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Effects of pregnancy and lactation on maternal bone status in mice artificially selected for larger skeletons

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Effects of pregnancy and lactation on maternal bone status in mice artificially selected for larger skeletons

by

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Abstract

Pregnancy and lactation are intensive physiological processes that require increased calcium demand from the maternal systems. This is mediated by changes to the maternal endocrine axes and may result in excessive bone resorption on the maternal skeleton. Pregnancy- and lactation-related changes in bone have yet to be investigated in a model with poorer bone quality and quantity. The Longshanks mouse (LS) is a mouse line selectively bred for increased tibia length. Although longer, the LS tibia is also weaker due to its altered microarchitecture of thinner and more widely spaced trabeculae. In this study, we sought to investigate the impacts of pregnancy and lactation on maternal bone microarchitecture using the LS as a model of a compromised skeletal phenotype with lower bone quality/quantity. Our study found that LS mice had increased bone quantity at postpartum and substantial decreases at mid-lactation when compared to Controls. The physiological response of increased bone postpartum thus depends on pre-pregnancy population characteristics and is possibly a mechanism to help protect maternal calcium reserves from excessive depletion. The differences in bone are induced by altered endocrine signaling in LS vs. CTL. The larger improvements in microarchitecture postpartum in the LS may be a result of increased OPG signalling, whereas bone resorption is likely increased in the LS during lactation by PTH and SOST action. This would allow increased mobilization of calcium stores and result in reduced maternal bone quantity/quality as seen in the LS mouse. Overall, this study demonstrated a possible protective response induced by pregnancy, resulting in increased bone volume postpartum, however lactational bone loss should be especially considered in populations with poor bone quality/quantity.

Table of Contents

List of Tables

List of Figures

List of Abbreviations & Nomenclature

LS Longshanks

CTL Control

H1 Hypothesis 1

H2 Hypothesis 2

BL Baseline/nulliparous sample

PTP Pregnancy timepoint/postpartum sample

LTP Mid-lactation timepoint

BV/TV% Trabecular volume over total endosteal volume percentage

Tb.Th Trabecular thickness

Ct.Th Cortical thickness

Ct.BMD Cortical bone mineral density

PROL Prolactin

TSH Thyroid-stimulating hormone

FSH Follicle-stimulating hormone

OPG Osteoprotegerin

SOST Sclerostin

PTH Parathyroid Hormone

PTPrP Parathyroid hormone-related peptide

OC Osteocalcin

RANKL Receptor activator of nuclear factor kappa-B ligand

RANK Receptor activator of nuclear factor kappa-B

ELISA Enzyme-linked immunosorbent assay

PLO Pregnancy- and lactation- associated osteoporosis

CHAPTER 1: INTRODUCTION

1.1 Overview of present study

Reproduction is a costly process where mothers transfer nutrients to offspring (1). Fetal ossification and lactogenesis require high levels of calcium from maternal reserves which may result in excessive bone resorption from the maternal skeleton (2). In response, maternal endocrine mechanisms are altered to protect the skeleton, resulting in microarchitectural changes in the maternal bone (3). Though pregnancy- and lactation- related changes in bone have been investigated in normal, healthy women and other animal models, none have explored these changes in a model with a lower quality skeleton phenotype (4–6).

The Longshanks mouse (LS) is a mouse line selectively bred for increased tibia length. Along with increased length, the LS has reduced bone volume with decreased, thin, and widely spaced trabecular bone in the proximal tibia when compared to the controls – indicators of a compromised skeletal phenotype and phenotypically similar to what is seen in human osteopenia (7).

Lower bone quality in LS mothers may restrict the available resources for fetal ossification and lactogenesis, and detrimentally affect the maternal skeleton. In this present study, we sought to investigate the impacts of pregnancy and lactation on maternal bone microarchitecture using the LS as a model with lower bone quality as seen in osteopenia-like phenotypes.

1.2 The maternal condition during pregnancy & lactation.

In mammals, maternal resources or nutrients derived from dietary intake and capital body stores are essential for fetal growth (8,9). Mothers who are capable of allocating more energy to growth during fetal development can promote nutritional investment in their offspring (10). Pregnancy and lactation are demanding processes and thus, require increased energy intake and

output (11). The mother must go through profound physiological adaptations, such as changes to her metabolism, cardiovascular, and endocrine systems, to meet the demands of pregnancy and lactation (12–14). This leads to severe energy expenditure which must be offset by high dietary energy intake and stores (15).

The energetic costs of lactation in mammals are exorbitantly higher than in pregnancy (1). Most mammals, including humans, are capital breeders, meaning that during gestation there is increased daily food intake, leading to increased maternal adiposity and resource stores (8,16). These fat and energy stores are mainly mobilized during lactation – likely to supplement the heavy energetic cost of lactogenesis (17). These adaptive maternal mechanisms during both pregnancy and lactation are necessary in order to provide sufficient glucose and other vital nutrients to the fetus (17).

Among mammals, another vital nutrient is calcium (14,18). Calcium is one of the most important minerals in the vertebrate body and a central component of building both fetal and postnatal, pre-weaned offspring (18,19). It is essential for proper muscle contraction, hormone function, and bone formation and maintenance (18). During pregnancy and lactation, the calcium homeostatic response includes increased intestinal and tubular absorption of calcium, and important changes to bone metabolism (19,20). These adaptations are upregulated to supply the fetus with calcium – the maternal skeleton serving as a storage unit for calcium (18,21). If dietary intake and absorption are not sufficient, then the maternal bone turnover will increase to release more calcium into maternal circulation for transport across the placenta (20). This can be achieved by activating several endocrine mechanisms; however, constant depletion becomes detrimental to the maternal skeleton (5,18,22). Specifically, the heavy calcium costs of

pregnancy and particularly, lactation can exhaust the maternal skeleton and cause metabolic bone diseases e.g., osteoporosis (23).

Calcium is one of the most abundant and essential minerals in the body and required for healthy maternal and fetal outcomes (18). Maternal control of calcium homeostasis must thus be altered to meet the demands of fetal ossification (18). Several maternal physiological systems are upregulated to increase serum calcium during pregnancy and lactation – most notably, bone metabolism.

1.2.1 Maternal Bone Metabolism.

Other than the obvious structural support the skeleton provides, the skeleton is a vital, dynamic organ for its essential roles in many important physiological processes such as hematopoiesis, mineral homeostasis, and metabolism (24). It is a specialized system that is constantly active through a continuous regenerative process of bone remodeling, carefully balanced between bone formation and bone resorption (24).

Normal bone remodeling is facilitated by crosstalk between several bone cell types, namely categorized into osteoblast or osteoclast lineages (25). Osteoblasts are differentiated mesenchymal precursors and are responsible for bone mineralization (25). These cells deposit collagen and minerals, such as hydroxyapatite, forming a complex matrix into mature lamellar bone (25). Hydroxyapatite is a mineral mainly composed of calcium and phosphate, and largely responsible for strong bone composite (24). Osteoclasts are derived from monocytic hematopoietic progenitor cells and are responsible for bone resorption (25). Osteoblasts recruit osteoclast precursors to the active remodeling site and enhance osteoclastogenesis (25). Once attached, osteoclasts will release hydrogen ions and initiate the dissolution of the mineralized matrix (25). Following this resorption, mononuclear cells move to prepare the dissolved

demineralized matrix for bone formation (25). Osteocytes, a terminally differentiated osteoblastic cell, are the most abundant cell involved in bone remodeling and have been more recently implicated in both bone formation and bone resorption (25,26). Osteocytes are unique in that their activity is mainly induced by mechanical stress sensation from bending or stretching of bone (26). Mechanosensation is then transduced into metabolic activity (26). Osteocytes can then secrete several anabolic and catabolic cytokines that act directly on osteoblasts and osteoclasts, thus influencing the ratio between bone formation and resorption (25,26). The skeleton is thus in a constant flux between bone resorption and bone mineralization to meet various physiological and biomechanical events (25).

Lactation and the mineralization of the fetal skeleton during gestation causes significant changes in the maternal bone metabolism and remodeling (18). In order to meet the demands of fetal ossification and lactation, mammalian maternal bone turnover is upregulated throughout pregnancy and increases dramatically in the third trimester where fetal bone mineralizes at a high rate (21,27). This leads to the deterioration of maternal bone volume and quality in some species (18).

In rats (*Rattus rattus*), maternal bone volume changes minimally during early gestation but by late gestation, the maternal skeleton suffers significant losses (28,29). De Bakker et al. (2017) reported that the tibia experienced more than a 40% loss in bone volume (28). In mice (*Mus musculus*), mothers experience signs of improved bone post-partum, with increases in bone mineral density (BMD) and bone volume (30). However, these improvements are not sustained. Mice showed similar significant losses in bone as other rodents during the nursing period (31,32). These severe lactational losses have been replicated in sheep, dogs, and several other mammals (33,34). Notably, these losses were primarily manifested in the trabeculae, whereas

cortical bone showed indices of improvement postpartum with increased cortical thickness and cortical area (18,27,30).

In humans, studies have reported consistent decreases in BMD throughout pregnancy (6,35,36). Shahtaheri et al. (1999) found that in early pregnancy, healthy women experienced a decrease in bone volume of 23.07% to 16.72%, mediated through decreased trabecular thickness, relative to non-pregnant controls (5). At late pregnancy, bone loss was fully restored with additional trabeculae but found that altered bone microarchitecture persisted postpartum despite bone recovery (5). Other studies have reported BMD losses of up to 45% with permanent loss in the long bones (37). Though the magnitude of loss in humans varies strongly between studies, there is a consensus that pregnancy causes bone loss and BMD alterations (18). These losses are exacerbated during lactation – a lactating mother will lose 1-3% of her BMD per month (38).

Despite the skeletal depletion from pregnancy and lactation, the healthy organism will typically recover its losses after a few months post-weaning. Ardeshirpour et al. (2007) found that in mice, by day 28 post-weaning, maternal BMD increased by 25% throughout the entire skeleton (39).

Given the high costs of fetal ossification and lactogenesis, premenopausal women with osteopenia or other bone conditions that increase bone brittleness or reduce its strength may risk having their condition exacerbated with pregnancy/lactation (38). These individuals also have increased susceptibility to bone fracture, particularly at birth and post-parturition (38,40,41). Because post-menopausal women are at highest risk for osteoporosis, premenopausal women will rarely receive routine evaluations of bone, such as BMD testing or bone biopsies (42). Some women develop pregnancy- and lactation-related osteoporosis but the lack of evaluations in

premenopausal women renders it difficult to assess if bone anomalies detected at post-parturition are a direct result of pregnancy and/or lactation (42).

Bone is a metabolically expensive tissue to maintain, but the significant alterations in bone physiology and quality in pregnancy and lactation indicates that bone is also a limited, and therefore expensive resource – particularly to the mother (18). Furthermore, constraints or challenges to normal maternal skeletal health, such as decreased bone quality, may be exacerbated by pregnancy and lactation, and lead to impaired fetal bone mineralization and decreased offspring quality (38). The mechanisms involved in bone remodeling are complex and not yet fully understood. However, it is clear that osteoblastic and osteoclastic activity is strongly modulated by the crosstalk of several hormonal axes (14).

1.3 Maternal endocrine control of bone metabolism.

Pregnancy and lactation involve numerous physiological and anatomical changes to the maternal systems which are tightly regulated by hormones. The exact mechanisms of maternal physiological processes are not well understood; however, with animal models and *in vitro* studies, there are insights into how hormones influence change in the maternal systems (14). In maternal bone metabolism, several endocrine axes are involved (14). Below are outlined several hormones that are significantly involved in calcium homeostasis during pregnancy and lactation.

1.3.1 PTH, OPG, SOST.

In humans, the skeletal changes seen in pregnancy and lactation are believed to be mainly regulated by parathyroid hormone-related peptide (PTHrP) (19). Endogenous PTHrP concentrations are very low in the absence of pathology or pregnancy/lactation (19,43). However, during pregnancy, PTHrP secretion from several maternal tissues (e.g., long bones,

uterine wall) as well as the placenta and the fetal parathyroid gland, is drastically increased (43). PTHrP stimulates placental calcium transport and indirectly inhibits osteoclasts (43).

In rodents, increased parathyroid hormone (PTH) is observed (19,44). PTH exerts its indirect anabolic effect through a few cascades – notably the OPG-RANKL-RANK system (Figure 1) (45). PTH promotes the binding of receptor activator of nuclear factor-kappa B ligand (RANKL) to osteoclastic cells, stimulating their differentiation and, in mature osteoclasts, their bone resorption activity (45). Osteoprotegerin (OPG) is a decoy receptor for RANKL and can inhibit this bone resorption by binding directly to RANKL, preventing its binding to its receptor, RANK (46). Osteoprotegerin is thus an anabolic mediator in bone remodeling.

OPG has also been shown to be targeted by the canonical Wnt signaling pathway through the stimulation of osteoblastogenesis, thereby promoting bone formation (46). Sclerostin (SOST) is a potent antagonistic regulator of the Wnt signaling pathway and thus, directly impacts OPG expression (46,47). SOST is expressed by osteocytes, and when overexpressed, upregulates RANKL and stimulates osteoclast formation and differentiation (47). Osteocytes are also a target cell for PTH, and recent transgenic models suggest that downregulation of SOST is required for the anabolic effects of PTH (45).

During pregnancy, rodents develop hyperparathyroidism and experience a fast rise in PTH concentrations as gestation progresses (19). By late gestation and postpartum, PTH levels were found to be tripled (48). High concentrations are sustained through lactation and drop off to normal levels 24-48 hours post-weaning (49). Similar patterns are seen with OPG in both rodent models and humans: marked increases at pregnancy and lactation before a sharp decline at weaning (50). Though SOST activity and concentrations during pregnancy and lactation remain unclear, recent evidence suggests that SOST may be increased at parturition (51).

1.3.2 FSH, PROL, & TSH.

The anabolic effect of estrogen on bone has been well-documented and is believed to be primarily responsible for bone loss in postmenopausal women (52,53). Follicle-stimulating hormone (FSH) promotes the secretion of estrogen and despite estrogen's positive impact on bone, FSH has been demonstrated to promote osteoclast differentiation and survival, independent of estrogen (54). FSH's direct action on osteoclasts is through numerous regulators, notably by increasing RANK expression (54).

Thyroid-stimulating hormone (TSH) is a hormone produced by the anterior pituitary gland and plays an essential role in regulating the development and function of the thyroid gland (55). Hyperthyroidism is associated with significant bone loss and recently, TSH has been hypothesized to affect bone formation mainly by direct action on osteoclasts (56). Abe et al. (2003) found that TSH inhibits osteoclast differentiation and survival through the inhibition of JNK phosphorylation – the same mechanism of estrogen on bone (56).

Prolactin (PROL) is a hormone secreted by the anterior pituitary and mainly acts to induce lactation (14). In lactating mothers, PROL produces an osteoclastic effect by directly acting on osteoblasts (57). Though the exact mechanisms are unclear, high concentrations of PROL decreased the expression of OPG and RANKL in several models (e.g., ovariectomized rodents, PROL-injected rats), indicating that PROL is a catabolic regulator on bone (57,58).

FSH remains low throughout pregnancy from negative feedback of the increasing levels of estrogen throughout gestation (59). In humans, FSH rises to above-normal levels after parturition and during lactation (59). Mice enter anestrus while lactating thus FSH remains low until post-weaning (60). Maternal TSH during pregnancy and lactation is not well-characterized in rodents (61). In humans, there is a marked decrease during the first trimester before a steady

rise that continues through lactation (61). PROL increases exponentially throughout gestation in humans before tapering off as the lactation period progresses (62). In rodents, PROL stays relatively low during pregnancy before there is a sharp surge at birth and a steady decline in lactation (63).

1.3.3 Osteocalcin, Insulin, & Leptin

Osteocalcin is a unique peptide secreted by osteoblasts and is involved in bone remodeling and energy metabolism (64). There are two different forms of osteocalcin – carboxylated and undercarboxylated (64). The fully carboxylated form of osteocalcin has Gla residues that have a high affinity for hydroxyapatite binding (64). The mechanisms of carboxylated osteocalcin remain poorly understood and the net effect of carboxylated osteocalcin is still debated (64,65). Carboxylated osteocalcin is believed to influence bone mineralization and in rodents, is used as a marker for bone formation (64,65).

Osteocalcin is decarboxylated during bone resorption from the acidic environment produced by osteoclastic activity (66). Undercarboxylated osteocalcin is then released into the bloodstream and considered its metabolically active form (66). Undercarboxylated osteocalcin directly enhances the expression of insulin genes thus stimulating insulin production – which has also been recently implicated in bone remodeling (64). Fulzele et al. (2010) found that insulin receptor signaling promoted osteoblast differentiation by suppressing Twist2. Twist2 antagonizes Runx2 – a transcription factor necessary for osteoblast differentiation (Figure 2) (67).

Osteocalcin is negatively regulated by leptin, a metabolic hormone produced by adipocytes and heavily involved in glucose metabolism (64). Leptin has been demonstrated to indirectly affect bone remodeling through sympathetic innervation with outputs that target

receptors on osteoblasts, inhibiting their proliferation and differentiation, and indirectly inhibiting osteocalcin as well (Figure 2) (68).

