ENERGY RESTRICTION EFFECTS ON ESTROGEN STATUS AND THE SKELETAL RESPONSE TO LOADING

A Dissertation

by

SIBYL NICHOLE SWIFT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Nutrition

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Co-Chairs of Committee,	Susan A. Bloomfield
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ABSTRACT

Energy Restriction Effects on Estrogen Status and the Skeletal Response to Loading.

(August 2010)

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Co-Chairs of Advisory Committee: Dr. Susan A. Bloomfield
Dr. Nancy D. Turner

Moderate energy restriction in young, exercising women attenuates the positive effects of exercise on bone density. Studies have shown that in the absence of adequate levels of circulating estrogen, there may not be enough functional estrogen receptor-α (ER-α) to respond adequately to loading. The experiment described in this document is significant because this model has not been explored under conditions of energy restriction (EnR) which are known to reduce circulating estrogen levels; it has been tested only in ovariectomized animals. The central hypothesis of this research is that reductions in estrogen due to EnR limit the ability of bone to respond to mechanical loading (LOAD) through a down-regulation of ER-α.

Study one determined which nutrient's (calcium or energy) restriction (-40%) had the greatest negative effects on the skeletal integrity of exercising female rats and whether exercise (EX) could mitigate these deleterious changes. EnR caused detrimental effects in many of the structural properties of bone; however EX attenuated losses in cancellous bone.

Study two ascertained whether EX maintained cancellous bone mass in female rats subjected to graded EnR (-20 or -40%) and whether changes in endocrine factors were related. EX preserved cancellous bone volume and osteoblast activity under both levels of EnR, in addition to total body lean mass and bone mineral content. A similar maintenance of serum insulin-like growth factor and estradiol occurred in the EX+EnR(40%) group suggesting that these changes may be related to the protective effects of EX.

Study three determined the effects of 40% EnR on bone formation rate to LOAD in young adult female rats and tracked alterations in ovarian function (estradiol). Although higher than non-loaded animals, the response of bone to LOAD in EnR animals was dampened in comparison to energy-replete animals.

The experiments described in this document are significant because these are the first experiments to explore the relationship between EnR and estrogen levels on cancellous bone response to LOAD. This is particularly important for physically active, energy restricted women because cancellous bone in these women will not experience the same effects of loading which can increase their risk for developing osteoporosis.

DEDICATION

To the love of my life, my husband, Joshua who made this possible.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Americans are a society known for their singular focus on striving to reach the pinnacle of success in their chosen field. It is not uncommon for individuals to make sacrifices and believe in the philosophy that they will do whatever it takes to achieve their goals. Military personnel and athletes consist of individuals that endure rigorous training on a daily basis to excel professionally. For a variety of reasons, weight-loss attempts are common in these groups. However, when combined with their exceptionally high level of activity, weight-loss elicits a much greater negative impact on their bone health compared to their sedentary counterparts. Therefore, these populations are at an increased risk for developing stress fractures and bone loss related to weight loss, overuse, energy deficiency, and menstrual disturbances[1-9]. Women in particular are at an increased risk (40-60% greater risk) for developing stress fractures or having lower bone mass compared to their male counterparts[1,2,6,8-10]. Another

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This dissertation follows the style of Journal of Bone and Mineral Research.

factor related to increased risk of developing stress fractures or bone loss is the higher incidence of eating disorders in female recruits (8%) and athletes (21%) compared to the general population (1-3%) [8,11,12]. Many studies involving female athletes have focused on low-estrogen levels and weight loss and their combined effects on bone mineral density (BMD) [3,4]. Therefore, female military personnel and athletes with high activity levels and a concurrent negative energy balance predispose themselves to a higher risk for bone loss.

Weight-loss achieved through dieting is attributed to having a negative impact on bone parameters. In both humans and animals subjected to energy restriction, serum bone formation markers such as procollagen carboxyl-terminal propeptide (PICP) [13], osteocalcin [14], and insulin-like growth factor-1 (IGF-1) [13,14] are typically suppressed while bone resorption markers like urinary cross-linked N-telopeptides of type 1 collagen (NTX) [13], collagen type-1 cross-linked C-telopeptide (CTX) [15], and deopypyridinoline (DPD) [14] are elevated. Energy restriction is also associated with reductions in total body bone mineral content (BMC) and density (BMD) [14,16-18]. Several animal studies have determined that the effects of energy restriction are also specific to sites that are clinically relevant to fracture risk. Regions of lower BMD after energy restriction were found in the proximal femur and femoral neck [19] and the tibia [19,20].

Previous studies have established that ovariectomized (OVX) rodents are an appropriate animal model for postmenopausal bone loss in humans [21,22]. In female subjects, energy restriction contributes to reductions in estrogen levels [19,20,23-26] which is

thought to be a mechanism for increased bone loss. In women and ovariectomized (OVX) rats, menopause and OVX trigger an increased rate of bone turnover where resorption exceeds formation [21,27-30]. This results in a marked loss of trabecular bone [31] which is not reversible when estrogen levels are returned to normal levels[32]. Another potential mechanism for estrogen deficiency-induced bone loss is the desensitization of bone to mechanical signals under conditions of estrogen deficiency which makes them less effective at maintaining normal bone mass [33,34].

In both human as well as rodent models it has been determined that reductions in circulating estrogen levels are associated with decreased levels of estrogen receptor alpha (ER-α) in bone [35-38]. When levels of ER-α are not sufficient, osteoblasts are less sensitive to mechanical loading [39] which is translated as disuse [37], reduces bone formation [40-42], and leads to bone loss [37].

The overall objectives of this research were to determine whether moderate energy restriction (40%) reduces circulating estrogen levels and limits the bone response

to exercise or mechanical loading and to determine if it does so through a reduction in the expression of ER-α in osteocytes and osteoblasts. The central hypothesis of this research is that reductions in estrogen due to energy restriction (-40%) limits the ability of bone to respond to mechanical loading through a down-regulation of the estrogen receptor alpha (ER-α).

The experiment being proposed in this document is significant because these are the first experiments to explore the relationship between energy restriction and estrogen levels on the expression of ER-α and the subsequent response of cancellous bone to mechanical loading. This is particularly important for physically active women that are energy restricted because cancellous bone in these women will not experience the same effects of loading. These deleterious changes related to energy restriction can ultimately increase their risk for developing osteoporosis.

CHAPTER II

REVIEW OF LITERATURE

Prevalence of Weight Loss in Highly Active Populations

Prevalence of Weight Loss in Military Personnel

Military personnel and athletes comprise two groups that are very well known for the rigors that they endure as part of their daily training regimen. Due to this excessively high activity level, weight-loss attempts in such physically active individuals impacts their bone health to a much greater magnitude than their sedentary counterparts. All recruits exhibiting significant weight loss in a very short period of time were at increased risk for developing stress fractures [(1)]. The prevalence rate among men versus women in the armed forces for stress fractures is 37% to 60% respectively [(9)]. During basic training, overuse injuries in the lower extremities accounted for 78% and 75% of women and men's injuries, respectively [(2)]. Of these injuries, a much higher incidence was attributed to new female recruits (10-12%) compared to their male counterparts (1-3%) [(10)]. Most recruits were not accustomed to daily physical exercise in their civilian lives, thus amplifying the physiological effects of basic training. Another factor related to increased risk of developing stress fractures is the higher incidence of eating disorders in female recruits (8%) compared to the general population (1-3%) [(11)]. This increased risk for female military personnel is attributed to the demand for a high level of fitness and the emphasis on meeting weight requirements for career advancement. Any indication of being overweight can require a soldier to weigh-in, which sensitizes them to their

physical appearance. In fact, military personnel can be deemed "non-promotable" if they are overweight, which means that they are not allowed to be assigned to "command positions" until the excess weight is lost [43]. The military believes that the repetitive motions of basic training will facilitate their recruits' learning. Unfortunately, when combined with weight loss these repetitive motions will increase the likelihood of repetitive motion injuries resulting from structural overload.

Prevalence of Weight Loss in Athletic Populations

Another subset of the population that is subject to scrutiny about their weight comprises athletes. Many athletes, both male and female, have cycled their weight to increase their competitive edge without any consideration for the long-term ramifications on their health. In order to enhance their athletic performance, many female athletes feel pressure to lose weight. Therefore, most studies involving female athletes focus on the correlations between low-estrogen levels and weight loss and their combined effects on BMD [3,4]. This combination of factors is known as the female athlete triad [44]. The effects of the related amenorrhea and disordered eating are often manifested later in life as an increased risk for osteoporosis. Bone loss in female athletes is often attributed to a combination of a chronic energy deficit and a subsequent weight loss that alters both serum IGF-1 and leptin levels [45]. Although weight loss may not be the desired outcome, a higher activity level requires an equivalent increase in food intake to reach energy balance that is difficult to achieve. Therefore, female athletes

with a high activity level and concurrent negative energy balance predispose themselves to a higher risk overuse injuries.

Impact of Weight Loss on Bone Health

The Impact of Weight Loss on Bone Health in Over-Weight Individuals

Body mass has consistently been shown as being positively correlated with BMD (46). Many studies have investigated the effects of weight loss in obese or postmenopausal women. Weight-loss (-17%) from a very low calorie diet (VLCD) caused significant rapid loss in obese women's total body BMD (-2.5%) [47]. It is interesting to note that bone loss attributed to weight loss in overweight and obese populations can be mitigated with the addition of aerobic exercise [17,48,49].

The Impact of Weight Loss on Bone Health in Normal-Weight Individuals

Normal-weight individuals exhibit a greater bone loss resulting from weight loss (>1%) than the overweight individuals (<1%) [50]. Evidence also suggests that there is a negative correlation between the number of times that dieters cycle their weight and their bone mineral content (BMC) values [51]. The risk of hip fracture was markedly increased with weight variability in middle-aged women over a 12 year period, which was further corroborated by the association between weight loss and hip fractures [52]. It was determined that a high dietary restraint (reduced energy intake) in young, exercising women attenuated the positive effects of exercise on bone [53]. In this particular population, weight loss achieved by diet alone (~10% body mass) can produce

significant reductions (~1%) in total body BMD [17,48,49,54,55] and increases in bone resorption markers like deoxypyridinoline (DPD) [55] and C-telopeptide of type I collagen (CTX) [17] after just 6 months.

Animal Models of Weight Loss and Its Impact on Bone Health

Animal studies allow for a more detailed investigation into the physiological changes in bone that occur with weight loss. Rats considered an acceptable surrogate model for human skeletal status in osteopenia and osteoporosis research [21]. They can even be used in exercise training protocols in place of human subjects as similar changes are seen in rats and humans' femur and tibia with exercise [56]. Weight loss in both lean and obese rats has been linked with significant reduction in BMD at clinically relevant sites of fracture such as the proximal femur and femoral neck [19], in addition to the tibia and distal femur [20]. Reductions in body weight (-30%) are also associated with negative alterations to cancellous microarchitecture (Tb.Th) and bone cell activity (Ob.N and Oc.N) in the femur and spine [57]. Weight loss in lean, non-obese rats is attributed to lower uterine weight [18] and estradiol levels [19,20] as well.

Specific Types of Nutrient Restriction and Effects on Bone Health

Nutrient Requirements for Optimal Bone Health

The nutrients that are currently the most commonly associated with bone health include, but are not limited to calcium, phosphorus, vitamin D, and protein. Calcium has been well established as a necessary nutrient for the achievement of optimal bone health.

Bone stores approximately 99% of the total body calcium as the hydroxyapatite crystal and therefore serves as the primary source of calcium when dietary intake is inadequate [58]. The current recommendation for all healthy adults between the ages of 19 and 50 years old is for the daily intake of 1000 mg of calcium to compensate for normal losses [59]. If dietary intake is insufficient, the calcium stored in bone will be released to maintain standard levels and intestinal calcium absorption decreases [58]. Almost 80-90% of the hydroxyapatite crystal in bone is constructed with calcium and the phosphorus that binds to it [60]. Vitamin D is essential to maintaining bone health because its active form, 1,25-dihydroxyvitamin D [1,25-(OH)2D] or calcitriol, is a steroid hormone that conserves both calcium and phosphorus levels by stimulating an increase in intestinal calcium absorption of both nutrients [58]. Currently, the recommendation for daily vitamin D intake for the same age range of the population as discussed for calcium is 200 international units (IU) [61]. Adequate protein intake is necessary for achieving and sustaining healthy bones during the constant remodeling that occurs in bone tissue. The current RDA for protein intake in healthy adults is 0.8 g/kg of body weight. The major protein in the organic matrix of bone is type I collagen that is synthesized when polypeptide chains are twisted into a triple helix arrangement. Collagen is then mineralized by calcium and phosphorus to produce bone tissue. Low-protein intake also impacts on bone health indirectly by decreasing calcium absorption in the small intestine by ill-defined mechanisms [62]. Compensatory elevations in parathyroid hormone (PTH) occur with decreased calcium absorption [62,63] and are related to a greater loss of BMD [64]. Maintaining sufficient protein intake is also

required to preserve levels of IGF-1 production which stimulate osteoblast function in bone [65]. Studies that have assessed the association between bone mass and protein intakes determined that a positive relationship exists not only for children, but also for both pre- and postmenopausal women as well as for men [66]. Inadequate intake of any of these nutrients could therefore result in a multitude of negative effects on bone tissue.

Effect of Calcium Restricted Diets on Bone Health (Humans)

It is well established that calcium is one of the major minerals required for laying down new bone. The Institute of Medicine currently recommends that adults age 19 – 50 years old consume 1,000 mg per day and increase this to 1,200 mg per day once they are over 50 years of age . For adequate absorption of calcium, it is recommended that the daily intake of vitamin D is 200 IU per day for individuals less than 50 years old, 400 IU per day for 50-70 years old, and 600 IU per day for anyone over the age of 70 [67]. Although most Americans in these age ranges are consuming the recommended levels of both calcium and vitamin D [68], the reduced food intake related to dieting can reduced these values [69]. Low calcium diets induce significant bone loss because they create a negative calcium balance which causes an increased secretion of parathyroid hormone [70]. Several studies have determined that there is a strong relationship between the consumption of at minimum the required levels of calcium and a greater bone mass in humans [71,72]. Furthermore, an increased risk for experiencing fractures is also strongly associated with a lower than recommended level of calcium intake in human subjects [71,73].

Effect of Calcium Restricted Diets on Bone Health (Rodents)

Previous studies have shown that very low calcium diets (-96%) do cause increases in whole body resorption and reduction in total body bone mass in mature female rats [74,75], but do not appear to affect body weight compared to age-matched controls [18,70,75-77]. These whole body effects include a decrease in total body BMC and BMD in female rats [70]. In addition, Shen et al. found that dietary calcium restriction (-90%) in female rats led to increased bone turnover and decreased cancellous bone volume (-27%) [76]. Seto et al. found that 90% reduction of calcium in young, male rats for 3 days caused significant defects to bone architecture with reduction in BV/TV (-14%), trabecular thickness (-15%), and trabecular number (-46%) [75]. Overall, inadequate intake of calcium produces negative alterations in cancellous microarchitecture which leads to an increased fracture risk in both humans and rodents.

Effect of Energy Restricted Diets on Bone Health (Humans)

As previously discussed, dieting or restricting calories is known to have negative consequences in bone. Deficiencies in energy-intake, calcium, and protein have all been investigated to determine both individually and in combination, what level of restriction causes the greatest negative effects on bone density. In human studies involving exercising subjects with high restraint scores, total body BMC was significantly lower than the age-matched control group [16]. One method used in assessing bone resorption due to energy-restriction is currently performed on blood samples which are tested for the following resorption and formation markers: urinary cross-linked N-telopeptides of

type 1 collagen (NTX), insulin-like growth factor-1 (IGF-1), and procollagen carboxyl-terminal propeptide (PICP). The effects of increasing levels of energy-restriction in women that ranged from 40 kcal/kg/lean body mass (LBM)/day to 10 kcal/kg/LBM/day was assessed with the previously mentioned markers [13]. Bone resorption was increased, as shown by increased blood concentration levels of NTX as dietary intake was reduced from 20- to 10/kcal/LBM/day. The blood concentration level of the bone formation marker PICP was reduced as energy-restriction increased from 40- to 30/kcal/LBM/day and continued as restriction was increased. IGF-1 levels fell significantly at 20/kcal/LBM/day, while estradiol levels increased initially at 30/kcal/LBM/day but steadily declined as energy-restriction increased. Another group found significant increases in another bone resorption marker, urinary collagen type-1 cross-linked C-telopeptide (CTX), after decreasing male and female subjects' calorie intake by 25% [15]. The negative consequences related to calorie restriction have also been detected by imaging clinically important sites of fracture such as the lumbar spine, total hip BMD and BMD at the femoral neck. Villareal et al. ascertained that mild energy restriction (-20%) over a year in human subjects resulted in significantly lower BMD at these sites [17].

Effect of Energy Restricted Diets on Bone Health (Rodents)

Energy restriction paradigms in older (16 months old) male rats have revealed that moderate energy restriction decreases total body BMD (assessed by DEXA scans) and resistance to fracture in femorae [14]. Those studies also provided information about

serum markers for bone turnover such as reduction in plasma osteocalcin (-20%), urinary deoxypyridinoline (-25%), and IGF-1 (-20%) which suggested that moderate energy restriction suppresses all modeling in bone [14]. Talbott et al. tested the effects of a 40% energy restricted diet in older female rats (10 months old) where they found significant decrements of ~2% of total body BMD [18]. Talbott et al. also assessed effects of moderate energy restriction in mature (20 wk old) and aged (48 wk old) female rats over 9 weeks in serum hormones and BMD (by radiography) of the tibia, femur and humerus [20]. In mature animals, only femoral BMD was decreased (-34%), but aged animals experienced significant reductions in BMD in tibia (-7%), femur (-35%) and humerus (-6%). These decrements to BMD in aged animals also corresponded to decreased peak load (-11%) compared to age-matched controls. Hawkins et al. examined the effects of a 40% energy restricted diet on mature, lean female rats over a 10 week period [19]. These animals exhibited significantly lower BMD at the tibia, distal femur, proximal femur, and femoral neck. It appears as though energy restriction negatively affects both older and mature animals' BMD at multiple sites, several which are clinically relevant to fracture risk.

Effect of Energy Restriction on Serum Metabolic and Estrogenic Hormones

Insulin-Like Growth Factor (IGF)-1

One of the most abundant growth factors in bone, insulin-like growth factor (IGF)-1 is regulated by growth hormones (GH) and parathyroid hormone (PTH) [78]. One of the key roles of IGF-1 in skeletal tissue is to stimulate both the activity of

osteoblasts and bone formation [78]. The effect of energy restriction on serum IGF-1 is well documented. The energy available in an organism's system will typically regulate the synthesis and availability of IGF-1 in the systemic circulation [79]. In adult humans who were subjected to acute periods of fasting, serum levels of IGF-1 were significantly reduced [80]. Decreased levels of IGF-1 are associated with negative outcomes for bone parameters. Significant decrements to IGF-1 levels [13,81,82] and an increase in bone resorption [13] are typical responses to reductions in energy availablility. Loucks et al. tested the effects of energy restriction (10, 20, and 30 kcal per kg of lean body mass per day) in combination with exercise which increased energy expenditure by 15 kcal per kg of lean body mass per day on serum markers of bone turnover [23]. They concluded that IGF-1 levels dropped suddenly between 20 and 30 kcal per kg of lean body mass per day. Sunters et al. determined that adequate levels of ER-α in response to strain are required for a full response of the IGF-1 receptor to IGF-1 [83]. Reijnders et al. ascertained that a single 4-point bending session elicited a 2-fold up-regulation in IGF-1 mRNA synthesis in osteocytes found in cortical bone at the midshaft tibia [84]. According to Reijnders et al, IGF-1 is involved in the process of translating the mechanical stimulation from loading into bone formation. In contrast to these findings, Fontana et al. observed no changes in serum IGF-1 levels in male and female subjects after chronic energy restriction (-20%) either with or without an exercise component for 1 and 6 years [85].

Leptin

The protein leptin is a product of the ob gene and is produced by multiple tissues but primarily by adipocytes [86,87]. Leptin plays a key role in regulating body mass by controlling both food intake and energy expenditure [86] which it accomplishes via its receptors in the hypothalamus [88]. Therefore, it is well established that leptin levels increase with food intake and decrease with food restriction. Leptin receptors are also present on osteoclasts, osteoblasts, and bone marrow stromal cells so leptin signaling appears to possess dual functions in terms of its ability to regulate bone mass [89]. Leptin exerts positive effects on bone formation through a peripheral regulation by increasing osteoblast proliferation which contributes to increased bone mass [90]. Central signaling through beta2-adrenergic (Adrb2) receptors on osteoblasts increases their expression of receptor activator of nuclear factor-kappaB ligand (RANKL), an osteoclast differentiation factor which increases bone resorption [91]. Weight loss via caloric restriction in overweight males and females over the course of 1 year resulted in significant reductions in serum leptin (-38%) [17]. In athletic populations, it has been determined that diet-induced weight loss resulted in significant decreases in serum leptin (-64%) which were correlated with changes in bone resorption markers [92]. Even recreational athletes experience reductions in serum leptin with energy restriction. Loucks et al. determined that leptin levels were significantly reduced in exercising women whose caloric intake was reduced by as little as 15 kcal/kg LBM/day [23]. In male rodents, both acute treatment (4 weeks) with a food restriction (-30%) protocol and chronic treatment (8 week) with a caloric restriction (-40%) protocol was associated with

reduced levels of serum leptin compared to ad lib-fed controls [57,93]. Similar results were found in a rat model for the female athlete triad. Female rodents were fed restricted diets (-30% food restriction) for 8 weeks. This treatment resulted in significantly reduced levels of serum leptin vs. control animals, several of which were lower than the minimal detectable level [25].

