Roles of P2X7 Receptors in Adipose and Skeletal Tissues of Mice

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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ROLES OF P2X7 RECEPTORS IN ADIPOSE AND SKELETAL TISSUES OF MICE

(Thesis format: Integrated-Article)

by

Kim Lee Beaucage

Graduate Program in Physiology

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology, specialization in Musculoskeletal Health Research

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

P2X7 is an ATP-gated ion channel in the plasma membrane of a number of cell types. Previous in vitro studies show that P2X7 plays a role in regulating the differentiation of osteoblasts and adipocytes from mesenchymal precursors, as well as cellular metabolism and energy homeostasis. Furthermore, P2X7 has been implicated in mediating the effects of mechanical stimuli in bone. Our objective was to investigate the roles of P2X7, mechanical vibration, and their possible interaction in regulating body composition in vivo. We developed a novel method to monitor body composition during growth and aging in mice using micro-computed tomography (Chapter 2). This method permits rapid and reproducible quantification of adipose, lean and skeletal tissues in a non-invasive manner. We then applied this methodology to monitor the body composition of wild-type and P2X7 knockout mice (Chapter 3). Loss of P2X7 function led to greater adiposity and ectopic lipid accumulation in older male mice. We next investigated the effects of low-magnitude, high-frequency vibration on wild-type and P2X7 knockout mice (Chapter 4). Under our conditions, vibration does not induce changes in body composition or bone microarchitecture. Together, our studies provide an effective tool for characterizing whole-body composition of mice and establish novel in vivo roles for the P2X7 receptor in regulating adipogenesis and lipid metabolism. Finally, our findings and those of others highlight the need to re-evaluate current thinking on the effects of low-magnitude, high-frequency vibration on body composition in vivo.
KEYWORDS

adiposity, bone mineral content (BMC), bone mineral density (BMD),
mechanotransduction, metabolism, microarchitecture, micro-computed tomography,
$P2rx7$, whole-body composition (WBC), whole-body vibration (WBV)
CO-AUTHORSHIP

Chapter 1 entitled “Introduction” was written by K.L. Beaucage with suggestions from Drs. S.J. Dixon, D.W. Holdsworth, M.W Grol, S.M. Sims and C.L. Pin.

Chapter 2 entitled “Quantitative micro-computed tomography for assessment of age-dependent changes in murine whole-body composition” was adapted from Beaucage et al., 2014. (Submitted), and reproduced here. The manuscript was written by K.L. Beaucage and Dr. S.J. Dixon with suggestions from S.I. Pollmann, Drs. S.M. Sims and D.W. Holdsworth. S.I. Pollmann assisted with analysis and interpretation of the experiments. All experiments were performed by K.L. Beaucage and were carried out in the laboratories of Drs. S.J. Dixon, S.M. Sims and D.W. Holdsworth.

Chapter 3 entitled “Loss of P2X7 Nucleotide Receptor Function Leads to Abnormal Fat Distribution in Mice” was adapted from Beaucage et al., 2014. Purinergic. Signal. 10(2):291-304, and reproduced here with permission from Springer Science + Business Media (see Appendix A). The publication was written by K.L. Beaucage and Dr. S.J. Dixon, with suggestions from Drs. S.M. Sims and D.W. Holdsworth. Under the supervision of K.L. Beaucage, summer student and fourth year thesis A. Xiao assisted with blinded analyses in Figure 3.1 d and e; he also assisted with some of the data collection for experiments involving mice at 12 months of age, data from which are a part of the means reported in Figures 3.1 b, 3.4 b, 3.5 c and d, 3.6 c-e, 3.7 b, 8, Tables 3.1-3.4. S.I. Pollmann assisted with whole-body composition analysis in Figure 3.2. M.W. Grol and R.J. Beach assisted with blinded analyses in quantifying the osmium positive staining shown in Figures 3.4 c, 3.5 e and 3.6 f. All other studies were performed by K.L. Beaucage. All experiments were carried out in the laboratories of Drs. S.J. Dixon, S.M.
Sims and D.W. Holdsworth.

Chapter 4 entitled “Low-Magnitude, High-Frequency Vibration Has No Effect on Murine Whole-Body Composition and Bone Microarchitecture” was written by K.L. Beaucage with suggestions from Drs. S.J. Dixon, M.W. Grol, S.M. Sims and D.W. Holdsworth. S.I. Pollmann assisted with whole-body composition analysis in Figures 4.1-4.3. M.W. Grol assisted in relieving K.L. Beaucage for a week of vibration studies, which contributed to the means of the cohorts 1-3 of the overall experiment. C.J.D. Norley assisted with high-resolution hind-limb imaging and processing. Under the supervision of K.L. Beaucage, summer student G.B. Blackler assisted with the high-resolution hind-limb image analyses, data from which are a part of the means shown in Figures 4.5-4.11. All experiments were performed by K.L. Beaucage and were carried out in the laboratories of Drs. S.J. Dixon, S.M. Sims and D.W. Holdsworth.

Chapter 5 entitled “Discussion” was written by K.L. Beaucage with suggestions from Drs. S.J. Dixon, S.M. Sims and M.W. Grol.
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LIST OF ABBREVIATIONS

3D three dimensional
A amplitude (peak-to-peak displacement)
aBMD areal bone mineral density
$a_{\text{max}}$ maximum acceleration
ALP alkaline phosphatase
ANOVA analysis of variance
ATP adenosine 5’-triphosphate
BMC bone mineral content
BMD bone mineral density
BMP bone morphogenetic protein
BMSCs bone marrow stromal cells
BMU basic multicellular unit
BSP bone sialoprotein
BV/TV bone volume fraction
BzATP 2’,3’-O-(4-benzoylbenzoyl)ATP
C/EBP CCAAT/enhancer-binding protein
CREB cAMP response element-binding protein
CIHR Canadian Institutes of Health Research
Col1 collagen type I
CT computed tomography
Ct.Ar cortical area
Ct.Th cortical thickness
DMP-1 dentin matrix protein-1
DXA dual-energy X-ray absorptiometry
EDTA ethylenediaminetetraacetic acid
f frequency
FAK focal adhesion kinases
FOV field of view
<table>
<thead>
<tr>
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<tr>
<td>GE</td>
<td>General Electric Corporation</td>
</tr>
<tr>
<td>g</td>
<td>gravitational acceleration</td>
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<td>HA</td>
<td>hydroxyapatite</td>
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<td>HR-MRI</td>
<td>high-resolution magnetic resonance imaging</td>
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<td>HR-PQCT</td>
<td>high-resolution peripheral quantitative computed tomography</td>
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<tr>
<td>HU</td>
<td>Hounsfield units</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
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<td>LRP5</td>
<td>low-density lipoprotein receptor-related protein 5</td>
</tr>
<tr>
<td>Ma.Ar</td>
<td>marrow area</td>
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<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
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<td>MEPE</td>
<td>matrix extracellular phosphoglycoprotein</td>
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<td>Micro-CT</td>
<td>micro-computed tomography</td>
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<td>MSCs</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
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<td>NFATc1</td>
<td>nuclear factor of activated T-cells, cytoplasmic 1</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NV</td>
<td>non-vibrated (sham)</td>
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<tr>
<td>OCN</td>
<td>osteocalcin</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>OPN</td>
<td>osteopontin</td>
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<tr>
<td>OSX</td>
<td>osterix</td>
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<td>P2</td>
<td>purinergic nucleotide receptors</td>
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<td>P2X</td>
<td>ATP-gated nonselective cation channels</td>
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<td>P2Y</td>
<td>G protein-coupled receptors for nucleotides</td>
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<td>PGE₂</td>
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<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ</td>
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pQCT  peripheral quantitative computed tomography
Pyk2  proline-rich tyrosine kinase 2
RANK  receptor activator of nuclear factor κB
RANKL  receptor activator of nuclear factor κB ligand
ROI  region of interest
rpm  revolutions per minute
PCR  polymerase chain reaction
RUNX2  runt-related transcription factor 2
SD  standard deviation
SEM  standard error of the mean
SOST  sclerostin
speCZT  GE eXplore speCZT dual-modality imaging system
SR-muCT  synchrotron radiation micro-CT
Tb.N  trabecular number
Tb.Th  trabecular thickness
Tb.Sp  trabecular spacing
TC  total cholesterol
TG  triglyceride
Ultra  GE eXplore locus Ultra imaging system
Vib  vibrated
WBC  whole-body composition
WBV  whole-body vibration
Wnt  wingless-related integration site family
WT  wild-type
CHAPTER ONE

INTRODUCTION
1.1 Chapter Summary

In this chapter, basic skeletal physiology will be described including an overview of bone structure, function and development. The different types of bone cells, and their functions as a team to build and maintain bones will be elaborated upon. This chapter will also describe the interactions between skeletal and adipose tissues, and their roles in systemic metabolic diseases. Additionally, purinergic receptor physiology with emphasis on a specific receptor (P2X7) will be discussed, along with the role of P2X7 in bone and adipose tissues. Furthermore, this Chapter reviews the possibilities for interplay among P2X7, bone and adipose tissues, and mechanical loading, and the potential to harness these effects to build bone and reduce adiposity in vivo. The rationale and specific objectives of the studies presented in Chapters 2-4 then follow.

1.2 Bone Physiology

1.2.1 Bone Structure and Function

In the past, bone was perceived to be a relatively inactive tissue that functioned solely to provide support for other organ systems within the body. However, studies conducted over the last few decades have challenged this perception. It is clear now that bone is a highly specialized and complex connective tissue that provides physical protection to vital organs, serves as a site for muscle attachment to support locomotion, contributes to regulation of calcium and phosphate mineral homeostasis and supplies a source of hematopoietic and mesenchymal stem cells (Clarke, 2008; Muruganandan, 2009). Adding to this complexity is the emerging idea that bone itself regulates energy
metabolism (Wei et al., 2014), emphasizing that there is still much to learn about this dynamic tissue.

Bone shares similarities to other connective tissues in that the cells are separated by extracellular matrix. However, bone is composed of both organic and inorganic components, which accounts for its extraordinary physical properties (Marieb et al., 2011). The organic portion of bone is primarily composed of type 1 collagen (ColI) fibers and proteoglycans, and makes up approximately 35% of bone mass (Marieb et al., 2011). The organization of collagen into parallel layers and angles provides bone with its tensile strength and flexibility, allowing it to resist forces such as twisting and stretching (Clarke, 2008; Marieb et al., 2011). Non-collagenous proteins, such as bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OPN), are also present and are thought to regulate the mineralization process (Clarke, 2008). The inorganic components of bone are more abundant than the organic portion (making up approximately 65% of bone mass), and consists of hydroxyapatite crystals $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ (Hadjidakis and Androulakis, 2006; Marieb et al., 2011). These crystals pack tightly around collagen in the extracellular matrix, contributing to the hardness and resistance to compressive loading in bone (Marieb et al., 2011).

Bones are present in a variety of shapes and sizes, which fit into four general categories: long bones, short bones, flat bones and irregular bones (Clarke, 2008; Marieb et al., 2011). In general, nearly every bone in the skeleton consists of an external layer of compact bone, an internal network of trabecular bone and bone marrow that resides within the trabecular spaces (Marieb et al., 2011). At the same time, the shape of each bone in the body is unique owing to differences in both formation and function. Flat
bones are thin and flattened, such as the cranial bones, sternum, ribs and scapula (Clarke, 2008). Long bones include most bones in the limbs and include the humerus, radius, ulna, femur, tibia and fibula. These bones are longer than they are wide and consist of a long shaft with two distinct ends. The hollow shaft forming the long axis of the bone is referred to as the diaphysis (Marieb et al., 2011). At both proximal and distal ends of the diaphysis, the long bones flare outward to form the cone-shaped metaphyses below the growth plate; and are then followed by the rounded bone ends above the growth plate referred to as the epiphyses (Clarke, 2008; Marieb et al., 2011).

The epiphyses and metaphyses are comprised of a trabecular network, while the diaphysis is made of dense compact bone. With the exception of the epiphyses, a connective tissue membrane called the periosteum covers the external surface of each bone (Marieb et al., 2011). The periosteum has two layers: a deep osteogenic layer that remodels the perimeter of the bone and a superficial outer layer that protects the bone by resisting tension due to bending forces. The periosteum is secured to the bone with thick collagenous perforating fibres, called Sharpey’s fibres. Within the bone, the inner surfaces are also lined with an osteogenic connective tissue membrane, called the endosteum. The endosteum covers the trabeculae and the central canal of osteons. Osteons (the Haversian system) are the long cylindrical structures running longitudinally and parallel throughout compact bone, which contribute structural strength and resistance to torsional stresses imposed upon compact bone (Marieb et al., 2011).
1.2.2 Skeletal Development

In vertebrate organisms, two distinct methods of bone formation have been described. The mechanism regulating the development of flat bones is termed intramembranous ossification, where mesenchymal cells directly differentiate into osteoblasts (Wagner and Karsenty, 2001). The second mechanism accounts for the development of the majority of bones in the axial and appendicular skeleton, and is referred to as endochondral ossification. In this process, mesenchymal cells form condensations and differentiate into chondrocytes (cartilage forming cells), which produce a cartilaginous template that defines the shape and site of future skeletal elements (Kronenberg, 2003; Wagner and Karsenty, 2001). The chondrocyte-directed matrix production regulates the size of the skeletal element being formed. Osteoblasts then differentiate from the perichondral cells adjacent to the hypertrophic chondrocytes to form the bone collar, while the cartilaginous template directs the invasion of vasculature and the formation of mineralized matrix along with chondrocyte apoptosis (Kronenberg, 2003). The mineralization process forms a primary ossification centre, where osteoblasts continue to accompany the vascular invasion of the newly forming bone. Chondrocytes continue to proliferate to lengthen the bone in a polarized manner, and the bone collar begins to form cortical bone (Kronenberg, 2003). Next, the secondary ossification centre forms at the bone ends through cycles of chondrocyte hypertrophy accompanied by vascular invasion and osteoblast activity, except that this forms the growth plate between the metaphyses and epiphyses (Kronenberg, 2003). Interestingly, human growth plates fuse after the completion of puberty (Emons et al., 2009), but growth plates in mice do not fuse and the mouse continues to grow throughout a lifetime (Bagi et al., 2011).
1.3 Bone Cells and Their Functions

1.3.1 Osteoblasts

As the cells responsible for bone formation, osteoblasts serve a vital role in bone growth and remodelling (Figure 1.1). In this regard, the functions of osteoblasts are controlled by various hormones and growth factors, which regulate formation, function and survival.

Osteoblasts develop from mesenchymal stem cells (MSCs), and cell lineage commitment is controlled by several master transcription factors and signalling cascades (Figure 1.2). Early stage commitment to the osteoblast lineage from MSCs is regulated by runt-related transcription factor-2 (RUNX2, known in past literature as Cbfa1). Mice lacking RUNX2 (Runx2\(^{-/-}\)) are incapable of bone formation due to the absence of osteoblasts; thus, these mice have only a cartilaginous skeletal template (Komori et al., 1997; Otto et al., 1997). Without proper ossification, maturation of the skeletal elements does not occur resulting in death of Runx2\(^{-/-}\) mice at birth due to respiratory failure (Komori et al., 1997; Otto et al., 1997). This genetically-modified mouse model clearly demonstrates the critical role of RUNX2 in regulating early osteoblast lineage commitment. Moreover, RUNX2 also plays a part in promoting the expression of bone matrix protein genes, alkaline phosphatase (ALP) activity (an early osteoblast marker), as well as in vitro mineralization of osteoblast-like cells and immature mesenchymal cells (Banerjee et al., 1997; Ducy et al., 1997; Harada et al., 1999).

Commitment to the late-stage mature osteoblast is regulated by the transcription factor Osterix (OSX). Similar to the Runx2\(^{-/-}\) mice, genetically-modified mice lacking the
Skeletal integrity depends on the maintenance of bone by teams of cells working closely together to turnover bone over a period of approximately 4 months. To initiate bone remodelling, an activation event of localized damage occurs such as microfractures, and this recruits osteoclast precursors to the surface area where the basic multicellular unit will form to remodel bone. Osteoclasts mature from these precursors and begin to adhere and resorb the bone. The activation and resorption phases together take approximately 3 weeks from beginning to end. The reversal phase then follows, where bone lining cells are recruited to the site by the osteoclasts and factors released from the bone matrix, and these precursors differentiate into osteoblasts. The osteoblasts secrete organic matrix called osteoid, which subsequently mineralizes during the formation phase to fill the resorbed area formed by the osteoclasts. A small subset of osteoblasts become embedded within the matrix, and will then terminally differentiate into mature osteocytes. Figure courtesy of Dr. M.W. Grol.
Figure 1.2 Signalling pathways involved in osteoblastogenesis and adipogenesis.

As both osteoblasts and adipocytes arise from the same mesenchymal progenitors (bone marrow stromal cells), several key transcriptional events direct the commitment toward either lineage. The osteoblast master transcription factor RUNX2 and the canonical Wnt transcriptional coactivator β-catenin are expressed in all stages of osteoblastogenesis to direct commitment during osteoblastogenesis. OSX appears at a later stage and directs the commitment of pre-osteoblasts towards mature osteoblasts. β-catenin also serves a dual role to inhibit PPARγ, the master transcription factor for adipogenesis. PPARγ also exhibits dual roles as it functions to also partially inhibit RUNX2, β-catenin and osteogenesis. In adipogenesis, C/EBPβ and C/EBPδ are present for the early commitment of pre-adipocytes, where at later stages PPARγ and C/EBPα synergize to enable the commitment towards the mature adipocyte.
OSX gene (\(Osx^{-/-}\)) develop a completely cartilaginous skeleton, and die at birth due to respiratory failure (Nakashima et al., 2002). However, these \(Osx^{-/-}\) mice exhibit expression of RUNX2 precursor cells, suggesting their differentiation is arrested and cannot progress past the early osteoblast without OSX. Furthermore, studies show that OSX is also critical for postnatal bone formation and growth (Zhou et al., 2010). Therefore, OSX is essential for mature osteoblast differentiation and the development and growth of healthy bones.

