INDEPENDENT AND COMBINED EFFECTS OF EXERCISE AND DIETARY CALCIUM AND PHOSPHORUS ON BONE

by

Michael Andrew Friedman

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Doctoral Committee:

Professor David H. Kohn, Chair
Professor Kenneth M. Kozloff
Professor Laurie K. McCauley
Professor Ronald F. Zernicke

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ABSTRACT

Bone fractures are costly injuries that are becoming more prevalent as the population ages. Increased fracture risk is often associated with decreased bone mass. Exercise is an intervention that can reduce fracture risk by increasing bone mass, tissue quality, and strength. Early in exercise programs, bone favors increasing tissue quality over increasing bone mass and strength, potentially increasing fracture risk. Exercise causes an increased demand for dietary minerals required to increase bone mass. Combining exercise with a mineral-supplemented diet may allow maintenance of increases in both tissue quality and bone mass early in an exercise program. This could lead to greater long-term increases in bone mass or greater rate of bone growth to reach peak bone mass. It was hypothesized that combining exercise with a calcium- and phosphorus-supplemented diet would increase bone mass and mineralization over exercise with a standard diet in adult mice.

Male, 4-month old mice performed daily treadmill exercise and were given either a standard or mineral-supplemented diet. Exercised mice taking a mineral-supplemented diet had greater cortical and trabecular bone mass after 3-4 weeks, compared to exercised mice on a standard diet. Greater bone mass was maintained after 8 weeks of treatment, as well as after an additional 8 weeks of non-exercise. Mice on the combined diet and exercise treatments had greater cortical bone mass and structural-level strength than non-exercised mice on the supplemented diet after 8 weeks. All

mice on the supplemented diet achieved the same peak bone mass and bone strength, and exercise allowed these mice to reach their peaks in less time.

Exercise increases mineral demand to increase bone mass and tissue quality. This work suggests standard dietary amounts of minerals are insufficient to maximize benefits from exercise. Increasing dietary mineral consumption with exercise can lead to greater bone mass and bone strength than exercise on a standard diet. Recommended values for dietary nutrients are determined based on non-exercise conditions, but this work suggests different nutrient amounts may be required for optimal bone health to meet different demands from exercise.

CHAPTER ONE

INTRODUCTION

BONE MASS AND FRACTURE RISK

As the population ages, non-traumatic fractures such as osteoporotic fractures are becoming increasingly prevalent and creating a greater health care burden, with costs expected to exceed $25 billion per year by 2025 [1]. Increased fracture risk is often attributed to decreased bone mass, but tissue quality factors. such as tissue age, architecture, mineralization, rate of bone turnover, collagen cross-linking, and microdamage accumulation also play important roles in determining fracture risk [2–8]. Clinical interventions for reducing fracture risk primarily focus on maintaining or increasing bone mass, but often do not fully consider the impact on bone tissue quality [9,10]. Better understanding of how both bone mass and tissue quality affect bone health may improve prevention, diagnosis, and treatment of non-traumatic fractures.

Weight-bearing exercise increases bone mass, structural (whole bone) strength, and tissue quality, making bone better able to resist fracture [11]. Exercise can have long-term benefits to bone health [12–18], making it beneficial to maximize bone mass accumulation early in life. Increases in cortical bone mineral content (BMC), cross-

sectional area, and stiffness that occur with exercise can remain months or even years of cessation of exercise [12–18]. Since bone mass continually declines with age throughout adulthood [19–21], it may be beneficial to accumulate bone mass earlier in life to maintain a higher level of bone mass in old age when weight-bearing exercise becomes more difficult to perform. Additionally, exercise offers many health benefits that may be indirectly related to bone fracture risk such as decreasing risk of falling. These numerous effects of exercise make it a recommended intervention for reducing the risk of osteoporotic fractures [9].

EXERCISE EFFECTS ON BONE

Bone responds to loading from exercise by increasing bone mass and bone strength to accommodate greater loads on bones and prevent damage from future loads [22]. Exercise is most beneficial earlier in life when bones are still growing and developing as it can increase bone mass and bone strength in children and adolescents [23]. Later in life, exercise continues to be beneficial by increasing bone strength and preventing loss of bone mass from aging.

Exercise can increase bone mass in humans [24–26], but it is unclear what effects exercise has at the tissue level. It is difficult to assess the effects of exercise on some properties of tissue quality in humans, such as collagen cross-linking and microdamage accumulation, because of the inability to collect bone tissue samples after a controlled exercise experiment. Animal models can be useful, as they can provide additional

insight into how interventions such as exercise affect bone mass and bone tissue quality by better allowing analysis of samples harvested at the end of an exercise program.

Rodent exercise models have shown increases in bone mass (cross-sectional area, BMC) and structural-level strength (yield force, ultimate force) after long-term exercise programs of 6-12 weeks [27–30]. However, short-term exercise, for a duration of 3 weeks, can slow growth, limiting increases in bone mass and structural-level strength, and even cause a reduction in strength compared to non-exercise controls [31,32]. Short-term exercise can still be beneficial, increasing tissue-level strength and quality (ultimate stress, microdamage resistance, and mineral-to-matrix ratio) [31]. The difference in bone's response to short-term exercise at the structural and tissue levels suggests that there may be a competition for minerals needed to increase structural-versus tissue-level strength and bone quantity versus bone quality. Bone seems to favor increasing tissue quality over increasing bone mass and structural-level strength in a short-term exercise program. The limits on bone adaptation seen in short-term exercise suggest that standard dietary amounts of mineral may be insufficient for optimal adaptation of bone in response to exercise.

Since long-term exercise programs show increases in bone mass and structural-level strength, short-term exercise effects that limit increases in bone mass and strength may only be transient. However, long-term exercise may cause a sustained increase in demand for minerals. For example, serum vitamin D_3 increases, serum parathyroid hormone decreases, and intestinal absorption of calcium and phosphorus increases in

rodents after exercise programs of at least 5 weeks [30,33,34]. This change in markers of bone metabolism with exercise suggests that exercise maintains increased demand for minerals even after lengthy exercise programs, which may ultimately be what leads to increases in bone mass and structural-level strength after lengthy exercise programs.

It may be beneficial to increase exercise intensity for maximizing increases in bone mass and bone strength. Bone adapts to higher-intensity loading during exercise by increasing bone formation to compensate [35]. Increasing bone formation rate could allow bones to have a more rapid increase in resistance to fracture. This increased bone formation rate could also require an increased dietary mineral supply to simultaneously maintain increases in bone mass and bone tissue quality with exercise.

DIETARY MINERAL REQUIREMENTS

Bone is composed of several minerals that are acquired only through dietary consumption, including calcium, phosphorus, and magnesium. The majority of bone mineral is made up of hydroxyapatite – a compound made of calcium and phosphorus. Calcium and phosphorus are important regulators of bone growth and mineralization. Threshold daily amounts of 700-1300 mg of calcium and phosphorus are needed from dietary sources and supplements to maintain optimal bone mass and bone strength [23,36,37]. Under healthy non-exercise conditions, dietary concentrations of calcium and phosphorus above the threshold do not lead to increases in bone mass and strength [23,38]. However, dietary mineral demands change with age. During skeletal growth, an increased supply of minerals from the diet is needed to meet the demands

of bone growth from modeling. Due to decreased ability to absorb dietary calcium, increased dietary calcium is also required with old age [36].

Intestinal absorption of calcium is not efficient, as only around 25% of dietary calcium is absorbed in adults [39]. Absorption of calcium can occur by active transport mediated by vitamin D or by passive transport mediated by diffusion. When blood calcium concentration is decreased and demand for calcium is increased, vitamin D_3 is released from the kidneys to increase intestinal absorption of calcium. Increasing dietary calcium leads to increased passive intestinal absorption of calcium. If demand for calcium is decreased and blood calcium concentration increased, then excess blood calcium can be filtered out by the kidneys and excreted as waste [36]. Dietary phosphorus is absorbed at a higher efficiency than calcium, but there is no method to increase active absorption. Instead, vitamin D_3 acts to increase active transport of both calcium and phosphorus across the intestine and into the blood [37]. Passive absorption of phosphorus can also occur by diffusion. Thus, the amount of phosphorus absorbed is dependent on dietary consumption and on how much demand for calcium affects blood concentration of vitamin D_3.

Changes in active intestinal absorption of calcium are usually sufficient to meet changing mineral demands from the body. However, there are some conditions where increased dietary calcium is required to increase passive intestinal absorption of calcium. Decreased ability to maintain calcium homeostasis with age causes an increased daily recommended value for calcium in men over age 70 and women over

age 50 [36]. Estrogen-deficient animals have shown benefits from increasing dietary calcium consumption on bone health. For example, adding calcium supplements to a diet can restore bone mass and strength lost following ovariectomy in rodents [40,41]. Exercise could be considered another condition where there is an increased demand for minerals that is not being met by changes in hormones that increase active intestinal absorption. Since exercise limits short-term increases in bone mass and structural-level strength in mice, adding mineral supplements to the diet during exercise may be a strategy to increase bone mass and structural-level strength early in an exercise program.

Combining exercise with a calcium- and phosphorus-supplemented diet can be one strategy to prevent short-term decreases in bone growth and to maximize long-term increases in bone mass from exercise. Bone mass has been increased by combining a lengthy exercise program with a calcium-supplemented diet in human children and adolescents [24,42–44]. However, there are many questions remaining about the effects of increasing dietary mineral supply during an exercise program. It is unclear if the benefits from combining exercise with a calcium-supplemented diet appear after short or long durations on the diet, or if benefits remain after the diet and exercise program has ended.

AIMS AND HYPOTHESES

Exercise increases bone mass and bone strength after lengthy programs of at least 6 weeks. After just 3 weeks of exercise, bone prioritizes increasing tissue quality over

increasing cortical area and longitudinal growth. Increasing dietary mineral supply

during an exercise program may increase mineral availability to bone, allowing for

simultaneous increases in tissue quality and tissue quantity. The experiments

conducted in this dissertation investigated effects of increasing dietary calcium and

phosphorus in exercising mice on bone mass, bone strength, and bone metabolism

markers. Bones were evaluated after short-term exercise, long-term exercise, and

exercise plus detraining to determine the timing of effects of exercise and dietary

mineral supply. Exercise intensity was also modified to evaluate the effects of

increasing loading frequency.

Global Hypothesis: Combining exercise with a mineral-supplemented diet will increase

blood mineral supply, bone mass, mineralization, and structural-level strength over

exercise with a standard diet. These increases will occur after short-term and long-term

exercise programs. Exercise will increase tissue quality, regardless of dietary mineral

supply.

Hypothesis 1: Combining exercise with a mineral-supplemented diet increases blood

calcium and phosphorus, cortical bone mass, and structural-level strength over exercise

with a standard diet. Exercise increases tissue-level strength and resistance to

microdamage, independent of dietary mineral supply.

> **Aim 1**: This hypothesis was tested in 16-week old male mice using an exercise
>
> program that consisted of daily running on a treadmill with either a standard diet
>
> or a calcium- and phosphorus-supplemented diet. Mice maintained the diet and

exercise program for 3 or 8 weeks. Cortical bone geometry, structural- and tissue-level mechanical properties, bone metabolism markers, and resistance to microdamage from fatigue loading were all measured. Differences in effects of 3-week versus 8-week exercise programs and relations between bone mass, bone metabolism, and mechanical properties were evaluated.

Hypothesis 2: Increasing exercise intensity by increasing treadmill speed will lead to increased frequency of loading on bones during exercise and increased bone growth. Combining high-speed exercise with a mineral-supplemented diet will lead to greater cortical bone mass than high-speed exercise on a standard diet or low-speed exercise on a supplemented diet.

> **Aim 2**: High-speed exercise was tested using 15-week old male mice exercising for 4 weeks. Exercise intensity was increased by increasing treadmill speed from 12 m/min (low-speed) used in aim 1 to 20 m/min (high-speed), and mice were also given either the standard or supplemented diet. Cortical geometry and trabecular architecture, structural- and tissue-level mechanical properties, and bone metabolism markers were measured. Differences between low- and high-speed exercised groups and differences in changes from baseline 15-week old mice were used to determine effects of increasing exercise intensity on bone health.

Hypothesis 3: Increases in cortical bone area and structural-level strength from combining a long-term exercise program with a mineral-supplemented diet will remain

after an equally-long period of non-exercise in the adult skeleton. These increases will be maintained after exercise if the diets remain unchanged.

> **Aim 3**: The 8-week diet and exercise program from aim 1 was again used in 16-week old male mice. Half the mice were sacrificed at the end of the 8-week program, and the other half remained in their cages for an additional 8 weeks of detraining. During detraining, all mice remained on the same diet they were given since day 1. Cortical geometry and trabecular architecture, structural- and tissue-level mechanical properties were measured at baseline, after 8 weeks of treatment, and after 8 weeks of treatment plus eight weeks of detraining. Differences between mice sacrificed immediately after exercise and mice sacrificed after detraining were used to determine long-term effects of exercise and the ability to maintain exercise changes after cessation of exercise.

Aim 1 is addressed in Chapters 2 and 3. Combining exercise and the supplemented diet led to the greatest cortical bone mass and structural-level strength of all groups, more than exercise or supplemented diet alone. This increase comes at no expense to tissue quality, as resistance to microcrack accumulation is higher with the combined exercise and supplemented diet than exercise alone after 3 weeks.

Chapter 4 addresses Aim 2 using 4 weeks of high-speed treadmill exercise. Mice on the high-speed exercise program had significantly lower body weight and tibia length than all other mice, regardless of diet. These differences came at no expense to cortical or

trabecular bone mass or mechanical properties. The supplemented diet significantly increased cortical bone mass only in exercised groups.

Aim 3 is addressed in chapter 5. After 8 weeks, exercised mice on the supplemented diet had significantly greater cortical bone mass, trabecular bone volume, and structural-level mechanical strength than exercised mice on the control diet. After 8 weeks of detraining, there were no significant effects of exercise for mice on the supplemented diet. All mice on the supplemented diet had significantly greater cortical and trabecular bone mass and structural- and tissue-level mechanical properties compared to mice on the control diet after 8 and 16 weeks. This study suggests the supplemented diet is more impactful on bone health and effective in maintaining benefits to bone long-term.

This body of work demonstrates that combining exercise with a mineral-supplemented diet increased cortical and trabecular bone mass and structural-level mechanical strength over exercise with the control diet in young adult mice. These increases come at no expense to tissue quality or other exercise effects on bone. The supplemented diet without exercise also increased cortical and trabecular bone mass and structural-level mechanical strength over the control diet without exercise. Combining exercise with the supplemented diet led to the same peak bone mass and mechanical strength, but in less time, as evidenced by differences in effects of diet after 3-four weeks, 8 weeks, and 16 weeks. This work sheds light on the types (structural versus tissue level) and timing of bone adaptation to exercise and how increasing dietary mineral supply

and bone mineral availability can increase the rate of change without negatively affecting bone health. Further work is needed to determine the optimal exercise intensity and duration and optimal amounts of dietary minerals and other nutrients required for maximizing increases in bone mass and mechanical strength.

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CHAPTER TWO

CALCIUM- AND PHOSPHORUS-SUPPLEMENTED DIET INCREASES BONE MASS AFTER SHORT-TERM EXERCISE AND INCREASES BONE MASS AND STRUCTURAL STRENGTH AFTER LONG-TERM EXERCISE IN MICE

ABSTRACT

Exercise has long-lasting benefits to bone health that may help prevent fractures by increasing bone mass, bone strength, and tissue quality. Long-term exercise of 6 to 12 weeks in rodents increases bone mass and bone strength. However, a three-week, short-term exercise program limits increases in bone mass and structural strength, compared to growth-related increases in non-exercised controls. Short-term exercise does increase tissue strength, suggesting that exercise may create competition for minerals that favors initially improving tissue-level properties over structural-level properties. It was therefore hypothesized that adding calcium and phosphorus supplements to the diet may prevent decreases in bone mass and structural strength during a short-term exercise program, while leading to greater bone mass and structural strength than exercise alone after a long-term exercise program. A short-term exercise experiment was done for 3 weeks, and a long-term exercise experiment was done for 8

weeks. For each experiment, male 16-week old C57BL/6 mice were assigned to 4 weight-matched groups – exercise and non-exercise groups fed a control or mineral-supplemented diet. Exercise consisted of treadmill running at 12 m/min, 30 min/day for 7 days/week. After 3 weeks, exercised mice fed the supplemented diet had significantly increased tibial tissue mineral content (TMC) and cross-sectional area over exercised mice fed the control diet. After 8 weeks, tibial TMC, cross-sectional area, yield force, and ultimate force were greater from the combined treatments than from either exercise or supplemented diet alone. Serum markers of bone formation (PINP) and resorption (CTX) were both decreased by exercise on day 2. In exercised mice, day 2 PINP was significantly positively correlated with day 2 serum Ca, a correlation that was weaker and negative in non-exercised mice. Increasing dietary mineral consumption during an exercise program increases bone mass after 3 weeks and increases structural strength after 8 weeks, making bones best able to resist fracture.

INTRODUCTION

Bone fragility fractures are common and costly injuries affecting more than 1.5 million people and costing $12-$18 billion in direct care each year [1]. These fractures are often attributed to reduced bone mass. Exercise has long-lasting benefits to bone health that may help prevent fragility fractures by increasing bone mass, structural-level (whole bone) strength, and tissue quality [2–6]. Animal models have shown increases in bone mass (cross-sectional area, bone mineral content) and structural-level strength (yield force, ultimate force) after long-term exercise programs of six-twelve weeks [7–10].

However, short-term exercise for a duration of three weeks can slow growth, limiting increases in bone mass and structural-level strength, and even causing a reduction in strength compared to non-exercised controls [11,12]. Short-term exercise can still be beneficial, increasing tissue-level strength and quality (ultimate stress, damage resistance, and mineral-to-matrix ratio) [11]. The difference in bone's response to short-term exercise at the structural and tissue levels suggests that there may be a competition for minerals needed to increase bone quantity versus bone quality and structural- versus tissue-level strength. Bone seems to favor increasing tissue quality over increasing bone mass and structural strength in a short-term exercise program. The limits on bone adaptation seen in short-term exercise suggest that standard dietary amounts of mineral may be insufficient for optimal adaptation of bone in response to exercise.

Calcium (Ca) and phosphorus (P) are important regulators of bone growth and mineralization. Threshold amounts of 700-1300 mg Ca and P per day are needed from dietary sources and supplements to maintain optimal bone mass and strength [13–15]. Under healthy non-exercise conditions, dietary concentrations of Ca and P above the threshold range offer no increases in bone mass and strength [13,16]. However, during skeletal growth, there is a greater dietary recommended value for minerals than during adulthood in order to meet the demands of bone growth from modeling [14]. Also, adding Ca supplements to a diet can restore bone mass and strength under abnormal conditions that reduce bone mass, such as following ovariectomy in rodents [17,18]. Since short-term exercise limits increases in bone mass and structural-level strength in

mice, adding mineral supplements to the diet during exercise may be a strategy to increase bone mass and structural-level strength without extending the duration of an exercise program.

Bone mass has been increased by combining exercise with a Ca-supplemented diet in human children and adolescents, but this only occurred after lengthy exercise programs [19–22]. There is also evidence to suggest long-term exercise may cause a sustained increase in demand for minerals. For example, serum vitamin D_3 increases, serum parathyroid hormone decreases, and intestinal absorption of Ca and P increases in rodents after exercise programs of at least five weeks [10,23,24]. This change in markers of bone metabolism with exercise suggests that exercise maintains increased demand for minerals even after lengthy exercise programs, which may ultimately be what leads to increases in bone mass and structural-level strength after lengthy exercise programs.

Combining a mineral-supplemented diet with a lengthy exercise program may be able to supply sufficient amounts of minerals to prevent short-term effects of exercise as well as lead to long-term greater increases in bone mass and structural strength than exercise with a standard diet. Thus, it was hypothesized that combining a mineral-supplemented diet with exercise would prevent decreases in bone mass and structural-level strength after 3 weeks of exercise, and increase bone mass and structural-level strength after 8 weeks of exercise, compared to exercise with a standard diet.

METHODS

Animals and Treatments – Three Weeks of Exercise x Mineral-Supplemented Diet

All animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals. Eighty male C57BL/6 mice, 27.0 ± 1.4 g mean body weight, were purchased from Charles River Laboratories (Wilmington, MA) at 14 weeks of age and given 2 weeks to acclimate. Starting on experiment day 1, at 16 weeks of age, mice were randomly assigned to one of 4 weight-matched groups – a non-exercise group fed the control diet (C), a non-exercise group fed the supplemented diet (D), an exercise group fed the control diet (CE), and an exercise group fed the supplemented diet (DE). The control diet consisted of an AIN-93G diet (TestDiet®, Richmond, IN) modified by adding dicalcium phosphate to contain 0.5% Ca and 0.5% P. The supplemented diet was modified by adding dicalcium phosphate and calcium carbonate to contain 2% Ca and 1% P. Ca, P, and Ca:P ratio were all increased to increase serum Ca by increasing passive intestinal Ca absorption [25–27]. The control diet contained 3.90 kcal/g with an energy distribution of 65.0% carbohydrates, 16.3% fat, and 18.7% protein while the supplemented diet had 3.69 kcal/g with an energy distribution of 63.0% carbohydrates, 17.2% fat, and 19.7% protein. All other nutrients were equivalent between the two diets. The short-term exercise program consisted of running on a 5° incline treadmill at 12 m/min, 30 min/day for 21 consecutive days [11,12]. Mice were gradually increased to a maximum speed of 12 m/min in the first 3 days of exercise. On day 22, at 19 weeks of age, mice were sacrificed, and left tibiae were harvested for analysis.

Animals and Treatments – Eight Weeks of Exercise x Mineral-Supplemented Diet

One-hundred twelve male, C57BL/6 mice, 25.9 ± 0.8 g mean body weight were purchased from Charles River Laboratories (Wilmington, MA) at 14 weeks of age and given 2 weeks to acclimate. Starting on experiment day 1, at 16 weeks of age, mice were randomly assigned to one of 4 weight-matched groups – a non-exercise group fed the control diet (C), a non-exercise group fed the supplemented diet (D), an exercise group fed the control diet (CE), and an exercise group fed the supplemented diet (DE). The control diet was the same as used in the 3-week exercise experiment. The supplemented diet was modified to contain 5% Ca and 1% P. The control diet contained 3.90 kcal/g with an energy distribution of 65.0% carbohydrates, 16.3% fat, and 18.7% protein while the supplemented diet had 3.39 kcal/g with the same energy distribution. All other nutrients were equivalent between the two diets.A greater amount of dietary Ca was used in the 8-week experiment to attempt to increase the magnitude of effects of diet and the power for detecting effects of diet. Exercise consisted of running on a 5° incline treadmill at 12 m/min, 30 min/day for 58 consecutive days. On day 59, at 24 weeks of age, mice were sacrificed, and left tibiae were harvested for analysis.

Cortical Geometry Measurements

Whole tibiae from 3- and 8-week groups were scanned with a voxel size of 18 μm using a GE/EVS MS-8 micro-CT specimen scanner and then analyzed with MicroView software (General Electric Health Care) and custom written Matlab (Math Works, Inc., Natick,

MA) scripts. A 90-µm thick transverse section from a standard site located 21.7% of the distance from the tibia-fibula junction to the proximal end of the tibia was chosen for measurement of cortical geometry metrics – tissue mineral content (TMC), volumetric tissue mineral density (TMD), cross-sectional area, and moment of inertia about the anterior-posterior axis. TMC and TMD were calculated using a fixed threshold and standard volume of interest. This section is located approximately at the center of the mechanical testing region. Another 90-µm thick transverse section at the fracture site was analyzed for cortical geometry measurements used in calculations of tissue-level mechanical properties (moment of inertia, distance from neutral axis).

Mechanical Testing

Structural- and tissue-level mechanical properties were measured in all 3- and 8-week experimental groups. Structural-level properties (force, deformation, stiffness, work) were measured from a 4-point bending to failure test (3-mm inner and 9-mm outer spans). Tibiae were loaded to failure with the medial side of the mid-diaphysis in tension under displacement control at 0.025 mm/sec at a data sampling rate of 30 Hz. Tissue-level mechanical properties (stress, strain, modulus, toughness) were estimated using beam bending theory with geometric measurements (moment of inertia about anterior-posterior axis, distance from centroid to medial side of the bone) from micro-CT data at the fracture site [28].

Serum Analysis

Fasting blood samples taken before daily exercise were collected by submandibular vein bleeding.   For the 3-week experiment, blood samples were collected on days 2 and 22. For the 8-week experiment, blood samples were collected 10 days before the start of the exercise program and on days 2, 30, and 59. Serum was isolated by centrifuge, and Ca and P concentrations were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). ELISAs were used to measure markers of bone formation and resorption – pro-collagen type I amino-terminal peptide (PINP) and carboxy-terminal collagen crosslinks (CTX) (Immunodiagnostic Systems, Inc., Scottsdale, AZ) on samples from day 22 of the 3-week experiment and days 2 and 59 of the 8-week experiment. All manufacturers' kit instructions were followed, including the use of the standards provided for obtaining standard curves.

Statistical Analysis

Data from the 3- and 8-week experiments were analyzed separately.   For each experiment, cortical geometry measurements, mechanical properties, and serum metabolite measurements were tested by Two-way ANOVA with Tukey's post-hoc tests to determine if the individual effects of diet or exercise were significant (p < 0.05) and if the combined treatments had a significant interactive effect. Paired t-tests were used to compare the concentrations of serum metabolites measured at different times. Simple linear regressions were run to develop correlations between measurements of

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cortical bone geometry, bone metabolism markers, and bone mechanical properties. Multiple linear regressions were also run to determine the relationship between measurements of cortical bone geometry and bone metabolism markers on bone mechanical properties.

RESULTS

Supplemented Diet Increased Cortical Bone without Affecting Mechanical Properties after Three Weeks

There were no significant differences in mean body weight between any of the groups on day 22 (28.9 ± 1.3 g for C group, 28.7 ± 1.4 g for D group, 28.1 ± 1.6 g for CE group, and 28.3 ± 1.6 g for DE group). Exercise had no effect on tibial cortical TMC, TMD, cross-sectional area, and moment of inertia about the anterior-posterior axis, but there was a significant effect of diet on TMC ($p < 0.001$, Two-way ANOVA, Figure 2.1) and cross-sectional area ($p < 0.01$, Two-way ANOVA, Figure 2.1). Mice on the combined diet and exercise protocol had significantly greater cortical TMC and area than mice that exercised while on the control diet (Figure 2.1). For mechanical properties at the structural level, there was a significant ($p < 0.05$ Two-way ANOVA, Figure 2.2) effect of exercise on ultimate deformation. Exercised mice on the control diet had significantly higher ultimate deformation than exercised mice on the supplemented diet and non-exercised mice on the control diet. At the tissue-level, there was a significant ($p < 0.05$, Two-way ANOVA, Figure 2.3) interactive effect between diet and exercise on ultimate

stress, and the exercised mice on the control diet had significantly greater ultimate stress than exercised mice on the supplemented diet.

Exercise and Supplemented Diet Combined Increased Cortical Bone and Mechanical Properties More Than Exercise or Diet Alone After Eight Weeks

Eight weeks of exercise coupled with a standard diet significantly increased TMC and TMD ($p < 0.05$ Two-way ANOVA, Figure 2.4). The supplemented diet had a significant main effect that increased all measurements of mineralization and cortical geometry – TMC, TMD, cross-sectional area, and moment of inertia ($p < 0.001$ Two-way ANOVA, Figure 2.4). Combining the supplemented diet and exercise led to a significant increase in tibial TMC, cross-sectional area, and moment of inertia compared to exercise alone ($p < 0.05$ Tukey's tests, Figure 2.4). However, for mice on the supplemented diet, exercise had no effect on TMD. Exercise did not affect cortical bone geometry for mice on the supplemented diet.

Exercise had a significant main effect that increased structural-level strength (yield force, $p < 0.001$ and ultimate force, $p < 0.05$) and deformation measures (yield deformation, $p < 0.01$ and pre-yield work, $p < 0.01$) (Figure 2.5). Deformation measures at the tissue-level (yield strain, $p < 0.001$ and pre-yield toughness, $p < 0.05$) were also significantly increased (Figure 2.6). The supplemented diet only had significant main effects at the structural-level, increasing yield force, ultimate force, stiffness, and pre-yield work ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.05$, respectively Two-way ANOVA,

Figure 2.5). Combining the supplemented diet with exercise led to significantly greater yield force, ultimate force, stiffness and pre-yield work than exercise alone ($p < 0.05$ Tukey's tests, Figure 2.5) and significantly greater yield force, ultimate force, pre-yield work, yield strain and pre-yield toughness than supplemented diet alone ($p < 0.05$ Tukey's tests, Figure 2.5, Figure 2.6). There were no differences in tissue-level mechanical properties between the exercise groups.

Exercise Decreased Serum Bone Metabolism Markers and Increased Serum Mineral Supply on Day 2

There were no significant effects of exercise or diet on day 22 serum CTX, PINP, or PINP/CTX ratio in the 3-week experiment (data not shown). Exercise had a significant main effect that decreased serum CTX and PINP in mice on day 2 of the 8-week experiment ($p < 0.001$, $p < 0.05$, respectively, Two-way ANOVA, Figure 2.7). There were no differences in CTX or PINP between the two exercise groups in either the 3- or 8-week experiment. However, the combined diet and exercise group had a significantly higher day 2 PINP/CTX ratio than the exercise-only group ($p < 0.05$ Tukey's test, Figure 2.7).

In the 3-week experiment, exercise had a significant main effect that increased day 2 serum Ca and P ($p < 0.01$, $p < 0.05$, respectively, Two-way ANOVA, Figure 2.8). For the 8-week experiment, exercise had a significant main effect that increased serum Ca on days 2 and 30, as well as serum P on day 2 ($p < 0.001$, $p < 0.05$, $p < 0.001$, respectively,

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Two-way ANOVA, Figure 2.9). The supplemented diet had a significant main effect that

increased serum Ca on days 2, 30, and 59 and increased serum P on day 2 ($p < 0.001$, $p <$

0.001, $p < 0.05$, $p < 0.05$, respectively, Two-way ANOVA, Figure 2.9). On day 2, serum Ca

was significantly higher in the combined supplemented diet and exercise group than in

the exercise-only group ($p < 0.05$ Tukey's test, Figure 2.9). From baseline to day 2, there

was a significant increase in serum Ca in the exercise-only group ($p < 0.05$, paired t-test,

Figure 2.9) and a marginally significant increase in serum Ca in the supplemented diet-

only and combined diet and exercise groups ($p = 0.0567$, $p = 0.0570$, respectively, paired

t-test, Figure 2.9). Serum P was significantly increased from baseline in these three

groups ($p < 0.05$, paired t-test, Figure 2.9). Both the supplemented diet and exercise

increased serum mineral supply, and combining the two increased serum Ca and P more

than either treatment alone.

