

# Bone development in growing female mice fed calcium and vitamin D at lower levels than is present in the AIN-93G reference diet



Jenalyn L. Yumol<sup>a</sup>, C. Brent Wakefield<sup>b</sup>, Sandra M. Sacco<sup>b</sup>, Philip J. Sullivan<sup>b</sup>,  
Elena M. Comelli<sup>b,c,d</sup>, Wendy E. Ward<sup>a,b,c,\*</sup>

<sup>a</sup> Department of Health Sciences, Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada

<sup>b</sup> Department of Kinesiology, Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada

<sup>c</sup> Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Ontario, Canada

<sup>d</sup> Joannah and Brian Lawson Centre for Child Nutrition, University of Toronto, Ontario, Canada

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## ABSTRACT

**Background:** The AIN-93G reference (REF) diet is used to allow the comparison within and between studies of different research groups but its levels of vitamin D (vit D) and calcium (Ca) may be higher than required for healthy bone structure and bone mineral density (BMD).

**Objective:** To determine if lower dietary levels of Ca (3.5, 3 or 2.5 g Ca/kg diet) at 1 of 2 levels of vit D (100 or 400 IU/kg diet) supports similar development of bone structure and BMD compared to AIN-93G reference (REF) diet in female CD-1 mice at 2 and 4 months of age.

**Methods:** Within a trial, weanling female mice (n = 12–15/group) were randomized to 1 of 4 diets until necropsy at 4 months of age: **Trial 1:** 100 IU vit D/kg + 3.5, 3 or 2.5 g Ca/kg diet or 1000 IU vit D/kg + 5 g Ca/kg diet (REF); and **Trial 2:** 400 IU vit D/kg + 3.5, 3 or 2.5 g Ca/kg diet or 1000 IU vit D/kg + 5 g Ca/kg diet (REF). At age 2 and 4 months, *in vivo* bone structure and BMD were assessed using micro-computed tomography ( $\mu$ CT) at the proximal and midpoint tibia. At age 4 months, lumbar vertebra 4 (L4) and mandible structure were analyzed *ex vivo*, femur strength at midpoint and neck was assessed and serum 25(OH)D<sub>3</sub> and PTH were quantified.

**Results:** For Trial 1 (100 IU vit D/kg), there were no differences in tibia structure at age 2 and 4 months nor L4 or mandible structure or femur strength at the midpoint or neck at 4 months of age despite lower serum 25(OH)D<sub>3</sub> among all groups compared to REF. For Trial 2 (400 IU vit D/kg), mice fed 2.5 g Ca/kg diet had lower ( $p < 0.05$ ) Ct.Ar/Tt.Ar and Ct.Th at the tibia midpoint compared to REF. Furthermore, Ct.Th. was greater in REF and 3.5 g Ca/kg diet compared to 2.5 g Ca/kg diet at age 2 but not 4 months of age. At L4, BV/TV was lower ( $p < 0.05$ ) in the 3 g Ca/kg diet group compared to REF at age 4 months. There were no differences among groups for serum 25(OH)D<sub>3</sub> or femur strength at the midpoint or neck. Serum PTH was not elevated compared to REF in either Trial.

**Conclusion:** Lowering both dietary vit D (100 IU/kg) and Ca (2.5 g/kg) in AIN-93G diet did not result in differences in bone development of female CD-1 mice at early adulthood. Translational relevance of bone studies conducted using the AIN-93G diet may be affected by its high vit D and Ca content.

## 1. Introduction

Rodent models are commonly used to investigate the effects of early life diet on bone mineral density (BMD) and bone structure, with the long term goal of developing dietary strategies to optimize bone development in humans (Ward et al., 2016; Kaludjerovic and Ward, 2010;

Fischer et al., 2017; Halloran et al., 2010). When investigating the potential beneficial effects of novel foods or food components, using a consistent reference diet such as the AIN-93G diet (Reeves, 1989, 1997; Reeves et al., 1993a, 1993b), reduces the variation when comparing findings within and between laboratories and ensures that observed effects are in fact due to the dietary intervention and not due to a

**Abbreviations:** ANOVA, analysis of variance; BMC, bone mineral content; BMD, bone mineral density; BV/TV, percent bone volume; Ca, calcium; Conn.Dn, connectivity density; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree anisotropy; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; ISO, isoflavones; L4, lumbar vertebrae 4; Ma.Ar, medullary area; PBM, peak bone mass; Ps.Pm, periosteum perimeter; PTH, parathyroid hormone; P, phosphorus; REF, AIN-93G reference diet; ROI, region of interest; SEM, standard error mean; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; vit D, vitamin D;  $\mu$ CT, micro computed tomography

\* Corresponding author at: Department of Kinesiology, Faculty of Applied Health Science, Brock University, 1812 Sir Isaac Brock Way, St. Catharines, Ontario L2S 3A1, Canada.

E-mail address: [wward@brocku.ca](mailto:wward@brocku.ca) (W.E. Ward).

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variation in the base diet.

The approach of using early life diet to set a trajectory for a long-term health outcome is generally referred to as ‘nutritional programming’. We have previously demonstrated that there is a window of opportunity during early life for novel food components, such as soy isoflavones, to favourably program bone outcomes at early adulthood in terms of higher BMD and improved bone structure and bone strength in female mice (Kaludjerovic and Ward, 2010; Dinsdale et al., 2012; Kaludjerovic and Ward, 2009; Kaludjerovic and Ward, 2015). While these studies have used the AIN-93G reference diet that is recommended for supporting growth, pregnancy and lactation, the levels of vitamin D (vit D, 1000 IU/kg) and calcium (Ca, 5 g/kg) in this diet may be higher than required for normal bone development, measured as BMD and bone structure in mice and rats (Glenn et al., 2014; Villa et al., 2016; Hunt et al., 2008). Findings from our group have demonstrated that normal bone development, measured as BMD and biomechanical bone strength, occurs with a significantly lower level of vit D (25 IU/kg) in mice fed an obesogenic diet, in inflammatory prone female mice or in healthy male mice (Glenn et al., 2014; Villa et al., 2016; Jahani et al., 2014). In these studies, dietary Ca was kept constant at 5 g/kg and diets were fed from weaning until 3 (Glenn et al., 2014; Jahani et al., 2014) or 7 months of age (Villa et al., 2016). In growing female Sprague-Dawley rats, Ca levels were manipulated by examining both lower and higher levels than in the AIN-93G reference diet, 1 through 7 g/kg. Vit D was kept constant at 1000 IU/kg. BMD, biomechanical bone strength and bone structure were assessed at the end of the 13 week feeding trial. Bone development was reported to be healthy if Ca intakes met or exceeded 2.5 g/kg (Hunt et al., 2008). This level is lower than the requirement established by the National Research Council of 5 g Ca/kg diet, the level of Ca in the AIN-93G diet (National Research Council, Committee on Animal Nutrition, Board on Agriculture, 1995).

