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Energy deficit increases hepcidin and exacerbates declines in dietary iron absorption following strenuous physical activity: a randomized-controlled cross-over trial

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ABSTRACT

Background: Strenuous physical activity promotes inflammation and depletes muscle glycogen, which may increase the iron regulatory hormone hepcidin. Hepcidin reduces dietary iron absorption and may contribute to declines in iron status frequently observed following strenuous physical activity.

Objectives: To determine the effects of strenuous physical activity on hepcidin and dietary iron absorption and whether energy deficit compared with energy balance modifies those effects.

Methods: This was a randomized, cross-over, controlled-feeding trial in healthy male subjects ($n = 10$, mean ± SD age: 22.4 ± 5.4 y, weight: 87.3 ± 10.9 kg) with sufficient iron status (serum ferritin 77.0 ± 36.7 ng/mL). Rest measurements were collected before participants began a 72-h simulated sustained military operation (SUSOPS), designed to elicit high energy expenditure, glycogen depletion, and inflammation, followed by a 7-d recovery period. Two 72-h SUSOPS trials were performed where participants were randomly assigned to consume either energy matched (±10%) to their individual estimated total daily energy expenditure (BAL) or energy at 45% of total daily energy expenditure to induce energy deficit (DEF). On the rest day and at the completion of BAL and DEF, participants consumed a beverage containing 3.8 mg of a stable iron isotope, and plasma isotope appearance was measured over 6 h.

Results: Muscle glycogen declined during DEF and was preserved during BAL (−188 ± 179 mmol/kg, *P*-adjusted < 0.01). Despite similar increases in interleukin-6, plasma hepcidin increased during DEF but not BAL, such that hepcidin was 108% greater during DEF compared with BAL (7.8 ± 12.2 ng/mL, *P*-adjusted < 0.0001). Peak plasma isotope appearance at 120 min was 74% lower with DEF (59 ± 38% change from 0 min) and 49% lower with BAL (117 ± 81%) compared with rest (230 ± 97%, *P*-adjusted < 0.01 for all comparisons).

Conclusions: Strenuous physical activity decreases dietary iron absorption compared with rest. Energy deficit exacerbates both the hepcidin response to physical activity and declines in dietary iron absorption compared with energy balance. This trial was registered at clinicaltrials.gov as NCT03524690. *Am J Clin Nutr* 2021;113:359–369.

Keywords: energy balance, exercise, hepcidin, inflammation, iron

Introduction

Hepcidin is a 25–amino acid peptide hormone that functions to reduce circulating iron concentrations by binding and signaling

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Data Availability: Data described in the manuscript and analytic code will be made available upon request.

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Abbreviations used: BAL, energy balance; CPK, creatine phosphokinase; CREBH, cyclic adenosine monophosphate response element binding protein-H; CRP, C-reactive protein; DEF, energy deficit; DLW, doubly labeled water; EPO, erythropoietin; IL-6, interleukin-6; Jak, Janus kinase; LDH, lactate dehydrogenase; PBRC, Pennington Biomedical Research Center; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Stat, signal transducer and activator of transcription; sTfR, soluble transferrin receptor; SUSOPS, sustained military operations; TIBC, total iron binding capacity; VO_{2peak}, peak oxygen uptake

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for the degradation of the cellular iron exporter ferroportin (1). Ferroportin is highly expressed in cells and tissues associated with iron transport, including reticuloendothelial macrophages and absorptive epithelial cells in the small intestine (2, 3). Therefore, hepcidin-mediated declines in ferroportin inhibit iron recycling and limit entry of dietary iron into portal circulation. Shortly after the discovery of hepcidin, Roecker et al. reported increased hepcidin concentrations in athletes following a marathon (4). Recent investigations have sought to determine whether the increase in hepcidin with strenuous physical activity is responsible for the declines in iron status that are frequently observed in physically active populations, such as manual laborers, military personnel, and endurance athletes (5).

Most studies have proposed that the increase in hepcidin with strenuous physical activity is due to an inflammatory response to repeated muscular contractions. These studies point to interleukin-6 (IL-6), as IL-6 is required for hepcidin induction and hypoferremia during inflammation through the Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway (6). The inflammation–hepcidin axis is thought to be an evolutionary adaptation of the host to restrict iron availability to pathogens (7–9). Glycogen depletion may also contribute to hepcidin activation through IL-6–dependent and -independent mechanisms. For example, IL-6 is synthesized and released by contracting skeletal muscle during prolonged physical activity in response to reductions in intramuscular glycogen (10–12). In addition, hepcidin is transcriptionally upregulated by gluconeogenic signals through the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A)/cyclic adenosine monophosphate response element binding protein-H (CREBH) signaling pathway (13). The PPARGC1A/CREBH pathway of hepcidin activation may be important in situations such as prolonged fasting and vigorous physical activity where glycogen stores are depleted and glucose must be synthesized de novo from nonhexose precursors in order to maintain blood glucose concentrations.

Military personnel experience periods of energy deficit during sustained combat and training operations (SUSOPS) due to increased energy expenditure and limited dietary intake. Such energy deficits during military operations reduce glycogen stores and activate gluconeogenesis (14). A previous study from our laboratory reported 245% and 33% increases in IL-6 and hepcidin, respectively, in volunteers participating in a 96-h SUSOPS that produced high energy expenditures (∼6000 kcal/d) and energy deficits of ∼50% total energy expenditure (15). Hepcidin concentrations post-SUSOPS were positively associated with total daily energy expenditure and the magnitude of energy deficit and negatively associated with energy intake. The primary objectives of this study were to determine the effects of SUSOPS on IL-6, hepcidin, and dietary iron absorption and whether energy deficit compared with energy balance modifies those effects. We hypothesized that SUSOPS would increase circulating concentrations of IL-6 and hepcidin and decrease iron absorption, and that energy deficit would exacerbate the increase in circulating concentrations of IL-6 and hepcidin and declines in iron absorption compared with energy balance.

Methods

The study conformed to the principles in the Declaration of Helsinki and was approved by the Institutional Review Board at the US Army Human Research Protections Office (Ft. Detrick). Participants provided written and voluntary informed consent (clinicaltrails.gov NCT03524690).

Participants

Healthy, recreationally active adult men (age 18–39 y, active duty military personnel) were recruited to participate in this study in April and September 2019. Inclusion criteria included no evidence of chronic illness, medication use, or musculoskeletal injury, and willingness to refrain from the following: *1*) pain relievers, nonsteroidal anti-inflammatory drugs, or other aspirin-containing products for 10 d before starting and for the duration of the study, *2*) alcohol and nicotine for the duration of the study, and *3*) vitamin and mineral supplements for at least 2 wk before starting and for the duration of the study. Exclusion criteria included musculoskeletal injuries, metabolic or cardiovascular abnormalities, history of any disease or abnormality of the gastrointestinal tract, anemia (hemoglobin $<$ 13 g/dL), blood donation within 4 mo of beginning the study, C-reactive protein (CRP) $>$ 5 mg/dL, abnormal prothrombin time/partial thromboplastin time test or problems with blood clotting, and history of complications with lidocaine.

Study design and procedures

This study was a 22-d, 2-trial, randomized cross-over study (**Figure 1**). Each trial consisted of a 72-h SUSOPS during which participants were either in energy deficit (DEF) or energy balance (BAL) followed by a 7-d recovery period. All aspects of the 2 trials were the same, including the timing of measures. The order in which participants completed the BAL and DEF trials was randomly assigned and balanced. Treatment order for each participant was randomly assigned using the random number generator feature in Microsoft Excel.

Prestudy baseline testing

Participants completed a 3-d diet record and a 3-d activity log during prestudy testing days. Peak oxygen uptake (VO_{2peak}) on a cycle ergometer and resting metabolic rate were measured during the prestudy testing period using standardized techniques and an indirect, open circuit respiratory system (True Max 2400, ParvoMedics).