During pregnancy, osteocalcin levels decline to below-normal levels before increasing during lactation (69). A marked decrease in insulin sensitivity is seen during early pregnancy and late pregnancy is characterized by insulin resistance (12). Leptin concentrations rise during pregnancy and return to baseline at parturition before hypoleptinaemia is developed during lactation (70).

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Bone metabolism is complex and is regulated by numerous pathways and hormones (18). Healthy fetal mineralization mandates alterations to the maternal bone metabolism via adjustments to several endocrine pathways (18). The maternal system must supply sufficient calcium to the fetus while also maintaining her own skeleton, protecting herself from over-depletion of resources (18). To date, however, nearly all studies describing endocrine control of bone metabolism during pregnancy and/or lactation have been conducted in humans and rodent models with healthy bone, or genetic knock out mice models (32,37,44,57,71–73).

In the maternal skeleton with compromised quantity or quality, these hormonal axes may be altered to meet the necessary resource investment into fetal ossification and self-maintenance, or the maternal physiological systems may over-deplete the maternal skeleton (18). This could potentially result in severely depleted maternal calcium stores, leading to pathological osteological conditions and/or insufficient resources – impairing fetal ossification (74). Despite the importance of healthy bone metabolism in pregnancy and lactation, for both mother and fetus, little is known regarding the potential impacts of pregnancy and lactation, and its associated mechanisms, in mothers with compromised bone health.

1.4 The Longshanks mouse

The Longshanks mouse (LS) is a mouse line selectively bred for increased tibia length relative to body mass. In 20 generations, in two independent Longshanks lines (hereafter LS1 and LS2), tibia length increased by 13-15% relative to random-bred mice from the same genetic background (CD-1, hereafter CTL), while body mass remained the same. In addition to its increased tibia length, the tibia shape in LS is altered relative to Controls, and exhibits characteristics of increased bone fragility, including increased whole bone rigidity and brittleness, as well as reduced work-to-fracture – a measure of the amount of energy required to break a long bone (75,76). Additionally, the LS has reduced bone volume with decreased, thin, and widely spaced trabecular bone in the proximal tibia when compared to the controls (75). Though both lines of LS underwent the same selective breeding protocol, LS2 shows greater trabecular bone loss whereas LS1 has decreased cortical thickness and density compared to its wildtype CTL (75). The decreased trabecular bone and cortical thickness, and increased rigidity/brittleness of the LS are phenotypically similar to what is seen in human osteopenia and thus, the LS is a potential model of compromised bone health (Figure 3) (75).

Besides tissue- and whole-bone level changes in the Longshanks skeleton, larger skeletons in the LS require more calcium during fetal ossification, as well as during the lactation period when the pups are already notably larger in Longshanks compared with Controls (75,77). Maternal calcium reserves are limited and therefore must be partitioned efficiently to increase reproductive success. Increased investments in LS from building and maintaining larger skeletons may further restrict the available maternal calcium/bone “budget” for fetal and pre-weaning development. In addition, LS have poorer bone condition and it is possible that the

maternal LS bone status is the constraining factor, thus limiting resources for the high demands of LS fetal ossification.

Due to the phenotypic differences in bone microarchitecture in the LS, the hormonal pathways that regulate bone physiology in LS are likely altered when compared to their random-bred counterparts, and these differences in the LS may further constrain the pathways needed for fetal ossification, thus resulting in insufficient resources.

1.5 Rationale

Maternal bone condition is a predictor of her offspring's health outcomes. Several hormonal pathways play significant roles in bone metabolism. Constraints on the maternal skeleton as a limited resource are likely exacerbated by pregnancy and lactation, resulting in the depletion of the maternal skeleton during fetal bone mineralization through the action of these hormonal pathways.

Despite the demonstrated role and importance of the maternal skeleton in the development of the fetal skeleton and during lactation, there is a large gap in our understanding of how lower bone quality, such as decreased bone volume or bone strength, influence both anabolic and catabolic bone metabolism in mothers during pregnancy and lactation. As well, the endocrine mechanisms involved in a compromised skeleton like that of the LS remain unclear. LS dams likely have altered hormonal pathways producing weakened skeletons. These different pathways may then influence how the maternal hormonal systems respond to increased resource demands for fetal ossification.

1.6 Study aims & hypotheses

Using the unique LS mouse model, this project aims to elucidate pregnancy and lactation-associated microarchitectural changes on compromised maternal bone. Furthermore, we are

interested in characterizing some of the underlying hormonal pathways that may be associated with bone quality changes via monitoring hormones throughout different time points.

The results of this study will not only help provide a better understanding of the extent of pregnancy- and lactation-related alterations in the maternal bone microarchitecture in the presence of existing lower bone quantity but also for understanding the hormonal mechanisms involved in bone homeostasis during pregnancy and lactation.

Main objective. The goal of the proposed research project is to investigate the impact of pregnancy and lactation on bone parameters in a model with altered/compromised bone phenotype.

H1: We hypothesized that pregnancy and lactation will differentially affect LS bone quality/quantity, when compared to CTLs. We predict that the LS females will have indicators of lower bone quantity than CTLs at postpartum and mid-lactation.

H2: We hypothesized that systemic endocrine indicators of bone quantity and metabolism will be altered in LS mothers, compared to CTL, reflecting LS compromised bone quality/quantity. We predict that LS females will have increased circulating levels of catabolic over anabolic hormones than the CTL, thus indicative of increased basal, postpartum, and mid-lactation LS bone resorption.

CHAPTER 2: METHODS

2.1 Experimental design

This study was cross-sectional in design, with three main timepoints used to evaluate bone-related metabolism and hormone concentrations during pregnancy and lactation in Longshanks and Control mice. The first timepoint was the nulliparous sample and defined as the baseline (BL) timepoint, i.e., to evaluate baseline differences in bone and hormonal variables among the three lines. The pregnancy timepoint (PTP) was set as day of parturition. The final timepoint was an approximately mid-lactation sample – set as day 12 of lactation (LTP).

To investigate **H1**, micro-computed tomography (μCT) scanning was used to visualize the bone microarchitecture and estimate bone quality/quantity for all females. The LS and CTL right tibia was scanned *ex* vivo, and trabecular and cortical parameters were assessed. Important hormones involved in bone metabolism were selected by literature search and described in Section 1.2. Differences among time-points in circulating hormone serum concentrations (**H2**) were measured by several immunoassays as described below. Bone microarchitectural and hormonal differences were thus compared within families (LS1, LS2, CTL) and within time-points (BL, PTP, LTP).

2.2 Animal samples

Procedures involving live mice were approved by the Health Sciences Animal Care Committee at the University of Calgary (protocol AC17-0026 and AC20-0180) and performed in accordance with best practices outlined by the Canadian Council on Animal Care. The original mouse lines were bred from a CD-1 stock (22). Three lines were established: the first two were selectively bred for increased tibia length (Longshanks 1 and 2)) and the last, was a random-bred

Control line. 16 breeding pairs were established for each line. Briefly, the original selective breeding process was as follows:

8-week-old mice were weighed and digitally radiographed to obtain body mass and tibia length, respectively. The top-ranked female and male within the family, i.e., those with the longest tibiae relative to body mass, were selected as breeders and mated at random with top-ranked individuals from 9 other litters within the line. This protocol was carried out until generation F22, at which point selective breeding ceased and the three lines were maintained through random non-sib mating (within line).

In the selection experiment, for reasons that remain unclear, but may involve stochastic variation and/or increasing inbreeding depression, we observed a secular trend in breeding success in the original random-bred Control line. Specifically, 12 generations after the selective breeding regimen was suspended (F22), Control breeding pairs showed increasing failure to breed rates, and/or produced unusually small litters for mice of a CD-1 background (3-5 pups).

Consequently, for this study, fresh CD-1 breeding pairs were thus purchased from Charles River Laboratories, Inc. (2023). These new CD-1 males and females were age-matched to their LS counterparts and left to acclimate to the same diet for at least two weeks before pairing.

117 breeding pairs were set up, 13 for each time point per line, e.g., for the CTL group, there were 13 different females set for each: BL, PTP, and LTP. All mice were left on a 12hr light/dark cycle at 20°C in individually ventilated cages. Mice had access to low-fat chow and water *ad libitum* (Pico-Vac Mouse Diet 5061, LabDiet, St. Louis, MO). The amount of food consumption and dam weight was measured daily to the nearest 0.01g until euthanasia to ensure

weight gain. Pregnancy was determined by daily plug checking after pairing. Once the vaginal plug was present, males and females were removed back to their cages.

On the day of parturition, litter sizes were recorded, and dams and pups were weighed to the nearest 0.01g for both PTP and LTP females. Pups were counted and weighed as a litter. For the nursing mothers in the LTP, the mother and pups were left undisturbed until P12 before euthanasia.

Females from each time point were anesthetized by isoflurane inhalation (induction at 5%, maintenance at 3%) and a terminal cardiac puncture was performed to collect a blood sample. They were then euthanized by cervical dislocation and pups, by decapitation. Specimens were frozen until the day of the post-pregnancy scan (see below).

2.3 Bone µCT scanning

All females were scanned by *ex vivo* µCT imaging. The frozen carcasses were left to thaw overnight at 4°C and then weighed to the nearest 0.01g. The right leg was placed into a custom leg scanning fixture in the µCT scanner (vivaCT 80; Scanco Medical, Brutisellen, Switzerland). The region of interest in our scans began 10 slices from the distal tip of the posterior side of the proximal tibial epiphysis. The right proximal tibia was scanned at an isotropic resolution of ~15.6µm (55 keV, 144 *M*a) for all mice. Each scan lasted approximately 30 minutes and captured ~671 slices or 10.4 mm. Total sample scan count for each time-point and mouse line is seen in Table 3.

Semi-automated slice-by-slice hand contours were used to outline the metaphysis of the right proximal tibia for every slice and began at 10 slices after the distal tip of the posterior side of the proximal tibial epiphysis (Figure 4). The script-defined contours use Snake algorithms to "snap" the contours to the regions of interest (78). The contours for each female included a

periosteal and an endosteal contour. Each scan included approximately 550-600 contours, thus extending approximately 8.74-9.36 mm towards the distal tibia. Each contour was user-checked and edited if necessary. For indicators of mineralization, greyscale μCT images were segmented using a constrained Gaussian filter (Gauss sigma = 1.2, Gauss support = 2) with a lower threshold of 225 mgHA/cm^3 and upper threshold of 1000 mgHA/cm^3.

Trabecular bone indices include the bone volume (BV, in mm^3), the total endosteal volume (TV, mm^3), and the bone volume ratio (BV/TV, %), and trabecular thickness (Tb.Th, mm). Cortical bone indices included cortical bone mineral density (Ct.BMD, mgHA/cm^3), and cortical thickness (Ct.Th, mm).

2.4 Serum analysis

After collection, the blood samples were left to clot at room temperature for 20-30 minutes before they were spun at 2,000g for 10 minutes in a cooled centrifuge. The isolated serum was then aliquoted into smaller aliquots and stored at -80°C until their use in serum analysis. All procedures and analyses were followed as instructor manual unless stated otherwise. Samples that showed an out-of-range result or did not pass outlier detection (See Section 2.5) were removed. The final count of sample size for each hormone is provided in (Table 4).

Quantification of FSH, LH, PROL, TSH. The aliquoted serum samples were diluted 2.5x with PBS and sent to a service provider to quantify these four hormones in a discovery assay using magnetic bead-based multiplex immunoassay (Custom Mouse Pituitary 4-Plex Custom Assay; Eve Technologies, Calgary, Canada.). The assay performed two replicates for each sample.

Quantification of Osteocalcin, OPG, SOST, PTH, Insulin, and Leptin. For the following hormones: Osteocalcin, OPG, SOST, PTH, Leptin, and Insulin, Enzyme-linked immunosorbent assay kits were ordered from several manufacturers (see below). The kits were followed as per the instruction manual. The samples were thawed on ice before usage. Multiple aliquots and running multiple assays per day allowed minimization of freeze-thaw cycles. A pilot run was performed before a full sample run on each kit to determine optimal dilution factors. The same eight serum samples from each line and time point were used for all the kits listed below. In total, each run included two plates with 72 samples and two replicates.

Osteocalcin. Carboxylated osteocalcin serum was quantified using an EIA kit from Takara bio (8x dilution, MK127; Mouse Gla-Osteocalcin High Sensitive EIA Kit, Takara Bio USA, Inc. USA). The stop and wash solution used was from a separate kit from the same manufacturer (MK021; Wash and Stop Solution for ELISA without Sulfuric Acid, Takara Bio USA, Inc. USA).

Osteoprotegerin. Osteoprotegerin serum concentration was assessed using an ELISA kit from RayBioTech (40x dilution, TNFRSF11B; Mouse OPG ELISA, RayBioTech Life, Inc. USA).

Sclerostin. Sclerostin serum concentration was quantified using a sandwich ELISA kit by LifeSpan Biosciences (10x dilution, LS-F23135; Mouse SOST/Sclerostin Sandwich ELISA kit, LifeSpan Biosciences, Inc. USA).

Parathyroid Hormone. A competitive ELISA kit was used to analyze PTH serum concentrations (10x dilution, LS-F5539; Mouse PTH/Parathyroid Hormone Competitive EIA ELISA kit, LifeSpan Biosciences, Inc. USA).

Insulin. A mouse insulin ELISA kit by Crystal Chem was used to quantify insulin concentration (90080; Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem, USA). 5uL of each sample with no diluent was used as per instructor manual.

Leptin. Leptin serum concentrations were quantified using a kit by RayBioTech (20x dilution, ELM-Leptin-1; Mouse Leptin ELISA. RayBioTech Life, Inc. USA).

2.5 Statistical analyses

For all statistical tests, significance was set as $p < 0.05$. Two-way ANOVAs were used to compare bone morphology and serum concentration data within timepoints and within family lines. Tukey's post-hoc (HSD) tests were used to test for statistical significance of pairwise differences in means among lines and control for multiple comparisons. Graphpad prism version 9.5.0 for Windows, Graphpad Software, San Diego, California USA., was used to perform the two-way ANOVAs and multiple comparison tests and to generate all figures. All other statistical analyses were performed in RStudio version 4.2.2, R Foundation for Statistical Computing platform, Vienna, Austria. The final sample count for each group and variable is found in (Table 1; Table 2; Table 3).

2.6 Outlier detection.

The residuals from the two-way ANOVAs were used to detect outliers prior to statistical analyses. To account for outliers who contributed to multiple hormone data sets (e.g., an entire mouse as an outlier across hormones), the residuals were standardized, and a mouse with a sum of standardized residuals greater or equal to |16.4| was removed. This means that the residuals across the ten hormones analyzed are on average below or above the fifth percentile (1.64 standard deviations) of the standard normal distribution. No full sample outliers were detected and therefore, each mouse is represented in the full sample (Table 4).

Individual data point outliers were identified using a cut-off of +2.5 standard deviations and -2.0 standard deviations was used. An uneven cut-off was set *a priori* to account for the pulsatile nature of circulating hormone levels. Two data points belonging to two different mice were removed in the PROL and osteocalcin datasets. Three data points for TSH and six points were removed from the FSH data. No outliers were detected in the OPG, SOST, insulin, and leptin datasets.

For the bone parameters data, the same procedure to account for outliers of an entire mouse across data points as the serum data with a sum of standardized residuals of 9.84 was used. No full sample outliers were detected. For single data point outliers, an even cut-off of +/- 2 standard deviations was used. Ct.BMD had the highest number of outliers detected – six were removed across the entire data set. BV/TV% had five outliers removed, Tb.Th had four points, and Ct.Th had three points removed.

For female body mass, there were no outliers detected, and for litter size, one data point was removed for LS2 at PTP due to the cannibalization of her litter.

2.6.1 Confounding variables

Litter size and female body mass were investigated as confounding variables in both the hormonal serum and bone parameter datasets. Scatter plots for each variable vs. the confounding variable, within a family line, were first visually assessed to determine a possible relationship. ANCOVAs were then performed for all variables with either litter size or female body as the covariate. The two-way ANOVAs and the corresponding ANCOVAs were then compared. Notably, there was minimal difference between the two analyses and thus, the results reported are from the two-way ANOVA only. Female body mass as a covariate for leptin was also analyzed further with a linear regression due to the fact that leptin is directly produced by

adipose tissue. The linear regression yielded similar conclusions to the ANOVA, thus for body mass, only the two-way ANOVA is reported.

CHAPTER 3: RESULTS

3.1 Summary statistics

Female body mass was not significantly different between any of the groups at baseline and pregnancy. In order to account for body mass as a covariate, body mass was measured for each female at the end of each time-point. The mean grams (SD) female body mass at BL of CTL, LS1, and LS2, respectively was 30.9 (3.0) g, 30.8 (2.8) g, and 28.3 (2.0) g. The pregnancy sample counterparts were 27.7% (42.7 (3.6) g), 28.6% (43.1 (3.8) g), and 32.6% (42.1 (3.3) g) heavier. At lactation, LS1 (55.7 (2.9) g) was only 8.1% (55.7 (2.9) g) and 8.4% (51.2 (2.6) g) heavier than LS2 and CTL, respectively (Table 1). As seen in previous studies, there is still a lack of difference in body mass between LS and CTL lines, specifically at BL and PTP.

