LACTATIONAL CHANGES IN BONE METABOLISM IN MICE LACKING PARATHYROID HORMONE (PTH)

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by

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ABSTRACT

The female skeleton loses a substantial amount of mineral content during lactation but then is fully restored after weaning. Very little is known about how this is regulated or accomplished. We studied calcium and bone metabolism during pregnancy and lactation in normal mice and in mutants that lack the gene encoding parathyroid hormone (PTH) to determine if PTH is required for the normal accretion of mineral during early pregnancy and the restoration of mineral after weaning.

We demonstrated that *Pth* null mothers were hypocalcemic and hyperphosphatemic as compared to normal. Some *Pth* null mothers had trouble lactating or died under anesthesia; both factors may have been due to hypocalcemia. To address this issue, midway through the project, all mice were placed on a 2% calcium chow instead of the standard 1% calcium chow. All genotypes (wild-type [WT], heterozygous [HET] and *Pth* null [HOM]) experienced similar gains in bone mineral content (BMC) during pregnancy and lost a comparable amount during lactation regardless of the chow they were fed. For the mice fed the standard chow, all genotypes showed a complete recovery in BMC within three weeks of weaning.

In summary, these preliminary results suggest that PTH is required for some aspect of normal calcium homeostasis during pregnancy and especially lactation but not to maintain mineral homeostasis and in particular, not to restore skeletal mineral content that was lost during lactation.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BMC	Bone Mineral Content
BMD	Bone Mineral Density
bp	Base pairs
Ca^{++}	Ionized Calcium
CaSR	Calcium-Sensing Receptor
DNA	Deoxyribonucleic Acid
DPA	Dual Photon Absorptiometry
DXA	Dual X-ray Absorptiometry
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
Gcm2	Glial Cells Missing Homolog 2
HCl	Hydrochloric Acid
HET	Heterozygote

HOM	Homozygote
KCl	Potassium Chloride
Mg^{++}	Magnesium Ion
$MgCl_2$	Magnesium Chloride
n	Number of observations
NaCl	Sodium Chloride
NaOAc	Sodium Acetate
NS	Non-significant
OPG	Osteoprotegerin
P	Probability
PCR	Polymerase Chain Reaction
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-Related Protein
RANKL	Receptor Activator of NF-κB Ligand
SDS	Sodium Dodecyl Sulfate

SE	Standard Error
SPA	Single Photon Absorptiometry
TAE	Tris Acetic Acid-EDTA
TE	Tris EDTA Buffer
WT	Wild-type

I Introduction

1.1 Calcium and Bone Homeostasis

In humans and other mammals, normal calcium and bone homeostasis includes the mechanisms whereby the concentration of calcium is tightly regulated in blood and extracellular fluid to control various biochemical processes (including muscle contraction, blood coagulation, nerve conduction), and whereby a sturdy and fully mineralized skeleton is maintained (1). In the adult, calcium and bone homeostasis is regulated in large part by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (the active form of vitamin D), calcitonin, and the sex steroids.

At the heart of calcium homeostasis is the need to control the blood calcium level, and this is regulated in large part by the calcium-sensing receptor (CaSR). This seven transmembrane-spanning, G protein-coupled receptor acts as the extracellular fluid compartment's thermostat for calcium and in particular regulates ionized calcium, which is the fraction of plasma calcium that is important for physiological processes (2). The CaSR does this through its presence in the chief cells of the parathyroid gland, the thyroidal C-cells, and the cells along the kidney tubules that are involved in calcium exchange (2). In response to an increase in ionized calcium, the CaSR modulates a decrease in the synthesis and secretion of PTH, an increase in the synthesis and secretion of calcitonin, and a reduction in urinary calcium reabsorption (2). This all occurs in an attempt to restore normocalcemia (2). On the other hand, in response to a decrease in ionized calcium, the CaSR is less activated by calcium ions. As a result, PTH synthesis and release escapes from inhibition, calcitonin synthesis and release is decreased, and

urinary calcium reabsorption is increased (2). These actions of the CaSR allow it to act as an effective moment-to-moment regulator of ionized calcium (2).

PTH is a major regulator of calcium homeostasis in the adult and it has several sites of action (3). It binds to its receptor on osteoblasts (bone forming cells) and osteoblast precursors; activation of the PTH receptor induces proliferation and differentiation, and reduces apoptosis of osteoblasts. In turn, activated osteoblasts stimulate the osteoclasts (bone resorbing cells) and osteoclast precursors to resorb bone and thereby release calcium and phosphate into the circulation. Thus, PTH indirectly stimulates bone resorption by acting on osteoblasts. PTH also acts on the kidney tubules to stimulate the reabsorption of calcium in the proximal tubules and to inhibit the reabsorption of phosphate in the distal tubules. It also enhances the synthesis of 1,25-dihydroxyvitamin D through stimulation of the enzyme 1-α-hydroxylase in the renal tubules of the kidneys, which converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (calcitriol) (3).

The mechanism by which PTH indirectly stimulates osteoclasts is not completely understood. If PTH stimulation of osteoblasts is sustained, osteoblasts in turn increase the secretion of receptor activator of NF-κB ligand (RANKL) and decrease the secretion of osteoprotegerin (OPG). RANKL is a type II membrane protein containing a cytoplasmic N-terminus and extracellular C-terminus. It is expressed in mature osteoblasts and has two known receptors; receptor activator of NF-κB (RANK) and OPG which are both members of the tumor necrosis factor receptor (TNFR) superfamily (4). RANK is a type I transmembrane protein while OPG lacks a transmembrane domain and

as a result is secreted. OPG functions as a decoy receptor to remove RANKL, whereas the absence of OPG permits RANKL to bind to RANK on osteoclasts. Thus, a net increase in RANKL/OPG enables RANKL to bind to its receptor on osteoclasts and osteoclast precursors, thereby stimulating osteoclastogenesis and osteoclast function leading to bone resorption (3,4). Short pulses of PTH increase the number of osteoblast cells by increasing the number of osteoprogenitor cells, decreasing the apoptosis of preosteoblasts and osteoblasts, increasing osteoblast proliferation, and possibly by converting the inactive bone lining cells to active osteoblasts (3). In turn, with a sustained PTH signal, there is an increase in osteoclast cell number and activity and these cells appear to take an active role, leading to bone loss (3). Factors including the dose of PTH, the mode of administration and the animal species determine whether the hormone is catabolic (net decrease in bone mass) or anabolic (net increase in bone mass) (3).

1,25-dihydroxyvitamin D plays a role in calcium homeostasis by increasing calcium absorption in the intestine through interaction with its nuclear vitamin D receptor (3,5). It also induces several proteins in the small intestine, including calcium binding protein calbindin-D9K, alkaline phosphatase, Ca^{2+}-ATPase, calmodulin and others, which facilitate calcium movement in the cytoplasm and transfer calcium from the basal lateral membrane into the circulation (5). Much of the calcium absorbed from the diet is actively absorbed through means that are dependent upon 1,25-dihydroxyvitamin D.

Calcitonin is another factor that influences calcium homeostasis. It is a peptide secreted primarily by the thyroidal C-cells but also by cells of the neuroendocrine system, mammary tissue, and placenta (6). Calcitonin acts on the skeleton by inhibiting the

activity of the osteoclasts so that less bone resorption takes place and, thereby, less calcium is released into the circulation (6). It also acts on the kidney by increasing the amount of calcium that is excreted in the urine (6). Although originally thought to be an important calcium regulatory hormone when first discovered, work in the past 20 years has indicated that it is largely vestigial in higher mammals, but can certainly have physiological effects in humans at pharmacological doses. Other effects of calcitonin have been observed including, anti-inflammatory actions, fracture and wound healing actions and antihypertensive actions, however the importance of these pharmacological aspects is yet to be determined physiologically (6).

Parathyroid hormone-related protein (PTHrP) is a second member of the PTH family. It is not likely to have a major role in the day-to-day maintenance of calcium homeostasis because of the fact that it is normally absent from the adult circulation (7). This hormone acts in many tissues to regulate development and function and as will be discussed in Section 1.3.2, it is an important factor during lactation.

PTHrP is widely expressed in fetal tissues, many epithelial surfaces, skeletal and heart muscle, distal renal tubules, hair follicles, brain and placenta (7). PTHrP and PTH share a common receptor (PTH/PTHrP receptor) and both have similar ranges of biological activities as they produce hypercalcemia, hypophosphatemia and cause an increase in 1,25-dihydroxyvitamin D production by the kidney (7).

1.2 Bone Mass Changes with Age

In trying to understand calcium and bone homeostasis during pregnancy and lactation, it is important to know how the skeleton adapts to age and other physiological stimuli. Despite common belief, the skeleton is not a static tissue but is a dynamic living organ that undergoes a continuous remodeling process such that bone is constantly being broken down while an equal amount of new bone is formed to replace it. It is through this constant remodeling process that bone strength is maintained; without remodeling, bone accumulates microdamage and becomes fragile over time. Childhood and early adolescence is a period characterized by longitudinal growth along with changes in skeletal size and shape (8). As shown in figure 1.1, when children grow, their bones are in a state of net bone gain in which bone formation exceeds bone resorption. Bone mass increases dramatically during growth and the amount of bone accumulated during this time may be important for the resistance to fractures in later life (8). When humans reach the approximate age of 20, they have attained their peak bone mass, and there is no further increase afterward. This level is maintained until menopause in women or older ages in men, after which there is a loss in bone mass due to decreases in various hormone levels.

Bone loss can also occur in adults as a result of such factors as prolonged inactivity or illness, estrogen withdrawal, weightlessness or corticosteroid treatment (4,9,10). In particular, when patients take doses of corticosteroids that exceed the equivalent of physiological amounts, at the molecular level, they experience an increase

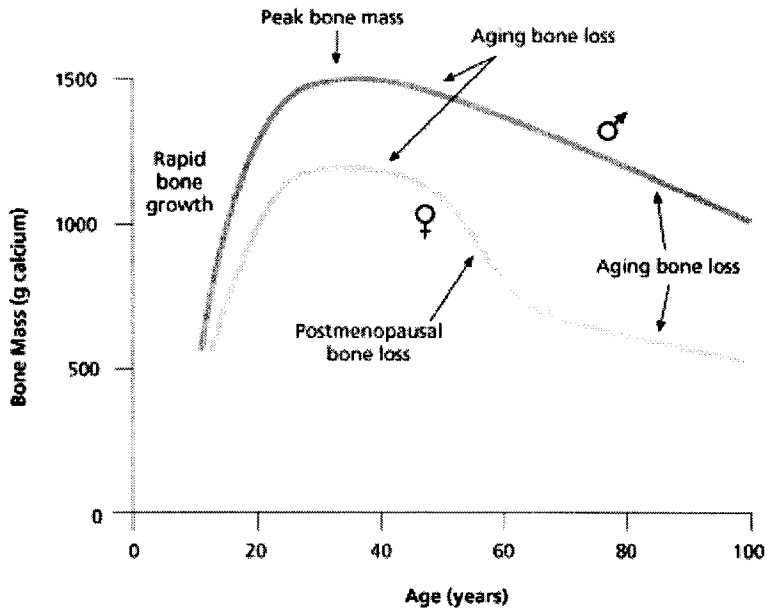


Figure 1.1: Relative changes in bone mass with age. As depicted, men and women experience slightly different changes in their bone mass as they age. In later life, the decrease in bone mass is more extreme for women (menopause) than men. (Reproduced from: Kassem J, Melton LJ, Riggs BL 1996 The type I/type II mode for involutional osteoporosis. Osteoporosis pp. 691–702).

in the number of apoptotic osteoblasts and osteocytes and bone biopsies have shown a decrease in trabecular bone mean wall thickness along with a decrease in bone volume (10). Parameters of bone resorption such as osteoclast-covered surface and osteoclast number have also been shown to be increased, meaning that bone resorption is occurring at a rate that exceeds bone formation and therefore, more bone is being broken down than new bone is being formed (10). As an example, bone loss as a result of steroid treatment occurs primarily in the trabecular bone (10). People taking steroids often have a low mineral apposition rate, a reduction in the trabecular wall thickness and a decrease in the number of osteoid seams leading to a decrease in bone volume (10). Any losses experienced by an adult due to steroid treatment are largely permanent, or are only partially recovered from.

1.3 Pregnancy and Lactation

For women, skeletal metabolism is significantly altered during pregnancy and lactation. This particular time period demonstrates how the skeleton can be used as a storehouse of mineral in order to provide to the fetus and neonate. Although pregnant and lactating women both experience similar demands in the amount of calcium needed to supply the fetus or neonate, the adjustments that must be made in each of these reproductive periods differs significantly from each other, as well as from the non-pregnant state. As explained in the next section, pregnant women meet the extra calcium requirements to provide to the developing fetus mainly by increasing the absorption of calcium from the diet (figure 1.2) (11). On the other hand, during lactation, nursing

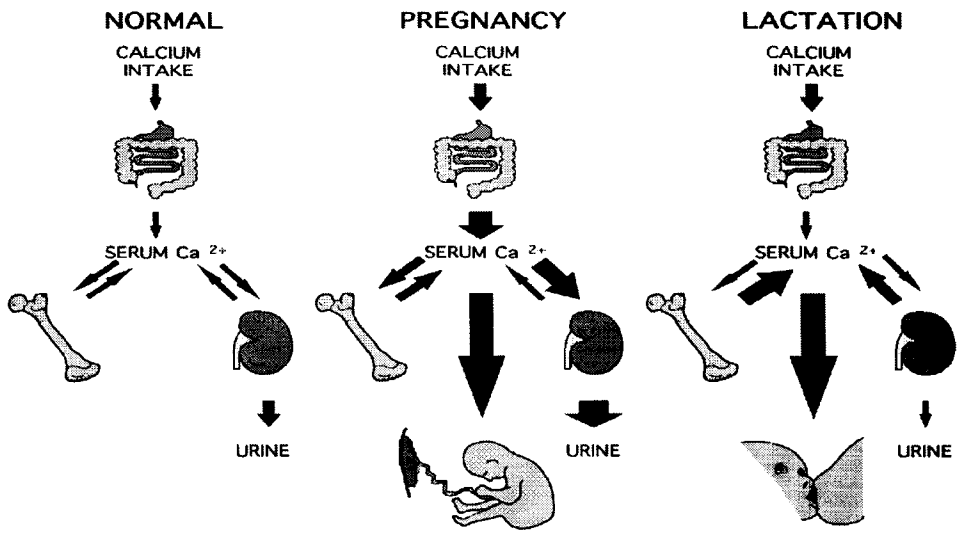


Figure 1.2: Schematic illustration of calcium homeostasis in human pregnancy and lactation, as compared to normal. The arrow thickness indicates a relative increase or decrease with respect to the normal and non-pregnant state. (Adapted from: Kovacs CS, Kronenberg HM 1997 Maternal-Fetal Calcium and Bone Metabolism During Pregnancy, Puerperium, and Lactation. Endocrine Reviews pp.832-872).

women markedly increase the rate of bone resorption and avidly reabsorb calcium in the kidneys to provide calcium for the milk and, thereby, to the nursing infant (11).

1.3.1 Pregnancy

During human pregnancy, calcium and other minerals are borrowed from the mother to mineralize the fetal skeleton (12). This places a potential strain on the mother's skeleton and her calcium homeostatic mechanisms, with the developing fetus gaining an average 30 g of calcium by term, but 80% of that during the third trimester (13,14). The fetal skeleton accretes about 250-300 mg of calcium daily during the final trimester (13).

Mothers experience changes during pregnancy in the levels of various minerals and calcitropic hormones including calcitonin, PTH, 1,25-dihydroxyvitamin D, and PTHrP (14). Total calcium levels are decreased significantly as pregnancy progresses, while ionized calcium (the physiologically important fraction) is observed to be constant throughout pregnancy. From early in pregnancy, serum phosphate levels are normal, calcitonin levels are increased and PTH levels fall to the low-normal range. This latter change may imply that PTH does not play a profound role during pregnancy because of these low levels or it may suggest that only low levels of PTH are required during this time while the mother prepares for the losses that occur later in pregnancy (14). Total 1,25-dihydroxyvitamin D levels are doubled during pregnancy and maintain this increase until term (13,14). Urinary calcium excretion is increased during pregnancy as compared to non-pregnancy.

In several studies, PTHrP has been observed at increased levels especially during late pregnancy (13). The exact source of production of PTHrP cannot be determined easily due to the fact that many fetal and maternal tissues produce the hormone (13). As a prohormone, it is quite possible that PTHrP may play diverse roles in the mother during pregnancy. The amino-terminal portion can stimulate skeletal calcium resorption and the renal 1α-hydroxylase enzyme; the midmolecular portions of the hormone stimulate placental calcium transfer, and the carboxy-terminal portion can inhibit osteoclastic bone resorption and could theoretically help in protecting the mother's skeleton from excessive resorption (13,15).

There is a doubling of intestinal calcium absorption during pregnancy in humans due to an increase in total 1,25-dihydroxyvitamin D. This occurs as the mother copes with the changes that normally occur during pregnancy. In fact, the mother's bone physiology adapts, presumably to meet the calcium requirements of the fetus while minimizing the danger of overwhelming her own needs. This finding was confirmed when investigators performed mineral balance and calcium kinetic studies using stable isotopes of calcium (^{48}Ca, ^{44}Ca, ^{42}Ca) in pregnant and non-pregnant women (16-18). Pregnant rats also experience a similar increase in absorption (19). It is thought that the increase in calcium absorption during pregnancy occurs not only because of the doubling of 1,25-dihydroxyvitamin D levels but also because of other hormones and factors which may influence intestinal calcium absorption. This appears to be the main way by which the mother adapts to the calcium demand of the fetus. In fact, it may be that the mother stores the excess calcium in preparation for the huge demand of the fetus during the third

trimester. This seems likely especially considering that intestinal calcium absorption increases in the first trimester, but that fetal demand for calcium is not present until well into the third trimester (13,19).

In humans, there are some data to show that increased bone resorption is present from early in pregnancy. In one particular study, 15 women had bone biopsies taken after they had terminated their pregnancies in the first trimester (20). It appeared as though there was increased bone resorption (including increased resorption surface), increased number of resorption cavities, and decreased osteoid. These findings were not observed in biopsies from non-pregnant controls or in biopsies from women at term who had cesarean-sections (20).

Human bone density data differ slightly from the data just mentioned in that there is very little or no loss in bone density observed during gestation. There are few studies which have examined changes in maternal bone mass during pregnancy due to the risks associated with fetal radiation exposure and of the studies conducted, the techniques used were far less precise or reproducible than dual x-ray absorptiometry (DXA) which is the current standard (19). DXA machines are used in measuring bone mass and bone density and provide rapid and precise measures of bone density. Of the outdated techniques, four human studies examined bone density during pregnancy using serial measurements by single photon absorptiometry (SPA) and/or dual photon absorptiometry (DPA) and found that there was no significant change in cortical or trabecular bone density (19). Presently, there are no human studies that have used DXA during pregnancy, however, several studies have used the modern technique before conception

and after delivery (12). Of the studies conducted, one found no change in bone density measurements in the lumbar spine at pre-conception or after 1-2 weeks post-delivery, while the other studies reported 4-5% decreases in bone density of the same region (post-partum measurement taken 1-6 weeks after delivery) (21-27). Normal bone density losses in women during lactation are around 1-3% per month and as such, it is extremely important that post-delivery measurements be taken as soon as possible (12). Also, these studies used baseline measurements that were taken from several to 18 months prior to pregnancy, which significantly affects the ability of these studies to detect true changes in bone density during pregnancy.