Linear Regression Analysis

For the 3-week experiment, cortical TMC and cross-sectional area were significantly

correlated with yield force ($r = 0.575$ and $r = 0.600$, respectively) in non-exercised mice

(Table 2.1). These correlations were weaker and not significant for exercised mice. Day

22 serum Ca, PINP, and CTX were not significantly correlated with cortical TMC, area,

and yield force in non-exercised mice. However, in exercised mice, day 22 serum Ca was

significantly negatively correlated with yield force ($r = -0.379$). For the 8-week

experiment, TMC and area were significantly positively correlated with yield force for

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both exercised and non-exercised mice (0.442 < r < 0.628). In exercised mice, day 2 serum Ca was significantly positively correlated with day 2 serum PINP, r = 0.516, while for non-exercised mice there was a negative non-significant correlation of r = -0.189 (Table 2.2). Day 2 serum PINP was significantly positively correlated with TMC, r = 0.447, in exercised mice while non-exercised mice had a non-significant less-positive correlation of r = 0.159. The opposite effect occurred in CTX as day 2 serum CTX was significantly positively correlated with TMC, r = 0.475, in non-exercised mice while exercised mice had a non-significant less-positive correlation of r = 0.316. Multiple linear regressions on the 3-week and 8-week experiment data did not reveal any further insight into the relations between cortical bone geometry, bone metabolism markers, and bone mechanical properties.

Synthesizing the main effects of diet and exercise on bone cortical geometry, metabolism markers, and mechanical properties (Table 2.3) shows that exercise increased serum mineral supply and decreased serum markers of bone turnover after one day. Exercise had no impact on cortical geometry after 3 or 8 weeks, while exercise increased both structural- and tissue-level mechanical properties after 8 weeks. The supplemented diet increased serum Ca for 8 weeks, but did not affect bone turnover. Most measurements of mineralization and cortical geometry were increased by the supplemented diet after 3 and 8 weeks. Structural-level mechanical properties were increased with the supplemented diet only after eight weeks. Thus, exercise increased mechanical properties without affecting cortical bone mass while the supplemented

diet increased cortical bone mass and structural-level mechanical properties for exercised and non-exercised mice.

DISCUSSION

Mice exercised for 3 weeks while fed the control diet did not increase cortical area, TMC, or structural strength (Figure 2.1, Figure 2.2). However, mice exercised for 3 weeks while fed the mineral-supplemented diet had significantly increased cortical area and TMC, compared to exercised mice fed the control diet (Figure 2.1). Extending the exercise protocol to 8 weeks, exercised mice fed the control diet had increased TMC, TMD, and mechanical properties (yield deformation, yield strain, and pre-yield work) compared to control mice (Figure 2.4, Figure 2.5, and Figure 2.6). Combining the supplemented diet with 8 weeks of exercise significantly increased cortical area, moment of inertia, TMC, and structural-level mechanical properties (yield force, ultimate force, stiffness, and pre-yield work) compared to exercise alone (Figure 2.4, Figure 2.5). Increasing dietary mineral consumption during exercise results in an early increase in cortical area and TMC that is maintained when continued for 8 weeks. These data suggest diets containing standard amounts of Ca and P may not be sufficient for maximizing increases in cortical bone size, mineralization and structural-level strength with exercise, even if the duration of the exercise program is extended.

Considering the main treatment effects of diet and exercise, the supplemented diet increased bone mass more than exercise, while exercise increased bone strength at the

structural-level more than the diet. Following 8 weeks of treatment, the supplemented diet had significant positive effects on a few measurements of structural strength, while exercise had significant positive effects on many measurements of structural strength and a few measurements of tissue strength (Figure 2.4, Figure 2.5, Table 2.3). After 8 weeks, the individual effects of diet and exercise were more pronounced when the supplemented diet and exercise were combined, as mice in that group had the greatest yield force, ultimate force, and pre-yield work of all groups. Thus, combined long-term treatments may best increase bone mass and strength.

Figure 2.10 shows a schematic illustration of how tibial cortical area changes over time in male C57BL/6 mice subjected to mineral-supplemented diet and/or exercise intervention. Exercise alone has no effect or causes a trend towards a decrease in cortical TMC and/or area after 3 weeks [11], but exercise increases TMC and TMD after 8 weeks. Adding the mineral-supplemented diet with exercise prevents decreases in TMC and/or area after 3 weeks, and leads to higher TMC and area than exercise alone after 8 weeks.

Consistent with other studies, long-term exercise had a significant positive effect on TMD, and mechanical properties at both the structural and tissue levels (Table 2.3) [29,30]. This 8-week exercise program increased structural-level strength without increasing cortical area, suggesting changes in bone tissue quality may be occurring to increase fracture resistance independent of bone quantity. Bones from exercised mice showed increased bending and fracture resistance (yield force, yield deformation, yield

strain, pre-yield work, pre-yield toughness) compared to bones from non-exercised mice (Table 2.3). Additionally, all exercised bones had increased post-yield strength (post-yield work and deformation) compared to the non-exercised bones (data not shown). Increases in TMC and area from combining the supplemented diet and exercise did not lead to loss of post-yield strength or increased brittleness. There may likely be tissue-level changes with exercise that increase mechanical strength independent of dietary mineral supply.

Exercise caused a quick reduction in bone turnover as there were significant negative effects on serum CTX and PINP on day 2 of the 8-week experiment (Figure 2.7). Combining the supplemented diet with exercise led to a higher PINP/CTX ratio on day 2 than exercise alone. This higher formation-favored state shows the supplemented diet only had an effect on bone metabolism when combined with exercise. The supplemented diet did not independently affect bone metabolism on any day measured, so changes in bone formation and/or resorption that could account for the increased TMC, TMD, area, and moment of inertia in non-exercised mice on the supplemented diet may be occurring at some intermediate time point not measured.

There were no group differences and no effects of diet or exercise on serum CTX and PINP on day 59 (Figure 2.7). Thus, if the diet and exercise program had been continued beyond the 8-week duration here, it seems unlikely that differences in bone mass between the groups would change. There was a decline in bone turnover markers for the non-exercising mice from day 2 to day 59 (Figure 2.7). This change in bone turnover

could be due to gradual decline from aging or signal a more acute shift in bone metabolism [31,32] The mice used in this study were approximately 4 months old at the start of the diet and exercise programs, and C57BL/6 mice have reduced bone growth after 5 months of age. It is possible that mice used in this study were of the age where skeletal growth and development plateaued sometime during the study, causing the change in bone turnover from day 2 to day 59.

On day 2 of the 3-week experiment, exercise significantly increased serum Ca and P (Figure 2.8). The 2% Ca supplemented diet alone did not have a significant effect on serum Ca or P. Therefore, dietary Ca was elevated to 5% in the 8-week experiment to increase serum mineral supply to increase the effects of diet. On day 2 of the 8-week experiment, both the supplemented diet and exercise significantly increased serum Ca and P, and the concentrations of Ca and P were further increased when the diet and exercise were combined (Figure 2.9). The increase in serum Ca and P with exercise indicates that exercise increased serum mineral availability independent of dietary supply. There could be separate mechanisms for increasing serum Ca and P with the supplemented diet or exercise, and they may work together to cause greater serum mineral concentrations when the supplemented diet and exercise are combined [14,33].

Serum P did not remain elevated with diet or exercise in measurements made beyond day 2 (Figure 2.9). Dietary P was increased in the supplemented diet to attempt to increase supply of serum P for increasing bone mass. Since increasing dietary Ca alone has little effect on serum Ca and bone mass [16,34,35], it was necessary to also increase

dietary P to increase passive intestinal absorption of Ca and total serum mineral supply. Additionally, increasing dietary P can increase TMC and bone structural strength when dietary Ca is also increased [25,26]. The amount of P in the supplemented diet here was lower than Ca which may explain why serum P was not elevated for the same duration or magnitude as Ca. The dietary P required for increasing bone mass may not be as high as Ca, so exercise may not cause as high an increase in demand for P as for Ca. To our knowledge, this is the first study to show exercise can have an effect on bone turnover and serum mineral levels within 24 hours in mice. With such early rapid changes in bone metabolism and mineral demand, increasing dietary mineral supply at the start of exercise programs may be necessary to increase PINP/CTX ratio to maximize gains in bone mass with exercise.

After 3 weeks, cortical TMC and cross-sectional area were significantly positively correlated with yield force in non-exercised mice (Table 2.1). These correlations were weaker and non-significant in exercised mice. This difference suggests increasing bone mass may not be as beneficial towards increasing structural strength with short-term exercise. There could be a change in tissue quality with exercise that causes other factors besides cortical bone mass to have a greater influence on yield force. After 8 weeks of exercise, cortical TMC and area were significantly positively correlated with yield force in both exercised and non-exercised mice (Table 2.2). Thus, for lengthier exercise programs, increases in bone mass could be more beneficial towards increasing structural strength.

There was a different relation between serum Ca and PINP, depending on whether or not the mice were exercised (Table 2.2). In mice exercised for 8 weeks, there was a significant positive correlation between day 2 serum Ca and day 2 serum PINP ($r = 0.516$,Table 2.2). PINP was a stronger predictor of cortical bone mass measurements (TMC, TMD, and area) in exercised mice than non-exercised mice. Increasing PINP early in an exercise program by increasing serum Ca may be most beneficial for increasing bone mass long-term in exercised mice. In non-exercised mice, day 2 serum Ca was non-significantly negatively correlated with PINP and CTX, suggesting increasing blood Ca supply has minimal effect on or may suppress bone turnover without exercise. Thus, increasing dietary Ca consumption is more impactful to bone turnover when combined with exercise.

Dietary mineral supply may affect the timing of exercise effects on bone. With the lower supply of minerals in the control diet, it is possible that exercised tibiae prioritized increasing some tissue-level mechanical properties over increasing cortical TMC and area after 3 weeks as ultimate stress was higher in the exercise-only group than in the combined supplemented diet and exercise group (Figure 2.2, Figure 2.3). Exercised mice on the supplemented diet may have prioritized increasing cortical area over tissue-level mechanical properties after 3 weeks. Differences in tissue-level mechanical properties between the exercise groups were not present after 8 weeks, suggesting mineral availability may only affect short-term changes to tissue-level properties.

Bone size, shape and architecture (cortical area, thickness, and section modulus) are contributing factors for preventing fractures [36]. Because fractures are a failure of bone to adapt to loading, increasing bone size, strength, and damage resistance may help prevent fractures. Short-term exercise can increase fatigue damage resistance in mice [11]. Exercise also increases mineral-to-matrix ratio and decreases carbonate/phosphate ratio possibly because exercise favors increasing tissue quality over increasing bone mass. If minerals are in short supply, increasing mineralization of existing tissue could be favorable to adding new tissue and increasing bone mass. This could increase tissue-level properties in a shorter time span. However, by not increasing bone mass in a short-term exercise program, bone would still be subjected to repetitive overloading that can lead to fractures. Since the mineral-supplemented diet increased bone mass in exercising mice after 3 and 8 weeks of exercise, dietary intervention also may be useful in preventing exercise-related fractures. Further work needs to be done to examine the effects of combining the mineral-supplemented diet with exercise on bone's resistance to fatigue damage and tissue level properties.

Most studies that examine dietary mineral requirements for optimal bone growth and bone mass accumulation do not involve exercise or only compare standard dietary Ca versus insufficient Ca [30,34]. As exercise creates an increased demand for minerals and alters bone metabolism, the threshold amounts of dietary minerals needed for optimal bone growth may be higher with exercise. Increasing dietary mineral consumption during an exercise program could increase blood mineral supply and allow greater

increases in cortical TMC and area in the short term, as well as increasing structural-level strength long term.

Acknowledgments
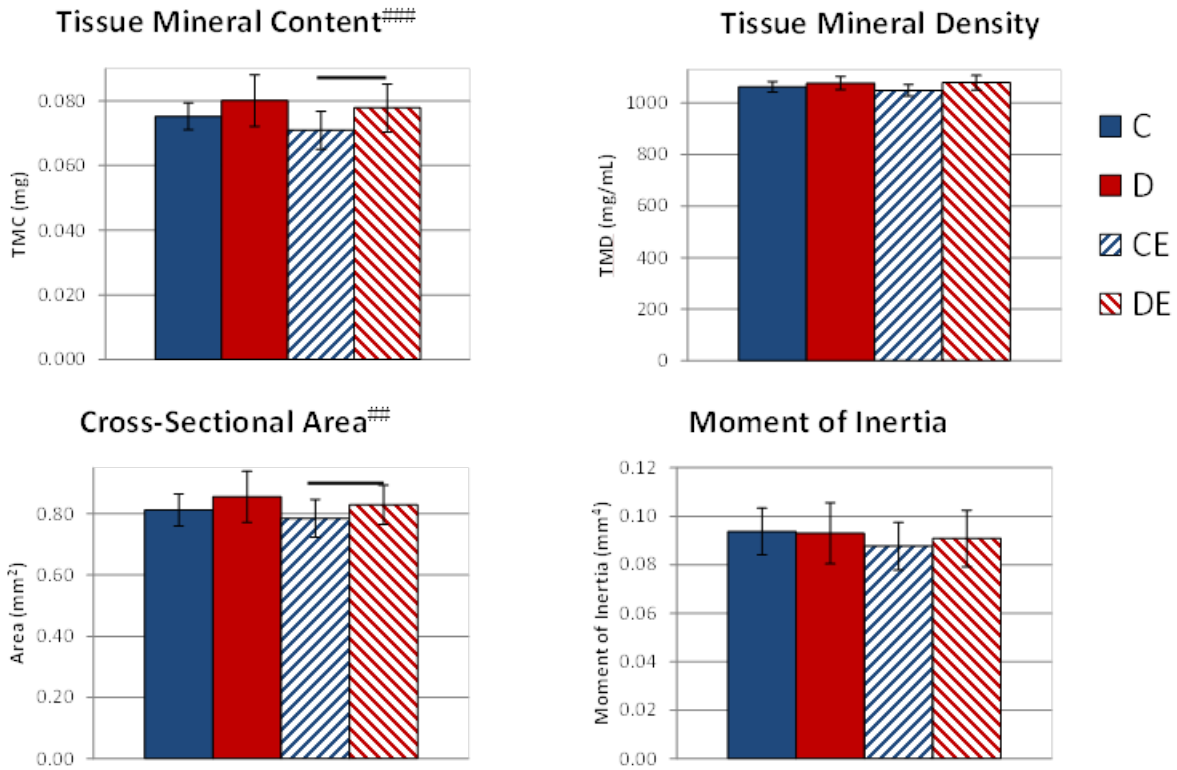
Figure 2.1. Mouse tibial cortical bone mineralization and cross-sectional geometric properties following 3 weeks of exercise x mineral-supplemented diet – tissue mineral content, volumetric tissue mineral density, cross-sectional area, and moment of inertia about the anteroposterior axis. Data shown as mean ± standard deviation. Exercised mice fed the control diet had significantly lower TMC and cross-sectional area compared to exercised mice fed the supplemented diet.

[#]Significant diet effect (##p < 0.01, ###p < 0.001, Two-way ANOVA).
Groups connected by horizontal bars are significantly different (p < 0.05, Tukey's test).

C – non-exercised mice fed the control diet
D – non-exercised mice fed the supplemented diet
CE – exercised mice fed the control diet
DE – exercised mice fed the supplemented

Figure 2.2. Tibial structural-level mechanical properties following 3 weeks of exercise x mineral-supplemented diet. Data shown as mean ± standard deviation. The exercised mice on the control diet had significantly greater ultimate deformation than exercised mice fed the supplemented diet and non-exercised mice on the control diet.

*Significant exercise effect ($p < 0.05$, Two-way ANOVA).

Groups connected by a horizontal bar are significantly different ($p < 0.05$, Tukey's test).

Figure 2.3. Tibial tissue-level mechanical properties following 3 weeks of exercise x mineral-supplemented diet. Data shown as mean ± standard deviation. Exercised mice on the control diet had significantly greater ultimate stress than exercised mice on the supplemented diet.

^Significant interactive effect ($p < 0.05$, Two-way ANOVA).

Groups connected by a horizontal bar are significantly different from each other ($p < 0.05$, Tukey's test).
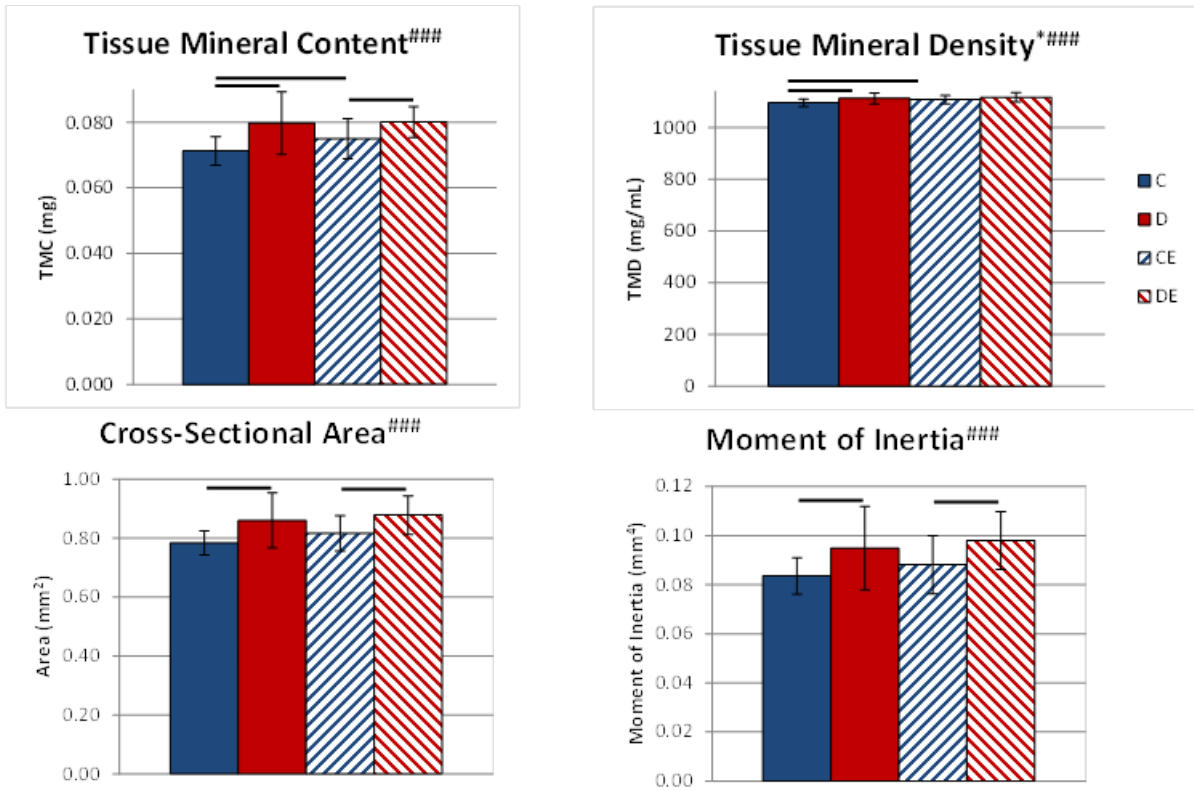
Figure 2.4. Tibial cortical bone mineralization and cross-sectional geometric properties following 8 weeks of exercise x mineral-supplemented diet. Data shown as mean ± standard deviation. The mineral supplemented diet significantly increased TMC, cortical area and moment of inertia in exercised and non-exercised mice, and vTMD in non-exercised mice.

*Significant exercise effect ($p < 0.05$, Two-way ANOVA).
#Significant diet effect (###$p < 0.001$, Two-way ANOVA).
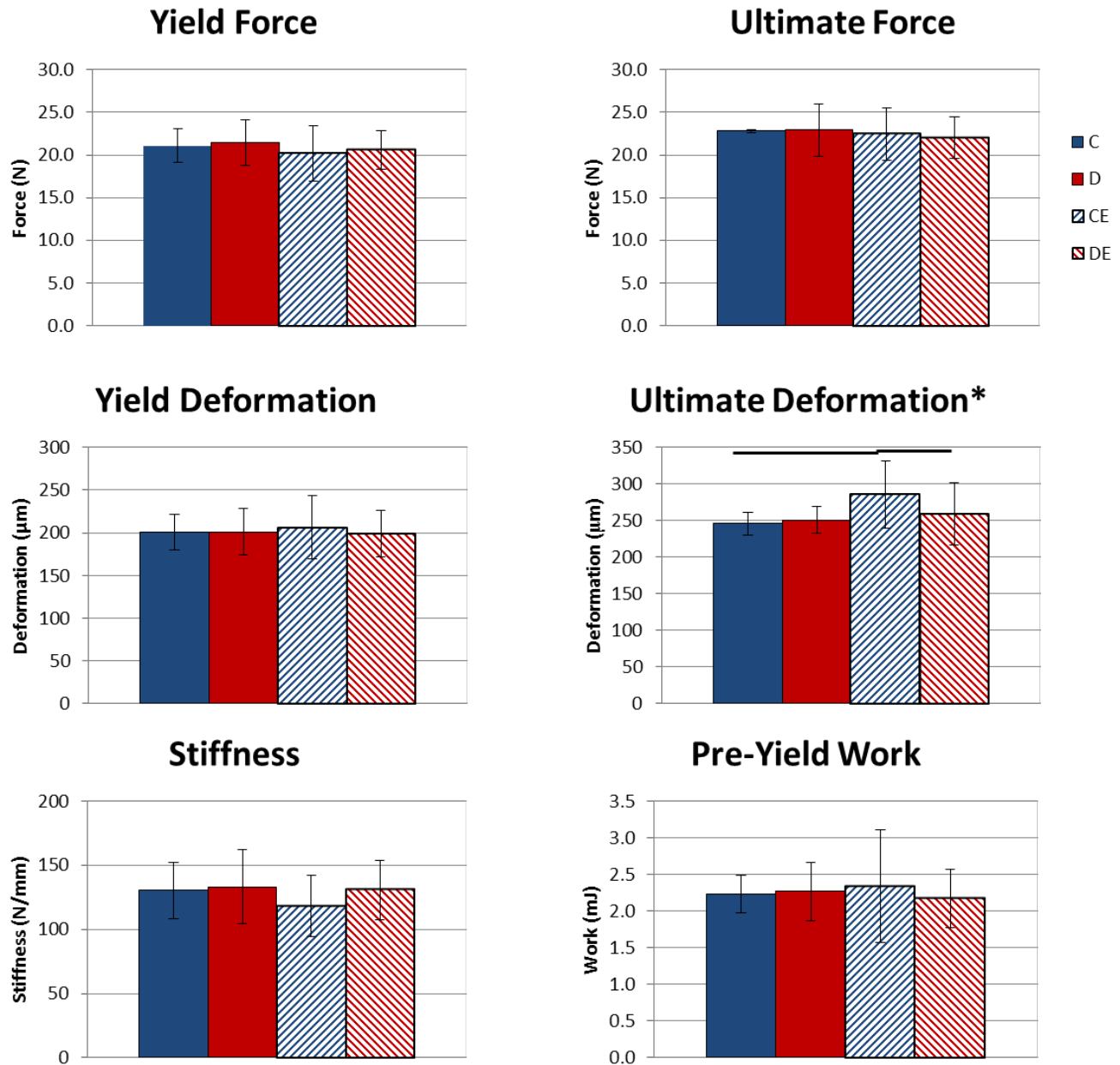Groups connected by a bar are significantly different from each other ($p < 0.05$, Tukey's test).
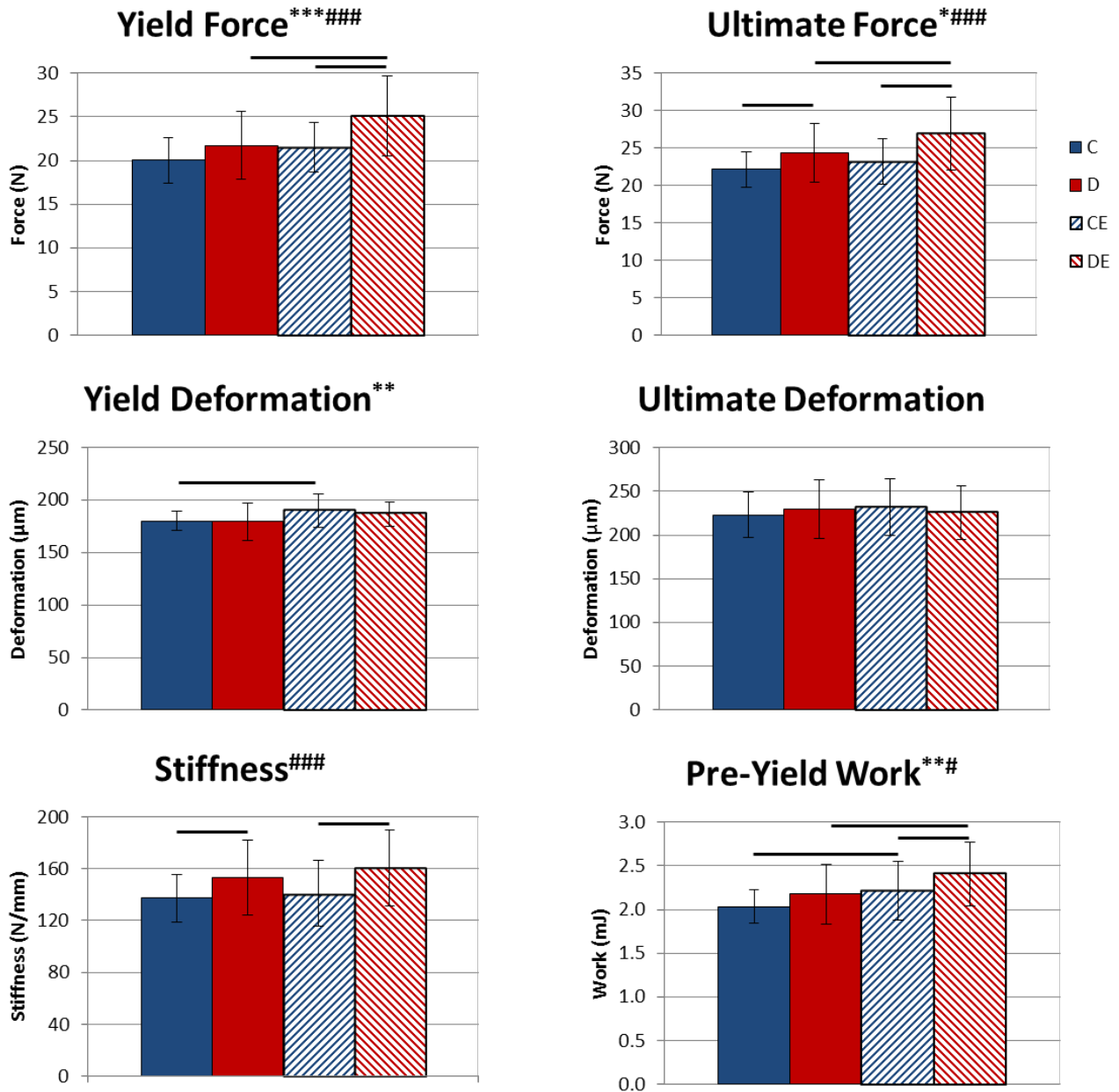
Figure 2.5. Tibial structural-level mechanical properties after 8 weeks of exercise x mineral-supplemented diet. Data shown as mean ± standard deviation. Combining the supplemented diet with exercise led to significantly greater structural strength than exercise or diet alone.

Significant exercise effect ($p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Two-way ANOVA).

#Significant diet effect (#$p < 0.05$, ###$p < 0.001$, Two-way ANOVA).