Glycogen normalization

Participants completed a muscle glycogen normalization protocol prior to both SUSOPS trials to limit the potential influence of baseline differences in muscle glycogen on the IL-6 and hepcidin response. Following an overnight fast, participants performed a 5-min warmup on a cycle ergometer at 50% of peak oxygen uptake (VO_{2peak}). After the warmup, participants completed repeated periods of 2 min of work at a mean ± SD 80 ± 5% VO_{2peak} followed by 2 min of recovery at 50 ± 5% VO_{2peak} for 50 min (i.e., 12 cycles). After completing the glycogen depletion protocol and until the beginning of each SUSOPS trial (days 1–2 and 11–12), participants were fed a controlled diet prescribed to maintain energy balance and providing ≥60% of total energy from carbohydrate to ensure adequate glycogen

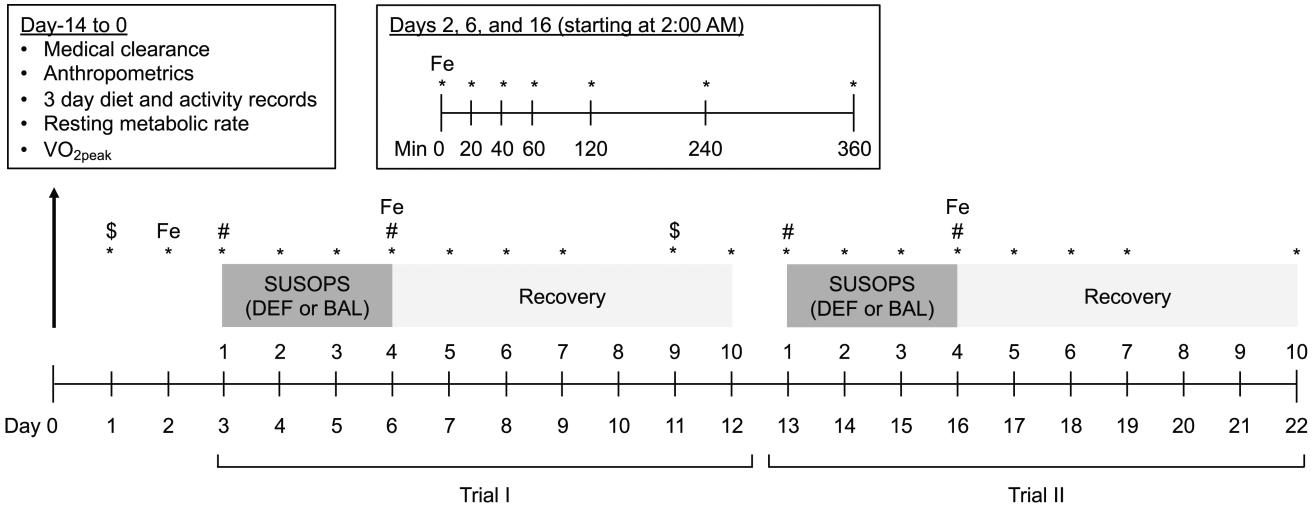

FIGURE 1 Overview of study design. Participants were randomly assigned to complete the SUSOPS trials in DEF or BAL. *blood draw; #muscle biopsy; $start of glycogen normalization and refeeding; Fe consumption of a beverage containing a stable iron isotope. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations; VO_{2peak}, peak oxygen uptake.

repletion and homogeneous glycogen concentrations within and between participants during both SUSOPS trials (DEF and BAL). Food and beverages were prepared and provided by study dietitians and consisted of commercial items.

SUSOPS DEF and BAL

The SUSOPS comprised a variety of military tasks designed to elicit high energy expenditure, glycogen depletion, and inflammation. Resting metabolic rate was multiplied by a factor of 1.3 to estimate energy expenditures for activities of daily living. Physical activity was prescribed at levels to expend ∼5000–6000 total kcal/d using the American College of Sports Medicine metabolic equations for steady-state exercise and the compendium of metabolic equivalents for physical activities (16). Total daily energy expenditure prescriptions were individualized to each participant's requirements and were held constant between SUSOPS DEF and SUSOPS BAL. Low-to-moderate intensity (30–65% VO_{2peak}) steady-state endurance-type exercise was the primary exercise modality. Participants performed 3 prolonged steady-state exercise bouts per day. Two of the 3 exercise bouts were ∼60–120-min load carriage exercise sessions, whereas the third was unloaded. A 120-min loaded steady-state road march was performed immediately prior to the end of the SUSOPS at 0000 on the evenings of day 5 and 15. The total distance covered was dictated by individual exercise prescriptions. The load carried was 33.5 ± 0.2 kg and comprised the basic uniform (∼5.3 kg), weapon and tactical equipment (∼11.2 kg), and rucksack (∼15 kg). During the remainder of each day, participants performed a number of military tasks to increase energy expenditure and simulate operational tasks. Sleep was restricted to 4 h/d beginning the evenings of days 2 and 12 and ending the evenings of days 5 and 15.

Plasma iron isotope appearance

To assess dietary iron absorption, plasma isotope appearance was determined on the day before the first trial (referred to as the "rest" day) and at the completion of each SUSOPS period (Figure 1). Participants consumed a stable iron isotope in the morning (∼02:00) after an 8-h fast. The timing was chosen such that the isotope was absorbed when hepcidin concentrations were expected to be the greatest (i.e., ∼3 h after completion of the final SUSOPS event (17), a 2-h loaded road march). An indwelling intravenous catheter was placed in the antecubital fossa (or distally) and a baseline blood sample was drawn before consuming the iron isotope (0 min). Participants then consumed a 300-mL drink containing 3.8 mg iron (representative of dietary iron in an iron-rich meal) as isotopically labeled $^{54}FeSO_4$ or $^{57}FeSO_4$. Venous blood samples were collected 20, 40, 60, 120, 240, and 360 min and 24, 48, and 72 h later to assess plasma isotope appearance.

Iron isotope preparation and sample analysis

Iron stable isotopes (^{57}Fe, 92.88% enrichment and ^{54}Fe, 98.37% enrichment) were purchased as iron (III) sulfate powder from Oak Ridge National Laboratory. The powder was dissolved in doubly distilled water and Fe^{3+} was reduced to Fe^{2+} by adding ascorbic acid at a molar ratio of 2:1 (ascorbic acid: iron) prior to use. Stable isotope concentrations were determined by inductively coupled plasma mass spectrometry (XSERIES II, Thermo Fisher Scientific). Plasma iron isotope appearance was calculated using isotope dilution as described previously (18, 19). Briefly, the amount of absorbed iron circulating in blood was calculated based on the amount of stable isotope administered, the amount of stable isotope detected in the blood, hemoglobin concentration, and blood volume, which were estimated based on participant height and weight.

Diets during SUSOPS DEF and BAL

Registered dietitians developed individualized daily menus for SUSOPS using Food Processor SQL (ESHA Research, Version 10.14). The diets during SUSOPS were derived primarily from components of US Military Meals Ready-to-Eat and

supplemental commercial food items to achieve prescribed macronutrient proportions. To limit the potential confounding effect of differing iron intakes, supplemental iron (ferrous sulfate drops, RxChoice) was added to an entree item with each of the meals during SUSOPS DEF to match total iron consumed during SUSOPS BAL. Water was allowed ad libitum. Participants received instructions from study dietitians on how to consume an ad libitum diet with consistent macronutrient distribution during the 2 recovery periods. Diet records were completed during the recovery periods (days 6–8 and 16–18).

