Role of calcitriol in regulating maternal bone and mineral metabolism during pregnancy, lactation, and post-weaning recovery

by

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science in Medicine

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Memorial University of Newfoundland

May 2017

St. John's Newfoundland and Labrador

ABSTRACT

Maternal physiology adapts throughout reproductive phases to meet the mineral needs of the fetuses and neonates. During pregnancy, intestinal calcium absorption doubles; conversely, during lactation, calcium is resorbed from the skeleton. Post-weaning, restoration of bone mineral content occurs, however the factors regulating these adaptations are unknown. Animals lacking the vitamin D receptor (VDR) or those deficient in vitamin D achieve post-weaning increases in bone mass, suggesting that calcitriol, the active form of vitamin D, is not required. An alternative explanation is that calcitriol may act through non-genomic receptors to have these effects. We used calcitriol-deficient *Cyp27b1* null mice to test whether calcitriol is required for upregulation of intestinal calcium absorption and skeletal recovery post-weaning. Compared to WT, and despite a rescue diet containing 2 % calcium, at baseline *Cyp27b1* nulls were hypocalcemic with markedly increased PTH and 30 % lower BMC; μCT displayed reduced femoral length, cortical thickness, and cortical area. During pregnancy serum calcium levels increased, PTH levels were reduced, and BMD increased by 45 %, so that all values became no different from WT. During lactation, serum calcium levels of *Cyp27b1* nulls decreased, PTH levels increased, BMC declined more markedly than in WT, and bone strength decreased; μCT analysis confirmed extensive endocortical resorption. Post-weaning, serum calcium of *Cyp27b1* null normalized, PTH levels remained high, BMC increased, and bone strength recovered; μCT confirmed recovery of cortical parameters. In comparison to WT mice, the *Cyp27b1* null females display very low levels of calcitriol throughout pregnancy and lactation. Intestinal calcium absorption is upregulated in the *Cyp27b1* null mice during pregnancy. In conclusion, calcitriol-independent mechanisms regulate mineral and bone metabolism during pregnancy and post-weaning; however, absence of calcitriol leads to greater transient bone loss during lactation.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to everyone who has supported me throughout my graduate program:

Dr. Christopher Kovacs, my supervisor, for providing such fascinating research that inspired my move across the country. You created an exceptional learning environment that challenged my academic potential and deepened my interest in reproductive science. I am also incredibly thankful for your help in applying for and winning scholarships, attending conferences, and your recommendations for further education.

Dr. Ann Dorward and Dr. Gary Paterno, my supervisory committee, for your insight throughout my project. Your valuable comments and criticisms were essential to the success of my thesis.

Dr. René St-Arnaud at McGill University in Montreal, Quebec, for your research efforts as well as the production and permission to use the *Cyp27b1* null mouse model.

Dr. Natalie Sims and Brett Tonkin at Melbourne University in Australia, for your guidance in my project and assistance with the research.

The Canadian Institutes of Health Research and Memorial University for generously funding my research.

Beth Kirby, the research assistant, for your expertise and endless patience. You were there to lend a hand for any problem that came my way, seemingly solving the unsolvable. The lab was a warm and welcoming place because of you.

Christine Wells, the research technician, for being my first best friend in Newfoundland. You were there for every single breakdown, breakthrough, and laugh that occurred, and the lab would not have been the same without you.

My fellow students of the Calcium Research Laboratory: Toby, Clare, Aaron, Kamal, Stacey, and Brittany, for your help, support, and steadfast friendship.

Dr. Anne Croy, my mentor at Queen's University, for introducing me to the research of Dr. Kovacs and for assisting me in finding a future career for which, I am truly passionate.

Nadia and Bob, my parents, for providing me with so many opportunities to pursue my goals. I look forward to my future with confidence and determination thanks to your constant belief in me. My sister Victoria, for entertaining every single one of my phone calls and standing in solidarity in being strong, independent women. My Nana Betty, Papa John, Nonna Maria, and Nonno Rino, for being my biggest fans. Finally to Neil and Hunter, for putting a smile on my face even on the most stressed of days. "To science!"

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1αOHase	1-α-Hydroxylase
1,25$(OH)_2$D	1,25-Dihydroxyvitamin D (Calcitriol)
1,25,OHD-26,23-lactone	26,23-lactone
7-DHC	7-Dehydrocholesterol
24OHase	24-Hydroxylase
24,25OHD	24,25-dihydroxyvitamin D
25OHD	25-Hydroxyvitamin D
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMPs	Bone Morphogenic Proteins
bp	Base Pairs
BRC	Bone Remodeling Compartment
Ca	Calcium
CaSR	Calcium Sensing Receptor
cm	Centimeter
Col1	Type 1 Collagen
Cyp27b1	Cytochrome P450 Family 27 Subfamily B Member 1
DBP	Vitamin D Binding Protein
dL	Decilitre
DNA	Deoxyribonucleic Acid

DXA ..... Dual X-ray Absorptiometry

ECM ..... Extracellular Matrix

EDTA ..... Ethylenediaminetetraacetic Acid

EIA ..... Enzyme Immunoassay

ELISA ..... Enzyme-linked Immunosorbent Assay

FGF23 ..... Fibroblast Growth Factor – 23

g ..... Gram

gf ..... Gram-force

GFR ..... Glomular Filtration Rate

GI ..... Gastrointestinal

HET ..... Heterozygote

hr ..... Hour

IACC ..... Institutional Animal Care Committee

IGF-1 ..... Insulin-like Growth Factor-1

IP ..... Intra-Peritoneal

IRMA ..... Immunoradiometric Assay

IU ..... International Unit

kD ..... Kilodalton

KO ..... Knock-Out

kV ..... Kilovolt

L ..... Litre

M ..... Mole

M-CSF ..... Cytokine Macrophage Colony Stimulating Factor

mA ..... Milliamps

MBCP ..... Membrane-Bound Calcium Pump

mg ..... Milligram

min ..... Minute

ml ..... Milliliter

mm ..... Millimeter

mmol ..... Millimole

mRNA ..... Messenger RNA

ms ..... Millisecond

MSCs ..... Mesenchymal Stem Cells

NAPI ..... Na-Pi Cotransport System

NFAT2 ..... Nuclear Factor for Activated T Cells 2

nmol ..... Nanomole

nm ..... Nanometer

OC ..... Osteocalcin

ON ..... Osteonectin

OPG ..... Osteoprotegerin

OPN ..... Osteopontin

p ..... Probability

P ..... Phosphorus

PCR ..... Polymerase Chain Reaction

pg ..... Picogram

P1NP ..... Procollagen Type 1 N-Terminal Peptide

PMCA……...…..Plasma Membrane Calcium Pump

PTH.....Parathyroid Hormone

PTHrP.....Parathyroid Hormone Related Peptide

RANKL.....Receptor Activator of Nuclear Factor-kappa β Ligand

ROI.....Region of Interest

RXR……..Retinoid X Receptor

s.....Second

SE.....Standard Error

TAE .....Tris Acetic Acid

TGF- ß……..……Transforming Growth Factor ß

UVB.....Ultraviolet B

VDR.....Vitamin D Receptor

wk..... Week

WT.....Wild-type

x g..... Times gravity

µCi .....Microcurie

µl ..... Microlitre

µm .....Micrometer

µmol .....Micromole

LIST OF APPENDICES

I. INTRODUCTION

1.1 Adult mineral and bone homeostasis

1.1.1 Purpose of the skeleton

Mineral and bone metabolism in the adult is a tightly regulated process. Through the interaction of several physiological compartments and hormones, serum mineral homeostasis and skeletal strength are maintained. Preservation of the integrity of bone is particularly important, due to the organ's numerous and essential roles in normal physiology to: provide internal support and protection for vital organs, enable locomotion through provision of attachment sites for muscles and tendons, host hematopoiesis in the bone marrow, and maintain a reservoir for inorganic ions, namely calcium and phosphate, for mobilization into circulation. [1] Bone also has a central role in mineral homeostasis through the flux of these minerals during formation and resorption.

1.1.2 Target organs

The main target organs involved in maintaining adult mineral and bone homeostasis include bone, the intestines, the parathyroid glands, and the kidneys. Blood serves as a form of communication among these organs. The constituents in blood, including the minerals and the hormones that are involved in regulating different target tissues, will first be described. The role of each of the target organs will then be explained in greater detail followed by an illustration of how each of these components fit together.

1.1.3 Serum mineral concentrations

1.1.3.1 Calcium

The adult human is comprised of approximately 1000 g of calcium, distributed in various forms throughout the body. The majority of this mineral (99 %) exists as the hydroxyapatite crystal in the mineral phase of bone, and the remaining 1 % is located in blood, extracellular fluid, and soft tissues. [2] Of the total calcium in blood, 50 % is free or ionized, 40 % is bound to albumin, and 10 % is bound to citrate or phosphate ions. [2] The free or ionized portion is biologically functional and is commonly used as an indicator of clinical calcium status. The normal range of extracellular ionized calcium is approximately 1.1-1.3 mM. [3] This level is tightly regulated because small decreases or increases disrupt the function of key organs and cells. Underlying this homeostasis is the Ca^{2+}-sensing receptor (CaSR), which keeps the ionized calcium level constant. [3] The parathyroid glands, kidneys, intestine, C-cells of the thyroid gland, and osteoblasts, osteocytes, and osteoclasts in bone all express this receptor, however, the most important effect of altering serum calcium is a result of its function in the parathyroid glands. [3] The typical human diet is either deficient or bordering on sufficient in calcium, so this mechanism is essential to ensure that the blood calcium stays within a narrow range.

1.1.3.2 Phosphorus

Similar to calcium, phosphorus is one of the most abundant minerals in the human body and is maintained in strict homeostasis. In the adult human, approximately 600-700 g of phosphorus are stored with calcium as the hydroxyapatite constituent of bone, 100-200 g are distributed in soft tissues, and trace amounts are located in extracellular fluids. [4] There are two forms of phosphorus in extracellular fluids: the organic form of phospholipids and the inorganic form of phosphate. [4]

The North American diet generally has much more phosphorus than needed; consequently we have to excrete the excess. There must be a phosphorus sensor, similar to the CaSR, however the phosphorus-sensing mechanism is poorly understood. Recently it has been suggested that inositol polyphosphate sensory domains are able to regulate phosphate intake, transport, and storage. [5]

1.1.4 Roles of the calcium and phosphorus-regulating hormones

Maintaining the concentrations of calcium and phosphorus within the set ranges requires the interaction of several target organs and several hormones that communicate among them. These include: parathyroid hormone (PTH), calcitriol ($1,25(OH)_2D$), fibroblast growth factor 23 (FGF23), parathyroid hormone related peptide (PTHrP), and estradiol.

1.1.4.1 Parathyroid hormone

Parathyroid hormone (PTH) is initially synthesized in the parathyroid gland as its nascent form of a pre-pro-peptide consisting of 115 amino acids. [6] The mature PTH peptide consisting of 84 amino acids is formed through removal of the pre- and pro-sequences. [7] This mature form is packaged into secretory vesicles within chief cells of the parathyroid gland from which it is secreted. [6] The main functions of PTH are to increase levels of serum calcium, lower serum phosphorus, stimulate production of calcitriol, and to control the degree to which the skeleton is forming or resorbing. The blood ionized calcium level is sensed by the CaSR at the plasma membrane of chief cells. [8] Calcium binds to the receptor, initiating signal transduction that leads to suppression of PTH secretion in the short term and reduced PTH mRNA transcription in the long term. [6] Conversely, a decline in extracellular calcium levels leads to reduced binding, disinhibition, and a rise in both PTH transcription and secretion. PTH then functions to restore

blood calcium levels to within the desired range of 1.1-1.3 mM through acting on bone, kidneys, and indirectly on the intestines.

Within bone, the dominant action of PTH is on osteoblasts, as the PTH receptor is located within these cells. PTH stimulates osteoblasts to form bone. Intermittent elevation increases osteoblast number and bone formation by attenuating apoptosis and increasing osteoblast life span. [9] However, if the stimulation of PTH is prolonged or of greater amplitude, then PTH causes bone resorption via the RANKL/OPG/RANK pathway (Figure 1). Specifically, PTH causes osteoblasts to make receptor activator nuclear factor-κB ligand (RANKL), which binds to RANK receptors on osteoclast precursor cells to promote their differentiation. [10] Simultaneously, PTH causes osteoblasts to reduce the expression of osteoprotegerin (OPG). [10] OPG acts as a "decoy" receptor for RANKL, such that a decrease leads to less competition and RANKL can bind more frequently to RANK. [11] The final result is that more osteoclasts are formed from precursors, recruited to the bone surface, and activated to resorb bone. [12]

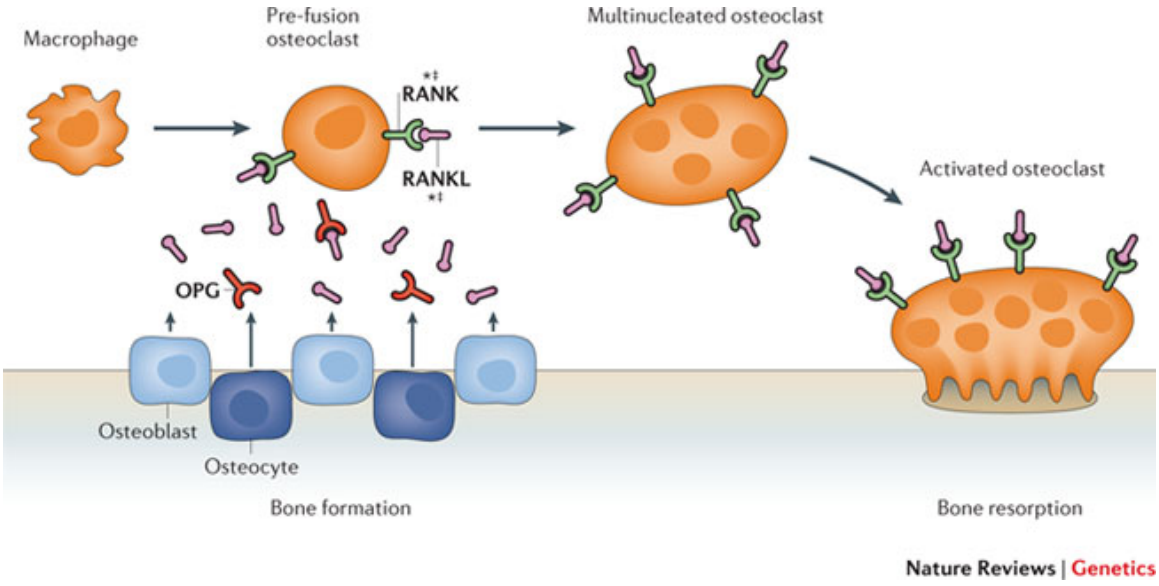


Figure 1: RANKL/OPG/RANK signalling pathway. [13] Osteoblasts make RANKL that binds to RANK on osteoclast precursor cells to promote their differefntiation. Osteoblasts and osteocytes also secrete OPG, a decoy receptor for RANKL that prevent binding of RANKL to RANK. *Reprinted by permission from Macmillan Publishers Ltd: NATURE REVIEWS GENETICS, copyright 2012 (Appendix A).*

PTH also plays a role in ensuring calcium balance through influencing renal function. PTH primarily stimulates renal calcium reabsorption in the proximal tubule and has a minor role in calcium excretion in the distal nephron.[2] Within the intestines, PTH has an indirect effect to maintain calcium balance that occurs via the kidneys, which will be discussed in section 1.1.6.

In addition to its major role of regulating calcium balance, PTH also affects the balance of phosphorus. PTH increases renal phosphate excretion by suppressing the expression of sodium-phosphate cotransporters NaPi2a and NaPi2c, thereby decreasing serum phosphate.[14] This will occur regardless of whether PTH is increased in response to low serum calcium or a high serum phosphate. A modest negative feedback loop is then evoked whereby low serum phosphate decreases PTH secretion and mRNA levels of PTH.[15,16] However, PTH is much more responsive to changes in calcium than it is to low phosphorus.

A variety of other mechanisms serve to regulate the secretion of PTH. Diminished levels of calcitriol result in reduced intestinal calcium absorption, leading to a compensatory increase in PTH secretion.[6] The mechanism underlying this regulation involves liganded VDR binding to negative regulatory elements in the promoter region of the PTH gene.[17] A similar effect results from dietary calcium restriction independent of vitamin D or calcitriol. FGF23 also has an inhibitory role in the secretion of PTH, through phosphorylating ERK1/2 and activating the MAPK pathway.[18]

1.1.4.2 Vitamin D metabolism

Calcitriol is the active form of vitamin D and has numerous biologically essential roles. It is synthesized from Vitamin D_3 as taken in from the diet through fortified dairy products and fish oils, or in the skin from 7-dehydrocholesterol (7-DHC) by ultraviolet radiation. [19] The conversion from biologically inert Vitamin D_3 to its active form involves two enzymatic modifications, first in the liver and then in the kidney (Figure 2). After D_3 synthesis, the vitamin is transported in general circulation by the vitamin D binding protein (DBP). [20] This ferries vitamin D to fat and muscle for storage, and to the liver where the first conversion reaction occurs. Here, vitamin D_3 is hydroxylated by cytochrome P450 enzymes to the prohormone 25-hydroxyvitamin D_3 (25OHD). [19] The second modification then occurs after transport by DBP to the kidney, where 25OHD is converted to 1,25-dihydroxyvitamin D_3 (calcitriol) and 24,25-dihydroxyvitamin D_3 (24,25OHD). [19] CYP27B1 1α-hydroxylase (1αOH) is present predominantly in the kidney and metabolizes the first reaction to calcitriol. This enzyme is also present in extrarenal sites including the placenta, monocytes, and macrophages. [21,22] In either location, calcitriol binds to the vitamin D receptor (VDR) to conduct its actions. [19] In contrast, a catabolic pathway also exists that serves to lower calcitriol. The enzyme CYP24A (24OH) limits the amount of calcitriol in target tissues by converting 25OHD to 24,25OHD, and catabolizing calcidiol into $1,24,25(OH)_3D$. [19] Both of these hydroxylated products are further degraded to water-soluble catabolites, although 24,25OHD and $1,24,25(OH)_3D$ have their own limited biological activity.

The entire process of vitamin D metabolism is tightly regulated, primarily at the level of the kidney (Figure 2). Substrate availability, amount of enzyme, cofactor availability, and enzymatic activity of CYP24A all control the action of 1αOH. [19] CYP24A is under stringent regulation by

calcitriol through negative feedback, as the enzyme can also hydroxylate calcitriol to form 1,24,25$(OH)_3$D and 1,25,OHD-26,23-lactone. Calcitriol may even be the preferred substrate for CYP24A. [23] Furthermore, numerous minerals and hormones also play a role in the metabolic pathway. PTH stimulates the production of calcitriol in the kidney, which in turn suppresses the production of PTH in a feedback loop. [19] PTH itself is suppressed by calcium via the CaSR, such that calcium has an inhibitory role in the production of calcitriol. Additionally, phosphate and FGF23 both inhibit calcitriol production. CYP24A is reciprocally regulated in contrast to CYP27B1, such that its activity is stimulated by phosphorus, calcium, calcitriol, and FGF23, and inhibited by PTH. [20] The dominant stimulator of CYP27B1 is normally PTH because in the absence of PTH, calcitriol is low. FGF23 however has an important negative influence because in the absence of FGF23, calcitriol levels are very high. Other hormones may also be involved, as calcitonin and prolactin have been reported to stimulate the production of calcitriol.[24,25]

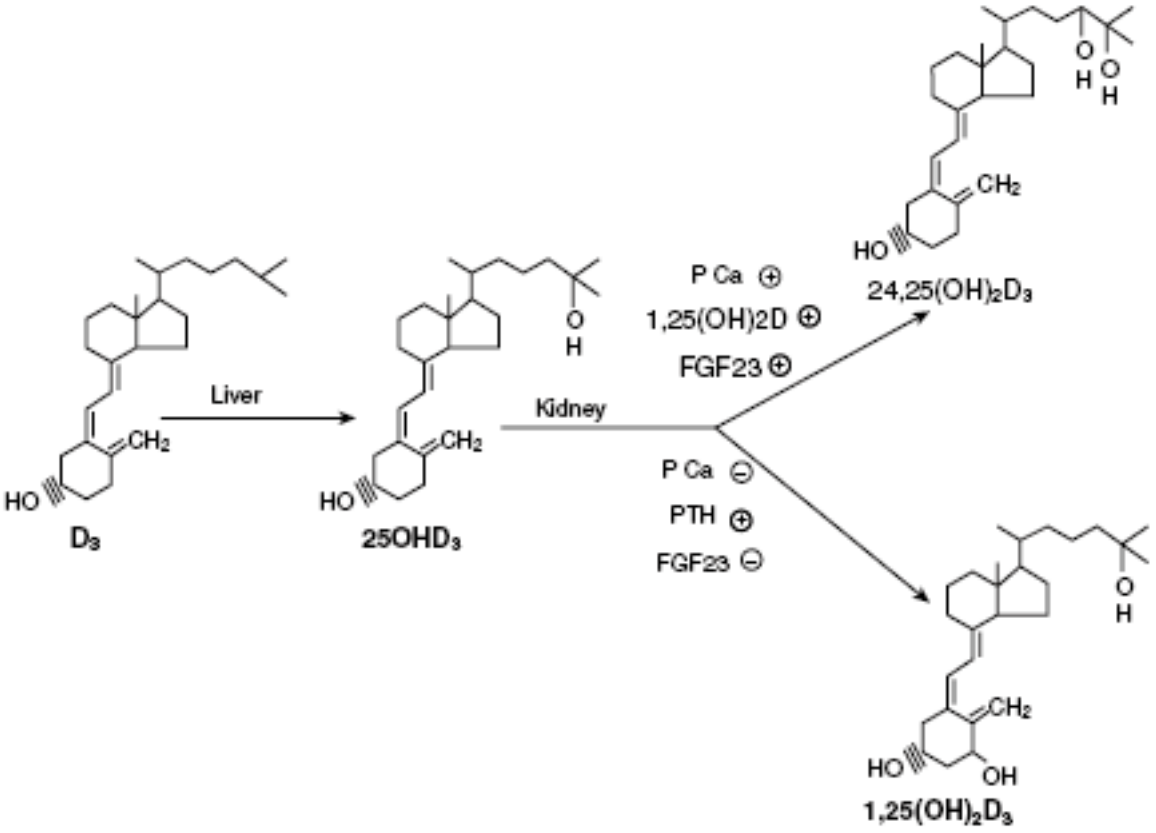


Figure 2: Vitamin D Metabolism. [19] Vitamin D undergoes two enzymatic modifications before it becomes biologically active as calcitriol. The liver converts vitamin D to 25OHD, and the kidney converts 25OHD to 1,25$(OH)_2$D (calcitriol) and 24,25OHD. Metabolic control is exerted primarily at the kidney. The action of 1αOH is controlled by substrate availability, amount of enzyme, cofactor availability, and enzymatic activity of CYP24A. Phosphate, calcium, and FGF23 also inhibit calcitriol production whereas PTH has a stimulatory effect. *Copyright, John Wiley and Sons (Appendix B).*

1.1.4.2.1 Calcitriol

Calcitriol is biologically active and has numerous roles in affecting mineral metabolism. Its classical actions are on bone, the intestines, and the kidneys to regulate serum calcium and phosphorus. However, the actions of calcitriol may extend beyond the skeleton to include regulation of the immune system, such that it may inhibit growth of cancer cells and protect against immune mediated disorders.[20]

The primary role of calcitriol is to stimulate intestinal calcium absorption. Calcitriol has been shown to stimulate both the active and passive pathways of calcium transport. [26] For the active pathway, calcitriol regulates the entire process, including entry of calcium across the brush border membrane, intracellular diffusion, and the energy requiring extrusion of calcium across the basolateral membrane. [19] In addition, calcitriol may also affect the rate of calcium intrusion and extrusion through the enterocyte, through stimulation of the plasma membrane calcium pump (PMCA). The actions of calcitriol may also be mediated by calbindin, the calcium binding protein, and TRPV6, a calcium selective channel. Intestinal phosphorus absorption is also stimulated by calcitriol, however the mechanism remains elusive.[19]

Calcitriol also has formative effects within bone, however this is predominantly indirect. Animal models of disrupted vitamin D physiology including vitamin D-deficient rats[27,28], vitamin D receptor knockout mice[29], and *Cyp27b1* knockout mice[30,31] all have impaired intestinal calcium absorption, leading to secondary hyperparathyroidism and rickets after weaning. Bypassing the need for calcitriol or its receptor with oral or parenteral calcium results in normalization of both the PTH level and the skeleton. [31-34] Moreover, additional studies in the *VDR* null teased this role

out to a greater extent. Knockouts of the vitamin D receptor just from intestinal cells results in the same phenotype as the global *VDR* knockout, whereas knockout of the *VDR* from osteocytes, osteoblasts, or osteoclasts does not cause the *VDR* null bone phenotype. [35,36] Furthermore, expressing the *VDR* in intestinal cells in the global *VDR* knockout rescues the bone phenotype. [35,37] These findings suggest that in regards to the skeleton, the main role of calcitriol is in delivery of mineral from upregulation of intestinal absorption. Any evidence supporting a direct role for calcitriol on bone cells is minimal. Double knockout models of both CYP27B1 and VDR fed a rescue diet have reduced bone volume, osteoblast number, and mineral apposition rate, indicating the skeletal phenotype was not completely prevented. [38]

The kidneys are also involved in calcitriol-mediated homeostasis. In the distal tubule, calcitriol enhances the actions of PTH on calcium transport through upregulation of the PTH receptor expression at both the mRNA and protein level. [39] Similar to the induction of calcium binding proteins and calcium selective channels in the intestine, calcitriol induces Calbindin-D_{28k} and TRPV5 in the distal tubule, thus increasing calcium reabsorption and return to the circulation. [19] A negative feedback loop exists to oppose this action, keeping calcium within a normal range. Through inhibiting CYP27B1 and inducing CYP24A, calcitriol moderates its own renal expression.[40]

1.1.4.2.2 Vitamin D receptor

Calcitriol is the hormonal ligand for the vitamin D receptor (VDR), which is to date the only known receptor for calcitriol. Belonging to a superfamily of nuclear receptors, VDR modulates gene expression through transcriptional regulation of target genes. Binding to DNA occurs as

heterodimers with the retinoid X receptor (RXR) on response elements within the gene.[41] The steroid hormone-binding domain is located from amino acid 182 to amino acid 427, providing high-affinity recognition.[42] The DNA-binding domain is a cysteine-rich region containing two zinc-fingers, and is located from amino acids 24-89.[42] Several coactivators are also involved in the transcriptional apparatus, such as SRC1, -2, and -3, and DRIP205.[43] Transcription factors YY1 and CCAAT, and SWI/SNF complexes that remodel chromatin additionally aid in the VDR-mediated transcription.[19] Once these proteins come together to form the complex, the DNA bends, serine-205 becomes phosphorylated, and the target gene is then activated or repressed (Figure 3).[43] The most powerfully regulated gene is the 24-hydroxylase enzyme that degrades vitamin D.[44]

The vitamin D receptor is widely expressed throughout the body. In addition to its appearance in classical target tissues of osteoblasts, enterocytes, and distal renal tubule cells, VDR expression has been found in parathyroid gland cells, skin keratinocytes, promyelocytes, lymphocytes, colon cells, pituitary gland cells, and ovarian cells.[43] Its widespread expression in these tissues is what has led to the theory that calcitriol has many extraskeletal roles, most of which remain unproven.[43]

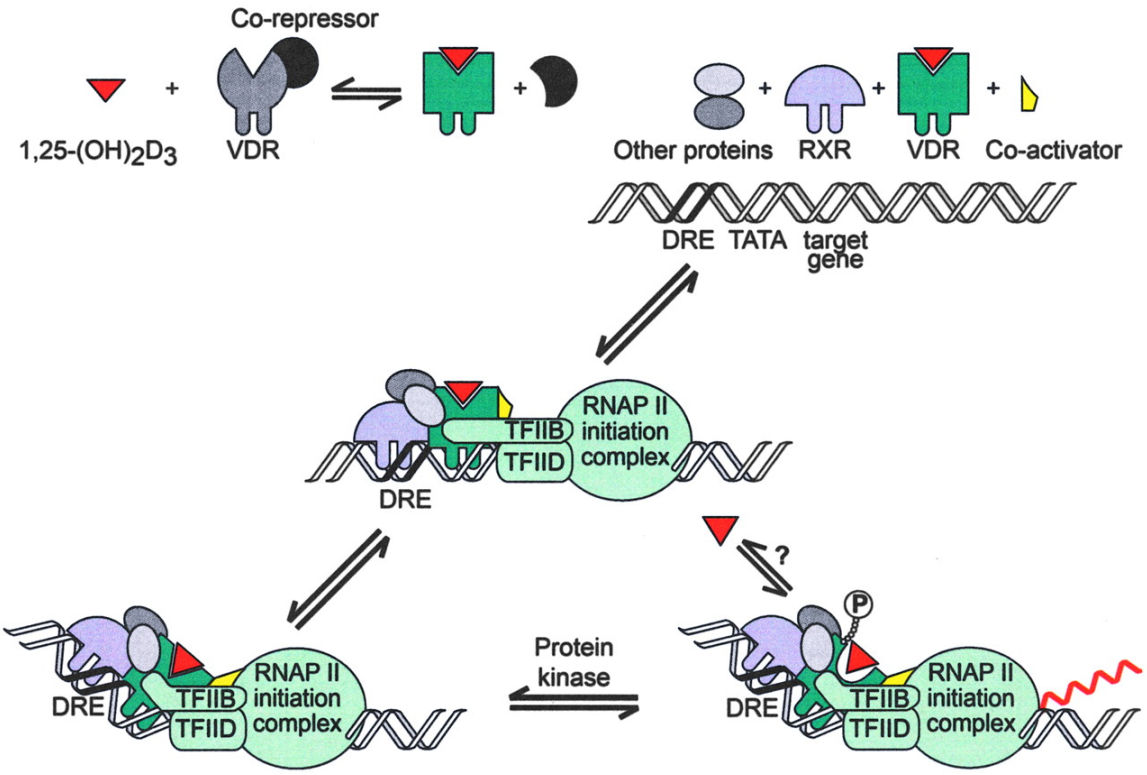


Figure 3: VDR modulation of gene expression by calcitriol. [43] VDR and its ligand form heterodimers with the retinoid X receptor (RXR) on VDR response elements (DRE) located in target genes. Together with co-activators and transcription factors, a complex forms that causes the DNA to bend. Phosphorylation occurs and transcription is either initiated or suppressed. *Copyright, Am J Clin Nutr (2004;80:1692S), American Society for Nutrition (Appendix C).*

1.1.4.3 Fibroblast growth factor 23

Fibroblast growth factor 23 (FGF23) belongs to the family of FGFs that regulate numerous developmental processes. Specific to FGF23 is its high expression in bone, particularly in osteoblasts, osteocytes, bone-lining cells, and osteoprogenitor cells. [45] mRNA expression is also detectable in the thymus, brain, and heart. [46] The full-length protein is 32 kD, however cleavage products of 12 and 20kD are also secreted. [47] In contrast to the majority of other FGFs, FGF23 is an endocrine factor that requires both the FGF receptors and a co-receptor αKlotho for activity. [47] The main function of FGF23 is to reduce serum phosphorus. This is achieved by reducing renal reabsorption through downregulation of the sodium-phophate cotransporters NAPI2a and NAPI2c, and by reducing intestinal absorption through suppression of NAPI2b. [48] The concentration of phosphate is then reciprocally regulated, as dietary intake of phosphate increases serum levels of FGF23. [49] In addition, FGF23 regulates calcitriol, such that high levels of calcitriol are measured in its absence. The inhibitory role of FGF23 is achieved through reducing expression of the anabolic enzyme CYP27B1 while increasing expression of the catabolic enzyme CYP24. [50] Thus, FGF23 has an indirect role in promoting a negative calcium balance by its actions to suppress intestinal calcium absorption. A negative feedback loop also exists with calcitriol, as injections of calcitriol result in increases of serum FGF23. [14] As calcitriol stimulates intestinal phosphorus absorption, FGF23 also has an indirect effect to lower serum phosphorus by reducing calcitriol. Lastly, FGF23 has a minor role in suppressing PTH. αKlotho is found in parathyroid cells where FGF23 inhibits PTH.[18]

1.1.4.4 Parathyroid hormone related protein

Parathyroid hormone related protein (PTHrP) is related to PTH, both derived from the same ancestral precursor through gene duplication.[51] They are structurally similar within the amino-terminal end enabling them to bind and stimulate the same PTHR1 receptor.[51] Beyond the first 13 amino acids however they share virtually no sequence homology.[52] PTHrP is normally absent from adult circulation apart from the progressive increase during pregnancy, coming from the placenta and breasts, and the more marked increase during lactation. Consequently PTHrP functions as a hormone in these time frames, but likely does not have endocrine function when it is undetectable. In the normal condition it is produced locally in certain tissues where it may have autocrine, paracrine, or intracrine functions.[53]

In humans, three different transcripts of the protein exist, containing 139, 141, or 173 amino acids. The physiological significance of these transcripts is uncertain, and in rodents, only the two shorter transcripts are found.[53] The physiological functions of PTHrP are diverse, as PTHrP mRNA has been found ubiquitously throughout body tissue. Some of the more well-documented local roles include that in the skeleton, smooth muscle, and mammary gland. In the skeleton, PTHrP stimulates growth of long bones during development through synchronizing chrondrocyte differentiation.[54,55] PTHrP is also critical in the cardiovascular system, as it functions to relax stretched smooth muscle cells in response to mechanical deformation, thus acting as a vasodilator.[53] In the mammary gland, the protein is important for branching morphogenesis.

The hormonal roles of PTHrP during pregnancy and lactation are of even greater importance as they contribute to the regulation of mineral homeostasis. Similar to the role of PTH, PTHrP

contributes to increased bone turnover. The amino-terminal portions of PTHrP stimulate bone loss through upregulation of osteoclasts, whereas the carboxyterminal portions inhibit osteoclast-mediated bone resorption. [55,56] PTHrP can also stimulate CYP27B1 but may be less potent than PTH. [51,57] Whether PTHrP accounts for the rise in calcitriol during pregnancy is uncertain. [56] The hormone has reciprocal roles in the kidneys, causing stimulation of calcium reabsorption but excretion of phosphorus. PTHrP is also particularly important for placental calcium transfer during pregnancy and regulating system calcium metabolism in the mammary gland during lactation.

1.1.4.5 Estradiol

Estradiol is the main circulating estrogen in premenopausal women. [58] It is synthesized in the ovaries from C19 metabolites of cholesterol. Estrogen receptor α (ERα) or β (ERβ) are transcriptionally activated by ligand binding with estradiol, and directly attach to estrogen response elements of target genes. [59] A nonclassical signaling pathway is also evoked by estradiol through activating cytoplasmic signaling cascades. [58] In this manner, the range of effects this hormone has on the body is vast and variable. Its main function is in the development and maintenance of female reproductive tissues, however estradiol also has profound effects on bone. Both ERα and ERβ have been detected in all skeletal cell types, with ERα having slightly higher expression. [60] Low levels of estradiol are responsible for linear bone growth, while higher levels drive the closure of the epiphyses and cease growth. [61] Additionally, estradiol affects skeletal maintenance through decreasing bone resorption, restraining the rate of bone remodeling, and assisting in maintaining a balance between bone formation and resorption. [58] These antiresorptive properties result from inhibition of RANKL and promotion of OPG

expression by osteoblasts. When estradiol is low, such as the case with ovarian failure, then RANKL increases, OPG decreases, bone resorption increases, and net bone loss occurs. [56]

1.1.5 Role of the intestines

Intestinal absorption of dietary calcium and phosphorus is crucial for maintaining skeletal homeostasis. For calcium, an average of 200 mg is absorbed per day. Two mechanisms are known to serve this purpose: a transcellular active transport process and a paracellular passive process. The active mechanism is located predominantly in the duodenum and upper jejunum and is regulated by calcitriol. [62] This route is responsible for a high proportion of calcium absorption when calcium intake is low, and in the growing young. [63] Movement across the absorptive enterocyte begins with entry through the calcium channel TRPV6, due to Ca^{2+} concentration differences between the intestinal lumen and cytosol. [64] The transport molecule $calbindin_{9k}$ then aids intracellular diffusion from the apical membrane to the basolateral membrane. [65] Here, extrusion of calcium is mediated by the Ca-ATPase. The sodium-calcium exchanger may also play a minor role in extrusion. However, studies on knockout mice of either TRPV6 or $calbindin_{9k}$ have shown increased intestinal calcium transport in response to *in vivo* administration of calcitriol, while double knockouts of both genes report impairments in absorption. [66,67] This suggests that the mechanisms explaining this route are still unclear, and that additional calcium channels or calbindins may also be involved in transport.

