The effect of calcium supplementation on bone calcium balance and calcium and bone metabolism during load carriage in women: a randomised controlled crossover trial

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

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Conflicts of interest disclosure

Anton Eisenhauer, Ana Kolevica, and Alexander Heuser are consultants for Osteolabs GmbH. The other authors have no competing interest to declare.

Ethics approval

This study was approved by the Ministry of Defence Research Ethics Committee (Ref: 1021/MoDREC/19).

Patient consent statement

Each participant had the study procedure and risks explained verbally and in writing before providing written informed consent.

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Not applicable. All material within this study is original.

Clinical trial registration: NCT04823156 (ClinicalTrials.gov)

Abstract

Calcium supplementation before exercise attenuates the decrease in serum calcium and increase in PTH and bone resorption. This study investigated the effect of calcium supplementation on calcium and bone metabolism during load carriage in women. Forty-eight women completed two load carriage sessions (load carriage 1 *n* = 48; load carriage 2 *n* = 40) (12.8 km in 120 min carrying 20 kg) 60 min after consuming either 1000 mg calcium (Calcium) or nothing (Control) in a randomised order. Pre- and post-exercise urine samples were analysed for calcium isotope ratio ($\delta^{44/42}$Ca). Fasted blood samples were taken before (pre-exercise), during (0, 20, 40, 60, 80, 100, 120 min), and after (+15, +30, +60, +90 min) exercise and analysed for markers of calcium and bone metabolism. There was no effect of load carriage or supplementation on urine $\delta^{44/42}$Ca ($p \geq 0.110$). Serum $\delta^{44/42}$Ca did not change with load carriage in Control ($p = 0.617$) but increased in Calcium ($p = 0.003$) and was higher at 120 min in Calcium *vs* Control ($p = 0.018$). Ionised calcium (iCa) decreased from pre-exercise to all exercise time-points ($p < 0.001$); iCa was higher in Calcium than Control throughout ($p < 0.001$). PTH increased from pre-exercise to 120 min in Control ($p < 0.001$) but decreased from pre-exercise to all time-points in Calcium ($p < 0.001$). PTH was higher in Control than Calcium from 0 to +90 min ($p < 0.001$). βCTX decreased from pre-exercise to 20 to +15 min in Control ($p \leq 0.004$); βCTX decreased from pre-exercise to 0 to +90 min in Calcium ($p < 0.001$). βCTX was lower in Calcium than Control from 20 to +90 min ($p \leq 0.036$). A 1000 mg calcium supplement before load carriage promotes bone calcium balance and prevents disruptions to bone and calcium homeostasis.

Key Words: Bone Metabolism, Calcium Metabolism, Supplement, Exercise, Female Health, Load carriage, Military

Lay summary

Strenuous exercise can place a high mechanical and metabolic demand on the skeleton and disturb calcium metabolism. Calcium supplementation before exercise can prevent this disturbance. This study investigated the effect of calcium supplementation on calcium and bone metabolism during load carriage exercise in women. Forty-eight women (military n = 38; civilian n = 10; average age 29 years) volunteered. Each participant completed a load carriage exercise session on two separate occasions in a randomised order; one with a 1000 mg calcium supplement (Calcium) 60 min before load carriage and one without (Control). Participants walked 12.8 km on the treadmill at 6.4 $km \cdot h^{-1}$ for 120 min whilst wearing a 20 kg rucksack. Venous blood samples were drawn pre-, during, and post-load carriage from an indwelling cannula. Venous blood samples were analysed for biochemical markers of bone and calcium metabolism. Calcium supplementation increased serum ionised calcium, lowered parathyroid hormone, and lowered bone resorption in the Calcium condition compared with the Control condition. Calcium supplementation had no effect on markers of bone formation. Consuming a 1000 mg calcium supplement 60 min before load carriage is protective of bone and calcium homeostasis.

Introduction

Military training is physically arduous and involves prolonged periods of load carriage.[1] The physical demands of military training[2] and load carriage[1] are greater in women than men, and women have a greater incidence of stress fractures in military training[3]. Military training places significant mechanical and metabolic demands on the skeleton,[4–9] and decreases in areal BMD in the axial skeleton [6,10] is indicative of disturbed calcium homeostasis (defined as a decrease in serum ionised calcium).[11] Serum calcium concentrations are tightly regulated and strenuous exercise disturbs calcium homeostasis; exercise decreases ionised calcium, stimulating release of PTH leading to increased bone resorption to maintain circulating concentrations of calcium.[11] Maintaining serum calcium through calcium infusion,[12,13] a high calcium meal,[14,15] or calcium supplementation[16–19] attenuates the increase in PTH and / or markers of bone resorption. In young men and women, 60 min load carriage exercise decreased serum ionised calcium, increased PTH and bone resorption,[20,21] and increased fractional calcium absorption and bone calcium balance,[21] demonstrating that military activities also disturb calcium homeostasis. It is unclear if acute calcium supplementation before load carriage exercise prevents the disturbance in calcium homeostasis in women.

There are six naturally occurring stable calcium isotopes in food and water (^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, and ^{48}Ca) and the ratio of two isotopes (*e.g.*, $\delta^{44/40}$Ca or $\delta^{44/42}$Ca) provides a measure of calcium isotopic composition of a biological sample.[22] Bone formation alters the relative abundance of light and heavy calcium isotopes and bone is isotopically lighter than soft tissue.[22] Using the model formulated by Skulan and DePaolo,[23] which describes the fractionation of calcium isotopes between soft and mineralized tissue, it is possible to quantitively determine net bone calcium loss and gain (bone calcium balance) through changes in the ratio of ^{42}Ca and ^{44}Ca ($\delta^{44/42}$Ca).[23,24] Bone formation favours the uptake of isotopically

lighter calcium isotopes—creating a positive bone calcium balance—that causes a shift in blood / urine composition towards an isotopically heavier state.[25] When bone resorption is dominant and calcium is lost from bone, the isotopic calcium composition of the blood / urine becomes lighter[24] and decreases in aBMD can occur. Blood is the central compartment from where calcium is exchanged[24] and blood and urine $\delta^{44/42}$Ca are strongly correlated.[26,27] Calcium homeostasis and calcium flux to and from the bone can be determined through changes in serum $\delta^{44/42}$Ca and non-invasively through urine $\delta^{44/42}$Ca[24–31] without the need for the expensive and logistically challenging dual-stable isotope technique. The measurement of $\delta^{44/42}$Ca provides a direct measurement of changes to bone calcium balance, whereas biochemical markers of bone resorption and formation infer acute bone turnover.[22,25,26] Naturally occurring calcium isotopes have been used to determine changes to bone calcium balance in bed rest studies[22,25,29,30] and kidney disease.[26]

The effect of load carriage and calcium supplementation on naturally occurring calcium isotopes is unknown. This study investigated the effect of calcium supplementation on calcium and bone metabolism in response to load carriage exercise in women, a population at increased risk of stress fracture during military training compared with men.[3] It was hypothesised that calcium supplementation would attenuate the decline in serum ionised calcium and increase in PTH and bone resorption, and prevent a negative bone calcium balance during load carriage.

