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Preservation of Bone Structure and Function by *Lithothamnion sp.* Derived Minerals

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Abstract Progressive bone mineral loss and increasing bone fragility are hallmarks of osteoporosis. A combination of minerals isolated from the red marine algae, *Lithothamnion sp*. was examined for ability to inhibit bone mineral loss in female mice maintained on either a standard rodent chow (control) diet or a high-fat western diet (HFWD) for 5, 12, and 18 months. At each time point, femora were subjected to μ-CT analysis and biomechanical testing. A subset of caudal vertebrae was also analyzed. Following this, individual elements were assessed in bones. Serum levels of the 5b isoform of tartrate-resistant acid phosphatase (TRAP) and procollagen type I propeptide (P1NP) were also measured. Trabecular bone loss occurred in both diets (evident as early as 5 months). Cortical bone increased through month 5 and then declined. Cortical bone loss was primarily in mice on the HFWD. Inclusion of the minerals in the diet reduced bone mineral loss in both diets and improved bone strength. Bone mineral density was also enhanced by these minerals. Of several cationic minerals known to be important to bone health, only strontium was significantly increased in bone tissue from animals fed the mineral diets, but the increase was large (5–10 fold). Serum levels of TRAP were consistently higher in mice receiving the minerals, but levels of P1NP were not. These data suggest that trace minerals derived from marine red algae may be used to prevent progressive bone mineral loss in conjunction with calcium. Mineral supplementation could find use as part of an osteoporosis-prevention strategy.

Keywords Bone · Bone mineral density · Bone mineral content · Calcium · Minerals · Osteoporosis · Red marine algae · Strontium · Trace elements

Abbreviations

AIN76A	American Institute of Nutrition 76A
ANOVA	Analysis of variance
BMD	Bone mineral density
GRAS	Generally regarded as safe
HFWD	High-fat western-style diet
μ-CT	Microcomputed tomography
P1NP	N-terminal propeptide of type I procollagen
TRAP	Tartrate-resistant acid phosphatase (5b)
2D	Two-dimensional
3D	Three-dimensional

Introduction

Osteoporosis is a condition characterized by low bone mass, low bone mineral content, and microarchitectural deterioration leading to enhanced bone fragility and consequent increase risk of bone fracture [1]. Although in the white population, men account for up to 30 % of the osteoporotic hip fractures [2], osteoporosis is widely regarded as a condition primarily affecting postmenopausal women [2, 3]. Genetic factors underlie susceptibility, but environmental variables (including diet) [4] also suggested to play a role. In particular,

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the "typical" Western-style diet, with its high content of saturated fat and sugar and low levels of calcium and vitamin D, is thought to be a contributor to bone fragility in susceptible individuals. This is well established based on epidemiological and interventional studies in humans [5, 6]. In addition, studies in experimental animals have directly demonstrated the deleterious effects of the Western-style diet on bone structure/function [7, 8] and age-related effects on the skeleton [9].

Since bone consists of a mineralized connective tissue, an adequate supply of inorganic minerals in the diet is critical throughout life. Calcium is the major cationic mineral incorporated into bone and a deficiency of calcium in the diet contributes to poor bone health [10–12]. In addition to calcium, other cationic minerals including boron, copper, iron, magnesium, manganese, potassium, selenium, silicon, strontium, and zinc have all been suggested as being important for the formation and maintenance of strong, healthy bones [13–17]. How these various trace elements support bone formation and maintenance is not fully understood. Incorporation into the mineralized bone is, no doubt, important. In addition, however, certain trace elements regulate osteoblast and osteoclast metabolism [17–20] and are critical to the formation of the organic bone matrix as well as to its mineralization. While an adequate supply of the essential trace elements throughout life is important to formation and maintenance of healthy bones, whether or not dietary supplementation with individual trace elements or with groups of trace elements can be an effective strategy for improving bone formation and preventing bone mineral loss with age remains to be seen. To date, supplementation studies with only a relatively few trace elements have been conducted, and the results, while suggestive, are inconclusive [13–15].

Based on this, we hypothesized that including a wide variety of trace minerals along with calcium in the diet throughout life would help to prevent bone loss that commonly occurs with age. To test this hypothesis, we utilized a mineral preparation derived from the red marine algae, *Lithothamnion sp*. Previous studies have shown that this mineral preparation reduced colon polyp formation in mice [21, 22] and also inhibited liver tumor formation [23] when included in the diet over the lifespan of the animals. As an unanticipated finding in the pilot study [24], our data suggested that mineral supplementation might also preserve bone structure, at least in female mice. However, in the pilot study, only a small number of animals were included, only a single time point (15 months) was examined, and only effects on long bones were assessed. In our pilot study, mineral supplementation was only included in a high-fat diet that is mineral deficient and known to promote bone loss and bone fragility [6, 7]. Here, we have utilized the same mineral preparation to directly assess effects on bone structure and function in cohorts of female C57/BL6 mice at three different time points (5, 12, and 18 months) in their lifespan. In this mouse strain, which is prone to early bone loss [9], mineral supplementation significantly increased bone mineralization at the early time point (5 months) and preserved bone structure throughout life. Most importantly, beneficial effects were seen in animals maintained for the entire study on a rodent chow designed for optimal health as well as in mice fed the high-fat diet.

Materials and Methods

Minerals The minerals used in this study were obtained from the skeletal remains of *Lithothamnion sp*., the red marine algae [21, 22] and consists entirely of the inorganic minerals (Marigot Ltd, Cork, Ireland). The product (GRAS 000028) contains approximately 12 % calcium and 1 % magnesium, but also has detectable levels of 72 other trace minerals. The mineral diets in this study made use of a single batch of the mineral product to avoid batch-to-batch variation.

Diets A standard rodent chow (AIN76A—control) and a variant of AIN76A, i.e., the high-fat Western diet (HFWD), were used in this study. The HFWD was prepared according to the formulation of Newmark et al. [25] and is designed to mimic food consumption patterns of individuals in Western society [26]. Both diets were used as is, or supplemented with *Lithothamnion sp*. derived minerals. The minerals were incorporated into the diet fed to the mice. The final concentrations of calcium in control and HFWD diets were 1.34 and 0.08 mg/kcal, respectively. With mineral supplementation, the control and HFWD diets contained 3.24 and 1.64 mg/kcal of calcium respectively. The slight increase in calcium in the supplemented HFWD as compared to the unsupplemented control diet reflects the fact that mice consume food based on kilocalories. The diets are designed, therefore, to provide a comparable level of consumed calcium in these two groups. Diets were provided ad libitum. Diets were formulated and provided by Research Diets Incorporated (New Brunswick, NJ, USA). The complete composition of each diet as fed is presented in Supplement Table 1. It should be noted that the control diet is formulated to contain a number of cationic minerals in addition to calcium that are known to be beneficial. All of these are included in the HFWD, as well. Supplement Table 2 provides comparative levels of important minerals in the four diets and shows the changes due to diet supplementation with the minerals.

Mice and Experimental Groups A total of 140 female C57BL/6 mice (Charles River, Portage, MI, USA) were put in four groups and started on either the control diet or the HFWD, both with and without the minerals beginning at 3 weeks of age. Diets were started at this age in order to observe early growth-related effects of the minerals on bone structure/function and subsequent effects on bone mineral

content over the entire 18-month period of study. Separate cohorts of mice were euthanized after 5, 12, or 18 months on their respective diet. For the 5- and 12-month periods, there were 10 female mice per diet group. For the 18-month period, there were 15 mice in each group. In addition to these cohorts of mice, five female mice were euthanized at the start of the study for baseline values. All of the procedures were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan.

Preparation of Skeletal Tissue and Microcomputed Tomography The right femora were carefully dissected free of associated connective tissue, immediately placed in sealed containers with lactated Ringer's solution, and frozen at −20 °C until use. Three-dimensional images of the femora in Ringer's solution were obtained using a microcomputed tomography (μ-CT) system (eXplore Locus SP, GE Healthcare Pre-Clinical Imaging, London, ON, Canada) as previously described and validated [24, 27]. The whole bone was scanned, and both trabecular and cortical regions of interest (ROIs) were reconstructed from the scans as described previously [28]. A more complete description of the μ-CT procedure can be found in the supplement under "Methodology."

A subset of caudal vertebrae (C8) were identified and carefully dissected. Upon dissection, the vertebrae were immediately placed in lactated Ringer's solution and frozen at −20 °C until use. The whole vertebrae were scanned, and ROIs through the cranial and middle isolateral surfaces were selected for analysis. μ-CT analysis was done exactly as with long bones.

Biomechanical Testing Long-bone mechanical properties were determined by loading the right femora to failure in four-point bending, using a customized testing fixture attached to a servohydraulic materials testing machine (858 Mini Bionix II; MTS Systems, Eden Prairie, MN, USA) [24, 29]. Complete description of biomechanical testing is included in the supplement under "Methodology."

Whole-bone mechanical properties of intact caudal vertebrae were also measured by compressing the vertebral body with a 3-mm diameter platen attached to a servohydraulic materials testing machine (858 Mini Bionix II; MTS Systems, Eden Prairie, MN, USA), as described previously [30].

Histological Evaluation The left femora were dissected, cleaned, and fixed in 10 % neutral buffered formalin for 24–48 h and then demineralized in a formic acid based decalcifier (Immunocal; Decal, Tallman, NY, USA) for 48 h. Tissues were processed for histology, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. Histological parameters were evaluated by a board-certified veterinary pathologist blinded to the experimental groups. Evaluation was performed using an Olympus BX45 light microscope at total magnifications ranging from ×40 to ×600.

