GENETIC DIFFERENCES CONTROL THE RESPONSE OF FEMUR AND LUMBAR SPINE TRABECULAR BONE MICROSTRUCTURE TO DIETARY CALCIUM RESTRICTION IN MICE

by

Xu Lan

A Thesis Submitted in Partial Fulfillment
Of the Requirements for a Degree with Honors
(Nutrition Science)

The College of Health and Human Sciences

Purdue University

My 2013

West Lafayette, Indiana

Abstract

Both dietary calcium and genetics influence bone density and structure. However, how genetics affect the adaptation response of bone parameters to dietary calcium (Ca) restriction is unknown. 11 inbred strains of mice were fed adequate (0.5%) or low (0.25%) Ca diets from 4-12 weeks of age and were evaluated for gene-by-diet interactions affecting bone structure at the femur and lumbar spine. We observed that genetics and diet main effects as well as gene-by-diet interactions significantly affected femur trabecular and cortical bone, and lumbar spine trabecular bone microCT parameters. There were no significant correlations between basal and adaptation parameters in femur or lumbar spine. Adaptation of lumbar spine trabecular bone parameters was smaller compared to adaptation observed in the femur trabecular bone. However, the adaptation parameters in these two bone sites were significantly and positively correlated. We conclude that (1) genetic controls of basal and adaptive bone parameters are distinct; (2) trabecular bone in lumbar spine is more resistant to calcium restriction than the trabecular bone in distal femur; (3) similar genetic forces affect the adaptation response in distal femur and lumbar spine trabecular bone. However, site-specific regulation may exist in bone structure adaptation response to calcium restriction in some of the inbred lines.

Introduction

Osteoporosis is a common bone disease in the US and the risk of having this disease is determined by the level of PBM (peak bone mass) and the subsequent rate of bone loss (Ilich & Kerstetter, 2000). Adequate calcium intake is crucial to the development of PBM in early adulthood and to minimize adult bone loss (figure 1). In human, the coordination of events occurring at the three-tissue axis of intestine, kidney and bone are necessary to control calcium metabolism (Fleet). When humans habitually consume a low calcium diet, the body undergoes a series of adaptive responses to maintain serum Ca level within a narrow range (figure 2). First, serum calcium level decreases and this is sensed by a calcium-sensing receptor in the parathyroid gland (Hebert, Brown, & Harris, 1997) leading to increased serum level of parathyroid hormone (PTH). PTH stimulates bone resorption, calcium reabsorption in kidneys, and the conversion of 25(OH) D (a very stable form of vitamin D) to 1, $25(OH)_2$ D (main active form of vitamin D) in the kidney. $1,25(OH)_2D$ affects calcium metabolism by increasing bone remodeling, calcium reabsorption in kidney, and active calcium absorption in the small intestine. When calcium intake is low and adaptation is inadequate, bone density and structure is compromised to maintain serum Ca level(Fleet).

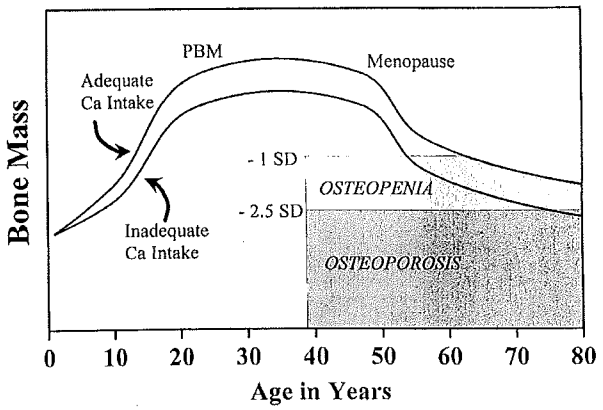


Figure 1. High peak bone mass (PBM) decreases the risk for osteoporosis later in life. Adequate calcium intake is crucial to the development of PBM in early adulthood and to minimize adult bone loss. According to the World Health Organization, the criteria for osteopenia is bone mineral density (BMD) between 1 SD and 2.5 SD below average peak young adult BMD, and criteria for osteoporosis is BMD ≥ 2.5 SD below. SD=standard deviation. (Ilich & Kerstetter, 2000)

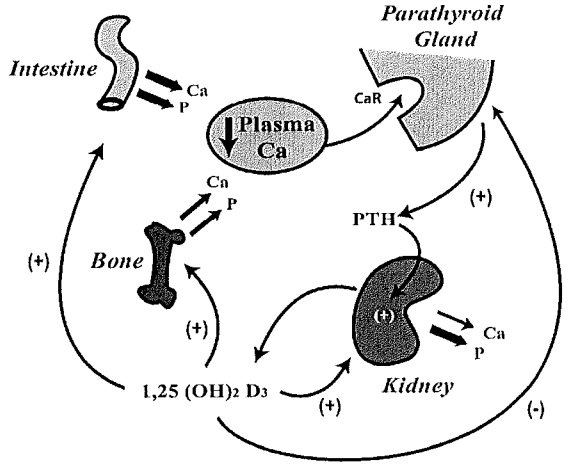


Figure 2: Low dietary calcium intake causes hormonal adaptation regulating events in intestine, kidney, and bone. When adaptation is inadequate, bone density and structure is compromised to main serum calcium level. (Fleet, Hong, & Zhang, 2004)

In addition to adequate dietary calcium intake, genetics also greatly influences bone density (Beamer, Donahue, Rosen, & Baylink, 1996). The simplest example of the role that genetics plays in bone health is seen when comparing racial groups. Racial differences in bone density and fracture risk, and race-by-diet interactions affecting bone mass has been reported in several studies (Barrett-Connor et al., 2005 miller 2005; Walker, Novotny, Bilezikian, & Weaver, 2008; Weaver et al., 2008). Barrett-Connor et al. reported that African Americans have higher areal bone mineral density than American whites, Native Americans, Hispanic and Asian (Barrett-Connor et al., 2005 miller 2005). Blacks were found to have greater skeletal calcium retention than whites across a range of calcium intakes (760-1981 mg Ca/d) and this is due to greater net calcium absorption and lower calcium excretion in the young black girls (Braun et al., 2007 Jackman 2013). A study in black and white adolescent girls on diets with high or low sodium concentrations found that both race and sodium intake significantly affected calcium retention. Also, 24-h urinary calcium excretion and 24-h sodium excretion were significantly affected by a race-by-diet interaction (Wigertz et al., 2005). However, we still lack of a clear understanding of the gene-by-diet interactions regulating bone parameters.

