter, salted	24	0.2	0.0	19.2	172	Peanut butter, smooth	34	7.8	8.8	16.6	216
Canadian bacon, Sysco	27	5.3	0.0	1.4	34	Jam, strawberry	42	0.1	28.6	0.0	115
Egg whites, Wholesome Farms, raw	55	6.0	0.0	0.0	30	Tortilla, plain	48	3.4	29.0	4.0	162
Orange juice, concentrate, unsweetened	316	2.1	34.1	0.2	142	*Breakfast total*		14.7	87.6	22.0	604
Milk, skim	165	5.6	8.2	0.1	56	**Morning snack**					
Breakfast total		25.8	115.3	22.1	766	Filled crackers, cheese and pepperoni	45	3.8	30.1	8.7	214
Lunch						Carbohydrate-fortified beverage	29	0.0	28.1	0.1	113
Pita bread, Sam's Choice, whole wheat	93	8.7	37.8	2.9	203	*Morning snack total*		3.8	58.2	8.8	327
Turkey breast, Block & Barrel, sliced	70	12.5	1.3	1.3	63	**Lunch**					
Lettuce, romaine, raw	28	0.3	0.9	0.1	5	Chili with beans	310	25.1	37.2	13.6	366
Tomatoes, raw	25	0.2	1.0	0.1	5	Cornbread	74	3.5	41.5	10.7	272
Mayonnaise, Sysco, regular	22.3	0.0	0.0	18.0	162	*Lunch total*		28.5	78.7	24.4	637
Cheese, Swiss	29	7.8	1.6	8.1	110	**Dinner**					
Pretzels, Rold Gold	53	4.7	42.3	2.3	206	Beef strips in savory tomato-based sauce	326	46.6	22.8	15.3	415
Lunch total		34.3	84.7	32.7	753	Potatoes, mashed, garlic	203	2.3	24.2	5.5	157
Snack						Dried fruit, cranberries	36	0.1	23.2	0.2	95
Raisins, seedless	38	1.2	30.1	0.2	114	Cheese spread, cheddar, plain	22	2.7	1.7	9.1	89
Crackers, Cheez-It, regular	26	2.6	15.1	6.6	131	Peppermint candy rings	15	0.0	14.5	0.0	56
M & M candies, milk chocolate	11	0.5	7.8	2.0	51	*Dinner total*		51.8	86.3	30.2	812
Pretzels, Rold Gold	29	2.6	23.1	1.3	113	**Evening snack**					
Snack total		6.8	76.2	10.0	408	Dried fruit, cranberries	36	0.1	23.2	0.2	95
Dinner						BBQ corn nuggets, bar	23	1.5	15.8	4.6	109
Chicken breast, sage lemon	95	19.7	0.6	6.8	146	Carbohydrate-fortified beverage	29	0.0	28.1	0.1	113
Rice, Uncle Ben's, Long Grain & Wild Rice Garden blend	170	4.8	39.6	1.0	193	*Evening snack total*		1.7	67.2	4.9	317
Vegetable mix, Sysco	157	1.7	9.3	0.5	43						
Pears, canned in juice	176	0.6	22.8	0.1	88	***Day total***		100.4	378.1	90.2	2699
Dinner roll, Rich's, whole wheat	65	7.2	34.3	3.1	181			14.9%	55.0%	30.1%	
Butter, salted	17	0.1	0.0	13.8	122						
Dinner total		34.2	106.6	25.2	773						
Day total		101.2	382.7	90.1	2700						
		15.0%	55.0%	30.0%							

Food provided in Phases 1 and 3 consisted of individualized meal plans designed to maintain energy balance. Foods provided in Phase 2 consisted of individualized US combat rations (Meals, Ready-to-Eat, MRE; menu 39; Ameriqual, Evansville, IN, USA; approximately two MREs/day) to maintain the same total energy intake and macronutrient distribution as in Phases 1 and 3. The sample diet presented is for an individual needing to consume 2700 kcal/day to maintain energy balance in Phases 1 and 3. PRO: calories derived from protein; CHO: calories derived from carbohydrates; FAT: calories derived from fat.