Other signalling pathways that also play important roles in developing bone include Wnt/\(\beta\)-catenin, Indian hedgehog and vascular endothelial growth factors, which are required for osteoblast differentiation (Kronenberg, 2003; Mak et al., 2006). Moreover, transcription factors such as nuclear factor of activated T-cells (NFAT)-c1, cytokines, growth factors and bone morphogenic proteins (BMPs) act to modify the actions of the aforementioned key players in osteoblastogenesis (Grol et al., 2009; Robling et al., 2006; Zhou et al., 2010). Thus, it is evident that multiple signalling pathways play a role in regulation of osteoblast differentiation and commitment. When osteoblasts mature, they begin to secrete non-mineralized osteoid consisting of matrix proteins expressing CoI, OCN, and ALP which is a key enzyme in mineralization to yield mature bone (Robling et al., 2006).

1.3.2 Osteocytes

As osteoblasts continue to secrete osteoid, a small subpopulation of osteoblasts becomes embedded within the non-mineralized bone matrix (Bonewald, 2011) (Figure 1.1). These osteoblasts then begin to undergo a dramatic transformation as they
terminally differentiate into osteocytes. The early osteocyte is marked by greater expression of E11, and, as the matrix matures and mineralizes, the osteocyte begins to express markers of mature osteocytes such as dentin matrix protein-1 (DMP-1), sclerostin (SOST) and matrix extracellular phosphoglycoprotein (MEPE) (Bonewald, 2011; Robling et al., 2006). The most abundant cell type in bone, osteocytes are characterized by long cytoplasmic processes (dendrites) that extend outward from the cell body (Bonewald and Johnson, 2008; Marks and Popoff, 1988). The osteocyte cell body lives within the mineralized matrix in cave-like lacunae, while these dendritic processes extend along tiny channels throughout the bone called canaliculi. The network of these dendritic processes are used for communication with neighbouring osteocytes via gap junctions, and are also believed to play a role in bone mechanotransduction (Bonewald, 2006; Robling et al., 2006). Additionally, canaliculi permit osteocytes to connect with cells lining the bone surface, including osteoblasts and osteoclasts (Bonewald and Johnson, 2008). In this regard, new bone produced by osteoblast has been shown to be maintained by the osteocytes and bone-lining cells (Robling et al., 2006). Additionally, the dendritic processes of osteocytes reach into the marrow cavity, allowing for not only recruitment of osteoclasts but also regulation of mesenchymal stem cell differentiation (Bonewald and Johnson, 2008). Consequently, these channels provide a means for communication, fluid flow and nutrient diffusion to cells deep within the dense matrix (Marks and Popoff, 1988).
1.3.3 Osteoclasts

Osteoclasts are multinucleated cells of hematopoietic origin that differentiate from progenitors of the monocyte-macrophage lineage (Kobayashi et al., 2009). Once matured, these specialized cells attach and spread on bone surfaces, and resorb bone by creating an acidic microenvironment through the secretion of acids and proteases (Robling et al., 2006) (Figure 1.1). Interestingly, osteoclast activity is controlled by cells of the osteoblast lineage and, during remodelling, bone resorbed by osteoclasts is replaced by the actions of osteoblasts (Martin et al., 2009). Osteoclast differentiation is initiated by mechanisms that are activated by macrophage-colony stimulating factor (M-CSF) and the transcription factor nuclear factor-κB ligand (NF-κB). Osteoblasts express receptor activator for nuclear factor-κB ligand (RANKL), and the binding of RANKL to its receptor, RANK in the monocyte-macrophage lineage initiates commitment to the osteoclast lineage. Osteoblasts also secrete osteoprotegerin (OPG), a decoy receptor for RANKL, acting to inhibit osteoclastogenesis and osteoclast function by blocking the interaction of RANKL with RANK (Kobayashi et al., 2009; Korcok et al., 2004). On the other hand, more recent studies have shown that osteoclasts can act to recruit osteoblasts and enhance osteogenesis by providing a source of soluble and membrane bound factors (Pederson et al., 2008; Zhao et al., 2006). Moreover, osteocytes have also been shown to communicate with and regulate osteoclast cells through RANKL expression (Xiong et al., 2011). Thus, crosstalk between bone cells regulates the fine balance maintaining the status quo in healthy adult bone.
1.3.4 Regulation of Bone Remodelling

Osteoclasts and osteoblasts have lifespans of a few weeks to months, respectively (Figure 1.1). On the other hand, the osteocyte life span can range from months to decades, depending on their anatomical location (Almeida and O'Brien, 2013). Osteoclasts have high activity levels over a short period of time due to the rapid nature of resorption, occurring over the course of a few weeks. The process of organic matrix secretion and mineralization by the osteoblast is an inherently longer process, taking upwards of a few months. Although the duration of these processes seem quite different, establishing equilibrium between the amount of bone resorbed and formed is critical for the maintenance of bone. When osteoclast activity exceeds that of osteoblast activity, the net result is bone loss due to increased bone resorption, resulting in reduced bone mass and strength (Almeida and O'Brien, 2013). Thus, the continual maintenance throughout a lifetime is imperative for proper structural integrity of bone.

Much of what we know about how bone maintains its structural properties at the microscopic level is attributed to the work of Harold Frost in the 1960s (Sims and Martin, 2014). Frost described two ways that bone cells work together to accomplish skeletal formation and/or renewal: bone modelling and bone remodelling (Robling et al., 2006). The addition or removal of bone can occur at four locations: trabeculae, Haversian (intracortical) envelopes, and endocortical and periosteal surfaces. Bone modelling is responsible for the spatial distribution and accumulation of bone during growth involving osteoclast activation and resorption, as well as osteoblast activation and formation, but not concomitantly at the same location. Once the skeleton reaches maturity after puberty
modeling contributes little to the maintenance of bone; unless the disease state of the bone or the mechanical loading of the bone has changed drastically (Robling et al., 2006).

The preventive maintenance and repair mechanism for damaged bone is referred to as bone remodelling, and involves a coordinated balance between bone formation and bone resorption by specialized osteoblasts and osteoclasts (Grol et al., 2009; Robling et al., 2006; Sims and Martin, 2014). Remodelling also allows access to ions for metabolic homeostasis (Parfitt, 1979). Remodelling is accomplished cyclically by the removal and replacement of areas of bone (for repair and preventive maintenance) through activation, resorption, reversal and formation phases in this respective sequence (Robling et al., 2006) (Figure 1.1). In this remodelling event, activation begins with localized damage to bone, which initiates the remodelling sequence by recruitment of osteoclasts (Parfitt, 1994; Sims and Gooi, 2008). Osteoclasts adhere to the bone surface and spread to begin resorption. This is followed by a reversal phase, whereby mononuclear lining cells (of unknown lineage) then prepare the bone surface by depositing a substance denoted as the cement (reversal) line. This thin layer of matrix allows for recruitment of osteoblast precursors to begin proliferation and differentiation into mature osteoblasts, filling the space that has been resorbed with newly formed bone (Parfitt, 1994; Sims and Gooi, 2008; Sims and Martin, 2014). Thus, teams of osteoblasts and osteoclasts temporarily arrange themselves to remodel bone in what is referred to as a basic multicellular unit (BMU) (Robling et al., 2006).

Trabecular and intracortical bone BMUs differ in structure and the process by which they replace bone (Sims and Martin, 2014), but both methods are the mechanistic bridges between cellular activity and bone morphology (Robling et al., 2006). In the
trabeculae, the BMU is located on the bone surface and is covered by a canopy of osteal macrophages (osteomacs) at the site of bone formation (Sims and Martin, 2014). Under this canopy, the surface is resorbed by osteoclasts and osteoblast precursors are recruited to form bone in the space that has been resorbed (Sims and Martin, 2014). Intracortical, periosteal and endocortical BMUs have a distinct three-dimensional structure, where the osteoclasts lead the way by resorbing bone and excavating a longitudinal tunnel (referred to as Howship’s lacunae) and defining the size and amount of bone that will be replaced. Osteoclast resorption initiates the remodelling cycle, recruiting osteoblasts to follow behind and refill the resorbed area through bone formation and subsequent mineralization of osteoid produced by osteoblasts (Grol et al., 2009; Robling et al., 2006). Thus, coupling between osteoclasts and osteoblasts in bone remodelling is controlled to ensure that bone is replaced in the region where it had been removed.

Remodelling by BMUs occurs continuously at multiple loci throughout the growth and maintenance of bones in the body (Robling et al., 2006). Bone remodelling is regulated by local and systemic factors, as well as mechanical stimuli (Grol et al., 2009). It is well known that in the absence of bone loading (a form of mechanical stimuli), bone strength and quality rapidly depreciate as seen in patients on bed rest for extended periods of time (attenuated muscle atrophy) (Prisby et al., 2008; Robling et al., 2006) and in astronauts (microgravity) (Robling et al., 2006; Rubin et al., 2001b). Thus, achieving a balance between the processes of resorption and formation is essential to maintain normal bone density and bone mass. A disruption in this equilibrium can lead to pathological bone phenotypes and metabolic bone diseases, such as osteoporosis (Rosen, 2000).

Therefore, the possibility of tipping the equilibrium favourably towards bone formation to
prevent or mitigate bone loss in osteoporosis may be possible by initiating bone remodelling events.

1.4 Stromal Cell Plasticity in the Skeleton

1.4.1 Bone Marrow Stromal Cell Plasticity

A reserve of bone marrow stromal cells (BMSCs) resides in the marrow cavity of adult long bones (Pittenger et al., 1999). These BMSCs are a mesenchymal progenitor cell population exhibiting multipotency, with the capacity to differentiate into other cell types, such as osteoblasts, adipocytes, chondrocytes and fibroblasts (Pittenger et al., 1999). BMSC differentiation is regulated by a multitude of signalling cascades alongside transcriptional events, which ensure that lineage determination is either inhibited or enhanced to obtain the desired specific cell-type (Harada, 2003). An imbalance in differentiation of osteoblasts from mesenchymal stem cells and osteoclasts from macrophage/monocyte precursors result in skeletal diseases such as osteoporosis and contribute to the pathogenesis of inflammatory autoimmune diseases such as rheumatoid arthritis (Soltanoff, 2009). The plasticity seen among cells derived from BMSCs is evident in diseases such as osteoporosis, where increased marrow adiposity is coupled with decreased bone formation (Rosen and Bouxsein, 2006). The factors that increase osteoblast differentiation at the expense of adipogenesis remain poorly understood. As such, research into the mechanisms governing BMSC plasticity is essential for understanding systemic degenerative bone diseases.
1.4.2 Adipocytes and Adipogenesis

The principal cell type responsible for formation and maintenance of adipose tissue is the adipocyte (also known as the fat cell). Adipogenesis is the process in which adipocytes are formed. The specific stages governing adipogenesis are determination and terminal differentiation. Just as osteoblast differentiation is regulated by several transcription factors, adipocyte differentiation is regulated by an adipocyte-specific transcription factor, peroxisome proliferator-activated receptor γ (PPARγ) (Figure 1.2). Playing a key role in energy metabolism, inflammation, cell proliferation, hyperlipidemia and gluconeogenesis, it is of no surprise that PPARγ is expressed in adipose tissue where it is responsible for adipogenesis and lipogenesis. Two members of the CCAAT/enhancer-binding protein (C/EBP) family, C/EBPβ and C/EBPδ, are expressed early in adipogenesis, both of which are involved in inducing PPARγ (Cao et al., 1991; Lefterova et al., 2008). There is increasing evidence that PPARγ and other PPARs play an important role in regulating obesity (Kersten, 2002). Additionally, studies have shown that PPARγ exhibits a reciprocal relationship with β-catenin, where by PPARγ suppresses β-catenin, and increased β-catenin levels inhibit PPARγ gene expression (Kawai et al., 2007; Moldes et al., 2003; Rosen and MacDougald, 2006). Furthermore, C/EBPα is another important transcription factor in late stages of adipogenesis (Cao et al., 1991; Lefterova et al., 2008). When co-expressed, PPARγ and C/EBPα synergize, promoting the differentiation of adipocytes even in the absence of exogenous PPAR activators (Tontonoz et al., 1994). Aside from master transcription factors, circulating blood glucose levels, insulin, LDL (cholesterol), triglycerides, adipocytokines (also referred to as adipokines such as leptin, adiponectin and resistin) undoubtedly play important roles.
as biomarkers of fat regulation and energy metabolism.

1.4.3 Relationship between Osteoblast and Adipocyte Determination

Studies have shown that $Ppar\gamma$-deficient embryonic stem cells fail to differentiate into adipocytes, and show enhanced osteogenesis (Akune et al., 2004). Interestingly, PPARγ and adipogenesis are inhibited by $\beta$-catenin (Rosen and MacDougald, 2006), which is in contrast to osteoblastogenesis where $\beta$-catenin plays a positive role (Figure 1.2). Furthermore, enhanced osteoblastogenesis and high bone mass has been demonstrated in $Ppar\gamma^{-/-}$ mice (Akune et al., 2004). Whereas PPAR-γ stimulates adipogenesis in the mesenchymal stem cells (MSCs) of bone marrow, it also acts to suppress osteoblastogenesis through partial inhibition of RUNX2 while enhancing osteoclastogenesis through enhancing gene expression in osteoclast precursors (Gourine et al., 2009). Thus, PPARγ is a master regulator of adipocyte differentiation whose expression is both necessary and sufficient for maintenance of the adipogenic phenotype and inhibition of osteoblastogenesis. Interestingly, it has previously been noted that increased rates of marrow adipogenesis in humans is associated with aging as well as chronic diseases such as diabetes mellitus and osteoporosis (Gourine et al., 2009). Thus, increased bone marrow adiposity seen in degenerative bone diseases suggests that cells of mesenchymal origin are not static in their determination and differentiation.

The plasticity seen among cells derived from MSCs is evident in diseases such as osteoporosis, where increased marrow adiposity is coupled with decreased bone formation. An imbalance in differentiation of osteoblasts from mesenchymal stem cells and osteoclasts from macrophage/monocyte precursors result in skeletal diseases such as
osteoporosis, and contribute to the pathogenesis of diseases such as rheumatoid arthritis (Soltanoff, 2009). The signalling pathways that regulate osteoblast-lineage commitment \textit{in vivo} have yet to be entirely understood. Therefore, understanding the factors that increase osteoblast differentiation at the expense of adipogenesis may be essential in the prevention and treatment of degenerative bone diseases, and is one of issues explored in my thesis.

\subsection*{1.4.4 Bone-fat Crosstalk}

Research over the past decade has been working towards revealing integrative signalling pathways referred to as crosstalk among cell and tissue types. The emergence of these signalling pathways will be crucial for understanding physiological properties of organs and tissues (Confavreux et al., 2009). Although bone and fat have been traditionally thought of as static and inert tissues, complex pathways link bone remodelling to fat metabolism (de Paula et al., 2010). As bone also has energy reserves of marrow fat, the determination of MSC lineage fate plays an important role in modulating the microenvironment of bone and its immediate energy metabolism (Lecka-Czernik, 2010).

The complexity of this crosstalk is apparent; adipocytes have been shown to secrete bone-active hormones (such as leptin and estrogens), and fat-mass has been shown to influence secretion of hormones from pancreatic beta cells (such as insulin) (Reid, 2008). Insulin has a central role in carbohydrate and fat metabolism, and has recently been shown to signal in osteoblasts by favouring osteoclast differentiation and resorption. The acidic environment created by this upregulated osteoclast activity has
been shown to activate OCN by decarboxylation (Wei et al., 2014). OCN is the most abundant non-collagenous protein in bone matrix (Patti et al., 2013), and is secreted exclusively by osteoblasts. OCN activation favours insulin secretion from pancreatic β-cells and enhancing insulin sensitivity in white adipose tissues, muscle and liver, contributing to whole-body glucose homeostasis (Wei et al., 2014). Moreover, circulating uncarboxylated fraction of OCN has been correlated with increased insulin sensitivity and reduced blood glucose and visceral fat, while both uncarboxylated and total OCN have been associated are increased with physical activity (Patti et al., 2013). Thus, the study of crosstalk between bone and fat is becoming a prominent area of skeletal biology, with important implications for metabolic diseases such as obesity, diabetes mellitus and osteoporosis (de Paula et al., 2010).

1.4.5 Skeletal Aging

Even with intricate and continuous self-renewal system inherent to bone, it cannot escape the effects of aging over a lifetime. Bone aging presents with an imbalance between the amount of bone resorbed by osteoclasts, and the amount of bone that osteoblasts can form to restore the resorption. This shift in the equilibrium results in both the loss of bone mass and strength (Almeida and O'Brien, 2013). At the morphological level, structural changes in bone such as reduced cortical bone thickness and density, accompanied by an increase in porosity contributes to the reduction of bone strength (Almeida and O'Brien, 2013; Halloran et al., 2002). At a histological level, a reduction in bone formation has been associated with inadequate numbers of osteoblasts due to apoptosis, and reduced osteocyte quantity (Almeida and O'Brien, 2013). Moreover,
decreased MSCs numbers in bone marrow are due in part to preferential adipogenic commitment over osteoblastogenesis, drastically increasing the number of adipocytes present with age (Almeida and O'Brien, 2013). Moreover, trabecular bone aging is associated with diminished bone volume and osteoclast numbers, reduced trabecular number (Tb.N) and thickness (Tb.Th), and increased spacing between trabeculae (Tb.Sp) (Almeida and O'Brien, 2013; Halloran et al., 2002). Taken together, the decline in functional bone contributes to the systemic bone loss observed in skeletal aging.

1.4.6 Diseases of Systemic Bone Loss

Musculoskeletal diseases are highly prevalent and pose a great burden, affecting millions of people globally. The prevalence of these diseases increases with age and becomes a greater concern with the aging population. In fact, among older age groups musculoskeletal diseases have been reported as the main cause of disability, imparting diminished quality of life due to reduced social function and mental health issues associated with pain and physical disability (Woolf and Pfleger, 2003).