While the aforementioned studies have altered the level of vit D or Ca, the effect of lowering both the level of dietary vit D and Ca in combination on BMD and bone structure has not been thoroughly studied. Previous literature has demonstrated that no effect on bone health occurs when Ca is lowered to 2.5 g/kg diet, at REF vit D level, (Hunt et al., 2008) and no differences in serum Ca and PTH occurs in the absence of vit D and 4 g Ca/kg diet (Anderson et al., 2007). By reducing both the levels of vit D and Ca, it is possible to avoid or attenuate compensatory mechanisms that may mask the effect of either low vit D or Ca when the other nutrient is provided in excess. Moreover, the implication of providing higher than required levels of vit D or Ca is that the potential positive effects of a dietary intervention to support healthy bone development may be diminished. Thus, a benefit of a dietary intervention to bone development could be masked due to a potential excess of vit D or Ca in the diet. Also of consideration is that humans are often not consuming these nutrients at recommended levels (Health Canada, 2009) and thus using a rodent diet that does not provide these nutrients in excess levels for bone health, may be more appropriate for extrapolating findings to humans.

When this study was designed, there was a paucity of studies in which both Ca and vit D were lowered in combination, so we used literature in which either Ca or vit D were lowered to guide our decisions as to which levels of vit D and Ca to study in combination. We had previously used a vit D level of 25 IU/kg diet in the CD-1 mouse model and shown that there was no detriment to BMD or bone strength (Jahani et al., 2014) but another study had shown that femur BMC and BMD were reduced at 25 IU/kg diet but not at 100 IU/kg diet compared to the level in REF diet (1000 IU/kg diet) (Fleet et al., 2008). Thus, we chose to study 100 IU as a level of vit D that was markedly lower than the REF diet but would likely support bone development. A level of 400 IU vit D/kg diet was selected as femur BMC and BMD were previously shown to be similar to mice fed REF diet (1000 IU vit D and 5 g Ca/kg diet) (Fleet et al., 2008) and we wanted to include a level of vit D that was in between the REF level and the 100 IU vit D/kg diet.

Within each of these levels of vit D (100 IU and 400 IU/kg diet), three different levels of Ca were provided. Based on the finding that dietary levels of Ca below 2.5 g Ca/kg diet compromised BMD, strength and structure of femurs in rats (Hunt et al., 2008), this level was studied as the lowest level of dietary Ca. But because this level of Ca had been studied in the context of a regular level of vit D and in rats, we chose to study two additional and slightly higher levels of Ca (3 and 3.5 g Ca/kg diet), in case the level of 2.5 g Ca/kg diet was insufficient to support healthy, normal bone development in mice.

The objective of this study was to determine if lower levels of vit D and Ca compared to the AIN-93G diet supports normal bone development at 2 and 4 months of age in female mice. Bone structure and BMD at the proximal and midpoint of the tibia were measured using *in vivo* micro-computed tomography ( $\mu$ CT) to assess longitudinal bone development within each mouse. Structure at other skeletal sites such as lumbar vertebra 4 (L4) and mandible were measured *ex vivo* at 4 months of age. Bone strength was examined at the midpoint and neck of the femur. Based on previous studies, we hypothesized that all of the combinations of Ca and vit D that were tested would support normal bone development as BMD, structure and/or strength was not compromised when Ca was higher than 2.5 g/kg diet or dietary vit D was 100 IU/kg diet or higher.

## 2. Methods

### 2.1. Animals and diets

This study was conducted in accordance with the Canadian Council of Animal Care and all experimental procedures have been approved by the Animal Care Committee at Brock University, St. Catharines, Canada. A total of twenty-two timed-pregnant CD-1 mice were purchased from Charles River Laboratories (St. Constant, QC, Canada) and fed AIN-93G diet (REF) and water *ad libitum* throughout pregnancy and lactation. Mice were housed under standard environmental conditions (12 h light:12 h dark cycle, room temperature of 23 °C). Considering that vit D can be endogenously produced with exposure to ultraviolet beta radiation, LED lighting with zero ultraviolet emissions were used in the mouse room.

Weaning female mice (21 days of age) (n = 118) were randomized to 1 of 4 color-coded diets (within each of the two trials) until necropsy at 4 months of age: **Trial 1:** AIN-93G REF diet containing 1000 IU vit D/kg + 5 g Ca/kg diet (REF) (TD.94045) or 1 of 3 experimental diets containing 100 IU vit D/kg + 3.5 (TD.160266), 3 (TD.160267), or 2.5 g Ca/kg diet (TD.160268) and **Trial 2:** AIN-93G REF diet containing 1000 IU vit D/kg + 5 g Ca/kg diet (REF) (TD.94045) or 1 of 3 experimental diets containing 400 IU vit D/kg + 3.5 (TD.160269), 3 (TD.160270), or 2.5 g Ca/kg diet (TD.160271). All diets were prepared by Envigo, Madison, WI. The level of Ca and vit D in the diets were confirmed by a third party (Maxxam Analytics, Mississauga, ON, Canada) (Table S1). Vit D was in the form of vitamin D<sub>3</sub>. Ca was included as calcium phosphate, monobasic, monohydrate (6.4 g calcium phosphate, monobasic, monohydrate/kg diet for all the diets) and as calcium carbonate (6.1 g calcium carbonate/kg diet for diet containing 3.5 g Ca/kg diet; 4.86 g calcium carbonate for diet containing 3 g Ca/kg diet; and 3.6 g calcium carbonate for 2.5 g Ca/kg diet).

Throughout the study, mice were housed 4 to 5 per cage, body weight was measured once weekly and food intake was measured 2 to 3 times each week. Food intake per mouse per day was calculated by dividing the total food consumed by the number of mice in a cage per day.

The right tibia of the offspring was scanned at 2 and 4 months of age using high resolution *in vivo*  $\mu$ CT (SkyScan 1176, Bruker microCT, Belgium) while mice were anesthetized with isoflurane. At 4 months of age, and immediately prior to euthanasia, blood samples were collected after a 12 h fast for serum 25(OH)D<sub>3</sub> and PTH analyses. Immediately after euthanasia, the lumbar vertebrae 4 (L4), right mandible and

femurs were removed for *ex vivo* analysis.

## 2.2. Bone structure and BMD

For all *in vivo* scans, the X-ray source was set with a voltage of 40 kV, amperage of 300 uA and an isotropic resolution of 9  $\mu\text{m}$ . A rotation step of 0.8° was applied over a 180° scanning frame and a 1 mm aluminum filter was used to reduce beam hardening effects (Sacco et al., 2017a). Images were then reconstructed with NRecon software, version v.1.9.6.10 (Bruker microCT, Belgium) and due to the variable curvature of bones and position during scanning, all images were rotated into the same plane using DataViewer v.1.5.1.3 (Bruker microCT, Belgium), which improves consistency when analyzing. The proximal tibia and tibia midpoint were the regions of interest (ROI) for trabecular and cortical bone analysis, respectively (Fig. S1). At the proximal tibia, the ROI was selected from a set reference point, the slice at which the primary spongiosa disconnects and the formation of the growth plate, and an offset of 75 and height of 70 slices were used to analyze trabecular bone. From the midpoint of the tibia, 50 slices towards the proximal end and 50 slices towards the distal end (total height = 100), represented the region of cortical analysis. Using a global threshold of 65 and 105, for trabecular and cortical bone, respectively, key outcomes were quantified using 2D and 3D analysis of the selected ROI (CT Analyzer v.1.14.4.1 + (64–114 bit), Bruker microCT, Belgium). Analysis of trabecular bone included BMD ( $\text{g}/\text{cm}^3$ ), percent bone volume (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N,  $\text{mm}^{-1}$ ), trabecular separation (Tb.Sp, mm), and degree anisotropy (DA, no unit) and connectivity density (Conn.Dn,  $\text{mm}^{-3}$ ). Analysis of cortical bone included cortical area fraction (Ct.Ar./Tt.Ar, %), cortical thickness (Ct.Th, mm), periosteum perimeter (Ps.Pm, mm), endocortical perimeter (Ec.Pm, mm), medullary area (Ma. Ar,  $\text{mm}^2$ ), and mean eccentricity (Ecc, no unit) of the defined ROI.

