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Bone structure is largely unchanged in growing male CD-1 mice fed lower levels of vitamin D and calcium than in the AIN-93G diet

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ABSTRACT

Background: Calcium (Ca) and vitamin D (vit D) in the AIN-93G diet may be higher than required for healthy bone development, and mask the potential benefit of a dietary intervention.
Objective: The objective was to determine if lower levels of Ca and vit D than is present in the AIN-93G diet supports bone development in growing male CD-1 mice.
Methods: Weanling male CD-1 mice were randomized to modified AIN-93G diets containing either 100 (Trial 1) or 400 (Trial 2) IU vit D/kg diet within one of two or three Ca levels (0.35, 0.30, or 0.25% Ca diet in Trial 1 or 0.35% or 0.25% in Trial 2) or the AIN-93G diet (1000 IU/kg vit D and 0.5% Ca) from weaning to 4 months of age (n = 13–15/group). At 2 and 4 months of age, BMD and structural properties of the tibia were analyzed in vivo. Structure of lumbar vertebra 4 (L4) and mandible, and femur strength were assessed ex vivo at age 4 months.
Results: There were no differences in tibia, L4, and mandible structure between the AIN-93G diet and the 0.35% Ca groups at either vit D level. A few structure outcomes were compromised with the 0.25 and/or 0.3% Ca diets but there were no differences in femur biomechanical strength compared to AIN-93G group in either Trial.
Conclusion: At 400 or 100 IU vit D/kg diet, Ca can be lowered to 0.35% without detriment to BMD or bone structure while bone strength is not altered at lower Ca (0.25%) compared to CD-1 mice fed AIN-93G diet. Because of genetic variation in CD-1 mice among different breeding facilities, results in CD-1 mice from other facilities may differ from the present study.

1. Introduction

Osteoporosis is characterized by low bone mineral density (BMD) and deterioration of bone structure resulting in an increased risk of fragility fractures (World Health Organization, 1994). Peak bone mass has been calculated as an influential factor for BMD later in life (Hernandez et al., 2003), and low BMD during childhood is a strong predictor of low BMD at young adulthood (Wren et al., 2014). While osteoporosis is typically diagnosed at later life stages, nutrition during early life has the ability to set a trajectory for better or poor bone health, and nutritional strategies to increase peak bone mass are often studied using preclinical models (Ward et al., 2016; Weaver et al., 2011).

Rodents are commonly used as preclinical models in nutritional intervention studies as micro-computed tomography (μCT) can be used to obtain detailed information regarding changes in BMD, bone structure and strength in response to diet interventions. These studies often incorporate standardized diets, such as the AIN-93G, to ensure nutritional consistency between and within research groups using animal models (Reeves, 1989; Reeves, 1997; Reeves et al., 1993a; Reeves et al., 1993b). While the level of calcium (Ca) in the AIN-93G diet was developed with consideration of supporting whole animal growth and

Abbreviations: BMD, bone mineral density; BV/TV, percent bone volume; Ca, calcium; Conn.D, connectivity density; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree anisotropy; Ec.Pm, endocortical perimeter; Ecc., eccentricity; L4, lumbar vertebra 4; Ma.Ar, medullary area; Ps.Pm, periosteal perimeter; ROI, region of interest; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; vit D, vitamin D; μCT, micro-computed tomography

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accrual of Ca in tibia while preventing kidney calcification, the current Ca level (0.5%) may be higher than required for bone development as bone structure was largely unchanged at lower levels of dietary Ca in both rat (Hunt et al., 2008) and mouse (Yumol et al., 2018) models. In weanling female Sprague-Dawley rats, the level of Ca in AIN-93G was studied at levels of 0.7 to 0.1% while holding vitamin D (vit D) constant (1000 IU/kg diet) over a period of 13 weeks (Hunt et al., 2008). No differences in tibia Ca and phosphorus content, breaking force, bending moment, and stress of the femur, and proximal tibia bone volume fraction and trabecular thickness, number and separation were observed among the groups fed 0.7 through 0.3% Ca (at 0.1% increments). Detrimental effects to structural properties of tibias, while few, were observed at 0.2% Ca, and all were compromised at 0.1% Ca (Hunt et al., 2008). This suggests dietary Ca can be decreased to less than half the current level in the AIN-93G diet (0.5%) before Ca content, biomechanical and structural properties of tibia are compromised in a rodent model. With the AIN-76A diet, Ca was adjusted to 0.25% until 24 weeks of age in female Sprague-Dawley rats and decreases in longitudinal tibia bone growth and reduced proximal tibial bone volume of the metaphyseal region compared to 0.5 and 1.0% Ca were observed (Peterson et al., 1995). Furthermore, increasing Ca to 0.5 or 1.0% at 24 weeks of age until 37 weeks did not substantially improve these outcomes. This suggests a non-reversible deleterious effect on to bone growth during early development compared to normal and high Ca levels (Peterson et al., 1995). These studies only used female rodent models, and considering there are differences in vit D regulated intestinal absorption of Ca between sexes (Song and Fleet, 2004), necessitates the need to study how variations of these nutrients affect the growing male rodent skeleton.

With regards to vit D, our laboratory has found that lower levels of dietary vit D (25 IU/kg) compared to 5000 IU vit D/kg had no effect on BMD and strength in several different male mouse models during development: healthy CD-1 mice (Jahani et al., 2014), C57BL/6 mice fed an obesogenic diet (Villa et al., 2016a) and an inflammatory prone mouse model (Glenn et al., 2014). We have also recently reported findings from female siblings of the mice included in the present study and that were fed the same diets (Yumol et al., 2018). When vit D and Ca were lowered to 100 IU vit D/kg and 0.35, 0.3 or 0.25% Ca, there were no differences in BMD and bone structure of the tibia, at 2 and 4 months of age (Yumol et al., 2018). Furthermore, at 4 months of age, bone structure of the lumbar vertebra (L4) and mandible and femur bone strength were unaltered (Yumol et al., 2018). Together, findings from these studies provide evidence for the need to re-evaluate the current levels of vit D and Ca in the AIN-93G diet for bone development. If these diets contain higher than required levels of vit D and Ca for bone health, studies investigating bone development may be conducted in supra-physiological conditions for vit D and Ca, and may confound results of nutritional intervention studies targeting bone outcomes such as BMD and bone structure. Given the aforementioned findings that male and female mice may have different responses to alterations in vit D in terms of Ca and bone biology, it was important to study males. Moreover, because of known differences in source of CD-1 outbred stock from different facilities (Aldinger et al., 2009), studying male siblings of previously studied females can identify potential sex-specific responses in terms of BMD, structure and strength to lower levels of dietary vit D and Ca.

Developing a diet that provides sufficient levels of vit D and Ca that support healthy bone structure and BMD enables researchers to be confident that their dietary interventions aimed at supporting bone health are not being tested in the context of excess vit D and Ca, which might mask a potential benefit of the test diet. This also relates to the human scenario as many North Americans do not consume vit D and Ca at recommended levels (Imamura et al., 2015; Health Canada (2006) Canadian Community Health Survey, Cycle 2.2, Nutrition, 2004; Bailey et al., 2010). Thus, the objective of this study was to determine, in male mice, if lower levels of vit D (100 or 400 IU vit D/kg) and Ca (0.35, 0.3, or 0.25%) support healthy bone development, assessed by measurement of BMD and bone structure of tibia using in vivo μCT at 2 and 4 months of age. To provide a more comprehensive assessment of bone development, other skeletal sites were assessed ex vivo at 4 months of age for structure (L4, mandible) and strength (femur midpoint, femur neck).

