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Dietary calcium intake and genetics have site-specific effects on peak trabecular bone mass and microarchitecture in male mice

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ABSTRACT

Trabecular bone (Tb) is used for rapid exchange of calcium (Ca) in times of physiologic need and the site-specific characteristics of Tb may explain why certain sites are more vulnerable to osteoporosis. We hypothesized that peak trabecular bone mass (PTBM) and Tb microarchitecture are differentially regulated by dietary Ca intake, genetics, or Gene-by-Diet (GxD) interactions at the distal femur and the fifth lumbar (L5) vertebra. Male mice from 62 genetically distinct lines were fed basal (0.5%) or low (0.25%) Ca diets from 4 to 12 wks of age. Afterwards, the right femur and L5 vertebra were removed and trabecular bone was analyzed by μCT. In mice fed the basal diet, bone volume fraction (BV/TV), trabecular number (Tb.N), and connectivity density (Conn.D) were significantly higher in the L5 vertebra than femur. Femur Tb had a weaker, more rod-like structure than the L5 vertebrae while mice fed the low Ca diet developed rod-like structures at both sites. Dietary Ca restriction also caused a greater relative reduction of Tb.N and Conn.D in the femur than L5 vertebra, i.e. it was more harmful to the integrity of Tb microarchitecture in femur. Genetics was a major determinant of Tb at both sites, e.g. heritability of BV/TV on the basal diet = 0.65 (femur) and 0.68 (L5 vertebra). However, while GxD interactions altered the impact of dietary Ca restriction on Tb parameters at both sites, the effect was not uniform, e.g. some lines had site-specific responses to Ca restriction. The significance of our work is that there are site-specific effects of dietary Ca restriction and genetics that work independently and interactively to influence the attainment of PTBM and Tb microarchitecture.

1. Introduction

Osteoporosis affects millions of individuals worldwide [1] and approximately 50% of osteoporotic fractures occur at trabecular bone-rich sites like the lumbar vertebrae and at the ends of long bones [2]. Consistent with the idea that trabecular bone-rich sites are sensitive to fracture, cadaver studies on the mechanical strength of trabecular bone from proximal femur [3,4] or the thoracic ninth vertebrae [5] show that bones with lower trabecular bone volume (BV/TV), fewer trabeculae, and reduced connectivity are more susceptible to structural failure. Trabecular bone may be more sensitive to fracture because it has a large surface-to-volume ratio that provides a rapidly exchangeable calcium (Ca) pool that is drawn upon in times of need [6]. For example, studies have shown that Ca is liberated from trabecular bone resulting in a decline in BV/TV during lactation [7,8] and in ovariectomized (OVX) mice [9] despite the fact that no significant change was observed at cortical bone-rich sites.

Previous studies suggest that some trabecular bone-rich sites are more sensitive to physiologic challenges. For example, OVX-induced bone loss in mature rats was greatest at the proximal tibia and distal femur (~75%), moderate in lumbar vertebrae (36%), and negligible in cranial and jaw bones (< 5%) [10]. In addition, while Shin et al. [11] found similar OVX-induced loss of BV/TV at the L4 vertebra and femur in rats, the main effect in the vertebra was reduced trabecular thickness while in femur it was a 6-fold reduction of trabecular number (Tb.N). Similarly, Liu et al. [12] found that lactation changed trabecular bone microarchitecture in vertebra from plates into rods while it reduced trabecular connectivity at the proximal tibia and distal femur. Thus, physiologic changes known to influence bone mass and fracture risk can have differential effects depending on the bone site examined.

Research shows that adequate dietary Ca intake is necessary for maximizing bone mass accumulation and structural development during growth [13]. However, some studies show that Ca supplements increase BMD more at the femoral neck than at the lumbar vertebra [14] and [15,16] others show that Ca fortified foods [17] increase BMC at the humerus and radius-ulna but not lumbar vertebra, femur, or tibia.

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Inconsistencies regarding the impact of dietary Ca on bone in clinical trials could result from a number of factors including the length of treatment, the level of Ca intake, pubertal status, or race. Carefully controlled studies in animal models over the whole growth period may be able to clarify whether there are site-specific effects of dietary Ca on trabecular bone.

In addition to environmental factors, previous studies have shown that distinct genetic determinants define trabecular characteristics at various skeletal sites [18–21]. We previously showed that the genetic diversity in inbred mouse lines can be used to identify the role of genetics in calcium and bone metabolism [22]. Using C57BL/6, C3H/HeN, and BALB/c inbred mouse lines, Buie et al. [23] found that the gain in L3 vertebral BV/TV from 6 to 12 wks of age was similar across lines but that tibial BV/TV gains were low in C57BL/6, intermediate in BALB/c, and high in C3H/HeN mice. In another study, C3H/HeJ mice had higher distal femur bone volume but lower L5-6 vertebral BV/TV and Tb.N than C57BL/6 mice [21]. Thus, in order to determine the site-specific impact of dietary Ca restriction on bone, we also need to consider the effect of genetics and gene x diet interactions on these responses.

Here we conducted a study to systematically examine how dietary Ca restriction affected the accumulation of trabecular bone mass at the femur and L5 vertebra of growing mice. In addition, we used a large population of inbred and recombinant inbred mouse lines to interrogate whether genetics affects how trabecular bone responds to low Ca intake at each site. We hypothesized that peak trabecular bone mass and microarchitecture at the distal femur and L5 vertebra are differentially regulated by dietary Ca restriction and the site-specific response to low Ca intake is regulated by genetics.

2. Materials and methods

2.1. Experimental design

To model the genetic diversity present in humans, we used a large, genetically-diverse population of mice for our experiment. This population included 11 common laboratory inbred mouse lines (129S1/SV1mJ (129S), A/J, AKR/J (AKR), C3H/HeJ (C3H),C57BL/6J (B6), CAST/EiJ (CAST), CBA/J (CBA), DBA/2J (DBA), PWK/PhJ (PWK), SWR/J (SWR), and WSB/EiJ (WSB)) and 51 BXD recombinant inbred (RI) mouse lines that are defined by a fixed recombination pattern of alleles from the B6 and DBA inbred mouse lines [24]. This population of mice encompasses three mouse subspecies (*Mus musculus domesticus*, *M.m. musculus*, and *M.m. castaneus*), includes classical inbred strains as well as more genetically divergent wild-derived inbred lines, and represents the parental strains of available genetic mapping resources [25]. Four-week-old male mice from the 62 lines were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). An equal number of mice from each line ($n = 8$ for all lines except BXD36 where $n = 4$) were randomly assigned to either a 0.5% (basal) Ca or 0.25% (low) Ca diet (AIN93G base with 200 IU vitamin D_3/kg diet, Research Diets, New Brunswick, NJ, USA). The dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca) [26] or to model the low level of dietary Ca intake seen in the U.S. population (0.25% Ca) [27,28]. Mice were group-housed (2–4 mice/cage) at the Purdue University animal facilities in conventional shoebox cages, maintained in rooms with UV-blocking filters over lights and a 12-hour light/dark cycle, and provided food and distilled water ad libitum. At 12 wks of age, at a point where Buie et al. have shown that mice reach peak trabecular bone mass [23], mice were fasted overnight. Right femora and L5 vertebrae were harvested and prepared for analysis as previously described [29]. Investigators were blinded to genotype and dietary treatment during allocation, animal handling, bone sample collection and endpoint measurements. All animal experiments complied with the ARRIVE guidelines and the Purdue Animal Care and Use Committee approved the experimental protocol.