Materials and Methods

Study design

This trial was registered as NCT04823156 (ClinicalTrials.gov) and the protocol has been published.[32] This was a non-blinded randomised controlled crossover trial. Following the completion of a screening visit to confirm eligibility (Pre-Screening; visit 1), each participant completed a load carriage exercise session on two separate occasions (visits 2 and 3) in a

randomised order; one with a calcium supplement (Calcium) (1000 mg) and one without (Control) (Figure 1A). Participants were randomised to trial order by the investigators with block randomisation and a block size of two. This study was approved by the Ministry of Defence Research Ethics Committee (Ref:1021/MoDREC/19). All laboratory testing was completed at the Army Human Performance Laboratory, Sandhurst, UK.

INSERT FIGURE 1 HERE

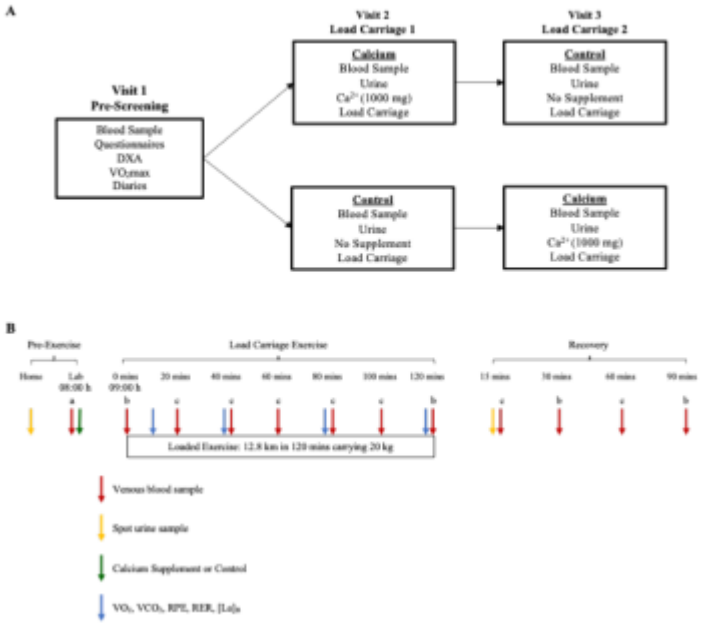

Figure 1. Overview of the study design (**A**) and load carriage exercise testing protocol (**B**).

Ca^{2+}, calcium supplement; Questionnaires: physical activity readiness questionnaire, menstrual history questionnaire, and Eating Attitudes Test – 26 item; Diaries: exercise and menstrual; DXA, dual-energy X-ray absorptiometry; $\dot{V}O_2max$, maximal rate of oxygen uptake. $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide production; RPE, rating of perceived exertion; RER, respiratory exchange ratio; $[La]_B$, blood lactate concentration. Calcium supplement: 1000 mg elemental calcium ingested 1 h prior to load carriage exercise. Control: No supplement provided.

[a]Procollagen type 1 N-terminal propeptide, bone-specific alkaline phosphatase, beta carboxy-terminal cross-linking telopeptide of type 1 collagen, albumin-adjusted calcium, ionised calcium, $\delta^{44/42}Ca$, phosphate, intact parathyroid hormone, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, 24,25-dihydroxyvitamin D, luteinising hormone, follicle stimulating hormone, oestradiol, testosterone, and sex hormone binding globulin were measured before load carriage.

[b]Procollagen type 1 N-terminal propeptide, beta carboxy-terminal cross-linking telopeptide of type 1 collagen, ionised calcium, albumin-adjusted calcium, phosphate, intact parathyroid hormone, sclerostin and osteocalcin

were measured at 0 and 120 min during load carriage, then 30 and 90 min after load carriage. $\delta^{44/42}Ca$ were measured at 120 minutes during load carriage.
[c]Procollagen type 1 N-terminal propeptide, beta carboxy-terminal cross-linking telopeptide of type 1 collagen, ionised calcium, albumin-adjusted calcium, phosphate, and intact parathyroid hormone were measured at 20, 40, 60, 80, and 100 min during load carriage, and 15 and 60 min post load carriage.

Participants

Regular and Reserve servicewomen in the UK Armed Forces and female civilians were invited to participate from July 2021 to March 2023. Inclusion criteria were: 18 to 36 years old; maximal rate of oxygen uptake ($\dot{V}O_{2max}$) of ≥35 $mL \cdot kg^{-1} \cdot min^{-1}$; eumenorrhoeic or using the combined oral contraceptive pill, an intrauterine system, or intrauterine device; body mass stable, defined as <5% change in self-reported body mass over the previous three months; BMI between 18 and 30 $kg \cdot m^{-2}$, and; not be pregnant (determined by pregnancy test) or lactating. Exclusion criteria were: disordered eating, defined as ≥20 on the Eating Attitudes Test–26 item (EAT-26); total 25(OH)D <30 $nmol \cdot L^{-1}$; whole-body BMD T-score <−1; oligomenorrhorea or amenorrhorea (<9 menstrual cycles in previous 12 months); current smoker or stopped smoking within the previous three months; taking medication known to affect bone or calcium metabolism (*e.g.*, treatment for thyroid disorders); history of heart, liver, or kidney disease, diabetes, or treated thyroid disorder; any bone injury in the previous 12 months; musculoskeletal injury that prevented exercise, or; haemoglobin <12 $g \cdot dL^{-1}$. Procedures and risks were explained verbally and in writing to participants before providing written informed consent.

Pre-screening

Participants completed a menstrual history questionnaire, the EAT-26 questionnaire, and declared current medication (including hormonal contraceptives), supplement use, and previous history of fractures. A single venous blood sample was taken for the measurement of haemoglobin and serum total 25(OH)D. Height (SECA 213, SECA, Germany), body mass

(SECA 869, SECA, Germany), and whole-body BMD, T-score, body fat, and lean mass were measured (Lunar iDXA™, GE Healthcare, USA). An incremental maximal exercise test was performed to determine $\dot{V}O_{2max}$. Participants ran to volitional exhaustion; the treadmill (ELG, Woodway, USA) started at 5 km·h^{-1} and increased by 1 km·h^{-1} per minute (1% incline). Participants wore a facemask (Hans Rudolph 7450, Cortex Biophysik, Germany) and expired and inspired gases were measured throughout using an online gas analyser (Metalyser 3B, Cortex Biophysik, Germany). Maximal rate of oxygen uptake was defined as the highest 30 s rolling average in recorded oxygen uptake ($\dot{V}O_2$). Participants were issued a menstrual diary to record menses dates until the end of the study for the scheduling of load carriage sessions.

Load carriage

Load carriage exercise was performed on the treadmill in the laboratory, separated by a minimum of two weeks. Participants not taking hormonal contraceptives started each testing session within the few days before or after menstruation. Participants using the combined oral contraceptive pill completed each testing session during the seven-day placebo phase; participants taking the combined oral contraceptive pill continuously or using an intrauterine system or intrauterine device were tested at any point. Participants rested and did not apply skin moisturiser in the 24-hours preceding each testing session.