LS1 litter size was significantly lower than CTL in the LTP group only. Litter size was recorded to investigate possible evolutionary implications of selective breeding as described in Section 4.5. PTP litter size count consists of pooled data from PTP females and day of parturition recorded for LTP females. LS1 had the lowest mean litter size of 10.1 (3.1) pups at LTP and CTL had the highest mean litter size of 14.1 (2.1) pups. There was no significant difference in litter size between CTL and LS2, and there were no differences between any of the groups at PTP (Table 2).

3.2 Maternal bone microarchitecture

LS1 gains more and lose more bone during pregnancy and lactation, respectively. At baseline, there were no differences in BV/TV% between any of the lines (Tukey's HSD, $p = ns$, $df = 92$). At pregnancy, both Longshanks' groups showed a significant increase – 81.9% in LS1 and 54.1% in LS2, relative to the BL females (Tukey's HSD, $p < 0.05$, $df = 92$). This increase

was not seen in the CTL group. All three lines experienced a significant decrease in relative bone volume at LTP, dropping below their respective baseline values (Tukey's HSD, $p < 0.05$, df = 92). LS groups experienced a larger percent change in bone volume between each time point with a significant increase and then a decrease (Tukey's HSD, $p < 0.05$, df = 92) (Table 3; Figure 5).

Despite no differences at baseline, LS groups had more bone volume than CTL at PTP – LS1 significantly surpassing CTL (Tukey's HSD, $p < 0.05$, df = 92). These differences were not sustained in LTP, with all groups having significantly lower bone quantity than their respective PTP counterparts (Tukey's HSD, $p < 0.05$, df = 92). Most notably, the percent differences between each time point were noticeably larger in the LS than the CTLs. LS females may be able to further increase skeletal stores during pregnancy than CTL in order to circumvent these larger drops during lactation.

CTL have higher bone quality and quantity at baseline than LS groups, but these differences are no longer present at lactation. CTL had significantly thicker trabeculae at BL than both LS groups (Tukey's HSD, $p < 0.05$, df = 94). The gap between all three groups closes at pregnancy, LS1 even surpassing CTL and LS2 groups (Tukey's HSD, $p < 0.05$, df = 94). LS groups gained trabecular thickness at pregnancy (Tukey's HSD, $p < 0.05$, df = 94); however, CTL trabeculae thickness did not change between BL and PTP (Tukey's HSD, $p = 0.92$, df = 94). At lactation, all three groups had a large decrease in thickness (Tukey's HSD, $p < 0.05$, df = 94). LS groups returned to their baseline values (Tukey's HSD, $p < 0.05$, df = 94), and CTL dropped significantly below baseline (Tukey's HSD, $p < 0.05$, df = 94) – to the same level as BL

LS groups, thus there were no differences between all the groups at lactation (Tukey's HSD, p = ns) (Table 3; Figure 5).

CTL had a thicker cortex at baseline than both LS groups (Tukey's HSD, $p < 0.05$, df = 94). CTL cortical thickness dropped by 15.8% at lactation, 11.7% for LS1, and 12.9% for LS2 (Tukey's HSD, $p < 0.05$, df = 94). All three groups had thicker cortical bone at pregnancy when compared to their nulliparous counterparts (Tukey's HSD, $p < 0.05$, df = 94); however, during lactation, there was no difference in cortical thickness between any of the groups (Tukey's HSD, p = ns, df = 94). CTL cortical BMD was significantly higher than both LS groups at all time points, despite LS groups showing a larger increase in cortical BMD at pregnancy (Tukey's HSD, $p < 0.05$, df = 94). There was no difference between PTP and LTP cortical BMD in both LS groups (Tukey's HSD, p = ns, df = 94). CTL returned to near baseline levels at lactation (Tukey's HSD, $p < 0.05$, df = 94). These changes were more substantial in the LS mice than in the CTL group. There were no differences between the groups at lactation (Tukey's HSD, p = ns, df = 94) (Table 3; Figure 6).

Consistent with previous findings, LS had lower bone quality and quantity at baseline, as seen by Ct.Th, Tb.Th, and Ct.BMD than CTL (Tukey's HSD, $p < 0.05$, df = 94). However, the differences between groups at PTP and LTP were substantially decreased (Tukey's HSD, $p < 0.05$, df = 94). Despite poorer bone quality/quantity at baseline than CTL, LS females were able to lessen or close the gap at postpartum. Thus, at LTP, though all lactating females had lower bone quantity/quality than the postpartum mice (Tukey's HSD, $p < 0.05$, df = 94), differences in cortical and trabecular thickness between groups were no longer observed (Tukey's HSD, p = ns, df = 94).

3.3 Maternal circulating hormones in serum

Females in the LS groups had higher PTH than CTL at LTP. At baseline and pregnancy, there was no significant difference in circulating PTH between any of the groups (Tukey's HSD, p = ns, df = 59). Despite the lack of significance at pregnancy, both LS groups had over 2x the PTH concentration of CTL. At LTP, LS1 and LS2 had 8x higher concentrations of PTH than CTL (Tukey's HSD, $p < 0.05$, df = 59). In LS groups, there was no difference between BL and PTP PTH (Tukey's HSD, LS1 p = 0.60, LS2 p = 0.10, df = 59). However, PTH concentrations are doubled in the LTP females compared to their BL counterparts. In LS1, the mean PTH in the BL females was 24.3 (19.0) pg/ml whereas, in the LTP females, the mean concentration was 67.8 (55.8) pg/ml (Tukey's HSD, $p < 0.05$, df = 59). LS2 groups had very similar concentrations of 27.3 (22.4) pg/ml at BL and 68.9 (46.0) pg/ml at LTP. Thus, in both LS groups, LTP females had significantly higher PTH concentrations when compared to the BL LS (Tukey's HSD, $p < 0.05$, df = 59). There was no significant difference between BL and LTP PTH concentrations in CTLs (Tukey's HSD, p = 0.89, df = 59) (Table 4; Figure 7).

LS groups have a larger change in OPG concentrations between each time point compared with CTL. There were no differences in OPG between the family lines at any time point (Tukey's HSD, p = ns, df = 60). In all three families, the PTP females had significantly higher OPG concentrations than both their BL and LTP complements (Tukey's HSD, $p < 0.05$, df = 60). In LS1, postpartum females had 42.7% higher concentrations than the mean LS1 BL females (Tukey's HSD, $p < 0.05$, df = 60). In LS2, PTP females had 51.8% higher OPG concentrations than their BL counterparts (Tukey's HSD, $p < 0.05$, df = 60), whereas, in CTL, there was no difference between PTP and BL females.(Tukey's HSD, p = ns, df = 60) (Table 4; Figure 7).

Serum SOST is higher in LS groups than in CTL. At baseline and lactation, there is no significant difference in SOST between any of the groups (Tukey's HSD, p = ns, df = 63). Despite this, LS groups may still have higher amounts of SOST at each time point – this difference heavily masked by the large variation around the mean. At BL, both LS groups have over double the SOST concentration of CTLs (Tukey's HSD, p = ns, df = 63). SOST in LS1 is significantly increased at pregnancy with a mean value of 1017.1 (576.7) pg/ml compared to BL (Tukey's HSD, $p < 0.05$, df = 63) and LTP LS1 concentrations of 566.8 (126.0) pg/ml and 451.7 (149.8) pg/ml, respectively (Tukey's HSD, $p < 0.05$, df = 63). LS2 also had a trend of increased SOST at pregnancy and a drop at lactation though it was not significant (Tukey's HSD, BL vs PTP p = 0.65, and PTP vs LTP p = 0.33, df = 63). Data spread of SOST around each mean is noticeably larger in LS groups, especially at pregnancy (Table 4; Figure 7).

FSH is highly variable in all groups. There was no significant difference between the lines at BL and LTP (Tukey's HSD, p = ns, df = 93). At pregnancy, CTL had a significantly higher FSH concentration than LS1, 727.8 (650.9) pg/ml and 287.0 (315.03) pg/ml (Tukey's HSD, $p < 0.05$, df = 93). LS2 had similar concentrations to LS1 with a mean of 181.424 (81.7) pg/ml (Tukey's HSD, p = 0.76, df = 93). Mean FSH concentration was lower at pregnancy for the LS2 groups when compared to BL counterparts – with a drop of 70% between BL and PTP (Tukey's HSD, $p < 0.05$, df = 93). A similar trend is seen in LS1 with a less severe drop of 37.3% (Tukey's HSD, p = 0.50, df = 93). In CTL, BL females had significantly lower concentrations than the PTP females (Tukey's HSD, $p < 0.05$, df = 93). The highest variation around the means was seen at baseline time points and the lowest was at lactation (Table 4; Figure 8).

TSH trends upwards at postpartum and mid-lactation in CTL but maintains constant in LS. Similar to FSH, there were no differences between the groups at any of the time points (Tukey's HSD, p = ns, df = 95). In CTL, there was a slight trend in the postpartum females of slightly increased but not significant TSH when compared to the baseline females (Tukey's HSD, p = 0.40, df = 95). Lactating CTL had significantly higher TSH concentrations with a mean value of 283.7 (163.3) pg/ml than the nulliparous females, 116.5 (76.9) pg/ml (Tukey's HSD, $p < 0.05$, df = 95). This trend was not seen in LS groups (Table 4; Figure 8).

PROL concentrations are higher in CTL than LS. Interestingly, there was no increase in prolactin at any time point except for between postpartum and nulliparous females in the CTL group (Tukey's HSD, $p < 0.05$, df = 108). When looking at within time points, both LS groups had significantly lower levels of prolactin at pregnancy: mean PROL in CTL was 57.4 (16.0) ng/ml whereas LS1 and LS2 concentrations were found to be 17.0 (12.8) ng/ml and 24.0 (16.0) ng/ml, respectively (Tukey's HSD, $p < 0.05$, df = 108). This difference in CTL may be sustained into lactation (Table 4; Figure 7).

In LS, osteocalcin drops during pregnancy. Both LS groups had lower mean osteocalcin in the postpartum sample when compared to the nulliparous mice (Tukey's HSD, $p < 0.05$, df = 61). These lowered levels were sustained in the lactation group. When looking at differences across lines at each time point, there was only a difference in the lactating mice sample where LS2 had significantly higher levels of osteocalcin than CTL (Tukey's HSD, $p < 0.05$, df = 61) (Table 4; Figure 9).

Insulin does not change throughout pregnancy and lactation. Mean insulin was consistent in all groups at all time points. The only significant difference is seen at baseline

where LS1 had higher insulin concentration than CTL, 92.1 (32.4) ng/ml and 134.9 (26.3) ng/ml, respectively (Tukey's HSD, $p < 0.05$, $df = 62$) (Table 4; Figure 9).

Leptin is significantly increased in LS groups at pregnancy and lactation. At baseline, there is no difference between the groups, however, at pregnancy, there is a substantial increase in LS groups (Tukey's HSD, $p < 0.05$, $df = 59$). Postpartum LS1 females had leptin concentrations 94.2% higher than the nulliparous sample, for LS2, 87.2% higher (Tukey's HSD, $p < 0.05$, $df = 59$). The difference between the CTL baseline and PTP was not significant but there was still an increase of 50.2% (Tukey's HSD, $p = 0.07$, $df = 59$). Lactating females had significantly lower leptin concentrations than the postpartum females for all three groups but were still markedly higher than the nulliparous sample (Table 4; Figure 9).

CHAPTER 4: DISCUSSION

4.1 Pregnancy improves maternal microarchitecture in both Longshanks and Control mice

Our study first aimed to investigate how compromised maternal skeletons are impacted by pregnancy and lactation. Despite lower bone quality in the LS, we found strikingly similar changes postpartum between all lines and these differences were more pronounced in the LS lines. There was either no difference or a significant increase in all trabecular and cortical parameters investigated between the nulliparous sample to the postpartum mice in all three groups.

In rats, pregnancy and lactation detrimentally affect the maternal skeleton, resulting in large losses in BV/TV%, Tb.Th and trabecular number, however in humans and mice, results are mixed (39,79,80). Gu et al. (2017) saw a significant increase in BV/TV% and trabecular number in the distal end of the femur in mice with a single pregnancy compared to non-pregnant controls (79). Our study corroborates these findings: we saw all three lines show significant increases in indicators of trabecular and/or cortical morphology in the postpartum sample compared to the BL mice.

The trends toward increases in bone quantity seen in this study suggest a possible protective response to the effects of pregnancy on the maternal skeleton. Though pregnancy-related changes are still unclear, lactation is strongly associated with negative impacts on the maternal skeleton (81). The increases in bone quantity seen in the present study may indicate possible augmented calcium mobilization and storage during pregnancy in preparation for the draining of maternal calcium reserves throughout lactation, thus protecting the maternal skeleton from substantial bone loss.

This potentially protective response has been seen in human studies. Shahtaheri et al. (1999) investigated microarchitectural changes in trans-iliac biopsies during early and late pregnancy (5). The authors found that mean relative bone volume fell during early pregnancies but rose significantly during late pregnancy (5). This early reduction in bone volume is likely the initial mobilization of calcium stores from the maternal skeleton in order to initiate early skeletogenesis in the fetus and in late pregnancy, the fetal skeleton undergoes mineralization at a high rate. The increase of bone tissue seen in late pregnancy by Shahtaheri et al. (1999) and shortly after pregnancy as demonstrated by the current study may thus be a preparatory response to the rapid changes associated with final fetal bone developments (5). Stated differently, increased trabecular bone seen at late pregnancy or shortly after parturition in all three lines may be a result of promoted bone deposition as a response to pregnancy to protect the maternal skeleton from significant calcium demands of fetal ossification and later, lactation.

In the trabecular compartment of the long bones found at the epiphyses, only ~20% of the volume is comprised of trabecular bone – the rest being marrow and fat (82). Trabecular bone turnover and rates of remodeling is higher than cortical bone and thus is hypothesized to be more important for mineral metabolism (83). The trabecular bone is also involved in transferring mechanical load to cortical bone (82,83). The large majority of bone mass is comprised of cortical bone, thick and dense, and mainly responsible for bone strength (82). As with trabecular bone, we also saw indicators of improvement in cortical bone characteristics. Notably, the increases in the cortical parameters were substantially larger than the increases in the trabecular indices. More specifically, all females in the postpartum (PTP) group had thicker cortical bone than their respective nulliparous samples with substantially increased cortical mineralization. These changes observed in cortical bone are strong indicators of improved bone strength.

The protective response is not only for the preservation of the maternal calcium stores during pregnancy, but it is also likely to help ease mechanical stresses on the maternal skeleton. Bone microarchitecture is a strong predictor of bone quality, independent of BMD. In humans, pregnancy significantly alters the musculoskeletal system, particularly around the spine, pelvic bones, and long bones of the leg (84). These bones are exposed to greater mechanical stresses and must be able to withstand increasing strains as weight gain and abdominal distension progress through pregnancy (84). Trabecular and cortical bone both have important roles in bone strength, however, cortical bone in particular bears the most mechanical strain (84,85). It is thus important for pregnant females to develop a strong trabecular network and cortical compartment during pregnancy to reduce the risk of sustaining musculoskeletal disorders, e.g., pelvic dislocation, osteitis pubis, etc. (84,85). In our study, all PTP females gained double to over triple their body weight by the day of parturition. Abdominal distension and substantial weight gain would undoubtedly cause an increased mechanical burden to the maternal skeleton, and thus require increased bone strength. This may explain why the postpartum mice had a markedly higher percent difference in cortical indices than trabecular indices when compared to mice in the baseline group.

In this study, we saw significant increases not only in overall BV/TV% but indications of trabecular and cortical microarchitecture alterations as well, especially in LS lines. All three lines showed trends toward increases in trabecular thickness postpartum. Shahtaheri et al. (1999) also observed pregnancy-related changes in trabecular thickness and spacing in humans, but additionally noted qualitative alterations in the trabeculae network (5). At late pregnancy, they found that the trabeculae were ordered in a more complex network, indicating improved bone quality (5). Though we did not analyze qualitative changes in the trabecular network, our

quantitative results of trabecular thickness consistent with some level of positive change in the proximal tibia microarchitecture.

Overall, our results consolidate that pregnancy causes microarchitectural changes in the proximal tibia of the dams. Thicker trabeculae and cortical bone postpartum, overall greater bone volumes (BV/TV%), and increased cortical mineralization are indicators of improved bone and may be adaptive to the cost of fetal mineralization & increased mechanical stress on the maternal skeleton during late pregnancy.

4.2 The magnitude of pregnancy-related changes in bone microarchitecture differs between populations.

Though all families showed the same direction of change in their microarchitecture, the LS lines experienced much greater magnitudes of difference in both cortical and trabecular indices when comparing the postpartum females and the nulliparous sample. Specifically, there were no differences in BV/TV% and trabecular thickness between BL and PTP CTL females whereas LS1 and LS2 postpartum females had 81.9% and 54.1%, respectively, more bone than their BL counterparts with 23.2% and 13.5% thicker trabeculae. Despite the lack of difference in the trabecular indicators in the CTL, postpartum CTL females did have significantly thicker cortical bone and mineralization than the BL CTL mice. However, the magnitude of the increase in the LS is still significantly more pronounced.

