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Calcium and vitamin D supplementation and bone health in Marine recruits: Effect of season☆

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ABSTRACT

Stress fractures are common overuse injuries caused by repetitive bone loading. These fractures are of particular concern for military recruits and athletes resulting in attrition in up to 60% of recruits that sustain a fracture. Army and Navy recruits supplemented with daily calcium and vitamin D (Ca + D) demonstrated improved bone strength and reduced stress fractures. The aim of the current study was to evaluate whether Ca + D supplementation improves measures of bone health in recruits undergoing United States Marine Corps initial military training (IMT), and whether the effect of supplementation on indices of bone health varied by season. One-hundred ninety-seven Marine recruits (n = 107 males, n = 90 females, mean age = 18.9 ± 1.6 y) were randomized to receive either Ca + D fortified snack bars (2000 mg Ca and 1000 IU vitamin D per day) or placebo divided into twice daily doses during 12 weeks of IMT. Anthropometrics, fasted blood samples, and peripheral quantitative computed tomography (pQCT) scans of the tibial metaphysis and diaphysis were collected upon entrance to- and post-training (12 weeks later). Half of the volunteers entered training in July and the other half started in February. Time-by-group interactions were observed for vitamin D status (25OHD) and the bone turnover markers, BAP, TRAP and OCN. 25OHD increased and BAP, TRAP and OCN all decreased in the Ca + D group ($p < .05$). Training increased distal tibia volumetric BMD (+1.9 ± 2.8%), BMC (+2.0 ± 3.1%), and bone strength index (BSI; +4.0 ± 4.0%) and diaphyseal BMC (+1.0 ± 2.2%) and polar stress strain index (SSIp; +0.7 ± 2.1%) independent of Ca + D supplementation ($p < .05$ for all). When analyzed by season, change in BSI was greater in the Ca + D group as compared to placebo in the summer iteration only (T*G; $p < .05$). No other effects of supplementation on bone tissue were observed. When categorized by tertile of percent change in BSI, recruits demonstrating the greatest changes in BSI and 25OHD entered training with the lowest levels of 25OHD ($p < .05$). Over all, these results suggest that Ca + D supplementation reduced some markers of bone formation and resorption and the decline in 25OHD over training in volunteers that started training in the summer was prevented by supplementation. Baseline 25OHD and trajectory may impact bone responses to IMT, but little effect of Ca + D supplementation was observed at the investigated doses.

Abbreviations: IMT, initial military training; BAP, bone specific alkaline phosphatase; BMC, bone mineral content; BMI, body mass index; Ca + D, calcium + vitamin D supplement; Crt Th, cortical thickness; CTX, c-telopeptide cross-links of type 1 collagen; OPG, osteoprotegerin; P1NP, procollagen 1 N-terminal peptide; PTH, parathyroid hormone; pQCT, peripheral quantitative computed tomography; RANKL, receptor activator of nuclear factor κB ligand; SSIp, stress strain index across the polar axis; TRAP, tartrate-resistant acid phosphatase; vBMD, volumetric bone mineral density

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1. Introduction

Stress fractures are prevalent during initial military training (IMT) with an 18 fold higher risk in United States recruits as compared to active duty service members [2]. Of those that sustain a fracture, 60% will attrite from training making these injuries particularly costly to the Department of Defense and recruits [3]. Stress fracture incidence rates are commensurate with training length and intensity [4], for example, Marine recruits (12 weeks of training) have the highest rates of stress fracture and Air Force recruits (7 weeks of training) have the lowest. While the etiology of stress fracture is not fully understood, contributing factors include age, sex, race, bone geometry, fitness level, nutrition, and hormonal status in women (e.g., amenorrhea). Overall fracture risk is, thus, determined by modifiable and non-modifiable factors, as well as interactions amongst these factors [5,6].

Previous research conducted during IMT suggests that supplemental calcium (2000 mg/day) and vitamin D (800–1000 IU/day) can reduce stress fracture incidence by 20% in female Navy recruits [7], and maintain circulating parathyroid hormone (PTH) and lead to greater increases in volumetric bone mineral density (vBMD) in male and female Army recruits [8]. With combination treatment, it is unknown whether benefits are due to calcium, vitamin D or their interaction. The primary role of PTH is to maintain circulating calcium within the physiological range, in part by increasing bone resorption, thus both vitamin D status (25OHD) and calcium intake can suppress PTH [9]. Inhibition of PTH and reduced bone resorption are positively related to lower osteoporotic risk in older adults [10]. Whether PTH suppression is responsible for a beneficial effect of calcium and vitamin D on bone health during military training remains unknown.

Several observational studies support a link between vitamin D status (25OHD) and stress fracture risk [11]. A comparable biomarker for calcium status does not exist, making it difficult to measure changes in this mineral and implications. Depending on the study, 25OHD at the start of training and/or at the time of stress fracture diagnosis is associated with fracture risk. Furthermore, the relationship between vitamin D status and risk of stress fracture is complicated by sex and race differences, seasonal variability in 25OHD, and magnitude of changes in 25OHD during IMT. In one case-control study in female Navy recruits, those that sustained a stress fracture of the tibia or fibula had pre-diagnostic mean 25OHD of 28 ng/ml as compared to controls whose mean 25OHD was 31 ng/ml [12]. The female recruits in the highest quintile of 25OHD status (mean 49.7 ng/ml) had half the risk of fracture (OR = 0.51) as compared to those in the lowest quintile of 25OHD status (mean 13.9 ng/ml). When whites/Caucasians and blacks/African Americans were analyzed separately, vitamin D status was significantly lower in Caucasian cases as compared to Caucasian controls, but the difference between African American cases and controls did not reach statistical significance. The authors concluded that circulating 25OHD levels of at least 40 ng/ml, achievable with 4000 IU/day of vitamin D, were required to reduce stress fracture risk. In a prospective study in over 1000 male Royal Marine recruits, of which 78 (7.2%) sustained at least one stress fracture, 25OHD < 20 ng/ml at the start of training was associated with a moderate increased risk (OR = 1.6) [13]. Similarly, in another prospective study in 800 male Finnish Military recruits, of which 22 (2.9%) sustained a stress fracture, those with 25OHD < 30 ng/ml were more likely to sustain a stress fracture (OR = 3.6) [14]. In these observational studies, either limited or no information was available regarding dietary calcium intake.

The purpose of the current study was to evaluate whether calcium and vitamin D supplementation results in improved measures of bone health in Marine recruits undergoing IMT. In addition, a secondary objective was to evaluate whether the effect of supplementation on indices of bone health varied by season. We hypothesized that supplementation with calcium and vitamin D would maintain circulating parathyroid hormone (PTH), improve 25OHD, and support greater increases in bone density and/or strength of the tibia as compared to placebo. In addition, supplementation would maintain 25OHD for recruits starting training in the summer and improve vitamin D status to a greater extent than placebo in recruits starting training in the winter. We hypothesized that the effect of supplementation on circulating 25OHD would be related to its impact on bone tissue.

2. Methods

2.1. Volunteers

This study was approved by the Institutional Review Boards at the US Army Research Institute of Environmental Medicine and Medical Research and Material Command and was conducted at Marine Corps Recruit Depot, Parris Island, SC (32.4°N latitude). Recruits were briefed regarding the study design and risks in a group setting without any uniformed personnel present, and with an ombudsman present to answer any questions regarding participation. Recruits were enrolled in the study after providing their free and informed written voluntary consent. Investigators adhered to US Army Regulation 70–25 and US Army Medical Research and Materiel Command Regulation 70–25 on the participation of volunteers in research. Potential volunteers were males and females between the ages of 18 and 42 who entered Marine recruit IMT at Parris Island during July 2015 and February 2016. Volunteer recruitment, enrollment, and study completion occurred between July 2015 and April 2016. Potential volunteers were excluded from the study if they were under 17 years of age, were pregnant or lactating women, had a history of kidney disease or renal calculi, or were allergic to any component of the food product bars. A total of 107 men and 90 women volunteered to participate (Fig. 1). Sample size estimates were determined using the change in circulating PTH previously observed in young adults undergoing IMT [8]. Anthropometric, demographic, dietary intake, biochemical and peripheral quantitative computed tomography (pQCT) data were collected within 1 week of arrival at IMT (pre-) and 12 weeks later at the end of training (post-). This trial was registered at clinicaltrials.gov under trial number NCT02636348.