Participants arrived at the laboratory (07:30h) after an overnight fast, had an indwelling cannula inserted in a vein in the antecubital fossa, and a baseline (pre-exercise) resting blood sample taken (08:00h) (Figure 1B). Participants remained fasted throughout the trial. For the Calcium trial, participants consumed a 1000 mg calcium supplement (08:00h) (2 × Calcichew 500 mg Chewable Tablets [calcium carbonate]) 60 min before exercise (09:00h); this dose and timing of calcium attenuates disturbances in calcium metabolism.[11] For the Control trial, participants

rested for 60 min before exercise. Sweat collectors (Macroduct Sweat Collector, ELITech Group, France) were attached to the forearm and remained in place for the exercise for the measurement of sweat calcium. Participants walked 12.8 km at 6.4 $km \cdot h^{-1}$ with a 1% incline for 120 min whilst wearing a 20 kg standard issue military backpack. This load carriage test is an annual fitness test completed by soldiers in the British Army. If a participant could not complete the full 120 min exercise bout, the mass was reduced by 5 kg when the next blood sample was drawn, and the mass carried in the first trial was matched in the second trial; all participants completed 120 min exercise at the required speed. Expired gases, heart rate, and rating of perceived exertion were measured for the first 10 min, then from min 35 to 40, 75 to 80, and 115 to 120 of exercise (Figure 1B). Expired gases were measured using a face mask (Hans Rudolph 7450, Cortex Biophysik, Germany) and heart rate was measured using a chest strap (Polar H10, Polar Oy, Finland) integrated through an online gas analyser (Metalyser 3B, Cortex Biophysik, Germany). Average $\dot{V}O_2$, respiratory exchange ratio, and heart rate were determined from the last three minutes of each recording period. Rating of perceived exertion was measured with the 6 to 20 Borg scale at the end of the recording period. Water was consumed *ad libitum* during exercise and each participant was provided bottled water of a known calcium concentration (11 mg·L). Water consumption and calcium consumption from water was measured by weighing participant's drinks bottles before and after exercise. Nude body mass after towel drying was measured before and immediately after exercise for the determination of sweat loss and calculation of total sweat calcium excretion; post-exercise body mass was adjusted for water consumed during exercise. The whole first urine void upon waking and an immediate post-exericse urine void was obtained for the measurement of calcium isotopes for the calculation of bone calcium balance. Where a participant could not produce an immediate post-exercise urine void, a sample was collected within 60 min of the cessation of exercise. Venous blood samples were obtained during load carriage at 0, 20, 40,

60, 80, 100, 120 min, and following load carriage at +15, +30, +60, and +90 min (Figure 1B). Blood samples were fractioned into serum and plasma for analysis of markers of bone formation, bone resorption and calcium metabolism, and sex steroid hormones. Whole blood samples were analysed immediately for blood lactate concentration (Lactate Pro 2, Arkray, Japan), ionised calcium (iCa), haemoglobin, and haematocrit (i-STAT Alinity, Abbott, USA; CHEM8+, Abbott, USA) (Figure 1B).

Biological sample collection and storage

Venous blood samples were drawn into vacutainers for the collection of serum, plasma, and whole blood (Becton Dickinson, USA). Serum and plasma vacutainers were left to stand at room temperature for 30 min before centrifugation at 3000 *g* 4°C for 10 min. Serum and plasma fractions were stored at −80°C until analysis. Urine voids were collected into a 1 L container; a 10 mL volume was aspirarted into a Monovette containing 15 mg·mL boric acid (Sarstedt, UK) and stored at −80°C until analysis. Sweat samples were transferred to 1.5 mL microvettes and stored at −80°C until analysis.

Calcium isotope measurement in blood, urine, and sweat

Serum, urine, and sweat samples were analysed for total calcium and the stable calcium isotopes ^{42}Ca and ^{44}Ca ($\delta^{44/42}Ca$, details in Supplementary Methods).[31] Calcium isotope measurements were performed on a Multicollector-Inductively Coupled Plasma-Mass Spectrometer (MC-ICP-MS) (Neptune plus, Thermo Fisher Scientific, Bremen, Germany). The long-term 2-sigmal standard deviation reproducibility of the Ca isotope value was 0.06‰.

Biochemical markers of bone metabolism, calcium metabolism, and sex steroid hormones

PINP, PTH, βCTX, osteocalcin, phosphate, luteinising hormone, follicle stimulating hormone, oestradiol, and sex hormone binding globulin were measured by the COBAS automated platform (Roche Diagnostics, Mannheim, Germany). The inter-assay coefficient of variations (CV) were ≤3% within their respective analytical working ranges. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to measure serum cortisol, testosterone, 25(OH)D3, 25(OH)D2, 24,25-dihydroxyvitamin D3 (24,25$(OH)_2$D3), 24,25-dihydroxyvitamin D2 (24,25$(OH)_2$D2), 1,25$(OH)_2$D3, and 1,25$(OH)_2$D2.[33] The measurement ranges were: 0–25 nmol·L^{-1} for 24,25$(OH)_2$D2 and 24,25$(OH)_2$D3, and 15–600 pmol·L^{-1} for 1,25$(OH)_2$D2 and 1,25$(OH)_2$D3. The intra-assay CV across the measuring range of the assays were: ≤7.7% for 24,25$(OH)_2$D2, ≤9.0% for 24,25$(OH)_2$D3, and ≤7.4% for 1,25$(OH)_2$D. The cumulative inter-assay CVs were: ≤10.6% for 24,25$(OH)_2$D2, ≤8.9% for 24,25$(OH)_2$D3, and ≤9.3% for 1,25$(OH)_2$D. Testosterone and cortisol were measured by the Waters Acquity Xevo TQ-XS mass spectrometer (Waters Corporation, USA); the measurement ranges of the assays were: 0.1–45 nmol·L-1 for testosterone and 1–1200 nmol·L^{1} for cortisol. The intra-assay CVs across the measuring range of the assays were: ≤7.2% for testosterone and ≤6.4% for cortisol. The cumulative inter-assay CVs were: ≤8.5% for testosterone and ≤5.6% for cortisol. Plate-based ELISA were used to analyse bone specific alkaline phosphatase (bone ALP) (MicroVue, Quidel, San Diego, CA, USA) and sclerostin (Biomedica, Vienna, Austria) in serum. Inter-assay CV across the measurement range was ≤5.8% for bone ALP and ≤8.5% for sclerostin. All sample analysis was carried out according to manufacturers' instructions and under Good Clinical and Laboratory Practice conditions. Changes in iCa, phosphate, PTH, PINP, βCTX, osteocalcin, and sclerostin were adjusted for changes in plasma volume with exercise[34] based on changes in haematocrit:[35] Expected concentration = [Haematocrit 2 (100–Haematocrit 1)/Haematocrit 1 (100–Haematocrit 2)] × C1. The expected concentration

was then used to adjust the measured concentration: adjusted concentration = C2–(expected concentration–C1).

Where:

Haematocrit 1: Pre-exercise haematocrit.

Haematocrit 2: Haematocrit in follow-up sample (exercise or recovery).

C1: Pre-exercise concentration.

C2: Concentration in follow-up sample (exercise or recovery).