2.2. Micro-computed tomography (μCT) evaluation

Femora and L5 vertebrae were analyzed using μCT (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) using settings reported elsewhere [22] with one exception. For all samples, the regions of interest were binarized using a global threshold (474.3 mg HA/cm^3 for femur and 559.6 mg HA/cm^3 for L5 vertebra). For the femur, the region of interest was defined as 56 slices starting from the first slice containing no evidence of distal growth plate. The L5 vertebra was selected for evaluation over the other lumbar vertebrae because it is the most common vertebra reported in the literature, particularly in genetic studies [30–32]. The entire L5 vertebra was scanned at a voxel size of 16 μm and trabecular bone was evaluated in a 50-slice region distal to the cranial growth plate. This region was chosen as it correlates well with the trabecular bone parameters of the entire L5 vertebra [33,34]. For both sites, we manually contoured trabecular bone every 10 slices with the outline 2–3 pixels away from the cortical bone, and the intermediate slices were interpolated with the contouring algorithm in the Scanco's 3D analysis software to create a volume of interest. We reported the measurements for bone volume fraction (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn.D, 1/mm^3) and structure model index (SMI) as recommended elsewhere [35]. SMI is an indicator of the shape of trabeculae; a value of 0 reflects trabeculae shaped like plates while a value of 3 reflects trabeculae shaped like cylindrical rods [35].

2.3. Statistical analysis

Statistical analysis was conducted using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC). Data points with a *Z*-score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. Supplemental Table S1 reports the number of mice in each genotype and dietary treatment group for each μCT parameter after outlier removal. In addition to the data obtained from each mouse on each diet, a parameter reflecting the response to dietary Ca restriction (RCR) was calculated as the percentage difference between the phenotypic value for an individual fed the 0.25% Ca diet and the line mean from the 0.5% Ca diet, standardized to the line mean from the 0.5% Ca diet [22]. Analysis of covariance (ANCOVA) was used to test the main effects and interaction effects on each parameter and their RCR while controlling for the covariate effects of body weight (BW) and/or femur length (FL) [36]. Repeated measure ANCOVA was used when site was a main effect in the model to account for within-subject covariance. When the residuals from ANCOVA were not normally distributed, the following transformations were used: BV/TV and Conn.D (√y), Tb.Th and Tb.Sp (log 10), and SMI ($[y]^2$).

Data were initially analyzed as a genetically diverse population and one-way ANCOVA was used to determine the impact of diet or site on the μCT parameters. Next, we used two separate approaches to assess whether there was a site-by-diet (SxD) interaction affecting μCT parameters. First, we used two-way, repeated-measure ANCOVA to examine the main effect of diet and site as well as their interaction. Second, we assessed the effect of site on the RCR parameters using one-way, repeated-measure ANCOVA.

Relationships between parameters were determined using Pearson's correlation tests. First, we investigated correlations among μCT parameters without regard for the genetic structure of our population. This analysis was performed separately for each site as well as for each diet group. Second, we examined the correlations between the basal femoral and vertebral μCT parameters. Third, we accounted for the genetic structure of our population by using covariate-corrected least square means (LSMeans) of each genetic line as measure of the parameter for each “genetic individual” ($n = 62$ mouse lines). We then used these line means to assess the correlation between parameters of “genetic individuals” fed the basal diet or the 0.25% Ca diet (LSMeans shown in

Supplemental Table S2). Finally, we used this data to assess the correlation between the μCT parameter of mice fed the basal diet and the RCR parameters.

Lastly, we used the genetic line (genotype) as a predictor variable. Initially we assessed the narrow-sense heritability (h^2) of each μCT parameter using the r^2 from a one-way ANCOVA (main effect = genotype); this was conducted separately for each bone site and for each diet group as well as for their RCR. Interactions among the main effects were investigated for each μCT parameter (i.e. genotype-by-diet (GxD), site-by-genotype (SxG) and GxDxS). The GxDxS interaction affecting each parameter was confirmed using a two-way ANCOVA of SxG interaction on RCR parameters. When site was a main effect, we used a general linear mixed model with the restricted maximum likelihood approach to estimate the main effects and their interactions while accounting for the within-subject covariance [37]. The covariance structure in this analysis was defined as compound symmetry (i.e. constant variance, constant off-diagonal matrix). This analysis assumes that the variance of each uCT parameter is the same at both sites.

The study was powered based on variance estimates for the effect of genetics on femoral μCT parameters in B6 mice ($n = 8$, 30% difference between dietary groups, SD = 20% of mean, $\alpha = 0.05$, power = 0.797). Differences were considered significant when $p \leq 0.05$. When a significant F statistic for an interaction was detected, post hoc pairwise comparisons were made using Tukey's HSD with $p \leq 0.05$ considered significant. Data are reported as LSMean ± SEM. For parameters that required data transformation, we report the back-transformed LSMean ± SEM for inclusion in tables and figures.

3. Results

3.1. Effect of dietary calcium restriction on trabecular characteristics in femur and vertebra of growing mice

In this genetically diverse population of mice, μCT analysis showed that the baseline values for all of the parameters except Tb.Sp were significantly different between femur and L5 vertebra (Table 1). For example, BV/TV was 18% higher and Conn.D was 23% greater in vertebra compared to femur. In addition, the SMI value showed that vertebral trabeculae were more plate-like than in femur (L5 vertebra = 1.3, femur = 2.3).

At each bone site, BV/TV, Tb.N, Tb.Th, and Conn.D were significantly lower, and Tb.Sp was significantly higher, in mice fed the low Ca diet (Table 1). In addition, the SMI for both femur and vertebra was higher, reflecting weaker, more rod-like trabeculae at both sites, because of inadequate Ca intake. A significant positive correlation existed between femoral and vertebral RCR for Tb.Th ($r = 0.63$, $p < 0.0001$) and BV/TV ($r = 0.55$, $p < 0.0001$). However, weaker correlations were seen for Tb.Sp ($r = 0.27$, $p < 0.0001$), Tb.N ($r = 0.26$, $p < 0.0001$) and Conn.D ($r = 0.17$, $p = 0.0004$).

ANCOVA revealed the existence of significant site-by-diet (SxD) interactions for Tb.N, Tb.Sp, and Conn.D (Fig. 1); a trend for a SxD interaction was seen for SMI ($p = 0.0658$). Consistent with the existence of SxD interactions, we observed significant differences in RCR at femur and spine for all of the μCT endpoints (Fig. 2). The negative femoral BV/TV RCR reflected a retardation in trabecular bone development that is due to loss of trabecular number and connections but the negative vertebral BV/TV RCR was due to a large change in the geometry of trabeculae (i.e. an SMI reflecting a more rod-like structure) as well as a 30% greater decrease of Tb.Th (Fig. 2).