Conversely, movement through the paracellular pathway occurs primarily when intake of calcium is normal or high, and increases relatively as the organism ages. [65] It is concentration-dependent, non-saturable, and occurs at a rate inversely proportional to that of intestinal

propulsion. [68] Tight junctions regulate the epithelial permeability, which affects the paracellular flow of calcium ions. [65] This mechanism can be utilized in the absence of calcitriol by administering a diet high in calcium. However, despite functioning in its absence, this process can also driven by calcitriol. In a study by Fujita and colleagues, the tight junction proteins claudin-2 and claudin-12 were shown to form paracellar Ca^{2+} channels in intestinal mucosa, and that the expression of these proteins were upregulated both *in vitro* and *in vivo* by calcitriol.[69]

For phosphorus, intestinal absorption also occurs via two mechanisms: one active, transcellular pathway, and one passive, paracellular pathway. The active pathway occurs mainly in the upper portion of the small intestine. [70] It requires a sodium-dependent phosphate transporter located at the apical membrane, NAPI2b that is regulated by calcitriol, the concentration of P_i in the intestinal lumen, and FGF23. [70] FGF23 controls this directly by suppressing NAPI2b, and indirectly by inhibiting calcitriol. The passive pathway is driven by electrochemical gradients and is usually sufficient to maintain homeostatic levels.

1.1.6 Role of the kidneys

The kidneys are responsible for calcium and phosphorus reabsorption and excretion, in addition to the formation of calcitriol. Maintaining a neutral balance of calcium involves eliminating approximately 200 mg per day, which is normally balanced by net intestinal calcium absorption. [71] Both complexed and ionized calcium are filtered by the glomerulus in amounts exceeding 10 g. [2] To balance these substantial amounts, approximately 98 % is reabsorbed along the nephron. The majority of this reabsorption (55 %) occurs along the proximal convoluted tubule. Approximately 10 % is then reabsorbed in the proximal straight tubule, 20 % in the loop of

Henle, 10 % in the proximal straight tubule, 10 % in the distal convoluted and connecting tubule, and 2 % in the collecting duct.[71]

Calcium reabsorption can occur through either a paracellular, passive movement or a transcellular, active route. The passive mode of transport is driven by an electrical or chemical gradient and occurs largely in the proximal convoluted tubule and the proximal straight tubule.[71]Active transport begins with passive flux across the apical membrane, mediated by TRPV5 and TRPV6 channels. [72] Diffusion through the cytosol is then buffered by Calbindin-D_{28K}. Compartmentalization of Ca^{2+} in intracellular organelles is also important here to maintain the intracellular concentration within a narrow range. [71] Lastly, active extrusion across the basolateral membrane occurs via the plasma membrane Ca^{2+}-dependent ATPase and the Na^{+}/Ca^{2+} exchanger.[71]

Multiple hormones influence calcium handling by the kidney. Both parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) increase the glomerular filtration rate and tubule reabsorption. [2] PTH stimulates CYP27b1 in the nephron, so that 25OHD is converted to calcitriol. This leads to an indirect action of PTH on the intestines for calcium absorption.[2] Calcitriol, estrogen, and dietary intake of calcium modulate the transcription of TRPV5 and TRPV6. [73] The trafficking and activity of these channels are stimulated by Klotho and calmodulin, respectively.[73]

The kidneys are also responsible for maintaining a balance of phosphorus. Of the 7 g filtered by the kidneys per day, approximately 80-90 % is reabsorbed along the nephron with the remainder

being eliminated in urine. [74] Similar to calcium metabolism, the majority of phosphorus is reabsorbed along the proximal tubule. [74] Passive mechanisms are unable to account for the movement of phosphorus across the kidney. Rather, Na-Pi cotransport systems (NAPI) mediate active transport across the cell membrane. [75] PTH, FGF23 and calcitriol are involved in regulating this process. Parathyroid hormone inhibits phosphorus absorption in the renal proximal tubule and decreases Na-Pi cotransport at the apical membrane. [76] FGF23 also has an inhibitory role, reducing the activity of NAPI transporters in renal epithelial cells. [77] This means that PTH, PTHrP, and FGF23 cause phosphorus excretion. Conversely, calcitriol has a stimulatory role, increasing renal recovery of phosphorus.[74]

The role of the kidneys in production of calcitriol is also critical for maintenance of mineral and bone homeostasis. The CYP27B1 1α-hydroxylase is predominantly located in this organ, and is thus the source of most of the calcitriol that becomes distributed throughout the body to exert its wide range of effects. [19] *Cyp27b1* expression in other tissues does not contribute much to the circulating level as anephric patients have low calcitriol levels.[78]

1.1.7 Role of bone

Bone plays a critical role in mineral homeostasis through modulating the flux of minerals via resorption and formation. To understand how this is accomplished, it is important to explain the structure and composition of bone tissue.

1.1.7.1 Bone structure and composition

Bone is a specialized form of connective tissue, containing cells, fibers, and ground substance. [79] It is characterized morphologically as cancellous or cortical. Cancellous bone, otherwise known as trabecular bone, is composed of a meshwork of trabeculae. It is predominantly found at the epiphyseal region of long bones, but also in flat bones and vertebral bodies. [1] Its sponge-like construction allows for a reduction of skeletal weight without a compromise in strength. Further, it has a high metabolic rate and responds quickly to changes in mechanical loading. However, this also renders cancellous bone more susceptible to damage from bone loss. [1] Conversely, the architecture of cortical bone is thick and dense. This arrangement provides it with maximal load-bearing capacity and strength, as a result of higher resistance to torsion and bending forces. [79] It is found largely at the diaphyses of long bones and accounts for 80 % of the weight of the human skeleton. Together, both forms contribute to overall bone strength.

Three cell types comprise bone tissue: osteoblasts, osteocytes, and osteoclasts. Each of these cells serves distinct skeletal functions and intercommunicates through direct cell contact or signaling molecules. [80] Osteoblasts are differentiated from mesenchymal stem cells (MSCs) by the action of several transcription factors and signaling cascades. Osteocytes are formed as a result of terminal differentiation of osteoblasts. Osteoclasts differentiate from the hematopoietic lineage and belong to the monocyte-macrophage family. Surrounding these cells lies the extracellular matrix (ECM), which makes up the preponderance of bone. In decreasing proportion, the ECM consists of mineral, collagen, water, noncollagenous proteins, and lipids. [81]

1.1.7.2 Osteoblasts

Osteoblasts make up approximately 4-6 % of the human skeleton. [82] They are mononuclear cells that form tight junctions with adjacent osteoblasts. Extensions of their plasma membrane allow for communication between each other and the surrounding medium. As mentioned, osteoblasts are derived from MSCs by the action of a multitude of cytokines. [83] Each has a role in regulating the expression of cell-lineage specific sets of transcription factors. Runx2 is the master switch for osteoblast differentiation, binding to the promoter regions of the majority of osteoblast-specific genes including type 1 collagen (Col1), alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON), and osteocalcin (OC). [84] Its importance to osteoblastogenesis is evidenced by *Runx2* knockout mice who completely lack osteoblasts. [85] Further, Runx2 regulates the expression of the zinc-finger-containing transcription factor Osterix, which interacts with the Nuclear factor for activated T cells 2 (Nfatc2) to control the transcription of target osteoblastic genes. [86] Numerous other transcription factors are involved in osteoblast differentiation, including bone morphogenic proteins (BMPs), transforming growth factor ß (TGF- ß), WNTs, hedgehogs, parathyroid hormone (PTH), insulin-like growth factor-1 (IGF-1), fibroblast growth factors (FGFs), and Notch.[83]

Primarily, osteoblasts function to synthesize the organic collagenous matrix and coordinate its mineralization. This process is maintained by the stages of differentiation: cellular proliferation, extracellular matrix deposition, matrix maturation, and mineralization. [87] Extracellular matrix components such as osteocalcin, osteopontin, and bone sialoprotein are initially deposited. [88] Subsequently, mature osteoblasts mineralize the matrix to form new bone.

1.1.7.3 Osteoclasts

Osteoclasts comprise the smallest percentage of bone cells, making up 1-2 % of the human skeleton.[82] They are large, multinucleated and highly motile, however they are usually seen apposed to the surface of bone. On the surface between the osteoclast and bone is an extensive ruffled border that consists of a series of finger-like cytoplasmic projections of the plasma membrane adjacent to bone.[89] Originating from hematopoietic stem cells, osteoclasts belong to the monocyte/macrophage family.[90] They are sufficiently developed in the presence of cytokine macrophage colony stimulating factor (M-CSF) and RANKL.[91] Respectively, these two ligands bind to the membrane receptors c-fms and RANK.

The primary functions of osteoclasts are resorption and degradation of mineralized bone matrix. Imperative for the resorptive function is close contact between the osteoclast and underlying bone matrix, mediated by integrin αvß3.[91] This creates a microenvironment isolated from the extracellular space for the secretion of hydrochloric acid and proteolytic enzymes, which in turn break down bone into its component minerals and small proteinaceous fragments. The compartment is acidified to a pH of 4.5 by a vacuolar H^+-ATPase and coupled chloride channel with a basolateral chloride-bicarbonate exchanger.[92] Resorption of bone is triggered by a polarization event. Acidified vesicles containing cathepsin K are transported via microtubules and actin to the sealing zone following osteoclastic bone recognition.[93] Attachment of these vesicles to the plasma membrane results in formation of the ruffled membrane containing the H^+ machinery. Exocytosis of cathepsin K couples the release of H^+ from this machinery, which functions to dissolve the matrix.[89] Type 1 collagen is degraded by cathepsin K, and its protein fragments are endocytosed into the surrounding intracellular fluid.[94] Release of the degradative

products may also be mediated by withdrawal of the osteoclast from the resorptive pit. A number of factors affect the secretion of acid and resulting bone resorption, including PTH and prostaglandin E_2 that increase secretion, while calcitonin decreases secretion.[95]

1.1.7.4 Osteocytes

In the adult skeleton, osteocytes constitute over 90-95 % of all bone cells, and are regularly dispersed throughout the mineralized matrix.[82] They form an extensive lacunocanalicular network, with the cell body being encased in a lacuna and dendrites traveling through bone in canaliculi.[82] It has been estimated that there are approximately 42 billion osteocytes in the average adult human skeleton, with 3.7 trillion dendritic projections from these cells.[96] With 23 trillion connections formed between these osteocytes and the cells on the bone surface, this order of magnitude is the same as current estimates of the neural network of the brain.[96] Osteocytes form as one of three possible destinies resulting from the terminal differentiation of an osteoprogenitor cell. By an unknown mechanism, some of these cells may also become lining cells or others may undergo apoptosis.[97]

Rather than merely acting as a passive placeholder in bone, osteocytes have numerous integral functions. They act as orchestrators of bone remodeling, sending signals of resorption or formation in response to mechanical strain. The mechanical load placed on bones drives interstitial fluid to flow through the unmineralized matrix surrounding osteocytes and their dendritic processes.[98] As a result, osteocytes produce signaling molecules that regulate the activity of osteoclasts and osteoblasts.[99] Various biomolecules are responsible for this intercellular communication, including nitric oxide, prostaglandins, bone morphogenic proteins,

and Wnts. [100] Osteoblasts specifically can be stimulated by prostaglandins and Wnts, and inhibited by nitric oxide and sclerostin. Opposingly, formation and activity of osteoclasts can be stimulated by M-CSF and RANKL. [101]

Another mechanism by which osteocytes modulate bone remodeling is through their programmed cell death. Osteocyte apoptosis can occur at sites of microdamage, where proapoptotic molecules are increased. At these loci, apoptotic osteocytes release apoptotic bodies that express RANKL and recruit osteoclasts to resorb bone. [102] Microdamage can also cause a dendritic process to break, similarly recruiting osteoclasts to break down bone as part of the initiation of repair. The attraction of osteoclasts here is probably mediated by a combination of fluid pressure changes within the broken canaliculus as well as a release of factors from the fluid. Necrosis may also be a mechanism whereby osteocytes orchestrate bone resorption, as targeted ablation of osteocytes has been demonstrated to activate osteoclasts in mice. [103] Through a single injection of diptheria toxin in mice, 70 % of osteocytes were eliminated from cortical bone, and they exhibited fragile bone and trabecular bone loss consistent with osteoclast activation.

Further research suggests that osteocytes can both add and remove mineral from its lacunocanalicular network. [104] Acting like osteoclasts, osteocytes can express genes of the hematopoietic lineage. This function is known as osteolytic osteolysis. Osteocytes can also act as osteoblasts, expressing genes of the mesenchymal lineage.

Osteocytes can also function as an endocrine gland through regulation of phosphate homeostasis. [104] Phex, Dmp1, and FGF23 are all highly expressed in osteocytes. As Phex and Dmp1

downregulate the expression of FGF23, osteocytes can increase reabsorption of phosphate by the kidney. [105] This role is critical for maintaining sufficient circulating phosphate and the inherent bone mineral content.

1.1.7.5 Bone remodeling

Taking together the various components of bone, it is clear that the organ is not a static structure but rather highly dynamic. Bone undergoes consistent remodeling with 25 % of trabecular and 3 % of cortical bone replaced each year. [106] This turnover is maintained by a delicate balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Such tight regulation is crucial for the repair of microdamages, replacement of old bone, and maintenance of calcium and phosphorus mobilization. [107] Any significant deviation from this balance could result in accelerated bone loss or bone gain. Therefore bone formation and bone resorption are normally coupled, such that increases to one results in increases to the other, or decreases to one causes decreases to the other. [107] This is accomplished by cross-communication between osteoblasts, osteoclasts, and osteocytes in a unique microenvironment known as a bone-remodeling compartment (BRC). [1] The outer lining of the BRC is comprised of flattened cells, which secrete regulatory factors into the confined compartment space separated from the bone marrow. Avoiding interference from growth factors secreted by blood cells in the marrow facilitates local regulation of the remodeling process. [107] Generally, bone resorption is accomplished within 2-3 wks while bone formation requires a time period of 2-3 months. The cycle for remodeling may be initiated by signals from osteocytes that induce the activation of the quiescent bone surface, or by the action of hormones as a result of systemic changes in homeostasis. [1]

1.1.8 Role of the parathyroid glands

The parathyroid glands regulate the balance of serum minerals by responding to changes in calcium and phosphorus. They are the main source of parathyroid hormone, and are therefore responsible for its downstream effects to maintain the normocalcemic state.[6] *In vivo*, PTH is secreted in response to small decreases in blood ionized calcium levels, and functions to raise the blood level of calcium by promoting bone resorption, reabsorption of calcium by the kidneys, and intestinal calcium absorption. PTH also functions to lower the levels of serum phosphorus by increasing renal phosphate excretion. These functions are further explained in section 1.2.4.1.

1.1.9 Interaction of target organs and the calciotropic and phosphotropic hormones

Now that the target organs and dominant calcium and phosphorus-regulating hormones have been identified, it is possible to explain how these components interact with each other to maintain serum mineral concentrations and long-term skeletal strength and mineral content (Figure 4). Firstly, PTH and FGF23 antagonistically regulate calcitriol. Parathyroid hormone is the main stimulator of calcitriol synthesis, and is released in response to low levels of serum calcium and high levels of serum phosphorus; and inhibited by high levels of calcium. It also stimulates FGF23. Fibroblast growth factor 23 is also released in response to high levels of serum phosphorus and inhibits the production and enhances the catabolism of calcitriol. In turn, calcitriol suppresses PTH production by the parathyroid glands and stimulates FGF23 production in bone. Calcitriol also evokes a pathway of self-regulation through inhibiting its own synthesis, in addition to being inhibited by high levels of phosphorus and calcium. These feedback loops are key to the maintenance of bone and mineral homeostasis.

Further, the calciotropic and phosphotropic hormones also interact with the target organs. The kidneys possess the CYP27B1 enzyme and are responsible for calcitriol synthesis. This conversion to the active form of vitamin D is modulated by the feedback loops previously mentioned, in addition to PTHrP that stimulates CYP27B1. The kidneys are also responsible for both mineral reabsorption and excretion. FGF23 and PTH reduce renal reabsorption of phosphate, while PTH stimulates renal reabsorption of calcium. The intestine is also a target organ in this complex interaction to regulate mineral homeostasis and bone metabolism. Its main function is calcium and phosphorus absorption, which is achieved through the action of calcitriol. The functions of bone are more extensive, with osteoblasts, osteoclasts, and osteocytes affecting the formation and resorption of bone, and creating a store of mineral and base. Estradiol and PTHrP exert their single function through acting on these bone cells, with estradiol inhibiting bone resorption and PTHrP promoting bone loss. Conversely, PTH and calcitriol can have both negative and positive effects on bone metabolism.

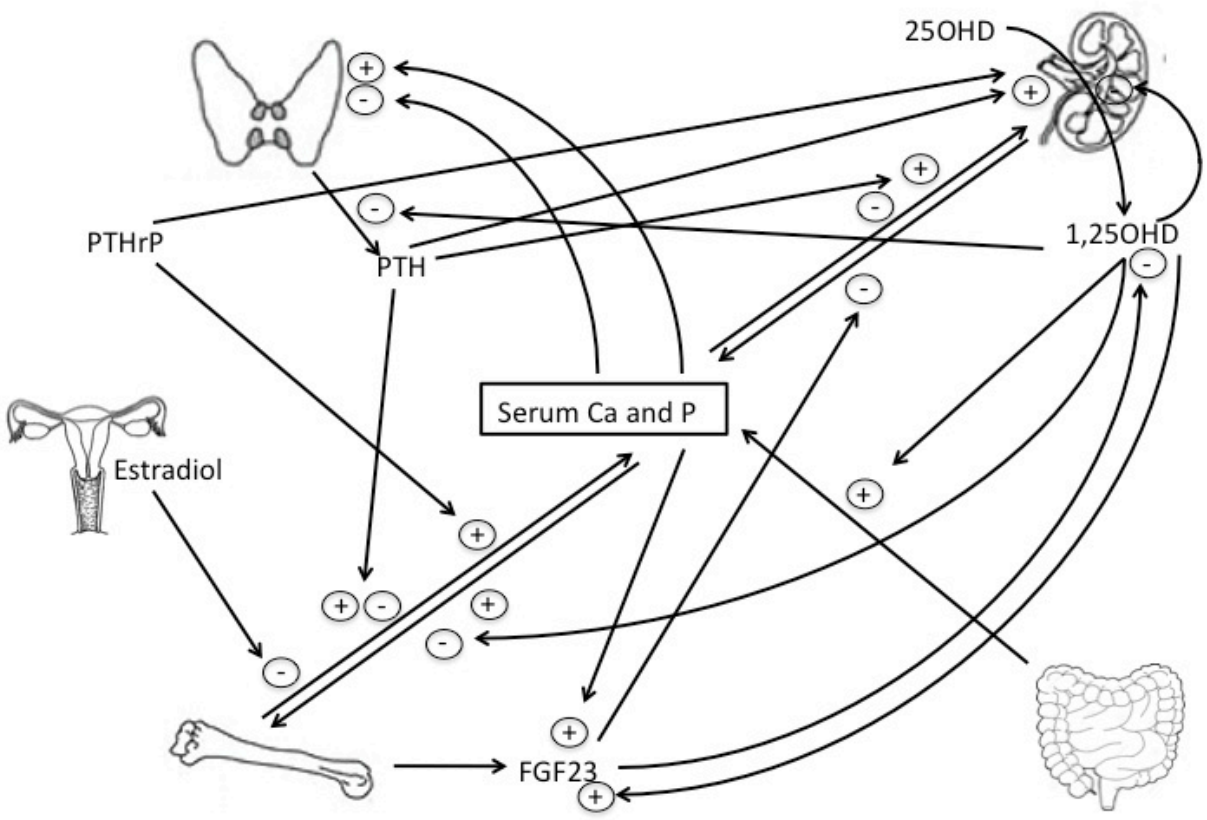


Figure 4: Interaction of the calcio/phosphotropic hormones and target organs in regulating bone and mineral homeostasis. Serum calcium and phosphorus are maintained within the desired range through the actions of PTH, calcitriol, FGF23, estradiol, and PTHrP on the parathyroid glands, kidneys, intestines, and bone. Feedback regulatory loops are also in operation to sustain the normal endocrinologic environment.

1.1.10 Human conditions of altered vitamin D metabolism

The physiological importance of vitamin D is evident in human disorders where calcitriol is rendered absent or nonfunctional. This is the case in a nutritional vitamin D deficiency, or in genetic conditions of inactivation of the *CYP27B1* gene or the *VDR*. Generally, these disorders cause impaired intestinal calcium and phosphorus absorption, which in turn causes insufficient mineralization of bone, leading to rickets in the growing child and osteomalacia in the adult. Rickets is characterized by decreased longitudinal growth, widening of the epiphyseal plate, and a bowing of tubular bones. [108] Children display slow developmental milestones in growth and walking. Osteomalacia is characterized by bone pain, muscular weakness, and difficulty walking. [108] There is also increased propensity to fracture due to the low quantity of mineralized bone. In either condition, tetany and convulsions may also be present in concordance with low concentrations of serum calcium.

1.1.10.1 Nutritional vitamin D deficiency

Dietary deficiency of vitamin D can lead to rickets or osteomalacia as there is inadequate substrate to be metabolized to calcitriol. Although there is no current agreement on the threshold for adequate intake of vitamin D, it has been defined previously as the serum 25OHD that maximally suppresses PTH, as an arguable minimum level of serum 25OHD (15, 50-75, 40, or 50 nmol/l), or as a recommended daily allowance of 600 IU/day for children and 800 IU/day for adults. [108] Several recent clinical trials in adults and children have shown that intestinal calcium absorption becomes maximal with a 25OHD level over 20 nmol/L, providing a strong argument for this as the threshold. [109-113] Certain populations are also more susceptible to vitamin D deficiency due to their age, latitude of habitat, skin color, type of clothing worn outdoors,

concomitant disorders, or drug usage. Children may be particularly vulnerable, as a result of insufficient levels of calcium in breast milk, or low sun exposure. [114] The elderly are also at increased risk, due to decreased dermal synthesis of vitamin D from UV-B irradiation. [108] Cultural habits of non-western immigrants who migrate to countries at higher latitudes similarly place this population at risk for poor calcium absorption, such as avoiding sunlight and wearing clothes that cover most of the skin, which may be compounded by a diet low in calcium. [108] Conditions of malabsorption also increase the likelihood of a vitamin D deficiency, including celiac disease, Crohn's disease, gastrectomy, gastric bypass, bowel resection, or pancreatitis. Lastly, certain drugs may have rachitic side effects, such as anticonvulsant therapy that increases the catabolism of calcitriol. [108] Treatment of this deficiency is largely met by a prescribed dose of vitamin D_3 and a calcium supplement.

1.1.10.2 Vitamin D-dependent rickets type 1

Inherited abnormalities of the *Cyp27b1* gene can also lead to rickets and osteomalacia as a result of altered vitamin D metabolism. In this condition, the conversion of 25OHD to calcitriol is prevented due to a deficit in the 1αOH enzyme. [115] Inheritance follows an autosomal recessive mode of transmission. [116] The *Cyp27b1* gene was mapped to chromosome 12q13.3, and several homozygous mutations have since been reported. Examples of these inactivating mutations include: Arg107His, Gly125Glu, Arg335Pro, and Pro382Ser. [115] The phenotype is heterogeneous, with symptoms ranging from hypotonia, weakness, growth failure, hypocalcemia, seizures, elevated PTH, and aminoaciduria. [115] Despite supplementation with vitamin D, patients retain low serum concentrations of calcitriol. [116] Treatment requires physiologic doses of

calcitriol or 1α-hydroxyvitamin D; a marketed form of vitamin D that gets hydroxylated in the liver to become calcitriol.

1.1.10.3 Vitamin D-dependent rickets type 2

Another rare genetic condition leading to rickets and osteomalacia is caused by inactivating mutations of the vitamin D receptor. In this condition, calcitriol is rendered nonfunctional due to resistance of target organs to the action of the active hormone. These inborn errors are also inherited in an autosomal recessive manner. [108] Mutations have been identified at both domains of the VDR. In the calcitriol-binding domain, a nonsense mutation was identified at nucleotide 970, changing tyrosine to a premature stop codon. [117] In the DNA-binding domain, missense mutations within exons 2 and 3 have been reported, which are responsible for encoding the zinc-finger structures. [42] These include a p.Gly30Asp mutation and a p.Arg70Gln mutation, both of which affect highly conserved amino acids essential for the function of DNA-binding. [42] Symptoms include severe rickets, seizures, hypocalcemia, secondary hyperparathyroidism, and scalp and body alopecia. [42] Further, these patients have very high serum levels of calcitriol. [118] Despite supplementation with calcitriol, rachitic symptoms remain. Treatment is not always effective and depends on the degree of hypocalcemia. If there is only limited hormone resistance, treatment with pharmacologic doses of calcitriol can upregulate calcium absorption and heal the symptoms of rickets. [108] If, however, there is complete abolishment of VDR function, no amount of calcitriol will have an effect, and so infusions of calcium and high oral doses are needed to overcome defective absorption of the mineral.[108]

1.1.11 Animal models of altered vitamin D metabolism

In addition to the human data on conditions of defective vitamin D metabolism, several animal models have been investigated. These are particularly useful for reproductive research as their gestational time is significantly shorter (~3 wks as compared to 9 months in humans). The animal models studied to date include vitamin D deficient rats, vitamin D deficient mice, *VDR* null mice, *Cyp27b1* null pigs and *Cyp27b1* null mice.

1.1.11.1 Vitamin D deficient rats

Multiple studies have investigated severely vitamin D deficient rats. [28,119-121] These animals were obtained as weanlings from a colony maintained on a diet that lacked vitamin D but contained 0.47 % calcium and 0.3 % phosphorus. [27] No exposure to UV light was permitted. At birth, the pups had normal skeletal lengths, morphology, ash weight, and mineral content of the ash. It is not until weaning that the pups begin to develop hypocalcemia, reduced mineralization, and rickets. These findings suggest that vitamin D is not required during fetal development to regulate calcium homeostasis, skeletal growth or mineralization. However, vitamin D becomes required at the time of weaning, when delivery of minerals is achieved through the intestines. [122]

1.1.11.2 Vitamin D deficient mice

Vitamin D deficient mice from two different genetic backgrounds have been studied in recent years. CD1 mice (outbred swiss mice from Charles River) were maintained on a diet containing 1.2 % calcium and 0.5 % phosphorus. Similar to the vitamin D deficient rats, they had normal concentrations of serum calcium and ionized calcium at birth. [123] Mice from the Swiss

background were also studied, however they had 20 % lower calcium levels than vitamin D-replete mice and normal phosphorus. [124]

1.1.11.3 *VDR* null mice

Three different models of *Vdr* null mice have been studied; one developed in Boston, the second by a group in Leuven, and the third by a group in Tokyo. The Boston model (Vdr^{Bos}) was engineered into C57BL/6 mice and has a deletion of the second zinc finger of the VDR DNA-binding domain resulting in no translated product. [29] Conversely, both the Leuven model (Vdr^{Leu}) and Tokyo model (Vdr^{Tok}) were engineered into CD1 mice and have a deleted exon encoding the first zinc finger of the VDR, such that a splice variant is secreted with normal ligand binding ability but no DNA binding ability. [122,125,126] Vdr^{Leu} and Vdr^{Tok} mice were backcrossed to C57BL/6 and showed no phenotypical differences at birth between null and WT fetuses. [126,127] Specifically, studies on Vdr^{Leu} mice showed normal serum calcium, phosphorus, PTH, renal excretion of calcium and phosphorus, and skeletal mineralization; and Vdr^{Tok} mice were described as having normal growth rate and behaviour. Vdr^{Bos} mice were backcrossed to Black Swiss and also had fetuses with normal serum mineral concentrations in addition to normal skeletal development. [128] It is upon weaning through early adulthood that the animals of all three models become afflicted by hypocalcemia, secondary hyperparathyroidism, hypophosphatemia, and rickets when on a standard chow (1 % calcium, 0.75 % phosphorus). It is at this time when the intestines become more dependent on active, calcitriol-dependent intestinal calcium absorption. However, use of a rescue diet allows for normal serum chemistries, skeletal morphology, and skeletal mineral content, bypassing the need for the VDR. [122]

1.1.11.4 *Cyp27b1* null pigs

Pigs unable to synthesize calcitriol were acquired from the "Hannover Pig Strain", which suffers from a naturally occurring null mutation of the 1α-hydroxylase. [129] The disease is inherited in an autosomal recessive manner. In the uterus, serum minerals are all normal. Symptoms present between four to six wks of age and closely resemble that of humans with vitamin D-dependent rickets type 1. Sows display a rachitic phenotype with hypocalcemia, hypophosphatemia, and - unphyiosological low concentrations of calcitriol in plasma, matching the human condition.

1.1.11.5 *Cyp27b1* null mice

The *Cyp27b1* gene consists of nine functional protein-coding exons, and two different models of *Cyp27b1* null mice have been studied (Figure 5). Dardenne and colleagues first engineered the model in 2001, inactivating the enzyme by deleting exon 8 encoding the heme binding domain, and part of introns 7 and 8. [30] That same year Panda and colleagues engineered a similar model, fully ablating the enzyme by deleting both the hormone binding domain encoded by exons 6 and 7, and the heme binding domain encoded by exon 8. [31] As with the previous animal models of defective vitamin D metabolism, *Cyp27b1* null mice are phenotypically normal at birth through most of the suckling period. At weaning, they have hypocalcemia that worsens with age, in addition to hypomineralization of bones and rickets. In response to the decreased blood calcium levels, the knockout mice have increased blood PTH levels, and this secondary hyperparathyroidism also becomes more pronounced with age. Further, as mediated by the low levels of extracellular calcium and phosphorus, the mice develop uterine hypoplasia and infertility when on a standard laboratory diet (1% calcium).

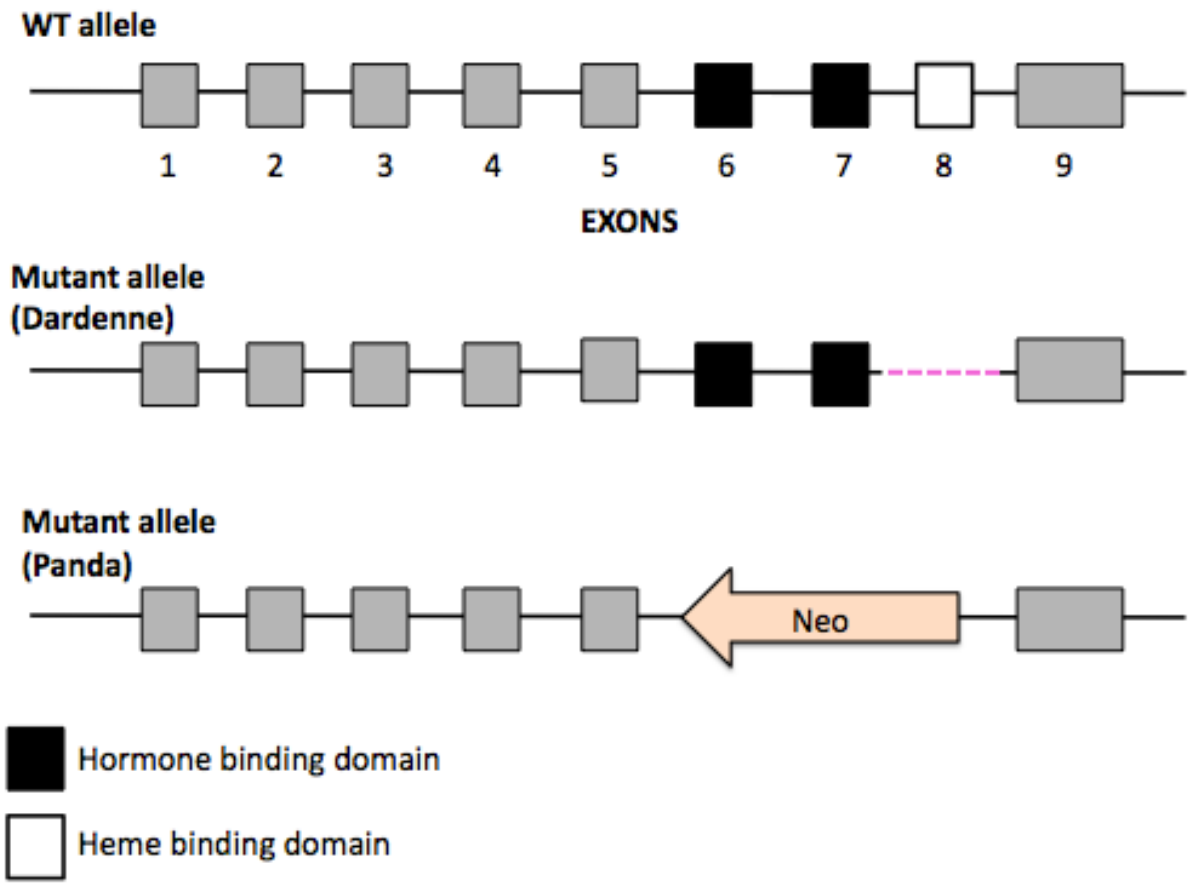


Figure 5: The *Cyp27b1* gene. Schematic representation of the 1αOH and the two mutant alleles studied to date. The WT allele is comprised of 9 exons, of which exons 6 and 7 encode the hormone binding domain while exon 8 encodes the heme binding domain. Dardenne inactivated the enzyme by cre-mediated excision of exon 8. Panda inactivated the enzyme by homologous recombination with a neomyocin resistance cassette replacing exons 6, 7, and 8.

Both models were later studied on a rescue diet of high calcium, high phosphorus, and high lactose at weaning. All groups claim that the diet rescued the phenotype of pseudo vitamin D deficiency rickets. In the first model by Dardenne and colleagues, mice were placed on this diet from 3 wks of age until harvest at 8.5 wks of age, at which point serum analyses, bone histology, histomorphometry, and biomechanical analysis were performed. [33] The high calcium diet corrected hypocalcemia and secondary hyperparathyroidism, and histomorphometry confirmed that rickets and osteomalacia was cured. The only difference the authors noted between genotypes was that the null mice gained weight less rapidly and that femur size remained smaller. The second model was also studied on the rescue diet at 3 wks of age until 15 wks, first by the original authors and then by Sun and colleagues. [38,130] Panda found that serum calcium, phosphorus, and PTH normalized to WT, in line with the initial paper stating that the rachitic phenotype was cured on the diet. However, they found several skeletal alterations in contrast to normal, including decreased femoral size, enlargement and distortion of the growth plate, reduced trabecular bone volume, reduced osteoid volume, and reduced osteoblast number. Panda and colleagues suggest that the observed skeletal abnormalities may be due to prolonged exposure to the rescue diet post-weaning and that bone formation may be more dependent on vitamin D as the animals age. The results by Sun and colleagues were again similar to previous findings indicating a reversal of rickets, as they observed normalization in both serum calcium and serum phosphorus at 3 months of age. In addition, they found a reversal in the defective reproductive phenotype.