Total daily energy expenditure

Doubly labeled water (DLW) was used to determine actual total daily energy expenditure and to verify the accuracy of estimated total daily energy expenditure during SUSOPS (DEF and BAL). Immediately before drinking the DLW, participants provided a urine sample to determine the natural abundance of ^{2}H and ^{18}O. A total of 120 g of DLW containing 10% $H_2{}^{18}O$ (~0.285 g $H_2{}^{18}O$/kg total body water) and 99% $^{2}H_2O$ (~0.15 g $^{2}H_2O$/kg total body water; Sigma-Aldrich) was administered on day 2 (~0000 after an 8-h fast). Urine samples were collected ~4 and 6 h after the DLW dosing for initial total body water determinations. One participant was randomly chosen to consume only locally available drinking water to control for natural changes in ^{2}H and ^{18}O abundance, and local water was analyzed to determine isotopic enrichments. The rate of disappearance of ^{2}H and ^{18}O for participants dosed with DLW was corrected for mean changes in background enrichments based on controls. Morning urine samples were collected daily during each SUSOPS period to determine elimination rates over time. Total body water was calculated by determining the regression line for the elimination of ^{2}H and ^{18}O and extrapolated to a maximum enrichment. Enrichments of ^{2}H and ^{18}O were determined using isotope ratio mass spectrometry (Finnigan Mat 252, Thermo Fisher Scientific). The ^{2}H and ^{18}O isotope elimination rates (k_H and k_O) were calculated by linear regression using the isotopic disappearance rates during each SUSOPS period. Thermic effect of food was estimated as 10% of DLW total daily energy expenditure (thermic effect of food = total daily energy expenditure × 0.1). Activity-induced energy expenditure was estimated by subtracting measured resting metabolic rate and thermic effect of food from total daily energy expenditure [activity-induced energy expenditure = total daily energy expenditure − (resting metabolic rate + thermic effect of food)].

Muscle biopsies

Percutaneous muscle biopsies were obtained from the vastus lateralis using a 5-mm Bergstrom needle with manual suction while the participant was under local anesthesia (1% lidocaine). The biopsy procedures were performed after an 8-h fast immediately before starting and within 30 min after completing each SUSOPS period (days 3, 6, 13, and 16). Glycogen concentration was determined in ~3 mg (dry weight) freeze dried muscle. Tissue was broken apart and visible connective tissue was removed. The tissue was then homogenized in water using a TissueLyser II with a 5-mm steel bead (Qiagen). Homogenates were boiled at 100°C for 5 min and centrifuged at 13,000 × *g* for 5 min at room temperature. Supernatants were removed and muscle glycogen concentrations were assessed using an endpoint colorimetric assay (Sigma-Aldrich).

Blood collection

Participants fasted ≥8 h before all blood draws. With the exception of blood collected for plasma iron isotope appearance, which started at ~2:00 AM, all blood was collected between 05:00 and 08:00. Hemoglobin was measured in whole, heparinized blood using a handheld iSTAT® point-of-care device and Chem8 + Cartridges (Abbott Point of Care; reference range: 12–17 g/dL). Serum insulin (reference range: 6–27 mIU/mL), testosterone (reference range: 160–726 ng/dL), CRP (high sensitivity; reference range: 0.2–11.0 mg/L), and serum ferritin (reference range: 28–365 ng/mL) were determined using an advanced automated immunoassay instrument (ImmuliteR 2000; Siemens Healthcare Diagnostic). Serum glucose (reference range: 70–110 mg/dL), free fatty acids (reference range: 0.1–0.6 mmol/L), glycerol (reference range: 0.03–0.19 mmol/L), myoglobin (reference range: 0–70 ng/mL), creatine phosphokinase (CPK; reference range: 38–333 IU/L), lactate dehydrogenase (LDH; reference range: 82–195 IU/L), serum iron (reference range: 50–160 μg/dL), and total iron-binding capacity (TIBC; reference range: 255–450 μg/dL) were determined using enzymatic and colorimetric measurements (Beckman Coulter DXC 600 Pro, Beckman Coulter). Transferrin saturation was calculated by dividing serum iron by TIBC. Erythroferrone (ERFE; Intrinsic Life Sciences; reference range: 0.16–10 ng/mL), erythropoietin (EPO; R&D Systems Inc.; reference range: 1.1–523 mIU/mL), serum hepcidin (high sensitivity, DRG International; reference range: 0.153–81 ng/mL), IL-6 (R&D Systems Inc.; reference range: 3.1–300 pg/mL), and soluble transferrin receptor (sTfR) (R&D Systems Inc.; reference range: 3.0–80 nmol/L) were determined using ELISAs. All assays were conducted by Pennington Biomedical Research Center (PBRC). PBRC follows good clinical practices and is accredited by the College of American Pathologists. All assays were run with standards and appropriate quality control material. In addition, PBRC runs external proficiency samples and results are compared with other laboratories across the country.

Statistical analysis

The primary outcomes for this study were IL-6, hepcidin, and dietary iron absorption. All other variables measured were secondary outcomes. Sample size calculations were derived from a previous study that found significant increases in hepcidin in male soldiers following a 7-d military training exercise (20). Using baseline hepcidin concentrations of 6.5 ± 3.5 ng/mL and an estimated increase in hepcidin of ~50% with training, it was estimated that 9 participants were sufficient to detect an increase in hepcidin with training at $\alpha = 0.05$ and power = 0.80. This sample size provides sufficient statistical power to detect large trial effects (power = 0.80, $\alpha = 0.05$, $d \geq 1.0$; GPOWER 3.1.9.7). To account for potential attrition, 13 participants were enrolled in the study. Statistical analyses were performed using SPSS version 25 (IBM Corp.). Data are presented as means ± SDs.