Estrogen

Estrogens are synthesized from cholesterol and are predominantly secreted by the ovaries although they can be synthesized in smaller amounts in other tissues (i.e. adipocytes, liver, adrenal glands, and breasts). There are three principal forms of estrogen found circulating in the plasma: beta-estradiol (the principal estrogen secreted by the ovaries), estrone, and estriol. Estrogen acts in a multitude of ways to regulate bone metabolism. Estrogen regulates the production of proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) to moderate bone resorption by osteoclasts [94]. Normal estrogen levels also inhibit the activation frequency of bone modeling units thereby maintaining the balance of remodeling by inhibiting osteoclast function [95]. Activation frequency is the number of new remodeling units activated in each unit of time. An enhanced activation frequency expands the area of bone that is currently being remodeled which increases cortical porosity and enlarges the resorption area on cancellous surfaces. Estrogen deficiency decreases the sensitivity of bone to mechanical strain signals and makes them less effective at maintaining normal bone mass [33,34].

In healthy young women, energy restriction is not only responsible for bone loss, but is also associated with reductions in estrogen levels. Loucks et al. found significant reductions in serum estrogen levels (-15%) after providing female subjects with a diet of 10 kcal/kg lean mass/day (energy available) for 5 days [23]. De Souza et al. determined that the negative consequences for bone are enhanced by the combination of estrogen and energy deficiency in women [24]. Several studies have investigated this paradigm using rodent models. Compared to adlib-fed controls, a 30% food restriction in female Sprague-Dawley rats resulted in a lower serum estradiol (62%) and ovarian weight (57%) [25]. Under conditions of chronic energy restriction (-40%), mature (20 wk old) female Sprague Dawley rats exhibited significantly lower serum estradiol values (40-50%) [20,26]. Hawkins et al. determined that 10 weeks of energy restriction (-40%) led to significant reductions in serum estradiol (-50%) in sedentary, mature female rats [19]. In both postmenopausal women and OVX rats, estrogen withdrawal has been associated with an increase in bone turnover where bone resorption has exceeded bone formation [28-30]. According to Lindberg et al., estrogen deficiency causes a marked increase in loss of trabecular bone [31]. Under conditions of estrogen and energy deficiency, exercise is unable to inhibit increases in bone turnover. The combination of estrogen and energy deficiency in premenopausal exercising women resulted in significant increases in a bone resorption marker (CTx) with a concurrent decline in a bone formation marker (P1NP). This is important because even years after resumption of normal menses (premenopausal women) or implementation of hormone replacement therapy

(postmenopausal women) bone loss incurred due to estrogen deficiency is not reversible [32].

Ovariectomy and Reductions in Estrogen Levels

Previous studies have established that ovariectomized (OVX) rodents are an appropriate animal model for postmenopausal bone loss in humans [21,22,96]. Both OVX and menopause trigger an increased rate of bone turnover where resorption exceeds formation [27]. In addition, they both exhibit an initial phase of rapid bone loss which is followed by a much slower phase and over time both appear to produce greater losses in cancellous, rather than cortical bone [27]. According to Lelovas et al. the normal effects of OVX (estrogen loss) in the skeleton involves bone resorption exceeding bone formation within as few as 2 weeks after OVX. This uncoupled remodeling reaches a steady-state within 90 to 270 days after OVX [21], depending on the bone site. In younger female mice, OVX negatively affected remodeling by decreasing Tb.N and increasing Tb.Sp at the distal femur [96]. In mature rats, OVX exerted similar effects on remodeling; osteoclast surface (OcS/BS,%) increased while Tb.N [97] and cancellous BV/TV decreased [97,98].

Impact of Estrogen Deficiency on the Expression of ER-α

In both human as well as rodent models it has been determined that reductions in circulating estrogen levels are associated with decreased levels of ER-α [37]. In a human model that compared women replete with ovarian steroids (before menopause or after

with hormone replacement therapy, HRT) to those that were deficient in ovarian steroids (post menopause or without HRT) they confirmed that estrogen deficiency resulted in reduced the number of osteocytes that contained immunodetectable ER-α by half, from 25% to -12% [36]. Ankrom et al. cultured osteoblasts from pre- and postmenopausal women to assess their ER-α levels and their function [99]. Cells cultured from postmenopausal women did not increase their production of hydroxyproline, a collagen synthesis marker, in response to treatment with estradiol while osteoblasts cultured from premenopausal women did increase their synthesis of collagen [99]. The results are similar when explored in a rodent model as well. Under conditions of estrogen deficiency such as OVX in rodents, there is an associated down-regulation of ER-α expression in bone. Zaman et al. determined that after OVX, the levels of ER-α expression are reduced in osteocytes found in cortical bone [38]. However, they determined that there was no apparent effect of OVX on ER-α expression in the secondary spongiosa due to the fact that the initial level of ER-α is low. In contrast, Lim et al. ascertained that there is an effect for OVX in rats' trabecular bone. Three weeks after OVX, ER-α expression was almost undetectable in cancellous bone at the distal tibia metaphysis [35].

The Effect of Exercise on Bone Remodeling

Bone Modeling/Remodeling in the Adult Skeleton

Bone is a dynamic tissue that senses changes in its environment and adapts its structure accordingly throughout an animal's life. Bone is comprised of either cortical

or cancellous bone. Cortical bone is a very compact material with very low porosity that is found in the diaphyses of long bones and surrounding cancellous bone tissue. The functional units of cortical bone are cylindrical structures called osteons. Cancellous bone, or spongy bone, is comprised of a latticework of interconnected trabeculae. Cancellous bone is typically located in the ends of long bones or within vertebrae and is highly vascular. Bone is constantly modified by the coordinated efforts of three major cell types: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are the cells responsible for bone formation while osteoclasts are responsible for resorbing bone. Osteoblasts originate from mesenchymal progenitors (stem cells) and osteoclasts are derived from mononuclear lineage. Osteocytes are former osteoblasts which were enclosed in newly formed bone and serve a unique function as mechanosensors.

Bone is constantly being turned over by processes of either modeling or remodeling. Modeling occurs most often in younger animals in response to the rapid changes associated with growth, but can occur in mature animals in response to higher than normal mechanical forces. The process of modeling occurs in one of two ways. The first involves the independent activation of osteoblasts and resulting bone formation, while the second entails independent activation of osteoclasts and subsequent bone resorption [100]. Therefore, osteoclasts and osteoblasts function independently from one another during modeling. The main function of modeling is to adapt the bone structure and shape quickly in response to rapidly changing demands (strains).

The key difference between modeling and remodeling is the coordination of osteoblast and osteoclast activity that occurs in remodeling. Remodeling occurs most

often in adult animals as the regulator of bone turnover throughout the animal's life and is coordinated by teams of cells that are known as the basic multicellular unit (BMU) [(100)]. BMUs are activated in response to chemical or mechanical signals and function to remove and replace packets of bone through the coordinated efforts of both osteoclasts and osteoblasts [(100)]. There are four phases in a remodeling cycle which include: activation, resorption, reversal, and formation [(101)]. The first phase, activation, occurs when pre-osteoclasts are recruited to the bone surface and seal themselves to it through binding by their integrin receptors to the bone matrix. This seal forms a resorbing area within which the osteoclasts create an acid environment by lowering the area's pH (4.0) to dissolve the mineral matrix and begins the second phase, resorption. This process creates cutting cones in cortical bone and forms Howship's lacunae on cancellous bone surfaces and ends with apoptosis of the osteoclasts. The third phase is known as reversal in which signals are sent out to pre-osteoblasts to recruit them to the surface of newly resorbed bone to begin laying down new bone. This step is key to maintaining the balance of bone resorption to formation to prevent bone loss from occurring. There are two theories on the mechanism for the coupling signal. The first involves the release of growth factors such as TGF-β, IGF-1, IGF-II, bone morphogenetic proteins (BMPs), platelet-derived growth factors (PDGF), and fibroblast growth factor (FGF) from osteoclasts when they undergo apoptosis that attract osteoblasts to the location. The second theory involves the changing strain levels ahead of and behind BMUs. Strains are lower in front of osteoclasts which attracts osteoclasts while strains are higher behind osteoclasts which attract osteoblasts. The final phase of the remodeling cycle is

formation which occurs in two steps. Osteoblasts synthesize bone matrix using calcium and phosphate ions. After completing the bone formation portion, osteoblasts have three potential fates: either become trapped in the mineralized matrix to serve as mechanosensors, undergo apoptosis, or stay on the bone surface and become bone-lining cells.

Rules for Bone Adaptation to Mechanical Loading

Bone adapts to mechanical strain according to a series of proven rules. An adaptation of bone to stimulus requires the mechanical stimulation to be dynamic, rather than static [102,103]. Additionally, the strain has to exceed predetermined strain intensity for it to be osteogenic. Physiological strain ranges from 200 to 2000 microstrain, therefore, this strain intensity must exceed 2000 microstrain [102]. Strain must be delivered at the appropriate frequency as well because the response of bone to the stimulation is reliant on the combined stimulus of both strain frequency and intensity. Furthermore, bone requires as few as 36 cycles to respond to loading and can become unresponsive above this limit [104]. Finally, the mechanical strain must be unusual because bone will adapt to usual loading patterns [102].

Effects of Running on Adult, Human Subjects' Bone Mass and Risk of Fracture

As previously discussed, aerobic exercise is capable of maintaining bone mass in humans even during periods of energy deficiency and/or weight loss [48]. The type of aerobic exercise that is performed is particularly important due to the type of mechanical

loading applied to the bone with each form. Participants in non-weight bearing sports such as road cycling are at a higher risk for developing osteopenia or osteoporosis than those involved in distance running due to lack of ground-reactive forces that are provided by weight-bearing exercises on the skeleton [105,106]. Magkos et al. corroborated this statement after investigating the differences in areal bone mineral density (aBMD) between male runners and swimmers [107]. Beshgetoor et al. determined that female runners experienced site-specific benefits of running compared to cycling, which was demonstrated by the runners' ability to maintain BMD at the lumbar spine rather than declines at this site like the cyclists [108]. Endurance swimmers whose type of aerobic exercise did not provide their skeleton with ground reactive forces like runners' did had significantly lower total body BMD (-7%) [107]. In a study that examined the effects of exercise type on site-specific changes in BMD, Duncan et al. determined that runners had higher femoral neck BMD than swimmers and a greater leg BMD compared to both swimmers and cyclists [109]. Similar results were obtained in several investigations of endurance runners compared to sedentary, age-matched controls. These studies observed higher total BMD values in the endurance runners compared to sedentary controls [110-113], and the BMD values were significantly correlated with fat-free mass, body mass, and training volume [111].

The Effects of Treadmill Running in Skeletally Mature Rodents' Bone Health

The effects of treadmill running have been tested in a multitude of rodent models to better understand the mechanisms through which exercise impacts bone health. Running typically causes expansion at both the periosteal and endosteal surfaces, as well as larger areas of bone growth on both surfaces [114]. This response is preferentially found in the tibia and the femur, rather than the vertebrae and is predominantly found in both cortical and cancellous bone but to differing degrees [114,115]. This is due to the fact that rats are tetrapedal animals and therefore experience greater mechanical forces on the tibia and femur as opposed to the lumbar spine [56]. Exercise effects mature rodents' bone geometry by increasing BMC [56,116] and BMD [117,118] in the proximal tibia, midshaft tibia and femur. Changes in cancellous microarchitecture related to treadmill exercise in mature female rats include an increased bone volume (+22%) [115,119] and induced higher bone formation rates [115,118-120] versus sedentary controls. In terms of bone cell activity in the secondary spongiosa of the proximal tibia, exercise decreases osteoclast number and resorbing surface while increasing osteoid surface and the amount of double-labeled surface area [119]. These changes are also responsible for positively influencing the structural integrity of bone which was assessed by mechanical testing at the femoral neck [119] and three-point bending of the tibia [121]. Not all endurance treadmill training elicits a positive response from bone. Over-training in the form of high intensity treadmill training has been determined to result in a decreased bone volume [122] and maximum force the bone could withstand [123]. Bennell et al. found no

significant difference between exercising vs. sedentary rats' histomorphometric parameters [121].

Resistance Exercise Effects on the Human Skeleton

The positive effects of resistance training on bone remodeling in young adults are well established. Lester et al. explored the effects of an 8 week resistance training program in young women and determined that resistance exercise in these previously inactive women significantly increased both vBMD at the distal tibia as well as biomarkers of bone formation (osteocalcin and bone-specific alkaline phosphatase, BAP) without affecting biomarkers of bone resorption (tartrate-resistant acid phosphatase, TRAP and CTx) [124]. In premenopausal women, resistance exercise has been demonstrated to elicit a site-specific rather than a total body response [125-127] which is important when attempting to maximize the positive effect of an exercise regimen on specific regions. This was supported by a review study published by Martyn-St. James et al. that determined that high-intensity resistance training performed by premenopausal women was able to increase BMD at the lumbar spine, but not at the femoral neck [128]. However, the strains generated by the resistance exercise must be sufficient to generate an osteogenic response. Several groups have tested the effects of a long-duration strength training program in premenopausal women and found no difference between the exercise or control groups [129,130]. Another consideration for maximizing the osteogenic response to exercise is the effect of concentric versus eccentric resistance training. Hawkins et al. found that eccentric rather than concentric resistance training yielded

significant increases in mid-femur BMD in premenopausal women [131]. Post-menopausal women represent another population of interest in terms of the effects of resistance training on bone outcomes. Nelson et al. treated postmenopausal women with a high-intensity resistance training regimen for 1 year and discovered that the exercise treatment not only increased BMD at the femoral neck (+1%), but also contributed to the maintenance of total body BMC whereas age-matched sedentary controls lost femoral neck BMD and total body BMC [132]. Several groups have investigated the effects of progressive strength training programs in postmenopausal women for 16-24 weeks and found that this population does not lose total body BMD [48], or BMD at the femoral neck or lumbar spine [48,133] which suggests that this regimen successfully maintained their BMD over time. There are also groups that have found no significant changes in bone parameters such as total body or regional BMD in postmenopausal women after the completion of a long-term strength training program [134,135]. It is important to consider the duration and intensity of these exercise protocols because these studies utilized programs that were of lower intensity and shorter duration than most.

Resistance Exercise Effects on the Rodent Skeleton

The osteogenic effects attributed to lifting a heavier weight for fewer repetitions compared to those associated with lifting a lighter weight for more repetitions is well established. This concept has been explored in young rats that climbed a vertical ladder carrying 150% of their body weight and found that the resistance training regimen was able to significantly increase BMD over the 6 week treatment compared to sedentary

controls [136,137]. However, when climbing was tested in young and mature female rats, it was discovered that loads of 150% of the animals' body weight were not effective at generating strains capable of increasing the bone formation rate [138]. In mature, male rats a squat-like exercise was determined to increase cancellous bone at the proximal tibia by stimulating an increased bone formation rate [139]. The effects of this squat-like resistance exercise with 65% of their body weight were tested in young, mature, and old male rats [140]. Buhl et al. ascertained that resistance exercise at this intensity did not elicit strains that were able to enhance the mechanical properties of the femur or alter the cancellous architecture of the proximal tibia in young or mature rats [140]. In rodent models of disuse, such as OVX or hindlimb suspension resistance exercise training has been found to significantly mitigate losses to bone properties. Hubal et al. utilized an OVX mouse model to test the effects of eccentric exercise training on bone mechanical properties [141]. After 8 weeks of high force eccentric contractions achieved through muscle stimulation, trained OVX rats demonstrated a significant increase in stiffness at the midshaft tibia compared to untrained OVX rats. In adult, male rats that were subjected to disuse achieved via hindlimb suspension, resistance training with flywheel technology effectively mitigated disuse-associated bone loss in cancellous bone at the distal femur [142]. Swift et al. investigated the effects of resistance exercise achieved through jump training with either low or high additional loads on bone parameters in mature, male rats [143]. The lower additional loads elicited a much greater bone formation (2-fold) and enhanced cancellous microarchitecture (both Tb.Th and BV/TV) at the proximal tibia. Additionally, the lower load resistance exercise increased

structural integrity which was demonstrated by an increased maximum force at the femoral neck [143]. Overall, it appears as though resistance training in rodents is able to increase bone formation in young and mature animals, while helping to preserve BMD in older animals.

Impact of Mechanical Loading on the Expression of ER-α

In the absence of adequate levels of circulating estrogen (which is required for ER-α expression), there may not be enough functional ER-α to process the cell-strain response [37]. Lee et al. expored the effects of ulnar loading in the high physiological range in a mouse ER-α knock-out (KO) model via strain gauges placed on the ulnar shaft [40]. In mice with functional ER-α there was a three-fold greater bone formation response on both the periosteal and endosteal surfaces at the midshaft compared to the ER-α KO mice. More specifically, this paradigm determined that ER-α is required for bone to adapt after periods of mechanical loading through an increase in the number of osteoblasts and in bone formation [41,42]. ER-α is not only required for osteoblast differentiation, it is essential for osteoblast sensitivity to mechanical loading. In an in vitro model using osteoblastic cell lines cultured under estrogen deficiency, it was discovered that osteoblasts in the early stage of differentiation were switching to differentiation pathways leading to adipocytes without adequate levels of ER-α [144]. Armstrong et al. found that ER-α, not estrogen, is necessary for the full expression of

bone cells' adaptive response to mechanical loading [39]. They theorized that in the absence of adequate levels of circulating estrogen which is required for ER-α expression, there may not be enough functional ER-α to process the cell-strain response. Using ER antagonists and ER-α-/- mice, Jessop et al. ascertained that functional ER-α are required for the proliferation of osteoblast-like cells in response to strain [145]. It is important to note that applying a load delivering strains that are too high can reduce ER-α expression [146]. According to Lanyon et al., when estrogen receptor function is diminished bone cells (i.e. osteocytes) translate their lower sensation of mechanical stimulation as a lack of use which leads to bone loss [37]. Therefore, decreased ER-α levels lead to significant decrements in the response of bone cells to mechanical stimulus and possibly explain the significant bone loss associated with postmenopausal osteoporosis [147].

CHAPTER III

RESTRICTION OF DIETARY ENERGY INTAKE HAS A GREATER IMPACT ON BONE INTEGRITY THAN DOES RESTRICTION OF CALCIUM IN EXERCISING FEMALE RATS

Introduction

Military personnel and athletes comprise two groups that are very well known for the rigors which they endure on a daily basis. These two groups utilize repetitive motions for their respective training programs which predispose them to a higher risk for overuse injuries, often manifested in the occurrence of stress fractures. New recruits are often not accustomed to daily physical exercise in their civilian lives when they begin basic training. This abrupt increase in their caloric output occurs simultaneously with a substantial caloric deficit, leading to a negative energy balance which amplifies the physiological effects of basic training. Weight-loss attempts in such a physically active population cause significant decrements to bone health that are much greater in magnitude than those evidenced in sedentary counterparts. Even after basic training, weight is a primary focus for servicemen and women. Any member of the military can be deemed ineligible for promotion if they are found to be over the weight limits; they are not allowed to further their education or be assigned to "command positions" until the excess weight is lost [(43)].

Studies involving female athletes typically focus their investigation on the correlations between low-estrogen levels and weight loss and their combined effects on

BMD [4]. Bone loss in female athletes is often attributed to a combination of a chronic energy deficit and a subsequent weight loss that alters their levels of IGF-1 and leptin [45]. Most studies investigate the effect of weight loss in either obese or postmenopausal women, or the combined effects of both. Dietary restraint in young, exercising women attenuates the positive effects of exercise on bone density in the high dietary restraint group [53]. Weight loss achieved through caloric restriction significantly reduces female subjects' total body BMD [47,54]. Even general reductions in body mass can decrease bone density. Compston et al. found that a 17% reduction in body mass during a 10-week protocol significantly reduced total body BMD by 2.5% [47]. Caloric restriction is usually accomplished through a reduction in total food intake which significantly reduces intake of total nutrients in addition to calories. Therefore, it must be considered that decrements to bone health may be attributable to deficits in key nutrients as well. Most data collected thus far are on sedentary populations, thus a real knowledge gap exists about the effect of energy and calcium restriction in exercising humans (military and athlete). However, this gap cannot be bridged in humans due to the invasive nature of data collection. Therefore, it is imperative to utilize an animal model to better understand how the restriction of these key nutrients impacts on bone parameters in exercising subjects.

Deficiencies in energy- and calcium-intake have been investigated to determine both individually and in combination, what level of restriction causes the greatest negative effects on bone density. Dieting or restricting calories is also known to have a multitude of negative consequences for geometric properties of bone [20] as well as an

increased incidence of the uncoupling of bone formation [148]. Although Illich et al. determined that individual correlations between protein, energy, and calcium intake and BMD exist in humans; no published study includes a side by side comparison of energy or calcium restriction with global food restriction [60]. Further, few if any data exist on how these altered nutrient intakes affect bone outcomes in regularly exercising animals. Our previous observations [93] indicate that restricting food intake by 40% in sedentary rodents results in significant reductions (-20%) in BMD at the proximal tibia. This study tested moderate (40%) restriction of calcium and of dietary energy, as well as all nutrients (global food restriction), in exercising female rats to determine which of these nutrients (calcium or energy) was the major contributor to bone alterations observed with global food restriction. Because alterations in diet composition can impact on acid-base balance, we measured net acid excretion and urine pH at the end of the dietary restriction period. More specifically, we wanted to ascertain if the increased protein content of the energy restricted diet would reduce the pH and increase the net acid excretion in the rats' urine ultimately altering bone cell activity. We hypothesized that the restriction of energy content of the diet will be the most detrimental to skeletal integrity and that alterations in the pH of this diet would enhance this effect.