Statistical analyses

The primary outcome was to compare exercise-induced changes in urine and serum bone calcium balance ($\delta^{44/42}Ca$) between the Control and Calcium conditions. Secondary outcomes were circulating measures of calcium and bone metabolism during load carriage. The required sample size was calculated using the R programming language (version 4.1.1) and the *'pwr'* package based on the null hypothesis that there was no difference in bone calcium balance between Control and Calcium conditions. Based on differences in calcium balance between women on a high (~1500 $mg \cdot d^{-1}$) and low (~300 $mg \cdot d^{-1}$) calcium diet (108.6 ± 127.9 vs −95.2 ± 24.6 $mg \cdot d^{-1}$, d = 2.21),[36] and men on a low (376 $mg \cdot d^{-1}$) and moderate (857 $mg \cdot d^{-1}$) calcium diet (10.0 ± 6.0 vs 0.1 ± 1.8 parts per ten thousand, d = 2.24),[28] it was anticipated that five per group were required to detect a difference in bone calcium balance between Control and Calcium with an α of 0.05 and a 1–ß of 0.90. The data from these trials were using chronic diets rather than acute supplementation and so we anticipated that the effect will be smaller. Therefore, a second sample size calculation was performed using PTH as an outcome. Based on a 18.9 to 26.8 $pg \cdot mL^{-1}$[14,16,19] higher serum PTH during exercise without calcium supplementation compared with calcium supplementation (calcium meal [1352 ± 53 mg *vs* 46

± 7 mg[14]] or calcium drink [1000 mg·L^{-1}[16] (3); 486 mg·L^{-1}[19]), it was anticipated that 26 were required to detect a difference in serum PTH concentration (d = 0.667) between the Control and Calcium trials with an α of 0.05 and a 1–ß of 0.90. To account for an estimated 20% study dropout and missing blood samples, we recruited 48 women.

All data were analysed using the R programming language (*v*.4.3.2). Baseline fasted total 25(OH)D, total 24,25$(OH)_2$D, total 1,25$(OH)_2$D, FSH, LH, oestradiol, sex hormone binding globulin, testosterone, and cortisol were compared between trials using paired sample *t*-tests or Wilcoxon signed-rank tests. Linear mixed effect models with restricted maximum likelihood estimation (*lme4 package v*.1.1-35.1) were used to examine treatment effects on serum $\delta^{44/42}$Ca, urine $\delta^{44/42}$Ca, urine calcium, iCa, PTH, phosphate, βCTX, PINP, sclerostin, and osteocalcin. Linear mixed effects models were also used to compare the physiological responses to exercise (blood lactate concentration, $\dot{V}O_2$, heart rate, respiratory exchange ratio, and rating of perceived exertion) between trials. Trial (Calcium *vs* Control), time, and their interaction were included as fixed effects. Random intercepts and slopes were assigned to each participant to account for within participant correlation for repeated measures and between participant differences in response to treatment; the correlation between intercept and slope was also included in the model. We did not adjust for the effect of sequence (trial by period interaction) as no carry-over effect was anticipated due to the short acting nature of calcium. No adjustment was made for baseline values due to the cross-over design. Significance of the fixed effects from each model were determined with Satterthwaite degrees of freedom (*lmerTest package v*.3.1-3). Normality of the residuals for each model were checked visually by plotting the residuals against the fitted values and from Q-Q plots. In the event of a significant main effect of time or significant trial × time interaction, pairwise comparisons with Holm-Bonferroni corrections and Kenward-Roger degrees of freedom were used to identify differences between

time-points (*emmeans package v*.1.8.9). Pooled data were used for main effects with no interaction, and each trial was analysed independently with a signifnt interaction effect. Sweat $\delta^{44/42}$Ca, sweat calcium concentration, sweat loss, and sweat calcium loss during exercise were compared between trials using paired-samples *t*-tests or Wilcoxon signed-rank tests. Effect sizes are presented as partial eta-squared (η_p^2) for main effects and interactions and paired Hedges' *g* for within-group paired comparisons (*effectsize package v*.0.8.6). Significance was accepted as $p < 0.05$.

Results

Participants

Of the 76 women who started the pre-screen, 48 women (military, *n* = 38; civilian, *n* = 10) completed the pre-screen and load carriage 1 (Control then Calcium *n* = 24, Calcium then Control *n* = 24) (Figure 2, Table 1). Eight women withdrew prior to load carriage 2 and 40 women completed load carriage 2 (Control then Calcium, *n* = 19, Calcium then Control, *n* = 21). Reasons for withdrawal included: not meeting all the inclusion criteria, military deployment, job relocation, sustained injury outside the study, changed hormonal contraception, and other commitments. There were no adverse events. The study ended upon completion of the final laboratory visit.

INSERT FIGURE 2 HERE

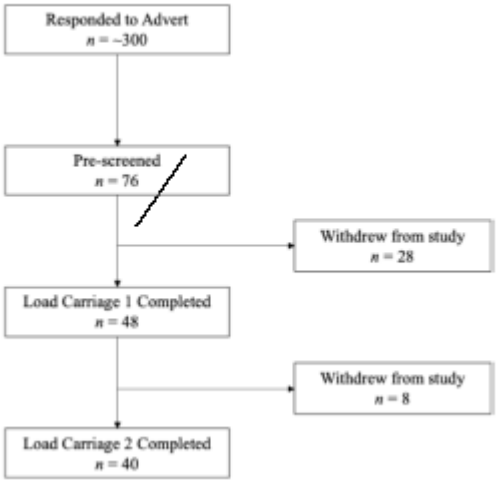

Figure 2. Participant flow through the study.

INSERT TABLE 1 HERE

Endocrine status

There were no differences in baseline vitamin D status, gonadotrophins, sex steroid hormones, or cortisol between the Control and Calcium trials ($p \geq 0.075$) (Table 2).

INSERT TABLE 2 HERE

Exercise

For women not taking hormonal contraceptives, the Control and the Calcium trials were performed at similar time post-menses (Control = 2 ± 5 days *vs* Calcium = 1 ± 4 days). Exercise started on average 64.6 ± 5.4 min after calcium ingestion in the Calcium trial. The calcium consumed during exercise through bottled water (Control = 9 ± 5 mg *vs* Calcium = 9 ± 5 mg) and the environmental temperature (Control = 22.7 ± 2.7°C *vs* Calcium = 22.7 ± 3.3°C, $p = 0.894$) was similar between trials. There was no difference between the Control and Calcium

trials for blood lactate concentration, $\dot{V}O_2$ ($L \cdot min^{-1}$), heart rate ($b \cdot min^{-1}$), respiratory exchange ratio, or rating of perceived exertion (main effects of trial, $p \geq 0.262$, $\eta_p^2 \leq 0.03$; trial × time interactions, $p \geq 0.184$, $\eta_p^2 \leq 0.02$) (Supplementary Table 1).