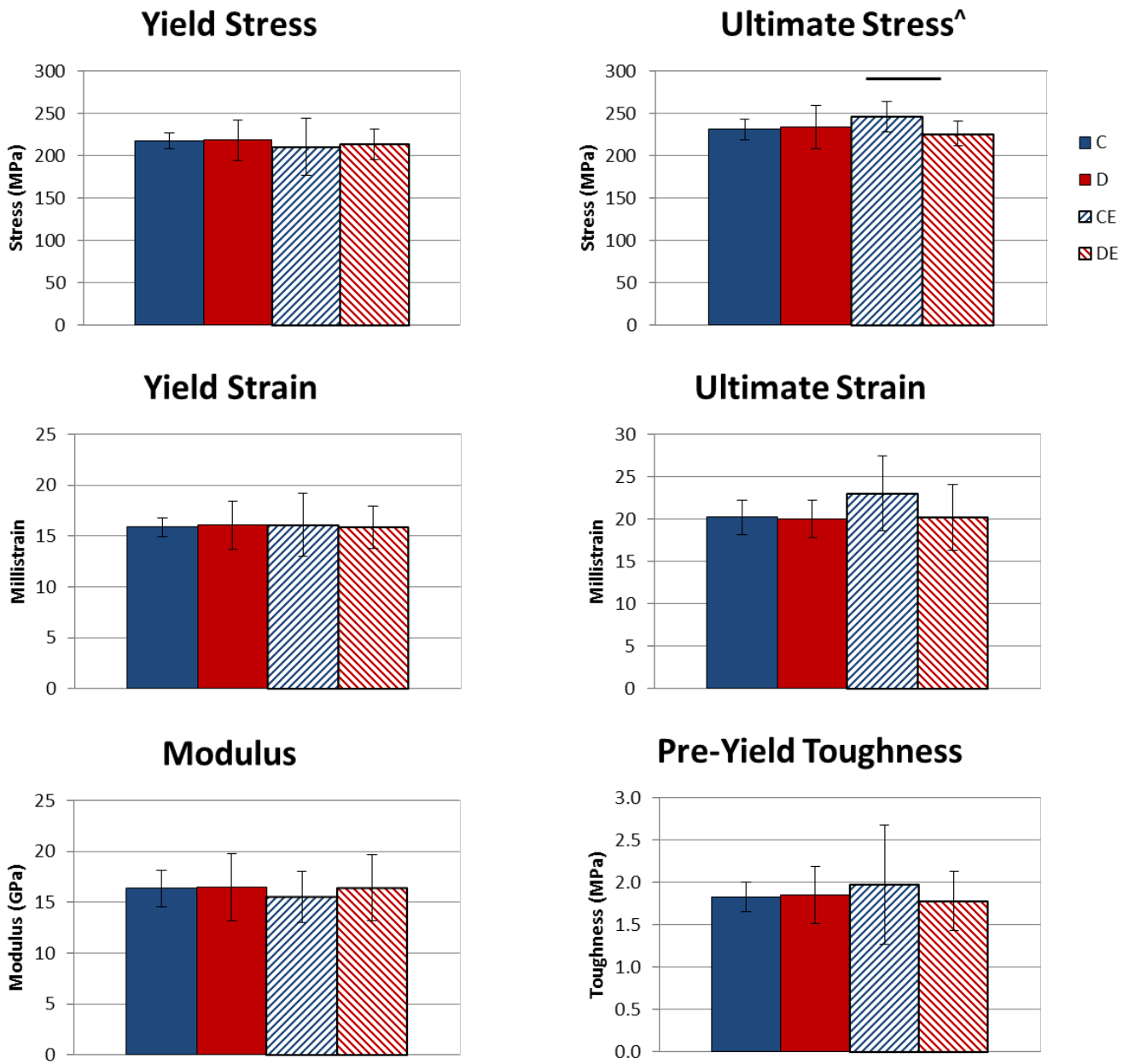Groups connected by a horizontal bar are significantly different from each other ($p < 0.05$, Tukey's test).
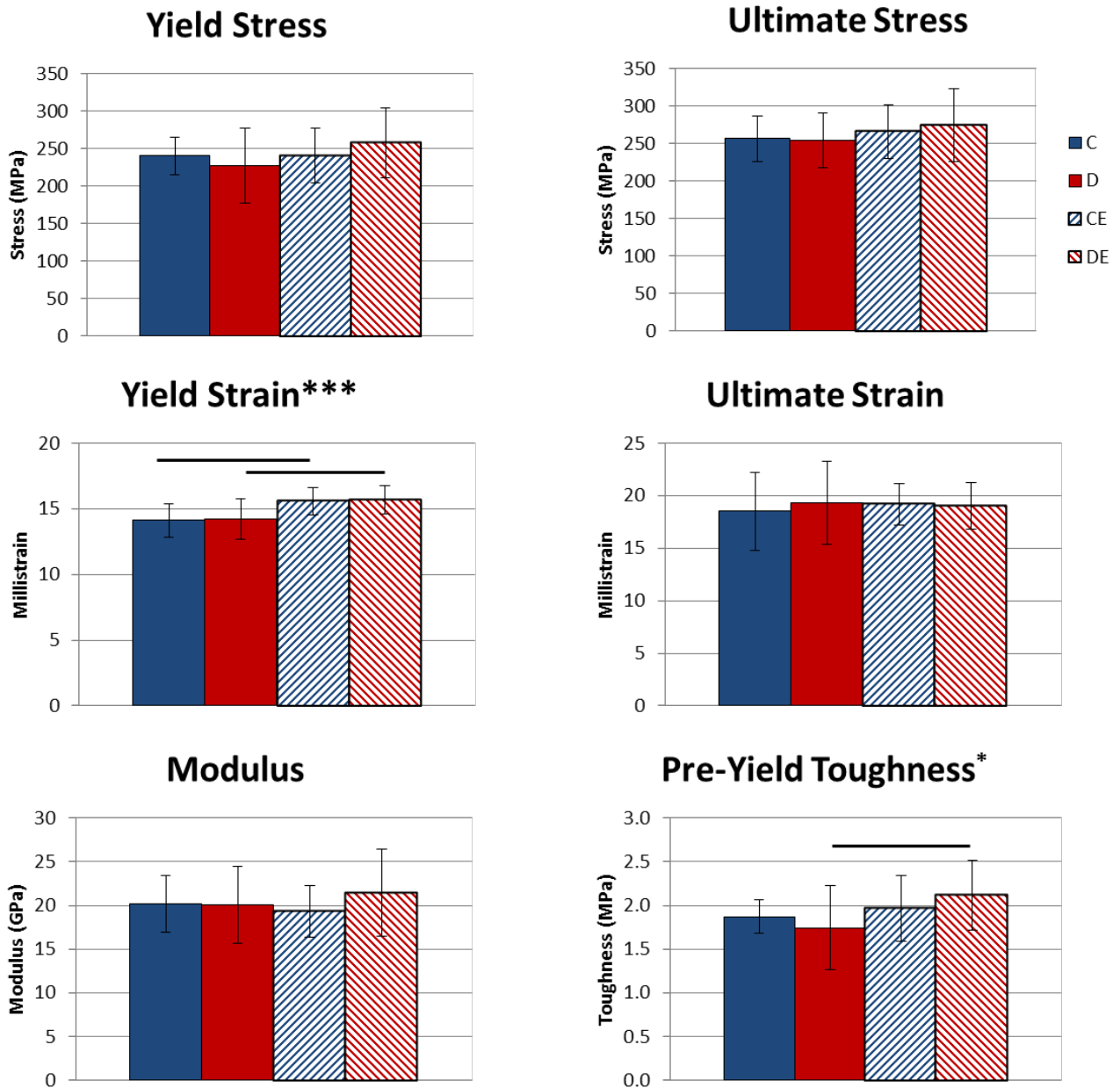
Figure 2.6. Tibial tissue-level mechanical properties following 8 weeks of exercise x mineral-supplemented diet. Data shown as mean ± standard deviation. Exercise significantly increased yield strain and pre-yield toughness. The supplemented diet did not affect tissue-level mechanical properties.

Significant exercise effect ($p < 0.05$, ***$p < 0.001$, Two-way ANOVA).

Groups connected by a horizontal bar are significantly different from each other ($p < 0.05$, Tukey's test).
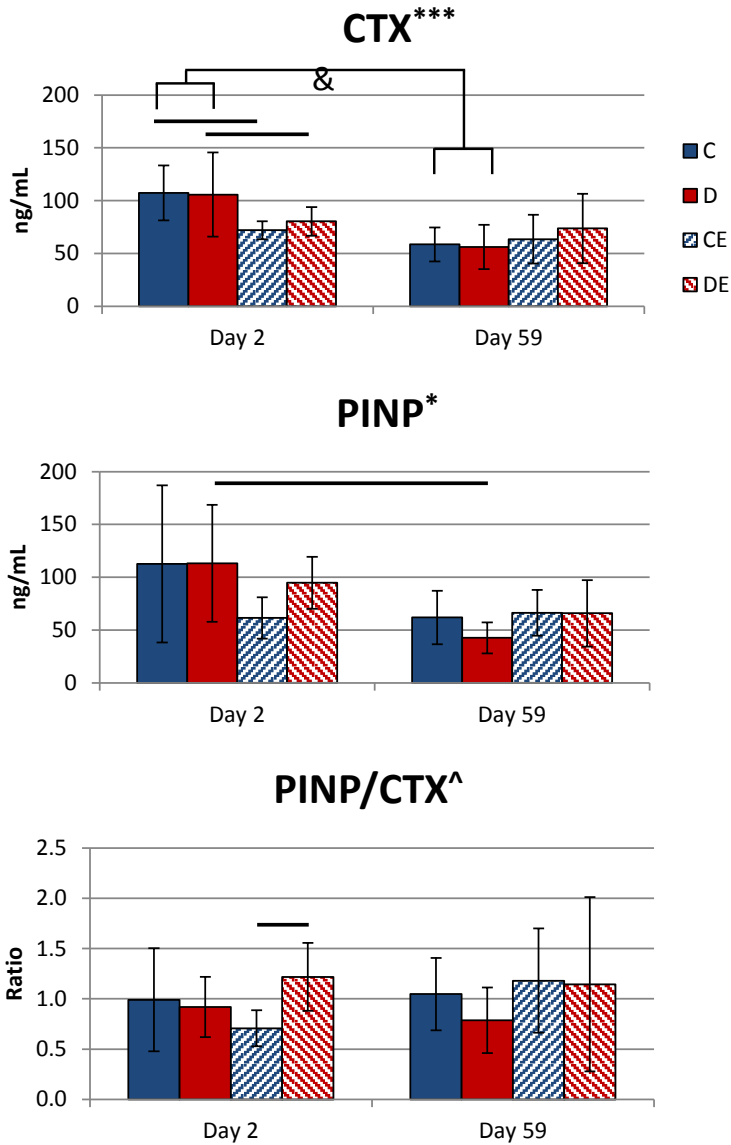
Figure 2.7. Serum CTX and PINP on Days 2 and 59 of the 8-week exercise x mineral-supplemented diet experiment. Data shown as mean ± standard deviation. CTX was significantly higher in non-exercise groups after one day of treatment and decreased to the same level as exercised mice by the end of the experiment. After 1 day of treatment, exercise with the supplemented diet caused a more formation-favored state than exercise with the standard diet.

Significant exercise effect on day 2 ($p < 0.05$, ***$p < 0.001$, Two-way ANOVA).
^Significant interactive effect of diet and exercise on day 2 ($p < 0.05$, Two-way ANOVA).
&Significantly different from day 2 values ($p < 0.05$, paired t-test).
Groups connected by a bar are significantly different ($p < 0.05$, Tukey's test).

Normalized Serum Ca
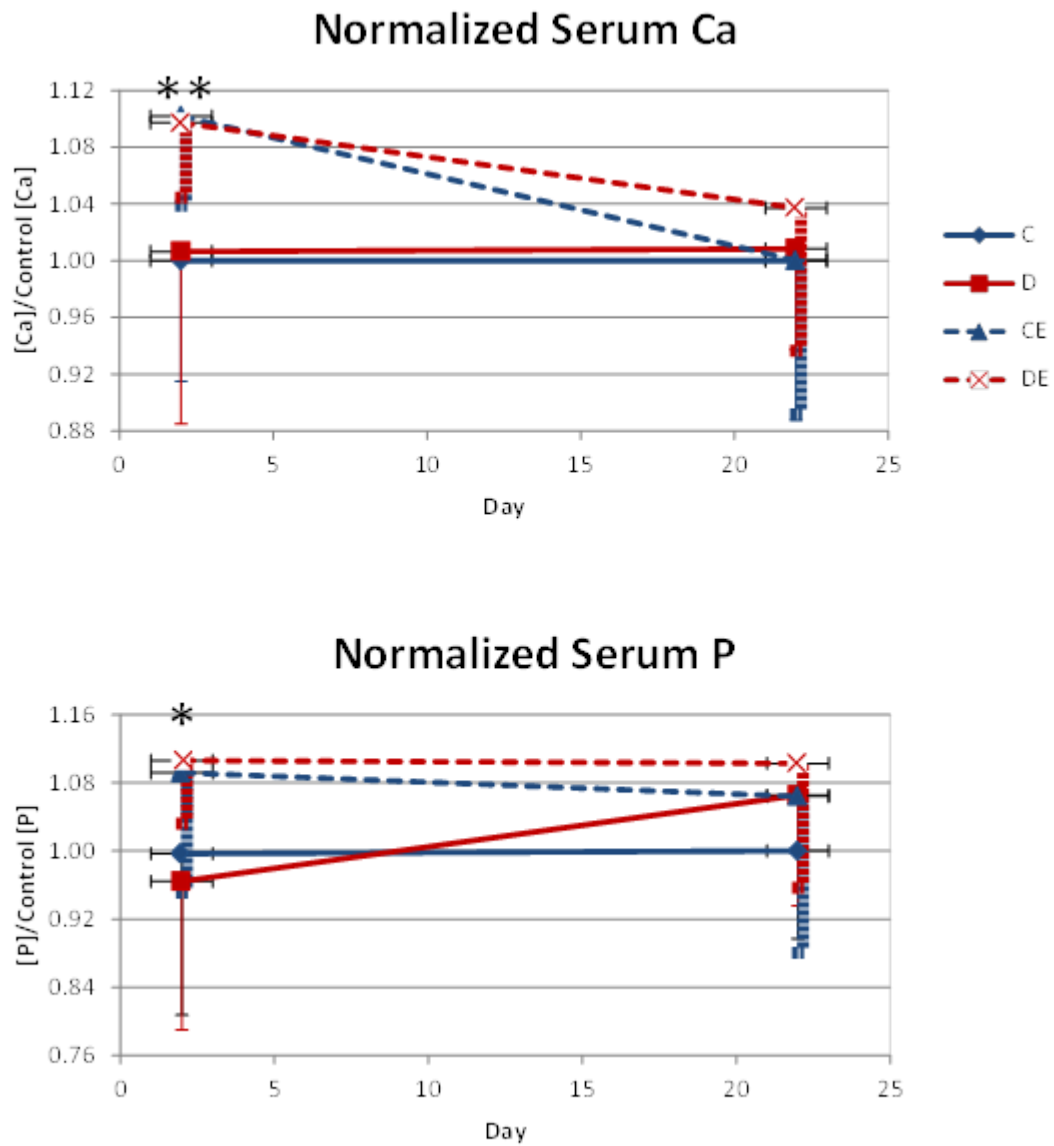
Normalized Serum P

Figure 2.8. Mean serum [Ca] and [P] on Days 2 and 22 of the 3-week exercise x mineral-supplemented diet experiment. Data is normalized to serum concentrations in non-exercised mice on the control diet at the same time point.

*Significant effect of exercise on Day 2 (*p < 0.05, **p < 0.01, Two-way ANOVA).

Normalized Serum Ca
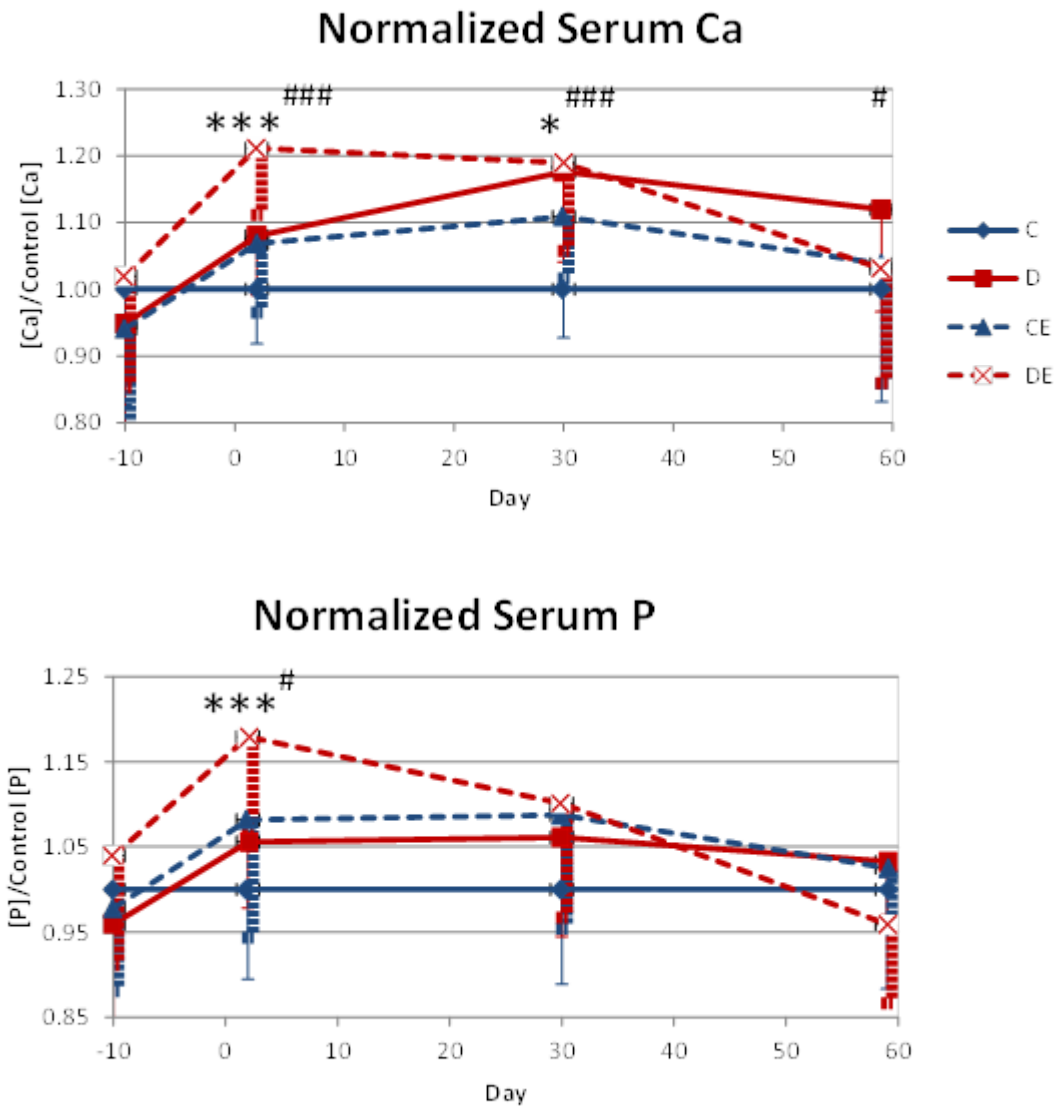
Normalized Serum P

Figure 2.9. Mean serum [Ca] and [P] 10 days before the experiment and on Days 2, 30, and 59 of the 8-week exercise x mineral-supplemented diet experiment. Data is normalized to serum concentrations in non-exercised mice on the control diet at the same time point. After one day of treatment, serum [Ca] and [P] were higher than pre-experiment ($0.041 < p < 0.057$, $3.3 \times 10^{-4} < p < 0.013$ paired t-test, respectively) for D, CE, and DE groups, and the magnitudes of the increases were highest for mice in the DE group.

Significant effect of exercise at respective times ($p < 0.05$, ***$p < 0.001$, Two-way ANOVA).
#Significant effect of diet at respective times (#$p < 0.05$, ###$p < 0.001$, Two-way ANOVA).

Group

C

CE

DE

Age (Weeks)   16   19   24
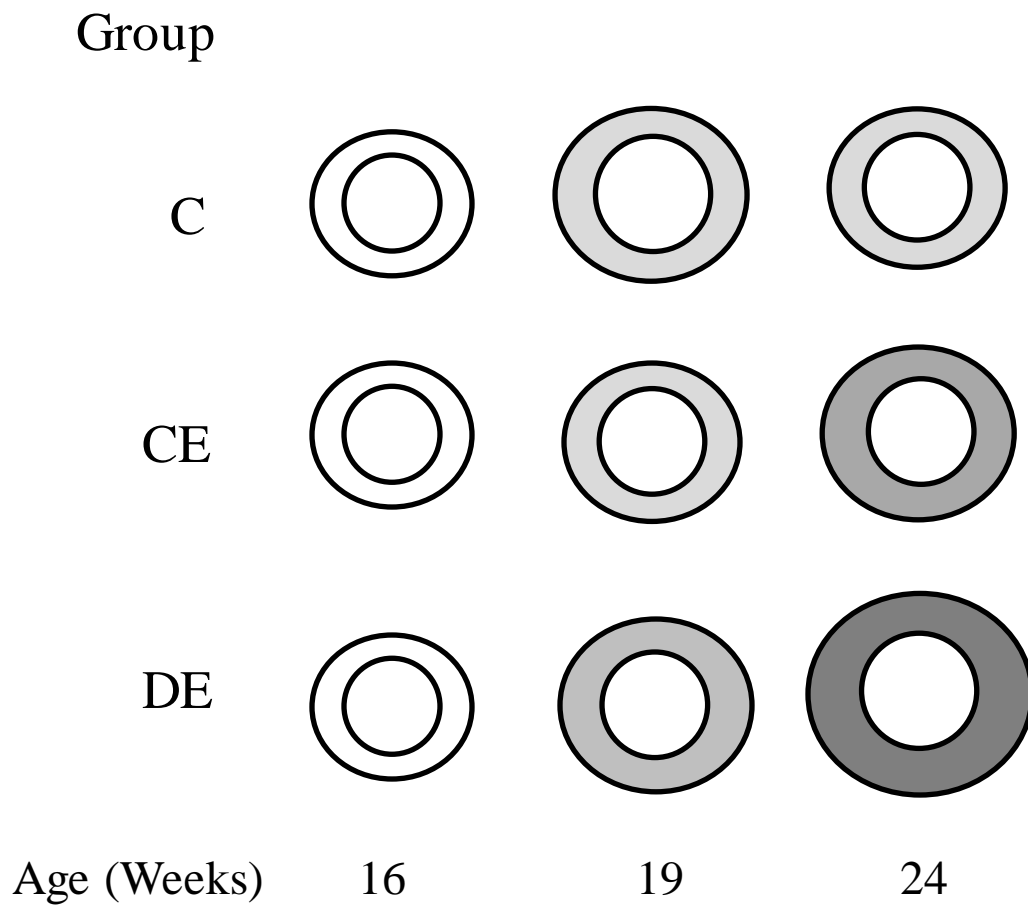
Figure 2.10. Representative mouse tibial cortical cross-sectional slices from mice age 16 [34], 19 and 24 weeks. Darker shading denotes greater TMC. Exercise alone (CE) limits increases in area and TMC at 19 weeks, but increases area and TMC from 19 to 24 weeks. Exercise with a mineral-supplemented diet (DE) has greater area and TMC at 19 weeks and 24 weeks compared to exercise alone.

Table 2.1. Correlation (r) matrices for blood biomarkers, tibial cortical bone mass, and tibial mechanical properties from the 3-week experiment. TMC and area were significantly positively correlated with yield force only in non-exercised mice. In exercised mice, day 22 serum Ca was significantly negatively correlated with yield force and yield stress.

*r is significant ($p < 0.05$).

Non-exercised Mice

	Day 22 PINP	Day 22 CTX	TMC	TMD	Area	Yield Force	Yield Def.	Yield Stress	Yield Strain
Day 22 Ca	-.050	.299	.101	.035	.067	-.063	-.392*	.025	-.192
Day 22 PINP		.235	.172	.079	.173	-.157	.101	-.082	-.054
Day 22 CTX			.344	.528*	.244	.040	-.068	.130	-.067
TMC				.531*	.974*	.575*	-.044	.210	.231
TMD					.342*	.226	-.094	.137	.001
Area						.600*	-.008	.205	.273

Exercised Mice

	Day 22 PINP	Day 22 CTX	TMC	TMD	Area	Yield Force	Yield Def.	Yield Stress	Yield Strain
Day 22 Ca	-.253	.355	.063	.142	.006	-.379*	-.310	-.506*	-.225
Day 22 PINP		.533*	.112	.360	.021	.037	-.021	.102	.048
Day 22 CTX			.229	.263	.197	.062	-.321	-.077	-.351
TMC				.644*	.943*	.141	-.123	-.365	-.015
TMD					.401*	-.024	-.245	-.221	-.216
Area						.174	-.069	-.373	.051

Table 2.2. Correlation (r) matrices for blood biomarkers, tibial cortical bone mass, and tibial mechanical properties from the 8-week experiment. In exercised mice, day 2 serum Ca was significantly positively correlated with day 2 PINP, and day 2 PINP was significantly positively correlated with TMC. These correlations did not occur in the non-exercised mice. TMC and area were significantly positively correlated with yield force for both exercised and non-exercised mice.

*r is significant (p < 0.05).

Non-exercised Mice

	Day 2 PINP	Day 2 CTX	TMC	TMD	Area	Yield Force	Yield Def.	Yield Stress	Yield Strain
Day 2 Ca	-.189	-.154	.013	.101	.108	-.312	.143	-.527	-.121
Day 2 PINP		.629*	.159	.067	.161	.183	-.066	-.001	-.272
Day 2 CTX			.475*	.120	.511*	.355	-.301	.180	-.160
TMC				.545*	.960*	.501*	-.351*	-.075	-.218
TMD					.405*	.288*	-.066	.082	-.133
Area						.442*	-.365*	-.086	-.210

Exercised Mice

	Day 2 PINP	Day 2 CTX	TMC	TMD	Area	Yield Force	Yield Def.	Yield Stress	Yield Strain
Day 2 Ca	.516*	.188	.128	.169	.145	.098	-.336	.347	.067
Day 2 PINP		.373	.447*	.235	.333	.006	-.291	-.088	.093
Day 2 CTX			.316	.183	.473*	.397	-.080	.239	-.146
TMC				.435*	.972*	.589*	-.187	.040	.075
TMD					.338*	.226	.142	.276	.184
Area						.628*	-.197	-.044	-.017

Table 2.3. Significant treatment effects of exercise, diet, and diet and exercise interaction (p < 0.05 Two-way ANOVA). Exercise increased serum mineral supply and decreased serum CTX and PINP on day 2, and was associated with increases in TMD and structural-level mechanical properties on day 59. Supplemented diet was associated with serum Ca and cortical bone geometry measurements at all time points studied, and the diet was associated with increased structural-level mechanical properties on day 59. Diet and exercise had an interactive effect on bone metabolism on day 2 and on tissue-level strength on day 22.

	Day 2	Day 22	Day 30	Day 59
Exercise Effects	↑Serum Ca, P ↓Bone Turnover (*CTX, PINP*)		↑Serum Ca	
				↑Cortical Bone (*TMD*)
		↑Structural Strength (*Ultimate Deformation*)		↑Structural Strength (*Yield F, Ultimate F, Yield Def, Pre-Yield Work*)
				↑Tissue Strength (*Yield Strain, Pre-Yield Toughness*)
Diet Effects	↑Serum Ca, P		↑Serum Ca	↑Serum Ca
		↑Cortical Bone (*TMC, Area*)		↑Cortical Bone (*TMC, Area, Moment of Inertia, TMD*)
				↑Structural Strength (*Yield F, Ultimate F, Stiffness, Pre-Yield Work*)
Diet x Exercie Interaction	Bone Turnover (*PINP/CTX*)	Tissue Strength (*Ultimate Stress*)		

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CHAPTER THREE

CALCIUM- AND PHOSPHORUS-SUPPLEMENTED DIET INCREASES TIBIAL CORTICAL BONE MASS AND RESISTANCE TO FATIGUE DAMAGE IN EXERCISED YOUNG ADULT MICE

ABSTRACT

Exercise has many benefits to bone at both the structural and tissue levels. With short-term exercise, tissue-level properties, such as mineral:matrix ratio and resistance to microdamage accumulation, increase before bone mass. Combining exercise with a calcium- and phosphorus-supplemented diet increases cortical tissue mineral content (TMC) and area after 3 and 8 weeks in exercised mice, while maintaining tissue-level mechanical properties. It was hypothesized that combining exercise with a mineral-supplemented diet for 3 weeks would increase cortical TMC and area while increasing resistance to microdamage accumulation. Male, 16-week old C57BL/6 mice were assigned to 5 groups – a baseline group, exercised groups fed a control (0.5% Ca, 0.5% P) or mineral-supplemented diet (5% Ca, 1% P), and non-exercised groups fed a control or mineral-supplemented diet for 3 weeks. The daily exercise was treadmill running at 12 m/min, 30 min/day. After sacrifice, right tibiae were fatigue-loaded ex vivo at 75 MPa peak stress, 2 Hz, for 21,600 cycles. Bones were stained and sections prepared for

histological analysis of microdamage. Exercised mice fed the supplemented diet had significantly greater cortical TMC and area than exercised mice fed the control diet. Both exercised groups had greater bending resistance before and after fatigue loading than baseline. Microcrack accumulation was lower in exercised mice fed the supplemented diet than exercised mice fed the control diet. In non-exercised mice, the supplemented diet did not affect bone mass or bending resistance and increased microcrack accumulation. The combined supplemented diet and exercise can increase bone mass and bone tissue quality after 3 weeks with no detriment to bone strength.

INTRODUCTION

Non-traumatic fractures such as osteoporotic and stress fractures can occur when bone is subjected to loading lesser than what might be expected to cause fracture. These injuries are indicative of a loss of bone mass and/or tissue quality that compromises bone strength and fracture resistance. Increased fracture risk is often attributed to decreased bone mass, but tissue quality factors, such as tissue age, architecture, mineralization, rate of bone turnover, collagen cross-linking, and microdamage accumulation also play an important role in determining fracture risk [1–7]. Clinical interventions for reducing fracture risk primarily focus on maintaining or increasing bone mass, but often do not consider the impact on bone tissue quality [8]. Better understanding of how tissue quality affects bone health may improve prevention, diagnosis, and treatment of non-traumatic fractures.

Exercise offers many health benefits and is a recommended intervention for reducing the risk of osteoporotic fractures [9]. Exercise can increase bone mass in humans [10–12], but it is unclear what effects exercise has at the tissue level. It is difficult to assess the effects of exercise on some properties of tissue quality in humans, such as collagen cross-linking and microdamage accumulation, because of the inability to collect bone tissue samples after a controlled exercise experiment. Animal models can be useful as they can provide additional insight into how interventions such as exercise affect bone mass and bone tissue quality by better allowing analysis of bone tissue samples harvested at the end of an exercise program. Long-term exercise programs of 6-12 weeks increase structural-level (whole bone) properties (cortical bone mineral content, area, yield force, and ultimate force) in rodents [13–17]. Exercise also increases tissue-level mechanical properties (yield strain, pre-yield toughness) after 8 weeks in mice [Chapter 2]. Short-term exercise of 3 weeks leads to prioritization of increasing tissue-level properties (ultimate stress, mineral:matrix ratio, resistance to microdamage accumulation) over structural-level properties [18]. Resistance to microdamage accumulation may be of particular importance in determining risk of stress fracture since accumulation of microdamage or decreased bone tissue remodeling to repair microdamage are major causes of these non-traumatic fractures [5,19].

Combining a mineral-supplemented (5% calcium, 1% phosphorus) with exercise for 8 weeks leads to greater cortical tissue mineral content (TMC), area, yield force, and ultimate force in mice than exercise on a standard diet [Chapter 2]. These increases come at no expense to tissue-level properties. Also, mice exercised while on a 5 %

calcium diet have greater formation-favoring bone metabolism (PINP/CTX ratio) after one day of exercise compared to exercised mice on a control diet (0.5% calcium, 0.5% phosphorus). Therefore, this combined diet and exercise treatment may have sufficient dietary mineral supply to maintain growth and increase bone mass while also increasing tissue quality. But it remains unclear what effects, if any, increasing dietary mineral supply has on tissue quality and if those effects are transient or long-term. Changes in mineral availability should only have short-term effects on bone mass and bone tissue quality as intestinal absorption can be modified over time to adapt to changing mineral demands [20]. Thus, it is particularly of interest to investigate changes in bone mass and bone tissue quality early in an exercise program. It was hypothesized that increasing dietary mineral supply of mice exercised for 3 weeks would increase cortical TMC, area and stiffness without affecting resistance to microdamage accumulation from fatigue loading.

METHODS

Animals and Treatments

All animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals. Sixty-four male C57BL/6 mice, 29.4 ± 1.2 g (mean ± standard deviation) body weight, were purchased from Charles River Laboratories (Wilmington, MA) at 14 weeks of age and placed in group housing. The mice were started on the control diet and were given 2 weeks to acclimate. Eight mice died or

were injured during this period and removed from the study. On experiment day 1, at 16 weeks of age, the remaining mice were randomly assigned to one of 3 weight-matched groups – a baseline group sacrificed on day 1 (B, n = 16), an exercised group fed the control diet (CE, n = 20), and an exercised group fed the supplemented diet (DE, n = 20). After 3 weeks, the exercised mice were sacrificed at age 19 weeks. Tibiae were harvested immediately after sacrifice for analysis. An additional 32 male C57BL/6 mice, 23.0 ± 1.1 g (mean ± standard deviation) body weight, were purchased from Charles River Laboratories at 14 weeks of age and placed in single housing. After 2 weeks acclimation, these mice were randomly assigned to one of 2 weight-matched groups (n = 16 per group) – a non-exercised group fed the control diet (C) and a non-exercised group fed the supplemented diet (D). At age 19 weeks, all mice were sacrificed, and tibiae were harvested for analysis.