Physiologically, these differences may help to resolve conflicting evidence regarding pregnancy and lactation-associated changes in bone (80,86,87). Though most studies note bone loss at some point during pregnancy and a protective effect pre-lactation in mice, a few have shown inverse results (87). Most studies only use changes in BMD and trabecular bone as a measurement of bone loss or gain (87). However, different mammal populations may have

innately varying bone remodeling activity and thus, will have varying directions and magnitudes of change in different areas of the bone (88). For example, younger pregnant women will typically see a positive relationship with BMD while older women will often see a detrimental relationship (88). Younger populations have increased bone remodeling activity whereas older populations typically have less active bone remodeling (88). These discrepancies may result from genetic differences in bone remodeling between populations and thus, result in different outcomes. Our study used adult mice all near the same age, therefore age is an unlikely explanation, however, the LS population has a distinctly different bone phenotype from the CTL.

The LS mouse model is phenotypically more similar to a human with compromised bone integrity. It is intuitive to believe that individuals with osteopenia or other related conditions may risk worsening their condition due to the expensive costs of pregnancy on the maternal skeleton. Contrary to this prediction, our results indicate that at postpartum, mice with previously fragile bones showed more improvements in bone microarchitecture than mice with normal bone microarchitecture. In the proximal tibia, the LS lines has significantly thinner trabeculae and cortical bone, with less cortical mineralization than CTLs – all indicators of more brittle, fragile bone as previously demonstrated by Farooq et al. (2017) (75). We can hypothesize that these physical alterations that LS sustained over the course of selective breeding for length caused changes to bone remodeling activity when compared to the random-bred CTL line, and thus, allowing each mouse line to exhibit different, in this case, larger relative changes in response to varying stressors.

To our knowledge, no studies have investigated pregnancy-related changes in bone in premenopausal women with pre-existing bone conditions or even with such substantive differences in bone phenotype as those demonstrated in LS and CTLs. However, To et al. (2003)

found that in humans, females with an initially low BMD had significantly less bone loss postpartum than individuals with normal pre-pregnancy BMD (89). These results imply that women with decreased BMD had a stronger protective mechanism during pregnancy than women with physiological BMD (89). Similar to the findings by To et al. (2003), because LS had a larger increase of bone postpartum than CTL, we hypothesize that in the LS female, pregnancy provokes a stronger protective response against bone loss than CTL.

Interestingly, despite some baseline differences between the LS and CTL lines, postpartum LS females had thicker trabeculae and more bone volume, surpassing postpartum CTL females, however, in the cortical indices, postpartum CTL females still had significantly higher cortical thickness and mineralization. This may be an example of possible different bone remodeling strategies between CTL and LS lines. The mechanism for such a stronger protective effect in LS may be more centered on trabecular remodeling rather than cortical remodeling. Notably, pregnancy-associated changes in microarchitecture have predominately been found in the trabecular compartment, thus it is expected that losses from lactation would mainly be trabecular, as seen by De Bakker et al. (2017) (28). Thus, LS females may induce a stronger protective response in the trabecular compartment rather than the cortical bone to account for the anticipated deficit from the costs of lactation.

Overall, our study provides additional evidence that there is a physiological response during pregnancy that leads to increased bone quantity, likely to protect the maternal skeleton in preparation for fetal ossification and lactation. More so, our study provides evidence that this response varies depending on population-specific pre-pregnancy characteristics.

4.3 Lactation is costly to the Longshanks and Control maternal skeleton

Mid-lactation was mainly characterized by significantly lower values of trabecular and cortical markers than the postpartum mice. The positive increases found in the postpartum mice were essentially lost and for most markers, returned to values similar to their respective nulliparous mice. BV/TV% effects were particularly severe, all lactating females having lower bone volume than the BL females.

The calcium costs of pregnancy and lactation are mainly attenuated by intestinal absorption, renal handling, and bone resorption (18,20). Pregnant rodents double their duodenal calcium absorption and reduce their renal calcium excretion (18,20). In lactation, these adaptive changes are similar, however, bone turnover is significantly upregulated (18). Lactating rats experience severe loss of bone mineralization, primarily in the trabecular compartment of the long bones (7,90). It is thus likely that the calcium costs of lactation exceed calcium intestinal absorption and reduced renal calcium excretion thus bone resorption must be upregulated to mobilize calcium stores and compensate for the exorbitant calcium requirements of lactation. We found supporting results with bone volume dropping by 35%-57.6% and trabecular thickness reductions of 16.9%-24.0% across groups. Though our study did not directly investigate calcium concentrations, these significant losses of cortical bone mineral we observed are likely from the extra calcium costs associated with milk production.

Intriguingly, these losses were exacerbated in the LS mice. By mid-lactation, most differences in microarchitecture disappeared between lines. Despite the substantial indicators of improved bone at pregnancy, lactating CTL and LS groups had similar trabecular and cortical thickness. Stated differently, LS mice gained more bone than CTL at pregnancy but also lost more bone by mid-lactation, thus any differences among lines were no longer observed except

for cortical mineralization where LS1 had the lowest mineralization. We had originally predicted (**H1**) that lactation would be particularly detrimental in the LS mice due to its compromised bone quality phenotype and the exacerbated costs of lactation to support development of pups with larger skeletons. However, the large gains in bone volume and thickness observed in the LS at pregnancy, possibly allowed LS to circumvent the severe loss at mid-lactation, thus possibly avoiding detrimental effects on the maternal skeleton. This is interesting because it implies that the potential protective effect of pregnancy was triggered by the LS mouse, in theory, to support maternal skeleton maintenance and alleviate the higher calcium costs of lactation.

The lactational losses in our study were mainly at trabecular sites. Cortical thickness and area in the lactating females were significantly decreased compared with the postpartum mice but were either a net positive difference or no difference at all when compared to their respective nulliparous counterparts. Our results thus supports the theory that lactation-associated changes in the bone microarchitecture are within the trabecular compartment as seen in other studies, likely due to the increased metabolic activity at trabecular bone sites rather than cortical bone (84).

Notably, cortical mineralization was only found to be lower than in postpartum females in the CTL group - there was no difference between postpartum and lactating in either LS line. Overall, cortical losses were higher in the lactating CTL mice. This observation further supports the possibility that CTL and LS have different bone remodeling strategies in pregnancy and lactation. Though CTL loses less trabecular bone, these mice lost relatively more cortical bone than LS. CTL thus mobilizes calcium stores from both trabecular and cortical bone, whereas the LS may be able to mitigate cortical bone loss by increasing trabecular bone stores during pregnancy.

To our knowledge, there is currently no study that investigates pregnancy and lactation-associated bone changes in a model with fragile and brittle skeletal phenotype, without genetic manipulation as seen in the LS. Here, we observed significant bone loss during lactation such that, despite a positive trend in pregnancy, lactating LS mice had significantly lower bone volume than their nulliparous sample. Notably, mice are weaned at around ~21-28 days, and in our sample, we only collected a mid-lactation point. Thus, it is likely that the bone volume and thickness will continue to decline until weaning, further depleting maternal calcium resources and likely to result in larger deficits in the maternal skeleton. It is valuable to consider the full length of lactation and its possible detrimental impact on the maternal skeleton, especially in those with prior fracture history and/or family history of osteoporosis. We thus speculate that women with fragile bones may have their condition exacerbated with lactation.

Lactation is associated with bone loss and this bone loss differs depending on the skeletal phenotypes of the population. In LS mice, increasing maternal stores during pregnancy may mitigate the over-depletion of the maternal skeleton during lactation.

4.4 Hormonal axes are altered in pregnancy & lactation in females with compromised bone health.

4.4.1 At baseline, LS and CTL mice differences may, in part, be due to subtle variances in hormone concentrations.

Perhaps surprisingly, there was mainly no significant difference in any of the hormone serum concentrations between the lines in the nulliparous mice. This would indicate that CTL and LS endocrine-controlled bone metabolism is still similar even after generations of selective breeding. However, there was a small trend towards increased catabolic hormone concentrations in the LS mouse lines. For example, sclerostin antagonizes the Wnt signaling pathway and thus

is directly involved in osteoclast formation. LS1 and LS2 mean sclerostin concentrations at BL were 56.6% and 58.9%, respectively, higher than CTL, although this difference was not statistically significant perhaps due to the wide variation in serum measurement levels. Similarly, when looking at PTH – another catabolic mediator, LS1 and LS1 had 37.9% and 44.7% higher, respectively, PTH concentrations than CTL. These subtle differences in hormones may imply higher levels of bone resorption in the LS and explain why LS mice showed significantly thinner trabeculae and cortical bone. Moreover, the OPG-RANKL-RANK system may be partly responsible for these patterns. There is no significant or discernible difference between OPG concentrations in the LS and CTL nulliparous mice. In principle, higher concentrations of SOST and PTH in LS could promote osteoclastic differentiation and formation by upregulating RANKL expression (47). With no differences in OPG concentration, the decoy receptor for RANKL, bone resorption would be favoured during remodeling (46). Bone resorption and bone formation are normally carefully balanced to maintain healthy bone homeostasis. Given the altered and weakened microarchitecture (thinner bone with reduced cortical mineralization) of the LS, we can hypothesize that bone resorption is promoted in the LS tibia and driven by a possible divergence of the OPG-RANKL-RANK system when compared to CTL.

Insulin, leptin, and osteocalcin have been recently implicated in a negative feedback loop in bone metabolism (Figure 2) (64). Osteocalcin, a marker for bone formation, is very slightly increased in LS compared to CTL – LS1 and LS2 were 10.2% and 22.3% higher than mean osteocalcin CTL concentration. Higher levels of osteocalcin are indicative of increased hydroxyapatite binding, thus lower maternal resource mobilization (65). Insulin was not different between the groups and leptin was noticeably decreased in LS. There was no significant difference between the families in leptin levels, however, CTL still had a 4.3x and 2.5x fold

leptin concentration increase than LS1 and LS2, respectively. The crosstalk and the relationship between these three hormones are still debated. Lee et al. (2007) first demonstrated that in osteocalcin knock-out mice, the mice were hyperglycemic with decreased blood insulin levels and pancreatic islet cell size (91). Since then, other studies have corroborated the fact that osteocalcin has important metabolic functions and is involved in the regulation of insulin (64,92,93). Hinoi et al. (2008) demonstrated that leptin can activate the sympathetic nervous system which enhances *Esp* expression in osteoblasts thus decreasing osteocalcin bioactivity (94). These findings along with others, such as leptin-deficient mice having high bone mass, supported the hypothesis that leptin indirectly exerts a net catabolic effect on bone mass (94,95). However, this hypothesis remains controversial. In contrast to Hinoi et al, Kalra, Dube, & Iwaniec (2009) observed that increased leptin expression suppressed pancreatic insulin secretion and increased osteocalcin release (96). In our study, it is possible that suppressed leptin levels could promote this endocrine loop thus possibly resulting in decreased cortical/trabecular thickness in the LS mouse (Figure 2).

There are few significant baseline differences in hormone serum data between LS and CTL mice. Nonetheless, we hypothesize that the OPG-RANKL-RANK system may be involved in producing the thinner trabeculae and cortical bone in the LS mouse through the actions of PTH, SOST, and OPG. Insulin, osteocalcin, and leptin crosstalk may also contribute to the microarchitectural differences observed in our study, however, it is important to note that any differences in insulin and leptin in our study may be due to metabolic variation and/or other physiological differences between the LS and CTL mice. Insulin is a broad-spectrum anabolic hormone involved in numerous physiological processes related to energy homeostasis (12). Leptin, another multi-faceted hormone, is essential to processes of metabolism, appetite

regulation, and neuroendocrine function (12). We did not evaluate the other essential physiological roles of insulin or leptin, however, due to the demonstrated roles of insulin and leptin in bone remodeling, we thus included insulin and leptin in our investigation.

4.4.2 "Protective" mechanisms & postpartum hormones in preparation for lactogenesis

Pregnancy was marked by a significant increase in many cortical and trabecular parameters (18). Consistent with this result, there was a significant increase in OPG in all three lines. High concentrations of OPG block osteoclastic activity by binding to RANK and promote osteoblastogenesis, thereby promoting bone formation (46). This result is consistent with human data where OPG markedly increases near the end term (97). This is likely because fetal ossification occurs at an increased rate in the latter half of gestation (98). Again, OPG was not different between the lines in the postpartum sample. However, the percent difference when looking at within lines is still markedly higher in the LS. Despite LS's weaker skeleton, LS mice may be eliciting a stronger response through OPG-RANKL-RANK signaling to increase maternal stores in preparation for the heavier costs of lactation in LS mice. In fact, Kram et al. (2020) recently demonstrated that OPG treatment could rescue low bone mass phenotype (99). Others have also reported similar results with OPG reversing osteoporotic bone in mice – which is phenotypically similar to LS mice bone microarchitecture (100). The significant increase in mean OPG concentration particularly in LS mice may thus be largely responsible for increased bone volume and thicker trabeculae/cortical bone or the potential protective effect of pregnancy in the LS bone.

The postpartum time-point was investigated in this study to capture the maternal endocrinology of late gestation where fetal ossification occurs. We may expect high concentrations of anabolic hormones to capture the increased bone volume as seen in the

postpartum mice, however, we observed higher concentrations of catabolic regulators, namely SOST, at the postpartum time point, especially in the LS lines. SOST was significantly increased only in LS1, however there still is a small increase in the CTL group and a more marked increase in LS2, with the postpartum group having 19.9% and 36.1% percent increase concentrations of SOST than the baseline mice, respectively. High concentrations of OPG *and* SOST are indicators of overall increased bone turnover (46). In late gestation when nearing term, maternal systems go through a cascade of physiological events in preparation for parturition and lactogenesis (101). In mice, lactogenesis begins before parturition and as the cardiac blood samples were taken the morning of the day of parturition, high concentrations of catabolic regulators would be expected to immediately localize these calcium stores for lactation.

Interestingly, prolactin was not increased at postpartum in the LS lines – only in the CTL mice when compared to the baseline cohort. Prolactin is essential for lactogenesis through stimulation of the mammary glands to synthesize milk (62). The lack of increase in prolactin in the LS mice at parturition is somewhat surprising and may be due to a couple of factors: 1) Prolactin concentration does not remain high throughout lactation and is activated by suckling (62). Suckling is associated with large spikes in maternal circulating prolactin and thus, the cardiac bleeds may have been taken before or after a feeding window (62). 2) Lactogenesis may be slightly delayed in the LS mouse. The higher concentrations of catabolic mediators of bone seen in our study (e.g., SOST, PTH) indicate higher bone resorption activity. This would promote the release of calcium stores into the circulatory system for lactogenesis. Thus, the calcium content for lactogenesis may be derived in greater proportion from maternal skeletal stores in the LS whereas CTL mice may be able to rely on calcium intestinal absorption or other maternal physiological processes for earlier lactogenesis.

TSH was not different between families at postpartum, however, all three groups at the postpartum time point had higher mean concentrations of TSH than the nulliparous mice, significantly so in LS1. This pattern is expected, as TSH levels rise during late gestation (102). FSH was also investigated and found to be significantly increased in the CTL compared to both LS lines in the postpartum sample. Estrogen and progesterone are at low concentrations at early gestation before rising in the latter half, FSH would thus be expected to be low at birth through negative feedback signaling (59). A large variation in FSH concentration was seen in both the nulliparous and postpartum mice. Estrus staging was not conducted in this study, but it is reasonable to assume this variation in the nulliparous mice is likely due to the females being at different stages of the estrus cycle thus causing large variation in sex hormone data.

We found that FSH was low at postpartum in the LS mouse, and this is consistent with the high estrogen levels required for parturition. However, postpartum CTL females had significantly higher concentrations than baseline CTL females. This is likely a result of the stochastic, pulsatory nature of the secretion of sex and metabolic hormones (103). Estrogen has been well established as a potent regulator of bone metabolism through various indirect and direct mechanisms by influencing the activity of osteoblasts, osteoclasts, and osteocytes (52). Overall, estrogen has a net positive effect on bone mass (52). FSH promotes estrogen production, however, has been observed to have a catabolic effect on bone regulation independent of estrogen (54). Our results of TSH and FSH are consistent with the results of previous studies, and given their low concentrations and lack of difference between lines, it is likely that the pregnancy-associated changes in the maternal bone microarchitecture are minimally affected by these hormones.

When compared with the baseline condition, mean osteocalcin concentration was significantly decreased in the postpartum mice in LS only. There was no significance in the CTL group, however, the mean concentration was still 21.1% lower. These observations indicate that osteocalcin was downregulated at postpartum. Consistent with this finding, leptin was significantly increased in LS only, while insulin remained unchanged for all three families. These observations taken together support that the osteocalcin, insulin, and leptin hormone crosstalk may be more active in the LS mouse when compared to the CTL line. The lack of change in insulin could possibly have been influenced by the substantial increase in leptin. High levels of leptin may have inhibited osteocalcin secretion and insulin production, thus favouring bone resorption in the LS.

The "protective" anabolic effect of pregnancy as witnessed in the microarchitectural data may have been partly produced by OPG in the LS mouse, however, when compared to the baseline mice, the postpartum mice had increased catabolic regulators of bone metabolism. This is likely due to the time of blood collection when bone resorption would be increased to mobilize calcium reserves for lactogenesis. In the LS mouse, this may be produced by stronger responses through increased OPG through the OPG-RANKL-RANK axis, and higher production of leptin in the Osteocalcin-Insulin-Leptin axis.