2. Methods

2.1. Animals and diets

The experimental protocol was approved by the Animal Care Committee at Brock University and all experimental procedures complied with the Canadian Council on Animal Care (AUP 16-03-02). Female, timed-pregnant CD-1 mice (n = 22) were purchased from Charles River Canada (St Constant, Quebec, Canada). Upon arrival, mice were caged individually in standard environmental conditions (12 h light:12 h dark cycle, room temperature of 23 °C), and acclimatized for one week. LED light was used in the housing room and was confirmed to produce no UVB radiation. All mothers were provided water and the AIN-93G diet ad libitum throughout pregnancy and lactation. At weaning until 4 months of age, male offspring (n = 13–15 group) were randomly assigned to one of two trials consisting of an AIN-93G diet (1000 IU vit D/kg and 0.5% Ca, TD.94045) (Envigo, Madison, Wisconsin) or in Trial 1, 100 IU vit D/kg and either 0.35 (TD.160266), 0.30 (TD.160267), or 0.25% (TD.160268) Ca, or Trial 2, 400 IU vit D/kg and either 0.35 (TD.160269), 0.30 (TD.160270), or 0.25% (TD.160271) Ca. Actual dietary levels of vit D and Ca were confirmed by a third-party laboratory (Maxxam Analytics, Mississauga, ON, Canada) (Table S1). Dietary vit D was measured using AOAC official method 982.29 in which the sample containing approximately 50 IU vit D was weighed and saponified with ethanolic potassium hydroxide to release the vitamin. The vitamin was subsequently extracted with petroleum ether and concentrated by rotovap. Interferences were removed by passing the extract through a silica SPE cartridge and using a preparative HPLC on a silica column. Analysis was performed by reversed-phase HPLC-UV using vit D_2 as an internal standard. Dietary Ca was measured using a modified version of AOAC official method 984.27 in which the diet sample was digested in nitric acid and hydrochloric acid, filtered, and analyzed by inductively coupled plasma spectrometry. All other dietary components were kept constant. Diets were colour coded to allow investigators involved with daily care and analyses to be unaware of dietary intervention assignments. Food intake was measured twice weekly, and body weight was measured once weekly using an electronic scale (Denver Instrument, MXX-5). Food intake was reported per mouse per day by dividing the total food consumed by the number of mice in the cage per day. In vivo μCT scans of the right tibia were performed at 2 and 4 months of age. Mice were sacrificed by cervical dislocation at 4 months of age after a 12 h fast, and blood was collected with serum separated and stored at −80 °C. L4, femurs and mandibles were excised, cleaned of surrounding soft tissue and stored in saline soaked gauze at −80 °C until analyses were performed.

2.2. Bone structure and BMD

2.2.1. In vivo μCT scanning of tibia

At 2 and 4 months of age, the right tibia were scanned using a high resolution in vivo μCT scanner (SkyScan 1176, Belgium) using methodology and analyses previously described (Yumol et al., 2018; Bouxsein et al., 2010; Sacco et al., 2017). The mice were anaesthetized in an induction chamber using isoflurane inhalant at a concentration of 3–5% dissolved in oxygen, transferred to the scanning bed of the μCT, and a nose cone was placed over the face of the mouse to continuously administer isoflurane (2.5–3.5%) for the duration of the scan. The mouse was positioned supine, with the right leg extended and secured using a

customized apparatus and the whole tibia placed in the center of the scan field (Sacco et al., 2017). To prevent movement and any other structures entering the field of view, the tail and the left leg was secured along the side of the scan bed. The following parameters were used to image the trabecular and cortical bone of the tibia: 9 μm isotropic voxel size, 40 kV, 300 μA, rotation step of 0.8 over a total rotation of 180°, 3350 ms exposure time, with a 1.0 mm Al filter, and no frame averaging that resulted in a scanning duration of approximately 16 min.

2.2.2. Ex vivo μCT scanning of L4 and mandible

Excised L4 and the right mandibular bone were scanned with a high resolution μCT scanner (SkyScan 1176, Bruker-microCT, Belgium). Samples were removed from storage in saline and then wrapped in parafilm to prevent the samples from losing moisture during scanning. Samples were placed in a polyethylene foam tube and mounted horizontally on the scan bed. The following scanning parameters were used for L4 and right mandibles: 9 μm isotropic voxel size, 45 kV, 545 μA, 0.25 mm Al filter, rotation step of 0.2° over a total rotation of 180°, and no frame averaging resulting in a scanning duration of 38 min.

2.2.3. Image reconstruction

All images were reconstructed using NRecon software (v.1.6.9.10, Bruker-microCT, Belgium). A Gaussian filter was applied to the images with smoothing, ring-artifact and beam hardening corrections, and defective pixel masking. Variable post-alignment compensation was calculated by NRecon and manually adjusted for all images.

2.2.4. Image analyses

To obtain the region of interest (ROI) for the trabecular bone at the proximal tibia, a transaxial slice was located at which the primary spongiosa of the proximal tibia metaphysis disconnects, and a “bridge” from the low-density cartilage of the growth plate is formed. From this identified slice, an offset of 0.63 mm was calculated based on the average distance required to eliminate the growth plate from the ROI. A height of 0.58mm that spanned towards the ankle was determined to represent the proximal metaphyseal trabecular bone for analysis. Global thresholding with a lower threshold of 65 was used to binarize bone tissue from non-bone tissue. The trabecular bone was manually segmented a few pixels away from the endocortical surface using a hand-drawn interpolated shape. For cortical bone of the tibia, an ROI was determined as 0.45 mm distally and proximally from the mid slice as calculated by the half way distance between the medial malleolus and the intercondylar eminence. Global thresholding with a lower threshold of 105 was used to binarize bone tissue from non-bone tissue. Automated processing was used to segment the cortical bone for analysis. The ROI of the vertebral body was delineated by the lower and upper cartilaginous end plates, which was located and delineated by manual drawing. Adaptive thresholding with a lower pre-threshold of 77 was set to binarize bone tissue from non-bone tissue. The ROI of the mandible began 0.09 mm inferior to the bifurcation of the roof of the roots of the first molar, which was defined as the slice at which the roots became visibly independent structures, and extended 0.81mm below this point. An interpolated hand-drawn shape was manually contoured excluding the dentin ligament surrounding each root, and the medial and lateral boarders were set at the width of the root. Each slice was then visually inspected. Adaptive thresholding with a lower pre-threshold of 87 was set to binarize bone tissue from non-bone tissue.

Trabecular structural measurements that were assessed using 3D analysis included the following: bone mineral density (BMD), percent bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), degree anisotropy (DA) and connectivity density (Conn. D). Cortical structural measurements that were analyzed using 2D analyses included cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), periosteal perimeter (Ps.Pm), endocortical perimeter (Ec.Pm), medullary area (Ma.Ar), and mean eccentricity (Ecc.) (CT Analyzer, v. 1.14.4.1 + (64–114 bit), SkyScan, Bruker-microCT, Belgium).