Materials and Methods

Animals and Experimental Design

Sprague-Dawley virgin, female rats were purchased from Harlan-Teklad and housed individually in a room with 12 hour light-dark cycles. Rats were aged to 4

months old at the beginning of the study, singly housed, and fed AIN-93M rat diet *ad libitum* for a 10-week acclimation period. Previous findings [149] revealed that adult female Sprague-Dawley rats experience significant declines in their cancellous vBMD at the proximal tibia metaphysis (PTM) within 4 weeks after switching from the vendor's Teklad 2018 rat chow to AIN-93M purified rat diet. During the acclimation period, peripheral quantitative computed tomography (pQCT) scans were performed at the PTM every 2 weeks to verify achievement of a steady-state bone mass, defined as two consecutive pQCT measurements with less than 10 mg/cm^3 change for both total and cancellous vBMD values. Upon completion of this acclimation period, rats were assigned to one of five groups by random block assignment based on cancellous vBMD values at day 0. To assess both early and late changes in cancellous and cortical bone geometry, pQCT scans were performed at day 0, week 4 and week 12. Just before each pQCT scan, urine was spot-collected and serum collected (from a leg vein) in anesthetized animals to assess urine pH and net acid excretion (NAE) and serum estradiol levels. At week 12, body composition and total body BMC was measured with dual energy x-ray absorptiometry (DEXA) scans. Calcein was injected subcutaneously (35 mg/kg) on days 9 and 2 prior to sacrifice to label mineralizing bone for analysis of dynamic histomorphometry. Anesthetized animals were then decapitated and tissues were harvested. Uteri were weighed after the removal of the ovaries and cervix, and any unusually high levels of fluid within the uterus were noted. Uterine weights are reported as relative uterine weight [uterine weight (g)/body weight (kg)]. Left tibiae were stored in 70% ethanol at 4°C until they were embedded for histomorphometric analysis.

Dietary Treatments

Two experimental control groups were fed AIN-93M *ad libitum* for the duration of the study and were subjected to the exercise treatment or served as sedentary controls restricted to cage activity (ADLIB-EX and ADLIB-SED, respectively). Nutrient restriction groups, all of whom were exercise trained for the duration of the study included 40% calcium-restriction (CAL-EX), 40% energy-restriction (ENE-EX) and 40% global food-restriction (ALL-EX). In order to isolate the effects of 40% restriction of calcium and energy, each restriction group's diet was formulated (Research Diets; New Brunswick, NJ) to provide 100% of all other nutrients while restricting only calcium or only energy (Table 1). Food intake for the ADLIB-EX group was averaged and equivalent weights of the calcium-restricted diet were fed to rats in the CAL-EX group. The rats in the ENE-EX group were fed 0.61 gm of the energy-restricted diet for every 1 gm of AIN-93M consumed by the ADLIB-EX group. The ENE-EX group's diet contained a higher density of all other nutrients in order to achieve 100% of the animals' daily requirements while restricting the weight of diet fed. The ALL-EX group was fed 0.60 gm of AIN-93M for every 1 gm of the ADLIB-EX group's average consumption from the previous week.

Table 1. Experimental variations of AIN-93M formulated to restrict either calcium or energy by 40% while providing 100% of all other nutrients.

Macronutrient (% kcal)	AIN-93M (D10012M)	40% Caloric Restriction (D01092702)	40% Calcium Restriction (D05090503)
Protein	15	24	15
Carbohydrate	76	60	76
Fat	9	16	9
kcal/gm	3.85	3.76	3.90
Mineral & vitamin (g/kg diet)			
Vit K	0.00075	0.00122	0.00075
Calcium	5.00	8.10	3.00
P	3.1	5.1	3.1
K	3.6	5.8	3.6
Mg	0.5	0.8	0.5
Vit D (IU per kg diet)	1000	1621.3	1000

Treadmill Exercise

All rats in the exercising groups were acclimatized to treadmill exercise 3 weeks prior to day 0 according to previously published protocol [150]. The first week of treadmill exercise consisted of five, 5-minute sessions, at 15 m/min on a 15% grade.

Over the next 2-week period, rats were exercised three times per week on a 15% grade for incrementally increased periods of time and speed. After the completion of the treadmill exercise acclimation period, rats exercised for three non-consecutive days per week on the treadmill. Sessions were 45 minutes long and were performed 25 m/min on a 15% grade to achieve approximately 60% of their maximal oxygen consumption [151]. Some rats needed mild stimulation to continue performing the prescribed exercise provided by a low-current electrical grid at the back of the treadmill belt or by short bursts of air from a modified air-gun. In our experience, most rats adapt well to treadmill running and easily avoid the shock grid after the first training session.

Peripheral Quantitative Computed Tomography (pQCT) Scans

In vivo measures of the tibial bone density and geometry were performed by pQCT scans (XCT Research M Stratec; Norland Corp.; Fort Atkinson, WI). We chose to measure vBMD at proximal and mid-shaft tibiae because these skeletal sites provide examples of mixed bone (cortical and cancellous) and purely cortical bone sites, respectively. A cone phantom scan was performed daily to calibrate the scanner. Immediately prior to being scanned, animals were anesthetized with a Ket/Med cocktail (ketamine 50 mg/kg, medetomidine 0.5 mg/kg). Slices (0.5 mm thick) were scanned at the metaphyseal (5, 5.5, and 6 mm from reference) and diaphyseal (1 slice at 50% total bone length) regions with a minimal voxel size of 100 μm. Total scan time was approximately 25 minutes for each rat. Data from the three contiguous metaphyseal region slices were averaged to get a single value for each variable at the proximal tibia.

Key outcome variables included cancellous and cortical volumetric bone mineral density (vBMD); total and cortical area; and total and cortical bone mineral content (BMC). *In vivo* coefficients of variation (CV's) from our laboratory using this method at the proximal tibia (with repositioning between scans) are ±2.13% for cancellous BMD, ±0.23% for cortical BMD, and ±1.95% for total area. Corresponding CV's at mid-diaphysis for the tibia are 0.86% for cortical BMD, 1.09% for cortical area, and 2.42% for marrow area.

Dual Energy X-Ray Absorptiometry (DEXA) Scans

Two days prior to sacrifice, DEXA whole body scans (GE-Lunar Prodigy Small Animal Program) were performed on rats' while anaesthetized to assess body composition (lean and fat mass) and total body BMC. Each rat was laid prone with its long axis aligned with the scan table's center line. Coefficients of variance for *in vivo* scans for lean mass, fat mass, and total body BMC are 1.07, 2.99, and 1.24%, respectively.

Cancellous Histomorphometry

Undemineralized proximal tibiae were serially dehydrated and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were microtomed either 8 µm thick for UV analysis of unstained sections at 20X (total bone surface, single- and double-labeled surface, and interlabel distances) or 4 µm thick for von Kossa staining and measurement at 20X of static histomorphometric properties (bone volume, osteoid

surface, and osteoclast surface). The region of interest began ~1.0 mm from the growth plate and encompassed a 6.0 mm^2 area within the endocortical edges. Mineral apposition rate (MAR; µm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS) for cancellous bone surfaces (BS) was calculated by using the formula MS/BS = [(single-labeled surface/2) + double-labeled surface]/surface perimeter x 100. Bone formation rate (BFR) was calculated with the formula: BFR = MAR X MS/BS. All histomorphometric analyses were performed using OsteoMeasure image analysis software (Version 2.31; Osteometrics, Inc.) interfaced with Optronics 3-chip color camera and an Olympus BX60 microscope with epifluorescent light (Leeds Instruments, Irving, TX). All nomenclature for cancellous histomorphometry followed previously established standards [(152)].

Analysis of Urine pH and Net Acid Excretion

Longitudinal measurements (baseline, week 4, and week 12) were performed on the animals' urine for pH and net acid excretion (NAE) to assess any differences between groups related to diet. Urine samples were tested in duplicate and analyzed for titratable acidity, ammonium, and bicarbonate according to previously published methods [(153)]. NAE was determined based on the results from these analyses as titratable acidity plus ammonium minus bicarbonate [(154)]. Prior to performing analyses on pH in urine using a TIM 856 Titralab pH electrode (PHC400-8), the electrode was calibrated using accepted standards.

Statistical Analyses

Data were analyzed using the SAS (version 9.1) program. Longitudinal data from weeks 0, 4 and 12 were analyzed using a two-way ANOVA with repeated measures for time. All end-point data were analyzed using a one-way ANOVA. Post-hoc analyses for all analyses were performed when significant main effects were detected with the LSM Error Test. ADLIB-SED group data were not included in these ANOVA's; unpaired t-tests were used to compare results for ADLIB-SED and ADLIB-EX animals in order to define the independent effect of exercise on outcome variables. Results are presented as mean ± standard error of the mean and, unless otherwise specified in the text, all significant changes or differences among mean values are significant at $p<0.05$. In some cases, comparisons with $p < 0.10$ are noted as trends.

Results

Energy and Food Restriction Negatively Affect Body Mass but Not Lean Weight

Most animals tolerated the nutrient restriction protocol with no ill effects. At week 10, one ENE-EX rat and one ALL-EX rat had reductions in body weight exceeding 30% of baseline body weight. For animal welfare reasons, the degree of energy or food restriction for these two rats was reduced from 40% to 30% restriction for the last 2 weeks of the experiment. No differences were found between SED and EX *adlib*-fed rats' actual energy or calcium intake (Table 2). ENE-EX and CAL-EX consumed significantly less energy (39%) and less calcium (-44%) than did ADLIB-EX, confirming efficacy of the dietary treatments. ALL-EX animals experienced a 37%

reduction in all nutrient intakes with the global food restriction treatment. Over 12 weeks, ADLIB-EX animals' mean body mass increased significantly and in parallel with that of the ADLIB-SED group (Fig 1, inset), confirming that these exercising female rats increased food intake to maintain energy balance. Exercise alone had no effect on lean mass in *adlib*-fed animals but did result in a 37% reduction in fat mass (Fig 2). However, mean body mass for both the ENE-EX and ALL-EX groups fell significantly by week 4; by week 12, these were 44% and 40% lower, respectively, than at baseline (Fig 1). All weight loss appears to be attributable to reduced body fat for both ENE-EX (-80%) and ALL-EX (-77%) groups, as lean mass was not affected by either energy or food restriction (Fig 2). Calcium restriction had no effect on body weight or fat or lean mass.

Table 2. The average energy (kcal) and calcium (g) intake per day throughout the 12 week experiment.

	ADLIB-SED	ADLIB-EX	CAL-EX	ENE-EX	ALL-EX
Avg Energy Intake/ Day (Kcal)	58.94 ± 0.70	57.56 ± 0.36[a]	54.03 ± 0.28[b]	35.13 ± 0.17[c]	36.21 ± 0.14[c]
%-Difference	*2.40*	---	*-6.14*	*-38.97*	*-37.09*
Avg Ca^{2+} Intake/Day (g)	0.77	0.75[a]	0.42[b]	0.76[a]	0.47[b]
%-Difference	*2.40*	---	*-44.40*	*+1.23*	*-37.09*

Values represent mean ± SEM. Different letters represent significant difference ($p<0.05$). %-Difference refers to dietary treatment versus exercising control group (ADLIB-EX).

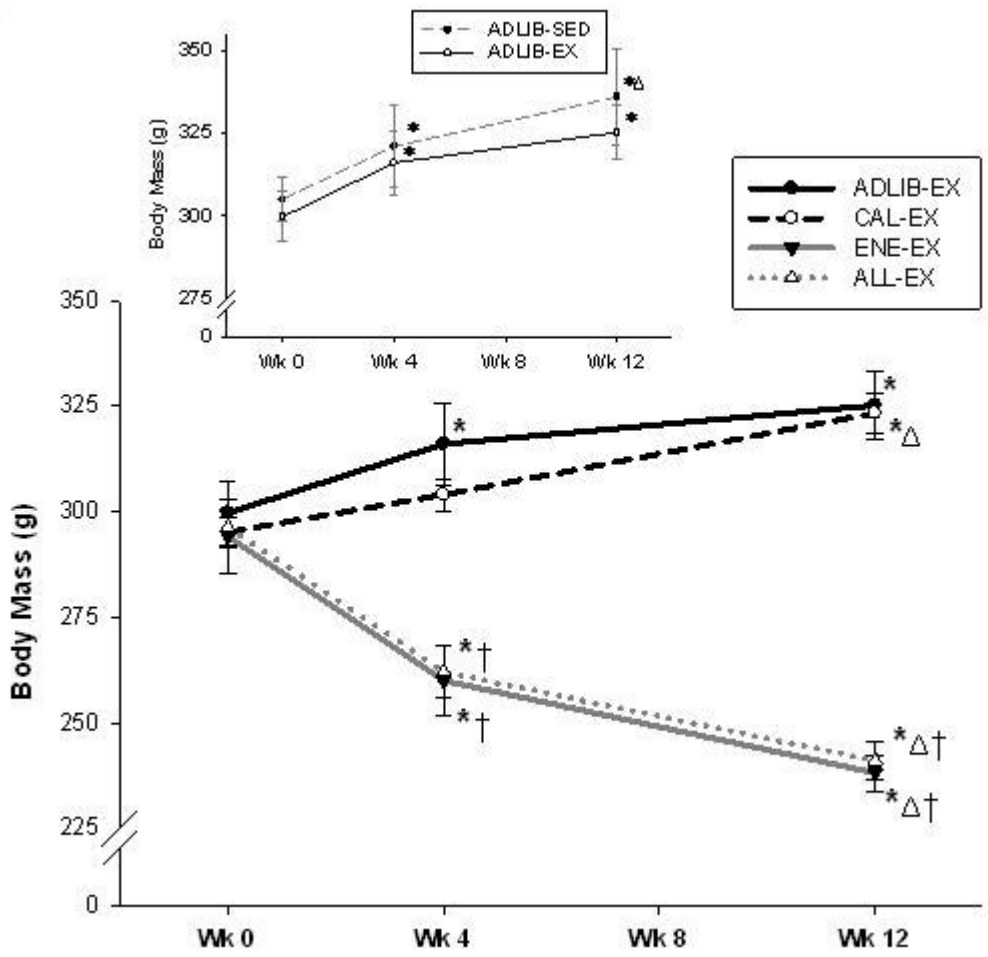


Figure 1. Body mass is negatively affected by 12 weeks of either 40% energy or 40% global food restriction but unaffected by 40% calcium restriction. *denotes difference vs. week 0, $^{\Delta}$difference vs. week 4, and †difference vs. both ADLIB and CAL-EX groups ($p<0.05$).

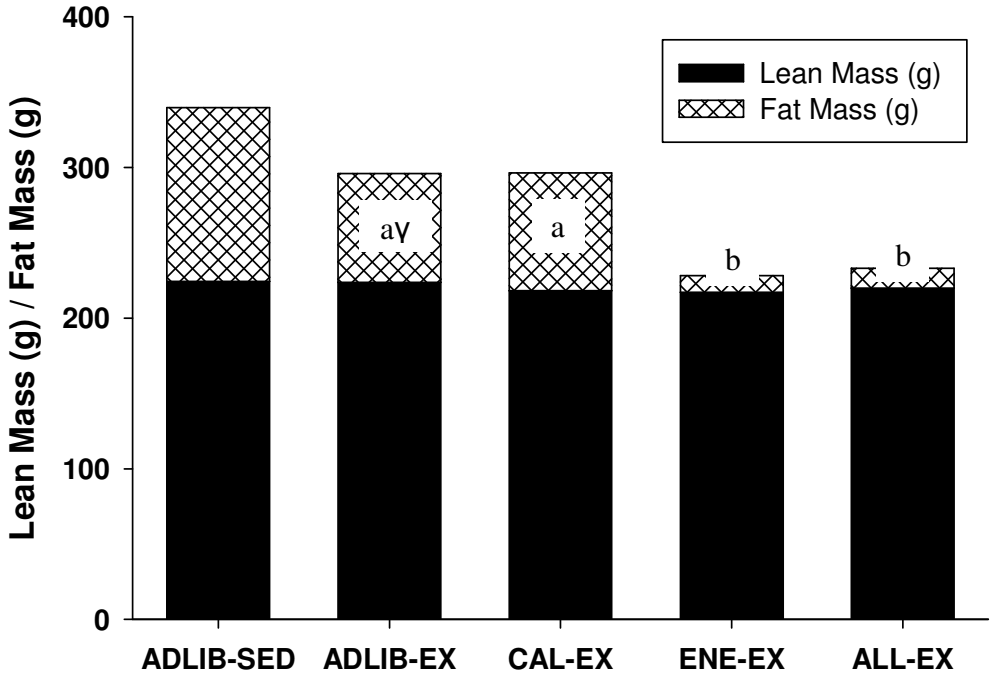


Figure 2. Fat mass, but not lean mass, in exercising female rats is negatively affected after 12 weeks of either 40% energy or global food restriction. Different letters represent significant difference ($p<0.05$). $^{\gamma}$ represents significant difference vs. ADLIB-SED.

Energy and Food Restriction Negatively Affect Both Total Bone Mass and Estrogen Status

Total body BMC was significantly lower in both ENE-EX (-17%) and ALL-EX (13%) groups, but was not affected by exercise alone nor by calcium restriction (Table 3). All food restriction and perhaps energy restriction affected estrogen status. Mean values for relative uterine weight (a bioassay for circulating estrogen) were lower for both ENE-EX (-27 %) and ALL-EX (-23%) groups versus that of ADLIB-EX animals

(Table 3). Dramatic declines in mean estradiol levels were apparent in ALL-EX (but not for ENE-EX rats) after Week 4 and Week 12 (-56%) (Fig 3), as compared to baseline values. Serum sample volumes were too low to allow for analysis of estradiol for ADLIB-SED rats.

Table 3. The effect of nutrient restriction on total body bone mineral content (BMC) and relative uterine weight in mature female rats.

PARAMETERS	ADLIB-SED	ADLIB-EX	CAL-EX	ENE-EX	ALL-EX
Total Body BMC (g)	10.44 ± 0.110	9.75 ± 0.12[a]	9.61 ± 0.09[a]	8.14 ± 0.10[b]	8.51 ± 0.11[b]
%-Difference	*+7.08*	---	*-1.39*	*-16.48*	*-12.67*
Relative Uterine Wt (g/kg BW)	1.56 ± 0.157	1.62 ± 0.241[a]	1.55 ± 0.161[a]	1.19 ± 0.087[b]	1.25 ± 0.125[b]
%-Difference	*-3.70*	---	*-2.90*	*-27.10*	*-23.26*

Values represent mean ± SEM. Different letters represent significant difference ($p<0.05$). %-Difference refers to dietary treatment versus exercising control group (ADLIB-EX).

Exercise Provides Only Short-Term Protection Against Loss of Cancellous vBMD With All Experimental Diets

Cancellous vBMD was significantly lower (-9%) in ADLIB-SED rats by the conclusion of the study, suggesting that some loss in cancellous bone is to be expected with normal aging in female rats (Table 4). Total vBMD for ADLIB-SED was significantly lower at both 4 (-6%) and 12 weeks (-8%) vs. week 0 (Fig 4). The ADLIB-

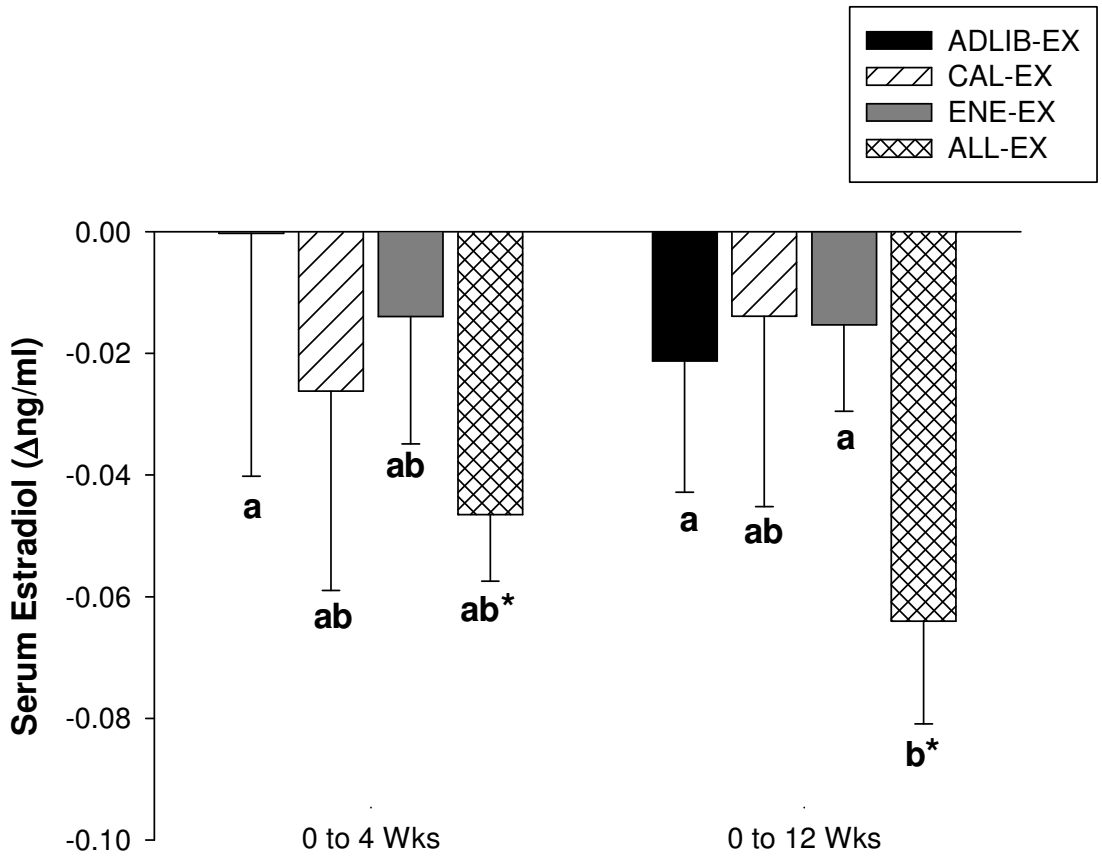


Figure 3. Global food restriction negatively impacts serum estradiol levels in exercising female rats over 12 weeks. Bars represent means ± SEM. *Denotes significant difference vs. week 0 ($p<0.05$). Letters signify difference between change scores, all values sig. for: $p<0.05$.