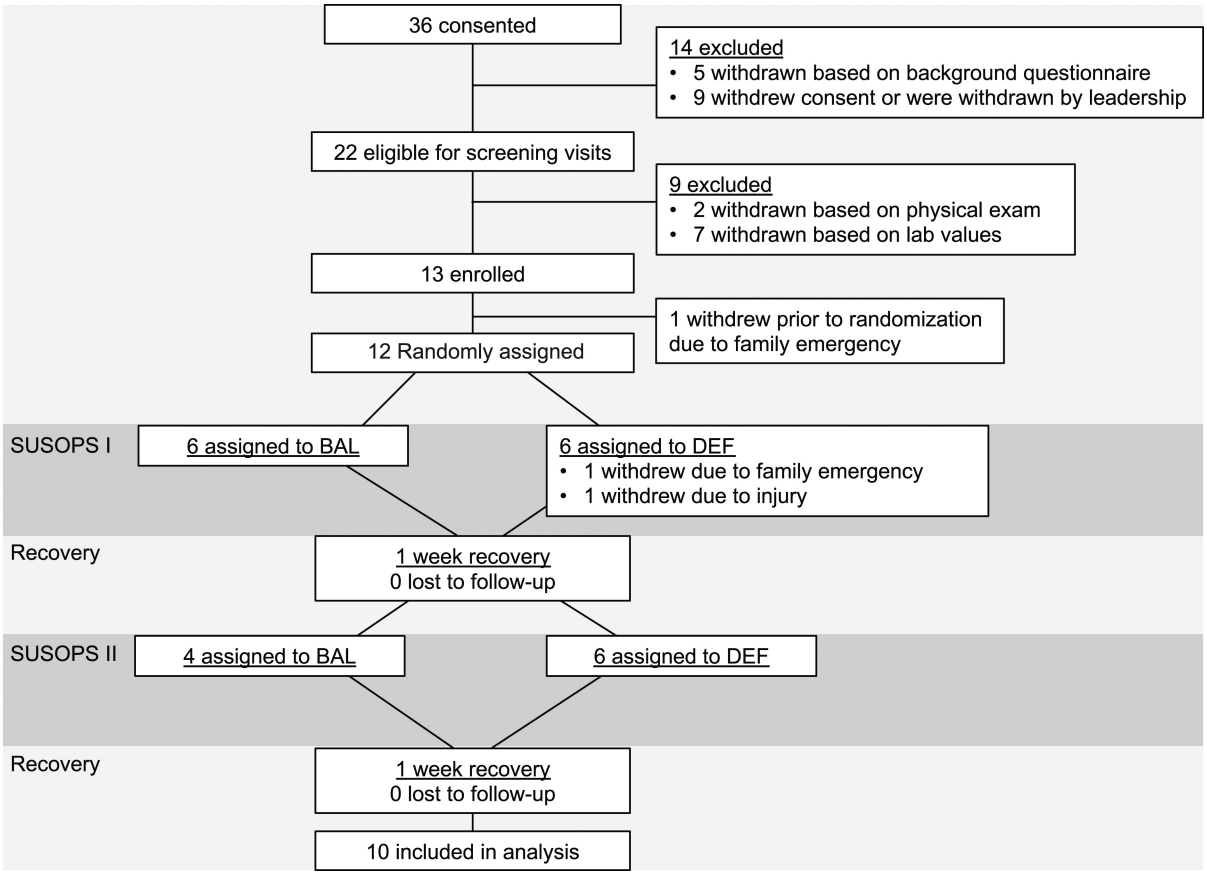

FIGURE 2 Participant flow chart. Thirty-six potential participants consented, 14 were excluded, 22 were eligible for screening visits, 9 were excluded following screening, 13 were enrolled, 1 discontinued participation prior to randomization, 12 were randomly assigned, and 10 completed the intervention. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations.

Shapiro-Wilk tests were used to determine normality of data. If normality was rejected ($P < 0.05$), log transformations were applied to normalize the data ($P > 0.05$). Differences in dietary intake for the controlled feeding days (rest, SUSOPS DEF, and SUSOPS BAL) were analyzed by 1-way ANOVA with Bonferroni correction for multiple comparisons. Paired Student *t*-tests were used to compare dietary intake during the recovery recall days and daily energy expenditure, thermic effect of food, activity-induced energy expenditure, energy balance, and energy deficit measured during SUSOPS DEF and SUSOPS BAL. General linear models with correlated errors were used to determine the main effects of the trial (i.e., DEF, BAL, and when relevant, rest; the trial included both SUSOPS and recovery when measurements were taken during recovery), time within trial (e.g., study day or minutes), and their interaction on biochemical measures and plasma iron isotope appearance. To test for carryover effects, a main effect of trial order (DEF first, BAL first) and an order-by-trial interaction were included in the model. No effects of trial order were observed, and these data are not shown. The residual maximum likelihood method was used to account for values that were missing completely at random on the dependent variable. If trial-by-time interactions were observed, a Bonferroni correction was applied for multiple comparisons. Statistical significance was set at $P < 0.05$. The P value was not adjusted for multiple endpoints, because the effectiveness of the multiple primary endpoints in the current study (IL-6, hepcidin, and dietary iron absorption) depended on the success of 2 or more primary endpoints (i.e., dietary iron absorption and hepcidin, dietary iron absorption and IL-6, or dietary iron absorption, hepcidin, and IL-6).

Results

Participants

Thirteen participants were enrolled, 12 were randomly assigned, and 10 completed the study (**Figure 2**). Baseline characteristics are shown in **Table 1**. Participants were young healthy males with sufficient iron status. With the exception of 1 participant who had a transferrin saturation of 14% (all other iron status indicators were in the normal range), none of the participants were iron deficient [ferritin < 20 ng/mL, sTfR > 32 nmol/L, and/or transferrin saturation < 20%, (21)] or anemic at baseline.

Dietary intake and physical activity

Dietary intake for the day preceding rest and during SUSOPS and recovery from SUSOPS are shown in **Table 2**. The

TABLE 1 Baseline participant characteristics[1]

	$n = 10$
Race and ethnicity, *n* (%)	
Non-Hispanic black	1 (10)
Non-Hispanic white	7 (70)
Hispanic	0
Other	2 (20)
Age, y	22.4 ± 5.4
Body weight, kg	87.3 ± 10.9
BMI, kg/m^2	27.0 ± 3.5
VO_{2peak}, mL/kg/min	40.5 ± 4.6
Biochemical measures[2]	
IL-6, pg/mL	1.9 ± 0.6
CRP, mg/L	1.2 ± 0.9
Hemoglobin, g/dL	15.4 ± 0.9
Ferritin, ng/mL	77.0 ± 36.7
Transferrin saturation, %	45.3 ± 22.0
sTfR, nmol/L	19.8 ± 2.3
Hepcidin, ng/mL	7.2 ± 1.6
ERFE, ng/mL	0.2 ± 0.1

[1] Values are means ± SDs or *n* (%). BMI, body mass index; CRP, C-reactive protein; ERFE, erythroferrone; IL-6, interleukin-6; sTfR, soluble transferrin receptor; VO_{2peak}, peak oxygen uptake.

[2] All baseline biochemical measures were measured on day 2.

macronutrient distribution of the diets (i.e., the percentage of kcals from carbohydrate, protein, and fat) was similar during rest, SUSOPS DEF, and SUSOPS BAL; however, during rest and SUSOPS DEF participants consumed approximately 50% less energy (rest: −3005 ± 166, SUSOPS DEF: −2922 ± 227 kcal/d), carbohydrate (rest: −472 ± 28, SUSOPS DEF: −450 ± 31 g/d), protein (rest: −76 ± 8, SUSOPS DEF: −68 ± 11 g/d), and fat (rest: −108 ± 8, SUSOPS DEF: −99 ± 9 g/d) per day compared with SUSOPS BAL (*P*-adjusted < 0.0001 for all comparisons). Dietary iron intake was similar during rest and SUSOPS DEF, but both were less than during SUSOPS BAL (rest: −6.2 ± 2.0, SUSOPS DEF: −7.1 ± 1.8 mg/d; *P*-adjusted < 0.0001 for both). However, after accounting for supplemental iron given with meals during SUSOPS DEF, DEF and BAL consumed an equivalent amount of total iron during SUSOPS (0.7 ± 0.8 mg/d; *P*-adjusted = 1.00). Participants consumed similar diets during recovery from SUSOPS DEF and SUSOPS BAL.

Total time exercising (SUSOPS DEF: 283 ± 47, SUSOPS BAL: 283 ± 48 min/d; $P = 0.95$), mean exercise intensity (SUSOPS DEF: 7.5 ± 0.3, SUSOPS BAL: 7.6 ± 0.3 average metabolic equivalents; $P = 0.19$), and mean effort (SUSOPS DEF: 66 ± 9, SUSOPS BAL: 67 ± 9% of VO_{2peak}; $P = 0.27$) did not differ between SUSOPS. Total daily energy expenditure and activity-induced energy expenditure remained the same during SUSOPS DEF and SUSOPS BAL (Table 2). Combined with diet, participants were in a −2047 ± 920 kcal/d deficit (−43 ± 9% energy deficit) during SUSOPS DEF, which differed from SUSOPS BAL (18 ± 20% energy deficit, $P < 0.001$).

Metabolic markers

Mean responses of clinical biomarkers during SUSOPS and recovery are shown in **Table 3**. Participants lost weight during SUSOPS DEF (−1.9 ± 1.1 kg) and maintained weight during SUSOPS BAL (0.5 ± 0.7 kg, *P*-adjusted < 0.0001). Muscle glycogen and metabolic parameters were consistent with increased gluconeogenesis during SUSOPS DEF compared with SUSOPS BAL. There was a trial-by-time interaction for muscle glycogen (*P*-interaction = 0.03), free fatty acids (*P*-interaction < 0.0001), and glycerol (*P*-interaction < 0.0001). Muscle glycogen declined during SUSOPS DEF and was preserved during SUSOPS BAL (−188 ± 179 mmol/kg, *P*-adjusted < 0.01). Circulating concentrations of free fatty acids and glycerol increased by 142% and 147%, respectively, during SUSOPS DEF, but were unchanged during SUSOPS BAL (free fatty acids: 0.42 ± 0.19 mmol/L, *P*-adjusted < 0.0001; glycerol: 0.043 ± 0.037 mmol/L; *P*-adjusted < 0.0001). EPO increased during SUSOPS (6.5 ± 2.1 mIU/mL; *P*-time < 0.0001) with no differences between DEF and BAL (*P*-trial = 0.30).