Osteoporosis is a common skeletal disorder, characterized by compromised bone quality (reduced bone mineral density), primarily caused by bone resorption exceeding bone formation resulting in an increased fracture risk (Pietschmann et al., 2009; Rosen, 2000). Age- and gender-specific factors in bone density play a role in predisposition to the disease. However, other lifestyle factors such as smoking, alcohol consumption, calcium and vitamin D intake, and bone loading (such as mobility and exercise) play critical roles in bone health throughout a lifetime (Pietschmann et al., 2009). The prevalence of osteoporosis is on the rise, with an estimated 40% of women expected to
experience an osteoporotic fracture in their lifetime (Melton et al., 1992). The limited therapeutic interventions available for treatment of osteoporosis are only marginally successful and primarily anti-resorptive in their properties (Akesson, 2003). Currently, no pharmacologic or non-invasive treatment options exist for the prevention of osteoporosis. This emphasizes the importance of elucidating the mechanisms that regulate bone remodelling to determine potential therapeutic targets for prevention and treatment.

Metabolic syndrome encompasses a myriad of diseases including obesity and dyslipidemia (Klop et al., 2013), has been associated with altered hormone levels, and is more prevalent with age (Dulloo and Montani, 2012). The global obesity epidemic is a contributing factor to musculoskeletal disease progression and prevalence. Interestingly, studies have begun to correlate the incidence of diabetic patients with increased risk of fracture due to decreased bone quality (type II diabetes) and reduced bone mineral density (type I diabetes) (Isidro and Ruano, 2010; Rakel et al., 2008). However, the signalling pathways that regulate osteoblast-lineage and adipocyte-lineage commitment in vivo have yet to be entirely elucidated. Therefore, understanding the factors that increase osteoblast differentiation at the expense of adipogenesis may prove to be an innovative approach to the prevention and treatment of musculoskeletal diseases including osteoporosis.

1.5 Purinergic Receptor Physiology

1.5.1 P2 Receptors

Purinergic receptors (purinoceptors) are a family of cell surface receptors activated by nucleotides or nucleosides (Burnstock, 1976), eliciting an array of
physiological responses. This family of receptors consists of the P1, P2X and P2Y subclasses (Fredholm et al., 1997). P1 receptors are a family of G protein-coupled receptors activated by extracellular adenosine. P2 receptors are activated by nucleotides and are subdivided into two classes: P2X (ATP-gated cation channels) and P2Y (G protein-coupled receptors). In mammals, eight P2Y receptor subtypes are known, P2Y_{1,2,4,6,11-14}, whereas seven members of the P2X family, P2X_{1-7}, are known to exist. With regards to the P2X subfamily, these receptors consist of either homo- or heterotrimeric channels that are permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\) (Khakh and North, 2006). Each P2X subunit possesses intracellular carboxyl and amino termini containing consensus binding motifs for protein kinases; two transmembrane domains, one involved in channel gating and the other that lines the ion pore; and a large extracellular loop that participates in ATP binding (Burnstock, 2007).

P2 receptors play important roles in regulating cellular physiology and pathology. Extracellular nucleotides such as ATP signal through P2 nucleotide receptors expressed in many cell types, including osteoblasts and osteoclasts (Dixon and Sims, 2000). It is well known that both osteoblasts and osteoclasts express multiple P2 receptor subtypes (Lenertz et al., 2011; Orriss et al., 2010). Nucleotides are released from cells constitutively and in response to mechanical stimulation, osmotic swelling, shear stress and inflammation (Bodin and Burnstock, 2001; Burnstock, 2007), and are thought to be key mediators in skeletal cell responses to mechanical stimuli (Dixon and Sims, 2000; Lenertz et al.; Panupinthu et al., 2008; Robling et al., 2006; Robling and Turner, 2009). Nucleotide release into the extracellular environment may occur through transporter and channel proteins, connexin or pannexin hemichannels, or vesicular exocytosis; although
the exact mechanisms have been debated (Bodin and Burnstock, 2001; Lazarowski et al., 2011). In bone, ATP released by mechanical stress (Romanello et al., 2001) is thought to bind to P2 receptors on cells of the osteoblast and osteoclast lineages, resulting in remodelling (Buckley et al., 2003; Dixon, 1998; Lenertz et al.; Li J, 2005) (Figure 1.3).

P2 receptors are known to play important roles in bone remodelling. For instance, in the P2Y family, P2Y₁ has been proposed to increase osteoclast formation and bone resorption, while P2Y₆ has been shown to enhance osteoclast survival (Korcok et al., 2005), and P2Y₂ has been shown to inhibit bone mineralization through obstruction of ALP activity (Orriss et al., 2010). Recent evidence suggests mice lacking functional P2Y₁₂ receptors have decreased resorptive activity in osteoclasts, and exhibited protection from bone loss (Su, 2012). Additionally, P2ry₁₃-deficient mice exhibit decreased osteoblast and osteoclast number, reduced bone turnover and increased marrow adipocytes (Biver et al., 2013). In the P2X family of subunits, P2X2 has been shown to increase bone resorption in osteoclasts, while P2X5 acts to increase osteoblast proliferation (Orriss et al., 2010). Thus, P2 receptors play an important role in bone remodelling.

1.5.2 P2X7 Nucleotide Receptors

Compared to the other P2X receptors, P2X7 has an extended intracellular C-terminal tail that provides a docking site for intracellular signalling molecules and is essential for the receptor’s ability to elicit pore formation and membrane blebbing (Garcia-Marcos et al., 2006; Grol et al., 2009). Pore formation can lead to cell death by triggering apoptosis due to massive uptake of Cl⁻ or excessive leakage of intracellular K⁺.
Figure 1.3 P2 receptors in bone.

This general schematic indicates the potential roles for P2 nucleotide receptor signalling in bone. Nucleotides are released by inflammation as well as mechanical stimulation of bone. ATP released into the extracellular environment from osteoblasts can act in an autocrine and/or paracrine fashion to signal through multiple cell-surface P2 receptors subtypes on osteoblasts and osteoclasts, regulating bone remodelling. Image courtesy of Dr. M.W. Grol.
Prolonged activation of P2X7 can provoke major cellular changes such as fragmentation of DNA or vesiculation and blebbing of the plasma membrane (Garcia-Marcos et al., 2006). Previous research from our laboratory has shown that membrane blebbing occurs in osteoblasts isolated from mouse calvarial cells upon activation of P2X7 receptors with ATP or a more potent P2X7 agonist \(2',3'\text{-O-}(4\text{-benzoylbenzoyl})\text{-ATP} \) (BzATP) (Panupinthu et al., 2007). \textit{In vivo}, osteoblast blebbing has been seen in bone healing studies (Olmedo et al., 1999), although the function of blebbing remains unknown. \textit{In vitro} pore formation in response to mechanical stimulation of osteoblasts seems to be mediated by P2 receptors, implicating a role for P2X7 receptor activation and pore formation \textit{in vivo} (Grol et al., 2009; Li et al., 2005).

### 1.5.3 Coupling P2X7 Activity in Bone Remodelling and Adiposity

As reviewed above, recent research in bone and adipose tissues has revealed complex pathways linking bone remodelling to lipid metabolism (de Paula et al., 2010; Ferron et al., 2010). P2X7 is known to play a key role in bone remodelling (Grol et al., 2009). Osteoblasts and osteoclasts have been shown to possess functional P2X7 receptors (Ke et al., 2003; Korcok et al., 2004; Naemsch et al., 2001; Panupinthu et al., 2008; Panupinthu et al., 2007). Mice deficient in P2X7 (\(P2rx7^{-/-}\)) display diminished periosteal bone formation, excessive trabecular bone resorption (Ke et al., 2003), and an impaired response to mechanical loading (Li et al., 2005), implicating P2X7 signalling in mechanotransduction.

Recent studies from our laboratory have illustrated that activation of P2X7 receptors results in enhanced osteoblast differentiation and matrix mineralization.
(Panupinthu et al., 2008). Interestingly, bone cells from $P2rx7^{-/-}$ mice displayed greater expression of adipogenic markers PPAR$\gamma$ and lipoprotein lipase in comparison to wild-type (WT) cells. Moreover, there was suppressed osteoblast differentiation in cultures of bone cells from $P2rx7^{-/-}$ mice (Panupinthu et al., 2008). This finding is important to understanding the mechanisms regulating differentiation, as osteoblasts and adipocytes arise from the same multipotent mesenchymal precursor and become terminally differentiated to either lineage due to the influence of regulatory transcription factors such as Osx and PPAR$\gamma$ (Akune et al., 2004). Furthermore, P2X7-induced osteogenesis required both production of lysophosphatidic acid (LPA) and cyclooxygenase activity (Panupinthu et al., 2008). ATP release in response to mechanical stimulation has been shown by others to lead to prostaglandin E$_2$ (PGE$_2$) production by osteoblasts. PGE$_2$ is a known anabolic factor in bone formation, the production of which is impaired in $P2rx7^{-/-}$ mice as well as the subsequent osteogenesis, even upon mechanical induction (Li et al., 2005; Panupinthu et al., 2008). Therefore, it appears that the activation of P2X7 receptors directs the commitment of BMSCs towards the osteoblast lineage, and away from the adipocyte lineage (Figure 1.4). In this thesis, I extend these in vitro observations by examining the effect of P2X7 on adiposity in vivo.

To date, there exists minimal literature on P2X7 in adipocytes. Given the association of P2X7 with lipid signalling pathways, this area of research is a fruitful avenue to pursue. Additionally, studies from our laboratory have found that P2X7 activation in osteoblast-like cells triggers Ca$^{2+}$-dependent stimulation of metabolic acid production that is dependent on glucose and phosphatidylinositol 3-kinase activity (Grol et al., 2012). Work from another lab has also shown that P2X7 is a key regulator of
Figure 1.4 The proposed role of P2X7 in osteogenesis and adipogenesis.

Previous work has suggested that P2X7 plays a role in upregulating osteogenesis and suppressing adipogenesis. Bone cells isolated from mice lacking functional P2X7 exhibit increased adipocyte markers and reduced osteogenic markers. Based on this work, P2X7 may play a role in the determination of lineage commitment from stromal cell populations.
aerobic glycolysis (Amoroso et al., 2012). Taken together, these studies reveal an emerging role for P2X7 in cellular metabolism.

1.5.4 P2X7 Expression and Polymorphisms in Humans

Human P2X7 receptor gene (P2RX7) polymorphisms represent a genetic risk factor for skeletal disease. Interestingly, P2RX7 is one of the most polymorphic P2 receptors, with 40 characterized nonsynonymous single nucleotide polymorphism (SNPs) (Di Virgilio and Wiley, 2002). Studies have shown that two of the most common polymorphisms in this gene result in a complete loss of function (as homozygous or in combination with other loss-of-function polymorphisms) (Fuller, 2009; Sluyter, 2011). The combination of these two variants is found in ~3% of human subjects, and has been found to be the most common cause of near-absence of P2RX7 function (Dao-Ung et al., 2004). Furthermore, several recent studies have established the association of loss-of-function polymorphisms in the P2RX7 receptor with lower bone mineral density (Wesselius et al., 2013), accelerated bone loss (Gartland et al., 2012) and increased osteoporosis risk (Husted et al., 2013; Wesselius et al., 2013). Studies have also shown that loss-of-function P2RX7 polymorphisms are associated with increased fracture risk and rates of bone loss in post-menopausal women (Grol et al., 2009; Jorgensen et al., 2012; Ohlendorff et al., 2007). Effects of these loss-of-function polymorphisms in humans are consistent with the skeletal phenotype of the P2rx7−/− mouse (Ke et al., 2003).

As aforementioned, few studies have investigated the role of P2X7 in adipocytes. One study has shown that human adipocytes express functional P2RX7 (Madec et al., 2011). In this same study, patients with metabolic syndrome showed enhanced expression
of \(P2RX7\) in adipocytes, suggesting a link between P2X7 and metabolic syndrome. Additionally, elevated \(P2RX7\) expression levels have been shown in obese patients and reduced levels have been shown in patients with type II diabetes mellitus (Glas et al., 2009). When challenging mice lacking functional \(P2rx7\) with a high-fat diet, these mice exhibited hyperglycemia and glucose intolerance (Glas et al., 2009), suggesting a role for P2X7 in metabolism. Moreover, increased rates of marrow adipogenesis in humans is associated with aging as well as chronic diseases such as diabetes mellitus and osteoporosis (Gourine et al., 2009). Currently, no studies exist that address the effects of P2X7 on the state of adiposity within the bone marrow. It will be important in future studies to investigate the role of P2X7 receptors in metabolic diseases affecting bone and adipose tissues.

1.6  Effects of Mechanical Stimulation on Bone

1.6.1  Mechanical Loading

In 1892, Julius Wolff published his “Law of Transformation of Bone”, where he reported that bone alters its inner architecture due to loading and, as a secondary effect, alters its shape, processes that can be modeled using mathematical laws (Wolff, 1986). This adaptability in response to functional loading is a unique property of the skeleton (Prisby et al., 2008; Robling et al., 2006; Skerry, 2008). As bones provide the ability for locomotion, they are designed to endure cyclic loading forces from common activities such as walking, to running and vigorous weight-lifting (Robling et al., 2006; Skerry, 2008). Load bearing bones of the axial and appendicular skeleton resist the deformations due to compressive, torsional or tensile forces (Duncan and Turner, 1995). Excessive
loading can result in failure, where bones fracture, most commonly due to torsional loading (Marieb et al., 2011).

Skeletal mechanical loading is critical for the development, growth and maintenance of bones involved in weight-bearing (Turner et al., 2009). Moreover, mechanical loading is also necessary for the normal functioning of joints (Liu et al., 2001). Mechanical loading has been shown to elicit anabolic responses in bone by altering the balance between resorption and formation (Ozcivici et al., 2010; Prisby et al., 2008). The osteogenic effect of loading results from increased activity of osteoblasts and decreased activity of osteoclasts, leading to increased bone formation rates and net bone formation (Judex and Zernicke, 2000). The local anabolic response to bone loading is apparent from studies in athletes such as tennis players, where their playing arms develop larger, denser bones relative to their non-playing arms (Jones et al., 1977; Turner et al., 2009). Thus, enhanced bone properties tend to be localized to the bones specifically involved in the loading events (Jones et al., 1977), as signal transmissibility becomes attenuated as it travels through the skeletal elements (Kiiski et al., 2008; Robling et al., 2006).

In the absence of mechanical loading, the skeleton experiences an absence of normal strain. This leads to accelerated bone turnover and loss of bone mass due to excessive resorption. This catabolic effect is evident in patients experiencing prolonged bed rest, those who are immobilized, and astronauts in microgravity (LeBlanc et al., 2007; Robling et al., 2006). Overuse can also damage the bone tissue, eliciting bone remodelling to replace the damaged bone. However, if damage occurs faster than it can be remodelled, then microcracks accumulate and subsequently stress fractures can occur.
1.6.2 Mechanotransduction

Mechanotransduction is the translation of mechanical stimulation into cellular responses (Grol et al., 2009; Robling and Turner, 2009). Various forms of mechanical stimulation include substrate stretching (tension), fluid shear stress, compressive loading and vibration. It has been suggested that bone cells have several mechanosensors at the cell surface that sense mechanical stimuli and initiate cellular responses through mechanotransduction (Turner et al., 2009). Putative mechanosensors, which may function individually or together, include: stretch-activated ion channels, focal adhesion kinases (FAK), proline-rich tyrosine kinase 2 (Pyk2), integrins, cell-cell adhesion elements (cadherins and gap junctions), primary cilia, and cytoskeletal elements (Papachroni et al., 2009; Turner et al., 2009). When stimulated, mechanosensors initiate cell signalling in a cell-type, duration and force-dependent manner.

Osteocytes have long been considered the key regulators of mechanotransduction in bone. This is due to their interconnectivity via processes and gap junctions, which are thought to allow them to regulate bone remodelling in response to mechanical loads (Bonewald and Johnson, 2008). The deformation of bones during loading can be sensed at the cellular level, whereby loading produces pressure gradients that drive extracellular fluid to flow through canaliculi (surrounding osteocyte processes) eliciting fluid shear stresses (Bonewald and Johnson, 2008). Shear stresses can elicit responses such as calcium influx via voltage- and mechanosensitive channels (Robling et al., 2006). Additionally, shear stress enhances ATP release into the extracellular environment, which
can then act on P2 receptors to initiate downstream signalling (Genetos et al., 2005; Robling et al., 2006) (Figure 1.2). SOST is also thought to play a role in mechanotransduction. SOST is constitutively expressed by osteocytes, and is an inhibitor of bone formation (Robling et al., 2008; Turner et al., 2009). SOST does this by binding low-density lipoprotein receptor-related protein 5 (LRP5) receptors, blocking Wnt binding and thus inhibiting Wnt signalling. Mechanical loading reduces SOST expression, thus increasing bone formation, whereas hind-limb unloading increases SOST expression and reduces bone formation (Robling et al., 2008; Turner et al., 2009).

Mechanotransduction is thought to also play a role in the process of osteoblasts becoming mature osteocytes (Bonewald, 2006). The role of osteoblasts in mechanotransduction, and their ability to respond to vibration, is less well studied than the osteocyte (Bonewald and Johnson, 2008). Osteoblasts can act independently of the osteocyte to mediate mechanotransduction, as evident in cell cultures (Robling et al., 2006). Mechanotransduction in osteoblasts is proposed to occur through mechanosensors such as stretch-activated ion channels, integrins and primary cilia. The mechanotransduction response is immediate, eliciting ATP and cytosolic calcium signalling, followed by production of other signalling molecules including nitric oxide (NO) and PGE₂, which in contrast to NO is anabolic for osteoblast activity and bone formation. This is followed by activation of transcription factors leading to bone formation (Figure 1.5).

BMSCs also have a role in mechanotransduction. The hydrostatic pressures within the marrow cavity produce fluid shear stresses on BMSCs, which can stimulate the release of NO, a strong inhibitor of bone resorption, likely via inhibition of RANKL
Figure 1.5 Mechanotransduction in bone.

This schematic illustrates the proposed model for how mechanical stimulation initiates a signalling cascade beginning with mechanosensors on cells of the osteoblast lineage, releasing intracellular ATP stores to the extracellular environment. Extracellular nucleotides then act in an autocrine/paracrine fashion to activate P2X (green) and P2Y (red) cell surface receptors on surrounding cells. Specifically, activation of P2X7 results in calcium influxes that further initiate the release of secondary messengers acting on other receptors (blue), activating transcription factors and gene expression resulting in enhanced bone formation.
expression (Robling et al., 2006). Thus, osteoclasts are believed to have an indirect response in mechanotransduction. Taken together, it is evident that all bone cell lineages play a role in mechanotransduction through a variety of possible mechanisms.