Diets and Exercise Program

The control diet consisted of an AIN-93G diet (TestDiet®, Richmond, IN) modified by adding dicalcium phosphate to contain 0.5% Ca and 0.5% P. The supplemented diet was modified by adding dicalcium phosphate and calcium carbonate to contain 5% Ca and 1% P. Ca, P, and Ca:P ratio were all increased to increase serum Ca by increasing intestinal Ca absorption [21–23]. The control diet contained 3.90 kcal/g with an energy distribution of 65.0% carbohydrates, 16.3% fat, and 18.7% protein while the supplemented diet had 3.39 kcal/g with the same energy distribution. All other

nutrients were equivalent between the two diets. The exercise program consisted of running on a 5° incline treadmill at 12 m/min, 30 min/day for 21 consecutive days [18,24]. Mice were gradually increased to a maximum speed of 12 m/min in the first 3 days of exercise.

Cortical Geometry Measurements

Whole tibiae were embedded in 1% agarose, placed in a 19 mm diameter tube, and scanned using a micro-CT specimen scanner (µCT100 Scanco Medical, Bassersdorf, Switzerland) with a voxel size of 12 µm (70 kVp, 114 µA, 0.5 mm AL filter, and integration time 500 ms). Scans were analyzed with Scanco IPL software. A 180-µm thick transverse section from a standard site located 21.7% of the distance from the tibia-fibula junction to the proximal end of the tibia was chosen for measurement of cortical geometry metrics - TMC, volumetric TMD, cross-sectional area, and moment of inertia about the anterior-posterior axis. This section is located approximately at the center of the mechanical testing region. Geometry was calculated using a fixed global threshold of 26% (260 on a grayscale of 0–1000) to separate bone from non-bone. Another 180-µm thick transverse section that visually matched the histological site was chosen for measurement of microdamage and was analyzed for cortical area that was used to normalize measurements of microdamage.

Fatigue Loading

Right tibiae were fatigued by cyclic 4-point bending (3-mm inner and 9-mm outer spans) at 2 Hz for 21,600 cycles at an average peak load of 75 MPa. For each tibia, individual cortical geometry measurements from the standard section were used to estimate the load required to produce 75 MPa using beam-bending theory [25]. Tibiae were cyclically loaded with the medial side of the mid-diaphysis in tension. During loading, bones were kept in a calcium-buffered saline solution to prevent dehydration and loss of mineral [26]. Loading was stopped before 21,600 cycles if peak deformation reached 425 microns.

Bending Resistance Measurements

One 75 MPa load-unload cycle was performed before and after cyclic loading for measurement of resistance to bending (stiffness, work done on bone). Stiffness (N/mm) was calculated as the slope of the linear line plotted through the first and last points of the loading portion of the load-unload curve. Work done during loading (mJ) was calculated as an estimate of the area between the loading and unloading portions of the load-unload curve, using the trapezoidal rule.

Histological Analysis

Tibiae were stained with 1% basic fuchsin, using a graded series of ethanol solutions. Staining the whole bone ensured that any damage that occurred during sectioning and preparing histology slides would not be stained [27]. Bones were embedded in Koldmount (Vernon-Benshoff Company, Albany, NY), and 150-200 μm-thick transverse cross-sections were cut on a low speed diamond saw while irrigated with Ca-buffered saline. Right tibial (fatigued) sections were taken from the mid-diaphysis at a location approximately in the mid-section of the 4-point loading configuration. Left tibial (non-fatigued) sections were taken from the same location as right tibial sections. Samples were then polished down to 90-120 μm to quantify microdamage under a laser scanning confocal microscope using a 60x oil immersion objective.

Microdamage was measured from medial and lateral sides of the sections, representing bone that was loaded in tension and compression, respectively. Microcracks were identified as distinguishable linear fluorescent cracks that spanned a depth of at least 15 μm. Diffuse damage was defined as areas of indistinguishable cracks with a depth of at least 15 μm. Total damage area was defined as total stain area from microcracks and diffuse damage. Average crack length, number of cracks, diffuse damage area, and total damage area were measured from right (fatigued) and left (non-fatigued) tibial sections. Measurements were normalized by cortical area. Individual fatigued bone microdamage measurements were further normalized by non-fatigued bone microdamage measurements from the contralateral tibia, giving a measurement

representative of how much the fatigue loading protocol propagates pre-existing microdamage and creates new microdamage.

Statistical Analysis

Cortical geometry measurements, bending resistance measurements, and microdamage measurements of fatigued vs. non-fatigued samples were tested by One-way ANOVA with Tukey's post-hoc tests using SPSS software (IBM, Armonk, NY) with a significance level of $p < 0.05$.

RESULTS

The Supplemented Diet Increased Tibial Cortical TMC and Area in Exercised Mice

There were no significant differences in mean body weight between any of the exercised groups at any time point measured (data not shown). Mice in both groups gained weight from 29.7 ± 1.1 g (mean $\pm$ SD) on day 1 to 32.8 ± 1.8 g when sacrificed at the end of 3 weeks. Exercised mice on the supplemented diet had significantly greater cortical TMC and area than exercised mice on the control diet ($p < 0.05$, Tukey's test, Figure 3.1). Additionally, exercised mice on the supplemented diet had significantly greater cortical area and moment of inertia, and significantly lower TMD than baseline mice.

Exercised Mice had Increased Bending Resistance Compared to Baseline

Both groups of exercised mice had significantly greater pre-fatigue and post-fatigue stiffness, compared to baseline (p < 0.05, Tukey's test, Figure 3.2). Percent loss of stiffness from pre-fatigue to post-fatigue was significantly higher in baseline than in both groups of exercised mice. There were no significant differences in work done during the pre-fatigue load-unload cycle. During the post-fatigue load-unload cycle, work done on bones from both groups of exercised mice was significantly lower than baseline.

The Supplemented Diet Prevented Increase in Crack Density from Fatigue Loading in Exercised Mice

Exercised mice on the supplemented diet had a significantly lower crack density ratio than exercised mice on the control diet (p < 0.05, Tukey's test, Figure 3.3). This ratio means there was a greater increase in crack density from the fatigue loading in exercised mice on the control diet than in exercised mice on the supplemented diet. When microdamage was classified as either occurring towards the periosteal or endosteal sides of the bone, there was significantly lower periosteal crack density ratio for exercised mice on the supplemented diet compared to exercised mice on the control diet (p < 0.05, Tukey's test, Figure 3.4). There were no significant differences in endosteal crack density ratio. No significant differences were detected in any other

microdamage measurements – diffuse damage density, total damage density (data not shown), and average crack length.

The Supplemented Diet Alone Had No Effects on Tibial Cortical Bone Geometry and Bending Resistance for Non-Exercised Mice

There were no significant differences in mean body weight between any of the non-exercised groups at any time point measured (data not shown). Mice in both groups gained weight from 23.0 ± 1.1 g (mean ± SD) on day 1 to 26.3 ± 1.4 g when sacrificed at the end of 3 weeks. There were no significant differences for any cortical bone geometry, mineralization, or bending resistance measurement for non-exercised mice (Figure 3.5, Figure 3.6). Both groups of non-exercised mice had no significant changes in stiffness or work done during the load-unload cycle from pre-fatigue to post-fatigue measurements. Crack density ratio and total damage density ratio (data not shown) were significantly higher in non-exercised mice on the supplemented diet than on the control diet ($p < 0.05$, t-test, Figure 3.7). There were no other significant differences in microdamage measurements between non-exercised groups. All body weight, cortical bone geometry, bending resistance measurements, and microdamage measurements (except TMD and average crack length) were significantly lower for non-exercised mice than baseline mice. Thus, no direct comparisons were made between exercised and non-exercised mice.

DISCUSSION

In this study, 3 weeks of exercise while on a mineral-supplemented diet led to increased fatigue damage resistance compared to exercise on a standard diet, as there was a lower ratio of microcrack density from fatigued tibiae to microcrack density from contralateral non-fatigued tibiae (Figure 3.3). Cortical TMC and area were greater in bones from exercised mice on the supplemented diet than bones from exercised mice on the control diet. However, the peak magnitude of the load during fatigue remained at 75 MPa for all bones, which normalized loads to bone size to account for differences in cortical area. Additionally, exercised mice on the supplemented diet had greater cortical area and moment of inertia (Figure 3.1) than baseline, but no differences in microdamage measurements (Figure 3.3). These results suggest differences in bone mass are not likely to be the cause of differences in fatigue damage resistance.

The difference in crack density between exercised groups on the different diets was driven by differences in crack density in bone closer to the periosteal edge (Figure 3.4). This was as expected because loading is highest near the surface of the bone [25]. Since exercise on a standard diet initially prioritizes increasing mineralization and tissue strength of existing tissue over adding new tissue [18], it may be possible that periosteal bone of exercised mice on the control diet is made up of older tissue that may be more susceptible to fatigue damage. Alternatively, at the end of 3 weeks of exercise, tibiae from exercised mice on the control diet may be beginning to add new periosteal bone tissue that is not as mineralized as in the combined supplemented diet and exercise group. However, this seems less likely as there were no significant differences in

cortical TMC, area, or moment of inertia between exercised mice on the control diet and baseline (Figure 3.1). There is not likely to be much new periosteal tissue in bones from exercised mice on the control diet at this time point [18].

Bones from both exercised groups had greater bending resistance than baseline, regardless of diet (Figure 3.2). For mice exercised while fed the control diet, increases in stiffness occurred despite no increases from baseline in cortical bone mass. Either exercise-induced adaptations increased tissue quality or the increased age of tissue may have allowed more time for increasing mineralization and cross-link maturity. Fatigue-loading caused a significantly greater loss of stiffness and increase in work in the bones of baseline mice than in bones from both exercised groups. This increase in work during loading suggests baseline bones were more likely to incur damage during load cycles at the end of the loading session [28]. It is possible that increasing the number of fatigue cycles may lead to significantly greater microdamage accumulation in baseline bones than in exercised bones. Thus, exercise affected tissue strength by increasing bending resistance before fatigue and increasing resistance to a loss of stiffness during fatigue. These improvements may also cause bones from exercised mice to have a greater fatigue life and be less likely to incur a non-traumatic fracture. The supplemented diet had no impact on bending resistance and did not appear to affect tissue strength. There were some limitations to testing of mechanical properties as bending resistance measurements did not include yield or failure testing in order to preserve mid-diaphyseal samples for measurement of microdamage.

Non-exercised mice had little to no new microcrack formation from fatigue loading, particularly for mice on the control diet that had a crack density ratio less than one (Figure 3.7). The stress that was able to induce microcrack formation in exercised mice was not sufficient to cause significant new damage in the non-exercised mice, suggesting peak stress during fatigue loading may not be a major factor in determining what causes microcracks. Perhaps other factors such as amount of pre-existing damage, bone turnover rate, tissue composition (mineral:matrix ratio, collagen cross-link status) may play a larger role in microdamage accumulation from repetitive loading.

The supplemented diet had different effects on bone mass and microdamage accumulation, depending on whether or not the mice exercised. For non-exercised mice, the supplemented diet did not increase cortical TMC or area after 3 weeks. Our previous work has shown that cortical TMC and area are increased in non-exercised mice after 8 weeks on a 5% Ca supplemented diet, but not after 3 weeks on a 2% Ca supplemented diet [Chapter 2]. This study showed no effect on cortical bone using a 5% Ca supplemented diet, suggesting the duration of time on the diet may be a greater factor than Ca content or Ca:P ratio in increasing cortical bone mass. Crack density ratio was higher in non-exercised mice on the supplemented diet than in non-exercised mice on the control diet. Although the fatigued/non-fatigued damage ratios were lower in non-exercised mice, this difference in effects of diet in exercised mice suggests that diet may have an opposite effect after 3 weeks when mice are not exercised.

It is also possible that non-exercised mice were not the correct age based on expected body weight for male C57Bl/6 mice [29]. Cortical TMC, area, moment of inertia, and pre-fatigue bending resistance were all significantly lower in both groups of non-exercised mice than in the baseline mice. Lower cortical bone mass suggests the non-exercised mice were either lower in age or had poorer nutrition [30,31]. This may help explain why there was more microdamage in the exercised mice bones if they were in fact older age since older bone is more susceptible to microdamage from fatigue loading [3]. Due to these concerns, no direct comparisons were made between exercised and non-exercised groups.

For bones from all groups, there was a large variance in the measurements of microdamage after fatigue which is not unexpected [2,18]. There may be many factors that could have led to the variation in microdamage, including global differences (location of histology section, distribution and localization of new tissue versus old tissue) and local differences (tissue composition or osteocyte activity). Data from this study suggest cortical TMC, area, pre-fatigue bending resistance, and post-fatigue bending resistance do not affect resistance to microdamage accumulation from fatigue loading. The mineral-supplemented diet increased resistance to microcrack accumulation in exercised mice, but it was unclear what changes occurred to cause this. More work needs to be done to further analyze tissue samples for differences in tissue composition that may be affecting resistance to microdamage.

In the first 3 weeks of an exercise program, exercise can favor improving bone tissue-level properties over increasing bone mass. Adding a supplemented diet with exercise may allow bone to increase both cortical bone mass and resistance to microdamage accumulation towards the beginning of an exercise program. This could optimize increases in bone mass to help prevent non-traumatic bone fractures during and after the exercise program.
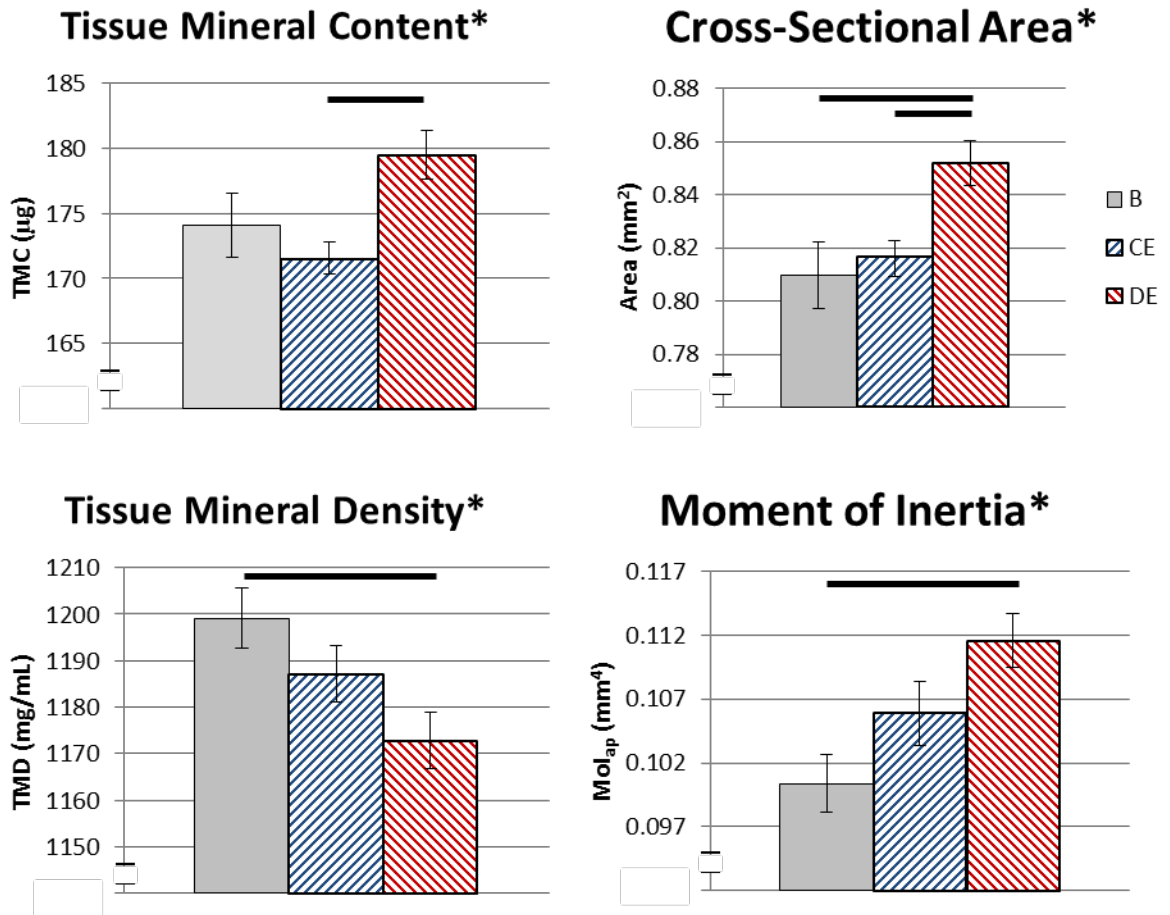
Acknowledgments

Figure 3.1. Mouse right tibial cortical bone mineralization and cross-sectional geometric properties (mean ± SEM) measured at the standard site used for calculation of load magnitude needed for fatigue loading. The supplemented diet significantly increased cortical TMC and area in exercised mice.

*p < 0.05, One-way ANOVA

Horizontal bar represents significant difference between groups, p < 0.05, Tukey's test.

B – baseline mice

CE – exercised mice fed the control diet
DE – exercised mice fed the supplemented

Figure 3.2. Right tibial mechanical properties before and after fatigue loading (mean ± SEM). Bending resistance and resistance to loss of strength from fatigue loading was significantly higher in the exercise groups, regardless of diet.

*p < 0.05, One-way ANOVA

^Significant difference between post-fatigue and pre-fatigue values, p < 0.05, t-test

Horizontal bar represents significant difference between groups, p < 0.05, Tukey's test.

B – baseline mice

CE – exercised mice fed the control diet
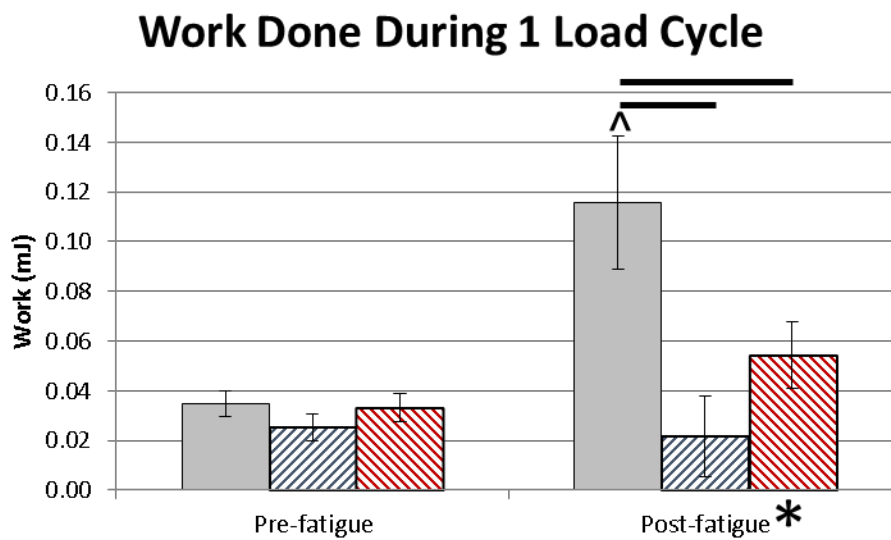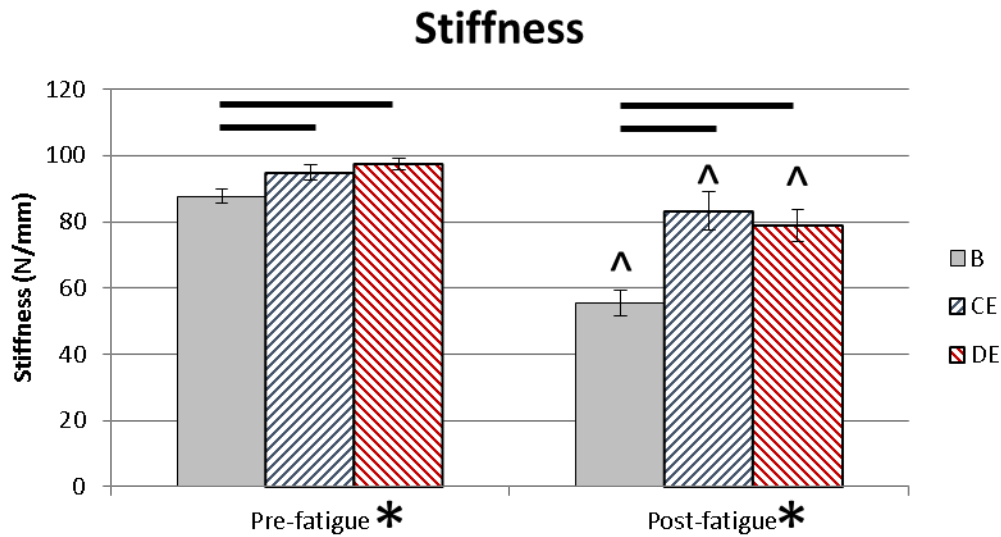
DE – exercised mice fed the supplemented

Figure 3.3. Ratio of fatigued/non-fatigued tibial microdamage measurements (mean ±
SEM). Compared to exercise alone, the combined supplemented diet and exercise
prevented an increase in microcracks with fatigue loading.

*p < 0.05, One-way ANOVA

Horizontal bar represents significant difference between groups, p < 0.05, Tukey's test.

B – baseline mice

CE – exercised mice fed the control diet
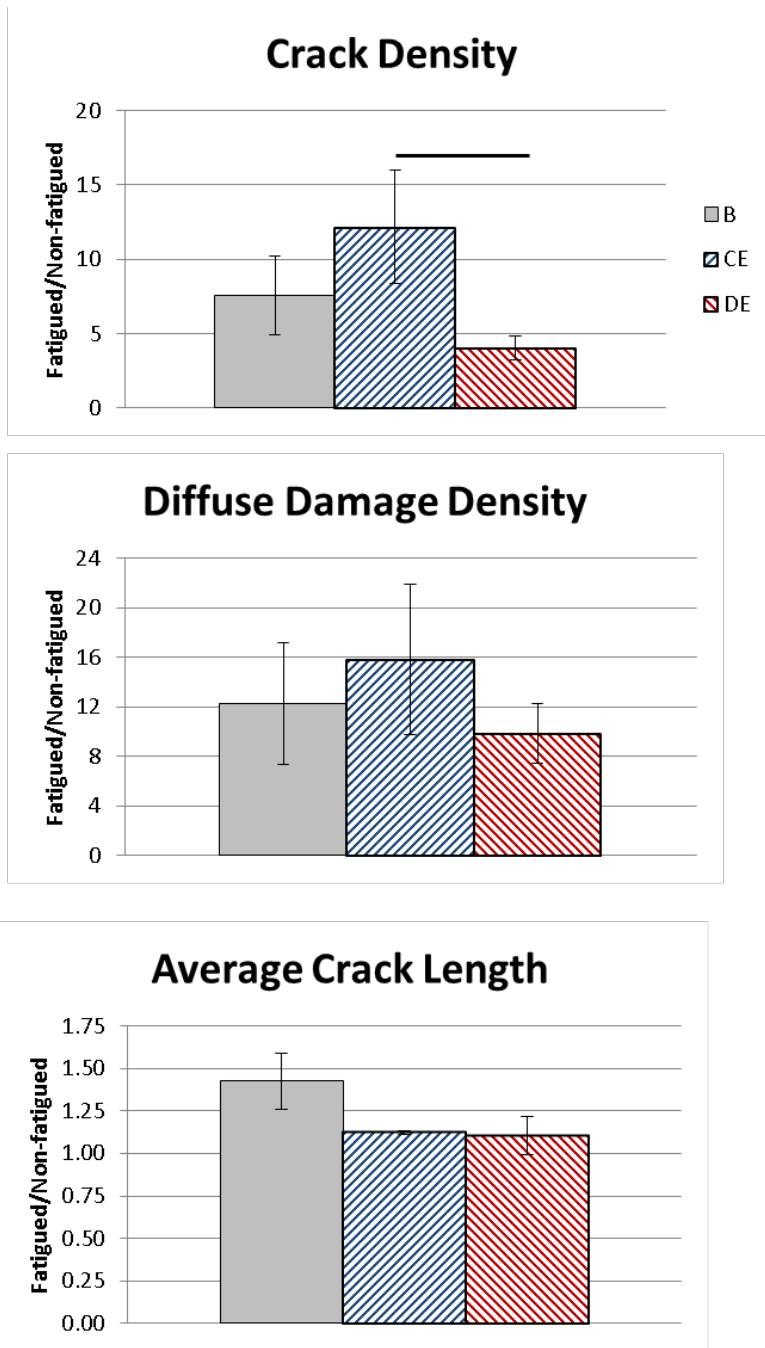
DE – exercised mice fed the supplemented
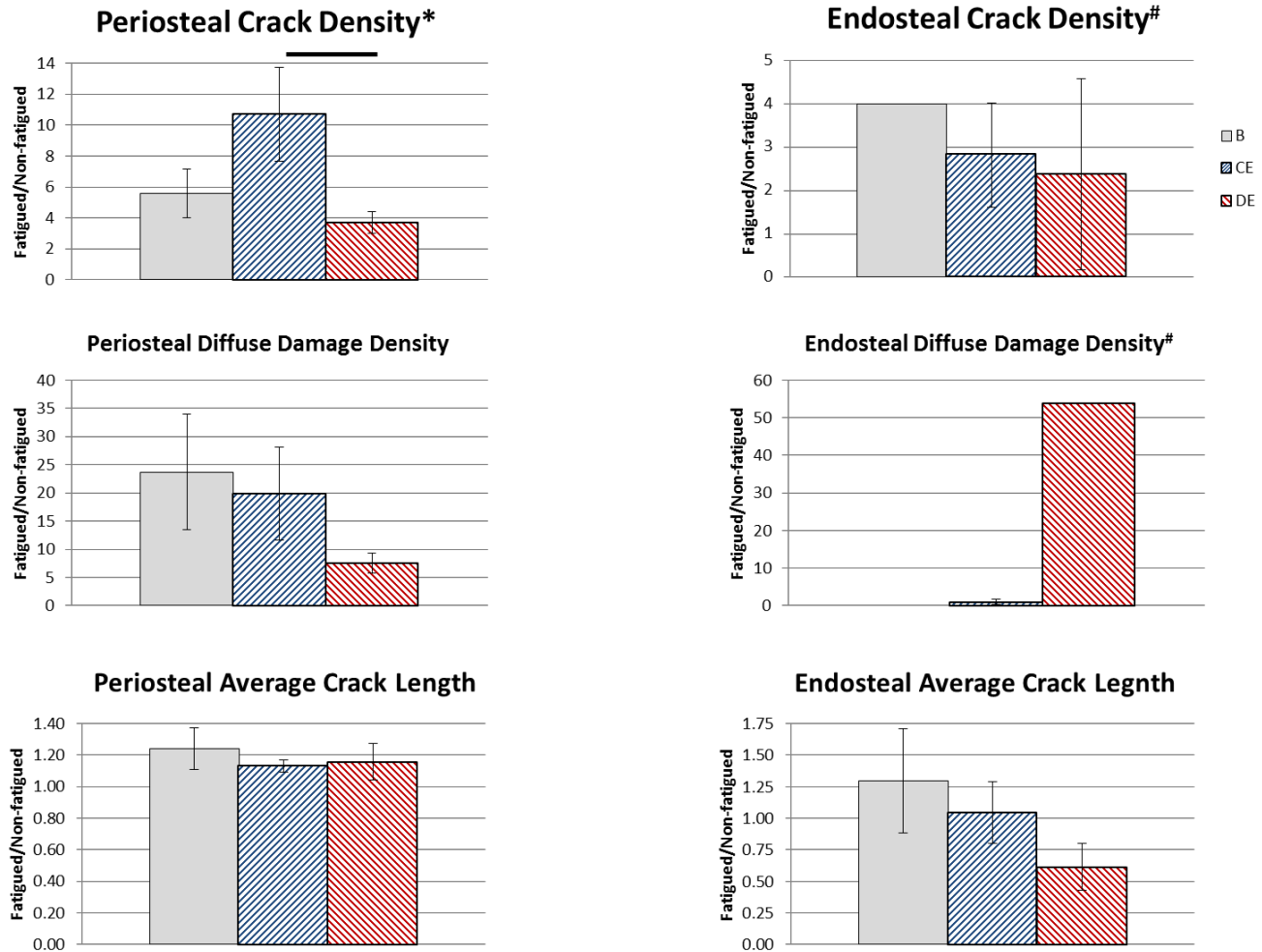
Figure 3.4. Ratio of fatigued/non-fatigued tibial microdamage measurements classified by periosteal or endosteal bone regions (mean ± SEM). Compared to exercise alone, the combined supplemented diet and exercise prevented an increase in periosteal microcracks with fatigue loading. There was little endosteal microdamage in non-fatigued bones, causing low sample sizes and inflated ratios.

*$p < 0.05$, One-way ANOVA

Horizontal bar represents significant difference between groups, $p < 0.05$, Tukey's test.

#Insufficient group sample sizes for statistical testing

B – baseline mice

CE – exercised mice fed the control diet

DE – exercised mice fed the supplemented
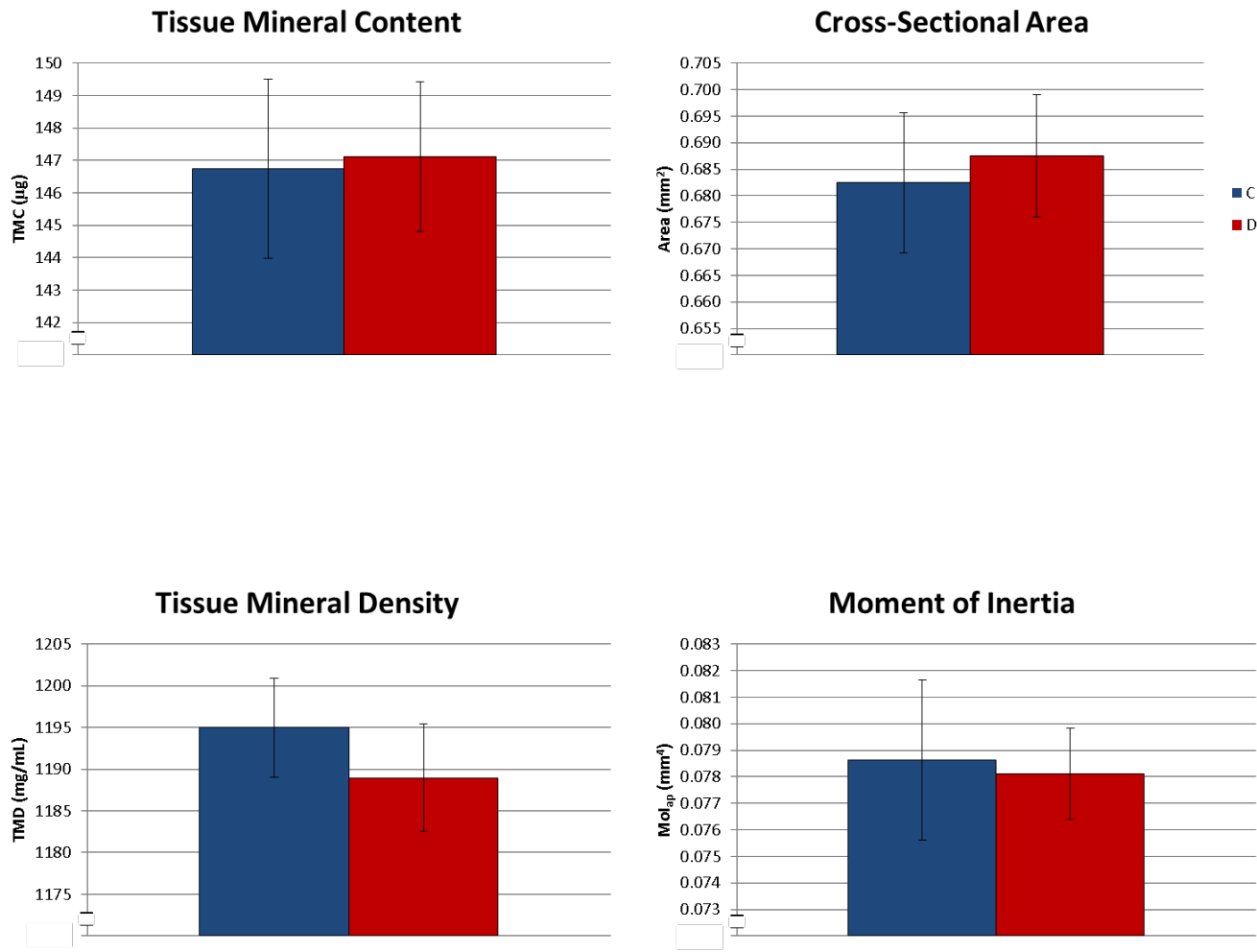
Figure 3.5. Mouse right tibial cortical bone mineralization and cross-sectional geometric properties from non-exercised mice at the standard site (mean ± SEM). There were no significant differences between these two groups.