SED group exhibited significant increases in both total and marrow area (Table 4), whereas our exercise paradigm appears to have suppressed these aging-related changes in bone geometry. Cancellous vBMD in the ADLIB-EX group was significantly lower at week 12 versus baseline value (-13%); it did not change significantly from weeks 4 to 12 (Table 4). Exercise did not independently affect cancellous vBMD, total BMC, or total vBMD at the PTM, even after 12 weeks of treadmill training. At week 12, total

vBMD was significantly lower for ENE-EX and ALL-EX, but not CAL-EX vs. week 0 (Fig 4). Total BMC at the PTM declined in all nutrient restricted groups vs. baseline by week 12 (Table 4).

Table 4. The effects of endurance exercise training and differing nutrient restriction diets on cancellous bone parameters at the proximal tibia metaphysis (PTM) and cortical bone parameters at the midshaft tibia as quantified by pQCT scans before, during, and after 12 weeks of intervention.

	ADLIB-SED		ADLIB-EX		CAL-EX		ENE-EX		ALL-EX	
	Δ0-4 Wks	Δ0-12 Wks	Δ0-4 Wks	Δ0-12 Wks	Δ0-4 Wks	Δ0-12 Wks	Δ0-4 Wks	Δ0-12 Wks	Δ0-4 Wks	Δ0-12 Wks
Proximal Tibia										
Total BMC (Δmg/mm)	+0.27 ± 0.11	+0.29 ± 0.31	-0.06 ± 0.29^{abc}	-0.21 ± 0.20^{abc}	+0.02 ± 0.24^{a}	-0.53 ± 0.15^{bcd}*	+0.11 ± 0.27^{ab}	-0.71 ± 0.17^{cd}*	-0.05 ± 0.22^{ab}	-0.77 ± 0.25^{d}*
Total Area (Δmg/mm^2)	+1.22 ± 0.22*	+1.63 ± 0.58*	+0.32 ± 0.54	+0.12 ± 0.58	+0.14 ± 0.69	-0.14 ± 0.41	+0.17 ± 0.54	-0.14 ± 0.40	+0.69 ± 0.38	+0.11 ± 0.52
Canc. vBMD (Δmg/mm^3)	-6.71 ± 6.25	-26.19 ± 15.14	-20.99 ± 19.67^{ab}	-38.69 ± 14.12^{bc}*	+0.41 ± 17.62^{ab}	-62.43 ± 10.75^{c}*	+8.27 ± 13.33^{a}	-70.10 ± 10.46^{c}*	-2.25 ± 11.03^{ab}	-62.15 ± 13.89^{c}*
Marrow Area (Δmg/mm^2)	+1.12 ± 0.18*	+1.58 ± 0.46*	+0.31 ± 0.47	+0.31 ± 0.58	+0.35 ± 0.54	+0.17 ± 0.34	+0.34 ± 0.47	+0.29 ± 0.43	+1.00 ± 0.44	+0.78 ± 0.44
Midshaft Tibia										
Cortical BMC (Δmg/mm)	+0.28 ± 0.05*	+0.56 ± 0.08*	+0.43 ± 0.11*	+0.50 ± 0.15*	+0.26 ± 0.12*	+0.43 ± 0.12*	+0.37 ± 0.10*	+0.41 ± 0.07*	+0.33 ± 0.06*	+0.37 ± 0.07*
Cortical Area (Δmg/mm^2)	+0.21 ± 0.04*	+0.35 ± 0.07*	+0.29 ± 0.09*	+0.25 ± 0.12*	+0.12 ± 0.11	+0.16 ± 0.12	+0.19 ± 0.09*	+0.16 ± 0.04	+0.19 ± 0.05*	+0.12 ± 0.05
Cortical vBMD (Δmg/mm^3)	-0.45 ± 4.00	+18.35 ± 4.90*	+7.41 ± 5.42^{a}	+30.62 ± 4.10^{bc}*	+19.38 ± 7.85^{abc}*	+41.70 ± 10.25^{d}*	+20.66 ± 5.85^{ab}*	+36.53 ± 5.47^{c}*	+13.52 ± 3.79^{a}*	+41.17 ± 3.85^{d}*

*Denotes significant difference vs. week 0 ($p<0.05$). Letters signify difference between change scores, all values sig. for: $p<0.05$. Unpaired t-tests within the same time point revealed no significant differences between ADLIB-EX and ADLIB-SED.

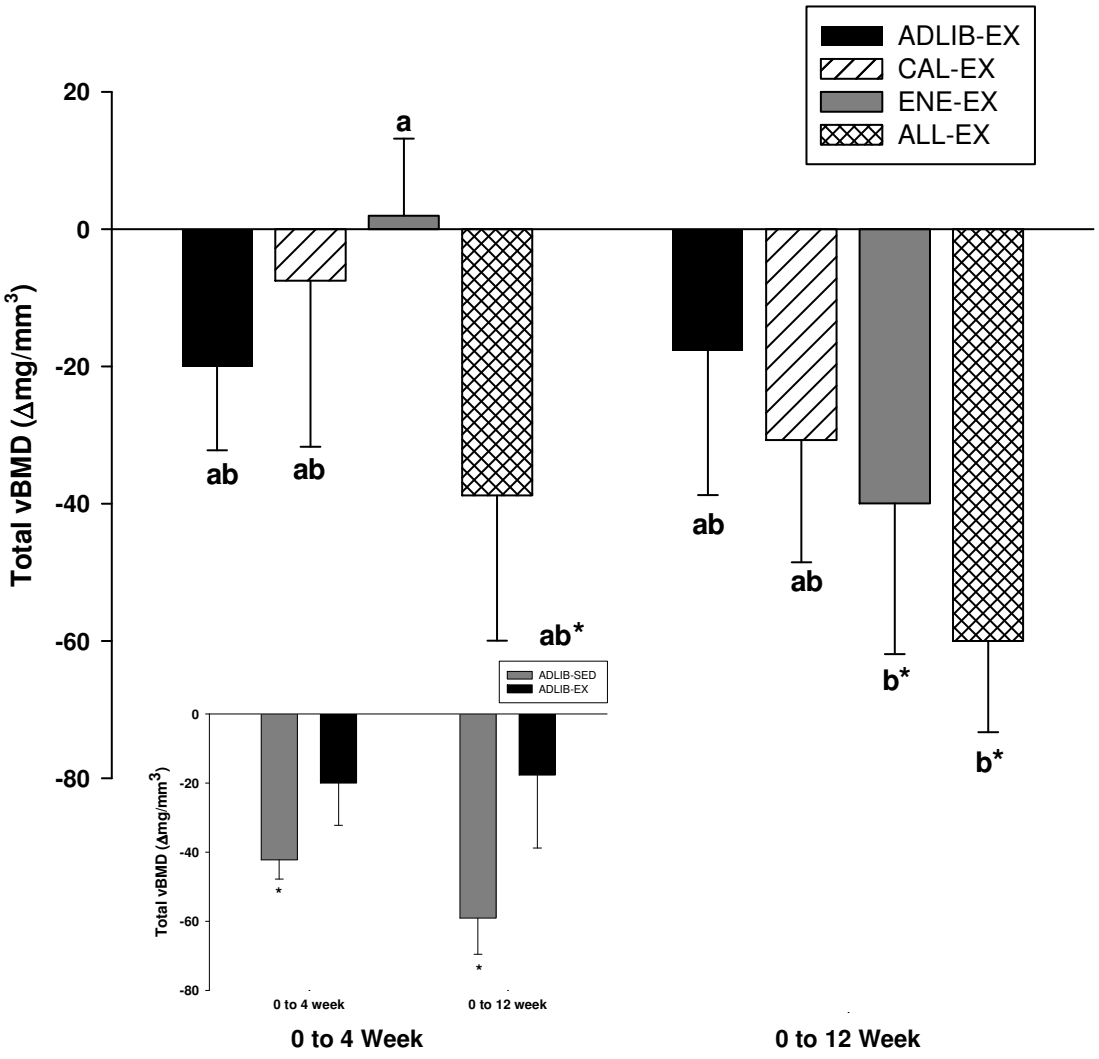


Figure 4. Endurance exercise training does not protect against the effect of nutrient restricted diets on total metaphyseal vBMD at the proximal tibia as quantified by pQCT before, during, and after 12 weeks of intervention. Bars represent means ± SEM. *Denotes significant difference vs. week 0 ($p<0.05$). Letters signify difference between change scores, all values sig. for: $p<0.05$.

Minor Effects of Nutrient Restriction at the Midshaft Tibia

At the tibial mid-diaphysis, a cortical bone site, although values for cortical area for both ENE-EX and ALL-EX were greater at week 4 vs. week 0, this effect was no longer apparent at 12 weeks (Table 4). Only *adlib*-fed animals (EX and SED) experienced increases in cortical area after 12 weeks of the treatment. Cortical BMC and vBMD increased in all groups regardless of exercise treatment or energy status by Week 12.

Differential Effect for Energy- vs. Global Food Restriction Revealed by Histomorphometric Analysis

Analysis of bone volume for the *adlib*-fed groups at the completion of the study revealed a trend for higher bone volume (+22%) with exercise (Table 5). Remodeling is suppressed for ADLIB-EX when compared to ADLIB-SED; both ObS/BS (-64%) and OcS/BS (-41%) are lower for ADLIB-EX in comparison to ADLIB-SED. Nutrient restriction in exercising animals exerted variant effects on cancellous bone microarchitecture. Bone volume (BV/TV, %) was reduced in the group restricted in energy only (-41%), but not in groups restricted in calcium (-22%) or all food (-20%) after 12 weeks compared to ADLIB-EX (Table 5). For osteoclast surface (OcS/BS, %)

Table 5. PTM cancellous bone microarchitecture and dynamic measures of bone formation activity after 12 weeks of endurance exercise and nutrient restriction.

Parameter	ADLIB-SED	ADLIB-EX	CAL-EX	ENE-EX	ALL-EX
Bone Volume (BV/TV, %)	18.65 ± 2.42	23.81 ± 3.71[a]	18.48 ± 2.08[ab]	14.11 ± 2.01[b]	19.10 ± 1.99[ab]
Osteoid Surface (OS/BS, %)	3.33 ± 0.90	3.24 ± 0.95	2.48 ± 0.54	2.24 ± 0.61	3.12 ± 1.03
Osteoblast Surface (ObS/BS, %)	0.23 ± 0.05	0.14 ± 0.04	0.14 ± 0.05	0.12 ± 0.06	0.12 ± 0.06
Osteoclast Surface (OcS/BS, %)	2.80 ± 0.53	1.98 ± 0.59[a]	2.67 ± 0.59[ab]	2.61 ± 0.53[ab]	4.17 ± 0.61[b]
Trabecular Thickness (TbTh, mm)	53.89 ± 3.30	65.15 ± 6.44[a]	55.36 ± 4.64[ab]	47.97 ± 5.28[b]	52.29 ± 4.11[ab]
Trabecular Separation (TbSp, mm)	255.19 ± 23.09	223.58 ± 31.27[a]	255.69 ± 21.50[ab]	304.21 ± 27.43[b]	226.38 ± 13.16[a]
Trabecular Number (TbN)	3.36 ± 0.25	3.57 ± 0.30[a]	3.30 ± 0.21[ab]	2.90 ± 0.16[b]	3.62 ± 0.15[a]
Bone Formation Rate (BFR/BS, µm/year)	0.10 ± 0.01	0.13 ± 0.03	0.08 ± 0.01	0.09 ± 0.02	0.07 ± 0.02
Mineralizing Surface (MS/BS, %)	7.13 ± 0.67	7.55 ± 1.31	5.91 ± 0.79	6.44 ± 0.75	6.70 ± 1.08
Mineral Apposition Rate (MAR, %)	1.38 ± 0.06	1.77 ± 0.24	1.27 ± 0.09	1.36 ± 0.13	0.89 ± 0.14

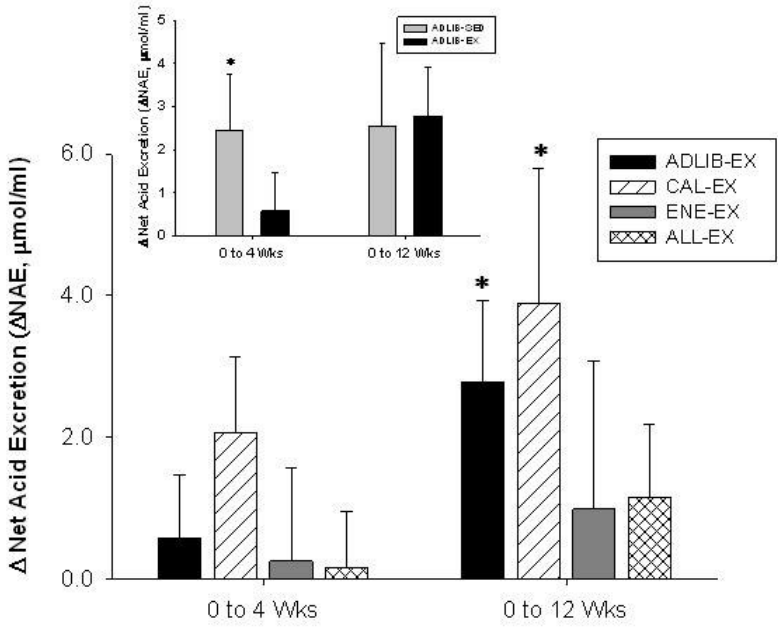
Values represent mean ± SEM. Different letters represent significant difference ($p<0.05$).

both the CAL-EX (+35%) and ENE-EX (+32%) groups are numerically higher versus ADLIB-EX while the ALL-EX group has a significantly higher OcS/BS (+111%). In terms of trabecular thickness (TbTh, mm), trabecular separation (Tb.Sp, mm), and trabecular number (Tb.N), the CAL-EX and ALL-EX groups were not different compared to ADLIB-EX. In contrast, ENE-EX is significantly lower compared to ADLIB-EX for both Tb.Th (-26%) and Tb.N (-19%) and larger for Tb.Sp (+36%).

Long-Term Nutrient Restriction Reduces Urine pH

In *adlib*-fed animals, exercise offers early protection from increased net acid excretion (NAE) and decreased urine pH observed in sedentary free-eating rats (Fig 5, inset). This effect of exercise disappeared by week 12. At the end of 12 weeks, ADLIB-EX was the only group with urine pH above the normal physiological level of 7.4 [32] (data not shown). By 12 weeks, NAE increased in both ADLIB-EX (16-fold) and CAL-EX (9-fold) groups (Fig 5A); urine pH declined significantly in all nutrient restriction groups by 12 weeks (Fig 5B).

A.



B.

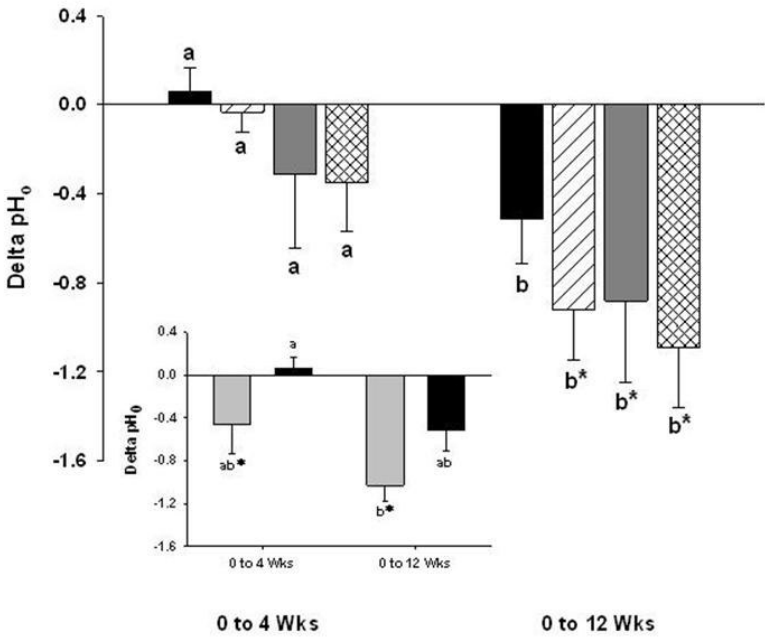


Figure 5. The effect of exercise and/or a 40% nutrient restricted diet on urine. A. Net Acid Excretion (NAE) and B. pH for Wk 0 to 4 and Wk 0 to 12. *Denotes significant difference vs. week 0 ($p<0.05$). Letters signify difference between change scores, all values sig. for: $p<0.05$.

Discussion

Moderate restriction of dietary energy resulted in deleterious bone alterations that were more similar to those observed in the 40% food restriction group than did restriction of calcium intake. The key difference between our paradigm and previous investigations is the addition of moderate treadmill exercise to both nutrient restriction paradigms. The comparison of differences between our two energy-replete groups provides useful insight into changes in bone related after chronic treadmill exercise. This study also included analyses of pH and net acid excretion in urine to explore whether metabolic acidosis may provide a mechanism for bone loss. Our findings do appear to rule out metabolic acidosis as a contributing cause to bone loss in our nutrient restricted groups. Although urine analyses indicated significant reductions in pH across all nutrient restricted groups, this is not correlated with significant changes in bone outcomes. Under conditions of chronic metabolic acidosis, bone cell activity is often altered in favor of bone loss [155,156]. Significant changes in osteoclast and osteoblast surface area were not detected with histomorphometric analysis in our paradigm which suggests low levels of bone turnover in all of our experimental groups.

In most adults attempting weight loss, the key component is the restriction of total food intake. Unfortunately, this usually means that intake of key nutrients for bone health such as calcium, protein, and energy is decreased as well. In rodent models using older, sedentary female rats (13 months old) a moderate food restriction over 4.5 months not only decreased body weight (-36%), but also uterine weight (-30%) compared to age-matched controls [148] which is similar to our findings in mature female rats. In

addition, this experiment found no effect for food restriction in cortical bone (which supports our findings) or serum IGF-1 or osteocalcin [148]. In our experiment, all groups' cortical BMC was greater after 12 weeks of exercise. In fact, our results more closely reflect those found in energy-replete, exercising animals [116,118,157] and seem to validate findings from Chen et al. that cortical bone is more sensitive than cancellous bone to stimulation by mechanical loading [118]. Another study by Banu et al. that utilizes the same model (sedentary, female rats) to assess changes in cancellous bone and found reductions in cancellous vBMD of both the L4 vertebrae (-30%) and the femoral neck (-5%) [158]. The addition of exercise to our moderate food restriction appears to have lessened the effect of food restriction on cancellous vBMD; there were no differences between the exercising groups at Week 12 (Table 4). In a protocol more similar to ours using sedentary, adult male rats Baek et al. performed histomorphometric analyses which suggest an effect in cancellous bone with a 30% food restriction for 6 weeks [93]. Although total vBMD at the proximal tibia (pQCT) decreased 4.7% compared to age-matched controls, there was no effect of food restriction at the midshaft tibia for vBMD or other geometric variables which corroborates our lack of effect in cortical bone. Food restriction (40%) has been shown to exert negative effects on cortical bone [158], more specifically on cortical BMC and area [27]. Histomorphometric analysis of the proximal tibia revealed decrements to MAR (-25%), MS/BS (-59%), BFR (-70), and osteoid surface (-33%) compared to age-matched controls [93], which mirrors our data with decrements of -50% (MAR), -11% (MS/BS), -46% (BFR) and -4% (osteoid surface) (Table 5). We observed some, but not all of the changes seen in these previous

experiments which appear to be related to the moderate treadmill exercise performed by our rats.

The major objective of this study was to test the relative importance of calcium and energy restriction in the context of regular endurance exercise training. Common wisdom is that calcium is one of the major minerals required for laying down new bone. Low-calcium diets are well accepted as a means for reducing bone mass and increasing bone resorption in rats [74,75] possibly above the loss resulting from estrogen deficiency alone in female rats [76]. Previous studies have shown that very low calcium diets do (-96% reduction) produce whole body effects such as a 100% increase in whole body resorption in mature female rats [74], but do not appear to affect body weight compared to age-matched controls [18,70,75-77] which corroborates our data which utilized a moderate calcium restriction paradigm (Fig 1). In addition, our paradigm did not appear to adversely affect either lean mass or body fat compared to the exercising controls, similar to previous findings [18] (Fig 2). Low calcium diets induce significant bone loss because they create a negative calcium balance which causes an increased secretion of parathyroid hormone [70]. Unlike previous calcium restriction paradigms [70] total body BMC from DEXA scans was not negatively affected when compared to the exercising controls (Table 3), but this previous study was not exercising their animals while they were restricted. This negative effect of calcium restriction in sedentary animals on bone appears to be localized to cancellous rather than cortical bone [17]. However, physical properties of the proximal tibia metaphysis assessed with pQCT scans in our study revealed no effect for calcium content of the diet for cancellous vBMD, total vBMD, or

cancellous BMC in our exercising animals (Table 4), which contradicts previous findings [70,75] in sedentary rats. Seto et al. found that 90% reduction of calcium in young, male rats for 3 days caused significant defects to bone architecture with reduction in BV/TV (-14%), trabecular thickness (-15%), and trabecular number (-46%) [75]. Non-significant increases in osteoclast surface area can explain at least in part the lower bone volume and highly spaced, smaller, less frequent trabeculae which corroborates previous findings [75,76] (Table 5). Dynamic histomorphometry indicates a trend for a lower bone formation rate, mineralizing surface, and mineral apposition rate in the calcium restricted group compared to the exercising controls (Table 5). Finally, urine pH was no longer at or above the physiological level at 12 weeks in calcium restricted rats, unlike the exercising controls' pH which could contribute to their higher osteoclast surface and lower bone formation rate.