3. Outcome measures

3.1. Primary outcomes

Military relevant measures of physical performance will serve as the primary study outcomes. A battery of performance tests, consisting of vertical jump, 3-repetition maximum (RM) deadlift, Wingate anaerobic cycle test, treadmill maximal cardiorespiratory fitness test (VO_{2max}), and timed weighted backpack march will be completed during each phase (days 4–5, 25–26; 46–47) (Table 4). Participants will be familiarized with the test battery in each phase prior to actual testing (days 1, 23, 44). The order and timing of the tests will be standardized (i.e., strength/power → anaerobic capacity → aerobic capacity). A snack will be provided at least an hour before performance testing (energy: 300 kcal; protein: 11.1 g; carbohydrates: 42.6 g; fat: 9.9 g). A warm-up will be completed before initiating testing, consisting of cycle ergometry for 5 min at a self-selected pace, 10 body-weight walking lunges, 10 dynamic walking hamstring stretches, 10 dynamic walking quadriceps stretches, 10 squat jumps, 10 arm circles, 10 arm swings, and 3 × 10 m jogs. A description of all physical performance tests is provided in Table 4.

3.2. Secondary outcomes

Secondary endpoints include body composition, whole-body and muscle protein turnover and their associated intracellular mechanisms, endocrine-, metabolic-, and safety-related biomarkers, as well as measures of cognitive function.

Anthropometrics and Body Composition. Height will be measured using a stadiometer (Harpenden Stadiometer, Holtain Company, UK) during screening visit 1 to the nearest 0.1 cm. Semi-nude body mass will be measured in duplicate after an overnight fast and morning void using a scale (GSE Inc. Model 450, GSE Scale Systems, Novi, MI, USA) during each screening visit and daily throughout the entire study to the nearest 0.1 kg. Body composition (fat, lean soft tissue, bone mineral, total body water [TBW], extracellular water [ECW], and intracellular water [ICW]), circumferences, and volume (3D optical scans, Fit3D, 3D Size Stream system Proscanner version 4.x [Fit3D, San Mateo, CA, USA], Size Stream SS20 [Size Stream, Cary, NC, USA]) will be measured after an overnight fast, proper hydration, and morning void once at the end of each phase (days 7, 28, and 49). Hydration status will be evaluated using an analysis of urine specific gravity (CLINITEK 500, Siemens Healthcare Diagnostics, Malvern, PA, USA) prior to body composition analyses.

Table 3
Sample schedule of low- and high-stress days during Phase 2 of the intervention.

	Low Day	High Day
0000	Sleep	Sleep
0100	Sleep	Sleep
0200	Sleep	Sleep
0300	Sleep	Sleep
0400	Snack, Rest	Snack, Rest
0500	Weighted Backpack March	Weighted Backpack March
0600	Weighted Backpack March	Weighted Backpack March
0700	Weighted Backpack March	Weighted Backpack March
0800	Breakfast, Rest	Breakfast, Rest
0900	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1000	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1100	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1200	Lunch, Rest	Lunch, Rest
1300	Operational Activities	Operational Activities
1400	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1500	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1600	Stretching, Yoga	Walk/Run/Elliptical/Bike/Weighted Backpack March
1700	Dinner, Rest	Dinner, Rest
1800	Walk/Run/Elliptical/Bike/Weighted Backpack March	Walk/Run/Elliptical/Bike/Weighted Backpack March
1900	Snack, Rest	Walk/Run/Elliptical/Bike/Weighted Backpack March
2000	Sleep	Walk/Run/Elliptical/Bike/Weighted Backpack March
2100	Sleep	Walk/Run/Elliptical/Bike/Weighted Backpack March
2200	Sleep	Walk/Run/Elliptical/Bike/Weighted Backpack March
2300	Sleep	Snack, Rest