Calcium isotopes

Calcium isotope data are presented in Figure 3. The $\delta^{44/42}Ca$ of the calcium supplement was 0.34‰. The first urine void collected upon waking was obtained at 06:20 ± 0:49 h for the Control trial and 06:15 ± 0:40 h for the Calcium trial. Post-exercise urine was collected 32 ± 20 min after exercise in the Control trial and 30 ± 16 min after exericse in the Calcium trial. There was no effect of calcium supplementation (main effect of trial, $p = 0.732$, $\eta_p^2 < 0.01$; trial × time interaction, $p = 0.293$, $\eta_p^2 = 0.03$) or load carriage exercise (main effect of time, $p = 0.110$, $\eta_p^2 = 0.03$) on urine $\delta^{44/42}Ca$. There was a trial × time interaction for urine calcium concentration ($p < 0.001$, $\eta_p^2 = 0.23$). Urine calcium concentration decreased in the Calcium ($p = 0.007$) and Control trials ($p < 0.001$), with a greater decrease in the Control trial. Urine calcium concentration was higher at the exercise 120 time-point in the calcium compared with Control trial ($p < 0.001$). There was a trial × time interaction for serum $\delta^{44/42}Ca$ ($p = 0.016$, $\eta_p^2 = 0.08$). Serum $\delta^{44/42}Ca$ did not change in the Control trial ($p = 0.617$) but increased in the Calcium trial ($p = 0.003$). Serum $\delta^{44/42}Ca$ was higher at the exercise 120 time-point in the Calcium compared with the Control trial ($p = 0.018$). There was no difference between trials for sweat $\delta^{44/42}Ca$, sweat calcium concentration, sweat loss, or sweat calcium loss (all $p \geq 0.121$) (Table 3).

INSERT FIGURE 3 HERE

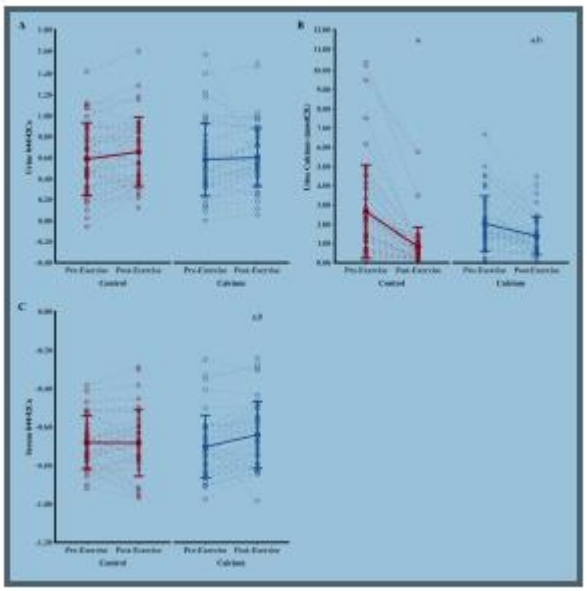

Figure 3. The effect of calcium supplementation on naturally occurring calcium isotopes in urine and serum in response to load carriage exercise. Data are mean ± SD; open circles represent individual data.

[a]$p < 0.05$ vs pre-exercise within trial; [b]$p < 0.05$ vs Control at the same time-point.

INSERT TABLE 3 HERE

Biochemical markers of calcium metabolism

Circulating markers of calcium metabolism data are presented in Figure 4. Examination of the residuals revealed that urinary calcium had a skewed distribution and ionised calcium, PTH, and adjusted PTH had long-tailed distributions and so these outcomes were log transformed. There was a trial × time interaction for ionised calcium ($p < 0.001$, $\eta_p^2 = 0.11$). Ionised calcium decreased from pre-exercise to exercise 120 and post-exercise 15 (both $p = 0.001$) in the Control trial. Ionised calcium increased from pre-exercise to exercise 20, 40, 60, 80, 100, 120,

and post-exercise 15, 30, 60, and 90 in the Calcium trial (all $p \leq 0.012$). Ionised calcium was higher in the Calcium than Control trial for exercise 20, 40, 60, 80, 100, 120, and post-exercise 15, 30, 60, and 90 ($p \leq 0.018$). There was a main effect of time ($p < 0.001$, $\eta_p^2 = 0.32$), but no main effect of trial ($p = 0.329$, $\eta_p^2 = 0.02$) or trial × time interaction ($p = 0.125$, $\eta_p^2 = 0.03$) for adjusted ionised calcium. Adjusted ionised calcium decreased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and post-exercise 15 (all $p < 0.001$). There was a trial × time interaction for phosphate ($p < 0.001$, $\eta_p^2 = 0.15$) and adjusted phosphate ($p < 0.001$, $\eta_p^2 = 0.07$). Phosphate increased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and decreased from pre-exercise to post-exercise 60 and 90 in the Control trial (all $p \leq 0.012$). Phosphate increased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, 120, and post-exercise 15, 30, 60, and 90 in the Calcium trial (all $p \leq 0.027$). Phosphate was higher in the Calcium than Control trial at exercise 100 and 120, and post-exercise 15, 60, and 90 (all $p \leq 0.040$). Adjusted phosphate increased from pre-exercise to exercise 20, 40, and 60, and decreased from pre-exercise to post-exercise 90 in the Control trial (all $p \leq 0.003$). Adjusted phosphate increased from pre-exercise to exercise 20, 40, 60, 100, and 120, and post-exercise 30 in the Calcium trial (all $p \leq 0.010$). Adjusted phosphate was not different between the Calcium and Control trials at any time-point (all $p \geq 0.117$). There was a trial × time interaction for PTH ($p < 0.001$, $\eta_p^2 = 0.27$) and adjusted PTH ($p < 0.001$, $\eta_p^2 = 0.26$). PTH increased from pre-exercise to exercise 120 and post-exercise 15 and decreased from fasted to post-exercise 90 in the Control trial (all $p \leq 0.013$). Adjusted PTH increased from pre-exercise to exercise 120 in the Control trial ($p = 0.011$). PTH and adjusted PTH decreased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and post-exercise 15, 30, 60, and 90 in the Calcium trial (all $p < 0.001$). PTH and adjusted PTH were higher in the Control compared with Calcium trial at exercise 0 20, 40, 60, 80, 100, and 120, and post-exercise 15, 30, 60, and 90 (all $p < 0.001$).

INSERT FIGURE 4 HERE

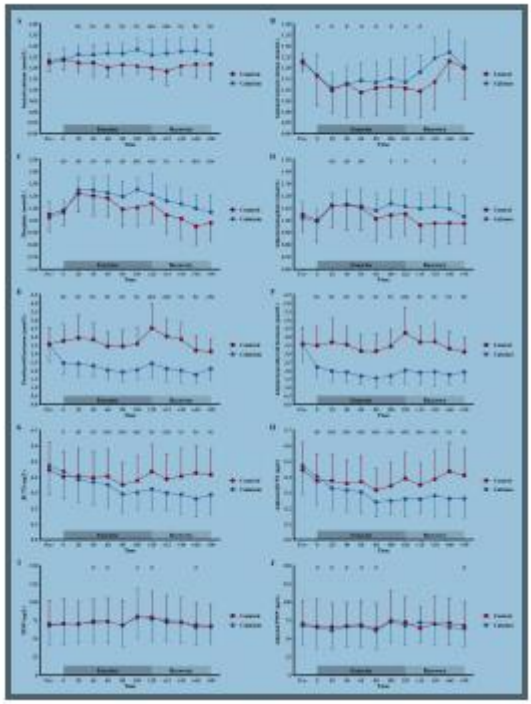

Figure 4. The effect of calcium supplementation on circulating markers of calcium and bone metabolism during load carriage exercise and recovery. Data are mean ± SD.