In studying changes in calcium metabolism in the skeleton it is often quite useful to examine bone turnover markers in both serum and urine to complement the static data collected from bone mineral content/density (BMC/BMD) measurements (28). Bone markers are proteins that are released during the process of bone formation or resorption, and hence measurement of serum or urine levels of these proteins reflects the relative activity of bone formation or resorption, respectively. These measurements are noninvasive, relatively inexpensive, can measure bone turnover changes over short intervals of time and can be assessed repetitively (28). In humans, the data on bone turnover markers is not consistent with the previously mentioned data from bone biopsies. Changes in bone turnover markers have shown that some of the resorption markers are low at the start of pregnancy but increase to values that are twice normal by the end of the third trimester (19). These markers include deoxypridinoline, pyridinoline, hydroxyproline. With regards to bone formation markers, osteocalcin in particular is low

during early pregnancy and often rises to normal levels by term (19). Procollagen I carboxypeptides and bone-specific alkaline phosphatase are other bone formation markers which are low at the beginning of pregnancy and remain at that level or rise to normal or above in the third trimester (19). Thus, the pattern of changes in bone markers suggests low turnover early in pregnancy and modestly increased turnover in late pregnancy. Although changes in bone markers indicate that bone turnover may be increased in humans in the third trimester, it appears that there are only minor changes in BMC during pregnancy (19). Numerous epidemiological studies have found that pregnancy has no long-term effect on the mineral content or fragility of the maternal skeleton (19).

Studies conducted on animals during pregnancy have shown that in rats, the parameters of bone formation and resorption are increased, thus indicating that bone is being remodeled (19). Ewes experience a 20% decrease in skeletal calcium and based on research from our lab, normal mice attain 10-20% increments in BMC during pregnancy (19,29).

Using data from both human and rats, it appears that bone turnover may be low during the first part of pregnancy but may be increased in the final trimester (19). The increase in bone turnover observed in late pregnancy correlates with the fetus accumulating a bulk amount of calcium from the mother (13,14,19). It may be possible that at the start of pregnancy, the maternal skeleton prepares itself through the decrease in bone turnover, for the transfer of calcium that is needed later in pregnancy for the

developing fetus. This is certainly the pattern observed in mice which gain a significant amount of BMC during pregnancy.

1.3.2 Lactation

During lactation, the maternal skeleton is rapidly resorbed in order to provide calcium and other minerals to create the calcium content of the milk to feed her nursing newborn (12). During this period, the mother may experience calcium losses of about 280-400 mg a day (13,14). Losses occur in both humans and animals. Women lose 5-10% of their BMC during six months of lactation, rats lose approximately 35% of their BMC during a three week period of lactation and normal mice experience a 30% decrease over the same three week interval (4,12,13,19,29). The rapid loss of BMC is mediated by PTHrP derived from the mammary glands in combination with low estrogen levels (13,30).

Minerals and calcitrophic hormones change during lactation and may be contributing in large part to the skeletal demineralization that occurs during this period. Ionized calcium is increased but remains within the normal range, serum phosphate is also increased and may even exceed the normal range (13). PTH on the other hand, has been observed to be reduced by 50% or more at the start of lactation and remains there until the time of weaning when it rises to normal and often even above normal postweaning (13). 1,25-dihydroxyvitamin D levels fall from the high levels of pregnancy to normal shortly after delivery and stay at that level during lactation (13).

With respect to calcium metabolism during lactation, calcium absorption in the intestine is equal to that of the non-pregnant state but decreased from that observed during pregnancy. This coincides with the decrease in 1,25-dihydroxyvitamin D levels from the high levels of pregnancy. During lactation, the amount of calcium lost in the urine is also reduced compared to the non-pregnant state (13).

The mechanisms responsible for the rapid loss of skeletal calcium content are not completely understood. Reduced estrogen levels are clearly important but are not likely to be the only factor responsible (12). Obviously, the intensity and duration of lactation are factors that influence the extent of bone loss (19). As an example, early resumption of menses (a marker for restoration of normal estradiol levels) or the use of an oral contraceptive during lactation can reduce skeletal losses, however, decreases in bone density will still occur if lactation is extended even after menses has resumed (19).

To determine how much of the increased bone resorption of lactation is attributed to low estradiol, one can consider what happens to calcium and bone metabolism in women who are at the reproductive age and are rendered severely estrogen deficient due to gonadotropin releasing hormone (GnRH) therapy for endometriosis and other conditions (12). Although irregular pulses of GnRH cause the pituitary gland to stimulate the ovaries, sustained administration of GnRH inhibits the pituitary and ovaries, leading to less estrogen being produced. After a woman has taken GnRH and been estrogen deficient for six months, she may experience trabecular bone losses of 1-4%, increased urinary calcium excretion and suppression of 1,25-dihydroxyvitamin D and PTH levels (19). In contrast, a woman who lactates exclusively for six months is not as

estrogen deficient, will lose more (5-10%) bone mineral density at trabecular and cortical sites and will have normal 1,25-dihydroxyvitamin D levels and reduced urinary calcium excretion. In looking at bone density changes alone, a women who lactates will completely restore all that she has lost post-weaning at a rate of 0.5-2% per month (12). On the other hand, women experiencing reduced estrogen levels due to GnRH analogue treatment will not completely recover from bone density losses after the treatment is stopped and normal estradiol levels have been restored (19). Observed differences that exist between a women who is estrogen deficient and one who is lactating may be due other factors such as PTHrP which acts synergistically with low estrogen levels to enhance bone resorption and by itself to stimulate reabsorption of calcium by the kidneys (12). One study examined the role of estrogen in mice during lactation and found that low estrogen and high PTHrP levels contributed to accelerated bone resorption during lactation (31). The researchers conducting the study also discovered that treating the lactating mice with high-dose estrogen (ie, 4-5 fold normal) lowered parameters of bone resorption and reduced the amount of bone lost by about 50-60% (31).

PTHrP is thought to play a significant role during lactation in regulating the demineralization of the skeleton (32). In response to suckling and signaling from the CaSR expressed in the lactating mammary tissue, PTHrP reaches the maternal circulation and stimulates resorption of calcium from the maternal skeleton, renal tubular reabsorption of calcium, and it may indirectly suppress PTH. PTHrP levels are significantly higher in the blood of lactating women than in nonpregnant controls, and are higher than the simultaneous PTH levels (30,33). The breast appears to be the main

source of the circulating PTHrP based on several lines of evidence. Levels have been observed in breast milk at concentrations 10,000 times the level observed in the blood of patients with hypercalcemia of malignancy and in normal human controls (14,33). Also, small increases in PTHrP have been observed as a result of suckling, a response mediated by prolactin (19). Other data exists supporting the role of PTHrP during lactation including a study involving conditional knockout mice which eliminated the PTHrP gene from mammary tissue at the onset of lactation. These mice displayed reduced levels of circulating PTHrP, 1,25-dihydroxyvitamin D, urinary cyclic adenosine monophosphate (cAMP) and bone turnover markers, and less BMC was lost during lactation (30). The researchers concluded the mammary-specific ablation of the PTHrP gene demonstrated that PTHrP is normally secreted into the maternal circulation from mammary tissue during lactation, and that it is required to stimulate osteoclastic bone resorption to induce the normal loss of bone mineral (30).

Calcitonin levels are elevated during the first six weeks of lactation and then fall to normal. Calcitonin appears to play a role during lactation because mice lacking the calcitonin gene lose approximately 50% or more of their BMC during three weeks of lactation, about twice that of their normal littermate sisters (34). This finding indicates that calcitonin does regulate lactational skeletal losses in some manner, and that in its absence the losses will more than double.

Calcium absorption in the intestine decreases during lactation to the non-pregnant rate from the increased rate of pregnancy and this corresponds to the fall in 1,25-dihydroxyvitamin D levels to normal (12).

Bone histomorphometry data collected from animals have shown that bone turnover is increased during lactation (19). Again, data are lacking for humans, but bone formation and resorption markers have been used to examine bone turnover (19). Markers for resorption (deoxypridinoline, pyridinoline, hydroxyproline and tartrate-resistant acid phosphatase) are elevated during lactation and are higher than the levels observed during the third trimester of pregnancy (19). Most studies involving bone formation markers have shown the levels to be higher during lactation than that observed during the final trimester. Overall, the changes in bone markers indicate that there is an increase in bone turnover during lactation, with the pattern (resorption >> formation) being consistent with the bone density data which show that there is a net loss of bone mass occurring (19).

Decreases in bone mass occur as a result of an increase in the rate of bone turnover accompanied by an imbalance between osteoclast and osteoblast activity (35). If there is an increase in the number and function of osteoclasts but not a corresponding increase in osteoblast number and function, then there is an increase in bone resorption. As a result, the normal "coupling" of bone resorption followed by bone formation is disrupted such that bone break-down occurs at a rate exceeding bone formation, and as a result, bone is lost (35).

1.4 Skeletal Recovery Post Weaning

Perhaps the most intriguing question regarding skeletal metabolism is how BMC lost during lactation is completely restored post-weaning. Lactation is the only

situation where an adult can lose a considerable amount of bone density and completely regain all that has been lost with no long-term consequences to the skeleton (12,13). If an adult were to experience a loss due to any of the factors mentioned earlier (prolonged inactivity or illness, estrogen withdrawal, weightlessness, steroids etc.) such a loss would largely be permanent and the individual would experience at most a partial regain in bone mass (4,9). Once lactation has ceased, the maternal skeleton is completely restored to its original mass within a few months by mechanisms that have remained unelucidated. Knowing that the adult skeleton has the ability to build bone mass after lactation, this mechanism (if understood) could potentially be adapted to treat people with osteoporosis or other disorders of low bone mass.

Among the few clinical studies that have examined the post-weaning phase, one study in particular looked at lactating women and compared them to non-lactating postpartum controls over the first year postpartum (36). During the first six months after delivery, serum PTH levels decreased, rose to normal at weaning and rose above normal after weaning (36). Whether this modest increase in circulating PTH means that the hormone is required for restoration of BMC remains unknown (36). A second study by Dobnig *et al.* stated that PTH was suppressed to 50% of control values immediately postpartum, but returned to normal values within 3-6 months postpartum, however the study did not extend past weaning (37). This increase may be a mechanism to stimulate calcium reabsorption by the kidneys as well as to stimulate osteoblasts to remineralize the skeleton (38). It is not clear as to how this increase in PTH is stimulated, however, one possible suggestion is that estrogen stimulates osteoblast activity and calcium uptake by

bone which leads to a decrease in serum calcium and in turn, an increase in serum PTH levels (38). However, this observation of PTH playing a role post-weaning remains suggestive but not conclusive.

Estrogen is known to play an important role in maintaining bone mass and its absence leads to bone loss during lactation and to osteoporosis after menopause. Therefore, it is a logical candidate to play a role during weaning when estrogen levels rebound to normal and bone is being restored (39). Trying to determine the contribution of estrogen is difficult due to the fact that no human or animal studies have manipulated the estrogen level independently of whether the female is lactating or weaning. The main effect of estrogen is to inhibit osteoclasts and thus it is unlikely to explain the full recovery of BMC that is achieved after weaning. With all of this in mind, it seems logical that estrogen may contribute to skeletal recovery post-weaning by inhibiting bone resorption but the hormone itself cannot explain recovery in full. Other factors likely act in concert with estrogen during this period.

PTHrP regulates skeletal demineralization during lactation but it is also possible that it is required for skeletal recovery post-weaning (30). Like PTH, pulses of PTHrP will stimulate bone formation whereas sustained high levels of PTH or PTHrP will stimulate bone resorption. Therefore, a change in the secretion pattern of PTHrP would enable it to play some role in BMC restoration. There are two main sources of PTHrP, the breast and the osteoblast, which could be involved in BMC restoration after weaning. The breast is a potent source of PTHrP during lactation and might remain so during recovery; however, since mammary tissue involutes quickly after lactation, it may be that

this is not a likely explanation. There are reports in humans showing that PTHrP levels are still increased after weaning and rat studies have shown PTHrP levels to be highest at the end of lactation and it may be that the elevated levels continue into weaning and contribute to skeletal recovery (19,40). Osteoblasts have been shown to produce PTHrP and an osteoblast-specific knockout of the PTHrP gene has shown that osteoblast-derived PTHrP is required to maintain bone mass in adult mice (41). Therefore, it is possible that PTHrP produced by osteoblasts may be required to stimulate the recovery after weaning and perhaps it is stimulated by loss of the hormonal milieu of lactation (high prolactin, high PTHrP and low estradiol in circulation) (42).

Calcitonin is also a potential suspect for contributing to skeletal recovery. It is a known inhibitor of osteoclastic bone resorption and research in our lab has confirmed the previously hypothesized role of calcitonin to protect the mother's skeleton from excessive resorption during lactation (4,34). We have shown that the calcitonin null mice completely restore their BMC to normal after weaning, even after experiencing a severe loss in BMC and as such, these observations confirm that calcitonin is not required for skeletal recovery after weaning. The studies do show, however, that the maternal skeleton has the ability to restore itself rapidly even when more than half of its mineral content has been lost (34).

It is obvious that there must be other factors which play a role during recovery after weaning. Given that PTH is a dominant hormone in regulating calcium and bone homeostasis in the adult and that it stimulates bone formation, it is an obvious candidate to examine to see if it is required for skeletal recovery after lactation.

1.5 Literature Concerning Absence of PTH During Pregnancy and Lactation

In planning to examine the role of PTH in skeletal recovery after weaning, it was realized that the model used would lack PTH during pregnancy and lactation. Therefore, it is important to briefly review the literature concerning the absence of PTH during the non-pregnant state as well as during pregnancy and lactation in both humans and animals.

Hypoparathyroidism is a disorder which occurs when PTH produced from the parathyroid gland is insufficient to maintain extracellular fluid calcium in the normal range or when adequate circulating concentrations of PTH are unable to function in tissues to maintain normal extracellular fluid calcium levels (43). A person suffering from hypoparathyroidism will have low blood calcium, high blood phosphorus and high urine calcium excretion (43). Some of the symptoms of hypoparathyroidism include, muscle cramps, tetany, seizures and the low blood calcium can be severe enough to lead to fatal arrhythmias (43).

When treating someone with hypoparathyroidism, the major goal is to restore serum calcium and phosphorus as close to normal as possible. The main pharmacological agents used are calcium supplements plus calcitriol to enhance intestinal absorption of calcium. One major drawback is that in the absence of PTH, the kidneys cannot avidly reclaim calcium and therefore, much of the supplemented calcium is lost in the urine. There is also a risk that the individual will develop kidney stones and possibly kidney failure from nephrocalcinosis (43). It is often a compromise in that the individual is treated with the minimum amount of calcium and calcitriol required so that the symptoms

of hypoparathyroidism are reduced but not eliminated and at the same time, the worsening of urinary calcium excretion is minimized (43).

There is a limited role for PTH during human pregnancy and it appears as though increases in 1,25-dihydroxyvitamin D and calcium absorption in the kidneys, both of which normally occur during pregnancy, will take place even if PTH is absent (14,19). There are two possibilities as to what a hypoparathyroid female may experience during pregnancy, in that the condition will either become worse or that it will improve. Both possibilities have been reported in the literature. During pregnancy, when there is such a significant loss of calcium from the mother to the fetus, it is possible that this may further impair the mother's ability to maintain a normal blood calcium and lead to more frequent and severe hypocalcemia unless the dose of calcium and calcitriol were increased to mimic the normal rise in calcitriol that occurs during pregnancy (12,14,19). Other case reports have shown that hypoparathyroid women had fewer symptoms of hypocalcemia and required less calcitriol and that the mother's condition improved during pregnancy. With the rise in 1,25-dihydroxyvitamin D, PTHrP and intestinal calcium absorption, the mother may be able to compensate for the low calcium associated with hypoparathyroidism through calcium supplementation. Thus, a hypoparathyroid woman who becomes pregnant, may have less hypocalcemia and require less supplemental calcium or calcitriol. In any case, any requirements that a hypoparathyroid woman may have for calcitriol during pregnancy, decrease substantially shortly after delivery when the mother breastfeeds because there is the possibility that hypercalcemia will occur unless the calcitriol dosage is reduced or discontinued (12,14). Thus, while the human

data leave uncertainty as to whether absence of PTH is clinically worsened or improved during pregnancy, it is very clear that PTH is not required for lactation.

Parathyroidectomized pregnant rats often exhibit signs of tetany at the end of pregnancy, during the time when the fetus obtains the bulk of maternal calcium it requires and in many situations, maternal death has occurred during the birthing process (19). In these animals, dietary intake and weight gain decline and serum 1,25-dihydroxyvitamin D decreases. These abnormalities, which are calcium related, can be prevented if the animals are fed a high-calcium, low-phosphorus diet (19). This data seems to suggest that rats develop secondary hyperparathyroidism at the end of gestation due to the decrease in maternal calcium and they may be more dependent on bone resorption and 1α-hydroxylase up-regulation both mediated by PTH (19). In the absence of parathyroid tissue, rats lose bone mineral during lactation (44). A group of investigators looked at parathyroidectomized rats and compared them to their siblings who had parathyroid glands and found that these animals still lost a comparable amount of bone mineral during lactation. The researchers stressed that PTH does not act alone in stimulating bone loss and that it must act in concert with other factors but that the hormone does play an important role in the process of skeletal loss during lactation (44).

1.6 PTH Knockout Mouse Model

In this project, I wished to explore the role of PTH in regulating calcium and bone metabolism during pregnancy, lactation and especially recovery post-weaning. Considering that the exact role of PTH during pregnancy and lactation in calcium and

bone metabolism is still not known, it seemed appropriate to study this using a knockout model with PTH absent.

The ideal model might have been to ablate the PTH gene at the time of weaning, but such a model was not feasible at the time this project started. A group of researchers at McGill University in Montreal generated a PTH knockout mouse model using homologous recombination in embryonic stem cells (42). This was a useful model for determining the precise role of PTH in calcium and bone metabolism. Mice deficient in PTH were created by replacing the entire coding sequence of PTH on exon 3 with the gene encoding neomycin resistance, however, due to the fact that these mice were not generated in our laboratory the details of the creation will not be discussed (42). *Pth* null mice are born normally, are fertile and live normal lifespans, however, they are hypocalcemic, hyperphosphatemic and show diminished cartilage matrix mineralization, decreased neovascularization, reduced expression of angiopoietin-1, and reduced metaphyseal osteoblasts and trabecular bone (42). Compared to their WT sisters, the null mice displayed abnormal vertebral column and skull formation and mineralization of both the skull and the bones were enhanced. Evidence suggests that the vertebral bodies were smaller and mineralization of the metacarpal and metatarsal bones were shorter (42). Additionally, and consistent with long standing hypoparathyroidism in humans, the BMC is higher in the mature adult null versus WT. This finding had the potential to confound our study since the *Pth* null mice would start out with a higher BMC and a normal absolute loss of BMC would be proportionately smaller as compared to WT.