3.2. The relationships between trabecular parameters is altered by diet and bone site

Several well-known relationships between trabecular parameters exist [38] and we observed these relationships among these parameters in the basal diet group at both sites (Supplemental Table S3 (unadjusted), S5 (body-size corrected)). We found similar relationships in mice receiving the 0.25% Ca diet (Supplemental Table S4, S6). There were also correlations between the μCT parameters of the two bone sites but these were generally weak to moderate (e.g. for femur vs. vertebra BV/TV, $r = 0.43$ in the basal Ca diet group; Supplemental Table S7). Within each site, there was a tight correlation between the basal and the 0.25% Ca diet values for each trabecular parameter (Supplemental Table S8). For example, the correlation in BV/TV between the two diet groups was 0.85 for femur ($p < 0.001$) and 0.88 for vertebra ($p < 0.001$). This shows that dietary Ca intake accounts for approximately 28% and 23% of the variation in femoral and vertebral BV/TV, respectively.

We next examined the relationship between the μCT parameters of mice fed the basal diet (basal values) and their RCR using LSMeans from each line as measure of the parameter for each "genetic individual" ($n = 62$; Supplemental Table S9). There was no correlation between basal and RCR values for BV/TV or Conn.D. However, a significant negative correlation was observed between the basal and RCR values at both sites for Tb.Sp (Fig. 3A, B) and Tb.N (Supplemental Table S9) and for vertebral Tb.Th (Fig. 3D) and SMI (Supplemental Table S9). This indicates that lines with a high basal phenotype were more sensitive to the low Ca diet during growth. However, because this relationship was not uniform across two sites, this suggests a site-specific impact of dietary Ca restriction on some μCT parameters.

3.3. Genetics regulates basal trabecular parameters as well as the ability to adapt to dietary Ca restriction at each bone site

Because our mouse population has a defined genetic structure reflected in the mouse lines, we investigated the influence of genetics on the basal μCT parameters or their RCR. Each μCT parameter was significantly influenced by genetics ($p < 0.0001$). The narrow sense heritability (h^2) was high for all of the μCT parameters in both femur ($h^2 = 0.56$ to 0.82) and vertebra ($h^2 = 0.59$ to 0.71) and these heritability estimates were not significantly reduced in mice fed the 0.25% Ca diet (Table 2). In contrast, the heritability of the RCR parameters

Table 1
Trabecular bone of mice fed a low Ca diet was significantly impaired compared to mice fed a basal Ca diet at both the femur and L5 vertebra.

μCT parameter	Femoral basal Ca diet	Femoral low Ca diet	Vertebral basal Ca diet	Vertebral low Ca diet
BV/TV	0.1973 ± 0.0038 ($n = 496$)	0.1704 ± 0.0036[a] ($n = 466$)	0.2346 ± 0.0029[c] ($n = 456$)	0.2124 ± 0.0029[a] ($n = 451$)
Tb.N (1/mm)	4.8016 ± 0.0401 ($n = 468$)	4.5204 ± 0.0400[a] ($n = 469$)	4.9046 ± 0.0029[c] ($n = 454$)	4.7754 ± 0.0029[a] ($n = 454$)
Tb.Th (mm)	0.0672 ± 0.0005 ($n = 467$)	0.0646 ± 0.0005[a] (n = 467)	0.0600 ± 0.0003[c] (n = 456)	0.0573 ± 0.0003[a] ($n = 454$)
Tb.Sp (mm)	0.2043 ± 0.0025 ($n = 465$)	0.2197 ± 0.0027[a] ($n = 464$)	0.2067 ± 0.0018 ($n = 451$)	0.2122 ± 0.0018[a] (n = 454)
Conn.D ($1/mm^3$)	96.4854 ± 2.4315 ($n = 471$)	82.6608 ± 2.2572[a] ($n = 469$)	128.3378 ± 1.9635[c] (n = 454)	123.2279 ± 1.9657[b] ($n = 453$)
SMI	2.2951 ± 0.0267 (n = 469)	2.4623 ± 0.0247[a] ($n = 473$)	1.3045 ± 0.0276[c] ($n = 452$)	1.4721 ± 0.0275[a] (n = 454)

Values are reported as LSmeans ± SEM (n) from one-way ANCOVA adjusted for BW and FL. Analysis of Tb.Th and SMI were adjusted only for FL.
[a] Significant at $p \leq 0.05$.
[b] A trend at $p \leq 0.10$ between the two diet groups.
[c] Significant at $p \leq 0.05$ between sites in the basal diet group.