We also sought to determine the effects of moderate energy restriction on bone parameters in exercising rats when all other nutrients were maintained at adequate levels. Energy restriction paradigms in older (16 months old) male rats have revealed that moderate energy restriction decreases total body BMD (assessed by DEXA scans) and resistance to fracture in femorae [14]. Those studies also provided information about serum markers for bone turnover such as reduction in plasma osteocalcin (-20%), urinary deoxypyridinoline (-25%), and IGF-1 (-20%) which suggested that moderate energy restriction suppresses all modeling in bone [14]. In terms of total body changes with 40% energy restriction, Talbott et al. tested similar diets to ours in young, growing animals (3 months old) and found that they gained 9% total body BMD assessed using DEXA scans

after 9 weeks of treatment [18]. When they tested this diet formulation in older female rats (10 months old) they found significant decrements of ~2% to total body BMD. These data for older rats corroborate our findings that moderate energy restriction in mature female rats reduced total body BMC significantly (-14%). Talbott et al. also assessed effects of moderate energy restriction in mature (20 wk old) and aged (48 wk old) female rats over 9 weeks in serum hormones and BMD (by radiography) of the tibia, femur and humerus [20]. In mature animals, only femoral BMD was decreased (-34%), but aged animals experienced significant reductions in BMD in tibia (-7%), femur (-35%) and humerus (-6%). These decrements to BMD in aged animals also corresponded to decreased peak load (-11%) compared to age-matched controls. We did not observe similar decrements to cancellous or cortical bone parameters in our moderate energy restriction study, but the key difference between our experiment and previous experiments is the addition of moderate treadmill running to our paradigm.

Our experiment utilized a moderate intensity treadmill training program, as opposed to more strenuous exercise training utilized by some investigators. Bourrin et al. [159], for example, found that high intensity treadmill training did increase trabecular number, but thinned the individual trabeculae and increased trabecular separation resulting in a decreased bone volume. Our exercising control group did not experience such deleterious effects attributed to the treadmill training regime. Although body weight was not negatively affected, sedentary animals continued to add weight after 4 weeks while exercising animals plateau. This is explained at 12 weeks by the slightly higher body fat and slight lower lean mass in sedentary animals which is similar to

findings from other groups [121]. Interestingly, total area changed significantly more in the sedentary control group when compared to all other exercising groups. This discovery may be explained by findings from Bourrin et al. [159] that suggest bone growth is delayed in exercising animals. Our results do show similarities to previous experiments that compared exercised and sedentary rats using histomorphometric analyses at the proximal tibia metaphysis. Bennell et al. [121] found no significant difference between exercising vs. sedentary rats' histomorphometric parameters which is comparable to findings in our experiment. However, trends in our data do show that bone volume was higher (22%) in exercising controls, similar to Yeh et al. [115] whose mature female rats completed 9 weeks of treadmill training.

There were several limitations to this study. Although an *ad lib*-fed, sedentary control group was utilized, this experiment did not include sedentary controls for each of the exercising, nutrient restriction groups. This would have provided insight into the exact effect of exercise during each of the nutrient restriction treatments.

The study was designed to elucidate which nutrient when restricted by 40% had the greatest negative effects on bone parameters in exercising female rats. It appears as though both 40% energy and global food restriction caused detrimental effects in many of the structural properties of bone assessed in this experiment. It was interesting to note that calcium restriction in these exercising rats had few negative effects on bone

outcomes which we attribute to the positive stimulus provided by the treadmill exercise. It is well known that under ideal conditions exercise provides an anabolic stimulus to bone while the catabolic effects related to nutrient restriction are also established. We conclude that exercise does appear to provide some anabolic stimulus to bone, even during the individual nutrients' restriction. In fact, negative changes in our experiment appear to be less severe with our moderate exercise protocol compared to previously published findings in sedentary animals. Our results stand apart from previous investigations involving nutrient restriction because this experiment was performed in exercising animals. Human studies investigating effects of calorie restriction-induced versus exercise-induced weight loss shows that calorie-restriction is associated with decrements to BMD at sites that are clinically relevant to fracture risk [(17)]. Our results suggest that the addition of moderate exercise to a diet may attenuate some of the losses in cancellous bone observed with weight loss.

CHAPTER IV

EXERCISE MODIFIES THE BONE RESPONSE TO GRADED REDUCTIONS IN ENERGY AVAILABILITY

Introduction

It is well established that highly active populations such as military personnel or athletes are at an increased risk for experiencing stress fractures. These groups' physical training entails a regimen that involves high impact, repetitive motions and a higher activity level than the general population which exerts a combined negative effect on total body bone mineral density (BMD) [160] resulting in an increased fracture risk. In addition, these particular groups' training increases their caloric output substantially such that they are unable or even unwilling to adjust their caloric intake to meet these increased needs. Previous studies have indicated that weight loss achieved via calorie restriction (-20%) alone is responsible for decreased bone mineral density in women [17]. Weight loss can be detrimental to bone health; in humans, each 10% reduction in body weight is associated with a 1% decrease in BMD [69]. Even small reductions in BMD can be clinically important because fracture risk is significantly increased with a 3% drop in BMD [161]. However, when exercise was utilized to increase energy expenditure (20%) in premenopausal women, it did not result in significant decrements to bone mineral density [17].

Bone loss in female athletes in particular is often attributed to a combination of chronic energy deficit and a subsequent weight loss that alters their levels of key

metabolic hormones (IGF-1 and leptin) [45,162]. Energy restriction has been also been linked with reductions in circulating estrogen levels in healthy, young women. In young adult women, an acute treatment (5 days) using an energy availability of 10 kcal/kg lean mass causes a 15% suppression in estradiol levels [23]. De Souza et al. determined that the negative consequences for bone are enhanced by the combination of estrogen deficiency and energy deficiency in women [24]. To further explore the effects of energy restriction on estrogen levels, several studies have investigated this paradigm using rodent models. Under conditions of chronic energy restriction (-40%), mature (20 wk old) female Sprague Dawley rats exhibited significantly lower serum estradiol values (-40%) [20]. Compared to *adlib*-fed controls, a 30% food restriction in female Sprague-Dawley rats resulted in a lower serum estradiol (-62%) and ovarian weight (-57%) [25]. It is important to note that this experiment utilized voluntary wheel running which makes it difficult to control for actual energy expenditure. Therefore, the combination of energy restriction and exercise in humans suggests that being in energy deficit attenuates the normal, positive response of bone to exercise.

No current study has investigated the effects of graded levels of energy availability in combination with endurance exercise on both endocrine factors as well as cancellous microarchitecture and formation indices in mature, female rats. The main purpose of this investigation was to determine whether energy restriction achieved via smaller reductions in caloric intake combined with increased energy expenditure alters the bone and endocrine response to graded energy deficits. In addition, we explored the role of endocrine factors reflecting reproductive function and energy metabolism

secondary to reduced energy restriction. We hypothesized that when combined with exercise, the impact of chronic reduced energy availability on cancellous bone formation would be dampened compared to sedentary animals subjected to the same reduced energy availability.

Materials and Methods

Animals and Experimental Design

Eighty-four female Sprague-Dawley rats were purchased from Harlan-Teklad and housed individually in a room with 12 hour light-dark cycles. Rats were aged to 5 months old at the beginning of the study, singly housed, and fed AIN-93M rat diet *ad libitum* for an 8-week acclimation period. Previous findings [93] revealed that adult female Sprague-Dawley rats experience significant declines in their cancellous vBMD at the proximal tibia metaphysis (PTM) within 4 weeks after switching from the vendor's Teklad 2018 rat chow to AIN-93M purified rat diet. Upon completion of this acclimation period, rats were assigned to one of seven groups (n=12/group) by random block assignment based on cancellous vBMD values and body weight at day 0, and then baseline controls (BC) were sacrificed. The remaining animals were assigned to either control groups (ADLIB-EX & ADLIB-SED) that were fed AIN-93M *ad lib* or to energy restricted (-10 and -30%), exercised groups (ER20-EX and ER40-EX, respectively) and sedentary energy-restricted group (ER20-SED and ER40-SED) that were fed modified AIN-93M with 20% and 40% less energy respectively, while 100% of all other nutrients were provided (Table 6). Body weights were recorded weekly to monitor animal health.

Serum was collected (from a leg vein) in anesthetized animals for ELISA assays of insulin-like growth factor (IGF-1) and leptin at day 0 and week 12. These collections were timed according to each rat's individual estrous cycle length to standardize collection points during the rats' metestrous or early diestrous phase of their cycle. At the same time points, body composition and total body BMC was measured with dual energy x-ray absorptiometry (DEXA) scans. Calcein was injected subcutaneously (35 mg/kg) on days 9 and 2 prior to sacrifice to label mineralizing bone for analysis of dynamic histomorphometry.

Table 6. The experimental variation of AIN-93M formulated to restrict energy by 10%, 20%, 30%, and 40% while providing 100% of all other nutrients.

Macronutrient (kcal%)	AIN-93M ADLIB-SED ADLIB-EX	10% Kcal Restriction ER20-EX	20% Kcal Restriction ER20-SED	30% Kcal Restriction ER40-EX	40% Kcal Restriction ER40-SED
Protein	15	16	18	21	24
Carbohydrate	76	73	70	66	60
Fat	9	10	12	13	16
kcal/gm	3.85	3.83	3.81	3.79	3.76

Anesthetized animals were then decapitated and tissues were harvested. Right proximal tibiae were cleaned of all tissue and stored in 70% ethanol at 4°C until processed for histomorphometric analysis. Uteri were weighed after the removal of the ovaries and cervix, and any unusually high levels of fluid within the uterus were noted. Uterine weights were recorded as a bioassay for estrogen activity.

Dietary Treatments

Two experimental control groups were fed AIN-93M *ad libitum* for the 12 week protocol and were subjected to the exercise treatment or served as sedentary controls restricted to cage activity (ADLIB-EX and ADLIB-SED, respectively). Rats in the ER20- and ER40-SED groups were fed 0.81 and 0.61 gm, respectively, of the specially formulated diet for every 1 gm of AIN-93M consumed by the average of both of the *adlib*-fed groups. Rats in the ER20- and ER40-EX groups were fed 0.91 and 0.71 gm of the specially formulated diet for every 1 gm of AIN-93M consumed by both of the *adlib*-fed groups. Energy density of the diets was reduced by decreasing the amount of corn starch. The exercising rats' treadmill running time was calculated to increase caloric expenditure by 10% per week in order to achieve a 20% and 40% energy deficit, respectively, for both groups. The energy restricted diets contained a higher density of all vitamins and minerals in order to achieve 100% of the animals' daily requirements as established by the National Research Council (163,164) (Table 6). Each animal's food intake was assessed over the course of the experiment to verify the actual level of energy available for each group. Energy available (EA) per week was quantified as total calories consumed over that week per animal minus any energy expended (kcal) via exercise during the same week per animal. EA was then normalized by each animal's body mass and represented as EA/g body mass.

Treadmill Exercise

All rats in the exercising groups were acclimatized to treadmill exercise for 3 weeks according to previously published protocol [165]. Over the 3 week ramping period, rats were exercised four times per week on a 15% grade for incrementally increased periods of time and speed. After the completion of the treadmill exercise acclimation period, rats exercised 4 days per week at 25 m/min on a 15% grade for 80-100 minutes per session. The exercise duration was determined by their weekly body weight in order to achieve a 10% increase in their weekly caloric expenditure. In addition, the treadmill exercise was performed at approximately 60% of their maximal oxygen consumption [151]. Some rats needed mild stimulation to continue performing the prescribed exercise provided by a low-current electrical grid at the back of the treadmill belt or by short bursts of air from a modified air-gun. Most rats adapted well to treadmill running and easily avoided the shock grid after the first training session.

Dual Energy X-Ray Absorptiometry (DEXA) Scans

Two days prior to sacrifice, DEXA scans (GE-Lunar Prodigy Small Animal Program) were performed on the rats' whole body while anaesthetized with a ketamine/medetomidine (2:1 ratio at 0.5 mL/kg body weight) cocktail to assess body composition (lean and fat mass) and total body BMC. Each rat was laid prone with its long axis aligned with the scan table's center line. Regions of interest (ROI) were drawn to exclude the animals' tails below the 3rd vertebrae. Coefficients of variance for *in vivo*

scans for lean mass, fat mass and total body BMC were 1.07, 2.99, and 1.24%, respectively, as determined from three repeat scans per animal.

Endocrine Assays

Serum leptin was measured by a rat ELISA according to the manufacture's protocol (Alpco Diagnostics, Salem, NH). The interassay coefficient of variation is less than 10% and the lowest detectable level is 10 pg/mL. Serum IGF-1 was measured using an EIA kit as suggested by the vendor (IDS Inc, Fountain Hills, AZ). The interassay coefficient of variation is 6% and the lowest detectable level is 2.8 ng/mL. Serum estradiol was measured using an estradiol double antibody RIA according to the vendor's instructions (Siemens, Plainfield, IN). The interassay coefficient of variation was less than 6% and the lowest level detectable is 1.4 pg/ml. All assays were performed at our Pennsylvania State University location.

Cancellous Histomorphometry

Undemineralized proximal tibiae were serially dehydrated and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were microtomed either 8 µm thick for UV analysis of unstained sections at 20X (total bone surface, single- and double-labeled surface, and interlabel distances) or 4 µm thick for von Kossa staining and measurement at 40X of static histomorphometric properties (bone volume, osteoid surface, osteoblast surface, osteoclast surface, and adiposite density). The region of interest began ~1.0 mm from the growth plate and encompassed a 6.0 mm^2 area within

the endocortical edges. Mineral apposition rate (MAR; µm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS) for cancellous bone surfaces (BS) was calculated by using the formula MS/BS = [(single-labeled surface/2) + double-labeled surface]/surface perimeter x 100. Bone formation rate (BFR) was calculated with the formula: BFR = MAR X MS/BS. All histomorphometric analyses were performed using OsteoMeasure image analysis software (Version 2.31; Osteometrics, Inc.) interfaced with Optronics 3-chip color camera and an Olympus BX60 microscope with epifluorescent light (Leeds Instruments, Irving, TX). All nomenclature for cancellous histomorphometry followed previously established standards [(152)].

Results

Equivalent Energy Availability Was Achieved in Sedentary and Exercising Energy-Restricted Groups

During the experimental period several animals were euthanized due to mammary tumor growths from the following groups: one from ADLIB-EX, one from ADLIB-SED, two from EE20-EX, and two from EE20-SED. Both a*d lib*-fed groups consumed similar levels of calories during the 12 week protocol (Fig 6) and had similar levels of EA/g body mass, with the exception of the first 3 weeks. During this time period all EX animals' treadmill running was ramping up and did not achieve its full intensity until the end of week three, hence their energy availability per gram body weight was higher than for SED rats. Over the course of the experiment, both of the *ad*

lib-fed groups' food intakes declined. When calculated as an average over the entire 12 weeks, both of the ER20 groups had -12% less EA/g body mass and both of the ER40 groups had -25% less EA/g body mass compared to their respective *ad lib*-fed controls.

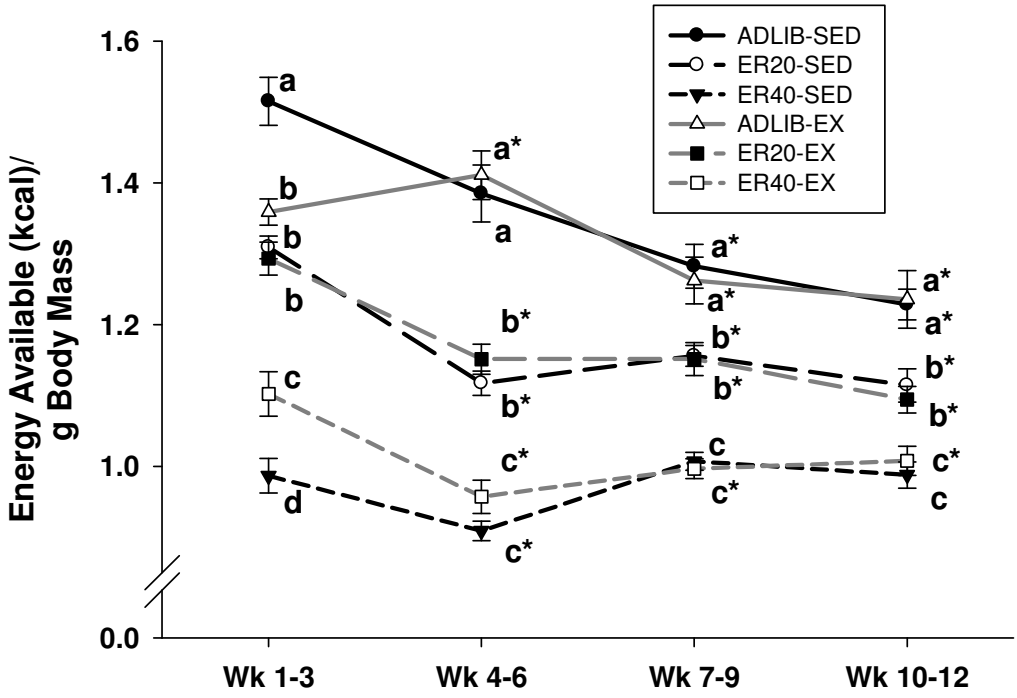


Figure 6. The energy available (kcal) per g body mass. Each time point reflects the average of three weeks. * denotes significant difference versus week 0 ($p<0.05$) within groups. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$) per time point.

Treadmill Exercise Reduces Energy Deficit Associated Reductions in Both Lean Mass and Total Body BMC

Both exercising and sedentary *adlib*-fed animals' body weight increased significantly over the duration of the 12 week protocol (+14% and +15%, respectively),

verifying that the exercising, *adlib*-fed rats increased their food intake to compensate for the increase in caloric expenditure (Table 7). Exercise training significantly altered the type of weight gained. The majority of mass gained by ADLIB-SED animals was due to increased fat mass (+47%), whereas ADLIB-EX rats had significant increases in lean mass (+12%) (Table 7). Mild energy restriction in ER20-SED resulted in a slightly lower increase in body mass that was achieved by an increase in lean mass and decrease in fat mass. More severe restriction of energy intake (ER40) resulted in reductions in body mass (-15%) achieved primarily through reductions in fat mass (-92%). The addition of exercise to mild energy restriction (ER20) preserved both lean and fat mass. However, EX was unable to protect lean mass against the detrimental effects of a more severe energy restriction (ER40).

All *adlib*-fed groups experienced significant gains in total body BMC after twelve weeks (Table 7). Interestingly, both of the ER20 groups' total body BMC increased over the course of the experiment. Severe energy restriction resulted in significantly lower total body BMC after 12 weeks (-7%).

Table 7. The longitudinal changes to body mass, body composition, and total body bone mineral content (BMC) with or without exercise and varying levels of caloric intake.

		Sedentary			Exercising		
		ADLIB	*ER20*	*ER40*	*ADLIB*	*ER20*	*ER40*
Body Mass (g)							
	Wk 0	275.42 ± 2.55	276.38 ± 2.66	281.32 ± 6.73	279.13 ± 3.78	287.64 ± 6.32	268.85 ± 3.95
	Wk 12	317.68 ± 4.23^{a*}	296.50 ± 4.37^{b*}	239.36 ± 3.65^{c*}	318.73 ± 7.36^{a*}	315.56 ± 6.20^{a*}	267.04 ± 5.67^{b}
	%-diff	*+15.34*	*+7.28*	*-14.92*	*+14.19*	*+9.71*	*-0.67*
Fat Mass (g)							
	Wk 0	43.33 ± 4.93	42.09 ± 3.80	48.63 ± 5.71	41.11 ± 3.41	38.56 ± 4.05	34.64 ± 31.13
	Wk 12	63.64 ± 4.27^{a*}	34.60 ± 3.91^{b}	4.13 ± 1.63^{d*}	49.45 ± 5.79^{b}	49.11 ± 4.71^{b}	18.36 ± 4.86^{c*}
	%-diff	*+46.87*	*-17.80*	*-91.51*	*+20.29*	*+27.36*	*-46.94*
Lean Mass (g)							
	Wk 0	228.89 ± 6.46	226.09 ± 3.74	224.75 ± 4.32	225.44 ± 4.09	231.33 ± 4.95	223.64 ± 5.72
	Wk 12	237.91 ± 3.79^{bc}	245.80 ± 4.52^{ab*}	211.25 ± 3.21^{d*}	253.18 ± 4.02^{a*}	250.67 ± 3.37^{a*}	233.86 ± 4.08^{c*}
	%-diff	*+3.94*	*+8.72*	*-6.01*	*+12.30*	*+8.36*	*+4.57*
Total Body BMC (g)							
	Wk 0	9.83 ± 0.22	9.65 ± 0.18	9.79 ± 0.24	9.63 ± 0.24	10.06 ± 0.28	9.61 ± 0.11
	Wk 12	11.11 ± 0.14^{a*}	10.44 ± 0.19^{b*}	9.09 ± 0.18^{d*}	11.11 ± 0.23^{a*}	11.03 ± 0.25^{a*}	9.91 ± 0.16^{c*}
	%-diff	*+13.02*	*+8.19*	*-7.15*	*+15.37*	*+9.64*	*+3.12*

Values are means ± standard error of the mean as change versus their respective baseline values. * denotes significant difference versus week 0 ($p<0.05$) within groups. Values not sharing are the same letter are significantly different from one another ($p<0.05$).