Body composition will be established using a 4-compartment model calculated from dual-energy x-ray absorptiometry ([DXA] Hologic DXA, Discovery A, Hologic, Marlborough, MA, USA) and bioelectrical impedance analysis ([BIA] Impedimed SFB7, Carlsbad, CA, USA; InBody S10, Cerritos, CA, USA; Jawon Cozy 930, Seoul, Korea; or similar) [40]. Multi-compartment methods (2, 3, and 4-compartment) and BIA will be used to calculate total body fat, fat-free mass (FFM), protein, hydration (TBW/FFM), and fluid distribution (ECW/ICW). Appendicular lean soft tissue mass as measured by DXA will be used as a proxy for total body skeletal muscle mass [25].

TBW will also be measured by deuterium (D_2O; Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA, Sigma-Aldrich, St. Louis, MO, USA) dilution once in each phase (days 7, 28, 49). Participants will provide a pre-dose urine sample and will then be orally dosed with D_2O-labeled water (99.9% D_2O) at 0.15 g/kg body mass, then the dose cup rinsed with 50 mL of unlabeled tap water and ingested. A second urine sample will be collected 4 h post-dose. The enrichment of D_2O in urine at 4 h will be used to determine TBW, as previously described [26–28].

Muscle and Whole-Body Protein Turnover. Muscle and whole-body protein turnover (protein synthesis [PS], protein breakdown [PB], and net protein balance) will be measured following an overnight fast at the end of each phase (days 7, 28, 49) by using the minimally-invasive pulse bolus stable isotope tracer injection technique [13] and the end-product method [29]. One intravenous catheter will be placed into a forearm vein on each arm, which will be used for blood sampling and bolus stable isotope administration. A blood sample will be drawn to establish background amino acid enrichment before the tracer injections. At the start of the 60-min tracer study (0 min; Fig. 1), a bolus injection of $^{2}H_5$ phenylalanine (35 μmol/kg) will be administered [30]. At the same time, participants will consume a single oral dose of ^{15}N alanine (333 μmol/kg), and their urine will be collected for the next 24 h. A bolus injection of ^{13}C 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 40 min, except at the 25-min mark, and at 10-min intervals for the remaining 20 min (10 total blood samples over ~60 min). Two muscle biopsies of the vastus lateralis will be obtained under local anesthesia (2% lidocaine with 0.5% bupivacaine) with a 5 mm Bergstrom needle and manual suction [31,32]. Approximately 250 mg of muscle tissue will be collected during each biopsy from the same incision at the 10-min and 60-mintue mark. Muscle samples will be snap frozen in liquid nitrogen and stored at −80 °C. Blood samples will be centrifuged, and serum will be stored at −80 °C. Muscle, blood, and urine samples will be analyzed for isotope enrichments using gas and liquid chromatography mass spectrometry [13].

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

$$FSR = \frac{[E_B(t_2) - [E_B(t_1)]}{\int_2^3 E_M(t)dt}$$

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 [13].

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

$$FBR = \frac{[E_M(t_2) - [E_M(t_1)\Big] \times \Big[\int_2^3 E_A(t) - E_M(t)dt\Big] - [E_M(t_3) - E_M(t_2)\Big] \times \Big[\int_1^2 E_A(t) - E_M(t)dt\Big]}{\Big[\int_2^3 E_M(t)dt \times \int_1^2 E_A(t)dt\Big] - \Big[\int_1^2 E_M(t)dt \times \int_2^3 E_A(t)dt\Big]} \times \frac{Q_M}{T_M}$$

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 [13]. Net muscle protein balance 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. [33]. Whole-body PS and PB will be calculated according to Stein et al. [34]:

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

Table 4
Description of physical performance tests.