C – non-exercised mice fed the control diet
D – non-exercised mice fed the supplemented

Stiffness
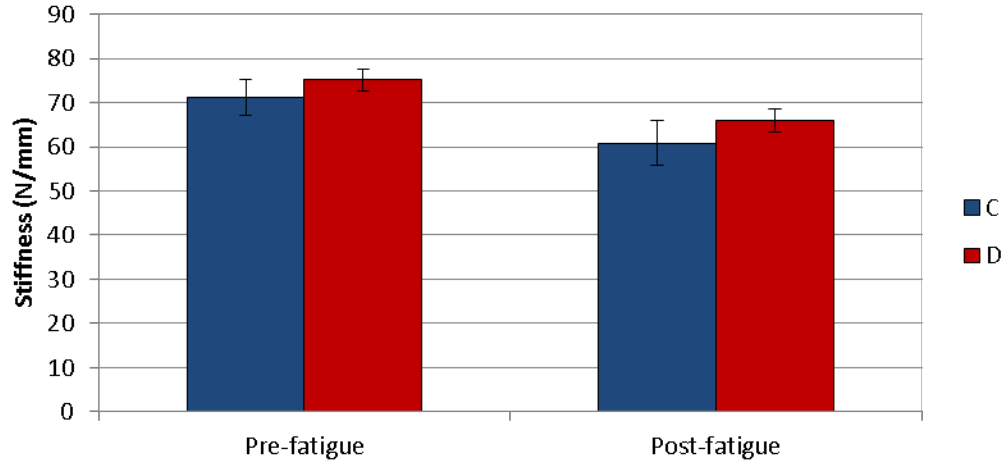
Work During 1 Load Cycle
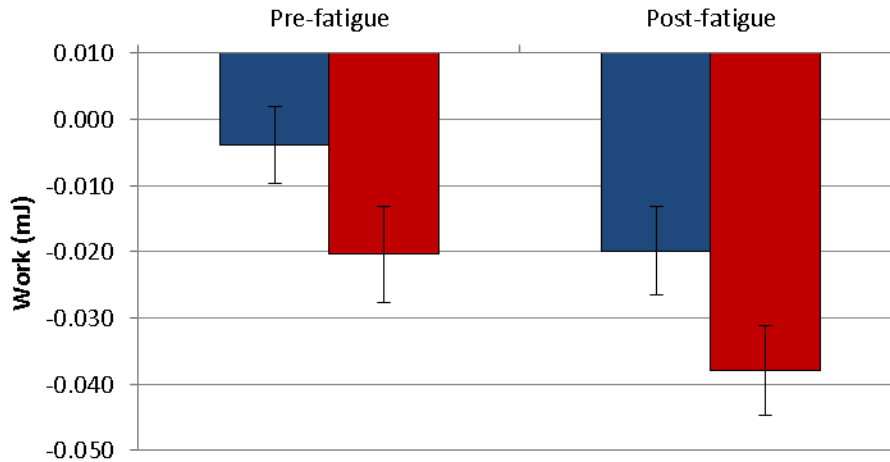
Figure 3.6. Right tibial mechanical properties before and after fatigue loading for non-exercise groups (mean ± SEM). There were no significant differences between these two groups.
C – non-exercised mice fed the control diet
D – non-exercised mice fed the supplemented

Figure 3.7. Ratio of fatigued/non-fatigued tibial microdamage measurements in non-exercised groups (mean ± SEM). For mice on the supplemented diet, fatigue loading increased crack density more than it did for mice on the control diet.

Horizontal bar represents significant difference between groups, p < 0.05, t-test.

C – non-exercised mice fed the control diet

D – non-exercised mice fed the supplemented

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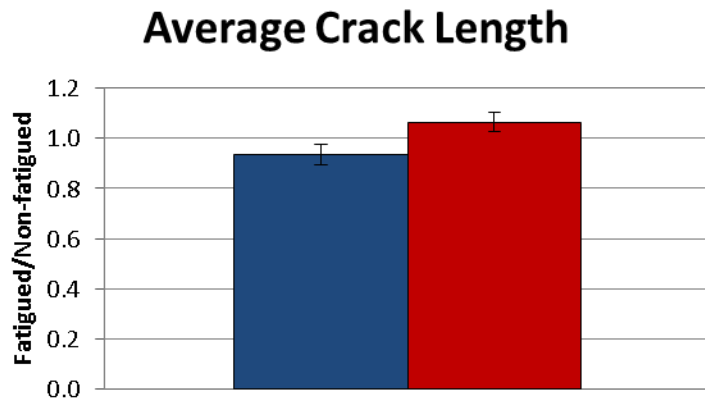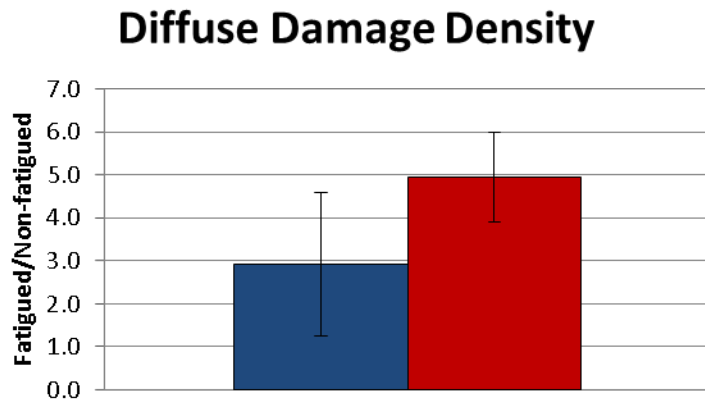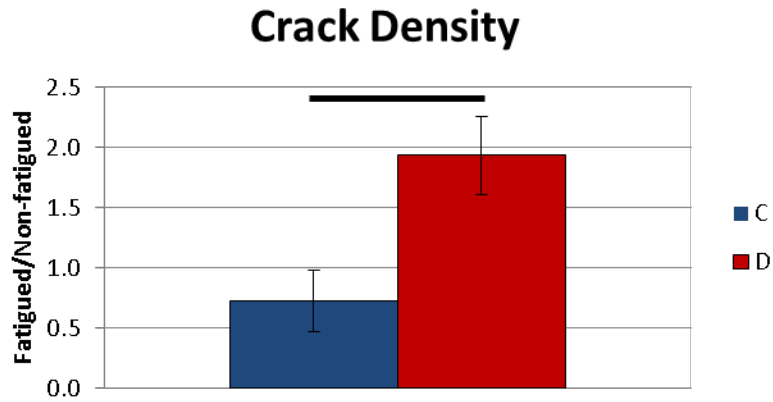[1]   Hui SL, Slemenda CW, Johnston CC. Age and bone mass as predictors of fracture in a prospective study. J Clin Invest 1988;81:1804–9.

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CHAPTER FOUR

CALCIUM- AND PHOSPHORUS-SUPPLEMENTED DIET INCREASES CORTICAL AND TRABECULAR BONE MASS AFTER FOUR WEEKS OF HIGH-SPEED TREADMILL EXERCISE IN ADULT MICE

ABSTRACT

Exercise has long-lasting benefits to bone health. Bone mass and the ability to exercise both decline with age, making it ideal to exercise earlier in life and to maximize gains in bone mass. Increasing strain on bone and frequency of loading during exercise can increase bone formation rate and cross-sectional area. Combining a short-term exercise program with a calcium- and phosphorus-supplemented diet increases cortical bone tissue mineral content (TMC) and area more than exercise alone in adult mice. It was hypothesized that combining high-speed running with a mineral-supplemented diet would lead to greater cortical TMC and area than high-speed running on a standard diet and low-speed running on a supplemented diet after 4 weeks. Male, 15-week old mice were assigned to 7 groups – a baseline group, non-exercised groups fed a control or supplemented diet, low-speed exercised groups fed a control or supplemented diet, and high-speed exercised groups fed a control or supplemented diet. Exercise consisted of 4 weeks of daily treadmill running for 20 min/day at 12 m/min or 20 m/min for low- and

high-speed exercise, respectively. High-speed exercised mice had significantly lower body weight and lower tibial length after 4 weeks. Cortical TMC and area were significantly higher in high-speed exercised mice on the supplemented diet than high-speed exercised mice on the control diet. Trabecular bone volume (BV) and bone density were significantly higher in all groups on the supplemented diet than groups on the control diet, regardless of exercise. For mice on the control diet, non-exercised mice had significantly lower trabecular BV than baseline, while both speeds of exercise prevented this decline. There were few effects of exercise or diet on mechanical properties as expected after only 4 weeks of treatment. For mice on the control diet, exercise significantly decreased serum PINP/CTX ratio on day 9 which may be preventing exercise from increasing bone mass or strength after only 4 weeks. For non-exercised mice on the supplemented diet, the serum PINP/CTX ratio on day 30 was significantly greater than for exercised mice, suggesting the supplemented diet may also lead to significantly greater bone mass in non-exercised mice if these interventions were extended beyond 4 weeks. Increasing exercise intensity can lower body weight while maintaining cortical and trabecular bone mass. A mineral-supplemented diet increases cortical and trabecular bone mass with high-speed exercise.

INTRODUCTION

Weight-bearing exercise increases bone mass, structural strength, and tissue quality, making bone better able to resist fracture [1]. Exercise can have long-term benefits to bone health [2–8], making it beneficial to maximize bone mass accumulation early in

life. Greater bone mass early in life is needed to maintain a higher level of bone mass in old age when bone mass declines while weight-bearing exercise becomes more difficult to perform [9–11].

Bone responds to loading from exercise by increasing bone mass and bone strength to accommodate greater loads on bones and prevent damage from future exercise loads [12]. Increasing loading on bones during exercise can increase bone mass since increasing magnitude of strain from loading increases bone cross-sectional area [13] and bone formation rate [14,15]. High-intensity exercise using increased treadmill speed may similarly be able to change loading on the bone by increasing loading frequency. Loading frequency is directly related to bone formation rate, and loads of greater strain magnitude are more impactful on bone formation rate when applied at a higher loading frequency [16].

Increasing bone formation rate may allow bone to reach greater peak bone mass or to achieve peak bone mass in a shorter time, allowing for a more rapid increase in resistance to fracture. This increased bone formation rate would require an increased dietary mineral supply to simultaneously maintain increases in bone mass, bone tissue quality and formation rate with exercise. Since exercise increases demand for dietary minerals, high-intensity exercise may be causing an even greater need for minerals that normal dietary amounts cannot provide. Combining high-intensity exercise with a calcium- and phosphorus-supplemented diet may be able to maximize bone mass by further increasing bone mass over standard, lower-intensity exercise. Under standard

exercise conditions (running at a speed similar to jogging), rodents exercised for 6-12 weeks have increased cortical bone mineral content, area, yield force, and ultimate force [8,17–20]. In young adult mice, combining exercise with a mineral-supplemented diet increases cortical tissue mineral content (TMC) and area compared to exercise with a standard diet after only 3 weeks of exercise [Chapter 2]. It was hypothesized that combining a mineral-supplemented diet with increasing exercise intensity by increasing treadmill speed would lead to greater cortical TMC and area than high-speed exercise on a standard diet after 4 weeks of exercise in young adult mice.

METHODS

Animals and Treatments

All animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals. One hundred thirty-eight male C57BL/6 mice, 26.3 ± 2.8 g mean body weight, were purchased from Charles River Laboratories (Wilmington, MA) at 13 weeks of age and placed in single housing to prevent fighting. The mice were started on the control diet and were given 2 weeks to acclimate. Starting on experiment day 1, at 15 weeks of age, mice were randomly assigned to one of 7 groups – a baseline group sacrificed on day 1 (B), a non-exercise group fed the control diet (C), a non-exercise group fed the supplemented diet (D), a low-speed exercise group fed the control diet (CE), a low-speed exercise group fed the supplemented diet (DE), a high-speed exercise group fed the control diet (CE+), and a high-speed exercise group fed the

supplemented diet (DE+). Mice were divided into groups of equal mean body weight and baseline serum Ca concentration. Baseline serum Ca was measured 5 days before experiment day 1. After 30 days of treatments, all mice from the experimental groups were sacrificed at 19 weeks of age, and left tibiae were harvested for analysis.

Diets and Exercise Program

The control diet consisted of an AIN-93G diet (TestDiet®, Richmond, IN) modified by adding dicalcium phosphate to contain 0.5% Ca and 0.5% P. The supplemented diet was modified by adding dicalcium phosphate and calcium carbonate to contain 5% Ca and 1% P. Ca, P, and Ca:P ratio were all increased to increase serum Ca by increasing intestinal Ca absorption [21–23]. The control diet contained 3.90 kcal/g with an energy distribution of 65.0% carbohydrates, 16.3% fat, and 18.7% protein while the supplemented diet had 3.39 kcal/g with the same energy distribution. All other nutrients were equivalent between the two diets. The low-speed exercise program consisted of running on a 5° incline treadmill at 12 m/min, 20 min/day for 29 consecutive days. Mice were gradually increased to a maximum speed of 12 m/min in the first 3 days of exercise. The high-speed exercise program consisted of running on a 5° incline treadmill at 20 m/min, 20 min/day. Mice were gradually increased to a maximum speed of 20 m/min in the first 8 days of exercise. Video analysis of mice running gave an estimated average frequency of 3.4 steps/second in mice running 12 m/min and 4.2 steps/second in mice running 20 m/min. Thus, the high-speed exercise

had greater loading frequency, and both exercise programs offered a sufficient number of load cycles/day to reach loss of mechanosensitivity such that any further increase in duration would not affect bone [24].

Cortical Geometry and Trabecular Architecture Measurements

Whole tibiae were embedded in 1% agarose, placed in a 19 mm diameter tube, and scanned using a micro-CT specimen scanner (µCT100 Scanco Medical, Bassersdorf, Switzerland) with a voxel size of 12 µm (70 kVp, 114 µA, 0.5 mm AL filter, and integration time 500 ms). Scans were analyzed with Scanco IPL software. A 180-µm thick transverse section from a standard site located 21.7% of the distance from the tibia-fibula junction to the proximal end of the tibia was chosen for measurement of cortical geometry metrics - TMC, volumetric tissue mineral density (TMD), cross-sectional area, and moment of inertia about the anterior-posterior axis. This section is located approximately at the center of the mechanical testing region. Geometry metrics were calculated using a fixed global threshold of 26% (260 on a grayscale of 0–1000) to separate bone from non-bone. Another 180-µm thick transverse section at the fracture site was analyzed for cortical geometry measurements used in calculations of tissue-level mechanical properties (moment of inertia, distance from neutral axis). Tibial scans were further analyzed for trabecular bone architecture. Proximal tibial metaphyseal sections immediately below the growth plate of 480-µm thick were analyzed in Scanco IPL software using freehand traced volumes of interest. Architecture metrics measured

were bone volume (BV), bone volume fraction (BV/TV), TMD, trabecular number, trabecular thickness, and trabecular separation. These metrics were calculated using a fixed global threshold of 18% (180 on a grayscale of 0–1000) to separate bone from non-bone.

Mechanical Testing

Structural- and tissue-level mechanical properties were measured in all groups. Structural-level properties (force, deformation, stiffness, work) were measured from a 4-point bending to failure test (3-mm inner and 9-mm outer spans). Tibiae were loaded to failure with the medial side of the mid-diaphysis in tension under displacement control at 0.025 mm/sec at a data sampling rate of 30 Hz. Tissue-level mechanical properties (stress, strain, modulus, toughness) were estimated using beam bending theory with geometric measurements (moment of inertia about anterior-posterior axis, distance from centroid to medial side of the bone) from micro-CT data at the fracture site [25].

Serum Analysis

Fasting blood samples taken before daily exercise were collected by submandibular vein bleeding. Blood samples were collected at baseline (day -4), after the first day of full speed running for the high-speed exercised mice (day 9) and on the final day (day 30).

Serum was isolated by centrifuge. Ca and P concentrations were measured by using the Calcium CPC LiquiColor test kit (Stanbio Laboratory, Boerne, TX) and the Phosphorus Liqui-UV kit (Stanbio Laboratory).   ELISAs were used to measure markers of bone formation and resorption – pro-collagen type I amino-terminal peptide (PINP) and carboxy-terminal collagen crosslinks (CTX) (Immunodiagnostic Systems, Inc., Scottsdale, AZ) – on samples from day 9 and day 30.   All manufacturers' kit instructions were followed, including the use of the standards provided for obtaining standard curves.

Statistical Analysis

Cortical geometry measurements, trabecular architecture measurements, and mechanical properties were tested by Two-way ANOVA with Tukey's post-hoc tests to determine if the individual effects of diet or exercise were significant ($p < 0.05$) and if the combined treatments had a significant interactive effect.   Student's t-tests were used to compare baseline to experimental groups.

RESULTS

High-Speed Exercise Prevented Weight Gain after Four Weeks

Exercise had a significant main effect that decreased body weight on day 29 ($p < 0.05$, Two-way ANOVA, Figure 4.1).   Both of the high-speed exercised groups and mice exercised at low-speed while fed the control diet did not gain weight after day 8. Mice

exercised at low-speed while fed the supplemented diet and both of the non-exercised mice groups continued gaining weight after day 8 and the body weights at day 29 of mice in these three groups were significantly higher than the body weights of mice in groups that did not gain weight during the same time ($p < 0.05$, Tukey's tests, Figure 4.1).

The Supplemented Diet Increased Tibial Cortical TMC and Area, and High-Speed Exercise Prevented an Increase in Tibial Length after Four Weeks

Exercise had a significant main effect on tibial length ($p < 0.05$, Two-way ANOVA, Figure 4.2). Four weeks of high-speed exercise prevented increases in tibial length for both groups of high-speed exercised mice. Tibial length was significantly higher in low-speed exercised mice on the control diet than high-speed exercised mice on the control diet ($p < 0.05$, Tukey's test, Figure 4.2). All groups except the 2 high-speed exercised mice groups had significantly greater tibial length than baseline ($p < 0.05$, t-test, Figure 4.2). There were significant main increasing effects of diet on cortical TMC and area after 4 weeks ($p < 0.05$, Two-way ANOVA, Figure 4.2). High-speed exercised mice on the supplemented diet had significantly greater TMC and area than high-speed exercised mice on the control diet ($p < 0.05$, Tukey's tests, Figure 4.2) and baseline mice ($p < 0.05$, t-test, Figure 4.2). Tissue mineral density was significantly greater than baseline in all groups except the non-exercised mice on the supplemented diet ($p < 0.05$, t-test, Figure 4.2).

The Supplemented Diet Increased Trabecular Bone Volume, and Both Speeds of Exercise

Prevented Loss of Trabecular Bone Volume for Mice on the Control Diet after Four Weeks

There were significant main effects of diet that increased trabecular BV, BV/TV, number, and thickness and decreased trabecular separation ($p < 0.05$, Two-way ANOVA, Figure 4.3). Each group on the supplemented diet had significantly greater trabecular BV, BV/TV, number, and thickness and significantly less trabecular separation than the control diet group subjected to the same exercise intensity ($p < 0.05$, Tukey's tests, Figure 4.3). Low-speed exercised mice on the control diet had significantly greater BV/TV than non-exercised mice on the control diet. High-speed exercised mice on the control diet had significantly greater trabecular number than non-exercised mice on the control diet. All groups on the supplemented diet had significantly greater BV and BV/TV than baseline, while the non-exercised mice on the control diet had significantly lower BV and BV/TV than baseline ($p < 0.05$, t-test, Figure 4.3). All groups on the control diet had significantly lesser trabecular number and significantly greater trabecular separation than baseline. Mice from all groups had significantly greater trabecular thickness than baseline. Low-speed exercised mice on the supplemented diet and all mice on the control diet had significantly greater TMD than baseline.

The Supplemented Diet Increased Yield Force, and Exercise Had No Effects on Structural-Level Mechanical Properties after Four Weeks

There was a significant main effect of diet that increased yield force ($p < 0.05$, Two-way ANOVA, Figure 4.4). On the supplemented diet, non-exercised mice and low-speed exercised mice had significantly greater yield force and pre-yield work than baseline ($p < 0.05$, t-test, Figure 4.4). Low-speed exercised mice on the control diet also had significantly greater yield force than baseline. There were no significant main effects of exercise or group differences for any structural-level mechanical property.

High-Speed Exercise Decreased Ultimate Stress, and the Supplemented Diet Had No Effects on Tissue-Level Mechanical Properties after Four Weeks

Exercise had a significant main effect that decreased ultimate stress, and pooled ultimate stress data from high-speed exercised mice was significantly lower than from non-exercised mice ($p < 0.05$, Two-way ANOVA, Figure 4.5). Ultimate stress was significantly lower in high-speed exercised mice on the supplemented diet than non-exercised mice on the supplemented diet ($p < 0.05$, Tukey's test, Figure 4.5). The non-exercised mice on the supplemented diet had significantly greater yield stress, ultimate stress, and Young's modulus than baseline ($p < 0.05$, t-test, Figure 4.5). There were no significant main effects of diet on tissue-level mechanical properties.

The Supplemented Diet Increased Serum Ca on Day 9 and Day 30 While Exercise Increased Serum Ca only on Day 30

Diet had significant main effects that increased serum Ca on day 9 and day 30, relative to the non-exercised mice on the control diet ($p < 0.05$, Two-way ANOVA, Figure 4.6). Exercise had a significant main effect that increased serum Ca on day 30. High-speed exercised mice had significantly greater serum Ca than non-exercised mice on day 30 ($p < 0.05$, Tukey's test, Figure 4.6). There were no significant differences between groups on the supplemented diet at the same time point. There were significant main effects of diet, exercise, and diet and exercise interaction on serum P on day 30. Both exercised groups on the supplemented diet had significantly lower serum P than the corresponding exercised groups on the control diet and lower serum P than the non-exercised mice on the supplemented diet.

Exercise Decreased PINP/CTX Ratio in Mice on the Control Diet on Day 9, and Mice on the Supplemented Diet on Day 30

Exercise had significant main effects that increased serum CTX and decreased PINP/CTX ratio on day 9 ($p < 0.05$, Two-way ANOVA, Figure 4.7). Both groups of exercised mice on the control diet had significantly higher day 9 CTX than the non-exercised mice on the control diet ($p < 0.05$, Tukey's test, Figure 4.7). Non-exercised mice on the control diet had significantly higher day 9 PINP/CTX ratio than non-exercised mice on the supplemented diet and both groups of exercised mice on the control diet. Exercise had

significant main effects on serum CTX, serum PINP, and PINP/CTX ratio on day 30. Diet

had a significant main effect that increased serum CTX on day 30. There was also a

significant diet and exercise interaction on the day 30 PINP/CTX ratio. In high-speed

exercised mice on the supplemented diet, day 30 serum CTX was significantly higher,

while day 30 CTX/PINP ratio was significantly lower compared to high-speed exercised

mice on the control diet. Both day 30 serum CTX and PINP were significantly lower for

high-speed exercised mice on the control diet than they were for that group on day 9 (p

< 0.05, t-test, Figure 4.7). Day 30 PINP/CTX ratio was significantly lower for non-

exercised mice on the control diet than it was for that group on day 9.

DISCUSSION

Four weeks of high-speed exercise prevented tibial growth and increases in yield force

from baseline (Figure 4.2,Figure 4.4). Longitudinal growth occurred only in the low-

speed exercised and non-exercised groups. High-speed exercised mice on the

supplemented diet had greater tibial cortical TMC and cross-sectional area than high-

speed exercised mice on the control diet, and all high-speed exercised mice had

increased cortical TMD from baseline. These data suggest that under the higher

frequency loading, longitudinal bone growth may have been limited in favor of

increasing cortical bone mass and mineralization. When the supplemented diet was

added to high-speed exercise, bone prioritized increasing cortical bone TMC and area

over restoring longitudinal growth. This prioritization may be a sign that the dietary

mineral supply was suboptimal for maximizing benefits from exercise.

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While for cortical bone there were no main effects of exercise on morphology, mineralization or mechanical properties, and few significant group differences between mice on different diets, trabecular bone was impacted more by the exercise and diet. The supplemented diet increased trabecular bone volume, independent of exercise (Figure 4.3). Exercise was more effective in mice on the control diet as both speeds of exercise increased BV and BV/TV relative to non-exercised mice. The speed of exercise did not affect trabecular bone properties. The different effects of diet and exercise on trabecular bone volume suggest that while BV and BV/TV are decreasing with age from 15 to 19 weeks, exercise without dietary intervention prevents this decline. Increasing dietary mineral supply increases bone volume, regardless of exercise.

Since combining exercise with the supplemented diet offered no further increases in trabecular bone volume over the supplemented diet alone, exercise may only be affecting trabecular bone when there is a smaller supply of dietary minerals. Mice on the supplemented diet had significantly greater serum Ca than non-exercised mice on the control diet on day 9 and day 30, while exercised mice on the control diet only had significantly greater serum Ca on day 30 (Figure 4.6). The greater serum supply of Ca from the supplemented diet may have led to greater increases in bone volume at earlier time points, which ultimately led to greater BV and BV/TV after 4 weeks. Exercise alone increased serum Ca only after day 9, and this increased mineral supply may be what prevented the decrease in BV and BV/TV from baseline in exercised mice on the control diet. There could be some peak bone volume achieved such that combining exercise with the supplemented diet did not increase BV beyond what was achieved with the

supplemented diet alone. If that is the case, it may be possible that extending the duration of the exercise program could lead to exercised mice on the control diet also reaching some peak bone volume.

Exercise may have only affected trabecular bone because changes in the response of bone to stimuli can be site specific [26]. The increased BV/TV, but not cortical area, TMC or TMD, in low-speed exercised mice on the control diet compared to non-exercised mice on the control diet may be a sign that this short-term exercise program was more effective on trabecular bone. Longer-term exercise for 8 weeks increases cortical TMC and area [Chapter 2] and may also have increased cortical TMC and area here if exercise had been continued for 4 more weeks. Changes in trabecular, but not cortical bone after a short exercise program could occur because trabecular bone metabolism can be more rapid than cortical bone metabolism.

There were few significant main effects of exercise or diet on structural-level and tissue-level mechanical properties as the supplemented diet increased only yield force, while exercise decreased ultimate stress (Figure 4.4, Figure 4.5). This lack of effect on mechanical properties is similar to what was seen after 3 weeks of exercise and supplemented diet treatments [31,Chapter 2]. After a short duration of an exercise program, bone appears to be in the process of changing tissue composition and increasing bone mass to achieve the changes seen after longer exercise programs of 6-12 weeks. Studying the effects of exercise after 4 weeks may not be adequate for understanding the full impact of exercise on mechanical properties. Also, testing failure

strength of bone may not be the best evaluation of short-term exercise effects on bone strength. Fatigue loading bones to determine fatigue life, measure buildup of microdamage, or measure resistance to loss of stiffness with fatigue may be better evaluations of the ability of short-term exercise to increase tissue quality.

There was no significant effect of exercise intensity on any measurements of cortical or trabecular bone, or mechanical properties. Increasing treadmill speed may not be impactful on bone if it does not lead to greater peak loads on bone, which may have occurred here. High-speed exercise did lead to greater average loading frequency, which increases bone formation rate [16]. Bone formation rate was not directly measured, but the bone metabolism markers CTX, PINP, and PINP/CTX can undergo changes that are indicative of increased bone formation [30,31,Chapter 2]. High-speed exercised mice on the control diet had significantly lower CTX and PINP on day 30 than on day 9 and lower day 30 CTX and PINP compared to low-speed exercised mice on the control diet (Figure 4.7). High-speed exercise also led to significantly greater serum Ca on day 30 and significantly lower tibia length for mice on the control diet (Figure 4.6). For mice on the control diet, this high-speed exercise program appears to be lowering bone turnover and preventing longitudinal growth, the opposite of what happens with longer-term exercise [20]. The supplemented diet appears to be preventing effects of high-speed exercise on tibial length and bone metabolism markers as there were no significant differences in any properties measured between the low-speed and high-speed exercised groups on the supplemented diet. More analysis of tissue composition

and tissue quality is needed to determine the full extent of effects of this high-speed exercise regimen and how dietary mineral supply interacts with exercise speed.

Both speeds of exercise significantly decreased PINP/CTX ratio for mice on the control diet on day 9, leading to a less formation-favored state of bone metabolism (Figure 4.7). Similar to exercise significantly decreasing PINP after one day of exercise [Chapter 2], lower PINP/CTX on day 9 suggests exercise may be continuing to limit bone growth for over a week. These are transient decreases as there are no significant differences in cortical bone mass, and trabecular bone volume is significantly greater in exercised mice after 4 weeks. Exercise did not affect bone metabolism markers for mice on the supplemented diet on day 9. This difference in effects on bone metabolism may be caused by the elevated serum Ca on day 9 seen in all mice on the supplemented diet and may be partially responsible for different effects of exercise on trabecular bone volume, depending on diet.

The opposite effect of diet occurred on day 30 as exercise significantly decreased the PINP/CTX ratio only for mice on the supplemented diet. One possible explanation is that exercised mice on the supplemented diet may be achieving the same peak bone mass as non-exercised mice on the supplemented diet, but at a faster rate. There would likely be some time point where bone metabolism has slowed down for exercised mice on the supplemented diet as was seen on day 30 in this study. Similarly, for non-exercised mice on the control diet, PINP/CTX is significantly lower on day 30 than on day 9, suggesting a decline in bone metabolism rate. Peak bone mass is expected to be

lowest in mice in this group, and this shift in bone metabolism may be a sign that these mice are also at or near peak bone mass. As there are many significant effects of exercise and diet on CTX, PINP, and CTX/PINP ratio on day 30, extending the duration of the exercise and diet treatments would likely lead to further changes in bone mass between the groups. Continuing these treatments until bone metabolism has reached some steady state would allow for the best evaluation of long-term effects of exercise intensity.