Cancellous Microarchitecture Appears to Be Protected by EX Under Conditions of -20% Energy Restriction

In *adlib*-fed animals EX did not have any independent effects on cancellous microarchitecture, but it did independently affect indices of bone formation (Table 8). ER20 stimulated an increase in these variables similarly regardless of exercise status and did not result in any differences vs. the *adlib*-fed groups. Although non-significant, ER20-EX (+40%) and ER20-SED (+61%) had numerically higher OS/BS compared to their respective control groups. ER40 negatively affected cancellous microarchitecture.

Both bone volume (-26%) and Tb.Th (-18%) for the ER40-SED group were significantly lower vs. ADLIB-SED. EX did not protect against decrements to bone volume with ER40 after 12 weeks. The addition of EX to 40% energy restriction prevented an increase in the adipocyte density in the marrow cavity. EX was unable to prevent thinning of trabeculae with ER40; values for Tb.Th for ER40-EX (-13%) were lower versus ADLIB-EX at week 12.

Table 8. The effect of treadmill exercise and/or varying levels of energy restriction on cancellous microarchitecture and bone cell activity at the proximal tibia.

	Baseline Control	Sedentary			Exercising		
		ADLIB	*ER20*	*ER40*	*ADLIB*	*ER20*	*ER40*
BV/TV (%)	29.08 ± 1.74	24.46 ± 1.20^{a}	21.63 ± 1.56^{ab}*	18.074 ± 2.54^{b}*	23.06 ± 1.69^{ab}*	25.910 ± 1.66^{a}	20.00 ± 1.52^{b}*
Tb.Th (µm)	48.11 ± 1.58	49.82 ± 2.00^{ab}	48.52 ± 2.10^{ab}	40.94 ± 1.69^{c}*	51.35 ± 1.60^{a}	52.26 ± 3.00^{a}	44.77 ± 2.19^{bc}
Tb.Sp (µm)	120.07 ± 8.14	155.34 ± 7.30^{a}	181.12 ± 10.89^{ab}*	206.56 ± 31.89^{b}*	167.2 ± 10.81^{ab}*	157.69 ± 11.48^{a}*	185.76 ± 11.47^{ab}*
Tb.N (1/mm)	6.06 ± 0.32	4.92 ± 0.17*	4.42 ± 0.18*	4.35 ± 0.51*	4.47 ± 0.26*	4.60 ± 0.35*	4.45 ± 0.23*
OS/BS (%)	1.63 ± 0.54	1.81 ± 0.24^{ab}	2.90 ± 0.60^{ac}	1.15 ± 0.22^{b}	2.62 ± 0.59^{abc}	3.68 ± 0.73^{c}	2.40 ± 0.50^{abc}
ObS/BS (%)	0.72 ± 0.33	0.62 ± 0.10^{ab}	0.98 ± 0.16^{ab}	0.48 ± 0.11^{b}	1.10 ± 0.36^{ab}	1.37 ± 0.37^{a}	0.99 ± 0.26^{ab}
OcS/BS (%)	2.26 ± 0.42	2.20 ± 0.81	2.14 ± 0.62	1.68 ± 0.57	1.87 ± 0.41	1.91 ± 0.47	1.83 ± 0.34
Adipocyte Density (#/mm)	78.57 ± 19.87	57.31 ± 17.00^{a}	126.87 ± 22.70^{a}	256.80 ± 50.69^{b}*	58.25 ± 19.34^{a}	122.84 ± 34.57^{a}	92.37 ± 22.03^{a}

Values are means ± standard error of the mean as change versus their respective baseline values. * denotes significant difference versus baseline value ($p<0.05$) within groups. Values not sharing are the same letter are significantly different from one another ($p<0.05$).

Treadmill Exercise Combined With 20% Energy Restriction Enhances Bone Formation at the Proximal Tibia of Mature Female Rats

In energy-replete animals, EX produced significant increases in MS/BS (+103%) vs. BC (Fig 7A); the much smaller increase in ADLIB-SED animals was not significant.

Mild energy restriction (ER20) resulted in higher MS/BS (+87% vs. BC rats), while more severe energy restriction (ER40) produced lower MAR (-29%) vs. BC (Fig 7B). The addition of EX to ER20 increased MS/BS (2-fold) and enhanced BFR compared to BC (+180%), and all other treatment groups (Fig 7C). The addition of EX to ER40 improved MS/BS compared to BC (+81%) and mitigated reductions in MAR vs. BC. Although non-significant, EX appears to have mitigated reductions in MAR under conditions of ER40 (Fig 7B).

Reduced Energy Availability Negatively Affects Estradiol Levels in Sedentary Animals Alone

In *adlib*-fed animals, uterine weight and serum estradiol were not affected by chronic treadmill exercise (Fig 8). Regardless of exercise treatment ER20 resulted in lower uterine weight vs. BC, but did not significantly reduce uterine weight compared to ADLIB groups (Fig 8A) at week 12. ER20 did result in lower serum estradiol (-26%) for ER20-SED (-26%) vs. ADLIB-SED (Fig 8B). More severe energy restriction (ER40) produced much lower estradiol levels (-63%) for ER40-SED (-63%) vs. adlib-fed animals. In sedentary animals, ER40 yielded significantly lower values for uterine weight vs. BC and ADLIB-SED (-36%). Serum estradiol was not affected by mild (ER20) or severe energy restriction (ER40) in exercising animals.

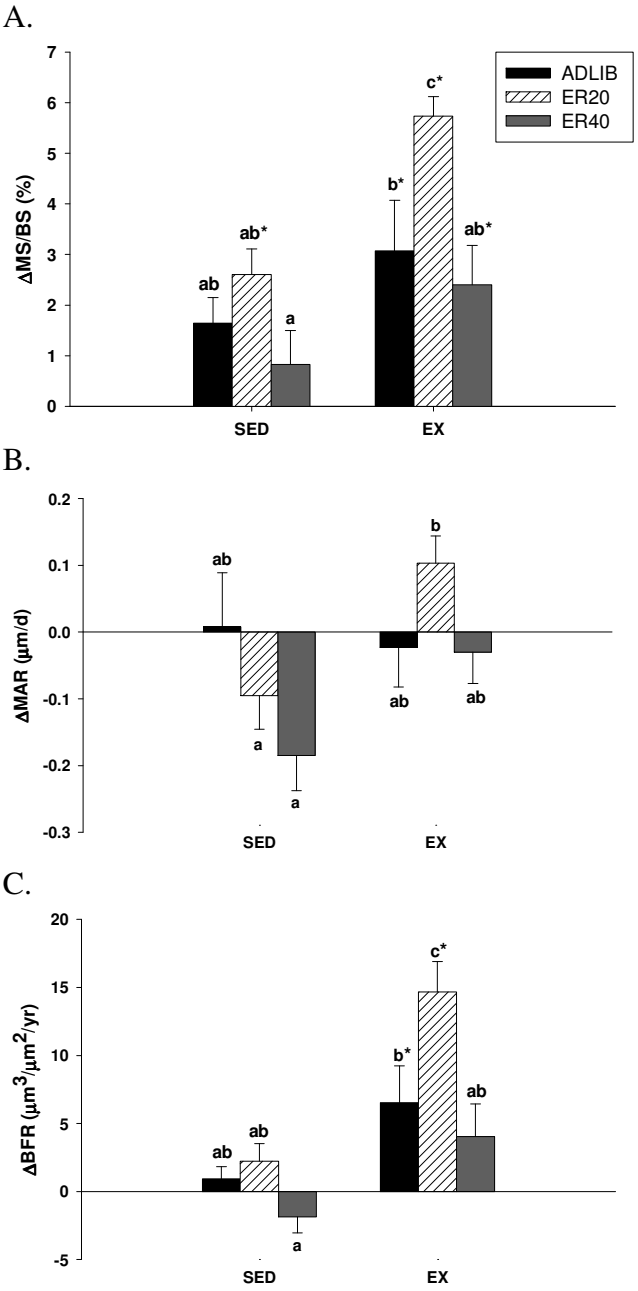
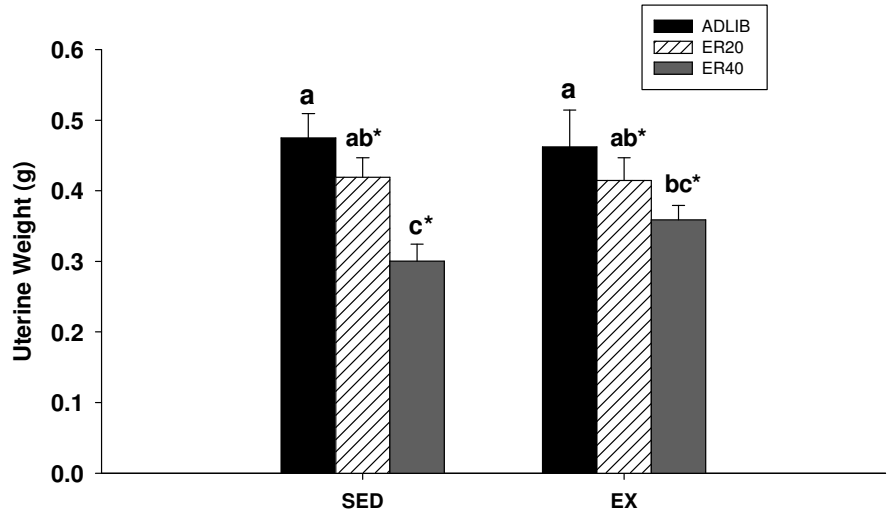


Figure 7. Treadmill exercise combined with ER20 enhances bone formation at the proximal tibia of mature female rats. A. Delta mineralized surface/bone surface (MS/BS, %). B. Delta mineral apposition rate (MAR, %). C. Delta bone formation rate/bone surface (BFR/BS, %). Change (delta) is calculated vs. baseline control groups. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

A.



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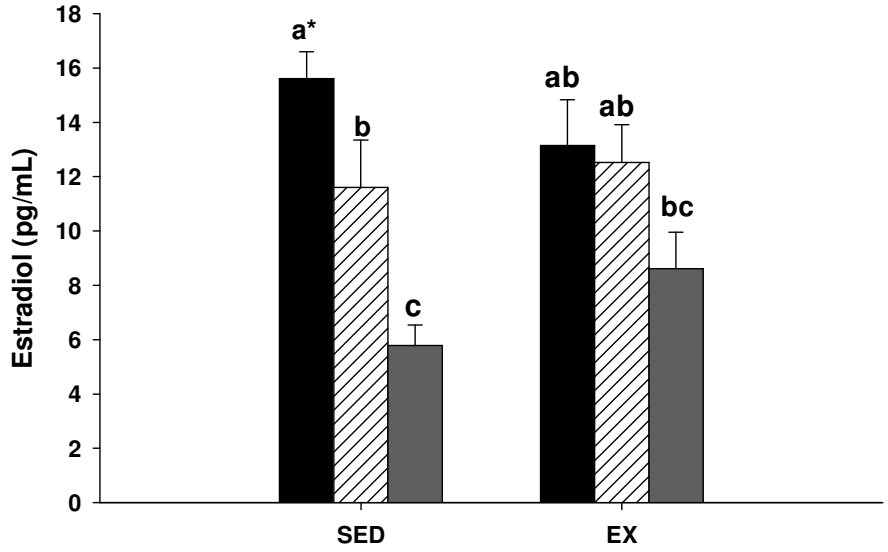
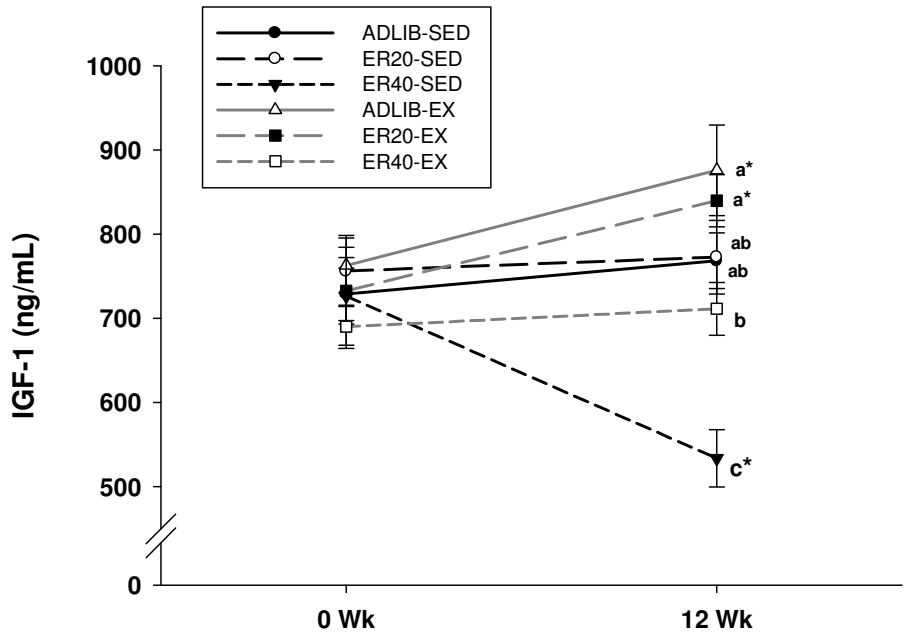


Figure 8. Although ER20 alone affects uterine weight, both levels of energy restriction (-20% and -40%) negatively affect estradiol levels in sedentary animals. A. Uterine weight (g). B. Estradiol (pg/ml). Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

Exercise Modifies Alterations in Endocrine Markers of Energy Metabolism With ER20, but Not With ER40

In *adlib*-fed animals 12 weeks of EX yielded significantly higher levels of serum IGF-1 versus week 0, but did not result in values higher than SED animals (Fig 9A). Serum leptin levels were however affected by EX; ADLIB-EX was significantly lower (-35%) vs. ADLIB-SED at week 12 (Fig 9B). Twelve weeks of ER20 did not result in significant alterations in serum IGF-1 levels vs. week 0 in SED animals. Serum leptin levels were lower for ER20-SED compared to the ADLIB-SED group (-68%) at week 12. Twelve weeks of ER40 in SED animals produced lower IGF-1 levels vs. week 0 (-19%) and compared to the ADLIB-SED group (-31%). More severe energy restriction (ER40) in SED animals resulted in significantly lower leptin levels (-84%) vs. the ADLIB-SED group. The addition of EX to ER20 led to higher serum IGF-1 (+14%) as well as serum leptin levels (+14%). When EX was added to ER40, both serum IGF-1 (-19%) and serum leptin (-70%) levels were lower compared to ADLIB-EX (-19%) by week 12. There was a correlation between IGF-1 and total body BMC for both SED (0.69, $p<0.0001$) and EX (0.58, $p<0.001$). Leptin values at week 12 correlate with fat mass for both EX (0.66, $p<0.01$) and SED (0.67, $p<0.01$).

A.



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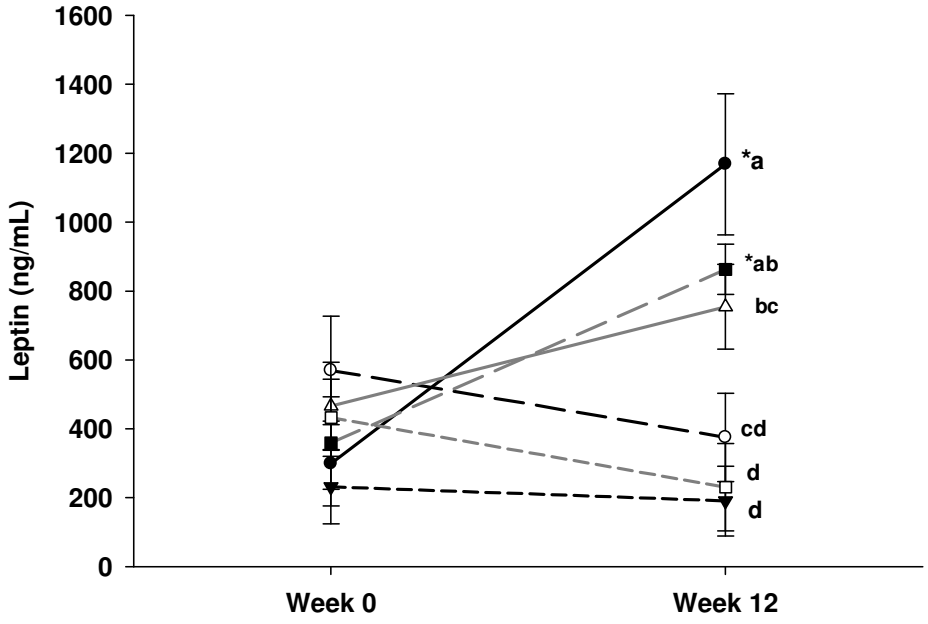


Figure 9. EX provides some protection for endocrine markers of bone metabolism (IGF-1 & leptin) against the effects of ER20, but is unable to mitigate decrements associated with ER40. A. Insulin-like growth factor (IGF)-1 (ng/ml). B. Leptin (ng/ml). Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

Discussion

The main purpose of this investigation was to determine whether the means of achieving graded reductions in energy availability (with or without exercise) alters the bone and endocrine response. We hypothesized that the addition of chronic treadmill exercise would lessen the negative impact of energy restriction on cancellous microarchitecture and formation indices at the proximal tibia. Both the exercising and sedentary groups achieved similar reductions in energy availability [energy consumed (kcal) – energy expended (kcal)] over the course of the 12 week experiment (Fig 6). The ER20 group had 12% less energy available and the ER40 groups had 25% less energy available vs. the *ad lib*-fed animals.

In accordance with our hypothesis, reducing energy availability with exercise in addition to restricting energy intake minimized loss of bone volume at the proximal tibia associated with ER40 found in their sedentary counterparts (Table 8). This change in bone volume is further explained by examining changes in individual parameters for cancellous microarchitecture. The addition of exercise limited the effects of ER40 on trabecular separation, but not trabecular thickness. In comparison to their respective control groups, trabecular separation was significantly higher (+33%) in sedentary animals subjected to ER40, but not for exercising animals (+11%). In contrast, 40% energy restriction and not exercise exerted the greatest effect on trabecular thickness. Both ER40 groups' trabecular thickness was significantly lower compared to both their respective control groups as well as BC. These data corroborate previous findings that utilized a 40% energy restriction in sedentary, adult female Sprague Dawley rats.

Moderate energy restriction (40%) significantly reduced bone volume (-35%) and trabecular thickness (-30%) compared to *ad lib*-fed controls [19]. Baek et al. tested the effects of a 30% food restriction on sedentary male rats' indices of bone formation and found significant reductions in MAR (-25%), MS/BS (-59), and BFR/BS (-70%). These findings are similar to values for our 40% energy restricted sedentary group, but not our 40% energy restricted exercising group. Therefore, the key difference between these studies and ours is the addition of a moderate exercise regimen.

The only other published data testing the combination of exercise training with reduced energy intake [25] used a voluntary wheel running model with female rats. All animals experienced significant estrous cycle disruption and concurrent reductions in serum estradiol after food restriction. There was no discernible effect for food restriction on tibia or femur areal BMD obtained from DEXA scans. The key difference between this experiment and ours is type of exercise. Treadmill running provides a controlled exercise regimen allowing for precise levels of energy expenditure, whereas voluntary wheel running distances are variable from day to day and with individual rats.

Overall, it appears as though exercise mitigated losses in total body bone mass related to ER40 by functioning to preserve lean mass. In energy-replete animals, exercise resulted in a greater accrual of lean mass (+12%) compared to sedentary controls (+4%) after 12 weeks (Table 7). This difference in lean mass was accentuated in ER40 groups; ER40-EX gained over 12 weeks (+5%) while ER40-SED animals lost (-6%). Similar changes occurred for total body BMC over 12 weeks, ER40-EX gained bone mineral (+3%) while ER40-SED lost (-7%). Our results suggest that when exercise

is combined with energy restriction it does not lead to reductions in lean mass as seen in sedentary animals. Several experiments in humans have determined that energy restriction leads to proportionate losses in lean mass that cannot be mitigated with aerobic exercise [165,166]. However, a few studies have determined that weight loss through implementation of a moderate exercise program rather than diet alone preserves lean mass [49], which corroborates our data.

The apparent protection against bone loss provided by our endurance running paradigm even during conditions of energy deficiency can possibly be explained by examining outcomes for serum IGF-1. Typically, synthesis of IGF-1 and its availability in the systemic circulation is related to the energy available in an organism's system [79]. Therefore, large reductions in energy available to an organism will lead to significant decrements to IGF-1 levels [13,81] and a concurrent decrease in bone formation [13]. In our exercising animals, 12 weeks of ER40 did result in significantly lower IGF-1 values versus *ad lib*-fed controls (Fig 9A). However, serum IGF-1 for ER40-EX was not significantly lower at 12 weeks compared to week 0. This is not the case for ER40-SED which was not only lower compared to its control group (-31%), but also reduced compared to week 0 (-19%). The preservation of IGF-1 under conditions of ER40 appears to be linked to the addition of the chronic treadmill exercise regimen to less severe energy restriction. Ihle & Loucks [13] tested the effects of graded levels of energy availability in exercising young women and found significant reductions in IGF-1 at a similar level of reduced energy availability as compared to our ER40 group. Nemet et al. found that reductions of energy intake (-33%) in adult males in conjunction with a

strenuous exercise regime over 7 days resulted in significant declines in total serum IGF-1 (-28%) [81].