Markers of muscle damage and inflammation

Time effects, but no trial or interaction effects, were observed for markers of muscle damage, such as myoglobin, CPK, and LDH (*P*-time < 0.0001 for all; Table 3). Post hoc comparisons for time revealed increased myoglobin, CPK, and LDH on day 3 of both SUSOPS trials compared with day 1 (*P*-adjusted < 0.0001). A trial-by-time interaction was observed for CRP (*P*-interaction < 0.01). CRP increased on day 3 of both SUSOPS trials compared with day 1. However, the increase in CRP was 59% greater during the DEF trial than during the BAL trial (2.6 ± 5.3 mg/L, *P*-adjusted < 0.0001). There was an effect of time for plasma IL-6 (*P*-time < 0.0001), but no trial (*P*-trial = 0.76) or interaction (*P*-interaction = 0.77) effects. Post-hoc comparisons for time demonstrated an increase in plasma IL-6 on day 2 of SUSOPS (11.1 ± 5.8 pg/mL) compared with day 1 of SUSOPS (2.6 ± 1.4 pg/mL; *P*-adjusted < 0.0001). A trial-by-time interaction was observed for plasma hepcidin (*P*-interaction = 0.001). Plasma hepcidin concentrations increased during SUSOPS DEF, but remained unchanged during SUSOPS BAL, such that hepcidin was 72% (7.8 ± 12.2 ng/mL, *P*-adjusted < 0.0001) greater on day 3 of SUSOPS and 59% (4.3 ± 10.4 ng/mL, *P*-adjusted < 0.01) greater on day 1 of recovery (day 4) during DEF compared with BAL.

Indicators of iron status

Trial-by-time interactions were observed for hemoglobin (*P*-interaction < 0.0001), ferritin (*P*-interaction = 0.01), serum iron (*P*-interaction < 0.0001), transferrin saturation (*P*-interaction < 0.0001), and sTfR (*P*-interaction = 0.05). There was a steady decline in hemoglobin during BAL beginning on day 2 of SUSOPS, reaching a 20% decrease on day 1 of recovery compared with day 1 of SUSOPS (−3.2 ± 0.7 g/dL, *P*-adjusted < 0.0001) before returning to pre-SUSOPS concentrations by day 3 of recovery (day 6) (Table 3). During DEF, there was an initial decline in hemoglobin on day 2 of SUSOPS, but hemoglobin rebounded to pre-SUSOPS levels on day 3, and then decreased again reaching a 15% reduction (−2.4 ± 0.8 g/dL, *P*-adjusted < 0.0001) on day 1 of recovery compared with day 1 of SUSOPS. Hemoglobin did not return to pre-SUSOPS levels until day 7 of recovery (day 10) following the DEF trial. Ferritin increased 33% on day 3 of SUSOPS DEF compared with day 1 (25.9 ± 12.7 ng/mL, *P*-adjusted = 0.02), but remained unchanged during SUSOPS BAL (−4.0 ± 8.9

TABLE 2 Dietary intake and total daily energy balance at rest and during and after 72-h simulated sustained military operations[1]

		SUSOPS			Recovery		
	Rest	DEF	BAL	*P* value	DEF	BAL	*P* value
Absolute intake							
Energy, kcal/d	2382 ± 279[a]	2515 ± 171[a]	5437 ± 377[b]	<0.0001	2467 ± 811	2379 ± 1061	0.79
Carbohydrate, g/d	361 ± 44[a]	382 ± 26[a]	833 ± 51[b]	<0.0001	255 ± 98	245 ± 127	0.83
Protein, g/d	63 ± 6[a]	71 ± 3[a]	139 ± 12[b]	<0.0001	108 ± 36	106 ± 49	0.90
Fat, g/d	74 ± 10[a]	84 ± 7[a]	183 ± 15[b]	<0.0001	114 ± 37	110 ± 50	0.77
Dietary iron, mg/d	15.5 ± 2.9[a]	14.6 ± 2.3[a]	21.8 ± 1.3[b]	<0.0001	10.6 ± 5.7	11.4 ± 6.1	0.66
Total iron, mg/d[2]	15.5 ± 2.9[a]	22.5 ± 1.4[b]	21.8 ± 1.3[b]	<0.0001	—	—	—
Relative intake							
Energy, kcal/kg/d	26.7 ± 2.4[a]	29.1 ± 3.0[a]	62.0 ± 6.0[b]	<0.0001	28.3 ± 9.6	26.4 ± 10.3	0.61
Carbohydrate, g/kg/d	4.0 ± 0.4[a]	4.4 ± 0.5[a]	9.5 ± 1.0[b]	<0.0001	2.9 ± 1.2	2.7 ± 1.3	0.67
Protein, g/kg/d	0.7 ± 0.1[a]	0.8 ± 0.1[a]	1.6 ± 0.1[b]	<0.0001	1.2 ± 0.4	1.2 ± 0.5	0.77
Fat, g/kg/d	0.8 ± 0.1[a]	1.0 ± 0.1[a]	2.1 ± 0.2[b]	<0.0001	1.3 ± 0.4	1.2 ± 0.5	0.59
Energy intake, %							
Carbohydrate	61 ± 0.7[a]	59 ± 0.6[b]	60 ± 1.0[b]	<0.0001	40 ± 8.0	40 ± 7.8	0.91
Protein	11 ± 0.3[a]	11 ± 0.8[a]	10 ± 0.2[b]	<0.0001	18 ± 3	18 ± 3.0	0.75
Fat	28 ± 0.6[a]	30 ± 0.7[b]	30 ± 1.0[b]	<0.0001	41 ±5.8	43 ± 7.1	0.96
Daily energy expenditure, kcal/d[3]	—	4573 ± 989	4775 ± 940	—	—	—	0.69
Resting metabolic rate, kcal/d	1905 ± 241	—	—	—	—	—	—
Thermic effect of food, kcal/d	—	457 ± 99	478 ± 94	—	—	—	0.70
Activity-induced energy expenditure, kcal/d	—	2201 ± 802	2383 ± 737	—	—	—	0.69
Energy balance, kcal/d	—	–2047 ± 920	689 ± 852	—	—	—	<0.001
Energy deficit, %	—	–43 ± 9	18 ± 20	—	—	—	<0.001

[1] Values are means ± SDs; $n = 10$. Dietary intake for the day preceding rest (day 1), average intake across the SUSOPS period during DEF and BAL, and average intake from 3-d food records completed on days 6–8 and 16–18 during recovery from SUSOPS DEF or BAL. Differences in dietary intake for the controlled feeding days (rest, SUSOPS DEF, and SUSOPS BAL) were analyzed by 1-way ANOVA. Bonferroni corrections were used for post hoc comparisons. Different letters indicate significant difference. Paired Student *t*-tests were used to compare dietary intake during the recovery recall days and energy balance during SUSOPS DEF and SUSOPS BAL. BAL, energy balance; DEF, energy deficit; SUSOPS, sustained military operations

[2] Differences between dietary and total iron during SUSOPS are due to supplemental iron provided during SUSOPS DEF (i.e., dietary iron and total iron were the same during rest, SUSOPS BAL, and recovery).