1.7 Project Description, Purpose and Hypothesis

In starting this project, the hypothesis was that PTH is required for normal mineral homeostasis during pregnancy and lactation and for restoration of the maternal skeleton after weaning.

I examined this hypothesis by studying *Pth* null mothers compared to WT sisters. Any disturbance in calcium and bone metabolism seen in the null mice could be attributed to a direct or indirect consequence of PTH. Wild-type (WT) mice have both copies of the PTH gene while heterozygous (HET) mice have one copy of the PTH gene and one copy of the neomycin resistant gene.

After mating heterozygous males and females together we were able to obtain WT, HET and *Pth* null offspring. At the start of the project we chose WT, HET and null sisters for subsequent study to compare them physiologically to determine if loss of PTH had any effect in the response to pregnancy, lactation and weaning. However, midway through the project, we focused solely on WT and null females for reasons that will be discussed in the Results section (Section III). The mice were studied through full cycles of pregnancy, lactation and weaning with monitoring of BMC, as well as various minerals in the serum and urine.

These studies were anticipated to provide definitive evidence about the role of PTH during lactation and post-weaning in mice, which in turn could lead to confirmatory studies in humans that lack PTH. Understanding how the skeleton restores itself may lead to new approaches to treating osteoporosis and other bone and mineral disorders.

II Materials and Methods

Specific details regarding protocols used for the experiments can be found in the Appendix.

2.1 Animal Husbandry

2.1.1 *Pth* null mice

The *Pth* gene knockout mice used in this study were obtained from Dr. Andrew Karaplis (McGill University, Montreal, Quebec, Canada) and were generated using homologous recombination in embryonic stem cells, as previously described (42).

These mice have been shown to be hypocalcemic from birth, but live apparently normal life spans, and are fertile (42). *Pth* mice were then back crossed into the outbred Black Swiss strain for at least four generations in order to be comparable to other knockout strains studied in our lab. The studies involved looking at the effects of pregnancy and lactation in these mice that lack PTH versus their WT sisters.

Colonies were maintained by mating heterozygous–deleted *Pth* males and females to produce wild-type (WT), heterozygous (HET), and *Pth* null mice. Genotypes were confirmed by polymerase chain reaction (PCR) of genomic DNA for detection of the native PTH allele versus the mutated allele. Animals were maintained in facilities operated by Animal Care Services of Memorial University of Newfoundland, in accordance with the Canadian Council on Animal Care (CCAC). All experimental procedures performed were approved by the Institutional Animal Care Committee (IACC) of Memorial University of Newfoundland.

2.1.2 Timed Matings

WT, HET and *Pth* null females were mated overnight with HET males. The presence of a vaginal seminal plug in the morning, after mating indicated gestational day 0.5. The length of the gestation period for mice is nineteen days. Adult mice were used for studies of pregnancy and lactation when they reached the age of twelve weeks, at which time they had reached sexual maturity and attained their peak bone mass.

2.2 Genotype Determination

2.2.1 Tagging Mice

For identification purposes, at weaning, mice were anaesthetized using Isoflurane™ (CDMV), and ears were tagged with a unique identifier.

2.2.2 Tail Clipping

At the time of tagging, at three weeks of age, tails were cut and placed in 1.5mL microcentrifuge tubes (Fisher Scientific) containing 500 µl lysis buffer (100mM Tris•HCl , pH 8.0 / 500 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0 / 0.2% SDS [sodium dodecyl sulfate] / 200 mM NaCl) containing 100 µg/mL proteinase K [Invitrogen]). The tubes were incubated at 55°C overnight in an isotherm oven (Fisher Scientific).

2.2.3 DNA Extraction

Following incubation of tails in an isotherm oven, the tubes were shaken and then centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred to fresh microcentrifuge tubes containing 0.5mL isopropanol and inverted to precipitate the DNA. Using small pipette tips the DNA precipitate was removed from each tube, placed into clean microcentrifuge tubes containing 0.5mL and shaken to dissolve the DNA. In the fumehood, 0.5mL of phenol/chloroform/isoamyl alcohol (100:100:1) was added to tubes which were then shaken. Next, the tubes were centrifuged for 2 minutes, the aqueous layer was removed and placed in fresh microcentrifuge tubes. Cold 0.12M sodium acetate (NaOAc) in ethanol (EtOH) was added, the tubes were inverted several times, and centrifuged for 10 minutes to pellet the DNA. The supernatant was decanted, pellets were washed with 70% EtOH and again centrifuged. Following this, the EtOH was removed and the pellets were air dried, resuspended in TE (Tris EDTA buffer) and stored at 4°C.

2.2.4 Conventional PCR (Polymerase Chain Reaction)

PCR was performed on mouse tail DNA to determine the presence of the gene of interest. The presence of the normal *Pth* gene was determined through amplification of a 520 base pair portion of exon 3 of PTH using sequence specific primers:

PTH1 (forward): 5'-GAG GCT TTG TAG TGG GTT TT
PTH2 (reverse): 5'-AGA GAA GTG GAA ATG AGT AG

(Unpublished data)

The presence of the *Pth* null gene was determined using primers specific for the 300 base pair portion of the neomycin resistant gene:

N1 (forward): 5'-GGA GAG GCT ATT CGG CTA TGA C
N2 (reverse): 5'-CGC ATT CGA TCA GCC ATG ATG G

(46).

The validity of the PCR technique was confirmed by Southern blotting using the originally described probe (43).

The PCR cocktail was composed of 10X PCR buffer (20mM Tris-HCl, pH8.4, 50mM KCl), dNTPs (0.2mM of each dATP, dCTP, dGTP, dTTP), primers ($PTH_{forward}$, $PTH_{reverse}$, $N_{forward}$ and $N_{reverse}$), 50mM $MgCl_2$, Taq DNA polymerase (0.02U/μL) and deionized water. 1.5μL (approximately 500ng) of the collected DNA samples were added to PCR tubes containing 49μL of the prepared cocktail. These tubes were then placed in the Peltier Thermal Cycler-Dual Alpha Blocks (PT-200 DNA Engine Thermal Cycler).

The following program conditions were applied for the conventional PCR for the PTH primers: denaturation at 94°C for 45 seconds, 40 seconds of annealing at 59°C, followed by one minute of elongation at 72°C. These steps were repeated 35 times, followed by 72°C for 7 minutes.

2.2.5 Gel Electrophoresis

PCR products were electrophoresed on a 1.2% agarose gel (2g agarose [Invitrogen], 10 ml of 10X TAE [pH 8.0, 0.12M EDTA, 0.40M Tris, 11.5% Glacial Acetic Acid] and 90 ml deionized water, 0.06% Ethidium Bromide [Invitrogen]). The

electrophoresis buffer consisted of TAE and deionized water. One microliter of Orange G (10mL 10X TAE, 500mg Orange G powder, 50mL glycerol) was added to each of the DNA samples in the PCR tubes as a loading dye. 10μL was transferred from each tube into the wells of the gel which was then run at 200V for approximately thirty minutes. Following this time, the gel was exposed to UV light and a picture was taken using Chemi-Imager Software and printed on Mitsubish Thermal paper (Perkin Elymer). The presence of a single 520 base pair (bp) band indicated that the mouse was wild-type (WT) for PTH, a band at 300 bp indicated that the mouse was null (homozygous [HOM]) for PTH while the presence of both bands indicated that the mouse was heterozygous (HET) for ablation of PTH (Figure 2.1).

2.3 Data Collection

2.3.1 Bone Densitometry

Total body bone mineral content (BMC) was measured using the PIXImus bone densitometer (Lunar). Mice were anaesthetized with Pentothal or a combination of Xylazine (Bayer)/Ketamine Hydrochloride (Bimeda-MTC) via an intraperitoneal injection. The mice were then placed on a slightly sticky specimen tray that immobilizes them for the 3-5 minutes required for the measurement to be completed. Following this, the mice awaken and are returned to their litters. Mice were scanned throughout full reproductive cycles for approximately 70-80 days. The entire reproductive period included pregnancy baseline (7-9 days), pregnancy (19 days), lactation (21 days), and post weaning (after 21 days post weaning). The pre-pregnancy baseline value was

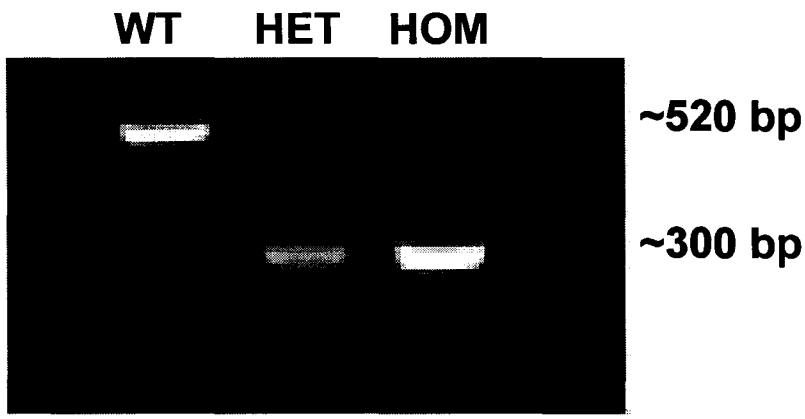


Figure 2.1: Genotyping by polymerase chain reaction (PCR). The PCR product is shown electrophoresed on an agarose gel, to demonstrate the typical results of a genotyping experiment. PTH1 and PTH2 are used to detect the wild-type allele; the PCR product is approximately 520 base pairs in length. N1 and N2 primers are used to detect the knockout (homozygous) allele; the PCR product is approximately 300 base pairs. Wild-type = WT, hetereozygous = HET and homozygous = HOM.

obtained by averaging the readings from the week prior to successful mating. Sample scans are shown in Figure 2.2.

Experiments in our lab have shown that when using DXA to take total body scans, the calcium dense head region is variable and cannot be fit completely on the scanner as pregnancy progresses. For these reasons, the head was not included in the scans. We were able to do this using a function in the computer program that excluded the head from the measurements. As a result, all body scans excluded the head region as an attempt to ensure that the values obtained were as accurate from mouse to mouse as they can possibly be. Regional measurements (spine, leg, tail) could be obtained after the total body scan was done. Using the computer, we are able to adjust the region of interest such that instead of looking at the whole body BMC, we could focus on the spine, tail or the hind limb. Other research in our lab has also shown that the fetal skeletons contribute a negligible amount to the mother's total body BMC at the end of pregnancy (29). The exact contribution of the fetuses was determined by obtaining readings before and after removal of fetuses by C-section. The entire litter accounted for $1.9 \pm 0.4\%$ of the total BMC (29). Therefore, when analyzing a mother's BMC during pregnancy we are confident that the values obtained reflect true changes in maternal BMC and are not significantly affected by the presence of the fetuses.

2.3.2 Ionized Calcium

Maternal whole blood was collected from the tail vein at baseline, day 18 of gestation, one week post-partum, three weeks post-partum and one and three weeks post-

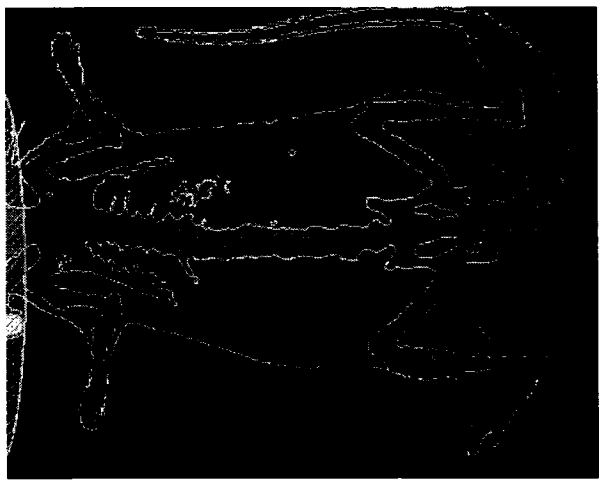


Figure 2.2: Representative scan of total body BMC. Measured at three weeks post-weaning in a mouse on the standard 1% calcium chow.

weaning. Samples were collected in 50 µl heparinized capillary tubes (Bayer) and analyzed immediately using a Chiron 634 Calcium/pH analyzer (Bayer) to measure the physiologically important fraction of calcium in blood.

2.3.3 Serum Collection

Maternal blood was collected from the tail vein at baseline, day 18 of gestation, one week post-partum, three weeks post-partum and one and three weeks post-weaning. Blood was collected using capillary tubes (Fisher Scientific), placed in 0.6mL microcentrifuge tubes (Fisher Scientific), centrifuged and the serum (top layer) was removed and stored at -20°C for later analysis.

2.3.4 Urine Collection

Upon first removal from the cage, mice usually promptly voided. Occasionally it was necessary to leave the mice in a clean cage to allow them to void. Maternal urine was collected at baseline, day 18 of gestation, one week post-partum, three weeks post-partum and three weeks post-weaning. The urine was collected using capillary tubes (Fisherbrand), placed in 0.6mL microcentrifuge tubes (Fisher Scientific) and stored at -20°C for later analysis.

2.4 Mineral Assays

2.4.1 Serum and Urine Analysis

Serum was analysed for calcium, magnesium and phosphorus using photometric assays (BioPacific Diagnostic Incorporated). Osteocalcin was also measured in serum as a marker of bone formation, using an Immunoradiometric Assay (IRMA).

Urine calcium, phosphorus and deoxypyridinoline (DPD) were also measured using photometric assay kits and corrected for with Creatinine-S (Diagnostic Chemicals Limited). Creatinine was measured on each urine sample and each calcium, phosphorus or DPD value was expressed relative to the concentration of creatinine in the sample, in order to correct for differences due to urine volume or concentration.

2.5 Ash Weight Analysis and Flame Atomic Absorption Spectroscopy

Following weaning and genotyping, three-week old pups were weighed, asphyxiated, placed into covered crucibles, and reduced to ash in a furnace for 48 hours at 500°C. The ash was then weighed, transferred to acid-washed vials, and dissolved in nitric acid. After five days, deionized water was added to each vial to form a 3% nitric acid solution. Samples were assayed on a Perkin-Elmer Corp. 2380 atomic absorption flame spectrophotometer to determine the absolute amount of calcium and magnesium. The ash residue is made up mostly of the heavy skeletal minerals, and hence the calcium and magnesium content of the ash is a direct measure of the absolute amount of calcium and magnesium within the skeleton.

Data for total ash weight, total calcium, magnesium content, and mineral content corrected for ash weight were collected.

2.6 Statistical Analysis

Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, Inc., Evanston, IL). ANOVA (analysis of variance) was used for the initial analysis; Tukey's test was used to determine which pairs of means differed significantly from each other. Two-tailed probabilities are reported and all data are presented as the mean ± SE (standard error). $P<0.05$ was considered statistically significant.

III Results

3.1 Success of Pregnancy and Lactation for the *Pth* null Females

As this project began, it was unexpectedly discovered that the *Pth* null had difficulties conceiving and often it would take several attempts before a *Pth* null female became pregnant, as compared to a WT female. There were sudden unexpected deaths during the 70 day reproductive cycles of *Pth* null mice with many during pregnancy and some during lactation, usually but not always in association with anesthesia (Table 3.1). After reviewing the literature, we discovered a very recent report indicating that *Pth* null mice have fewer pregnancies and offspring rates, that some appeared not to lactate well, and that these problems were attenuated when the mice were fed a calcium enriched chow (45). We also noticed that even when the mother did lactate successfully, she would cull her litter to a much smaller size by eating pups and in many cases she would cull her litters down to 3 or 4 pups compared to the normal 6-12 pups that their wild-type siblings would nurse. Based on all of this we switched all mice from the standard 1% calcium chow (Prolab®, Animal Specialties and Provisions, Quakertown, PA, USA) to the 2% calcium enriched chow that was used by other researchers (45).

We did not have the opportunity to measure PTH or 1,25-dihydroxyvitamin D in these studies. PTH has already been shown to be absent in *Pth* null mice (42) and 1,25-dihydroxyvitamin D is expected to be low due to absence of PTH's effect to stimulate the 1α-hydroxylase. Normally during pregnancy 1,25-dihydroxyvitamin D is nearly doubled (13,14) due to mechanisms that appear to be independent of PTH, since PTH levels are normally low throughout pregnancy. Such factors as PTHrP, prolactin, placental

	WT		*Pth* null	
	Standard Chow	**Calcium Enriched Chow**	**Standard Chow**	**Calcium Enriched Chow**
Number of Mice studied	14	9	7	9
Deaths during Pregnancy	1 (first trimester)	1 (third trimester)	1 (first trimester)	3 (third trimester)
Anesthetic Related Deaths during Pregnancy	0	1	1	2
Deaths Unrelated to Anesthetic during Pregnancy	1	0	0	1
Deaths during Lactation	2	1	1	1
Anesthetic Related Deaths during Lactation	2	1	1	1
Deaths Unrelated to Anesthetic during Lactation	N/A	N/A	N/A	N/A
Mice that did not Lactate and/or Culled their Litters	3	1	3	1

Table 3.1: Maternal Complications and Deaths for WT and *Pth* null females during pregnancy and lactation. Anesthetic related deaths normally occurred within several minutes of the mouse being injected (overdose). Deaths unrelated to anesthetic occurred randomly and most often on days when the mouse was not injected with anesthetic, and therefore the death was not associated with the drug. When the females culled their litters it was sometimes to the point where there were no pups left in the litter and when the mothers failed to lactate (due to stress) the pups died of starvation.

lactogen and others may stimulate the 1α-hydroxylase. Whether 1,25-dihydroxyvitamin D levels will increase normally during pregnancy in the absence of PTH is unclear because such measurements have not been done in pregnant aparathyroid humans nor have we yet done them in pregnant *Pth* null mice. If in fact the normal doubling of 1,25-dihydroxyvitamin D levels during pregnancy requires the presence of PTH, then 1,25-duhydroxyvitamin D levels will be low in *Pth* null mice and will explain many of the calcium related problems that they experience during pregnancy.

The effect of the 1% versus 2% calcium chow was immediately apparent. Figure 3.1 shows that when fed the 1% calcium chow diet, 100% of the *Pth* nulls were lactating 24-48 hours post-partum while 33% were still lactating with live pups at three weeks post-partum. This is compared to the WT and HET siblings, 100% of whom were lactating at 24-48 hours post-partum and 62% and 73% of whom were lactating after three full weeks, respectively. In contrast, the null females were more successful during lactation when fed the 2% calcium chow, 100% of the mothers were lactating immediately after delivery while 67% were still lactating after three weeks of lactation. For the WT females on the 2% calcium chow 100% were lactating with live pups at 24-48 hours post-partum while 86% were still lactating three weeks later. Thus, the 2% calcium chow prevented the difficulties in initiating and maintaining lactation, also, the unexpected maternal deaths during pregnancy and lactation were very infrequent on the 2% calcium chow.