1.6.3 Low-Magnitude, High-Frequency Vibration

Mechanical loading was first thought to produce an anabolic effects in bone at only high magnitudes, similar to what is experienced during vigorous physical activity (Rubin et al., 2001b). Conversely, studies over the last few decades have built a growing body of evidence demonstrating that low-magnitude stimuli can promote osteogenic effects in bone. With the immediate need for non-pharmacological interventions to mitigate bone loss, the advent of low-magnitude, high frequency whole-body vibration (WBV) has become a popular form of anabolic mechanical stimulation in bone (Rubin et al., 2001b). In the last decade alone, WBV has become wide-spread due to commercialization of these devices marketed towards athletic training, and as a potential prevention/treatment method for musculoskeletal diseases (Muir et al., 2013).

WBV is accomplished by the subject (a human or animal) standing on a vibrating platform (Fritton et al., 1997). Vibration is a mechanical form of oscillatory motion that moves continuously back and forth about an equilibrium point (Halliday et al., 2008). In vivo vibration studies in animals and humans often report vibration with a sinusoidal function. The movement of vibration platforms can be controlled by altering frequency and/or amplitude (both of which affect acceleration). Frequency is measured by the number of oscillations per second, expressed in hertz (Hz) (Lorenzen et al., 2009). Peak-to-peak amplitude is the maximum distance between the displacements on either side of
the equilibrium point. The maximum acceleration (rate of change in velocity over time) is dependent on frequency and amplitude. For a sinusoidal vibration, the maximum acceleration is given by the following equation:

\[ a_{\text{max}} = \frac{A(2\pi f)^2}{9.81} \]

where \( a_{\text{max}} \) is maximum acceleration in g

\( A \) is amplitude (0.5 of peak-to-peak displacement) in m

\( f \) is the frequency in Hz

Moreover, in the term ‘low magnitude, high-frequency vibration’, low-magnitude refers to acceleration (<1 g, where \( g = 9.98 \text{ m/s}^2 \)) and high-frequency refers to frequencies in the range of 20 – 90 Hz (Lau et al., 2010). It is imperative that studies report these variables using consistent terminology for comparison among studies.

It is important to note that, although use of WBV devices is commercialized and popular, the safety of these devices needs further study. Safety is a critical consideration, especially for those suffering with musculoskeletal diseases or injuries, and those who are infirm or elderly (Chan et al., 2013; Kiiski et al., 2008). Although vibration may be delivered with low acceleration, the signal can either become amplified (due to resonance) or attenuated (damped). Not surprisingly, transmissibility through skeletal tissues is dependent on the frequency, amplitude, maximal acceleration and location of measurement in the skeleton. To date, studies have shown that short daily exposures to vibration at low amplitudes are safe, but caution should be exercised with usage (Kiiski et al., 2008; Muir et al., 2013).
1.6.4 Effects of Whole-Body Vibration

Low-magnitude, high-frequency WBV has been previously shown to increase bone formation (Garman et al., 2007; Hwang et al., 2009; Rubin et al., 2001a; Xie et al., 2006; Xie et al., 2008), reduce fat formation (Luu et al., 2009; Rubin et al., 2007), increase lean muscle tissue (Verschueren et al., 2004; Xie et al., 2008) and improve balance (Gusi et al., 2006) (Figure 1.6). Low-magnitude, high-frequency vibration is estimated to produce microstrain that is several orders of magnitude below that which is experienced during vigorous physical activity in humans (Rubin et al., 2002). In vivo studies have demonstrated that brief vibration treatments with frequencies ranging from 20-50 Hz over differing time courses (days, weeks, months) and can increase bone quantity and quality relative to controls (Judex et al., 2004, Rubin et al., 2002). Furthermore, hind-limb unloaded mice showed a significant reduction in the rate of bone formation (Rubin et al., 2001b). This effect in hind-limb unloaded mice was rescued by brief, intermittent vibration, which increased bone formation rates to control values. Vibration studies have tested a range of frequencies, amplitudes and accelerations for their impact on bone (Judex et al., 2005; Rubin et al., 2001b). However, despite the body of research showing a desirable effect of vibration on the skeleton, more recent studies have not supported this notion. Studies by other groups, using a variety of vibration dose parameters, do not show improved skeletal properties using WBV (Castillo et al., 2006; Cheung and Giangregorio, 2012; Lynch et al., 2011; Manske et al., 2012). Therefore, further studies are required to investigate the controversial results supporting the current paradigm.

Additionally, a multitude of studies have reported the use of whole-body vibration
Figure 1.6 General overview of vibration-induced mechanotransduction.

Osteoprogenitors, osteoblasts, osteoclasts and osteocytes are responsive to a variety of mechanical stimuli, including strain, fluid shear stress and vibration. The shaded triangle illustrates the strength of the vibration stimulus sensed at the animal, organ and cellular level. The mechanisms by which cells convert these stimuli into biological responses are referred to as mechanotransduction. Studies have shown that low-magnitude, high-frequency vibration can increase bone and muscle formation while reducing fat formation. However, the cellular mechanisms responsible for the changes seen with whole-body vibration are unknown. Given the clinical implications, understanding how whole-body vibration affects bone at the morphological and cellular level is of emerging interest.
as an anabolic factor in the musculoskeletal system, however, there remains to be a consensus on the ideal parameters to which tissues respond in an anabolic fashion. The proper anabolic dose of vibration frequency, amplitude, acceleration, and duration and repetitions of vibration exposure remains to be determined. Moreover, the cellular and molecular mechanisms underlying the response of bone to mechanical stimuli remain largely unknown. Taken together, this indicates the urgent need for further research on this form of mechanical stimulation as a training or treatment intervention.

1.6.5 The Relationship between Mechanical Stimulation, Bone Formation and P2X7

It is well established that mechanical loading is an strong anabolic stimulus for bone formation (Robling et al., 2006). As described above, mechanical stimulation leads to the release of nucleotides into the extracellular environment, which can then act as autocrine/paracrine regulators of osteoblast and osteoclast function through P2 receptor activation (Grol et al., 2009). Both bone cell types express multiple subtypes of P2X and P2Y receptors, and it has been hypothesized that P2 receptor signalling is important in mechanotransduction (Dixon and Sims, 2000) (Figure 1.3). A signalling cascade initiated by nucleotide release can elicit the formation of secondary signalling molecules such as NO, PGE2, and LPA from osteoblasts. Research from our laboratory has shown that osteoblasts produce LPA and PGE2 in response to P2X7 activation (Panupinthu et al., 2008). Both NO and PGE2 are known as important biochemical mediators in mechanical loading (Robling et al., 2006). Secondary signalling along with P2 receptor activation can activate transcription factors (such as NFAT, CREB and β-catenin) leading to changes in
gene expression, resulting in enhanced osteoblast differentiation and bone formation (Figure 1.5).

Studies have also shown that mechanical loading promotes osteoblast differentiation at the expense of adipogenesis (David et al., 2007). This is consistent with other work indicating that mechanical stimulation inhibits adipocyte differentiation from mesenchymal stem cells, favouring an osteogenic state even under the influence of an adipogenic environment (Sen et al., 2008). Furthermore, mouse calvarial cells exhibit pore formation in response to fluid shear stress (Li et al., 2005), suggesting involvement of P2X7. Additionally, the presence of P2X7 on osteoclasts can induce osteoclast apoptosis (Grol et al., 2009; Ke et al., 2003). Decreasing osteoclast function and increasing osteoblast function by mechanical stimulation and P2X7 activation may prevent or slow the detrimental effects of degenerative bone diseases. Whole-body vibration has been reported to enhance bone formation by reducing osteoclast function and increasing osteoblast function (Xie et al., 2006); it has also been shown to reduce adipogenesis (Luu et al., 2009; Rubin et al., 2007). Thus, whole-body vibration may cause release of ATP that subsequently activates P2X7, with consequent effects in bone and adipose tissues. P2X7 receptors themselves may be a target for future pharmacological and mechanical therapies.

1.7 Rationale, Hypotheses and Objectives of the Research

Given roles for P2X7 in the determination of cell fate and in mechanotransduction, we hypothesized that P2X7, mechanical vibration, and their possible interaction influence body composition in vivo. In this thesis, I developed a rapid
and reproducible method to characterize whole-body composition during growth and aging of mice, which has not been described previously. The goal was to use this and other methodologies to characterize a genetically modified mouse model with loss of \( P2rx7 \) function. I then investigated the response of mice to whole-body vibration and the possible role of P2X7 in this process. The three specific objectives were proposed, as described below.

### 1.7.1 Quantitative Micro-Computed Tomography for Assessment of Age-Dependent Changes in Murine Whole-Body Composition

**Rationale** – Micro-computed tomography (micro-CT) is the gold-standard imaging modality used routinely to quantify radio-opaque tissue mass in small animal models, which also allows for accurate quantification of soft tissue volumes. Recent advances in micro-CT permit for *in vivo* imaging and quantification of whole-body composition (WBC), including total body, adipose, lean and skeletal tissue parameters that previously could only be evaluated post-mortem. In addition, there is a large demand for quantitative phenotyping of genetically modified mouse models. Consequently, there is a need to develop high-throughput methods for analysing WBC of mice. This is of particular importance for growth and aging studies, where mice must be scanned safely and repeatedly at several ages. However, a quantitative and effective method has yet to be validated for the repeated imaging of animals and quantification of WBC parameters.

**Specific Objective 1** – To characterize changes in whole-body composition of mice during growth and aging using a novel, high-throughput method for analysis of micro-CT images.
1.7.2 Loss of P2X7 Nucleotide Receptor Function Leads to Abnormal Fat Distribution in Mice

*Rationale* – Expression of P2X7 receptors has been reported in adipocytes, yet little is known about their role in this tissue. Recent studies have also shown that the P2X7 receptor regulates metabolism and energy homeostasis in other cell types. Coupling this to previous research from our laboratory indicating that adipogenic markers are upregulated in cells lacking P2X7, a further investigation into the *in vivo* effects of *P2rx7* loss-of-function is necessary. As whole-body composition of skeletal, lean and adipose tissues change with age, we investigated an aging mouse model to potentially provide relevance to age-related diseases such as osteoporosis and obesity.

*Specific Objective 2* – To assess the role of P2X7 in adipocyte distribution and lipid accumulation *in vivo.*

1.7.3 Low-Magnitude, High-Frequency Vibration has No Effect on Murine Whole-Body Composition and Bone Microarchitecture

*Rationale* – Recent evidence from our laboratory demonstrates that activation of P2X7 regulates both osteoblast differentiation and adipogenesis. Moreover, the signalling molecules regulating mesenchymal stem cell differentiation in response to mechanotransduction have yet to be elucidated. We propose that ATP is released upon mechanical stimulation, leads to the activation of P2X7 receptors, modulating osteogenesis and adipogenesis. Mechanical stimulation through WBV has been shown by some authors to have favourable effects on bone, adipose and muscle tissues. To
investigate vibration in a preclinical model, we studied its effects on WBC in wild-type and $P2rx7$ loss-of-function mice.

Specific Objective 3 – To investigate the effects of whole-body vibration on body composition and bone microarchitecture, and the possible role of P2X7 in these processes.
1.8 References


CHAPTER TWO

QUANTITATIVE MICRO-COMPUTED TOMOGRAPHY FOR
ASSESSMENT OF AGE-DEPENDENT CHANGES
IN MURINE WHOLE-BODY COMPOSITION

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1 This Chapter has been reproduced from:
Quantitative micro-computed tomography for assessment of age-dependent changes in
murine whole-body composition. [submitted], with some modifications.
2.1 Chapter Summary

Micro-computed tomography (micro-CT) is used routinely to quantify radiopaque tissue mass in small animal models. Our goal was to evaluate repeated *in vivo* micro-CT imaging for monitoring whole-body composition in studies of growth and aging in mice. Male mice from 2-52 weeks of age were anesthetized and imaged using an eXplore Locus Ultra and/or eXplore speCZT scanner. Images were reconstructed into 3D volumes, signal-intensity thresholds were used to classify each voxel as adipose, lean or skeletal tissue, and tissue masses were calculated from assumed densities. Images revealed specific changes in tissue distribution with growth and aging. Quantification showed biphasic increases in total CT-derived body mass, lean and skeletal tissue masses, consisting of rapid increases to 8 weeks of age, followed by slow linear increases to 52 weeks. In contrast, bone mineral density increased rapidly to a stable plateau at ~14 weeks of age. On the other hand, adipose tissue mass increased continuously with age. A micro-CT-derived total mass was calculated for each mouse and compared with gravimetrically measured mass, which differed on average by less than 3%. Parameters were highly reproducible for mice of the same age, but variability increased slightly with age. There was also good agreement in parameters for the same group of mice scanned on the eXplore Locus Ultra and eXplore speCZT systems. This study provides reference values for normative comparisons; as well, it demonstrates the usefulness of *in vivo* single-energy micro-CT scans to quantify whole-body composition in high-throughput studies of growth and aging in mice.
2.2 Introduction

Mouse models are increasingly important in biomedical research due to their ease of genetic manipulation, ability to mimic human diseases, small body size, and relatively affordable costs (Rosenthal and Brown, 2007). When using animal models, it is important to have reference values for normative comparisons. Mouse strains often used in research today include C57BL/6, BALB/c, 129 substrains, C3H and DBA/2 mice (Beck et al., 2000; Buie et al., 2008; Crawley et al., 1997; Hankenson et al., 2008). Descriptions of the most commonly used C57BL/6 mouse include masses of wet and dry individually-isolated bones, and bone mineral density (BMD) and bone mineral content (BMC) of specific bones (e.g. tibia and femur) obtained using destructive or imaging methods (Brochmann et al., 2003; Glatt et al., 2007; Sheng et al., 1999; Somerville et al., 2004). Although many parameters can be measured post-mortem, longitudinal in vivo studies of growing and aging mice were not possible until the advent of non-invasive imaging modalities. It is now important to establish safe, effective and reproducible approaches for assessing whole-body composition in vivo and to establish normal reference values for growing and aging mice.

Several in vivo imaging modalities exist for determining skeletal phenotypes in animals, each with differing strengths and weaknesses. Dual-energy X-ray absorptiometry (DXA) is a simple approach to determine areal BMD (aBMD) and is used for clinical diagnosis and monitoring the progression of bone diseases such as osteoporosis (Pisani et al., 2013). Determination of fat content from the scans is also possible (Halldorsdottir et al., 2009; Pietrobelli et al., 1996; Senn et al., 2007; Sjogren et al., 2001). However, DXA does not distinguish between cortical and trabecular bone, and
is known to overestimate or underestimate the density of large or small bones, respectively (Judek et al., 2003). When using small animal models such as mice, the accuracy of measurement is critical; therefore, CT methods are preferred. High-resolution peripheral quantitative computed tomography (HR-pQCT) is a method used clinically to determine BMD in peripheral long bones of human patients (Cheung et al., 2013) and has also been used to quantify BMD and bone microarchitecture in rodents (Bagi et al., 2006; Beamer et al., 1996; Richman et al., 2001). High-resolution magnetic resonance imaging (HR-MRI) can be used to quantify soft and hard tissues. Although radiation is not an issue in this method, high-resolution images for bone morphometry require long scan times (Donnelly, 2011). Another CT method to determine BMD is synchrotron radiation micro-CT (SRmuCT). Although SRmuCT yields images with high spatial resolution and excellent signal-to-noise ratio, it has more commonly been used for ex vivo rather than in vivo studies (Donnelly, 2011; Ito, 2005; Ito et al., 2003; Martin-Badosa et al., 2003; Raum et al., 2007). Furthermore, a limitation of this modality is that few synchrotron facilities are available (Donnelly, 2011). Therefore, the widely available technology of micro-computed tomography (micro-CT) has become a popular choice for assessment of BMD and bone microarchitecture (Kazakia et al., 2008).

Micro-CT is the gold standard for morphologic assessment and quantification of radio-opaque tissues in small samples (Donnelly, 2011; MacNeil and Boyd, 2007), including evaluation of skeletal anatomy, abnormalities and morphometry in rodent models (Ford-Hutchinson et al., 2003; Hankenson et al., 2008). Moreover, micro-CT has several advantages: it is designed specifically for imaging and analysis of small samples at high-resolution; some systems have the capacity to perform scans of whole animals
such as small rodents; *in vivo* scans can be obtained rapidly without sacrificing animals; and longitudinal studies with repeated scanning of animals are possible with low radiation exposure (Glatt et al., 2007; Granton et al., 2010; Judex et al., 2003). In addition, advanced micro-CT imaging allows for accurate quantification of soft-tissue volumes. This permits assessment of whole-body composition (WBC) *in vivo*, including total body, adipose, lean and skeletal tissue parameters (Granton et al., 2010), which previously could only be evaluated post-mortem using the other imaging modalities mentioned above. Thus, advanced *in vivo* micro-CT imaging is an emerging technique to monitor growth including normal and abnormal skeletogenesis in rodents (Guldberg et al., 2004). However, advanced image analysis can be quite time consuming and tedious, as large data sets can take hundreds of hours to quantify using conventional approaches. Therefore, as micro-CT has become a preferred imaging modality for quantification of WBC in small animals, the need for high-throughput methods of analysis becomes increasingly urgent.