[3] $n = 9$, 1 participant served as study control for doubly labeled water.

ng/mL, *P*-adjusted = 1.00); ferritin concentrations following SUSOPS DEF were 43% greater on day 3 (27.3 ± 33.2 ng/mL, *P*-adjusted < 0.0001) and 40% greater on day 1 of recovery (24.3 ± 36.0 ng/mL, *P*-adjusted < 0.0001) compared with BAL. There was an initial decline in serum iron (−43.3 ± 30.4 μg/dL, *P*-adjusted < 0.01) and transferrin saturation (−12.8 ± 10.2%, *P*-adjusted < 0.01) on day 2 of SUSOPS BAL compared with day 1, whereas concentrations were maintained during SUSOPS DEF (serum iron: −14.1 ± 30.4 μg/dL, *P*-adjusted = 1.00; transferrin saturation: −4.1 ± 9.6%, *P*-adjusted = 1.00). Serum iron and transferrin saturation declined during recovery from both SUSOPS DEF and SUSOPS BAL compared with day 1 of SUSOPS. sTfR declined on day 2 of SUSOPS BAL and continued to decline to day 1 of recovery compared with day 1 of SUSOPS (−0.37 ± 0.18 nmol/L, *P*-adjusted < 0.0001). sTfR was maintained during SUSOPS DEF, but then declined on day 1 of recovery compared with day 1 of SUSOPS (−0.26 ± 0.24 nmol/L, *P*-adjusted < 0.01) before returning to pre-SUSOPS levels. ERFE did not change during either SUSOPS trial or recovery.

Plasma iron isotope appearance

Trial, time, and interaction effects were observed for plasma iron isotope appearance ($P < 0.0001$ for all). Plasma iron isotope appearance peaked 120 min after ingesting the stable iron isotope following rest and mean appearance at 120 min was 74% lower following SUSOPS DEF and 49% lower following SUSOPS BAL compared with rest (**Figure 3**A). Participants absorbed 21.6 ± 8.7% of the 3.8-mg iron dose at rest, 12.9 ± 6.8% at the end of SUSOPS BAL, and 7.2 ± 3.7% at the end of SUSOPS DEF (*P*-trial < 0.0001, Figure 3B). There were trial, time, and interaction effects for hepcidin ($P < 0.0001$ for all). Hepcidin increased 360 min after ingestion of the stable iron isotope following rest (12.2 ± 8.2 ng/mL, *P*-adjusted < 0.0001) and following SUSOPS DEF (7.0 ± 7.9 ng/mL, *P*-adjusted < 0.0001) compared with 0 min, but did not increase following SUSOPS BAL (2.8 ± 4.2 ng/mL; *P*-adjusted = 0.71, Figure 3C). Trial and time effects ($P < 0.0001$ for both) were observed for serum iron, but no interaction (*P*-interaction = 0.17, Figure 3D). Mean serum iron concentrations were greater at the end of rest compared with SUSOPS DEF (38 ± 23 μg/dL; *P*-adjusted < 0.0001) and SUSOPS BAL (53 ± 31 μg/dL; *P*-adjusted < 0.0001) and at the end of SUSOPS DEF compared with SUSOPS BAL (14.6 ± 21 μg/dL; *P*-adjusted < 0.0001). Serum iron concentrations increased 360 min following ingestion of iron compared with 0 min (54 ± 27 μg/dL; *P*-adjusted < 0.0001). There was an effect of trial (*P*-trial < 0.0001), but no time (*P*-time = 0.27) or interaction effect (*P*-interaction = 1.00) for serum ferritin (Figure 3E). Serum ferritin was greater following SUSOPS DEF compared with rest (18 ± 30 ng/mL; *P*-adjusted < 0.0001) and SUSOPS BAL (23 ± 33 ng/mL; *P*-adjusted < 0.0001). Trial ($P < 0.0001$) and time ($P = 0.02$), but no interaction ($P = 0.15$) effects were found for ERFE.

TABLE 3 Clinical biomarkers during and after 72-h simulated sustained military operations[1]

	DEF								BAL								*P* value		
	SUSOPS, d			Recovery, d					SUSOPS, d			Recovery, d							
	1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10	Trial	Time	TxT
Body weight, kg	88.3 ± 10.8	87.5 ± 10.6*	86.4 ± 10.8†*	87.3 ± 10.2*	88.2 ± 10.4	88.5 ± 10.7	88.6 ± 10.6	88.7 ± 10.7	88.1 ± 10.9	88.6 ± 10.8	88.6 ± 11.3	89.3 ± 10.3	88.7 ± 10.4	88.4 ± 10.3	88.5 ± 10.3	88.8 ± 10.4	<0.0001	<0.0001	<0.0001
Muscle glycogen, mmol/kg	495.1 ± 107.6	—	—	247.5 ± 75.1†*	—	—	—	—	498.4 ± 162.7	—	—	448.2 ± 174.7	—	—	—	—	0.02	0.002	0.032
Insulin, mIU/mL	10.1 ± 4.3	—	7.0 ± 3.4	—	11.2 ± 6.8	—	15.1 ± 12.2	15.7 ± 20.3	10.6 ± 5.4	—	7.1 ± 2.5	—	12.1 ± 8.4	—	12.9 ± 7.8	10.7 ± 6.4	0.62	0.02	0.62
Glucose, mg/dL	89.5 ± 8.9	—	84.1 ± 8.0	—	91.4 ± 11.8	—	93.1 ± 16.2	92.5 ± 12.3	92.2 ± 11.7	—	89.0 ± 9.1	—	93.0 ± 12.2	—	91.4 ± 11.9	89.6 ± 10.4	0.65	0.09	0.23
FFA, mmol/L	0.35 ± 0.08	—	0.86 ± 0.16†*	—	0.29 ± 0.10	—	0.20 ± 0.09	0.17± 0.13†	0.36 ± 0.15	—	0.44 ± 0.09	—	0.32 ± 0.15	—	0.22 ± 0.12	0.28 ± 0.19	0.03	<0.0001	<0.0001
Glycerol, mmol/L	0.032 ± 0.006	—	0.079 ± 0.040†*	—	0.036 ± 0.005	—	0.030 ± 0.000	0.036 ± 0.010	0.031 ± 0.003	—	0.036 ± 0.011	—	0.034 ± 0.011	—	0.038 ± 0.014	0.032 ± 0.004	<0.01	<0.0001	<0.0001
Testosterone, ng/dL	503.3 ± 102.0	—	439.5 ± 95.9	—	440.3 ± 118.2	—	518.1 ± 95.2	524.3 ± 116.3	497.5 ± 144.8	—	422.3 ± 107.1	—	465.8 ± 104.2	—	509.8 ± 122.2	521.2 ± 116.9	0.94	<0.0001	0.88
EPO, mIU/mL	7.28 ± 0.9	—	—	13.82 ± 1.7	—	—	—	—	6.79 ± 1.3	—	—	13.15 ± 2.5	—	—	—	—	0.30	<0.0001	0.85
Myoglobin, ng/mL	33.0 ± 8.9	—	47.2 ± 16.6	—	29.1 ± 13.0	—	26.6 ± 11.0	32.1± 16.5	30.1 ± 6.3	—	46.3 ± 20.9	—	30.6 ± 10.5	—	28.7 ± 10.5	30.4 ± 11.4	0.53	<0.0001	0.89
CPK, IU/L	126.9 ± 80.8	—	951.9 ± 1161.0	—	316.8 ± 251.7	—	157.7 ± 130.3	171.5 ± 195.6	135.2 ± 94.8	—	782.5 ± 666.1	—	200.4 ± 114.9	—	151.9 ± 120.2	149.5 ± 153.8	0.39	<0.0001	0.96
LDH, IU/L	121.2 ± 19.4	—	166.8 ± 47.2	—	147.1 ± 34.0	—	129.6 ± 25.3	127.9 ± 19.9	118.5 ± 14.0	—	157.7 ± 39.4	—	146.7 ± 24.5	—	136.6 ± 23.5	124.7 ± 22.6	0.52	<0.0001	0.44
CRP, mg/L	1.13 ± 0.9	3.38 ± 2.2*	7.83 ± 5.0†*	4.78 ± 3.5†	3.40 ± 2.2	1.94 ± 1.4	1.44 ± 1.0	1.27 ± 0.9	1.11 ± 0.7	2.18 ± 1.3	5.23 ± 3.4†	4.67 ± 3.6†	4.35 ± 3.3†	2.95 ± 2.0	2.10 ± 1.4	1.46 ± 1.1	0.06	<0.0001	<0.01
IL-6, pg/mL	2.85 ± 1.6	11.2 ± 6.5	3.6 ± 2.4	4.35 ± 4.1	5.15 ± 7.2	8.03 ± 7.8	1.57 ± 0.3	2.46 ± 2.2	2.29 ± 1.2	11.1 ± 5.3	3.70 ± 1.3	4.86 ± 6.4	5.70 ± 8.6	7.70 ± 6.8	5.96 ± 5.7	1.15 ± 1.4	0.76	<0.0001	0.77
Hepcidin, ng/mL	8.40 ± 2.2	11.91 ± 4.7	14.95 ± 10.1†*	13.73 ± 8.9†*	4.61 ± 1.8	3.95 ± 0.6	3.84 ± 0.8	5.52 ± 3.0	8.42 ± 2.6	11.08 ± 5.1	7.20 ± 3.4	9.47 ± 4.7	6.11 ± 3.7	4.79 ± 1.9	3.81 ± 1.3	4.80 ± 1.8	<0.0001	<0.0001	0.001
Hemoglobin, g/dL	15.7 ± 0.7	14.7 ± 1.1†	15.2 ± 1.0*	13.3 ± 1.0†	14.2 ± 0.8†	14.5 ± 0.9†	14.6 ± 1.4†*	15.3 ± 0.6*	16.1 ± 0.7	14.7 ± 0.8†	14.4 ± 0.8†	12.9 ± 1.0†	14.7 ± 0.9†	15.4 ± 0.9	15.2 ± 1.4	15.4 ± 0.7	0.10	<0.0001	<0.0001
Ferritin, ng/mL	77.3 ± 34.4	87.2 ± 35.1	103.2 ± 41.9†*	98.5 ± 38.0*	87.7 ± 27.9	73.6 ± 25.0	64.2 ± 20.7	65.0 ± 33.8	79.9 ± 33.7	77.0 ± 31.4	75.9 ± 31.6	74.1 ± 31.9	82.0 ± 33.6	79.8 ± 32.7	66.9 ± 29.7	57.5 ± 23.9	<0.0001	<0.0001	0.01
Serum iron, μg/dL	115.2 ± 35.8	101.1 ± 17.7*	125.5 ± 35.9*	128.5 ± 20.4	53.0 ± 11.4†	55.4 ± 10.5†	65.2 ± 24.3†	64.3 ± 21.6†*	101.7 ± 35.5	58.4 ± 15.8†	88.0 ± 14.8	130.9 ± 34.4	58.7 ± 16.5†	67.7 ± 25.0†	57.3 ± 13.7†	108.6 ± 38.1	0.11	<0.0001	<0.0001
Transferrin saturation, %	35.8 ± 11.5	31.7 ± 4.9*	40.6 ± 10.3*	46.0 ± 5.2	17.5 ± 3.4†	18.1 ± 4.2†	21.9 ± 10.0†	19.2 ± 7.1†*	31.9 ± 12.0	19.1 ± 5.4†	30.8 ± 6.9	47.2 ± 14.8†	19.0 ± 6.7	22.2 ± 10.0	18.6 ± 4.8†	32.6 ± 11.7	0.22	<0.0001	<0.0001
sTfR, nmol/L	20.7 ± 3.9	19.4 ± 4.0	18.9 ± 3.3	17.6 ± 2.5†	18.4 ± 3.9†*	19.8 ± 3.8	20.3 ± 3.8	24.0 ± 4.5†*	21.4 ± 3.7	19.0 ± 3.4†	18.1 ± 4.0†	17.0 ± 2.5†	19.7 ± 4.2	20.1 ± 3.6	20.7 ± 3.6	22.0 ± 3.4	0.29	<0.0001	0.05
ERFE, ng/mL	0.91 ± 1.5	0.90 ± 1.4	1.06 ± 1.4	1.10 ± 1.2	1.49 ± 1.3	1.04 ± 1.3	1.01 ± 1.5	0.90 ± 1.3	0.88 ± 1.5	0.98 ± 1.3	1.06 ± 1.4	1.06 ± 1.2	1.39 ± 1.5	1.04 ± 1.4	0.93 ± 1.4	1.05 ± 1.4	0.84	0.03	1.00