2.3. Biomechanical strength testing

Bone strength was analyzed in regions rich in cortical and trabecular bone, at the femur midpoint and neck, respectively, as previously described (Yumol et al., 2018). The left femur was rehydrated in 1× PBS and total length and midpoint thickness were measured using digital calipers. 3-point bending was performed at the femur midpoint using a Materials Testing System (Model 4442, Instron Corp., Norwood, MA, USA) and the associated software (Bluehill 2, Instron Corp., Norwood, MA, USA). Femurs were place on two supports of the bending jig (6 mm span) with the femur midpoint directly below the crosshead. Femur neck fracture was analyzed for peak load in which the femur was securely placed in a customized holder exposing the femur head. For both tests, the cross head was lowered at a rate of 2 mm/min until fracture occurred (Fonseca and Ward, 2004).

2.4. Biochemical analyses

LC-MS/MS was used to determine serum 25(OH)D_3 concentrations and was performed by the Analytical Facility for Bioactive Molecules of the Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, ON, Canada). Serum PTH was measured using a mouse PTH 1-84 ELISA kit (REF 60-2305) (Immunotopics Inc., San Clemente, CA, USA) as per manufacturer's instructions.

2.5. Statistical methods

A sample size of 8 or 15 per group was determined, and based on a previous intervention in male CD-1 mice in which an effect of a diet intervention on main outcomes, peak load of L2 and femur midpoint peak load, was observed (peak load of L2: sample size of 8 per group with two tailed alpha of 0.05, power of 80; peak load of femur midpoint: sample size of 15 per group with two-tailed alpha = 0.05, power of 80) (Kaludjerovic and Ward, 2013). All statistical analyses were performed using SPSS software. Outliers were defined by 3 interquartile rule and removed before data analysis. Body weights and food intake were analyzed using a repeated measures ANOVA with 2 factors, age and diet. Tibia BMD and bone structure measures at 2 and 4 months of age were analyzed using a mixed ANOVA with 2 factors of age and diet as a between-subject factor. All data for each group at each time point was checked for normality and data that failed this test were transformed. For Trial 1, Ct.Th at 4 months and DA at 2 months, while for Trial 2, Ct.Th at 2 months and Tb.Sp. at 2 and 4 months were transformed using log10. Normality was subsequently checked and confirmed for these outcomes. All ex vivo bone structure and serum outcomes were analyzed using a one-way ANOVA. Statistical significance was determined with $p < 0.05$.

3. Results

3.1. Trial 1: AIN-93G or 100 IU vit D/kg and 0.35, 0.30, or 0.25% Ca diets

3.1.1. Serum 25(OH)D_3 and PTH

All groups fed modified diets, 100 IU vit D/kg + 0.35% Ca group (4.61 ± 0.23 ng/mL), 100 IU vit D/kg + 0.3% g Ca group (4.74 ± 0.83 ng/mL) and 100 IU vit D/kg + 0.25% Ca group (4.89 ± 0.52 ng/mL), had significantly lower serum 25(OH)D_3 than the AIN-93G group (11.03 ± 0.91 ng/mL) ($p < 0.001$). There was no difference in serum PTH among groups ($p > 0.05$), AIN-93G (253.10 ± 43.27 pg/mL), 100 IU vit D/kg + 0.35% Ca group (234.10 ± 30.64 pg/mL), 100 IU vit D/kg + 0.3% Ca group (252.30 ± 34.99 pg/mL) and 100 IU vit D/kg + 0.25% Ca group

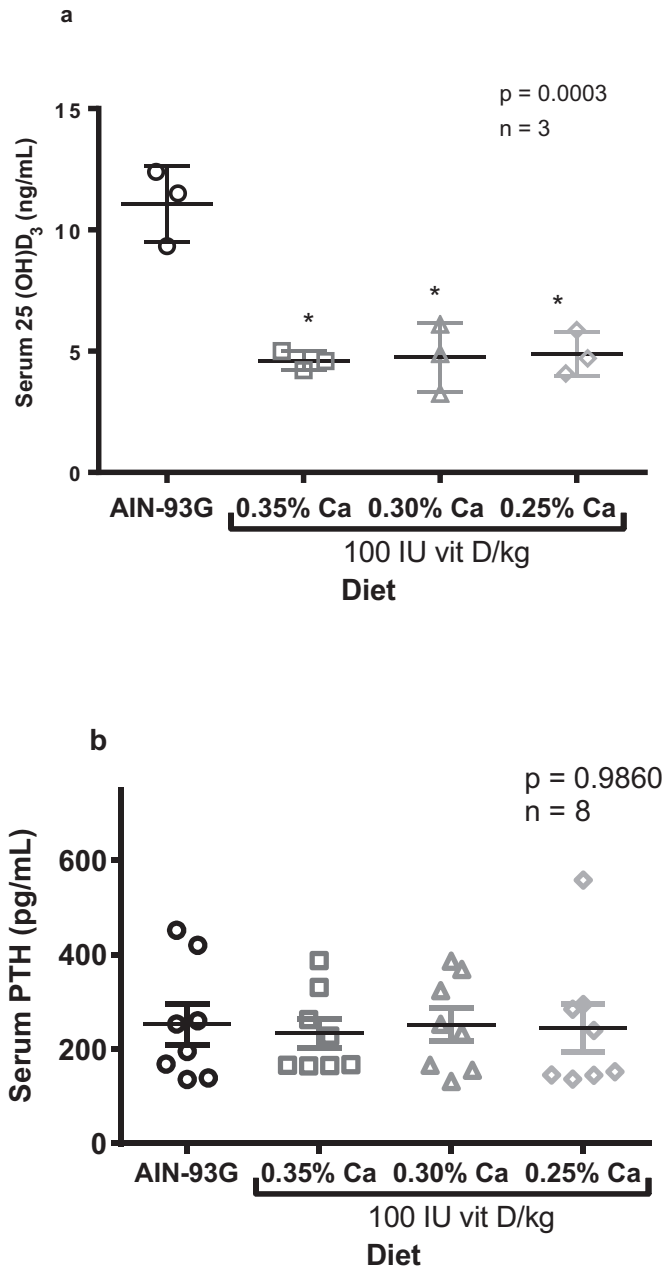

Fig. 1. Trial 1. Dietary effects of lower vit D and Ca on serum 25(OH)D_3 (n = 3/group) (a) and serum PTH (n = 8/group) (b) in male CD-1 mice. Mean ± SEM. *Represents a significant difference from the AIN-93G group, $p < 0.05$.

(244.60 ± 50.48 pg/mL) (Fig. 1).

3.1.2. Body weight and food intake

There were no differences in body weight among groups over the course of the trial, however, there was a significant effect for age as body weight increased with age for all groups ($p < 0.001$) (Fig. 2). There were no differences in average food consumption per day among groups (AIN-93G, 4.2 ± 0.1 g; 100 IU vit D/kg + 0.35% Ca group, 4.2 ± 0.1 g; 100 IU vit D/kg + 0.3% Ca group, 4.0 ± 0.1 g; and 100 IU vit D/kg + 0.25% Ca group, 4.0 ± 0.1 g) ($p > 0.05$).