4.4.3 Hormonal differences in the cost of lactation in Longshanks vs. Controls.

By mid-lactation, many circulating hormones returned to levels similar to that of their nulliparous counterparts. Prolactin concentration in lactating mice was not different from postpartum or baseline mice in all families – again, likely due to the timing of feeding variation during bleeding. Once lactating, mice will enter anestrus thus FSH and estrogen will be low, and mean FSH concentrations were found to be low at similar levels in all families.

We continued to observe differences in LS and CTL endocrine profiles involved with bone metabolism. OPG and SOST concentrations were both significantly decreased in the LS mouse compared to the postpartum sample, there was still a percent decrease in the CTL albeit not significant. PTH, however, was significantly increased in the LS1 when compared to nulliparous mice and LS2 in both the nulliparous and postpartum samples. Lactating mice had significantly lower bone volume and decreased trabecular/cortical thickness. Increased PTH concentrations would promote RANKL binding to osteoclasts, thus stimulating their bone resorption activity (45). Low concentrations of OPG would allow increased RANKL binding to its receptor RANK, leading to a net result of amplified bone resorption. This could possibly explain the substantial decreases in the microarchitectural indices (e.g., BV/TV%, Tb.Th) experienced by the LS mouse during lactation (46).

Leptin was decreased in lactating mothers when compared to postpartum mice. In the LS, leptin concentrations were still higher than in the nulliparous mice whereas CTL returned to baseline levels. LS1 were significantly heavier than the other two families but despite this, when female mass was analyzed as a covariate, there was little difference in the hormonal and microarchitectural results. These large fluctuations of leptin concentration throughout the study with very little difference in body weight observed suggest that leptin is heavily involved in other maternal physiological processes. The controversial findings in the literature and our results render it unclear if leptin is involved in pregnancy and lactation-associated microarchitectural differences in the LS and CTL mice (68). Decreased osteocalcin concentrations would indicate decreased bone formation which is thus consistent with the bone loss experienced during lactation. With decreased leptin in the lactating mice, we might hypothesize that by mid-lactation, leptin signaling is still attenuating osteocalcin concentrations.

Alongside no changes in mean insulin concentration, the positive regulator of osteocalcin, bone formation continues to be reduced in the lactating mothers.

An interesting result in this study, as described in Section 4.3, was that trabecular indices were more heavily diminished at the mid-lactation than cortical indices, particularly at the mid-lactation point. These uneven results may be caused by different hormone actions on cortical and trabecular bone. For example, estrogen has been demonstrated to be protective of cortical bone in humans, and osteocalcin null rat models had improved trabecular bone with no changes to cortical bone (104,105). More recently, leptin has been shown to negatively affect trabecular bone while having a positive effect on cortical bone (106). This may explain why cortical mineralization was sustained with less severe decreases in cortical thickness. Looking at the lactation point, leptin was significantly increased in the LS mice compared to the CTL mice and when comparing the cortical microarchitectural results, we observed that lactating CTL had less cortical mineralization than the CTL postpartum females, whereas there was no change in the LS. Similarly, the percent decrease in cortical thickness is larger in the CTL mice than in the LS. Leptin may thus be more involved in bone metabolism in the LS mouse than in the CTL, producing differential effects on the cortical and trabecular compartments.

Given our results and as predicted by **H2**, it is clear that the endocrine axes involved in bone metabolism are altered in the Longshanks mice during lactation when compared to the Controls. In LS, PTH may have increased bone resorption through RANKL signaling and leptin may be inhibiting bone formation.

4.5 Decreased Longshanks litter size as a trade-off for increased fetal ossification.

Over the course of selective breeding for increased tibia length, LS lines had a marked decrease in litter size in unpublished observations. Larger skeletons in the LS require more

calcium during fetal ossification, however maternal calcium reserves are limited and therefore must be partitioned efficiently to increase reproductive success. Limitations from fixed energy budgets produce inevitable trade-offs, thus the increased bone volume may have forced LS to differentially allocate resources to increasing ossification at the expense of litter size – an offspring quantity vs. quality trade-off.

In our study, however, only LS1 had significantly lower litter sizes in the pooled PTP and LTP day 0 count. LS2 was still noticeably lower than CTL albeit not significant. Additionally, LS2 had one litter removed due to the complete cannibalization of her litter. Thus, though we did not find decreased litter size in the LS compared to the CTL, this may be due to the small sample sizes within the mouse lines used in this study likely causing new and stochastically driven proportions of allele frequencies in each mouse line, potentially leading to phenotypic alterations in both bone physiology and fertility. Without active selective breeding, it is therefore possible that litter size was altered in unpredictable ways in one or more of the mouse lines. Despite this, the decreased litter size pattern was still noticeable thus, we may still speculate about the costs of pregnancy and lactation in an evolutionary context.

Decreased litter size is, by definition, an indication of reduced evolutionary fitness (107). However, it may be necessary to free up maternal energy stores and reallocate these resources to other physiological processes or expensive tissues (107). In our study, we found that LS mice had a significantly stronger "protective" effect from pregnancy, but these gains were lost by mid-lactation. LS is thus mobilizing more calcium stores during pregnancy and lactation than the CTL mice. This may imply that the calcium demands in the LS mouse are higher despite having smaller litter sizes. Stated differently, the fetal ossification of a pup may be more demanding and costly in the LS than in a CTL dam. With a constrained resource budget, the higher calcium cost

of fetal ossification in the LS suggests that the energy must be offset elsewhere, in this case, we hypothesize the decreased litter size. It is likely that if a post-weaning timepoint was investigated, we would continue to see declines in LS maternal microarchitecture. The maternal system thus must balance the heavy costs of lactogenesis and the resources necessary for the self-maintenance of her own skeleton. In unpublished observations, we found that there were no differences in average P0 pup mass, however LS had increased total volume of bone invested by dams in their litter (Appendix B). The decreased litter sizes would allow this increased investment of bone per pup in the LS females without the potential pathological depletion of her own calcium resources.

Our study focused on the endocrine changes during pregnancy and lactation in the LS that would regulate maternal resources, however, the nutrient or resource itself would be the mineral, calcium. A few studies have reported a possible association between calcium and litter size. In rats, hypocalcemia has been associated with decreased litter size (108). In 2012, Schmidt and Hood found that in white-footed mice (*Peromyscus leucopus*), females who were fed low-calcium diets had decreased bone volume and produced smaller litters (109). As well, Schmidt and Hood (2012) observed that with reduced calcium intake, mothers allocated less calcium per individual offspring (109). Given that both LS and the low calcium white-footed mice both have decreased bone quantity, we suggest that calcium is a limiting resource in the maternal skeleton that may be forcing the reallocation of resources from litter size to fetal ossification.

The mechanisms of this possible trade-off would likely be through endocrine control. Our results demonstrate that several endocrine axes involved in bone homeostasis in the LS are altered from that of the CTLs. In particular, mean concentrations of catabolic hormones were noticeably higher in the LS mice throughout all three time points. Elevated catabolic hormones

would promote osteoclastic activity, thus enhancing bone resorption and calcium release from the maternal skeleton. LS is mobilizing more calcium through endocrine signaling which allows increased fetal ossification and more bone per pup, thus diminishing the available resource for litter size. This is evidently a costly process that has manifested in the LS bone microarchitecture, with diminished bone volume mid-lactation.

Though we are unable to determine a possible litter size vs. bone volume trade-off, increased bone investment per pup in LS and increased circulating catabolic hormones as observed in this study may be indicative of a bone volume trade-off at the expense of litter size in the LS.

4.6 Clinical implications for human skeletal health in pregnancy and lactation

Our results highlight the varying impacts of pregnancy and lactation on the bone which could be used to create health guidelines for specialized clinical considerations in premenopausal women with weakened skeletons. There is a critical lack of research and understanding of pathological bone loss associated with pregnancy and lactation, such as pregnancy-related and lactation-related osteoporosis (PLO) (38). PLO is rare and has only been reported in case reports and series, however has great physiological and psychological burdens, such as increased fracture and affecting working ability, in patients (38). The ethics involved, the lack of research, and poor awareness among clinicians, severely limit our knowledge of the possible risk factors, mechanisms, prognosis, and treatment (38). A large gap in the research involves a lack of BMD screening of premenopausal women, thus it is unclear if PLO is developed in women with a healthy bone phenotype and/or PLO exacerbates prior bone anomalies e.g., low BMD, and osteopenia (42).

LS is phenotypically similar to populations with reduced bone quantity and increased rigidity/fragility. Increased catabolic systemic hormones as seen in this study may be involved in the mechanisms seen in PLO. LS mothers experienced high lactational bone loss that likely would have continued until weaning. This would exacerbate the reduced bone quantity and subsequently, increase fragility and thus the risk for pain and fracture – as seen in PLO, therefore lactational bone loss and its associated transient osteopenia should be considered in populations of weaker, more fragile skeletons to avoid the risk of skeletal pain and fracture.

Currently, there are no standardized treatment protocols for PLO, however recommendations include lactation cessation combined with pharmacological agents, including hormone-based therapy (38). As previously mentioned, OPG has been demonstrated to rescue reduced bone volume phenotype and also prevent complete lactational bone loss in mice (99). In our study, OPG concentrations at postpartum were markedly higher in the LS than CTL, we thus hypothesize that particularly in LS mice, the potential protective effect observed at postpartum is driven, in part, by increased serum OPG. Given our results, OPG could also be a potential target for therapeutics in an altered skeletal phenotype as seen in LS and women with osteopenia/PLO.

CHAPTER 5: CONCLUSIONS

The main aim of this study was to investigate the impact of pregnancy-related and lactation-related changes on bone microarchitecture using a mouse model with increased bone fragility. Both Longshanks lines showed trends towards increases in bone quality at postpartum, however, had substantial decreases at mid-lactation when compared to Controls, particularly in the trabecular compartment. Given these results, we conclude that the physiological response of increased bone postpartum varies depending on pre-pregnancy population characteristics (thus inducing a stronger response in LS) and is possibly a mechanism to help protect maternal calcium reserves from excessive depletion experienced during rapid fetal ossification and lactation.

The differences in LS vs. CTL maternal microarchitecture may be due to altered endocrine axes as seen in this study. More specifically, the larger improvements in LS bone postpartum may be induced by increased OPG signalling, allowing LS to increase larger amounts of maternal reserves than CTL despite its baseline reduced bone quantity/quality. Bone resorption is likely increased in the LS during lactation. Consistent with the diminished bone volume and quality in the maternal microarchitecture as seen in the LS, lactating LS females had higher concentrations of PTH and leptin and low concentrations of OPG and osteocalcin, than the CTL counterparts.

Increased mobilization of maternal stores implies that fetal ossification is particularly costly in the LS, and thus may have resulted in decreased litter size observed in the previous generations. Further studies require increasing sample sizes to better assess litter size differences and continuing our study by investigating postweaning-associated changes in the maternal skeleton to assess skeletal recovery.

CHAPTER 6: LIMITATIONS & FUTURE DIRECTIONS

6.1 Limitations

6.1.1 Skeletal phenotyping by µCT scanning.

µCT scanning is currently considered the "gold" standard when investigating bone microarchitecture of rodents, however there are still considerations to be noted, such as scan time, isotopic resolution, and selection of region of interest. (110). The scans in our study were scanned at a resolution of 15.6µm. Isotropic voxel size is an essential consideration in µCT scanning (110). Mice trabeculae typically fall within the range of 20 to 60µm, thus it is recommended that the ratio of voxel size and object size is at least equal to two (110). Scanning at high resolutions, however, significantly increases scan times. Because LS has thinner trabeculae than that of the CTL mice, it may be possible that not all trabeculae in the LS were captured in our analysis, or trabecular thickness may be over-estimated with larger voxels placed on thinner bone.

The region of interest in our scans began 10 slices from the distal tip of the posterior side of the proximal tibial epiphysis. Because the LS tibia is longer than the CTL, the CTL scan would encompass more of the diaphysis region, thus cortical measurements, especially, would be affected. More specifically, the LS tibia was likely under-sampled whereas the CTL tibia was over-sampled. In future analyses, it would be beneficial to investigate if a percentage of the bone length or choosing another landmark in the diaphysis as the set endpoint of the region of interest significantly alters the trabecular and cortical parameters as presented in this study.

6.1.2 Hormone analysis by Immunoassays – Biological and pre-analytical variability.

Though enzyme immunoassays are the most frequently used method for quantifying circulating hormone concentrations, the results are prone to be skewed from several points of

variability and thus reproducibility may be questioned (103,111). Intra-assay and inter-assay coefficients of variation are reported in Appendix D. Many hormones analyzed in this study (e.g., insulin, leptin) are secreted in a pulsatile pattern that follows circadian rhythms and/or highly contingent on animal behaviours, such as feeding and stress (103). The hormones were all sampled in the morning within 2 hours, and thus this would reduce variability associated with circadian rhythms. Though mice are most active between dusk and dawn, it is possible that some females may have fed just prior to their sample collection (112). This would cause a spike in serum glucose and subsequently, insulin and other metabolic hormones (113). FSH is also prone to high biological variability due to its variation during the estrus cycle (114). In our study, the most variability was seen at baseline, and this is likely due to females being at different stages of their cycle. Future analysis should consider fasting mice prior to sample collection and vaginal cytology for estrus staging.

Notably, our pregnancy time point was set as day of parturition, however parturition in itself is an intensive process that requires several physiological changes that may have been captured in our hormonal data (115). Our pregnancy time point is thus more accurately a parturition or early lactation time point. It would be interesting to investigate a late gestation time-point in future studies. Fetal ossification occurs exponentially at approximately E16 during gestation, it is likely that maternal bone turnover would also be increased to meet this sudden demand (98). In a pilot study, we did try to investigate this time-point longitudinally with a saphenous vein bleed, but very low blood volume outputs raised animal welfare concerns and we thus opted for a cross-sectional design in this present study instead.

6.1.3 Other possible mechanistic explanations of changes in the microarchitectural data.

Another key limitation of this study is that the changes in the bone microarchitecture observed may be a result of and/or influenced by other mechanisms, rather than solely systemic endocrine control. Our study measured circulating serum data, thus limiting us to endocrine mechanisms. It is possible that the changes associated with pregnancy and lactation in the bone microarchitecture may be a result of paracrine and/or autocrine signalling (116,117). For example, osteoblasts, osteoclasts, and osteocytes can secrete molecules that influence the differentiation and activity of each other without systemic involvement (116,117).

Additionally, we did not investigate mechanical loading. The increased mechanical stresses on bone during pregnancy may have influenced osteoblastic and osteoclastic activity through osteocyte mechanosensation. This would induce microarchitectural changes in the maternal skeleton (26). However, in humans and rodents, several studies have corroborated that load-bearing during pregnancy is predominantly at the vertebral sites (84,118–120). For example, De Bakker et al. (2017) reported that during pregnancy in rats, the proximal tibia had a lower proportion of total load applied to the bone, however had severe deterioration of the microarchitecture, conversely at the lumbar vertebral site, the trabecular bone bore a substantially greater share of total applied load with minimal trabecular bone loss (119). These results along with others suggest that the changes to the trabecular microarchitecture in the long bones, particularly the tibia, are associated with a more metabolic role in pregnancy whereas the vertebra may be more involved in maintaining structural integrity from increased mechanical loading (120). These findings alongside our investigation indicate that the bone loss seen at mid-lactation compared to postpartum females in LS and CTL is likely predominantly from upregulated bone metabolism, rather than mechanical-induced remodeling. Nonetheless, the long

bones still receive some level of increased mechanical stress with pregnancy and thus, future analysis of indicators of biomechanical properties in LS vs. CTL mothers would be interesting and help further illustrate the microarchitectural changes on compromised bone in pregnancy and lactation.

6.1.4 Litter sizes – Embryo loss & cannibalization.

This study did not account for possible embryo loss or resorption. Mothers were removed daily from their cages for weigh-ins. Chernoff et al. (1988) found that in CD-1 mice, restraint stress significantly increased embryo and fetal mortality (121). In our study, day of parturition was approximately around D18-19 of gestation among all families. This is earlier than the typical 19-22 gestation length seen in CD-1s and may be partially due to increased stresses during pregnancy (122).

Genetic differences, possibly from inbreeding-associated genetic drift, between CTL and LS lines may have affected labour and cause decreased sensitivity to environmental stresses in LS lines, resulting in fewer embryos lost in LS. Future experiments could include ultrasound examinations to monitor these possible losses and/or uterine dissections at E10 to count embryos in the uterine horn. This will determine if the observed reduced litter size is likely due to other problems, e.g., labour. During lactation, the mothers and pups were only measured on day 0 and day 12 of pre-weaning to reduce stress, however, cannibalization may have occurred in this timeframe and thus, final litter sizes may not be accurate.

We were also interested in the possible evolutionary implications of our study with the observation of lower litter sizes in the LS. Though litter size was only decreased in LS1 compared to CTL, this may have been due to differential embryo loss, stress, or stochastic

selection. A larger sample size would be more representative of the mean litter size in the LS and CTL mice populations and allow us to better investigate this potential trade-off.