*Ex vivo* analysis occurred at the body of L4 and inter-radicular septum of the first molar (Fig. S2). Prior to scanning, the samples were wrapped in parafilm to prevent moisture loss. Sequentially, the samples were placed in a polystyrene foam tube and secured to the scan bed using tape. Scanning parameters included a voltage of 45 kV, and an amperage of 545 uA with an isotropic resolution of 9  $\mu\text{m}$ . A rotation step of 0.2° was applied over a scanning frame of 180° and a 0.25 mm aluminum filter was used to reduce beam hardening (Sacco et al., 2017b). An adaptive threshold of 77 and 87 was used for L4 and mandible, respectively. Images were reconstructed, reoriented and analyzed using the same programs as discussed in the *in vivo* procedures. With regards to L4, the top and bottom slices of the body were identified and 80 slices from the top and bottom set the boundaries for the ROI. Due to the ROI drawn, cortical bone parameters of the L4 include Ct.Ar and Ct.Th. The ROI of the mandible bones included 90 slices which started from 10 slices inferior to the bifurcation roof of the first molar roots, defined by the slice number where the roots became visibly independent structures. The main trabecular outcome measured was BV/TV.

## 2.3. Bone strength

Biomechanical bone strength was analyzed at the femur midpoint and femur neck as previously reported (Fonseca and Ward, 2004). Prior to strength testing, left femurs were hydrated in 1 × PBS and weight, length and width measurements were measured. Width measurements were analyzed at the femur midpoint in the anteroposterior and mediolateral directions. Testing was performed using a Materials Testing System (Model 4442, Instron Corp., Norwood, MA, USA) and the associated software (Bluehill 2, Instron Corp., Norwood, MA, USA). At the femur midpoint, 3 point bending was performed. Femurs were placed on two supports of the bending jig with a span of 6 mm such that the femur midpoint was directly below the crosshead. To determine the peak load for the femur neck fracture, the femur was securely placed in

a customized holder with the proximal head directly below the crosshead. For both tests, the crosshead was lowered at a rate of 2 mm/min until fracture occurred (Fonseca and Ward, 2004).

## 2.4. Serum parathyroid hormone and 25-hydroxyvitamin D

Serum PTH concentration was analyzed using a commercially-available assay (Mouse PTH 1-84 ELISA, Immunotopics, REF 60-2305) and according to the manufacturers' instructions. Serum 25(OH)D<sub>3</sub> concentration was determined *via* liquid chromatography-mass spectrometry at the Analytical Facility for Bioactive Molecules of the Center for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, ON).

## 2.5. Statistical analyses

All data are presented as mean  $\pm$  SEM and statistical analyses were performed using IBM SPSS Statistics 24. Within each trial, differences in tibia BMD and bone structure were determined using a 2 (age) by 4 (treatment) mixed analysis of variance (ANOVA). A repeated measures ANOVA for body weight was used to compare intervention groups. A one-way ANOVA was used to examine *ex vivo* bone outcomes of L4 and mandible, biomechanical strength of the femur midpoint and femur neck, as well as serum PTH and serum 25(OH)D<sub>3</sub> concentration. Differences were considered significant at  $p < 0.05$  and a Bonferroni correction (Bonferroni *post hoc* test) was used to identify specific differences between groups.

## 3. Results

### 3.1. Trial 1: intervention diets containing 100 IU vit D/kg and 3.5, 3 or 2 g Ca/kg

#### 3.1.1. Body weight and food intake

There was no effect on body weight due to the diets ( $p > 0.05$ ) (Fig. 1a). There was a main effect of age ( $p < 0.001$ ) as body weight increased from weaning to 4 months of age. No significant diet  $\times$  age interaction ( $p > 0.05$ ) was observed (Fig. 1a). Additionally, there were no differences in average food consumption per day between the groups ( $p > 0.05$ ), REF ( $3.1 \pm 0.3$  g), 100 IU vit D/kg + 3.5 g Ca/kg diet group ( $3.7 \pm 0.3$  g), 100 IU vit D/kg + 3 g Ca/kg diet group ( $3.3 \pm 0.4$  g) and 100 IU vit D/kg + 2.5 g Ca/kg diet group ( $3.7 \pm 0.3$  g).

#### 3.1.2. *In vivo* tibia structure

There was no main effect of diet ( $p > 0.05$ ) (Table 1, Fig. 2, Fig. S3). Developmental changes were observed for all trabecular and cortical bone structure outcomes measured. There was an effect of age ( $p < 0.05$ ) at the proximal tibia and midpoint of the tibia, respectively, and an increase in tibia length with age ( $p < 0.05$ ) was also observed. Furthermore, there was no significant diet  $\times$  age interaction ( $p > 0.05$ ). BMD of the proximal tibia did not differ among dietary intervention groups (Table 1).

#### 3.1.3. *Ex vivo* structure of L4 and mandible

There were no significant differences in trabecular and cortical bone outcomes of L4 among the diet groups ( $p > 0.05$ ) (Table 2, Fig. 2, Fig. S3). There were no significant differences in trabecular bone structure of the inter-radicular septum among groups at 4 months of age ( $p > 0.05$ ) (Table 2).

#### 3.1.4. Biomechanical strength testing

There were no significant differences among groups for femur weight, length or width (anterioposterior or mediolateral) ( $p > 0.05$ ) (Table 3). There were also no differences in biomechanical strength properties at the femur midpoint and peak load at the femur neck

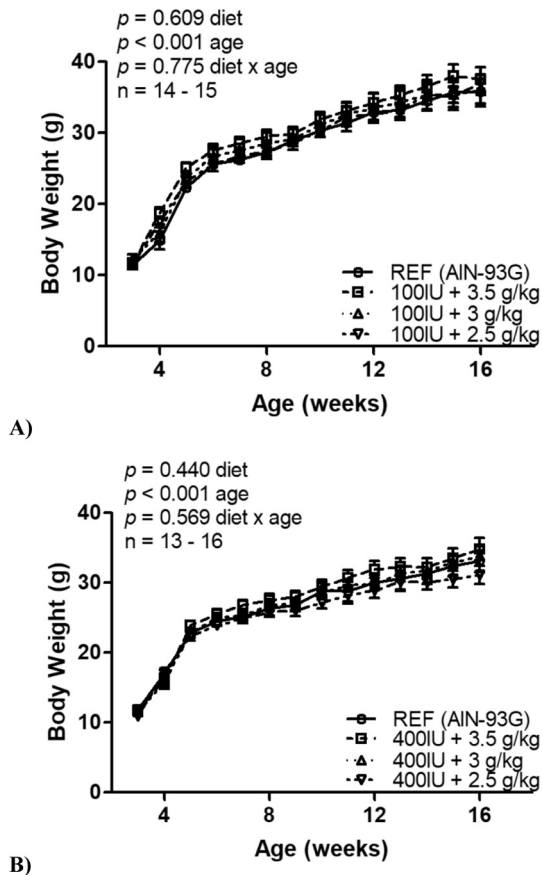


Fig. 1. Bodyweight of female CD-1 mice in A) Trial 1 (100 IU vit D/kg) and B) Trial 2 (400 IU vit D/kg). Data are reported as mean  $\pm$  SEM and no significant differences ( $p > 0.05$ ) were reported among dietary interventions.

among diet groups ( $p > 0.05$ ) (Table 3).