3.1.3. In vivo tibia structure

At the proximal tibia, there was a significant main effect for diet where the AIN-93G group had greater Tb.Th (mm) compared to the 0.25% Ca group ($p < 0.05$). There was a main effect for age where BV/TV (%), Tb.Th (mm), and Tb.Sp (mm) were greater at 4 months of age, while Conn.D (mm^3), Tb.N (mm^{-1}), and DA (no units) were greater at 2 months of age ($p < 0.05$). At the midpoint of the tibia, there was a main effect for diet where the AIN-93G group had greater Ct.Th compared to the 0.30 and 0.25% Ca groups ($p < 0.05$). A main effect for age was observed where tibia length (mm), Ct.Ar/Tt.Ar (%), Ct.Th (mm), Ps.Pm (mm), and Ecc. (no units) were greater at 4 months of age, while Ec.Pm (mm)and Ma.Ar (mm^2) were greater at 2 months of age ($p < 0.05$) (Table 1, Fig. 3).

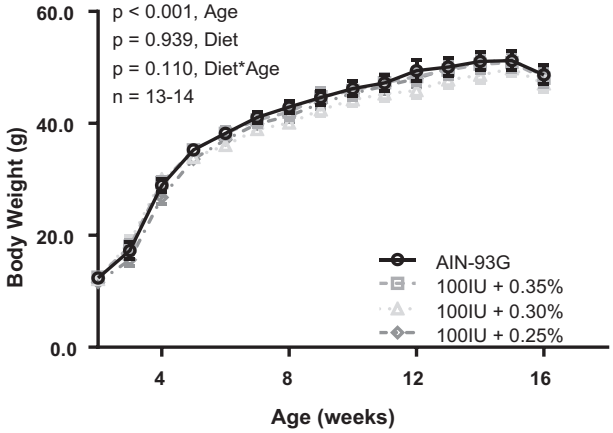

Fig. 2. Trial 1. Body weight of male CD-1 mice (n = 13–14/group) fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca. Data are reported as mean ± SEM. There were no differences ($p > 0.05$) among dietary groups.

3.1.4. Ex vivo structure of L4 and mandible

No difference in trabecular or cortical structure among dietary intervention groups at L4 or mandible ($p > 0.05$) (Table 2, Fig. 3).

3.1.5. Biomechanical strength testing

There was no difference in femur midpoint or femur neck strength among the dietary intervention groups ($p > 0.05$) (Table 3).

3.2. Trial 2: AIN-93G or 400 IU vit D/kg and 0.35 or 0.25% Ca diets

The group fed 0.30% Ca diet was removed from the study as one cage housing 5 males from this group demonstrated aggressive behaviour causing injury; the sample size was too small to include the group in the final analyses.

3.2.1. Serum 25(OH)D_3 and PTH

There were no differences in serum 25(OH)D_3 among groups ($p > 0.05$), AIN-93G (8.68 ± 3.00 ng/mL), 400 IU vit D/kg + 0.35% Ca group (11.01 ± 4.01 ng/mL) and 400 IU vit D/kg + 0.25% Ca group (12.65 ± 3.46 ng/mL). There were also no differences in serum PTH levels among groups ($p > 0.05$), AIN-93G (252.60 ± 44.18 pg/mL), 400 IU vit D/kg + 0.35% Ca group (256.80 ± 35.04 pg/mL) and 400 IU vit D/kg + 0.25% Ca diet group (268.30 ± 36.91 pg/mL) (Fig. 4).

3.2.2. Body weight and food intake

There were no differences in body weight among groups over the course of the trial, however, there was a significant effect for age as body weight increased with age for all groups ($p < 0.001$) (Fig. 5). There were no differences in average food consumption per day among groups (AIN-93G, 4.2 ± 0.2 g; 400 IU vit D/kg + 0.35% Ca group, 4.3 ± 0.1 g; and 400 IU vit D/kg + 0.25% Ca group, 4.5 ± 0.1 g) ($p > 0.05$).

Table 1
Trial 1: Structure of the proximal and midpoint tibia of male CD-1 mice at 2 and 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Age (month)	Diets				Mixed ANOVA, p-values		
		AIN-93G n = 14	100 IU vit D/kg 0.35% Ca n = 14	100 IU vit D/kg 0.30% Ca n = 14	100 IU vit D/kg 0.25% Ca n = 13	Diet	Age	Diet × age
Tibia length, mm	2	18.22 ± 0.11	18.35 ± 0.13	18.28 ± 0.13	18.10 ± 0.12			
	4	19.05 ± 0.15	19.29 ± 0.15	18.97 ± 0.12	19.03 ± 0.17	0.553	< 0.001	0.234
Tibia (trabecular)								
BMD, g/cm^2	2	0.249 ± 0.01	0.225 ± 0.01	0.219 ± 0.01	0.225 ± 0.01			
	4	0.246 ± 0.016	0.224 ± 0.01	0.225 ± 0.01	0.228 ± 0.01	0.321	0.733	0.845
BV/TV, %	2	20.70 ± 1.73	16.27 ± 1.64	15.22 ± 1.15	15.65 ± 1.67			
	4	21.62 ± 2.66	17.63 ± 2.27	17.47 ± 1.57	17.87 ± 1.22	0.166	0.011	0.850
Tb.Th., μm	2	75.50 ± 0.00[a]	70.20 ± 0.00	71.50 ± 0.00	68.10 ± 0.00[b]			
	4	91.60 ± 0.00[a]	86.70 ± 0.00	85.40 ± 0.00	82.70 ± 0.00[b]	0.013	< 0.001	0.841
Tb.Sp., mm	2	0.210 ± 0.01	0.237 ± 0.02	0.249 ± 0.01	0.216 ± 0.00			
	4	0.280 ± 0.03	0.312 ± 0.03	0.292 ± 0.02	0.251 ± 0.01	0.384	< 0.001	0.150
Tb.N, mm^{-1}	2	2.707 ± 0.17	2.289 ± 0.20	2.113 ± 0.14	2.269 ± 0.18			
	4	2.325 ± 0.27	2.002 ± 0.24	2.032 ± 0.16	2.159 ± 0.13	0.353	< 0.001	0.198
DA, no units	2	2.000 ± 0.07[a]	2.066 ± 0.08[a]	2.042 ± 0.06[a]	2.096 ± 0.06[a]			
	4	1.861 ± 0.05[b]	1.906 ± 0.07[b]	1.863 ± 0.06[b]	1.961 ± 0.08[b]			< 0.001
Conn.Dn, mm^{-3}	2	147.503 ± 10.40	126.536 ± 14.02	112.038 ± 8.54	120.659 ± 11.68			
	4	114.967 ± 13.24	107.188 ± 11.55	99.405 ± 7.96	100.515 ± 9.58	0.310	< 0.001	0.570
Tibia (cortical)								
Ct.Ar/Tt.Ar, %	2	63.64 ± 1.14	62.53 ± 0.75	62.20 ± 0.92	61.07 ± 0.87			
	4	67.91 ± 0.96	67.58 ± 1.00	67.22 ± 1.24	66.75 ± 1.01	0.653	< 0.001	0.334
Ct.Th, mm	2	0.2469 ± 0.01[a]	0.2360 ± 0.00	0.2256 ± 0.00[b]	0.2257 ± 0.00[b]			
	4	0.2774 ± 0.01[a]	0.2691 ± 0.01	0.2559 ± 0.00[b]	0.2635 ± 0.00[b]	0.006	< 0.001	0.428
Ps.Pm, mm	2	5.22 ± 0.07	5.06 ± 0.06	4.99 ± 0.07	5.10 ± 0.08			
	4	5.45 ± 0.06	5.28 ± 0.04	5.33 ± 0.10	5.38 ± 0.09	0.275	< 0.001	0.590
Ec.Pm, mm	2	3.28 ± 0.08	3.24 ± 0.07	3.27 ± 0.06	3.40 ± 0.08			
	4	3.11 ± 0.07	3.03 ± 0.08	3.13 ± 0.08	3.23 ± 0.09	0.306	< 0.001	0.802
Ma.Ar, mm^2	2	0.5513 ± 0.06	0.5918 ± 0.02	0.5797 ± 0.02	0.6222 ± 0.03			
	4	0.5513 ± 0.02	0.4988 ± 0.04	0.5045 ± 0.05	0.5218 ± 0.05	0.916	0.004	0.393
Ecc, no units	2	0.7308 ± 0.01	0.7208 ± 0.01	0.7283 ± 0.01	0.7273 ± 0.01			
	4	0.7391 ± 0.01	0.7231 ± 0.01	0.7423 ± 0.01	0.7377 ± 0.01	0.804	0.002	0.436

Values are mean ± standard error of the mean (SEM).
[a] and [b] signify a significant difference between groups ($p < 0.05$).