Both high-speed and low-speed exercised mice on the supplemented diet had significantly lower day 30 serum P than non-exercised mice and exercised mice on the control diet (Figure 4.6). It may be possible that serum P was being depleted to use for increasing bone mineralization as both groups with lower serum P were the only groups with significantly higher cortical TMC than baseline (Figure 4.2). Rodents subjected to treadmill exercise can increase demand for P as well as Ca [30]. Although the supplemented diet has twice the P as the control diet, this still may not be sufficient for the increased mineral demands from exercise at this time point. Exercise did not decrease serum P for mice on the control diet, but this may be due to the difference in Ca in the diets as the control diet had equal amounts of Ca and P, while the supplemented diet had 5 times more Ca than P. The Ca:P ratio in the supplemented diet may provide an adequate amount of Ca, but not P. Further analysis of tissue composition of these bones may be needed to determine if there were any differences in bone Ca and P content from the diets or exercise treatments. Additionally, mice were not assigned to groups of equivalent baseline serum P. Initial differences in serum P

may have been a factor in the significant group differences seen on day 30, though only the exercised mice on the supplemented diet had a decrease in serum P from day 9 to day 30.

One significant main effect of exercise that did not translate into differences in cortical or trabecular bone properties was the lower body weight for all high-speed exercised mice and for low-speed exercised mice on the control diet (Figure 4.1). Although mice in these groups had lower body weight, they did not have lower tibial bone mass or mechanical strength, as might be expected [31,32]. When bone mass was normalized by weight, the high-speed exercised mice on the supplemented diet had significantly higher tibial length, cortical TMC, area and TMD than all other mice on the supplemented diet (data not shown). Thus, even though exercise did not directly affect most measurements of bone mass or mechanical strength for mice on the supplemented diet, the high-speed exercise allowed for these mice to reach the same bone mass and strength while at a lower body weight. This high-speed exercise regimen may be offering additional health benefits not measured, such as preventing increases in fat mass, increasing lean body mass, and improving cardiovascular health, at no expense to bone health.

After 4 weeks of high-speed treadmill exercise, exercised mice had lower body weight, and the exercise prevented an increase in tibial length, regardless of dietary mineral supply. These changes in growth did not come at the expense of tibial cortical bone mass, trabecular bone volume, or mechanical properties. High-speed exercised mice on

the supplemented diet had the greatest cortical TMC and area and trabecular BV and BV/TV after 4 weeks. Combining a high-intensity exercise program with a mineral-supplemented diet may best enable peak bone mass to be reached and prevent fractures.
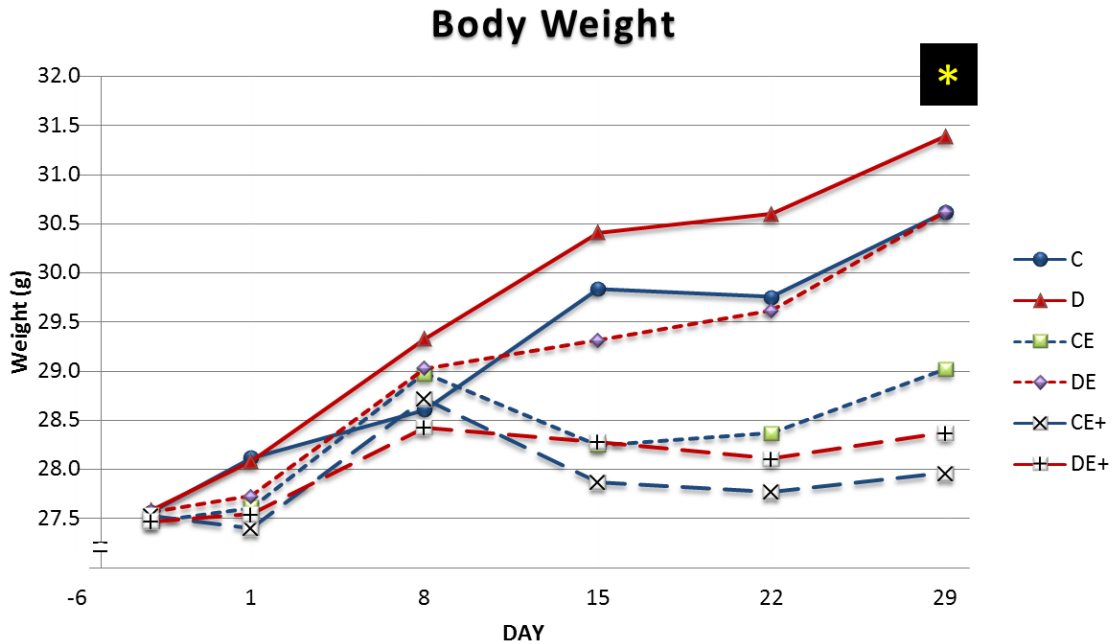
Acknowledgments

Figure 4.1. Mouse body weight (mean ± SD). High-speed exercise limited gains in body weight after four weeks. On day 29, both of the high-speed exercised groups and the low-speed exercised group on the control diet had lower body weight than the non-exercised groups and the exercised group on the supplemented diet.

*Significant exercise effect (p < 0.05, Two-way ANOVA).
C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
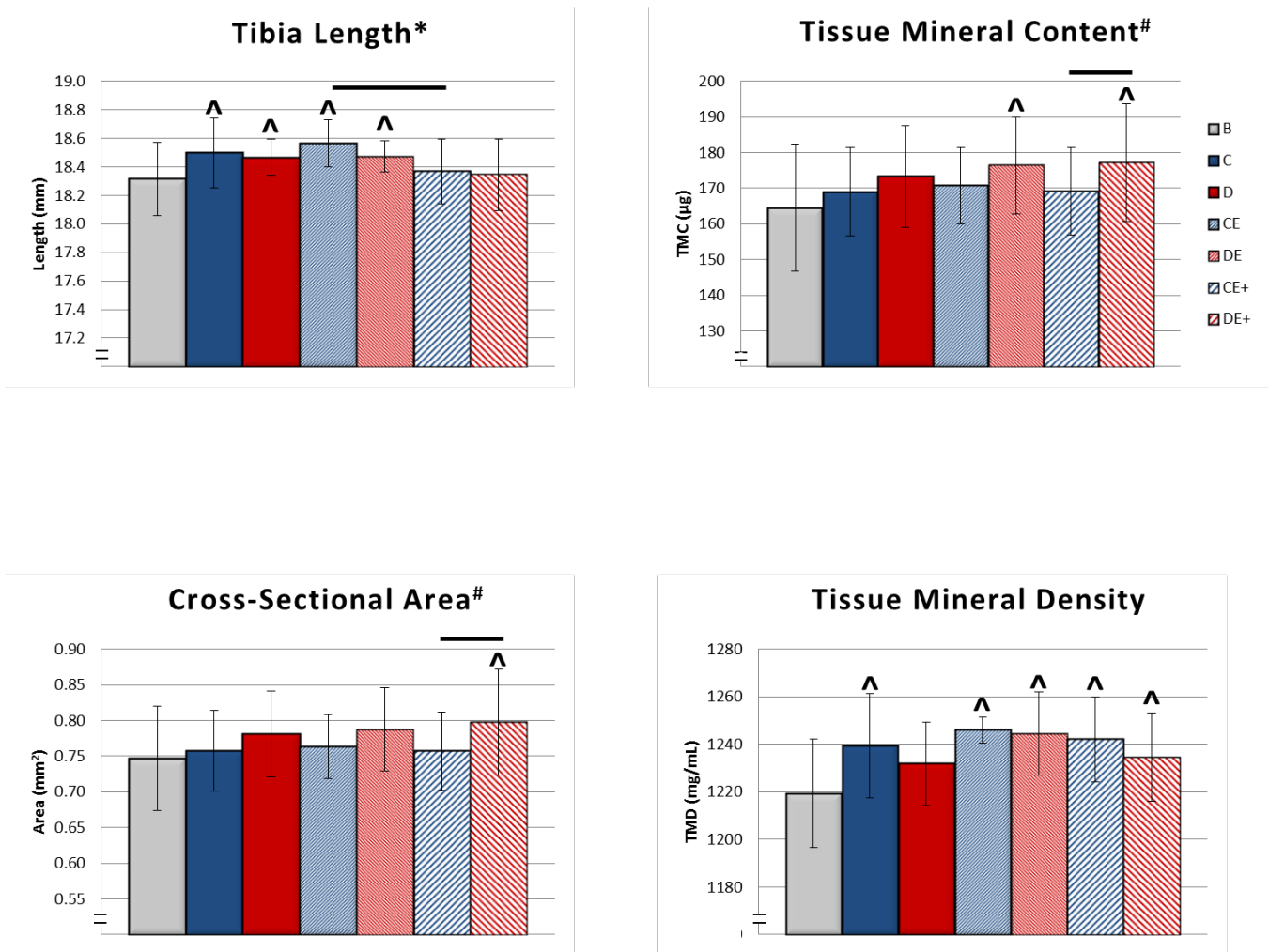
Figure 4.2. Mouse tibial cortical bone cross-sectional geometric properties and mineralization (mean ± SD). Tibial length increased from baseline for all groups except the high-speed exercised groups. High-speed exercised mice fed the supplemented diet had significantly greater TMC and cross-sectional area compared to high-speed exercised mice fed the control diet.

#Significant diet effect ($p < 0.05$, Two-way ANOVA).
*Significant exercise effect ($p < 0.05$, Two-way ANOVA).
^Significantly different from baseline ($p < 0.05$, t-test).
Horizontal bar represents significant difference between groups ($p < 0.05$, Tukey's test).

B – baseline mice, C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
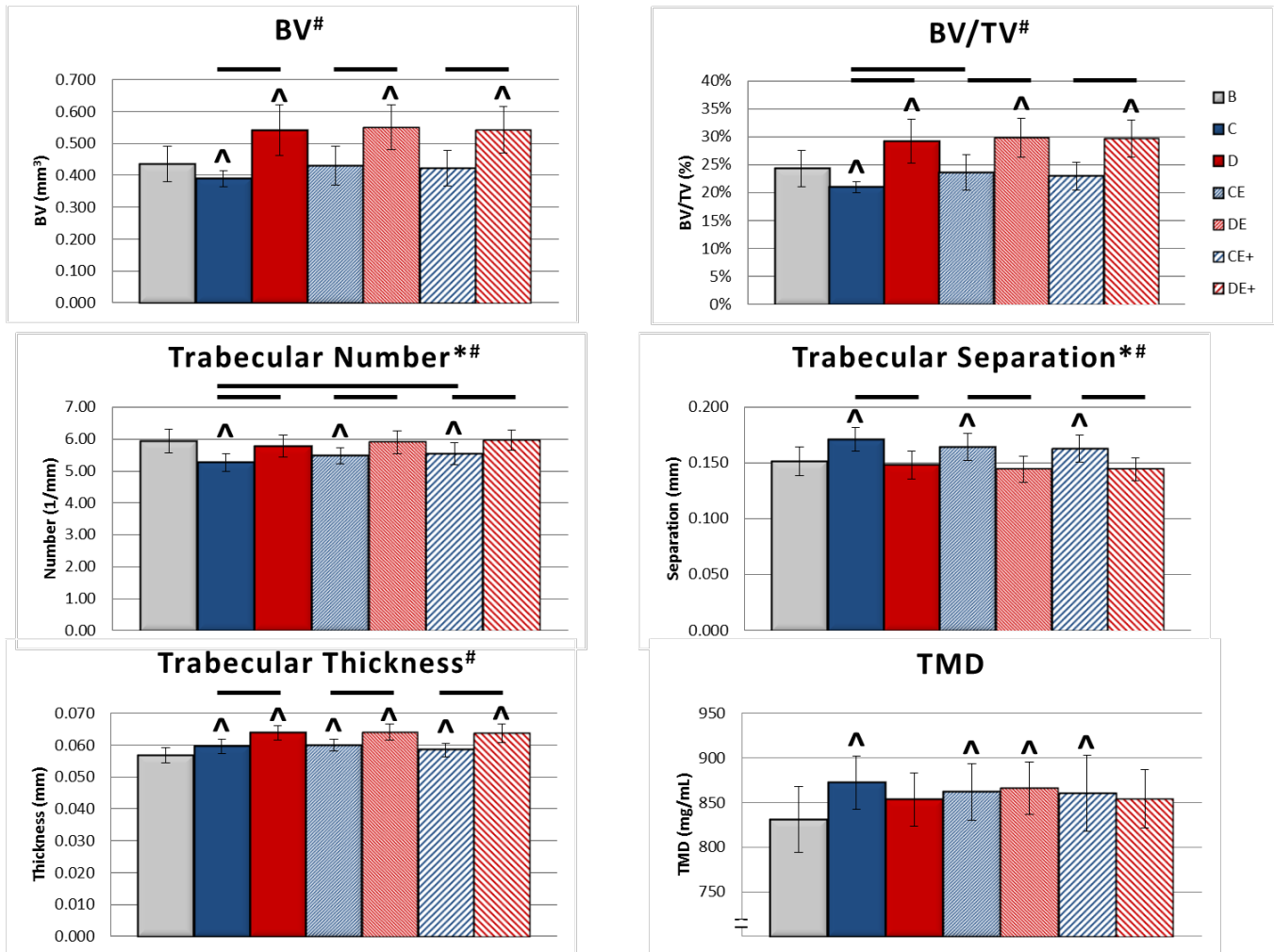
Figure 4.3. Proximal tibial trabecular architecture (mean ± SD). Diet had a significant main effect on every property except tissue mineral density (TMD), increasing trabecular bone volume (BV), bone volume/total volume (BV/TV), number, and thickness and decreasing separation. For mice on the control diet, both speeds of exercise prevented a significant decrease in BV and BV/TV from baseline.

#Significant diet effect (p < 0.05, Two-way ANOVA).
*Significant exercise effect (p < 0.05, Two-way ANOVA).
^Significantly different from baseline (p < 0.05, t-test).
Horizontal bar represents significant difference between groups (p < 0.05, Tukey's test).
B – baseline mice, C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
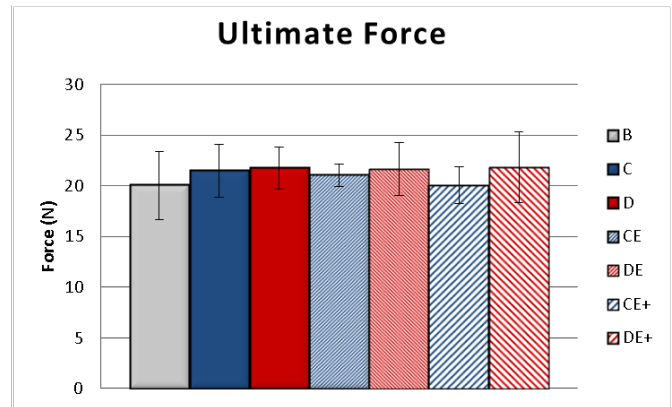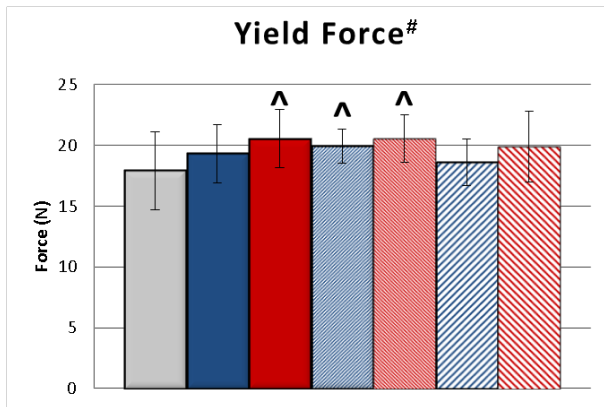
Figure 4.4. Structural-level tibial mechanical properties (mean ± SD). There was a significant main effect of diet on yield force, and non-exercised mice on the supplemented diet and mice exercised at low intensity on the standard and supplemented diets had significantly greater yield force and pre-yield work than baseline.

#Significant diet effect ($p < 0.05$, Two-way ANOVA).
^Significantly different from baseline ($p < 0.05$, t-test).
B – baseline mice, C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
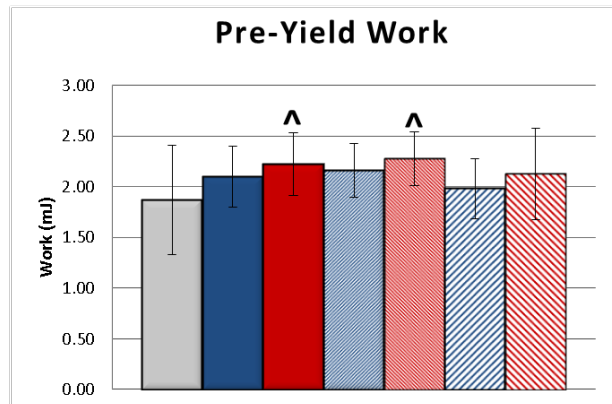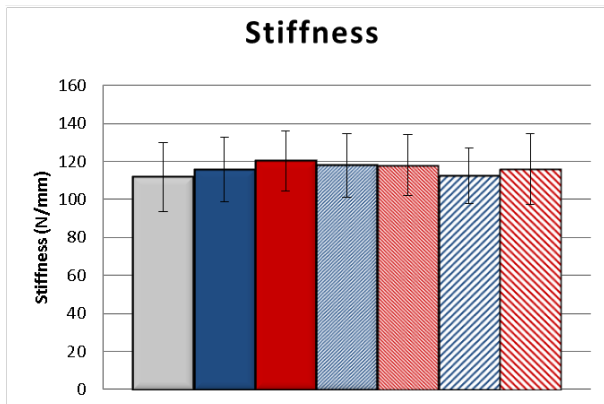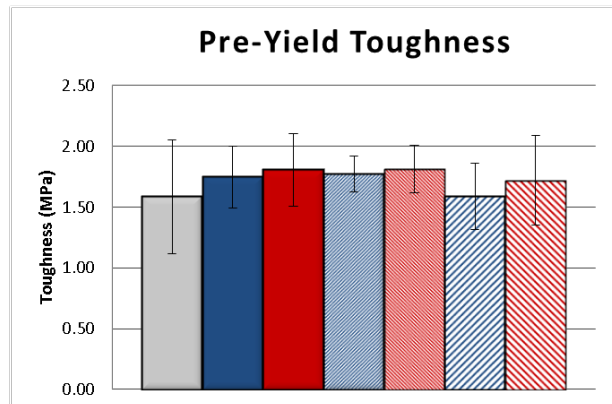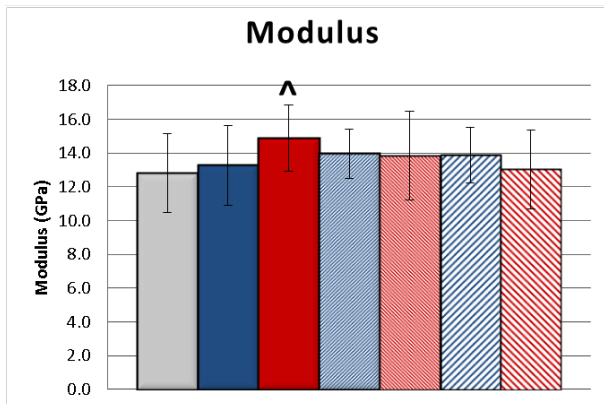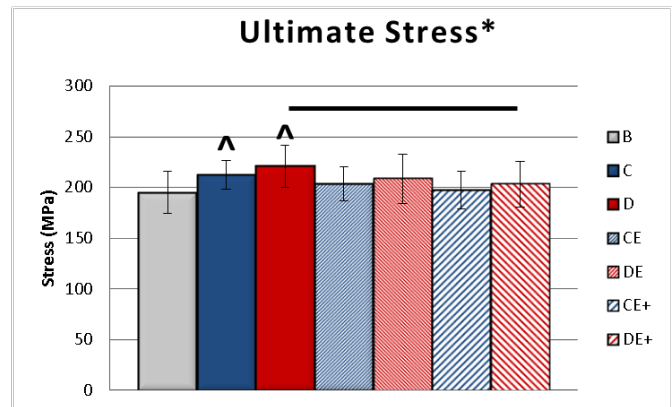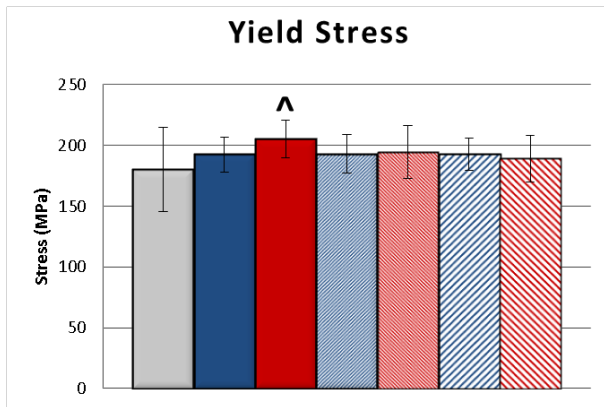
Figure 4.5. Tissue-level tibial mechanical properties (mean ± SD). Yield stress, ultimate stress, and modulus were all significantly greater than baseline in non-exercised mice on the supplemented diet. Exercise had a significant decreasing main effect on ultimate stress, and high-speed exercised mice on the supplemented diet had significantly lower ultimate stress than non-exercised mice on the supplemented diet.

*Significant exercise effect ($p < 0.05$, Two-way ANOVA).
^Significantly different from baseline ($p < 0.05$, t-test).
Horizontal bar represents significant difference between groups ($p < 0.05$, Tukey's test).
B – baseline mice, C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
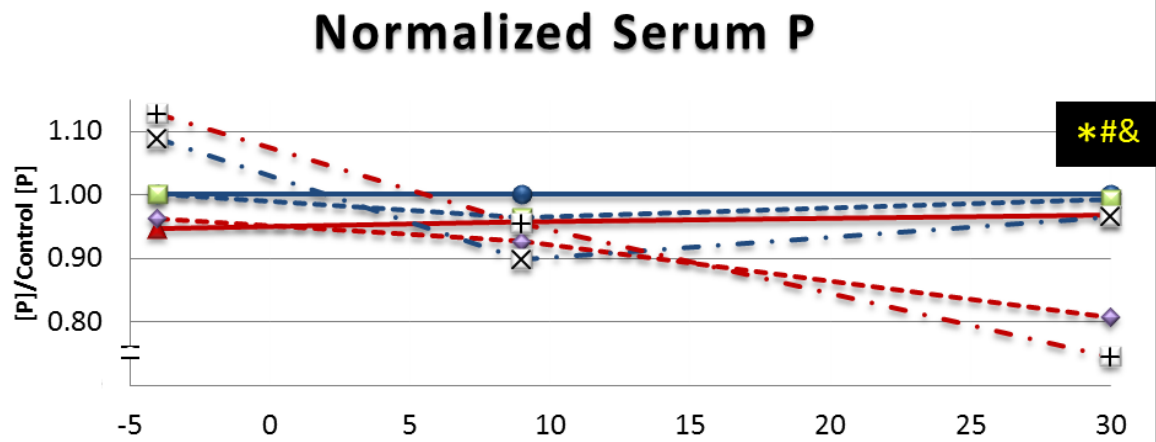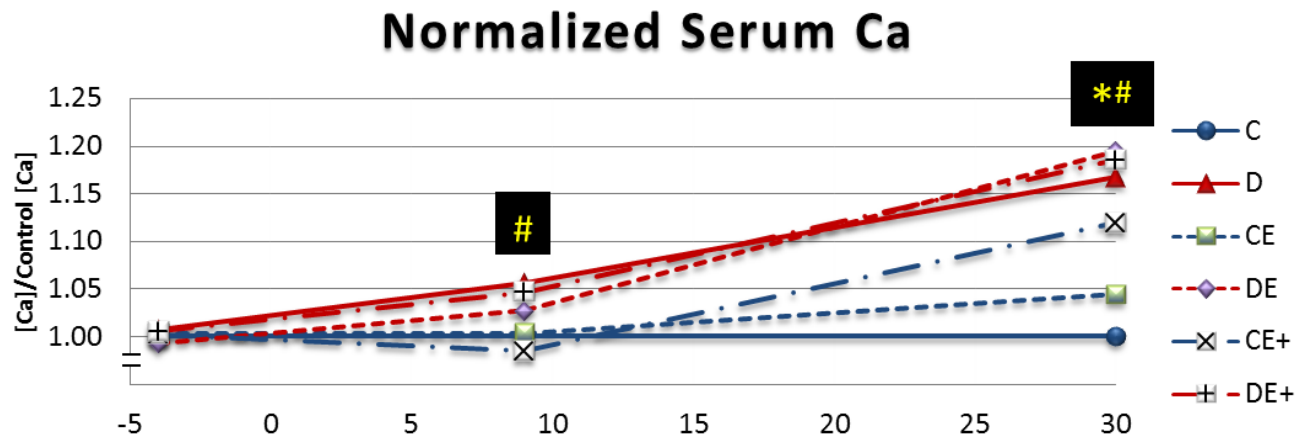
Figure 4.6. Mean serum [Ca] and [P] on days -4, 9, and 30. Data is normalized to serum concentrations in non-exercised mice on the control diet at the same time point. The supplemented diet significantly increased serum Ca on days 9 and 30. Exercise only significantly increased serum Ca on day 30. Serum P was significantly decreased in exercised mice on the supplemented diet only after day 30.

#Significant diet effect on that day (p < 0.05, Two-way ANOVA).
*Significant exercise effect on that day (p < 0.05, Two-way ANOVA).
&Significant diet and exercise interaction on that day (p < 0.05, Two-way ANOVA).
C – non-exercised mice fed the control diet, D – non-exercised mice fed the supplemented diet, CE – low-speed exercised mice fed the control diet, DE – low-speed exercised mice fed the supplemented, CE+ – high-speed exercised mice fed the control diet, DE+ – high-speed exercised mice fed the supplemented diet
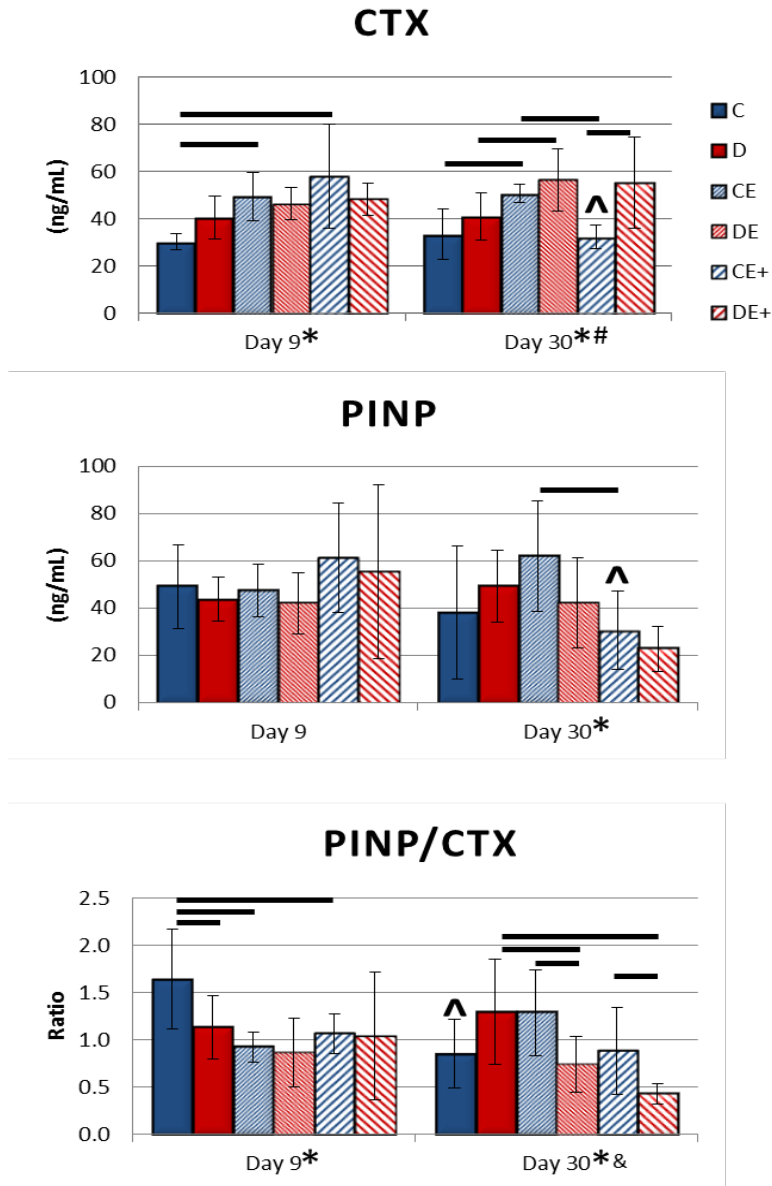
Figure 4.7. Serum CTX and PINP (mean ± SD) on day 9 and day 30. Exercise significantly increased CTX on day 9 and day 30, regardless of treadmill speed. High-speed exercise significantly decreased PINP on day 30, compared to low-speed exercise. For mice on the control diet, exercise significantly decreased PINP/CTX on day 9, causing a less formation-favored state of bone metabolism. Similarly, for mice on the supplemented diet, exercise significantly decreased PINP/CTX on day 30.

#Significant diet effect (p < 0.05, Two-way ANOVA).
*Significant exercise effect (p < 0.05, Two-way ANOVA).
^Significant difference between day 9 and day 30 measurement (p < 0.05, t-test).
Horizontal bar represents significant difference between groups (p < 0.05, Tukey's test).
&Significant diet and exercise interaction on that day (p< 0.05, Two-way ANOVA).