Tartrate-Resistant Acid Phosphatase and N-Terminal Peptide of Type I Procollagen Blood was obtained at the time of necropsy from each animal. Tartrate-resistant acid phosphatase (TRAP) and N-terminal peptide of type I Procollagen (P1NP) were assessed in serum samples using commercially available enzyme-linked immunosorbant assays (ELISAs) (Immunodiagnostic Systems, Inc., Fountain Hills, AZ, USA). TRAP is produced by osteoclasts and macrophages and can be detected in serum. The ELISA used here measures TRAP 5b, the form specific to osteoclasts [31]. TRAP 5b is thought to be a measure of osteoclast number rather than activity [32]. P1NP is a measure of osteoblast function. Type I collagen is the major collagenous protein in bone [33].

Levels of Individual Trace Elements in Long Bones Following μ-CT and biomechanical testing, the long bones (one femur and tibia from all animals in each group) were "pooled" by group and time point and analyzed for levels of trace metals. Bones were "pooled" in order to have a sufficient amount of material to obtain a detectable signal. Bones were digested in a concentrated nitric acid solution (10 ml) for approximately 30 min, after which they were cooled to room temperature. Concentrated hydrochloric acid (10 ml) was added, and the sample was digested for an additional 15 min. After cooling and dilution with distilled water, levels of individual trace elements were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) except fluoride, which was determined by Association of Analytical Communities 984.37 assay. Bone preparation and assays were done on a fee-for-service by Advanced Laboratories, Inc. (Salt lake City, UT, USA).

Statistical Evaluation Data from μ-CT analysis, biomechanical testing and biochemical evaluations were obtained for individual mice. The data were presented as group averages and standard deviations at each time point. Differences among groups were compared for statistical significance using ANOVA followed by paired group comparisons (two tailed). Differences were considered significant at the $p < 0.05$ value. While assessing the trace elements, individual "pooled" samples could not be analyzed statistically, the data (strontium only) were subjected to two-way factorial ANOVA to determine if the effect of diet or time was significant. Data from the three time points were then grouped together and analyzed. Differences were considered significant at the $p < 0.05$ value.

Results

Animal Weight and Survival Data Animals were regularly monitored and weighed biweekly throughout the study period. At the initiation of the study, 3-week-old animals had an

average weight of 14.5±1 g. Over the 18-month period of dietary intervention, animals gradually gained weight on both diets. Animals on the HFWD gained more weight than animals on the control diets, but the mineral supplement had no effect on weight gain in either diet. The progression in weights is provided in Supplement Table 3 at all time points (5, 12, and 18 months).

Over the course of the 18-month maintenance period, there were 14 premature deaths. This included two animals between 6 and 12 months and 12 mice between months 13 and 18. The majority of the latter deaths occurred in months 15–17. Necropsies were done on all the animals that died prematurely and bones from these mice were included in their corresponding diet/time groups. Of these animals, two were on the control diet, two were on the mineral-supplemented control diet, six were on the HFWD, and four were on the mineral-supplemented HFWD.

Bone Mineral Density Initially, we assessed bone mineral density (BMD) by μ-CT in trabecular and cortical ROIs of the femora. Femoral results are shown in Table 1, where it can be seen that in both the control diet and the HFWD, trabecular BMD fell significantly from baseline level. The drop could be seen as early as the 5-month time point and continued throughout the study. By the 18-month time point, trabecular BMD in mice on the control diet was 69 % of the baseline value and in the HFWD was only 45 % of the baseline level. In mice that received the minerals in either diet, BMD values at the 5-month time point were substantially increased as compared to baseline level (35 and 16 % increase). From thereon, BMD values fell. However, at both the 12- and 18-month time points, trabecular BMD values were higher in mice with the minerals than in those without (trabecular BMD was enhanced by minerals up to 82 % at 18 months in HFWD). This was observed in both diets. Thus, the decrease in trabecular BMD that occurred over time was clearly mitigated in both diets by supplementation with the *Lithothamnion sp*. derived minerals (Table 1).

In contrast to trabecular findings, cortical BMD increased significantly in both diets between baseline and 5 months of age (Table 1). Between the 5- and 18-month time points, BMD values declined slightly. Similar trends were observed in both diets, but the decline with time was more severe in the HFWD. Mineral-supplementation had little effect on BMD values in control diet mice, but preserved BMD in the HFWD (up to 24 % increase at 18 months).

Although the primary focus of the study was on long bones, effects of mineral supplementation on vertebrae were also assessed. For this, a subset of caudal vertebrae from mice on both the HFWD and the control diet was analyzed at the 12-month time point. An additional subset of caudal vertebrae from mice on the HFWD was also analyzed at the 18-month time point. It can be seen from Table 1 that (similar to what was observed in long bones), mineral supplementation dramatically increased trabecular bone BMD in both diets. Increases ranged from 19 % at 12 months in the control diet to 43 and 46 % increases at 12 and 18 months in the HFWD. Also similar to what was seen in long bones, there was little improvement in cortical bone BMD in the control diet but increases of 29 and 22 % were seen in the HFWD at the 12 and 18-month time points, respectively, with minerals inclusion in the diet as compared to the HFWD alone.

Femoral Bone Structure Given the significant effect of mineral supplementation on femoral BMD, a wide range of μ-CT parameters were assessed. Trabecular bone volume, number and thickness are shown for each of the three time points in Fig. 1a. To summarize, a rapid loss of trabecular bone was observed such that a decline from the beginning of the study (3 weeks of age) was apparent in these animals as early as the 5-month time point. At the fifth month, trabecular number was reduced by 67 and 64 % in the control and HFWD groups relative to what was seen at the initiation of the study. Trabecular bone loss was reduced in the presence of the minerals. With the addition of minerals, corresponding declines were only 17 and 34 %. Trabecular bone loss continued over the 18-month observation period, but at all time points, there was less bone loss in the mice receiving the minerals than in mice receiving unsupplemented diets (Fig. 1a). The results were similar in both diets. While the largest changes were in trabecular number as opposed to thickness, the minerals beneficially influenced trabecular thickness as well. A loss of trabecular number concomitant with reduced trabecular thickness was associated, as expected, with an increase in trabecular space (Supplement Tables 4–6) and decreased BV/TV (Fig. 1a). For example, bone volume was reduced to 85 % in the HFWD group at 18 months from baseline but the mineral-containing HFWD enhanced bone volume up to 50 % at 18 months. A representative 3D μ-CT image of the trabecular region (distal metaphysis) from the femur of a female mouse in each diet group is shown (Fig. 1c). Histological images from distal femoral condyles from mice on the HFWD with or without the minerals (hematoxylin and eosin-stained sections of decalcified bone) at the 18-month time point are shown (Fig. 1d). The histological images are consistent with what was observed by μ-CT, i.e., increased bone preservation within the trabecular region in the presence of the minerals. We did not attempt to quantify trabecular bone by morphometry because of limitations inherent in two-dimensional representation of trabeculae in a histological section. Additional histological findings included increased adipose tissue and decreased hematopoietic tissue in some sections of mice fed a HFWD in comparison to mice on the supplemented HFWD.

Cortical bone properties, including marrow area and cortical area, are shown in Fig. 1b. Although the changes in cortical bone properties were not as dramatic as those

Table 1 Bone mineral density in femora and vertebrae

Femoral BMD (mg/cc)				
Diet group	Baseline	5 months	12 months	18 months
Trabecular				
	174±18			
Control		156±12*	131±21*	120±38*
Control+minerals		234±15[c]	158±35	142±40
HFWD		164±54	146±11*	79±29*
HFWD+minerals		201±11[a,b]	171±18[a,b]	143±30[a]
Cortical				
	151±6			
Control		347±16	309±36	303±27
Control+minerals		378±25[c]	302±31	299±28
HFWD		314±36	287±30	260±34
HFWD+minerals		339±16	322±21	322±47[a]

Vertebral BMD (mg/cc)		
	12 months	18 months
Trabecular		
Control	619±63	Not done
Control+minerals	737±83[c]	Not done
HFWD	482±34	452±46
HFWD+minerals	687±107[a]	659±108[a]
Cortical		
Control	961±30	Not done
Control+minerals	942±14	Not done
HFWD	731±25	763±146
HFWD+minerals	941±33[a]	928±43

Femoral data are based on 5 mice at baseline (3 weeks of age), 10 mice at 5 and 12 months, and 15 mice at 18 months in each diet group. Vertebral data are based on six mice at 12 months in each diet group and six mice in each of the two high-fat diets at 18 months. Values are means and standard deviations. Statistical significance was determined by ANOVA followed by paired group comparisons. "*" are placed to show the statistically significant drop in the BMD relative to baseline. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant increase relative to the HFWD group, and "b" shows statistically significant increase relative to control; "c" is placed on the control+minerals group and shows significant increase relative to the control (p <0.05). Data from baseline and 5 months in all groups and in control diets at 18 months for the vertebrae are not available

observed in trabecular bone, mice on the HFWD had a larger marrow area value and smaller cortical area value than did mice on the control diet. The deleterious consequences of the HFWD were prevented by the minerals (Fig. 1b). A representative μ-CT image of the cortical region (mid-diaphysis) from the femur of a female mouse in each diet group is shown (Supplement Figure 1). Tables 4, 5, and 6 in the supplementary material present all of the femoral μ-CT parameters assessed at the three time points for both trabecular and cortical ROIs.