Pre-, pre-exercise.

[a]$p < 0.05$ vs pre-exercise within trial for Control; [b]$p < 0.05$ vs pre-exercise within trial for Calcium; [c]$p < 0.05$ for Calcium vs Control; [d]$p < 0.05$ vs pre-exercise for Calcium and Control pooled.

Biochemical markers of bone resorption and bone formation

Circulating markers of bone resorption and bone formation are presented in Figure 4 and 5. Examination of the residuals revealed that βCTX and PINP had long-tailed distributions and so these outcomes were log transformed. There was a trial × time interaction for βCTX ($p < 0.001$, $\eta_p^2 = 0.41$) and adjusted βCTX ($p < 0.001$, $\eta_p^2 = 0.35$). βCTX decreased from pre-exercise to exercise 20, 40, 60, 80, 100, and post-exercise 15 in the Control trial (all $p \leq 0.008$). βCTX decreased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and post-exercise

15, 30, 60, and 90 in the Calcium trial (all $p \leq 0.001$). Adjusted βCTX decreased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and post-exercise 15 and 30 in the Control trial (all $p \leq 0.004$). Adjusted βCTX decreased from pre-exercise to exercise 0, 20, 40, 60, 80, 100, and 120, and post-exercise 15, 30, 60, and 90 in the Calcium trial (all $p < 0.001$). βCTX was higher in control than calcium at exercise 60, 80, 100, and 120, and post-exercise 15, 30, 60, and 90 (all $p \leq 0.002$). Adjusted βCTX was higher in the Control than Calcium trial at exercise 20, 40, 60, 80, 100, and 120, and post-exercise 15, 30, 60, and 90 (all $p \leq 0.043$). There were main effects of time (both $p < 0.001$, $\eta_p^2 \geq 0.17$), but no main effects of trial (both $p \geq 0.433$, $\eta_p^2 \leq 0.02$) or trial × time interactions (both ≥ 0.288, $\eta_p^2 = 0.02$) for PINP and adjusted PINP. PINP increased from pre-exercise to exercise 40, 60, 100, and 120, and decreased from fasted to post-exercise 60 (all $p \leq 0.030$). Adjusted PINP decreased from pre-exercise to exercise 0, 20, 40, 60, and 80, and post-exercise 90 (all $p \leq 0.047$). There were main effects of time (both $p < 0.001$, $\eta_p^2 \geq 0.12$), but no main effects of trial (both $p \geq 0.409$, $\eta_p^2 \leq 0.01$) or trial × time interaction (both $p \geq 0.219$, $\eta_p^2 \leq 0.03$) for sclerostin and adjusted sclerostin. Sclerostin increased from pre-exercise to exercise 120 and decreased from pre-exercise to post-exercise 90 (both $p \leq 0.002$). Adjusted sclerostin increased from pre-exercise to exercise 120 ($p = 0.006$). There was no effect of calcium supplementation (main effect of trial, $p = 0.920$, $\eta_p^2 < 0.01$; trial × time interaction, $p = 0.760$, $\eta_p^2 = 0.01$) or load carriage exercise (main effect of time, $p = 0.272$, $\eta_p^2 = 0.03$) on osteocalcin. There was a main effect of time ($p = 0.005$, $\eta_p^2 = 0.11$) but no effect of calcium supplementation (main effect of trial, $p = 0.375$, $\eta_p^2 = 0.03$; trial × time interaction, $p = 0.255$, $\eta_p^2 = 0.03$) for adjusted osteocalcin. Adjusted osteocalcin increased pre-exercise to exercise 120 ($p = 0.036$).

INSERT FIGURE 5 HERE

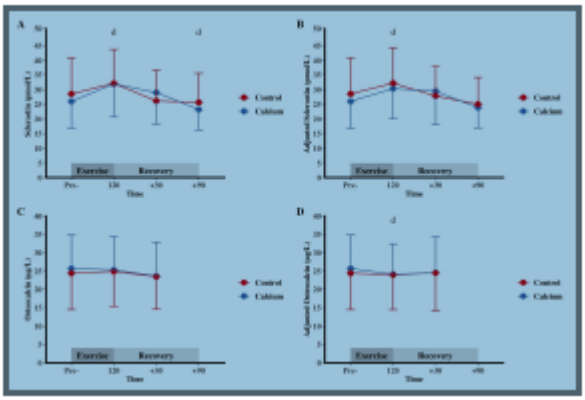

Figure 5. The effect of calcium supplementation on circulating markers of bone metabolism during load carriage exercise and recovery. Osteocalcin was not measured at the post-exercise 90-min time-point. Data are mean ± SD.

Pre-, pre-exercise.

[a]$p < 0.05$ vs pre-exercise within trial for Control; [b]$p < 0.05$ vs pre-exercise within trial for Calcium; [c]$p < 0.05$ for Calcium vs Control; [d]$p < 0.05$ vs pre-exercise for Calcium and Control pooled.

Discussion

A loaded march (12.8 km in 2 h carrying 20 kg) decreased circulating ionised calcium and increased PTH in women. A 1000 mg calcium supplement 1 h before exercise prevented the decrease in circulating serum ionised calcium, suppressed PTH, decreased bone resorption, and may improve bone calcium balance. Load carriage is a fundamental military activity and is incorporated into military training and testing, with the exercise in this study a test of fitness in the British Army. Women have recently been allowed to enter the most arduous military combat roles in the UK and other nations, but being a woman and in a combat role increase the risk of developing stress fractures, likely due to high volumes of weight-bearing exercise including prolonged load carriage.[3] The data in this study provides new insight into the bone and calcium metabolic disturbances during arduous load carriage in women, and highlights the

potential of an acute nutritional strategy to attenuate these disturbances. The participants in this study were homogenous and so these results are likely specific to young active women.

Calcium isotopes

Serum $\delta^{44/42}$Ca did not change in the Control trial but increased in the Calcium trial. Naturally occurring calcium isotopes have been used to determine changes to bone calcium balance in bed rest[22,25,29,30] and kidney disease;[26] our study is the first to use these isotopes to measure the effect of exercise and calcium supplementation. Bone formation favours the uptake of lighter calcium isotopes from the soft tissue into bone, creating a heavier isotopic composition of soft tissue (blood); during bone resorption, lighter calcium isotopes are released from bone shifting soft tissue to a lighter isotopic composition.[22,23] The higher post-exercise serum $\delta^{44/42}$Ca in the Calcium trial indicates a shift towards a relatively heavier calcium isotope compared with pre-exercise values and with exercising without calcium supplementation. This increase in $\delta^{44/42}$Ca could arise from two sources: changes in the exchange of lighter calcium isotopes between bone and circulation (increased uptake into, or reduced release from, bone), or an increase in calcium absorption from the intestine due to the effects of the isotopically heavier calcium supplement (0.34‰). Load carriage exercise in women has previously shown to increase gut calcium absorption, decrease urinary calcium excretion, and, when exercise was coupled with calcium intake >700mg·d^{-1}, a shift towards a more positive bone calcium balance.[21] The post-exercise blood sample was 3 h after the supplement and some gut calcium absorption was still likely to be occuring, however, a decrease in βCTX in the Calcium trial shows a decrease in bone resorption, and the shift towards a heavier composition of the serum is likely due to be a combination of increased gut calcium absorption and increased bone calcium balance. A limitation of our isotope method is the inability to determine the amount of calcium absorbed during exercise. The unchanged serum $\delta^{44/42}$Ca in the Control trial, despite a