### 3.1.5. Biochemical analyses

Serum 25(OH) $D_3$  concentrations were significantly lower in all of the 100 IU vit D/kg dietary groups compared to the REF group ( $p < 0.05$ ) (Table 4). Serum PTH concentrations did not differ among diet groups ( $p > 0.05$ ).

## 3.2. Trial 2: intervention diets containing 400 IU vit D/kg and 3.5, 3 or 2 g Ca/kg

### 3.2.1. Body weight and food intake

Similar to Trial 1, diet had no significant effect on body weight ( $p > 0.05$ ) while there was an effect of age due to growth ( $p < 0.001$ ) (Fig. 1b). Additionally, no diet  $\times$  age interaction was present ( $p > 0.05$ ) (Fig. 1b). There were no differences in average food consumption per day among groups (REF,  $3.3 \pm 0.2$  g; 400 IU vit D/kg + 3.5 g Ca/kg diet group,  $3.2 \pm 0.2$  g; 400 IU vit D/kg + 3 g Ca/kg diet group,  $3.1 \pm 0.5$  g; and 400 IU vit D/kg + 2.5 g Ca/kg diet group,  $3.1 \pm 0.2$  g) ( $p > 0.05$ ).

### 3.2.2. In vivo tibia structure

There were no main effects in trabecular and cortical bone structure among diet groups (Table 5, Fig. 3, Fig. S4). Developmental effects occurred in both trabecular and cortical bone outcomes ( $p < 0.05$ ), with a few significant diet  $\times$  age interactions ( $p < 0.05$ ). At the midpoint of the tibia, there was an effect of diet at 2 months of age in which the REF group had greater Ct.Ar/Tt.Ar than the 400 IU vit D/kg + 2.5 g Ca/kg diet group ( $p < 0.05$ ). REF and 400 IU vit D/kg + 3.5 g Ca/kg diet groups also had greater Ct.Th compared to the

400 IU vit D/kg + 2.5 g Ca/kg diet group ( $p < 0.05$ ). At 4 months of age, in the REF and 400 IU vit D + 3.5 g Ca/kg diet groups, BV/TV and BMD were lower compared to 2 months ( $p < 0.05$ ). Furthermore, an increase in tibia length with age ( $p < 0.05$ ) was also observed. There was a significant diet  $\times$  age interaction ( $p < 0.05$ ) in which BMD was lower at 4 months, compared to 2 months, in the REF and 400 IU vit D/kg + 2.5 g Ca/kg diet groups (Table 5).

### 3.2.3. Ex vivo structure of L4 and mandible

For both the L4 and mandible, there were no significant differences in trabecular and cortical structure among the diet groups ( $p > 0.05$ ), with the exception of a lower BV/TV ( $p < 0.05$ ) in the 400 IU vit D + 3 g Ca/kg diet group compared to REF (Fig. 3, Fig. S4, Table 6).

### 3.2.4. Biomechanical strength testing

There were no significant differences among groups for femur weight, length or width (anterioposterior or mediolateral) ( $p > 0.05$ ) (Table 7). There were also no differences in biomechanical strength properties at the femur midpoint and peak load at the femur neck among diet groups ( $p > 0.05$ ) (Table 7).

### 3.2.5. Biochemical analyses

Serum 25(OH) $D_3$  concentration was similar between REF and all of the dietary intervention groups receiving 400 IU vit D/kg ( $p > 0.05$ ). Serum PTH was significantly greater in the REF group compared to the 400 IU vit D/kg + 2.5 g Ca/kg diet group ( $p < 0.05$ ). (Table 4).

## 4. Discussion

This study demonstrates that lowering both vit D and Ca in the REF diet, to 100 or 400 IU vit D/kg in combination with 3.5, 3 or 2.5 g Ca/kg, has little to no effect on bone development in terms of BMD, bone structure and bone strength from weaning to 4 months of age in female CD-1 mice. In comparison to mice fed the REF diet, when vit D was lowered to 100 IU/kg, there were no structural differences at any of the sites of interest, including tibia, lumbar spine and mandible. Also, peak load at the femur midpoint and femur neck did not differ from the REF diet or among groups.

When vit D was lowered to 400 but not 100 IU/kg diet, the combination with 2.5 g Ca/kg had modest effects on trabecular or cortical bone structure at the proximal and midpoint tibia, respectively. Few interactions were observed between dietary intervention and age; however, results were inconsistent and may be due to the multiple comparisons performed. With respect to L4 and the mandible, dietary intervention had no effect on bone development, with the exception that the 400 IU vit D + 3 g Ca/kg diet group had lower BV/TV ( $p < 0.05$ ) at L4 compared to the REF group. The fact that these same differences were not evident in both the trials, and particularly that this effect was not observed at the lower level of vit D (100 IU/kg diet) and that bone strength outcomes did not differ among groups suggest that these are not biologically meaningful differences. Moreover, while serum PTH levels in the 100 IU trial were not significantly elevated compared to the REF group, serum PTH tended to be higher in the intervention groups and this aspect requires further investigation in a study of longer duration. The lower serum PTH in the 400 IU vit D + 2.5 g Ca/kg diet group compared to REF group does not make biological sense and may be a result of Ca absorption adaptations. That serum PTH is not elevated even in the context of markedly lower levels of vit D suggests 2.5 g Ca/kg diet may be adequate for passive calcium absorption and/or this low level of vit D (100 IU vit D/kg) can regulate the expression of calcium channels for active absorption (Lieben et al., 2010). Interestingly, when vit D was lowered to 100 IU/kg, serum 25(OH) $D_3$  concentration was significantly lower compared to REF group with no effect on bone structure or BMD. The serum 25(OH) $D_3$  levels in the present study are within the range of normal serum 25(OH) $D_3$  levels reported in another study in mice that used the same gold