Test	Performance Metric(s)	Description of Test	Outcome Variables	Rest Time Following Test	Days Assessed
Vertical Jump	Lower-body power	Participants will stand with heels flat on the ground and dominant side closest to the Vertec (Jump USA, Sunnyvale, CA, USA). They will reach up as high as possible to determine vertical reach. A Tendo™ unit (Tendo Sports Machines, Trenchin, Slovak Republic) will be attached to the participant's waist during the assessment. The Tendo unit consists of a transducer that measures velocity defined as linear displacement over time. The participant will jump from a standing position by flexing both knees and hips rapidly to move downward, and then extend their knees and hips rapidly while swinging up their dominant arm to touch the highest possible vane on the Vertec. Participants will complete three jumps, allowing for 60–90 s rest between each jump.	Jump height, average power, partial average power, peak power, average velocity, peak velocity, peak force	5 min	Phase 1: 4 Phase 2: 25 Phase 3: 46
3-Repetition Maximum (RM) Deadlift	Lower- and upper-body muscular strength; lower-body muscular power	A trap bar deadlift will be performed in accordance with the US Army Combat Fitness Test [39]. A Tendo™ unit will be attached to one end of the bar to measure bar velocity and power. Participants will begin by completing three warm-up sets (8–10 repetitions at ~50% 1-RM, 1 min rest; 6–8 repetitions at ~65% of 1-RM, 2 min rest; 4–6 repetitions at ~75–80% of 1-RM, 3 min rest). The bar will then be loaded with ~85–90% of estimated 1-RM. Loads will be determined using a prediction equation based off of 5-RM deadlift completed during the familiarization session (5-RM/0.87 = 1-RM) [40]. Participants will stand in the middle of the trap bar with their feet about shoulder width apart. The participant will bend at the knees and hips, reach down and grasp the center of the handles. Arms should be positioned fully extended, back flat, head in a neutral position, head and eyes to the front or slightly upward, shins almost perpendicular to the ground, and heels in contact with the ground. The participant will stand up and lift the bar by extending the hips and knees until in an upright stance. They will pause slightly at the top of the movement, and the test administrator will signal to the participant that the concentric portion of the movement is complete. By flexing the hips and knees slowly, the participant will lower the bar to the ground, maintaining a neutral spine. The weight plates must touch the ground but may not bounce. If the participant fails to complete three continuous repetitions under control, they will retest at a lower weight. If they successfully complete three repetitions, additional weight may be added, and they will retest after at least 3 min of rest. A true 3-RM should be achieved after 3–5 attempts.	3-RM weight; for each repetition: average power, partial average power, peak power, average velocity, peak velocity, peak force	10 min	Phase 1: 4 Phase 2: 25 Phase 3: 46
Wingate Anaerobic Cycle Test	Anaerobic capacity	Participants will be positioned on an electronically-braked cycle ergometer (Excalibur Sport, Lode, The Netherlands). Seat height will be adjusted so that the knee is almost in full extension (approximately 5–10° of knee flexion) when the pedal is positioned at the lowest point. Participants will grasp the handlebars and remain seated for the entire test. They will begin by pedaling for 5 min at 50 W, maintaining a cadence between 60 and 90 RPM. Thirty seconds before the test, the participant will increase their cadence to 90 RPM. Once the test begins, a fixed resistance will be added to the bike and the participant will begin to pedal maximally for 30 s, trying to maintain the cadence throughout the test. The fixed resistance will be determined from body mass, cycle cadence, and a torque factor determined off the participant's performance during the familiarization test. The cycle settings and fixed	Torque, peak power, time to peak power, mean power, rate of fatigue, fatigue slope, total work	60 min	Phase 1: 4 Phase 2: 25 Phase 3: 46

(*continued on next page*)

Table 4 (*continued*)