2.2. Intervention

Volunteers were block randomized by race (Caucasian, African American, or other) and sex by the study team to either placebo or calcium and vitamin D (Ca + D) bar as previously described [8]. A second study arm was initiated that provided calcium and vitamin D or placebo treatments as 8 pills per day, but was not included in the final analysis due to poor compliance with pills. Volunteers and all research personnel conducting data collection and/or analysis were blind to the group assignment. Bars were identical to those utilized in a prior randomized controlled trial (RCT) in Army recruits and were manufactured and labeled by the Combat Feeding Directorate (CFD; Natick Soldier Research Development and Engineering Center, Natick, MA) with a 3-letter code to indicate the study groups. The study key was maintained independent of the study team by the CFD. Bars contained 130–140 kcal, 23–25 g carbohydrate, 5 g fat, and 1–2 g protein. Separate biochemical analyses (Covance Laboratories, Madison, WI) were completed on composites of 5 bars from both the intervention and placebo bar lots from both the summer and winter study iterations. Placebo bars contained ≤20 mg of incidental calcium and < 1.4 IU vitamin D3. Ca + D bars from the summer iteration contained 945 mg Ca and 518 IU vitamin D3; bars from the winter iteration contained 1123 mg Ca and 469 IU vitamin D3. The doses of calcium (as calcium carbonate) and vitamin D (as vitamin D3) were chosen based on prior work reporting a reduction in stress fracture incidence in Navy recruits and greater increases in distal tibia vBMD over the course of Army IMT [7,8]. The placebo and Ca + D bars were identical in taste and appearance. Bars were similar to an existing military ration item and conformed to all ration standards for safety and stability, and were

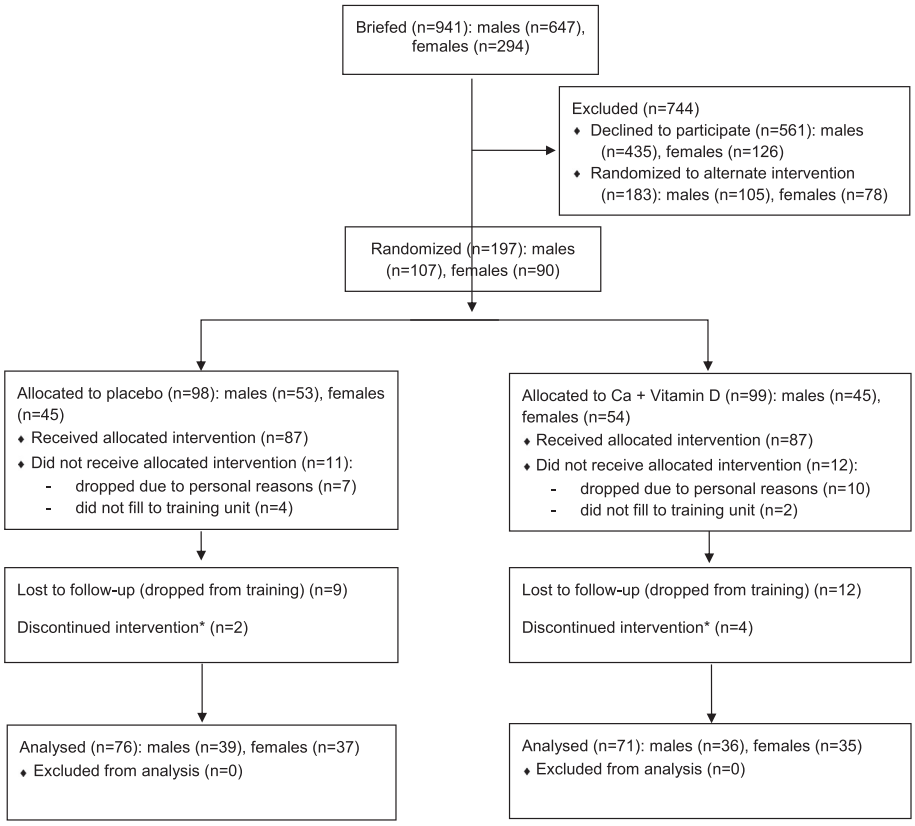

Fig. 1. Study flow diagram.

individually labeled with volunteer ID numbers and packaged into one-week allotments (14 bars each). Volunteers were provided the bars weekly and instructed to consume 2 bars per day, one either mid-morning or mid-afternoon, and the other in the hours after dinner. Empty wrappers and uneaten bars were collected from each volunteer during the weekly bar exchanges in order to determine compliance.

2.3. IMT

Marine Corps recruit IMT is a 12 week training course consisting of physical training, didactic military instruction and military training exercises. Physical training includes running, marching, obstacle courses, and strength training. Military-specific physical training includes rappelling, weapons training, marching and standing in formation, and tactical exercises. Recruits consume three self-selected meals in a cafeteria-style dining facility, and additional snacking or consumption of dietary supplements is strictly prohibited.

2.4. Anthropometrics

Anthropometric data were collected while fasted in the morning with volunteers wearing their physical training uniform, which consists of light weight shorts and a short-sleeved shirt. All measures were collected pre- and post- IMT except for height which was collected at the pre- time point only. For height and weight, volunteers removed their footwear. Height was measured to the nearest 0.1 cm using a stadiometer (Creative Health Products, Plymouth, MI). Weight was measured to the nearest 0.1 kg using a calibrated digital scale (Belfour Scales, Saukville, WI) and body mass index (BMI) was calculated as body wt (kg)/ht $(m)^2$. Body fat percentage was calculated using Jackson and Pollock's 3-site skinfold method using calipers [15]. For females the sites were tricep, suprailiac, and abdomen; for males the sites were tricep, subscapula, and chest, consistent with the American College of Sports Medicine Guidelines for Exercise Testing and Prescription [16]. Skinfold measurements were made in duplicate to the nearest mm; if the measures differed by > 2 mm a third measurement was taken.

2.5. Dietary intake

In order to estimate habitual nutrient intake for the 6 months preceding training, and for the 12 weeks of training, 2014 Block Food Frequency Questionnaires (FFQ) were administered pre- and post-IMT under the supervision of registered dietitians. Questionnaires were developed and analyzed by NutritionQuest (Berkeley, CA) utilizing National Health and Nutrition Examination Survey (NHANES) dietary recall data (2007–2008 and 2009–2010), the US Department of Agriculture's Food and Nutrient Database for Dietary Studies (FNDDS 5.0), and the Nutrient Database for Standard Reference (SR27).

2.6. Blood collection and circulating biomarkers

Fasting blood samples were collected from the antecubital vein of volunteers pre- and post- IMT. Blood was allowed to clot at room temperature and then centrifuged for the collection of serum which was then frozen and shipped to USARIEM and the Pennington Biomedical Research Center (PBRC, Baton Rouge, LA) for biochemical assays. Enzyme-linked immunosorbent assays (ELISA) were performed at USARIEM using a Dynex DS-2 immunoassay system, and included the following: bone alkaline phosphatase (BAP, Quidel), osteocalcin (OCN, ALPCO), tartrate-resistant acid phosphatase (TRAP, Quidel), C-telopeptide cross-links of type I collagen (CTX, Immunodiagnostic Systems), and procollagen I N-terminal propeptide (P1NP, MyBioSource). Sclerostin (SOST), Dickkopf-related protein 1 (DKK1), osteoprotegerin (OPG), and soluble RANK ligand (sRANKL) were measured on a Luminex MAGPIX system using kits from EMD Millipore. Intact parathyroid hormone (PTH) was assayed on a Siemens Immulite

2000 system. Inter-assay coefficients of variation (CV) were as follows: BAP 2.0%, OCN 7.2%, OPG 5.4%, TRAP 5.0%, CTX 4.7%, P1NP 6.9%, sclerostin 12.2%, sRANKL 10.3%, and PTH 3.9%. All samples were run in duplicate, and samples were re-assayed in the event of poor agreement between the replicates. Calcidiol (25-hydroxyvitamin D; (25OHD)) and calcitriol (1,25-dihydroxyvitamin D; ($1,25(OH)_2D$)) were measured using radioimmunoassay (Diasorin Inc., Stillwater, MN) at the PBRC chemistry laboratory, which is accredited by the College of American Pathologists (CAP) and routinely participates in inter-laboratory standardization testing. The inter-assay CV for 25OHD was 6.7%.