The data collected for this project will be presented in two separate sections because data obtained from the 1% and 2% calcium diets are not comparable. Neither

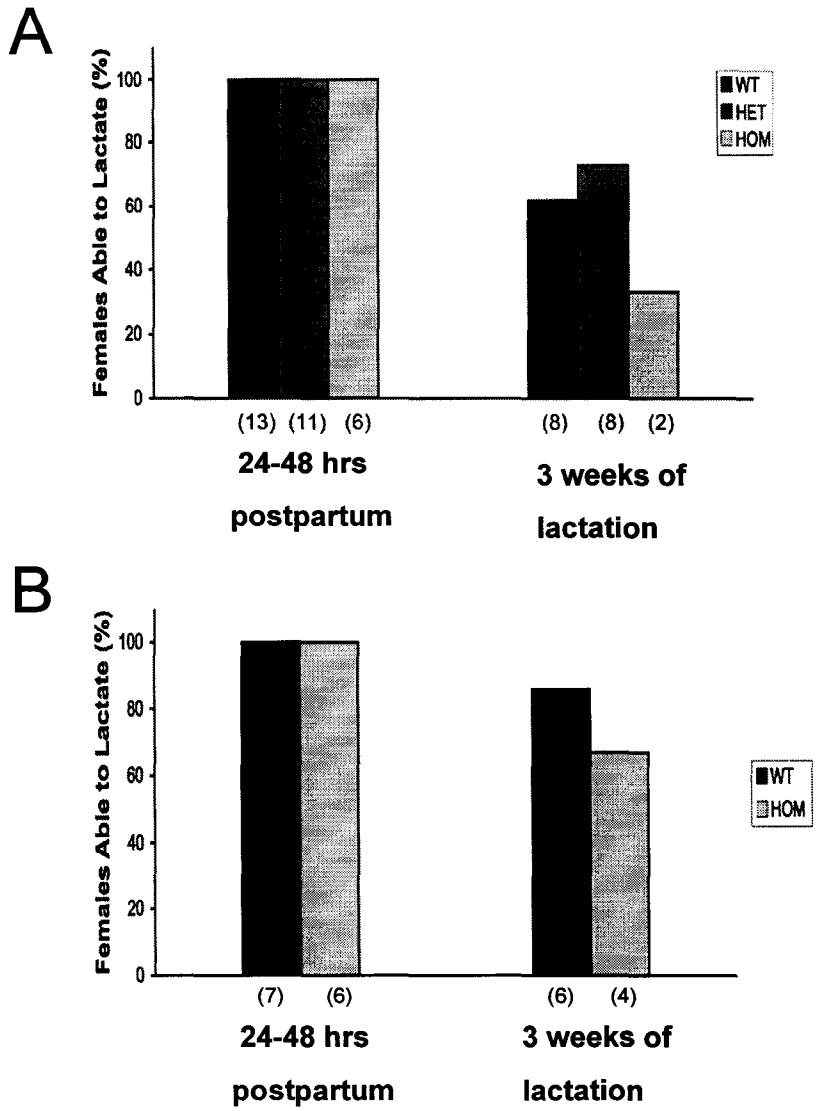


Figure 3.1: Percentage of mice that successfully lactated after delivery when fed the standard (A) or the calcium enriched (B) chow. When fed the standard chow, all genotypes lactated 24-48 hours. Most WT and HET mice were lactating 21 days postpartum, while most *Pth* null (HOM) mice were not. In some cases the mother had died suddenly, while in other cases the mother appeared not to lactate well and ate her pups. When fed the calcium enriched chow, all WT and *Pth* null (HOM) mice lactated 24-48 hours postpartum while only 86% of the WT mice and 67% of the *Pth* null (HOM) mice were lactating 21 days postpartum. The numbers in parentheses indicate the numbers of mice studied.

section is complete in and of itself and further research is required to confirm the findings.

Results from Mice Fed Standard Chow (1% Calcium, 0.75% Phosphorus)

3.2 Bone Densitometry

As shown in Figure 3.2, the *Pth* null mothers nursed far fewer pups compared to their WT and HET sisters. The *Pth* null mice aggressively culled their litters to a few pups (or none) whereas the WT and HET litters were not culled by us to match these numbers. The number of pups nursed during lactation likely had an impact on relative bone density changes between WT and *Pth* null. Figure 3.3 shows that when mice were fed the standard 1% calcium chow, all genotypes experienced a similar gain during pregnancy of approximately 13% of total body BMC relative to pre-pregnancy values. The BMC values fell during lactation to a trough of approximately 86% and all values returned to baseline within three weeks, with no significant differences among the time to recovery by genotype. The number of pups nursed averaged ten for WT, six for HET and four for *Pth* null mothers.

Reviewing the numbers in parentheses, one can see that while six *Pth* null mice were studied to the end of pregnancy, only two were studied during lactation and weaning. The remaining mice were lost to sudden maternal deaths during lactation, or did not lactate.

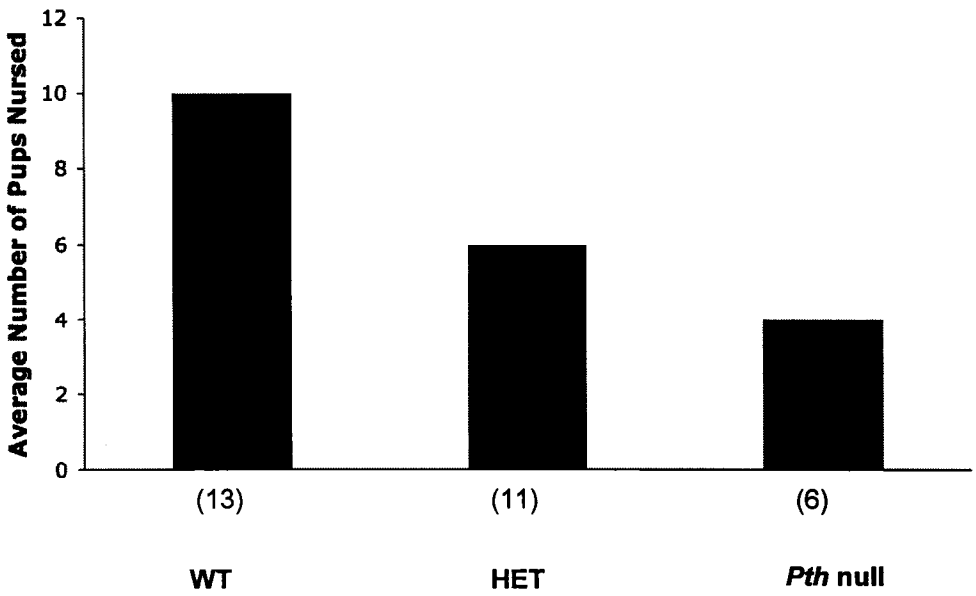


Figure 3.2: Average number of pups nursed by WT, HET and *Pth* null (HOM) mothers when fed the standard 1% calcium chow. The *Pth* null mothers nursed far fewer pups than their WT and HET siblings. The numbers in parentheses indicate the numbers of mice studied.

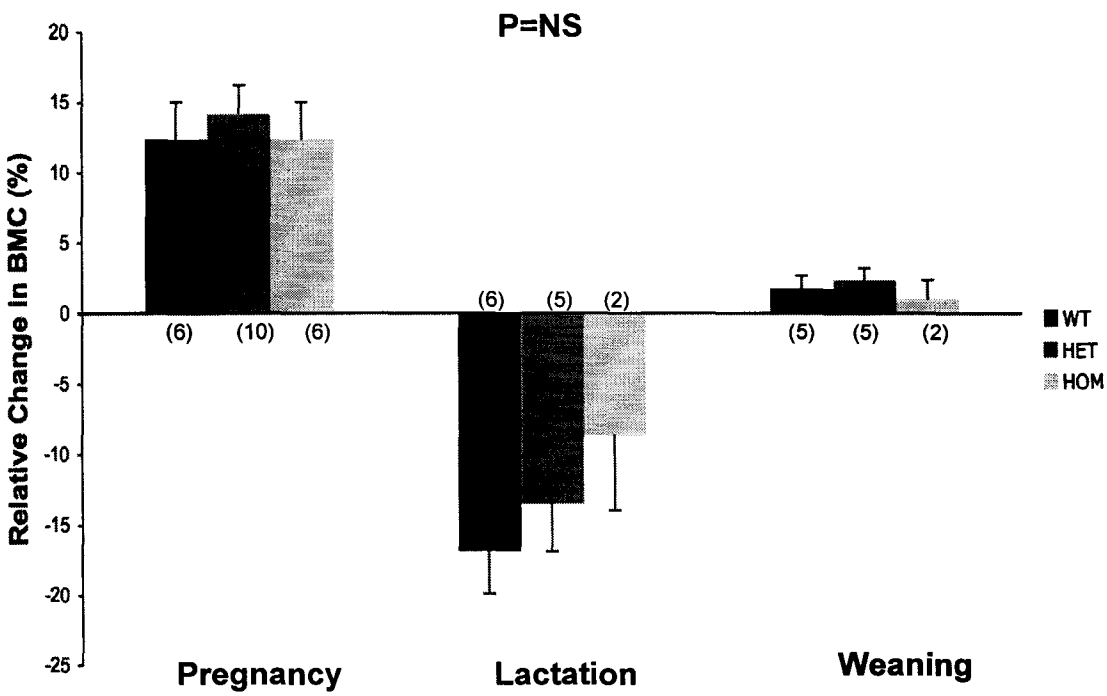


Figure 3.3: Total Body BMC (mean ± SE) in WT, HET and *Pth* null mothers at day 18 of pregnancy, day 19 of lactation and 21 days post-weaning, expressed as a percent from baseline (pre-pregnancy) BMC. The number of pups nursed averaged ten for WT females, six for HET females and four for *Pth* null (HOM) females. The numbers in parentheses indicate the numbers of mice studied.

3.3 Whole Blood Ionized Calcium

As displayed in Figure 3.4, *Pth* null mice were hypocalcemic at pre-pregnancy baseline compared to their WT sisters, however, the ionized calcium levels did not change significantly for any of the genotypes at any time point studied.

Fewer samples were obtained from *Pth* nulls, in fact, in several cases only one *Pth* null was studied. The low numbers are due to deaths and failure to lactate. In response to this, we decided to leave the few remaining *Pth* nulls alone during lactation so as to not disturb them from their pups.

3.4 Mineral Physiology

Maternal serum levels of magnesium and phosphorus were measured because regulation of calcium metabolism is often linked to phosphorus metabolism, and to a lesser extent, magnesium metabolism.

3.4.1 Serum Magnesium

Results shown in Figure 3.5 indicate that serum magnesium is slightly decreased during pregnancy in each of the genotypes, similar to what we have observed to occur in normal mice, although the changes were not statistically significant.

3.4.2 Serum Phosphorus

As expected, *Pth* null mice were hyperphosphatemic at the pre-pregnancy baseline compared to their WT sisters, however, results in Figure 3.6 show that serum

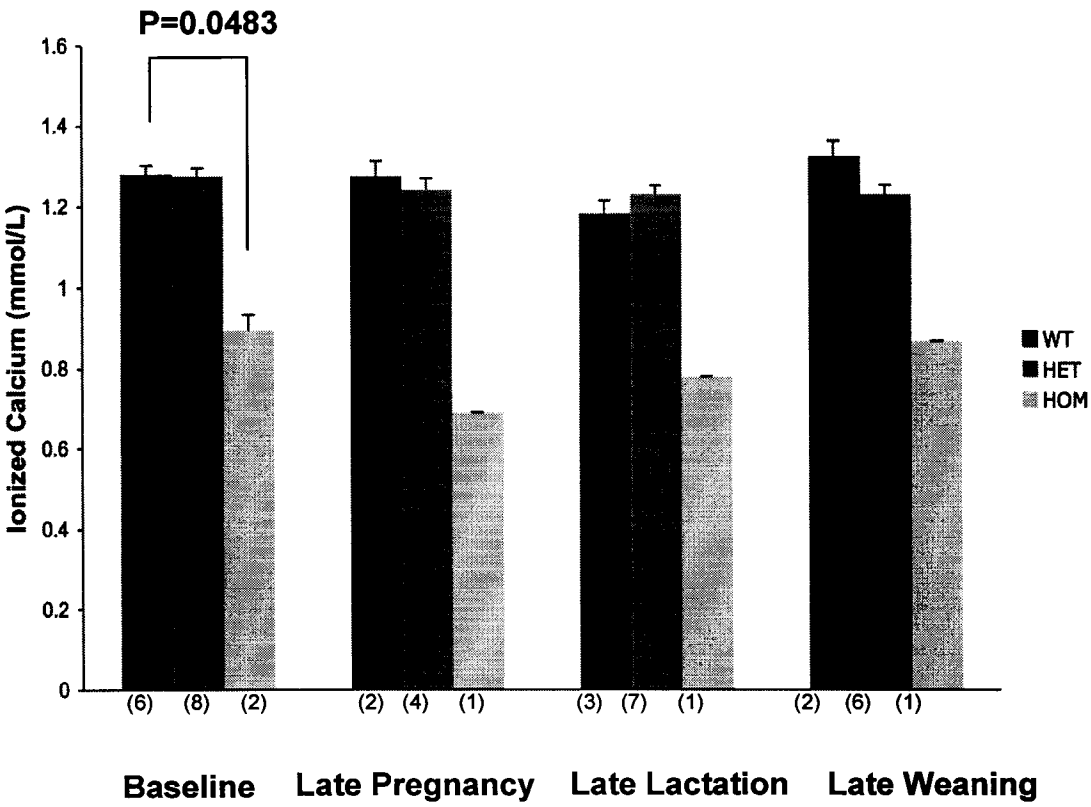


Figure 3.4: Maternal whole blood ionized calcium (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). At baseline, the *Pth* null (HOM) females were hypocalcemic compared to their WT and HET sisters. There was no significant change in ionized calcium during pregnancy, lactation and weaning in any of the genotypes. The numbers in parentheses indicate the number of mice studied.

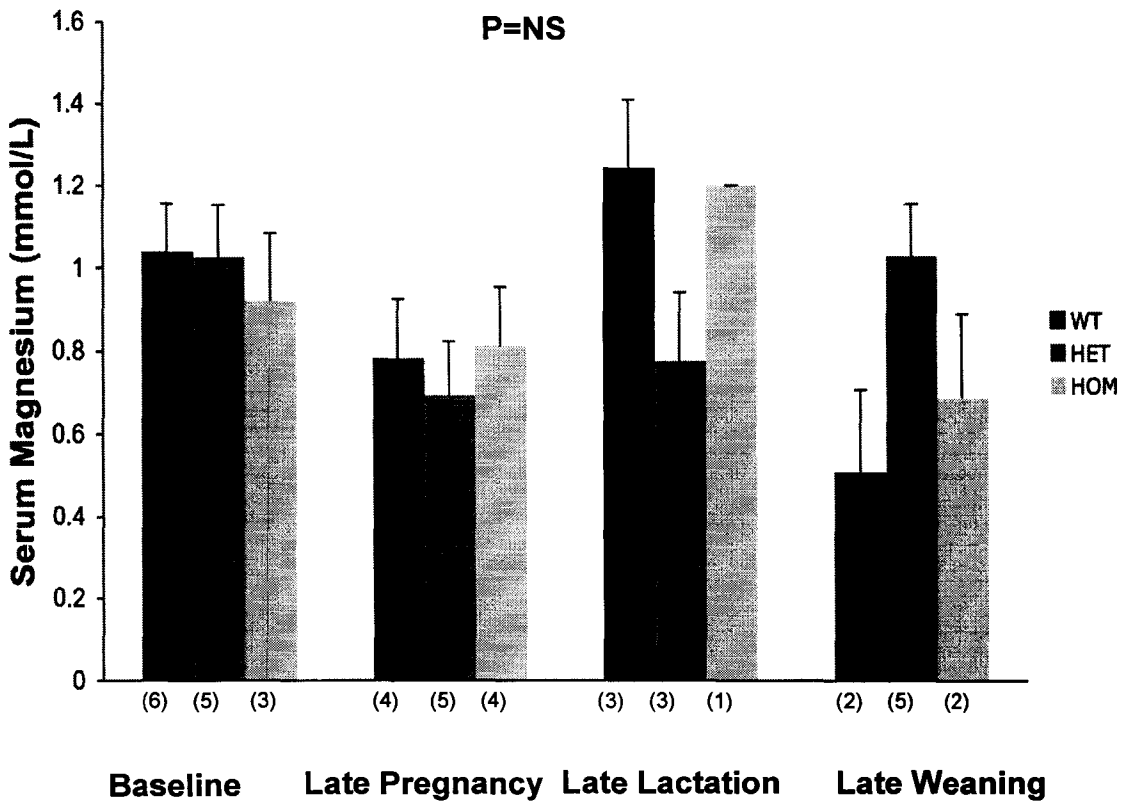


Figure 3.5: Maternal serum magnesium levels (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Serum magnesium declined non-significantly during pregnancy in all genotypes, however, there was no significant difference between any of the genotypes. The numbers in parentheses indicate the numbers of mice studied.

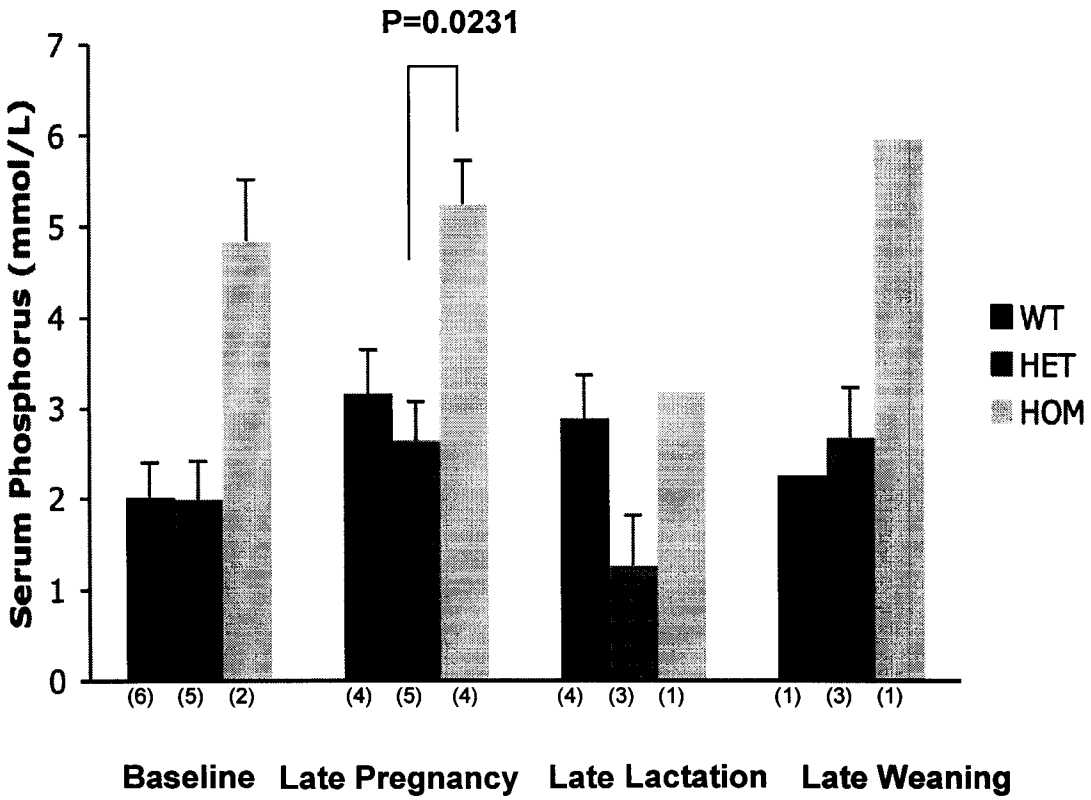


Figure 3.6: Maternal serum phosphorus (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Serum phosphorus was elevated in *Pth* null (HOM) mice, and did not change significantly during pregnancy or lactation. The numbers in parentheses indicate the numbers of mice studied.

phosphorus did not change significantly over the reproductive period for WT, HET of *Pth* null females.