3.2.3. In vivo tibia structure

There were no differences in trabecular structure of proximal tibia among groups at 2 and 4 months of age. There was an interaction effect where BV/TV (%) was greater at 2 versus 4 months of age for the 0.35% Ca group, and Tb.Th (mm) was greater at 4 compared to 2 months of age for all dietary groups ($p < 0.05$). Significant main effect for age revealed Tb.N (mm^{-1}) and Conn. D (mm^{-3}) were greater at 2 compared to 4 months, and Tb.Sp (mm) was higher at 4 versus 2 months

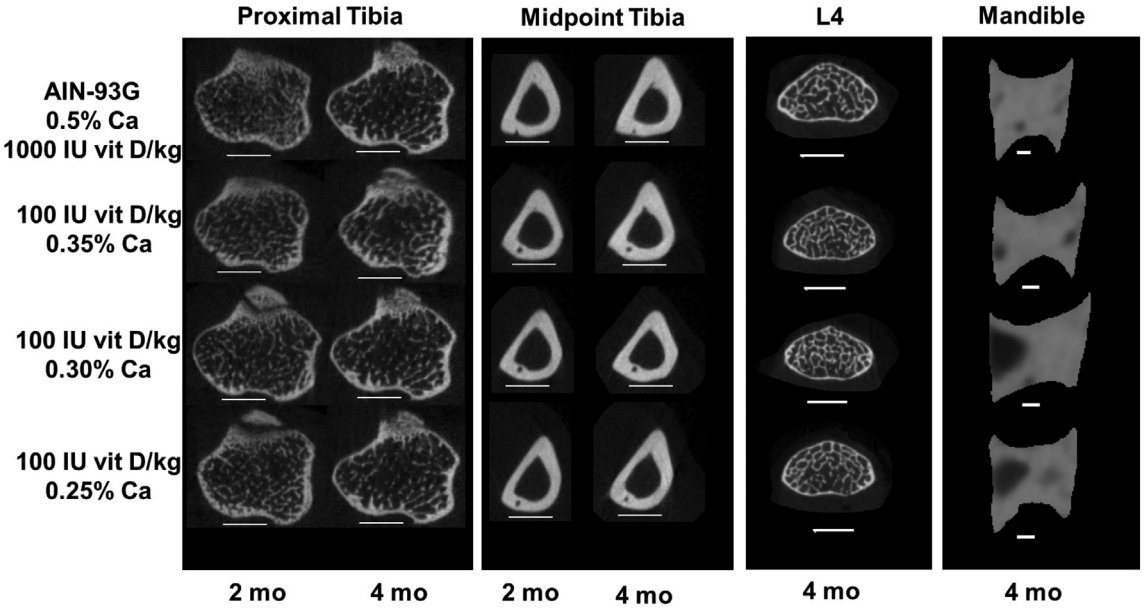

Fig. 3. Trial 1. Grey scale images of the proximal and midpoint tibia, 4th lumbar vertebra (L4), and mandible bone of male CD-1 mice. White lines are 1 mm for the proximal and midpoint tibia and L4, and white lines represent 0.1 mm for the mandible.

Table 2
Trial 1: Structure of the L4 and mandible of male CD-1 mice at 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Diets				One way - ANOVA
	AIN-93G	100 IU vit D/kg 0.35% Ca	100 IU vit D/kg 0.30% Ca	100 IU vit D/kg 0.25% Ca	
	n = 8	n = 8	n = 8	n = 8	p-Value
L4 (trabecular)[a]					
BV/TV, %	27.646 ± 1.48	28.167 ± 1.15	27.122 ± 1.53	25.648 ± 1.69	0.657
Tb.Th., μm	71.60 ± 0.01	72.60 ± 0.01	73.20 ± 0.01	72.60 ± 0.01	0.603
Tb.Sp., mm	0.208 ± 0.01	0.211 ± 0.01	0.225 ± 0.01	0.235 ± 0.02	0.356
Tb.N, mm^{-1}	3.867 ± 0.21	3.878 ± 0.14	3.700 ± 0.18	3.527 ± 0.21	0.516
DA, no units	1.853 ± 0.04	1.816 ± 0.07	1.756 ± 0.04	1.745 ± 0.06	0.441
Conn.Dn, mm^{-3}	188.534 ± 21.61	164.417 ± 10.47	150.991 ± 13.84	150.064 ± 14.84	0.293
L4 (cortical)[a]					
Ct.Ar, mm^2	0.459 ± 0.01	0.446 ± 0.01	0.427 ± 0.01	0.436 ± 0.02	0.204
Ct.Th, μm	90.90 ± 0.01	91.40 ± 0.01	89.80 ± 0.01	89.00 ± 0.01	0.760
Mandible[a]					
BV/TV, %	77.59 ± 2.00	76.54 ± 2.88	77.14 ± 1.74	77.33 ± 4.22	0.995

[a] Values are mean ± standard error of the mean (SEM).

($p < 0.05$). At the midpoint of the tibia, the 0.25% Ca group had lower Ct.Th than the 0.35% Ca group ($p < 0.05$). There was a significant effect for age where Ec.Pm (mm) and Ma.Ar (mm^2) were greater at 2 months compared to 4 months, and tibia length (mm), Ps.Pm (mm), Ct.Th (mm) were greater at 4 months ($p < 0.05$) (Table 4, Fig. 6).

3.2.4. Ex vivo L4 and mandible structure

There were no differences in trabecular or cortical structure among dietary groups for the L4 and the mandible ($p > 0.05$) (Table 5, Fig. 6).

3.2.5. Biomechanical strength testing

There were no differences in femur midpoint or femur neck strength among the dietary intervention groups ($p > 0.05$) (Table 6).

4. Discussion

This study shows that tibia BMD, structure and length as well as structure of L4 and mandible, and strength of femurs in male CD-1 mice is unchanged when fed the AIN-93G diet containing 100 or 400 IU vit D/kg and 0.35% Ca from weaning to 4 months of age. Moreover, only a few differences in bone structure were observed at lower levels of Ca emphasizing that markedly lower levels of both vit D and Ca than are present in the AIN-93G diet supports development of healthy bone structure and BMD in male CD-1 mice. Specifically, trabecular thickness in the 0.25% Ca diet group and cortical thickness in the 0.30% and 0.25% Ca groups were decreased compared to the AIN-93G diet group in Trial 1 (100 IU vit D/kg diet). While in Trial 2 (400 IU vit D/kg diet), cortical thickness was significantly less in the 0.25% Ca group compared to the 0.35% Ca group. However, peak load at the femur midpoint and femur neck was unaltered in both trials. Moreover, yield load and energy to yield load as well as peak load at femur midpoint were similar among groups. Our finding that serum PTH concentrations was similar among groups suggests that none of the groups were challenged with low serum Ca homeostasis, even when serum $25(OH)D_3$ was reduced among all groups receiving 100 IU vit D/kg diet compared to the AIN-93G group.