1.2 Adaptations during pregnancy and the role of calcitriol

Mineral and bone metabolism is significantly challenged during pregnancy due to the incredible demand from the developing fetus and placenta. A full-term fetus requires sufficient mineral to be provided throughout pregnancy, 80 % of which is accreted during the third trimester in humans. [131] For calcium, an average of 30 g is accumulated by birth. [132] For phosphorus and magnesium, 20 g and 0.80 g are obtained by term, respectively. [133] The proportional demand for mineral is even greater in rodents, due to their shorter gestational period and larger litter size. A mouse delivers more than 95 % of the mineral content to their fetuses within the last 5 days of pregnancy. [56]

A plethora of changes in calciotropic hormone levels occur during this time period as compared to the normal adult state (Table 1). During normal human pregnancy, serum phosphate and magnesium remain constant, while serum calcium decreases as the fetus grows. [134] Calcium in serum is bound principally to albumin, so the observed declines during pregnancy generally represent the reductions in the albumin-bound fraction and is therefore physiologically unimportant. Measurements of ionized calcium indicate that the pre-pregnancy level is maintained throughout gestation, confirming that there is no real change in the physiologically relevant level of calcium in the blood. [134] In contrast, PTH experiences a significant decrease during the first trimester, then a recovery to its normal value by term. [135] Calcitriol, although stimulated by parathyroid hormone, has relative excursions distinct from PTH. Total calcitriol levels double or triple early in pregnancy and maintain this increase until term. [56] Levels of free calcitriol are increased in all three trimesters, as indicated by a modest increase in vitamin D binding protein and a decline in serum albumin. [56] To modulate this increase, the maternal kidney

1α-hydroxylase is upregulated, as a result of hormonal stimulators such as estradiol, prolactin, and placental lactogen. [136] PTHrP levels are also increased in the circulation during pregnancy due to multiple tissues in the mother and the fetus, and may contribute to the stimulation of calcitriol synthesis. [136] In addition, PTHrP may also suppress PTH during this time. The mineral physiology of rodent pregnancy is largely similar to that of human pregnancy, however rats have low ionized calcium and increased PTH concentration. The increase of serum calcitriol levels in mice and rats may also be greater than that measured in humans, with measurements of two to seven-fold increases.[56]

Table 1: Changes in serum concentrations of humans and rodents during pregnancy compared to the non-pregnant, non-lactating mature female

	Human [56]	**Rat** [56]	**Mouse** [56]
Serum calcium	Low	Low	Normal
Ionized calcium	Normal	Low	Normal
Phosphorus	Normal	Normal	Normal
Magnesium	Normal	Normal	Normal
PTH	Low or low-normal	Increased	Low or normal
Calcitriol	Increased	Increased	Increased
PTHrP	Increased	Increased	Increased

The source of the increased concentrations of calcitriol during pregnancy has been a cause of debate, whether it is due to contribution from the placenta or maternal kidneys. Previous studies have indicated that *in vitro*, cultures of both full-term and young (8-12 wks gestation) placental tissue express Cyp27b1 and synthesize calcitriol. [21] Rats that have had 5/6 of their kidneys removed had a relatively normal increase in calcitriol during pregnancy. [137] The authors concluded that the pregnancy-induced increase in calcitriol could only be due to the placenta; however, the result is still compatible with the remaining kidney increasing calcitriol production. This is made more evident by studies in completely nephrectomized rodents, which had a greatly reduced amount of tritiated calcitriol in the maternal circulation after tritiated 25OHD was administered to the fetuses. [138,139] Whether that calcitriol was synthesized in the placenta or other maternal tissues is unclear. Additionally, maternal renal *Cyp27b1* mRNA expression is 35-fold higher than placental *Cyp27b1* expression. [140] Human data are compatible with the maternal kidneys and not the placenta being the dominant source of calcitriol in the maternal circulation because anephric women do not have higher amounts of calcitriol in the circulation during pregnancy. [78] Thus, the data are most consistent with the role of the kidney being more important for pregnancy-stimulated calcitriol than the placenta.

To meet the need for calcium delivery, the mother relies primarily on increased efficiency of intestinal absorption, with minor contributions from skeletal mineral. Intestinal calcium absorption is doubled from as early as 12 wks gestation in humans, whereas in rats does not double until day 20-22 of their 24 day gestation. [56,141] The extent of resorption that occurs during this time however is less certain. Skeletal resorption can certainly be marked when dietary intake is insufficient to meet the combination of maternal and fetal demand for this mineral. Human

data indicate that a positive calcium balance occurs by mid pregnancy, though there is a lack of data on bone mass, structure, and mineralization at this time point. Markers of bone formation and resorption are both low in the first trimester and increase by term, suggesting that bone turnover may be increased in the third trimester. [141] This pattern is consistent with a demand for mineral late in pregnancy and the resulting need to resorb from the maternal skeleton. Most dual x-ray absorptiometry (DXA) studies have only been done before and after pregnancy to avoid fetal radiation exposure, thus any information on bone mineral content during pregnancy is limited. Generally these small studies show 0 % to 5 % decreases in lumbar spine BMC from 1-8 months before pregnancy to 1-6 wks after delivery. [142-144] One large study found BMD to be reduced by 1.8 % at the lumbar spine, 3.2 % at the total hip, and 2.4 % at the whole body from baseline to 15 days postpartum. [145] It is unclear whether these small changes are due to pregnancy or bone loss from the onset of breastfeeding. Another study measured DXA of the radius during pregnancy, and found no change across the three trimesters. [146] Overall the results suggest that BMC declines minimally or has no change as a result of upregulated bone resorption. Bone loss may be more likely to occur in women who do not meet the calcium absorption requirements for themselves and their fetus.

Animal models may be better suited than humans to estimating the changes in BMC during pregnancy as measurements can be assessed throughout gestation. In mice, bone mineral content (BMC) may increase or decrease depending on the genetic background and skeletal site. [134] Outbred mice of the Black Swiss strain have a progressive 10-20 % increase in whole body BMC, no change in lumbar spine, and a 10-15 % gain in hind limb. [140,147,148] C57BL/6 mice show a 5 % decrease in whole body, a 15 % decline in lumbar spine, and a 10-20 % gain in the hind

limb BMC. [149] Conversely, CD1 mice show no significant changes in BMC at any region. [150] In rats, histomorphometric parameters of both bone formation and bone resorption are increased, however, bone mass remains constant. [134] This assessment is complicated by only comparing pre-pregnancy to end of pregnancy, such that increases in bone mass early in pregnancy followed by bone resorption later on would not be measured.

Pregnancy also leads to physiological changes in kidney function. Both human and animal data show an increase in creatinine clearance and glomular filtration rate (GFR). [134] From wk 12 of human gestation, the 24-hr urine calcium excretion is increased, largely due to increased intestinal absorption of calcium. [134] When participants are fasted however, these values remain normal or low.

It is unclear how big of a role calcitriol plays during pregnancy. Supporting a major role for the hormone is its function in stimulating intestinal calcium absorption and deposition of mineral to the skeleton through increasing calcium channels TRPV5/6, transport molecule calbindin$_{9k}$, Ca^{2+}-ATPase and the sodium-calcium exchanger. [56] Concentrations of calcitriol increase two to three-fold in human pregnancy and two to seven-fold in rodent pregnancy. However, human and animal data are also compatible with calcitriol not being the main stimulator during pregnancy. Previous human studies have indicated that intestinal calcium absorption increases in the first trimester while calcitriol does not markedly increase until the third trimester. [141] This could suggest that calcitriol is not required for early upregulation of intestinal calcium absorption, but more for the peak demand later in pregnancy. Studies in severely vitamin D-deficient rats[27,120,151,152], *Cyp27b1* null pigs[129,153], and *VDR* null mice[147,154] also suggest that calcitriol is

not required to invoke the adaptations of pregnancy or that other hormones may be able to compensate when calcitriol is absent. Vitamin D deficient rats demonstrate an increase in calcium transport in the intestine, and mice lacking the vitamin D receptor also demonstrate increased duodenal calcium absorption. [120,147,155] Severely vitamin D deficient rats have shown both increases and decreases in bone mineralization during pregnancy. [152,156] *VDR* null mice undergo increases of 55 % in bone mineral content (BMC) as measured by DXA, in addition to having a marked reduction in secondary hyperparathyroidism, normalized serum calcium, and increased renal calcium excretion. [147] Another study of *VDR* nulls demonstrate increased serum calcium, an increase in trabecular BMD of the femur, increased femoral ash weight, and reduced osteoid and osteoclast paramaters as assessed by histomorphometry. [157] It was noted that both of the VDR studies used a calcium-enriched diet at the time of mating to restore fertility. Although this may have partially confounded the results to increase bone mass and modulate the improvements to mineral homeostasis, the enriched diet was found to not completely account for the full increase in BMC during pregnancy. [147] Collectively this suggests that vitamin D, calcitriol, or its receptor is not required for normal intestinal and skeletal adaptations of reproduction. In its absence, additional factors such as prolactin, placental lactatogen, estradiol, or calcitonin may be partially compensating. [158-165]

1.3 Adaptations during lactation and the role of calcitriol

Lactation is similarly characterized by significant calcium demand and numerous adaptations to the normal adult state. During nine months of breastfeeding, 30 g of calcium is transferred from the mother to the neonate. [166] Per day, the average calcium loss into milk is 200-210 mg. [167] In

rodents, maternal milk output increases proportionally with number of pups in the litter and their weight.[56]

Calcium, phosphorus, and the calciotropic hormones adapt to the increased calcium demands of the lactating mother (Table 2). In humans, serum calcium is unchanged or slightly increased. [168] Similarly, ionized calcium levels are normal or somewhat increased. Measurements of serum phosphate indicate that levels are increased, due to increased renal phosphate absorption and skeletal resorption of the mineral. [141] Parathyroid hormone levels are decreased by 50 % during breastfeeding, as measured by intact PTH assays. This level rises to normal by weaning. Calcitriol levels of lactating women decrease from pregnancy to normal levels. PTHrP levels are also significantly increased during this time, with expression upregulated in lactating mammary tissue. [141] Lastly, estradiol levels decrease as a result of delivering the placenta and from high prolactin levels suppressing ovarian function. [56] Despite similarities to human lactation, there are several key differences within the mineral physiology of rodents during this time. In rats, the levels of both serum calcium and ionized calcium are largely variable, with animals measured to be hypocalcemic, normocalcemic, and hypercalcemic, due to differences in strains, litter sizes, and diet. [56] Specifically, rodents will become hypocalcemic with larger litter sizes or reduced calcium content of the diet. Measurements of serum phosphorus are also variable in both rats and mice. Another major difference is in the levels of calcitriol, where the 2-fold increase seen in pregnancy is also observed during lactation. [169] Estradiol is likely suppressed in rodents but this is unclear due to the sensitivity of available assays.

Table 2: Changes in serum concentrations of humans and rodents during lactation compared to the non-pregnant, non-lactating mature female

	Human [56]	**Rat** [56]	**Mouse** [56]
Serum calcium	Normal	Variable	Normal
Ionized calcium	Normal to slightly increased	Low	Normal
Phosphorus	Increased	Variable	Variable
PTH	Low	Increased	Low to normal
Calcitriol	Normal	Increased	Increased
PTHrP	Increased	Increased	Increased
Estradiol	Low	Low?	Low?

In contrast to the main adaptation of increased intestinal calcium absorption during pregnancy, intestinal calcium absorption is normal during lactation in women as compared to the non-pregnant, non-lactating mature female. Calcium demand is largely met by an increase in bone resorption with moderate contribution from renal conservation of calcium. [141] Rodents however have a greater calcium demand due to their larger litter sizes and shorter duration of time lactating (21 days). So in addition to the skeletal and renal contributions, they also maintain an increased rate of intestinal calcium absorption, similar to what was achieved during pregnancy.

Mobilization of skeletal calcium is a result of increased trabecular bone resorption by osteoclasts and mineral resorption in both trabecular and cortical bone by osteolytic osteolysis. [56] As a result, there are drastic changes to skeletal microarchitecture and strength. Histomorphometric studies reveal increases in osteoblast number, osteoblast surface, and osteoid thickness, and even greater increases in osteoclast number and resorptive surfaces. [56] This leads to reduced mineralized tissue volumes, thinning of trabeculae, and decreased trabecular number. Furthermore, osteocyte lacunae are enlarged, leading to objective loss of bone. The strength, stiffness, and toughness of vertebrae, tibia, and femora are all adversely affected by these changes to the skeleton. Hormonal

regulators of these adaptations include upregulation of PTHrP and low estradiol levels. In humans, losses of 3-10 % have been measured at trabecular sites, with smaller losses at cortical sites from the 2-6 months of lactation. [170] Despite any supplemental calcium intake, this negative calcium balance remains while breastfeeding. [171] Multiple randomized trials comparing 1,800 mg to 800 mg calcium intake, 2,400 mg to 1,200 mg, 1,400 mg to <300 mg and a 1 g supplement to controls demonstrated that BMC declined to the same extent in both groups. [172-175] Relative losses in 3 months or 6 months of lactation were also not affected by maternal calcium intake. [176,177] It is noted however that prolonging the period of breastfeeding results in even greater bone loss. [178] These results all show that in humans, increasing calcium intake above the recommended daily amount does not reduce the amount of BMC lost during lactation. Conversely, bone loss in rodents is suppressible by a high calcium diet. In the C57BL/6 strain of mice, losses of 25% or more from the lumbar spine during the three wks of lactation are normal. [149] The effect of diet is shown when lactating mice fed a 2 % calcium diet had blunted BMC losses in comparison to mice fed a 1 % calcium diet. [140] However, bone loss was still not prevented. Taken together with the human data, these results confirm that bone loss during lactation is hormonally dependent rather than simply a function of diet.

As mentioned, rodents maintain an increased rate of intestinal calcium absorption during lactation that is accompanied by an increase in calcitriol, while both levels fall in humans. Rats, and likely mice, upregulate duodenal expression of calbindin$_{9k}$, Ca-ATPase, and VDR to moderate this adaptation. [56] The kidneys also participate in the adaptations to provide milk to the neonate in both humans and rodents, as this period is characterized by a reduction in GFR. Urine calcium excretion has been measured as low as 50 mg over a time period of 24 hrs. [141] The

reduction in urine calcium excretion, accompanied by increased urine phosphorus excretion, is consistent with the actions of PTHrP on kidney tubules and thereby implies that PTHrP is likely responsible for these changes in urine mineral handling. [56]

Prior animal models provide insight into the role of calcitriol during lactation. It is again unclear how big of a role calcitriol plays, as the hormone acts both directly and indirectly to promote deposition of skeletal mineral, however bone loss is similar despite absence of VDR or vitamin D deficiency. *VDR* null mice raised on a standard 1 % calcium diet and switched to a 2 % calcium diet prior to mating resorbed their skeleton normally during lactation as compared to their WT sisters. [147] Additionally, serum calcium and phosphorus remained normal throughout this time. The same *VDR* null mice were also studied on a 2 % calcium diet since the time of weaning so as to prevent acquisition of the rachitic phenotype. [179] During lactation, the knockout mice resorbed a similar amount of bone as measured by microCT and ash weight, and lost a similar amount of femoral bone strength. This suggests that the resorption of bone occurs independently of the vitamin D receptor. Vitamin D-deficient mice from the CD1 background also had normal serum calcium and ionized calcium during lactation, in addition to normal nutritional and calcium content in their milk. [123] Only moderate differences in these mice were noted, with lower serum phosphorus and 15 % lower lactose content in their milk. Vitamin D-deficient mice in the NIH Swiss background had opposing results, with mild hypocalcemia but normal phosphorus compared to vitamin D-replete mice. [124] Studies in vitamin D-deficient rats are slightly different, as they were more likely to become hypocalcemic during lactation regardless of the calcium content of their diet. [152,155,156,180-182] Although they resorbed a normal amount of calcium from their femora, the vitamin D-deficient rats had widened osteoid seams,

and increased osteoblast surface, osteoclast number, and resorptive surface. [181] Overall, these results indicate that calcitriol, the VDR or vitamin D is not required for bone resorption during lactation.

The importance of calcitriol during lactation is further challenged by studies showing that intestinal calcium absorption is unaltered by vitamin D deficiency. In one study, vitamin D deficient rats on a 0.47 % calcium diet showed a doubling of intestinal calcium absorption despite having lower serum calcium and phosphorus, having no difference compared to WT. [180] Although it is clear that calcitriol is able to stimulate intestinal absorption of calcium, it is possible that other hormones are able to compensate in its absence, just as in pregnancy.

1.4 Adaptations during post-weaning recovery and the role of calcitriol

The time period following lactation is remarkable because it is the only time during adult life where the skeleton undergoes complete reversal following bone loss. Bone turnover continues to be increased during this time, but is uncoupled to favor bone formation. Specifically, bone formation is increasing out of proportion to any change in bone resorption. There are drastic changes to the bone microenvironment, due to a reduction in osteoclast number and activity prompted by apoptosis of osteoclasts, a substantial decrease in RANKL and RANK expression, and a marked increase in osteoblast precursors, osteoblast number, and osteoid surface. [56] Osteolytic osteolysis also ceases, with osteocytes now expressing genes of the mesenchymal lineage and functioning to restore the mineralized matrix. [56] Calciotropic hormones are also altered, with decreases measured in PTH and calcitriol and an increase in calcitonin. As a result of this combined activity, the human skeleton regains lost mineralization in 3 to 12 months post-

weaning. [142,170,172,173,176,178,183-185] Several studies have found that not only is the reduction in bone loss reversed after lactation has ceased, but there may actually be a protective effect on peak bone mass, bone mineral content, and fracture risk. [186-199] In rodents, the recovery of skeletal mineral occurs within 2 to 4 wks.[140,147-150,152]

There is extensive evidence supporting this post-weaning recovery in both humans and rodents. Human data are largely based on DXA, and show a complete reversal of the lactational losses in bone density, with sooner recovery for shorter intervals of breastfeeding. [56] Femoral structural analysis of data from DXA scans shows that decreases in cross-sectional area, cortical thickness, and bending strength during lactation were all reversed by 6 months post-weaning. [200] In mice, serial studies using DXA, and cross-sectional studies using skeletal ash weight indicate a complete recovery of skeletal mineral content. [56] Histomorphometric analysis demonstrates recovery of bone volumes, trabecular thickness, and mineral content within two to four wks post-weaning in the mouse and after four to eight wks in the rat. [56] MicroCT has also been used to assess this recovery, revealing full vertebral normalization of bone volumes, trabecular number and thickness, mineralization, stiffness, and reversion from a rod-like back to a plate-like structure. [150,201] However, persistent alterations have been found in the microarchitecture of tibiae and femora using microCT as compared to virgin mice or pre-pregnancy values. [156,201] Despite failure of these sites to fully recover the trabecular microarchitecture, biomechanical testing shows that strength and stiffness still return to normal. [149]

The factors underlying this recovery are largely unknown. Studies investigating intestinal calcium absorption have observed relative increases that could facilitate restoration of skeletal

mineralization, however the levels are still much lower than those achieved during pregnancy, and calcitriol is downregulated. [202] Severely vitamin D-deficient rats improve bone mass and architecture after lactation, and *VDR* null mice also increase BMC to 50 % higher than baseline. [147,156] PTH also rises to normal following lactation, suggesting it could be involved with restoration of mineral to the skeleton, however *Pth*-null mice fully restore their skeleton above baseline BMC post-weaning. [140] *Ctcgrp* null mice show the most remarkable change during lactation with losses of 55 % from lumbar spine BMC, but fully regain it within 18 days post-weaning. [148,203] Mice with osteoblast-specific ablation of *Pthrp* also lactate normally and fully recover BMC after weaning. Together these studies suggest that vitamin D, VDR, PTH, calcitonin, and osteoblast-derived PTHrP are not required to achieve bone formation and post-weaning recovery in the maternal skeleton.

One study found that a diet of sufficient calcium is required for normal skeletal recovery, as this was prevented in rats with a diet low in calcium but normalized three wks later once placed a normal diet. [204] Another possible factor could be increased weight bearing post-weaning. Rats forced to stand upright to reach their food during lactation and 6 wks following demonstrated greater increments in femoral and tibial bone mass. [56] Upregulation of estradiol to normal levels may also contribute to skeletal recovery, as demonstrated in humans by an earlier resumption of menses in coordination with an earlier increase in BMC following lactation. [142] However, osteoblast function is not strongly reliant on estradiol, suggesting that it is not responsible for the rapid recovery of bone post-weaning. Collectively, these factors may help contribute to greater BMD achievements after weaning, but cannot account for the full recovery in mineralization.

Understanding what regulates this phenomenon may enable discovery of new treatments for bone loss. The bone recovery seen post-weaning is so unusual because of its rapidity and relative completeness, in contrast to other causes of bone loss that are followed by slow and partial, if any, recovery. For example, astronauts in space experience bone loss, however have only slow and partial recovery upon return to earth. After prolonged stays in space, BMD losses of 2.5-10.6 % in the lumbar vertebrae and 3-10 % in the femur have been documented. [205] Although follow-up studies from return to earth are limited, one study measured losses of 4.5 % trabecular bone after 6 months exposure to microgravity, and still a 2.55 % decrease present after 6 months recovery time. [206] Similarly, bed rest also induces significant bone loss. With 90 days of bed rest, bone loss of 6 % in the distal tibia has been reported, along with reduction in cortical bone thickness and density of greater than 2 %.[207] Moreover, the use of GnRH analog treatment for six months causes estrogen-deficiency bone loss in women of reproductive age that is not recovered more than a year after estrogen status has returned to normal. [56] Numerous other conditions induce active resorption of bone, such as osteoporosis or Paget's disease. Although an extensive amount of research has been conducted, the available treatments are still less effective than the full recovery seen following weaning.

1.5 Possible non-VDR mediated actions of calcitriol

The classical role of calcitriol in regulating mineral metabolism has been explained in depth. It binds stereospecifically to the vitamin D receptor to activate or repress target genes. In the adult, calcitriol has an antirachitic effect on bone, increases the efficiency of intestinal calcium absorption with increased demand for calcium as a result of growth, and enhances calcium transport in the kidney. As previously described, the regulatory systems invoked during

pregnancy, lactation, and post-weaning recovery may only partially depend on vitamin D or VDR, as they persist despite knockout animal models. Still, the question remains whether calcitriol is required.

Although the only known receptor for calcitriol is the vitamin D receptor, non-VDR mediated effects of calcitriol have been extensively suggested throughout the literature. One example of these putative non-genomic actions of calcitriol is in post-weaning skeletal recovery of *Vdr* null mice. Although they undergo substantial increases in BMC and intestinal calcium absorption during pregnancy, resorb the skeleton normally during lactation and recover fully post-weaning, the knockout mice were found to have high circulating levels of calcitriol in comparison to their WT sisters. [128,147] Therefore, it remains conceivable that calcitriol is able to act through alternative signalling mechanisms to mediate the normal skeletal and intestinal adaptations of reproduction.

Further research is provided by Lai and colleagues, who comment on the range of biological effects calcitriol has on the body. [208] They suggest that such varied effects as transcaltachia (the rapid stimulation of intestinal calcium absorption), phosphoinositide turnover, and the induction of protein kinase C, phospholipase A_2, guanylate cyclase and alkaline phosphatase cannot be explained by VDR-mediated gene expression. A possible specific calcitriol membrane coupled receptor explaining transcaltachia was isolated from the intestinal membranes of chicks, and named the calcitriol-membrane-associated rapid response to sterioid. [209] This receptor was found to mediate rapid responses to calcitriol, including phospholipase A_2 activation, membrane fluidity, and phospholipase C activation, which leads to the activation of protein kinase C and

intracellular calcium release (Figure 6). [210] These responses occurred either within seconds, or 10 to 60 minutes. Comparatively, hours are required for the genomic actions of calcitriol to occur. [211] However, the existence of this specific receptor is still up for debate, as evidence suggests that calcitriol can bind to an alternative ligand pocket on the VDR to mediate these rapid non-genomic responses. [212]

Another hypothetical pathway for the alternative signalling mechanisms is through VDR participation in signal transduction, rather than the classical route of transcriptional regulation (Figure 6). Buitrago and colleagues indicated this non-transcriptional participation by showing that calcitriol stimulated rapid survival/proliferation responses in skeletal muscle cells. [212] Specifically, calcitriol activated MAPKs and phosphorylated Src. This effect was abolished when VDR expression was reduced, suggesting its interaction in the pathway. [212]

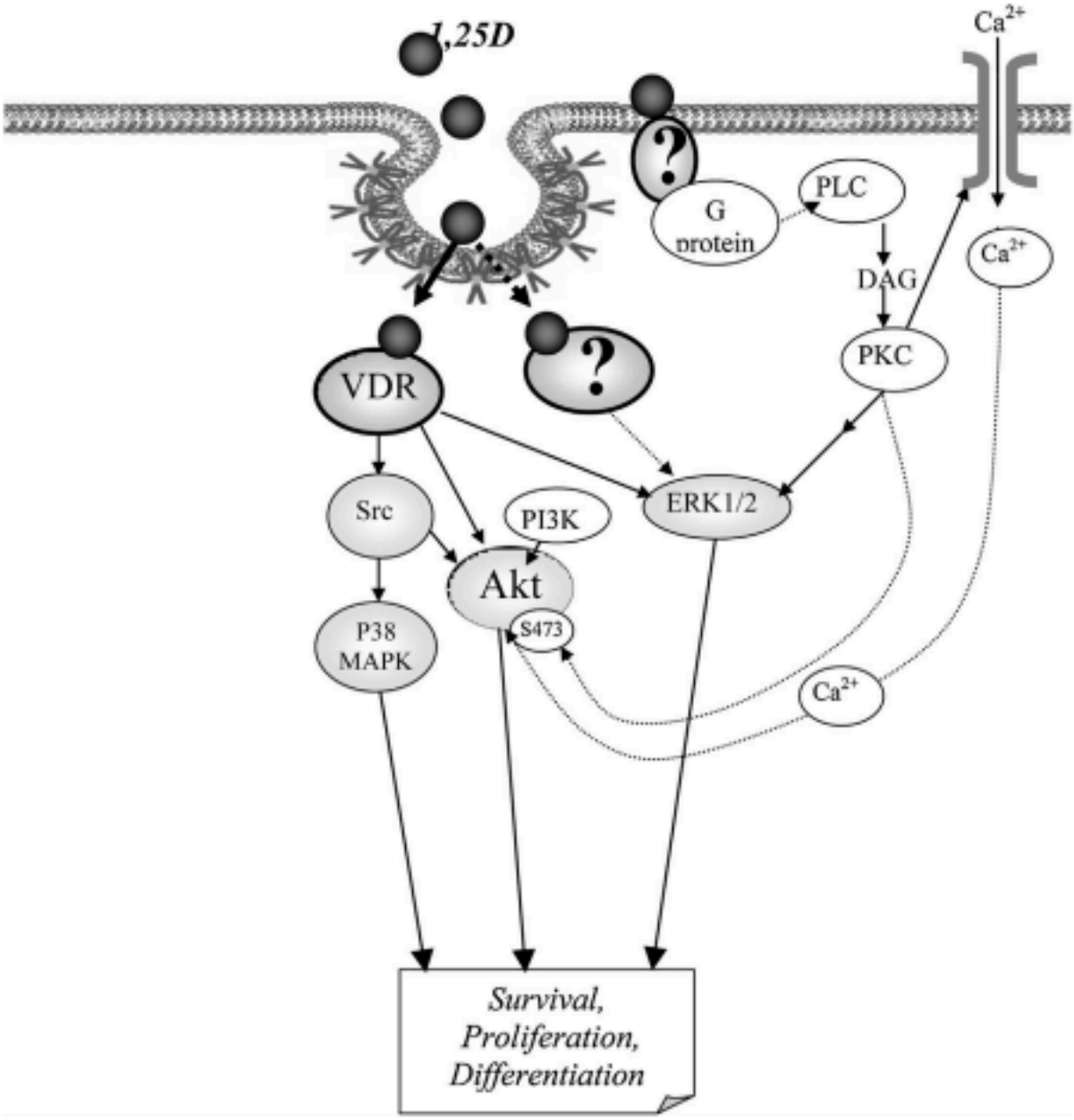


Figure 6: Hypothetical alternative signalling mechanisms of calcitriol in skeletal muscle cells. [212] Calcitriol may act through an unknown membrane receptor to mediate rapid responses via PLC/PKC, which could contribute to intracellular calcium release. Calcitriol may also act through the VDR in an alternative signalling pathway via phosphorylation of Src and MAPK activation to induce cellular survival, proliferation, and differentiation. *Copyright, Elsevier (Appendix D).*

1.6 Rationale, hypothesis, and objectives

Calcitriol has numerous essential roles to regulate bone and mineral metabolism in the adult. However, vitamin D and VDR are not required for the intestinal and skeletal adaptations to pregnancy, lactation, and post-weaning. It is unknown whether calcitriol can act independently of the vitamin D receptor to regulate these changes during reproductive cycles.

Our hypothesis is that calcitriol is required for upregulation of intestinal calcium absorption during pregnancy and post-weaning, and to enable normal recovery of bone mass post-weaning, such that the high levels seen in the *Vdr* null mice are acting through alternative signalling mechanisms to have these effects. If correct, then loss of calcitriol would impair intestinal calcium absorption and bone metabolism during the reproductive cycle and would lead to effects that loss of VDR does not. This would also promote further study to identify the other mechanistic pathways through which calcitriol acts. If the data lead to rejection of my hypothesis, this would confirm that calcitriol is not required for regulating intestinal and bone metabolism during reproduction, and would underscore the need to identify factors that do regulate intestinal calcium absorption and bone metabolism during pregnancy, lactation, and post-weaning.

We tested our hypothesis by examining a mouse model with the *Cyp27b1* gene ablated. These knockout mice lack the enzyme that synthesizes calcitriol. Studies were performed comparing sister *Cyp27b1* null and WT pairs raised on a rescue diet from the time of weaning. Rigorously comparing WT and *Cyp27b1* null mice that were first degree relatives of each other and mated to the same males allowed for stronger comparisons where differences could be attributable to

genotype rather than genetic drift from breeding. The research aimed to investigate (1) bone mineral content by DXA, (2) blood and urine biochemistry, (3) skeletal microarchitecture of femurs and lumbar vertebrae by μCT, (4) cortical bone strength by 3-point bend test, and (5) intestinal calcium absorption at pre-pregnancy baseline, end pregnancy, end lactation, days 7, 14, 21, and 28 of post-weaning recovery, and virgin age-matched controls to the recovery day 28 samples. Through contrasting the phenotypes and looking for evidence of compensation in the absence of calcitriol, it should become apparent whether or not calcitriol contributes importantly to the regulation of skeletal and mineral homeostasis during the reproductive cycle.

II. MATERIALS AND METHODS

2.1 Mouse model

Cyp27b1 null mice were provided by Dr. René St-Arnaud at McGill University in Montreal, Quebec. The Cyp27b1 enzyme was inactivated by Cre-mediated excision of the gene in embryonic stem cells. Homologous recombination with the targeted-1αOH locus and wild-type locus between two loxP sites flanking exon 8 deleted the heme-binding domain. Effectively, this created a null allele.[30] Three genotypes result from mating heterozygous males and females together: wild type with both normal *Cyp27b1* alleles (*Cyp27b1*$^{+/+}$), heterozygous (HET or *Cyp27b1*$^{+/-}$) with one normal *Cyp27b1* allele and one *Cyp27b1* allele ablated, and null with both *Cyp27b1* alleles ablated (*Cyp27b1*$^{-/-}$).

2.2 Genotyping

2.2.1 Animal identification

At 21 days of age, experimental mice were weaned from their mothers and separated into cages based on sex, with a maximum of 4 mice per cage. Mice were briefly anesthetized under isoflurane, and their right ear was crimped with a tag denoting mouse strain and number.

2.2.2 Tail sample collection

While still under the anesthetic from ear tagging, a 0.5 cm section of tail was cut with a sterile razor blade and placed into a 1.5 ml eppendorf tube labeled with the mouse identification. To digest the tail section, 300 µl of cell lysis solution and 1.5 µl of proteinase K was added to each tube, and samples were placed in the incubator (Fisher Scientific, Burlington, ON) at 55ºC

overnight. Bleeding was stopped by applying a small amount of pressure to the tail. Mice recovered from anesthesia in a separate clean cage.

2.2.3 DNA extraction

The following day, DNA extraction was conducted as per the Qiagen Puregene® Core Kit A (Qiagen, Toronto, ON). Digested tail samples were removed from the incubator and 100 µl of protein precipitation solution was added to each tube. Samples were mixing using a vortex for 20 s on high speed, and then centrifuged for 3 mins at 16,000 x g. Following precipitation of the proteins, 300 µl of isopropanol was pipetted into a clean 1.5 ml microcentrifuge tube, and the supernatant from the previous step was decanted. Tubes were inverted 50 times to precipitate the DNA, and then centrifuged for 1 min at 16,000 x g to attain a pellet. After centrifugation, the supernatant was discarded and 300 µl of 70 % ethanol was added. To wash the DNA pellet, the tubes were inverted several times and centrifuged for 1 min at 16,000 x g. The supernatant was again decanted and the tube was drained on a piece of absorbent paper, and excess ethanol was removed with a pipette. The ethanol was further allowed to evaporate by leaving the tubes open for 5 mins. Following air dry, 100 µl of DNA hydration solution was added, and samples were vortexed for 5 s. Lastly, samples were incubated at 65 ºC for a time period of 1 hr to solubilize the DNA.

2.2.4 Polymerase chain reaction (PCR)

PCR was carried out on the DNA collected from mice tails using a three-primer system. The forward primer detected both the WT and null allele, while primers 2 and 3 separately detected

the *Cyp27b1* null allele and *Cyp27b1* WT allele, respectively. Specifically, the primer sequences were as follows:

Cyp27-1 (forward): 5' - AAT TCC CGT GTC CCA GAC AGA GAC ATC C – 3'

Cyp27-2 (reverse): 5'- GGT CAT GGG CTT GAT AGG AAG AGC ACC – 3'

Cyp27-3 (reverse): 5'- GGG TGG GGA ATG TGA AGA AGA GGA TCT G – 3'

As a result of primer 2 annealing to the DNA template downstream to primer 3, the *Cyp27b1* null mice were identified by a larger transcript than the WT mice. Specifically, the product of two primers for the null *Cyp27b1* allele was 350 bp, whereas a 250 bp product resulted from two primers for the WT *Cyp27b1* allele (Figure 7).

The PCR cocktail was made with 10x PCR reaction buffer, deoxyribose nucleotide tri-phosphates (dNTPs – dATP, dTTP, dCTP, dGTP), primers (*Cyp27-1, Cyp27-2, Cyp27-3*), $MgCl_2$, Taq DNA polymerase, and distilled water (Invitrogen, Burlington, ON). A 19.5 µl aliquot of this cocktail was pipetted into each labeled reaction tube with 2 µl of DNA. Tubes were then placed in the PCR machine.

The entire PCR program consisted of 7 steps to amplify the respective DNA sequences, which is described as follows: Step 1: 94°C for 5 mins to initialize the reaction, Step 2: 94°C for 30 s to denature the DNA, Step 3: 58°C for 30 s to anneal the primers, Step 4: 72°C for 30 s to synthesize and elongate a complementary strand, Step 5: return to step 2 and repeat for 35 cycles for continued amplification, Step 6: 72°C for 10 mins to elongate any remaining strands and Step 7: 4°C for storage of the reaction.

2.2.5 Gel electrophoresis

Gel electrophoresis was performed to separate and analyze the DNA fragments from the PCR program using a 1.2 % agarose gel. Specifically, the gel was made with 100 ml 10X TAE (0.12 M EDTA, 0.40 M Tris, 11.5 % Glacial Acetic Acid, pH 8.0), 900 ml deionized water, 1.2 g agarose (Invitrogen), and 10 µl SYBR Safe DNA Gel Stain (Invitrogen). The TAE was diluted to a 1X buffer solution, and the agarose was dissolved in 100 ml by microwaving at two separate intervals of 1 min. The gel stain was added to the solution and swirled. The solution was then poured into the casting tray and left for 30 mins to polymerize. During this time, 4 µl of 6x loading dye (10 mM Tris, 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol, 60 mM of 0.5 M EDTA, 1.5 M dH_2O) was added to each of the 21.5 µl PCR products.

Following solidification of the gel, 800 ml of running buffer (720 ml deionized water and 80 ml 10X TAE) was added to the gel electrophoresis chamber and the gel inserts were carefully removed. 15 µl of PCR product and the loading dye mix was injected into each well of the gel. After loading, the samples were run at 200 V for 20 mins.

The bands were visualized under UV light using the Kodak Gel Doc System. The results were imaged using Bio-Rad Image Lab version 5.1. The presence of a single band at 250 bp indicated the mouse was WT, a single band at 350 bp indicated the mouse had two copies of the mutated allele and was therefore *Cyp27b1* null, and the presence of both bands indicated the mouse was heterozygous for the ablated allele ($Cyp27b1^{+/-}$) (Figure 8).