In the present study, we report the first use of micro-CT to monitor and repeatedly quantify WBC in groups of mice during growth and aging. WBC analysis was enabled by in-house-designed software, which quantifies whole-body parameters from scanned images, based on threshold density values of specific tissues and phantom calibrators. Although others have previously reported densities and characteristics of specific bones (Glatt et al., 2007; Miller et al., 2007; Somerville et al., 2004), there have been no previous studies using *in vivo* micro-CT to quantify changes in WBC of mice during growth and aging. In the present study, cohorts of mice were scanned at 3-week intervals from 2 to 8 weeks of age and from 8 to 26 weeks of age, with additional groups scanned
at 39 and 52 weeks of age. Outcome values included: total body mass (g); adipose, lean
and skeletal tissue masses (g) and mass percents (%); bone mineral density (BMD, mg
HA/cm³); and bone mineral composition (BMC, mg HA). Femur and tibia lengths were
measured using anatomical markers, and maximum BMD of femoral mid-diaphyses was
quantified. Variability and internal validity of the scanning protocol were assessed. Our
findings establish that non-invasive single-energy micro-CT is an accurate and effective
tool for characterizing the WBC of mice during growth and aging.
2.3 Materials and methods

2.3.1 Animals

Mice, maintained on a mixed genetic background of commonly used strains (C57BL/6 × 129/Ola × DBA/2), were housed in standard cages and maintained on a 12-hour light/dark cycle, with water and standard mouse chow available ad libitum (2018 Teklad Global 18% protein rodent diet, Harlan Laboratories, Indianapolis, IN, USA). This study was conducted in accordance with the policies and guidelines of the Canadian Council on Animal Care and was approved by the Animal Use Subcommittee of The University of Western Ontario, London, Canada. Four cohorts of mice were studied. For cohort 1, the same mice were scanned at 2, 5 and 8 weeks of age. The mice in cohort 2 were scanned at three-week intervals from 8 to 26 weeks of age, inclusive. Cohort 3 mice were studied at 39 weeks of age. Finally, the mice in cohort 4 were scanned at 52 weeks of age (Table 2.1).

2.3.2 Micro-computed tomography (micro-CT) image acquisition

Whole-body composition of mice between 2 and 52 weeks of age was assessed using micro-CT. Mice were anesthetized with isoflurane (Forane, catalog # CA2L9100, Baxter Corporation, Mississauga, ON, Canada) and imaged using an eXplore Locus Ultra micro-CT scanner and/or an eXplore speCZT scanner (GE Healthcare Biosciences, London, ON, Canada). A calibrating phantom composed of air, water and cortical bone-mimicking epoxy having a bone mineral equivalent of 1100 mg/cm³ (SB3; Gammex, Middleton, WI, USA) (White, 1978) was scanned together with the animals. On the eXplore Locus Ultra scanner, 1000 projection images were obtained over a single 16-
Whole-body scans using micro-computed tomography were performed on four cohorts of male mice. In cohort 1, the same mice were scanned at three-week intervals (2, 5 and 8 weeks of age). Cohort 2 mice were scanned at three week-intervals (from 8-26 weeks of age). Cohort 3 was scanned only at 39 weeks and cohort 4 only at 52 weeks of age. All cohorts were scanned using the eXplore Locus Ultra system, and cohort 1 was also scanned using the eXplore speCZT system.

Table 2.1 Mouse cohorts.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>2-8</td>
<td>8-26</td>
<td>39</td>
<td>52</td>
<td>2-52</td>
</tr>
<tr>
<td>Number of mice (n)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>31</td>
</tr>
</tbody>
</table>
second rotation (80 kVp, 55 mA tube current, 16 ms exposure). On the eXplore speCZT scanner, 900 projection images were obtained over a single 5-minute rotation (90 kVp, 40 mA tube current, 16 ms exposure). While still anesthetized immediately after scanning, mice were gravimetrically weighed using a portable electronic precision scale (Acculab VICON VIC-511; Sartorius Group, Germany).

Data sets were reconstructed into 3D volumes from the X-ray projection data with nominal isotropic voxel spacing of 154 µm (eXplore Locus Ultra) and 50 µm (eXplore speCZT) using a cone-beam filtered backprojection algorithm. The 50 µm volumes from the eXplore speCZT were then spatially-averaged to 100 µm (to improve the signal-to-noise characteristics of the volume) and 150 µm (to compare with data from the eXplore Locus Ultra). The reconstructed data were linearly rescaled into Hounsfield units (HU), with the voxel gray-level of air being -1000 HU and water 0 HU.

2.3.3 Analysis of whole-body composition

Using MicroView software (GE Healthcare Biosciences), three signal-intensity thresholds (-200, -30 and 190 HU) were used to classify each voxel as adipose (-200 to -31 HU), lean (-30 to 189 HU), or skeletal tissue (≥190 HU), respectively for the 154 and 150 µm scans. A different set of global thresholds was determined using MicroView for classification of adipose, lean and skeletal tissue for the 100 µm scans (-275, -40 and 250 HU, respectively). The signal-intensity thresholds were determined visually by applying thresholds to CT-derived data from several mice at each age to properly classify tissues based on observed anatomy. In-house-designed software was used to calculate tissue masses from assumed tissue densities of 0.95 (adipose), 1.05 (lean) and 1.92 (skeletal).
g/cm³, as listed by the International Commission on Radiation Units and Measurements (ICRU, 1989). The software computed the tissue masses first by calculating the volume of voxels (in cm³) for each of adipose, lean, and bone tissue using the signal-intensity thresholds described above (Beaucage et al., 2014; Granton et al., 2010). Once the volume of each tissue was calculated, its contributing mass was computed as the product of the tissue volume and the corresponding tissue density. The sum of all tissue masses yielded a CT-derived estimate of whole-body mass. In addition, the software was used to calculate BMD and BMC. Briefly, BMD was computed as the ratio of the average HU value of the skeletal region of interest (ROI) to the measured HU value of the SB3 calibrator, multiplied by the known hydroxyapatite (HA)-equivalent density of the SB3 (1100 mg/cm³). Thereafter, the program automatically computed BMC as the product of the BMD (mg HA/cm³) and the total volume of the skeletal ROI used in the BMD calculation.

2.3.4 Hind-limb long-bone length

Bone lengths were determined using the line measurement tool in MicroView. Femurs were measured from the base of the lateral femoral condyle to the tip of the greater trochanter. Tibial lengths were measured from the base of the medial malleolus to the tip of the intercondylar eminence.

2.3.5 Determination of maximum bone mineral density

The maximum value of BMD was determined for an ROI that encompassed the mid-diaphysis of the left femur. The ROI was 33% of the total femur length along the long
axis and spatially encompassed the entire mid-diaphysis in the other axes. In-house-designed software was used to determine the maximum BMD value for each femur. For consistency, when assessing these BMD values in Cohort 1 mice using 100 µm eXplore speCZT scans, we used the same threshold value as above for skeletal tissue (250 HU). Similarly, when assessing the BMD in skeletally mature mice (Cohorts 2-4) using 154 µm eXplore Locus Ultra scans, we used the same threshold value as above for skeletal tissue (190 HU). For each ROI, a histogram of BMD values was generated, plotting the gray level frequency versus the gray level from the indicated skeletal thresholds to the maximum value. The maximum BMD was reported as the 95th percentile of this histogram.

2.2.6 Statistical analyses

Data are presented as means ± standard deviation (S.D.). Differences between two groups were assessed using the Student’s t-test. Differences among three or more groups were evaluated by two-way analysis of variance followed by a Bonferroni multiple comparisons test. Curves were fit to growth data (total body mass, lean and skeletal tissue mass, BMD and BMC) by non-linear least squares regression using two-phase association in GraphPad Prism software version 5 (GraphPad Software Inc., La Jolla, CA, USA). Curves were fit to growth data for adipose tissue mass by non-linear least squares regression using an exponential growth equation in GraphPad Prism. Differences were accepted as statistically significant at \( p < 0.05 \). All \( n \) values represent the number of mice used in each group.
2.4 Results

2.4.1 Imaging whole-body composition of mice during growth and aging

A total of 31 male mice, divided into four cohorts, were assessed using *in vivo* quantitative micro-CT at specific ages (Table 2.1). Scans obtained using the eXplore Locus Ultra micro-CT were reconstructed with nominal isotropic voxel spacing of 154 \( \mu \)m and rescaled into Hounsfield units. An in-house-designed program was used to assign each voxel as adipose, lean or skeletal tissue, based on threshold values. Representative mid-coronal images demonstrate the pattern of changes in whole-body composition during growth and aging (Figure 2.1). At 2 weeks of age, the mice had a large amount of adipose tissue (blue) relative to their body size, which appeared to be primarily subcutaneous fat. At this age, the skeleton was still developing, as evident by the larger relative size of the appendicular skeletal elements and skull relative to overall body size, as well as incomplete mineralization of caudal vertebrae. By 5 weeks of age, we saw a marked reduction in subcutaneous adipose tissue accompanied by a corresponding increase in the proportion of lean tissue. At 52 weeks of age, mice exhibited a marked increase in visceral adiposity at the expense of lean tissue. These examples illustrate that 3D micro-CT images with appropriate thresholding can reveal the localization of tissue types within the body and changes in their distribution with growth and aging.

2.4.2 Quantification of whole-body composition during growth and aging

Adipose, lean and skeletal tissue volumes were converted into tissue masses by multiplying each volume by the respective tissue densities of 0.95, 1.05, and 1.92 g/cm\(^3\) (ICRU, 1989). Time course studies revealed dramatic changes in body composition.
Figure 2.1 Representative micro-CT images of whole-body composition showing total, adipose, lean, and skeletal tissues.

Cohorts of male mice at several ages were anesthetized for whole-body scans using the eXplore Locus Ultra micro-CT. Scans were reconstructed with nominal isotropic voxel spacing of 154 µm and rescaled into Hounsfield units using the same protocol for all ages. An in-house-designed program was used to assign each voxel as adipose, lean or skeletal tissue, based on threshold values. Each row shows the same mid-coronal slice from a single representative mouse at each age, indicated on the left. Images were overlaid with the identified skeletal tissue (shown in white as surface-rendered images of the highest quality with no decimation). Second and third columns show adipose tissue (blue) and lean tissue (red), respectively. High-density particles in the abdomen of mice from 5-52 weeks of age arose from material in the animal chow. Scale bar represents 1 cm at each age point. Images are representative of at least 7 mice at each age point.
during growth, and more modest changes during aging (Figure 2.2). Total CT-derived body mass showed an initial rapid increase from 2 to 8 weeks of age, followed by a slow linear increase to 52 weeks of age (Figure 2.2 A). Lean and skeletal tissue masses and BMC displayed a pattern similar to that of total body mass (Figure 2.2 C, 2 E, 2 F). In contrast, BMD rose rapidly until 11-14 weeks and then plateaued (Figure 2.2 B). Unlike other parameters, adipose tissue mass increased continuously, with the rate of increase escalating with age (Figure 2.2 D). The percent change in whole-body composition parameters was calculated between 2 and 52 weeks of age (Table 2.2). We found that lean and skeletal masses increased 300-350%, whereas adipose tissue mass showed a larger relative increase of over 460%. BMD doubled and BMC increased 7-fold, reflecting both the increase in BMD and growth in skeletal mass over time.

We next assessed the variability in whole-body composition parameters among mice at each age. Figure 2.3 illustrates the range of values observed for each individual mouse. Variability was also quantified as the coefficient of variation (Table 2.3). Variability in CT-derived total body mass was relatively constant at each age (Figure 2.3 A), with coefficients of variation ranging from 7 to 9%. On the other hand, coefficients of variation for BMD and BMC were larger at 2 weeks of age decreasing to 1-2% and 4-5% in older age groups, respectively (Figure 2.3 B and C). Adipose tissue mass showed greatest variability (Figure 2.3 D), with coefficients ranging from 16% at 2 weeks of age to 26-33% in older mice. Variability of lean tissue mass was relatively constant (Figure 2.3 E), with coefficients ranging between 4 and 6%; whereas variability in skeletal tissue mass decreased with aging (Figure 2.3 F), with the coefficient of variation declining from 10% at 2 weeks to 3% at 52 weeks.
Figure 2.2 Quantification of changes in whole-body composition with mouse age.

Cohorts of male mice were imaged as described in the legend to Figure 2.1. In-house-designed software was used to calculate tissue masses from assumed tissue densities of 0.95 (adipose), 1.05 (lean), and 1.92 (skeletal) g/cm$^3$, respectively. Total body mass ($A$) exhibited an initial rapid increase from 2 to 8 weeks of age, followed by a slow linear increase to 52 weeks. Bone mineral density (BMD, $B$) also showed a rapid increase until 11-14 weeks of age, but in this case was followed by a plateau. Bone mineral content (BMC, $C$) and lean and skeletal tissue masses ($E$, $F$) showed patterns similar to $A$. In contrast, adipose tissue mass ($D$) displayed a distinct pattern, increasing continuously with the rate of increase rising with age. Data points for 8-week-old mice in cohorts 1 and 2 are plotted overlaid in each graph. Data are means ± S.D., $n \geq 7$ mice per age group. Curves were fit by non-linear least squares regression.
Table 2.2 Percent change of whole-body composition parameters with growth and aging.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 weeks of age Mean ± S.D.</th>
<th>52 weeks of age Mean ± S.D.</th>
<th>Percent increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-derived total body mass (g)</td>
<td>7.8 ± 0.2</td>
<td>36.3 ± 1.2</td>
<td>364</td>
</tr>
<tr>
<td>Adipose tissue mass (g)</td>
<td>1.8 ± 0.3</td>
<td>10.1 ± 2.6</td>
<td>463</td>
</tr>
<tr>
<td>Lean tissue mass (g)</td>
<td>4.8 ± 0.2</td>
<td>21.3 ± 1.2</td>
<td>341</td>
</tr>
<tr>
<td>Skeletal tissue mass (g)</td>
<td>1.2 ± 0.1</td>
<td>4.8 ± 0.1</td>
<td>306</td>
</tr>
<tr>
<td>BMD (mg HA/cm³)</td>
<td>171.2 ± 6.8</td>
<td>339.6 ± 6.3</td>
<td>98</td>
</tr>
<tr>
<td>BMC (mg HA)</td>
<td>105.8 ± 14.5</td>
<td>850.9 ± 37.0</td>
<td>704</td>
</tr>
</tbody>
</table>

Data represent means ± S.D. of the indicated parameters obtained from scans on the eXplore Locus Ultra of mice at 2 weeks of age and 52 weeks of age (n = 8 and 7 mice, respectively). All parameters were significantly greater at 52 weeks of age compared to their values at 2 weeks of age (p < 0.05 determined using unpaired two-tailed Student’s t-test).
Figure 2.3 Variability of whole-body composition values.

Images of male mice were obtained and analyzed as described in the legends to Figures 1 and 2. Data points (black circles) represent values of the indicated parameter for each individual mouse. Data show the vertical scatter for the age indicated numerically on the x-axis (horizontal scatter was introduced for clarity only). Eight mice were imaged at each age except 52 weeks, where n = 7 mice. Bars indicate mean ± S.D. for each age. Data for 8-week-old mice are presented for both cohorts 1 and 2. A-F illustrate parameters of whole-body composition in absolute values. G-I illustrate adipose, lean and skeletal tissue masses as a percentage of the total CT-derived body mass.
Table 2.3 Coefficients of variation in whole-body composition values at selected ages.

<table>
<thead>
<tr>
<th>Coefficient of Variation (%)</th>
<th>2 weeks of age</th>
<th>26 weeks of age</th>
<th>52 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-derived total body mass</td>
<td>7.6</td>
<td>8.5</td>
<td>9.0</td>
</tr>
<tr>
<td>BMD</td>
<td>4.0</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>BMC</td>
<td>13.7</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Adipose tissue mass</td>
<td>15.8</td>
<td>32.6</td>
<td>25.8</td>
</tr>
<tr>
<td>Lean tissue mass</td>
<td>4.3</td>
<td>5.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Skeletal tissue mass</td>
<td>10.0</td>
<td>4.1</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Male mice were scanned and images were analyzed as described in the legends to Figures 1 and 2. Coefficients of variation were calculated as the ratio of the S.D. to the mean, expressed as a percentage. Eight mice were imaged at each age except 52 weeks, where $n = 7$ mice.
When tissue masses were expressed as a percentage of total body mass, a large decrease in adipose tissue mass was apparent from 2 to 5 weeks of age, with a corresponding increase in lean tissue mass (Figure 2.3 G, H). In contrast, the percentage of skeletal tissue remained relatively constant with growth and aging (Figure 2.3 I).

### 2.4.3 Length of long bones during growth and aging

When characterizing skeletal phenotypes of mice, the assessment of bone length has been a measurement frequently reported in the literature (Beamer et al., 1996; Brodt et al., 1999; Glatt et al., 2007; Somerville et al., 2004). Therefore, to establish external validity, we determined the lengths of hind-limb long bones using our micro-CT data sets. Using the line measurement tool in MicroView software, the right and left tibiae and femurs were measured in the scanned volume of each mouse. Tibial lengths were determined from the base of the medial malleolus to the tip of the intercondylar eminence (Figure 2.4 A, B). Femoral length was measured from the base of the lateral femoral condyle to the tip of the greater trochanter (Figure 2.4 D, E). Both bones showed rapid growth – over 2 to 8 weeks of age, tibial length increased from 10.5 to 16.3 mm and femoral length increased from 8.7 to 15.3 mm. Thereafter, there was only a slight increase in length to 17.3 mm for the tibia and 16.7 mm for the femur at 52 weeks of age. There were no significance differences between left and right bones at any age point (Figure 2.4 C, F).
Figure 2.4 Lengths of tibiae and femurs in growing and aging mice.

Right and left tibiae and femurs were measured using the line measurement tool in MicroView from base of the medial malleolus to tip of the intercondylar eminence (for tibiae) and base of the lateral femoral condyle to tip of the greater trochanter (for femurs). Right and left bone lengths were overlaid (C, F), revealing no significant differences between right and left limb lengths ($p > 0.05$, determined by two-way repeated measures ANOVA). Data are means ± S.D. Eight mice were imaged at each age except 52 weeks, where $n = 7$ mice.
2.4.4 Maximum bone mineral density

As regional variations in BMD occur within skeletal tissue, the maximum BMD value within a defined ROI better reflects the extent of compact bone mineralization. Therefore, we assessed maximum BMD of an ROI comprised of the mid-diaphyseal region of the femur. Maximum BMD values were reported as the 95th percentiles of the densities. These values displayed a rapid increase from 2 to 11 weeks of age, followed by a slow linear increase to 52 weeks of age (Figure 2.5). Values for the 99th percentile showed a similar pattern as the 95th percentile, reaching 1102 ± 38 mg HA/cm³ at 39 weeks of age, comparable to previously reported values for cortical bone BMD in mice of 1100 to 1200 mg HA/cm³ (Entezari et al., 2012; Windahl et al., 1999). These findings reveal that maximum BMD increases during growth in mice.