decrease in serum ionised calcium and increased PTH, suggests PTH prevented any further decrease in serum ionised calcium through reduced renal excretion of calcium by the kidney, rather than drawing calcium from the skeleton or intestine. This supposition is supported by the unchanged βCTX and greater decrease in urine calcium concentration with exercise in the Control compared with Calcium trial; although, the higher βCTX in the Control compared with Calcium trial supports bone turnover being higher to rescue decreasing iCa, which was not reflected in the isotope measures. The shift towards a heavier isotopic composition of the serum within the Calcium but not Control trial suggests the 1000 mg calcium supplement acutely improved gut calcium uptake and bone calcium balance during exercise but exercise without calcium did not result in a negative bone calcium balance.

Despite differences in serum $\delta^{44/42}$Ca between Control and Calcium trials, no differences were observed in urine $\delta^{44/42}$Ca between trials or as a result of exercise. A bed rest study detected lower urine $\delta^{44/42}$Ca compared with pre-bed rest after 10 days,[25] but we did not detect acute changes. The post-exercise urine sample was obtained as quickly as possible post-exercise to align with the 120 min exercise blood sample. While the isotopic composition of serum and urine are comparable,[26,27] previous studies have used blood and urine samples obtained at rest and the change in isotopic composition of the serum induced by exercise might not have been reflected in the urine within 1 h post-exercise. The change in serum but not urine $\delta^{44/42}$Ca composition at the 120 min exercise was likely due to the natural delay from the kidneys and generation of urine; future exercise studies should consider obtaining a urine sample at > 1 h post-exercise to capture serum $\delta^{44/42}$Ca changes within the urine. Future research would also benefit from exploring changes in serum and urine $\delta^{44/42}$Ca during exercise recovery. No differences in sweat loss volume, sweat calcium loss, sweat calcium concentration, or sweat $\delta^{44/42}$Ca were observed between Control and Calcium trials. Our study is the first to measure

calcium isotopes in sweat, and no change in sweat $\delta^{44/42}$Ca —despite a change in serum $\delta^{44/42}$Ca—and the small dermal calcium loss supports that calcium sweat loss is not a primary determinant of decreased serum calcium.[34]

Biochemical markers of calcium metabolism

Ionised calcium decreased and PTH increased in the Control trial, and ionised calcium increased and PTH decreased in the Calcium trial. The suppressed PTH in the Calcium trial partly explains the higher bone calcium balance with exercise. Exercise has shown to decrease circulating ionised calcium and increase PTH in walking in older men and women,[12,18,37] and in cycling,[13,14,16,17,34,38] rowing,[15] and load carriage[20,21] in young adult men and / or women. The increase in PTH we report here (approximately one third) is smaller than some of the increases previously reported (approximately double)[11] and the limited increase in PTH could explain why we did not observe a decrease in bone calcium balance during exercise in the Control trial. Differences between our study and others could be due to mode of exercise, however, previous load carriage studies in men and women have also shown PTH can more than double following 60 mins load carriage.[20,21] These load carriage studies employed shorter duration and higher intensity exercise than our study (best effort[20] and exercise at 65% $\dot{V}O_{2max}$[21]), and treadmill running data show that PTH only increases at exercise intensities above 65% $\dot{V}O_{2max}$;[39] the smaller increase in PTH in our study compared with others could be due to the lower exercise intensity. Our trial did not include a no exercise control condition and so the effect of exercise alone vs circadian rhythms cannot be determined, however, the circadian rhythm of PTH (and βCTX) involve early morning peaks[40] and so the effect of exercise alone on PTH was likely underestimated.

The mechanisms for the decrease in circulating calcium during exercise is unclear but could be due to increased muscle calcium uptake or changes in the 'miscible' calcium pool.[11,21] Maintenance of circulating ionised calcium during exercise with infusion of calcium gluconate attenuates the increase in PTH.[12,13] Consumption of a 1000 mg calcium supplement[16,17] or high calcium meal (~1000 to 1300 mg)[14,15] 20 min to 2 h before exercise has also shown to attenuate the decrease in ionised calcium and / or increase in PTH in high-intensity cycling and rowing. Our data provide evidence that calcium supplementation prevents disturbances to calcium homeostasis in women during military load carriage. Parathyroid hormone secretion is regulated by serum ionised calcium; a decrease in ionised calcium stimulates PTH production and an increase in calcium inhibits PTH production and stimulates PTH breakdown and inactivation.[41] Parathyroid hormone maintains circulating calcium by increasing reabsorption of calcium from the kidney, increasing calcium absorption from the small intestine—by increasing $1,25(OH)_2D$ production—and by mobilising calcium from the bone.[41] We were unable to determine the how much calcium was absorbed from the intestine with our methods but the lower urine calcium concentration in the Control *vs* Calcium trial was likely due to the higher PTH increasing calcium reabsorption at the kidney.[12,21] The lower phosphate in the Control trial could also be due to inhibited renal reabsorption of phosphate with higher PTH.[41] The implications of decreasing PTH with calcium supplementation in an acute bout of load carriage exercise for long-term bone injury risk and adaptation are not clear; intermittent increases in PTH are osteogenic with sustained higher PTH resulting in bone loss.[11,21,41] Higher PTH has been associated with increased stress fracture risk in military training[42] and British Army basic training increases PTH in women but not men,[7] consistent with the higher risk of stress fracture in women. A daily vitamin D and calcium supplement (1000 to 2000 $mg \cdot d^{-1}$) during military training has also shown to prevent the increase in PTH[8] and βCTX,[43] augment tibial adaptations,[8] and reduce stress fractures incidence.[44]