Controlling environmental factors and calcium intake are difficult in human populations. In addition, human populations have tremendous genetic diversity. As a result, we chose to use inbred mouse lines to study the independent and interacting effects of dietary calcium intake and genetics on bone density and structure. The gene and diet interactions observed across inbred mouse lines will give insights into the genes controlling adaptation of bone to dietary Ca stress in human populations. 11 inbred mouse lines were fed adequate or low Ca diets and bone microstructure in the femur and the lumbar spine were examined using microcomputed tomography (μCT). We chose to analyze distal femur and lumbar spine because both are trabecular bone-rich sites. We also examined midshaft femur which is mainly consist of cortical bone. Our hypothesis is that there is diversity in the impact that dietary calcium restriction has on the loss of bone structure in both femur and lumbar spine.

Methods

Experimental Design

Four week old, male mice were obtained from The Jackson Labs (Bar Harbor, ME): 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). These 11 common laboratory inbred strains were chosen to encompass three mouse subspecies (*Mus musculus domesticus, M.m.musculus, and M.m. castaneus)*, to include both classical inbred strains and more genetically divergent wild-derived inbred lines, and to represent parental strains of available genetic mapping resources (Roberts, Pardo-Manuel, Wang, McMillan, & Threadgill, 2007).

laboratory rabbits under calcium deprivation, significant differences in bone porosity was observed between control and calcium deficiency groups in the vertebral body but not in the femoral head (Wu, Boyd, Fix, & Burr, 1990). This suggests that vertebrae are more vulnerable to bone loss than the femur. However, Liu et al. examined the site-specific changes in bone parameters during and after lactation in CD1 mice, and found that after mice had completed lactation, no difference was seen in lumbar vertebra L3 bone microstructure compared to nulliparous mice but distal femur bone microstructure was significantly lower (Liu, Ardeshirpour, VanHouten, Shane, & Wysolmerski, 2012). This observation indicates that in mice lumbar spines are more resistant to bone loss induced by pregnancy and lactation (Liu et al., 2012), and this is consistent to what we observed in trabecular bone loss induced by calcium restriction.

There were significant, positive correlations between the adaptation parameters for BV/TV in femora and spine. This suggests that although we didn't see a statistically significant effect of genetics on adaptation of lumber spine trabecular bone, it is still under the influence of the same genetic forces that are controlling adaptation in distal femur trabecular bone. However, the relationship of adaptation between femur and lumbar spine does not account for all the variation between the traits. For instance, B6, A/J, C3H and CBA lines had different BV/TV adaptation patterns between femora and spine. This suggests that in some inbred lines, femora and lumbar spines are under similar genetic influences, while in other lines, site-specific regulation exists in the adaptive response of bone structure to low dietary calcium intake. The mechanisms that underline these site-specific adaptation responses are unknown and need future investigation. One possible explanation is that bone resorption rates are affected by site-specific sensitivity to serum $1,25(OH)_2$ D and PTH levels.

Mice were fed an AIN93G semi-purified diet containing 200 IU vitamin D/kg diet and either 0.5% (adequate) or 0.25 % Ca (low) from 4 wk to 12 wks of age (Research Diets, New Brunswick, NJ). The calcium levels in the diets were chosen to maintain Ca homeostasis (0.5 % Ca) or elicit an adaptive response in serum 1, 25 $(OH)_2D$ (0.25 % Ca). Mice were given food and water *ad libitum* and were maintained in rooms with UV blocking filters over lights with a 12 h light/dark cycle. At 12 wks of age mice were killed. The spine and hindlimbs were harvested, some muscle was removed, and the remaining tissue was preserved in 10% neutral buffered formalin for 2 weeks, and then stored in 70% ethanol.

Preparation of bone samples

The vertebra right above the hip bones was separated (L4 or L5, depending upon the inbred line) and the muscle surrounding the spine was removed completely. The right hindlimb was separated into femur and tibia and the muscle was completely removed from the femur using a scalpel. After muscle was removed bones were transferred back to 70% ethanol for storage.

Bone Phenotyping

The trabecular bones in the vertebral body and the distal femur, and the cortical bone in midshaft femur were analyzed using microcomputed tomography (μCt 40, Scanco Medical, Bassersdorf, Switzerland). Samples were immersed in 70% ethanol and scanned in a turntable cylindrical poly-ether-imide (PEI) vials (15x75) using a multiplex scanning method. A multiplex sample holder was made to have three scanning chambers stack on one another.

Images were obtained using a cubic voxel size of 16 μm, X-ray tube potential of 55 kVp, and X ray intensity of 145 μA, and 300 ms integration time. The measurements include bone volume density (BV/TV, %), trabecular number (Tb. N, 1/mm), trabecular spacing (Tb. Sp, mm), and

trabecular thickness (Tb. Th, mm) in the trabecular bone, and cortical bone volume fraction (Ct.Ar/Tt.Ar, %) and cortical thickness (Ct. Th, mm) in the cortical bone. All images were reconstructed using the Scanco software and measures obtained were based on 3D model-independent algorithms.

Lumber spine: Three vertebrae were placed on one toothpick. In each scanning chamber, 6 toothpick sets were loaded, with five toothpick groups set around the perimeter of the chamber and one in the middle. When scanning, toothpick sets and pipette markers were immobilized with cotton and immersed in 70% ethanol. Using this scanning method, 54 samples maximum can be scanned at one time.

The region of interest (ROI) for trabecular bone was set 0.8 mm from the growth plate and 50 slices were reconstructed starting from the first slice containing no evidence of growth plate or primary spongiosa. Contours to delineate the trabecular bone region were drawn manually. The bone tissue was segmented using a single global threshold of 240.

Femur: The ROI for trabecular bone was set at 1 mm from the growth plate. 94 slices were scanned (1.5 mm) and 56 slices (1 mm) were reconstructed starting from the first slice containing no evidence of growth plate or primary spongiosa. Contours to delineate the trabecular bone region were drawn manually.

The ROI for cortical bone was placed at 50% of the length of the bone (midshaft), 15 slices (0.24 mm) were scanned and reconstructed. A single global threshold value was set.

Statistical Analysis

The outliers were determined by normalizing each line/diet group to its mean and calculating a z score for each value. Those datapoints with a z score in the extreme 2.5 % of either end of the distribution within a line/diet group were removed. Summary statistics for the raw data were calculated after outlier removal. The data for the entire study population was examined for adherence to a normal distribution by fitting it to a two-way ANOVA model (using line, diet, and interaction effects) and testing the residuals with the Anderson-Darling test for normality.

Previous studies have shown that bone parameters are tightly correlated to indices of body size (Lang et al., 2005 Mack 2005). The presence of body size-associated covariate effects on all phenotypes was assessed by Pearson's correlations using body weight (BW) and femur length (FL). Significant, independent confounding effects of BW and/or FL were then removed by linear regression and the resulting residual values were used in subsequent analyses (Lang et al., 2005 Mack 2005). In the lumbar spine, BV/TV, Tb.N and Tb.Sp were affected by both BW and FL, and Tb.Th was only affected by FL. In the distal femur, phenotypes affected by BW and FL were Tb.N and Tb.Sp. Tb.Th was only affected by BW and BV/TV was only affected by FL. In midshaft femur, both Cort.Th and Ct.Ar/Tt.Ar were affected by BW. After transformation and co-variate correction, each phenotype was assessed for the presence of main effects (line, diet) and a line by diet interaction by two-way ANOVA. When significant effects were detected, post-hoc comparisons were made using Tukey-Kramer. Statistical significance was set at $P<0.05$.