The findings of the present study suggest that the current level of vit D in the AIN-93G (1000 IU/kg) diet is higher than required for healthy bone development in male CD-1 mice. Moreover, these findings are in agreement with the results from a parallel study that was conducted in the female siblings to these mice, in which the identical diets used in this study were fed (Yumol et al., 2018). In that study, female siblings had no differences in BMD and bone structure of the tibia at 2 or 4 months of age when vit D was lowered to 100 IU/kg and Ca to 0.25% (Yumol et al., 2018). Furthermore there were no differences in L4 and mandible bone structure and femur strength at 4 months of age at these lower levels of vit D and Ca (Yumol et al., 2018). Another study, also showed that low vit D intake (50, 100, 200, or 400 IU/kg) in male mice (C57BL/6) from weaning until 14 weeks of age resulted in no differences in BMC and BMD of the femur, measured using DXA, and lower BMC and BMD were only observed when vit D was fed at 25 IU/kg (Fleet et al., 2008). Considering that no difference in BMD, structure at

Table 3
Trial 1: Biomechanical strength properties at the femur midpoint and femur neck of male CD-1 mice at 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Diets				One way - ANOVA
	AIN-93G	100 IU vit D/kg 0.35% Ca	100 IU vit D/kg 0.30% Ca	100 IU vit D/kg 0.25% Ca	
	n = 12	n = 13	n = 10	n = 12	p-Value
Femur midpoint[a]					
Whole femur weight, g	0.1220 ± 0.0027	0.1237 ± 0.0031	0.1200 ± 0.0033	0.1218 ± 0.0049	0.919
Whole femur length, mm	16.84 ± 0.13	16.72 ± 0.11	16.65 ± 0.20	16.37 ± 0.42	0.570
Width (anteroposterior), mm	1.54 ± 0.03	1.51 ± 0.03	1.56 ± 0.04	1.52 ± 0.03	0.807
Width (mediolateral), mm	2.16 ± 0.02	2.14 ± 0.03	2.15 ± 0.04	2.09 ± 0.08	0.683
Yield load, N	17.22 ± 0.49	16.01 ± 1.02	16.69 ± 1.72	15.17 ± 1.09	0.596
Energy to yield load, mJ	0.64 ± 0.02	0.67 ± 0.07	0.62 ± 0.09	0.53 ± 0.05	0.484
Peak load, N	35.00 ± 1.74	34.95 ± 2.30	36.23 ± 2.93	33.47 ± 1.30	0.845
Energy to peak load, mJ	6.66 ± 0.65	7.79 ± 0.71	7.30 ± 0.79	7.28 ± 0.59	0.696
Femur neck[a]					
Peak load, N	21.25 ± 1.11	24.31 ± 0.76	22.23 ± 1.61	22.03 ± 1.85	0.377

[a] Values are mean ± standard error of the mean (SEM).

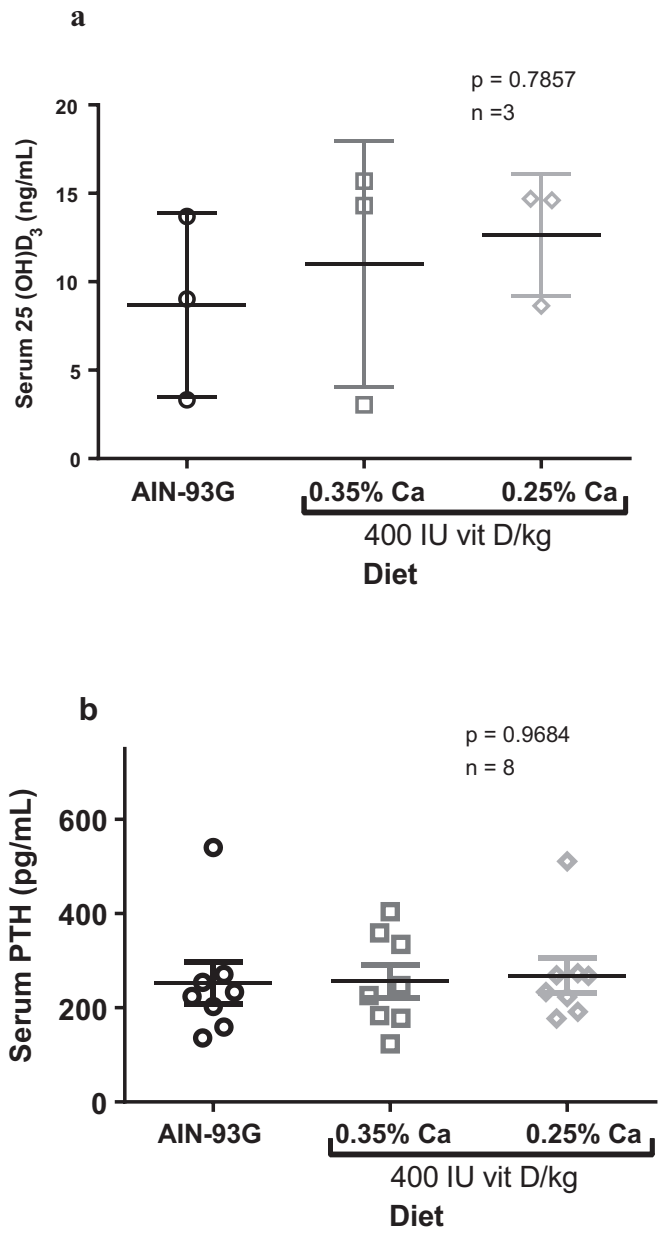

Fig. 4. Trial 2. Dietary effects of lower vit D and Ca on serum 25(OH)D_3 (n = 3/group) (a) and serum PTH (n = 8/group) (b) in male CD-1 mice. Mean ± SEM. There were no differences ($p > 0.05$) among dietary groups.