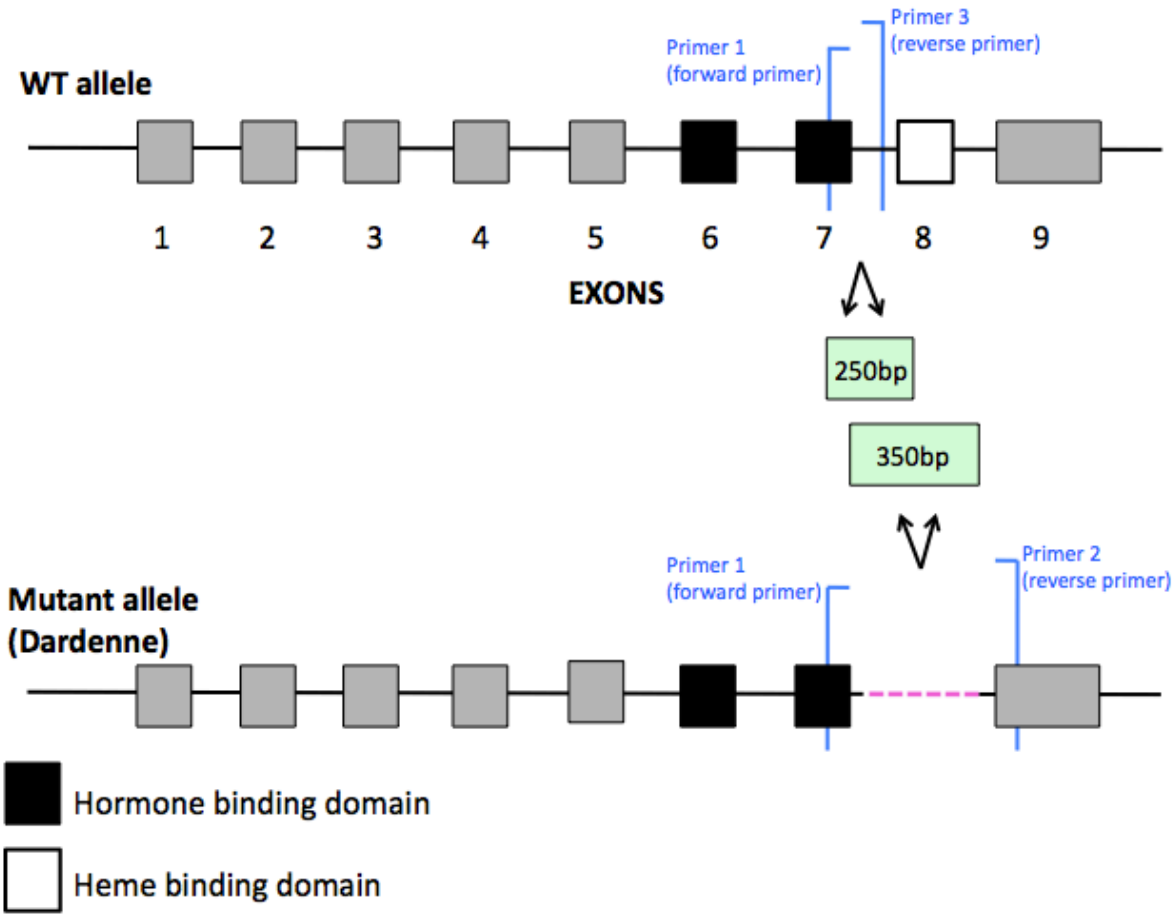


Figure 7: Schematic of the alignment of primers used to distinguish the retained and deleted portions of the *Cyp27b1* gene. HET, WT, and *Cyp27b1* null mice were identified by PCR using a 3-primer system. Primer 1 detected both the WT and null allele, primer 3 detected the WT allele, and primer 2 detected the null allele.

Figure 8: Genotyping by PCR and gel electrophoresis. Sample PCR products run on a 1.2 % agarose gel. The presence of both 250 bp and 350 bp bands represents a heterozygous mouse, the presence of a single 250 bp band indicates a WT mouse, and the presence of a single 350 bp band indicates a null mouse.

2.3 Animals

2.3.1 Rescue diet and water

Both WT and *Cyp27b1* null mice were placed on a rescue diet (TekLad TD.94112; Harlan Teklad, Madison, WI) at weaning to allow for normal conception rates and a normal phenotype in the nulls. [33,38,130] This consisted of 2 % calcium, 1.25 % phosphorus, and 20 % lactose. Tap water was triple filtered using a 0.2, 1, and 5 μm component system and was available *ad libitum*. The filtration system removes dirt, sediment, sand, rust, and scale particles. Dissolved calcium was likely not filtered out using this method.

2.3.2 Scheduled mating and litter sizes

Light and dark cycle was used for housing mice, with light from 08:00 to 20:00. Mice utilized for generating the colony were not scanned for BMC, and therefore could be mated as early as 8 wks of age. HET by HET matings were established to generate a colony of all genotypes according to Mendelian genetics. Virgin first-degree relative pairs of WT and *Cpy27b1* null females were selected for study at 10 wks of age, at which time bone mass has reached a relatively stable plateau. Baseline measurements on experimental mice were conducted at this time. Consequently, females were placed with heterozygous breeding males at approximately 16:00 hrs on Thursdays. After mating overnight, mice were manually checked for presence of a vaginal mucus plug at approximately 9:00 hrs the morning after mating. The presence of a plug indicated that the female had mated and may have conceived; if so this was gestational day 0.5. However, a plug was not always evident because it was too small or had already fallen out. Mice were placed back in original group housing cages and mated again the following Thursday until pregnancy was visually confirmed. After visual confirmation mice were placed into single

housing cages. Normal gestation for these mice was 18.5 days. Lactation lasted 21 days, and was ensured by weaning the pups from the mother on this day. Litter sizes were counted at the time of weaning. Mice used as virgin controls were left unmated and harvested at the same time as day 28 of recovery to coincide with mated mice.

2.4 Reproductive cycles

Full reproductive cycles lasted a minimum of 82 days (Figure 9). Pre-pregnancy was defined as the 14 days prior to being placed with a breeder male and after the female had reached 10 wks of age. Pregnancy spanned 18.5 days and lactation occurred over 21 days. Weaning was defined as the day in which the suckling pups were manually removed from the mother, and post-weaning spanned 28 days, even if BMC had recovered prior to that point. Recovery was analyzed at 4 stages for experimental purposes: 7 days, 14 days, 21 days, and 28 days.

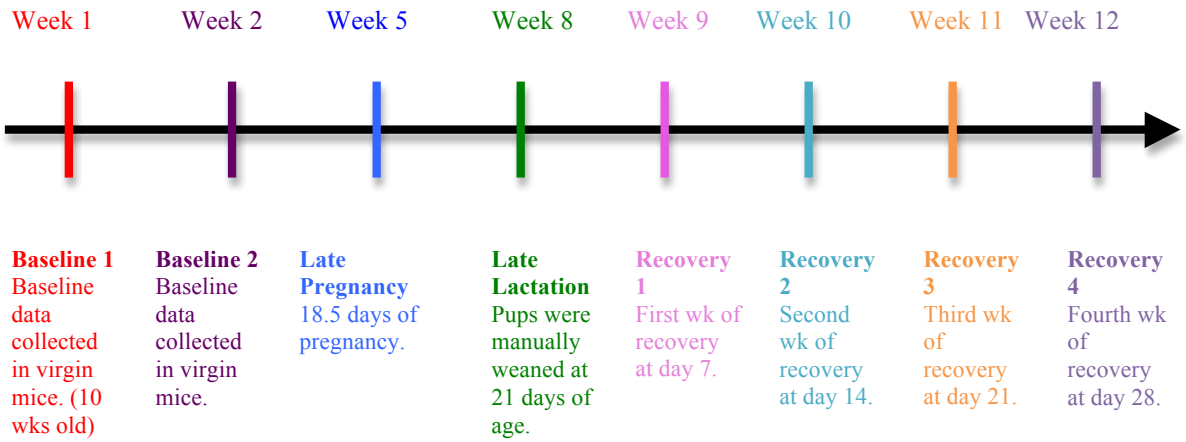


Figure 9: Schematic time line of data collection throughout the reproductive cycle in mice. Samples of serum, urine, and bone were collected in addition to DXA scans at various time points throughout the reproductive cycle.

2.5 Data collection and storage

2.5.1 Litter sizes

Litter numbers were counted at the time of weaning (21 days post-partum) for both WT and *Cyp27b1* null mice. This number was used rather than the number of pups initially birthed by the mother, since mice often cull their litters over the first several days. If the WT or null mothers however were nursing different numbers of pups, I was prepared to cull the litters after birth.

2.5.2 Urine

Prior to every BMC scan, each mouse was placed into a separate clean and empty cage in order to void. Urine was collected with a pipette into 0.6 ml microcentrifuge tubes and stored at -20 ºC until time of assay. Maternal urine samples were collected at baseline, day 18.5 of gestation, day 21 of lactation, and weekly for the 4 wks of recovery.

2.5.3 Blood

Blood samples of serum were taken by bleeding the tail vein for longitudinal sampling and by cardiac puncture for terminal samples. Tail vein blood was taken after each BMC scan while the mouse was still under anesthetic (single intraperitoneal injection of a 20 uL mixture of 50 mg/ml ketamine and 20 mg/ml xylazine). A small section of tail was cut with a razor blade, and the blood was massaged out and collected into a 0.6 ml microcentrifuge tube. A small amount of pressure was applied to the tail to stop the bleeding. Sample was then centrifuged for approximately 7 mins to separate the serum from packed red cell volume. The serum was pipetted into a newly labeled 0.6 ml microcentrifuge tube and stored in the -20 ºC freezer. Alternately, cardiac puncture was performed prior to euthanasia in order to obtain larger blood

volumes. For this procedure, mice were temporarily anesthetized under isoflurane, and then held firmly in one hand to prevent limb or head movement. Sterile 1 cc syringes were attached to 20 gauge needles and skin was pierced beneath the mouse ribcage at a slight angle to penetrate the heart. Blood from the heart was then drawn into the syringe and emptied into 1.5 ml microcentrifuge tubes. The mother was then killed by cervical dislocation. Samples were subsequently spun on a microcentrifuge for approximately 7 mins at 16000 x g to separate out serum from clotted blood. Serum was then pipetted into a newly labeled 1.5 mL microcentrifuge tube and stored in the -20ºC freezer until time of assay.

Plasma was collected from mice used in the intestinal calcium absorption experiments. Blood samples were taken by cardiac puncture as described above. Following cervical dislocation, blood from the syringe was emptied into heparinized tubes. Samples were kept in a -20ºC freezer designated for radioactive samples until analysis.

2.5.4 Bone

Femur, tibia, fibula, and lumbar spine were harvested at specific time points of the mouse reproductive cycle (baseline, lactation, recovery 1, and recovery 4) in addition to virgin age-matched controls to the recovery 4 mice (Figure 9). Mice were initially anesthetized with isoflurane and then killed by cervical dislocation. Hair and flesh were removed using forceps and scissors. A coronal incision was cut across the ventral torso to expose the vertebral column. The lumbar vertebrae were isolated using a complete transverse incision at the pelvic line and cervical line, including extra thoracic vertebrae to avoid errors. The heads of the femurs were then carefully separated from the ileum and pubis through the application of slight pressure to

the joint with scissors. Tibia and fibula were isolated from the femur using scissors. Storage methods differed depending on the intended future analysis, which will be explained in each corresponding section.

2.6 Bone mineral content

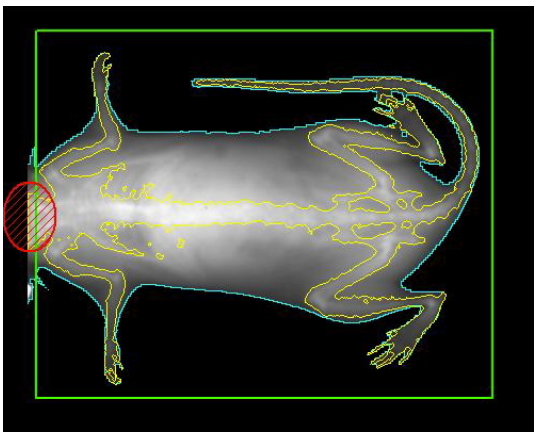
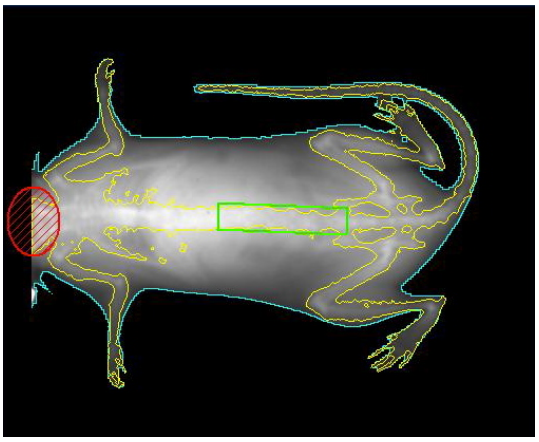
BMC was measured using the PIXImus 2 DXA. To ensure standardization of measurements, a quality control scan was performed with a "phantom" mouse (BMD=0.0630 g/cm, % fat =11.9 %) to calibrate the machine. As stated by the manufacturer, the precision of the DXA machine is 3.31 % for BMC (GE Lunar PIXImus, Madison, WI). Excess calcium from the diet found in the gut could artificially increase the apparent whole body and lumbar spine BMC reading as DXA measures calcium regardless of whether it is found in bone or outside the skeleton. To minimize this inaccuracy, all mice were fasted 18 hrs prior to DXA scans.

Mice were prepared for the scans with anesthesia to minimize movement of the head and limbs. Initial anesthesia was induced using isoflurane poured onto paper towel in a small glass chamber. This was followed by an intraperitoneal (IP) injection of a 20 uL mixture of Ketamine (50 mg/ml) and xylazine (20 mg/ml). Once anesthetized, mice were placed on holding trays with a specific orientation to allow for precise BMC measurement and reproducibility. Spines were manually straightened, arms were extended perpendicular to body, paws were placed flat, and the tail was curved around the left side of the torso. For all scans, the head was excluded due to a large amount of variability within the region, in addition to keeping the ear tag from confounding the scan. Thus, only the body and limbs were included in the BMC measurements.

Scans were analyzed for whole body (minus head and neck), lumbar spine, and hind limb BMC measurements in grams (Figure 10). Lunar PIXImus software allowed for the region of interest (ROI) to be adjusted to isolate the lumbar spine, hind limb, and to exclude an area of head or ear tag scanned.

Bone mineral content was measured using the previously mentioned scheme at baseline, late pregnancy, late lactation, and 4 wks of recovery (Figure 9). A minimum of 2 baseline scans were performed on each mouse one week apart to ascertain an average pre-pregnancy value and confirm that BMC had reached a steady plateau. Further baseline scans were performed if there was a BMC difference >5% or if the mouse did not become pregnant after three weeks. BMC values were reported by absolute value, and as relative changes to the initial averaged baseline value prior to mating. To calculate the relative changes for recovery, the fourth recovery BMC measurement was used.

BMC measurements were collected using two different methodologies. In the first, mice were fasted overnight and left in their original cage to be scanned the following day. In the second revised analysis, mice were changed to a new cage in attempts to limit coprophagia, and similarly fasted overnight.

A)  B) 

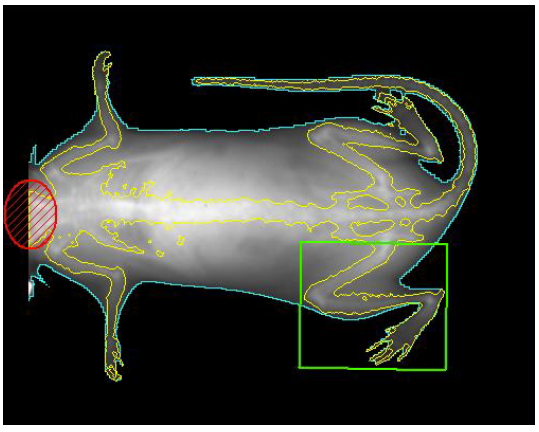
C) 

Figure 10: Representative scans of whole body, lumbar spine, and hind limb BMC measurements. DXA scans were taken at the main reproductive time points. In each scan, the green box identifies the region of interest. A) Representative scan of whole body BMC. The red circle is used to exclude the head and neck, due to a large amount of variability in this region. B) Representative scan of the lumbar spine, as identified by the 6 vertebrae selected. C) Representative scan of the hind limb, including the femur, tibia, and fibula.

2.7 Mineral assessment

2.7.1 Serum and urine total calcium measurement

The test principle of the total calcium assay is that calcium reacts with Arsenazo III in an acid solution. [213] The calcium-Arsenazo complex develops a blue-purple colour with a maximal absorbance at 650 nm. Results read at this wavelength are directly proportional to the total concentration of calcium in the sample. Both serum and urine were measured at the main time points (Figure 9) using samples from the first DXA set. Reportable range of the kit (Sekisui Diagnostics, Charlottetown, PEI) was 0.01 mmol/L to 3.75 mmol/L. For urine, the assay was first performed using a saline dilution of 1:10. Values were read lower than the reportable range, so the kit was repeated using undiluted urine. Serum samples were also measured undiluted. All samples were measured in duplicate if possible and followed kit instructions. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Piscataway, NJ) was used to read the absorbance at 650 nm. Final urine results were corrected for by the concentration of creatinine (mmol Ca/mmol Creatinine). Deionized water was used as a blank.

2.7.2 Serum and urine inorganic phosphate measurement

The test principle of the phosphorus assay is that inorganic phosphorus reacts with ammonium molybdate in the presence of sulfuric acid to produce an unreduced phosphomolybdate complex. [214] The concentration of inorganic phosphorus in each sample is directly proportional to the increase in absorbance at 340 nm. Serum and urine were measured at the main time points (Figure 9) using samples from the first DXA set. Reportable range of the kit (Sekisui Diagnostics, Charlottetown, PEI) was 0.03 mmol/L to 6.46 mmol/L. For urine, the assay was initially performed using a saline dilution of 1:10. Samples measured outside of the reportable

range were then repeated either with a dilution factor of 1 or 100. Serum samples were measured undiluted. All samples were measured in duplicate if possible and followed kit instructions. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech) was used to read the absorbance at 340 nm. Final urine results were corrected for by the concentration of creatinine (mmol P/mmol Creatinine). Deionized water was used as a blank.

2.7.3 Urine creatinine measurement

The test principle of the creatinine-S assay is that creatinine reacts with picrate in an alkaline environment to produce a creatinine-picrate Janovsky complex. [215] The concentration of creatinine in each sample is directly proportional to the rate of increase in absorbance at 510 nm. This assay was performed as a calculation basis for normalizing the values of all other urinary metabolites. Urine samples at the main time points of the reproductive cycle were tested (Figure 9) using samples from the first DXA set. Reportable range of the kit (Sekisui Diagnostics, Charlottetown, PEI) was 4 μmol/L to 1945 to μmol/L. All samples were measured in duplicate using a saline dilution of 1:10, and followed the procedure in the kit. A spectrophotometer (Ultraspec 2000; Pharmacia Biotech) was used to read the absorbance at 510 nm. Deionized water was used as a blank.

2.8 Hormone physiology

2.8.1 Serum parathyroid hormone (PTH)

Mouse serum PTH was measured using a Mouse Intact PTH Enzyme-linked ImmunoSorbent Assay (ELISA) Kit (Immunotopics Inc, San Clement, CA.). The detection limit of the assay was 4 pg/ml. The test principle applies two-site ELISA methodology to determine serum PTH

concentration. [216] Intact PTH contains 84 amino acids. The C-terminal portion is recognized by an anti-mouse PTH antibody anchored to the well surface and is biotinylated for capture. The N-terminal portion is recognized by a horseradish peroxidase (HRP)-labeled anti-mouse antibody, and forms a sandwich complex with the capture antibody. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of PTH in the sample. Serum samples from the first DXA set were measured at baseline, late pregnancy, late lactation and recovery 1. Experiments followed the kit procedure in duplicate. Results were first read at 450 nm in a microplate spectrophotometer (Epoch; Biotek, Winooski, VT) and analyzed using Gen5 software (Version 2.04.11, BioTek Instruments Inc). A large number of the PTH values for the *Cyp27b1* null mice were above the detection limit of the reader. As per the kit protocol, the plate was read again at 620 nm to extend the analytical range to the highest standard, and these values were used for analysis.

2.8.2 Serum calcitriol

Mouse serum calcitriol was measured using a 1,25-Dihydroxy Vitamin D Enzyme Immunoassay (EIA) kit intended for human samples but validated in mice (Immunodiagnostic Systems, Inc, Gaithersburg, MD). The manufacturer's limit of the assay for human serum was 6 pmol/L. The assay was performed over two days, using immunoextraction to purify calcitriol in serum then quantification by EIA. The methodology was based on competitive binding of either free calcitriol or calcitriol linked to biotin for a limited amount of highly specific sheep anti-calcitriol binding sites. [217] The amount of complexed biotin bound to the anti-sheep antibody was inversely proportional to the concentration of calcitriol. The assay was carried out in single, using 120 µl of undiluted serum measured at baseline, late pregnancy, and late lactation. Kit instructions

followed the alternative sample preparation and remaining immunoextraction and assay procedure. Results were read at 450 nm in a microplate spectrophotometer (Epoch; BioTek) and analyzed using Gen5 software.

2.8.3 Serum fibroblast growth factor 23 (FGF23)

Mouse serum FGF23 was measured using an FGF23 ELISA Kit (Kainos Laboratories, Tokyo, Japan). The detection limit of the assay was 3 pg/ml. The test principle is a two-step ELISA.[218] Generally, FGF23 is captured by an immobilized FGF23 antibody at the plate surface of the well; the HRP-labeled FGF23 antibody then forms a sandwich complex with the first antibody. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of FGF23 in the sample. Serum samples from both DXA sets were measured at baseline, late pregnancy, late lactation and recovery 1. Experiments followed the kit procedure in duplicate. Results were read at 450 nm in a microplate spectrophotometer (Epoch; Biotek) and analyzed using Gen5 software.

2.9 Markers of bone turnover

2.9.1 Serum procollagen type 1 N-terminal peptide (P1NP)

Procollagen type 1 N-terminal propeptide (P1NP), a bone formation marker, was measured in the serum of WT and *Cyp27b1*$^{-/-}$ mice at baseline, late pregnancy, late lactation, and recovery 1 using samples from both DXA sets. An EIA kit for P1NP (Inter Medico, Markham, ON.) was used for analysis. The detection limit has not been reported. The assay is based on the principle that type 1 collagen is synthesized during bone formation, to comprise the majority of the organic component in bone matrix.[219] During the synthesis of collagen, the amino- and

carboxyterminal ends of the procollagen molecule release propeptides that are secreted into blood circulation. Enzyme immunoassay is therefore able to detect these propeptides. In this manner, the serum concentration of P1NP reflects the rate of bone formation. Specifically, the kit is a competitive EIA that uses a polyclonal rabbit anti-P1NP antibody to coat the inner surface of the microtitre wells. Experiments followed the procedure in the kit in duplicate. Results were read at 450 nm in a microplate spectrophotometer (Epoch; Biotek) and analyzed using Gen5 software, with colour intensity being inversely proportional to the concentration of P1NP.

2.9.2 Urine deoxypyrodinoline

Deoxypyrodinoline (DPD), a bone resorption marker, was measured in the urine of WT and *Cyp27b1*$^{-/-}$ mice at baseline, late pregnancy, late lactation, and recovery 1 using samples from the first DXA set. An EIA kit for DPD (Quidel, San Diego, CA.) was used for analysis. The detection limit of the assay was 1.1 nmol/L. The assay is based on the principle that type 1 collagen of bone is crosslinked by pyridinoline (PYD) and deoxypyridinoline, which provide rigidity and strength. [220] DPD is formed from an oxidation reaction of the amino acid lysine, and is released into circulation when bone is resorbed. As DPD is unaffected by diet and excreted unmetabolized in urine, it is a suitable candidate for the assessment of bone resorption. In this manner, elevated levels of urinary DPD indicate elevated bone resorption levels. The kit is a competitive EIA that uses a monoclonal anti-DPD antibody to coat the inner surface of the microtitre wells. Experiments followed the kit procedure in duplicate. Results were read at 405 nm in a microplate spectrophotometer (Epoch; Biotek) and analyzed using Gen5 software, with colour intensity being inversely proportional to the concentration of DPD. Final results were corrected for by the concentration of creatinine (nmol DPD/mmol Creatinine).

2.10 Micro-computed tomography (μCT)

WT and *Cyp27b1* null mice were killed by cervical dislocation at baseline, late lactation, recovery 1, recovery 4, and virgin mice age-matched to the recovery 4 mice. Initially for mice at recovery 4, the top three lumbar vertebrae were harvested together and surrounding tissue was removed using a sterile blade and scissors. Tibias were similarly harvested with removal of surrounding tissue, and the fibula was kept on to allow for easier orientation analysis. Both lumbar vertebrae and tibias were stored together in scintillation vials filled with formalin until analysis.

Femurs were also collected for μCT, and these were harvested with surrounding muscle tissue attached, to avoid damaging the sample. Femurs were stored with the hind limbs in 70 % ethanol, due to the preference of the St. Vincent's Institute of Medical Research. Samples were analyzed by Brett Tonkin and guidance was provided by Dr. Natalie Sims at the University of Melbourne. It was performed using a high resolution μCT scanner (Skyscan 1076, Kontich, Belgium). Images were acquired using 9 mm voxel resolution, 0.5 mm aluminum filter, 50 kV voltage, and 100 mA current. The exposure time was set at 2600 ms, the rotation set at 0.5 degrees, and frame averaging = 1. Reconstruction and analysis of the images was performed using Skyscan software programs NRecon (version 1.6.3.3), DataViewer (version 1.4.4), and CT Analyzer (version 1.12.0.0). Femoral trabecular analysis region of interest (ROI) was performed 15 % above the distal end of the femur toward the femora mid-shaft, with a 12.6 % ROI and threshold values set as 40-255. Cortical analyses were performed 35 % above the distal end of the femur toward the femora mid-shaft, with a 12.6 % ROI and threshold values set as 85-255. Reconstruction limits were set at low: 0.000 and high: 0.138.

2.11 3-Point bend test

Tibias from both WT and *Cyp27b1* null mice were harvested at baseline, late lactation, recovery 1, and recovery 4. Adjacent tissue was removed in addition to the fibula that was broken off. Specimens were kept frozen at -20ºC until the night before testing. To thaw, tibias were placed at room temperature in 1X PBS. Cortical bone strength was tested using a 3-point bend fixture attached to an Instron Series 3340 electromechanical single column test instrument (Instron, Norwood, MA) together with the Instron Series IX/s software package. The platform was labeled to place each tibia evenly on the same location, and each tibial end was fixed. The crosshead was lowered so that the 10 N load cell was located approximately 2-3 cm above the midshaft of the tibia. The machine was balanced prior to each analysis, after which point the start button was activated and the crosshead descended at 10 mm/min. The descent of the crosshead continued until the tibia bone was broken. The software displayed numerous biomechanical parameters, of which maximum load (gf), displacement (μm), maximum strain (gf/mm^2), maximum strain (%), and slope (gf/mm) were manually recorded.

2.12 Intestinal calcium absorption

Intestinal ^{45}Ca absorption assay was performed according to previously described methods. [221] The radioactivity accumulated in the blood after oral gavage was used as an indirect measure of intestinal calcium absorption. *Cyp27b1* null and WT mice were studied at baseline, day 17.5 of pregnancy, and recovery 1. Day 17.5 of pregnancy was selected instead of 18.5 days so as to avoid premature birth while allowing sufficient time for intestinal absorption to reach its peak value. Four heterozygous females were used and averaged as quenching controls. All mice were fasted 18 hrs prior to analysis. Experimental mice were given 0.2 ml of a solution containing 8

ml of buffer (10 mM Tris-acetate pH 7.5 and 0.5 mM $CaCl_2$) and 1 µl ^{45}Ca via gastric gavage. In this manner each mouse received 0.5 µCi ^{45}Ca. Mice were held firmly at the scruff of the neck and feeding needle attached to filled syringe was slid along the roof of the oral cavity. The feeding needle was inserted into the esophagus, where the contents of the syringe were released. Feeding needle was then removed and mouse was placed in a cage specific for radioactive studies. After a 45 min waiting period following administration of the ^{45}Ca, experimental mice were temporarily anesthetized with isoflurane and blood was collected by cardiac puncture. Blood was injected into a heparinized tube and mice were then immediately killed via cervical dislocation. All blood samples were stored at -20 ºC until analysis. To determine the radioactivity accumulated, an aliquot of 100 µl of whole blood from each wild type and *Cyp27b1* null mouse were counted with 10 ml scintillation fluid in a multi-purpose scintillation counter (Beckman Coulter, U.S.A).

2.13 Statistical analysis

StatPlus 5.8.3.8 for Macintosh (AnalystSoft Inc, Vancouver, BCF) was used for data analysis. Differences among means were calculated by one-way ANOVA (analysis of variance) with post-hoc tests (unstacked), and the Tukey-Kramer test was used to determine which differences were statistically significant. All data are presented with ± standard error (SE). On the graphs, significant differences are marked, and the number of samples studied is indicated in parentheses on the x-axis.

2.14. Animal care approval

All studies involving live mice were performed with the prior approval of the Institutional Animal Care Committee of Memorial University.

III. RESULTS

3.1 Litter sizes

To measure changes in BMC throughout the mouse reproductive cycle, it is extremely important to have the WT and null mothers well-matched for litter size, especially during lactation. The number of pups nursed during this time period will determine the amount of calcium required, and therefore the amount of bone resorbed from the maternal skeleton and the amount of calcium absorbed from the intestines. The breeding pairs involved in this study proved to be well-matched (Figure 11) with litter sizes of 5.2 ± 0.7 and 5.1 ± 0.7 for WT and *Cyp27b1* null, respectively (p=NS). The litters were not culled as both types of dams nursed the same number of pups on average.

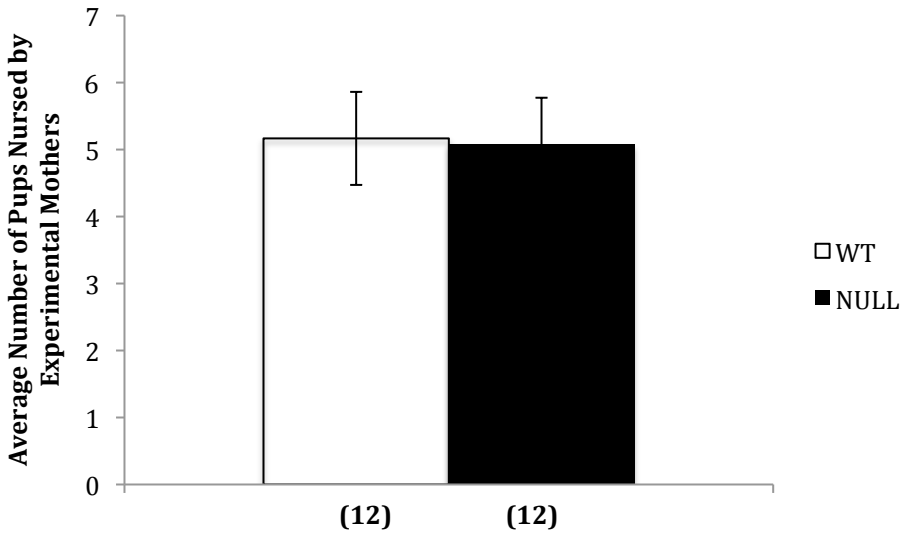


Figure 11: Average number of pups nursed by experimental mothers. At weaning, the litter sizes were equal between WT and Null mothers. Values are means ± SE and sample sizes are indicated in parentheses.

3.2 Bone mineral content

3.2.1 First analysis of BMC excursion

Despite prior studies showing that the rescue diet allowed the *Cyp27b1* null mice to develop normally without rickets, at baseline *Cyp27b1* nulls had 30 % lower whole body BMC than WT (0.417 ± 0.028 g and 0.563 ± 0.017 g, respectively) (Figure 12). [33,38,130] Absolute changes of whole body BMC of both genotypes throughout the reproductive period are shown in Figure 13. The whole body measurement is largely an indicator of cortical bone. WT mice do not show any statistically significant changes across the reproductive cycle. During pregnancy, *Cyp27b1* null mice significantly increase their BMC, and then experience a significant decrease during late lactation. Between genotypes, the BMC increase in the nulls during pregnancy results in a similar value to WT (0.628 ± 0.023 g in WT and 0.556 ± 0.025 g in *Cyp27b1* null). The *Cyp27b1* null females lose more BMC than WT during lactation, such that the null BMC value is significantly lower than WT (0.567 ± 0.027 g in WT and 0.412 ± 0.031 g in *Cyp27b1*$^{-/-}$). The relative changes in whole body BMC are shown in Figure 14. It is shown that both genotypes had BMC increases from baseline to late pregnancy, with an 11.9 ± 1.9 % increase in WT and 43.6 ± 13.5 % increase in null. Although the null mice have resorbed more bone during lactation, both genotypes are at a similar degree below baseline at late lactation. Subsequently, WT and null recover to a value above pre-pregnancy. However, none of these relative changes in whole body BMC were statistically significant between genotypes or between time points.

The lumbar spine has greater contents of trabecular bone in comparison to the whole body. Despite this difference, the lumbar spine showed similar absolute and relative changes in BMC (Figure 15 and 16). Greater lactational losses in this region were expected based on previous

studies showing that more bone is resorbed from the spine as compared to the appendicular skeleton.[56] However, excursions are likely blunted due to the enriched diet reducing skeletal resorption, and to the artifactual increases in BMC caused by any calcium remaining in the gut overlying the spine. As a result, none of these changes reach statistical significance. Both genotypes gained BMC during pregnancy as compared to baseline, resorbed bone during lactation, and recovered to a value above baseline after weaning.

The hind limb is intermediate between the whole body and lumbar spine in terms of the relative content of trabecular bone. Its response to the reproductive periods was largely similar to that observed in the whole body and hind limb regions. WT mice do not show any statistically significant changes across the reproductive cycle (Figure 17). The *Cyp27b1* nulls experience a significant increase in hind limb BMC during pregnancy, followed by a significant decrease to late lactation and a significant recovery post-weaning. Between genotypes, the *Cyp27b1* null has a significantly lower baseline value (0.078 ± 0.002 g in WT and 0.059 ± 0.005 g in *Cyp27b1*$^{-/-}$). Both genotypes have similar BMC during pregnancy (0.094 ± 0.004 g in WT and 0.080 ± 0.005 g in *Cyp27b1*$^{-/-}$), while the greater lactational BMC loss in the nulls compared to WT is evidenced by trough points of 0.053 ± 0.004 g and 0.074 ± 0.004 g, respectively. Figure 18 depicts the relative changes in hind limb BMC. There were no significant differences between the genotypes or within genotypes across the reproductive period. It is noted however that similar to whole body, the relative 50 % increase in hind limb BMC for *Cyp27b1*$^{-/-}$ as compared to the 20 % increase in WT brought both genotypes to an equal value from baseline to late pregnancy.

In summary, BMC of *Cyp27b1* null females was lower than WT at baseline, increased during pregnancy to become equivalent to WT, declined to baseline during lactation, and increased post-weaning to a value higher than pre-pregnancy.

3.2.2 Revised analysis of BMC

Following the previous analysis, it was noted that the drops in BMC during lactation were less than expected, especially in the lumbar spine where it normally drops to 15-20 % below baseline in WT mice. [56] We wondered if retention of calcium in the gut was obscuring the decline in BMC and realized that the mice were eating their calcium-rich feces. Also, prior studies in the Kovacs laboratory on *Pth* null mice indicate that the excursion in BMC is less on the enriched diet as compared to the normal diet. [140] Therefore, to reduce potential confounding variables, the mice were studied again, this time after not only fasting them but also putting them into clean cages so that there would be less of an opportunity for them to eat feces as a rich source of calcium.

These data were also analyzed by comparing both absolute changes (Figure 19) and relative changes (Figure 20) in BMC. Similar to the first analysis, *Cyp27b1* null mice have significantly lower whole body BMC than WT at baseline and late lactation, while none of the relative changes in whole body BMC reached statistical significance. Within the lumbar spine, the null absolute BMC was lower than WT at baseline and late pregnancy, however not statistically significant, again comparable to the first analysis. The hind limb also showed several similarities, with the nulls significantly increasing their absolute BMC from baseline to late pregnancy, losing BMC during lactation, and recovering post-weaning. However, there were several important differences noted from the first data set. Primarily, the decline in lumbar spine BMC during

lactation disappeared with the cage changes. Figure 19B shows that the nulls actually experience a significant increase in lumbar spine BMC from baseline to late lactation. The excursions that occurred in the hind limb region were also much more pronounced than the first analysis. Both genotypes showed significant differences between the relative amounts of BMC gained at late pregnancy to the relative amount lost at late lactation. The null also lost significantly more BMC from baseline to late lactation (-18.67 ± 6.64 %) than the WT (3.49 ± 3.92 %). Lastly, the knockout females had a significant increase between the relative BMC lost during lactation to the relative BMC gained during recovery.

Of note, the sample sizes for each of the figures are displayed on the x-axis. The sample size intended for each genotype using the original methodology and the new methodology of placing the mice in clean cages was 12 and 12 for WT and null. However, due to unanticipated deaths during delivery of anesthesia for DXA scans and intentional harvesting of mice at late lactation in the second methodology for histomorphometry experiments, numbers did not turn out consistent across the reproductive cycle. Furthermore, early parturition in both genotypes prevented these mice from being available for DXA scans at the time point of late pregnancy, so numbers were notably lower at this time point.