Table 1. Summary of statistically significant effects influencing bone uCT parameters. Ct.Ar/Tt.Ar = cortical area fraction; Ct.Th = average cortical thickness; BV/TV = bone volume fraction; Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness

ANOVA (p values)				
Sites	Parameters	Gene	Diet	Gene-Diet Interaction
Distal Femur	BV/TV	<0.001	0.05	<0.001
	Tb.N	<0.001	0.5	0.02
	Tb.Sp	<0.001	0.38	0.05
	Tb.Th	<0.001	<0.001	<0.001
Midshaft Femur	Ct.Ar/Tt.Ar	<0.001	<0.001	0.82
	Ct.Th	<0.001	<0.001	0.01
Lumbar Spine	BV/TV	<0.0001	0.45	0.31
	Tb.N	<0.0001	0.91	0.04
	Tb.Sp	<0.0001	0.77	0.23
	Tb.Th	<0.0001	0.01	<0.001

Results

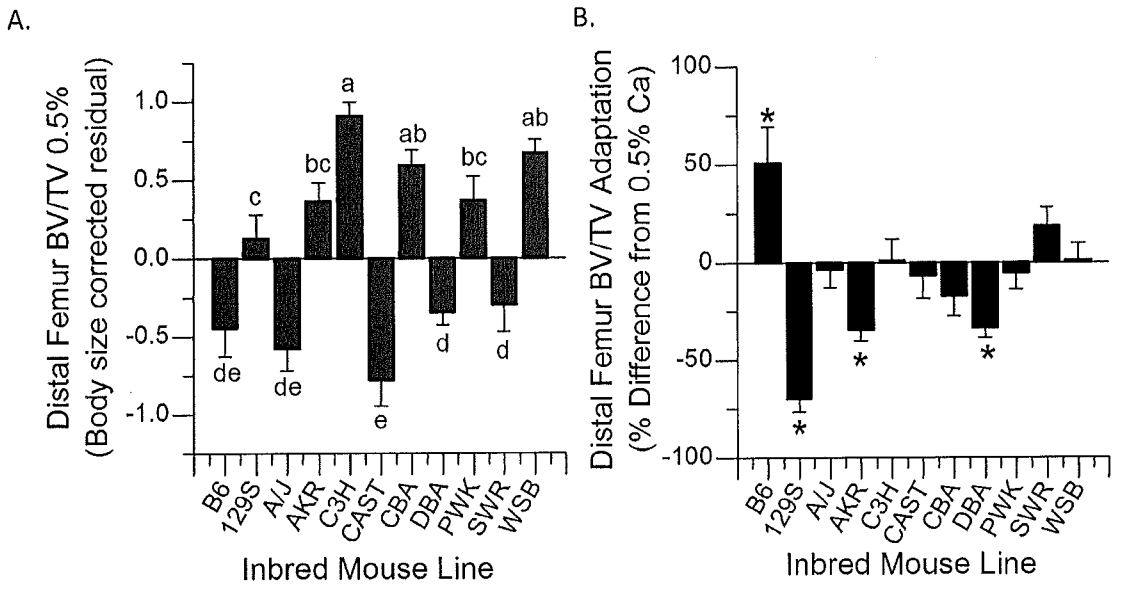
Distal femur bone parameters and their adaptation to dietary calcium restriction are variable across the 11 inbred mouse lines.

In the distal femur, BV/TV was significantly affected by both genetic and diet main effects as well as by gene-by-diet interactions (table 1). On 0.5% Ca diet, there was significant variation in BV/TV across 11 inbred lines, with C3H, CBA and WSB having the highest BV/TV, and B6, A/J and CAST having the lowest (figure 3A). Similar levels of variation were observed across the lines for Tb.N, Tb.Sp and Tb.Th when mice were fed the 0.5% Ca diet (table 1).

The adaptive response to low calcium diet varied significantly among the 11 inbred mouse lines. Dietary calcium intake significantly affected BV/TV in the distal femur in some, but not all of the lines. BV/TV increased significantly in B6 mice in response to low calcium diet, while a

significant decrease was noted for 129S, AKR and DBA mice (figure 3B and supplementary table 5). Dietary calcium intake restriction also significantly affected Tb.Th, but not Tb.N or Tb.Sp (table 1). The adaptive response seen for distal femur Tb.Th was similar to what we observed for BV/TV, i.e. a significant increase in B6 mice and a significant decrease in 129S, AKR and CBA mice (see supplementary table 5).

Figure 3. Genetic diversity in (A) basal femur BV/TV and (B) adaptation of femur BV/TV to low Ca intake among 11 inbred mouse lines. (A) Body size corrected basal femur BV/TV. Lines labeled with the same letter are not significantly different ($p<0.05$). (B) Adaptation of femur BVTV to low Ca intake. *Adaptation significantly differs from 0 ($P<0.05$).



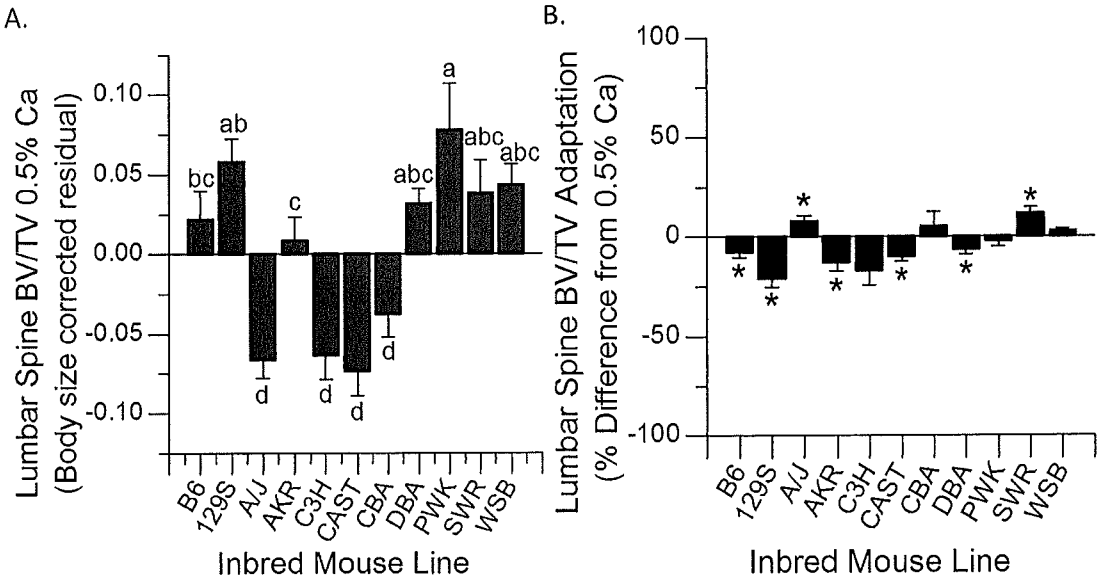
Lumbar spine bone parameters and their adaptation to dietary calcium restriction are variable across the 11 inbred mouse lines.