2.7. pQCT

Volunteers underwent a pQCT scan (XCT 2000 or 3000, Stratec Medizintechnik GmbH, Pforzheim, Germany) of the non-dominant tibia both pre- and post-IMT using methods previously described [3]. Briefly, tibia length (cm) was measured using a flexible tape measure from the palpated medial malleolus to the tibial plateau prior to positioning the lower leg horizontally within the X-ray gantry. An initial scout view scan was performed to identify the distal aspect of the tibia, and a reference line was placed at the growth plate. Scans were then acquired at 4% (metaphysis) and 14 and 66% (diaphysis) of the approximated tibia length. The 4% site was chosen in order to evaluate the trabecular envelope and the 14 and 66% sites were selected in order to examine the cortical envelope. Slice thickness was 2 mm and voxel size was set at 0.4 mm with a scanning speed of 20 mm/s. The total, trabecular and cortical tibia properties were determined using detection thresholds of 169 mg/cm^3, 650 mg/cm^3 and 710 mg/cm^3, respectively. The parameters assessed at the 4%-site included total vBMD, BMC and cross-sectional area (CSA). Bone parameters assessed at the 14 and 66%-sites included cortical vBMD, BMC, and thickness (Crt Th). As an estimate of compressive strength at the 4% site, the bone strength index (BSI) was calculated as the product of the total bone cross-sectional area (CSA) and the total density squared [26]. To estimate the diaphyseal torsional strength, the stress strain index (SSI) was calculated for the polar (SSIp) axes as previously reported [26,27]. Pre- and post-IMT scans for each individual were completed on the same machine. For each volunteer, the reference line for the post-scan was placed automatically by the XCT software (Stratec, version 6.2) using the pre- scan image. Images were visually inspected and excluded from analysis if movement was high (Visual Inspection Rating System score > 3.3) [17]. Calibration of the pQCTs was checked daily using the manufacturer provided cone and cortical phantoms. Short term precision for pQCT variables in our laboratory was determined by performing three scans on 15 subjects at both the distal metaphyseal and diaphyseal sites, each measurement was separated by a day. The mean CV for measurements at the 4% tibia site were: 0.006% (Tot vBMD), 0.006% (Tot BMC), and 0.007% (Tot BSI). Mean CV for measurements at the 14% site were: 0.003% (Ct vBMD), 0.005% (Ct BMC), 0.008% (Ct Th), and 0.01% (SSIp). Mean CV for measurements at the 66% site were: 0.002% (Ct vBMD), 0.007% (Ct BMC), 0.009% (Ct Th), and 0.007% (SSIp).

2.8. Statistical analyses

Data are presented as means ± standard deviation (SD). To evaluate the change in each bone parameter and blood biomarker from pre- to post-IMT, we used linear mixed models. A random intercept was included for each study participant to account for within individual correlation between the pre- and post-IMT training measurements. Independent variables included time, treatment group (placebo or Ca + D) and the interaction. The base model only included the independent variables, and additional models were run and included the following covariates: race (white/Caucasian, black/African American, other), season in which the recruit started training (summer or winter), and BMI (kg/m^2; continuous) at baseline. As adjustment for confounders did not change statistical outcomes, all data are presented as unadjusted means. Percent change in each bone tissue variable was also calculated (post-pre/pre *100) and univariate ANOVA was utilized to evaluate group differences. As unadjusted bone strength at the 4% site was the most robust change observed over the course of training, we divided volunteers into tertiles based upon percent change in BSI in order to identify variables that were associated with change in bone strength. We then compared continuous variables between the first (bottom) and third (top) tertiles using *t*-tests, and categorical variables were compared using chi-squared tests. All analyses were conducted using the Statistical Package for Social Sciences software (SPSS, version 24; Chicago, IL). Statistical significance was set at $p < .05$.

3. Results

3.1. Volunteer characteristics

Of the 197 recruit volunteers who enrolled in the study, 147 completed the follow-up visit at the end of training. Reasons for withdrawal from the study are detailed in Fig. 1. There were no demographic or anthropometric differences between those who completed the trial and those who did not. The volunteers who completed the study were primarily white, non-Hispanic young adults. There were no between group differences in any demographic or nutrient intake variable when comparing the placebo and Ca + D groups. However, BMI was lower in the Ca + D group as compared to placebo at baseline (Table 1, $p < .05$). Both groups contained an equal distribution of volunteers that started training in the summer and winter and half of the volunteers in each group were female (Table 1). Mean compliance did not differ between placebo (97.9 ± 3.1%) and Ca + D treatment (96.3 ± 7.6%).

3.2. Dietary intake and anthropometric changes

There were no differences in calcium and vitamin D intake between groups at baseline (Table 1), and the percent of volunteers who met the recommended dietary allowances (RDA) for these nutrients prior to coming to training did not differ. Baseline intakes of calcium and vitamin D did not differ by group (Table 1). Consistent with the experimental design, total calcium and vitamin D intakes increased in the Ca + D group over training ($p < .001$), but not in the placebo group (Table 1, T*G, $p < .001$).

Body weight ($p = .001$) and BMI ($p = .001$) decreased in the placebo group over training, whereas, no changes were observed in the Ca + D group (Table 1; T*G, $p < .05$). Body fat % decreased in both groups over training but to a greater extent in the placebo group compared to Ca + D (Table 1; T*G, $p < .05$). Absolute weight and body fat % did not differ between groups at either time point. BMI was higher in the placebo group at baseline ($p < .05$), but was similar between groups post-training.

3.3. Circulating biomarkers of bone metabolism

Ca + D supplementation resulted in increased 25OHD as compared to placebo (T*G; $p < .05$), even after controlling for season and race (Table 2). Change in 25OHD was negatively associated with starting 25OHD ($R = -0.77$, $p < .001$) and a trend was observed between change in body fat % and change in 25OHD ($R = -0.18$, $p = .06$). The active vitamin D metabolite, $1,25(OH)_2D$ did not change in either group over the course of training. Markers of bone turnover, BAP ($p < .001$) and TRAP5b ($p < .001$), decreased over training with Ca + D, but not in the placebo (Table 2; T*G). The OPG:RANKL ratio more than doubled in both groups over the course of training due to increases in OPG and decreases in RANKL (Table 2; $p < .001$) and OC decreased in both groups ($p < .01$). In addition, two inhibitors of the bone anabolic Wnt signaling pathway, SOST and DKK1, both decreased with training (Table 2; $p < .001$).

Table 1
Volunteer characteristics.

		Total group		Summer		Winter	
	Time point	Placebo bars (n = 76)	Ca + D bars (n = 71)	Placebo bars (n = 37)	Ca + D bars (n = 37)	Placebo bars (n = 39)	Ca + D bars (n = 34)
Sex, n (%)	Pre						
Male		39 (51)	36 (51)	22 (59)	23 (62)	17 (44)	13 (38)
Female		37 (49)	35 (49)	15 (41)	14 (38)	22 (56)	21 (62)
Race, n (%)	Pre						
White/Caucasian		57 (75)	51 (72)	28 (76)	26 (70)	29 (74)	25 (74)
Black/African American		16 (21)	14 (20)	6 (16)	8 (22)	10 (26)	6 (15)
Other		3 (4)	6 (8)	3 (8)	3 (8)	0 (0)	3 (8)
Age, y	Pre	18.9 ± 1.6	18.8 ± 1.5	18.7 ± 1.8	18.3 ± 1.0	19.2 ± 1.4	19.3 ± 1.7
Ht, cm	Pre	168 ± 8	168 ± 9				
Wt, kg	Pre	69.4 ± 9.7	67.3 ± 12.0	68.6 ± 8.0	68.1 ± 12.3	72.1 ± 10.4	68.1 ± 13.3
	Post	67.9 ± 8.3$^{\#\#}$	67.2 ± 10.7	67.6 ± 7.1	64.3 ± 11.2	70.0 ± 8.6$^{\#\#}$	66.6 ± 11.1$^{\#\#}$
BMI, kg/m^2	Pre	24.3 ± 2.5	23.4 ± 2.7$^{¥}$	23.7 ± 2.2	23.2 ± 2.7	25.3 ± 2.4	24.3 ± 2.8
	Post	23.7 ± 1.9$^{\#\#}$	23.4 ± 2.2	23.3 ± 1.6	23.3 ± 2.1	24.6 ± 1.8$^{\#}$	23.8 ± 2.3$^{\#}$
Body fat, %	Pre	17.9 ± 7.2	16.2 ± 6.1	16.0 ± 7.1	14.8 ± 6.0	19.8 ± 7.1	19.2 ± 5.1
	Post	13.4 ± 5.6$^{\#}$	12.9 ± 4.8$^{\#}$	12.3 ± 4.5$^{\#\#\#}$	12.4 ± 4.1$^{\#\#\#}$	14.1 ± 6.5$^{\#\#\#}$	14.1 ± 4.9$^{\#\#\#}$
[1]Dietary calcium, mg/day	Pre	1157 ± 678	1201 ± 620	1135 ± 564	1274 ± 641	1115 ± 648	1201 ± 588
	Post	1146 ± 552	3131 ± 700$^{\#\#\#, ¥}$	886 ± 337$^{\#}$	2651 ± 415$^{\#\#\#, ¥}$	1354 ± 597	3588 ± 684$^{\#\#\#, ¥}$
[1,2]Meeting RDA for calcium, n (%)	Pre	27 (40)	31 (53)	15 (44)	14 (48)	12 (36)	17 (57)
	Post	32 (45)	64 (100)$^{¥}$	6 (18)	33 (100)$^{¥}$	26 (70)	31 (100)$^{¥}$
[1]Dietary vitamin D, IU/day	Pre	264 ± 190	288 ± 194	274 ± 185	283 ± 190	248 ± 171	308 ± 216
	Post	262 ± 187	1209 ± 203$^{\#\#\#, ¥}$	169 ± 92$^{\#\#}$	1142 ± 162$^{\#\#\#, ¥}$	350 ± 217	1275 ± 240$^{\#\#\#, ¥}$
[1,2]Meeting RDA for vitamin D, n (%)	Pre	5 (6)	8 (14)	2 (6)	3 (10)	3 (6)	5 (10)
	Post	4 (6)	64 (100)$^{¥}$	0 (0)	33 (100)$^{¥}$	4 (11)	31 (100)$^{¥}$

Only subjects that completed the study are included. Data are means ± SD or n (%). Compared to pre- within a treatment group; $^{\#\#\#}p < .001$, $^{\#\#}p < .01$, $^{\#}p < .05$. Compared to placebo group within time point; $^{¥}p < .05$. [1]Dietary intake information was excluded if implausible energy intake was reported (< 300 or > 4500 kcal/d for females and < 800 or > 5000 kcal/d for males, n = 13 pre, n = 1 post) as previously reported [1]. [2]RDA = Recommended daily allowance (calcium = 1300 mg/day < 19 years of age ≥1000 mg/day; Vitamin D = 600 IU/day). $^{¥}$Compared to placebo group ($p < .05$).