Vertebral Bone Structure Figure 2 demonstrates effects of *Lithothamnion sp*. derived minerals on vertebral bone structure (from a subset of vertebrae). Similarly, as seen in femoral bone, where the major effect of the minerals was on the both trabecular region and the cortical region, in the vertebral bone, both trabecular and cortical properties were improved in the mineral-treated mice. In contrast to femoral bone, where the improvement in trabecular structure was a reflection of changes in both number and thickness, in the vertebral bone, the minerals' effect was primarily on trabecular thickness and volume. Consistent with data from long bones, however, was the fact that improvement with minerals occurred in mice on either diet. Also consistent, results were more impressive in mice on the HFWD than in mice on the control diet. Representative μ-CT images of vertebrae from both the control and HFWD groups at the 12 month time point are shown in Fig. 2c. All of the vertebral μ-CT parameters assessed at the two time points are presented in Supplemental Tables 7 and 8.

Biomechanical Properties of Femora and Vertebrae Biomechanical properties of long bones were determined by testing femora to failure in four-point bending. These measurements, which primarily reflect cortical bone mechanical properties

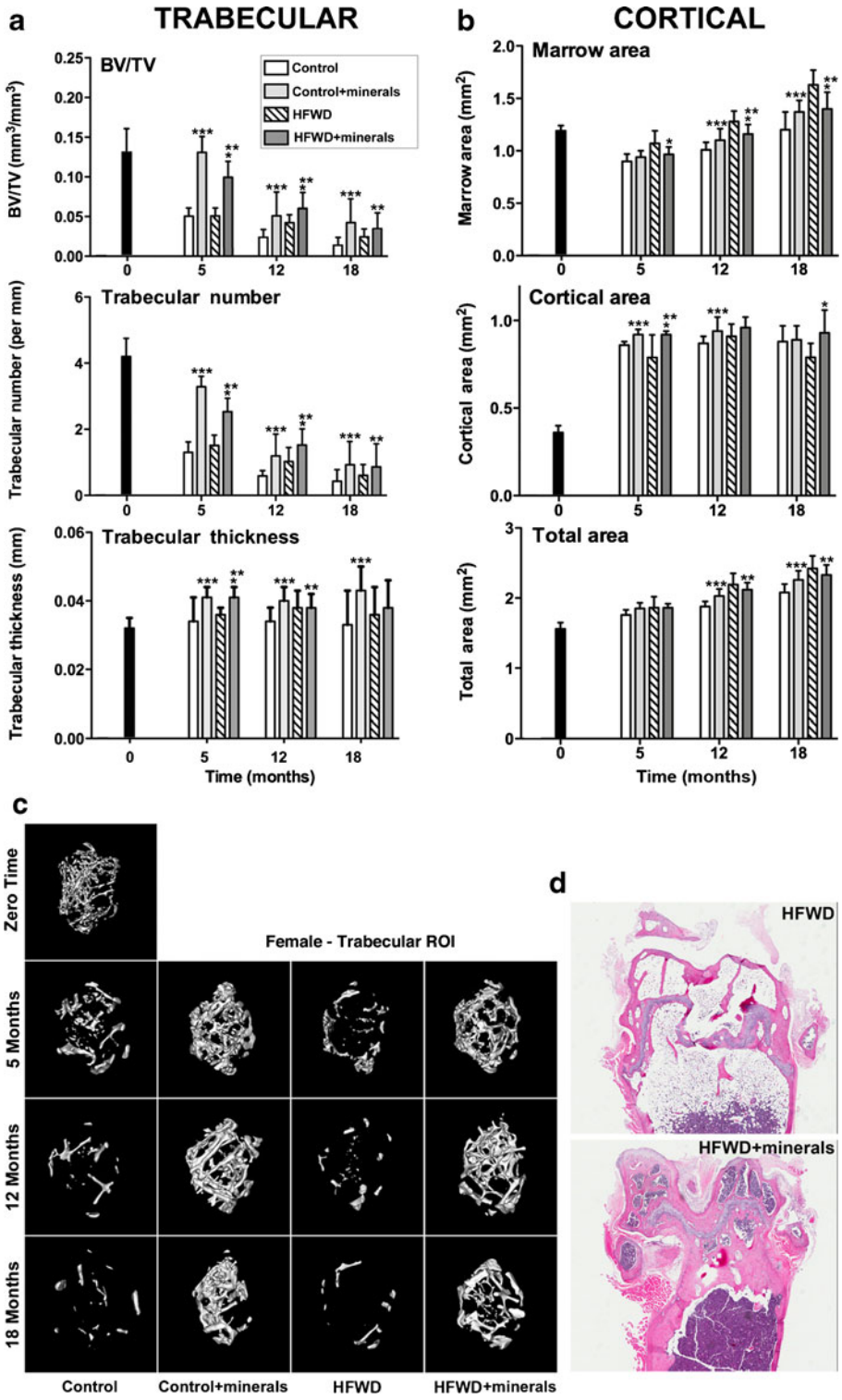

Fig. 1 Structural features of femoral bone. **a** Trabecular/ **b** cortical μ-CT parameters: Data are based on 5 mice at baseline (3 weeks of age), 10 mice at 5 and 12 months, and 15 mice at 18 months in each diet group. Values are means and standard deviations. Statistical significance of each parameter was assessed by ANOVA followed by paired group comparisons. Statistical significance at the $p < 0.05$ level is indicated by *asterisks*. *Single asterisk* by the HFWD + minerals indicates statistically significant improvement relative to HFWD alone; *double asterisk* by the HFWD+minerals indicates statistically significant improvement relative to control; *triple asterisk* by the control+ minerals indicates statistically significant improvement relative to control. **c** μ-CT images: a representative 3D μ-CT image of the trabecular region from the femur of a female mouse in each diet group is shown. **d** Histological images: hematoxylin and eosin-stained sections of decalcified bone (distal femoral condyles) from a mouse (at 18 months) in two high-fat diet groups are shown (bars=200 μm)

[34], are presented in Fig. 3a. Mice on the HFWD demonstrated an increase in ductility compared to mice on the control diet which can be seen by the decrease in stiffness and the increase in displacement ratio. The displacement ratio was especially sensitive to diet. Consistent with the decrease in bone stiffness, mice on the HFWD demonstrated an increase in maximum load value as compared to mice on the control diet [8]. In mice treated with the minerals, biomechanical properties were improved. Specifically, bone stiffness was enhanced (36 % at 5 months, 3 % at 12 months, and 32 % at