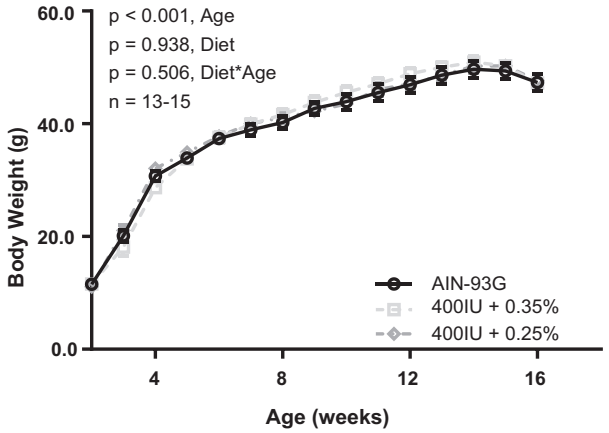

Fig. 5. Trial 2. Body weight of male CD-1 mice (13–14/group) fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca. Data are reported as mean ± SEM. There were no differences ($p > 0.05$) among dietary groups.

multiple sites (tibia, L4, mandible) or bone strength was observed at either level of vit D, in combination with a Ca level of 0.35%, provides further evidence for reducing the level of vit D in the AIN-93G diet when using the CD-1 mouse model. Interestingly, a separate experiment using male Sprague-Dawley rats examining the effects of diets containing 0.25% or 0.5% Ca at either 400, 1000, or 10,000 IU vit D/kg diet, resulted in a lower femur BMD in the 0.25% Ca groups at any level of vit D (Fleet et al., 2008). While the present study found no changes in BMD and only a few structural outcomes were compromised at this level of Ca (0.25%), the differences between these results may suggest CD-1 mice are more resilient to lower dietary Ca levels than Sprague-Dawley rats. The bone phenotype of mice in response to Ca intake differs among inbred strains (Replogle et al., 2014; Tordoff et al., 2007). For example, in a study of eleven genetically diverse inbred strains of male mice fed a modified AIN-93G diet containing 200 IU vit D/kg and 0.5% or 0.25% Ca from 4 to 12 weeks of age, different mouse strains had specific and differing responses to a low Ca diet in terms of femur BMD, bone volume fraction and cortical area fraction (Replogle et al., 2014). This suggests that genetic background significantly influences BMD and structural phenotype, and the ability of each strain to adapt to a low Ca diet. Furthermore, these findings provide justification for investigating the specific vit D and Ca requirements of mouse models. While inbred models provide invaluable information regarding genetic background and trait loci for bone researchers, the CD-1 mouse has been used previously in toxicology (Lai et al., 2009) and cancer research (Manenti et al., 2003), and to study the effects of early diet on bone development (Ward et al., 2016). The findings of the present study provide the framework for the development of a modified AIN-93G diet containing levels of Ca and vit D that support bone health in growing male CD-1 mice without providing excess that may mask beneficial effects of dietary interventions. Moreover, the positive effects of food components that can program bone health may prove to be greater in the context of a diet that does not contain excess Ca and vit D.

Previous studies by our group have investigated the programming effects of maternal vit D on BMD and bone structure in male offspring in different mouse models (Jahani et al., 2014; Villa et al., 2016a; Glenn et al., 2014). Mothers consumed either low (25 IU/kg) or high (5000 IU/kg) vit D and male offspring were weaned to a diet containing either the low or high maternal levels of vit D. Interestingly, low dietary vit D consumption in the male offspring diet, even when exposed to a low vit D maternal diet, resulted in no effect on BMC and BMD measured by DXA in male CD-1 mice (Jahani et al., 2014). Furthermore, while maternal exposure elicited significant improvements in trabecular number and trabecular separation of femurs in offspring, exposure to the high vit D diet after weaning did not provide benefits to structure in male C57BL/6 mice using the same experimental design (Villa et al., 2016a). These studies were investigating the programming effects of vit D and used a higher level of vit D than the AIN-93G diet. Considering that multiple studies found no difference in BMD and or bone structure between 25 and 5000 IU vit D/kg, these results call into question the vit D requirements for these mouse models and/or what Ca level would be appropriate (Jahani et al., 2014; Villa et al., 2016a; Glenn et al., 2014; Yumol et al., 2018; Villa et al., 2016b).

A limitation of this study may be the relatively short duration of the intervention. Considering osteoporosis occurs later in life, studying mice into older age (18–24 months) when BMD and structure decline, vit D and Ca at these lower levels may alter bone outcomes. Based on the findings that there is variation in BMD among strains of inbred mice (Replogle et al., 2014), the findings of this study may be limited to the CD-1 mouse, as other mouse models may respond differently. Because the CD-1 outbred stock has a higher degree of genetic heterogeneity,

Table 4
Trial 2: Structure of the proximal and midpoint tibia of male CD-1 mice at 2 and 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Age (months)	Diets			Mixed ANOVA, p-values		
		AIN-93G n = 14	400 IU vit D/kg 0.35% Ca n = 14	400 IU vit D/kg 0.25% Ca n = 13	Diet	Age	Diet × age
Tibia length, mm	2	17.946 ± 0.10	17.940 ± 0.10	17.840 ± 0.10			
	4	18.756 ± 0.12	18.871 ± 0.11	18.668 ± 0.16	0.602	< 0.001	0.616
Tibia (trabecular)							
BMD, g/cm^2	2	0.238 ± 0.01	0.277 ± 0.02	0.246 ± 0.01			
	4	0.250 ± 0.02	0.255 ± 0.02	0.246 ± 0.01	0.396	0.610	0.054
BV/TV, %	2	18.512 ± 2.02	26.266 ± 3.30[a]	19.651 ± 1.48			
	4	21.383 ± 2.38	22.272 ± 2.60[b]	20.637 ± 1.50			0.026
Tb.Th., μm	2	74.400 ± 0.01[a]	80.900 ± 0.00[a]	75.300 ± 0.00[a]			
	4	91.100 ± 0.01[b]	85.000 ± 0.00[b]	85.300 ± 0.00[b]			0.014
Tb.Sp., mm	2	0.221 ± 0. 02	0.186 ± 0.01	0.207 ± 0.01			
	4	0.267 ± 0.03	0.252 ± 0.02	0.244 ± 0.01	0.441	< 0.001	0.331
Tb.N, mm^{-1}	2	2.452 ± 0.24	3.117 ± 0.25	2.515 ± 0.14			
	4	2.291 ± 0.21	2.567 ± 0.25	2.224 ± 0.13	0.192	0.001	0.212
DA, no units	2	1.970 ± 0.05	1.876 ± 0.05	2.021 ± 0.05			
	4	1.860 ± 0.05	1.844 ± 0.07	1.996 ± 0.06	0.067	0.222	0.683
Conn.Dn, mm^{-3}	2	144.920 ± 14.12	184.067 ± 15.81	147.724 ± 9.76			
	4	126.755 ± 9.46	150.785 ± 16.21	129.761 ± 10.25	0.121	0.004	0.656
Tibia (cortical)							
Ct.Ar/Tt.Ar, %	2	64.054 ± 0.77	64.963 ± 0.85	62.256 ± 0.92			
	4	67.815 ± 0.90	68.169 ± 0.84	67.062 ± 1.07	0.333	< 0.001	0.136
Ct.Th, μm	2	24.220 ± 0.01	24.910 ± 0.01[a]	22.840 ± 0.00[b]			
	4	26.960 ± 0.01	27.490 ± 0.01	26.140 ± 0.01			0.019
Ps.Pm, mm	2	5.0727 ± 0.07	5.097 ± 0.06	5.084 ± 0.09			
	4	5.235 ± 0.09	5.270 ± 0.09	5.266 ± 0.08	0.966	< 0.001	0.963
Ec.Pm, mm	2	3.163 ± 0.10	3.224 ± 0.06	3.319 ± 0.11			
	4	2.998 ± 0.09	3.081 ± 0.08	3.107 ± 0.09	0.583	< 0.001	0.565
Ma.Ar, mm^2	2	0.565 ± 0.02	0.560 ± 0.02	0.592 ± 0.03			
	4	0.417 ± 0.06	0.541 ± 0.03	0.500 ± 0.05	0.349	0.006	0.227
Ecc, no units	2	0.745 ± 0.01	0.739 ± 0.01	0.758 ± 0.01			
	4	0.743 ± 0.01	0.736 ± 0.01	0.761 ± 0.01	0.212	0.901	0.864

Values are mean ± standard error of the mean (SEM).
[a] and [b] signify a significant difference between groups ($p < 0.05$).