Table 2
Circulating bone biomarkers pre- and post- Marine initial military training (IMT) and by season.

		Total group		Summer		Winter	
	Time point	Placebo (n = 72)	Ca + D (n = 69)	Placebo (n = 37)	Ca + D (n = 35)	Placebo (n = 35)	Ca + D (n = 34)
PTH, pg/ml	Pre	31.0 ± 12.8	28.5 ± 13.8	29.9 ± 9.6	27.5 ± 13.5	28.6 ± 15.4	30.3 ± 14.3
	Post	29.8 ± 13.5	28.1 ± 15.8	26.9 ± 13.3	26.7 ± 17.1	29.8 ± 13.7	31.2 ± 14.3
25OHD, ng/ml	Pre	29.9 ± 10.9	25.6 ± 7.4	34.9 ± 8.5	29.4 ± 6.5$^{¥}$	25.7 ± 11.2	22.7 ± 7.1
	Post	29.6 ± 7.0	27.8 ± 5.5$^{\#}$	32.4 ± 6.1$^{\#\#}$	29.7 ± 4.7$^{¥}$	27.8 ± 7.1$^{\#\#}$	26.7 ± 6.1$^{\#\#}$
1,25D, pg/ml	Pre	79.9 ± 24.4	80.1 ± 26.0	86.6 ± 16.9	83.5 ± 16.9	73.5 ± 28.9	81.5 ± 35.2
	Post	76.4 ± 18.2	78.0 ± 18.0	71.6 ± 15.5$^{\#\#\#}$	77.5 ± 14.4$^{\#\#\#}$	78.9 ± 20.2	81.3 ± 21.9
P1NP, pg/ml	Pre	29.9 ± 21.6	22.9 ± 25.1$^{¥}$	34.5 ± 25.0	27.5 ± 31.6	22.1 ± 15.7	20.9 ± 13.2
	Post	30.4 ± 23.2	21.7 ± 14.5$^{¥}$	29.2 ± 18.1$^{\#}$	23.2 ± 16.7$^{\#}$	28.1 ± 27.6$^{\#\#}$	22.8 ± 11.6$^{\#\#}$
BAP, U/L	Pre	29.6 ± 10.3	34.1 ± 11.5	28.7 ± 10.0	36.4 ± 12.9$^{¥}$	31.0 ± 10.7	34.3 ± 9.8
	Post	28.2 ± 7.2	28.5 ± 9.1$^{\#\#\#}$	27.2 ± 6.4	29.2 ± 10.2$^{\#\#\#}$	29.9 ± 7.8	25.2 ± 7.6$^{\#\#\#}$
TRAP, U/L	Pre	3.9 ± 1.3	3.7 ± 1.0	3.66 ± 1.31	3.73 ± 1.17	3.97 ± 1.29	3.63 ± 0.97
	Post	4.2 ± 1.5$^{\#\#, ¥}$	3.2 ± 0.8$^{\#\#\#}$	3.92 ± 1.02	3.26 ± 0.90$^{\#\#\#, ¥}$	4.51 ± 1.67$^{\#}$	3.20 ± 0.77$^{\#, ¥}$
CTX, ng/ml	Pre	1.0 ± 0.3	0.9 ± 0.3	1.0 ± 0.4	1.0 ± 0.3	0.9 ± 0.3	0.9 ± 0.3
	Post	0.8 ± 0.3$^{\#\#\#}$	0.7 ± 0.3$^{\#\#\#}$	0.8 ± 0.3$^{\#\#\#}$	0.8 ± 0.2$^{\#\#\#}$	0.8 ± 0.3$^{\#\#\#}$	0.7 ± 0.4$^{\#\#\#}$
OCN, ng/ml	Pre	11.7 ± 4.5	13.2 ± 4.2$^{¥}$	9.2 ± 3.8	13.8 ± 3.5$^{¥}$	12.7 ± 4.5	14.1 ± 5.2
	Post	10.0 ± 3.6$^{\#\#\#}$	10.1 ± 3.7$^{\#\#\#}$	8.6 ± 3.3$^{\#}$	11.2 ± 2.9$^{\#\#\#, ¥}$	10.1 ± 3.8$^{\#\#\#}$	10.1 ± 4.7$^{\#\#\#}$
OPG, pg/ml	Pre	234 ± 59.3	225 ± 54.3	220 ± 55	222 ± 48	254 ± 59	238 ± 62
	Post	279 ± 80.2$^{\#\#\#}$	277 ± 62.6$^{\#\#\#}$	250 ± 81$^{\#\#\#}$	262 ± 67$^{\#\#\#}$	317 ± 65$^{\#\#\#}$	303 ± 50$^{\#\#\#}$
sRANKL, pg/ml	Pre	28.8 ± 38.1	36.8 ± 35.1	40.2 ± 48.1	19.4 ± 13.5	24.2 ± 22.3	36.5 ± 45.6
	Post	18.8 ± 32.2$^{\#\#\#}$	27.6 ± 32.7$^{\#\#\#}$	27.2 ± 39.5$^{\#\#\#}$	12.9 ± 11.5$^{\#\#}$	16.4 ± 22.1$^{\#\#\#}$	24.9 ± 41.8$^{\#\#\#}$
OPG:RANKL	Pre	14.3 ± 9.2	14.9 ± 13.9	10.7 ± 7.8	18.1 ± 14.9	15.6 ± 9.8	14.7 ± 14.5
	Post	32.0 ± 40.0$^{\#\#\#}$	35.2 ± 33.6$^{\#\#\#}$	32.2 ± 55.8$^{\#\#\#}$	41.5 ± 43.0$^{\#\#\#}$	34.5 ± 21.5$^{\#\#\#}$	32.4 ± 19.5$^{\#\#\#}$
SOST, pg/ml	Pre	1260 ± 356	1277 ± 390	1141 ± 312	1428 ± 348	1374 ± 364	1097 ± 374
	Post	1051 ± 281$^{\#\#\#}$	1050 ± 401$^{\#\#\#}$	964 ± 216$^{\#\#\#}$	1260 ± 349$^{\#\#\#}$	1170 ± 303$^{\#\#\#}$	818 ± 334$^{\#\#\#}$
DKK1, pg/ml	Pre	1427 ± 490	1330 ± 423	1578 ± 425	1488 ± 425	1289 ± 514	1140 ± 340
	Post	1217 ± 388$^{\#\#\#}$	1213 ± 350$^{\#\#\#}$	1292 ± 410$^{\#\#\#}$	1274 ± 382$^{\#\#\#}$	1162 ± 359	1148 ± 305

Data are unadjusted means ± SD; analyzed using linear mixed models with a random intercept for each study participant. Fixed factors included time, intervention/group, time*group interaction, race and season. Compared to pre- within treatment group; $^{\#\#\#}p < .001$, $^{\#\#}p < .01$, $^{\#}p < .05$. Compared to placebo group within time point $^{¥}p < .05$.

When summer and winter were analyzed separately, seasonal differences in biomarkers were observed. As expected, baseline vitamin D status was lower in recruits that started training in the winter (23.6 ± 8.9 ng/ml) as compared to summer (31.8 ± 8.4 ng/ml, $p < .001$). In the winter 25OHD increased over the course of training in both groups (Table 2; $p < .01$). In the summer, a time-by-group interaction was observed ($p < .01$) as 25OHD declined in the placebo group (−3.2 ng/ml, $p = .001$), but was maintained in the Ca + D group (Table 2). The active vitamin D metabolite, $1,25(OH)_2D$, declined in both groups during the summer ($p < .001$), but no changes were observed in the winter. Seasonal differences were also observed with respect to pre- to post- changes in P1NP. In the summer, P1NP decreased in both groups ($p < .05$), whereas it increased in the winter ($p < .01$). However, P1NP was higher at the pre- time point in the summer (31.3 ± 27.9 pg/ml) as compared to winter (21.2 ± 14.4 ng/ml, $p < .01$), but did not differ between seasons post-training (27.3 ± 19.8 vs. 25.0 ± 21.8 ng/ml, respectively). P1NP was also highly correlated with 25OHD both pre- ($R = 0.94$, $p < .01$) and post-training ($R = 0.80$, $p < .05$).