Test	Performance Metric(s)	Description of Test	Outcome Variables	Rest Time Following Test	Days Assessed
		resistance will remain the same throughout the entire study, although a decrease in body mass is expected in Phase 2. Verbal encouragement will be given. Power output will be recorded by the Lode Ergometry Manager software (version 10.11.0, Lode B.V., Lode, The Netherlands).			
Treadmill Maximal Cardiorespiratory Fitness Test (VO_{2max})	Aerobic capacity	Peak oxygen uptake (VO_{2peak}) will be measured using a graded exercise test and an indirect open circuit respiratory system (ParvoMedics TrueOne 2400, East Sandy, UT, USA) on a treadmill (Track Master TMX425CP, Full Vision, Inc., Newton, KS, USA). The test will be performed at ambient indoor temperature (20–22 °C) and humidity conditions (30–80%). Participants will be fit with a mouthpiece, headgear, nose clip, and heart rate (HR) monitor. Participants will begin by completing a 5-min warm-up on the treadmill. Then, participants will run for 4 min at a pace predetermined during familiarization at a 0% grade. At 4 min, the grade will be increased to 2%, followed by an additional 2% every 2 min thereafter until volitional exhaustion. Verbal encouragement will be given. HR and ratings of perceived exertion ([RPE] 6–20 Borg scale) [41] will be recorded during each stage. VO_{2max} criteria in Phase 1 will include a plateau in VO_2 with an increase in work rate; maximum respiratory exchange rate (RER) $\geq$ 1.10; maximum HR no less than 10 bpm below age-predicted maximum (220-age); RPE of $\geq$19. Due to the intervention in Phase 2, it is unlikely that all subjects will achieve a maximal response; thus, O_2 uptake data obtained from tests will be referred to as VO_{2peak}, or the highest recorded O_2 consumption. The maximum HR obtained during VO2max test during Phase 1 will be used as a reference point to determine workloads and intensities for the exercise bouts during Phase 2.	VO_{2peak}, RPE, HR, maximal HR, RER	N/A	Phase 1: 4 Phase 2: 25 Phase 3: 46
Timed Weighted Backpack March	Aerobic endurance	Load carriage is an essential aerobic-based military task. Soldiers are expected to carry a standard fighting load of 31.3 kg (68.9 lbs) and move at a rate of 4 km/h (2.5 miles/hour) in an ideal situation. Participants will be fit with a 31.3 kg weighed backpack and will be required to march a 4 km (2.5 miles) course as fast as possible. On command, they will start the test, and a stopwatch will be used to record the time to reach each 0.5-mile increment until the full course has been completed. The participant will be instructed to finish the course in the quickest time possible while walking or running. The participant will not be permitted to listen to music, and no verbal encouragement will be given during the test.	Time to complete each 0.5 mile	N/A	Phase 1: 5 Phase 2: 26 Phase 3: 47

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

where N_{EX} is urinary urea nitrogen excretion and N_{IN} is dietary nitrogen intake during the 24-h urine collection period. Net whole-body protein balance will be calculated as the difference between whole-body PS and PB.

Molecular Signaling Studies. A sample of muscle collected at each time point will be used to explore potential mechanisms by which testosterone regulates muscle mass and the metabolic response to the simulated operational stress. To assess these signaling pathways, a global gene array analysis using Illumina next-generation sequencing (Illumina Inc., San Diego, CA, USA) will be used. Total ribonucleic acid (RNA) will be isolated from muscle using the Trizol/ethanol precipitation. Quantity and quality of RNA will be assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Total RNA (500 ng) 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 $> \pm$ 1.5-fold compared with baseline values and a value of $p < 0.05$.

Following RNA-Sequencing, identified target pathways will be further assessed using Western blot. Briefly, muscle will be homogenized in ice buffer (1:10 wt/volume) 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis using precast Tris·hydrochloric acid gels (Bio-Rad, Hercules, CA, USA). Proteins will be transferred to polyvinylidene fluoride 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, Hercules, CA, USA) and Image Lab software (Bio-Rad, Hercules, CA, USA). To confirm equal protein loading per well, a normalizing protein will be assessed.