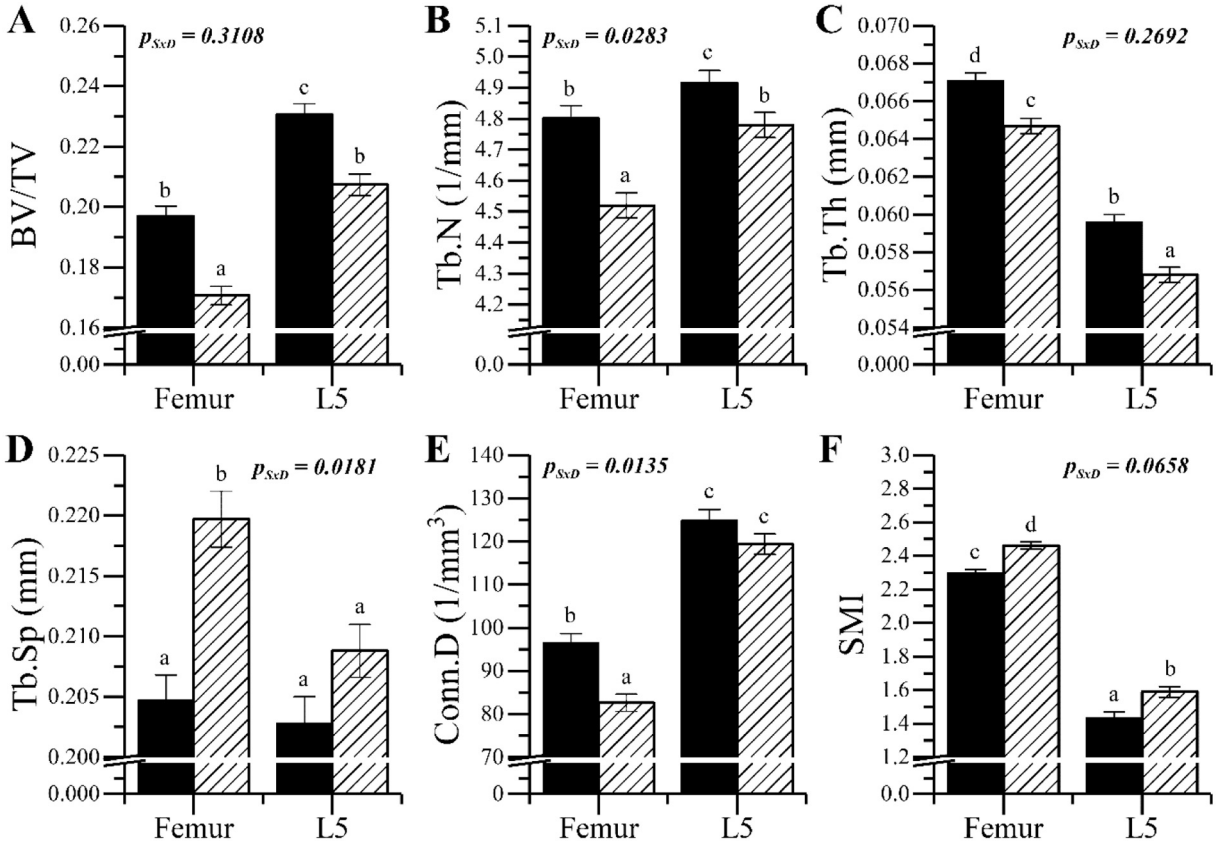

Fig. 1. Site-by-diet (SxD) interactions affecting (A) BV/TV, (B) Tb.N, (C) Tb.Th, (D) Tb.Sp, (E) Conn.D, and (F) SMI. Symbols represent the LSmeans ± SEM from two-way, repeated measure ANCOVA. Filled bar = basal (0.5%) Ca diet group. Hatched bar = 0.25% Ca diet. Values with different letter superscripts are significantly different from one another (Tukey's HSD, $p \leq 0.05$). p_{SxD} = p-value for SxD interaction.

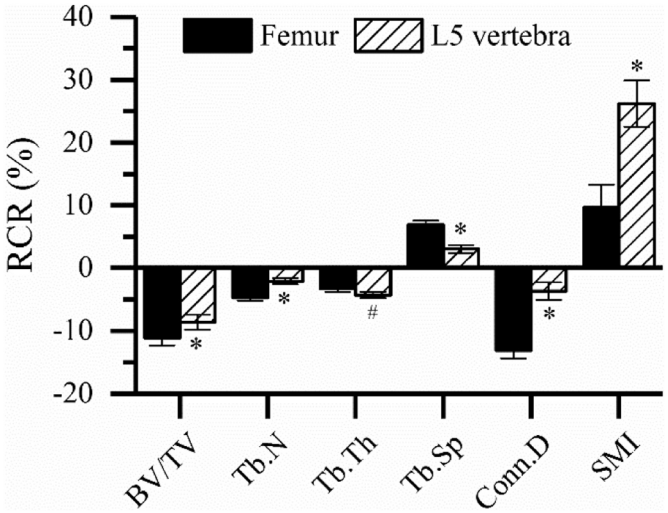

Fig. 2. Site-specific differences in the response to dietary Ca restriction (RCR) for trabecular bone μCT in the distal femur and L5 vertebae. RCR for each μCT parameter in a mouse was calculated using the equation ($X_{low\ Ca\ diet} - X_{line\ mean,\ basal\ Ca\ diet}/X_{line\ mean,\ basal\ Ca\ diet}$)*100. Bars = Mean ± SEM (n = 436–445). Repeated measures one-way ANCOVA was conducted; femur length, but not body weight, was significantly correlated with BV/TV RCR, Tb.Th RCR and Conn.D RCR and was used as a covariate in these analysis. *Significant at $p \leq 0.05$ and #a trend at $p \leq 0.10$.

were more moderate than basal estimates both in the femur (h^2 = 0.28 to 0.44) and the vertebra (h^2 = 0.30–0.58).

Two-way ANCOVA revealed a genotype-by-diet (GxD) interaction affecting BV/TV, Tb.Th, Tb.N, Conn.D, and SMI in the femur and L5 vertebra. In addition, there was a trend towards an interaction for Tb.Sp (p = 0.0685). Consistent with the two-way ANCOVA analysis, a significant genotype effect was observed for all of the RCR parameters at both sites. The Z-scores for μCT RCR parameters revealed significant variation across lines at both sites (representative data for BV/TV RCR and Conn.D RCR are shown in Fig. 4; data for other parameters are shown in Supplemental Fig. S1).

Finally, we tested whether complex interactions between diet, genetics, and site were affecting μCT parameters using two different approaches: three-way ANCOVA on trabecular parameters and site-by-genotype (SxG) interactions on RCR parameters. Three-way ANCOVA showed that a site-by-diet-by-genotype interaction affected BV/TV (p = 0.0073), Tb.Th (p = 0.0137), Conn.D (p = 0.0111), and SMI (p = 0.0108). A representative example of this complex interaction is illustrated for Conn.D in Fig. 5; data from other significant parameters are shown in Supplemental Fig. S2. Z-scores from the baseline and the 0.25% Ca group varied across the 62 genetic lines and the patterns of variation differed between the femur (Fig. 5A.a.) and the vertebra (Fig. 5A.b.). For example, the low Ca diet reduced Conn.D equally at both sites in BXD20 mice, while BXD56 mice were resistant to Ca restriction at both sites (Fig. 5B.a.). In contrast, we observed differential tissue responses for BXD28 (femur only) and BXD98 (L5 only) (Fig. 5B.b.). This demonstrates the complexity of how diet and genetics interact to influence trabecular bone in each site during growth. Consistent with this, a significant SxG interaction was observed for all of the RCR parameters (data not shown).