Consistent with the results obtained with seasonal stratification, when volunteers were stratified by pre-IMT 25OHD (< 20 ng/ml vs ≥ 20 ng/ml), those starting training with 25OHD < 20 ng/ml exhibited an increase in 25OHD over training regardless of treatment group (+7.9 ng/ml, $p < .001$). In contrast, a time-by-group interaction ($p < .01$) was observed for those who started training with 25OHD ≥ 20 ng/ml such that 25OHD declined in the placebo group (−2.7 ng/ml, $p < .01$) but was maintained over training with Ca + D treatment.

3.4. pQCT

Several bone parameters changed over the course of training with the most robust changes observed at the 4% site (Fig. 2). At this site, total BMC, total vBMD and BSI all increased independent of treatment group (Table 3; all $p < .05$). At the 14% site, cortical BMC, thickness, and SSIp all increased during training whereas only BMC and SSIp increased at the 66% site in both groups (Table 3; all $p < .05$). No time-by-group interactions were observed for any pQCT parameter.

When summer and winter iterations were analyzed separately, 4% BSI increased to a greater extent in the Ca + D group (+4.50 ± 2.9) as compared to placebo (+2.7 ± 2.9 mg/mm*4) in the summer (Table 3; T*G; $p = .02$), but no such interaction was observed in any other bone tissue variable in the summer or the winter. In addition, Ct BMC ($p = .02$) and SSIp ($p < .01$) at the 66% site increased in both groups in the summer, but did not change in the winter (Table 3).

When volunteers were divided into tertiles based on percent change in BSI over training, there were no differences in sex, race, age, or treatment group in tertile 3 as compared to tertile 1. Also, neither dietary nor total intakes of calcium and vitamin D differed by tertile. However, those in tertile 3 exhibited a greater percent loss in body fat percentage (−32.0 ± 15.2%) compared to those in the lowest tertile (−20.3 ± 21.7%; $p < .05$), whereas starting body fat % did not differ between tertile 3 and 1. In addition, tertile 3 contained a greater proportion of volunteers that started training in the winter (62% vs. 42%), had lower vitamin D status at both the start (23.9 ± 7.9 vs. 31.3 ± 9.8 ng/ml) and end of training (26.4 ± 6.5 vs. 30.5 ± 6.6 ng/ml), but exhibited a greater increase in 25OHD (+3.9 ± 25.7 vs. −9.9 ± 31.3 ng/ml) over training as compared to tertile 1. Change in 25OHD was positively correlated with change in BSI when BSI was treated as a continuous variable ($R = 0.31$, $p = .001$). A trend toward lower $1,25(OH)_2D$ in tertile 3 as compared to tertile 1 was also observed ($p = .06$). Volunteers in tertile 3 exhibited lower P1NP at the pre- time point and higher OCN at post- as compared to those in tertile 1 (Table 4; $p < .05$ for both); no other markers of bone turnover differed at either time point.

In order to determine whether training and treatment effects on BSI and bone turnover markers varied according to vitamin D status, volunteers were stratified by starting 25OHD (< 20 ng/ml vs ≥ 20 ng/ml) (Supplemental Table 1). Of the 115 volunteers with valid pre- and post-pQCT scans of the 4% tibia, n = 27 (23.5%) presented with low 25OHD at the start of training. A time-by-starting status interaction was observed ($p < .05$) where BSI increased to a greater extent in those with low starting 25OHD (+6.13, $p < .001$) compared to those with higher starting 25OHD (+4.09, $p < .001$). However, time-by-treatment group interactions were not observed for BSI in either vitamin D status group. Since the proportion of African American volunteers was greater in the group with low as compared to higher 25OHD status ($p < .01$), volunteers were further stratified by major race classifications (White/Caucasian or Black/African American, Supplemental Tables 2 and 3). In these stratified analyses, BSI changes did not differ by starting vitamin D status group (Supplemental Tables 2 and 3).

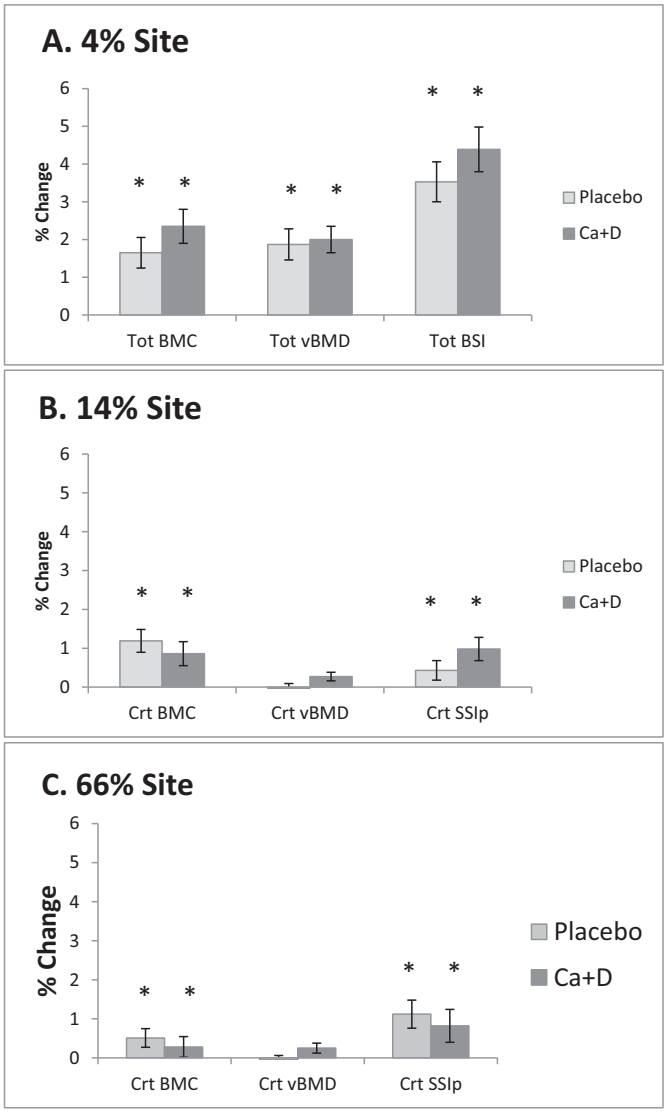

Fig. 2. Percent change in pQCT bone parameter pre- to post- Marine IMT. Data are unadjusted means ± SD. *Main effect of time ($p < .05$).

4. Discussion

The main finding of this randomized double-blind, placebo-controlled trial is that daily calcium (2000 mg) and vitamin D (1000 IU) supplementation improved vitamin D status and reduced some markers of bone turnover in Marine Corps recruits undergoing 12 weeks of IMT

Table 3
Bone changes pre- and post- Marine IMT and by season.