Finally, microRNA regulating identified pathways by RNA-Sequencing will also be assessed as a potential mechanism contributing to testosterone and EDef-induced alterations in muscle mass and metabolism. Equal amounts of total RNA will be synthesized into complimentary deoxyribonucleic acid for analysis using a TaqMan® microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Individual probes or microarrays will be used to assess changes in microRNA expression.

Endocrine, Metabolic, and Safety Biomarkers. Blood samples will be collected after an overnight fast on several occasions throughout the study to assess endocrine function (days 1, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 29, 32, 38, 44, 49). Blood samples will be centrifuged, and serum and plasma will be stored at −80 °C. All blood samples will be analyzed for total testosterone, free testosterone (determined by calculation [35]), luteinizing hormone, follicle-stimulating hormone, sex hormone binding globulin, insulin-like growth factor-1, growth hormone, estradiol, insulin, cortisol, glucagon, interleukin-6, and catecholamines (epinephrine, norepinephrine), according to manufacturer's instructions. Blood samples collected at the beginning/end of each phase (days 1, 7, 28, 49) will also be analyzed for metabolic and safety parameters, including free-fatty acids, glucose, glycerol, lactate, β-hydroxybutyrate, prostate specific antigen, creatinine, triglycerides, total cholesterol, high-density lipoproteins, low-density lipoproteins (determined by calculation [36]), potassium, uric acid, albumin, calcium, magnesium, iron, creatine phosphokinase, alanine aminotransferase, alkaline phosphatase, and complete blood count with differential. If a participant's testosterone concentrations are not within normal reference range (300–1000 ng/dL) at the end of the study, a blood sample will be obtained by venipuncture and analyzed for testosterone concentration every 90 days until levels have returned to within normal reference range to assure full recovery of endogenous testosterone production.

Cognitive Function, Personality, Mood, and Sleep. A battery of cognitive performance tests and questionnaires will be administered to assess cognition, personality, mood, and sleep habits (Table 5). Assessments will be conducted on a personal computer. Daily spontaneous motor activity, sleep, and circadian rhythms will be assessed throughout the study using an actigraph (Readiband™, Fatigue Science, Honolulu, HI, USA). The wrist-worn ReadiBand™ has been validated in comparison to polysomnography and shows concordance of 90% or greater in terms of sleep-scoring accuracy [37]. The ReadiBand™ will be worn continuously (24 h/day) on the dominant wrist during each phase. Sleep will not be restricted during Phases 1 or 3. During Phase 2, participants will be allowed 8 h of sleep between 2000–0400 on low stress days and 4 h of sleep between 0000 and 0400 on high stress days (Table 3). Sleep outside these defined periods will not be permitted.

Table 5
Description of cognitive function, personality, mood, and sleep tests.