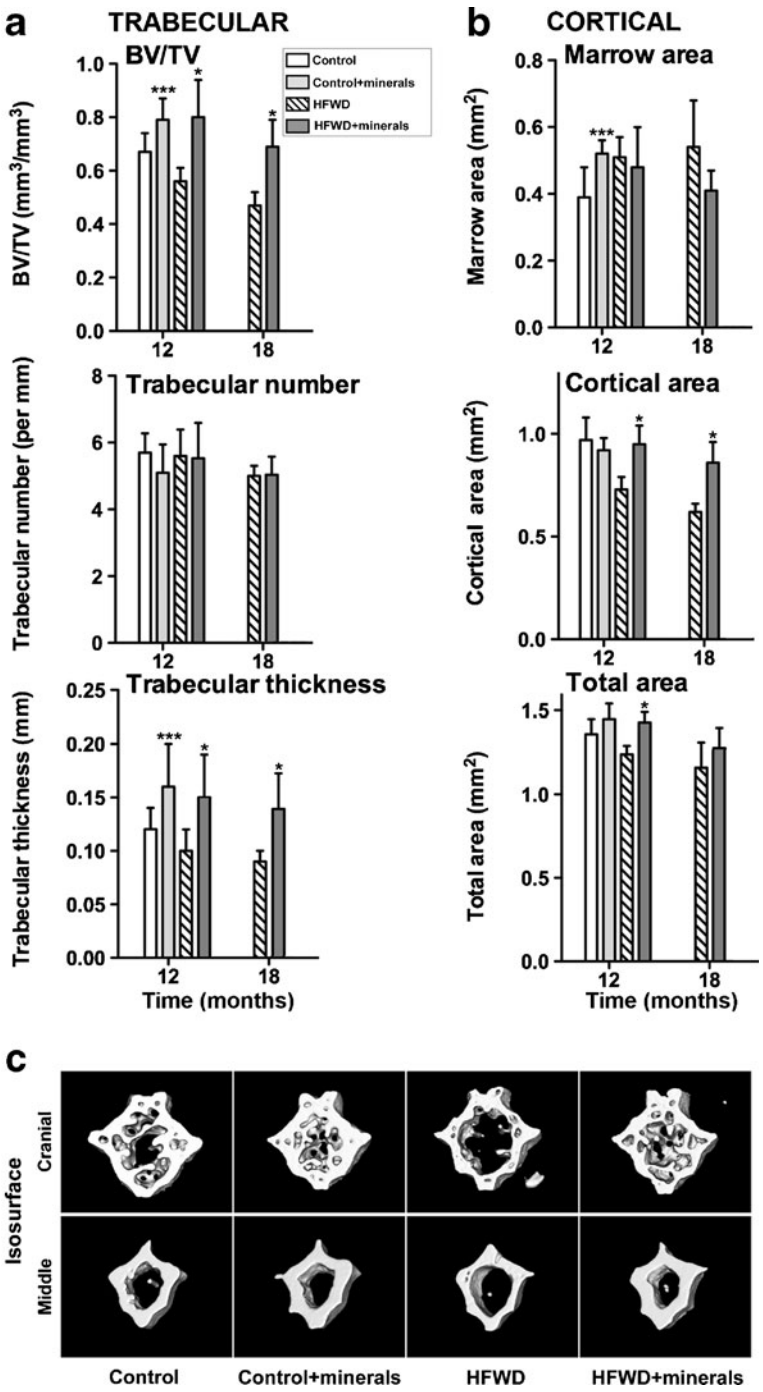

Fig. 2 Structural features of vertebral bone. **a** Trabecular/ **b** cortical μ-CT parameters: Data are based on 6 mice at 12 months in each diet group and 6 mice in each of the two high-fat diets at 18 months. Values are means and standard deviations. Statistical significance of each parameter was assessed by ANOVA followed by paired group comparisons. Statistical significance at the $p < 0.05$ level is indicated by *asterisks*. *Single asterisk* by the HFWD+minerals indicates statistically significant improvement relative to HFWD alone; *double asterisk* by the HFWD+minerals indicates statistically significant improvement relative to control; *triple asterisk* by the control+ minerals indicates statistically significant improvement relative to control. **c** μ-CT images: representative 3D μ-CT images of the cranial and middle region from a C8 vertebra of a mouse in each diet group at 12 month time point

18 months) in mice on the HFWD with minerals as compared to the HFWD alone mice.

Biomechanical properties were also assessed in a subset of vertebrae. Vertebrae were tested for resistance to compression loads. Results in the compression-damage assay are a reflection of both stiffness and bone strength and depend on both trabecular and cortical properties [30]. As seen in Fig. 3b, both stiffness and strength were substantially improved in mice receiving *Lithothamnion sp*. derived minerals. Improvement was observed in both diets. Stiffness was increased by 48 and 49 % at 12 months in the two supplemented diets as compared to the respective unsupplemented diet groups and by 30 % at 18 months in the mineral-supplemented HFWD as compared to HFWD alone. Similarly, maximal load was increased by 12 and 32 % at the 12-month time point in mineral-supplemented diets as compared to unsupplemented diet groups and by 51 % at the 18-month time point in the supplemented HFWD compared to the HFWD alone.

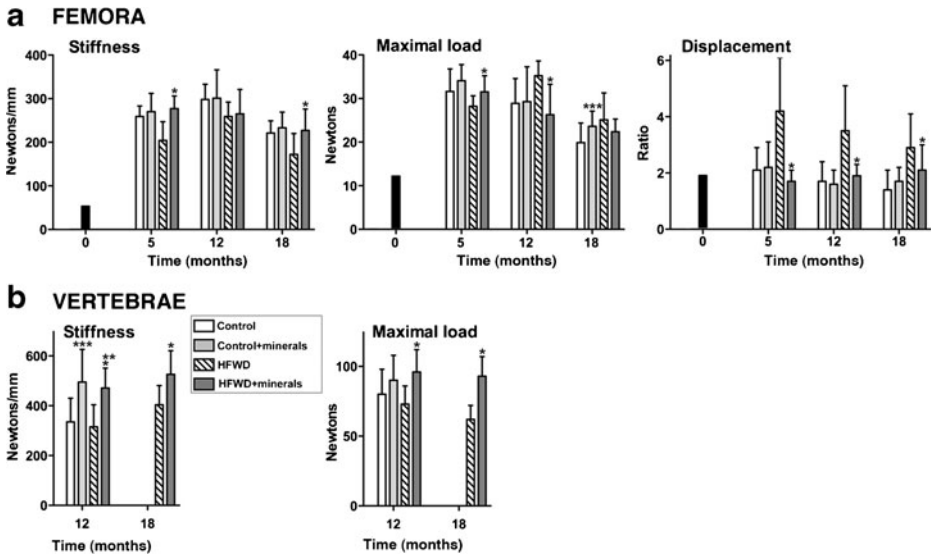

Fig. 3 Biomechanical properties. **a** Femora: data are based on 5 mice at baseline (3 weeks of age), 10 mice at 5 and 12 months, and 15 mice at 18 months in each diet group. **b** Vertebrae: data are based on six mice at 12 months in each diet group and six mice in each of the two high-fat diets at 18 months. Values are means and standard deviations. Statistical significance of each parameter was assessed by ANOVA followed by paired group comparisons. Statistical significance at the $p < 0.05$ level is indicated by *asterisks*. *Single asterisk* by the HFWD+minerals indicates statistically significant improvement relative to HFWD alone; *double asterisk* by the HFWD+minerals indicates statistically significant improvement relative to control; *triple asterisk* by the control+minerals indicates statistically significant improvement relative to control

Levels of Individual Trace Elements in Bone Following μ-CT analysis and biomechanical testing, bones were assessed for levels of 41 individual elements. To summarize, 28 of the 41 different trace elements assessed (including three that are known to be important for bone, i.e., boron, copper, and selenium) were below detectable limits (0.5 μg/g) (Supplement Table 9). Among other minerals that play a role in bone health (including calcium, magnesium, potassium, iron, zinc, manganese, and silicon), bone levels of each were largely unaffected by diet or (in the case of iron and manganese) actually higher in bones of mice on the high-fat diet (Supplement Table 9). In contrast, and perhaps most interesting, strontium levels were dramatically increased (up to 10-fold) in bones of mice on the mineral-supplemented diets relative to mice on the respective, unsupplemented diets. This was seen on both diets at all three time points (Fig. 4). Interestingly, bone calcium levels in mice on the control diet and HFWD with or without the minerals were similar at all the time points (Fig. 4) and calcium was present as the major mineral in the bone. For example, there was a difference in the level of calcium of 12 % at 5 months, 7 % at 12 months, and 9 % at 18 months in bones of mice on the HFWD as compared to the HFWD with minerals, although there

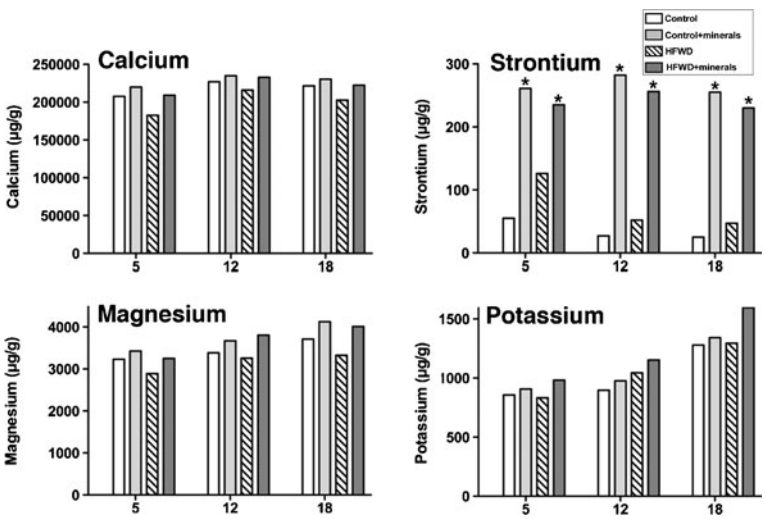

Fig. 4 Strontium levels in bone; comparison with calcium, magnesium, and potassium. Long bones from all mice in a group were pooled together, digested, and analyzed by ICP-OES as one sample per diet group (10 mice per group at 5 and 12 months and 15 mice at 18 months). *Asterisk* is placed on diet groups with minerals and indicates statistically significant increase in the strontium level (using two-way factorial ANOVA) than the diet groups without minerals ($p < 0.05$)

was 95 % less calcium in the unsupplemented high-fat diet, itself. In mice on the control diet with minerals (where the dietary calcium level was twice that of control), bone calcium levels were 4 % higher at 5 months, 3 % at 12 months, and 4 % at 18 months than in bones of mice on the control diet without supplementation. As shown in Fig. 4 minimal differences in magnesium and potassium were also observed between mice with or without the minerals.

Serum TRAP and P1NP TRAP and P1NP levels were assessed as measures of bone turnover and bone formation, respectively. TRAP levels were consistently higher in mice on either diet with the minerals than in mice from groups without the minerals (Table 2). This was not seen with P1NP. In fact, levels of P1NP were slightly lower in mice from supplemented groups than in mice from the respective unsupplemented diet group (Table 2).

Discussion

This study demonstrates that bone structure and function in female mice are preserved by minerals obtained from marine red algae (*Lithothamnion sp*.) in a long-term dietary intervention study. The findings may be particularly significant since bone mineral loss was prevented in mice maintained on a rodent chow (control) as well as on a HFWD. Previous studies have demonstrated that the Western-style diet contributes to bone loss in people [4, 5], and other studies have shown that a high-fat diet leads to bone mineral loss in rodents and other experimental animals [7, 8]. The control diet is routinely used for optimal health maintenance in mouse care. The current study confirmed more rapid and, ultimately, greater bone deterioration in mice on the HFWD, but, consistent with a previous study [9], demonstrated that significant bone mineral loss also occurred in mice on the rodent chow. Of interest, while both trabecular and cortical bone loss occurred in the HFWD, it was primarily trabecular bone that was lost in control diet mice. The minerals induced early bone built up (i.e., between 3 weeks of age and 5 months) and retarded subsequent bone mineral loss over an 18-month period. Bone mineral preservation was observed in both cortical and trabecular regions. However, the effects of the minerals were much greater in the trabecular bone. Thus, mineral addition had its greatest effect where bone loss was most rapid and severe.