2.4.5 Internal validity, precision and reproducibility of scanning protocols

To assess reproducibility of the scanning protocol, we examined two separate cohorts of mice at 8 weeks of age (C1 and C2). Both cohorts were scanned using the eXplore Locus Ultra micro-CT. Images were reconstructed with isotropic voxel spacing of 154 µm, rescaled into Hounsfield units and subjected to whole-body composition analysis. Similar values were obtained for the two cohorts of mice (Figure 2.6). For example, the differences in gravimetric and CT-derived total body masses were only 2.9% and 2.5%, respectively. This similarity provided justification for combining data from the two cohorts of mice to obtain the time courses illustrated in Figure 2.2. Moreover, there was no significant difference between total body masses measured gravimetrically and using micro-CT.
Figure 2.5 Maximum bone mineral density.

Mice from 2 to 8 weeks of age were scanned on the eXplore speCZT system at 50 µm and re-binned to 100 µm for these analyses. Mice from 11 to 52 weeks of age were scanned on the eXplore Locus Ultra system at 154 µm nominal voxel spacing, as described in the legends of Figures 1 and 2. Left femurs were cropped from the whole-body images and axes were reoriented. A region of interest (ROI) that encompassed the mid-diaphysis in all three spatial directions was cropped from each of the volumes. The ROI, which included only cortical bone, was then used to determine the maximum value of BMD. Data are means ± S.D., $n = 7$ mice per age group. The curve was fit by non-linear least squares regression.
Figure 2.6 Reproducibility of whole-body composition determinations.

Two separate cohorts of 8-week-old male mice were imaged as described in the legend to Figure 1 and then weighed to determine gravimetric total body mass. Data were subsequently analyzed to determine CT-derived total body mass and adipose, lean and skeletal tissue masses as described in the legend to Figure 2. Symbols represent values of the indicated parameter for each individual mouse (filled black circles represent animals in cohort 1, and open circles represent cohort 2). Bars are means ± S.D. Eight mice were imaged in each cohort. There were no significant differences between values of gravimetric and CT-derived total body masses, providing validation of the micro-CT-derived tissue mass measurements. In addition, there were no significant differences in masses of adipose, lean or skeletal tissue between cohorts 1 and 2 ($p > 0.05$, determined by two-way ANOVA).
Next, the relationship between gravimetric and CT-derived total body mass was examined in greater detail. We compared the gravimetric to the CT-derived mass at all ages (Table 2.4). The computed total mass corresponded well with the weight of each mouse. The CT-derived masses were consistently less than the gravimetrically measured masses, differing by < 3%. Although small, this difference was significant in mice ≥ 17 weeks of age. These data provide internal validation of the accuracy of our micro-CT-derived tissue mass measurements.

As described above, whole-body composition was assessed using the eXplore Locus Ultra micro-CT scanner at 154 μm resolution. However, the growing mice in cohort 1 (age 2-8 weeks) had smaller, less mineralized bones. Therefore, to confirm the accuracy of measurements made using the eXplore Locus Ultra scanner, cohort 1 was also scanned at higher resolution using the eXplore speCZT scanner. Images produced using the eXplore speCZT scanner at 50 μm isotropic voxel spacing were spatially-averaged to 100 and 150 μm. There were small, but in some cases, statistically significant differences in the CT-derived measures of total mass, BMD, BMC, and adipose, lean and skeletal tissue mass obtained using each scanner (Figure 2.7). CT-derived total body mass, BMC and lean tissue mass showed the least differences among scan conditions (Figure 2.7 A, C and E). Scans obtained using the eXplore Locus Ultra appeared to underestimate adipose tissue mass relative to those obtained using the eXplore speCZT scanner (Figure 2.7 D and G), while slightly overestimating lean tissue mass (Figure 2.7 E and H). Interestingly, images obtained using the eXplore speCZT scanner with 100 μm voxel spacing, appeared to underestimate BMD relative to the other conditions (Figure 2.7 B), while overestimating skeletal tissue mass (Figure 2.7 F and I).
Table 2.4 Gravimetric and CT-derived total body masses of mice at each age.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Cohort</th>
<th>Gravimetric Mass (g)</th>
<th>CT-derived Mass (g)</th>
<th>n</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>8.0 ± 0.2</td>
<td>7.8 ± 0.2</td>
<td>8</td>
<td>1.9</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>19.5 ± 0.5</td>
<td>19.1 ± 0.6</td>
<td>8</td>
<td>1.7</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>24.3 ± 0.5</td>
<td>23.9 ± 0.5</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>23.6 ± 0.6</td>
<td>23.3 ± 0.6</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>1 + 2</td>
<td>24.0 ± 0.4</td>
<td>23.6 ± 0.4</td>
<td>16</td>
<td>1.6</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>22.7 ± 0.5</td>
<td>22.2* ± 0.5</td>
<td>8</td>
<td>2.2</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>23.1 ± 0.5</td>
<td>22.8 ± 0.5</td>
<td>8</td>
<td>1.5</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>24.0 ± 0.4</td>
<td>23.4* ± 0.4</td>
<td>8</td>
<td>2.3</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>24.8 ± 0.8</td>
<td>24.2* ± 0.7</td>
<td>8</td>
<td>2.4</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>25.8 ± 0.7</td>
<td>25.1* ± 0.7</td>
<td>8</td>
<td>2.8</td>
</tr>
<tr>
<td>26</td>
<td>2</td>
<td>26.2 ± 0.8</td>
<td>25.5* ± 0.8</td>
<td>8</td>
<td>2.9</td>
</tr>
<tr>
<td>39</td>
<td>3</td>
<td>33.2 ± 0.9</td>
<td>32.6* ± 0.9</td>
<td>8</td>
<td>2.1</td>
</tr>
<tr>
<td>52</td>
<td>4</td>
<td>37.2 ± 1.3</td>
<td>36.3* ± 1.2</td>
<td>7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Male mice were imaged as described in the legend to Figure 1 and then weighed to determine gravimetric total body mass. Data were subsequently analyzed to determine CT-derived total body mass. For 8-week-old mice, data are shown for cohorts 1 and 2, individually and combined. Data are means ± S.D. n equals the number of mice scanned. The percent differences between means are shown in the right column. * indicates significance difference between gravimetrically and micro-CT-derived total body masses (p < 0.05, assessed by two-way repeated measures ANOVA with Bonferroni’s post hoc test).
Figure 2.7 Comparison of whole-body composition values determined using different scanners and voxel spacing.

Cohort 1 mice were scanned at 2, 5 and 8 weeks of age on both the eXplore Locus Ultra and eXplore speCZT micro-CT scanners. Images were reconstructed to 154 μm for the eXplore Locus Ultra (green), and 150 μm and 100 μm for the eXplore speCZT (red and blue, respectively), and rescaled into Hounsfield units. Each voxel was then assigned as adipose, lean or skeletal tissue, and tissue masses were calculated. A-F illustrate parameters of whole-body composition in absolute values. G-I illustrate adipose, lean and skeletal tissue masses as a percentage of the CT-derived total body mass. Data are means ± S.D., n = 8 mice in each cohort. a indicates significant difference between the Ultra and speCZT 150 μm scans, b indicates significant difference between the Ultra and speCZT 100 μm scans, and c indicates significant difference between the speCZT 150 μm and speCZT 100 μm scans (p < 0.05, determined by two-way repeated measures ANOVA with Bonferroni’s post hoc test).
Taken together, these data establish that non-invasive single-energy micro-CT is a valid, precise and reproducible tool for characterizing WBC of mice during growth and aging.
2.5 Discussion

BMD and composition are commonly reported measures of bone quantity and quality; these can be used to infer skeletal strength, fracture risk and disease state (Donnelly, 2011; Dougherty, 1996; Kazakia et al., 2008). Invasive methods have traditionally been used to determine BMD and composition, requiring post-mortem studies of whole bones in small animals. Destructive testing can be used to assess microarchitecture, mechanical tests to determine strength and elastic modulus, and ash measurements to reveal chemical composition (Donnelly, 2011; Kazakia et al., 2008). These methods are time consuming, destructive and require sacrificing the animal. Therefore, rapid, quantitative and non-invasive alternatives are desirable. Imaging modalities such as micro-CT have become a preferred avenue for measuring BMD and microarchitecture of isolated bones, in addition to assessing skeletal growth in vivo (Bouxsein et al., 2010; Guldberg et al., 2004).

In the present study, we investigated changes in whole-body composition with growth and aging in mice. Using non-invasive micro-CT, we were able to repeatedly scan cohorts of mice over several ages. Our results provide valuable reference data for researchers using mouse models of development, aging and disease. The whole-body masses measured using gravimetric and CT methods align well with previous reports of age-related changes in gravimetric whole-body mass of mice. Our mice more than doubled their body mass between 2 and 5 weeks of age, and exhibited ~50% increase between 4 and 8 weeks. Previous studies of growing male C57BL/6 mice reported a more modest ~30% increase between 1 to 2 months (Glatt et al., 2007), and a comparable ~47% increase in body mass (Somerville et al., 2004). Moreover, between 4 and 52 weeks of age, we observed an ~2.3-fold increase in body mass similar to previous reports.
of ~2.0 to 2.2-fold increase in body mass over the same period (Glatt et al., 2007; Halloran et al., 2002; Somerville et al., 2004).

To the best of our knowledge, the present study is the first to report CT-derived whole skeleton data for mice. Previous studies have reported CT-derived BMD for individual bones (vertebra, femur, tibia) (Amblard et al., 2003; Beamer et al., 1996; Buie et al., 2008; Martin-Badosa et al., 2003; Richman et al., 2001). We found that whole-body BMD values increase until 11 to 14 weeks of age, then plateau. Glatt and colleagues showed using DXA that whole-body aBMD increased rapidly up to 4 months of age in C57BL/6 mice, followed by a slower increase up to 12 months of age (Glatt et al., 2007). Bonkowski et al. measured aBMD and BMC in young (6-7 weeks), adult (7-10 months) and aged (28-32 months) mice. In keeping with our findings, they reported a large increase associated with growth (young-adult) and only small changes associated with aging (adult-aged) (Bonkowski et al., 2006).

Our micro-CT methodology offers the ability to quantify adipose and lean tissue components of the mouse in addition to measuring the skeletal tissues. It is known that the C57BL/6 mouse has a propensity to gain weight with age compared to other mouse models (Brochmann et al., 2003). Our data confirm other work using DXA showing a gradual increase in adipose tissue mass percent between 1 and 5 months of age, sharply increasing thereafter up to 12 months of age (Glatt et al., 2007). Interestingly, our work shows the proportion of adipose tissue at 2 weeks of age is high relative to body mass, whereas lean tissue is quite low. These values change drastically at 5 weeks of age, at which time adipose tissue mass has dropped and lean tissue mass has increased. Adiposity is important for early survival through thermal regulation by brown adipose
tissue up to ~10 days of age (Xue et al., 2007). Thereafter, mice begin to develop insulation in the form of white adipose tissue until weaning at 21 days; white adipose tissue then quickly diminishes by 2 months of age (Kozak et al., 2010; Xue et al., 2007). In keeping with these previous studies, our data showed pronounced subcutaneous adiposity at 2 weeks of age followed by a drastic reduction at 5 weeks, possibly reflecting changes in the requirement for thermal insulation. Furthermore, the replacement of adipose by lean tissue may reflect increased energy demands due to ambulation and dietary changes post-weaning at 3 weeks of age.

We also found that the maximum value of BMD increases rapidly until 11 weeks of age, and is then followed by a slow linear increase. Maximum BMD values represent the degree of mineralization of compact cortical bone. These values were found to increase with age and, in older mice, were comparable to values previously reported for cortical bone BMD in mice (Entezari et al., 2012; Windahl et al., 1999). As we are reporting maximum BMD values, it is important to note that these are often higher than values reported in other studies quantifying mean BMD in ROIs that include both compact bone and marrow spaces.

In the present study, we provide several measures to evaluate the internal and external validity of our protocol. First, we measured the lengths of the tibia and femur to compare them with published values. Although seemingly straightforward, considerably different measurement methods have been previously reported. Many early studies measured mouse bone lengths using calipers or micrometer screw gauges (Somerville et al., 2004); others have used conventional radiographs, pQCT (Beamer et al.), micro-CT (Amblard et al., 2003) or a combination of manual and imaging techniques (Bagi et al.,
2006). However, precisely defined anatomical beginning and end points for measurements are not always described. We selected identifiable anatomical landmarks to consistently measure the long bones. Our findings correlate well with data from previous reports. Somerville et al. showed similar changes in tibial length with age in male C57BL/6 mice (Somerville et al., 2004). Somerville’s study showed an ~20% increase in tibia length between 1 and 3 months of age, followed by a ~2.8% increase between 3 and 12 months of age (Somerville et al., 2004). Our data revealed a ~24% increase in tibia length between 1 and 3 months of age and an ~3.4% increase between 3 and 12 months of age. Femur lengths of our male mice showed similar patterns to that of other work in male C57BL/6 mice, with only small increases in bone length after 2 months of age (Glatt et al., 2007). Specifically, Glatt and coworkers showed an ~33% increase in femur length between 1 and 3 months of age, followed by a ~3.9% increase between 3 and 12 months (Glatt et al., 2007). Our data revealed ~30% increase in femur length between 1 and 3 months of age, followed by a ~6.5% increase between 3 and 12 months. These findings help validate our methodology and establish its reproducibility.

As a measure of internal validity, we compared CT-derived total body masses to gravimetrically measured masses at each age. These values were in good agreement, with the CT protocol underestimating total body weight by less than 3%. This finding helps establish the accuracy of our imaging and analysis methods for determining tissue masses.

To assess reproducibility, we scanned two separate cohorts of mice, both at 8 weeks of age, at different times using the same scanner and scanning protocol. These data revealed that the two cohorts of mice had nearly identical distributions of tissue and total
body weights. Stability and reproducibility of the eXplore Locus Ultra micro-CT system itself has been previously shown (Du et al., 2007), further solidifying this assessment. In addition, we scanned one cohort of mice at 2, 5 and 8 weeks of age using two micro-CT systems. We found that there was good agreement between WBC outputs from scanning on both systems, although it is evident that machine-specific performance parameters (e.g., resolution and noise) can influence measurements. Taken together, our findings establish the validity and reproducibility of this approach for quantifying whole-body composition in studies of growth and aging in mice.

Our protocol offers a number of advantages. First, mice are anesthetized by isoflurane inhalation, which is less invasive with reduced adverse effects compared to anesthetic agents that are injected intraperitoneally. Mice recover quickly and repeated use of isoflurane is possible without harm. Scout scans can be used to determine precise positioning of animals prior to full scanning. Scans are non-invasive, rapid (as short as 16 seconds) and have low-radiation exposures. To establish the radiation safety of micro-CT scanning, a recent study demonstrated no significant effect on cardiac and pulmonary tissues in mice that were purposely delivered multiple exposures at triple the typical dose of radiation over a 6-week period (Detombe et al., 2013). Thus, repeated scans can be used to monitor changes over time in longitudinal studies without concern for adverse dose-related effects. Moreover, analysis using this whole-body composition method is high-throughput. In addition, our study shows that repeated long term imaging of mice (such as those in cohort 2, which were scanned every three weeks for a total of four months) is safe.

There are also some limitations of our protocol that should be noted. To allow for
accurate measurement of WBC and maintain an acceptably low radiation dose, the volumes required spatial averaging to lower resolutions (100 and 150 μm) to diminish noise levels in the images. Thus, in younger mice with smaller skeletons, skeletal mass and BMD may be underestimated due to under-mineralization and partial volume effects arising from increased isotropic voxel spacing. In addition, to quantify microarchitecture within the trabecular network of mouse bones, higher scan resolutions are required, as trabeculae are approximately 30-50 μm in width (Bouxsein et al., 2010). Thus, our in vivo scans at 50 μm are not capable of imaging small trabecular struts in mouse bones. Therefore, assessments of mouse bone microarchitecture must be performed at a scan resolution of ≤20 μm as post-mortem specimen scans.

Another limitation is that the earliest age that can be assessed is 2 weeks. It is not possible to obtain in vivo data from animals younger than this, as their bones are not sufficiently mineralized to perform quantifiable analyses at these resolutions. In contrast, micro-CT measurements of ex vivo murine fetal skeletons correlate well with standard histological techniques in mice as young as gestational day 17-19 (Oest et al., 2008). Furthermore, assessments of whole-mouse skeletons as young as 2 days of age are feasible when scanning post-mortem and with higher scan resolutions (Guldberg et al., 2004). To the best of our knowledge, in vivo micro-CT scanning prior to 2 weeks of age in mice has yet to be reported.