In lumbar spines, BV/TV was significantly affected by genetic background ($p<0.05$) (table 1), with 129S, PWK, SWR and WSB having the highest BV/TV, and A/J, C3H, CAST and CBA

having the lowest (Figure 4A). A similar level of variability can be observed for Tb.N, Tb.Sp and Tb. Th (table 1).

There was no significant change in BV/TV in response to low dietary calcium intake and this was also true for Tb.N and Tb.Sp (table 1). Only lumbar spine Tb.Th was significantly affected by both genetic background and dietary calcium main effects as well as by gene-by-diet interactions. Analysis of Tb.Th adaptation parameters indicated that Tb.Th significantly decreased in 129S, AKR, C3H and DBA (see supplementary table 5).

Figure 4. Genetic diversity in (A) basal lumbar spine BV/TV and (B) adaptation of lumbar spine BV/TV to low Ca intake among 11 inbred mouse lines. (A) Body size corrected basal lumbar spine BV/TV. Lines labeled with the same letter are not significantly different ($p<0.05$). (B) Adaptation of lumbar spine BVTV to low Ca intake. *Adaptation significantly differs from 0 ($P<0.05$).



Correlation analysis between baseline and adaptation parameters

There was no significant correlation between baseline and adaptation data for either BV/TV or Tb.Th in the distal femur (figure 5 and table 2). Similar results were also observed in the lumbar spine for BV/TV and Tb.Th (table 2).

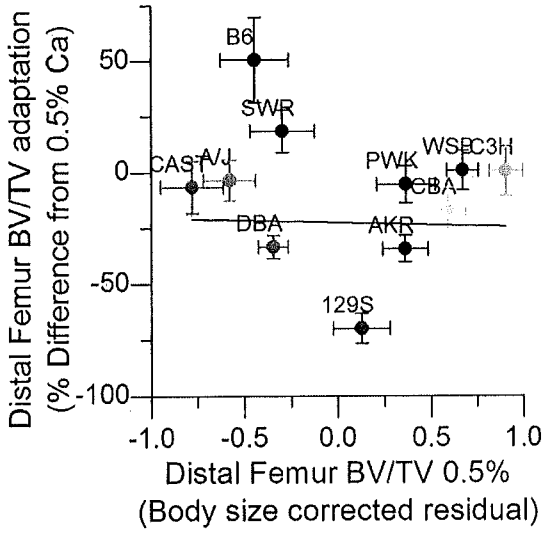


Figure 5. Basal and adaptation of BV/TV in the distal femur to calcium restriction was not significantly correlated.

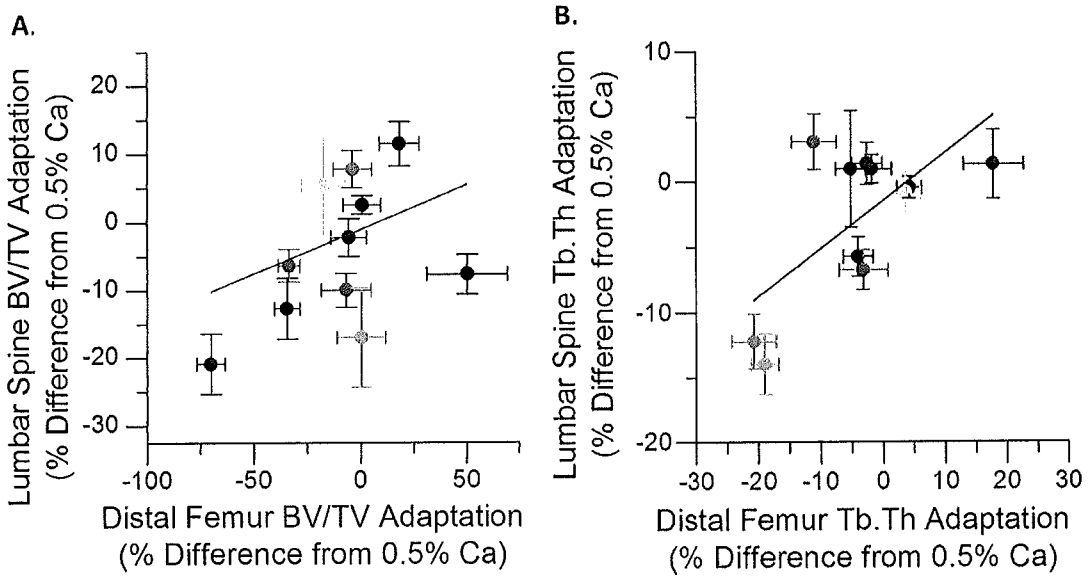
Table 2. Summary of correlation analysis between basal and adaptation parameters in distal femur or lumbar spine, and between distal femur and lumbar spine for BV/TV or Tb.Th.

Correlation analysis	*r*	*p*
Distal Femur 0.5% Ca and adaptation		
BV/TV	-0.34	0.31
Tb.Th	-0.50	0.12
Lumbar spine 0.5% Ca and adaptation		
BV/TV	-0.29	0.40
Tb.Th	-0.47	0.14
Distal Femur and lumbar spine		
BV/TV Adaptation	0.29	0.01
Tb.Th Adaptation	0.53	<0.0001

Correlation analysis between femur and lumbar spine adaptation parameters

There was a significant, positive correlation between femur and spine values for BV/TV adaptation ($r = 0.29$, $p = 0.01$) and for Tb.Th adaptation ($r = 0.53$, $p < 0.0001$) (Figure 6).

Figure 6. BV.TV and Tb.Th adaptation to calcium restriction in femur and lumbar spine is significantly and positively correlated. (A) BV/TV adaptation parameters. (B) Tb.Th adaptation parameters



Midshaft femur

Ct.Ar/Tt.Ar was significantly affected by genetic backgrounds and diet main effects (table 1). Ct.Th was significantly affected by both genetics and diet main effects as well as by gene-by-diet interactions (table 1).

Discussion

In this study, we examined how dietary calcium and genetic background each regulates bone microstructure in the femur and lumbar spine among eleven inbred mouse lines. On a diet with

adequate calcium content, we observed variation in trabecular and cortical bone parameters among 11 inbred lines in both femora and lumbar spines (table 1). Previous studies have identified genetic variability in bone density and structures among inbred mouse lines (Beamer et al., 1996; Turner et al., 2000). Our data confirm previous findings showing that on adequate calcium diet, C3H mice have higher femur cortical thickness and total femur density than DBA or B6 mice (Beamer et al., 1996; Turner et al., 2000), and C3H mice lumbar spine trabecular bone have lower BV/TV and higher Tb.Th than B6 mice (Turner et al., 2000). Our data extend these earlier studies in two important ways. First, we have examined 11 inbred lines that represent the full diversity of the mouse genome. Second, we have conducted this comparison on a controlled diet rather than a chow diet that can vary over time.