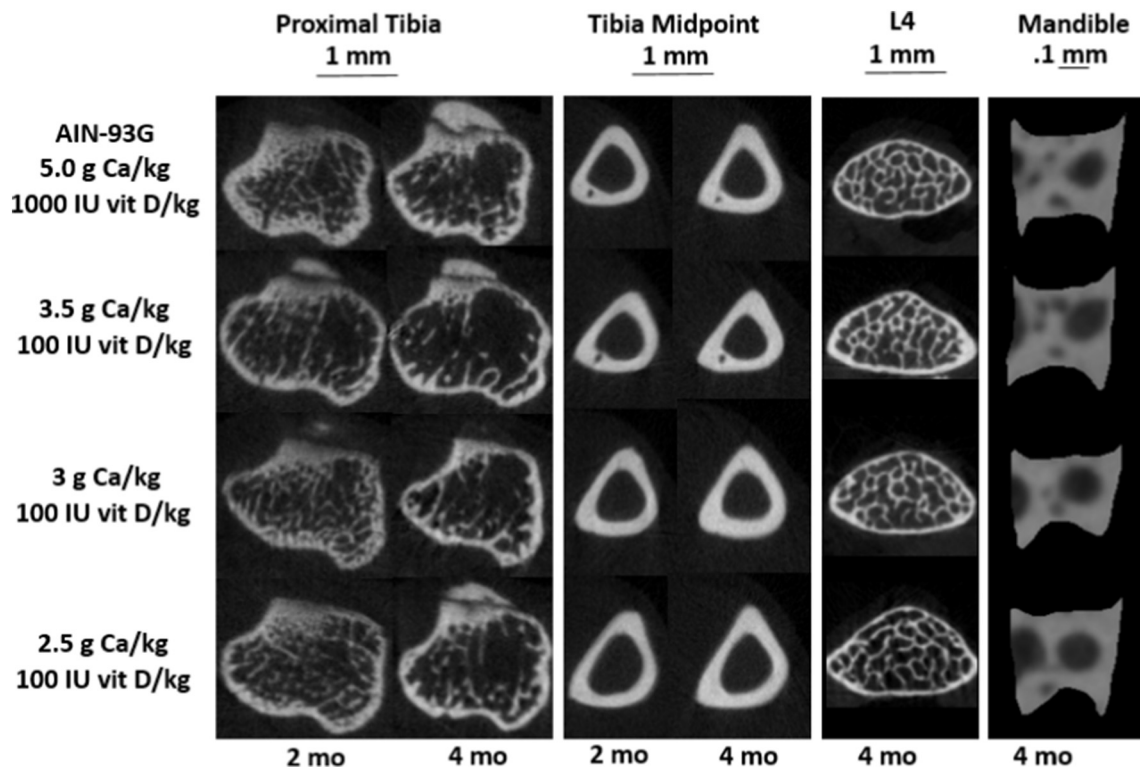


**Table 1**

Trial 1 (100 IU vit D/kg diet): *in vivo* analysis of trabecular and cortical bone morphology, of the proximal and midpoint tibia, respectively, in female CD-1 mice.

	Age (months)	Dietary intervention				Mixed ANOVA, <i>p</i> -values		
		100 IU vit D/kg						
		REF n = 15	3.5 g Ca/kg n = 15	3 g Ca/kg n = 14	2.5 g Ca/kg n = 12	Diet	Age	Diet × age
Tibia length <sup>a</sup> , mm	2	17.40 ± 0.12	17.57 ± 0.11	17.62 ± 0.10	17.41 ± 0.13	NS	0.000	NS
	4	18.77 ± 0.12	18.81 ± 0.10	18.90 ± 0.09	18.74 ± 0.13			
Tibia (trabecular) <sup>a</sup> BMD, g/cm <sup>2</sup>	2	0.208 ± 0.008	0.200 ± 0.007	0.206 ± 0.007	0.219 ± 0.010	NS	0.000	NS
	4	0.177 ± 0.009	0.163 ± 0.005	0.174 ± 0.012	0.182 ± 0.010			
BV/TV, %	2	13.87 ± 1.35	13.12 ± 0.99	13.88 ± 1.03	15.76 ± 1.75	NS	0.000	NS
	4	10.25 ± 1.35	9.77 ± 1.07	11.19 ± 1.85	11.52 ± 1.47			
Tb.Th., mm	2	0.073 ± 0.002	0.073 ± 0.002	0.071 ± 0.001	0.074 ± 0.002	NS	0.000	NS
	4	0.089 ± 0.002	0.088 ± 0.002	0.090 ± 0.003	0.089 ± 0.002			
Tb.Sp., mm	2	0.317 ± 0.017	0.323 ± 0.016	0.304 ± 0.014	0.292 ± 0.014	NS	0.000	NS
	4	0.431 ± 0.009	0.442 ± 0.015	0.441 ± 0.018	0.431 ± 0.007			
Tb.N, mm <sup>-1</sup>	2	1.879 ± 0.154	1.789 ± 0.108	1.932 ± 0.117	2.081 ± 0.172	NS	0.000	NS
	4	1.122 ± 0.125	1.096 ± 0.106	1.205 ± 0.148	1.259 ± 0.137			
DA, no units	2	2.162 ± 0.091	2.070 ± 0.056	2.255 ± 0.075	2.013 ± 0.063	NS	0.000	NS
	4	1.808 ± 0.044	1.987 ± 0.071	2.053 ± 0.099	1.891 ± 0.120			
Conn.Dn, mm <sup>-3</sup>	2	117.10 ± 9.43	95.92 ± 7.58	101.18 ± 6.15	118.88 ± 9.34	NS	0.044	NS
	4	93.17 ± 8.16	93.79 ± 9.06	110.16 ± 18.59	88.88 ± 13.21			
Tibia (cortical) <sup>a</sup> Ct.Ar/Tt.Ar, %	2	59.48 ± 0.82	58.91 ± 0.58	58.75 ± 0.76	58.82 ± 0.74	NS	0.000	NS
	4	67.97 ± 0.87	66.29 ± 0.77	67.25 ± 0.77	66.63 ± 0.80			
Ct.Th, mm	2	0.204 ± 0.003	0.209 ± 0.003	0.207 ± 0.004	0.204 ± 0.003	NS	0.000	NS
	4	0.247 ± 0.004	0.245 ± 0.002	0.249 ± 0.004	0.245 ± 0.004			
Ps.Pm, mm	2	7.387 ± 0.150	7.645 ± 0.110	7.595 ± 0.127	7.590 ± 0.171	NS	0.000	NS
	4	7.090 ± 0.151	7.502 ± 0.144	7.336 ± 0.116	7.342 ± 0.147			
Ec.Pm, mm	2	2.966 ± 0.071	3.048 ± 0.053	3.015 ± 0.062	3.039 ± 0.072	NS	0.000	NS
	4	2.596 ± 0.070	2.775 ± 0.067	2.678 ± 0.050	2.715 ± 0.064			
Ma.Ar, mm <sup>2</sup>	2	0.518 ± 0.025	0.558 ± 0.017	0.553 ± 0.021	0.545 ± 0.026	NS	0.000	NS
	4	0.417 ± 0.023	0.472 ± 0.022	0.447 ± 0.020	0.454 ± 0.022			
Ecc, no units	2	0.666 ± 0.012	0.666 ± 0.010	0.671 ± 0.013	0.692 ± 0.013	NS	0.000	NS
	4	0.655 ± 0.011	0.655 ± 0.015	0.644 ± 0.018	0.655 ± 0.017			

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.



**Fig. 2.** Trial 1 (100 IU vit D/kg diet): representative grayscale transaxial images of the right tibia, L4 and mandible of female CD-1 mice. No visual differences among dietary groups were observed.

**Table 2**

Trial 1 (100 IU vit D/kg diet): *ex vivo* analysis of trabecular and cortical bone morphology of L4 and bone volume fraction of the mandible in female CD-1 mice.