The ability of our exercise paradigm to lessen several of the deleterious effects related to ER40 appears to be linked to altered endocrine responses. One key difference between our model and the paradigm used by Ihle & Loucks is the length of treatment. Their human study was completed over a 5 day period while our protocol lasted for 12 weeks to allow for assessment of the chronic, rather than acute effect of the treatment. There may be a threshold for energy availability below which exercise is unable to mitigate reductions in IGF-1. Zanker et al. tested the effects of either energy balance or energy deficit (-50%) in male endurance runners and found that reductions in IGF-1 occurred despite the long-distance running [82]. The lower level of energy available to their subjects abolished the positive effects related to exercise. Therefore, the threshold for positive effects of exercise on IGF-1 disappears when energy is restricted by more than 40%. Our data suggest that treadmill running does provide sufficient anabolic stimulation to mitigate bone loss in rats subjected to chronic energy deficits (-40%). Our endurance training protocol not only maintained lean body mass, but also buffered the IGF-1 response against a long-term energy restriction which was manifested through an apparent protection of total body BMC and cancellous bone volume.

Several studies have investigated energy restriction for varying durations in sedentary, female rats. Compared to *adlib*-fed controls, a 30% food restriction in sedentary, female Sprague-Dawley rats resulted in a lower serum estradiol [25]. In addition, all of the energy restricted rodents exhibited either disrupted estrous cycles or

became completely acyclic. Under conditions of chronic energy restriction (40%), mature, sedentary female Sprague Dawley rats exhibit lower serum estradiol values (-40 to -50%) [20,26]. Hawkins et al. determined that 10 weeks of energy restriction (-40%) led to significant reductions in serum estradiol (-50%) in sedentary, mature female rats [19]. In our experiment, ER40 resulted in significantly lower uterine weight compared to *ad lib*-fed controls in both sedentary and exercising female rats (-36 and -22%, respectively) (Fig 8A). This global effect for ER40 was not apparent for circulating estradiol levels. There was no difference between EX rats' estradiol levels (Fig 8B), however, SED rats' estradiol levels decreased proportionately after both ER20 and ER40 (-26 and -63%, respectively).

An interesting observation resulting from this experiment is that our lower level of reduced energy availability when achieved with treadmill exercise and a small reduction in energy intake increased IGF-1, BFR, and MS/BS. ER20-EX not only had higher MS/BS and BFR/BS compared to BC, they were significantly greater versus ADLIB-EX (Fig 7A and 7C). There were no differences in serum IGF-1 levels between both *ad lib*-fed groups and their respective ER20 groups (Fig 9a), which suggests that

ER20 did not impact IGF-1. Serum leptin was significantly lower for ER20-SED compared to ADLIB-SED, but this difference was not apparent between ADLIB-EX and ER20-EX (Fig 4B). This difference could relate to the differences between the accumulation of fat mass (Table 7) for each ER20 group. ER20-EX accumulated fat (+27%) over the 12 week protocol, while ER20-SED lost fat (-18%). The exercising rats with milder reductions in energy availability exhibited no sign of change in uterine weight, nor in serum estradiol. Therefore, the end result was little endocrine change by treatment with 20% energy restriction. Additionally, 20% energy restriction had virtually no impact on total skeletal mass as reflected by total body BMD (Table 7).

There were a few limitations to this experiment. To achieve the desired levels of energy restriction in this experiment the composition of the diets' macronutrients were adjusted. Therefore, it is possible that the altered composition might have contributed to several of the changes that occurred in this experiment. The diets were formulated to account for this change in composition. In fact, each of the diets contains the same number of total grams protein and fat. Contrary to our expectations, serum IGF-1 levels for ER40-EX decreased between 0 and 4 weeks. However, 0-12 week values showed an increase of 46% which suggests that the ER40-EX animals consumed more food the last

week. Unfortunately, due to the high volume of measurements requiring anesthesia occurring during the last week of the study, EX groups were not able to complete all days of scheduled exercise treatment. This smaller reduction in energy availability may have also contributed to the surprising outcomes for our estradiol data. The seemingly protective effect that EX provided for estradiol levels could be related to the additional energy intake. In human subjects, there is a threshold for energy availability (20 kcal/kg lean body mass) below which reproductive hormone disruption occurs [23]. Therefore, the lack of any apparent disruption to serum estradiol levels in our experiment is related to the increased energy availability for the EX groups.

In conclusion, our findings suggest that achieving a moderate (-25%) reduction in energy availability with combined exercise mitigates the deleterious effects on cancellous bone microarchitecture and formation indices seen with energy restriction alone. In addition, a mild reduction in energy availability (-12%) did not produce any harmful effects to bone. These findings are particularly important for long term bone health in highly active populations such as military personnel and athletes who may frequently experience periods of reduced energy availability.

CHAPTER V

MECHANICAL LOADING INCREASES ESTROGEN RECEPTOR ALPHA EXPRESSION IN OSTEOCYTES AND OSTEOBLASTS DESPITE CHRONIC ENERGY RESTRICTION

Introduction

In both human as well as rodent models it has been determined that reductions in circulating estrogen levels are associated with decreased expression of estrogen receptor-alpha (ER-α). In a human model that compared estrogen-replete women (before menopause or after with hormone replacement therapy, HRT) to those that were estrogen-deficient (post menopause without HRT) estrogen deficiency resulted in lower ER-α protein levels (-12%) [36]. In rats, ovariectomy (OVX) reduces ER-α mRNA expression in trabecular bone [35].

In the absence of adequate levels of circulating estrogen, there may not be enough functional ER-α to process the cell-strain response. In knock-out mice lacking functional ER-α bone formation on both periosteal and endosteal surfaces of the ulna following bouts of mechanical loading are reduced three-fold. More specifically, this paradigm determined that ER-α is required for bone to adapt after loading via an increase in the number of osteoblasts and in bone formation [41,42].

Part of the impaired response to mechanical loading may derive from an impact on osteoblast differentiation. Bone marrow stromal cells can differentiate into either pre-osteoblasts or adipocytes and this is regulated by a variety of factors. In an in vitro

model using osteoblastic cell lines cultured under estrogen deficiency, it was discovered that osteoblasts in the early stage of differentiation were switching to differentiation pathways leading to adipocytes without adequate levels of ER-α [144]. ER-α is not only required for osteoblast differentiation, it is essential for osteoblast sensitivity to mechanical loading. Lanyon et al. found that ER-α, not estrogen, is necessary for the full expression of bone cells' adaptive response to mechanical loading [39]. They theorized that in the absence of adequate levels of circulating estrogen which is required for ER-α expression, there may not be enough functional ER-α to process the cell-strain response.

Previous studies have explored the function of ER-α during mechanical loading only in models with severely reduced estrogen levels (e.g., post-OVX). This model has not been explored in response to intermediate reductions in circulating estrogen, such as that observed following chronic dietary energy restriction. Chronic energy restriction (-40%) in female rodents not only results in significantly lower estradiol levels, but also negatively affects geometric properties of bone [20]. Contrary to other mechanical loading models, we propose to utilize a simulated resistance training paradigm that demonstrates robust increases in cancellous bone formation during conditions of disuse [167,168]. The objective of this study was to determine the bone response to mechanical loading in energy-restricted rodents and whether there is a simultaneous down-regulation of bone cell estrogen receptor alpha (ER-α). Our hypothesis was that 12 weeks of a 40% energy restriction in female rats will cause a reduction in ER-α positive osteoblasts and osteocytes. Our second purpose was to discern whether the reduction in ER-α

expression in osteocytes and osteoblasts is associated with a significant attenuation of the bone formation response to mechanical loading. We hypothesized that the reduced expression of ER-α would be associated with an attenuated bone formation response to standardized loading via high-force muscle contractions in energy restricted female rats.

Materials and Methods

Animals

Sixty female Sprague-Dawley rats were purchased from Harlan-Teklad and housed individually in a room with 12 hour light-dark cycles. Rats were 5 months old at the beginning of the study, moved to single housing, and fed AIN-93M rat diet *ad libitum* for an 8-week acclimation period. Previous findings [93] revealed that adult female Sprague-Dawley rats experience significant declines in their cancellous vBMD at the proximal tibia metaphysis (PTM) within 4 weeks after switching from the vendor's Teklad 2018 rat chow to AIN-93M purified diet, hence the need for this prolonged acclimation period. Rats were then assigned to one of five groups by random block assignment based on body weight at day 0, and then baseline controls (BC) were sacrificed. The remaining animals were assigned to either control groups (ADLIB-SHAM and ADLIB-LOAD) that were either fed AIN-93M *ad libitum* or to energy restricted groups (ER40-SHAM and ER40-LOAD) that were fed 0.61 gm of the specially formulated diet for every 1 gm of AIN-93M consumed by the *ad lib*-fed rats. The energy restricted diet contained a higher density of all other nutrients in order to achieve 100% of the animals' daily requirements (Table 9). After the 12 week dietary

intervention, animals in the LOAD groups were subjected to an acute loading using stimulated muscle contractions for 3 sessions every 3rd day. The SHAM groups were

Table 9. AIN-93M Mature Rodent Diet (D10012M) and modification to 40% caloric restriction (D01092702).

	AIN-93M (D10012M)	40% Caloric Restriction (D01092702)
Macronutrient (kcal%)		
Protein	15	24
Carbohydrate	76	60
Fat	9	16
kcal/gm	3.85	3.76
Vitamin/Mineral (g/kg diet)		
Phosphorus	3.1	5.1
Calcium	5.0	8.1
Potassium	3.6	5.8
Magnesium	0.5	0.8
Vitamin K	0.00075	0.00122
Vitamin D (IU/kg diet)	1000	1621

anesthetized for the same duration as the LOAD animals, fine wires were inserted, but no muscle contractions were performed. Calcein was injected subcutaneously (35 mg/kg) on days 9 and 2 prior to sacrifice to label mineralizing bone for analysis of

dynamic histomorphometry. Upon completion of the experimental treatment, anesthetized animals were decapitated and tissues harvested. Uteri were weighed after the removal of the ovaries and cervix as a bioassay for estrogen activity. Whole left femorae were paraffin-embedded for immunohistochemical (IHC) staining for ER-α in osteoblasts and osteocytes. Left proximal tibia were serially dehydrated then embedded in methylmethacrylate for analysis of static (osteoid surface, bone volume, and osteoblast surface) and dynamic (bone formation rate, mineral apposition rate, and mineralizing surface) histomorphometry because it is a cancellous bone site known for being responsive to estrogen [33].

Dietary Treatment

Standard AIN-93M diet was purchased from Research Diets, Inc. (New Brunswick, NJ) and used for the 100% intake groups. It is critical to this protocol to use a purified rat diet to minimize phytoestrogen intake, which can have independent effects on bone, and to assure tight control over other nutrient content. Further, AIN-93M was designed to better match laboratory rat dietary requirements than the standard rat diets, such as 2018 Harlan Teklad, which our rat vendor uses (Table 9). A variation on the AIN-93M was formulated to allow for the reduced energy intake this design requires (Table 9) while ensuring that protein, calcium, and all other vitamins/minerals intake remains constant; this is achieved by increasing the density of protein and vitamin/mineral mixes. Hence, the only deficiency experienced by the restriction group is energy content of the diet. Two experimental control groups were fed AIN-93M *ad*

libitum for the duration of the study (ADLIB-LOAD and ADLIB-SHAM). Rats in both the ER40-LOAD and ER40-SHAM groups were fed 0.61 gm of the specially formulated diet for every 1 gm of AIN-93M consumed by the *ad lib*-fed groups to achieve a 40% deficit in caloric intake for both groups.

In Vivo *Muscle Stimulation*

At the start of week 12 of the experimental protocol, animals in the LOAD groups were exposed to 3 standardized bouts of muscle contractions as described previously [167] using a rodent-sized dynamometer to test for responsiveness to loading. Loading occurred every third day over a 9-day period for a total of three sessions. After the rat was anesthetized with inhaled isofluorane, was placed in right lateral recumbency on the dynamometer platform, its foot secured to the servomotor shoe, and percutaneous fine wire electrodes were inserted high on the lateral upper leg to stimulate the sciatic nerve. Once the electrodes were connected to a Grass Instruments stimulus isolation unit (Model SIU5; Astro-Med, Inc; W. Warwick, RI) the first step was to optimize isometric contraction torque production by the posterior crural muscles. Next, 4 sets of 5 eccentric contractions were performed at the appropriate intensity. Contraction intensity can vary between sessions; therefore, the peak isometric and eccentric torque was optimized prior to each loading session. This was performed with 3-5 contractions while adjusting stimulation voltage. Contractions were performed over a 40° arc using an angular velocity of 200°/s at 180 Hz. The stimulus duration was 2000 ms with the first 1000 ms being isometric, followed by 1000 ms eccentric. This protocol results in

lower peak eccentric contraction torque production (75% of maximum eccentric torque), thus minimizing the potential for muscle injury while still enabling an adequate stimulus for bone adaptation. Bout duration for this training procedure was 30 minutes, including induction and recovery from anesthesia. Animals in the SHAM groups were anesthetized and percutaneous electrodes placed on the same 3 days when the other two groups were subjected to stimulated muscle contraction loading.

Cancellous Histomorphometry

Undemineralized proximal tibiae were serially dehydrated and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were microtomed either 8 µm thick for UV analysis of unstained sections at 20X (bone formation rate, single- and double-labeled surface, and interlabel distances) or 4 µm thick for von Kossa staining and measurement at 40X of static histomorphometric properties (bone volume, osteoid surface, osteoblast surface, trabecular thickness, trabecular separation, and trabecular number). The region of interest began ~1.0 mm from the growth plate and encompassed a 6.0 mm^2 area within the endocortical edges. Mineral apposition rate (MAR; µm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS) for cancellous bone surfaces (BS) was calculated by using the formula MS/BS = [(single-labeled surface/2) + double-labeled surface]/surface perimeter x 100. Bone formation rate (BFR) was calculated with the formula: BFR = MAR X MS/BS. All histomorphometric analyses were performed using OsteoMeasure image analysis software (Version 2.31; Osteometrics,

Inc.) interfaced with Optronics 3-chip color camera and an Olympus BX60 microscope with epifluorescent light (Leeds Instruments, Irving, TX). All nomenclature for cancellous histomorphometry followed previously established standards [152].

Immunohistochemical Stain for ER-α

Whole left femur were stored in 4% paraformaldehyde for 24 hours at room temperature before being transferred to a sodium citrate solution to decalcify the bone specimens for 2 weeks during which they were stored in a cold room. Samples were paraffin-embedded before following instructions from Santa Cruz Biotechnology, Inc. for applying the ER-α antibody at a dilution range of 1:300. At the distal femur metaphysis, total osteoblasts and osteocytes that stain positive for ER-α were quantified at 40X using an Olympus BX60 microscope with epifluorescent light (Leeds Instruments, Irving, TX).

Statistical Methods

The data is reported as mean ± standard error of the mean (SEM) for each group. Statistical differences between the four experimental groups were evaluated using a two-way ANOVA (SAS 9.1 statistical package), except for energy consumed and body mass, which were analyzed by three-way ANOVA with repeated measures on time. Analysis of the effects of diet, load and the interaction of both factors were performed and the difference between individual groups was determined using the Least Squares Means method. A one-way ANOVA was utilized to assess differences between each treatment

group and baseline control histomorphometry values. P values less than 0.05 were considered statistically significant.

Results

The Dietary Energy Restriction Protocol Did Effectively Restrict Caloric Intake by 40% for Both ER40 Groups, Resulting in Decreased Body Mass and Uterine Weight

Over the course of the 12 week energy restriction protocol, both of the 40% energy restricted groups (ER40) consumed 40% less calories than their *ad lib*-fed control groups (Fig 10). Starting at week 0, both of the ER40 groups' food intake was significantly lower than the ADLIB groups. Unexpectedly, at week 4 the ADLIB-LOAD group consumed less total calories than the ADLIB-SHAM group. However, their food intake was the same as ADLIB-SHAM when averaged over the duration of the 12 week energy restriction protocol.

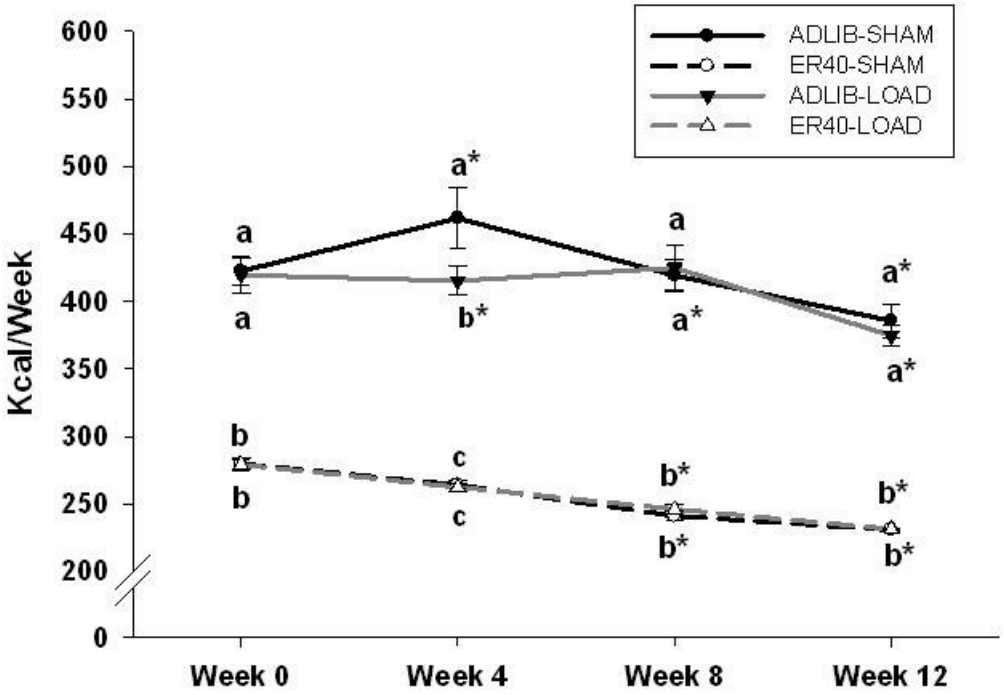


Figure 10. Dietary treatment effectively reduced energy intake (kcal/week) vs. *ad lib*-fed groups. Group means not sharing the same letter within time-point are significantly different from one another ($p<0.05$). *denotes significant difference versus week 0 values ($p<0.05$).

There was no significant difference between either ADLIB group's body mass over the 12 week protocol (Fig 11). By week 4 and through week 12, both ADLIB groups' body mass was significantly greater than week 0 values. There was an increasingly detrimental effect of ER40 on body mass in both ER40 groups. By 4 weeks, body mass was 11% lower vs. *ad lib*-fed groups. Chronic exposure to ER40 led to significant reductions in body mass: -20% by 8 weeks and -24% by 12 weeks versus *ad lib*-fed groups. Body mass for both ER40 groups was significantly lower from week

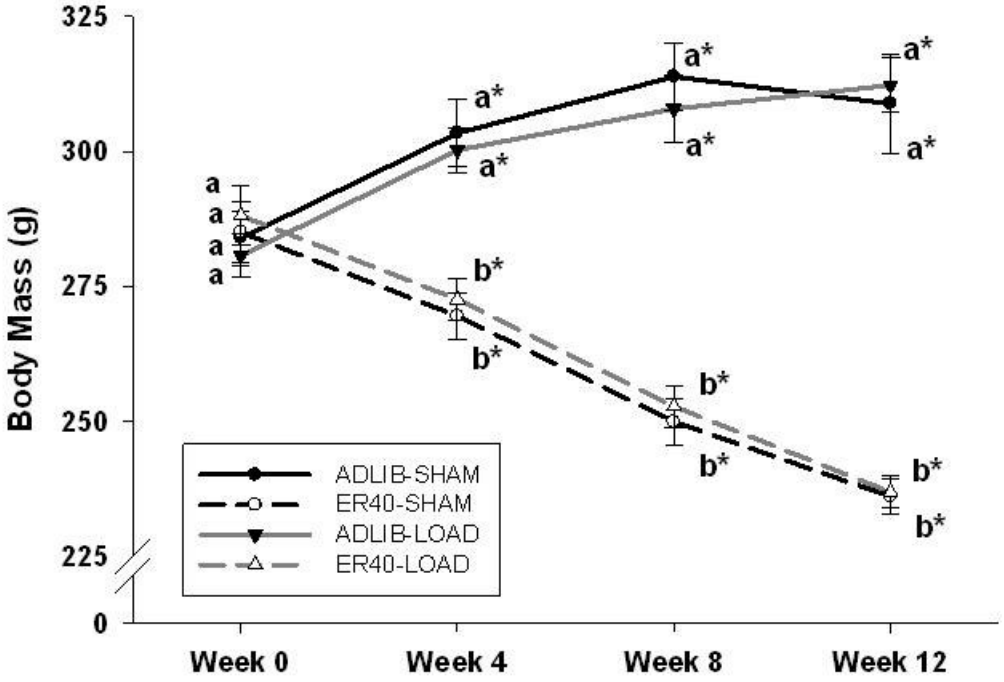


Figure 11. Body weight is adversely affected by 40% energy restriction. Groups not sharing the same letter within time-point are significantly different from one another ($p<0.05$). *denotes significant difference versus week 0 values ($p<0.05$).