Biochemical markers of bone resorption and bone formation

In the Control trial, bone resorption—measured by βCTX—decreased during exercise. Calcium supplementation decreased βCTX during exercise to a greater extent than the Control trial and kept βCTX suppressed longer. The decrease in βCTX demonstrates that exercise and calcium supplementation decreased type I collagen degradation. This finding supports the serum $\delta^{44/42}$Ca data suggesting that calcium supplementation increased bone calcium balance during exercise through a decrease in bone breakdown. A decrease in βCTX could be consistent with adaptive remodelling in response to mechanical loading[5,7,9] and calcium supplementation might promote adaptation to exercise, but longer term data are needed. The decrease in βCTX could also be due to circadian effects—with the peak of βCTX occurring early in the morning and then decreasing[40]—or because the increase in PTH in the Control trial was only small compared with previous studies.[11] These data are in contrast to other studies that show an increase in βCTX when ionised calcium decreases and PTH increases during exercise,[12,13,15–18,20,37,38] whereas we observed a decrease in βCTX in the Control trial; an increase in βCTX would be expected to lower serum $\delta^{44/42}$Ca. Differences between our study and previous studies could be due to differences in exercise intensity and / or modality. Our study used moderate-intensity exercise which decreases βCTX,[39] whereas most other studies used higher intensity exercise which can increase βCTX.[39] Exercise mode also appears to influence the βCTX response, with low impact prolonged activities generally causing the biggest increase in βCTX[45] and our data involved high-impact load carriage whereas previous studies used low-impact walking,[12,18,37] cycling,[13,16,17,19,38] or rowing.[15] Our data agree with calcium infusion[13] and supplementation[14,15,19] studies showing that calcium decreases the βCTX response to exercise, although some studies show calcium supplementation attenuates the decrease in ionised calcium and / or increase in PTH without changing βCTX.[16–18] The impact

of acutely decreasing the βCTX response to exercise on long-term bone injury and adaptation is not clear. Military training studies show decreased,[5,7,9,46] increased,[4,43] and unchanged[6,8] βCTX over 8 to 44 weeks training in men and / or women, with some of these studies also showing adaptive bone formation at the tibia[4–9,43,46] demonstrating a complex relationship between βCTX and skeletal adaptation.

Circulating PINP—a marker of type I collagen synthesis[47]—increased during exercise and decreased during recovery, with no difference between groups. Osteocalcin—a bone matrix protein synthesised by mature osteoblasts[47]—did not change with exercise. Osteocalcin may not be a sensitive indicator of bone formation with exercise, and PINP and osteocalcin reflect different processes. Sclerostin—a glycoprotein secreted by osteocytes[48]—increased immediately following exercise and decreased during recovery. The mechanism for the increase in post-exercise sclerostin is unclear, but a decrease in sclerostin (as seen in recovery) can promote bone formation through disinhibition of the Wnt signalling pathway,[48] which is consistent with new bone formation in the tibia in military training.[4–9] Acute exercise typically increases markers of bone formation[45], however, military training studies report increased[7,9,43] or unchanged[4–6,8,46] PINP production, increased,[9] decreased[7–9,46] or unchanged[4] osteocalcin, and increased[9], decreased[4,7,46], or unchanged[6] sclerostin in men and women. Nevertheless, these data confirm that calcium supplementation influences the bone resorption, but not bone formation, response to load carriage.

Limitations

Our study was unblinded and we did not use a placebo in our control condition, but we do not anticipate these limitations affecting our findings as our primary outcome was urinary and circulating markers of calcium metabolism. Feeding can affect some of the biochemical

markers in this study[40] but the small supplement compared with control condition was unlikely to result in any feeding effect. Although our participants met strict inclusion criteria, it is likely there was varied load carriage experience between participants. Accordingly, some of our participants decreased their load carriage mass mid-trial due to perceived inability to complete the task, however, both trials were matched for mass carried and the cross-over design also means participants completed both control and calcium conditions. We did not have a measure of habitual calcium intake of our participants, which can affect the calcium absorption of the supplement, however, our randomised controlled crossover design means variations in habitual intake between participants is well balanced between trials. We did not have a non-exercise condition and so we are unable to exclude effects of circadian rhythms. Finally, our trials were completed fasted, and these data may not reflect the metabolic responses following a meal.

Conclusions

An arduous military load carriage exercise task decreased circulating ionised calcium and increased parathyroid hormone in physically active women. A 1000 mg calcium supplement 1 h before exercise maintained circulating ionised calcium, suppressed parathyroid hormone, decreased bone resorption, and may improve bone calcium balance. These changes in bone metabolism and bone calcium balance with calcium supplementation may help protect skeletal health in servicewomen and longer-term studies are warranted.

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Author Contributions

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Thomas O'Leary: Conceptualization, Funding Acquisition, Data Curation, Formal Analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – original draft.

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TABLES

Table 1. Participant characteristics.

Characteristic	Mean ± SD or n (%)
Age (years)	29 ± 4
Body mass (kg)	65.3 ± 8.5
Height (m)	1.67 ± 0.06
Body mass index ($kg \cdot m^{-2}$)	23.3 ± 2.2
Hormonal contraceptive use	
None	28 (58%)
Mirena coil	9 (19%)
Combined oral contraceptive pill	9 (19%)
Copper coil	2 (4%)
EAT-26 score	4 ± 4
Haemoglobin ($g \cdot dL^{-1}$)	14.0 ± 1.1
Whole-body areal bone mineral density ($g \cdot cm^{-2}$)	1.23 ± 0.09
Body fat (%)	25.9 ± 5.9
Lean mass (kg)	45.9 ± 5.0
T-score	1.4 ± 0.9
Maximal oxygen uptake ($mL \cdot kg^{-1} \cdot min^{-1}$)	45 ± 6
Total 25(OH)D ($nmol \cdot L^{-1}$)	78.7 ± 24.1

EAT-26: Eating Attitudes Test – 26 item; Total 25(OH)D: total 25-hydroxyvitamin D.

Table 2. Baseline fasted endocrine status before exercise. Data are mean ± SD or median (25^{th} percentile, 75^{th} percentile).

Outcome	Control	Calcium
Total 25(OH)D ($nmol \cdot L^{-1}$)	73.2 ± 24.1	68.4 ± 30.7
Total $24,25(OH)_2D$ ($nmol \cdot L^{-1}$)	5.2 ± 2.3	4.9 ± 3.6
Total $1,25(OH)_2D$ ($pmol \cdot L^{-1}$)	107.1 ± 31.8	105.5 ± 31.6
FSH ($IU \cdot L^{-1}$)	5.0 ± 2.7	4.6 ± 2.4
LH ($IU \cdot L^{-1}$)	7.5 ± 5.2	6.6 ± 5.4
Oestradiol ($pmol \cdot L^{-1}$)	168 (112, 532)	186 (108, 523)
SHBG ($nmol \cdot L^{-1}$)	66 (52, 110)	68 (54, 101)
Testosterone ($nmol \cdot L^{-1}$)	0.95 ± 0.46	0.90 ± 0.35
Cortisol ($nmol \cdot L^{-1}$)	436 (363, 858)	463 (341, 558)

Total $24,25(OH)_2D$, total 24,25-dihydroxyvitamin; FSH, follicle stimulating hormone; LH, luteinising hormone; SHBG, sex hormone binding globulin.

Table 3. The effect of calcium supplementation on sweat calcium during load carriage exercise. Data are mean ± SD or median (25^{th} percentile, 75^{th} percentile).

	Control	**Calcium**
Sweat $\delta^{44/42}Ca$	-0.09 ± 0.20	-0.06 ± 0.20
Sweat Calcium Concentration ($mmol \cdot L^{-1}$)	0.70 (0.50, 1.06)	0.83 (0.47, 1.46)
Sweat Loss (mL)	1,131 ± 459	1,122 ± 300
Sweat Calcium Loss (mg)	32 (21, 60)	33 (22, 64)

Missing sweat $\delta^{44/42}Ca$ data: Control n = 18, Calcium n = 13.

Missing sweat calcium concentration and sweat calcium loss data: Control n = 12, Calcium n = 4.

Missing sweat loss data: Control n = 5, Calcium n = 3.