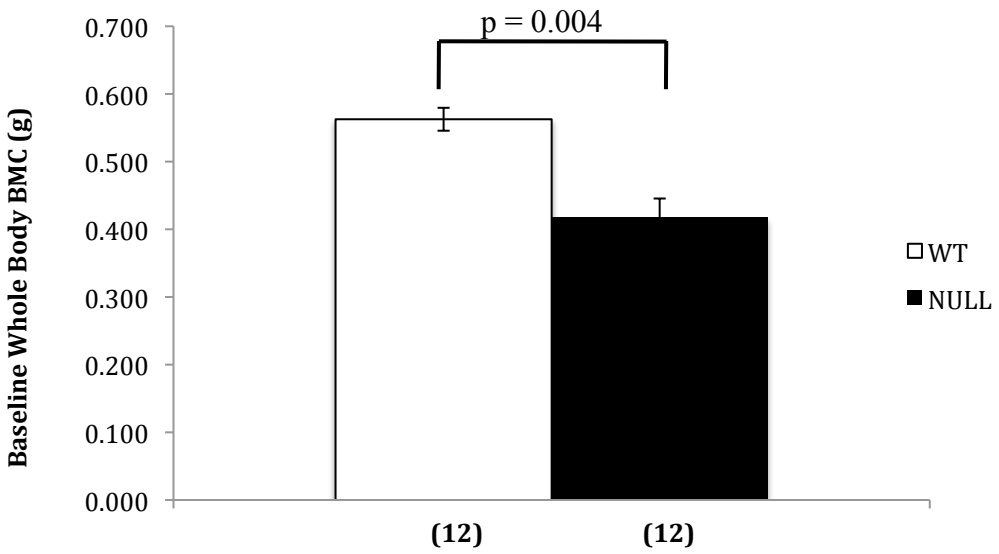


Figure 12: Whole body BMC in WT and *Cyp27b1*$^{-/-}$ mice at baseline. *Cyp27b1* null mice have significantly less BMC than WT at baseline. Values are means ± SE and sample sizes are indicated in parentheses.

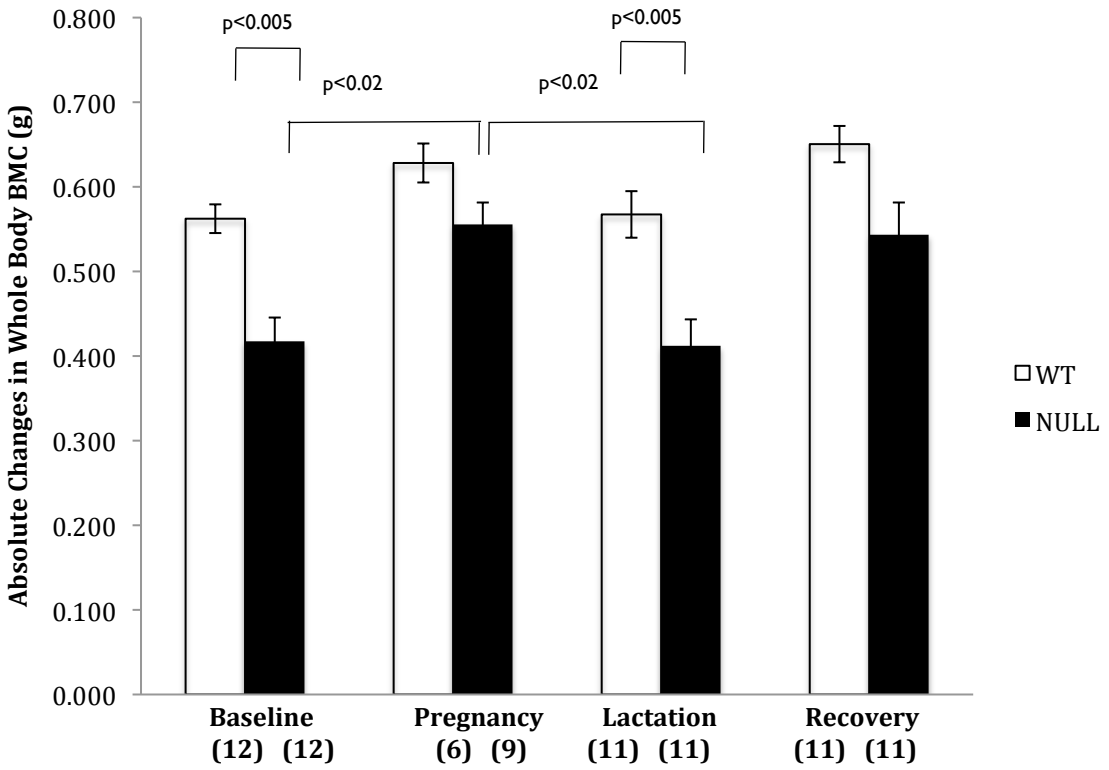


Figure 13: Absolute changes in whole body BMC throughout the reproductive cycle. The BMC was significantly reduced in *Cyp27b1*$^{-/-}$ as compared to WT at baseline and late lactation. The null BMC increases significantly from baseline to pregnancy, and decreases significantly at late lactation. Values are means ± SE and sample sizes are indicated in parentheses.

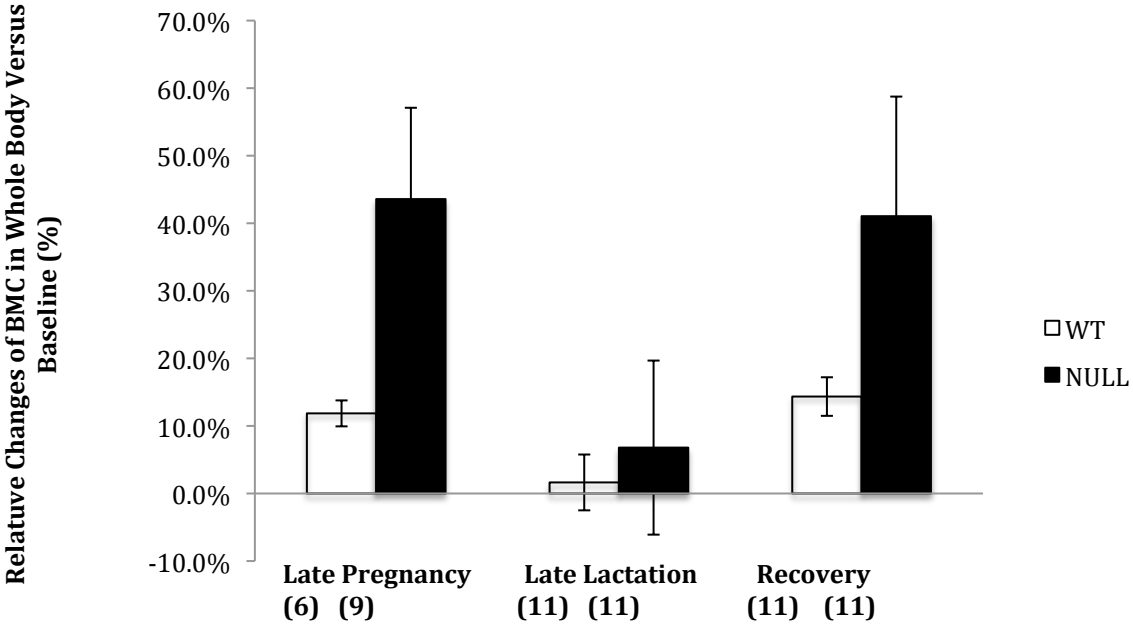


Figure 14: Relative changes in whole body BMC versus baseline throughout the reproductive cycle. There were no significant differences between WT and *Cyp27b1*$^{-/-}$ at any time point throughout the reproductive cycle. In general, both genotypes experience BMC increases during pregnancy, losses during lactation, and return their BMC values to slightly above baseline after weaning. Due to their lower baseline value, *Cyp27b1* nulls experience greater relative excursions compared to WT. Values are means ± SE and sample sizes are indicated in parentheses.

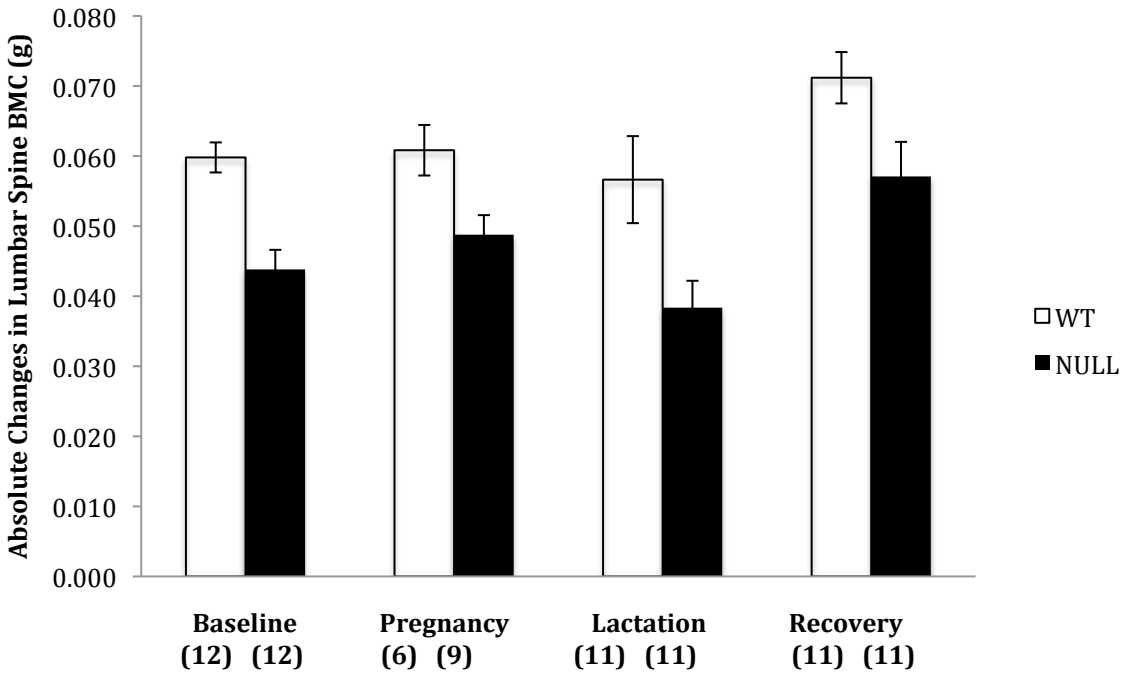


Figure 15: Absolute changes in lumbar spine BMC throughout the reproductive cycle. No significant difference was noted between either genotype at any time point. Values are means ± SE and sample sizes are indicated in parentheses.

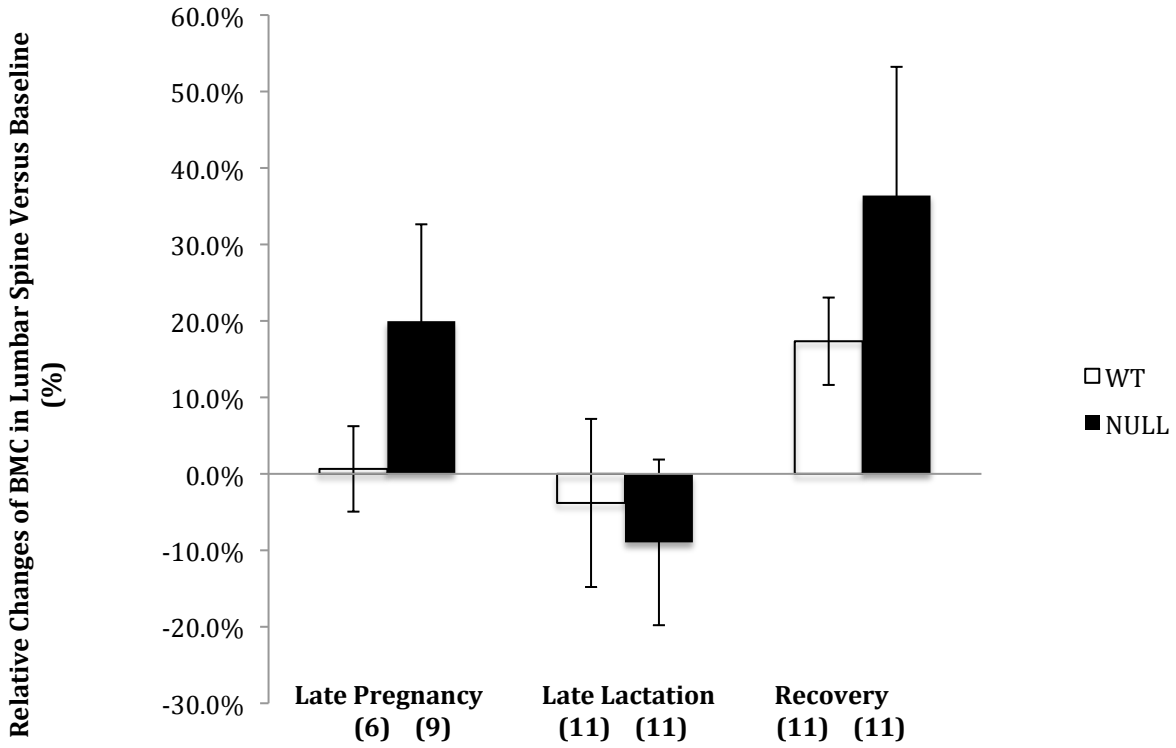


Figure 16: Relative changes in lumbar spine BMC versus baseline throughout the reproductive cycle. There were no significant differences between WT and *Cyp27b1*$^{-/-}$ at any time point throughout the reproductive cycle. The lumbar spine showed the greatest relative decrease in BMC during late lactation, and then recovered post-weaning. Values are means ± SE and sample sizes are indicated in parentheses.

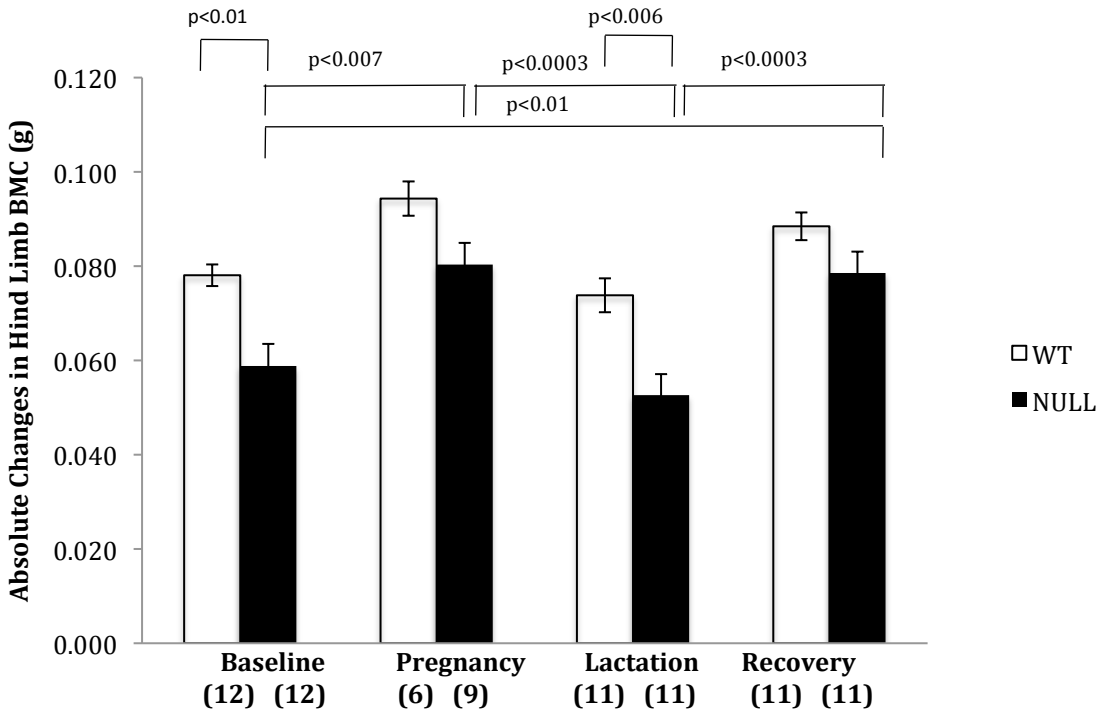


Figure 17: Absolute changes in hind limb BMC throughout the reproductive cycle. The BMC was significantly reduced in *Cyp27b1*$^{-/-}$ as compared to WT at baseline and late lactation. The null BMC significantly increases from baseline to pregnancy, decreases significantly at late lactation, and significantly increases post-weaning to a value higher than what was seen pre-pregnancy. Values are means ± SE and sample sizes are indicated in parentheses.

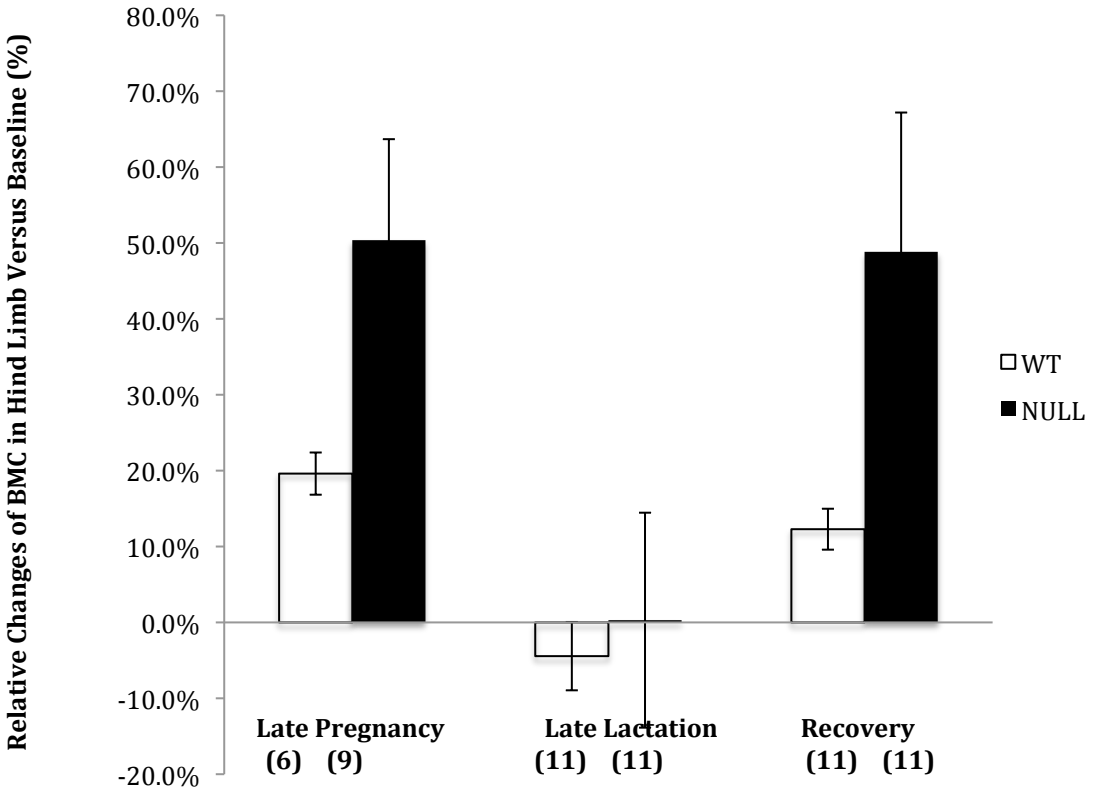


Figure 18: Relative changes in hind limb BMC versus baseline throughout the reproductive cycle. There were no significant differences between WT and *Cyp27b1*$^{-/-}$ at any time point throughout the reproductive cycle. The hind limb showed the greatest relative increase in BMC during pregnancy, followed by a decline during lactation, and an increase during recovery. Values are means ± SE and sample sizes are indicated in parentheses.

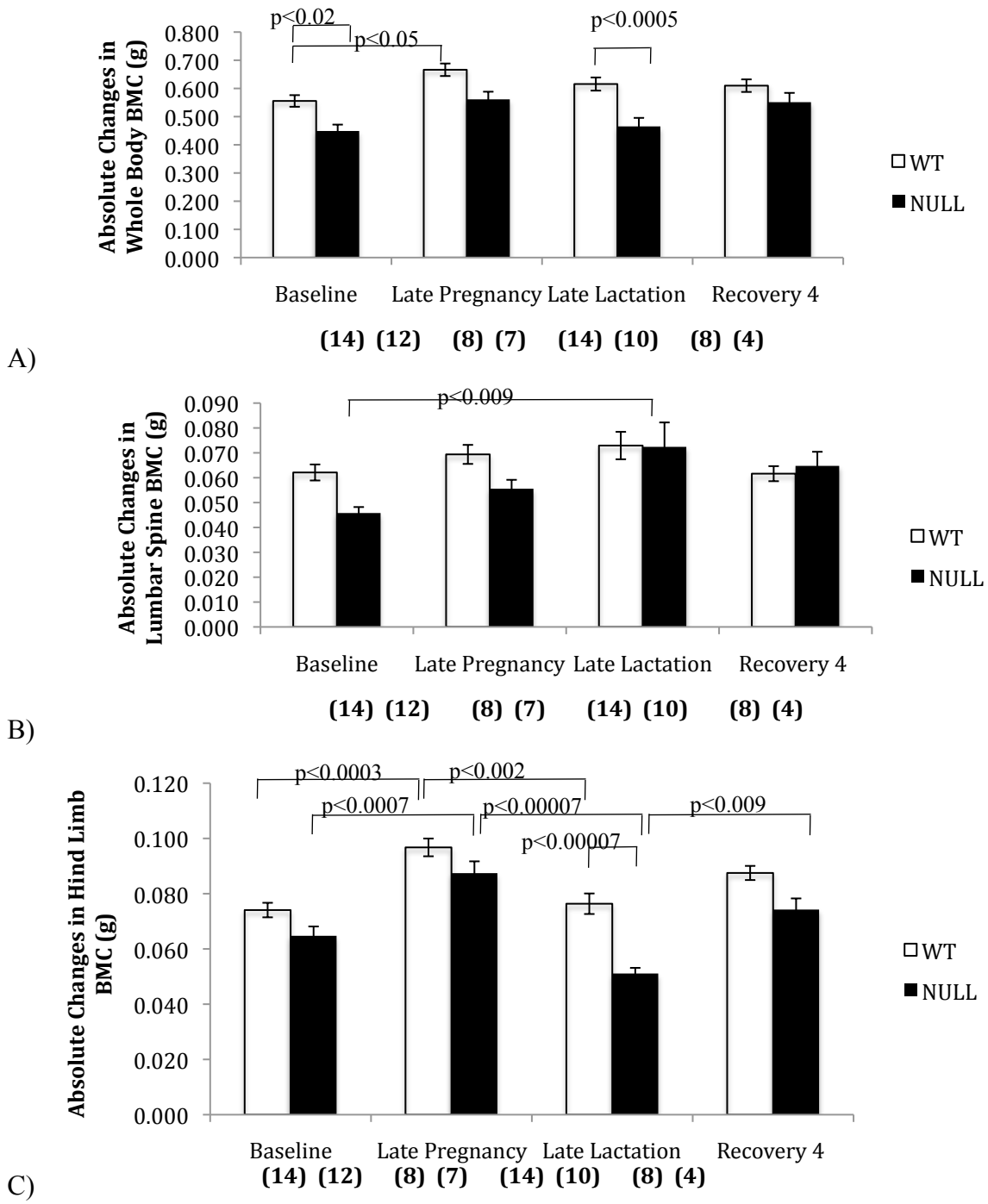


Figure 19: Revised analysis of absolute changes in BMC throughout the reproductive cycle. Whole body BMC (A) was significantly lower in *Cyp27b1*$^{-/-}$ than in WT at baseline and late lactation. WT significantly increase BMC from baseline to late pregnancy. In B) lumbar spine, null BMC significantly increases from baseline to late lactation. Both genotypes display significant increases in hind limb BMC (C) from baseline to late pregnancy, followed by a decline in late lactation. Null BMC is significantly lower than WT at late lactation, and increases significantly post-weaning. Values are means ± SE and sample sizes are indicated in parentheses.

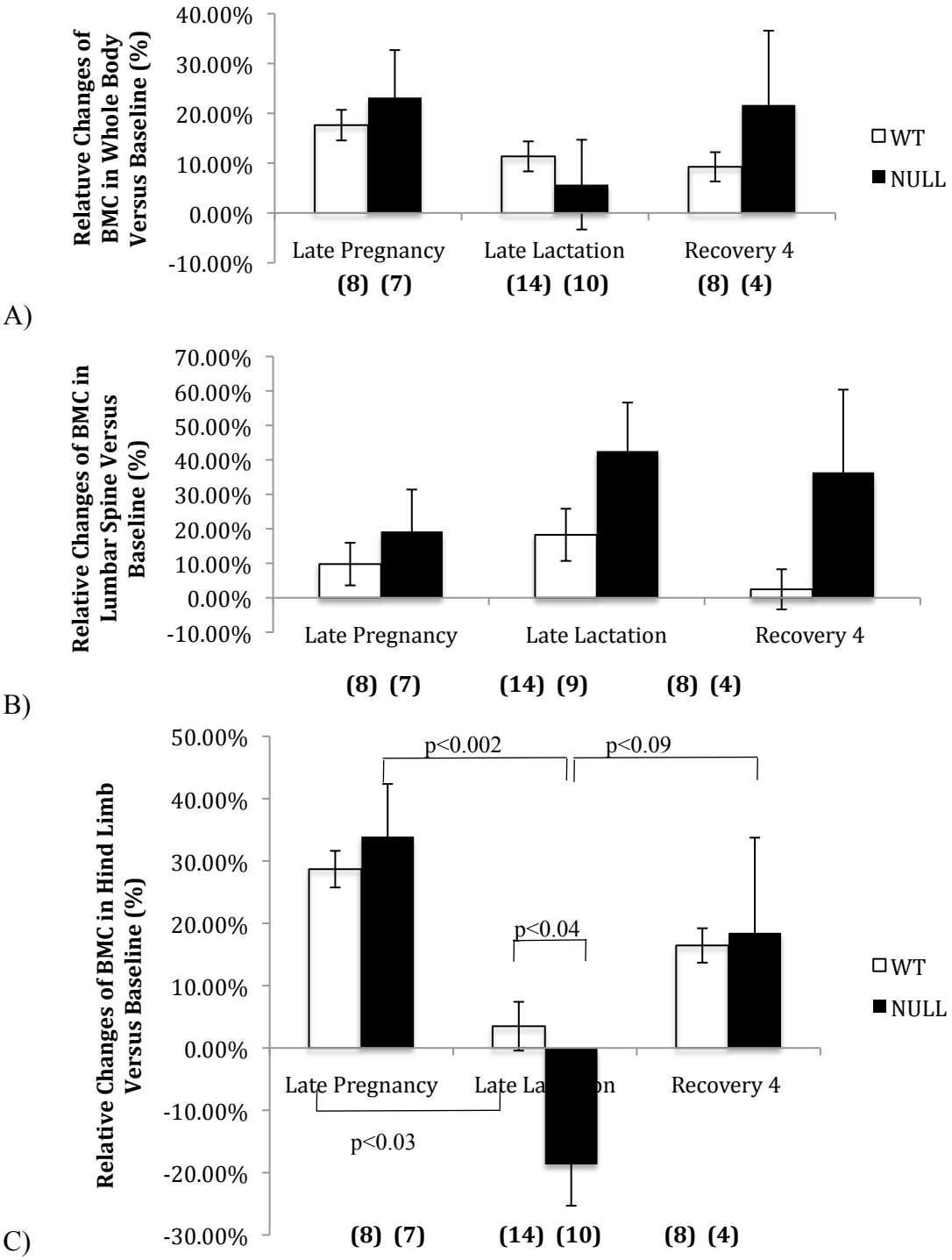


Figure 20: Revised analysis of relative changes in BMC versus baseline throughout the reproductive cycle. There were no significant differences between WT and null at any time point in A) whole body or B) lumbar spine. In C) hind limb, BMC is significantly reduced in both genotypes from late pregnancy to late lactation, then increases significantly in the null post-weaning. The relative change at late lactation in *Cyp27b1*$^{-/-}$ is significantly reduced in comparison to WT. Values are means ± SE and sample sizes are indicated in parentheses.

3.3 Mineral assessment

Mineral analysis of serum and urine was next assessed, as the levels of calcium and phosphorus are impacted by delivery from the intestine, bone, kidneys, and parathyroid gland.

3.3.1 Serum and urine total calcium measurement

Normally, serum calcium concentrations in the mouse do not change throughout the reproductive period, and this was the case for WT mice (Figure 21). However, there were significant changes in serum calcium concentrations in the null females. Despite the rescue diet, *Cyp271* nulls have significant hypocalcemia at baseline (1.98 ± 0.03 mmol/L in WT and 1.69 ± 0.12 mmol/L in *Cyp27b1*$^{-/-}$). Serum calcium levels are rescued during pregnancy and post-weaning recovery, but worsened when the dam was lactating (2.07 ± 0.05 mmol/L in WT and 1.11 ± 0.12 mmol/L in *Cyp27b1*$^{-/-}$).

Urine calcium in the fasted mouse is also normally unchanged throughout the reproductive cycle. In this experiment, urine calcium excretion was similar between WT and null mothers (Figure 22). The mean levels did drop approximately 50 % during lactation from baseline in both genotypes, even though statistical significance was not achieved.

To summarize, WT mice displayed constant levels of serum calcium, whereas the *Cyp27b1* null had hypocalcemia at baseline and late lactation that was rescued by pregnancy and post-weaning recovery. Both genotypes had a non-significant decline in urine calcium during lactation.

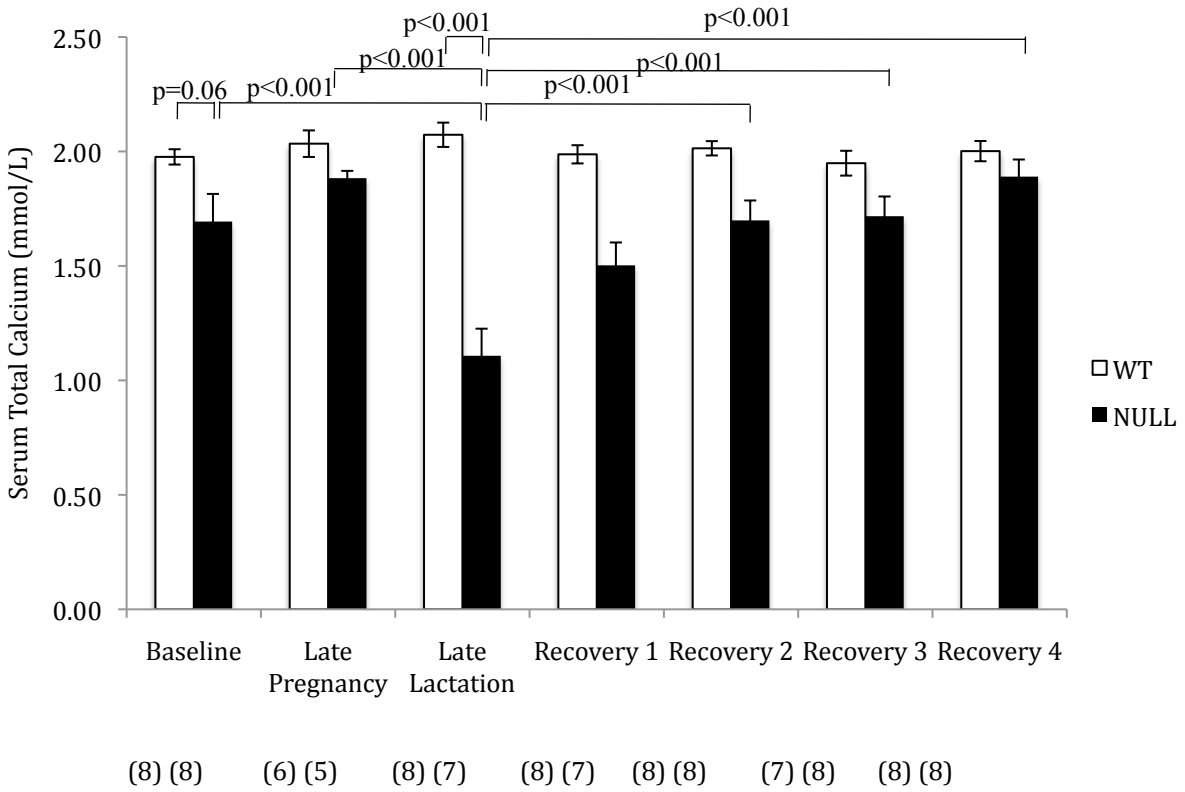


Figure 21: Serum total calcium in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. *Cyp27b1* nulls were hypocalcemic at baseline. The serum calcium concentrations increased during pregnancy such that it was no different from WT, dropped significantly in the null mice during lactation, and gradually improved to equal WT during post-weaning recovery. Values are means ± SE and sample sizes are indicated in parentheses.

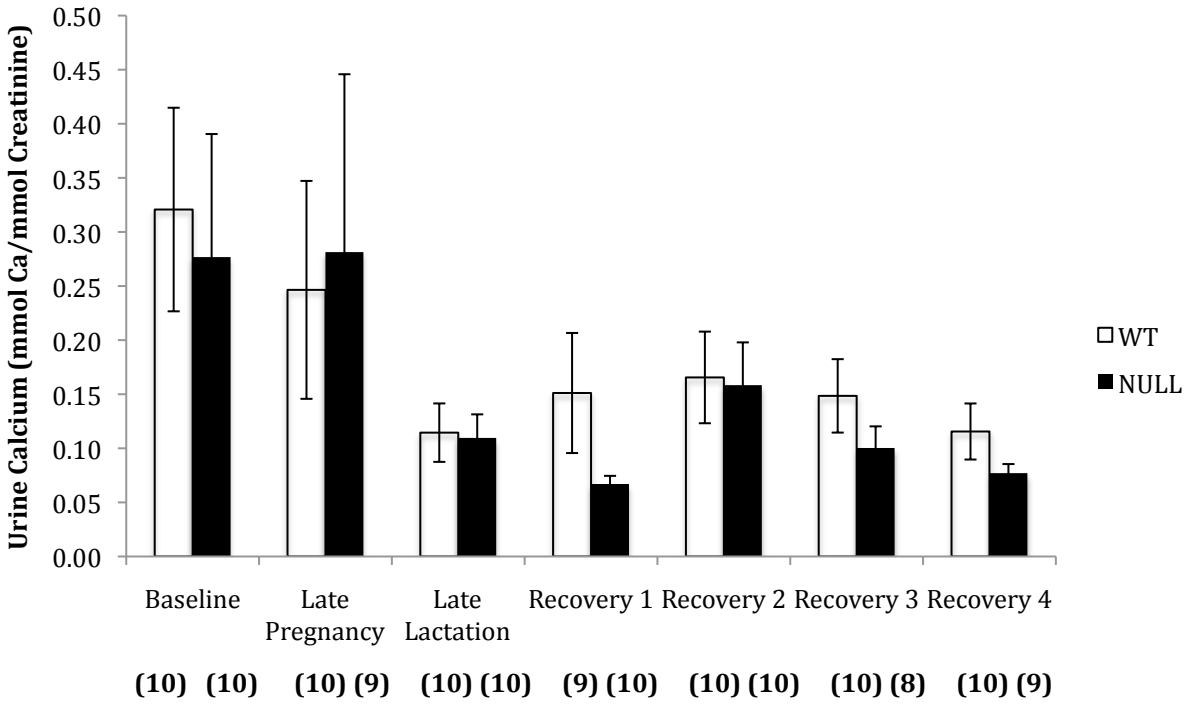


Figure 22: Urine total calcium in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. *Cyp27b1* null and WT mice had similar urine calcium excretion at all time points throughout the reproductive period. Values are means ± SE and sample sizes are indicated in parentheses.

3.3.2 Serum and urine inorganic phosphorus measurement

In the normal condition, serum phosphorus increases during mid-lactation due to increased skeletal resorption that exceeds the capacity of the kidneys to excrete. Observing the late lactation time point rather than mid-lactation will have missed this normal change. In this experiment, the WT had constant levels of serum phosphorus throughout the reproductive cycle (Figure 23). *Cyp27b1* nulls experience a significant decrease in serum phosphorus from baseline to late lactation.

In the unfasted mouse, urine phosphorus normally increases during lactation due to the effect of PTHrP to promote excretion and due to the increased renal filtered load of phosphorus. These changes are more marked at mid-lactation rather than the late lactation time point we measured. Findings in this experiment (Figure 24) show that the WT experienced a doubling of urine phosphorus during lactation but it did not reach statistical significance. The null also did not show any statistically significant changes throughout the reproductive cycle.