In addition to the impact of genetics on baseline bone phenotypes, we also found that adaptation of bone volume and trabecular bone parameters to dietary calcium restriction were significantly affected by genetics in both femora and spines. However, there was no significant correlation between the baseline and adaptation parameters in either distal femora or lumbar spine. This suggests that there are distinct genetic controls influencing the baseline bone parameters and the adaptation response to low dietary calcium intake. The variation controlling bone structure adaptation response may be explained by genetic variation controlling the response of calcium absorption, serum $1,25(OH)_2D$, serum PTH, or other factors related to calcium metabolism and bone regulation to dietary Ca restriction. There are other lines of evidence that also support the existence of gene-by-diet interactions affecting bone or calcium metabolism. Previously, Chen et al. reported that when dietary calcium intake is low, serum PTH significantly increased in B6 but not in C3H (Chen & Kalu, 1999). In humans, black adolescent girls, aged 11-15 years, have higher rates of calcium absorption and retention, and

absorb calcium more efficiently at low calcium intakes compared to white girls (Weaver et al., 2008). In addition, there is a tendency for African Americans to have lower serum 25(OH) D levels and similar or higher PTH levels, which reduce the sensitivity to resorptive effects of PTH (Walker et al., 2008 Weaver 2013). Our data provides a foundation for future studies investigating the mechanism and the responsible genes that control bone parameters adaptation.

Gene-by-diet interactions among inbred mouse lines correspond to race-by-diet interactions in human beings. Our data shows that inadequate calcium intake causes low bone density and structure in some of the inbred strains, but does not affect others (see supplementary table 5). This suggests that some inbred strains are more resistant to calcium restriction compared to others. Similarly in humans, African Americans were found to have higher bone density than other racial groups (Walker, Novotny et al. 2008 Weaver 2013). This suggests that inadequate calcium intake negatively influences development of PBM and increases bone fracture risk in some racial groups or individuals, but not others. Thus, those individuals sensitive to low Ca diets may have higher dietary Ca requirements than others. Further understanding in the gene-by-diet interactions regulating bone density and structure will inform personalized dietary Ca requirements for osteoporosis prevention.

In contrast to what we observed for femur, there were no significant gene-by-diet interactions affecting BV/TV and Tb.Sp in lumbar spine. We believe that this is due to the much smaller scale of bone adaptation in spine compared to that observed in the femur. As a result our small sample size was insufficient to see significant differences in spinal adaptation to dietary Ca restriction. Our data also indicate that trabecular bone in lumbar spine is more resistant to dietary calcium restriction than the trabecular bone in distal femur (figure 3B and 4B). Site-specific regulation of bone density and structures have been reported, but with inconsistent results. In

Supplementary Table 1. Raw characteristics of Femur Microarchitecture of 3 Month Old Mice from 11 Inbred Lines Fed either 0.25% or 0.5% Ca Diets

	B6	129S1	A/J	AKR	C3H	CAST	CBA	DBA	PWK	SWR	WSB
Ct.Ar/Tt.Ar (%)											
0.25% Ca	0.455 (0.027)	0.565 (0.026)	0.577 (0.01)	0.621 (0.023)	0.666 (0.021)	0.546 (0.012)	0.659 (0.022)	0.631 (0.007)	0.544 (0.009)	0.534 (0.027)	0.543 (0.014)
0.5% Ca	0.465 (0.014)	0.585 (0.026)	0.59 (0.012)	0.636 (0.01)	0.677 (0.018)	0.559 (0.012)	0.678 (0.029)	0.64 (0.006)	0.547 (0.008)	0.549 (0.024)	0.561 (0.009)
Ct.Th (mm)											
0.25% Ca	0.208 (0.011)	0.251 (0.024)	0.239 (0.005)	0.308 (0.015)	0.32 (0.017)	0.208 (0.007)	0.296 (0.012)	0.251 (0.007)	0.216 (0.005)	0.229 (0.017)	0.221 (0.01)
0.5% Ca	0.205 (0.008)	0.279 (0.022)	0.0239 (0.007)	0.33 (0.011)	0.332 (0.014)	0.224 (0.006)	0.302 (0.013)	0.255 (0.005)	0.213 (0.006)	0.247 (0.015)	0.227 (0.011)
BV/TV (%)											
0.25% Ca	5.94 (1.98)	2.43 (1.53)	2.18 (0.58)	9.54 (2.44)	11.53 (3.69)	0.72 (0.24)	3.89 (1.25)	1.62 (0.45)	4.45 (1.04)	4.01 (0.85)	4.5 (0.96)
0.5% Ca	3.95 (1.79)	8.04 (2.61)	2.26 (0.38)	14.51 (3.93)	11.47 (1.59)	0.77 (0.37)	5.48 (1.8)	2.23 (0.46)	4.72 (2.04)	3.38 (1.19)	4.47 (1.15)
Tb.N (1/mm)											
0.25% Ca	3.29 (0.38)	2.44 (0.43)	2.5 (0.15)	3.99 (.26)	3.46 (0.35)	2.1 (0.49)	2.39 (0.26)	2.36 (0.22)	3.13 (0.61)	3.3 (0.33)	3.28 (0.27)
0.5% Ca	3.12 (0.12)	3.61 (0.25)	2.45 (0.19)	4.11 (0.23)	3.51 (0.36)	2.5 (1.22)	2.49 (0.46)	2.61 (0.36)	2.75 (0.4)	3.03 (0.64)	3.26 (0.5)
Tb.Sp (mm)											
0.25% Ca	0.305 (0.03)	0.412 (0.088)	0.425 (0.035)	0.254 (0.018)	0.284 (0.04)	0.501 (0.098)	0.419 (0.047)	0.419 (0.049)	0.357(0.072)	0.308 (0.03)	0.313 (0.022)
0.5% Ca	0.316 (0.02)	0.282 (0.021)	0.413 (0.03)	0.246 (0.017)	0.281 (0.039)	0.419 (0.209)	0.423 (0.082)	0.394 (0.055)	0.39 (0.063)	0.362 (0.09)	0.319 (0.055)
Tb.Th (mm)											
0.25% Ca	0.052 (0.006	0.041 (0.005)	0.04 (0.002)	0.051 (0.004)	0.061 (0.007)	0.041 (0.003)	0.058 (0.007)	0.037 (0.002)	0.042 (0.003)	0.041 (0.004)	0.041 (0.002)
0.5% Ca	0.044 (0.006)	0.052 (0.007)	0.038 (0.003)	0.063 (0.008)	0.063 (0.003)	0.04 (0.007)	0.065 (0.009)	0.038 (0.002)	0.044 (0.004)	0.042 (0.003)	0.042 (0.003)

Data are expressed as mean (± standard deviation).