The present study is the first characterization of changes in WBC during growth and aging of mice using micro-CT. Our findings provide critical reference data for other investigators using mice as disease models. Additionally, we show marked differences in WBC values during a period of rapid growth between 2 and 5 weeks of age, highlighting
the importance of collecting data at precise ages when comparing groups within this range. Moreover, our study presents an in-depth analysis of WBC using an in-house-designed micro-CT protocol. This innovative methodology facilitates precise, long-term, repeated assessment of adipose, lean and skeletal tissues \textit{in vivo} in growing and aging mice. Furthermore, our non-invasive method minimizes the use of research animals, as the mice can be scanned repeatedly. In addition, the 3D volumes enabled us to precisely measure growing long bones using consistent anatomical markers. Our findings establish that non-invasive single-energy micro-CT is an accurate and effective tool for high-throughput high-content characterization of WBC in mouse models.
2.6 References


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CHAPTER THREE

LOSS OF P2X7 NUCLEOTIDE RECEPTOR FUNCTION LEADS TO ABNORMAL FAT DISTRIBUTION IN MICE

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3.1 Chapter Summary

The P2X7 receptor is an ATP-gated cation channel expressed by a number of cell types. We have shown previously that disruption of P2X7 receptor function results in downregulation of osteogenic markers and upregulation of adipogenic markers in calvarial cell cultures. In the present study, we assessed whether loss of P2X7 receptor function results in changes to adipocyte distribution and lipid accumulation in vivo. Male P2X7 loss-of-function (KO) mice exhibited significantly greater body weight and epididymal fat pad mass than wild-type (WT) mice at 9 months of age. Fat pad adipocytes did not differ in size, consistent with adipocyte hyperplasia rather than hypertrophy. Histological examination revealed ectopic lipid accumulation in the form of adipocytes and/or lipid droplets in several non-adipose tissues of older male KO mice (9-12 months of age). Ectopic lipid was observed in kidney, extraorbital lacrimal gland and pancreas, but not in liver, heart or skeletal muscle. Notably, lacrimal gland and pancreas from 12-month-old male KO mice had greater numbers of adipocytes in perivascular, periductal and acinar regions. As well, lipid droplets accumulated in the renal tubular epithelium and lacrimal acinar cells. Blood plasma analyses revealed diminished total cholesterol levels in 9- and 12-month-old male KO mice compared to WT controls. Interestingly, no differences were observed in female mice. Moreover, there were no significant differences in food consumption between male KO and WT mice. Taken together, these data establish novel in vivo roles for the P2X7 receptor in regulating adipogenesis and lipid metabolism in an age- and sex-dependent manner.
3.2 Introduction

Global epidemics of metabolic syndrome diseases represent major health and economic burdens, with incidences on the rise (Dixon, 2010; Hassan et al., 2012). Obesity (central adiposity), dyslipidemia, hypertension, type 2 diabetes mellitus and cardiovascular diseases are hallmarks of metabolic syndrome (Dulloo and Montani, 2012). Recent studies have shown that, in addition to lipid storage, adipose tissue regulates other tissues and systems throughout the body. In particular, adipose is an active endocrine organ (Hassan et al., 2012; Shuldiner et al., 2001) that is involved in cross-talk with other tissue types such as bone (Ducy et al., 2000; Ferron et al., 2010). Moreover, it plays important roles in local and systemic inflammation (Jacobi et al., 2012; Suganami et al., 2012) and energy metabolism (Kopecky et al., 2004; Lee et al., 2007; Medina-Gomez, 2012).

Extracellular nucleotides, such as ATP, signal through purinergic (P2) receptors on the membrane of most cell types. Two subfamilies of P2 receptors exist: P2X (ATP-gated nonselective cation channels) and P2Y (G protein-coupled receptors) (Burnstock, 2007). There are seven known subtypes of P2X receptors, P2X1-7. P2X7 is of emerging interest as a potential therapeutic target in a number of diseases. This receptor is unique in that it is activated by relatively high concentrations of ATP. Moreover, P2X7 signaling can cause the formation of large nonselective membrane pores and induce dynamic membrane blebbing (Burnstock, 2007; Panupinthu et al., 2008). This receptor plays important roles in chronic pain (Hughes et al., 2007; Sorge et al., 2012), apoptosis (Coutinho-Silva et al., 1999; Gu et al., 2011; Surprenant et al., 1996), bone remodeling (Grol, 2009; Ke et al., 2003; Li J, 2005), immune responses (Dubyak, 2012; Miller et al., 2011; Sakowicz-Burkiewicz et al., 2013; Solle et al., 2001) and the function of exocrine
and endocrine organs (Burnstock and Novak, 2013; Novak, 2008).

Genetically modified mice that lack functional P2X7 receptors (KO) present with an osteopenic phenotype owing to diminished periosteal bone formation and excessive trabecular bone resorption (Ke et al., 2003). Moreover, these mice exhibit an impaired skeletal response to mechanical loading (Li J, 2005). In vitro evidence has since demonstrated that activation of P2X7 receptors in bone-forming osteoblasts results in cell-autonomous enhancement of differentiation and matrix mineralization (Panupinthu et al., 2008). Moreover, cultures of bone cells from P2X7 KO mice, which include mesenchymal progenitors, display greater expression of adipogenic markers compared to cultures from wild-type (WT) mice. This upregulation of adipogenic markers was accompanied by downregulation of osteogenic markers and suppression of osteoblastic differentiation (Panupinthu et al., 2008). Osteoblasts and adipocytes both arise from mesenchymal stem cells and become terminally differentiated through expression of master transcription factors such as osterix and peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)) (Ducy et al., 1997; Muruganandan et al., 2009; Rosen et al., 2000). Therefore, evidence points to P2X7 playing a critical role in directing differentiation towards the osteoblast lineage and away from the adipocyte lineage.

The P2X7 receptor has also been shown to regulate cellular metabolism. Activation of P2X7 on osteoblast-like cells dramatically increases the production of metabolic acid (Grol et al., 2012). This P2X7-induced increase in proton efflux is dependent on the activity of phosphatidylinositol 3-kinase and the presence of glucose. It has also been shown that heterologous expression of P2X7 in HEK293 cells promotes aerobic glycolysis, and increases levels of glycolytic enzymes and glycogen stores.
(Amoroso et al., 2012). Therefore, P2X7 activation enhances cellular energy metabolism, suggesting a role for this receptor in energy homeostasis.

Expression of functional P2X7 receptors has been reported in adipocytes purified from murine white adipose tissue (Sun et al., 2012), as well as cultured adipocytes from human visceral and subcutaneous adipose tissues (Madec et al., 2011). However, little is known about the role of P2X7 receptors in adipose tissue. Recent studies have reported that ablation of $P2rx7$, the gene encoding P2X7, does not affect body and gonadal fat pad weights in mice up to 4 months of age (Sun et al., 2012). In the present study, we report that loss of P2X7 function leads to increased adiposity and lipid accumulation in older male mice. P2X7 KO mice were found to have increased body and epididymal fat pad weights, and reduced total plasma cholesterol levels compared to WT mice at 9 months of age. Ectopic lipid deposits were observed in the kidneys, pancreas and extraorbital lacrimal glands. There were no significant differences in food consumption by KO and WT mice at any age. These results point to heretofore unrecognized roles for the P2X7 receptor in adipogenesis and lipid metabolism.
3.3 Materials and methods

3.3.1 Animals

The P2X7 KO mouse was obtained from Pfizer (Solle et al., 2001). Although a splice variant (with C-terminal truncation) is detectable in some tissues of the Pfizer KO mouse, it is inefficiently trafficked to the cell surface and displays greatly diminished receptor function (Masin et al., 2012). Mice were maintained on a mixed genetic background (129/Ola × C57BL/6 × DBA/2) by crossbreeding of heterozygous mice. Genotypes were identified by PCR. Mice were housed in standard cages and maintained on a 12-hour light/dark cycle, with water and standard mouse chow (2018 Teklad Global 18% protein rodent diet, Harlan Laboratories, USA) available ad libitum. All aspects of this study were conducted in accordance with the policies and guidelines of the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario, London, ON, Canada.

Food intake and body composition of 2, 6, 9 and 12-month-old WT and KO mice were assessed. For these studies, mice were housed individually. Mice and food were weighed using a portable electronic scale (Acculab VICON VIC-511; Sartorius Group, Germany). Food intake was determined by subtracting the weight of food remaining after each week from the initial amount of food provided.

3.3.2 Micro-computed tomography (micro-CT)

Mice were anesthetized with isoflurane (Forane, catalog # CA2L9100, Baxter Corporation, Mississauga, Canada) and imaged using an eXplore Locus Ultra micro-CT scanner or an eXplore speCZT scanner (GE Healthcare Biosciences, London, ON,
Canada). Scans were analysed as described previously (Granton et al., 2010). Briefly, a calibrating phantom composed of air, water and cortical bone-mimicking epoxy with a mineral value of 1.1 g/cm³ (SB3; Gammex RMI) (Gulam et al., 2000) was scanned together with the animals. On the eXplore Locus Ultra, 1000 projection images were obtained over a single 16-second rotation (80 kVp, 55 mA tube current, 16 ms exposure). On the speCZT scanner, 900 projection images were obtained over a single 5-minute rotation (90 kVp, 40 mA tube current, 16 ms exposure). Each of the eXplore Locus Ultra and speCZT data sets were reconstructed into 3D volumes with an isotropic voxel spacing of 154 µm and 150 µm, respectively, and scaled into Hounsfield units (HU).

Using MicroView software (GE Healthcare Biosciences), three signal-intensity thresholds (-200, -30 and 190 HU) were used to classify each voxel as adipose, lean or skeletal tissue, respectively. Custom software was used to calculate tissue masses from assumed tissue densities of 0.95 (adipose), 1.05 (lean), and 1.92 (skeletal) g/cm³. The computed total mass corresponded well with the measured weight of each mouse (within ± 2%). In addition, the software was used to calculate bone mineral density (BMD) and bone mineral content (BMC). Briefly, BMD was obtained as the ratio of the average HU value of the skeletal region of interest to the measured HU value of the SB3 calibrator, multiplied by the known density of the SB3 (1.1 g/cm³). Thereafter, the program automatically computed BMC as the product of the BMD and the total volume of the skeletal region of interest used in the BMD calculation.
3.3.3 Histology and histomorphometry

Mice were fasted 6 hours prior to euthanasia by CO$_2$ (100%) asphyxiation. Mice were weighed and organs harvested, weighed and prepared for histological analyses using standard protocols. The following organs/tissues were examined: epididymal/retroperitoneal fat pads, heart, liver, spleen, pancreas, kidneys, salivary glands (submandibular and sublingual), extraorbital lacrimal glands, Harderian glands and skeletal muscle (quadriiceps). All tissues were fixed in 10% neutral buffered formalin (pH 7.0). Some samples were then processed, paraffin-embedded and blocks were sectioned at 5 µm thickness using a Leica RM2255 microtome (Leica Microsystems, Wetzlar, Germany). Sections were mounted on positively charged glass slides (Superfrost plus microslides; VWR International, Mississauga, ON, Canada) and stained with hematoxylin and eosin (H&E, Harris hematoxylin and eosin; Surgipath, Leica Microsystems).

Adipocyte number and diameter in fat pads were determined using Image Master software (Photon Technology International Inc., London, ON, Canada). Adipocyte density was calculated as the number of adipocytes within the field of view (FOV) divided by the area of the FOV (1.30 mm$^2$). Adipocyte diameter was obtained by measuring the maximum diameter of each adipocyte within the same FOV.

After harvest, other samples were manually cut into small (2 × 2 × 2 mm) portions in preparation for staining with osmium tetroxide (modified from (Carson, 1996)) to reveal lipid deposits. Briefly, tissue samples were fixed in 10% neutral buffered formalin (pH 7.0) overnight, washed and stained with osmium tetroxide (VWR International, Mississauga, ON, Canada) 1% in water for 2 h with intermittent agitation.
Samples were then washed with water for 15 min × 2, differentiated with 5% periodic acid solution in water (Alfa Aesar, Ward Hill, MA, USA) for 30 min, agitating periodically and washed twice more with running water for 30 min each. Samples were then processed, paraffin-embedded, sectioned and stained with H&E. Osmium tetroxide staining was quantified using a Leica DM1000 microscope coupled to a DFC295 digital camera and Leica Application Suite software (version 3.8.0). Brightness and contrast were adjusted by the same amounts for all images of each tissue type (both WT and KO). A blinded observer then quantified the area of tissue stained with osmium tetroxide using Image Master software. For all images of each tissue, brightness and threshold values for the RGB channels were adjusted to select for osmium tetroxide staining (black), but not hematoxylin or eosin staining. To quantify total tissue area, brightness and RGB thresholds were adjusted to select for the total area of stained tissue (including osmium tetroxide, hematoxylin and eosin). The area of osmium-positive staining was reported as a percentage of total tissue area.

3.3.4 Blood plasma chemistry and tissue lipid analyses
Mice were fasted for 6 h prior to euthanasia by CO₂ asphyxiation. Cardiac puncture was performed immediately post-mortem to obtain plasma samples for analysis of glucose, triglyceride (TG) and total cholesterol (TC) levels. Blood, treated with EDTA anticoagulant, was sedimented for 10 min at 10,000 rpm. Plasma was frozen at -20°C, and contents were analyzed using a Cobas Mira S autoanalyser (Metabolic Phenotyping Laboratory, Robarts Research Institute, London, ON, Canada). In addition, immediately following cardiac puncture, some tissues were harvested, snap frozen in liquid nitrogen,
and stored at -20°C. Tissues were homogenized and lipids extracted using the Folch technique (Folch et al., 1957). Total cholesterol and triglycerides were then quantified using enzymatic, colorimetric assays described previously (Burnett et al., 1997).

3.3.5 Statistical Analyses

Data are presented as means ± SEM or as medians and ranges. Differences between two groups were assessed using $t$ tests. Differences among three or more groups were evaluated by two-way analysis of variance followed by a Bonferroni multiple comparisons test. For variables with non-normal distribution, comparisons were performed using a nonparametric Mann-Whitney U-test. Differences were accepted as statistically significant at $p < 0.05$. All $n$ values represent the number of mice used in each group. For histological studies, at least 2 sections were examined of each tissue from each mouse.
### 3.4 Results

#### 3.4.1 Age- and sex-dependent effects of loss of P2X7 receptor function on body mass and composition

At 9 months of age, male P2X7 KO mice appeared larger than male WT mice (Figure 3.1 a). WT and KO mice were sacrificed and weighed at 2, 6, 9 and 12 months of age. Interestingly, the total body weight of male KO mice was significantly greater than WT at 9 months of age (Figure 3.1 b, Table 3.1). In contrast, there were no significant differences in the total body weight of female mice in any age group (Table 3.2). The significant difference in body weight of male mice at 9 months of age was confirmed independently in a second cohort of mice (11 WT and 12 KO mice). When both cohorts were combined, the body weights of WT and KO mice were 39.5 ± 1.7 g and 48.9 ± 1.3 g, respectively (means ± SEM, n = 21 and 22 mice, respectively, *p* < 0.05, *t* test).

To determine tissue changes underlying the greater body weight of KO mice, whole-body composition was analysed using micro-CT. Live mice were anaesthetized and scanned. Images were reconstructed at an isotropic voxel size of 150 or 154 µm and masses of adipose, lean and skeletal tissues determined using MicroView software and an in-house designed analysis program (Figure 3.2 a). Micro-CT confirmed greater total body mass in a third cohort of KO mice at 9 months of age, and revealed significantly greater adipose tissue mass in these mice compared to WT controls (Figure 3.2 b,c). Using this approach, no significant differences in lean tissue mass, skeletal tissue mass, bone mineral content or bone mineral density were detected at any age (Figure 3.2 d-g).
Figure 3.1 Body weight of male wild-type (WT) and P2X7 knockout (KO) mice.

a Image depicting the greater body size of a P2X7 KO male mouse in comparison to a WT male mouse at 9 months of age. b Two, 6, 9 and 12-month-old WT and KO mice were sacrificed and weighed to compare total body mass. Greater body weight was seen only at 9 months of age in KO mice compared to WT controls. Data are means ± SEM, n = 10 mice of each genotype and age. *Indicates significant difference between KO and WT (p < 0.05), determined by two-way analysis of variance and Bonferroni post hoc test.
Table 3.1 Total body and tissue mass for male wild-type (WT) and P2X7 knockout (KO) mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>2 months WT</th>
<th>2 months KO</th>
<th>9 months WT</th>
<th>9 months KO</th>
<th>12 months WT</th>
<th>12 months KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>24.3 ± 0.6</td>
<td>25.7 ± 0.4</td>
<td>37.0 ± 2.5</td>
<td>44.7 ± 1.5*</td>
<td>40.8 ± 2.6</td>
<td>43.3 ± 1.7</td>
</tr>
<tr>
<td>Fat pads</td>
<td>ND</td>
<td>ND</td>
<td>1.939 ± 0.248</td>
<td>2.782 ± 0.150*</td>
<td>1.999 ± 0.234</td>
<td>2.308 ± 0.190</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.244 ± 0.008</td>
<td>0.261 ± 0.013</td>
<td>0.373 ± 0.027</td>
<td>0.445 ± 0.023</td>
<td>0.346 ± 0.015</td>
<td>0.378 ± 0.016</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>0.227 ± 0.015</td>
<td>0.219 ± 0.010</td>
<td>0.343 ± 0.015</td>
<td>0.365 ± 0.009</td>
<td>0.246 ± 0.015</td>
<td>0.248 ± 0.015</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.146 ± 0.005</td>
<td>0.126 ± 0.008</td>
<td>0.247 ± 0.016</td>
<td>0.262 ± 0.029</td>
<td>0.287 ± 0.038</td>
<td>0.35 ± 0.040</td>
</tr>
<tr>
<td>EO lacrimal glands</td>
<td>0.034 ± 0.005</td>
<td>0.056 ± 0.009*</td>
<td>0.055 ± 0.005</td>
<td>0.050 ± 0.007</td>
<td>0.051 ± 0.005</td>
<td>0.043 ± 0.004</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.056 ± 0.005</td>
<td>0.083 ± 0.0202</td>
<td>0.078 ± 0.007</td>
<td>0.093 ± 0.010</td>
<td>0.062 ± 0.005</td>
<td>0.092 ± 0.009*</td>
</tr>
<tr>
<td>Liver</td>
<td>1.377 ± 0.054</td>
<td>1.46 ± 0.096</td>
<td>1.700 ± 0.007</td>
<td>2.145 ± 0.102*</td>
<td>1.788 ± 0.120</td>
<td>1.840 ± 0.066</td>
</tr>
<tr>
<td>Heart</td>
<td>0.221 ± 0.017</td>
<td>0.254 ± 0.027</td>
<td>0.258 ± 0.012</td>
<td>0.291 ± 0.010*</td>
<td>0.341 ± 0.030</td>
<td>0.351 ± 0.022</td>
</tr>
<tr>
<td>Harderian gland</td>
<td>0.032 ± 0.005</td>
<td>0.028 ± 0.003</td>
<td>0.056 ± 0.005</td>
<td>0.056 ± 0.006</td>
<td>0.040 ± 0.006</td>
<td>0.039 ± 0.003</td>
</tr>
</tbody>
</table>