6.1.5 The LS model.

Both mice and humans experience bone mass and microarchitecture changes during pregnancy but differ in magnitude and direction. Rodents exhibits a state of hyperthyroidism during pregnancy and experience severe bone losses during lactation. In humans, there is a much more attenuated response. Though humans also experience bone loss with pregnancy, it may result from other mechanisms rather than the hormones explored in this study. Notably, our study investigated PTH, however in humans, PTHrP is upregulated in pregnancy while PTH is suppressed (18). Nevertheless, LS is particularly useful in studying bone-related changes with pregnancy/lactation due to the lack of research and understanding of the effect of pregnancy and lactation on a compromised, osteopenia-like skeletal phenotype with reduced bone volume.

6.2 Future directions

6.2.1 Fetal ossification.

This study was strictly focused on maternal physiology and bone microarchitecture; however, fetal ossification should also be investigated to determine if the altered pregnancy/lactation associated changes in LS maternal bone morphology and hormonal axes seen in this study differentially affect fetal mineralization compared to CTLs. The findings in this study imply that LS lose more bone during lactation and by assessing bone quantity in the pups, we could determine if bone investment in LS is thus greater, perhaps due to LS' larger skeletons. Additionally, by investigating the relationship between fetal ossification and maternal systems, we may be able to further probe the anecdotal reduced litter size observation in the LS, that is,

larger fetal skeletons may limit the number of offspring that a mother can carry relative to her available bone tissue.

6.2.2 Calcium.

Calcium and other calciotropic minerals should be quantified and investigated in future studies. Mechanistic investigations were focused on endocrine control, however by evaluating the mineral itself, this would elucidate if these hormonal fluctuations seen in this study are, in fact, associated with changes in maternal circulating calcium rather than e.g., increased hormone concentration from other biological variability, such as feeding and physical activity (123,124). This would also help confirm if the increased catabolic hormone concentrations in the LS compared to CTL did indeed result in larger amounts of calcium being released from the LS maternal skeleton into circulation.

6.2.3 Recovery after lactation.

Post-weaning recovery in female bone quantity/quality is well documented in humans and rodents; yet, this has not been investigated in an altered phenotype with reduced bone quantity and increased fragility as seen in the LS (81). Because LS induced a stronger potential protective effect at pregnancy, it may be possible that LS may also have stronger mechanisms for recovery in order to further avoid complete depletion and help maintain the maternal skeleton, or the costs of lactation in the LS may severely impact the maternal skeleton without proper recovery. This is an important due to the lack of research in individuals with less and weaker bone. With the known bone loss experienced in lactation and potentially without proper homeostatic recovery, individuals with e.g., osteopenia may develop pathological conditions post-weaning. Investigating this gap in research will provide a more comprehensive understanding of the costs associated with pregnancy and lactation in weaker bone.

CHAPTER 7: TABLES & FIGURES

7.1 Tables

	Line		
	Control	**LS1**	**LS2**
BL	30.852 (3.024) n = 13	30.797 (2.751) n = 12	28.388 (2.016) n = 12
PTP	42.667 (3.548) n = 12	43.110 (3.821) n = 12	42.101 (3.290) n = 11
LTP	50.993 (3.670) [1] n = 13	55.713 (2.944) [C,2] n = 12	51.151 (2.551) [1] n = 12

Table 1. Mean dam body mass (g) and sample sizes among lines at each time point (BL = baseline, PTP = postpartum, LTP = mid-lactation). Dam weight is shown as mean (SD). Superscripts denote significant differences in means ($p < 0.05$) between a given group and time-point: [C]Control, [1]Longshanks-1, [2]Longshanks-2.

	Line		
	Control	**LS1**	**LS2**
PTP	13.524 (2.294) n = 21	10.417 (2.765) n = 24	12.522 (2.313) n = 23
LTP	12.556 (2.128) [1] n = 9	10.818 (2.359) [C,2] n = 11	12.308 (2.496) [1] n = 12

Table 2. Mean litter size (pups) with sample count among lines at each postpartum (PTP) and mid-lactation (LTP) time-point. Postpartum time-point includes pooled P0 count from mid-lactation females. Litter size is shown as mean (SD). Superscripts denote significant differences in means ($p < 0.05$) between a given group and time-point: [C]Control, [1]Longshanks-1, [2]Longshanks-2.

	CTL			LS1			LS2		
	BL	**PTP**	**LTP**	**BL**	**PTP**	**LTP**	**BL**	**PTP**	**LTP**
BV/TV (%)	0.104 (0.021) n =11	$0.114^{1,2}$ (0.029) n =11	0.074^{2} (0.014) n = 10	0.105 (0.026) n = 11	$0.191^{C,2}$ (0.038) n = 11	0.081^{2} (0.012) n = 12	0.085 (1.131) n = 13	0.131^{1} (0.015) n = 10	$0.049^{C,1}$ (0.018) n =12
Tb.Th (mm)	$0.076^{1,2}$ (0.004) n = 12	0.077 (0.004) n = 10	0.064 (0.003) n = 10	0.063^{C} (0.005) n = 11	0.082 (0.008) n = 12	0.063 (0.002) n = 13	0.064^{C} (0.005) n = 13	0.074 (0.003) n = 9	0.063 (0.003) n = 13
Ct.Th (mm)	$0.263^{1,2}$ (0.010) n = 11	0.285^{1} (0.018) n = 11	0.240 (0.021) n = 11	$0.216^{C,2}$ (0.018) n = 11	0.261^{C} (0.017) n = 12	0.233 (0.019) n = 13	$0.239^{C,1}$ (0.023) n = 12	0.271 (0.011) n = 10	0.236 (0.015) n = 12
Ct.BMD ($mgHA/cm^3$)	$945.671^{1,2}$ (30.481) n = 11	$1002.241^{1,2}$ (11.251) n = 10	966.385^{1} (31.435) n = 11	$822.616^{C,2}$ (15.628) n = 9	$925.555^{C,2}$ (22.245) n = 12	$933.245^{C,2}$ (22.245) n = 12	$857.597^{C,1}$ (11.290) n = 11	$958.120^{C,1}$ (10.051) n = 10	954.470^{2} (16.826) n =12

Table 3. Mean trabecular and cortical bone data from the tibial metaphysis for all three lines at baseline (BL), postpartum (PTP), and mid-lactation (LTP). Data presented as mean (SD). Superscripts denote significant differences in means ($p < 0.05$) between a given group and time-point within a bone variable: ^{C}Control, ^{1}Longshanks-1, ^{2}Longshanks-2.

	CTL			LS1			LS2		
	BL	**PTP**	**LTP**	**BL**	**PTP**	**LTP**	**BL**	**PTP**	**LTP**
PROL (ng/mL)	25.228 (24.460) n = 13	57.351[1,2] (15.950) n = 11	48.381 (40.998) n = 10	14.639 (17.406) n = 11	17.002[C] (12.774) n = 12	28.839 (28.839) n = 13	10.198 (10.322) n = 12	23.970[C] (15.970) n = 11	27.634 (18.117) n = 13
FSH (pg/mL)	277.058 (121.709) n = 12	727.773[1] (650.895) n = 12	407.683 (67.786) n = 9	458.339 (491.261) n = 10	287.020[C] (315.039) n = 13	348.324 (108.126) n = 12	613.156 (181.424) n = 11	181.424 (81.748) n = 10	383.430 (98.233) n = 13
TSH (pg/mL)	116.462 (76.899) n = 12	183.143 (110.551) n = 12	283.693 (163.299) n = 10	127.121 (94.479) n = 9	294.622 (224.716) n = 13	229.432 (107.461) n = 13	150.575 (79.569) n = 11	240.987 (119.545) n = 12	184.586 (60.910) n = 12
PTH (ng/mL)	15.109 (13.703) n = 7	12.418 (12.203) n = 7	8.217[1,2] (6.541) n = 8	24.332 (19.004) n = 8	38.055 (18.851) n = 8	67.757[C] (55.842) n = 8	27.298 (22.406) n = 7	28.033 (21.403) n = 8	68.871[C] (45.962) n = 7
OPG (pg/mL)	171.341 (69.033) n = 7	237.069 (48.901) n = 7	205.549 (39.468) n = 8	174.618 (56.846) n = 8	304.287 (71.823) n = 8	166.509 (51.580) n = 8	146.369 (57.421) n = 7	303.977 (87.338) n = 8	142.236 (37.138) n = 8
SOST (pg/mL)	245.833 (78.522) n = 6	384.750[1] (127.766) n = 6	183.689 (93.986) n = 7	566.763 (125.976) n = 8	1017.063[C] (576.652) n = 8	451.675 (149.764) n = 8	597.686 (256.404) n = 7	745.929 (583.213) n = 7	504.471 (125.470) n = 7
Insulin (uiU/mL)	92.133[1] (32.362) n = 8	117.093 (66.727) n = 7	101.623 (59.594) n = 8	134.875[C] (26.282) n = 8	102.800 (14.291) n = 8	111.058 (14.291) n = 8	111.673 (16.871) n = 8	111.800 (42.214) n = 8	121.163 (31.828) n = 8
Leptin (pg/mL)	522.775 (163.200) n = 7	1049.472[1,2] (213.019) n = 7	251.422[1,2] (72.713) n = 8	122.662 (41.427) n = 8	2131.191[C] (864.173) n = 8	1165.136[C] (242.535) n = 8	207.923 (58.242) n = 7	1618.172[C] (661.592) n = 8	813.518[C] (560.241) n = 7
Osteocalcin (ng/mL)	13.923 (2.649) n = 8	10.989 (2.238) n = 8	8.775[2] (2.469) n = 7	15.497 (3.437) n = 8	10.486 (3.270) n = 8	11.055 (2.504) n = 8	18.025 (3.268) n = 7	11.067 (1.001) n = 8	12.726[C] (2.225) n = 8

Table 4. Mean circulating hormone data from serum of all three lines at baseline (BL), postpartum (PTP), and mid-lactation (LTP). Data presented as mean (SD). Superscripts denote significant differences in means ($p < 0.05$) between a given group and time-point within a hormone variable: [C]Control, [1]Longshanks-1, [2]Longshanks-2.

7.2 Figures

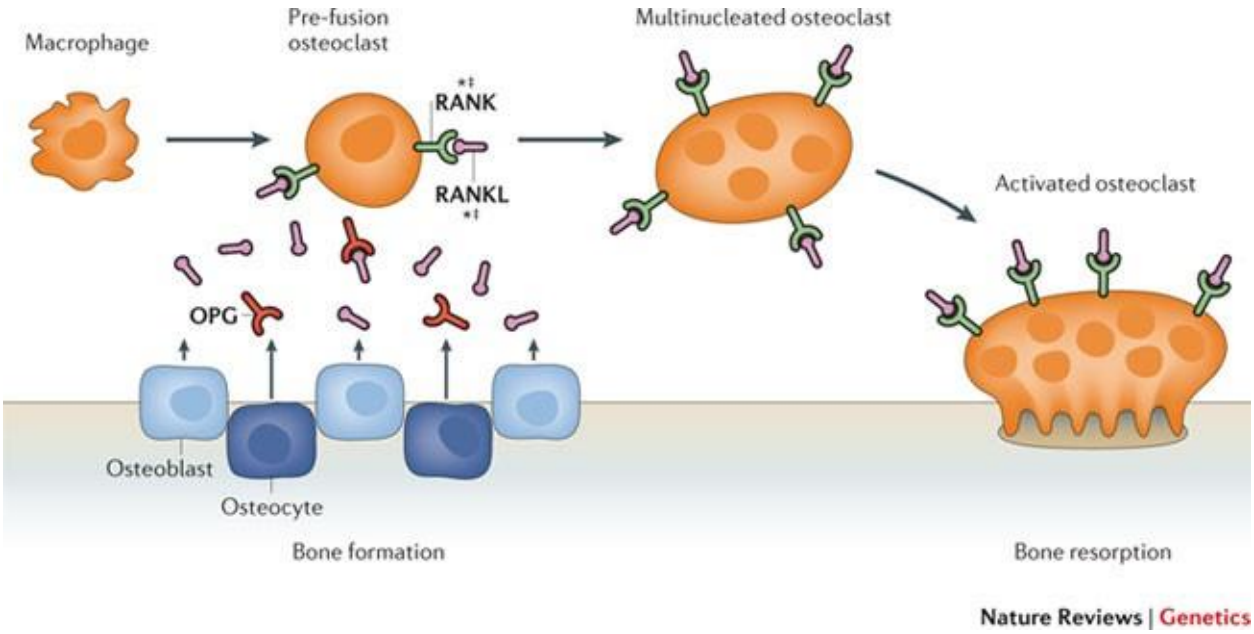

Figure 1. Proteins identified as genome-wide-significant through genome-wide association studies (GWASs) are indicated in bold font and with a bold outline. RANK is encoded by tumour necrosis factor receptor superfamily, member 11a (TNFRSF11A), its ligand RANKL is encoded by TNFSF11, and the decoy receptor OPG is encoded by TNFRSF11B. To generate activated osteoclasts, RANKL is secreted by osteoblasts and osteocytes in bone, and these bind to its natural receptor, RANK, on the surface of pre-fusion osteoclasts. To fine-balance this activation system, osteoblasts and osteocytes also secrete OPG, which is a natural decoy receptor for RANKL and prevents binding of RANKL to RANK. *Indicates the relevance of the gene to human monogenic skeletal disease. ‡Indicates genes with evidence arising from mouse knockouts. Reuse from "Genetics of osteoporosis from genome-wide association studies: advances and challenges." By Richards, J., Zheng, HF. & Spector, T. *Nat Rev Genet* **13**, 576–588 (2012). Copyright 2012 by Springer Nature. Reuse with permission.

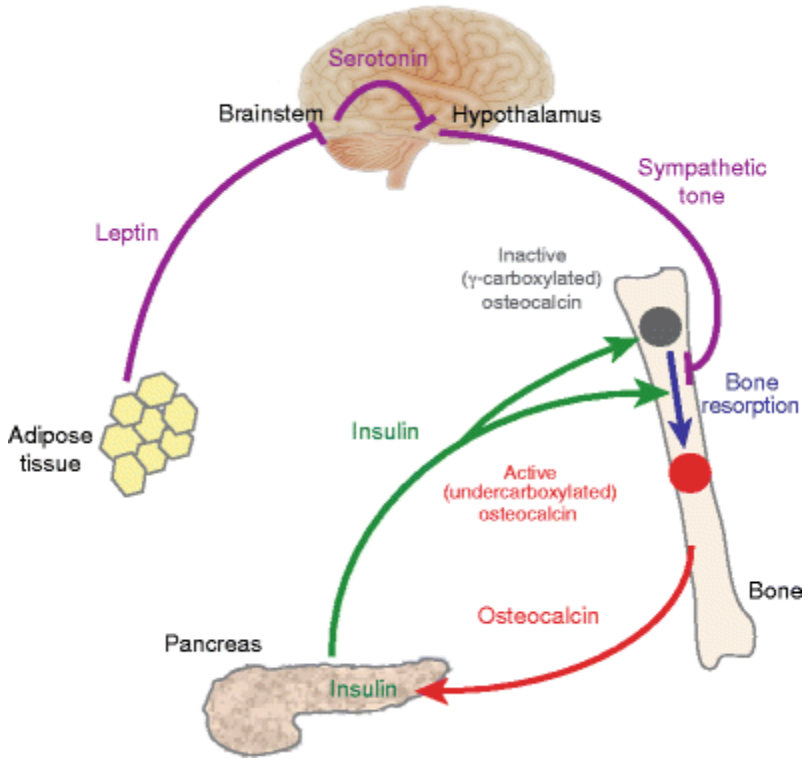

Figure 2. Schematic representation of the cross-talk between bone and energy metabolism. Carboxylated osteocalcin is secreted by osteoblasts and binds to the bone matrix. It is then released and activated through decarboxylation during bone resorption. Released undercarboxylated, i.e. active, osteocalcin reaches pancreatic beta cells to increase their proliferation and enhance the production and release of insulin. In turn, insulin stimulates osteocalcin production by osteoblasts and favours its activation through an osteoblast-dependent activation of bone resorption. In contrast, leptin secreted by adipocytes and signalling to brainstem neurons inhibits the production of brain serotonin, which itself is a negative regulation of sympathetic output, and this results in an increase of sympathetic tone. The sympathetic tone is a negative regulator of bone resorption, and this leptin-dependent regulatory mechanism inhibits osteocalcin activation. Reuse from "The role of osteocalcin in the endocrine cross-talk between bone remodelling and energy metabolism." By Ducy, P. *Diabetologia* **54**, 1291–1297 (2011). Copyright 2011 by Springer Nature. Reuse with permission.