Test	Description of Test	Days Assessed
Cognitive Function		
Balloon Analogue Risk Task [42]	Objective is to keep a simulated balloon inflated without popping (30 trials). 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 a risk-learning component as some balloon colors pop with less inflation and others with more, while a third category is unpredictable. Designed to measure willingness to take risks versus "play it safe".	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Scanning Visual Vigilance Task [43]	Participants scan a computer screen to detect the occurrence of infrequent, difficult to detect stimuli that appear randomly on a computer screen for 2 s. Upon detection, the participant will press a button as rapidly as possible. Detection accuracy and response time are recorded, as are false alarms. Assesses visual vigilance.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Psychomotor Vigilance [44]	Test requires participants to sustain attention and respond rapidly and accurately to a series of numerical time-count stimuli that appear on a computer screen by pressing a button. Reaction time and response accuracy as well as response lapses are scored. Test of vigilance and visual reaction time.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Match to Sample [45]	Participant views an 8 × 8 matrix of a red and green checkerboard for 4 s, followed by a variable delay. After the delay, the original sample matrix and a second matrix that differs slightly (in that the color sequence two squares are reversed) are presented. The participant has 15 s to select the matching matrix. Assesses short-term spatial memory and pattern recognition skills.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
N-Back Test [46]	Participants monitor the identity or location of a series of stimuli (letters) and indicate when the presented stimulus is the same as the one presented "n" trials back (e.g., 0, 1, 2, or 3). Measures response time and accuracy to test working memory.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Mood and Personality		
Provoked Aggression [47]	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., Goleta, CA, USA). 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 a limb. A gel is rubbed onto the skin at the electrode site to enhance conductance. 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.	Phase 1: 6 Phase 2: 27 Phase 3: 32, 38
Buss-Perry Aggression Questionnaire [48]	Statements (29-item) are ranked along a 5-point continuum from "extremely uncharacteristic of me" to "extremely characteristic of me." Results are shown in terms of scores on 4 scales: physical aggression, verbal aggression, anger, and hostility.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Evaluation of Risks Scale [49]	Assesses willingness to take risks through participant responses to 24 items on a visual analog scale.	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Profile of Mood States Questionnaire [50]	An inventory of subjective mood states (65-item). Results consist of six mood sub-scale scores (tension, depression, anger, vigor, fatigue, and confusion).	Phase 1: 2, 4, 6 Phase 2: 12, 14, 17, 22, 27 Phase 3: 32, 38
Sleep		
Modified Pittsburg Sleep Quality Index [51]	Self-rated questionnaire that assesses sleep quality and disturbances on a nightly basis. Generates scores on subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, and sleep disturbances.	Daily
Sleep Monitoring (Actigraphy)	Assesses spontaneous motor activity, circadian rest/activity cycles, and sleep using a watch-sized, wrist-worn device	Daily

3.3. Treatment allocation, randomization, and blinding

Following Phase 1 testing, participants will be randomized to receive either a single intramuscular injection of testosterone undecanoate (TEST; 750 mg, standard pharmaceutical dose [12]) 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 day SUSOPS. A randomization scheme will be determined using a block design ($n = 4$) and age stratification (<29 years or ≥ 29 years) in a 1:1 (TEST:PLA) ratio. Randomization will be done by a biostatistician with no direct study affiliation, and the randomization schedule will be given to the pharmacist, who 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.

3.4. Sample size estimation

Physical performance is the primary study endpoint for this study. The intended total EDef during the 20-day SUSOPS will be ~43,380 kcal, which, based on a previous meta-regression of data generated from military field studies, should elicit a ~9% reduction in total body mass and ~7% decline in lower-body physical performance [2]. Based on the results from OPS I [8], we expect body mass loss in TEST to be ~50% less than PLA, such that lower-body muscular strength and power declines from baseline are attenuated by 50% in TEST relative to PLA. Thus, the sample size necessary to detect differences of that magnitude between treatments is 15 per group with 90% power. To account for possible attrition (5% attrition in OPS I prior to randomization), 16 participants will be assigned to each group (32 total participants). Enrollment will stop once 32 participants have completed the study.

3.5. Statistical analysis

All data analyses will be based on the intention-to-treat principle using SAS (SAS Institute version 9.4, Cary, NC, USA) or SPSS statistical software (IBM Statistics, Chicago, IL, USA), unless otherwise noted. Between group comparisons for all baseline, pre-study variables and Phase 1, Phase 2, and Phase 3 TDEI, TDEE, EIEE, total sleep, percent and absolute EDef will be assessed using two sample Student's *t*-tests. Primary analysis of physical performance, body composition, muscle and whole-body protein homeostasis, endocrine, metabolic, and safety biomarkers, and cognitive function will be performed using a mixed-effect linear model. Treatment (TEST and PLA), phase (Phase 1, Phase 2, and Phase 3), 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. All analyses will be considered 2-tailed, with $\alpha = 0.05$ considered statistically significant.