Table 2 Serum TRAP and P1NP levels

TRAP (U/ml)			
Diet group	5 months	12 months	18 months
Control	1.1±0.1	17.5±2.6	10.3±0.7
Control+minerals	1.4±0.2	18.0±2.0	19.6±2.3[c]
HFWD	1.5±0.2	16.4±2.1	15.1±1.5
HFWD+minerals	1.5±0.2	26.3±3.8	24.4±2.1[a,b]
P1NP (ng/ml)			
		12 months	18 months
Control		24.5±0.9	21.3±1.8
Control+minerals		22.2±2.6	21.4±2.0
HFWD		26.4±2.3	32.7±5.9
HFWD+minerals		19.6±1.5	19.0±2.6

Baseline TRAP=1.2±0.5 U/ml. Values are means and standard deviations. Statistical significance was determined by ANOVA followed by paired group comparisons. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant increase relative to the HFWD group, and "b" shows statistically significant increase relative to control; "c" is placed on the control+minerals group, and shows significant increase relative to the control (p <0.05). P1NP data at baseline and at 5 month are not available

While the consequences of bone mineral loss—increased susceptibility to fracture (especially in the head of the femur and the vertebral discs)—are usually seen in later life, bone mineral loss typically begins during young adulthood and progresses over time [35]. Our findings suggest that inclusion of a minerals combination such as the one used here may provide a strategy for retarding progressive bone mineral loss. To the extent that BMD is a measure of resistance to fracture [36], reduced susceptibility to fracture could be expected. One might assume that effectiveness would be seen even with individuals who are consuming a "healthy" diet.

At this point, we do not know which minerals present in the mineral combination contribute to its beneficial effects. Calcium is, undoubtedly, important, but it should be noted that while mice on the mineral-supplemented control diet received a calcium dose equivalent to twice the level of control animals, and mice on the mineral-supplemented HFWD received an amount of calcium comparable to that received by control diet mice. In spite of this, mice on either diet with the minerals demonstrated several features that distinguished them from control mice. At the same time, bone calcium levels varied by only a few percent among the different groups. Although even the small differences in bone calcium level could be crucial to bone loss in high-fat diet mice, the implication is that while calcium is a critical component of the mineral preparation, other trace elements in it also appear to play important role in maintaining bone structure and function, in conjunction with calcium.

Among the cationic minerals that have been suggested previously as important to bone health are boron, copper, iron, magnesium, manganese, selenium, silicon, strontium, and zinc [13–17]. How the different minerals contribute to bone strength is not known, and it is, perhaps, best to not speculate beyond noting that small amounts of these trace minerals are incorporated into the bone matrix along with calcium [37, 38]. Strontium may be particularly important to the overall beneficial activity of the mineral combination. With each of the other minerals present in the mineral preparation, bone levels

were either below detectable limits, comparable in all groups or actually lower in the presence of the HFWD. In contrast, the bone level of strontium was significantly increased (up to 10-fold) in mice receiving the minerals derived from marine red algae in either diet relative to the unsupplemented groups. Increased strontium was seen at 5 months in mice on either supplemented diet and persisted over the 18-month period of the study. Previous studies have convincingly demonstrated the beneficial effects of strontium on bone structure [17, 39]. In Europe, a strontium-containing pharmaceutical (strontium ranelate) is an approved therapeutic for prevention of bone mineral loss and bone fragility [40]. A study in rats has suggested that long-term treatment with strontium ranelate increases bone mass, architecture, and fracture resistance [41]. The exact mechanism of action is still not fully understood. Strontium salts are more acid insoluble than comparable calcium salts [42], and slower demineralization of strontium salts as compared to calcium salts may be part of the effect. This may be especially important for individuals consuming a typical Western diet as this diet is known to produce an acidic environment [43].

Incorporation of trace elements such as strontium into the bone matrix may not be the entire explanation. *In vitro* studies have shown that strontium and several other trace elements influence the function of both osteoclasts and osteoblasts [17–20], leading to increased bone matrix synthesis and turn over. In this regard, however, it should be noted that we consistently saw increased TRAP activity but no increase in P1NP in the serum of mice on the mineral-supplemented diets relative to control mice. Without seeing increases in both P1NP and TRAP, it is difficult to postulate increased bone cell metabolism and increased bone turnover as the major mechanism. Additional experiments will be required to address this issue.

Finally, it should also be considered that the beneficial effects on bone may be secondary to other, more global actions of the minerals. An attractive (alternative) hypothesis is that the combination of minerals functions to help control systemic inflammation, a known risk factor for bone mineral loss [44]. In support of this, we observed in the same animal model that mice on the mineral-supplemented diets had fewer colonic polyps than control mice [21, 22] and that liver tumor formation was almost completely absent in these animals [23]. Associated with both findings were reduced inflammatory lesions throughout the intestinal tract. Confounding the issue, however, is that inflammatory changes in the gastrointestinal tract did not appear until late — 12 months and beyond. In contrast, diet-induced changes in bone structure/function were seen within 5 month of starting the diet. The relative contribution of systemic changes versus bone-specific effects will require further study.

Ultimately, whether mineral supplementation will have a similar effect in humans as shown here with rodents is not known and controlled clinical studies will be necessary to address this question. Although the focus of this study was on the role of trace minerals in preservation of bone structure/function rather than on the source of minerals, per se, the natural product used here has already been examined in a previous small-scale clinical study related to osteoarthritis pain [45]. There is no reason why a study designed to assess biomarkers of bone health could not be undertaken with this natural product or with the minerals as present in it. Furthermore, while our data suggest an important role for strontium (among the trace elements and in the presence of calcium) in the beneficial activity of the natural product, the reality is that marine algae accumulate many different minerals from seawater. There are, undoubtedly, multiple trace elements present in the mineral product at amounts below detectable level. Any and all of these might be contributing to the beneficial activity of the natural product. Until the mechanisms of action of the mineral product are clearly defined, it will be difficult to elucidate which minerals are most important, singly or in combination.

In conclusion, this study shows that minerals obtained from marine red algae promote early (3 weeks to 5 months) bone mineral build-up and preserve bone mineralization over an 18-month period in female C57BL/6 mice. This occurs in mice on either a high-fat diet or a standard rodent chow. These findings support further effort to determine if mineral supplementation might provide an approach for maintenance of bone structure/function in the face of age and high-fat diet-related events that tend to reduce bone mineral content.

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Disclosure of Conflict

All authors state that they have no financial or personal conflict of interest (no disclosures).

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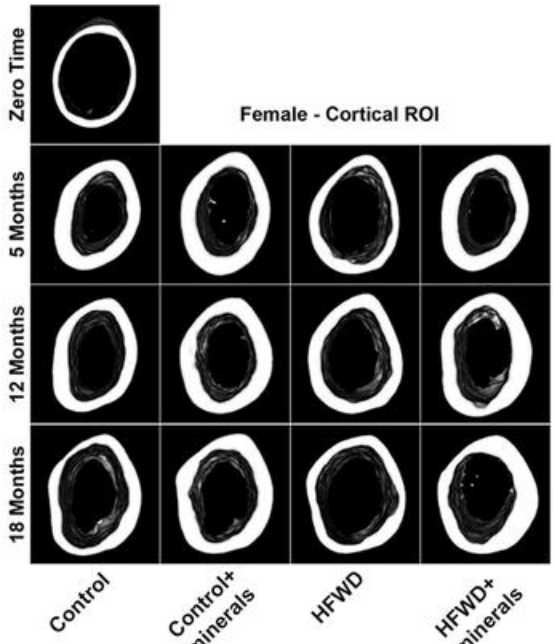

Supplementary Figure 1: A representative 3D μ-CT image of the cortical region of interest (mid -diaphysis) from the femur of a female mouse in each diet group.

SUPPLEMENTAL METHODOLOGY

Micro-computed tomography (μ-CT). Three-dimensional images of the femora in Ringer's solution were obtained using a μ-CT system (eXplore Locus SP, GE Healthcare Pre-Clinical Imaging, London, Ontario, Canada) as previously described and validated [24,27]. Measurements were taken at an operating voltage of 80 kV and 80 mA of current with an exposure time of 1600 ms using the Parker method scan technique, which rotates the sample 180 degrees plus a fan angle of 20 degrees. The effective voxel size of the reconstructed image was 18 x 18 x 18 μm^3. Images were globally thresholded and used to quantify parameters related to bone density, geometry, and morphology.

Whole bone was scanned and regions of interest (ROI) were reconstructed from it. Femoral trabecular (ROI) were selected by using a spline function to manually select a two dimensional (2D) (key frame contour) region encompassing only the trabecular bone within the distal metaphysis. A key frame contour was selected on every 10 frames starting at the distal growth plate and continuing proximal until the ROI depth was reached. The ROI depth was standardized to a percentage of the overall femur length (10%). After the completion of the key frame contours, additional contours were generated by interpolating contours between key frames. A 3D ROI was then generated from all the contours. The trabecular ROIs were assessed both densitometrically (BMD and tissue mineral density) and morphologically (bone volume fraction, surface-to-volume ratio, trabecular thickness, number, and spacing) [28]. Cortical ROIs were selected within the mid-diaphysis of the femur. Specifically, femoral ROIs were selected by locating the center point between the greater trochanter and the distal growth plate. A cylindrical ROI was centered around this point encompassing the entire cortical cross-section with the depth of the ROI being standardized to 18% percent of the overall femur length. Cortical ROIs were assessed both densitometrically (BMD and tissue mineral density) and geometrically (mean thickness, cross-sectional area, bending moments of inertia, and endosteal and periosteal perimeters) [28].