	Dietary intervention				One way – ANOVA <i>p</i> -value
	100 IU vit D/kg				
	REF n = 10	3.5 g Ca/kg n = 10	3 g Ca/kg n = 9	2.5 g Ca/kg n = 10	
L4 (trabecular) <sup>a</sup>					
BV/TV, %	25.51 ± 1.75	22.88 ± 1.06	25.39 ± 2.27	23.31 ± 1.37	NS
Tb.Th., mm	0.079 ± 0.001	0.077 ± 0.002	0.078 ± 0.002	0.078 ± 0.002	NS
Tb.Sp., mm	0.300 ± 0.020	0.307 ± 0.014	0.288 ± 0.020	0.305 ± 0.011	NS
Tb.N, mm <sup>-1</sup>	3.232 ± 0.190	2.975 ± 0.102	3.229 ± 0.199	2.991 ± 0.116	NS
DA, no units	1.814 ± 0.069	1.767 ± 0.033	1.775 ± 0.030	1.705 ± 0.053	NS
Conn.Dn, mm <sup>-3</sup>	140.04 ± 12.01	127.31 ± 9.54	101.18 ± 6.15	118.89 ± 9.34	NS
L4 (cortical) <sup>a</sup>					
Ct.Ar, mm <sup>2</sup>	0.465 ± 0.009	0.480 ± 0.021	0.468 ± 0.024	0.483 ± 0.013	NS
Ct.Th, mm	0.097 ± 0.002	0.097 ± 0.003	0.097 ± 0.004	0.099 ± 0.002	NS
Mandible <sup>a</sup>					
BV/TV, %	90.91 ± 2.17	93.28 ± 1.02	89.79 ± 2.35	93.10 ± 1.77	NS

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

standard technique, LC-MS, as used in our study (Kaufmann et al., 2014).

Our findings are consistent with the results obtained from lowering vit D (Glenn et al., 2014; Villa et al., 2016) or Ca (Hunt et al., 2008) independently. Specifically, each of these studies show that lower levels of vit D or Ca than in the AIN-93G diet supports bone health in growing female mice or rats. Glenn et al. (2014) observed that female 129/SvEvIL-10 KO mice fed low (25 IU/kg) or high (5000 IU/kg) vit D levels had no differences in femur or vertebral bone mineral content (BMC), BMD, or bone strength at 3 months of age. Using the same study design, in a C57BL/6J mouse model, Villa et al. (2016) studied female mice at age 7 months and demonstrated that when females were fed an obseogenic diet, BMD, BMC, strength and structure of the femur and L2 were not compromised at 25 IU vit D/kg compared to a supplemental level of vit D (5000 IU/kg). A study in growing female Sprague-Dawley rats modified dietary Ca to contain a range of levels, from 1 to 7 g Ca/kg (at 1 g increments) and measured bone outcomes at 4 months of age (Hunt et al., 2008). This study showed that a dietary Ca level above 2 g/kg diet does not alter bone development. This statement was based on both structural outcomes and strength properties of tibia and the fact that differences are observed when Ca level drops below 2 g/kg diet, but are normal between 2 and 3 g/kg diet (Hunt et al., 2008).

It has been identified that both strain and species of rodents play an integral role in establishing minimal nutritional requirements of vit D and Ca for bone development. Replogle et al. (2014) examined multiple

inbred strains of mice and showed differences in adaptation to low Ca (2.5 g/kg) by measuring femur BMD and structure. With respect to different species, there is evidence that mice and rats adapt differently to low levels of Ca (2 g/kg), characterized by Ca absorption and the rate of bone loss (Wolinsky and Guggenheim, 1974). While previous research has investigated changes in bone development by lowering vit D to as low as 0 IU/kg diet while keeping the Ca level at the reference level (5 g/kg/day) (van der Meijden et al., 2015) or lowering Ca to 1 g/kg diet while keeping the vit D level at reference level (1000 IU/kg) (Hunt et al., 2008), there is limited research in which both vit D and Ca are altered in combination. We studied CD-1 mice to be consistent with our previous and ongoing studies in which we report the effects of early life diet on BMD, bone structure and biomechanical bone strength at ages that represent critical stages of bone developmental (Dinsdale et al., 2012; Kaludjerovic and Ward, 2009; Kaludjerovic and Ward, 2015; Sacco et al., 2017a; Ward et al., 2007). CD-1 mice are used because they are an outbred stock and differences in genetic variation may be relatable to variation in human populations (Aldinger et al., 2009). However, whether bone development in other strains of mice responds similarly to the levels of vit D and Ca used in the present study requires further study.

In determining the levels of vit D and Ca in the AIN-93G diet, levels were established to promote rapid growth leading to maximum body size at maturity and it was assumed that these levels would also be adequate for reproduction, lactation, and maintenance (National

**Table 3**

Trial 1 (100 IU vit D/kg diet): biomechanical strength testing at the femur midpoint and femur neck.

	Dietary intervention				One way – ANOVA <i>p</i> -value
	100 IU vit D/kg				
	REF n = 13	3.5 g Ca/kg n = 14	3 g Ca/kg n = 14	2.5 g Ca/kg n = 13	
Femur midpoint <sup>a</sup>					
Weight, g	97.0 ± 3.1	101.4 ± 2.3	97.0 ± 3.1	94.7 ± 2.6	NS
Length, mm	16.55 ± 0.11	16.56 ± 0.11	16.57 ± 0.10	16.45 ± 0.13	NS
Width (anteroposterior), mm	1.38 ± 0.04	1.41 ± 0.02	1.44 ± 0.02	1.44 ± 0.02	NS
Width (mediolateral), mm	1.77 ± 0.05	1.82 ± 0.04	1.76 ± 0.03	1.77 ± 0.05	NS
Yield load, N	14.38 ± 0.79	14.17 ± 0.54	14.24 ± 0.73	14.09 ± 0.59	NS
Energy to yield load, mJ	0.56 ± 0.09	0.52 ± 0.03	0.47 ± 0.05	0.47 ± 0.03	NS
Peak load, N	28.64 ± 1.58	28.66 ± 1.07	30.24 ± 1.34	29.34 ± 1.19	NS
Energy to peak load, mJ	5.33 ± 0.72	5.09 ± 0.38	5.58 ± 0.45	5.58 ± 0.45	NS
Femur neck <sup>a</sup>					
Peak load, N	20.09 ± 0.87	21.75 ± 1.10	19.56 ± 1.00	19.67 ± 0.75	NS

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

**Table 4**

Serum PTH and 25(OH)D<sub>3</sub> concentrations for Trial 1 and Trial 2. Mean ± SEM and no significant differences (*p* < 0.05) is reported between dietary interventions.

	Dietary intervention				One-way ANOVA <i>p</i> -value
	REF	3.5 g Ca/kg	3 g Ca/kg	2.5 g Ca/kg	
Trial 1: 100 IU vit D/kg					
25(OH)D <sub>3</sub> , ng/mL (n = 3) <sup>a</sup>	15.49 ± 4.32	5.79 ± 0.83*	5.02 ± 0.31*	5.66 ± 1.05*	0.0322
PTH, pg/mL (n = 6–11) <sup>a</sup>	185.09 ± 35.88	224.37 ± 23.40	233.34 ± 36.15	234.97 ± 76.41	NS
Trial 2: 400 IU vit D/kg					
25(OH)D <sub>3</sub> , ng/mL (n = 3) <sup>a</sup>	13.40 ± 1.97	16.97 ± 0.69	12.50 ± 1.70	14.53 ± 2.43	NS
PTH, pg/mL (n = 6–8) <sup>a</sup>	296.39 ± 39.03	299.87 ± 75.31	198.28 ± 52.38	116.73 ± 26.00*	0.0483

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

\* Significant difference compared to REF group (*p* < 0.05).