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CHAPTER FIVE

CALCIUM- AND PHOSPHORUS-SUPPLEMENTED DIET INCREASES BONE MASS AND MECHANICAL STRENGTH AND PREVENTS LOSS OF BONE MASS AND MECHANICAL STRENGTH WITH AGING IN ADULT MICE

ABSTRACT

Exercise has long-lasting benefits to bone mass and structural strength even after cessation of exercise. Combining a lengthy exercise program with a calcium- and phosphorus-supplemented diet increases cortical bone tissue mineral content (TMC), area, yield force, and ultimate force more than exercise alone in adult mice. It was hypothesized that combining exercise with a mineral-supplemented diet would lead to greater cortical TMC, area, yield force, and ultimate force immediately after a lengthy exercise program and after an equally long period of non-exercise (detraining) in adult mice. Male, 16-week old mice were assigned to 9 weight-matched groups – a baseline group, exercise and non-exercise groups fed a control or mineral-supplemented diet for 8 weeks, exercise + detraining and non-exercise groups fed a control or mineral-supplemented diet for 16 weeks. Exercise + detraining consisted of 8 weeks exercise followed by 8 weeks non-exercise. The daily exercise program consisted of running on a treadmill at 12 m/min, 30 min/day. All mice gained body weight at the same rate from

30.8 ± 1.4 g (mean ± SD) on day 1 to 39.4 ± 3.4 g after sixteen weeks. After 8 weeks, all mice fed the supplemented diet had greater tibial cortical TMC and area, trabecular bone volume/tissue volume (BV/TV), yield force, and ultimate force than all mice fed the control diet. Exercise only increased cortical TMC and area and only did so in mice on the supplemented diet. After 16 weeks, all mice fed the supplemented diet maintained greater tibial cortical TMC and area, trabecular BV/TV, yield force, and ultimate force than all mice fed the control diet. Exercise + detraining had no effects on mice on the supplemented diet. For mice on the control diet, exercise + detraining prevented a decrease in yield force, ultimate force, yield stress and ultimate stress from 8 to 16 weeks. Non-exercised mice on the control diet had decreased bone mass from baseline to after 8 weeks and decreased trabecular bone, structural strength and tissue strength from 8 weeks to 16 weeks. These negative effects likely occurred as a result of increasing mouse body weight and/or age. Long-term use of dietary mineral supplements may help increase and maintain bone mass in adult mice. Exercise while on a standard diet prevents loss of trabecular bone and bone mechanical strength with weight gain and/or aging.

INTRODUCTION

Weight-bearing exercise offers many benefits to bone health that may help reduce fracture risk. Exercise increases bone mass, structural-level (whole bone) strength, and tissue quality, making bone better able to resist fracture [1]. There is also evidence that exercise has long-term benefits to bone health, even after cessation [2–5]. Increases in

cortical bone mineral content, cross-sectional area, and structural-level strength (stiffness) can remain after months or even years of inactivity following exercise [2–8]. Since bone mass continually declines with age throughout adulthood [9–11], it may be beneficial to accumulate bone mass earlier in life to maintain a higher level of bone mass in old age when weight-bearing exercise becomes more difficult to perform.

Combining exercise with a calcium- and phosphorus-supplemented diet can be one strategy to maximize bone mass early in adulthood. In young adult mice, combining exercise with a mineral-supplemented diet increases cortical tissue mineral content (TMC), area, yield force, and ultimate force more than exercise alone [Chapter 2]. If these increases in bone mass and structural-level strength can also be maintained after stopping exercise, then combining exercise with a mineral-supplemented diet may be a better method of accumulating bone mass earlier in life than just exercise on a standard diet. Thus it was hypothesized that increases in cortical TMC, area, yield force, and ultimate force after 8 weeks of a combined supplemented diet and exercise program in mice would remain after 8 weeks of detraining, giving the greatest long-term benefits to bone mass and structural strength.

METHODS

Animals and Treatments

All animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals. One hundred seventy-six male C57BL/6 mice, 30.2 ± 1.2 g mean body weight, were purchased from Charles River Laboratories (Wilmington, MA) at 14 weeks of age and placed in single housing to prevent fighting. The mice were started on the control diet and were given 2 weeks to acclimate. Starting on experiment day 1, at 16 weeks of age, mice were randomly assigned to one of 5 weight-matched groups – a baseline group sacrificed on day 1 (B), a non-exercise group fed the control diet (C), a non-exercise group fed the supplemented diet (D), an exercise group fed the control diet (CE), and an exercise group fed the supplemented diet (DE). After 8 weeks, each experimental group was divided into 2 weight-matched groups of equal number of mice. One group was sacrificed immediately at age 24 weeks, and the other group was allowed to continue for an additional 8 weeks. Mice that continued for weeks 9-16 remained on the same diets as during weeks 1-8, but discontinued exercise. At the end of week 16, all remaining mice were sacrificed at age 32 weeks. Left tibiae were harvested immediately after sacrifice for analysis.

Diets and Exercise Program

The control diet consisted of an AIN-93G diet (TestDiet®, Richmond, IN) modified by adding dicalcium phosphate to contain 0.5% Ca and 0.5% P. The supplemented diet was modified by adding dicalcium phosphate and calcium carbonate to contain 5% Ca and 1% P. Ca, P, and Ca:P ratio were all increased to increase serum Ca by increasing

intestinal Ca absorption [12–14]. The control diet contained 3.90 kcal/g with an energy distribution of 65.0% carbohydrates, 16.3% fat, and 18.7% protein while the supplemented diet had 3.39 kcal/g with the same energy distribution. All other nutrients were equivalent between the two diets. The exercise program consisted of running on a 5° incline treadmill at 12 m/min, 30 min/day for 56 consecutive days [15,16]. Mice were gradually increased to a maximum speed of 12 m/min in the first 3 days of exercise.

Cortical Geometry and Trabecular Architecture Measurements

Whole tibiae were embedded in 1% agarose, placed in a 19 mm diameter tube, and scanned using a micro-CT specimen scanner (μCT100 Scanco Medical, Bassersdorf, Switzerland) with a voxel size of 12 μm (70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms). Scans were analyzed with Scanco IPL software. A 180-μm thick transverse section from a standard site located 21.7% of the distance from the tibia-fibula junction to the proximal end of the tibia was chosen for measurement of cortical geometry metrics - TMC, volumetric tissue mineral density (TMD), cross-sectional area, and moment of inertia about the anterior-posterior axis. This section is located approximately at the center of the mechanical testing region. Geometry metrics were calculated using a fixed global threshold of 26% (260 on a grayscale of 0–1000) to separate bone from non-bone. Another 180-μm thick transverse section at the fracture site was analyzed for cortical geometry measurements used in calculations of tissue-

level mechanical properties (moment of inertia, distance from neutral axis). Tibial scans were further analyzed for trabecular bone architecture.   Proximal tibial metaphyseal sections immediately below the growth plate of 480-µm thick were analyzed in Scanco IPL software using freehand traced volumes of interest. Architecture metrics measured were bone volume (BV), bone volume fraction (BV/TV), TMD, trabecular number, trabecular thickness, and trabecular separation. These metrics were calculated using a fixed global threshold of 18% (180 on a grayscale of 0–1000) to separate bone from non-bone.

Mechanical Testing

Structural- and tissue-level mechanical properties were measured in all groups. Structural-level properties (force, deformation, stiffness, work) were measured from a 4-point bending to failure test (3-mm inner and 9-mm outer spans). Tibiae were loaded to failure with the medial side of the mid-diaphysis in tension under displacement control at 0.025 mm/sec at a data sampling rate of 30 Hz. Tissue-level mechanical properties (stress, strain, modulus, toughness) were estimated using beam bending theory with geometric measurements (moment of inertia about anterior-posterior axis, distance from centroid to medial side of the bone) from micro-CT data at the fracture site [17].

Statistical Analysis

Cortical geometry measurements, trabecular geometry measurements, and mechanical properties were tested by Two-way ANOVA with Tukey's post-hoc tests to determine if the individual effects of diet or exercise were significant ($p < 0.05$) and if the combined treatments had a significant interactive effect. Student's t-tests were used to compare baseline to experimental groups and to compare week 8 to week 16 experimental group values.

RESULTS

All Mice Continuously Gained Body Weight throughout the Study

There were no significant differences in mean body weight between any of the groups at any time point (Figure 5.1). Mice started at an above average weight of 30.8 ± 1.4 g (mean ± SD) at 16 weeks of age on day 1. All mice continuously gained weight in the first 14 weeks, ending with an overall average of 39.4 ± 3.4 g after 16 weeks.

The Combined Supplemented Diet and Exercise Increased Tibial Cortical TMC and Area While the Control Diet Decreased Area from Baseline after Eight Weeks

There were significant main effects of diet on cortical TMC, area, and moment of inertia about the anterior-posterior axis after 8 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.2). Exercise also had significant main effects on TMC and area. Eight weeks of exercise on the supplemented diet led to the greatest cortical TMC, area, and moment of inertia of

all groups (p < 0.05, Tukey's tests, Figure 5.2). TMC and area increased from baseline only when the supplemented diet and exercise were combined (p < 0.05, t-test, Figure 5.2). Exercised mice on the control diet had decreased cortical area from baseline. Non-exercised mice on the control diet had decreased cortical TMC and area from baseline. The supplemented diet prevented these decreases in non-exercise mice. TMD increased from baseline only in exercised mice.

The Supplemented Diet Increased Tibial Cortical TMC and Area after Sixteen Weeks, Regardless of Exercise

There were significant main effects of diet on cortical TMC, area, and moment of inertia, but no main effects of exercise after 16 weeks (p < 0.05, Two-way ANOVA, Figure 5.2). Non-exercised mice on the supplemented diet had significantly greater TMC, area, and moment of inertia after 16 weeks than after 8 weeks on the diet (p < 0.05, t-test, Figure 5.2). All mice sacrificed after 16 weeks had no loss of cortical bone geometry measurements compared to mice that were sacrificed immediately after 8 weeks of exercise. TMD was significantly greater than baseline in all groups after 16 weeks (p < 0.05, t-test, Figure 5.2), and there were no significant differences between the 16-week groups.

The Supplemented Diet Increased Tibial Trabecular BV, BV/TV, Number and Thickness after Eight Weeks

There were significant main effects of diet on BV, BV/TV, trabecular number, thickness, and separation after 8 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.3). Exercise had a significant main effect on BV/TV and trabecular thickness. Exercised mice on the supplemented diet had the greatest trabecular thickness of all groups and had greater BV, BV/TV, trabecular number, trabecular thickness and less trabecular separation than exercised mice on the control diet ($p < 0.05$, Tukey's tests, Figure 5.3). Both groups on the supplemented diet had significantly greater BV, BV/TV, and trabecular thickness than baseline mice ($p < 0.05$, t-test, Figure 5.3). The control diet had the opposite effect, as all mice on it had significantly lower BV, BV/TV, trabecular number and greater trabecular separation than baseline, regardless of exercise state. TMD in both groups on the supplemented diet was significantly greater than baseline mice.

The Supplemented Diet Increased Tibial Trabecular BV, BV/TV, Number and Thickness after Sixteen Weeks

For all groups except the non-exercised mice on the supplemented diet, BV, BV/TV, and trabecular number were significantly lower after 16 weeks than after 8 weeks ($p < 0.05$, t-tests, Figure 5.3). There were also significant main effects of diet on BV, BV/TV, TMD, trabecular number, thickness, and separation after 16 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.3). Exercise had no significant main effects on trabecular architecture

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at this time point. Exercised mice on the supplemented diet had significantly greater BV, BV/TV, trabecular thickness and less trabecular separation than exercised mice on the control diet ($p < 0.05$, Tukey's tests, Figure 5.3). Both groups on the supplemented diet had significantly greater BV, BV/TV, TMD and trabecular thickness than baseline mice ($p < 0.05$, t-tests, Figure 5.3). The control diet had the opposite effect, as all mice fed this diet had significantly lower BV, BV/TV, and trabecular number with greater trabecular separation than baseline. Trabecular thickness was significantly greater than baseline for all groups and greater than it was after 8 weeks for the non-exercise groups. Trabecular separation was higher after 16 weeks than after 8 weeks for all mice on the control diet. TMD in non-exercised mice on the supplemented diet was significantly greater than it was for mice on the same treatment for 8 weeks and greater than it was for non-exercised mice on the control diet after 16 weeks.

The Supplemented Diet Increased Tibial Yield Force, Ultimate Force, Stiffness, and Pre-Yield Work after Eight Weeks

There were significant main effects of diet on yield force, ultimate force, stiffness, and pre-yield work after 8 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.4). Exercised mice on the supplemented diet had significantly greater yield force, ultimate force, and stiffness than exercised mice on the control diet ($p < 0.05$, Tukey's tests, Figure 5.4). Non-exercised mice on the supplemented diet had significantly greater yield force, ultimate force, stiffness, and pre-yield work than non-exercised mice on the control diet. All

groups on the supplemented diet had significantly greater stiffness and lower ultimate deformation than baseline (p < 0.05, t-test, Figure 5.4). Exercised mice on the control diet also had significantly lower yield force, ultimate force, and ultimate deformation than baseline.

The Supplemented Diet Increased Tibial Yield Force, Ultimate Force, Yield Deformation, Stiffness, and Pre-Yield Work after Sixteen Weeks

There were significant main effects of diet on yield force, ultimate force, yield deformation, stiffness, and pre-yield work after 16 weeks (p < 0.05, Two-way ANOVA, Figure 5.4). There were no significant main effects of exercise, but diet and exercise had a significant interactive effect on yield force, ultimate force, ultimate deformation, and pre-yield work. Exercised mice on the supplemented diet had significantly greater stiffness and pre-yield work than exercised mice on the control diet after 8 weeks exercise plus 8 weeks detraining (p < 0.05, Tukey's tests, Figure 5.4). Non-exercised mice on the supplemented diet had significantly greater yield force, ultimate force, yield deformation, ultimate deformation, stiffness, and pre-yield work than non-exercised mice on the control diet. Non-exercised mice on the control diet had significantly lower yield force, ultimate force, yield deformation, ultimate deformation, and pre-yield work after 16 weeks than after 8 weeks (p < 0.05, t-test, Figure 5.4). Exercised mice on the control diet did not show this decline from 8 to 16 weeks even though exercise was discontinued after 8 weeks.

The Supplemented Diet and Exercise Did Not Affect Tibial Tissue-Level Mechanical Properties after Eight Weeks

There were no significant main effects of diet or exercise on tissue-level mechanical properties, but there was a significant diet and exercise interaction on ultimate strain after 8 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.5). There were also no significant group differences for any tissue-level mechanical property measured after 8 weeks. Non-exercised mice on the supplemented diet had significantly lower yield strain and ultimate strain and greater Young's modulus than baseline ($p < 0.05$, t-test, Figure 5.5). Exercised mice on the control diet also had significantly lower yield strain and ultimate strain than baseline. Non-exercised mice on the control diet had significantly lower ultimate strain than baseline.

The Supplemented Diet Increased Tibial Yield Stress, Ultimate Stress, and Pre-Yield Toughness after Sixteen Weeks

There were significant main effects of diet on yield stress, ultimate stress, and pre-yield toughness after 16 weeks ($p < 0.05$, Two-way ANOVA, Figure 5.5). There was a significant diet and exercise interaction on yield stress, ultimate stress, and pre-yield toughness as well. Non-exercised mice on the supplemented diet and exercised mice on the control diet had significantly greater yield stress, ultimate stress, and pre-yield

toughness than non-exercised mice on the control diet (p < 0.05, Tukey's tests, Figure

5.5). For non-exercised mice on the control diet, these tissue-level mechanical

properties were all significantly lower after 16 weeks than after 8 weeks. Exercised mice

on the control diet did not show this decline from 8 to 16 weeks even though exercise

was discontinued after 8 weeks.

DISCUSSION

In this study, 8 weeks of exercise while on a mineral-supplemented diet led to the

greatest tibial cortical TMC, area, and moment of inertia out of all the groups (Figure

5.2). For all mice, there were no significant decreases in cortical bone geometry

measurements from 8 to 16 weeks, even when exercise was discontinued. Non-

exercised mice on the supplemented diet also had significantly greater tibial cortical

TMC, area, and moment of inertia than non-exercised mice on the control diet. These

properties significantly increased from 8 to 16 weeks in mice on the supplemental diet,

but not exercised, such that there were no differences in cortical bone measurements

between the 2 groups on the supplemented diet. Thus, the data suggest combining

exercise with the supplemented diet leads to some peak cortical bone mass at a faster

rate than with the supplemented diet without exercise. Without exercise, mice on the

supplemented diet were still able to reach the same elevated bone mass, but it took

longer than 8 weeks. It is unclear if non-exercised mice on the supplemented diet have

reached peak bone mass or if they would continue to accumulate bone if allowed more

time on the diet. For all mice on the control diet, there was a significant decline in

cortical area from baseline to 8 weeks, but no change from 8 to 16 weeks. Exercise had little effect on mice on the control diet, as there were no significant group differences between the two control diet groups at either time point.

The supplemented diet also significantly increased tibial BV, BV/TV, trabecular number and thickness, and decreased trabecular separation relative to the control diet after 8 and 16 weeks (Figure 5.3). Similar to with cortical bone, exercise was less impactful than diet on trabecular bone properties. There were significant main effects of exercise on BV/TV and thickness after 8 weeks. Exercise also significantly increased trabecular thickness in mice on the supplemented diet compared to non-exercised mice on the supplemented diet. However, this difference was transient as non-exercised mice on the supplemented diet had greater trabecular thickness after 16 weeks than after 8 weeks. Based on the cortical bone data, which suggests both groups on the supplemented diet achieve the same peak bone mass but at different rates, it may be possible that the same result occurred in the trabecular bone, but at a different time point. Since trabecular bone has more rapid turnover, this may have led to the supplemented diet groups reaching peak BV, BV/TV, and trabecular number earlier than 8 weeks. Similarly, exercised mice did not have significantly greater BV and BV/TV than non-exercised mice after 8 weeks. It is possible that exercised mice reached peak bone volume at some time point earlier than 8 weeks, and non-exercised mice caught up. Exercise can increase BV/TV after only 4 weeks of training [Chapter 4].

Just as it did for cortical bone, the control diet had detrimental effects on trabecular

bone as BV, BV/TV and trabecular number were significantly lower than baseline after

eight weeks (Figure 5.3). However, unlike cortical bone, trabecular bone properties

continued to decline after 16 weeks. This may be caused by the ability for more rapid

bone turnover to occur in the proximal tibial trabecular bone than in the mid-diaphyseal

cortical bone. If the treatments had detrimental effects on bone mass, they are likely to

be apparent in trabecular bone earlier than in cortical bone. Alternatively, the decline in

trabecular bone could be an effect of lower regular loading at the proximal tibia from

daily cage activities.

Mice on the supplemented diet had significantly greater structural-level strength (yield

force, ultimate force, stiffness, and pre-yield work) after 8 weeks compared to mice on

the control diet (Figure 5.4). These differences are to be expected since the

supplemented diet also significantly increased cortical bone mass. Exercise had no

significant main effects on structural strength after 8 weeks despite significantly

increasing cortical TMC and area. It may be possible that the greater cortical bone mass

in the combined supplemented diet and exercise group consists of newer, less

mineralized tissue that does not contribute to structural-level strength. Compositional

analysis would be needed to determine if there were differences in tissue quality. Mice

on the supplemented diet had increased stiffness and decreased ultimate deformation

from baseline. Thus, the bones appeared to have increased brittleness with age and/or

body weight in the first 8 weeks. There were no changes in any structural-level

mechanical property from 8 to 16 weeks for non-exercised mice on the supplemented

diet. While non-exercised mice on the supplemented diet had greater cortical bone mass after 16 weeks than after 8 weeks, this did not translate to changes in structural-level strength.

After detraining, the magnitude of differences in structural-level mechanical properties between the 2 exercised groups decreased such that there was no longer a significant difference in yield force and ultimate force after 16 weeks. It could be possible that exercise-induced changes to the tibia were still going on after exercise stopped, leading to the prevention of loss of mechanical strength in exercised mice on the control diet. For exercised mice on the supplemented diet, perhaps the combined treatments accelerate the rate of implementation of changes on bone mass and structural-level strength. Thus, bones from these mice may have already completed exercise adaptations after 8 weeks while bones from exercised mice on the control diet may be still undergoing adaptations during the detraining period. Bone can still adapt to exercise after loading stops, leading to improvements not noticed if mice are sacrificed immediately at end of the exercise program [19,20].

Exercised mice on the control diet had significantly lower yield force and ultimate force than baseline after 8 weeks (Figure 5.4). These decreases did not happen for non-exercised mice on this diet. However, there was a decline in structural strength from 8 to 16 weeks for these mice as yield force, ultimate force, yield deformation, ultimate deformation, and pre-yield work were all significantly lower after 16 weeks than after 8 weeks or at baseline. This loss of mechanical strength occurred despite no changes in

cortical bone mass measurements. Trabecular bone volume declined in non-exercised mice on the control diet from 8 to 16 weeks, but that is unlikely to affect mechanical properties as proximal tibial trabecular bone is located outside the testing region during the 4-point bending failure test to measure mechanical properties. Decreases in structural strength without changes in bone mass could be the result of changes in tissue quality [16]. For mice on the control diet, tissue-level mechanical properties also displayed a similar effect as structural-level mechanical properties, as there were no significant differences between the 2 groups on the control diet after 8 weeks, followed by significantly greater yield stress, ultimate stress, and pre-yield toughness in the exercised mice after 16 weeks (Figure 5.5).

Although exercise did not prevent a decline in structural-level strength from baseline to 8 weeks, exercised mice on the control diet did not have the same decrease in structural-level and tissue-level strength from 8 to 16 weeks as non-exercised mice. This occurred even though the exercised mice were going through detraining from 8 to 16 weeks and thus, had the same activity level as non-exercised mice during that time period. Exercise increases cortical bone tissue quality without increasing bone mass, and that may be what caused bones from exercised mice on the control diet to have greater mechanical strength after 16 weeks without having greater cortical bone mass [16].

It was an unexpected result for exercise to not increase cortical bone mass either immediately after 8 weeks of exercise or even after 8 weeks of detraining. Lack of

exercise effects may have been due to the high mouse body weights. The constant

increase in body weight of the mice was an unintended consequence of the treatments.

Greater body weight may have caused greater loading on bones during daily cage

activities. This would make the exercise loads less impactful since the bones would

already be accustomed to higher loading on a regular basis. Alternatively, it may be

possible that there were some benefits of exercise for mice on the control diet that

occurred at some time point not examined. Nevertheless, if there were increases in

cortical bone at some time point not measured, those increases were not maintained

after 8 weeks of detraining. Exercise also did not cause any significant differences for

trabecular bone properties of mice on the control diet. The high body weight of the

mice may also play a role in preventing exercise from affecting trabecular bone, or the

timing of when the mice were sacrificed for analysis may not be in line with when

exercise changes are present. Additionally, the mice were single housed in this study as

opposed to group housing previously [Chapter 2]. Singly housed mice have lower

cortical TMC, which can result in decreased structural-level strength and may have been

a factor in the lack of exercise effects on cortical bone [21].

Non-exercised mice on the control diet had a decline of cortical bone mass from

baseline to 8 weeks, a decline of trabecular bone volume from baseline to 8 weeks and

from 8 to 16 weeks, a decline of structural strength from 8 to 16 weeks, and a decline of

tissue strength from 8 to 16 weeks. It was unclear if these decreases were effects of

aging or effects of increasing body weight [22]. The choice of diets used may have been

a major factor in the large weight gain seen for all mice. Although the diets were not

high in fat (16.3% calories from fat), the high carbohydrate content (65% calories from carbohydrates) may have been just as effective in causing excessive body weight gain. Decreases in bone mass and mineralization seen with the non-exercise control and supplemented diets compared to baseline are similar to changes seen when Ca supplements are added to high-fat diets in diet-induced obesity [23]. Also, obesity has greater effects on trabecular bone than on cortical bone[22], similar to how decreases in trabecular bone volume occurred as body weight increased in non-exercised mice on the control diet. If the treatments used in this study did induce obesity, causing negative effects on bone mass and bone strength, the supplemented diet may have helped to prevent or attenuate some of the detrimental effects.

The properties measured in this study make it difficult to differentiate effects of aging from weight gain as all groups were of the same age and all gained weight at the same rate. But it seems unlikely for negative effects of age to occur in mice that are aging from 16 weeks old at baseline to 32 weeks old at the end of 16 weeks of treatment. Perhaps some threshold body weight was reached during the study where mice became obese and started having negative effects on bone. Alternatively, major changes in housing and diet may cause changes in bone mass and strength. However, because of the prolonged decreases in bone properties over 16 weeks, it is unlikely to be solely due to acclimation to a new housing environment, different diet, or single housing. Previous studies done using mice of a similar age, same acclimation period, and the same control diet did not show a decrease in tibial cortical bone mass or bone strength after 3 weeks [Chapter 3] or 4 weeks [Chapter 4] on the diet in single housing. Because bone can

adapt to changes in loading and dietary factors in a few weeks to a month, changes in bone mass and bone strength seen from 8 to 16 weeks most likely occurred after the mice had become fully adjusted to their new environment.

To our knowledge, this is the first study to show long-term consumption of a mineral-supplemented diet leads to increases and maintenance of cortical bone, trabecular bone, structural-level strength, and tissue-level strength in adult mice. These increases occurred without any detected detrimental effects on bone or overall health. The supplemented diet maintained most increases in structural-level strength for exercised mice and all increases in structural-level strength for non-exercised mice from 8 to 16 weeks. This diet also prevented loss of tissue-level strength in non-exercised mice from 8 to 16 weeks. These data suggest long-term consumption of a mineral-supplemented diet may be beneficial in preventing loss of bone structural-level and tissue-level strength with age and/or weight gain. Exercise was also beneficial as it allowed mice on the supplemented diet to achieve peak cortical bone mass in less time and prevented loss of trabecular bone and bone mechanical strength from 8 to 16 weeks in mice on the control diet. Long-term use of dietary mineral supplements may help increase and maintain bone mass with weight gain and/or aging in adult mice.

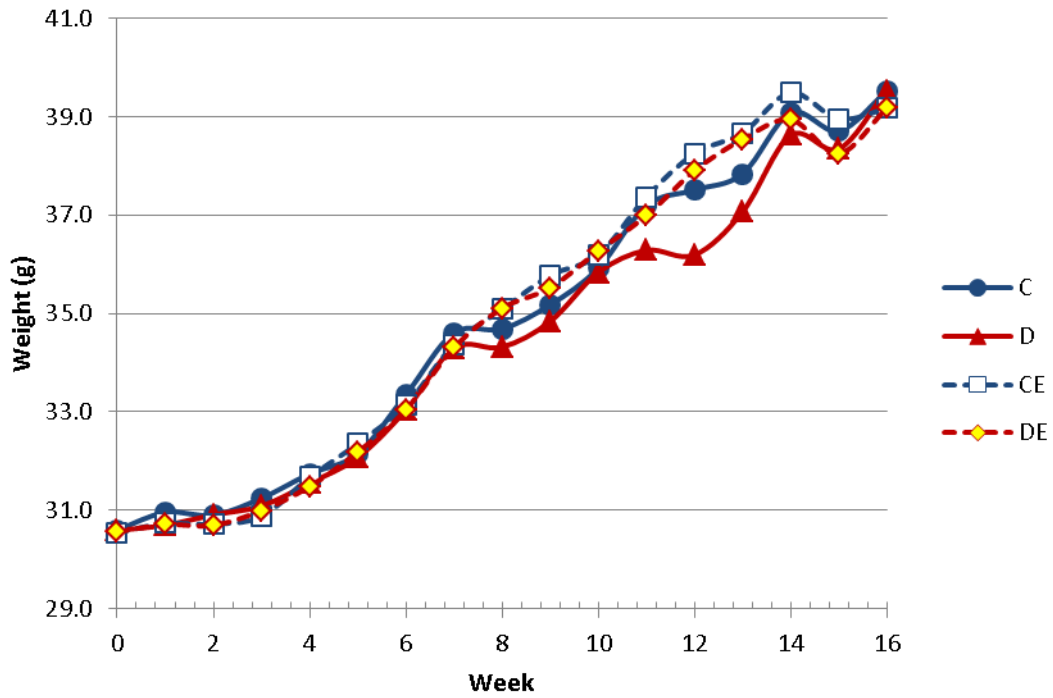**Acknowledgments**

Body Weight

Figure 5.1. Mouse body weight (mean). All mice started at above average weight for mice of this background strain and gender. Weight increased throughout the study for all groups.

Tissue Mineral Content

Tissue Mineral Density

Cross-Sectional Area

Moment of Inertia
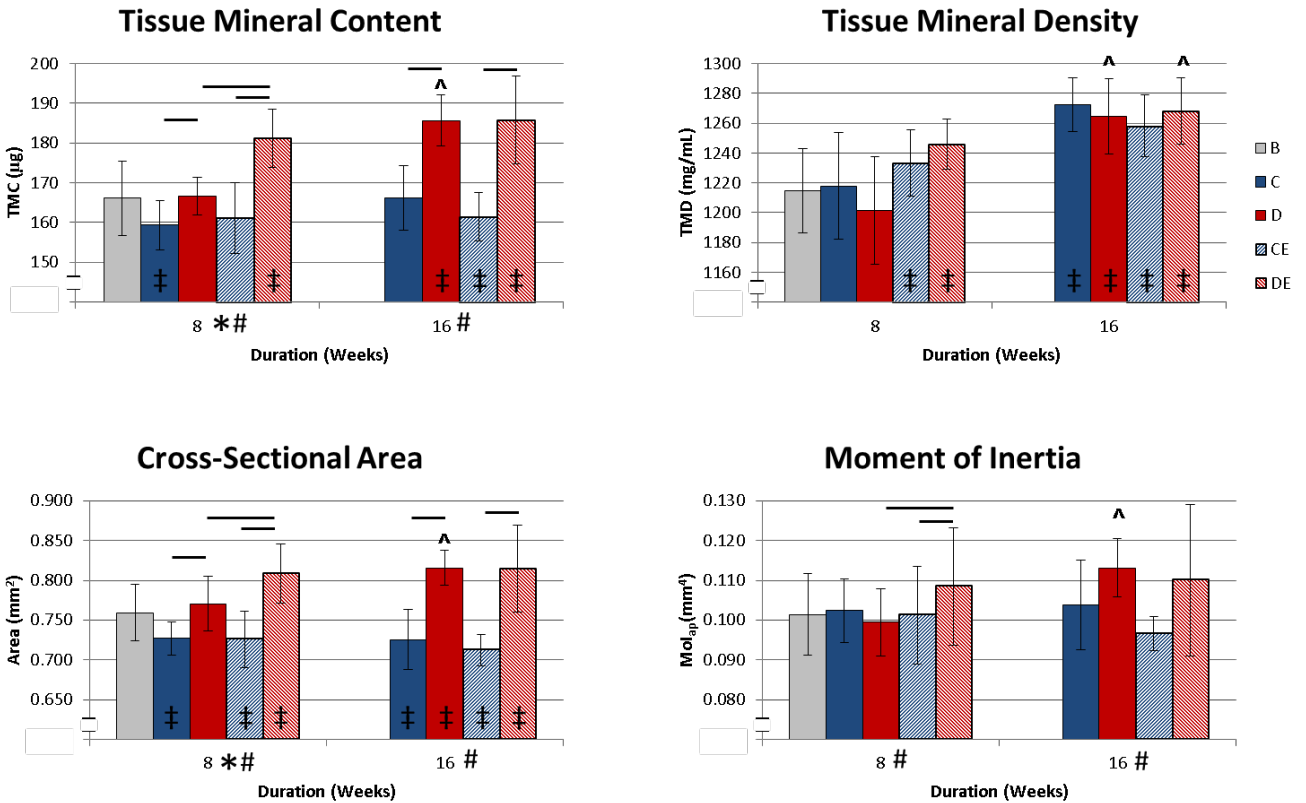
Figure 5.2. Tibial cortical cross-sectional geometric properties (mean ± SD). After 8 weeks of exercise, mice on the combined supplemented diet and exercise regimen had the greatest TMC and area. After 8 weeks of detraining, bone mass was maintained. Non-exercised mice on the supplemented diet had no change in bone mass from baseline to 8 weeks but had greater cortical area and TMC after 16 weeks than after 8 weeks. Mice on the control diet had lower area than baseline at both time points measured.