A subset of caudal vertebrae (C8) were identified and carefully dissected. Upon dissection, the vertebrae were immediately placed in lactated Ringer's solution and frozen at -20^{o}C until use. Whole vertebrae were scanned and ROIs through the cranial and middle isolateral surfaces were selected for analysis. μ-CT analysis was done exactly as indicated above with long bones.

Biomechanical testing. Long-bone mechanical properties were determined by loading the left femora to failure in 4-point bending, using a customized testing fixture attached to a servohydraulic materials testing machine (858 Mini Bionix II; MTS Systems, Eden Prairie, MN). All femora were loaded at a constant displacement rate of 0.5 mm/s [24,29]. Femora were loaded in the anterior-posterior direction so that the posterior side of the bone was in tension and the anterior side was in compression. Load-displacement curves were analyzed using MATLAB software (version R2008b; The Mathworks Inc., Natick, MA) to determine yield load, failure load, stiffness, energy to failure, and displacement ratio. Yield load was defined as the elastic limit before which permanent deformation occurred as measured by the secant method (secant stiffness differed by 10% from the initial tangential stiffness). Ultimate load (max load) was the load at which the bone catastrophically failed. Stiffness was defined as the slope of the linear region of the pre-yield load-displacement curve. Energy to failure was determined with numerical integration as the area under the load-displacement curve up to the point at which the bone failed. A displacement ratio was calculated as the ratio of ultimate displacement to yield displacement to characterize the relative magnitudes of elastic and plastic deformation.

Whole-bone mechanical properties of intact caudal vertebrae were measured by compressing the vertebral body with a 3 mm diameter platen attached to a servohydraulic materials testing machine (858 Mini Bionix II; MTS Systems, Eden Prairie, MN), [30]. In these compression tests, the cranial and caudal endplates of the caudal vertebrae were not altered prior to testing. Compression tests were conducted at a displacement rate of 0.05mm/sec. Mechanical properties included failure load and stiffness. Failure load was defined as the highest load preceding a rapid decrease in the measured load.

Supplement Table 1. Composition of the four diets

	1-AIN76A (Control)		2-Control+minerals		3-HFWD		4-HFWD+minerals	
	gm%	**kcal%**	**gm%**	**kcal%**	**gm%**	**kcal%**	**gm%**	**kcal%**
Protein	20.3	20.8	19.1	20.8	24.4	20.5	22.9	20.5
Carbohydrate	66	67.7	66	67.7	48.5	41.8	45.7	41.8
Fat	5	11.5	5	11.5	20	37.8	18.8	37.8
Total	91.3	100	86	100	92.9	100	87.5	100
kcal/gm	3.9		3.67		4.76		4.49	
INGREDIENTS	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Casein (80 Mesh)[a]	200	800	200	800	240	960	240	960
DL-Methionine	3	12	3	12	0	0	0	0
L-Cystine	0	0	0	0	3.6	14	3.6	14
Corn starch	150	600	150	600	100	400	100	400
Maltodextrin 10	0	0	0	0	75	300	75	300
Sucrose	500	2000	500	2000	310.418	1242	310.418	1242
Cellulose (BW200)	50	0	50	0	20	0	20	0
Corn oil	50	450	50	450	200	1800	200	1800
Ethoxyquin	0.01	0	0.01	0	0.01	0	0.01	0
Mineral Mix for AIN76A[b]	35	0	35	0	0	0	0	0
Mineral Mix (without Calcium Phosphate)[c]	0	0	0	0	21	0	21	0
Monosodium phosphate	0	0	0	0	7.98	0	7.98	0
Monopotassium phosphate	0	0	0	0	7.91	0	7.91	0
Calcium carbonate (40% calcium)	0	0	0	0	0.88	0	0.88	0
Mineral supplement (12% calcium)	0	0	62	0	0	0	62	0
Vitamin Mix for AIN76A	10	40	10	40	0	0	0	0
Vitamin Mix (without Vit. D3 or Folic Acid)	0	0	0	0	12	48	12	48
Choline bitartrate	2	0	2	0	1.2	0	1.2	0
Folic acid	0	0	0	0	0.00023	0	0.00023	0
Vitamin D3 (100,000 IU/g)	0	0	0	0	0.0012	0	0.0012	0
TOTAL	1000.01	3902	1062.06	3902	1000.05	4764	1062.05	4764

[a]Amount of essential minerals in Casein added to all diets (amount per 100 gm of Casein): calcium (25 mg), magnesium (2 mg), copper (0.01 mg), Iron (0.4 mg), manganese (0.014 mg), potassium (2.5 mg), selenium (0.03 mg), sodium (5.2 mg), zinc (4.2mg).

[b]Amount of essential minerals in mineral mix for low fat diets with/without mineral supplement (used at 35 gm/kg diet): calcium (5.2 gm), magnesium (0.5 gm), chromium (2 mg), copper (6 mg), Iron (45 mg), manganese (59 mg), potassium (3.6 g), selenium (0.16 mg), sodium (1.0 gm), zinc (29 mg).

[c]Amount of essential minerals in mineral mix for western-style diets with/without mineral supplement (used at 21 gm/kg): magnesium (0.6 gm), chromium (2.4 mg), copper (7.2 mg), Iron (54 mg), manganese (71 mg), potassium (4.3 g), selenium (0.19 mg), sodium (1.2 gm), zinc (35 mg).

Diets were formulated by Research Diets Incorporated (New Brunswick, NJ).

Supplement Table 2. Minerals concentration in diets (mg/kg)
(as present in the diets from all sources)

Mineral	Control	Control+Minerals	HFWD	HFWD+Minerals
Calcium	5250	11950	410	7360
Magnesium	504	1362	605	1457
Sodium	1010	1088	1212	1279
Potassium	3605	3402	4326	4081
Copper	6.02	5.74	7.2	6.9
Chromium	2	1.93	2.4	2.3
Iron	46	53	55	61
Manganese	59	58	71	69
Selenium	0.22	0.44	0.26	0.48
Strontium	<0.05	40	<0.05	40
Zinc	37	36	45	43

Diets were formulated and mineral calculations were provided by Research Diets Incorporated (New Brunswick, NJ).

Supplement Table 3. Average weight by diet group at 5, 12 and 18 months

Group	Weight (gm)		
	5 months	12 months	18 months
Control	25 ± 2	33 ± 2	35 ± 4
Control + minerals	24 ± 2	31 ± 5	32 ± 5
HFWD	28 ± 5	46 ± 6	47 ± 6
HFWD + minerals	31 ± 4	44 ± 8	49 ± 7

Values are means and standard deviations. Zero time (3 weeks of age) average weight was 14.5±1 gm.

Supplement Table 4. Micro-CT analysis of cortical and trabecular regions of femora from **female** mice in four diet groups at **five** months

	CONTROL	CONTROL + minerals	HFWD	HFWD + minerals
Cortical				
Bone mineral content (mg)	3.9 ± 0.2	4.2 ± 0.2[c]	3.6 ± 0.3	4.0 ± 0.2[a]
Bone mineral density (mg/cc)	347 ± 16	378 ± 25[c]	314 ± 36	339 ± 16
Tissue mineral density (mg/cc)	1136 ± 24	1140 ± 12	1030 ± 77	1123 ± 15[a]
Mean thickness (mm)	0.215 ± 0.007	0.225 ± 0.007[c]	0.188 ± 0.030	0.225 ± 0.008[a, b]
Polar moment of inertia (mm^4)	0.133 ± 0.008	0.147 ± 0.011	0.142 ± 0.024	0.147 ± 0.013
Endosteal perimeter (mm)	3.6 ± 0.2	3.8 ± 0.1	3.9 ± 0.2	3.7 ± 0.1[a]
Periosteal perimeter (mm)	5.0 ± 0.2	5.2 ± 0.2	5.1 ± 0.3	5.1 ± 0.1
Marrow area (mm^2)	0.90 ± 0.07	0.94 ± 0.06	1.07 ± 0.12	0.94 ± 0.07[a]
Cross sectional area (mm^2)	0.86 ± 0.02	0.92 ± 0.03[c]	0.79 ± 0.13	0.92 ± 0.02[a, b]
Total area (mm^2)	1.76 ± 0.07	1.85 ± 0.08	1.86 ± 0.16	1.86 ± 0.06
Trabecular				
Bone mineral content (mg)	0.51 ± 0.04	0.74 ± 0.06[c]	0.59 ± 0.15	0.65 ± 0.05[b]
Bone mineral density (mg/cc)	156 ± 12	234 ± 15[c]	164 ± 54	201 ± 11[a, b]
Tissue mineral density (mg/cc)	577 ± 55	596 ± 30	527 ± 20	581 ± 13[a]
Bone volume fraction (mm^3/mm^3)	0.05 ± 0.01	0.13 ± 0.02[c]	0.05 ± 0.01	0.10 ± 0.02[a, b]
Surface to volume ratio (mm^2/mm^3)	60.0 ± 10.0	49.6 ± 3.7[c]	55.6 ± 3.5	49.6 ± 4.0[a, b]
Trabecular thickness (mm)	0.034 ± 0.007	0.041 ± 0.003[c]	0.036 ± 0.002	0.041 ± 0.003[a, b]
Trabecular number (1/mm)	1.36 ± 0.25	3.30 ± 0.28[c]	1.47 ± 0.29	2.50 ± 0.31[a, b]
Trabecular spacing (mm)	0.67 ± 0.09	0.26 ± 0.03[c]	0.66 ± 0.12	0.37 ± 0.06[a, b]

Each femur was subjected to micro-CT at two ROIs—cortical (mid-diaphysis) and trabecular (distal metaphysis). With each bone, ten cortical parameters and eight trabecular parameters were assessed. Values are means and standard deviations. Statistical significance was determined by ANOVA followed by paired group comparisons. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant improvement relative to the HFWD group, "b" shows statistically significant improvement relative to CONTROL; "c" is placed on the CONTROL+minerals group, and shows significant improvement relative to the CONTROL ($p < 0.05$). Data are based on 10 female mice in each diet group at 5 months.