[1] Values are means ± SDs; $n = 10$. Effects of trial, time, and trial-by-time interactions were analyzed using linear mixed models. To test for carryover effects, a main effect of trial order and order-by-trial interaction was included in the model; no effects were observed, and these data are not shown. Bonferroni corrections were used for post hoc comparisons. †Different from day 1 SUSOPS within a trial ($P < 0.05$). *Trial difference within a timepoint ($P < 0.05$). BAL, energy balance; CPK, creatine phosphokinase; CRP, c-reactive protein; DEF, energy deficit; EPO, erythropoietin; ERFE, erythroferrone; FFA, free fatty acids; LDH, lactate dehydrogenase; sTfR, soluble transferrin receptor; SUSOPS, sustained military operations.

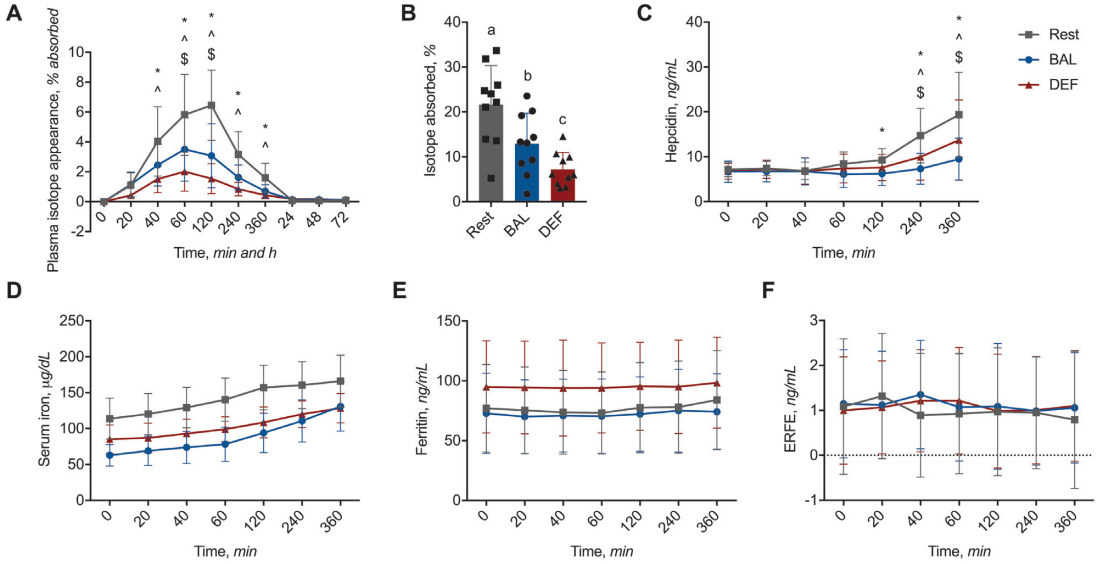

FIGURE 3 Time course of plasma iron isotope appearance, hepcidin, erythroferrone, and iron status indicators following consumption of a beverage containing an oral iron isotope. Time course of plasma iron isotope appearance (A). Data represent the percentage of isotope absorbed at a given time point of the total fraction that was absorbed, P-trial < 0.0001, P-time < 0.0001, P-interaction < 0.0001. Total amount of isotope absorbed (B). Time course of hepcidin (C), P-trial < 0.0001, P-time < 0.0001, P-interaction < 0.0001; serum iron (D), P-trial < 0.0001, P-time < 0.0001, P-interaction $= 0.17$; ferritin (E), P-trial < 0.0001, P-time $= 0.27$, P-interaction $= 1.00$; and ERFE (F), P-trial < 0.0001, P-time $= 0.02$, P-interaction $= 0.15$, after consuming an oral iron isotope following rest, SUSOPS BAL, and SUSOPS DEF. Values are means ± SD; $n = 10$. Effects of trial, time, and trial-by-time interactions were analyzed using linear mixed models. To test for carryover effects, a main effect of trial order and order-by-trial interaction was included in the model; no effects were observed, and these data are not shown. Bonferroni corrections were used for post hoc comparisons. *Rest different from BAL, $P < 0.05$; ^ rest different from DEF, $P < 0.05$; $BAL different from DEF, $P < 0.05$. Different letters indicate significant difference. BAL, energy balance; DEF, energy deficit; ERFE, erythroferrone.