4. Discussion

There are several important findings from our study. First, we show

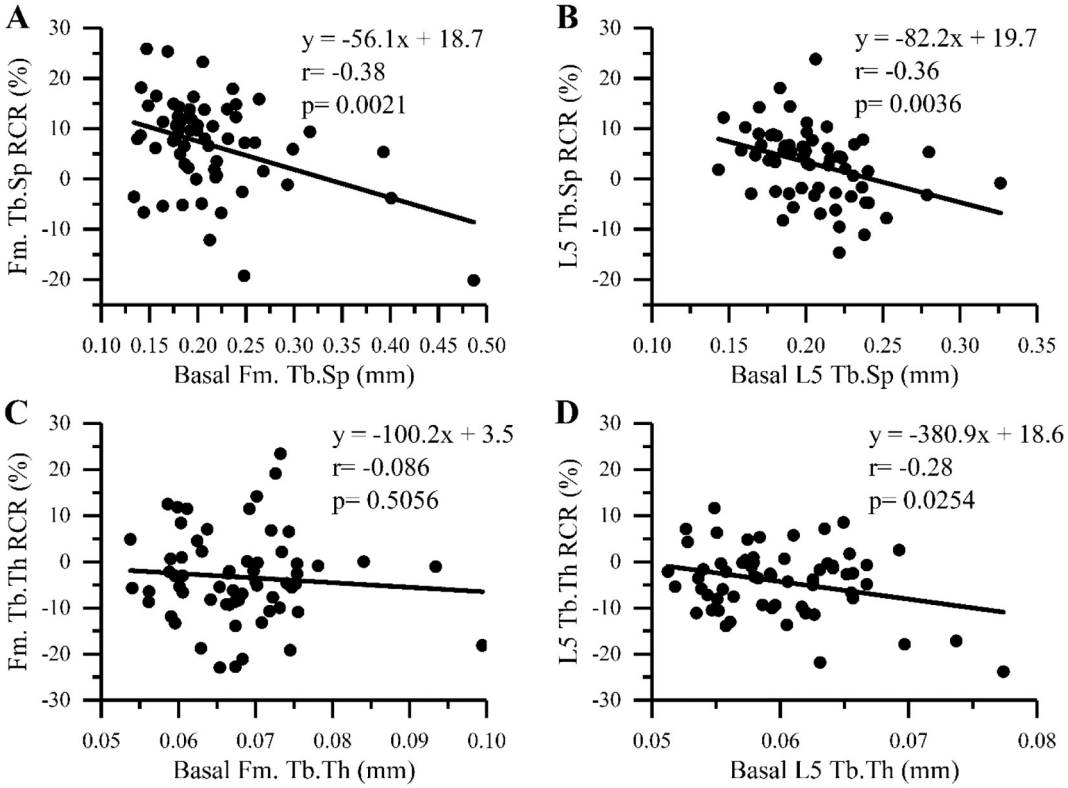

Fig. 3. Correlations between the μCT parameters and their corresponding response to dietary Ca restriction (RCR) for (A) femoral Tb.Sp, (B) vertebral Tb.Sp, (C) femoral Tb.Th, and (D) vertebral Tb.Th (D). Pearson's correlation tests were conducted on body size-corrected LSMeans (n = 62 genetic lines). A slope and an intercept for each linear correlation are reported. x = the μCT parameter of mice fed the basal diet, y = the corresponding RCR, r = correlation coefficient. p = p value of the regression line. Fm. = femur; L5 = L5 vertebra.

Table 2
Heritability estimates (h^2) of trabecular parameters and their RCR[a].

μCT parameter	Diet	Femoral h^2	Vertebral h^2
BV/TV	0.50%	0.65	0.68
	0.25%	0.63	0.64
	RCR	0.37	0.31
Tb.N	0.50%	0.79	0.70
	0.25%	0.80	0.71
	RCR	0.32	0.30
Tb.Th	0.50%	0.56	0.59
	0.25%	0.58	0.51
	RCR	0.44	0.36
Tb.Sp	0.50%	0.82	0.71
	0.25%	0.84	0.69
	RCR	0.33	0.30
Conn.D	0.50%	0.74	0.69
	0.25%	0.70	0.69
	RCR	0.28	0.36
SMI	0.50%	0.58	0.69
	0.25%	0.56	0.67
	RCR	0.41	0.58

Values were r^2 from one-way ANCOVA (main effect = genotype) adjusted for FL with the exception of the analysis of L5 vertebral Tb.N, Conn.D, and their RCR where BW was the significant covariate.

[a] RCR = response to dietary Ca restriction.

that dietary Ca is a critical factor regulating the development of peak trabecular bone mass, even in a genetically diverse population. Thus, this study overcomes a weakness of other mouse studies of inbred mice, where the effects may not be generalizable, and addresses a question that cannot be ethically tested in human children. Second, we show that there are site-specific differences in the impact of inadequate dietary Ca on trabecular bone. Finally, we report that genetics has a significant impact on trabecular bone at both spine and femur and that these genetic effects influence how trabecular bone responds to low Ca intake. Thus, our data reveal that while maintaining an adequate dietary Ca intake is a good general strategy for maximizing peak bone mass, they also show that the benefit of adequate Ca is not uniform across individuals or across bone sites.

Other groups had attempted to determine the impact that low Ca intake has on trabecular bone microarchitecture in controlled animal studies using Ca restriction in the context of low protein diets [39] or with very low dietary Ca intake [40]. However, our study is unique in that it captures the effect of a modest, and human relevant, dietary Ca inadequacy (i.e. 50% of the requirement [27,28]) on attainment of trabecular bone during growth. We found that dietary Ca restriction attenuated trabecular bone accrual in both femur and vertebra but that the impact on trabecular bone microarchitecture was distinct at each site, i.e. lower Tb.N and Conn.D in the femur but reduced Tb.Th with increased SMI in the vertebra. Reduced Tb.N and Conn.D has a greater consequence on the mechanical integrity of trabecular bone than does an equal reduction in Tb.Th and the shape of trabeculae. This because as trabeculae and their interconnectivity are lost bone structure is significantly weakened [12,41,42]. Thus, the reduction of femoral Tb.N and Conn.D we observed due to low dietary Ca intake could make this site more sensitive to future fracture compared to the vertebra. Fortunately, it may be possible to restore bone strength following a period of diet-compromised bone development provided a substantial number of trabeculae are still intact [42]. For example, although lactation induced the thinning of trabeculae and the perforation of trabecular plates in the vertebrae of mice, this effect was reversed because of the improved calcium balance that results from weaning [12,43]. In contrast, weaning did not improve the loss of Tb.N and connectivity that was caused by lactation in femur.

Another unique feature of our study is that we used 62 lines of mice to represent the high level of genetic diversity observed in humans. This