Research Council, Committee on Animal Nutrition, Board on Agriculture, 1995). Although the AIN-93G diet was not intended to provide nutrients in excess, this aspect was not a focus in the formulation process (Reeves et al., 1993a, 1993b). While it is recognized that the formulation of a REF diet is dependent on the specific objective of a study, findings from this study suggest that the current AIN-93G diet provides excessive levels of vit D and Ca required for normal development of bone structure and BMD, and as discussed previously, may attenuate or mask the effect of dietary intervention. Previously, determining Ca requirements for mice was largely based on preventing kidney calcification, commonly observed with the precursor AIN-76 diet. In the development of the AIN-93 diet, Reeves et al. (1993b) compared the current level of Ca (5 g/kg) to both a lower and higher level, 3 and 6.7 g/kg, respectively, and there was no difference in Ca content of tibia. The lack of difference also supports the hypothesis that

the current level of Ca may be in excess of that required for healthy bone development. However, with the development of more sophisticated methods such as μCT to image 3-dimensional bone structure, we can more comprehensively evaluate levels of Ca and vit D required for bone development without providing excess of these nutrients. While most women in Canada do not consume the recommended levels of vit D and Ca (Health Canada, 2009), it is arguable that the standard rodent diet should reflect this in order to allow for generalizability to humans.

Strengths of the study included the ability to study the same mice at two stages of bone development to reduce variability, the number of skeletal sites assessed provided a comprehensive assessment of skeletal effects, and the fact that both cortical and trabecular bone were studied. Limitations included the fact that mice were not studied into later adulthood and the reality that other strains of mice may not necessarily respond similarly to the CD-1 stock. However, it is important to

**Table 5**

Trial 2 (400 IU vit D/kg diet): *in vivo* analysis of trabecular and cortical bone morphology, of the proximal and midpoint tibia, respectively, in female CD-1 mice.

	Age (months)	Dietary intervention				Mixed ANOVA, <i>p</i> -values		
		REF n = 15	3.5 g Ca/kg n = 13	3 g Ca/kg n = 13	2.5 g Ca/kg n = 14	Diet	Age	Diet × age
Tibia length <sup>a</sup> , mm	2	17.21 ± 0.13	17.44 ± 0.10	17.36 ± 0.14	17.24 ± 0.11	NS	0.000	0.020
	4	18.36 ± 0.09	18.75 ± 0.11	18.50 ± 0.13	18.71 ± 0.14			
Tibia (trabecular) <sup>a</sup>						NS	0.000	0.017
BMD, g/cm <sup>2</sup>	2	0.220 ± 0.011	0.227 ± 0.008	0.202 ± 0.008	0.218 ± 0.010			
	4	0.188 ± 0.012	0.199 ± 0.005	0.178 ± 0.008	0.209 ± 0.009			
BV/TV, %	2	16.53 ± 1.71	14.05 ± 0.93	13.04 ± 1.30	15.20 ± 1.73	NS	0.019	0.000
	4	11.71 ± 1.58	13.47 ± 0.56	10.78 ± 1.02	15.19 ± 1.35			
Tb.Th., mm	2	0.079 ± 0.001	0.073 ± 0.002	0.073 ± 0.001	0.075 ± 0.001	NS	0.000	NS
	4	0.094 ± 0.003	0.092 ± 0.003	0.090 ± 0.003	0.092 ± 0.001			
Tb.Sp., mm	2	0.302 ± 0.022	0.303 ± 0.025	0.320 ± 0.020	0.298 ± 0.021	NS	0.000	NS
	4	0.411 ± 0.023	0.403 ± 0.017	0.430 ± 0.014	0.383 ± 0.015			
Tb.N, mm <sup>-1</sup>	2	2.086 ± 0.190	1.917 ± 0.107	1.793 ± 0.159	1.914 ± 0.167	NS	0.000	0.004
	4	1.247 ± 0.153	1.460 ± 0.048	1.190 ± 0.106	1.618 ± 0.130			
DA, no units	2	2.133 ± 0.081	2.055 ± 0.065	2.056 ± 0.074	2.043 ± 0.076	NS	NS	NS
	4	2.261 ± 0.134	1.839 ± 0.071	2.180 ± 0.108	2.040 ± 0.090			
Conn.Dn, mm <sup>-3</sup>	2	112.85 ± 11.49	104.43 ± 8.04	111.33 ± 12.27	126.91 ± 10.03	NS	0.000	NS
	4	64.62 ± 7.46	67.48 ± 4.80	61.87 ± 7.74	73.48 ± 5.68			
Tibia (cortical) <sup>a</sup>						0.046	0.000	0.046
Ct.Ar/Tt.Ar, %	2	61.53 ± 0.67	60.33 ± 0.82	59.84 ± 0.97	57.28 ± 0.93*			
	4	68.85 ± 0.57	67.43 ± 1.05	66.44 ± 0.85	66.43 ± 1.00			
Ct.Th, mm	2	0.214 ± 0.003	0.208 ± 0.003	0.204 ± 0.003	0.196 ± 0.004	NS	0.000	NS
	4	0.249 ± 0.003	0.248 ± 0.004	0.240 ± 0.002	0.244 ± 0.006			
Ps.Pm, mm	2	7.368 ± 0.088	7.571 ± 0.145	7.444 ± 0.141	7.633 ± 0.123	NS	0.000	NS
	4	7.237 ± 0.089	7.346 ± 0.164	7.307 ± 0.149	7.347 ± 0.095			
Ec.Pm, mm	2	2.892 ± 0.050	3.065 ± 0.076	2.981 ± 0.075	3.101 ± 0.071	NS	0.000	NS
	4	2.611 ± 0.040	2.723 ± 0.090	2.632 ± 0.048	2.710 ± 0.062			
Ma.Ar, mm <sup>2</sup>	2	0.494 ± 0.014	0.521 ± 0.024	0.513 ± 0.025	0.559 ± 0.023	NS	0.000	NS
	4	0.413 ± 0.011	0.445 ± 0.027	0.428 ± 0.014	0.454 ± 0.017			
Ecc, no units	2	0.704 ± 0.010	0.682 ± 0.003	0.703 ± 0.010	0.699 ± 0.015	NS	0.000	NS
	4	0.704 ± 0.014	0.648 ± 0.013	0.683 ± 0.013	0.678 ± 0.016			

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

\* Significant difference compared to AIN-93G group (*p* < 0.05).