3.4.3 Serum Osteocalcin

Results from serum osteocalcin diplayed in Figure 3.7 show that the levels did not change significantly over the reproductive period for any of the genotypes. Again, low numbers are an issue and further investigation is required to confirm these findings but based on what we have found, there was no significant change in the osteocalcin levels despite the shift from bone resorptive state of lactation to bone formative state of weaning.

3.4.4 Urinary Deoxypyridinoline (DPD)

Urinary deoxypyridinoline (DPD) does not change significantly over the reproductive period (figure 3.8). DPD was corrected for with creatinine and the units are expressed as nmol/L DPD per mmol/L creatinine. This differs from what our lab and other labs have previously found, that DPD normally increases during lactation. In this case, it may be an issue of low sample numbers and clearly additional studies are needed.

3.4.5 Ash Weight Analysis and Flame Atomic Absorption Spectroscopy

We also measured the content of calcium and magnesium in three week old pups at the time of weaning, in order to determine if maternal genotype affected the amount of

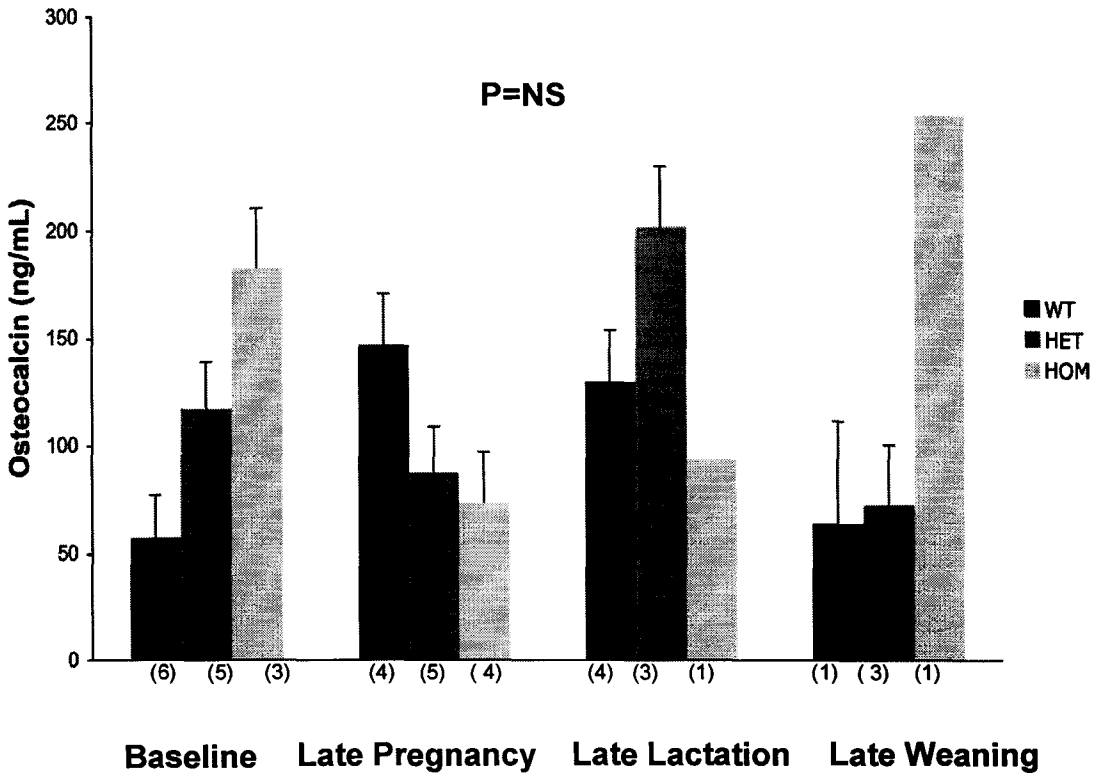


Figure 3.7: Maternal serum osteocalcin (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). There was no significant change in serum osteocalcin during pregnancy, lactation and weaning in any of the genotypes. The numbers in parentheses indicate the number of mice studied.

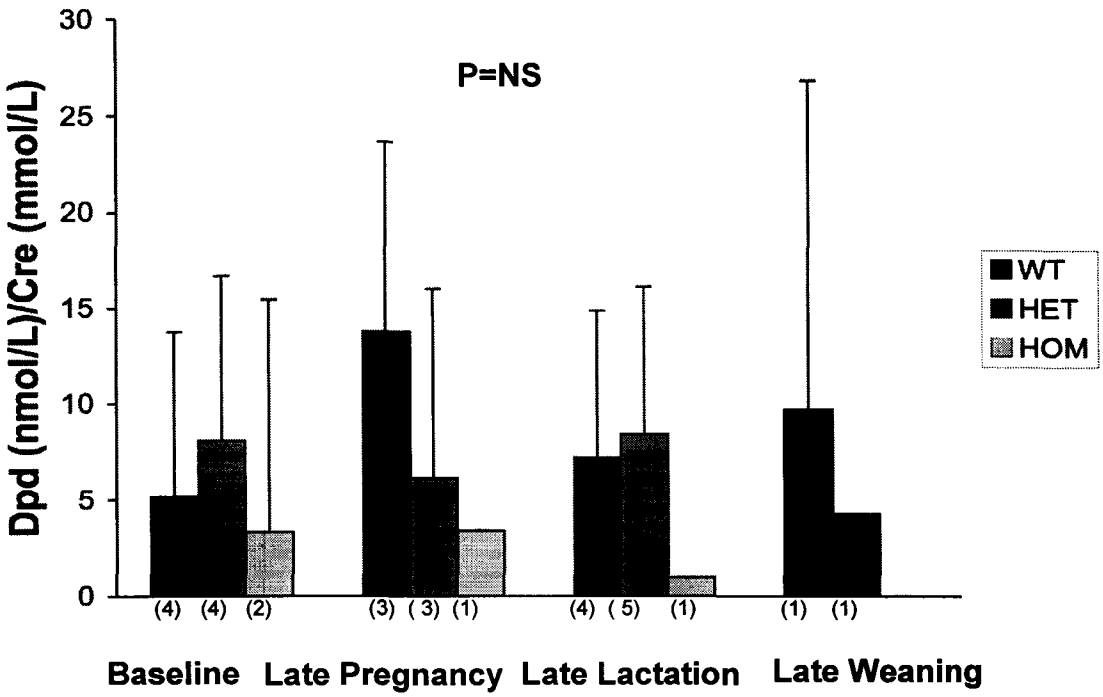


Figure 3.8: Maternal urinary deoxypyridinoline (DPD) corrected for creatinine (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Urinary calcium was elevated significantly (P=0.0395) in WT mice during pregnancy compared to baseline. There was no significant difference between any of the stages for the *Pth* null (HOM) females. The numbers in parentheses indicate the numbers of mice studied.

mineral accreted by the neonatal skeletons. The ash weights were corrected to account for the wet weight of each pup.

As shown in Figure 3.9, there was no significant difference among the ash weights of WT, HET or HOM pups of HET dams. For the pups of HOM dams, as shown in the same figure, there was also no significant difference between either of the genotypes. Also, by comparing the results from HET dams and *Pth* null dams, it is evident that maternal absence of PTH did not affect the net accretion of mineral by the pups.

As displayed in Figure 3.10, there was no significant difference in skeletal calcium content in any pups from HET dams nor among pups of *Pth* null dams. The calcium content of the pups was unaffected by whether the mother was *Pth* null or not. Similarly, for skeletal magnesium, there were no significant differences in magnesium content among the pups of HET mothers or null mothers and again no differences were seen across the maternal genotypes either (Figure 3.11).

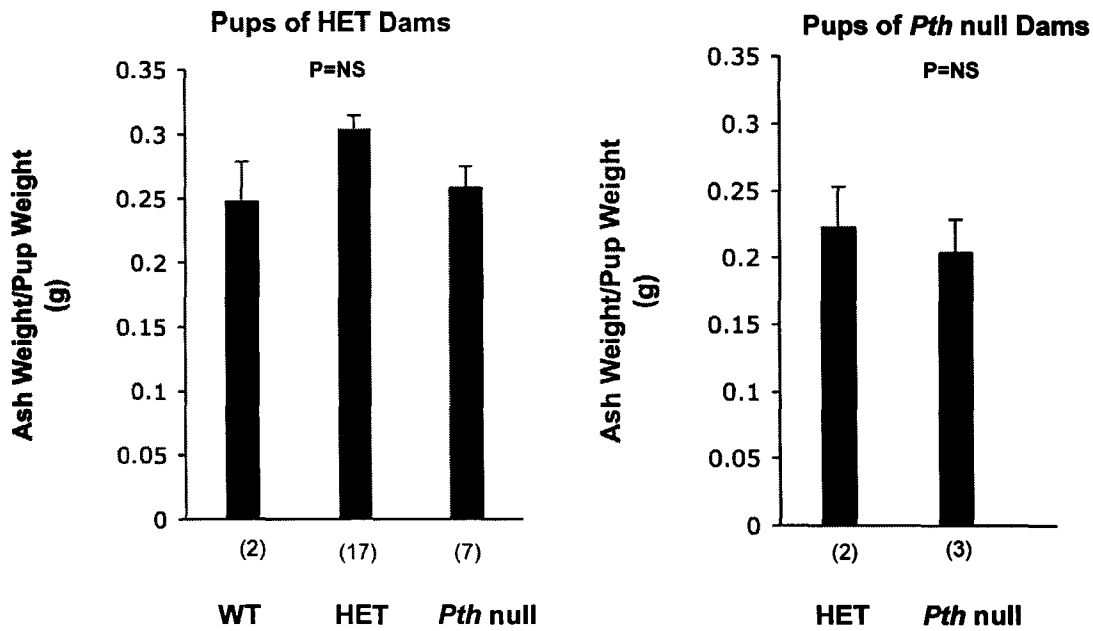


Figure 3.9: Skeletal mineral (mean ± SE) present in pups following 21 days of lactation (HET and *Pth* null Dams). Results were calculated as pup ash weight (g)/ initial pup wet weight (g). There was no significant difference in skeletal mineral between any of the genotypes of either mothers. The numbers in parentheses indicate the numbers of pups studied.

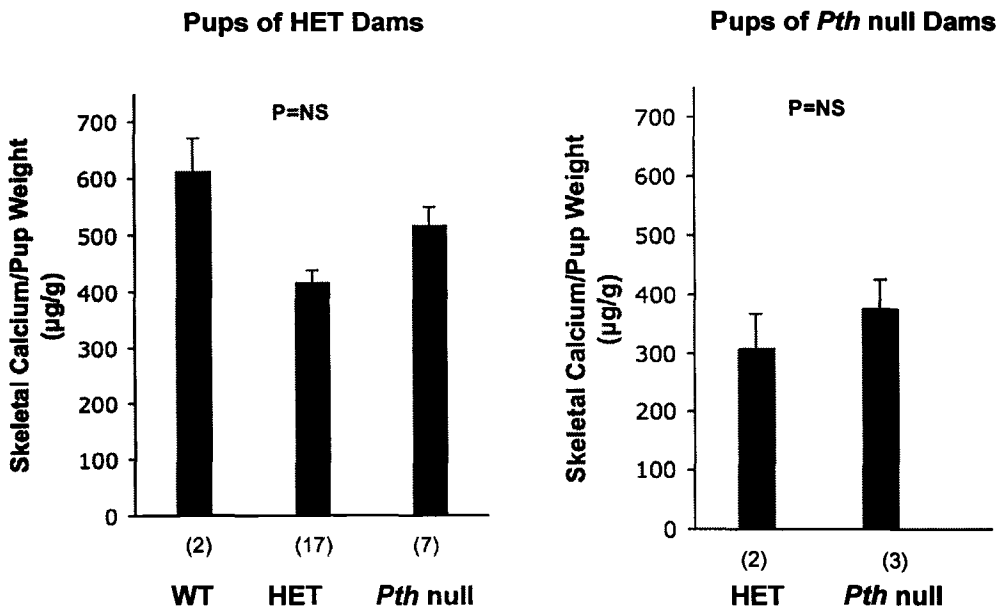


Figure 3.10: Skeletal calcium (mean ± SE) present in pups following 21 days of lactation (HET and *Pth* null Dams). Results were calculated as skeletal calcium(µg)/pup wet weight (g). There was no significance difference in skeletal calcium for any of the genotypes of either mothers. The numbers in parentheses indicate the numbers of pups studied.

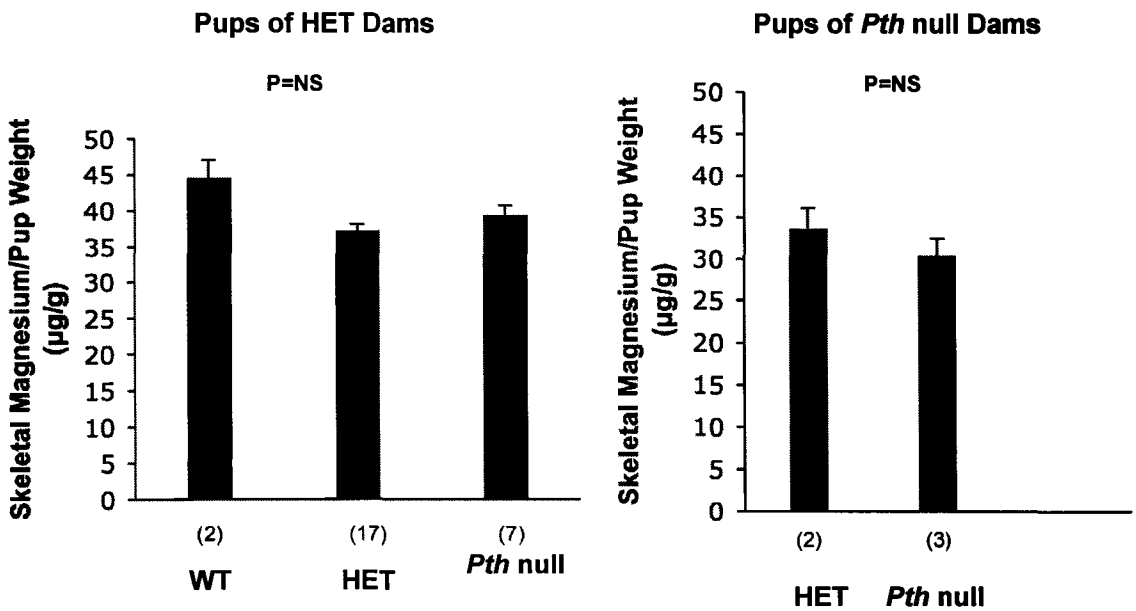
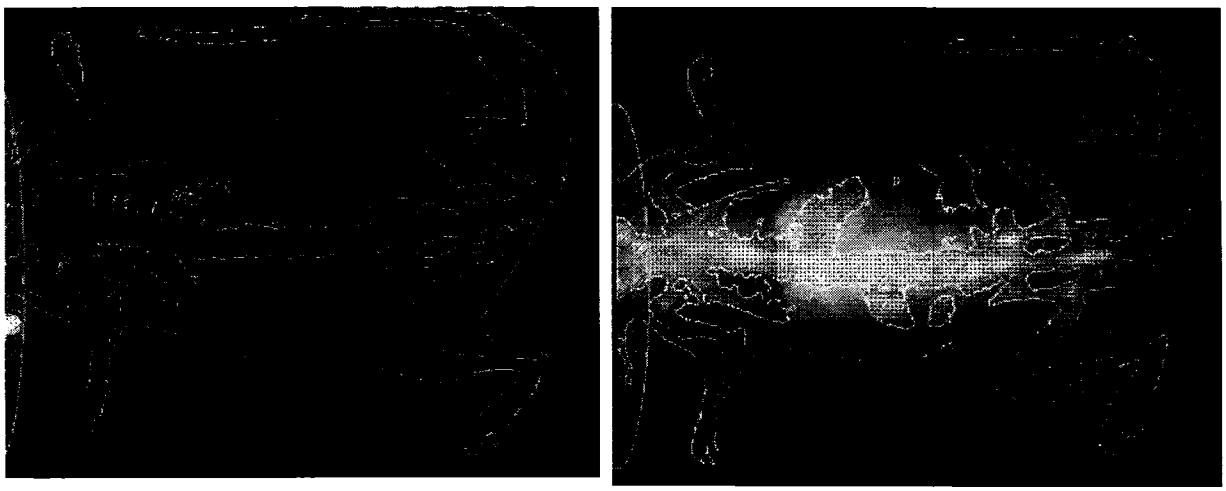


Figure 3.11: Skeletal magnesium (mean ± SE) present in pups following 21 days of lactation (HET and *Pth* null Dams).
Results were calculated as skeletal magnesium/pup wet weight. There was no significance difference in skeletal magnesium for any of the genotypes of either mothers. The numbers in parentheses indicate the numbers of pups studied.

Results from Mice Fed Calcium Enriched Chow (Lactose, 2% Calcium, 1.25% Phosphorus)

Due to time constraints, halfway through my two year Masters program, we decided to switch the studies to focus solely on WT and *Pth* null females all of whom were fed a calcium enriched chow, containing lactose, 2% calcium and 1.25% phosphorus (Harlan Teklad, Madison, Wisconsin, USA). A drawback with using the enriched diet was that the excess calcium would constipate the mice so that when they were scanned, we would get an extremely high total body BMC reading due to radio opaque, calcium rich feces, as displayed in Figure 3.12. To deal with this situation, we initially were limited to using only the hind limb region of the scan to examine changes in BMC since this region does not include the bowels. Of course, this is not as ideal as using the whole body scan and especially less ideal than using the spine, which is where the most profound changes occur during lactation. We later discovered that if the chow was removed from the cages for several hours prior to scanning, the intestines would become completely free of the excess calcium and a whole body scan could be taken. This discovery was made late in the project and up to that point only hind limb scans had been taken, and therefore, only hind limb data are available to be shown in this section.

With the mice on the calcium enriched diet we also began culling WT litters to ensure that the numbers of pups nursed equaled that of the null mice. However, none of the mice fed the standard chow had had their litters culled.