Ct.Ar/Tt.Ar = cortical area fraction (diaphysis); Ct.Th = average cortical thickness (diaphysis); BV/TV = bone volume fraction (distal epiphysis); Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness

Supplementary Table 2. Corrected Residual of Femur Microarchitecture of 3 Month Old Mice from 11 Inbred Lines Fed either 0.25% or 0.5% Ca Diets

	B6	129S1	A/J	AKR	C3H	CAST	CBA	DBA	PWK	SWR	WSB
Ct.Ar/Tt.Ar											
0.25% Ca	-0.13 (0.02), 8	-0.03 (0.02), 8	-0.01 (0.01), 7	0.004 (0.02), 8	0.07 (0.02), 7	0.02 (0.01), 8	0.05 (0.03), 7	0.03 (0.01), 7	-0.01 (0.01), 8	-0.06 (0.03), 7	-0.002 (0.02), 7
0.5% Ca	-0.13 (0.01), 7	-0.01 (0.03), 8	0.01 (0.02), 7	0.02 (0.01), 7	0.08 (0.02), 7	0.03 (0.01), 6	0.07 (0.02), 8	0.05 (0.01), 7	-0.01 (0.01), 7	-0.04 (0.02), 7	0.01 (0.01), 7
Ct.Th											
0.25% Ca	-0.05 (0.02), 7	-0.02 (0.02), 8	-0.02 (0.01), 7	0.02 (0.02), 8	0.05 (0.02), 8	0.01 (0.004), 8	0.01 (0.02), 7	-0.02 (0.01), 7	-0.01 (0.01), 7	-0.03 (0.02), 8	0.01 (0.01), 7
0.5% Ca	-0.06 (0.01), 8	0.01 (0.02), 8	-0.02 (0.01), 8	0.04 (0.01), 7	0.06 (0.02), 8	0.02 (0.01), 6	0.03 (0.01), 7	-0.01 (0.01), 8	-0.01 (0.01), 7	0.02 (0.01), 7	0.01 (0.01), 8
BV/TV											
0.25% Ca	0.12 (0.46), 7	-0.96 (0.52), 8	-0.70 (0.32), 8	0.11 (0.41), 8	0.94 (0.34), 8	-0.79 (0.37), 7	0.33 (0.33), 7	-0.74 (0.34), 7	0.45 (0.24), 7	-0.03 (0.24), 7	0.62 (0.20), 6
0.5% Ca	-0.44 (0.49), 7	0.13 (0.40), 7	-0.58 (0.37), 7	0.36 (0.35), 8	0.91 (0.24), 7	-0.78 (0.42)	0.59 (0.26), 7	-0.34 (0.21)	0.37 (0.42), 7	-0.30 (0.45), 7	0.67 (0.24), 8
Tb.N											
0.25% Ca	0.005 (0.44), 8	-0.75 (0.32), 7	-0.51 (0.28), 7	0.47 (0.45), 7	0.45 (0.28), 7	-0.40 (0.57), 7	-0.08 (0.34), 8	-0.34 (0.33), 7	0.17 (0.47), 8	0.31 (0.35), 7	0.44 (0.18), 6
0.5% Ca	-0.23 (0.28), 6	0.10 (0.24), 7	-0.61 (0.24), 8	0.41 (0.26), 7	0.44 (0.42), 7	-0.05 (1.23), 6	-0.01 (0.40), 8	-0.10 (0.37), 8	-0.17 (0.37), 7	-0.04 (0.65), 8	0.54 (0.43), 8
Tb.Sp											
0.25% Ca	-0.04 (0.08), 7	0.24 (0.16), 8	0.22 (0.10), 8	-0.13 (0.14), 7	-0.20 (0.12), 8	0.19 (0.23), 7	0.01 (0.14), 7	0.10 (0.13), 8	0.005 (0.17), 8	-0.12 (0.10), 7	-0.15 (0.05), 6
0.5% Ca	0.04 (0.10), 7	-0.02 (0.07), 7	0.20 (0.09), 8	-0.09 (0.08), 7	-0.18 (0.14), 8	-0.10 (0.63), 7	0.01 (0.17), 8	0.03 (0.14), 8	0.08 (0.16), 7	0.04 (0.25)	-0.18 (0.14), 8
Tb.Th											
0.25% Ca	0.10 (0.11), 8	-0.18 (0.12), 8	-0.18 (0.06), 7	-0.02 (0.07), 7	0.24 (0.11), 8	0.06 (0.06), 7	0.12 (0.13), 8	-0.28 (0.09), 7	0.004 (0.08), 8	-0.15 (0.09), 8	0.01 (0.06), 6
0.5% Ca	-0.08 (0.14), 8	0.06 (0.14), 7	-0.19 (0.09), 8	0.18 (0.14), 8	0.27 (0.06), 8	0.02 (0.16), 7	0.26 (0.16), 8	-0.21 (0.06), 8	0.05 (0.9), 7	-0.13 (0.07)	0.02 (0.06), 8

Body size corrected residual data are expressed as mean (standard deviation), n.

Ct.Ar/Tt.Ar = cortical area fraction (diaphysis); Ct.Th = average cortical thickness (diaphysis); BV/TV = bone volume fraction (distal epiphysis); Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness

In conclusion, our study indicates that there are distinct genetic controls influencing baseline bone parameters and adaptation response to low dietary calcium intake. Trabecular bone in lumbar spine is more resistant to dietary calcium restriction than the trabecular bone in distal femur. However, the same genetic forces are affecting the adaptation response in the distal femur and lumbar spine trabecular bone. Site-specific regulations exist in the bone structure adaptation response to calcium restriction in some of the inbred lines. The responsible genetics factors and mechanisms that regulate bone structure adaptation responses need further investigation. Our study gains insight to race-by-diet interactions in human populations and advances the development of personalize dietary calcium requirement for achieving high PBM and preventing bone fractures.

Acknowledgements:

First and foremost, I would like to express my sincere gratitude to my mentor Dr. James Fleet for his wisdom, patience, advice and guidance through my honors project. I thank Perla Reyes-Fernandez, who has been working side by side with me during most of this project, for giving me technique support, guidance and encouragement. I am also grateful to Rebecca Replogle for statistical analysis and technical support. I also thank Fa Wang and John Replogle for their help during this process.

This work was supported by NIEHS grant ES019103 awarded to Dr. James Fleet and by Purdue University College of Health and Human Sciences Honors Program.