Raw data for ERFE are shown but statistics were performed on log-transformed data. ERFE was lower following rest compared with SUSOPS DEF (−0.09 ± 0.63 ng/mL; P-adjusted < 0.0001) and SUSOPS BAL (−0.13 ± 0.56 ng/mL; P-adjusted < 0.0001).

Discussion

Those who frequently engage in strenuous physical activity are vulnerable to declines in iron status (5). Declines in iron status are associated with reduced physical performance and cognitive impairments (22), highlighting the importance of understanding the mechanisms by which iron status declines with physical activity. The major finding of this study was that 72 h of strenuous physical activity decreased dietary iron absorption compared with rest in nonanemic individuals with sufficient iron status. Moreover, findings indicate that energy deficit during strenuous physical activity increased hepcidin and diminished iron absorption compared with energy balance.

Few studies have directly examined the effects of physical activity on iron absorption using radioactive or stable iron isotopes. The first such study was conducted prior to the discovery of hepcidin and compared iron absorption in 8 male elite distance runners to 8 male blood donors with depleted iron stores (23). Using radiolabeled iron ($^{59}FeSO_4$) and measuring its incorporation into red blood cells, the authors found ∼50% (although statistically nonsignificant) reduction in iron absorption in runners compared with blood donors (runners 16 ± 13%, blood donors 30 ± 33%). A reduction in iron absorption is consistent with findings from the current study. However, recently, Moretti et al. conducted a cross-over intervention during which 10 iron-sufficient recreational male runners completed a 16-d control phase of no running followed by a training phase during which participants ran 8 km on alternate days for 22 d (24). On day 1 of the control phase and day 5 of the training phase participants were given a test beverage containing $^{57}FeSO_4$ to determine oral iron absorption and intravenous ^{58}Fe citrate to determine erythroid iron utilization. Whole blood was collected 16 days later to determine isotope incorporation into red blood cells. Despite an increase in IL-6, plasma hepcidin decreased and oral iron absorption increased with training compared with rest. The authors attribute these findings to the increased erythropoietic demand of exercise, which is consistent with previous studies that have demonstrated that erythropoiesis strongly suppresses hepcidin (25). In the current study, hepcidin increased with energy deficit during physical activity, but not energy balance, despite similar increases in IL-6 and EPO. The apparent discrepancy between findings from the current study and the study described above may be due to the length of the training program. Given the short duration of physical activity in the current study, it is unlikely that the observed increase in EPO produced a meaningful increase in erythropoiesis (26). Future studies should consider the interaction between erythropoiesis and gluconeogenic stimuli on the hepcidin response and its contribution to dietary iron absorption and iron status during short- and longer-term physical activity.

The increase in hepcidin in the current study is likely due to energy deficit, and not sleep deprivation, as a time-dependent increase in hepcidin has been observed in humans following

an 18-, 42-, and 66-h fast, but not prolonged (50 h) sleep deprivation (27). The increase in hepcidin with energy deficit suggests that the PPARGC1A/CREBH pathway of hepcidin activation may contribute to the observed increase in hepcidin with physical activity. In this model, PPARGC1A and CREBH would stabilize CREBH binding and transactivate the hepcidin (*HAMP*) promoter in response to gluconeogenic stimuli during energy deficit (28, 13). CREBH, encoded by *CREB3L3*, is not only upregulated in response to gluconeogenic stimuli in energy-depleted conditions, but also by the unfolded protein response during endoplasmic reticulum stress and the acute-phase inflammatory response (28, 13). While most markers of inflammation and muscle damage increased with physical activity regardless of energy status, the slightly higher concentrations of CRP with energy deficit suggest a greater inflammatory response compared with energy balance. Likewise, the increase in ferritin with physical activity during energy deficit (and following ingestion of the oral iron isotope), but not energy balance, likely indicate a heightened acute phase response and not an improvement in iron stores, as changes in ferritin tend to reflect changes in inflammatory status and CRP. Thus, it is possible that the acute-phase response and gluconeogenic stimuli may cooperate to upregulate hepcidin during physical activity, particularly when energy stores are depleted. These findings do not necessarily rule out effects of other mediators, such as the mammalian target of rapamycin (29), on the hepcidin response to exercise and cellular iron metabolism.

Interestingly, although there was an increase in hepcidin with energy deficit during physical activity, hepcidin concentrations were not different when the oral iron isotope was ingested and remained the same 2 h postingestion. It is also noteworthy that the decrease in iron absorption during energy balance occurred despite no change in hepcidin with physical activity. This may be due, in part, to the timing of the blood draws and the transient nature of the IL-6 and hepcidin response. IL-6 and hepcidin both have short half-lives of several minutes (30, 31). Muscle-derived IL-6 peaks immediately following exercise and rapidly declines to baseline concentrations (17). Immune cells produce a more sustained increase in IL-6 to repair tissue damage, but the response is lower in magnitude than the initial peak in IL-6 from muscle. Alternatively, Zimmermann et al. reported that hepcidin is a modest predictor of dietary iron absorption, suggesting that other factors may influence iron absorption (19). Whether the decline in iron absorption during energy balance is dependent on hepcidin warrants further study.

Previous studies demonstrate that circulating concentrations of hepcidin are lowest in the early morning and increase throughout the day (27). Thus, it is likely that the increase in hepcidin following rest reflects diurnal variation (32, 33) and not an increase in response to ingestion of 3.8 mg iron. This would be consistent with studies demonstrating that hepcidin increases following ingestion of larger doses of iron (e.g., > 40 mg iron) (6, 19, 34). The increased amplitude of the hepcidin response to diurnal variation following rest likely reflects that the participants had a relatively low iron requirement. In contrast, the suppressed amplitude of the hepcidin response following physical activity perhaps indicates an increased requirement for iron. Although the current study was not designed to produce robust changes in iron status, in general, iron status tended to decline with physical activity in both conditions. This is reflected by a decrease in transferrin saturation during recovery from physical activity and lower serum iron concentrations following ingestion of the oral iron isotope.

The strengths of this study include the cross-over design, the use of stable isotopes to determine dietary iron absorption, and the tightly controlled nature of all study conditions, including diet and exercise. This study has several limitations. First, the study included only 10 participants. Although the study employed a cross-over design, findings should be replicated with a larger sample size. Secondly, the numerous study outcomes increase the risk of making a type I error. Lastly, iron-sufficient male participants were chosen in the current study because low iron stores suppress the hepcidin response to physical activity, even in the presence of inflammation (35); however, future studies should include a more diverse study sample, including females and individuals with a range of iron stores.

To our knowledge, this is the first randomized controlled trial to demonstrate the relationship between physical activity–induced declines in iron absorption, the hepcidin response, and energy status. A major finding from the current study was that energy deficit during physical activity increased hepcidin concentrations and diminished iron absorption compared with energy balance, suggesting that interventions to maintain energy balance may be an effective strategy to prevent the decline in iron status with physical activity. These findings may be important for designing and implementing policies to prevent and treat iron deficiency in military personnel, endurance athletes, and potentially other populations that experience negative energy balance, such as in areas where malnutrition and infection are common.

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