A B

Figure 3.12: Representative scans of total body BMC. Measured at three weeks post-weaning in a mouse on the standard 1% calcium chow (**A**) and at four weeks post-weaning in a mouse on the 2% calcium enriched chow (**B**). As shown in **B**, when mice are fed the calcium enriched diet, the excess calcium constipates the animals and results in radio opaque feces that artifactually inflate the BMC reading. On the other hand, if the mouse is switched back to the 1% calcium diet for several hours prior to the scan being taken, the image looks indistinguishable from A because the radioopaque feces have cleared the abdomen (data not shown).

3.5 Bone Densitometry

As shown in Figure 3.13, there was an increase in hind limb BMC during pregnancy to a peak of approximately 11% in WT females and 6% in *Pth* nulls relative to pre-pregnancy baseline. The BMC values fell approximately 3.5% below pre-pregnancy baseline during lactation in both genotypes. Preliminary results indicate that the WT females recover to their pre-pregnancy baseline hind limb BMC within three weeks post-weaning. Due to the fact that no *Pth* null females survived after reaching their trough point, there is no post-weaning data available. It is important to keep in mind that these results are based upon hind limb BMC readings and that whole body but especially spine BMC readings are required to be certain what excursions in BMC occur during lactation and recovery in mice.

3.6 Whole Blood Ionized Calcium

Figure 3.14 shows that at pre-pregnancy baseline, *Pth* null mice were hypocalcemic compared to their WT sisters. However, there was no significant change in ionized calcium for either of the genotypes at any time point. When data from the mice fed the 1% calcium chow (Figure 3.4) was compared to data from the mice fed the 2% calcium chow (Figure 3.14), the *Pth* null females on the standard 1% calcium chow had slightly lower ionized calcium at the peak of pregnancy, trough of lactation as well as during late weaning.

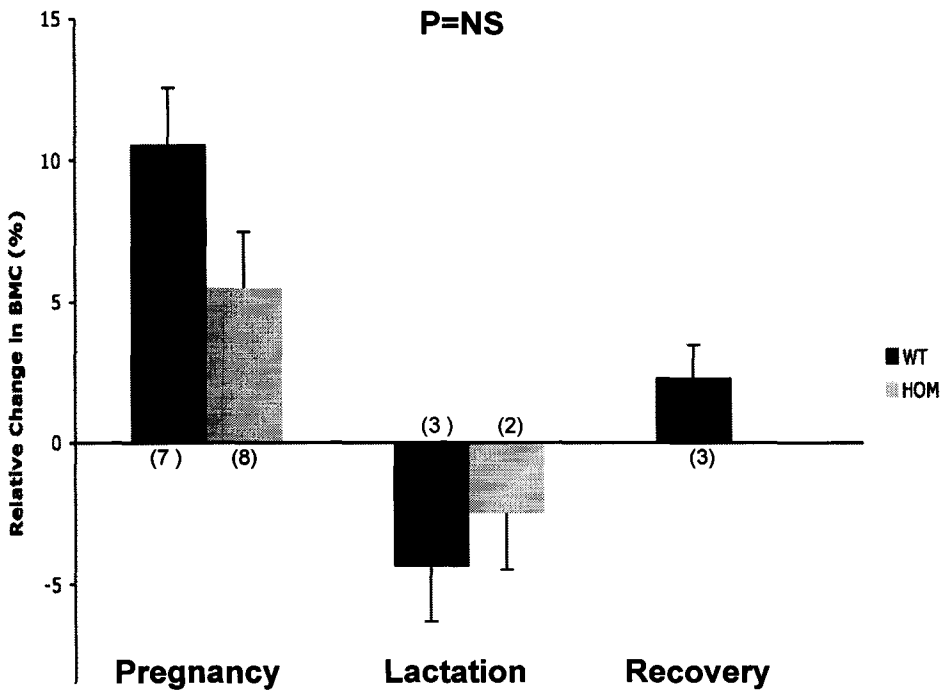


Figure 3.13: Hind Limb BMC (mean ± SE) in WT and *Pth* null (HOM) mothers at day 18 of gestation and day 19 of lactation, and 21 days post weaning, expressed as a percent from pre-pregnancy baseline BMC. The numbers in parentheses indicate the number of mice studied.

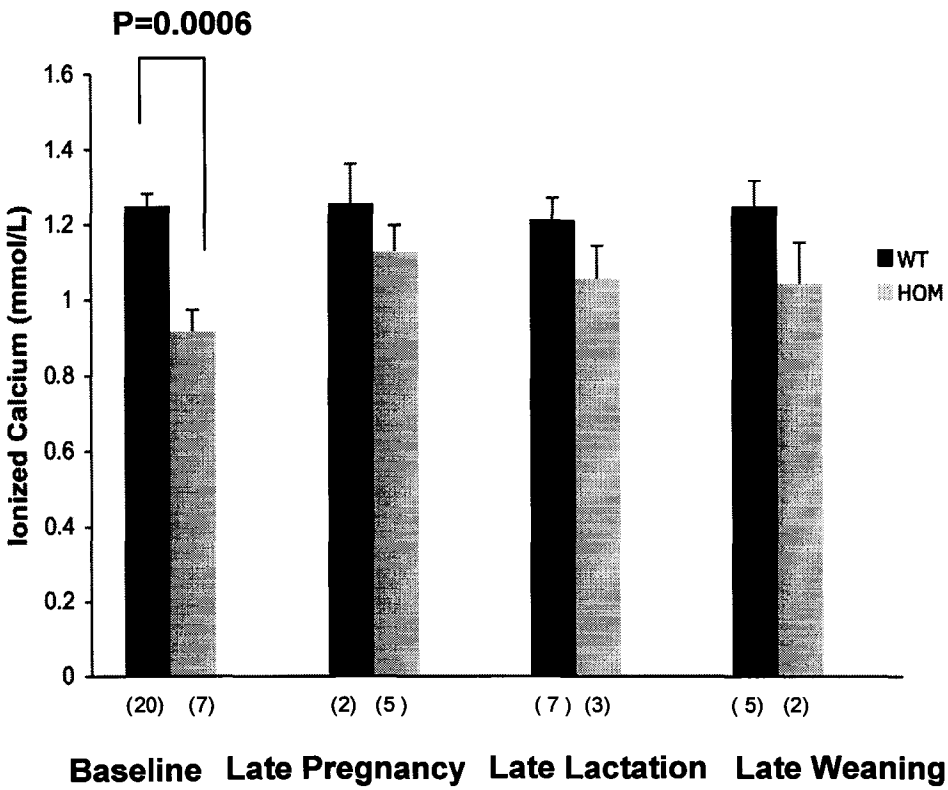


Figure 3.14: Maternal whole blood ionized calcium (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). At baseline, the *Pth* null (HOM) mice were hypocalcemic compared to their WT sisters. There was no significant change in ionized calcium during pregnancy, lactation and weaning in either of the genotypes. The numbers in parentheses indicate the number of mice studied.

3.7 Mineral Physiology

Maternal serum levels of magnesium and phosphorus were measured and ash of three week old pups was analysed to measure the content of calcium and magnesium in the skeleton.

3.7.1 Serum Magnesium

Results from Figure 3.15 indicate that serum magnesium was decreased non-significantly during late weaning in *Pth* nulls, however, there was no obvious change in serum magnesium for either of the genotypes at any of the stages studied.

3.7.2 Serum Phosphorus

Pth null females were hyperphosphatemic compared to their WT sisters, however, results show that serum phosphorus did not change significantly over the reproductive period in either the WT or *Pth* null females (Figure 3.16).

3.7.3 Urine Calcium

Figure 3.17 shows that urine calcium was elevated significantly (P=0.0395) in WT mice during pregnancy compared to baseline however, there was no significant difference for the HOM females at any of the stages.

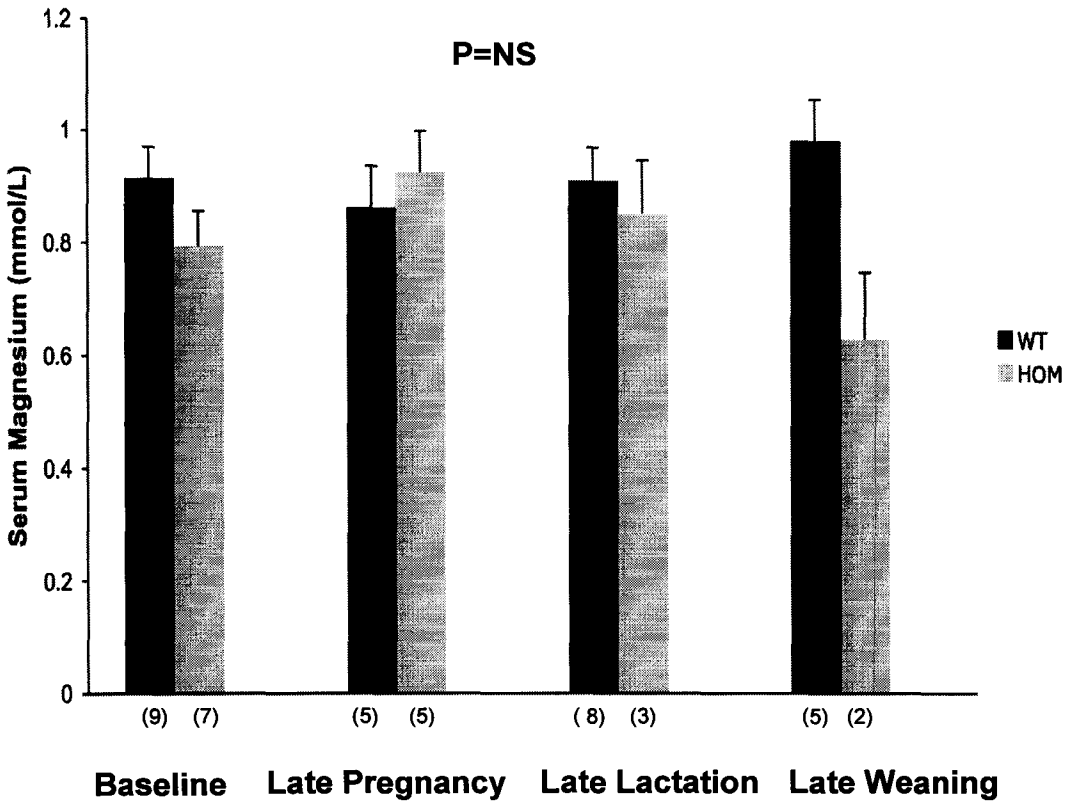


Figure 3.15: Maternal serum magnesium levels (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Serum magnesium declined non-significantly during late weaning in the *Pth* null (HOM) females, however, there was no significant difference between either of the genotypes at any stage. The numbers in parentheses indicate the numbers of mice studied.

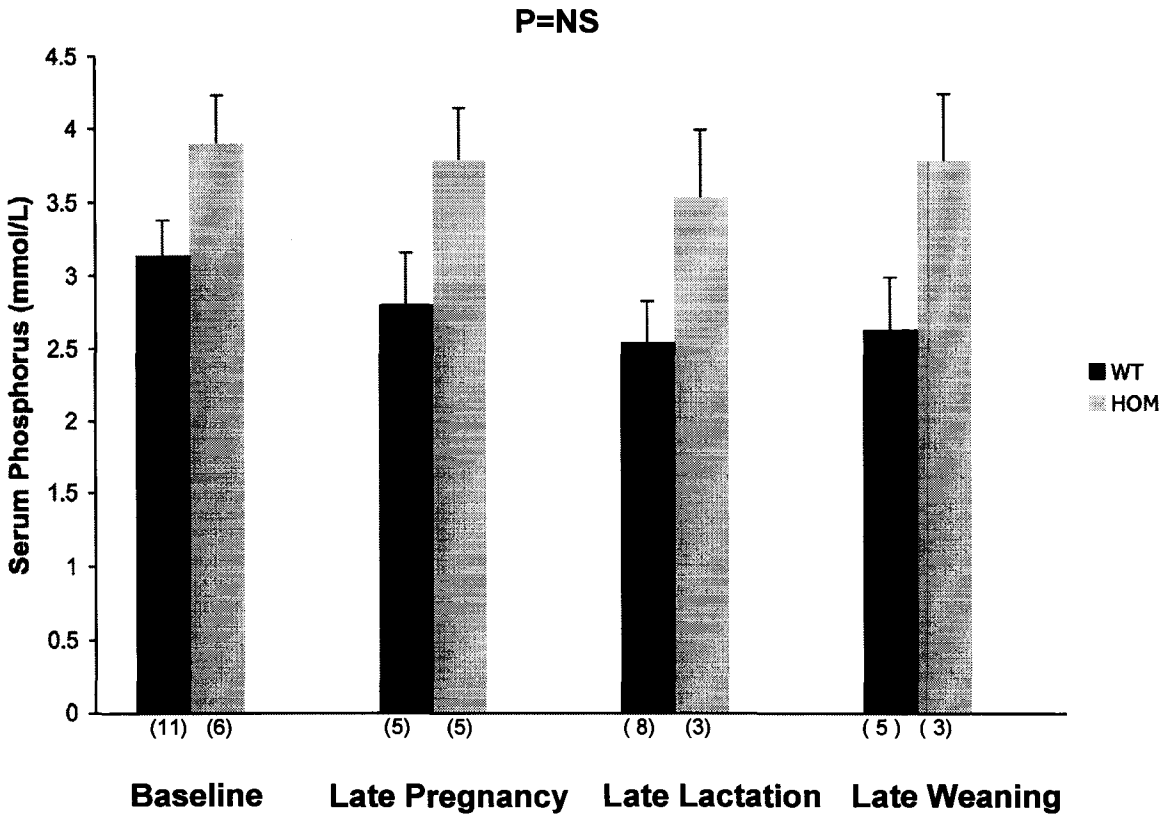


Figure 3.16: Maternal serum phosphorus levels (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Serum phosphorus was elevated in the *Pth* null (HOM) females and did not change significantly during pregnancy, lactation or weaning. There was no significant difference between the genotypes at any stage. The numbers in parentheses indicate the numbers of mice studied.

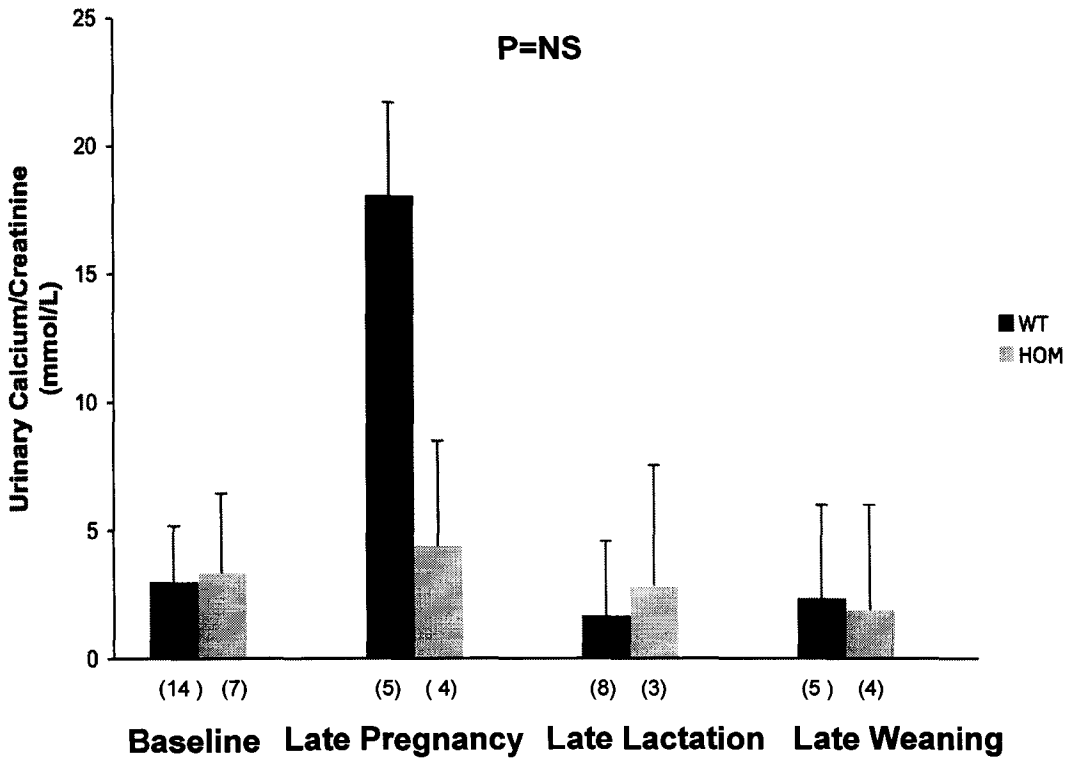


Figure 3.17: Maternal urinary calcium corrected for creatinine (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Urinary calcium was elevated significantly (P=0.0395) in WT mice during pregnancy compared to baseline. There was no significant difference between any of the stages for the *Pth* null (HOM) females nor was there a significant difference between the genotypes. The numbers in parentheses indicate the numbers of mice studied.

3.7.4 Urine Phosphorus

As displayed in Figure 3.18, urinary phosphorus was non-significantly elevated in both WT and *Pth* null females during pregnancy compared to baseline, however, there was no significant difference between the genotypes at any other stage.

3.7.5 Ash Weight Analysis and Flame Atomic Absorption Spectroscopy

As shown in Figure 3.19, there was a small but significant difference between the ash weights of the WT pups compared to HET pups of WT dams (P=0.0022) but there was no significant difference between HET and *Pth* null pups of *Pth* null dams. There was also no obvious difference in total skeletal calcium (Figure 3.20) or magnesium (Figure 3.21) in pups of WT of *Pth* null dams.

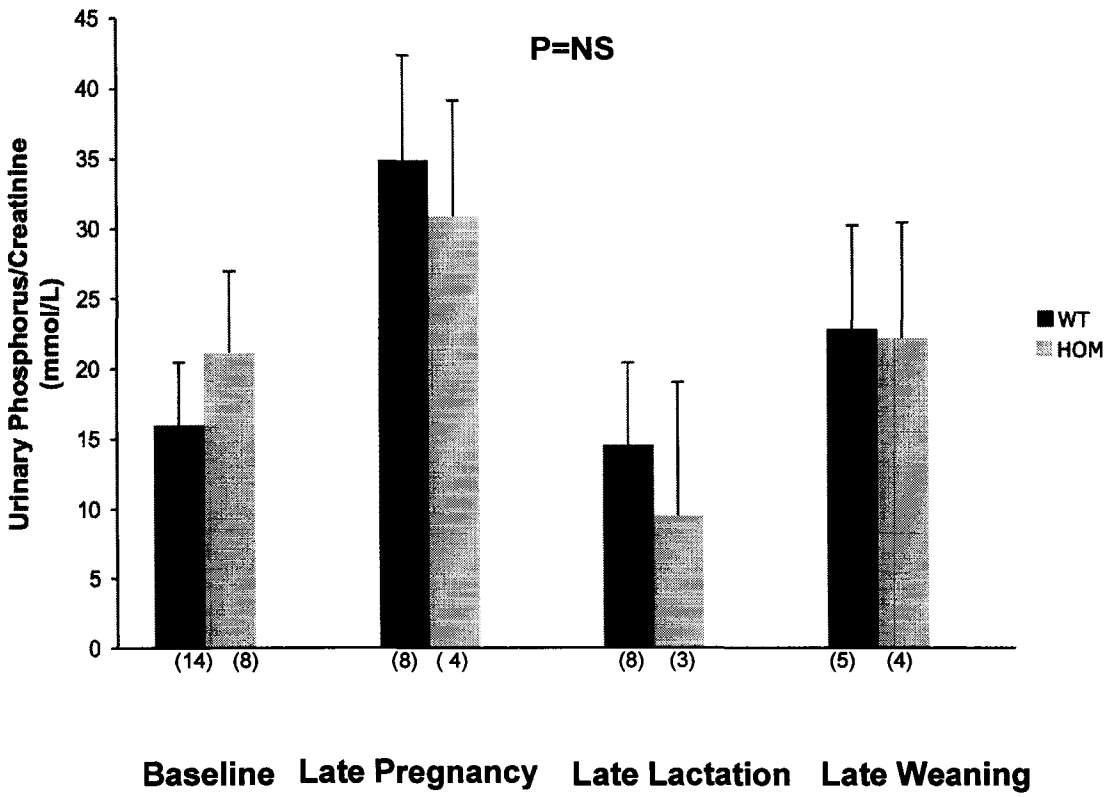


Figure 3.18: Maternal urinary phosphorus corrected for creatinine (mean ± SE) over the entire reproductive period (baseline [pre-pregnancy], pregnancy, lactation and weaning). Urinary phosphporus was non-significantly elevated in both WT and *Pth* null (HOM) females during pregnancy compared to baseline, however, there was no significant difference between the genotypes at any stage. The numbers in parentheses indicate the numbers of mice studied.