Supplementary Table 3. Raw characteristics of Lumbar Spine Microarchitecture of 3 Month Old Mice from 11 Inbred Lines Fed either 0.25% or 0.5% Ca Diets

	B6	*129S1*	*A/J*	*AKR*	*C3H*	*CAST*	*CBA*	*DBA*	*PWK*	*SWR*	*WSB*
BV/TV (%)											
0.25% Ca	0.287 (0.026), 8	0.287 (0.046), 8	0.195 (0.014), 8	0.304 (0.044), 8	0.157 (0.039), 8	0.094 (0.007), 7	0.137 (0.027), 8	0.215 (0.015), 7	0.311 (0.023), 7	0.319 (0.024), 7	0.248 (0.008), 6
0.5% Ca	0.311 (0.029), 8	0.363 (0.055), 8	0.181 (0.021), 8	0.349 (0.045), 8	0.189 (0.037), 8	0.105 (0.020), 7	0.130 (0.040), 8	0.230 (0.018), 8	0.318 (0.082), 8	0.286 (0.056), 8	0.242 (0.029), 7
Tb.N (1/mm)											
0.25% Ca	5.567 (0.415), 8	5.389 (0.500), 7	4.340 (0.419), 8	4.728 (0.496), 8	3.442 (0.421), 8	3.359 (0.258), 8	2.940 (0.174), 8	4.597 (0.375), 8	5.229 (0.235), 7	5.853 (0.436), 7	4.544 (0.313), 7
0.5% Ca	6.028 (0.197), 8	5.682 (0.636), 8	4.136 (0.335), 7	4.830 (0.306), 8	3.483 (0.202), 7	3.550 (0.268), 7	3.170 (0.343), 8	4.530 (0.294), 8	5.207 (1.012), 8	5.322 (0.501), 8	5.066 (0.372), 7
Tb.Sp (mm)											
0.25% Ca	0.172 (0.015), 8	0.202 (0.046), 8	0.232 (0.025), 8	0.205 (0.022), 8	0.295 (0.040), 8	0.296 (0.022), 8	0.336 (0.015), 7	0.218 (0.017), 8	0.191 (0.013), 7	0.171 (0.014), 7	0.214 (0.018), 7
0.5% Ca	0.159 (0.006), 8	0.174 (0.026), 8	0.243 (0.031), 8	0.196 (0.018), 8	0.280 (0.023), 8	0.288 (0.016), 7	0.332 (0.036), 8	0.222 (0.015), 8	0.180 (0.027), 7	0.186 (0.020), 8	0.189 (0.016), 7
Tb.Th (mm)											
0.25% Ca	0.061 (0.004), 8	0.063 (0.004), 8	0.055 (0.001), 8	0.071 (0.005), 8	0.062 (0.003), 8	0.048 (0.002), 8	0.063 (0.004), 8	0.057 (0.002), 7	0.067 (0.008), 8	0.066 (0.002), 7	0.061 (0.003), 7
0.5% Ca	0.060 (0.004), 8	0.071 (0.005), 8	0.055 (0.002), 8	0.083 (0.006), 8	0.066 (0.005), 8	0.048 (0.005), 7	0.061 (0.005), 7	0.060 (0.002), 8	0.067 (0.008), 8	0.065 (0.004), 8	0.060 (0.002), 7

Data are expressed as mean (± standard deviation), N total.

BV/TV = bone volume fraction; Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness

Supplementary Table 4. Corrected Residual of Lumbar Spine Microarchitecture of 3 Month Old Mice from 11 Inbred Lines Fed either 0.25% or 0.5% Ca Diets

	B6	*129S1*	*A/J*	*AKR*	*C3H*	*CAST*	*CBA*	*DBA*	*PWK*	*SWR*	*WSB*
BV/TV (%)											
0.25% Ca	0.008 (0.03), 8	0.018 (0.05), 8	-0.047 (0.027), 8	-0.01 (0.049), 8	-0.084 (0.041), 8	-0.075 (0.032), 7	-0.027 (0.043), 8	0.017 (0.035), 7	0.081 (0.027), 7	0.082 (0.022), 7	0.023 (0.018), 6
0.5% Ca	0.022 (0.050), 8	0.058 (0.042), 8	-0.066 (0.033), 8	0.009 (0.042), 8	-0.063 (0.043), 8	-0.073 (0.041), 7	-0.037 (0.042), 8	0.032 (0.027), 8	0.078 (0.077), 7	0.038 (0.059), 8	0.043 (0.035), 7
Tb.N (1/mm)											
0.25% Ca	0.599 (0.497), 8	0.402 (0.635), 7	-0.222 (0.471), 8	-0.445 (0.694), 8	-1.097 (0.440), 8	-0.840 (0.38), 8	-0.727 (0.412), 8	0.492 (0.417), 8	0.535 (0.388), 7	1.33 (0.383), 7	-0.015 (0.465), 7
0.5% Ca	0.982 (0.422), 8	0.491 (0.528), 8	-0.536 (0.461), 7	-0.602 (0.267), 8	-1.163 (0.263), 7	-0.711 (0.517), 7	-0.58 (0.383), 8	0.395 (0.401), 8	0.419 (0.97), 7	0.7 (0.52), 8	0.711 (0.417), 7
Tb.Sp (mm)											
0.25% Ca	-0.03 (0.025), 8	-0.011 (0.034), 8	0.003 (0.026), 8	0.013 (0.034), 8	0.065 (0.039), 8	0.051 (0.028), 8	0.045 (0.023), 7	-0.041 (0.022), 8	-0.025 (0.027), 7	-0.060 (0.011), 7	-0.010 (0.028), 7
0.5% Ca	-0.039 (0.023), 8	-0.015 (0.020), 8	0.022 (0.036), 8	0.020 (0.013), 8	0.056 (0.026), 8	0.047 (0.031), 7	0.051 (0.036), 8	-0.033 (0.025), 8	-0.036 (0.028), 6	-0.039 (0.021), 8	-0.048 (0.022), 7
Tb.Th (mm)											
0.25% Ca	-0.005 (0.005), 8	-0.003 (0.003), 8	-0.009 (0.001), 8	0 (0.006), 8	-0.002 (0.003), 8	-0.004 (0.004), 8	0.003 (0.004), 8	-0.004 (0.004), 7	0.008 (0.008), 8	0.002 (0.003), 8	0.004 (0.002), 7
0.5% Ca	-0.007 (0.005), 8	0.003 (0.004), 8	-0.008 (0.004), 8	0.01 (0.006), 8	0.002 (0.006), 8	-0.004 (0.004), 7	0.002 (0.004), 7	0 (0.003), 8	0.006 (0.006), 7	0.002 (0.005), 8	0.004 (0.003), 7

Data are expressed as mean (± standard deviation), N total.