3.6. Data management and monitoring

Study participants will be assigned unique subject identification (ID) numbers. Study subject ID numbers will be used on all data collection instruments, including questionnaires, data collection forms, biological specimen tubes, and computer records. All forms will be kept under lock and key, or password-protected if computerized, and under the control of the principal investigator and project manager. Most data are automatically uploaded from the instruments that measure the endpoint. All self-report inventories and questionnaires will be completed in REDCap (Vanderbilt University, Nashville, TN, USA) via surveys [38]. Data will be exported from REDCap for analysis. A master list linking the participants' names and ID numbers will be kept in a password-protected computer file with access restricted to the principal investigator and study navigator. All data are entered into an integrated and automated data management system that has been validated and undergoes quality assurance by the PBRC Research Computing Core. All data are backed up daily. Biological samples that are moved off-site for analysis will not contain any personally identifiable information and will be labeled with only the unique subject ID numbers. Staff at these sites will not have access to the master list at any time.

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 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. There is more than minimal risk for participating in this trial, and any adverse events will also be monitored. 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. Adverse events will be reported to the principal investigator, project manager, chair of the PBRC IRB, chair of the study DSMB, and Safety Officer throughout the trial. Examples of adverse events include but are not limited to: 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. Serious adverse events 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, a permanent disability or incapacity, a clinically significant abnormal laboratory or diagnostic test result, or 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. An adverse event or experience is defined as any health-related unfavorable or unintended medical occurrence that happens after randomization.

In accordance with the Declaration of Helsinki, 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.

4. Ethical considerations

Written informed consent will be obtained from all study participants. Any protocol modifications will be conveyed to investigators, the IRB, and trial registries, regulators, and participants. Recruitment for this study is ongoing; it commenced in September 2019 and is expected to conclude in July 2021.

All participants are assured of their confidentiality both verbally and in the informed consent form. The clinical facilities are limited to the staff of the institution and participants, which is enforced through stringent security measures. Medical records are stored in locked areas. Access to these areas is limited to the clinical staff, director of the facilities, and the onsite principal investigator. Participants' medical records are filed according to ID numbers, but lab reports also contain participant names as a mandatory criteria for lab certification. All forms on the chart display the ID number. Electronic data storage is similarly restricted with only the principal investigator and authorized persons having access to databases containing confidential clinical records, i.e., those containing name or other identifying information.

If requested, participants will be provided a summary results sheet at the completion of the study. The summary results will include body composition and physical performance tests, and available lab work results. The study results will be disseminated at national and international conferences and published in peer-reviewed journals.

5. Conclusions

The OPS II trial aims to determine whether a single dose of long-acting testosterone undecanoate (750 mg) safely and steadily maintains normal testosterone concentrations and enhances military relevant measures of performance, while attenuating muscle and total mass loss, without impairing cognitive function, during, and in recovery from a simulated, multi-stressor SUSOPS.

Author contributions

Alyssa N. Varanoske: methodology, investigation, visualization, writing – original draft, writing – review and editing; Melissa N. Harris: methodology, software, validation, investigation, resources, data curation, project administration; Callie Hebert: methodology, software, validation, investigation, resources, data curation, project administration; Emily E. Howard: investigation; Neil M. Johannsen: methodology, software, validation, resources; Steven B. Heymsfield: conceptualization, methodology, investigation, resources, supervision; Frank L. Greenway: supervision; Lee M. Margolis: conceptualization, methodology; Harris R. Lieberman: conceptualization, methodology; David D. Church: methodology, investigation, data curation; Arny A. Ferrando: conceptualization, methodology, validation, resources, supervision, funding acquisition; Jennifer C. Rood: conceptualization, methodology, resources, data curation, writing – review and editing, supervision, funding acquisition; Stefan M. Pasiakos: conceptualization, methodology, resources, supervision, funding acquisition, writing – original draft, writing – review and editing. All authors read and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare that they have no competing interests. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

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