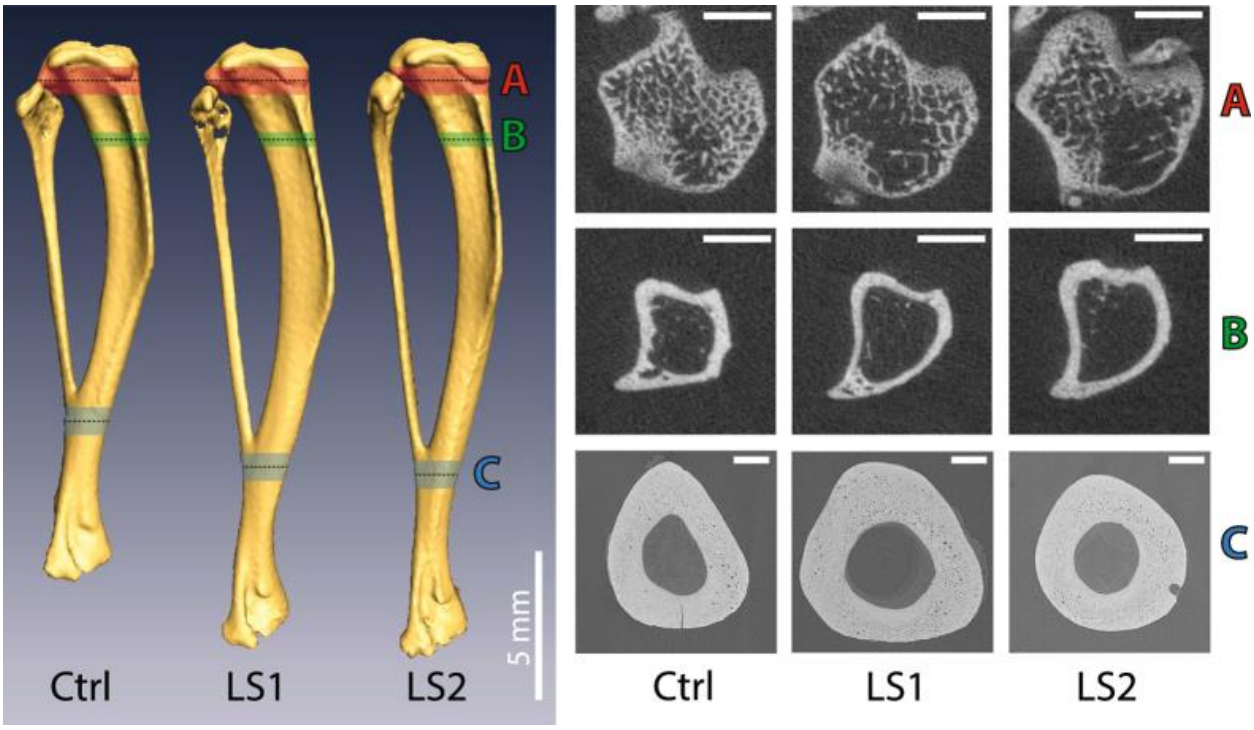

Figure 3. Representative tibiae and cross-sections. Whole tibiae are shown on the left, with the location and size of the stacks used for µCT analyses of trabecular bone (A, red), proximal cortical bone (B, green), and distal diaphysis cortical bone (C, blue). The black dashed lines indicate the middle of each stack, where the transverse sections shown on the right were taken. Scale bar for A and B = 1 mm, for C = 250 µm. Abbreviations: Ctrl: Control, LS1: Longshanks 1, LS2: Longshanks 2. Reuse from "Cortical and trabecular morphology is altered in the limb bones of mice artificially selected for faster skeletal growth" By Farooq, S., Leussink, S., Sparrow, LM., Marchini, M., Britz, HM., Manske, SL., Rolian, C. *Sci Rep* **7**, 10527 (2017). Copyright 2017 by Authors. Reuse with permission.

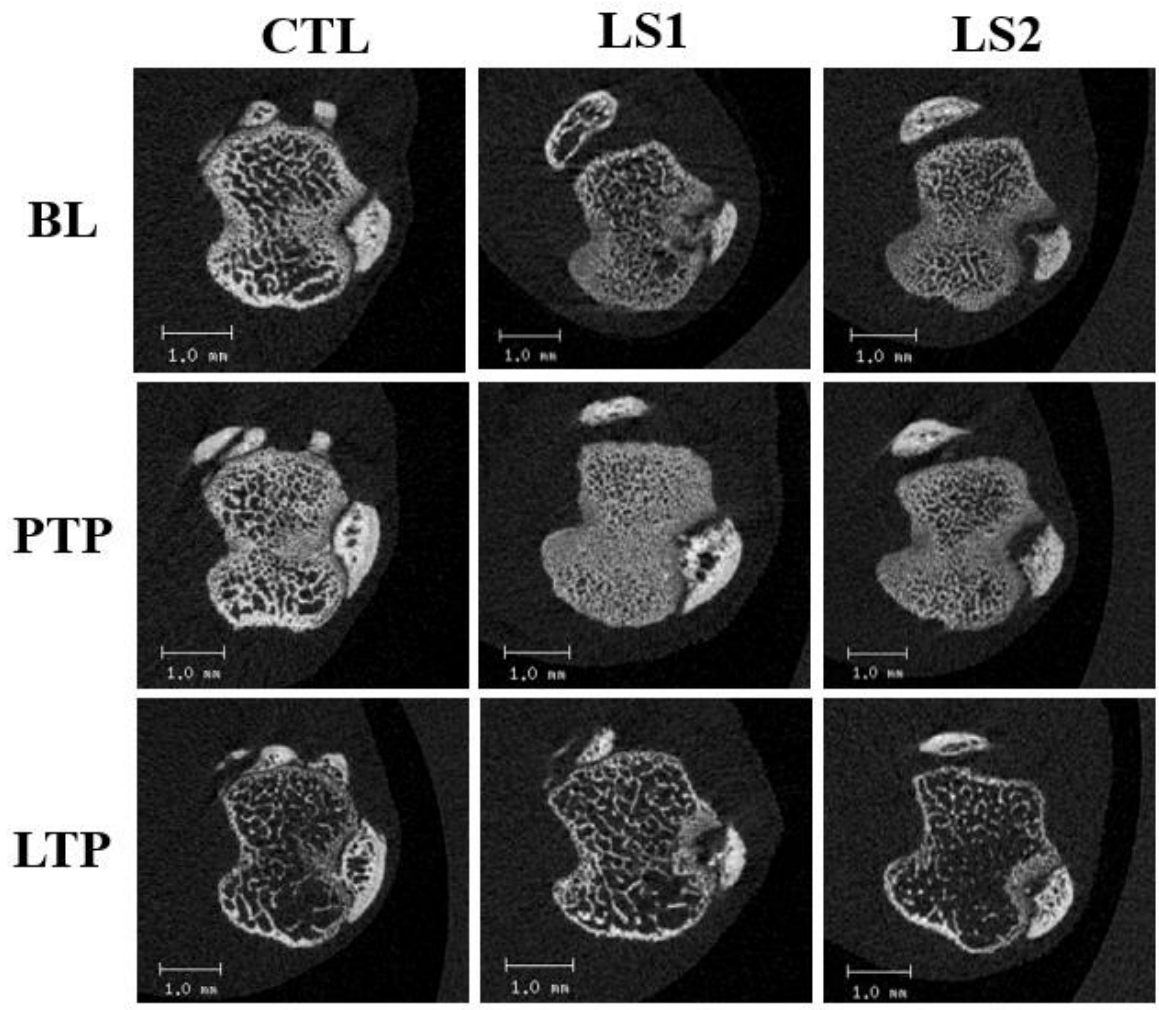

Figure 4. Representative right promixal tibiae cross-sections depicting approximate start location (10 slices from the distal tip of the posterior side of the proximal tibial epiphysis) of μCT analyses of trabecular bone in the nulliparous (BL), postpartum (PTP), and lactating (LTP) females. CTL: Control, LS1: Longshanks 1, LS2: Longshanks 2. Scale bar set as 1 mm.

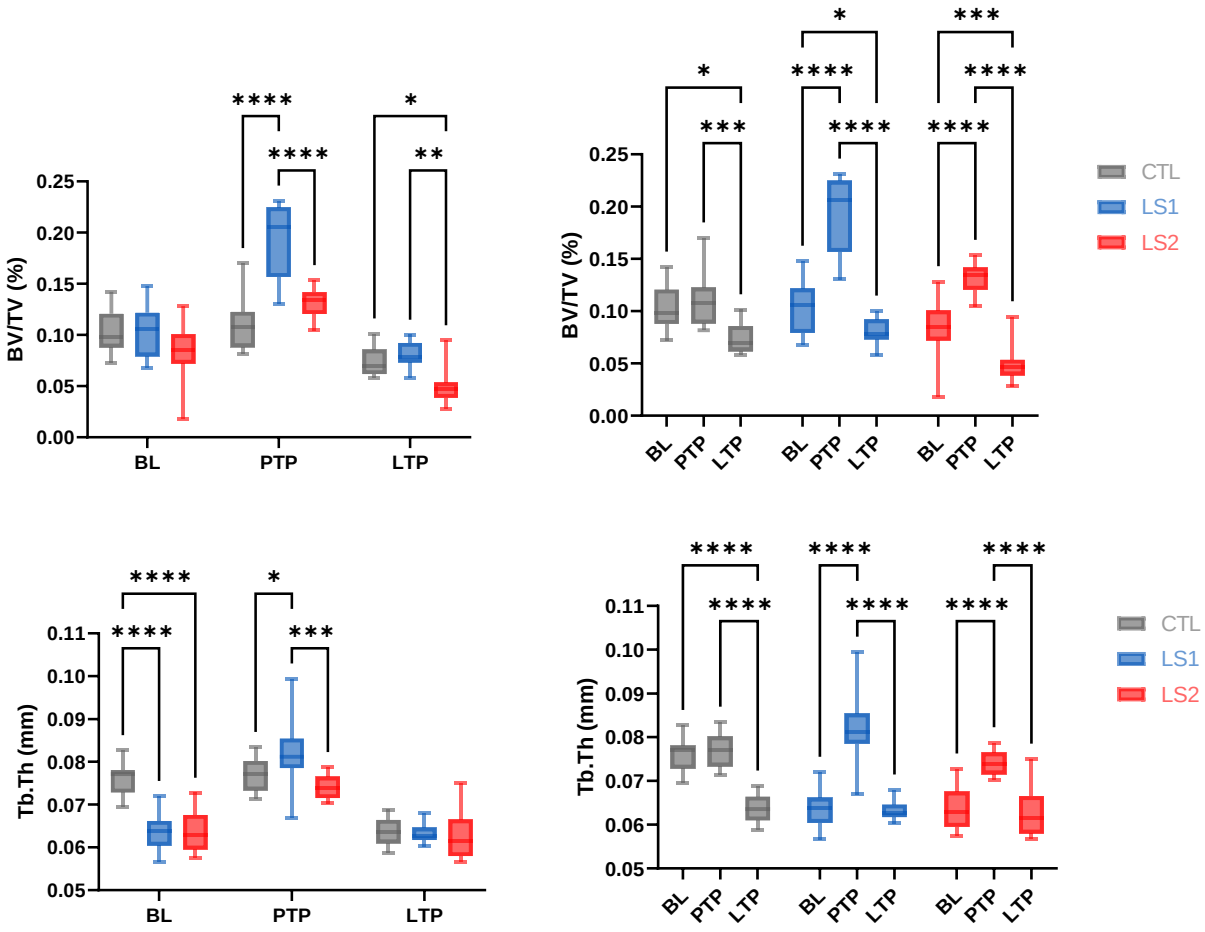

Figure 5. Box plot graphs representing trabecular bone microarchitectural data at baseline (BL), postpartum (PTP), & mid-lactation (LTP) for all three lines. Box plot graphs to the left represent comparisons between lines at each time-point. Box plot graphs on the right depict comparisons between each time-point within lines. **P<0.05.*

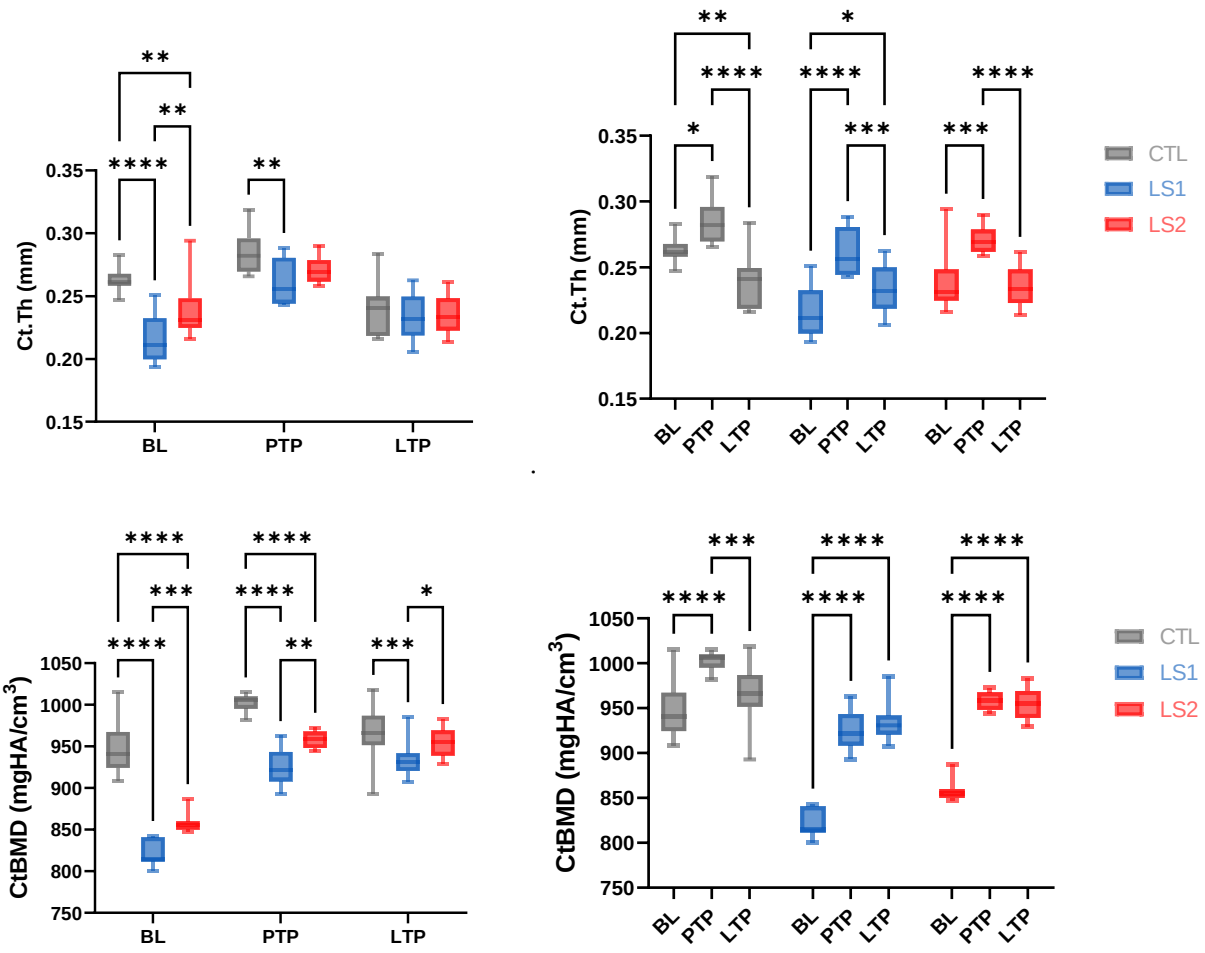

Figure 6. Box plot graphs representing cortical bone microarchitectural data at baseline (BL), postpartum (PTP), & mid-lactation (LTP) for all three lines. Box plot graphs to the left represent comparisons between lines at each time-point. Box plot graphs on the right depict comparisons between each time-point within lines. **P<0.05.*

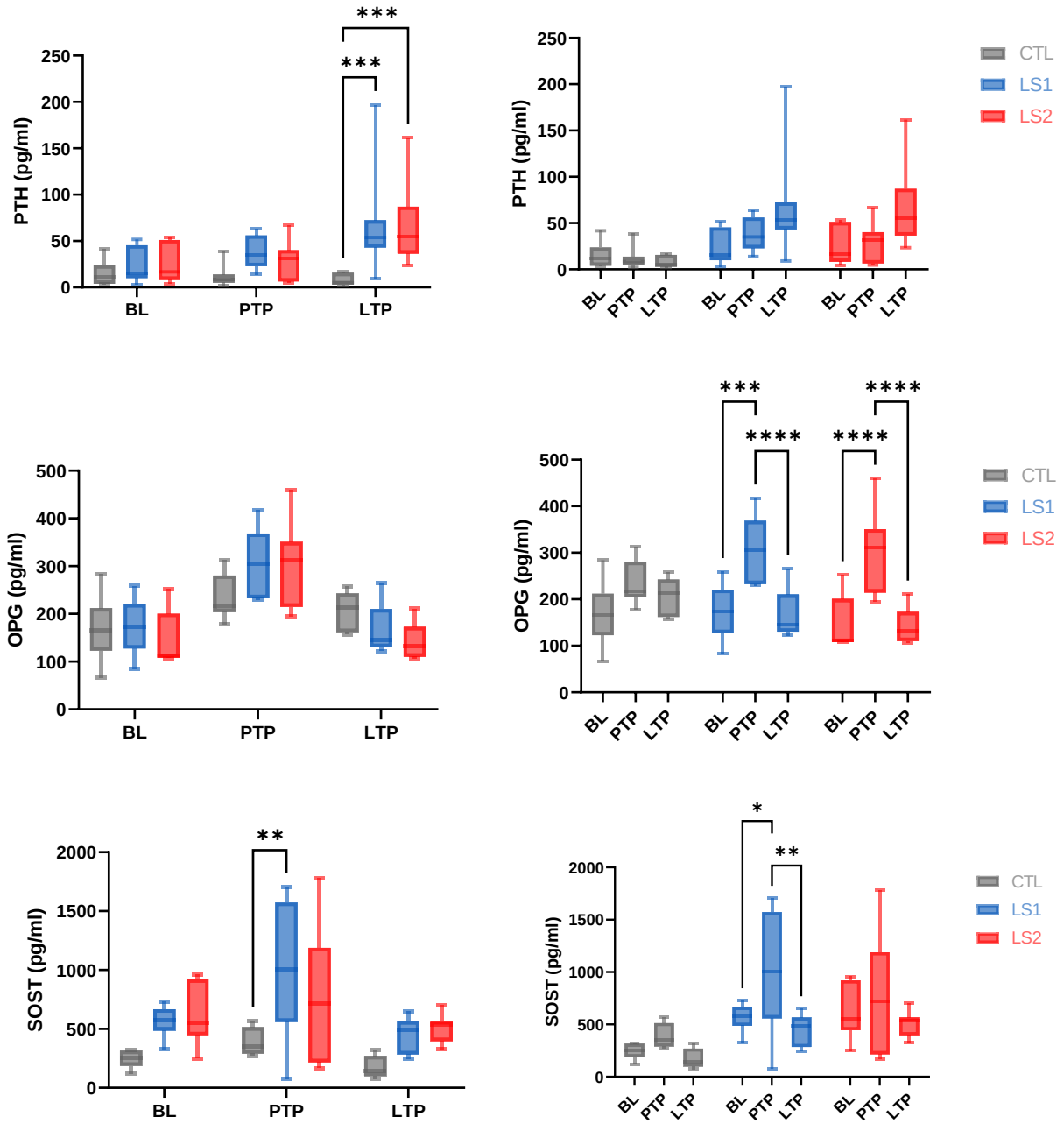

Figure 7. Box plots graphs representing circulating hormone concentrations of parathyroid hormone (PTH), osteoprotegerin (OPG), & sclerostin (SOST) in serum at baseline (BL), postpartum (PTP), & mid-lactation (LTP) for all three lines. Box plot graphs to the left represent comparisons between lines at each time-point. Box plot graphs on the right depict comparisons between each time-point within lines. *$P<0.05$.