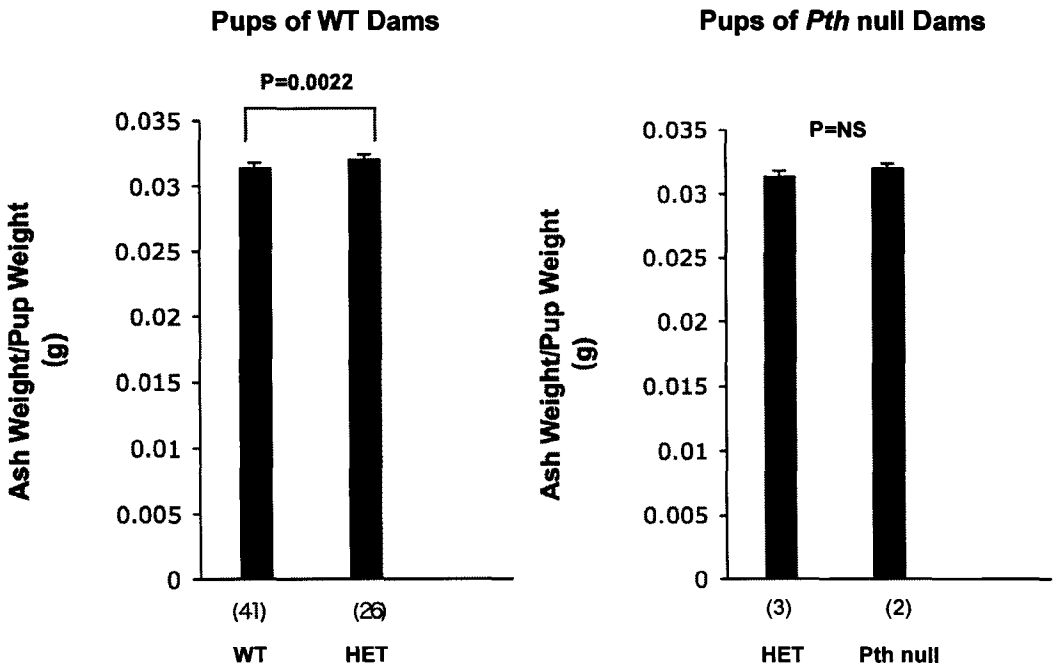


Figure 3.19: Skeletal mineral (mean ± SE) present in pups following 21 days of lactation (WT and *Pth* null Dams). Results were calculated as pup ash weight (g)/initial pup wet weight (g). There was a significant difference in skeletal mineral between WT and HET pups (P=0.0022) of WT dams but no significant difference between HET and *Pth* null pups of *Pth* null dams. The numbers in parentheses indicate the numbers of pups studied.

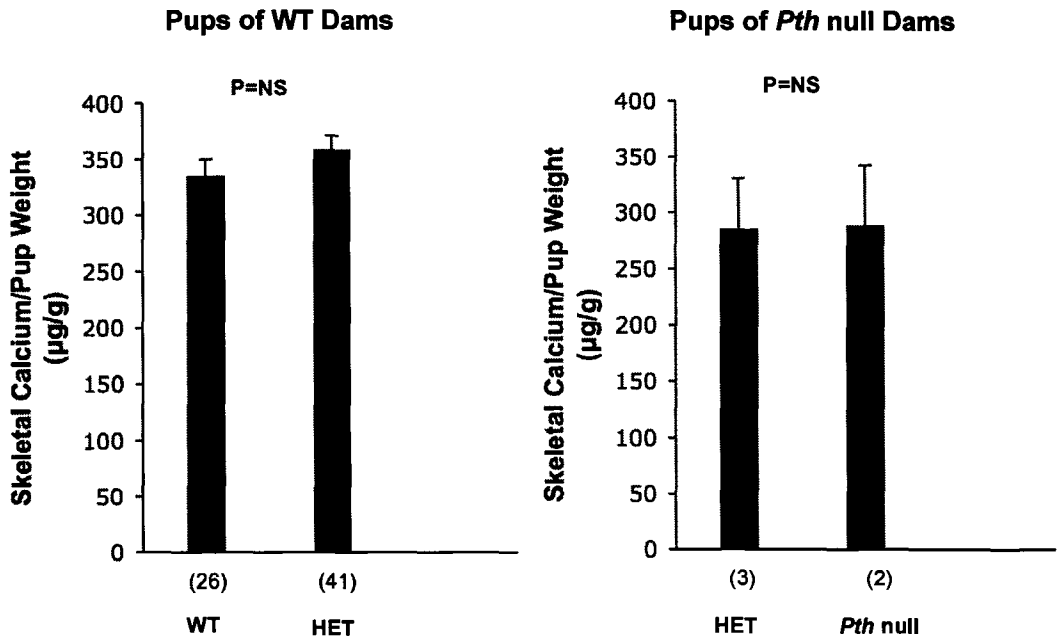


Figure 3.20: Skeletal calcium (mean ± SE) present in pups following 21 days of lactation (WT and *Pth* null Dams). Results were calculated as skeletal calcium/pup wet weight. There was no significant difference in skeletal calcium between pups of either mothers. The numbers in parentheses indicate the numbers of mice studied.

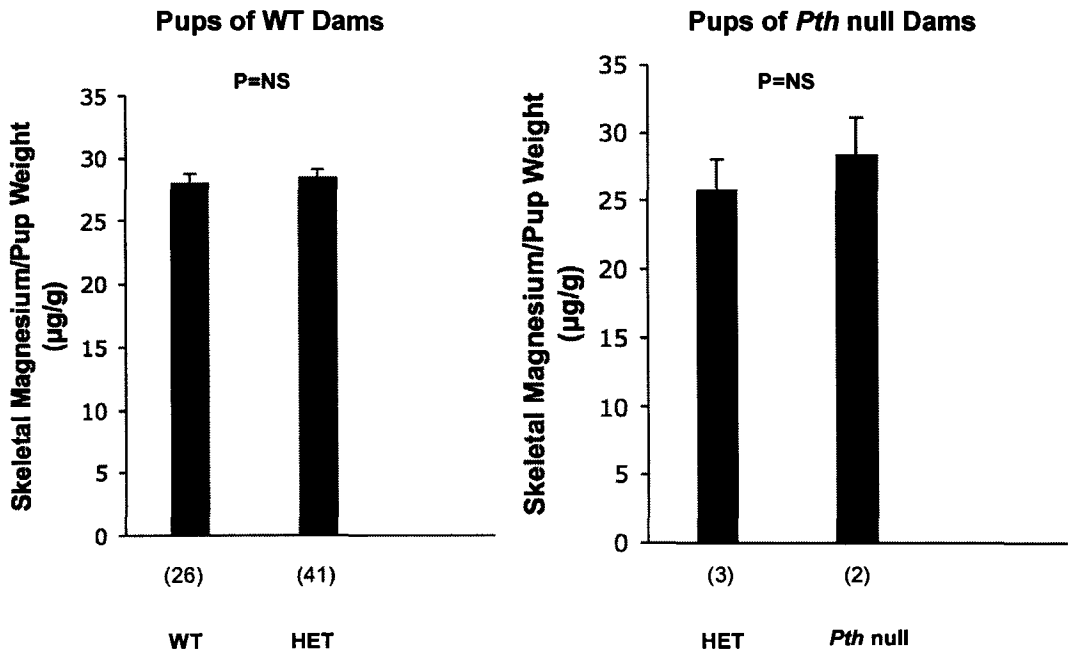


Figure 3.21: Skeletal magnesium (mean ± SE) present in pups following 21 days of lactation (WT and *Pth* null Dams). Results were calculated as skeletal magnesium/pup wet weight. There was no significance difference in skeletal magnesium between pups of either mothers. The numbers in parentheses indicate the numbers of mice studied.

IV Discussion

These studies involved measuring several parameters of calcium and bone metabolism to determine whether PTH is required for regulating mineral homeostasis during pregnancy and lactation and whether PTH is needed for remineralization of the skeleton post-weaning. We examined the maternal effects of PTH deletion through measuring BMC changes, ionized calcium and various calciotropic hormones in both serum and urine. We also measured skeletal development of three week old pups after they had been weaned.

4.1 Trouble-shooting Problems Encountered During the Course of the Project

Any research project can and usually does have unexpected problems, perhaps especially when working with animals. This project was no exception. We dealt with every obstacle encountered to the best of our abilities within the limited amount of time available for a Masters project.

One major obstacle that we faced during the first year of the project was the fact that there were some sudden maternal deaths during pregnancy or at the start of lactation, many of which may have been due to hypocalcemia, with or without exposure to anesthesia. These deaths were more commonly observed during pregnancy rather than lactation regardless of the chow the mice were fed and most deaths were anesthetic related however there were deaths unrelated to anesthesia as well (Table 3.1). In most cases, the animals died within minutes of being given anesthetic, while in few cases, the mice were found dead several hours after they had been injected. In the first instance,

one simple explanation is that the mice were given too large a dose of anesthetic and that this led to their death by stopping respiration. In the latter instance, death may have resulted due to late complications of anesthesia. The fact that the mice were hypocalcemic to begin with and then were given anesthesia may have increased the risk of hypothermia and cardiac arrhythmias.

A related issue with this project was that originally we scanned the mice every other day over a full 70 day reproductive period, including pre-pregnancy baseline, pregnancy, lactation and weaning/recovery. If the *Pth* nulls are more susceptible to sudden death due to anesthesia, the frequent scanning and anesthesia meant that the mice were too frequently exposed to the risk of sudden death. To deal with these issues, all mice were placed a 2% calcium chow instead of the standard 1% calcium chow to reduce the effects of severe hypocalcemia. The original anesthetic used in our lab for this protocol was methohexital sodium which gave 10-15 minutes of anesthesia; however, this anesthetic was discontinued by the manufacturer just prior to the start of my project. Therefore, at the start of this project, the anesthetic used to anaesthetize the mice prior to scanning was Penthothal® (Abbott Laboratories, Vaughan, Ontario, Canada) which was eventually changed to a combination of Ketamine Hydrochloride (Pfizer Canada Inc. Kirkland, Quebec, Canada) and Xylazine (Bayer Inc. Toronto, Ontario, Canada). Penthothal® caused more prolonged anesthesia which in turn, may have caused maternal deaths. Ketamine Hydrochloride/Xylazine proved to be better in that it was faster acting and in most cases, mice recovered within 1-2 hours and there were also fewer maternal deaths while using this anesthetic. Unfortunately, since the diet was also changed at the

same time, it is difficult to know how much of the improvement in maternal mortality was due to the change in anesthetic. In addition, we also started to scan the mice less frequently and only scanned them at the significant time-points (pre-pregnancy baseline, peak of pregnancy, trough of lactation and weaning/recovery) and not every other day.

In any case, when using anesthetic, these problems and in particular the deaths are not surprising and are often unavoidable. The change in anesthetic and in the scanning schedule did appear to reduce the number of maternal deaths previously encountered. In addition, we did try several things to reduce the number of deaths, including placing the animal under a heat lamp while monitoring the temperature to ensure that they did not become hypothermic or overheated. We also tried to avoid returning the mice to Animal Care (cooler environment) until they had fully recovered from the anesthetic.

The apparent improvement on the 2% calcium chow may have resulted from several factors. We speculate that the extra calcium ingested by the *Pth* null mice from the 2% calcium chow may reduce or eliminate the problems caused by low 1,25-dihydroxyvitamin D levels. This in turn may improve calcium absorption in the intestines as more calcium is available. 1,25-dihydroxyvitamin D normally doubles during pregnancy (13,14) and even hypoparathyroid individuals may experience some increase because of effects of PTHrP, prolactin and placental lactogen to stimulate the 1α-hydroxylase (19). However, despite all of this 1,25-dihydroxyvitamin D could still be low in *Pth* nulls and this would cause reduced intestinal calcium absorption. 1,25-dihydroxyvitamin D mediated absorption is active and not affected by dietary calcium content. On the other hand, passive absorption of calcium will be increased by a higher

calcium content in the diet. Therefore, the 2% calcium diet should increase the passive absorption of calcium and this in turn would reduce the likelihood of hypocalcemia and maternal deaths in the *Pth* nulls.

Another problem which arose early in the course of the project was that many *Pth* null females experienced other problems during lactation. It appeared as though either the *Pth* null mothers simply could not lactate or that they would reject their pups and cull their litters to zero. It may be that because the *Pth* null mothers are hypocalcemic they simply cannot mobilize sufficient calcium to produce milk, and their pups die of starvation. Another possibility is that the mother cannot cope with the calcemic stress of nursing a large number of pups and to deal with this stress she culls her litter. However, it is also possible that the female simply rejects and eats her pups, something commonly observed in mice and other rodents as a way to deal with non-specific stress. A similar issue is that in many cases, when returning a lactating mother to her pups after she had recovered from anesthetic, she would neglect her young and they would die or she would sometimes eat them. In order for the data collected to be comparable, steps were taken in the latter half of this project to ensure that all mice were nursing the same number of pups. Twenty-four hours after delivery all litters were culled down to 3-4 pups, which was the average number of pups *Pth* null females nursed. Culling the litters would make the results more comparable between females of different genotypes by eliminating variability due to mothers nursing different numbers of pups.

With regards to the problems experienced by the *Pth* null mice during lactation, it would be interesting to know if the pups contributed to these problems in any way. As

will be discussed in a little more detail in the Future Directions Section, it would be useful to conduct cross-fostering experiments so that the pups of the WT and *Pth* null mothers would be switched shortly after birth. This would examine the possibility that the problems during lactation might be due to abnormal fetuses and not actually because of the mother or her genotype.

All of the problems mentioned, including the low reproduction rates and problems during lactation for the *Pth* nulls slowed the progress in the first year of the project and led to fewer completed reproductive cycles than expected. By changing the diet, we hoped to have more success in obtaining data from mice during the reproductive period. Unfortunately, as mentioned earlier in this report, a problem then arose with the 2% calcium chow in that it constipated the mice and rendered the spine BMC readings invalid because of radio opaque feces. This was initially dealt with by performing scans of the hind limb only. Eventually the problem was solved when it was discovered that placing the mice on the regular 1% calcium chow overnight would clear the bowels and allow the whole body and spine BMC scans. However, this solution was discovered too late to impact on the data collection reported here.

As can be observed in many of the figures, the sample size for the *Pth* nulls is often low due to a combination of the problems discussed. However, because of time constraints, we were unable to obtain sufficient or definitive numbers on the 2% calcium chow. Any data obtained from mice on the 2% calcium chow were presented separately from the data collected from the mice on the 1% calcium chow due to the fact that the two sets of data are not comparable.

4.2 Limitations in Using Knockout Models

In scientific research today, when looking at genetic diseases, genes and their functions, and the many other scientific research questions it is often most practical to use a gene knockout model in which all or part of a gene has been removed or inactivated through genetic manipulation. Of interest to our lab is that knockout models can be used to examine the role of various genes during skeletal development, however, these models are not useful in instances where the phenotype is severe enough to lead to fetal or neonatal death (46). Using animals in experimental research has enabled scientists to go beyond what previous years of research allowed them to do. As an example, engineering of the mouse genome has changed the way biomedical research is done due to the fact that the animal can be used for a wide range of experiments (46).

In spite of the fact that conventional and transgenic knockout models are very useful there can also be severe limitations with choosing to use either of these models. Altering the genetics of the germline of a mouse or any other animal may answer the research questions at hand but there may also be severe developmental consequences, which in turn complicate the analysis. One major consequence is that the alteration in the genome is present from conception and this prevents studying the role of the gene in adults and also, the gene is deleted in one or both of the alleles in the genome (46). When assessing the phenotype of an animal that has been genetically modified, other factors may alter the phenotype, including compensatory effects by one gene upon the removal or overexpression of another, toxic effects due to inappropriate gene expression etc. (47).

The genetic background is an important factor when interpreting data and it is crucial to know whether the phenotype observed is due to the targeted mutation or as a result of the genetic background (46). Also, it is known that bone mineral density (BMD) can vary between inbred strains of mice and as a result genetic background can interfere with the interpretation of data (46). However, this problem can be dealt with by backcrossing the mouse lines to a single genetic background for several generations and then studying animals from the same litter of the various genotypes (wild-type, heterozygous, and homozygous) to account for any influence the genetic background may have on the various phenotypes (47). In this project, we focused on comparisons between sisters from a predominantly Black Swiss backcrossed background.

When assessing the results obtained from knockout models and trying to extrapolate the results from mouse models to human bone, one has to be aware of the fact that there are many important differences between the two species (46). There is a postural difference between mice and humans and as a result, the biomechanical loading patterns are different. Rodents, unlike humans, rarely experience remodeling in their cortical bone, and human adults have fused growth plates with no linear growth, while in rats, there is no fusion of growth plates and the bone grows throughout adult life (46). In spite of the fact that these differences exist between mouse and human bone, mouse models are still quite useful and significant knowledge can be gained if the models are used appropriately (46).

In the case of studying pregnancy and lactation, there are several similarities and differences between humans and mice. As mentioned previously, mice experience a gain

in total body BMC during pregnancy, an increase that is not observed in humans. During pregnancy, humans provide a considerable amount of calcium to the fetus, in fact, 21g of calcium is transferred to the developing fetus, 80% of which occurs during the third trimester (19). In mice, 12mg of calcium is delivered to each fetus over the last four to five days of pregnancy. The calcium losses occur more abruptly in the mouse and in relation to body size, they experience a greater loss compared to humans. During lactation, both humans and mice lose BMC from their trabecular bone however, the magnitude and speed of loss differ. Lactating rats lose up to 35% of their BMC during 21 days of lactation while females only lose 3-8% after 2-6 months of lactation (19). Complete recovery in BMC occurs in humans several months after lactation has ceased and in approximately three weeks for mice (19).