In summary, both WT and null siblings show constant levels of serum phosphorus throughout reproduction except for a decrease during late lactation in the null. Urine phosphorus increases in both genotypes during lactation but does not reach statistical significance.

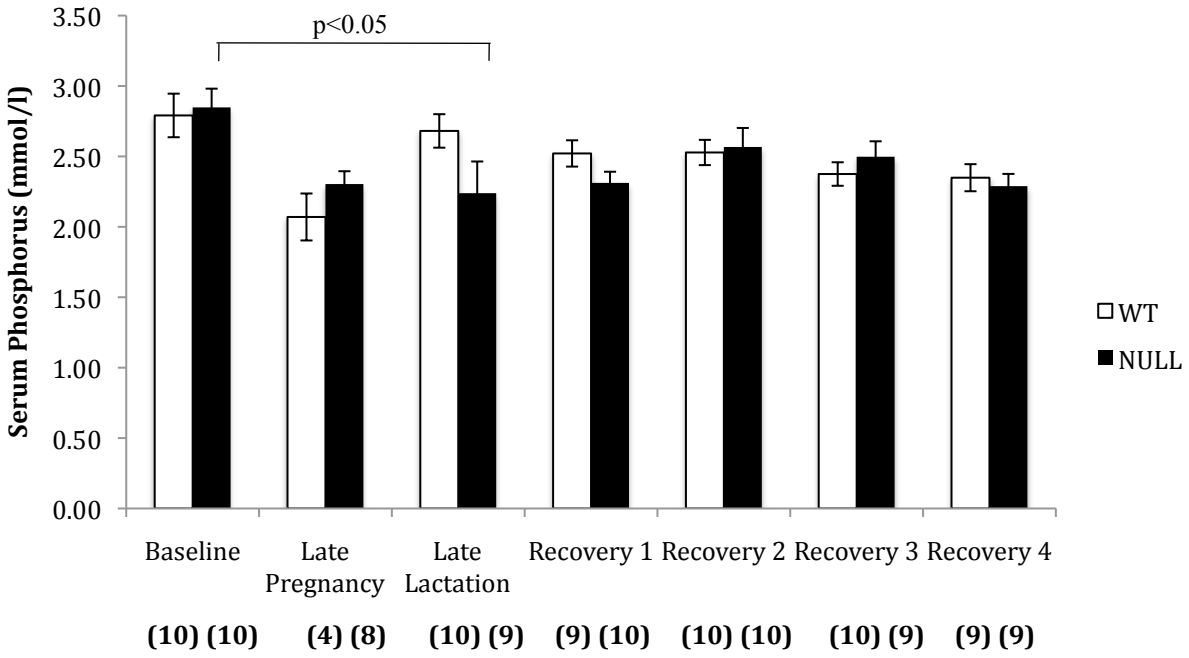


Figure 23: Serum phosphorus in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. There were no significant differences in serum phosphorus levels between both genotypes throughout reproduction. Values are means ± SE and sample sizes are indicated in parentheses.

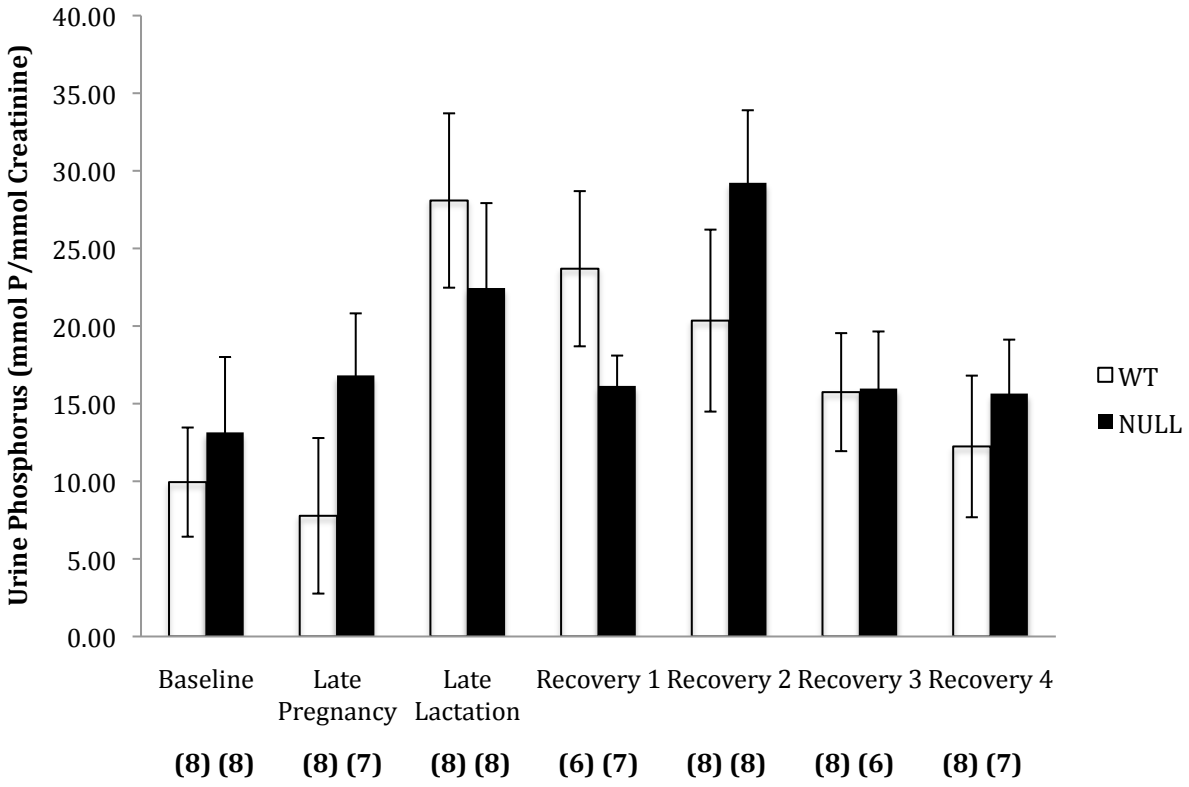


Figure 24: Urine phosphorus in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. *Cyp27b1* null and WT mice had similar urine phosphorus excretion at all time points throughout the reproductive period. Values are means ± SE and sample sizes are indicated in parentheses.

3.4 Hormone physiology

Hormonal analysis of serum was performed to gain a better understanding of the physiological role in regulating calcium and phosphorus homeostasis and the observed changes to the skeleton during reproduction.

3.4.1 Serum PTH

PTH usually decreases during pregnancy and lactation, and published research reports that PTH is normal in *Cyp27b1* null mice maintained on the rescue diet. [33,38] WT mice display low values during pregnancy and lactation, followed by a doubling post-weaning. *Cyp27b1* nulls display marked secondary hyperparathyroidism (Figure 25). At baseline, PTH levels are increased (2,815 ±1,292 ng/ml vs. 8.4 ± 3.9 ng/ml), then reduced at pregnancy to a level numerically higher than WT but not significantly different. During lactation, PTH increased to 5,500 ± 680 ng/ml, and remained high post-weaning.

Since the diet was not changed, it is evidently something about pregnancy alone that caused PTH to normalize during pregnancy, and whatever this is disappears during lactation.

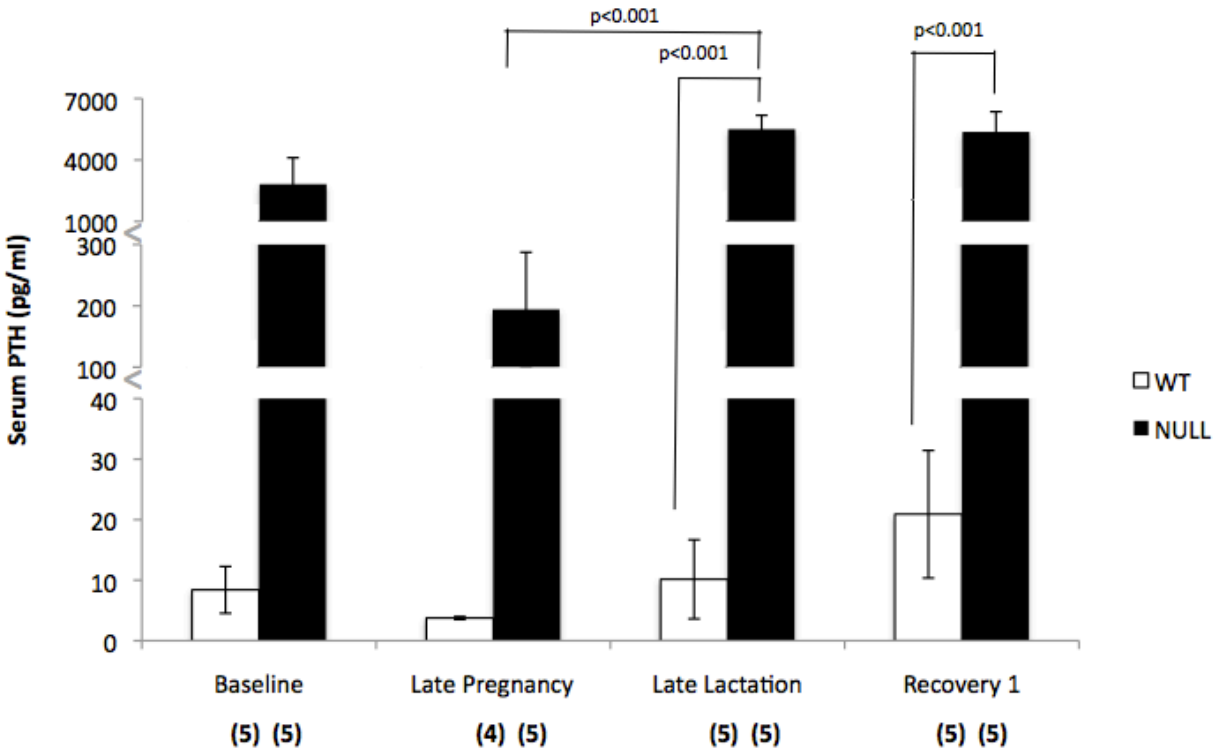


Figure 25: Serum PTH in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. *Cyp27b1* nulls display marked secondary hyperparathyroidism at baseline, lactation and post-weaning; however, the PTH levels are similar to WT during pregnancy. Values are means ± SE and sample sizes are indicated in parentheses.

3.4.2 Serum calcitriol

In rodents, calcitriol levels normally increase during pregnancy and lactation. Serum calcitriol rose >5-fold in WT mice during pregnancy, and remained high during lactation (Figure 26). This increase was absent in *Cyp27b1* nulls. The null calcitriol level is lower at baseline than WT (12.18 ± 1.04 pmol/L vs. 46.41 ± 13.81 pmol/L), although not statistically significant. This value of 12 pmol/L is indistinguishable from the detection limit of human sera (6 pmol/L) and may represent the detection limit for mouse sera under these conditions (detection limit in mouse serum has not been reported). The null baseline value then showed an apparent but statistically non-significant increase during pregnancy.

In summary, WT mice experienced the expected several-fold increase in calcitriol during pregnancy and lactation. In contrast, the nulls had low values throughout the reproductive cycle that increased non-significantly during pregnancy.

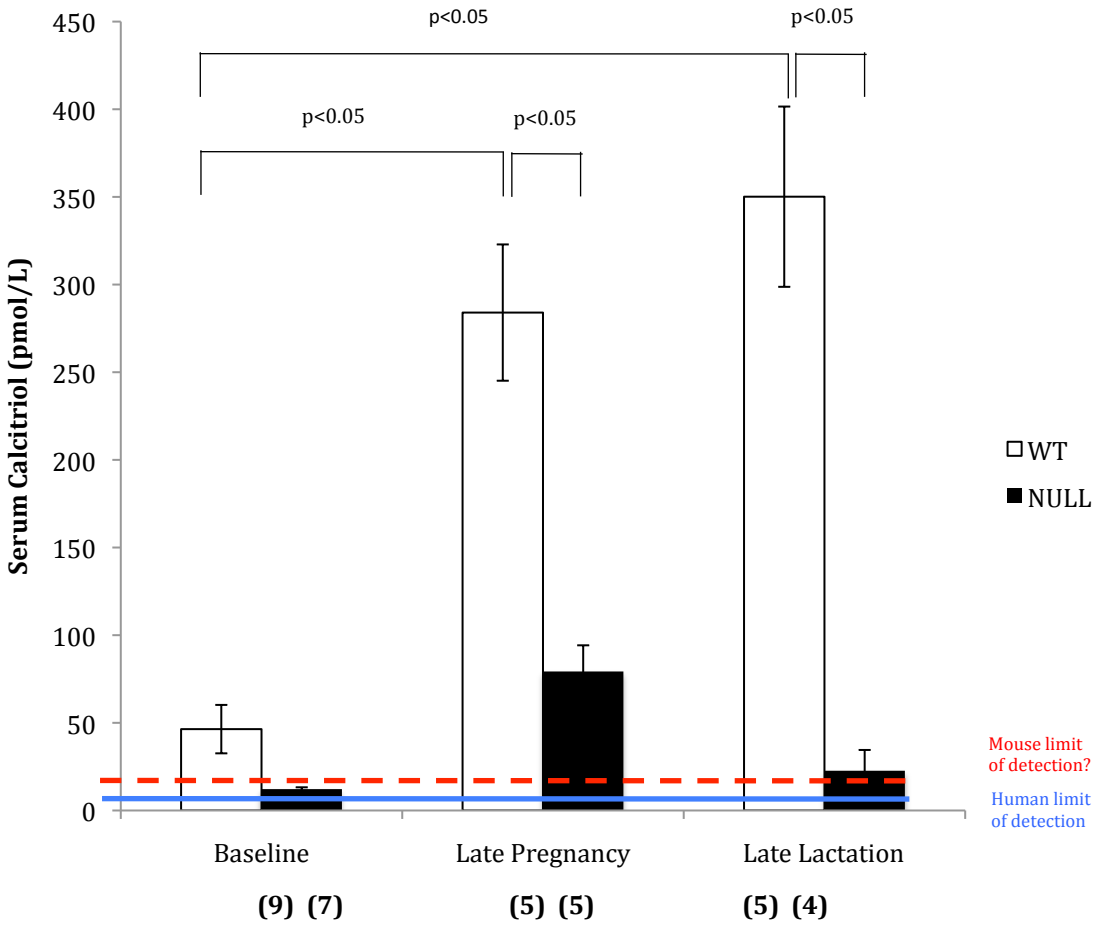


Figure 26: Serum calcitriol in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. *Cyp27b1*$^{-/-}$ mice have a trend towards lower calcitriol levels at baseline than WT. Serum calcitriol rose >5 fold in WT mice during pregnancy while this increase is absent in the nulls. The knockout mice also display low calcitriol levels at late lactation while the WT remains high. Values are means ± SE and sample sizes are indicated in parentheses.

3.4.3 Serum FGF23

FGF23 inhibits calcitriol synthesis, and calcitriol in turn stimulates FGF23 synthesis. It was therefore expected that FGF23 levels would increase during pregnancy in the WT mouse, while the knockout would be deficient throughout reproduction. A significant increase in FGF23 was observed in the WT during pregnancy, followed by a significant decrease in late lactation and recovery 1 (Figure 27). Basal FGF23 was lower in the *Cyp27b1* null and the rise during pregnancy was significantly blunted (Figure 27). Between genotypes, FGF23 levels are significantly lower in the *Cyp27b1*$^{-/-}$ than the WT at late pregnancy (196.48 ± 51.55 pg/ml vs. 696.56 ± 34.99 pg/ml), late lactation (38.91 ± 24.18 pg/ml vs. 347.71 ± 110.35 pg/ml), and recovery 1 (12.85 ± 4.19 pg/ml vs. 306.09 ± 60.37 pg/ml).

In summary, FGF23 levels were increased in WT during pregnancy, while the rise was blunted in the *Cyp27b1* null.

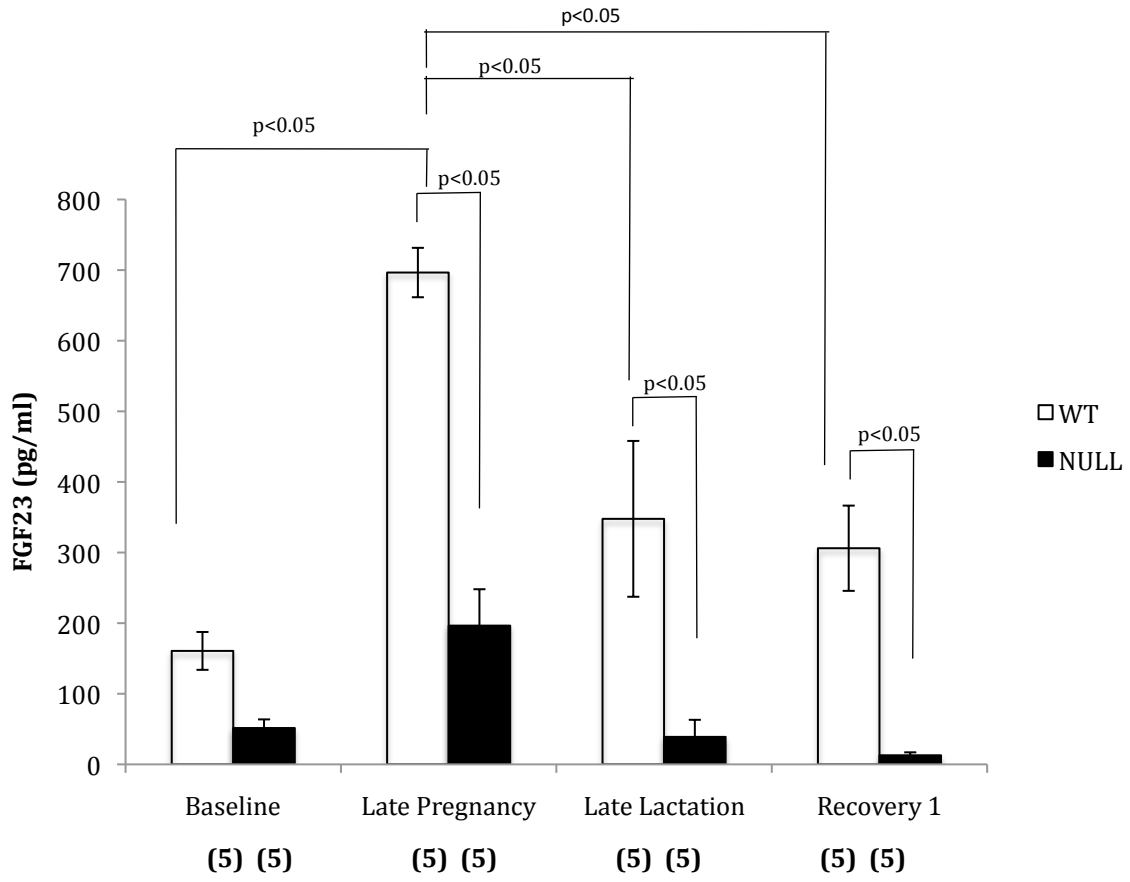


Figure 27: Serum FGF23 in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. FGF23 was increased in WT during pregnancy. In the *Cyp27b1* null, basal FGF23 was lower and the rise during pregnancy was not significant. Values are means ± SE and sample sizes are indicated in parentheses.

3.5 Markers of bone turnover

The P1NP serum biomarker and DPD urine biomarker were measured to indicate changes in the rate of bone formation and resorption, respectively.

3.5.1 Serum P1NP

P1NP levels are not significantly changed in WT throughout the reproductive cycle, however show a non-significant decrease and increase during pregnancy and post-weaning, respectively. This pattern is mirrored in the P1NP levels of the null siblings. The peak of P1NP at recovery 1 in *Cyp27b1*$^{-/-}$ is significantly greater than its values at late lactation, late pregnancy, and baseline. Between genotypes, P1NP is significantly increased in the *Cyp27b1* null over WT at recovery 1 (44.12 ± 4.56 ng/ml in WT and 173.68 ± 28.14 ng/ml in *Cyp27b1*$^{-/-}$), indicative of increased bone formation, but no different from WT at the earlier time points (Figure 28).

Both genotypes show a general trend of decreased bone formation during pregnancy, followed by increased bone formation during lactation and recovery. This increase is greater and reaches statistical significance in the *Cyp27b1* null.

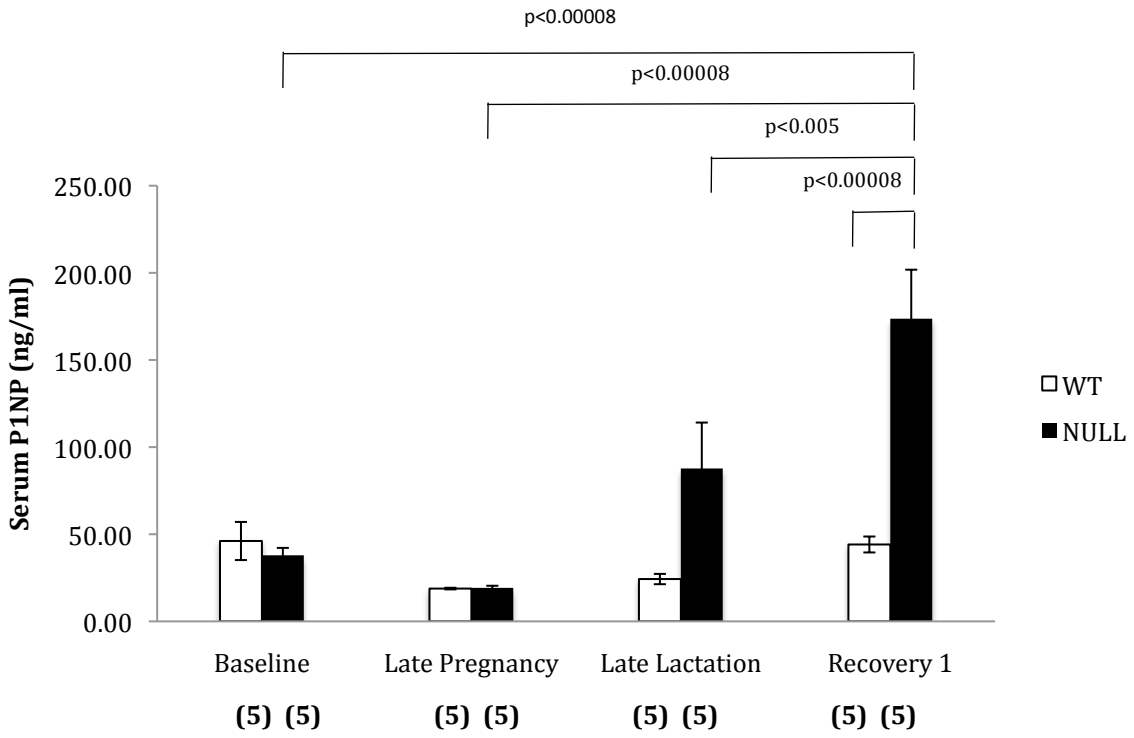


Figure 28: Serum P1NP in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. The bone formation marker P1NP rose significantly during post-weaning recovery in the *Cyp27b1* null. WT showed the same pattern of serum P1NP changes during the reproductive period, however these changes were not significant. Values are means ± SE and sample sizes are indicated in parentheses.

3.5.2 Urine DPD

DPD normally increases early to mid lactation, and then subsides to normal by late lactation. WT mice are consistent with normal at baseline and late pregnancy; however, they maintain increased bone resorption at the end of lactation (Figure 29). *Cyp27b1* null mice have a trend of decreased bone resorption at late pregnancy and late lactation. Between genotypes, the null has higher DPD than WT at every time point, although this does reach statistical significance.

No significant differences are noted between the urine DPD levels of WT and *Cyp27b1* null mice at any time point.

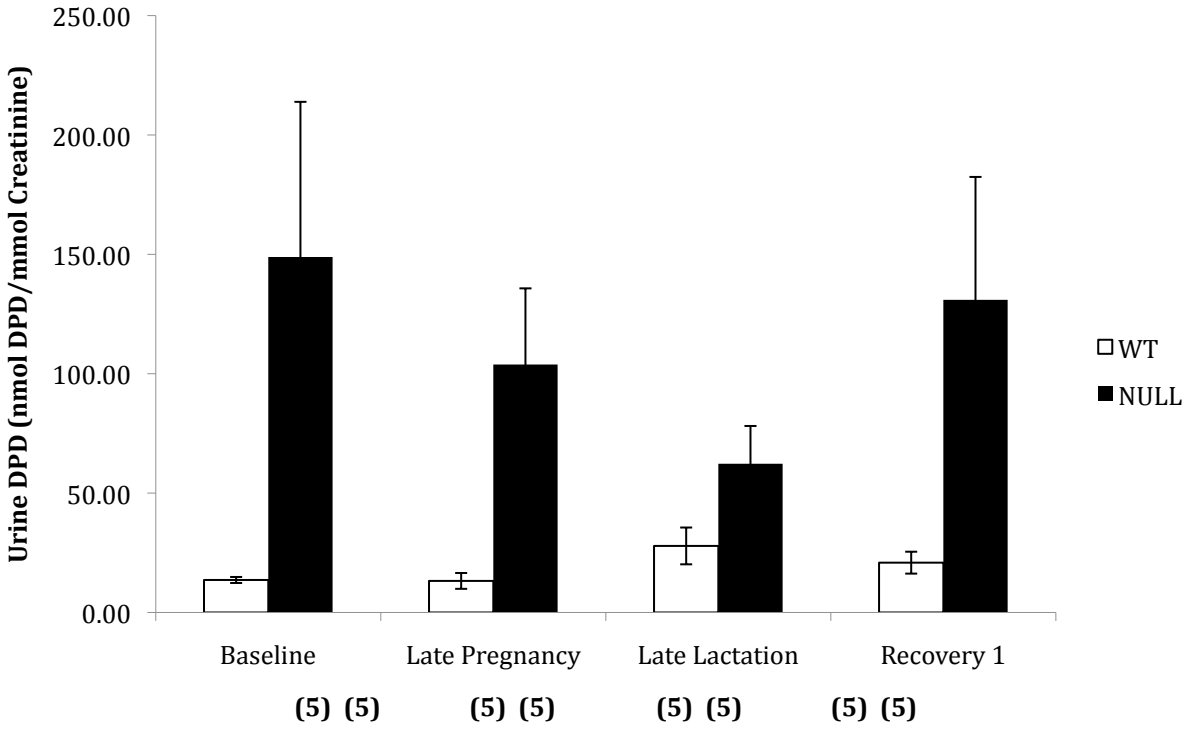


Figure 29: Urine DPD in WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. WT and *Cyp27b1*$^{-/-}$ mice do not have any significant differences between them at any time point. Values are means ± SE and sample sizes are indicated in parentheses.

3.6 Micro computed tomography

Micro-computed tomography was used to assess skeletal microarchitecture on *Cyp27b1* null and WT femurs. Although DXA provides a measure of bone mineralization, this is only 2-dimensional and no information is provided on thickness or structure of the skeleton. Further, interference from calcium outside the skeleton may have falsely increased measurements of the mineral content. Analyzing the bone microarchitecture could determine if there were any alterations in the trabecular or cortical structure between the two genotypes.

Throughout reproduction and in comparison to virgin controls age-matched to mice harvested at the recovery 4 time point (Figure 9), there were several notable micro architectural differences in the femora within and between genotypes. It is noted that the late pregnancy time point is not represented, so comparisons are made between the time points of baseline and late lactation for all the analyses. Femoral length remains constant in the WT mice throughout reproduction and the virgin controls have similar measurements (Figure 30). In the null animals, there appears to be an effect of recovery whereby bone length is significantly improved at recovery 1 and recovery 4 as compared to baseline. The null virgin controls display a femoral length in between that of baseline and late lactation. No statistically significant differences in femoral length were found between WT and *Cyp27b1* null mice.

Trabecular bone volume shows a pattern of lower BV/TV in late lactation, recovery 1, and recovery 4 compared to baseline and the virgin controls in WT mice (Figure 31). Surprisingly, the WT mice have no detectable difference in trabecular bone volume between late lactation and recovery 4. This effect of lactation and recovery is also the same in null siblings. Between

genotypes there are no significant differences. The changes in trabecular bone volume are mostly mirrored by the alterations in trabecular number (Figure 34). Both WT and null display reduced trabecular number at late lactation, recovery 1, and recovery 4 in comparison to the virgin controls, with no differences between genotypes. Trabecular separation is in line with the previous results, showing the opposing pattern as trabecular bone volume and trabecular number (Figure 32). WT mice display a trend of increased separation at late lactation, recovery 1, and recovery 4 compared to baseline and virgin controls, although this is not statistically significant. *Cyp27b1* null show this same pattern, reaching statistical significance in comparison to the virgin controls. Between genotypes there are no significant differences.

The changes in trabecular thickness are separate from the aforementioned adaptations, with only the WT experiencing a significant decrease from baseline to late lactation (Figure 33). There is a trend towards increased trabecular thickness during recovery, however this does not reach statistical significance nor the thickness measured pre-pregnancy. The femoral trabecular thickness of virgin controls is also similar to the recovery values. The *Cyp27b1* nulls show a similar pattern to WT, however none of the changes measured are significant. There are also no differences measured between genotypes.

Looking next at the changes to cortical bone, it can be seen that the WT have significantly reduced cortical thickness at recovery 1 and recovery 4 in comparison to virgin controls (Figure 35). The nulls experience even greater loss of cortical thickness but fully recover this by the recovery 4 time point. The null virgin controls have the highest cortical thickness; significantly increased in comparison to all the reproductive time points measured. Further evidence for the

loss of cortical bone in the nulls is shown by their significantly reduced thickness in comparison to WT at late lactation and recovery 1. Cortical area remains constant in the WT throughout reproduction and is equal to the femoral cortical area of virgin controls (Figure 36). The knockout mice show a trend of reduced cortical thickness at late lactation and recovery 1, followed by improvement at recovery 4 to a value higher than pre-pregnancy; however, this is not statistically significant. In the null virgin control, cortical area is significantly greater than all the reproductive time points measured. Between genotypes, cortical area is lesser in the null than their WT siblings at late lactation.

Marrow area does not change throughout reproduction in WT mice, although the virgin controls have a small but non-significant decrease compared to the earlier time points (Figure 37). *Cyp27b1* null mice show a significant increase in marrow area at late lactation and recovery 1 in comparison to the virgin controls, corroborating the loss of cortical bone seen in the changes to cortical thickness and area. No significant differences are present between genotypes. Finally, endocortical perimeter (Figure 38) and periosteal perimeter (Figure 39) are not modified by reproduction, and there are no significant differences noted within or between genotypes.

To summarize, the assessment of skeletal architecture using micro-computed tomography revealed a significant increase in femoral length of the nulls after a reproductive cycle, permanent loss of trabecular bone in WT and null mice, and loss followed by recovery of cortical bone in the nulls.

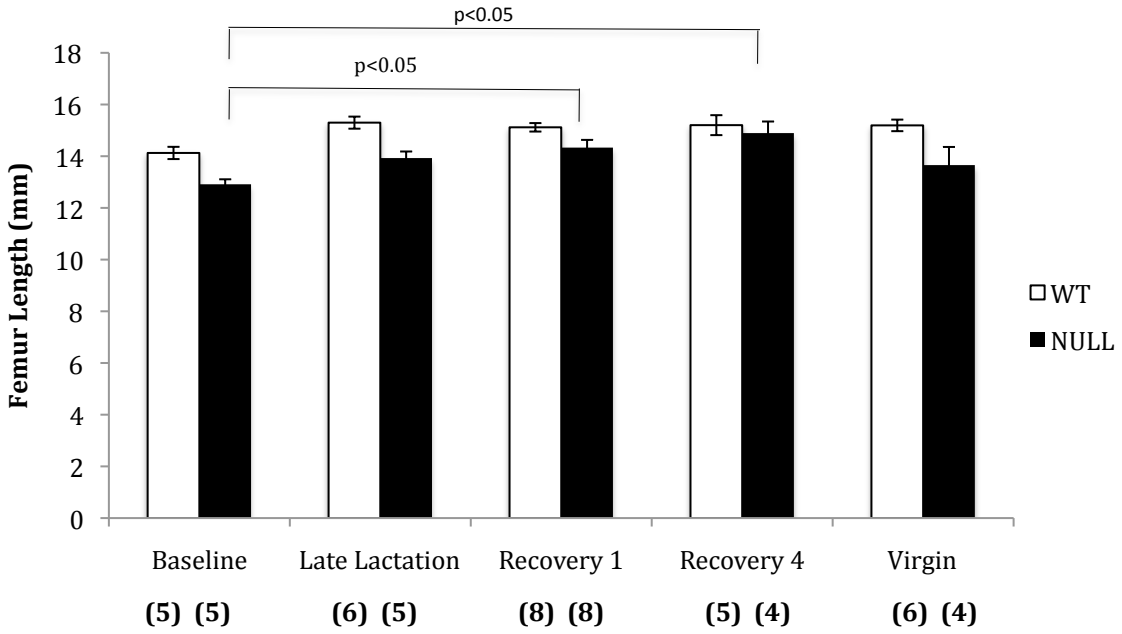


Figure 30: Femoral length of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Null mice have improved bone length at recovery 1 and recovery 4 compared to baseline. Values are means ± SE and sample sizes are indicated in parentheses.

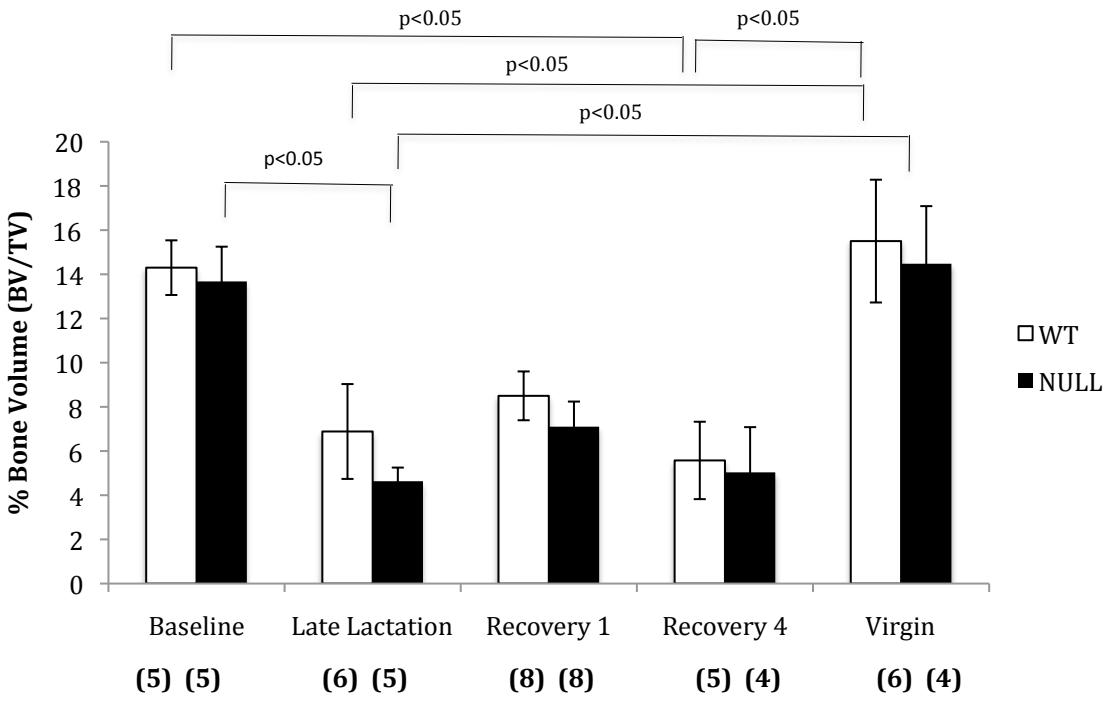


Figure 31: Percent femoral bone volume of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Both genotypes have lower BV/TV in late lactation as compared to the virgin controls. The knockout mice also have reduced bone volume in comparison to baseline, while the WT show a decrease from baseline to recovery 4 such that the femurs have significantly lower volume compared to the virgin age-matched controls. Values are means ± SE and sample sizes are indicated in parentheses.