4 through week 12 compared to their values at week 0. After 12 weeks, the uterine weight for the ER40-SHAM group was significantly lower (-18%) versus ADLIB-SHAM (Fig 12). Uterine weight was numerically lower for ER40-LOAD (-13%) versus ADLIB-LOAD.

LOAD Enhances Indices of Bone Formation in Cancellous Bone

Twelve weeks of energy restriction significantly altered cancellous microarchitecture at the proximal tibia. ER40-SHAM had significantly lower bone volume (BV/TV) (-46%) and trabecular thickness (Tb.Th) (-14%) and higher trabecular

separation (Tb.Sp) (+21%) compared to ADLIB-SHAM (Table 10). There was no apparent effect for energy restriction on any of the indices of bone formation (osteoid surface, OS/BS and osteoblast surface, ObS/BS) or resorption (osteoclast surface, OcS/BS) (Table 10).

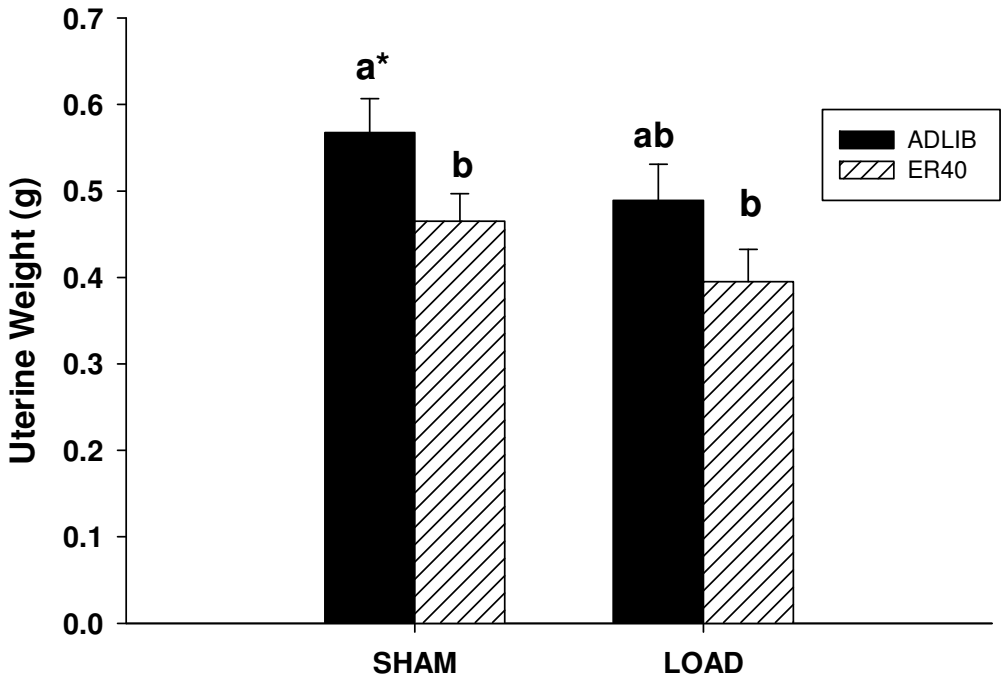


Figure 12. Uterine weight is significantly lower due to 40% energy restriction in SHAM loaded groups. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

LOAD did positively impact indices of bone formation and resorption. ADLIB-LOAD animals had significantly larger OS/BS (+8-fold) and ObS/BS (+9-fold) and smaller OcS/BS (-56%) compared to ADLIB-SHAM.

Bone Formation Response to LOAD Is Dampened With ER40

In SHAM loaded animals, ER40 exerted negatively affected MAR (-31%) vs. ADLIB (Fig 13B). The response to LOAD was reduced by ER40 for MS/BS (-18%), MAR (-24%), and BFR/BS (-36%), but was still significantly higher vs. both SHAM groups (Fig 13A, B, & C). In *ad lib*-fed animals, LOAD stimulated significantly higher MAR (+57%) and BFR/BS (+3.4-fold) when compared to baseline control values.

Up-Regulation of ER-Alpha Expression in Osteoblasts and Osteocytes with LOAD

ER40 did not diminish any LOAD-induced increases in ER-α expression. There was no independent effect for ER40 on the expression of ER-α in either osteoblasts or osteocytes (Fig 14A & B). However, the LOAD groups had higher numbers of both ER-α positive osteoblasts (+3-fold) and osteocytes (+6-fold) compared to the SHAM groups.

Table 10. The effect of energy restriction and/or load on cancellous microarchitecture and indices of bone formation and resorption.

	Baseline Control	SHAM		LOAD	
		ADLIB	ER40	ADLIB	ER40
Bone Volume/Total Volume	32.81 ± 1.25	29.46 ± 1.68[a]	23.06 ± 1.51[b*]	27.42 ± 1.51[a*]	27.76 ± 1.24[a*]
Adipocyte Density	39.19 ± 13.02	49.19 ± 14.07	109.33 ± 25.20*	71.60 ± 21.88	72.57 ± 30.23
Trabecular Separation	112.64 ± 6.59	134.40 ± 8.63[a]	162.11 ± 8.09[b*]	147.87 ± 8.33[ab*]	142.30 ± 6.10[ab*]
Trabecular Thickness	54.13 ± 0.94	55.50 ± 3.34[a]	47.65 ± 1.92[b*]	54.72 ± 2.09[a]	54.22 ± 2.06[ab]
Trabecular Number	6.07 ± 0.22	5.35 ± 0.27	4.84 ± 0.17*	5.01 ± 0.21*	5.13 ± 0.16*
Osteoid Surface	1.14 ± 0.23	2.29 ± 0.74[a]	1.23 ± 0.24[a]	20.63 ± 2.73[b*]	15.49 ± 3.02[b*]
Osteoblast Surface	0.32 ± 0.13	0.50 ± 0.17[a]	0.39 ± 0.09[a]	5.22 ± 1.02[b*]	4.59 ± 0.70[b*]
Osteoclast Surface	1.60 ± 0.13	1.85 ± 0.51[a]	1.48 ± 0.39[ab]	0.81 ± 0.22[bc]	0.53 ± 0.10[c*]

Values are group mean ± standard error of the mean. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). Values without letters mean that group means were not significantly different from one another. * denotes significant difference versus baseline control values ($p<0.05$).

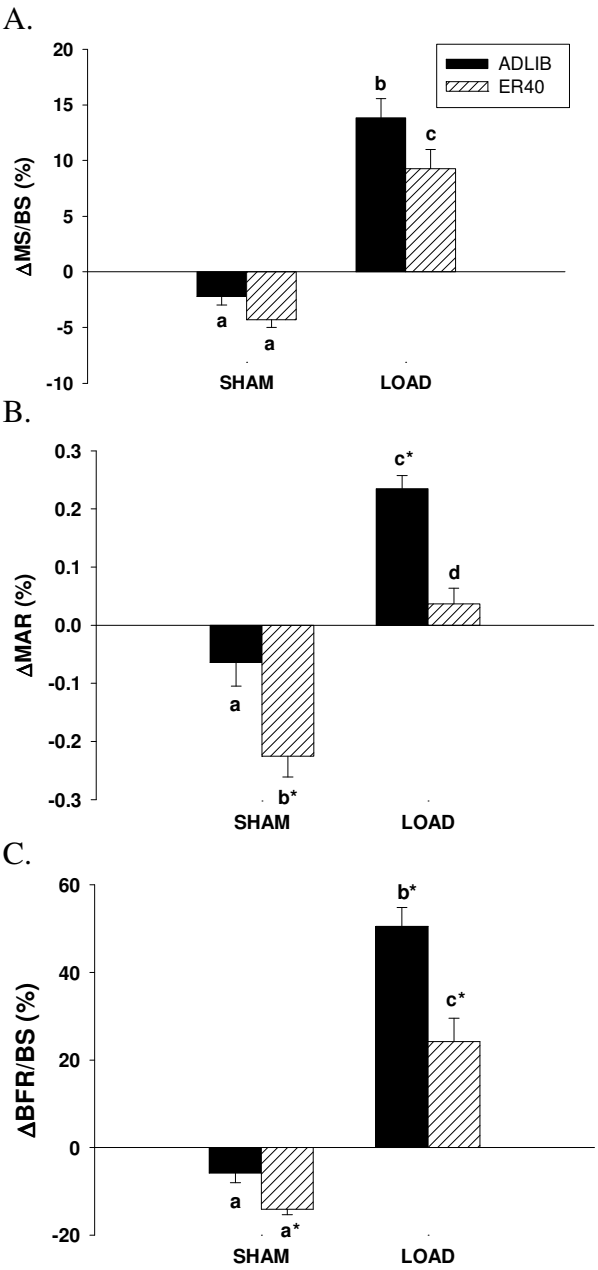
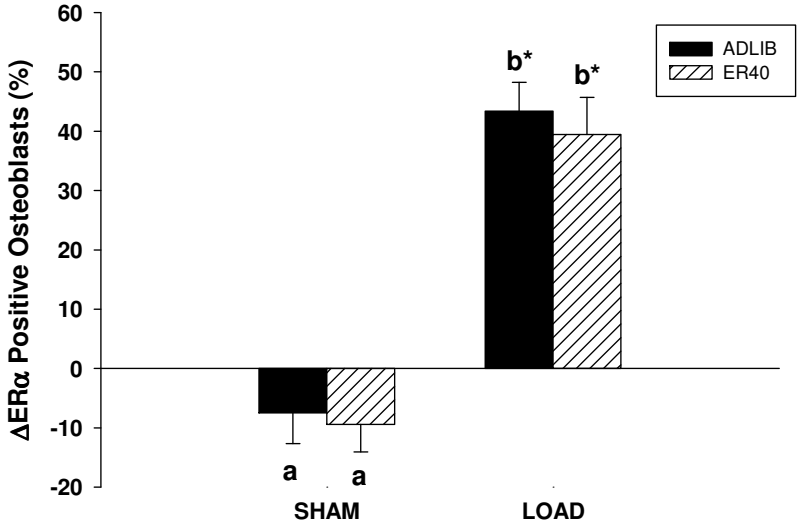


Figure 13**.** Response of proximal tibia cancellous bone to mechanical loading (MS/BS, MAR, and BFR/BS) in energy restricted animals is dampened vs. energy-replete animals. A. Delta mineralized surface/bone surface (MS/BS, %). B. Delta mineral apposition rate (MAR, %). C. Delta bone formation rate/bone surface (BFR/BS, %). Change is calculated as delta vs. baseline control groups. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

A.



B.

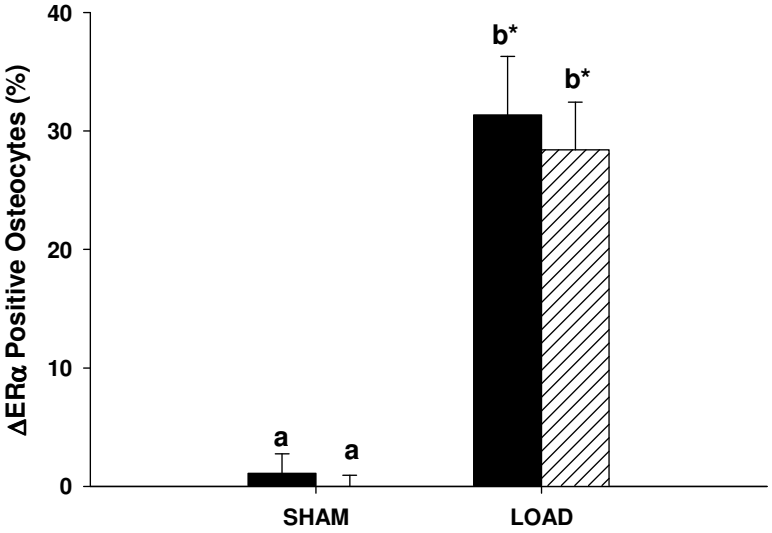


Figure 14. Energy restriction (-40%) does not dampen estrogen receptor alpha (ER-α) expression in the distal femur in response to mechanical loading (LOAD). A. Delta ER-α positive osteoblasts (%). B. Delta ER-α positive osteocytes (%). Change is calculated as delta vs. baseline control groups. Groups not sharing the same letter for each variable are significantly different from one another ($p<0.05$). *denotes significant difference versus baseline control values ($p<0.05$).

Discussion

The objective of this study was to determine whether energy restriction (-40%) would limit the bone response to mechanical loading (LOAD) through a down-regulation of ER-α in female rats' osteoblasts and osteocytes. In contrast with our hypothesis, ER40 did not induce a down-regulation of ER-α expression in osteoblasts or osteocytes (Fig 14A & B) in response to LOAD. In fact, an acute period of LOAD stimulated an enormous response in ER-α expression, with no effect for ER40. Therefore, the mechanism for the negative effects of energy restriction on cancellous microarchitecture and bone formation does not appear to be related to altered expression of estrogen receptor alpha.

Negative effects of food and energy restriction on bone properties of rodents are well documented. Older female rats exhibit declines in total body BMD as well as femur vBMD after 10 weeks of moderate energy restriction [(20)]. Restricting all food intake by 40% in older female rats significantly decreases cancellous bone area at the proximal tibia [(158)]. Similar results are seen in male animals. Calorie restriction (40%) in 14-wk old male mice results in significant reductions in Tb.Th and number of osteoblasts, while increasing the number of osteoclasts [(57)]. Restricting all food by 30% in adult male rats impairs bone formation, resulting in lower total vBMD at the proximal tibia [(93)]. Restricting energy by 40% produces greater losses in lean female rats' BMD at the tibia, distal femur, proximal femur, and femoral neck compared vs. control animals [(19)]. The current study's ER40-SHAM group experienced similar alterations to cancellous microarchitecture; cancellous bone mass and trabecular thickness were

significantly lower and trabecular separation significantly higher vs. values in ADLIB-SHAM animals (Table 10).

The negative effects of ER40 on uterine weights which serve as biomarkers of circulating estrogen levels are well established. Nine weeks of ER40 resulted in lower serum estradiol in aged (48 wk old) female rats [20] as well as significantly reduced uterine weights (-31%) compared to age-matched controls [148]. In mature, lean female rats 10 weeks of ER40 reduced serum estradiol vs. baseline controls [19]. Although we were unable to measure serum estradiol, uterine weights are an accepted bioassay for circulating estrogen and were reduced for both ER40 groups (Fig 12).

Under conditions of estrogen deficiency such as OVX in rodents, there is an associated down-regulation of ER-α expression in osteocytes found in cortical bone [38]. There is also an effect for OVX in rats' trabecular bone. Three weeks after OVX, ER-α mRNA expression was almost undetectable in cancellous bone at the distal tibia metaphysis [35]. The results are similar when explored in a human model as well. In women who are ovarian steroid deficient, the number of osteocytes containing ER-α decreases by half, from 25% to 12% [36]. It is important to note that unlike our experiment, these studies utilized subjects with severely reduced or zero estrogen levels. Our model is the first to our knowledge to explore the effects of a moderate reduction in circulating estrogen levels, as observed after physiological energy restriction which is commonly used by dieters. This moderate decrease in circulating estrogen rather than zero estrogen, might explain the differences between our previous data and the apparent lack of effect of chronic energy restriction on ER-α in this study.

It is well established that bone is unable to respond normally to mechanical strain without functional levels of ER-α. Lee et al. examined the effects of ulnar loading in the high physiological range on the bone formation response of mature, female ER-$\alpha^{+/+}$ and ER-$\alpha^{-/-}$ mice [40-42]. Mice with fully functional ER-α exhibited significantly higher bone formation responses on both periosteal and endosteal surfaces, while these responses to loading in ER-α KO mice were diminished 3-fold [40-42]. Functional ER-α are required for the proliferation of osteoblast-like cells in response to strain [169]. In addition, the activation of the Wnt/B-catenin signaling pathway in response to dynamic strain requires functional ER-α [39]. It is important to note that not all strains that are applied to bone elicit a positive response. In fact, applying a load delivering strains that are too high can reduce ER-α expression [146]. This is why it is significant that our model of mechanical loading is more closely related to resistance training (rather than an external loading paradigm) because it utilizes muscle strains that are closer to the physiological range (75% of peak isometric torque).

The effect of energy restriction on serum insulin-like growth factor (IGF)-1 is well documented. The energy available in an organism's system will typically regulate

the synthesis and availability of IGF-1 in the systemic circulation [79]. Significant decrements to IGF-1 levels [13,81] and an increase in bone resorption [13] are typical responses to reductions in energy availability. Under conditions of energy restriction similar to those in our experiment, Loucks et al. determined that IGF-1 was lower even when energy restriction was combined with endurance exercise in female subjects [23]. Sunters et al. determined that adequate levels of ER-α in response to strain are required for a full response of the IGF-1 receptor to IGF-1. This would not have been a factor for the dampened bone formation response in our experiment because we were unable to detect significant reductions in ER-α level in the ER40-LOAD group. A single 4-point bending session of tibial bone in rodents can elicit a 2-fold up-regulation in IGF-1 mRNA synthesis in cortical bone osteocytes [84]. IGF-1 is involved in the process of translating the mechanical stimulation from loading into bone formation [84]. Therefore, even a slight reduction of IGF-1 resulting from the chronic restriction of energy intake in our paradigm could be related to a decrease in the response of cancellous bone to loading. Future experiments that explore this relationship are

warranted to determine the exact role that reductions in IGF-1 related to prolonged energy restriction plays in this reduced response to mechanical loading.

There were a few limitations to this experiment. It would be desirable to measure serum estrogen and IGF-1 directly. Estradiol data would have provided valuable insight into the actual levels of circulating estrogen and how they related to the expression of estrogen receptor alpha in osteocytes and osteoblasts. Serum IGF-1 data would have helped to explain the observed detriments to the bone formation response to loading that were related to chronic energy restriction.

In conclusion, these data demonstrate that three sessions of mechanical loading are capable of stimulating increases in bone formation activity even in animals subjected to prolonged moderate energy restriction. Chronic energy restriction does limit the bone formation response, but not the up-regulation of ER-α after mechanical loading. These data suggest that functional ER-α expression is necessary but not sufficient for the full bone formation response to mechanical loading.

CHAPTER VI

CONCLUSIONS

The overall purpose of the first experiment was to determine which nutrient's moderate restriction, caused the greatest negative effect to cancellous bone microarchitecture and bone formation. Calcium or energy was restricted by 40% in exercising rats for a side-by-side comparison with an *ad libitum*-fed group and a 40% food restriction group that were both exercising as well. The second project sought to elucidate whether treadmill running would provide ample stimulation during mild (20%) and moderate (40%) energy restriction to mitigate the effects of graded energy restriction observed in sedentary, control animals. Body composition, uterine weight, measurements of endocrine markers of energy (IGF-1 and leptin) and estrogen status, and dynamic and static histomorphometry were performed to evaluate the effectiveness of treadmill exercise in mitigating the negative consequences of chronic energy restriction. The third project was completed to determine whether reductions in estrogen due to energy restriction (-40%) limits the ability of bone to respond to mechanical loading through a down-regulation of the estrogen receptor alpha (ER-α). Uterine and

total body weights, static and dynamic histomorphometry at the proximal tibia metaphysis, and immunohistochemistry at the distal femur metaphysis were utilized to assess the role that ER-α played in the response of cancellous bone to the acute mechanical loading paradigm.

The results from these investigations demonstrate that moderate energy restriction (40%) elicits the greatest negative effects on cancellous bone microarchitecture and bone formation in exercising rats; calcium restriction (40%) does not produce any detectable damage to cancellous microarchitecture or bone formation; treadmill running does provide sufficient anabolic stimulation to mitigate bone loss in rats subjected to chronic, mild energy deficits (20%); the estrous cycle, uterine weight, and estradiol levels appear to be disrupted with 40% energy restriction; there is an apparent threshold found at 20% energy restriction that is associated with a positive effect on bone formation rate for both exercising and sedentary rats; 40% energy restriction in combination with exercise mitigates losses in cancellous microarchitecture

that were detected in their sedentary counterparts; a brief period of mechanical loading is able to mitigate losses in cancellous bone volume related to 40% energy restriction; chronic energy restriction does limit the bone formation response, but not the up-regulation of ER-α after mechanical loading.

These studies suggest that deficits to bone outcomes associated with moderate (40%) energy restriction are not fully restored with exercise. The mechanism for the suppressed bone formation with energy restriction is not conclusively related to a down-regulation in estrogen receptor alpha protein. Another possible explanation for the decrements to bone outcomes associated with moderate energy restriction are reductions in insulin-like growth factor (IGF)-1. Further research defining the combined effect of energy restriction and mechanical loading on IGF-1 levels is necessary to define the exact role that IGF-1 might have on bone outcomes. This data could potentially assist in mitigating bone loss associated with energy restriction in exercising individuals.

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PUBLICATIONS:

1. Swift SN, Swift JS, Greene ES, De Souza MJ, Bloomfield SA 2010 Energy restriction effects on cancellous bone microarchitecture: impact of endurance exercise. Med Sci Sports Exerc **42**(5):S15.
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3. Swift JM, Gasier HG, Wiggs MP, Swift SN, Hogan HA, Fluckey JD, Bloomfield SA 2010 Increased training loads do not magnify cancellous bone gains with rodent jump resistance exercise. Bone [submitted].
4. Swift S, Greene ES, De Souza MJ, Bloomfield SA 2009 Thresholds for bone loss with energy restriction change with exercise status. FASEB J **23**:720.9.