In doing scientific research and especially in working with knockout models, there is always the possibility of confounding factors which could influence the results. For this project in particular, there are several such factors, including the fact that the *Pth* null mice have a slightly higher bone mass than their WT sisters to begin with. This in itself could effect the bone density findings. The *Pth* null mice also experienced hypocalcemia and hyperphosphatemia both of which were not observed in the WT siblings. Both of these factors could potentially influence and conflict the results if they are not taken into consideration in data analysis.

If all of the appropriate considerations are taken into account, using knockout models is scientific research is very useful and reliable. It is important however, that one

is aware of these limitations so that they did not assume the results obtained are strictly due to the alteration to the genome.

4.3 Project Summary

The problems encountered with the *Pth* null females during the first year slowed the progress of the project tremendously. With the time constraints, it was decided that we would make several changes to the project with the hope that any problems during pregnancy and lactation would be avoided. However, as mentioned previously, the drawback was that the data obtained from mice on the 1% calcium chow could not be compared to the data obtained from mice on the 2% calcium chow and as such the data have been kept separate. Overall, the impact of these events is that far fewer *Pth* null females were studied through complete reproductive periods than originally planned, and the data set is not conclusive in its present form.

Results from the mice fed the standard 1% calcium chow indicate that *Pth* null mice experienced changes in total body BMC during pregnancy, lactation and weaning that were similar to their WT and HET siblings. For mice fed the 2% calcium chow, results indicate that both WT and *Pth* null mice experience an increase in hind limb BMC during pregnancy and a decrease during lactation. WT females recovered to their pre-pregnancy baseline within three weeks post-weaning and there is no such data regarding the *Pth* null females at this point in time. It is important to keep in mind that this data is based on hind limb BMC values and if spine scans were available, the results would be more accurate and reliable.

After changing the chow, it appeared that mice were more successful during pregnancy and lactation and that there was a reduction in the number of maternal deaths. It appeared that PTH was required in mice during lactation because in the absence of PTH, the animals were prone to sudden death. Pregnant hypoparathyroid rats on a low calcium diet or vitamin D deficient often become hypocalcemic, can show signs of tetany and often die during late pregnancy and especially lactation. In humans, there does not appear to be any obvious problems during late pregnancy and especially lactation because PTHrP seems to completely take over as evidenced by hypoparathyroid women who often experience improvements in their hypocalcemic symptoms (19). Therefore, a difference between rodents and humans is that humans do not need PTH to support lactation but rodents do, given that many *Pth* null mice did not lactate and that there were some maternal deaths in late pregnancy and early lactation which were not related to anesthetic use. Some possible explanations for this could be the fact that animals normally have larger litter sizes (six to twelve fetuses) while humans normally have only one fetus. Also, the gestational period in rats and mice is quite short and this requires that the animals provide a large bulk of calcium to several fetuses over a few days, compared to humans who do this over a full trimester. It may be that lactation overwhelms the mice such that they need to use both PTH and PTHrP to break down bone, whereas humans only need PTHrP.

One finding from this project which is of particular importance especially for the continuation of the work is the fact that when fed the standard 1% calcium chow, mice have far more problems during pregnancy and lactation compared to those fed a 2%

calcium chow. The supplemented calcium appeared to decrease the presumed hypocalcemia-induced sudden deaths and diminished the problems experienced by the mice due to absence of PTH.

Absence of PTH had its expected impact on serum chemistry as the *Pth* null females were both hypocalcemic and hyperphosphatemic. There was no significant change in serum chemistries (calcium, magnesium and phosphorus) over pregnancy and lactation, whether WT or *Pth* null. Serum magnesium was slightly decreased during pregnancy in mice fed the 1% calcium chow and this is similar to what has been observed in normal mice. A possible explanation for this could be that fetal demand for magnesium exceeds the mother's ability to maintain the level in her circulation. For mice fed the 2% calcium chow, there did not appear to be any decrease in serum magnesium during pregnancy as was expected. To account for this, it may be that when the mice fed the 2% calcium chow become constipated, this has an effect of allowing more transit time to absorb nutrients. It may be that these mice absorb more and excrete less magnesium, and therefore there is more in circulation and this is why the levels are not decreased during pregnancy.

Pth null mice had lower urinary calcium excretion during pregnancy compared to WT mice. This differs from the normal situation where in hypoparathyroidism is associated with an increase in urinary calcium excretion due to loss of the effect of PTH to stimulate calcium reabsorption. One possible explanation for the low urinary calcium excretion could be that the animals are either not ingesting or not absorbing enough calcium and are severely hypocalcemic, to the point that the filtered load of calcium is

low. In other words, the *Pth* null mice had lower urinary calcium excretion because pregnancy overwhelms their ability to maintain normocalcemia and normal perfusion of the kidneys by calcium. The findings from the ash weight and atomic absorption spectroscopy analysis show that the pups are able to get what they need from their mothers regardless of the maternal genotype and it does not appear as though the pups are at any major disadvantage. There was no significant difference between the ash weights, the total skeletal calcium content or the total skeletal magnesium content between pups of WT, HET or *Pth* null dams fed the 1% calcium chow. There was a significantly lower ash weight of WT versus HET pups of WT dams fed the 2% calcium chow, although there was no significant difference in the calcium or magnesium content of the ash. Although statistically significant, the differences appear to be trivial.

Pth null mice experience an increase in bone density as they age to the point that their skeletons become overmineralized. This is interesting considering the fact that unpublished data from our lab shows the *Pth* null fetus to have an undermineralized skeleton and compared to WT siblings, in particular, they have lower ash weights as well as lower total skeletal calcium and magnesium. Mice lacking both the parathyroids and PTH (*Hoxa3* null) have an even lower ash weight and calcium and magnesium content than their WT and HET siblings (50). This confirms that the *Hoxa3* null skeleton is undermineralized in the absence of PTH. The combination of this data confirms a role for PTH because in its absence, fetal mice experience a lower ash weight and less calcium and magnesium in their skeletons. At some point postnatally, the absence of

PTH leads to a reversal of this, such that adult *Pth* null mice have overmineralized skeletons.

Although the results presented are not definitive, it does appear that absence of PTH increases the risk of maternal hypocalcemia and death during pregnancy and lactation, but that PTH may not be required for the loss of mineral during lactation or for its restoration afterward. Experiments must now be continued with mice on the 2% calcium chow (removed several hours before scanning) with less frequent scanning and anesthesia to obtain conclusive results.

V Future Directions

After dealing with the many obstacles that arose during the course of this project, there was very little time to redo many of the experiments. In many cases, the sample sizes analyzed were extremely small and more mice have to be studied in order to make the results more reliable. The project will be continued taking into account all of the discoveries made during the course of this project. All mice will be fed the calcium enriched chow, scanned less frequently and taken off the chow several hours prior to scanning so that whole body scans can be obtained in addition to regional measurements (spine and hind limb). The anesthetic used will be the same as the one used in the latter half of this project (Ketamine Hydrochloride/Xylazine) as it appears to be successful in keeping the mice under for a very short period of time and there do not appear to be any major problems during recovery from the anesthetic.

This project can be expanded to look at other aspects of calcium and bone metabolism such as other calciotropic hormones including PTHrP, calcitriol, and sex steroids such as estradiol in the knockout mice. These hormones have not yet been measured simply due to time constraints.

We are still not certain as to why the *Pth* null mothers experience so many problems during lactation. It is not clear as to whether the mothers cull their litters to reduce the calcemic stress of nursing a large number of pups, or whether they simply reject their pups and eat them. It would be useful to examine these issues in more detail by adjusting the litter size so that the demands on the mother during lactation are varied (eg. four, six, eight pups in various experimental groups). This will allow us to examine

how litter size affects the mother's skeleton during lactation with regards to the amount of bone mineral content she loses. This further experimentation could clarify the uncertainties.

Still related to this issue of maternal problems during lactation, it would be interesting to determine if the problems experienced by the *Pth* nulls could actually be due to abnormal fetuses. This can be looked at by performing cross-fostering experiments whereby the *Pth* null mice would nurse WT pups and vice versa. This would allow us to determine whether or not it was actually the pups who were causing the problems during lactation completely independent of the mother and/or her genotype. However, due to the fact that many of the *Pth* nulls failed to go through full periods of lactation, we did not attempt these experiments over the course of my Masters project. We felt that it was more beneficial to leave lactating *Pth* null mothers with their pups because of the fact that successful lactation for these mice was rare. However, it would be useful to conduct these experiments when the project continues to gain a better understanding of whether or not the fetuses contribute in any way to the problems during lactation.

Adjusting the litter size or doing these cross-fostering experiments, presents other problems because the *Pth* null mice often cull their litters early (during the birthing process or in the first few hours afterward). Thus, there will be few pups to cross-foster and no large litters to cull to lower numbers.

It would be useful to look at the skeleton of these mice specifically during lactation and recovery to gain a better understanding of how the bones are affected by

lactation and weaning in terms of the extent of bone loss and regain. The information obtained could be included with what we have already observed from bone density data. We could examine markers of bone formation and resorption for evidence of what may be going on to allow the *Pth* null mice to lose less BMC during lactation if, in fact, they truly do lose less bone. The skeleton could be looked at on a more molecular level to determine if there are any major differences between the structure of the bones of the null mice compared to their WT siblings. For example, it would be helpful to examine the activity of the osteoblasts and osteoclasts to determine what is going on as bone is being remodeled and also to determine the extent to which the osteoblasts and osteoclasts influence the remodeling process in these mice. The skeleton could be tested for mechanical strength using three-point bend equipment to determine whether the femurs and vertebra from mice of either genotype are stronger than those of the other. If the absence of PTH leads to a weaker maternal skeleton during lactation or recovery, it will be determined using this technique. Bone tissue can be stored and used for later analysis, including, bone histomorphometry where tetracycline can be administered *in vivo* at different stages, after which the animal will be sacrificed and the bone harvested. The distance between the bands of tetracycline within the bone will indicate the rate of mineralization. Staining techniques are also available that allow for measurement of other parameters such as the number of osteoblasts and osteoclasts, the periosteal width etc. as well as examination of gene regulation within the skeleton during these key stages. In this respect, it will also be useful to look at the neonates at the time of weaning to

determine if there is any effect due to absence of maternal PTH on neonatal growth, mineral homeostasis and skeletal strength.

This further analysis would provide definite answers as to what happens to the *Pth* null female during lactation and weaning. The additional experiments would provide information to include in the lactation and recovery story as we presently understand it in these mice based on bone density data.

Finally, it would be advantageous to look at conditional knockout mice models where PTH can be knocked out of the parathyroids at the start of lactation or the onset of recovery. Knocking out PTH at a specific time during reproduction ie, at the start of lactation, may also eliminate maternal deaths previously observed due to hypocalcemia. In this case, to eliminate PTH from the parathyroids at the start of lactation, one could use the reverse tetracycline transactivator system whereby the enzyme Cre (bacterial recombinase) is driven to cleave out PTH. Upon administration of tetracycline the promoter in turn drives the Cre enzyme to cleave out the gene. Cre does this by binding to the lox P sites, cutting the sites in half and then splicing them together and once the target DNA is excised, it is degraded. Using this specific targeting strategy would be useful because it would disrupt the PTH gene from the parathyroids only.

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DNA Extraction From Tissues

1. Put tissue (e.g. tail tip) in Eppendorf tube containing 0.5mL lysis buffer. Buffer is stored at room temperature; proteinase K is added shortly before use.

2. Incubate overnight at 55°C.

3. Next morning, shake horizontally by hand for 2 to 3 minutes, and spin in a micro centrifuge for 10 minutes to precipitate hair.

4. Pour supernatant into fresh Eppendorf tubes containing 0.5mL isopropanol, and invert to precipitate DNA.

5. Pick DNA up with small pipette tips and put it into Eppendorf tubes containing 0.5mL water.

6. Shake by hand for 5 minutes to dissolve DNA into a viscous solution.

7. Add 0.5mL of phenol/chloroform/isoamyl alcohol (100:100:1) and shake vigorously for approximately 1 minute. A turbid, milky solution should result.

8. Spin for 2 minutes at maximum speed in a micro centrifuge.

9. Carefully aspirate the supernatant and place it in fresh Eppendorf tubes. Add 1mL of 25:1 EtOH:NaOAC solution. Invert several times until DNA comes out of solution.

10. Spin for 10 minutes at maximum speed to precipitate DNA. A tiny pellet should be seen.

11. Discard supernatant by pouring it off, then add 1mL of 70% EtOH.

12. Pour off the EtOH and dry the pellet.

13. Resuspend the DNA pellet in 50-250μL TE (Tris-EDTA). Store at 4°C.

Calcium Assay
(Diagnostic Chemicals Limited)

Reagents:

Calcium Reagent
Calcium Standard

Procedure:

1. Into separate test tubes, pipette 20 µL of deionized water, standard, or serum to be assayed.
2. Add 2.0 mL of reagent and mix.
3. Incubate for 30 seconds at 18-26°C.
4. Determine the absorbance of the standard and of each unknown at 650 nm using the deionized water sample as the reagent blank.

Calculation:

To determine the concentration of calcium, perform the following calculations:

Calcium (mmol/L) = $A/A_s \times$ concentration of the standard

A= absorbance of the unknown
A_s= absorbance of the standard

Example:

A= 0.631
A_s= 0.496

Concentration of the standard= 2.5mmol/L (or 10mg/dL)

Calcium (mmol/L) = 0.631/0.496 × 2.5mmol/L
= 3.2mmol/L

Calcium (mg/dL) = 0.631/0.496 × 10mg/dL
= 12.7mg/dL

Principle:

$$2 \text{ Arsenazo III} + Ca^{++} \rightarrow \text{Ca-Arsenazo Complex}^{++} \text{ (blue-purple)}$$

Aresenazo III reacts with calcium in an acid solution to form a blue-purple complex. The color development has a maximum absorbance at 650nm and is proportional to the calcium concentration in the sample.

Magnesium Assay
(Diagnostic Chemicals Limited)

Reagents:

Magnesium Reagent
Magnesium Standard

Procedure:

1. Into separate test tubes, pipette 20μL of deionized water, standard or serum to be assayed.
2. Add 3.0mL of reagent and mix.
3. Incubate for 5 minutes at 18-26°C. Determine the absorbance of the standard and each of the standard and of each unknown at 520nm using the deionized water sample as the reagent blank.

Calculations:

To determine the concentration of magnesium, perform the following calculations:

Magnesium (mmol/L) = $A/A_s \times$ concentration of the standard

A= absorbance of the unknown
A_s= absorbance of the standard

To convert to mg/dL, multiply the answer from the above equation by 2.4.

Example:

Absorbance of the unknown = 0.448
Absorbance of the standard = 0.176
Concentration of the standard = 1.0 mmol/L (or 2.4 mg/dL)

Magnesium (mmol/L) = $0.448/0.176 \times 1.0$ mmol/L
= 2.5 mmol/L

Magnesium (mg/dL) = 2.5 mmol/L $\times 2.4$
= 6.0 mg/dL

Background of test:

Magnesium, along with potassium, is the most abundant intracellular cation. Magnesium is a coenzyme required for the metabolism of carbohydrates, lipids, and proteins. The method used to measure magnesium is atomic spectrophotometry, employing xylidyl blue-1. The technique proceeds as follows:

$$\text{Xylidyl blue-1} + Mg^{++} \rightarrow \text{Mg-xylidyl blue complex (red)}$$

The absorbance increase, at 520 nm, of the red complex formed is directly proportional to the concentration of magnesium in the serum.

Phosphorus Assay
(Diagnostic Chemicals Limited)

Reagents:

Phosphorus Blank Reagent (R1)
Phosphorus Molybdate Reagent (R2)
Phosphorus Standard

Procedure:

1. Into separate test tubes, pipette 10 µL of deionized water, standard, or serum to be assayed.
2. Add 1.0 mL of Blank Reagent (R1) and mix.
3. Add 200 µL of Molybdate Reagent (R2) and mix.
4. Incubate for 10 minutes at 18-26°C.
5. Determine the absorbance of the standard and of each unknown at 340 nm using the deionized water sample as the reagent blank.

Calculation:

To determine the concentration of inorganic phosphate, perform the following calculations:

Inorganic Phosphorus (mmol/L) = $A/A_s \times$ concentration of the standard

A= absorbance of the unknown
A_s= absorbance of the standard

Example:

A= 0.149
A_s= 0.262
Concentration of the standard= 2.0mmol/L (or 6.2 mg/dL)

Inorganic Phosphorus (mmol/L) = 0.149/0.262 × 2.0 mmol/L
= 1.1 mmol/L

Inorganic Phosphate (mg/dL) = 0.149/0.262 × 6.2 mg/dL
= 3.5 mg/dL

Principle:

Inorganic Phosphorus + Ammonium Molybdate $+H_2SO_4$

↓

Unreduced Phosphomolybdate complex

The reaction of inorganic phosphorus with ammonium molybdate in the presence of sulfuric acid (H_2SO_4), produces an unreduced phosphomolybdate complex. The absorbance of this complex at 340nm is directly proportional to the inorganic phosphorus concentration.

Creatinine-S Assay
(Diagnostic Chemicals Limited)

Reagents:

Creatinine Base Reagent (R1)
Creatinine Picrate Reagent (R2)
Creatinine Standard

Procedure:

1. Prepare the required volume of creatinine working reagent (one volume of R2 and four volumes of R1). Mix well before using.
2. Into separate test tubes, pipette 100 µL of deionized water, creatinine standard, or serum to be assayed. Urine samples require pre-dilution with 0.9% saline.
3. Add 2.0 mL of creatinine working reagent and incubate for 20 seconds.
4. Record the absorbance of the standard at 510nm at 20 seconds (A_{S1}) and at 80 or 140 seconds (A_{S2}). Also record the absorbance of each unknown at 510nm at 20 seconds (A_{S1}) and 80 or 140 seconds (A_{S2}).

Calculation:

To determine the concentration of creatinine, perform the following calculations:

Creatinine (µmol/L) = A_2-A_1/A_{S2}-A_{S1} × concentration of the standard

A_2= final absorbance of the unknown
A_1= initial absorbance of the unknown
A_{S2}= final absorbance of the standard
A_{S1}= initial absorbance of the standard

Example:

A_2= 0.117
A_1= 0.057
A_{S2}= 0.051
A_{S1}= 0.016

Concentration of the standard= 354µmol/L (or 4.0mg/dL)

Creatinine (μmol/L) = 0.117-0.057/0.051-0.016 × 354μmol/L
= 607μmol/L

Creatinine (mg/dL) = 0.117-0.057/0.051-0.016 × 4.0mg/dL
= 6.9mg/Dl

Principle:

Creatinine + alkaline picrate → creatinine-picrate complex

At an alkaline pH, creatinine reacts with picrate to form a complex. The rate of increase in absorbance at 510nm due to formation of the creatinine-picrate complex is directly proportional to the concentration of creatinine in the sample.

Creatinine measurements are used in the diagnosis and treatment of renal diseases, in monitoring renal dialysis, and as a calculation basis for measuring other urine analytes.