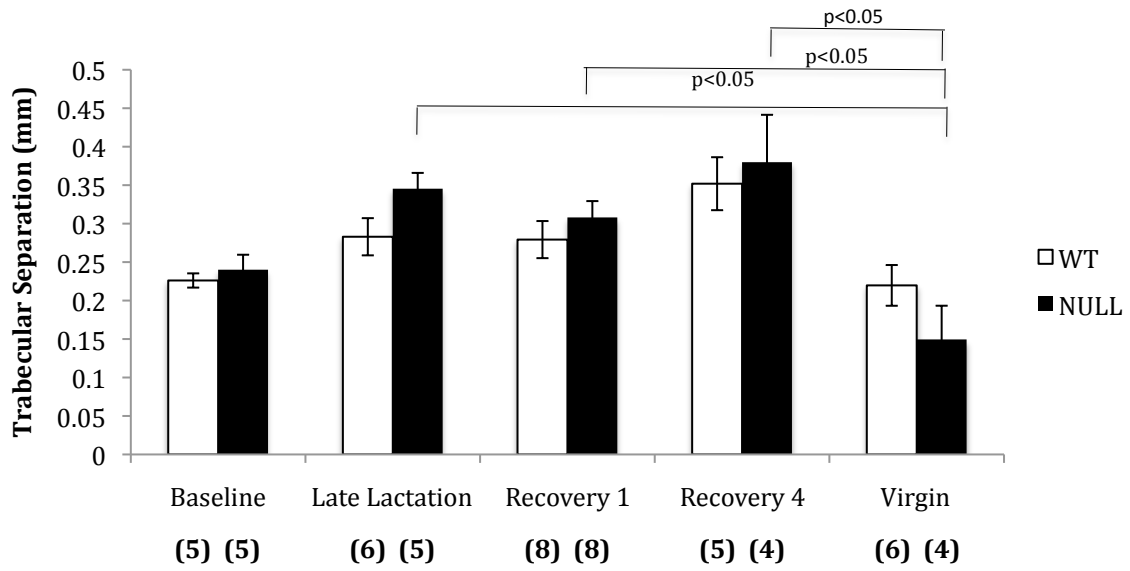


Figure 32: Femoral trabecular separation of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Null mice have increased trabecular separation at late lactation, recovery 1, and recovery 4 as compared to the virgin controls. Values are means ± SE and sample sizes are indicated in parentheses.

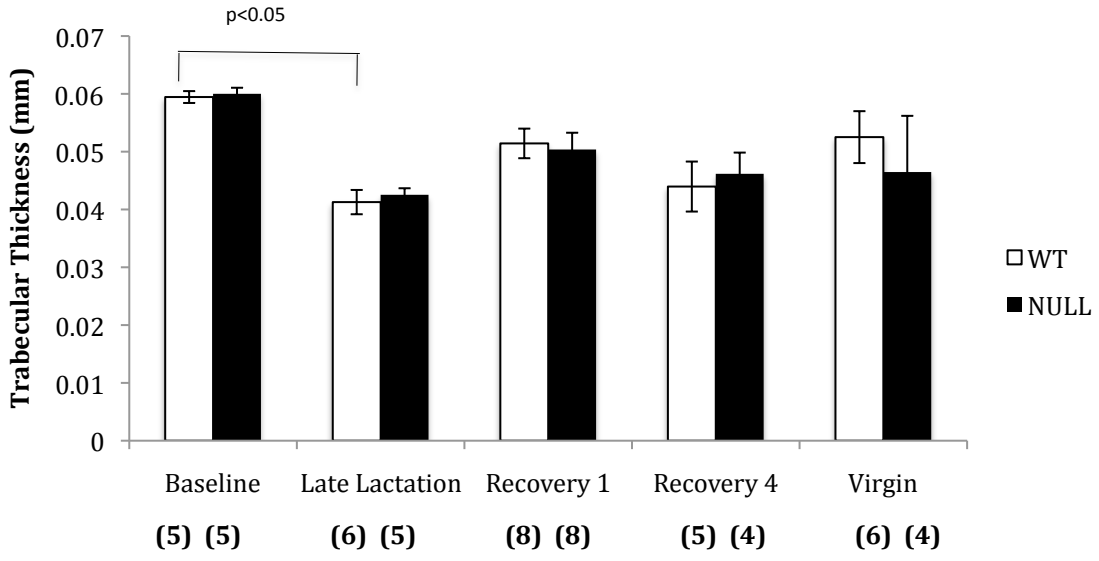


Figure 33: Femoral trabecular thickness of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. WT mice have reduced trabecular thickness from baseline to late lactation. Values are means ± SE and sample sizes are indicated in parentheses.

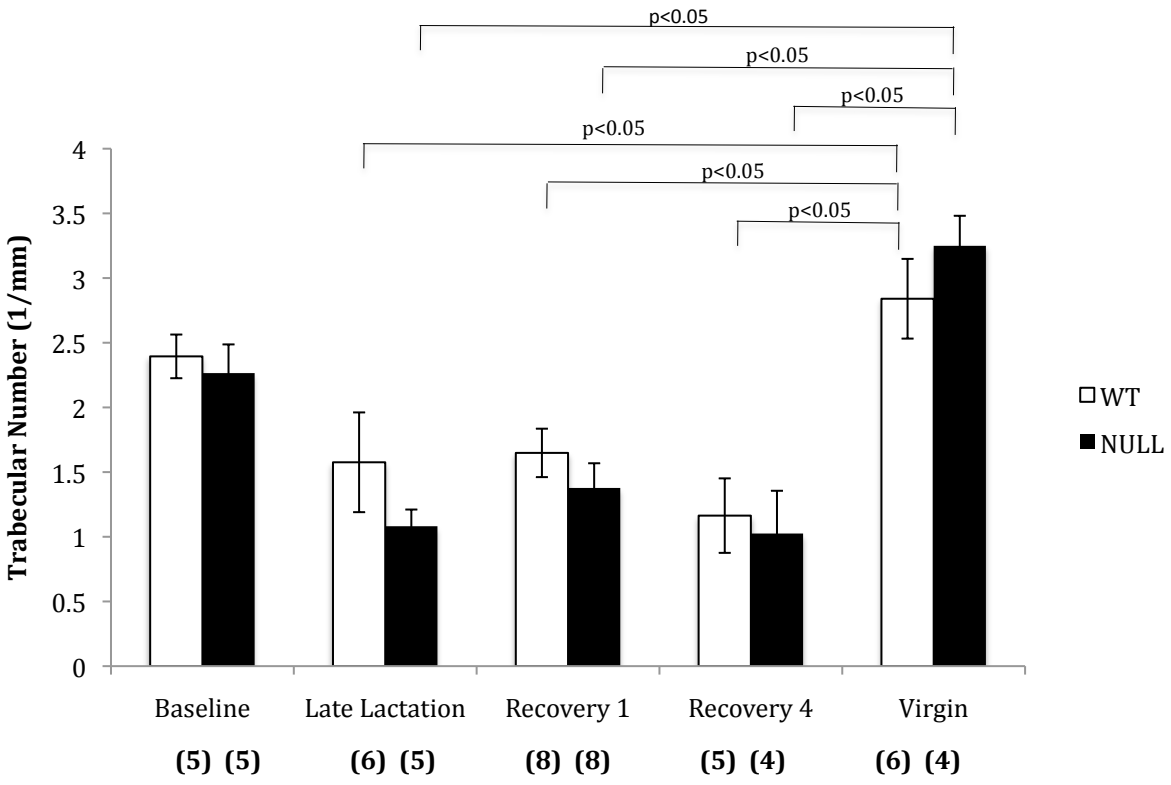


Figure 34: Femoral trabecular number of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Both genotyes display reduced trabecular number at late lactation, recovery 1, and recovery 4 as compared to virgin controls. Values are means ± SE and sample sizes are indicated in parentheses.

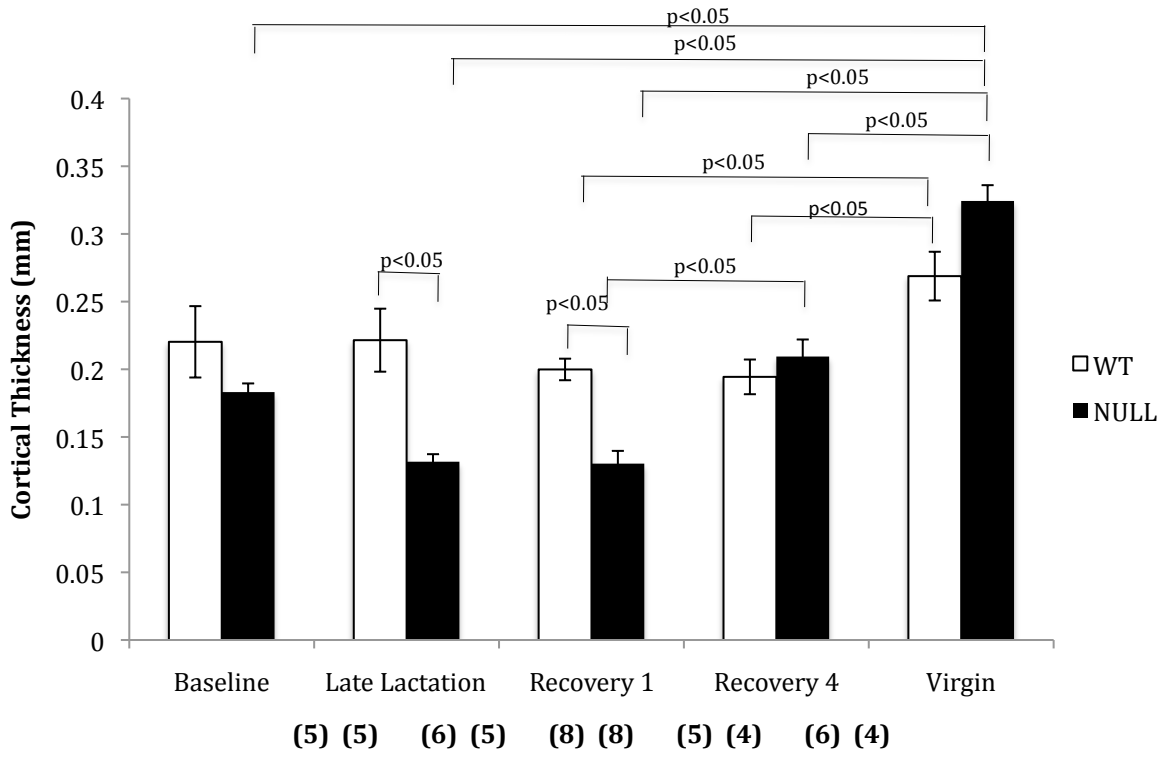


Figure 35: Femoral cortical thickness of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. WT mice have reduced cortical thickness at recovery 1 and recovery 4 in comparison to virgin controls. Null mice have reduced cortical thickness in comparison to virgin controls at all time points measured. Null cortical thickness is lesser than WT at late lactation and recovery 1. Values are means ± SE and sample sizes are indicated in parentheses.

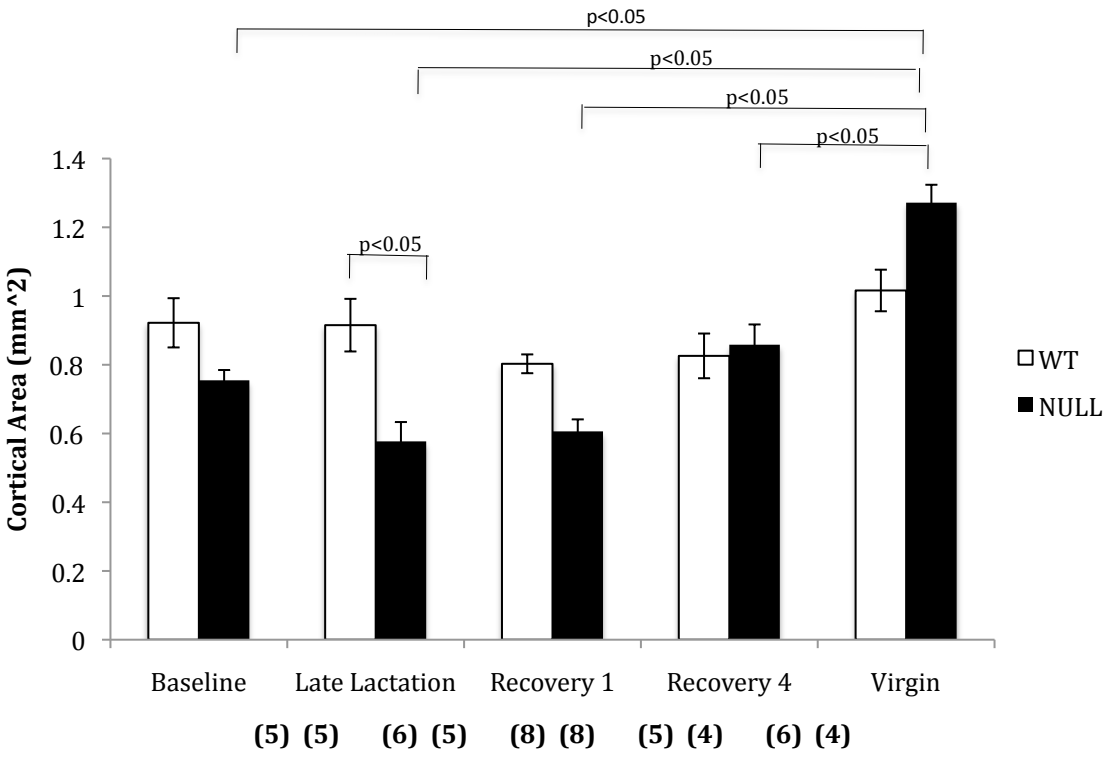


Figure 36: Femoral cortical area of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Cortical area is reduced compared to virgin controls at all time points in the null. The knockout mice also have lesser cortical area than WT at late lactation. Values are means ± SE and sample sizes are indicated in parentheses.

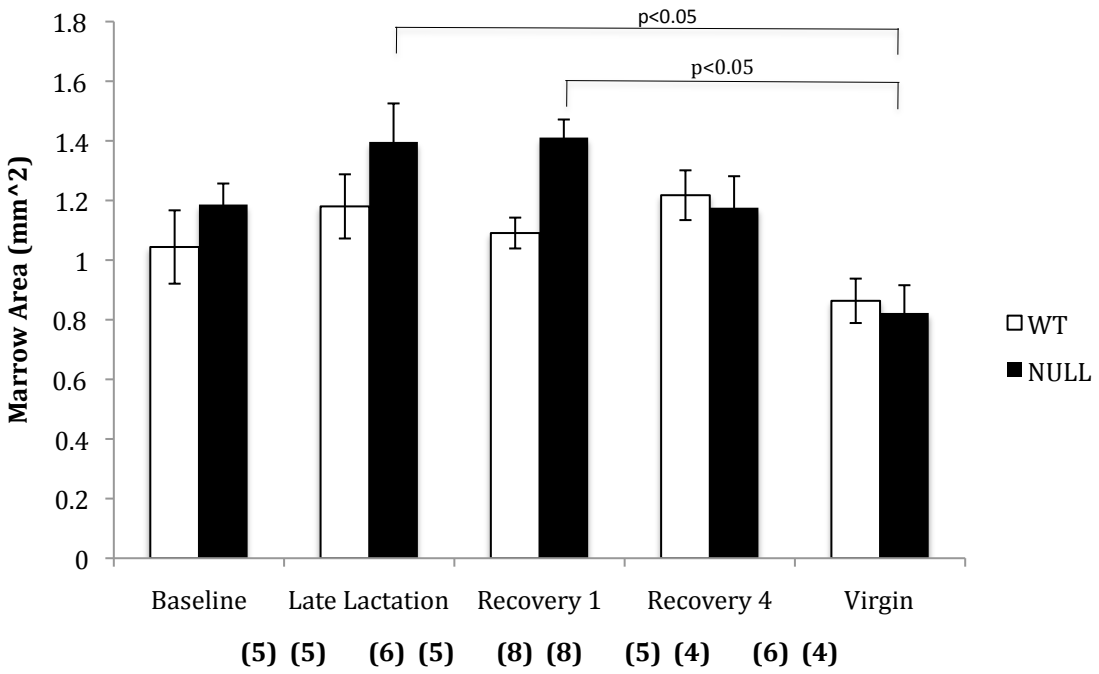


Figure 37: Femoral marrow area of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. Marrow area is increased compared to virgin controls at late lactation and recovery 1 in the null. Values are means ± SE and sample sizes are indicated in parentheses.

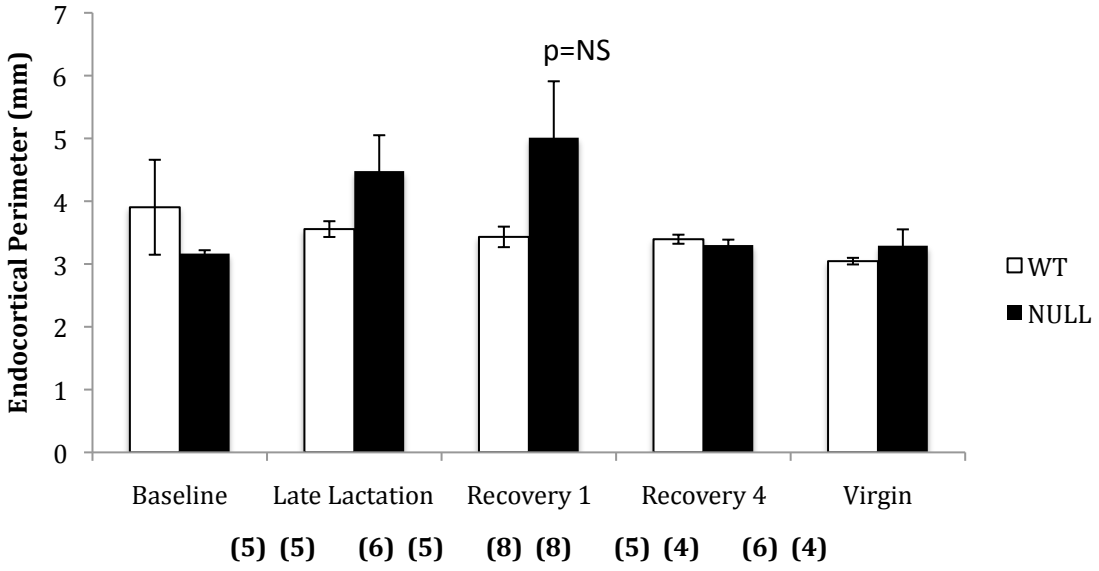


Figure 38: Femoral endocortical perimeter of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. No significant differences were measured at any time point between or within genotypes. Values are means ± SE and sample sizes are indicated in parentheses.

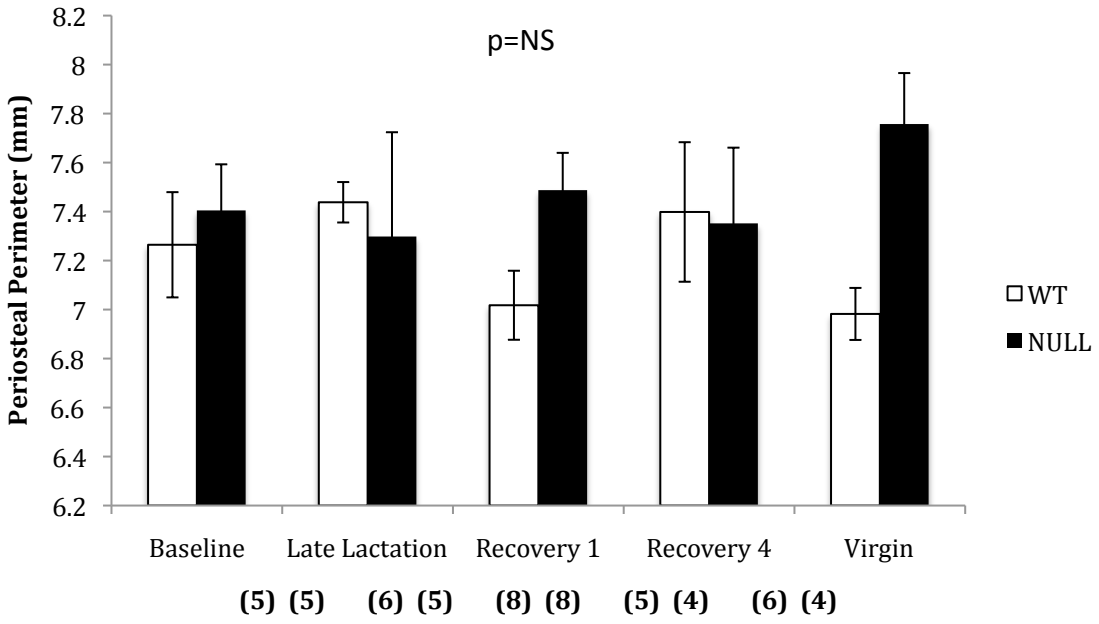


Figure 39: Femoral periosteal perimeter of WT and *Cyp27b1*$^{-/-}$ mice throughout the reproductive cycle. No significant differences were measured at any time point between or within genotypes. Values are means ± SE and sample sizes are indicated in parentheses.

3.7 3-Point bend test

A standard 3-point bend test was performed to assess the changes to cortical bone strength throughout the reproductive period. Tibias from normal and knockout mice were used to assess this at baseline, late lactation, recovery 1, and recovery 4. It was predicted that there would be decreased bone strength during lactation based on our results depicting BMC loss and μCT changes in trabecular and cortical parameters of the femora. BMC increases and improved cortical parameters then predict that there would be improved bone strength after recovery. As expected, *Cyp27b1* null and WT tibias have weaker bone at late lactation, which recovers fully by recovery 4 (Figure 40 to 43).

The ultimate load of force required to break tibias in WT was significantly decreased from baseline to late lactation, then significantly increased by recovery 4 (Figure 40). *Cyp27b1* null tibia take longer to regain their strength, displaying significantly reduced force required to break at late lactation and recovery 1 in comparison to baseline and recovery 4. The difference in speed of recovery is further evidenced by the lower ultimate load in the null at recovery 1 in comparison to WT (0.82 ± 0.05 gf vs. 1.86 ± 0.09 gf).

The significant changes seen in energy absorption (Figure 43) mirror those of the ultimate load of force required to break tibias. WT mice absorbed less energy prior to breakage at late lactation compared to baseline, which then increased by recovery 4. *Cyp27b1* null tibia show reduced energy absorbed from baseline to late lactation and recovery 1, then an increase by recovery 4. Between genotypes, *Cyp27b1* null shows reduced energy absorption prior to breakage compared to WT at recovery 1, confirming the increased length of time for strength recovery.

Tibial stiffness remains relatively constant in the WT throughout reproduction (Figure 42). Consistent with the indication of weaker bone in null tibia at recovery 1, knockout tibia display reduced stiffness in comparison to their baseline value (4.29 ± 0.38 gf/mm vs. 7.89 ± 0.50 gf/mm). The null also has reduced stiffness at recovery 1 in comparison to WT (8.89 ± 0.36 gf/mm).

Displacement of bone from its starting position prior to breakage is another measure provided by the by 3-point bend test and is an inverse measure of cortical bone strength. WT mice have constant displacement before tibial breakage throughout the reproductive cycle (Figure 41). In the null, displacement is significantly increased at late lactation and recovery 1 compared to recovery 4. Between genotypes, displacement is higher in the null at recovery 1 (0.88 ± 0.08 μm) and recovery 4 (0.63 ± 0.08 μm) as compared to WT (0.54 ± 0.05 μm at recovery 1 and 0.55 ± 0.08 μm at recovery 4) (Figure 41).

These results show that in both WT and *Cyp27b1* mice, cortical bone strength decreases during lactation and increases post-weaning, consistent with bone loss followed by recovery and the previous DXA and cortical μCT data. A vertebral crush test is needed to assess trabecular parameters.

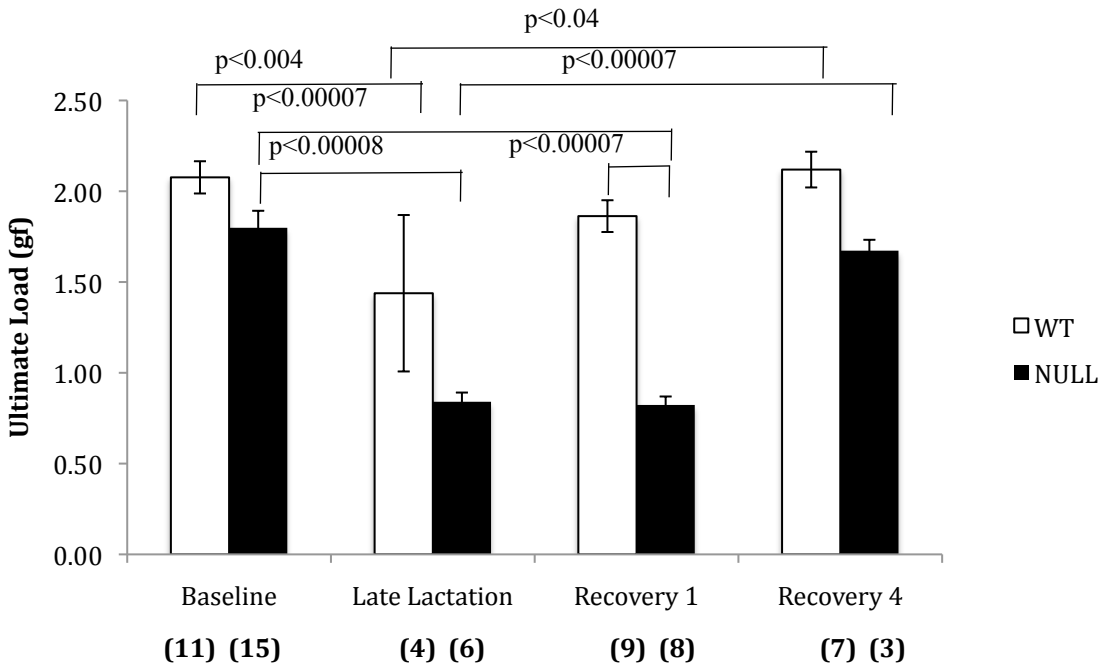


Figure 40: Ultimate load until failure using a standard 3-point bend test fixture throughout the reproductive cycle. Ultimate load required to cause tibia breakage showed a significant decrease from baseline to late lactation in both WT and *Cyp27b1* null mice, which significantly increased in both genotypes by recovery 4. *Cyp27b1* null tibias also required significantly reduced load to cause breakage at recovery 1 in comparison to WT. Values are means ± SE and sample sizes are indicated in parentheses.

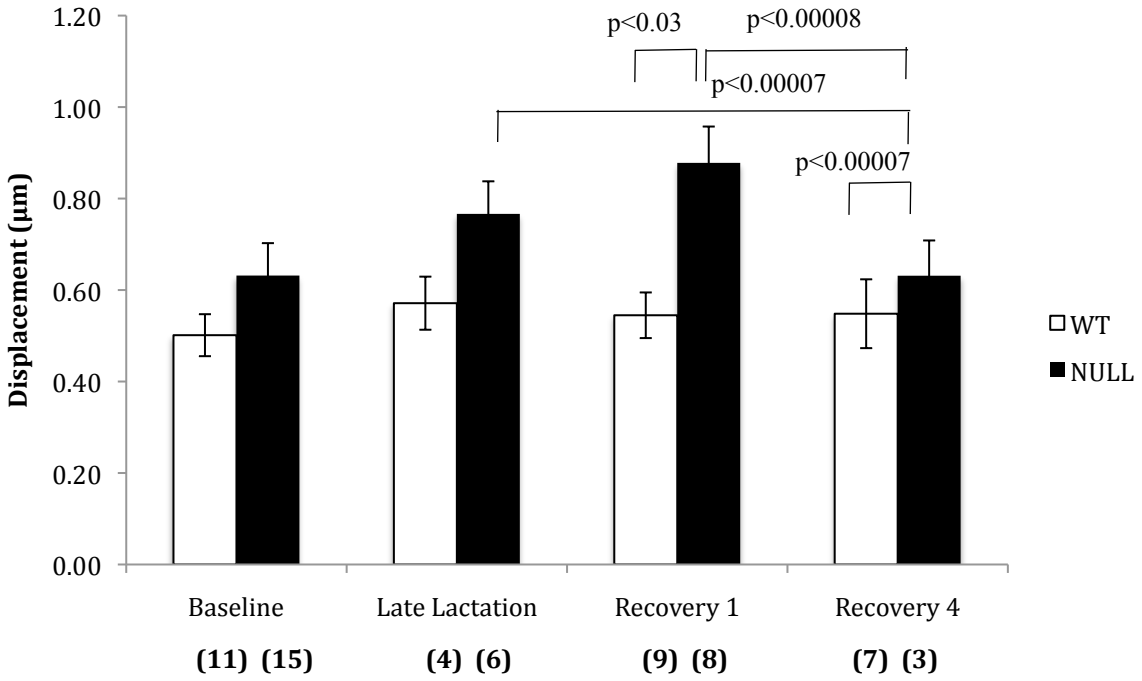


Figure 41: Tibial displacement before breakage using a standard 3-point bend test fixture throughout the reproductive cycle. Null tibias display significantly increased displacement before breakage at Recovery 1 and Recovery 4 in comparison to WT. This distance before breakage is also significantly reduced at Recovery 4 in comparison to Recovery 1 and Late Lactation in the *Cyp27b1* null. Values are means ± SE and sample sizes are indicated in parentheses.

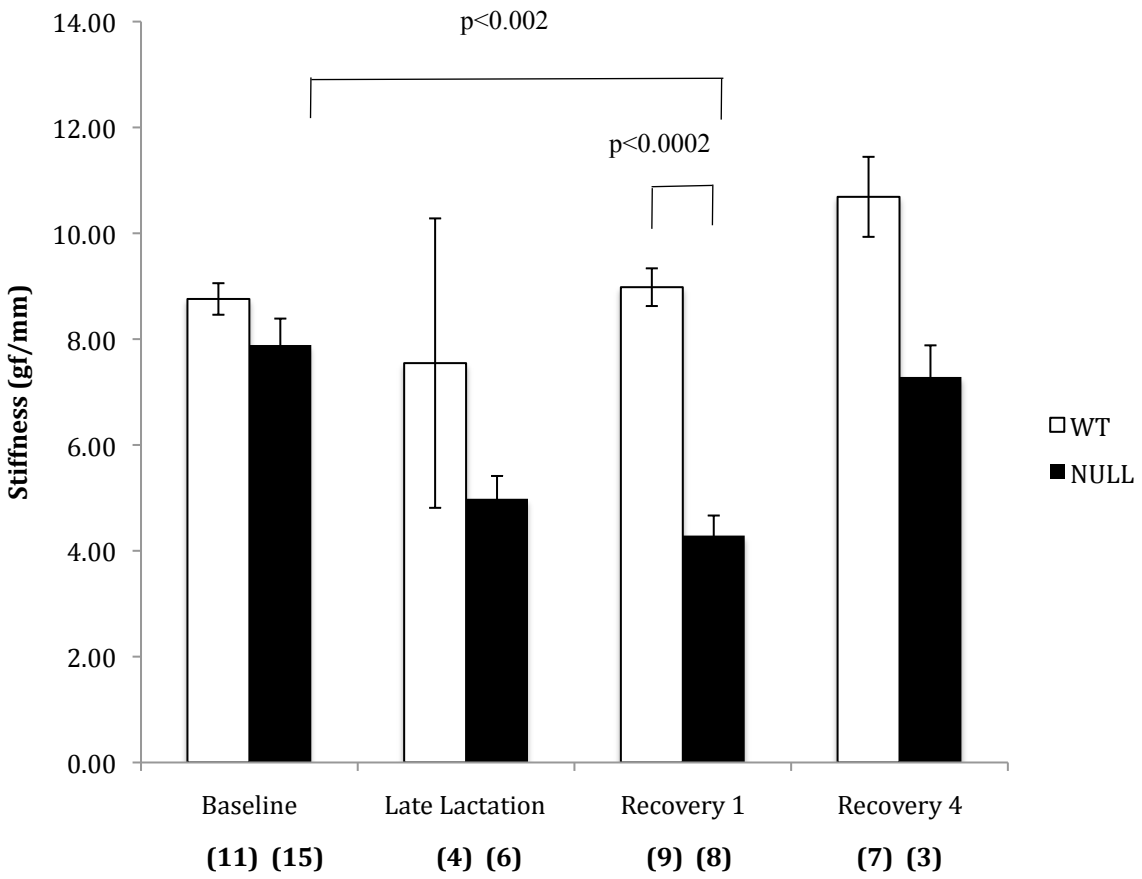


Figure 42: Tibial stiffness using a standard 3-point bend test fixture throughout the reproductive cycle. *Cyp27b1* null tibiae have significantly reduced stiffness at Recovery 1 in comparison to baseline. There is also significantly reduced stiffness in the null tibia in comparison to WT at Recovery 1. Values are means ± SE and sample sizes are indicated in parentheses.

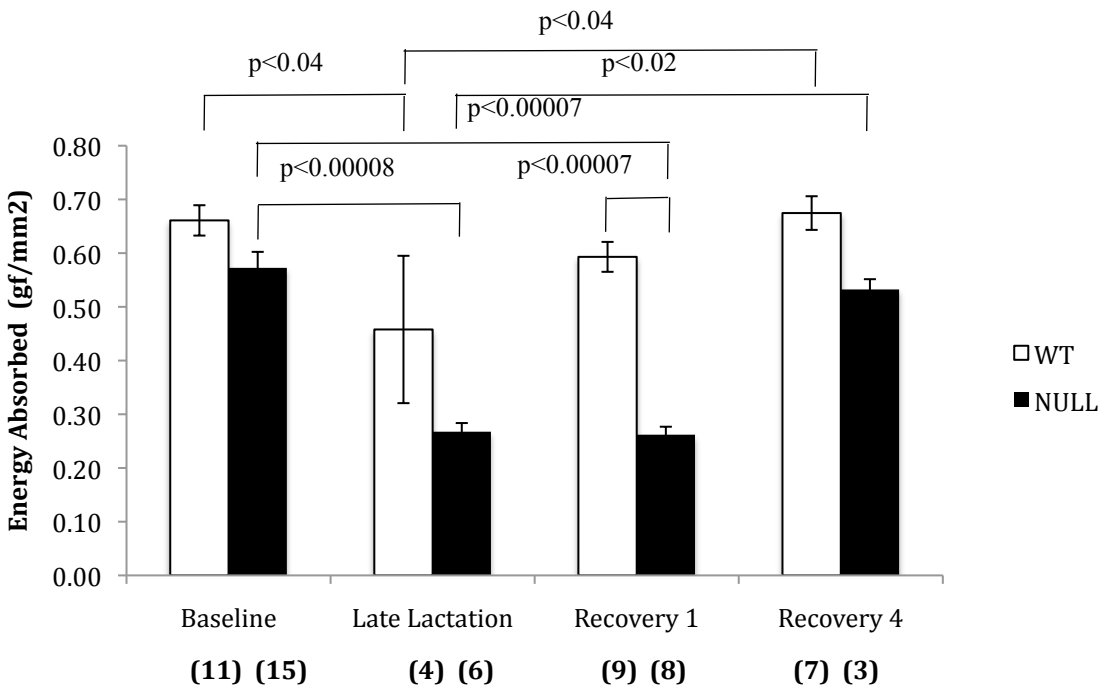


Figure 43: Energy absorbed by tibias using a standard 3-point bend test fixture throughout the reproductive cycle. Both *Cyp27b1* null and WT tibia absorbed significantly less energy prior to breakage at Late Lactation in comparison to Baseline, followed by a significant increase to Recovery 4. Null tibia recover this energy absorption later than WT, displaying significantly less energy absorbed at Recovery 1 in comparison to WT, and in comparison to its baseline value. Values are means ± SE and sample sizes are indicated in parentheses.

3.8 Intestinal calcium absorption

Normally, intestinal calcium absorption doubles during pregnancy in humans and rodents. This increase has been assumed to be caused by the increase in calcitriol; however, vitamin D deficient animals and *VDR* null mice still upregulate intestinal calcium absorption during pregnancy. ^{45}Ca injection via gastric gavage was performed and blood ^{45}Ca levels were analyzed as an indirect measure of intestinal calcium absorption. This was necessary to determine if *Cyp27b1* null mice have reduced absorption at baseline compared to the WT mice, and if despite the absence of calcitriol, null mice were able to upregulate intestinal calcium absorption during pregnancy and post-weaning. In this experiment, the amount of radioactivity absorbed into the bloodstream was found to be constant across baseline, late pregnancy, and recovery 1 in the WT (Figure 44). In the *Cyp27b1* null, the blood levels of radioactivity were significantly higher at late pregnancy than recovery 1. No significant differences between genotypes were measured.

Cyp27b1 null mice have increased instestinal calcium absorption during pregnancy, whereas WT mice had similar intestinal calcium absorption at all time points throughout the reproductive period.