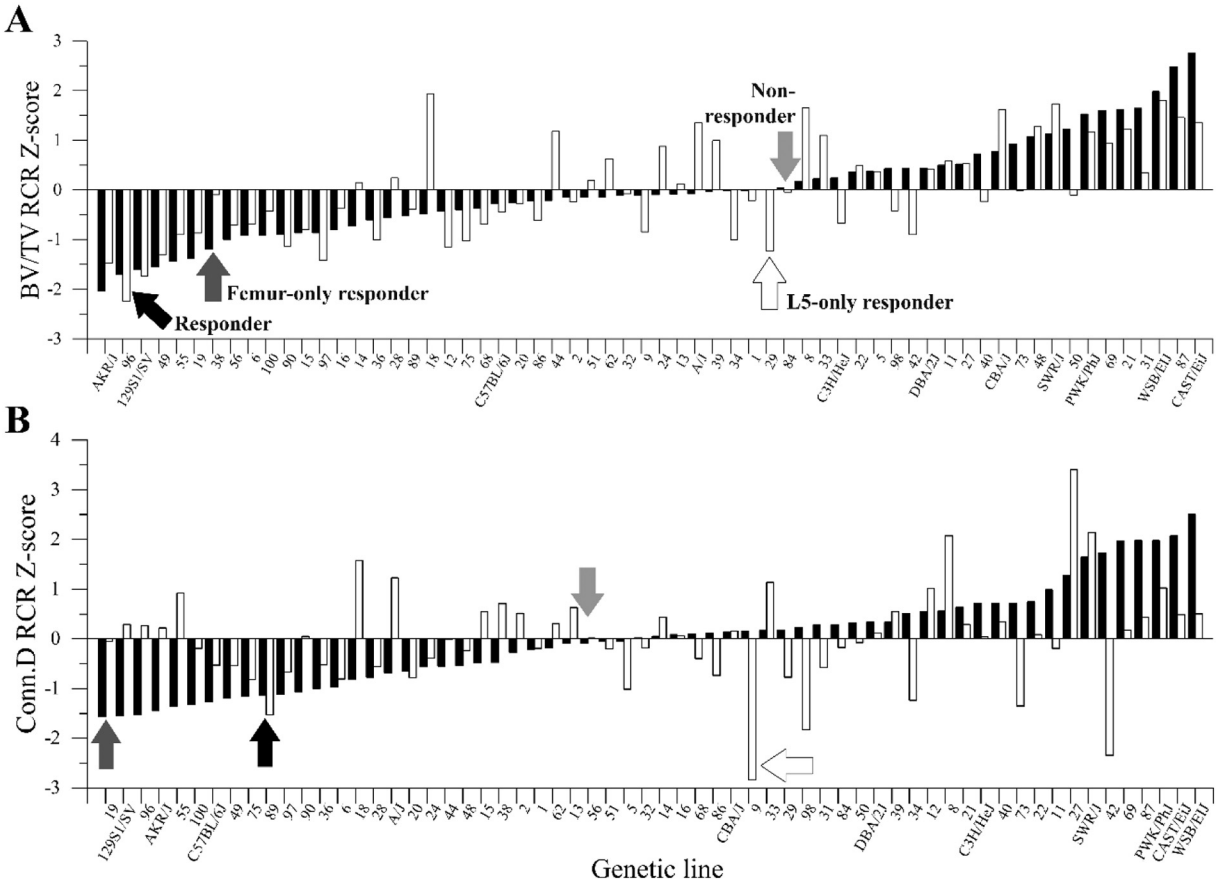

Fig. 4. Site-by-genetic interactions affect the response of trabecular parameters to dietary Ca restriction (RCR). (A) BV/TV RCR, (B) Conn.D RCR. Data are presented as *Z*-scores. The x-axis reports the 51 BXD RI lines as numbers along with abbreviations for the 11 inbred mouse lines. Filled bar = distal femur. Open bar = L5 vertebra. Arrows identify four mouse lines that represent the site-specific responses to low dietary Ca intake at each bone site. Light grey arrow = neither site responds. Black arrow = both sites respond. Dark grey arrow = femur-only responder. White arrow = L5 vertebra-only responder.

allowed us to address whether genetics modulates the response of each skeletal site to low dietary Ca intake. Here, we found significant genotype-by-diet-by-site interactions affecting trabecular parameters (Fig. 5). This extends our previous report in 11 inbred mouse lines, where we identified significant genetic effects on the response of BMD and trabecular parameters to dietary Ca restriction in the distal femur [22]. The complex interaction among diet, genetics, and site was seen in two ways: in the pattern of variation of the femoral and vertebral RCR parameters across lines (Fig. 4 and Supplemental Fig. S1) and in the differential heritability estimates for the RCR parameters between the two sites (e.g. Conn.D: L5 vertebra = 0.36, femur = 0.28; Table 2). Collectively, this indicates that genetics influences the site-specific effect of Ca insufficiency on the development of trabecular bone during growth.

Our study is the first to carefully test the independent and interactive effects of dietary Ca restriction and genetics on trabecular bone mass accrual and microarchitecture at two clinically relevant skeletal sites. There are many strengths to our study. First, we conducted our study under strictly controlled environmental conditions that included the use of a well-defined, semi-purified experimental diet. Second, by coupling the controlled dietary intervention to a large, genetically diverse population of mice, we could assess the effects of site and diet on trabecular bone in ways that are difficult to do in humans. Finally, we could take advantage of the genetic structure of our population to identify relationships between genetics and the site-specific response of trabecular bone to low Ca intake. Nonetheless, there were certain limitations for our study. First, we only measured trabecular parameters at the 12 wks of age. Therefore, we do not know what happens in each stage of growth or whether low dietary Ca intake delays, rather than blocks, peak bone mass accretion. Furthermore, our study was conducted only in male mice. Previously, we showed that female C57BL/6 mice have a more robust physiologic response to dietary Ca restriction, and are more responsive to changes in serum 1,25-dihydroxyvitamin D, than males [44]. As a result, our current findings may not apply directly to trabecular bone mass accrual in females. Lastly, we did not test for mechanical properties of our bone samples. Future studies using finite element analysis may provide more insight in this matter.

The critical finding of our study is that dietary Ca deprivation during growth leads to site-specific differences in the development of trabecular bone microarchitecture at the femur and L5 vertebra. In addition, we report for the first time that genetics has a site-specific influence on how trabecular bone responds to low dietary Ca intake. The significance of our work is that dietary Ca restriction and genetics work both independently and interactively to influence the attainment of peak trabecular bone mass and its microarchitecture. Furthermore, these interactions are not uniform across bone sites. In the future, it will be important to identify the genetic variants that underlie the response to low dietary Ca intake at each skeletal site.

Declaration of Competing Interest

None.

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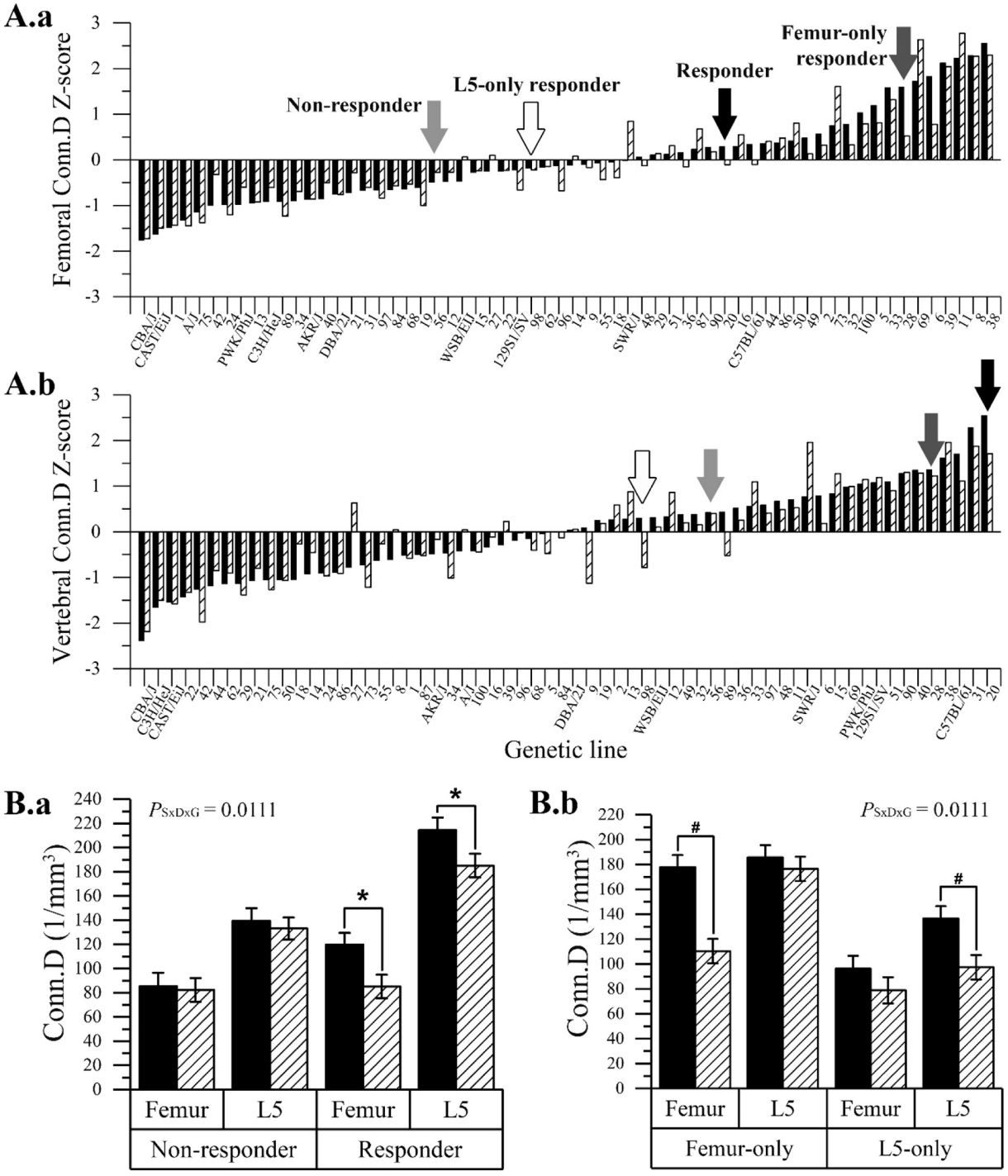