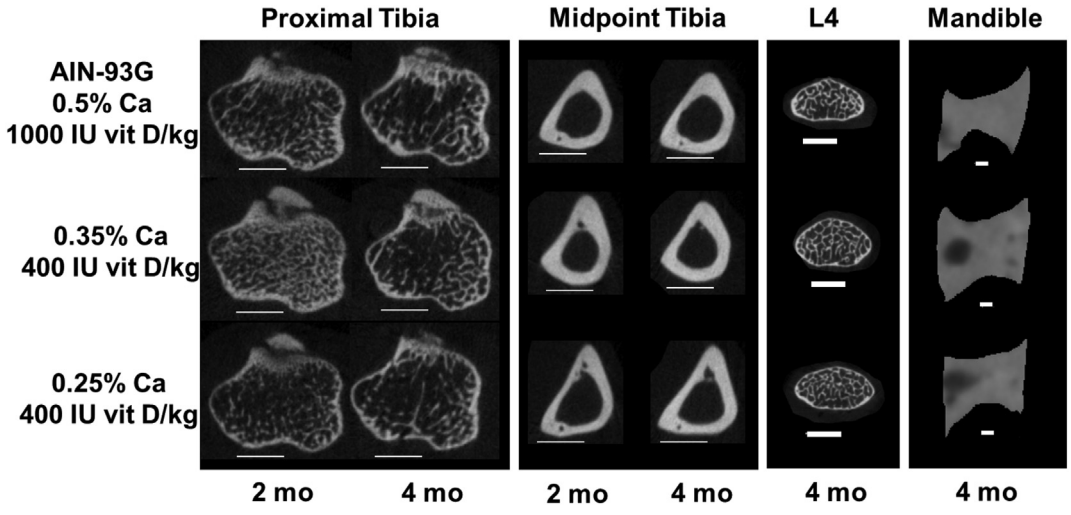

Fig. 6. Trial 2. Grey scale images of the proximal and midpoint tibia, 4th lumbar vertebra (L4), and mandible bone of male CD-1 mice. White lines are 1 mm for the proximal and midpoint tibia and L4, and white lines represent 0.1 mm for the mandible.

creating larger within group variability compared to inbred strains, a larger sample size may be required compared to studies using an inbred strain. Additionally, the CD-1 mouse has been shown to have genetic variations among colonies from different facilities which could result in different findings if these experiments were repeated in CD-mice from a different facility (Aldinger et al., 2009). However, the genetic variation of an outbred stock may better reflect the variability of responses that would be seen in a human population (French et al., 2015). Strengths of this study include the use of in vivo μCT which provides not only an analysis of BMD but also the structure of bone. Considering trabecular and cortical bone structure have specific growth patterns from 2 to 4 months of age, imaging structure within each mouse at these points decreases variability within groups and provides a better understanding of BMD and bone structural changes (Sacco et al., 2017). This provides

Table 5
Trial 2: Structure of the L4 and mandible of male CD-1 mice at 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Diets			One way - ANOVA
	AIN-93G	400 IU vit D/kg 0.35% Ca	400 IU vit D/kg 0.25% Ca	
	n = 8	n = 8	n = 8	p-Value
L4 (trabecular)[a]				
BV/TV, %	28.487 ± 1.7	29.491 ± 2.37	27.364 ± 0.95	0.682
Tb.Th., μm	74.100 ± 0.00	73.100 ± 0.00	72.000 ± 0.00	0.417
Tb.Sp., mm	0.211 ± 0.01	0.211 ± 0.01	0.216 ± 0.01	0.790
Tb.N, mm^{-1}	3.839 ± 0.20	4.011 ± 0.27	3.800 ± 0.11	0.723
DA, no units	1.877 ± 0.07	1.762 ± 0.03	1.884 ± 0.04	0.207
Conn.Dn, mm^{-3}	171.873 ± 16.47	195.559 ± 19.21	160.161 ± 9.49	0.253
L4 (cortical)[a]				
Ct.Ar, mm^2	0.469 ± 0.02	0.4543 ± 0.01	0.4529 ± 0.01	0.652
Ct.Th, μm	94.300 ± 0.00	91.900 ± 0.00	9.130 ± 0.00	0.504
Mandible[a]				
BV/TV, %	73.90 ± 5.90	77.14 ± 2.61	71.50 ± 3.79	0.639

[a] Values are mean ± standard error of the mean (SEM).

an opportunity to use a within subject measures statistical design to test tibia outcomes at 2 and 4 months of age and therefore, increasing the power of the statistical model. Additionally, measuring bone structure using μCT provides detailed information regarding the structure in a non-destructive manner. Considering the interdependent physiological effects of Ca and vit D, reducing the levels of both nutrients provides evidence for a combined reduction of these nutrients in CD-1 mice for future studies. Lastly, the assessment of multiple skeletal sites, including the appendicular and axial skeleton as well as mandible, provides a broader understanding of how the skeletal system will respond to these dietary levels.

5. Conclusion

In conclusion, findings from the present study suggest that the current level of vit D and Ca is higher than required in the AIN-93G diet to achieve healthy BMD, bone structure and strength in growing male CD-1 mice. This has implications for researchers studying the effects of dietary interventions in rodent studies as use of the AIN-93G may mask or attenuate potential benefits of a dietary intervention. This aspect requires future investigation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bonr.2018.100191.

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Conflict of interest and funding disclosure

CB Wakefield, JL Yumol, 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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Table 6
Trial 2: Biomechanical strength properties at the femur midpoint and femur neck of male CD-1 mice at 4 months of age fed the AIN-93G diet or modified diets containing lower levels of vit D and Ca.

	Diets			One way - ANOVA
	AIN-93G	400 IU vit D/kg 0.35% Ca	400 IU vit D/kg 0.25% Ca	
	n = 13	n = 11	n = 13	p-Value
Femur midpoint[a]				
Whole femur weight, g	0.1165 ± 0.0038	0.1205 ± 0.0022	0.1172 ± 0.0018	0.665
Whole femur length, mm	16.29 ± 0.20	16.35 ± 0.16	16.31 ± 0.10	0.110
Width (anteroposterior), mm	1.51 ± 0.02	1.50 ± 0.03	1.55 ± 0.03	0.250
Width (mediolateral), mm	2.19 ± 0.03	2.17 ± 0.03	2.20 ± 0.04	0.419
Yield load, N	16.32 ± 0.65	16.55 ± 1.21	15.73 ± 0.60	0.629
Energy to yield load, mJ	0.57 ± 0.04	0.63 ± 0.06	0.54 ± 0.03	0.217
Peak load, N	33.70 ± 2.29	35.95 ± 1.86	34.39 ± 2.14	0.217
Energy to peak load, mJ	6.25 ± 0.87	7.10 ± 0.52	7.00 ± 0.62	0.678
Femur neck[a]				
Peak load, N	22.18 ± 1.39	23.22 ± 1.56	21.89 ± 0.90	0.388

[a] Values are mean ± standard error of the mean (SEM).

EM Comelli holds the Lawson Family Chair in Microbiome Nutrition Research at the University of Toronto.

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