Supplement Table 5. Micro-CT analysis of cortical and trabecular regions of femora from **female** mice in four diet groups at **twelve** months

	CONTROL	CONTROL + minerals	HFWD	HFWD + minerals
Cortical				
Bone mineral content (mg)	4.1 ± 0.2	4.4 ± 0.4	4.1 ± 0.3	4.6 ± 0.3[a, b]
Bone mineral density (mg/cc)	309 ± 36	302 ± 31	287 ± 30	322 ± 21
Tissue mineral density (mg/cc)	1129 ± 14	1119 ± 23	1060 ± 13	1105 ± 18[a, b]
Mean thickness (mm)	0.210 ± 0.011	0.218 ± 0.019	0.199 ± 0.010	0.218 ± 0.014[a]
Polar moment of inertia (mm^4)	0.152 ± 0.013	0.173 ± 0.017[c]	0.190 ± 0.036	0.188 ± 0.015[b]
Endosteal perimeter (mm)	3.8 ± 0.1	4.0 ± 0.2[c]	4.2 ± 0.2	4.1 ± 0.2[b]
Periosteal perimeter (mm)	5.0 ± 0.1	5.3 ± 0.1[c]	5.5 ± 0.4	5.5 ± 0.2[b]
Marrow area (mm^2)	1.01 ± 0.07	1.10 ± 0.11[c]	1.28 ± 0.10	1.16 ± 0.09[a, b]
Cross sectional area (mm^2)	0.87 ± 0.04	0.94 ± 0.08[c]	0.91 ± 0.07	0.96 ± 0.06[b]
Total area (mm^2)	1.88 ± 0.07	2.03 ± 0.10[c]	2.19 ± 0.16	2.12 ± 0.10[b]
Trabecular				
Bone mineral content (mg)	0.44 ± 0.07	0.53 ± 0.09[c]	0.58 ± 0.04	0.64 ± 0.07[a, b]
Bone mineral density (mg/cc)	131 ± 21	158 ± 35	146 ± 11	171 ± 18[a, b]
Tissue mineral density (mg/cc)	598 ± 30	587 ± 22	564 ± 33	569 ± 22[b]
Bone volume fraction (mm^3/mm^3)	0.02 ± 0.01	0.05 ± 0.03[c]	0.04 ± 0.01	0.06 ± 0.02[a, b]
Surface to volume ratio (mm^2/mm^3)	60.1 ± 7.1	50.8 ± 4.8[c]	53.8 ± 8.7	53.5 ± 5.0[b]
Trabecular thickness (mm)	0.034 ± 0.004	0.040 ± 0.004[c]	0.038 ± 0.005	0.038 ± 0.004[b]
Trabecular number (1/mm)	0.52 ± 0.16	1.25 ± 0.67[c]	1.01 ± 0.30	1.49 ± 0.47[a, b]
Trabecular spacing (mm)	2.13 ± 0.84	1.08 ± 0.81[c]	1.04 ± 0.36	0.69 ± 0.21[a, b]

Each femur was subjected to micro-CT at two ROIs—cortical (mid-diaphysis) and trabecular (distal metaphysis). With each bone, ten cortical parameters and eight trabecular parameters were assessed. Values are means and standard deviations Statistical significance was determined by ANOVA followed by paired group comparisons. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant improvement relative to the HFWD group, "b" shows statistically significant improvement relative to CONTROL; "c" is placed on the CONTROL+minerals group, and shows significant improvement relative to the CONTROL ($p < 0.05$). Data are based on 10 female mice in each diet group at 12 months.

Supplement Table 6. Micro-CT analysis of cortical and trabecular regions of femora from **female** mice in four diet groups at **eighteen** months

	CONTROL	CONTROL + minerals	HFWD	HFWD + minerals
Cortical				
Bone mineral content (mg)	4.0 ± 0.4	4.4 ± 0.5[c]	3.6 ± 0.4	4.5 ± 0.6[a, b]
Bone mineral density (mg/cc)	303 ± 27	299 ± 28	260 ±34	322 ± 47[a]
Tissue mineral density (mg/cc)	1105 ± 25	1132 ± 29[c]	1076 ± 33	1140 ± 41[a, b]
Mean thickness (mm)	0.201 ± 0.034	0.191 ± 0.017	0.159 ± 0.014	0.205 ± 0.022[a]
Polar moment of inertia (mm^4)	0.181 ± 0.019	0.204 ± 0.030[c]	0.195 ± 0.030	0.213 ± 0.029[b]
Endosteal perimeter (mm)	4.1 ± 0.2	4.4 ± 0.1[c]	4.7 ± 0.2	4.4 ± 0.2[a, b]
Periosteal perimeter (mm)	5.4 ± 0.2	5.5 ± 0.2[c]	5.7 ± 0.2	5.6 ± 0.2[b]
Marrow area (mm^2)	1.20 ± 0.17	1.37 ± 0.11[c]	1.63 ± 0.14	1.40 ± 0.16[a, b]
Cross sectional area (mm^2)	0.88 ± 0.09	0.89 ± 0.08	0.79 ± 0.08	0.93 ± 0.13[a]
Total area (mm^2)	2.08 ± 0.12	2.26 ± 0.13[c]	2.42 ± 0.18	2.33 ± 0.14[b]
Trabecular				
Bone mineral content (mg)	0.43 ± 0.13	0.50 ± 0.11	0.34 ± 0.14	0.44 ± 0.09[a]
Bone mineral density (mg/cc)	120 ± 38	142 ± 40	79 ± 29	143 ± 30[a]
Tissue mineral density (mg/cc)	556 ± 70	645 ± 24[c]	604 ± 38	610 ± 40[b]
Bone volume fraction (mm^3/mm^3)	0.01 ± 0.01	0.04 ± 0.03[c]	0.02 ± 0.01	0.03 ± 0.02[b]
Surface to volume ratio (mm^2/mm^3)	62.2 ± 15.7	47.6 ± 7.1[c]	57.8 ± 10.2	54.7 ± 8.9
Trabecular thickness (mm)	0.033 ± 0.010	0.043 ± 0.007[c]	0.036 ± 0.008	0.038 ± 0.008
Trabecular number (1/mm)	0.33 ± 0.24	0.93 ± 0.73[c]	0.52 ± 0.27	0.85 ± 0.73[b]
Trabecular spacing (mm)	2.62 ± 1.25	1.70 ± 1.21	3.23 ± 3.27	1.42 ± 0.59[a, b]

Each femur was subjected to micro-CT at two ROIs—cortical (mid-diaphysis) and trabecular (distal metaphysis). With each bone, ten cortical parameters and eight trabecular parameters were assessed. Values are means and standard deviations. Statistical significance was determined by ANOVA followed by paired group comparisons. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant improvement relative to the HFWD group, "b" shows statistically significant improvement relative to CONTROL; "c" is placed on the CONTROL+minerals group, and shows significant improvement relative to the CONTROL ($p < 0.05$). Data are based on 15 female mice in each diet group at 18 months.