Fig. 5. Representative data from Conn.D for the complex Site-by-Diet-by-Genetics interaction regulating trabecular parameters. (A) Z-score from each genetic line of femoral Conn.D (A.a.) and vertebral Conn.D (A.b.) are shown. The x-axis reports the 51 BXD RI lines as numbers along with abbreviations for the 11 inbred mouse lines. Black bars = 0.5% Ca diet group. Hatched bars = 0.25% Ca diet group. Arrows identify four mouse lines that are representative of the types of responses to low dietary Ca intake at the two sites. Light grey arrow = no response to diet at either site. Black arrow = lower response to diet at both sites. Dark grey arrow = a response to diet a just the femur. White arrow = a response to diet at just the L5 vertebra. (B) Bar charts showing the Conn.D values for lines identified with arrows in panel (A). Bars represent LSMeans of Conn.D from each group. Black bars = 0.5% Ca diet group. Hatched bars = 0.25% Ca diet group. (B.a.) A non-reponder line (BXD56) and a responder line (BXD20). (B.b.) A L5-only responder line (BXD98) and a femur-only responder line (BXD28). p_{SxDxG} = *p*-value for SxDxG interaction. *P*-values for pairwise comparisons between diet groups within each site and line: $^{*}p < 0.005$, $^{\#}p < 0.05$.

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Author's roles

Study design: JCF; Study conduct: JCF; Bone analysis: PRF and KC; Data management, QTL analysis, and statistical analysis: KC; Data interpretation: JCF, PRF, and KC; First draft of manuscript: JCF and KC; Revision of manuscript and approval of the final version of the manuscript: JCF, PRF and KC. JCF takes responsibility for the integrity of the data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.05.011.

References

[1] G. Ballane, J.A. Cauley, M.M. Luckey, G. El-Hajj Fuleihan, Worldwide prevalence and incidence of osteoporotic vertebral fractures, Osteoporos. Int. 28 (2017) 1531–1542.

[2] O. Johnell, J.A. Kanis, An estimate of the worldwide prevalence and disability associated with osteoporotic fractures, Osteoporos. Int. 17 (2006) 1726–1733.

[3] T.E. Ciarelli, D.P. Fyhrie, M.B. Schaffler, S.A. Goldstein, Variations in three-dimensional cancellous bone architecture of the proximal femur in female hip fractures and in controls, J. Bone Miner. Res. 15 (2000) 32–40.

[4] S. Nawathe, H. Akhlaghpour, M.L. Bouxsein, T.M. Keaveny, Microstructural failure mechanisms in the human proximal femur for sideways fall loading, J. Bone Miner. Res. 29 (2014) 507–515.

[5] A.J. Fields, S. Nawathe, S.K. Eswaran, M.G. Jekir, M.F. Adams, P. Papadopoulos, T.M. Keaveny, Vertebral fragility and structural redundancy, J. Bone Miner. Res. 27 (2012) 2152–2158.

[6] B. Clarke, Normal bone anatomy and physiology, Clin. J. Am. Soc. Nephrol. 3 (Suppl. 3) (2008) S131–S139.

[7] G.M. Ellinger, J. Duckworth, A.C. Dalgarno, M.H. Quenouille, Skeletal changes during pregnancy and lactation in the rat: effect of different levels of dietary calcium, Br. J. Nutr. 6 (1952) 235–253.

[8] E. Lozupone, A. Favia, Distribution of resorption processes in the compacta and spongiosa of bones from lactating rats fed a low-calcium diet, Bone 9 (1988) 215–224.

[9] D.W. Dempster, R. Birchman, R. Xu, R. Lindsay, V. Shen, Temporal changes in cancellous bone structure of rats immediately after ovariectomy, Bone 16 (1995) 157–161.

[10] X.L. Liu, C.L. Li, W.W. Lu, W.X. Cai, L.W. Zheng, Skeletal site-specific response to ovariectomy in a rat model: change in bone density and microarchitecture, Clin. Oral Implants Res. 26 (2015) 392–398.

[11] Y.H. Shin, D.C. Cho, S.H. Yu, K.T. Kim, H.J. Cho, J.K. Sung, Histomorphometric analysis of the spine and femur in ovariectomized rats using micro-computed tomographic scan, J. Korean Neurosurg. Soc. 52 (2012) 1–6.

[12] X.S. Liu, L. Ardeshirpour, J.N. VanHouten, E. Shane, J.J. Wysolmerski, Site-specific changes in bone microarchitecture, mineralization, and stiffness during lactation and after weaning in mice, J. Bone Miner. Res. 27 (2012) 865–875.

[13] C.M. Weaver, C.M. Gordon, K.F. Janz, H.J. Kalkwarf, J.M. Lappe, R. Lewis, M. O'Karma, T.C. Wallace, B.S. Zemel, The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations, Osteoporos. Int. 27 (2016) 1281–1386.

[14] J.P. Bonjour, A.L. Carrie, S. Ferrari, H. Clavien, D. Slosman, G. Theintz, R. Rizzoli, Calcium-enriched foods and bone mass growth in prepubertal girls: a randomized, double-blind, placebo-controlled trial, J. Clin. Invest. 99 (1997) 1287–1294.

[15] T. Chevalley, J.P. Bonjour, S. Ferrari, D. Hans, R. Rizzoli, Skeletal site selectivity in the effects of calcium supplementation on areal bone mineral density gain: a randomized, double-blind, placebo-controlled trial in prepubertal boys, J. Clin. Endocrinol. Metab. 90 (2005) 3342–3349.

[16] X.M. Ma, Z.W. Huang, X.G. Yang, Y.X. Su, Calcium supplementation and bone mineral accretion in Chinese adolescents aged 12–14 years: a 12-month, dose-response, randomised intervention trial, Br. J. Nutr. 112 (2014) 1510–1520.

[17] S. Iuliano-Burns, L. Saxon, G. Naughton, K. Gibbons, S.L. Bass, Regional specificity of exercise and calcium during skeletal growth in girls: a randomized controlled trial, J. Bone Miner. Res. 18 (2003) 156–162.

[18] C.H. Turner, Y.F. Hsieh, R. Muller, M.L. Bouxsein, D.J. Baylink, C.J. Rosen, M.D. Grynpas, L.R. Donahue, W.G. Beamer, Genetic regulation of cortical and trabecular bone strength and microstructure in inbred strains of mice, J. Bone Miner. Res. 15 (2000) 1126–1131.