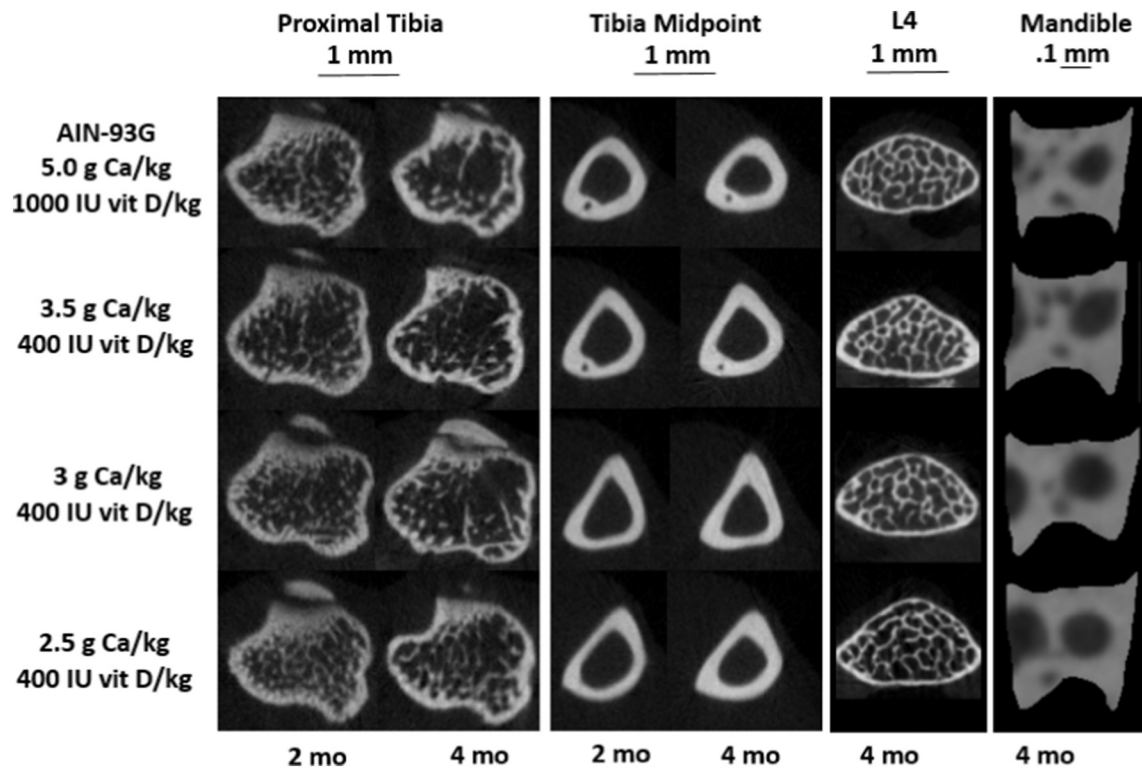


Fig. 3. Trial 2 (400 IU vit D/kg diet): representative grayscale transaxial images of the right tibia, L4 and mandible of female CD-1 mice. No visual differences among dietary groups were observed.

Table 6

Trial 2 (400 IU vit D/kg diet): *ex vivo* analysis of trabecular and cortical bone morphology of L4 and bone volume fraction of the mandible in female CD-1 mice.

	Dietary intervention				One way – ANOVA p-value
	400 IU vit D/kg				
	REF n = 9	3.5 g Ca/kg n = 10	3 g Ca/kg n = 10	2.5 g Ca/kg n = 10	
L4 (trabecular) <sup>a</sup>					
BV/TV, %	29.27 ± 1.69	26.74 ± 1.62	22.16 ± 1.29*	24.06 ± 1.68	0.043
Tb.Th., mm	0.080 ± 0.002	0.077 ± 0.002	0.074 ± 0.001	0.076 ± 0.002	NS
Tb.Sp., mm	0.258 ± 0.011	0.260 ± 0.017	0.302 ± 0.013	0.292 ± 0.014	NS
Tb.N, mm <sup>-1</sup>	3.646 ± 0.158	3.474 ± 0.235	2.982 ± 0.158	3.172 ± 0.168	NS
DA, no units	1.748 ± 0.035	1.848 ± 0.068	1.862 ± 0.084	1.717 ± 0.067	NS
Conn.Dn, mm <sup>-3</sup>	150.76 ± 12.52	132.88 ± 19.22	131.63 ± 14.64	136.01 ± 11.68	NS
L4 (cortical) <sup>a</sup>					
Ct.Ar, mm <sup>2</sup>	0.484 ± 0.015	0.452 ± 0.012	0.460 ± 0.016	0.476 ± 0.022	NS
Ct.Th, mm	0.100 ± 0.002	0.094 ± 0.002	0.095 ± 0.001	0.098 ± 0.003	NS
Mandible <sup>a</sup>					
BV/TV, %	90.84 ± 1.83	88.82 ± 2.33	90.14 ± 2.35	87.11 ± 2.21	NS

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

\* Significant difference compared to AIN-93G group (*p* < 0.05).

consider that the AIN-93G diet was developed for all rodents, regardless of strain or rodent species. Whether males respond similarly should also be reported. Due to limited availability of serum, assessment of serum markers such as serum Ca and 1,25(OH)<sub>2</sub>D<sub>3</sub> were not measured and such measures would be useful. Moreover, we did not adjust the level of phosphorus (P) in the REF diet such that the Ca to P ratio was lower than the REF diet and may increase the risk of kidney calcification (Reeves, 1997; Reeves et al., 1993b). Of note is that a previous study that tested dietary Ca levels both above and below the REF diet (and below the levels studied in the present study) observed no differences in the Ca level in kidney when dietary P level was kept constant (Hunt et al., 2008). However, the effect of a lower ratio of Ca to P to kidney health when both Ca and vitamin D levels are lowered requires further

investigation.

### 5. Conclusion

In summary, the lack of effect on bone structure and strength suggests there may be an opportunity for the AIN-93G diet to be re-evaluated to contain levels of Ca and vit D adequate for bone development without being in excess. Given that outcomes of bone structure, BMD and strength were largely similar to those fed AIN-93G diet suggests that the current levels of vit D and Ca in the reference rodent diet are higher than that required for bone development in female CD-1 mice. Therefore, we recommend that investigators studying potential benefits of novel dietary interventions to support bone health carefully consider



**Table 7**

Trial 2 (400 IU vit D/kg diet): biomechanical strength testing at the femur midpoint and femur neck.

	Dietary intervention				One way – ANOVA <i>p</i> -value
	400 IU vit D/kg				
	REF n = 14	3.5 g Ca/kg n = 13	3 g Ca/kg n = 14	2.5 g Ca/kg n = 12	
<b>Femur midpoint<sup>a</sup></b>					
Weight, g	100.4 ± 3.1	101.9 ± 3.5	97.8 ± 2.6	101.7 ± 4.1	NS
Length, mm	16.22 ± 0.10	16.61 ± 0.13	16.34 ± 0.13	16.43 ± 0.15	NS
Width (anterioposterior), mm	1.42 ± 0.02	1.44 ± 0.03	1.44 ± 0.02	1.46 ± 0.03	NS
Width (mediolateral), mm	1.87 ± 0.04	1.87 ± 0.03	1.84 ± 0.03	1.87 ± 0.03	NS
Yield load, N	16.03 ± 0.87	14.78 ± 0.89	15.14 ± 0.77	15.09 ± 1.02	NS
Energy to yield load, mJ	0.61 ± 0.06	0.72 ± 0.19	0.57 ± 0.05	0.57 ± 0.05	NS
Peak load, N	31.47 ± 1.33	31.69 ± 1.77	28.54 ± 0.87	28.82 ± 1.82	NS
Energy to peak load, mJ	5.93 ± 0.41	5.53 ± 0.59	4.88 ± 0.42	5.42 ± 0.72	NS
<b>Femur neck<sup>a</sup></b>					
Peak load, N	18.85 ± 1.00	19.36 ± 1.25	17.61 ± 1.12	19.01 ± 1.18	NS

<sup>a</sup> Values are mean ± standard error of the mean (SEM), NS = not significant.

the level of Ca and vit D used in the control diet to avoid potential masking of an effect of a dietary intervention. Because responses may be strain specific, it may be important for a research group to characterize bone development at multiple levels of Ca and vit D in the strain typically used in the research program, prior to testing a dietary intervention. Future studies can also investigate the effects of the lowered levels of vit D and Ca over a longer period of time to analyze later life stages, particularly during aging when BMD declines and bone structure is compromised.

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### Conflict of interest and funding disclosure

JL Yumol, CB Wakefield, SM Sacco, PJ Sullivan, EM Comelli and WE Ward have no conflicts of interest.

### Transparency document

The Transparency document associated with this article can be found, in online version.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bonr.2018.05.004>.

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