*Significant exercise effect (p < 0.05, Two-way ANOVA)

#Significant diet effect (p < 0.05, Two-way ANOVA)

&Significant diet and exercise interaction (p < 0.05, Two-way ANOVA)

-Significant group difference (p < 0.05, Tukey's test)

‡Significantly different from baseline (p < 0.05, t-test)

^Significantly different from after 8 weeks (p < 0.05, t-test)

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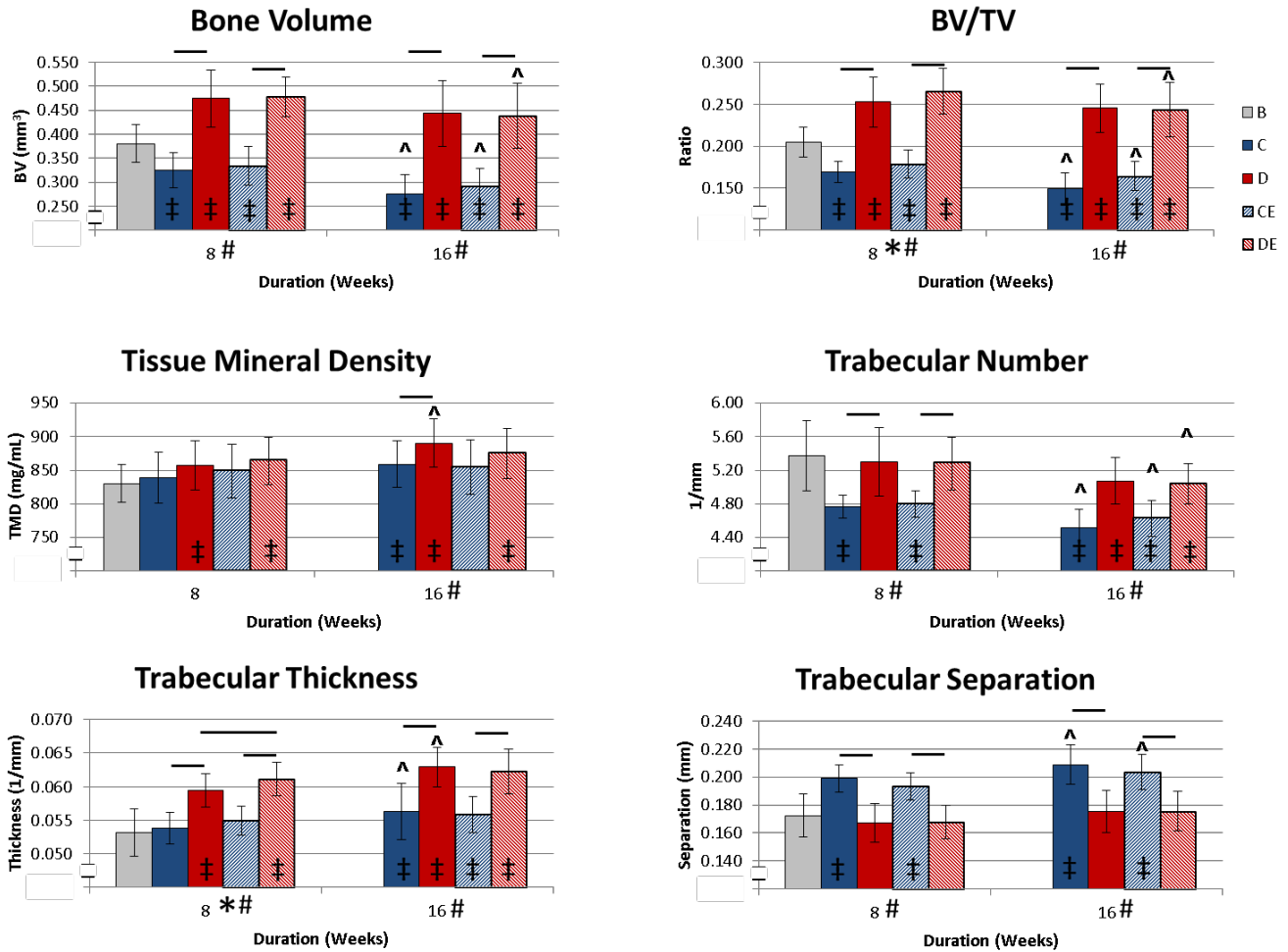
Figure 5.3. Proximal tibial trabecular architecture (mean ± SD). After the first 8 weeks, the control diet decreased most measurements of trabecular bone while the supplemented diet prevented decreases or increased these measurements. After 16 weeks, the non-exercise supplemented diet group maintained BV, BV/TV, and trabecular number while all other groups had lower values than after 8 weeks.

*Significant exercise effect (p < 0.05, Two-way ANOVA)

#Significant diet effect (p < 0.05, Two-way ANOVA)

&Significant diet and exercise interaction (p < 0.05, Two-way ANOVA)

-Significant group difference (p < 0.05, Tukey's test)

‡Significantly different from baseline (p < 0.05, t-test)

^Significantly different from after 8 weeks (p < 0.05, t-test)
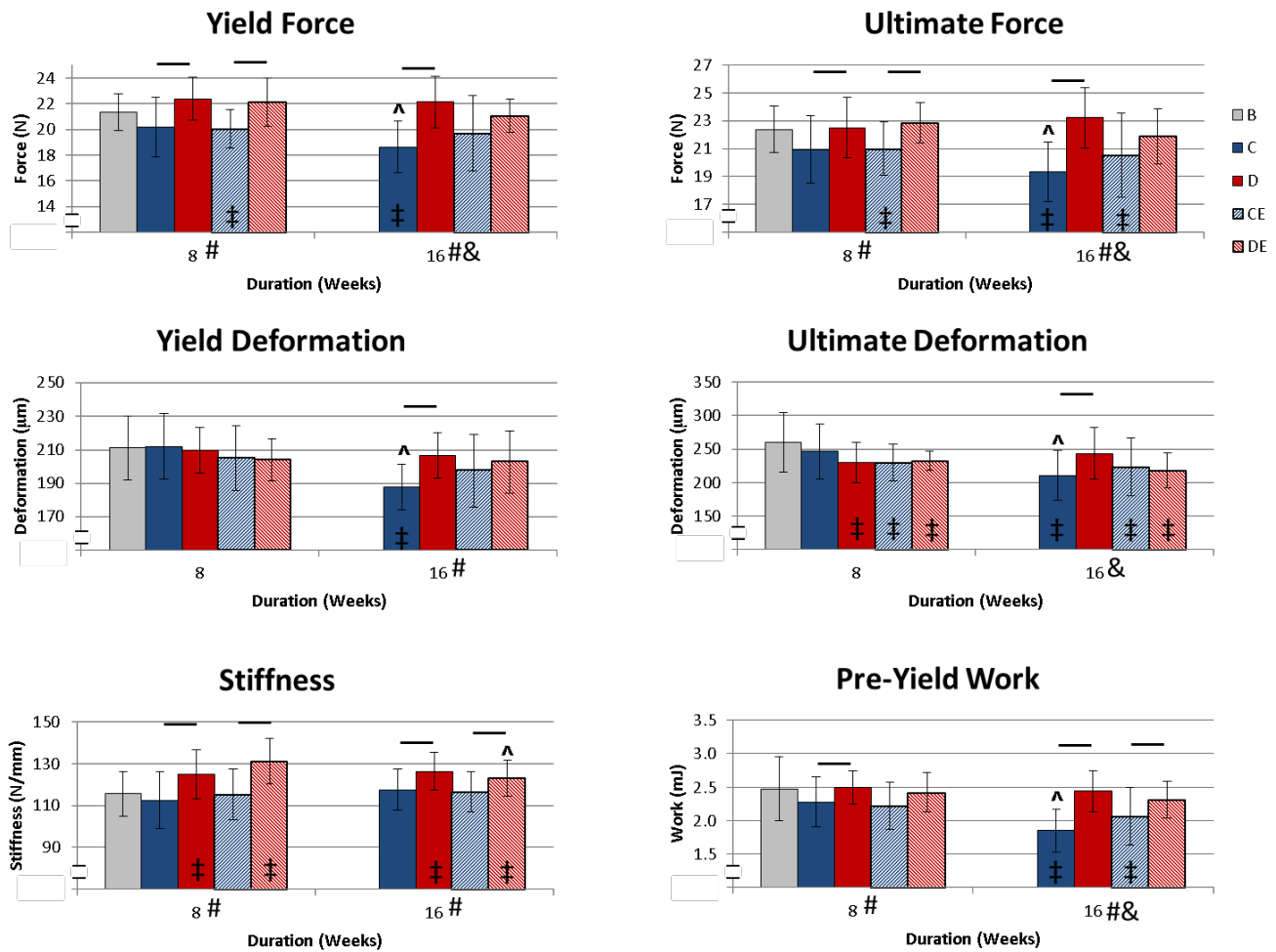
Figure 5.4. Structural-level tibial mechanical properties (mean ±SD). Groups on the supplemented had greater yield force, ultimate force, and stiffness than groups on the control diet after 8 weeks. Exercise on the control diet prevented loss of structural strength from 8 to 16 weeks. The supplemented diet also prevented loss of strength from 8 to 16 weeks.

*Significant exercise effect (p < 0.05, Two-way ANOVA)

#Significant diet effect (p < 0.05, Two-way ANOVA)

&Significant diet and exercise interaction (p < 0.05, Two-way ANOVA)

-Significant group difference (p < 0.05, Tukey's test)

‡Significantly different from baseline (p < 0.05, t-test)

^Significantly different from after 8 weeks (p < 0.05, t-test)

133
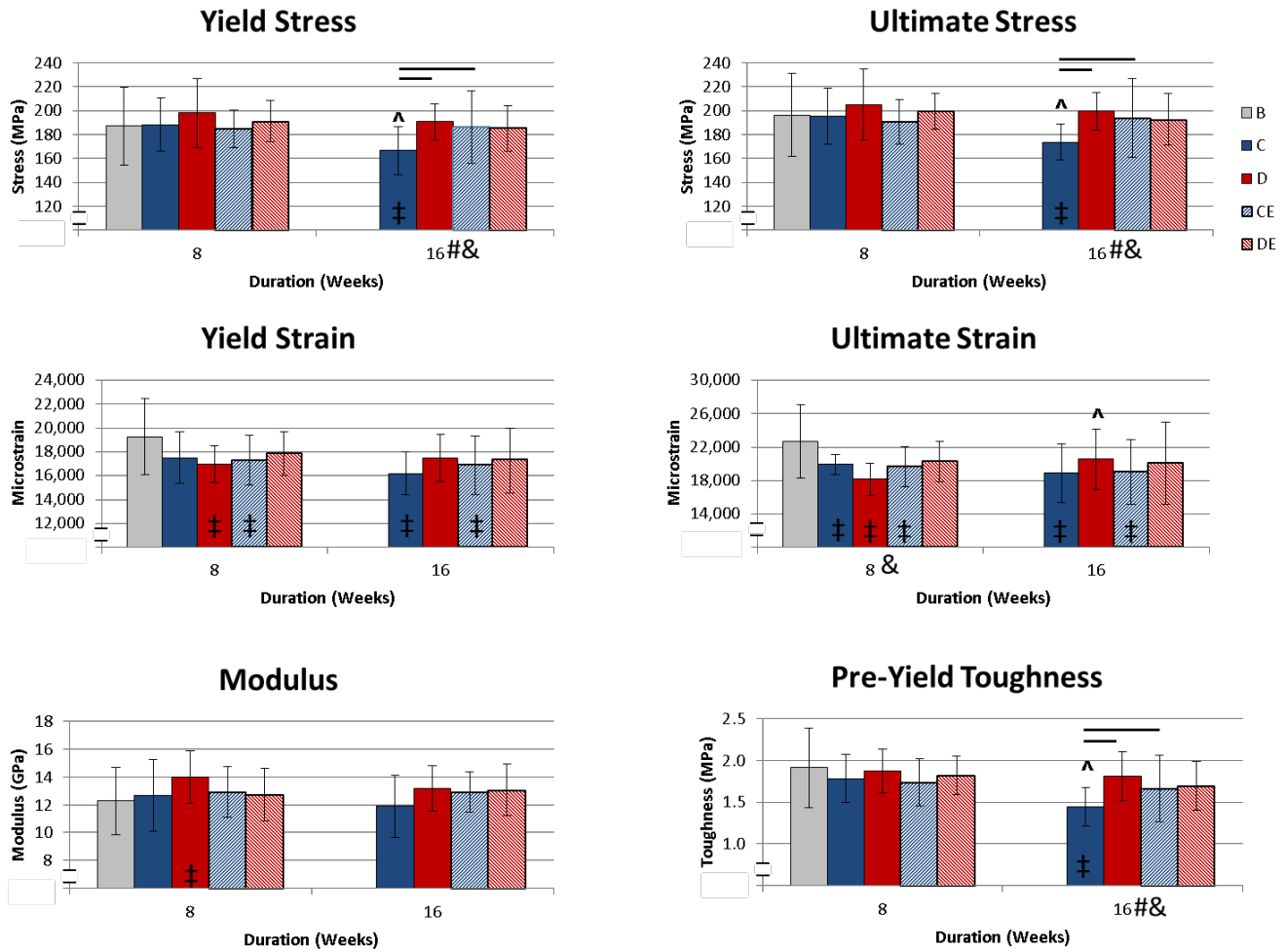
Figure 5.5. Tissue-level tibial mechanical properties (mean ± SD). Diet and exercise had no effects on tissue strength after 8 weeks. There was decreased yield stress, ultimate stress, and pre-yield toughness in the non-exercise control group from 8 to 16 weeks. Both exercise and the supplemented diet prevented this decrease in strength.

*Significant exercise effect (p < 0.05, Two-way ANOVA)

#Significant diet effect (p < 0.05, Two-way ANOVA)

&Significant diet and exercise interaction (p < 0.05, Two-way ANOVA)

-Significant group difference (p < 0.05, Tukey's test)

‡Significantly different from baseline (p < 0.05, t-test)

^Significantly different from after 8 weeks (p < 0.05, t-test)

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CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

EFFECTS OF COMBINING EXERCISE WITH A MINERAL-SUPPLEMENTED DIET

Conventional understanding is that long-term exercise is beneficial for increasing bone mass and bone strength. What is not well understood is the temporal adaptation of bone in response to exercise, in particular adaptation early in an exercise program. Results presented in Chapters 2-4 showed that with a standard diet, exercise does not increase cortical bone mass or bone strength after 3-4 weeks. Exercised mice on the control diet did not show significant increases in bone mass and bone strength compared to non-exercised mice on the control diet until after 8 and 16 weeks, respectively.

The supply of minerals available for increasing bone mass and mineralization may be a factor in the amount of time it takes for exercise-induced changes to occur and in the magnitude of exercise effects. Combining the mineral-supplemented diet with exercise increased both the rate and magnitude of changes in bone mass and bone strength, leading to the significant increases seen after both short- (Chapters 2-4) and long-term (Chapters 2, 5) exercise programs. The long-term effects of combining the

supplemented diet with exercise suggest that increasing mineral supply better allows bone to increase both bone mass and bone strength after any duration of treatment. However, since there were no effects of diet on markers of bone metabolism after 8 weeks of exercise (Chapter 2), it is unlikely that tibiae from mice on the control diet would have increased bone mass to catch up to the tibiae from mice on the supplemented diet if the exercise program were extended for an even lengthier duration.

The supplemented diet was not expected to affect bone mass for non-exercised mice since the control diet had a sufficient amount of calcium for achieving peak bone mass [1–3]. However, the supplemented diet had increased concentrations of both calcium and phosphorus. Since bone mineral is comprised of a combination of calcium and other minerals, it may be necessary to increase additional dietary minerals beyond calcium for increasing bone mass from diet alone. Also, the supplemented diet had an increased ratio of calcium to phosphorus. Dietary recommendations for humans call for a 1:1 ratio of calcium to phosphorus, but this may not be the optimal ratio for intestinal absorption of minerals and for increasing bone mass and mineralization. The supplemented diet used in this work consistently increased serum calcium concentration, regardless of exercise or time point measured – from day 2 to day 59. It may be important to have a constantly elevated supply of dietary minerals to maintain an elevated supply of minerals in the blood.

There are some limitations to using this mouse model that may make some of the

results difficult to translate to humans. Mice require a much higher concentration of

calcium in the diet than humans. If the standard 0.5% calcium from the mouse diet was

applied to adult humans who have a daily recommended value of 1000 mg calcium,

then it would take eating 200 g (0.44 lb) of food to obtain the recommended amount of

calcium. This is far less food than is typically consumed, meaning the recommended

human diet has a lower than 0.5% calcium content. There may be differences in how

calcium and/or other nutrients are digested and metabolized in the mouse that also

account for the higher concentration of calcium required for maintaining bone health.

Additionally, this mouse model only used healthy adult male mice. It is unclear if similar

results would be found using female mice or mice with a disease affecting bone health

such as osteopenia.

TIMING OF CHANGES TO BONE WITH EXERCISE AND DIETARY INTERVENTIONS

The results of this work showed the different effects of exercise and dietary minerals on

bone mass and bone strength in a young adult mouse model. With a standard dietary

amount of calcium and phosphorus, non-exercised mice had increased tibia length and

decreased trabecular bone volume after 4 weeks (Chapter 4). After 8 weeks, cortical

area was also decreased (Chapter 5), and after 16 weeks, yield force and yield stress

were decreased (Chapter 5). Exercised mice on the control diet also had increased tibia

length after 4 weeks, but exercise prevented the decrease in trabecular bone volume

(Chapter 4). After 8 weeks, exercise either increased (Chapter 2) or had no effect

(Chapter 5) on cortical bone mass and bone strength, and after 16 weeks, exercise prevented a decrease in yield force and yield stress (Chapter 5).

When the supplemented diet was combined with exercise, mice had increased cortical area after 3 weeks (Chapters 2, 3), increased trabecular bone volume after 4 weeks (Chapter 4), and increased yield force after 8 weeks (Chapters 2, 5), compared to exercised mice on the control diet. For each measurement of bone mass and structural-level strength, exercised mice on the control diet never caught up to exercised mice on the supplemented diet, even after 16 weeks. Non-exercised mice on the supplemented diet had the same or lower cortical area and yield force and lower trabecular bone volume after 8 weeks (Chapters 2,5). There were no differences in any bone mass or bone strength measurement after 16 weeks.

Thus, all mice on the supplemented diet ended up with greater cortical and trabecular bone mass after 3-4 weeks and greater structural-level strength after 8 weeks, compared to all mice on the control diet. Combining exercise with the supplemented diet caused mice to reach their maximum bone mass and structural-level strength in only 8 weeks, compared to 8-16 weeks needed for non-exercised mice. In this work, dietary mineral supply was a more impactful factor on peak bone mass and bone strength. Exercise increased the rate at which these peaks were achieved for mice on the supplemented diet and prevented some loss of bone mass and bone strength for mice on the control diet.

Although the supplemented diet was more impactful than exercise in the model used in this work, it is possible that other variables such as the starting age of the mice being near the end of skeletal growth or the low-impact type of exercise may have also been important. One difficulty encountered with using mice was the inability to directly increase exercise load magnitude for studying increasing exercise intensity. It is difficult to measure loading on bones of mice to determine the effects of increasing treadmill speed on bone. Also, directly increasing loading by attaching weights to mice is difficult as the animals would continuously attempt to remove harnesses where weights could be attached. Thus, the exercise model used here consisted of aerobic treadmill running where the only parameter modified was treadmill speed.

An additional concern with the mouse model is that mouse bones have limited ability to undergo intracortical remodeling. This type of remodeling that exists in humans would allow for repair of microdamage and could affect how increased minerals supplied from the diet are utilized. Loading during exercise is highest near the periosteal surface of bone, and the exercise regimen was not designed to be high-impact. Thus, it is possible that intracortical remodeling would not have been very impactful for this type of exercise. Despite some limitations, it still remains beneficial to use mice for examining tissue samples for mechanical properties and tissue quality measurements after exercise and diet interventions.

The results from this work challenge the conventional theory that exercise is more beneficial than dietary calcium for bone health. Timing of effects of changing diet and

exercise can be different and need to be accounted for when studying effects on bone health. However, after short and long durations, combining the mineral-supplemented diet with exercise always increased cortical bone mass compared to exercise alone. The dietary recommended value for calcium is calculated based on what is required for optimal bone health without exercise. The increased bone mass from combining the supplemented diet with exercise suggests there may be different requirements of dietary calcium for optimal bone health with exercise.

Additionally, this work gives new insights into the effects of increasing dietary phosphorus along with dietary calcium. Although increasing only dietary calcium beyond standard amounts has not been shown to increase bone mass or bone strength, there may be potential for increasing bone mass by simultaneously increasing dietary calcium and dietary amounts of other nutrients.

FUTURE WORK

This work demonstrates there can be a difference in dietary requirements for maximizing bone mass with exercise compared to non-exercise. Combining a mineral-supplemented diet with exercise increases bone mass in children and adolescents [4–7]. This is consistent with the increases in bone mass seen from combining the supplemented diet with exercise in mice in this work, suggesting diet and exercise effects from the mouse model may translate into similar effects in humans. Future work

should aim to continue to modify the supplemented diet used to maximize gains in bone mass and bone strength with exercise and look to transition to human studies.

Additionally, increasing dietary minerals beyond the recommended amounts may be beneficial without exercise, if the minerals are increased in the right ratios for increasing intestinal absorption. Current literature suggests adding calcium supplements to diets of healthy humans can be more harmful than beneficial, increasing the risk of cardiovascular disease [8,9]. Subjects with lower bone mass and higher risk of fracture did not have increased risk of disease with calcium supplements. More work needs to be done to determine the mechanisms increasing the risk of cardiovascular disease and how individuals with increased demand for calcium respond differently to calcium supplementation.

Future studies should investigate the mechanisms involved in increasing bone mass with the supplemented diet and the effects of increasing dietary minerals on tissue composition and other tissues such as non-loaded bones (vertebrae), muscle, and fat. The timing of changes in bone in response to exercise loading and increasing dietary mineral supply should also be further examined.

Further Modifications to Current Diet and Exercise Model

The supplemented diet with 5% calcium and 1% phosphorus increased bone mass and bone strength at all time points studied, with and without exercise. However, only

serum calcium concentration remained elevated after 8 weeks on the diet. Serum

phosphorus concentrations for mice on the supplemented diet returned to the same

level as for mice on the control diet after as little as nine days (Chapter 4). Since there

are no known mechanisms for increasing phosphorus absorption independent of

calcium absorption [10], dietary phosphorus may have been a limiting factor that

prevented further increases in bone mass or bone strength from occurring with the

supplemented diet. The 5:1 dietary calcium-phosphorus ratio is higher than the 5:3

calcium-phosphorus ratio in bone minerals. In Chapter 2, the 3-week study was done

using a supplemented diet with 2% calcium and 1% phosphorus, leading to similar

increases in cortical bone mass as were seen after 3-4 weeks on the 5% calcium diet in

Chapters 3 and 4. However, the 2% calcium diet did not increase serum calcium or

phosphorus concentrations at any time point. Thus, it may be ideal to increase dietary

calcium and phosphorus while using a lower calcium-phosphorus ratio such as a diet

with 5% calcium and 3% phosphorus. There would also need to be some method for

evaluating the bones' mineral composition, such as Raman spectroscopy. Measuring

carbonate-phosphate ratio in bone tissue could give better insight into the effects of

changing dietary phosphorus content on bone composition.

Future studies investigating mineral supply and demand should measure serum vitamin

D_3 concentrations. Vitamin D_3 is expected to increase with increased mineral demand

from exercise and decrease when serum calcium concentration is elevated, such as with

the supplemented diet. It remains unclear how combining exercise with the

supplemented diet would affect serum vitamin D_3 concentration. Understanding how

this crucial hormone reacts to conflicting signals could provide valuable insight into how exercise affects mineral demand. The only reliable assays for measuring mouse serum vitamin D_3 currently available are radioimmunassays that were unavailable to be used in this work.

Long-term benefits of exercise were evaluated in Chapter 5. The long-term effects of the supplemented diet on non-exercised mice were unexpected as increasing dietary calcium beyond standard amounts should not increase bone mass long term without exercise [2,11]. Future studies should also look at returning mice to the control diet after some lengthy period on the supplemented diet to determine if increases in bone mass remain and how long they last.

The mouse model used in this work only examined effects on bone and only analyzed tibiae, one of the bones that would receive the highest amount of loading from treadmill exercise. It would be important to also evaluate lesser-loaded bones such as vertebrae to determine if exercise and diet effects were localized onto the highest loaded bones. For mice on the supplemented diet, it would provide valuable insight into where minerals are ultimately being transported when the serum concentrations of calcium remain elevated for such a lengthy period of time.

The exercise model used in this work consisted only of aerobic exercise (running on a treadmill). It was difficult to study effects of increasing exercise intensity and increasing exercise loads on bone using treadmill exercise. Future work should examine effects of combining the supplemented diet with resistance exercise. This would better allow for

direct quantification of loads being applied to bones and for studying the effects of increasing exercise loads. Treadmill exercise increases load frequency and load magnitude on bones in the legs that are already frequently being loaded by daily activities. Resistance exercise in humans could be used to better isolate the effects of exercise loading by studying effects on the humerus and ulna.

Additionally, non-bone tissues such as muscle and fat could be examined to provide further insight into the interactive effects of exercise and diet. Non-exercised and low-speed exercised mice on the supplemented diet had increased bone mass, but did not have any differences in body weight compared to mice on the control diet. It is unclear which tissues were affected to account for these differences in weight. Increasing bone mass would likely lead to increased muscle mass to handle the higher loading required to move heavier bones. Elevating dietary calcium may also be disrupting intestinal absorption of other nutrients such as fat, which could lead to lower fat mass for mice on the supplemented diet [12,13]. Weight distribution is also important in obese humans who lose weight, where there is often accompanying loss of bone mass. Exercise and increasing dietary calcium each can attenuate bone loss, but it remains unclear if there are further benefits from combining exercise and a mineral-supplemented diet [14].

Timing of Changes from Exercise and Diet

Better understanding of the timing of effects on bone from exercise or dietary changes could be helpful in designing exercise and diet regimes for maximizing bone mass and

bone strength. Using this same mouse model, in vivo micro-CT could help to better determine changes in trabecular and cortical bone with time. There were conflicting results in this work as exercise increased cortical area after 8 weeks only in one of two experiments. Tracking changes in bone mass for each mouse over time could give a more accurate depiction of changes than comparing to separate baseline groups or groups sacrificed periodically. In vivo micro-CT would also be useful in determining the earliest time point where differences in bone mass can be detected.

Since the supplemented diet increased serum calcium after as little as one day of treatment and increased cortical area after 3 weeks in exercised mice, there may be some time point earlier than 3 weeks when effects of the supplemented diet on bone mass become significant. Human PTH (hPTH (1-34)), a bone anabolic agent, can increase BMD in as little as one week and had long-term increases in cortical bone mass and bone strength in adult mice [15]. The supplemented diet showed similar long-term anabolic properties and may have similar acute effects as well. Further investigation on the timing and rate of changes with the diet needs to be done to determine if it is the optimal anabolic agent for increasing bone mass and strength in the least amount of time.

Short-term exercise prevents increases in bone mass after 3 weeks (Chapter 3), and high-speed exercise prevents increases in tibia length after 4 weeks (Chapter 4). Structural-level and tissue-level strength and bending resistance were not affected by these reductions in growth. Future work should investigate the mechanisms leading to

bone's utilization and prioritization of minerals and other nutrients with exercise. Understanding which cells and genes are activated in response to exercise loading that prioritize increasing existing tissue strength over new bone formation could help design therapies that could accelerate bone's adaptation to loading.

The starting age of intervention is likely to be a major factor directing how exercise and diet affect bone health. Since there are different requirements for dietary calcium depending on age and since exercise can have different effects at different ages, it would be necessary to investigate increasing dietary mineral supply with exercise in subjects of different ages. Exercise had few effects on bone mass and mainly affected bone strength and bending resistance, increasing or preventing loss of strength with age. The mice used in this work were 15-16 weeks old, representing an approximately 18 year-old human. Although the mouse bones were still growing, they were likely of an age near the end of skeletal development. Using younger mice could have led to greater peak bone mass from combining exercise with the supplemented diet. Alternatively, using an older mouse population where skeletal growth had ended would also be important to determine if benefits from the supplemented diet could be gained even in old age.

This work showed a significant increase in day 2 serum calcium and phosphorus and decrease in day 2 serum bone metabolism markers from exercise (Chapter 2). Fasting blood samples were collected before daily exercise. Future work could examine the timing of changes in serum minerals and metabolism markers immediately after

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exercise and before and after feeding. This would help to understand the timing of changes in bone metabolism and the interactions between changes in exercise and diet. For example, the timing of when food is consumed in relation to when daily effects of exercise on mineral demand occur could be important. It would then be helpful to know how long after exercise there is an increased demand for minerals to give a better understanding on when to consume dietary mineral supplements for maximum effect.

Other Modifications to the Diet

The supplemented diet used in this work was designed to increase mineral supply available for bone. This was achieved by increasing calcium, phosphorus, and calcium-phosphorus ratio to help increase passive intestinal absorption. Increasing passive absorption means blood mineral concentrations could remain elevated, regardless of if there are any changes in active absorption that are intended to decrease blood mineral levels. In addition to modifying amounts and ratio of calcium and phosphorus, other nutrients could be adjusted to help increase absorption of minerals and increase blood mineral concentrations. Vitamin D could be added to the diet, which would increase active intestinal absorption of calcium and phosphorus. By consuming vitamin D, there would be increased absorption of dietary minerals, regardless of hormone levels of parathyroid hormone and vitamin D_3.

Increasing dietary protein content may also be beneficial for increasing bone mass [16]. Dietary protein is thought to aid in absorption and transport of dietary minerals and

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could be a mechanism to increase absorption of minerals without increasing dietary mineral supply [12]. Also, if optimal amounts of calcium, phosphorus, and nutrients that help transport these minerals can be achieved, this may lead to other nutrients becoming the limiting factor for increasing bone mass. Most likely, protein would be the next nutrient that needs to have consumption increased in order to provide nutrients for building the organic matrix of new bone tissue and for increasing muscle mass to accommodate heavier bones. These modifications to the supplemented diet and exercise program could be applied to mice, larger animals (rats), and humans. Differences in how nutrients are absorbed and metabolized in different animals suggest future studies involving dietary modifications should primarily use humans.

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