[19] I. Sabsovich, J.D. Clark, G. Liao, G. Peltz, D.P. Lindsey, C.R. Jacobs, W. Yao, T.Z. Guo, W.S. Kingery, Bone microstructure and its associated genetic variability in 12 inbred mouse strains: microCT study and in silico genome scan, Bone 42 (2008) 439–451.

[20] Y. Zhang, J. Huang, Y. Jiao, V. David, M. Kocak, E. Roan, D. Di'Angelo, L. Lu, K.A. Hasty, W. Gu, Bone morphology in 46 BXD recombinant inbred strains and femur-tibia correlation, Sci. World J. 2015 (2015) 728278.

[21] M.H. Sheng, D.J. Baylink, W.G. Beamer, L.R. Donahue, K.H. Lau, J.E. Wergedal, Regulation of bone volume is different in the metaphyses of the femur and vertebra of C3H/HeJ and C57BL/6J mice, Bone 30 (2002) 486–491.

[22] R.A. Replogle, Q. Li, L. Wang, M. Zhang, J.C. Fleet, Gene-by-diet interactions influence calcium absorption and bone density in mice, J. Bone Miner. Res. 29 (2014) 657–665.

[23] H.R. Buie, C.P. Moore, S.K. Boyd, Postpubertal architectural developmental patterns differ between the L3 vertebra and proximal tibia in three inbred strains of mice, J. Bone Miner. Res. 23 (2008) 2048–2059.

[24] J.L. Peirce, L. Lu, J. Gu, L.M. Silver, R.W. Williams, A new set of BXD recombinant inbred lines from advanced intercross populations in mice, BMC Genet. 5 (2004) 7.

[25] A. Roberts, F. Pardo-Manuel de Villena, W. Wang, L. McMillan, D.W. Threadgill, The polymorphism architecture of mouse genetic resources elucidated using genome-wide resequencing data: implications for QTL discovery and systems genetics, Mamm. Genome 18 (2007) 473–481.

[26] NRC, Nutrient Requirements of Laboratory Animals, National Academy Press, Washington, DC, 1995.

[27] Wallace TC, Reider C, Fulgoni VL, 3rd. Calcium and vitamin D disparities are related to gender, age, race, household income level, and weight classification but not vegetarian status in the United States: analysis of the NHANES 2001–2008 data set. J. Am. Coll. Nutr. 2013;32: 321–30.

[28] R.P. Heaney, Nutritional factors in osteoporosis, Annu. Rev. Nutr. 13 (1993) 287–316.

[29] P.C. Reyes Fernandez, R.A. Replogle, L. Wang, M. Zhang, J.C. Fleet, Novel genetic loci control calcium absorption and femur bone mass as well as their response to low calcium intake in male BXD recombinant inbred mice, J. Bone Miner. Res. 31 (2016) 994–1002.

[30] M.L. Bouxsein, T. Uchiyama, C.J. Rosen, K.L. Shultz, L.R. Donahue, C.H. Turner, S. Sen, G.A. Churchill, R. Muller, W.G. Beamer, Mapping quantitative trait loci for vertebral trabecular bone volume fraction and microarchitecture in mice, J. Bone Miner. Res. 19 (2004) 587–599.

[31] S.M. Tommasini, T.G. Morgan, M. van der Meulen, K.J. Jepsen, Genetic variation in structure-function relationships for the inbred mouse lumbar vertebral body, J. Bone Miner. Res. 20 (2005) 817–827.

[32] W.G. Beamer, K.L. Shultz, L.R. Donahue, G.A. Churchill, S. Sen, J.R. Wergedal, D.J. Baylink, C.J. Rosen, Quantitative trait loci for femoral and lumbar vertebral bone mineral density in C57BL/6J and C3H/HeJ inbred strains of mice, J. Bone Miner. Res. 16 (2001) 1195–1206.

[33] B.K. Philip, P.J. Childress, A.G. Robling, A. Heller, P.P. Nawroth, A. Bierhaus, J.P. Bidwell, RAGE supports parathyroid hormone-induced gains in femoral trabecular bone, Am. J. Physiol. Endocrinol. Metab. 298 (2010) E714–E725.

[34] S.H. Windahl, A.E. Borjesson, H.H. Farman, C. Engdahl, S. Moverare-Skrtic, K. Sjogren, M.K. Lagerquist, J.M. Kindblom, A. Koskela, J. Tuukkanen, P. Divieti Pajevic, J.Q. Feng, K. Dahlman-Wright, P. Antonson, J.A. Gustafsson, C. Ohlsson, Estrogen receptor-alpha in osteocytes is important for trabecular bone formation in male mice, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 2294–2299.

[35] M.L. Bouxsein, S.K. Boyd, B.A. Christiansen, R.E. Guldberg, K.J. Jepsen, R. Muller, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography, J. Bone Miner. Res. 25 (2010) 1468–1486.

[36] D.H. Lang, N.A. Sharkey, A. Lionikas, H.A. Mack, L. Larsson, G.P. Vogler, D.J. Vandenbergh, D.A. Blizard, J.T. Stout, J.P. Stitt, G.E. McClearn, Adjusting data to body size: a comparison of methods as applied to quantitative trait loci analysis of musculoskeletal phenotypes, J. Bone Miner. Res. 20 (2005) 748–757.

[37] R.I. Jennrich, M.D. Schluchter, Unbalanced repeated-measures models with structured covariance matrices, Biometrics 42 (1986) 805–820.

[38] I.H. Parkinson, N.L. Fazzalari, Interrelationships between structural parameters of cancellous bone reveal accelerated structural change at low bone volume, J. Bone Miner. Res. 18 (2003) 2200–2205.

[39] C. Fournier, R. Rizzoli, P. Ammann, Low calcium-phosphate intakes modulate the low-protein diet-related effect on peak bone mass acquisition: a hormonal and bone strength determinants study in female growing rats, Endocrinology 155 (2014) 4305–4315.

[40] M. Ferretti, F. Cavani, A. Smargiassi, L. Roli, C. Palumbo, Mineral and skeletal homeostasis influence the manner of bone loss in metabolic osteoporosis due to calcium-deprived diet in different sites of rat vertebra and femur, Biomed. Res. Int. 2015 (2015) 304178.

[41] X.E. Guo, C.H. Kim, Mechanical consequence of trabecular bone loss and its treatment: a three-dimensional model simulation, Bone 30 (2002) 404–411.

[42] M.J. Silva, L.J. Gibson, Modeling the mechanical behavior of vertebral trabecular bone: effects of age-related changes in microstructure, Bone 21 (1997) 191–199.

[43] L. Ardeshirpour, P. Dann, D.J. Adams, T. Nelson, J. VanHouten, M.C. Horowitz, J.J. Wysolmerski, Weaning triggers a decrease in receptor activator of nuclear factor-kappaB ligand expression, widespread osteoclast apoptosis, and rapid recovery of bone mass after lactation in mice, Endocrinology 148 (2007) 3875–3886.

[44] Y. Song, J.C. Fleet, 1,25 dihydroxycholecalciferol-mediated calcium absorption and gene expression are higher in female than in male mice, J. Nutr. 134 (2004) 1857–1861.