		Total group		Summer		Winter	
	Time point	Placebo (n = 58)	Ca + D (n = 57)	Placebo (n = 33)	Ca + D (n = 31)	Placebo (n = 25)	Ca + D (n = 26)
4%							
Tot BMC, mg/mm	Pre	360.4 ± 74.6	358.8 ± 71.6	358.9 ± 61.0	364.4 ± 73.1	362.4 ± 90.8	352.2 ± 70.5
	Post	366.0 ± 76.1$^{\#\#\#}$	367.0 ± 73.3$^{\#\#\#}$	362.2 ± 60.5$^{\#\#\#}$	370.9 ± 74.4$^{\#\#\#}$	371.0 ± 93.9$^{\#\#\#}$	362.4 ± 73.2$^{\#\#\#}$
Tot vBMD, mg/cm^3	Pre	343.4 ± 39.3	336.9 ± 44.8	342.9 ± 37.0	340.3 ± 40.0	344.1 ± 43.0	333.0 ± 50.5
	Post	349.7 ± 39.7$^{\#\#\#}$	343.2 ± 43.6$^{\#\#\#}$	347.0 ± 39.1$^{\#\#\#}$	346.4 ± 37.8$^{\#\#\#}$	353.2 ± 41.0$^{\#\#\#}$	339.5 ± 50.3$^{\#\#\#}$
Tot BSI, mg/mm*4	Pre	125.3 ± 35.5	122.9 ± 37.4	123.6 ± 27.1	125.3 ± 33.6	127.5 ± 44.7	120.0 ± 42.0
	Post	129.4 ± 36.6$^{\#\#\#}$	127.9 ± 38.7$^{\#\#\#}$	126.3 ± 27.9$^{\#\#\#, ¥}$	129.8 ± 34.1$^{\#\#\#, ¥}$	133.5 ± 45.9$^{\#\#\#}$	125.7 ± 44.0$^{\#\#\#}$
14%							
Crt BMC, mg/mm	Pre	211.9 ± 40.6	209.7 ± 40.8	212.1 ± 37.4	209.7 ± 39.2	211.6 ± 45.6	209.7 ± 43.2
	Post	214.5 ± 41.6$^{\#\#\#}$	211.4 ± 40.7$^{\#\#\#}$	215.0 ± 38.9$^{\#\#\#}$	211.5 ± 39.2$^{\#\#\#}$	213.7 ± 46.0$^{\#\#}$	211.3 ± 43.1$^{\#\#}$
Crt vBMD, mg/cm^3	Pre	1130 ± 22	1123 ± 22	1131 ± 19	1119 ± 20	1128 ± 26	1127 ± 24
	Post	1130 ± 22	1126 ± 21	1131 ± 20	1121 ± 19	1128 ± 25	1131 ± 21
Crt Th, mm	Pre	2.86 ± 0.50	2.81 ± 0.49	2.85 ± 0.50	2.82 ± 0.42	2.87 ± 0.53	2.79 ± 0.56
	Post	2.90 ± 0.51$^{\#\#\#}$	2.82 ± 0.48$^{\#\#\#}$	2.90 ± 0.53$^{\#\#}$	2.84 ± 0.42$^{\#\#}$	2.90 ± 0.49$^{\#}$	2.81 ± 0.55$^{\#}$
SSIp, mm*3	Pre	1589 ± 431	1587 ± 378	1598 ± 397	1594 ± 408	1577 ± 486	1580 ± 351
	Post	1595 ± 432$^{\#\#\#}$	1603 ± 387$^{\#\#\#}$	1603 ± 397$^{\#}$	1608 ± 422$^{\#}$	1584 ± 487$^{\#\#}$	1598 ± 354$^{\#\#}$
66%							
Crt BMC, mg/mm	Pre	351.8 ± 57.1	351.9 ± 62.1	351.9 ± 50.8	350.0 ± 65.5	351.7 ± 65.0	353.8 ± 59.9
	Post	353.7 ± 58.2$^{\#}$	352.9 ± 62.2$^{\#}$	354.4 ± 51.4$^{\#}$	352.1 ± 66.5$^{\#}$	352.9 ± 66.6	353.6 ± 59.2
Crt vBMD, mg/cm^3	Pre	1121 ± 26	1117 ± 23	1114 ± 18	1110 ± 20	1129 ± 33	1124 ± 23
	Post	1120 ± 25	1120 ± 21	1114 ± 18	1112 ± 19	1128 ± 31	1127 ± 22
Crt Th, mm	Pre	4.36 ± 0.53	4.32 ± 0.60	4.32 ± 0.42	4.29 ± 0.61	4.41 ± 0.65	4.36 ± 0.60
	Post	4.35 ± 0.54	4.31 ± 0.59	4.32 ± 0.42	4.30 ± 0.61	4.40 ± 0.67	4.33 ± 0.59
SSIp, mm*3	Pre	2662 ± 715	2687 ± 659	2710 ± 662	2715 ± 684	2604 ± 784	2660 ± 646
	Post	2691 ± 714$^{\#\#}$	2710 ± 672$^{\#\#}$	2750 ± 653$^{\#\#}$	2744 ± 712$^{\#\#}$	2619 ± 789	2677 ± 643

Data are unadjusted means ± SD and were analyzed using linear mixed models with a random intercept for each study participant. Fixed factors included time, intervention group, time-by-group interaction, race and sex, and baseline BMI as a covariate. Compared to pre- within a treatment group; $^{\#\#\#}p < .001$, $^{\#\#}p < .01$, $^{\#}p < .05$. Time-by-group interaction; $^{¥}p < .05$.

and these effects varied by season. In contrast, effects of Ca + D on bone tissue were limited; BSI improved in the Ca + D group to a greater extent than placebo, but only in the summer iteration. Otherwise, skeletal parameters were largely unaffected by supplementation. This was the first Ca + D supplementation trial in Marine Corps recruits, and the first in military trainees comparing the effects of supplementation between two seasons. While several changes in tibial pQCT measures were observed from pre- to post- training, effects of Ca + D treatment on bone changes were only observed in recruits that started training during the summer and were limited to one parameter at the distal tibia. When volunteers were divided into tertiles based on percent increase in distal tibia bone strength, lower baseline vitamin D status and greater increases in 25OHD were observed in those with the greatest increase in bone strength over training. In addition, those who exhibited the greatest increase in bone strength were more likely to start training in the winter and lose a greater proportion of body fat.

The observed decreases in BAP and TRAP over training in the Ca + D group may indicate a reduction in bone remodeling with supplementation, which in other environments is generally considered beneficial for bone tissue as higher rates of turnover result in temporal weakness due to the lag between bone resorption and mineralization [18]. However, in the current training environment, both modeling and remodeling processes are occurring and the precise effects of supplementation on each of these could not be determined in this study. Other bone biomarker changes were consistent across treatment groups and these relationships held when dadta were analyzed by season. In support of bone formation observed by pQCT, the anti-anabolic peptides, SOST and DKK1 both decreased in both groups during training. However, the biomarker of bone formation, OCN, decreased in both groups during training, while the formation marker, P1NP, did not change in either group. These results suggest that the biomarkers of bone formation may not be reflective of the new bone formation that occurred during IMT as indicated by the pQCT results. Changes in biochemical markers of bone resorption and osteoclast differentiation were similarly variable. CTX decreased in both groups and the OPG:RANKL ratio increased in both groups. These results are in contrast to a prior report in which Ca + D supplementation increased the OPG:RANKL to a greater extent compared to placebo [8].

Seasonal differences in vitamin D status are well known, with the nadir in vitamin D status occurring around February and the apex around July depending on latitude [19]. Season and baseline 25OHD are known to influence the effectiveness of vitamin D supplementation to increase 25OHD, such that larger increases are expected in those with low starting 25OHD and/or in the winter [8,19]. In the current study, 25OHD status increased in both groups in the winter iteration when starting status was lowest, whereas 25OHD declined in the summer iteration in the placebo group and was maintained in the Ca + D group. Similarly, when volunteers were stratified by baseline 25OHD (< 20 ng/ml vs ≥ 20 ng/ml), an effect of treatment was only observed in those who started training with higher 25OHD. In these individuals, Ca + D treatment prevented a decline in 25OHD, whereas those who started training with 25OHD < 20 ng/ml experienced an improvement in 25OHD independent of treatment. In contrast to our findings, Rees et al. demonstrated that 1000 IU/d vitamin D resulted in an increase in 25OHD in older adults (aged 45–75 y) regardless of season, although this trial provided supplementation over the course of one year [20]. In a 20 wk. vitamin D dose-response study, Heaney and colleagues reported that 500–1000 IU/d maintained, but did not significantly increase, 25OHD in healthy males who began supplementation in the fall [19]. The authors hypothesized that contributions from endogenous tissue stores were likely required to maintain vitamin D status with this level of supplementation, and much higher levels, i.e. 3800 IU/d are required to maintain 25OHD in the absence of cutaneous synthesis [22]. These findings are consistent with the results of our study and suggest that higher levels of vitamin D supplementation may be required to increase vitamin D status during 12 weeks of IMT depending on season.

In recruits who began training in the winter, the observed increase

Table 4
Predictors of change in distal tibia bone strength index during Marine IMT.