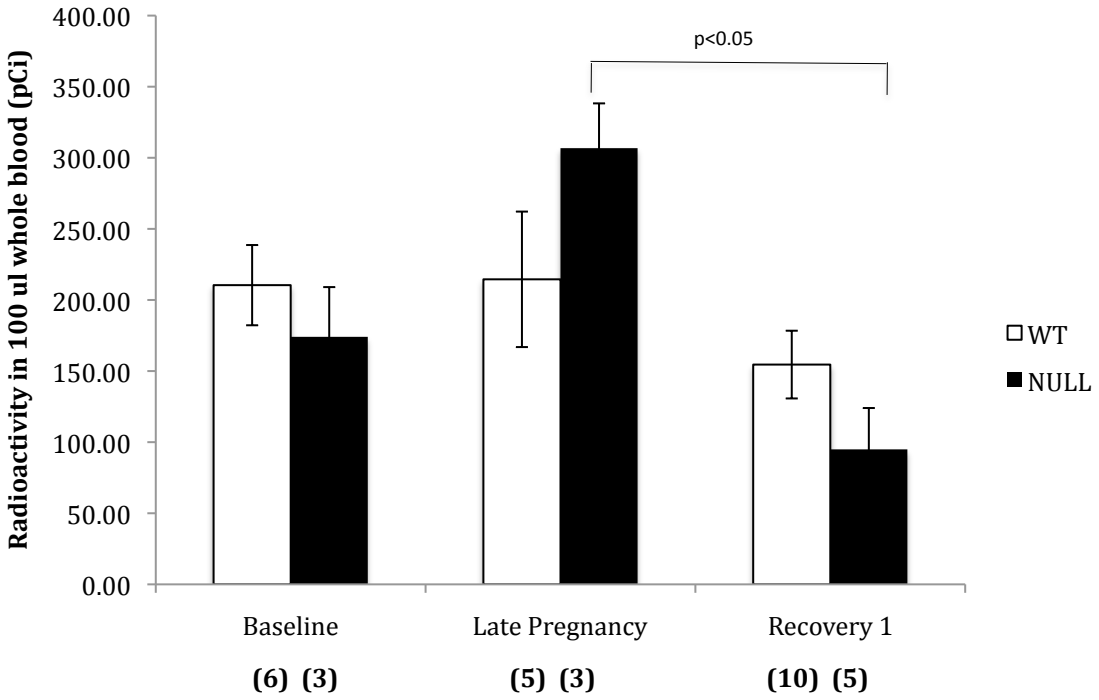


Figure 44: Radioactivity in 100 µl whole blood after injection of ^{45}Ca throughout the reproductive cycle. The *Cyp27b1* null had significantly higher radioactivity absorbed into circulation at late pregnancy compared to recovery 1. There were no significant differences in blood radioactivity between the genotypes at any time point. Values are means ± SE and sample sizes are indicated in parentheses.

IV. DISCUSSION

Calcitriol is the active form of vitamin D and plays a critical role in regulating calcium and bone metabolism in the child and adult, primarily through its actions to increase intestinal calcium absorption. Previous studies suggest that vitamin D and VDR are not required to regulate the adaptations to bone and intestines throughout reproduction. [27,120,147,151,152,154] However, these studies had several limitations including variable extent of skeletal recovery in vitamin D deficient rats, and uncertainty as to whether the very high calcitriol concentrations in the circulation of *Vdr* null mice are biologically active or not. It is possible that calcitriol is acting through non-genomic receptors to invoke the regulatory systems during pregnancy, lactation, and post-weaning recovery. [128,147,208-212] Our hypothesis was that calcitriol is required for upregulation of intestinal calcium absorption during pregnancy and post-weaning, and to enable normal recovery of bone mass post-weaning. If so, the high levels of circulating calcitriol would have enabled the *Vdr* null to have a normal skeletal and intestinal response during the reproductive cycle; therefore, we predict that inhibition of calcitriol synthesis would lead to a different bone and intestinal phenotype than loss of Vdr protein, with a reduced potential for BMC recovery after the demands of pregnancy and lactation. We tested this hypothesis by examining BMC and other bone phenotypes, biochemical measurements, and intestinal calcium absorption of calcitriol-deficient *Cyp27b1* null female mice during one reproductive cycle with matched litter sizes.

Compared to WT, at baseline *Cyp27b1* nulls were hypocalcemic with markedly increased PTH and 30 % lower BMC; μCT displays a trend of reduced femoral length, cortical thickness, and cortical area. During pregnancy in *Cyp27b1* nulls, serum calcium increased to normal values,

PTH substantially reduced to approach WT values, and BMC increased 45 % so that all values became equivalent to WT. During lactation in *Cyp27b1* nulls, serum calcium dropped, PTH increased to similar values seen prior to pregnancy, BMC declined to baseline, and cortical bone strength decreased; μCT confirmed extensive endocortical resorption in addition to loss of trabecular number and thickness. During post-weaning in *Cyp27b1* nulls, serum calcium normalized again, PTH remained at very high levels, BMC increased to 40 % above pre-pregnancy values, and cortical bone strength recovered to pre-pregnancy levels. Although there was recovery of cortical bone, μCT shows that both WT and *Cyp27b1* nulls still had reductions in femoral trabecular bone volume at 4 wks post-weaning. Whether these changes would resolve or persist is presently unknown. In comparison to WT mice, the *Cyp27b1* null females display low (probably undetectable) levels of calcitriol and significantly lower levels of FGF23 throughout the reproductive cycle. Blood ^{45}Ca levels indicative of intestinal calcium absorption were increased during pregnancy in the *Cyp27b1* null, while WT mice displayed constant levels throughout the reproductive period.

4.1 Influence of the rescue diet on baseline phenotype

In order to support fertility, *Cyp27b1* null females and WT females derived from heterozygous *Cyp27b1*$^{+/-}$ breeders were fed a 2 % calcium "rescue" diet from the time of weaning. At baseline, *Cyp27b1* null mice have reduced BMC, reduced femoral cortical thickness and area, hypocalcemia, and marked secondary hyperparathyroidism. These changes occur despite the "rescue diet", suggesting that the diet does not maintain normal mineral homeostasis and bone mass in mice that have reached 10 wks of age. This is largely in contrast to previous literature.[33,38,130] Dardenne studied the same model we used, and found that the rescue diet corrected

hypocalcemia and secondary hyperparathyroidism, and histomorphometry confirmed that rickets and osteomalacia was cured. [33] There are a number of reasons as to why our results may be in contrast to the rescued rachitic phenotype shown by these authors. Primarily, our mice were studied at an older age, beginning at 10 wks as compared to 8.5 wks. Also, some of the methodologies used to analyze the bone and mineral metabolism were different. Dardenne performed serum biochemical measurements of calcium and phosphorus using a Monarch automated analyzer, whereas we utilized a spectrophotometer. Histomorphometry was used rather than μCT to analyze the microarchitecture of bone, and they did not measure BMC in any way. Moreover, the model went through some years of subsequent inbreeding in their facility before being sent to us, and so the mice may have drifted genetically from what was originally created. Although we backcrossed the mice into C57BL/6 as soon as we received them, the mice we studied may be genetically distinct from the original strain that Dardenne studied.

Two other groups used a different and independently created *Cyp27b1* null model, and found that the enriched diet fully rescued or prevented the rachitic phenotype. As explained in section 1.1.11.5, both Panda and Sun used a model that had the *Cyp27b1* gene fully ablated by deletion of both the hormone binding domain encoded by exons 6 and 7, and the heme binding domain encoded by exon 8. [38,130] In contrast, our model (the same one originally reported by Dardenne) only had exon 8 deleted, with part of introns 7 and 8 (Figure 5). These differences in engineering conceivably could have led to the differences seen in serum calcium, phosphorus, and PTH. There were also several differences in their methodologies, including an autoanalyzer for serum calcium and phosphorus, and computer-assisted image analysis of hematoxylin and eosin staining or histochemical staining rather than μCT. However, Panda did find several skeletal

alterations with reduced trabecular volume, reduced osteoid volume, and reduced osteoblast number, similar to our findings. Thus, these changes in bone volumes and osteoblast numbers suggest that the enriched diet did not fully prevent the rachitic phenotype. These mice were studied at 15 wks. They suggest that it is possible that the mice become more dependent on calcitriol with age, and that prolonged exposure to the diet results in skeletal abnormalities. Furthermore, although Panda and Sun studied pairs of female siblings, only Sun generated the colony and experimental mice by mating heterozygous males and females together. Panda maintained their colony by mating null males to heterozygous females, which could have created null mice that drifted genetically from WT. These mice were also maintained on a mixed genetic background with contributions from C57BL/6 and BALB/c strains, which may have resulted in considerable variation in the mouse phenotype. [222,223]

To summarize, Dardenne, Panda, and Sun all found normal levels of serum calcium and phosphorus in *Cyp27b1* mice on the rescue diet, with no or modest changes to the skeleton. The most striking difference was the PTH level, which was equivalent to WT levels on the rescue diet as measured by both Dardenne and Panda, whereas our studies show significant secondary hyperparathyroidism. Dardenne used the same mouse intact PTH ELISA kit as the one we employed (Immunotopics), whereas Panda used a two-site immunoradiometric assay (IRMA) (Immunotopics). It is also critical to note that Sun did not measure PTH, and it is therefore possible that the level could have been increased. The diet may be ruled out as a potential cause for these differences, as the same manufacturer and formula was used in the previous studies (TD.96348, Teklad) as was in ours, except ours had a green colour coding (TD.94112, Teklad). Our studies analyzed different details, using DXA as reflective of mineralization and μCT for

structural information, in comparison to the histomorphometry or image analysis of staining. Histomorphometry would have shown increased surface area and thickness of unmineralized osteoid, which is pathognomonic of rickets. A limitation of histomorphometry, however, is that it examines a sample (one or a few sections) of the tibia or femur, or the vertebrae, and assumes that the rest of the bone has the same structure. MicroCT examines all of the bone in the region of interest, mitigating the error from selective sampling. We still plan to do histomorphometry, but for the purposes of completing a Masters degree, the work is beyond the time possible. Nonetheless, we may have detected a phenotype that slowly emerges as the mice get older that Dardenne stopped too early to detect, and numerous other reasons may compensate for the differences seen with Panda and Sun. From our results, the diet does not normalize mineral and skeletal homeostasis because serum calcium, PTH, and bone mass are not normal by 10-12 wks of age. Age-related changes in the permeability of the intestines to passive calcium absorption may mean that the rescue diet becomes insufficient with age, or it could be that there is still a role for calcitriol in older mice to regulate bone metabolism directly or influence intestinal calcium absorption independent of diet.

4.2 Adaptations to reproduction

A variety of experiments were conducted to determine if the loss of calcitriol synthesis would impair intestinal calcium absorption and skeletal metabolism despite the presence of the enriched calcium diet. Contrary to our initial hypothesis and to their impaired mineral homeostasis and reduced bone mass at baseline, the *Cyp27b1* null females had significantly improved bone metabolism (BMC and cortical bone strength) post-weaning and efficiency of intestinal calcium absorption was increased during pregnancy. We expected to be studying *Cyp27b1* null females

that were phenotypically indistinguishable from WT females because of use of the rescue diet, but instead the mice had several deficits at baseline. Having lower BMC, reduced femoral cortical thickness and area, hypocalcemia and secondary hyperparathyroidism, may have actually been more beneficial to the research because it revealed that pregnancy and post-weaning caused improvements in bone mass and mineral homeostasis independent of the constant presence of the rescue diet. In fact, this turned out to be the case as pregnancy improved certain parameters, including serum calcium, BMC, and secondary hyperparathyroidism.

4.2.1 *Cyp27b1* null response to pregnancy

Pregnancy is characterized by significant demand on the mother to provide calcium for fetal development. In my experiments, it was observed that pregnancy improved the *Cyp27b1* null female dam. BMC increased during pregnancy, such that the low bone mass of the *Cyp27b1* null females relative to WT females at baseline became equal to WT females during pregnancy. Hypocalcemia and secondary hyperparathyroidism were also rescued at this time point. This likely indicates increased intestinal calcium absorption via calcitriol-independent mechanisms that resulted in mineralization of osteoid and suppression of PTH release. The ^{45}Ca in blood increased during pregnancy in the *Cyp27b1* null compared to baseline, suggesting an improvement in intestinal calcium absorption during pregnancy. These results are very similar to what was seen in the *Vdr* null female mice; despite the absence of a functional receptor for calcitriol, the *Vdr* null females also showed BMC increases of 58 % and a reduction in PTH during pregnancy. [147] The *Vdr* nulls were studied using a different methodology for intestinal calcium absorption (duodenal loop method), but did show a significant increase from baseline to pregnancy.

4.2.2 *Cyp27b1* null response to lactation

During lactation, milk production places further demands on the mother's calcium resources. In mice, the dam mitigates against the greater demand by increasing intestinal calcium absorption and resorption of the skeleton. Humans accomplish this by relying on increased resorption of the skeleton, while intestinal calcium absorption is normal. Reducing the calcium intake of mice increases skeletal resorption whereas increasing calcium intake has the opposite effect. *Cyp27b1* null female mice lost a significant amount of bone in the hind limb as compared to pregnancy using the revised analysis of BMC. Both the first and revised analysis also showed that the null had significantly lower BMC compared to WT at this time in whole body and hind limb. These losses were actually greater than what the *Vdr* nulls experienced, as their BMC at late lactation was equal to WT. [147] The 3-point bend test corroborated a loss of cortical bone strength in the null tibiae during this time. μCT results provide further insight into the loss of bone during lactation. The adaptations to trabecular bone are largely the same between genotypes, with both displaying lower bone volume, reduced trabecular number, and increased trabecular separation during this time. However, the *Cyp27b1* null female mice experienced greater changes to cortical bone than their WT counterparts. Loss of calcitriol results in significantly lower cortical thickness and cortical area. This may suggest that calcitriol provides a protective effect on cortical bone during lactation. The significantly increased marrow area and trend toward increased endocortical perimeter indicates that the changes to cortical bone occur largely due to endocortical resorption in these areas rather than a reduction in periosteal apposition.

One possible explanation for the BMC, cortical bone strength, and structural changes in the skeleton of the *Cyp27b1* null female mice is that *Cyp27b1* null mice have had less calcium

absorbed through the diet, so they resorb more bone in compensation. It is possible that intestinal calcium absorption was not upregulated during lactation. Alternatively, there could have been no difference in intestinal calcium absorption, but that loss of calcitriol directly leads to increased PTH secretion and consequently, bone resorption. Loss of calcitriol could also have affected milk production, creating an increased drain on the supply that results in compensatory bone resorption. Measurement of the calcium content of milk in *Cyp27b1* null and WT mice could determine if this is the case or not.

Despite significant resorption of bone seen in the knockout during lactation, *Cyp27b1* nulls became markedly hypocalcemic. Secondary hyperparathyroidism accompanied the decrease in serum calcium. Urine calcium excretion decreased in both genotypes throughout lactation, suggesting that calcium was not being lost in the urine but rather being used to produce milk. These results are indicative of the maternal challenge to provide adequate mineral to their growing pups. Serum phosphorus also decreased in the null at late lactation, however, a non-significant increase of the mineral was measured in the urine. WT dams are carrying WT and heterozygous pups while *Cyp27b1* null dams are carrying heterozygous and null pups. Both dams carried similar numbers of pups, but the possibility that the different genotypes of the pups somehow influenced the maternal responses to pregnancy and lactation has not been excluded. The dams will consume their own urine and feces, which is a source of calcium, and unavoidable. Since WT and *Cyp27b1* null dams had similar urine calcium excretion, and consumed the same diet and water, the calcium available to consume was therefore similar between the two groups.

Intestinal calcium absorption was not measured during lactation, however it is critical to know whether it is reduced in the *Cyp27b1* nulls at this time point. If so, it could imply that placental factors, such as placental lactogen, are responsible for upregulating intestinal calcium absorption during pregnancy, and that these factors are lost during delivery. However if intestinal calcium absorption is upregulated equal to WT, then loss of calcitriol may be leading to effects on milk production, parathyroid glands, or bone loss independent of intestinal calcium delivery. A future direction for this project is to measure intestinal calcium absorption and milk calcium content in the WT and *Cyp27b1* null females during lactation, to address this specific hypothesis.

4.2.3 *Cyp27b1* null response to post-weaning recovery

Following the 3 wks of lactation, there is clear evidence in *Cyp27b1* null females for improvements in bone and mineral metabolism despite the absence of calcitriol. An increase in BMC was measured by DXA in both genotypes, to a value higher than what was measured at baseline. This recovery of bone mass was measured in whole body, lumbar spine, and hind limb in both the initial data set and the revised protocol with mice placed in clean cages, to avoid the confounding factor of coprophagia during the pre-assay fasting period. Collectively, this likely indicates calcitriol-independent stimulation of new bone formation. This is similar to the recovery seen in *Vdr* null female mice, which also showed increased BMC to a value higher than pre-pregnancy baseline. [147]. MicroCT suggests that there may also be a boost of longitudinal growth in the nulls during this time. Cortical bone increased in both genotypes, as evidenced by the increase in cortical thickness and cortical area. However, the changes to trabecular bone did not recover within the 4 wks of study, as shown by the consistent decrease in bone volume at the fourth wk post-weaning. These site-specific changes in bone microarchitecture may be explained

by the properties of long bones. Similar to previous research, our results suggest that long bones may have lasting alterations in trabecular structure after lactation. [156,201] It has been proposed that different skeletal sites may resorb bone using different mechanisms. [201] The rods and plates of long bones are perforated and broken during lactation, impairing the ability to restore microarchitecture. Conversely, bone loss in the spine may occur through thinning and perforation of trabecular plates, allowing for retained connectivity and a filling of the perforations after lactation. Performing microCT on the spine would help provide further evidence of this theory, and is a future direction for this project. Despite trabecular bone not recovering post-weaning, the 3-point bend test shows a gain in bone strength in the null female tibiae from late lactation to recovery. This test is largely a measurement of cortical bone properties, which did recover to normal. Thus, long-term bone strength may be normal after lactation in the *Cyp27b1* null female because of recovery of cortical bone parameters and despite permanent changes in trabecular architecture.

In contrast to the hypocalcemia that developed during lactation, serum minerals normalized during the post-weaning interval, with calcium and phosphorus being equal between the 2 maternal genotypes. The blood ^{45}Ca experiments further suggest that intestinal calcium absorption is equivalent to WT post-weaning despite the absence of calcitriol. It may be suggested that intestinal calcium absorption is maintained by the influence of other hormones when calcitriol is absent. An alternative explanation is that intestinal calcium absorption was similar during both lactation and recovery, and that the main problem for the null mice was the drain of calcium into milk. Shutting off milk production may have allowed serum calcium to

normalize and facilitate bone recovery without implying any change to the rate of intestinal calcium absorption. Again, measuring the calcium content of milk should prove informative.

Of interest, PTH still remained significantly higher in the *Cyp27b1* nulls as compared to WT at the recovery 1 time point. Serum calcium was no different between WT and null 1 wk following weaning; however, the levels continued to rise in the null until the fourth wk. It is possible that PTH may have normalized in the null after 4 wks post-weaning, or it could also be the case that hyperparathyroidism is a persistent mechanism to compensate for the loss of calcitriol. Perhaps the unknown factors that helped the *Cyp27b1* null mice during pregnancy were wearing off, consequently reverting to the baseline state of elevated PTH. This may include intestinal calcium absorption dropping back to lower values. In the long run, such high PTH levels will be deleterious to bone.[10]

Taking into consideration all of the changes in PTH levels measured throughout the reproductive cycle, it suggests that pregnancy is a key time frame in which calcitriol-independent alterations in bone metabolism occur which rescue the null. During lactation, the loss of calcitriol may compromise the *Cyp27b1* null mice to resorb more bone and lose proportionately more bone strength. The recovery of bone mass during the post-weaning interval is also indicative of temporary calcitriol-independent effects. After all, the *Cyp27b1* nulls have low bone mass at baseline but are increasing their bone mass significantly during post-weaning. Something is clearly different about the post-weaning interval (with active bone formation occurring) as compared to pre-pregnancy (when bone mass was reduced compared to WT).

4.3 Bone mineral content

BMC was analyzed using two different methodologies; in the first method mice were fasted overnight and left in original cages to be scanned the following day, and in the second method mice were changed to new cages and similarly fasted overnight. Both analysis strategies showed that BMC of *Cyp27b1* null females was lower than WT at baseline, increased during pregnancy to become equivalent to WT, declined to baseline during lactation, and increased post-weaning to a value higher than pre-pregnancy. The revised analysis was performed because we noticed that the drops in BMC during lactation were less than expected, especially in the lumbar spine, and wondered if this was due to retention of calcium in the gut from the mice eating their calcium-rich feces. Rather, the revised method showed reduced losses in the lumbar spine during lactation, and greater losses in the hind limb. If coprophagia was really having an effect on the BMC, it was expected that placing mice into clean cages would have exaggerated the losses in lumbar spine rather than reducing them. Furthermore, the differences in hind limb seem to be independent from changing to new cages, as any intestinal retention of calcium would not be measured in this region. Therefore, these changes are thought to be unrelated to the variable of coprophagia and more in line with general variation. It is noted that the majority of the biochemical assays on serum and urine included samples taken from both the first and revised method. However, because changing to new cages likely had no direct effect on the BMC, this should also not have affected the mineral physiology.

4.4 intestinal calcium absorption

^{45}Ca in blood increased during pregnancy in the *Cyp27b1* null compared to recovery, suggesting that intestinal calcium absorption is upregulated during this time. WT mice had similar levels of

intestinal calcium absorption throughout the reproductive period, and there were no differences between genotypes at any time point. Contrary to our hypothesis, this suggests that calcitriol is not required for upregulation of intestinal calcium absorption during pregnancy. The data do raise several questions however, as WT mice did not show the same increase in intestinal calcium absorption during pregnancy, and neither genotype maintained an increase post-weaning.

A variety of possible reasons may explain these unexpected results. The setting of the experiment did not allow for calculation of absolute calcium absorption. The original investigators of the oral gavage technique reported both the gavage results and the ^{45}Ca results, which were consistent with narrow confidence intervals. [221] Similar to subsequent investigators however, we have only reported the blood ^{45}Ca results as the gavage results were too variable and therefore uninterpretable. [26,224-226] In this manner, blood ^{45}Ca still provides an estimation of both active and passive mechanisms across the entire gastrointestinal tract. As a future direction for the project, modifying the gavage protocol to accurately assess the radioactivity in the intestines may provide a more direct measure. Additionally, intestinal calcium absorption should be different on a 0.47 % calcium diet vs. a normal 1 % calcium diet vs. an enriched 2 % calcium "rescue diet". It is possible that excess calcium in the diet may be greatly increasing passive absorption, which is maintained during all of the three time points measured. As a future direction, measurement of the changes in relevant gene expression within the duodenum would help to provide further confirmation of the increase in intestinal calcium absorption during pregnancy.

Lastly, the rise in BMC and marked decline in PTH during pregnancy are independent confirmation that calcium delivery through the intestines must have improved during pregnancy, as indicated by the rise in ^{45}Ca in blood. Again this implies that calcitriol is not required for upregulation of intestinal calcium absorption during pregnancy.

4.5 Confirmation of calcitriol absence in the *Cyp27b1* null mouse model

The aforementioned results all suggest that skeletal mineralization and formation occur independently of calcitriol during pregnancy and post-weaning. It was therefore pertinent to this research to confirm that calcitriol was in fact absent in our *Cyp27b1* null mouse model throughout the entire time period of reproduction. Calcitriol had been measured previously by our collaborators at McGill who created the mouse model, and in their publication they reported "not detectable" levels in the null at both 3 wks and 8 wks of age. [30] That is, they described the result as "not detectable" but did not report a numerical value. Calcitriol was also measured in the other model by Panda, who similarly reported "undetectable" without stating a numerical result. [38] Consequently, what value either of the assays actually yielded is unknown. In this thesis work, the detection limit of the EIA kit that we used was 6 pmol/L in humans. Calcitriol levels in the *Cyp27b1* null mouse at baseline were near the human detection limit with a mean of 12 pmol/L. This could indicate that 12 pmol/L is the actual limit of detection in mice, and that calcitriol is undetectable in our knockout model. Conversely, it could also indicate that a low level of calcitriol can be formed despite the absence of *Cyp27b1*. It is likely that the former is correct, because the assay uses mouse antibodies and is designed for assay of human serum; the detection limit for mouse serum has not been reported. Therefore, a non-specific increase in the apparent limit of detection is likely whenever mouse serum is used in an assay using antibodies

raised in mice. It is far less likely that the mice are still making calcitriol despite absence of the only gene known to be involved in calcitriol formation; however, the possibility exists as the full annotation of gene and protein functions continues in mice and humans. [30,31,56]

The level of calcitriol in the *Cyp27b1* null is not statistically lower than WT at baseline, although it was numerically lower; about one quarter that of WT (12 pmol/L vs. 46 pmol/l). The lack of statistical significance is likely due to the enriched diet suppressing expression of calcitriol in the basal, non-pregnant state, and due to the low sample size. Previous research found a significant difference between the two genotypes at baseline, however these mice were on a diet of regular chow and the WT mice had greater levels of calcitriol (150 pmol/L) than found in our study (46 pmol/L). [30] A larger sample size in our study would likely have helped to differentiate what really is a true difference between WT and *Cyp27b1* null calcitriol levels at baseline.

4.6 Source of the rise in maternal calcitriol during pregnancy

Calcitriol normally increases two- to five-fold during pregnancy, and as previously described; a persistent belief has been that the placenta is the source. [21,137] Measuring calcitriol was, therefore, also critical to elucidate the source of calcitriol during the pregnancy-induced rise, whether it came from the fetus and placenta or maternal kidneys. Importantly, the assay indicated that calcitriol rose significantly as expected in WT during pregnancy (46 pmol/L to 284 pmol/L) and lactation (350 pmol/L), and this increase was absent in *Cyp27b1* nulls. This suggests that the pregnancy-related increase in calcitriol comes from the maternal kidneys and not the placentas. If the placentas were a significant source of calcitriol, then the presence of *Cyp27b1*$^{+/-}$ placentas should have resulted in a large increase in serum calcitriol in the *Cyp27b1* null mothers mated to

HET males. There was a numerical increase in mean calcitriol of pregnant, null females, in the assay from 12 pmol/L to 79 pmol/L that was not statistically significant. This could mean either that there was no real change in calcitriol, or that the placentas do contribute a small amount of calcitriol to the maternal circulation, but not enough to explain the normal two- to five-fold increase in pregnant WT mice. This was not a main endpoint of the study and so we were not rigorously testing the idea. Should we wish to confirm this further, the rise in calcitriol during pregnancy could be analyzed more fairly by comparing *Cyp27b1* null and WT mothers that each bore only heterozygous pups, by mating *Cyp27b1* null and WT mice to WT and null males, respectively.

4.7 FGF23 during pregnancy

There is minimal research on FGF23 during pregnancy, other than prior studies showing that the serum concentration doubles in the mother. [56,140] Our results show that the changes in FGF23 largely mirror the changes in calcitriol, rather than any fluctuations in serum phosphorus. During pregnancy, the WT mice experience a significant rise in FGF23 while this is blunted in *Cyp27b1* nulls. The WT levels decrease at late lactation and recovery 1, however, they still remain significantly higher than the FGF23 serum concentrations of the knockout females. This confirms that the increase in FGF23 may be largely driven by calcitriol during pregnancy.

4.8 Non-VDR mediated actions of calcitriol

Our hypothesis stated that calcitriol is required for upregulating intestinal calcium absorption during pregnancy and post-weaning, and to enable normal recovery of bone mass post-weaning, such that the high calcitriol levels in the *Vdr* null mice are able to act through non-VDR

receptors to have these effects. Contrary to that hypothesis, our results indicated that *Cyp27b1* null mice had significantly improved bone and mineral metabolism during both pregnancy and post-weaning despite the deficiency of calcitriol. Serum calcium increased, PTH substantially reduced, intestinal calcium absorption increased, and BMC increased during pregnancy. They lost a normal amount of bone during lactation (more than normal in the hind limb), but this was followed by a normalization of serum calcium, BMC increase, femoral length increase, and recovery of bone strength and cortical parameters during post-weaning. Bone mass achieved by 4 wks of post-weaning actually exceeded the pre-pregnancy value. These results are largely similar to what was seen with loss of the VDR in *Vdr* null mice. Mice lacking calcitriol's receptor upregulated intestinal calcium absorption, mineralized osteoid, and increased BMC during pregnancy, lost a normal amount of bone mass during lactation, and recovered BMC post-weaning to a value significantly higher than prior to pregnancy. [147] Overall, our data suggest that calcitriol was not acting through alternative signalling mechanisms to upregulate bone formation in the *Vdr* null. Instead, the similar phenotypes of *Cyp27b1* null and *Vdr* null mice during the reproductive cycles suggest that calcitriol's actions were lost in both, and that the adaptations to pregnancy, lactation, and post-weaning were achievable despite loss of calcitriol's actions. The high serum calcitriol levels observed in the *Vdr* null females are likely a response to absence of the receptor but do not imply retained biological activity of calcitriol in the absence of its receptor. Calcitriol is likely not required to regulate intestinal and bone metabolism during reproduction, and our results do not support the existence of alternative signalling mechanisms for calcitriol action.

4.9 Limitations

Although animal models provide excellent starting points for research, their limitations must be recognized with respect to the elementary findings and applications to the etiology of human disease. All mammals experience bone loss during lactation followed by a full recovery post-weaning; however, there are clear differences in their physiological adaptations to pregnancy compared with humans. Mice are polytocous whereas humans generally nurse singletons, such that there are enhanced skeletal losses and recovery in mice. Rodents therefore upregulate intestinal calcium absorption during lactation while humans do not need this mechanism to obtain adequate calcium. Comparisons of the recovery process between species may therefore be made with caution, and any results require translation and verification in humans. Additionally, it may be questioned whether the results found here are specifically due to the loss of calcitriol, or whether indirect effects come into play, such as genetic ablation of the *Cyp27b1* gene and compensation by marked hyperparathyroidism. There were also significant differences in the mouse phenotype using the enriched diet as compared to previous research as discussed, which could have affected the results. In the future, conditional mouse models of loss of calcitriol during post-weaning recovery may be useful for teasing out the specific role of calcitriol during this time point. It also would have been useful to analyze the diet and confirm its contents to further investigate why it was not adequate to prevent osteomalacia and secondary hyperparathyroidism in the *Cyp27b1* null mice. However by the time this became apparent, the batch of diet had all been consumed.

The enriched diet was necessary for the mice to become pregnant promptly but it confounded the situation. WT mice did not show the normal magnitude of excursions in bone mineral content

during lactation, particularly that losses during lactation were significantly blunted. Performing further DXA measurements using mice placed in clean cages the night prior eliminated the influence of coprophagia in artificially raising the late lactation values. These scans were largely similar to the initial measurements and suggests that the blunted BMC losses during lactation result from the enriched diet. Previous research in the Kovacs laboratory have also shown this effect, using PTH WT and null mice in the Black Swiss strain. [140] On a normal diet with 1 % calcium, WT and null mice gained BMC during pregnancy, lost during lactation to 15 % to 18 % lower than their baseline values, and restored the skeleton above baseline post-weaning. However on the enriched diet, the lactational decline was reduced while the gains achieved during pregnancy and recovery were unchanged. This indicates that increased availability of dietary calcium reduces skeletal resorption during lactation in mice, unlike in women who have been shown to lose a similar amount of BMD during lactation regardless of calcium intake. [171] It is also noted that DXA does not provide any 3-dimensional information about structure, and so the information provided will differ from the 3-dimensional technique of μCT.

Due to the short time period of a Masters degree, it wasn't possible to repeat experiments when trends were discovered that might have been resolved with larger sample sizes. The intestinal calcium absorption experiments were performed closer to the end of the project and involved numerous attempts at standardizing the protocol. Further experiments here are necessary to elucidate the effects on intestinal calcium absorption with greater accuracy, especially by looking at lactation. As a result of the short time frame, there are numerous other limitations due to a lack of data. μCT of the skeleton during pregnancy was not assessed, such that any changes in skeletal microarchitecture between baseline and late lactation are unknown. Furthermore, the

femur was the only bone assessed for microCT. Analysis of the lumbar vertebrae should also be performed. Similarly, time did not permit the analysis of histomorphometry, which would provide critical information on trabecular structure. It is possible that the *Cyp27b1* null mice had significant osteomalacia during the whole experiment, as would have been evident with the measurement of osteoid and cell numbers.

4.10 Future considerations

4.10.1 Calcitriol assay for use in mouse research

Our EIA kit used to measure calcitriol in the *Cyp27b1* null and WT mice yielded a baseline value of 12 pmol/L for the null mice, while the limit of detection for human samples was 6 pmol/L. Although it is likely that calcitriol is absent in the *Cyp27b1* null female mice, we cannot be certain based on the sensitivity of the assay. Finding a more sensitive calcitriol assay for use in mouse research, such as high performance liquid chromatography, would help to confirm that calcitriol is undetectable in the knockout model.

4.10.2 Measurement of calcitriol during pregnancy

Our results show that calcitriol rose significantly in the WT mothers during pregnancy while this rise was absent in the *Cyp27b1* nulls, consistent with prior literature showing that calcitriol comes from the maternal kidneys during pregnancy with little or no contribution from placentas. We cannot, however, completely exclude a contribution from the placenta. The WT mothers would have a litter of WT and heterozygous pups, while the null mothers would have a litter of null and heterozygous pups, and this could imbalance the potential fetal and placental contribution of calcitriol to the maternal circulation. In order to make a proper comparison

between mothers and evaluate the placentas as a significant source of calcitriol during pregnancy, WT dams should be mated to *Cyp27b1* null studs and *Cyp27b1* null dams should be mated to WT studs. This would allow for all mothers to only have litters with heterozygous pups, and any difference between the two genotypes of mice in the levels of calcitriol achieved during pregnancy could be attributed to the maternal kidneys rather than the contribution of placentas. A further test could involve including *Cyp27b1* null dams mated to *Cyp27b1* null studs, which would mean that all fetuses and placentas would lack that ability to make calcitriol. Comparing the calcitriol level of *Cyp27b1* null mothers bearing only *Cyp27b1* null pups to *Cyp27b1* null mothers bearing only heterozygous pups would help identify the placental contribution to calcitriol in the maternal circulation, if any.

4.10.3 Improvements to study power

Other laboratory members are currently performing experiments to address the limitations of this research due to lack of data. As mentioned, the blood ^{45}Ca values suggest that intestinal calcium absorption was upregulated in the *Cyp27b1* null during pregnancy, but a larger sample size and more direct measure of radioactivity accumulated in the intestine will help to provide confirmation. These studies will also be performed during the time period of lactation. Studies are also being done now to measure the calcium content of milk. In the future, femur and lumbar vertebrae are to be collected at late pregnancy and analyzed by microCT. Once collected, all of the vertebral samples will be analyzed by microCT and histomorphometry will also be performed. Another experiment to complete will involve intestinal gene expression to assess whether intestinal calcium absorption was actually upregulated during pregnancy.

4.10.4 Cell culture experiments to identify factors involved in post-weaning recovery

Our results indicate that calcitriol is not required for the normal recovery of bone mass post-weaning. This would suggest that other factors are able to regulate mineral and bone metabolism during the reproductive cycle. As the known calciotropic hormones have already been investigated in the Kovacs laboratory for their role in the recovery phase to no avail, the next step would be to broaden the spectrum of search. Genome-wide microarrays on bone and marrow during post-weaning may help to identify the differentially regulated genes that stimulate recovery following lactation. Cell culture experiments would then be able to narrow the list of candidate genes and determine if they stimulate osteoblast proliferation, differentiation, or mineralization *in vitro*.

4.11 Conclusions

My research hypothesis stated that calcitriol is required for upregulating intestinal calcium absorption during pregnancy and post-weaning, and to enable normal recovery of bone mass post-weaning, such that the high calcitriol levels in the *Vdr* null mice are able to act through alternative signalling mechanisms to have these effects. However, my studies in *Cyp27b1* null mice largely refute that hypothesis. During pregnancy and post-weaning, *Cyp27b1* null mice experienced significant improvements in bone and mineral metabolism, confirming that these adaptations to reproduction are achieved independent of calcitriol, or at least in a low calcitriol setting, given the limitations of the human-based calcitriol assay. Rather than being involved in the recovery phase, calcitriol may still serve a protective role against endocortical resorption during lactation, as the *Cyp27b1* null mice experienced greater losses of cortical bone during this period. The effects on bone during pregnancy and recovery are largely similar to what was seen

with loss of the VDR, further confirming that calcitriol was not acting through alternative signalling pathways in *Vdr* null mice, at least for its direct actions to promote deposition of skeletal mineral. Nonetheless, before the intestinal calcium absorption experiments are redone with adjusted methodology, we cannot completely exclude the possibility of non-VDR mediated actions of calcitriol on the intestine.

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