Supplement Table 7. Micro-CT analysis of cortical and trabecular regions of C8 vertebrae from **female** mice in four diet groups at **twelve** months

	CONTROL	CONTROL + minerals	HFWD	HFWD + minerals
Cortical				
Bone mineral content (mg)	0.44 ± 0.05	0.43 ± 0.03	0.34 ± 0.03	0.43 ± 0.03[a]
Bone mineral density (mg/cc)	961 ± 30	942 ± 14	731 ± 25	941 ± 33[a]
Tissue mineral content (mg)	0.41 ± 0.05	0.41 ± 0.03	0.30 ± 0.03	0.40 ± 0.03[a]
Tissue mineral density (mg/cc)	1056 ± 23	1050 ± 15	962 ± 13	1044 ± 25[a]
Mean thickness (mm)	0.31 ± 0.04	0.27 ± 0.01	0.22 ± 0.02	0.28 ± 0.04[a]
Inner perimeter (mm)	2.38 ± 0.28	2.86 ± 0.14[c]	2.68 ± 0.14	2.70 ± 0.33
Outer perimeter (mm)	5.05 ± 0.38	5.31 ± 0.22	5.22 ± 0.31	5.28 ± 0.28
Marrow area (mm^2)	0.39 ± 0.09	0.52 ± 0.04[c]	0.51 ± 0.06	0.48 ± 0.12
Cortical area (mm^2)	0.97 ± 0.11	0.92 ± 0.06	0.73 ± 0.06	0.95 ± 0.09[a]
Total Area (mm^2)	1.36 ± 0.09	1.45 ± 0.09	1.24 ± 0.05	1.43 ± 0.06[a]
Volume (mm^3)	0.46 ± 0.05	0.46 ± 0.02	0.47 ± 0.03	0.46 ± 0.02
Volume of bone (mm^3)	0.39 ± 0.05	0.39 ± 0.02	0.31 ± 0.02	0.38 ± 0.02[a]
Trabecular				
Bone mineral content (mg)	0.40 ± 0.07	0.55 ± 0.08[c]	0.30 ± 0.03	0.40 ± 0.06[a]
Bone mineral density (mg/cc)	619 ± 63	737 ± 83[c]	482 ± 34	687 ± 107[a]
Tissue mineral content (mg)	0.36 ± 0.08	0.51 ± 0.09[c]	0.25 ± 0.03	0.38 ± 0.07[a]
Tissue mineral density (mg/cc)	808 ± 28	858 ± 49	711 ± 15	794 ± 45[a]
Bone volume fraction (mm^3/mm^3)	0.67 ± 0.07	0.79 ± 0.08[c]	0.56 ± 0.05	0.80 ± 0.14[a]
Surface to volume ratio (mm^2/mm^3)	17.3 ± 2.7	13.1 ± 3.3[c]	20.1 ± 3.0	14.8 ± 6.3
Trabecular thickness (mm)	0.12 ± 0.02	0.16 ± 0.04[c]	0.10 ± 0.02	0.15 ± 0.05[a]
Trabecular number (1/mm)	5.7 ± 0.3	5.1 ± 0.8	5.6 ± 0.8	5.5 ± 1.1
Trabecular spacing (mm)	0.06 ± 0.01	0.04 ± 0.01[c]	0.08 ± 0.02	0.03 ± 0.02[a, b]
Volume (mm^3)	0.65 ± 0.07	0.75 ± 0.05[c]	0.62 ± 0.03	0.59 ± 0.04
Volume of bone (mm^3)	0.44 ± 0.08	0.60 ± 0.07[c]	0.35 ± 0.04	0.47 ± 0.07[a]

Each C8 vertebra (from a subset) was subjected to micro-CT at two ROIs—cortical (middle isosurface) and trabecular (cranial isosurface). With each vertebra, twelve cortical parameters and eleven trabecular parameters were assessed. Values are means and standard deviations. Statistical significance was determined by ANOVA followed by paired group comparisons. "a" and "b" are placed on the HFWD+minerals group: "a" shows statistically significant improvement relative to the HFWD group, "b" shows statistically significant improvement relative to CONTROL; "c" is placed on the CONTROL+minerals group, and shows significant improvement relative to the CONTROL ($p < 0.05$). Vertebral data are based on 6 female mice at 12 months in each diet group.

Supplement Table 8. Micro-CT analysis of cortical and trabecular regions of C8 vertebrae from **female** mice in two diet groups at **eighteen** months

	HFWD	HFWD + minerals
Cortical		
Bone mineral content (mg)	0.32 ± 0.03	0.39 ± 0.06[a]
Bone mineral density (mg/cc)	763 ± 146	928 ± 43
Tissue mineral content (mg)	0.28 ± 0.03	0.36 ± 0.06[a]
Tissue mineral density (mg/cc)	960 ± 74	1028 ± 47
Mean thickness (mm)	0.21 ± 0.04	0.25 ± 0.06
Inner perimeter (mm)	2.78 ± 0.31	2.48 ± 0.24
Outer perimeter (mm)	4.91 ± 0.25	4.90 ± 0.41
Marrow area (mm^2)	0.54 ± 0.14	0.41 ± 0.06
Cortical area (mm^2)	0.62 ± 0.04	0.86 ± 0.10[a]
Total area (mm^2)	1.16 ± 0.15	1.27 ± 0.12
Volume (mm^3)	0.43 ± 0.07	0.41 ± 0.05
Volume of bone (mm^3)	0.29 ± 0.02	0.35 ± 0.04[a]
Trabecular		
Bone mineral content (mg)	0.32 ± 0.04	0.42 ± 0.07
Bone mineral density (mg/cc)	452 ± 46	659 ± 108[a]
Tissue mineral content (mg)	0.25 ± 0.04	0.37 ± 0.07[a]
Tissue mineral density (mg/cc)	768 ± 46	846 ± 58
Bone volume fraction (mm^3/mm^3)	0.47 ± 0.05	0.69 ± 0.10[a]
Surface to volume ratio (mm^2/mm^3)	21.9 ± 2.7	15.1 ± 3.4[a]
Trabecular thickness (mm)	0.09 ± 0.01	0.14 ± 0.03[a]
Trabecular number (1/mm)	5.0 ± 0.3	5.0 ± 0.5
Trabecular spacing (mm)	0.11 ± 0.01	0.06 ± 0.02[a]
Volume (mm^3)	0.70 ± 0.05	0.64 ± 0.07
Volume of bone (mm^3)	0.33 ± 0.04	0.44 ± 0.07

Each C8 vertebra (from a subset) was subjected to micro-CT at two ROIs—cortical (middle isosurface) and trabecular (cranial isosurface). With each vertebra, twelve cortical parameters and eleven trabecular parameters were assessed. Values are means and standard deviation. Statistical significance was determined by paired group comparisons ($p<0.05$). "a" is placed on the HFWD+minerals group: "a" shows statistically significant improvement relative to the HFWD group. Vertebral data are based on 6 female mice each of the two high-fat diets at 18 months.

Supplement Table 9A. **Bone Mineral Analysis at 5 months in long bones of female mice(µg/g)**

5 months

	CONTROL	CONTROL+ minerals	HFWD	HFWD+ minerals
Fluoride	0.83	1.06	0.85	0.88
Barium	4.32	5.2	7.58	3.4
Boron	<0.5	<0.5	<0.5	<0.5
Calcium	207400	219900	182600	209100
Copper	<0.5	<0.5	<0.5	<0.5
Iron	105	81	315	100
Lanthanum	0.99	0.75	1.2	0.66
Magnesium	3232	3425	2891	3250
Manganese	1.93	1.77	6.3	1.56
Phosphorus	99597	105564	88548	98629
Potassium	856	907	832	982
Selenium	<0.5	<0.5	<0.5	<0.5
Silicon	3.1	<0.5	2.91	<0.5
Strontium	54.6	261	126	235
Zinc	129	151	246	149

The long bones (one femur and tibia from each animal in the group) were "pooled" and analyzed for levels of trace metals found in the mineral-rich product to give a single value at each time point. Some of these elements were recorded below detectable levels when their concentration level found below 0.5µg/g. The levels of individual trace elements were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) except Flouride which was done by AOAC 984.37 assay.

Supplement Table 9 B **Bone Mineral Analysis at 12 and 18 months in long bones of female mice (µg/g)**

	12 months				18 months			
	CONTROL	**CONTROL+ minerals**	**HFWD**	**HFWD+ minerals**	**CONTROL**	**CONTROL+ minerals**	**HFWD**	**HFWD+ minerals**
Fluoride	6.1	8.31	4.9	5.86	6.35	7.06	6.83	6.13
Aluminum	0.98	3.46	2.04	2.79	<0.5	<0.5	<0.5	<0.5
Antimony	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Arsenic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Barium	3.84	5.43	5.21	3.03	3.06	3.92	4.45	2.41
Beryllium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Bismuth	<0.5	<0.5	2.62	<0.5	<0.5	<0.5	<0.5	<0.5
Boron	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Calcium	226800	234900	215900	232800	221400	230300	202500	222400
Cadmium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Chromium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cobalt	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Copper	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Iron	213	120	374	113	107	126	301	85.2
Lanthanum	<0.5	<0.5	0.58	<0.5	<0.5	<0.5	<0.5	<0.5
Lead	<0.5	<0.5	<0.5	<0.5	<0.5	0.95	1.39	1.03
Lithium	0.52	0.89	<0.5	0.63	1.98	1.35	0.83	0.78
Magnesium	3386	3671	3258	3803	3712	4122	3324	4010
Manganese	1.47	1.46	3.95	1.89	1.58	1.29	3.47	1.24
Mercury	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Molybdenum	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Nickel	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Niobium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Phosphorus	118400	119800	110400	116300	105500	112200	97980	108400
Potassium	897	977	1044	1153	1279	1342	1295	1595
Selenium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Silicon	0.57	<0.5	0.57	<0.5	0.71	<0.5	0.61	<0.5
Silver	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Sodium	11890	11220	11780	12500	15090	15940	14460	16200
Strontium	27.31	282	52.3	256	24.48	255	46.64	230
Sulfur	3297	3228	3237	3398	3153	3175	2917	3115
Tellurium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Thallium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Thorium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Tin	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Titanium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Tungsten	<0.5	0.566	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Vanadium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Yttrium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Zinc	157	197	246	191	150	174	219	174
Zirconium	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

The long bones (one femur and tibia from each animal in the group) were "pooled" and analyzed for levels of trace metals found in the mineral-rich product to give a single value at each time point. Some of these elements were recorded below detectable levels when their concentration level found below 0.5µg/g. The levels of individual trace elements were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) except Flouride which was done by AOAC 984.37 assay.