		Tertile 1 (n = 38)	Tertile 2 (n = 40)	Tertile 3 (n = 37)
BSI, mg/mm*4	Pre	134 ± 32	118 ± 27	130 ± 46
	Post	134 ± 32	121 ± 28	140 ± 49
BSI, % change		0.23 ± 1.37	3.12 ± 0.74	8.33 ± 3.85$^{\#\#}$
Group, n (%)	Placebo	22 (58)	19 (48)	17 (46)
	Ca + D	16 (42)	21 (52)	20 (54)
Age, y		18.8 ± 1.6	19.0 ± 2.1	19.0 ± 1.2
Sex	Male	21 (55)	15 (38)	20 (54)
	Female	17 (45)	25 (62)	17 (46)
Race	White/Caucasian	24 (63)	29 (73)	25 (68)
	Black/African American	10 (26)	9 (23)	11 (30)
	Other	4 (11)	2 (4)	1 (2)
Season, n (%)	Summer	22 (58)	28 (70)	14 (38)
	Winter	16 (42)	12 (30)	23 (62)$^{\#\#}$
BMI, kg/m^2	Pre	24.1 ± 2.7	23.6 ± 2.6	24.6 ± 2.4
	Post	23.9 ± 1.9	23.6 ± 2.0	23.7 ± 2.1
Body fat, %	Pre	16.7 ± 7.3	18.1 ± 6.7	17.3 ± 6.1
	Post	12.7 ± 5.3	14.1 ± 4.9	11.8 ± 5.1
Body fat, % change		−20.3 ± 21.7	−19.1 ± 16.5	−32.0 ± 15.2$^{\#}$
[1]Diet Ca, mg/d	Pre	1302 ± 763	1184 ± 477	1116 ± 592
	Post	1163 ± 533	1016 ± 464	1005 ± 430
[1]Diet vit D, IU/d	Pre	319 ± 236	260 ± 135	274 ± 189
	Post	267 ± 171	212 ± 135	242 ± 163
Compliance, %		97.9 ± 2.8	97.1 ± 6.0	93.5 ± 9.6$^{\#}$
Total Ca intake, mg/d	Post	1993 ± 1255	2027 ± 1126	2040 ± 997
Total Vit D intake, IU/d	Post	649 ± 539	709 ± 531	716 ± 455
Pre vitamin D status, n (%)	< 20 ng/ml	8 (21.1)	7 (17.5)	12 (32.4)
	≥ 20 ng/ml	30 (78.9)	33 (82.5)	25 (67.6)
25(OH)D, ng/ml	Pre	31.3 ± 9.8	30.0 ± 9.5	23.9 ± 7.9$^{\#}$
	Post	30.5 ± 6.6	31.6 ± 5.4	26.4 ± 6.5$^{\#}$
25(OH)D, ng/ml Change		−9.9 ± 31.3	−7.5 ± 20.1	3.9 ± 25.7$^{\#}$
1,25(OH) $_2$D, pg/ml	Pre	86.0 ± 27.4	80.5 ± 21.7	76.8 ± 24.7
	Post	76.0 ± 20.4	73.0 ± 13.5	80.7 ± 20.8
PTH, pg/ml	Pre	31.1 ± 9.5	27.6 ± 12.4	25.5 ± 12.6
	Post	31.7 ± 17.2	27.8 ± 13.2	27.9 ± 13.1
OCN, ng/ml	Pre	11.9 ± 5.0	10.9 ± 3.8	14.0 ± 4.7
	Post	9.4 ± 3.5	9.2 ± 3.0	11.5 ± 4.4$^{\#\#}$
P1NP, ug/l	Pre	38.5 ± 38.1	24.0 ± 15.6	20.1 ± 14.9$^{\#}$
	Post	33.2 ± 27.7	22.1 ± 11.3	24.5 ± 21.2
BAP, ug/l	Pre	29.7 ± 9.5	31.7 ± 9.1	30.9 ± 10.9
	Post	27.1 ± 6.9	29.0 ± 7.6	27.6 ± 8.5
CTX, ng/ml	Pre	0.9 ± 0.3	0.9 ± 0.3	1.0 ± 0.4
	Post	0.8 ± 0.3	0.8 ± 0.2	0.8 ± 0.3
TRAP, U/L	Pre	3.47 ± 0.85	3.75 ± 1.06	3.98 ± 1.50
	Post	3.60 ± 0.78	3.46 ± 1.09	3.71 ± 1.28
DKK1, pg/ml	Pre	1425 ± 514	1461 ± 528	1285 ± 408
	Post	1131 ± 436	1218 ± 363	1208 ± 269
OPG, pg/ml	Pre	241 ± 52	216 ± 51	244 ± 58
	Post	286 ± 73	272 ± 71	277 ± 78
RANKL, pg/ml	Pre	42.0 ± 59.7	29.6 ± 23.8	22.8 ± 20.4
	Post	32.0 ± 50.6	19.3 ± 23.9	14.0 ± 15.7
OPG:RANKL	Pre	13.8 ± 9.3	11.6 ± 10.2	18.9 ± 16.7
	Post	26.7 ± 26.6	38.7 ± 58.1	40.5 ± 34.1
SOST, pg/ml	Pre	1256 ± 430	1224 ± 376	1316 ± 312
	Post	1055 ± 320	1105 ± 305	1011 ± 344

Data are unadjusted means ± SD and were analyzed by independent t-tests for differences between tertile 1 and tertile 3. Categorical data were analyzed by Chi square; $^{\#\#}p < .01$, $^{\#}p < .05$. [1]Dietary intake information was excluded if implausible energy intake was reported (< 300kcals or > 4500kcals, n = 13 pre, n = 1 post) as previously reported [1].

in 25OHD is likely due to release from endogenous fat stores as volunteers in the winter iteration lost more body fat than those who started training in the summer. This observation is consistent with prior reports in which fat loss was associated with increases in 25OHD even in the absence of sunlight [21]. Consistent with this hypothesis, change in 25OHD tended to correlate with change in fat mass (p = .06). Interestingly, volunteers with the greatest change in bone strength were more likely to start training in the winter, when starting 25OHD was lowest, yet experienced the greatest change in 25OHD over the course of training. However, when volunteers were stratified by starting 25OHD < 20 ng/ml or ≥ 20 ng/ml, the change in BSI did not differ by starting vitamin D status. These data suggest both direction and magnitude of change in 25OHD trajectory rather than baseline status cutoffs may be important factors relating vitamin D status to bone health during IMT and this relationship may be related to tissue level availability of 25OHD.

Optimal calcium and vitamin D status impact bone health directly by supporting mineralization, and indirectly by suppressing the PTH-1-alpha hydroxylase axis. The majority of vitamin D signaling occurs via binding of $1,25(OH)_2D$ to the vitamin D receptor (VDR), nuclear translocation, and subsequent binding to the vitamin D response element in the promoter region to regulate gene expression. Circulating $1,25(OH)_2D$ is produced via renal activation of 25OHD by the 1-alpha hydroxylase enzyme CYP27B1 and is in general catabolic to bone as it increases osteoclastic bone resorption. The main endocrine function of $1,25(OH)_2D$ is to maintain circulating calcium concentrations by increasing intestinal calcium absorption, renal reabsorption and bone resorption. In addition to intestinal and renal cells, the VDR is expressed in osteoblasts and osteocytes where it regulates bone turnover and interacts with signaling arising from mechanical stimuli such as load bearing exercise [7,8]. $1,25(OH)_2D$ signaling in osteoblasts results in RANKL-induced osteoclastogenesis and increased bone resorption in order to release calcium into circulation; in this way activation of the PTH-1alpha hydroxylase axis over time weakens bone matrix in order to preserve serum calcium. In vitro studies in cultured cells also indicate that exogenous $1,25(OH)_2D$ treatment is anti-anabolic as pre-treatment of osteoblasts with $1,25(OH)_2D$ prior to pulsating fluid flow abolishes the anabolic nitric oxide response to mechanical loading [23,24].

Thus, Ca + D supplementation during IMT may support bone health by increasing circulating calcium and preventing the PTH-induced activation of vitamin D. In support of this, a prior study in which Army recruits were provided the same level of calcium and vitamin D supplementation as the current study, PTH increased in the placebo group and not in the Ca + D group over the 8 weeks of training [8]. While we did not observe a similar increase in PTH following training here, this may be due to the short half-life of PTH; supplementation may have prevented perturbations in the PTH-1-alpha-hydroxylase axis towards the beginning of training which would not have been detected 12 weeks later. Acute increases in PTH following exercise have been reported by others, and this increase can be prevented by providing calcium before or during the exercise [25,26]. Consistent with downstream effects of PTH suppression, in the current study Ca + D treatment decreased TRAP and BAP suggesting that supplementation suppressed bone turnover. Of note, the dose of calcium used in the Army study as well as the current study (2000 mg/d) was chosen based on the investigation by Lappe and colleagues, and results in a total intake that is likely higher than required to maintain PTH during IMT. In the current study, total mean calcium intake from diet and supplemental sources was just over the tolerable upper limit (UL; 3000 mg/d) for recruits under 19 yr and 125% of the UL (2500 mg/d) for those 19 yr and older. As little to no measurable effect of this level of supplementation was observed on bone tissue outcomes, the current study does not support benefit of intakes at this level.

The imaging results of the current study are of similar or greater magnitude to the bone responses observed after Army training. Previously documented in Army recruits undergoing 8 weeks of IMT,

distal tibial BSI increased by approximately 1–3% depending on treatment group [8]. In a separate study utilizing high resolution pQCT to evaluate changes in tibial (micro)architecture in female Army recruits, total vBMD of the distal tibia also increased by approximately 2%, which translated into a 2.4% increase in stiffness and failure load [27]. In the current study we report that distal tibial BSI increased by 3.3% in the placebo group and 4% in the Ca + D group whereas total vBMD increased by approximately 1.8% in both groups. Cadaveric studies indicate that BSI explains 75–85% of the variation in failure load suggesting that even small changes will have clinical significance [28]. Also consistent with these prior reports, more robust bone changes occurred at the distal metaphyseal site as compared to the diaphysis [8,27]. Thus, the current findings are of similar or greater magnitude to the bone responses observed after Army training. As Marine recruit training is 50% longer than Army training (12 vs 8 weeks), the changes in bone appear to be commensurate with the length of training and the number of bone loading cycles during training.