BV/TV = bone volume fraction; Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness

Supplementary Table 5. Statistical test for adaptation parameters for BV/TV and Tb.Th in distal femur and Lumbar spine

		B6	*129S1*	*A/J*	*AKR*	*C3H*	*CAST*	*CBA*	*DBA*	*PWK*	*SWR*	*WSB*
Distal Femur	BV/TV Adaptation (%)											
	mean (standard deviation), N total	50.43 (50.30), 7	-69.88 (18.87), 8	-3.63 (25.66), 8	-34.25 (16.69), 8	0.63 (32.26), 8	-6.57 (31.07), 7	-17.14 (27.25), 7	-33.33 (12.53), 6	-5.43 (21.83), 7	18.43 (25.05), 7	0.83 (21.46), 6
	p	0.0380	<0.0001	0.7000	0.0007	0.9600	0.6000	0.1500	0.0013	0.5400	0.1000	0.9300
	Tb.Th Adaptation (%)											
	mean (standard deviation), N total	17.85 (13.92), 8	-20.74 (9.97), 8	4.23 (5.21), 7	-18.95 (5.99), 7	-3.16 (10.93), 8	3.60 (6.41), 7	-11 (10.07), 8	-4.01 (6.35), 7	-5.18 (6.75), 8	-1.80 (8.96), 8	-2.56 (5.88), 6
	p	0.01	0.0006	0.08	0.0002	0.44	0.19	0.02	0.15	0.07	0.59	0.33
Lumbar Spine	BV/TV Adaptation (%)											
	mean (standard deviation), N total	-7.66 (8.33), 8	-20.87 (12.62), 8	7.79 (7.87), 8	-12.71 (12.71), 8	-16.97 (20.83), 8	-9.99 (6.55), 7	5.31 (21.02), 8	-6.37 (6.35), 7	-2.25 (7.26), 7	11.52 (8.57), 7	2.53 (3.33). 6
	p	0.035	0.0023	0.027	0.025	0.055	0.0068	0.5	0.038	0.44	0.012	0.12
	Tb.Th Adaptation (%)											
	mean (standard deviation), N total	1.32 (7.44), 8	'-12.22 (5.93), 8	-0.45 (2.37), 8	-13.98 (6.64), 8	-6.73 (4.33), 8	-0.87 (5.01), 8	3.07 (5.99), 8	-5.72 (4.03), 7	0.98 (12.62), 8	0.96 (2.86), 7	1.37 (4.2), 7
	p	0.63	0.0006	0.6	0.0006	0.0032	0.64	0.19	0.0095	0.83	0.41	0.42

Reference

Barrett-Connor, E., Siris, E. S., Wehren, L. E., Miller, P. D., Abbott, T. A., Berger, M. L., . . . Sherwood, L. M. (2005). Osteoporosis and fracture risk in women of different ethnic groups. *J Bone Miner Res, 20*(2), 185-194.

Beamer, W. G., Donahue, L. R., Rosen, C. J., & Baylink, D. J. (1996). Genetic variability in adult bone density among inbred strains of mice. *Bone, 18*, 397-403.

Braun, M., Palacios, C., Wigertz, K., Jackman, L. A., Bryant, R. J., McCabe, L. D., . . . Weaver, C. M. (2007). Racial differences in skeletal calcium retention in adolescent girls with varied controlled calcium intakes. *Am J Clin Nutr, 85*(6), 1657-1663.

Chen, C., & Kalu, D. N. (1999). Strain differences in bone density and calcium metabolism between C3H/HeJ and C57BL/6J mice. *Bone, 25*(4), 413-420.

Fleet, J. C. (2006). Molecular Regulation of Calcium Metabolism. In C.M.Weaver & R.P.Heaney (Eds.), *Calcium in Human Health* (pp. 163-190). Totowa, NJ: Humana Press. (Reprinted from: NOT IN FILE).

Fleet, J. C., Hong, J., & Zhang, Z. (2004). Reshaping the way we view vitamin D signaling and the role of vitamin D in health. *Nutrition Research Reviews, 17* 241-248.

Hebert, S. C., Brown, E. M., & Harris, H. W. (1997). Role of the Ca(2+)-sensing receptor in divalent mineral ion homeostasis. *J Exp.Biol, 200 (Pt 2)*, 295-302.

Ilich, J. Z., & Kerstetter, J. E. (2000). Nutrition in bone health revisited: a story beyond calcium. [Review]. *J Am Coll Nutr, 19*(6), 715-737.

Lang, D. H., Sharkey, N. A., Lionikas, A., Mack, H. A., Larsson, L., Vogler, G. P., . . . McClearn, G. E. (2005). Adjusting data to body size: a comparison of methods as applied to quantitative trait loci analysis of musculoskeletal phenotypes. *J.Bone.Miner.Res., 20*(5), 748-757.

Liu, X. S., Ardeshirpour, L., VanHouten, J. N., Shane, E., & Wysolmerski, J. J. (2012). Site-specific changes in bone microarchitecture, mineralization, and stiffness during lactation and after weaning in mice. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. *Journal of Bone and Mineral Research, 27*(4), 865-875. doi: 10.1002/jbmr.1503

Roberts, A., Pardo-Manuel, d. V., Wang, W., McMillan, L., & Threadgill, D. W. (2007). The polymorphism architecture of mouse genetic resources elucidated using genome-wide resequencing data: implications for QTL discovery and systems genetics. *Mamm.Genome., 18*(6-7), 473-481.

Turner, C. H., Hsieh, Y. F., Muller, R., Bouxsein, M. L., Baylink, D. J., Rosen, C. J., . . . Beamer, W. G. (2000). Genetic regulation of cortical and trabecular bone strength and microstructure in inbred strains of mice. [Research Support, U.S. Gov't, Non-P.H.S.

Research Support, U.S. Gov't, P.H.S.]. *Journal of Bone and Mineral Research, 15*(6), 1126-1131. doi: 10.1359/jbmr.2000.15.6.1126

Walker, M. D., Novotny, R., Bilezikian, J. P., & Weaver, C. M. (2008). Race and diet interactions in the acquisition, maintenance, and loss of bone. [138/6S-II/1256S pii]. *J Nutr, 138*(6), 1256S-1260S.

Weaver, C. M., McCabe, L. D., McCabe, G. P., Braun, M., Martin, B. R., Dimeglio, L. A., & Peacock, M. (2008). Vitamin D status and calcium metabolism in adolescent black and white girls on a range of controlled calcium intakes. *J Clin Endocrinol Metab, 93*(10), 3907-3914.

Wigertz, K., Palacios, C., Jackman, L. A., Martin, B. R., McCabe, L. D., McCabe, G. P., . . . Weaver, C. M. (2005). Racial differences in calcium retention in response to dietary salt in adolescent girls. [Clinical Trial Randomized Controlled Trial Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *American Journal of Clinical Nutrition, 81*(4), 845-850.

Wu, D. D., Boyd, R. D., Fix, T. J., & Burr, D. B. (1990). Regional patterns of bone loss and altered bone remodeling in response to calcium deprivation in laboratory rabbits. [Research Support, U.S. Gov't, P.H.S.]. *Calcified Tissue International, 47*(1), 18-23.