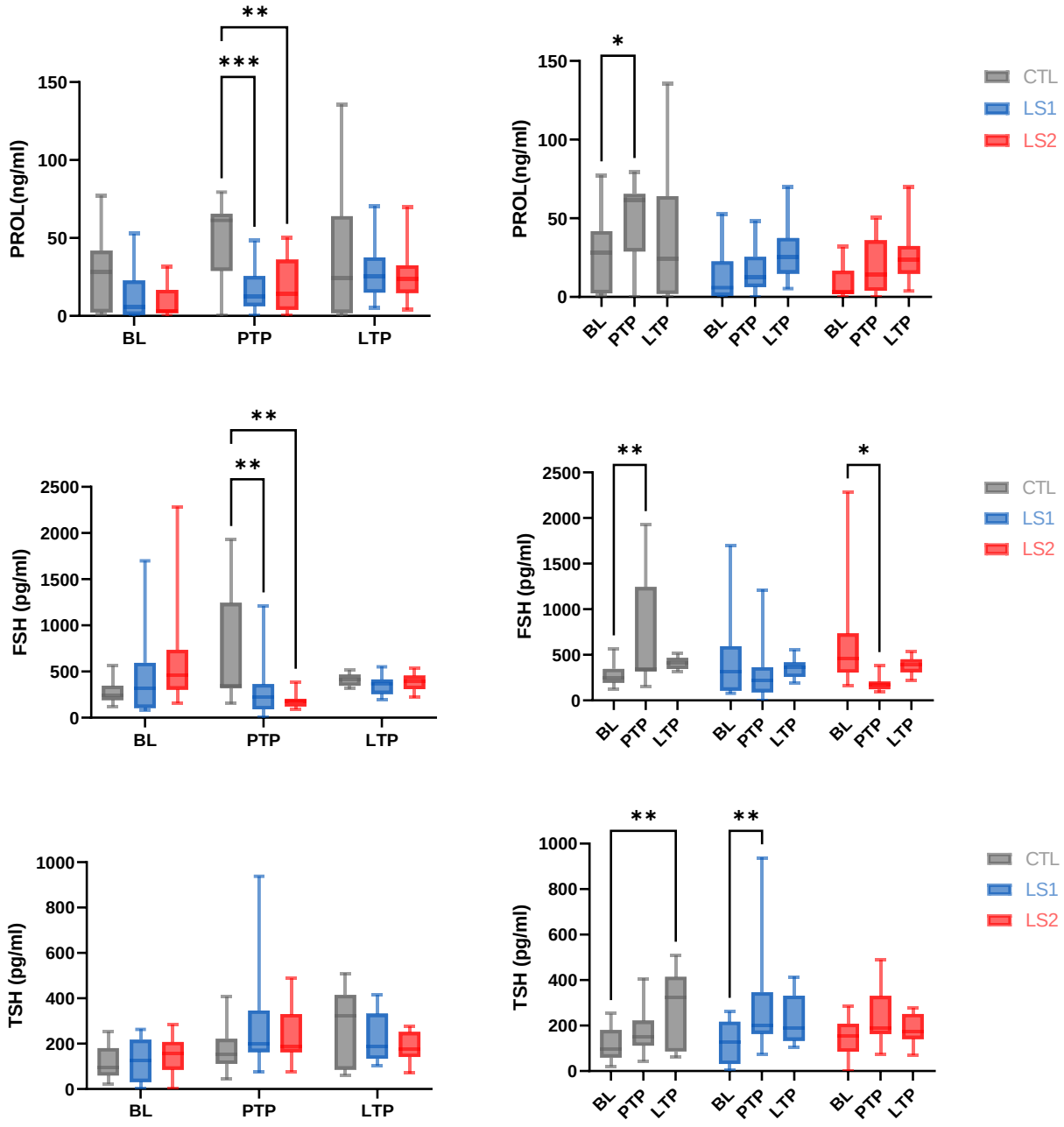

Figure 8. Box plots graphs representing pituitary hormone concentrations of prolactin (PROL), follicle-stimulating hormone (FSH), & thyroid-stimulating hormone (TSH) in serum at baseline (BL), postpartum (PTP), & mid-lactation (LTP) for all three lines. Box plot graphs to the left represent comparisons between lines at each time-point. Box plot graphs on the right depict comparisons between each time-point within lines. *$P<0.05$.

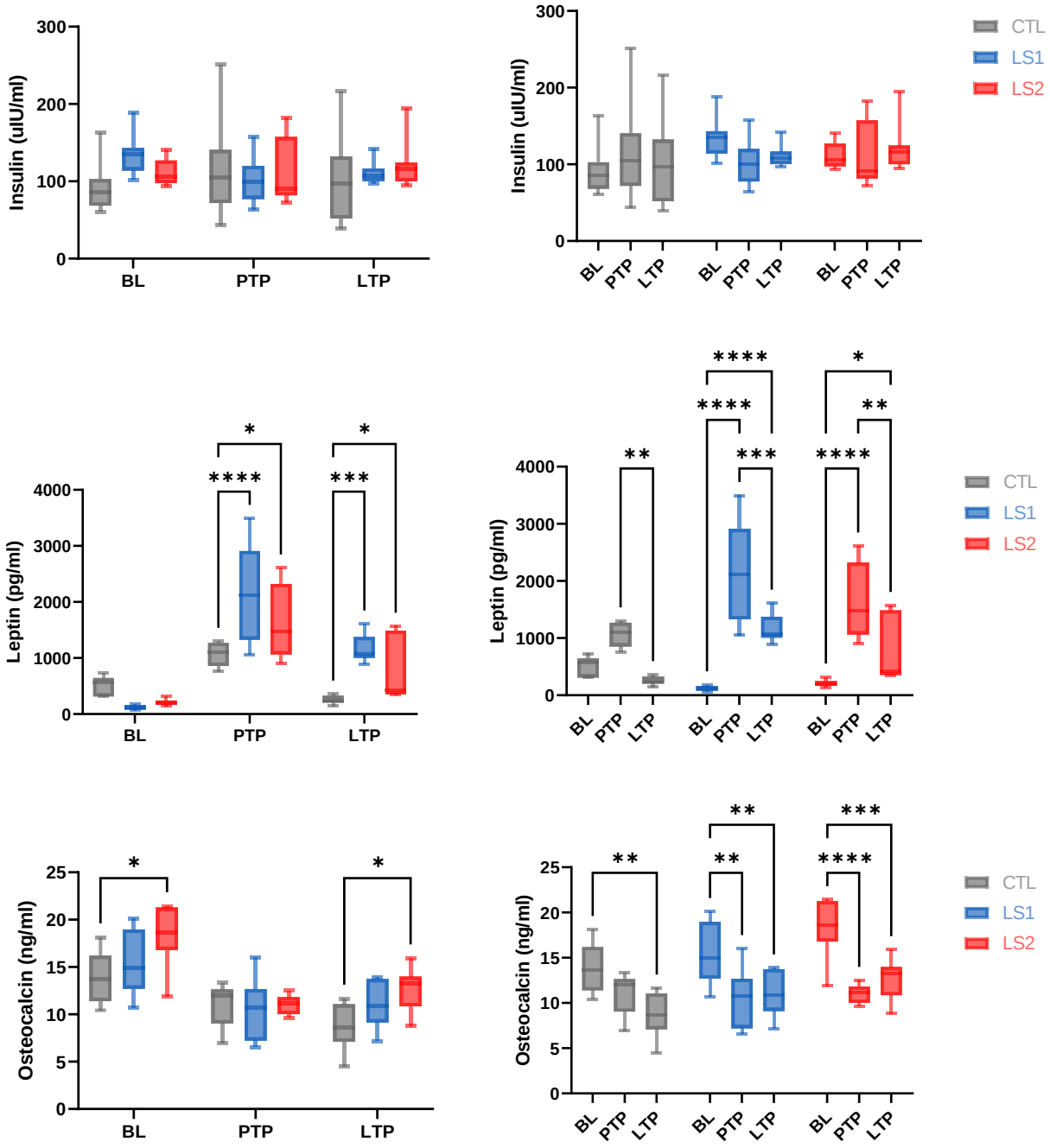

Figure 9. Box plots graphs representing circulating hormone concentrations of insulin, leptin, & osteocalcin in serum at baseline (BL), postpartum (PTP), & mid-lactation (LTP) for all three lines. Box plot graphs to the left represent comparisons between lines at each time-point. Box plot graphs on the right depict comparisons between each time-point within lines. *$P<0.05$.

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Figure 2.

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APPENDIX B: Unpublished observations

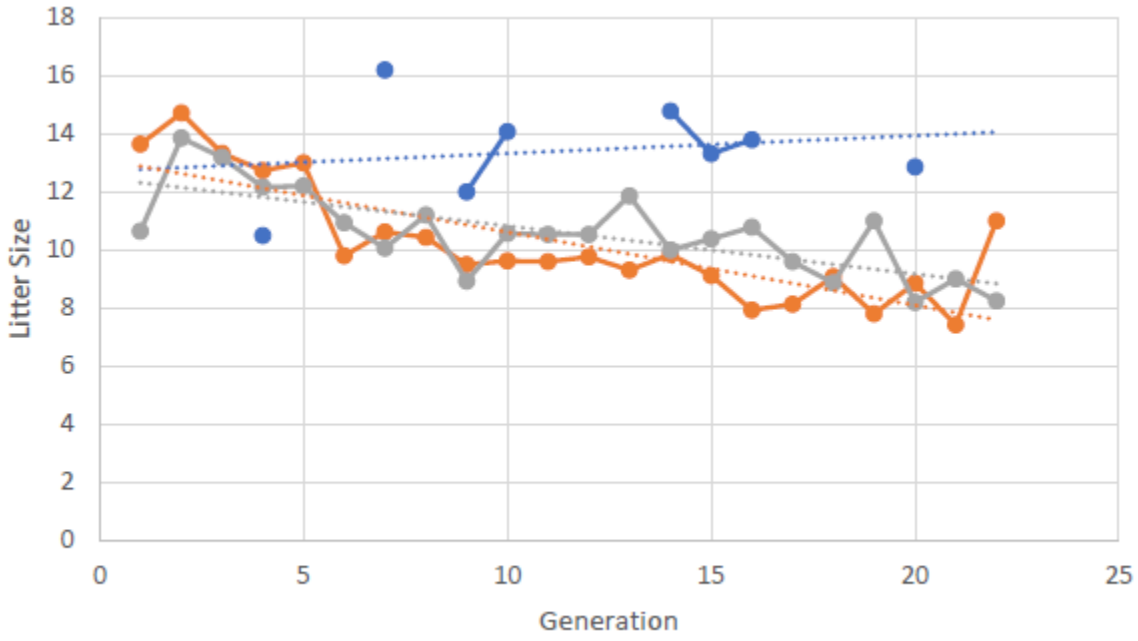

Figure 1. Two lines of F20 mice with increased tibia length by artificial selection (LS1 as orange, LS2, as grey) litter size over 20 generations compared to Control mice (blue). Control mice were only measured for tibia length, body mass, and litter size at the generations shown.

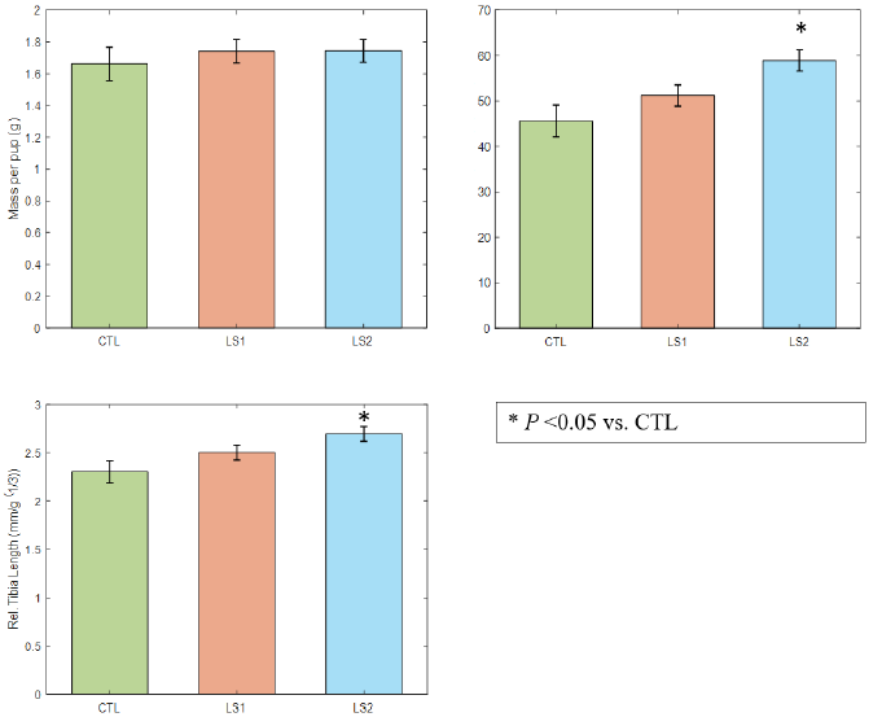

Figure 2. Bar graphs representing lease-squared mean mass and bone volume per pup, and relative tibia length after controlling for litter size of P0 pups of all three lines (CTL = green, LS1 = orange, LS2 = blue. **P<0.05 vs CTL.*

APPENDIX C: Certificate of Animal Use Protocol

UNIVERSITY OF CALGARY

Certificate of Animal Use Protocol Approval

To: Campbell Rolian

RE: Renewal 2 for ACC Study #AC17-0026

Title: Investigating the developmental genetic basis and functional consequences of increased limb bone length in a selectively bred mouse population

Effective: 3/29/2019 Expires: 3/28/2020

Sponsoring Agency(s):
Burroughs Wellcome Fund
Natural Sciences and Engineering Research Council
Hunter Family Foundation

The Animal Care Committee, having examined the Animal Use Protocol, approves of the procedures described therein and certifies with the applicant that they are in accordance with the principles outlined in the current Guidelines of the Canadian Council on Animal Care.

Sincerely, Date: March 18, 2019

Simon Hirota, HSACC

APPENDIX D: Coefficients of Variation (CV) for ELISAs

Hormone	Kit	Intra-assay CV (%)	Inter-assay CV (%)
Osteocalcin	MK127; Mouse Gla-Osteocalcin High Sensitive EIA Kit, Takara Bio USA, Inc. USA	<5.3	<4.6
OPG	TNFRSF11B; Mouse OPG ELISA, RayBioTech Life, Inc. USA	<10	<12
SOST	LS-F23135; Mouse SOST/Sclerostin Sandwich ELISA kit, LifeSpan Biosciences, Inc. USA	<6.1	<6.4
PTH	LS-F5539; Mouse PTH/Parathyroid Hormone Competitive EIA ELISA kit, LifeSpan Biosciences, Inc. USA	<10	<12
Insulin	90080; Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem, USA	<10	<10
Leptin	ELM-Leptin-1; Mouse Leptin ELISA. RayBioTech Life, Inc. USA	<10	<12

Table 1. Intra-assay and inter-assay coefficients of variation as reported by the manufacturer from each immunoassay kit instruction manual.