This study had many strengths owing to the randomized, double-blind placebo-controlled design including block randomization to equally distribute volunteers by race and sex between the two study groups. Also, the doses of calcium and vitamin D chosen were based on prior work showing a reduction in stress fracture incidence in female Navy recruits [7]. However, despite the randomization, BMI at the start of training was lower in the Ca + D group as compared to placebo. BMI was thus included as a covariate in the adjusted models in order to account for this difference. In addition, while change in 25OHD was positively associated with change in distal tibial strength, whether this is causal cannot be definitively established. However, given the known importance of vitamin D to skeletal health, it is reasonable to postulate that the trajectory of vitamin D status would impact bone adaptation to mechanical loading and in vitro pulsatile flow studies with osteoblasts support this notion [23,25]. Finally, the pre- post- study design does not provide full resolution of the biomarker responses to IMT. For example, we could not ascertain the shape of the curve for PTH over the course of training. However, this is the first study to evaluate the effects of Ca + D supplementation on bone responses to IMT in recruits starting training in summer and winter. These findings provide further insight into relationships between vitamin D status and bone health during training, however there were limited benefits to bone tissue from the dose of Ca + D supplementation provided in this study and these. varied by season. Future studies characterizing the acute and intermediate responses of PTH, bone biomarkers, and bone tissue changes to training and Ca + D supplementation would be useful to determine the nature of these relationships over time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.03.021.

References

[1] Lutz LJ, Gaffney-Stomberg E, Scisco JL, Cable SJ, Karl JP, Young AJ, McClung JP: Assessment of dietary intake using the healthy eating index during military training. U.S. Army Med. Dep. J. 2013:91–97.
[2] B.H. Jones, S.B. Thacker, J. Gilchrist, C.D. Kimsey Jr., D.M. Sosin, Prevention of lower extremity stress fractures in athletes and soldiers: a systematic review, Epidemiol. Rev. 24 (2) (2002) 228–247.
[3] K.E. Friedl, R.K. Evans, D.S. Moran, Stress fracture and military medical readiness: bridging basic and applied research, Med. Sci. Sports Exerc. 40 (11 Suppl) (2008) S609–S622.
[4] B.R. Waterman, B. Gun, J.O. Bader, J.D. Orr, P.J. Belmont Jr, Epidemiology of Lower Extremity Stress Fractures in the United States Military, Mil. Med. 181 (10) (2016) 1308–1313.
[5] A.A. Wright, J.B. Taylor, K.R. Ford, L. Siska, J.M. Smoliga, Risk factors associated with lower extremity stress fractures in runners: a systematic review with meta-analysis, Br. J. Sports Med. 49 (23) (2015) 1517–1523.
[6] J.M. Jacobs, K.L. Cameron, J.A. Bojescul, Lower extremity stress fractures in the military, Clin. Sports Med. 33 (4) (2014) 591–613.
[7] J. Lappe, D. Cullen, G. Haynatzki, R. Recker, R. Ahlf, K. Thompson, Calcium and vitamin d supplementation decreases incidence of stress fractures in female navy recruits, J. Bone Miner. Res. 23 (5) (2008) 741–749.
[8] E. Gaffney-Stomberg, L.J. Lutz, J.C. Rood, S.J. Cable, S.M. Pasiakos, A.J. Young, J.P. McClung, Calcium and vitamin D supplementation maintains parathyroid hormone and improves bone density during initial military training: a randomized, double-blind, placebo controlled trial, Bone 68 (2014) 46–56.
[9] P.R. Ebeling, Vitamin D and bone health: epidemiologic studies, Bonekey Rep. 3 (2014) 511.
[10] Vasikaran S, Eastell R, Bruyere O, Foldes AJ, Garnero P, Griesmacher A, McClung M, Morris HA, Silverman S, Trenti T et al: Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. Osteoporos. Int. 2011, 22(2):391–420.
[11] D. Dao, S. Sodhi, R. Tabasinejad, D. Peterson, O.R. Ayeni, M. Bhandari, F. Farrokhyar, Serum 25-hydroxyvitamin D levels and stress fractures in military personnel: a systematic review and meta-analysis, Am. J. Sports Med. 43 (8) (2015) 2064–2072.
[12] A.A. Burgi, E.D. Gorham, C.F. Garland, S.B. Mohr, F.C. Garland, K. Zeng, K. Thompson, J.M. Lappe, High serum 25-hydroxyvitamin D is associated with a low incidence of stress fractures, J. Bone Miner. Res. 26 (10) (2011) 2371–2377.
[13] T. Davey, S.A. Lanham-New, A.M. Shaw, B. Hale, R. Cobley, J.L. Berry, M. Roch, A.J. Allsopp, J.L. Fallowfield, Low serum 25-hydroxyvitamin D is associated with increased risk of stress fracture during Royal Marine recruit training, Osteoporos. Int. 27 (1) (2015) 171–179.
[14] J.P. Ruohola, I. Laaksi, T. Ylikomi, R. Haataja, V.M. Mattila, T. Sahi, P. Tuohimaa, H. Pihlajamaki, Association between serum 25(OH)D concentrations and bone stress fractures in Finnish young men, J. Bone Miner. Res. 21 (9) (2006) 1483–1488.
[15] A.S. Jackson, M.L. Pollock, Practical assessment of body composition, Phys. Sportsmed. 13 (5) (1985) 76–90.
[16] W.R. Thompson (Ed.), ACSM's Guidelines for Exercise Testing and Prescription, 8 edn, Lippincott Williams & Wilkins, 2010.
[17] R.M. Blew, V.R. Lee, J.N. Farr, D.J. Schiferl, S.B. Going, Standardizing evaluation of pQCT image quality in the presence of subject movement: qualitative versus quantitative assessment, Calcif. Tissue Int. 94 (2) (2014) 202–211.
[18] D. Ruffoni, P. Fratzl, P. Roschger, R. Phipps, K. Klaushofer, R. Weinkamer, Effect of temporal changes in bone turnover on the bone mineralization density distribution: a computer simulation study, J. Bone Miner. Res. 23 (2008) 1905–1914.
[19] A.R. Webb, L. Kline, M.F. Holick, Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin, J. Clin. Endocrinol. Metab. 67 (2) (1988) 373–378.
[20] J.R. Rees, L.A. Mott, E.L. Barry, J.A. Baron, R.M. Bostick, J.C. Figueiredo, R.S. Bresalier, D.J. Robertson, J.L. Peacock, Lifestyle and other factors explain one-half of the variability in the serum 25-hydroxyvitamin D response to cholecalciferol supplementation in healthy adults, J. Nutr. 146 (11) (2016) 2312–2324.
[21] H.G. Gasier, E. Gaffney-Stomberg, C.R. Young, D.C. McAdams, L.J. Lutz, J.P. McClung, The efficacy of vitamin D supplementation during a prolonged submarine patrol, Calcif. Tissue Int. 95 (3) (2014, Sep) 229–239.
[22] R.P. Heaney, K.M. Davies, T.C. Chen, M.F. Holick, M.J. Barger-Lux, Human serum

25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol, Am. J. Clin. Nutr. 77 (1) (2003) 204–210.
[23] A.R. Wijenayaka, M. Prideaux, D. Yang, H.A. Morris, D.M. Findlay, P.H. Anderson, G.J. Atkins, Early response of the human SOST gene to stimulation by 1alpha,25-dihydroxyvitamin D3, J. Steroid Biochem. Mol. Biol. 164 (2016) 369–373.
[24] H.M. Willems, E.G. van den Heuvel, G. Carmeliet, A. Schaafsma, J. Klein-Nulend, A.D. Bakker, VDR dependent and independent effects of 1,25-dihydroxyvitamin D3 on nitric oxide production by osteoblasts, Steroids 77 (1–2) (2012) 126–131.
[25] K.L. Shea, D.W. Barry, V.D. Sherk, K.C. Hansen, P. Wolfe, W.M. Kohrt, Calcium supplementation and parathyroid hormone response to vigorous walking in post-menopausal women, Med. Sci. Sports Exerc. 46 (10) (2014) 2007–2013.
[26] D.W. Barry, K.C. Hansen, R.E. van Pelt, M. Witten, P. Wolfe, W.M. Kohrt, Acute calcium ingestion attenuates exercise-induced disruption of calcium homeostasis, Med. Sci. Sports Exerc. 43 (4) (2011) 617–623.
[27] Hughes JM, Gaffney-Stomberg E, Guerriere KI, Taylor KM, Popp KL, Xu C, Unnikrishnan G, Staab JS, Matheny RW, Jr., McClung JP et al: Changes in tibial bone microarchitecture in female recruits in response to 8weeks of U.S. Army Basic Combat Training. Bone 2018, 113:9–16.
[28] S.A. Kontulainen, J.D. Johnston, D. Liu, C. Leung, T.R. Oxland, H.A. McKay, Strength indices from pQCT imaging predict up to 85% of variance in bone failure properties at tibial epiphysis and diaphysis, J. Musculoskelet. Neuronal Interact. 8 (4) (2008) 401–409.