Data are means ± SEM in grams. Data are based on at least 7 animals per group. ND = not determined. Mass of the single organ is reported for kidney, pancreas, spleen, liver and heart. Mass of both right and left organs is reported for fat pads, salivary glands (submandibular and sublingual glands), extraorbital (EO) lacrimal glands and Harderian glands. *indicates significant difference from WT ($p < 0.05$), based on unpaired two-tailed $t$ test.
Table 3.2 Total body and tissue mass for female wild-type (WT) and P2X7 knockout (KO) mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>2 months</th>
<th></th>
<th>9 months</th>
<th></th>
<th>12 months</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Total</td>
<td>18.11 ± 0.58</td>
<td>18.62 ± 0.35</td>
<td>23.56 ± 0.77</td>
<td>25.30 ± 0.66</td>
<td>27.91 ± 1.43</td>
<td>26.76 ± 0.55</td>
</tr>
<tr>
<td>Fat pads</td>
<td>ND</td>
<td>ND</td>
<td>0.264 ± 0.026</td>
<td>0.305 ± 0.059</td>
<td>0.675 ± 0.188</td>
<td>0.585 ± 0.069</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.156 ± 0.011</td>
<td>0.165 ± 0.006</td>
<td>0.207 ± 0.008</td>
<td>0.211 ± 0.007</td>
<td>0.179 ± 0.019</td>
<td>0.192 ± 0.017</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>0.144 ± 0.009</td>
<td>0.133 ± 0.007</td>
<td>0.185 ± 0.010</td>
<td>0.182 ± 0.008</td>
<td>0.154 ± 0.007</td>
<td>0.150 ± 0.016</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.128 ± 0.012</td>
<td>0.132 ± 0.015</td>
<td>0.180 ± 0.010</td>
<td>0.211 ± 0.017</td>
<td>0.161 ± 0.007</td>
<td>0.192 ± 0.017</td>
</tr>
<tr>
<td>EO lacrimal glands</td>
<td>0.026 ± 0.006</td>
<td>0.025 ± 0.008</td>
<td>0.033 ± 0.006</td>
<td>0.028 ± 0.004</td>
<td>0.027 ± 0.004</td>
<td>0.026 ± 0.004</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.063 ± 0.009</td>
<td>0.058 ± 0.007</td>
<td>0.070 ± 0.005</td>
<td>0.075 ± 0.004</td>
<td>0.081 ± 0.004</td>
<td>0.070 ± 0.007</td>
</tr>
<tr>
<td>Liver</td>
<td>1.036 ± 0.047</td>
<td>1.156 ± 0.046</td>
<td>1.178 ± 0.078</td>
<td>1.315 ± 0.048</td>
<td>1.178 ± 0.053</td>
<td>1.097 ± 0.115</td>
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<tr>
<td>Heart</td>
<td>0.153 ±0.005</td>
<td>0.1583 ± 0.012</td>
<td>0.192 ± 0.014</td>
<td>0.202 ± 0.013</td>
<td>0.206 ± 0.013</td>
<td>0.187 ± 0.010</td>
</tr>
<tr>
<td>Harderian gland</td>
<td>0.031 ± 0.004</td>
<td>0.023 ± 0.002</td>
<td>0.037 ± 0.004</td>
<td>0.039 ± 0.005</td>
<td>0.039 ± 0.005</td>
<td>0.051 ± 0.011</td>
</tr>
</tbody>
</table>

Data are means ± SEM in grams. Data are based on at least 7 animals per group. ND = not determined. Mass of the single organ is reported for kidney, pancreas, spleen, liver and heart. Mass of both right and left organs is reported for fat pads, salivary glands (submandibular and sublingual glands), extraorbital (EO) lacrimal glands and Harderian glands.
Figure 3.2 Micro-CT analysis of whole-body composition of male WT and P2X7 KO mice.

a Top left panel (Total) is a reconstructed micro-CT mid-coronal image of a live male 6-month-old WT mouse. Image was overlaid with the identified skeletal tissue (shown in white as a surface-rendered image in the top right panel). Lower panels are images of the same slice showing lean tissue in red and adipose tissue in blue. b-g Data are means ± SEM of the indicated parameters. Nine-month-old KO mice exhibit significantly greater total body mass and adipose tissue mass than WT controls. In contrast, lean tissue mass, skeletal mass, bone mineral content and bone mineral density were not significantly different at any age. *Indicates significant difference between KO and WT (p < 0.05) at the same age, determined by two-way analysis of variance and Bonferroni post hoc test (n = 6-8 mice of each genotype at each age).
3.4.2 Morphometric analysis of adipocytes from fat pads of WT and P2X7 KO mice

Since P2X7 is expressed in adipocytes (Madec et al., 2011) and the difference in total body mass was associated with a significant increase in adipose tissue, we examined the fat pads in WT and KO mice. Epididymal fat pads of WT and KO male mice (9 and 12 months of age) were isolated and weighed post-mortem. Fat pad mass was significantly greater in KO than in WT male mice at 9 months of age (Figure 3.3 a,b), with no significant difference observed at 12 months of age (Table 3.1). In addition, no significant differences were observed in the mass of female parametrial fat pads (analogous to the epididymal fat pads in male mice) at either 9 or 12 months of age (Table 3.2). Thus, these data show the same age- and sex-dependence as the differences in body weight and composition described above.

Histological assessments of epididymal fat pads in 9- and 12-month-old male mice revealed similar adipocyte morphology in WT and KO mice (Figure 3.3 c). Adipocyte size and density were determined by quantifying the largest diameter and counting the number of adipocytes per field of view (five fields of view analysed for 2 sections from each mouse fat pad). These parameters did not differ significantly in WT and KO mice of either age group (Figure 3.3 d,e). The similar size and density of adipocytes in WT and KO mice, together with the greater fat pad mass in 9-month-old male KO indicate that the difference in fat pad mass is due to adipocyte hyperplasia, rather than hypertrophy.
Figure 3.3 Morphometric analysis of adipocytes in fat pads of male WT and P2X7 KO mice.

Epididymal fat pads were isolated post-mortem. a Image depicting epididymal fat pads from a 9-month-old WT and KO mouse. Scale bar represents 1 cm. b Mass of epididymal fat pads was significantly greater in P2X7 KO than in WT male mice at 9 months of age. Data are means ± SEM, n = 10 mice of each genotype. Combined mass of right and left fat pads is reported. *Indicates significant difference between KO and WT (p < 0.05), determined by t test. c Representative histological sections of epididymal fat pads from 9-month-old male WT and P2X7 KO mice stained with H&E, with no marked differences in appearance. d,e Sections of fat pads from 9- and 12-month-old mice were analyzed for adipocyte density and size. The number of adipocytes and largest diameter of each adipocyte in each field of view were measured. Data for 9-month-old mice are shown. For both 9- and 12-month-old mice, there was no significant difference between WT and KO in adipocyte density or size (p > 0.05, t test). Data are means ± SEM, n = 5 mice of each genotype and age with 2 sections and 5 fields of view per section analysed for each mouse.
3.4.3 Histological differences in the kidneys of WT and KO mice

In addition to fat pads, several tissues from 2-, 9- and 12-month-old male and female mice were isolated post-mortem, weighed and assessed histologically. Histological differences were noted in specific tissues of older male mice using H&E staining. No differences were apparent in 2-month-old male mice or female mice at any age.

P2X7 is expressed in the collecting ducts of adult mouse kidney (Hillman et al., 2005). We found that, at both 9 and 12 months of age, kidney tissue exhibited spherical vacuole-like spaces (~3-12 µm in diameter; yellow arrowheads), which did not stain with H&E (Figure 3.4 a). There were markedly greater numbers of these spaces in male KO than in WT mice. We next examined tissues stained with osmium tetroxide, which specifically labels lipids and lipid-rich structures for optical microscopy (Belazi et al., 2009). A population of spherical structures stained positively with osmium tetroxide in kidney tissues only from KO mice (Figure 3.4 b, black stain), consistent with the presence of lipid droplets in some renal tubular epithelial cells. Image analysis revealed that osmium tetroxide staining was significantly greater in kidneys from KO than from WT mice (Figure 3.4 c). Based on size and shape, these osmium-stained structures appear to correspond to a subset of the spherical vacuole-like spaces identified in sections stained with H&E. Despite these histological alterations, there was no significant difference in the mass of the kidneys at any age in both males and females (Tables 3.1 and 3.2).
Figure 3.4 Histology of kidneys in male WT and P2X7 KO mice.

a Representative histological sections of cortico-medullary region of the kidney from 12-month-old male WT and P2X7 KO mice stained with H&E. At both 9 and 12 months of age, KO kidney exhibited multiple spherical structures (~3-12 µm in diameter, yellow arrowheads), which failed to stain with H&E. b Staining with osmium tetroxide revealed that these spherical structures were filled with lipid (black). Images shown are from 12-month-old male mice; similar results were found in 9-month-old male mice (not shown). Scale bar represents 50 µm for all images. Images are representative of 8-11 male mice of each genotype and age (9 and 12 months). c For 12-month-old mice, the area that stained positively with osmium tetroxide was determined using image analysis and expressed as a percentage of the total tissue area. Data are medians and interquartile ranges (IQR, boxes) with whiskers representing the 10th and 90th percentiles; n = 5 mice of each genotype with 2 sections per mouse. At least 2 fields of view per section were analysed. *Indicates a significant difference between KO and WT (p < 0.05), determined by one-tailed Mann-Whitney U-test.
3.4.4  **Histological differences in the pancreases of WT and KO mice**

In exocrine pancreas, P2X7 is expressed in ducts and may regulate secretion (Novak, 2008). Pancreases were isolated from 2-, 9- and 12-month-old WT and KO mice. Striking histological differences were observed in male mice at 12 months of age; no differences were observed in younger mice or in female mice. Pancreatic tissues from 12-month-old male KO mice exhibited multiple large, membrane-bound structures (~10-30 µm in diameter), resembling adipocytes (Figure 3.5 a,b). The intracellular vacuole in these cells failed to stain with H&E. Staining with osmium tetroxide confirmed the presence of lipids (arrowheads) (Figure 3.5 c,d). These adipocytes were located in the exocrine pancreas predominantly among ducts and vasculature, as well as in boundaries between pancreatic lobes and sporadically among acini. No adipocytes were observed associated with pancreatic islets. By comparison, few adipocytes were seen in pancreatic tissues from age- and sex-matched WT mice. Quantification confirmed that osmium tetroxide staining was significantly greater in the pancreatic tissues of KO compared to WT mice (Figure 3.5 e). No differences were noted in the appearance of acini in WT and KO pancreases. There were also no significant differences between WT and KO in the mass of the pancreas at any age in male and female mice (Tables 3.1 and 3.2).

3.4.5 **Histological differences in the extraorbital lacrimal glands of WT and KO mice**

Exocrine pancreas and extraorbital lacrimal glands are serous glands that exhibit similar histological appearances. P2X7 has been reported in both the acinar and ductal cells of rat lacrimal gland (Hodges et al., 2009). As observed for pancreas, we found a greater number of adipocytes in the extraorbital lacrimal glands of male mice at 12 months of
**Figure 3.5 Histology of pancreas in male WT and P2X7 KO mice.**

Images are representative histological sections at low magnification (a and c), with higher magnification images from the same fields shown in b and d. a,b Representative histological sections of pancreas from 12-month-old male WT and P2X7 KO mice stained with H&E. KO pancreas exhibited multiple large membrane-bound spherical structures (~10-30 µm in diameter, yellow arrowheads), which failed to stain with H&E, resembling adipocytes. These structures were located predominantly among ducts and vasculature, as well as in boundaries between pancreatic lobes. c,d Staining with osmium tetroxide confirmed the identity of these cells as adipocytes (yellow arrowheads). Images are representative of 10 male mice of each genotype. e The area that stained positively with osmium tetroxide was determined using image analysis and expressed as a percentage of the total tissue area. Data are medians and IQR (boxes) with whiskers representing the 10th and 90th percentiles; n = 5 mice of each genotype with 2 sections per mouse. At least 2 fields of view per section were analysed. *Indicates a significant difference between KO and WT (p < 0.05), determined by one-tailed Mann-Whitney U-test.
age (Figure 3.6), with no differences in younger or in female mice. These adipocytes were observed among the ducts and vasculature, as well as at the boundaries between lobes. In addition, small lipid droplets (~1 µm in diameter) accumulated in the basal aspect of lacrimal acinar cells to a much greater extent in KO than in WT mice (Figure 3.6 d,e). As in kidney and pancreas, osmium tetroxide staining was significantly greater in the extraorbital lacrimal glands of KO compared to WT mice (Figure 3.6 f). In males, the mass of the extraorbital lacrimal glands was significantly greater in KO than in WT mice at 2 months of age (Table 3.1). However, there were no significant differences in older males or in females (Table 3.2).

3.4.6 Histological differences in the salivary glands of WT and KO mice

P2X7 expression has been reported previously in both ducts and acinar cells of mouse submandibular gland (Nakamoto et al., 2009; Pochet et al., 2007). Interestingly, in our study the submandibular salivary gland displayed multiple spherical structures that did not stain with H&E (Figure 3.7 a). Like the kidney tissues, a greater number of these structures were observed in glandular tissue from older male KO mice than from WT mice. However, in contrast to the tissues described above, these spherical structures in the submandibular gland did not stain with osmium tetroxide (Figure 3.7 b), revealing the absence of lipid. In addition, these structures appeared to be located centrally within acini of the submandibular gland, but were absent in the adjacent sublingual gland. Moreover, there were no significant differences in the mass of the salivary glands at any age in both males and females (Tables 3.1 and 3.2).
Figure 3.6 Histology of extraorbital lacrimal glands in male WT and P2X7 KO mice.

Images are representative histological sections at low magnification (a and c), with higher magnification images from the same fields shown in b, d and e. a,b Representative histological sections of extraorbital lacrimal glands from 12-month-old male WT and P2X7 KO mice stained with H&E. Similar to pancreas, adipocyte-like cells (yellow arrowheads) were observed among ducts and vasculature, as well as in boundaries between lobes in glands from KO mice. c,d Osmium tetroxide staining confirmed the presence of adipocytes in the KO tissues. e Higher magnification images of regions outlined by yellow rectangles in d (inset). These images reveal markedly greater accumulation of small lipid droplets in the basal cytoplasm of acinar cells in the KO mice. Images are representative of 10 male mice of each genotype. f The area that stained positively with osmium tetroxide was determined using image analysis and expressed as a percentage of the total tissue area. Data are medians and IQR (boxes) with whiskers representing the 10th and 90th percentiles; n = 5 mice of each genotype with 2 sections per mouse. At least 2 fields of view per section were analysed. *Indicates a significant difference between KO and WT (p < 0.05), determined by one-tailed Mann-Whitney U-test.
Figure 3.7 Histology of salivary glands in the male WT and P2X7 KO mice.

a Representative histological sections of salivary glands from 12-month old male WT and P2X7 KO mice stained with H&E (SM: submandibular gland, SL: sublingual gland). At both 9 and 12 months of age, multiple spherical structures, which failed to stain with H&E (yellow arrowheads), were observed in the submandibular but not sublingual gland. b Staining with osmium tetroxide revealed that these spherical structures did not contain lipid (images shown are from 12-month-old male mice). Scale bar represents 50 um for all images. Images are representative of 8-11 male mice of each genotype and age (9-12 months).
3.4.7 Histology of spleen and other tissues of interest in WT and KO mice

P2X7 is highly expressed in cells of the immune system and therefore in organs such as the spleen (Sun et al., 2012). Spleens were isolated post-mortem, weighed and assessed histologically at 9 and 12 months of age. H&E revealed greater basophilic staining coupled with megakaryocyte hyperplasia in male KO compared to WT mice at 12 months of age (Figure 3.8 a). These histological differences were accompanied by significantly greater mass of the spleen (Figure 3.8 b). Osmium tetroxide did not reveal lipid staining in the spleen (not shown).

Several other tissues were isolated post-mortem and assessed by histology. Livers and hearts from male KO mice had significantly larger masses than WT at 9 months of age; a difference not seen at 12 months of age. No histological differences between WT and KO were apparent in these tissues or in skeletal muscle at either age. No other histological or weight differences were seen in males (Table 3.1). In females, no significant differences were seen in the mass of any of the tissues examined (Table 3.2). Furthermore, no histological differences were seen in any female tissues at any age (data not shown).

3.4.8 Food consumption, blood plasma chemistry and tissue lipid analyses

To assess whether the phenotype seen in older male mice was the result of differences in diet, food consumption was measured by weighing food once weekly. No significant differences in average daily food consumption were seen between male WT and KO mice at any age (Figure 3.9), suggesting that the phenotype may be due to metabolic differences.
Figure 3.8 Histology of spleen in male WT and P2X7 KO mice.

a Representative histological sections of spleen from 12-month-old male WT and P2X7 KO mice stained with H&E. Images revealed greater basophilic staining coupled with megakaryocyte hyperplasia in the KO compared to the WT spleen. No differences between WT and KO were observed in spleen sections stained with osmium tetroxide (not shown). b The mass of the spleen of was significantly greater in KO than in WT at 12 months of age only. Data are means ± SEM, n = 10 mice of each genotype (*p < 0.05, t test).
Figure 3.9 Food consumption by male WT and P2X7 KO mice.

Food consumption was measured by weighing food once weekly. No significant differences were seen at any age between the WT and KO mice. Data are the mean consumption per day and are expressed as means ± SEM, $p > 0.05$, determined by two-way analysis of variance and Bonferroni post hoc test, $n \geq 6$ mice of each genotype and age.
To investigate the metabolic phenotype, blood plasma samples were obtained by cardiac puncture and assayed for glucose, triglyceride and total cholesterol. In comparison to WT, male KO mice had significantly reduced plasma cholesterol levels at both 9 and 12 months of age. No significant differences in plasma triglyceride or glucose levels were seen at either age for WT and KO mice (Table 3.3). Furthermore, no significant differences in any plasma parameters were seen in female WT and KO mice at 9 and 12 months of age (Table 3.4). Selected tissues were isolated post-mortem to determine whether histological changes were accompanied by changes in tissue levels of triglycerides or total cholesterol. KO mouse heart tissue had significantly reduced tissue cholesterol levels in comparison to WT controls at 9 months of age; whereas, there were no significant differences in triglyceride or cholesterol levels in other tissues (Table 3.5).
Glucose, triglyceride, and total cholesterol (mmol/L) were measured in blood plasma samples obtained by cardiac puncture. In comparison to WT, KO mice had significantly reduced plasma cholesterol levels both at 9 and 12 months of age. No significant differences in plasma triglyceride or glucose levels were seen between WT and KO mice at either age. Data are means ± SEM, *$p < 0.05$ compared to corresponding WT based on unpaired two-tailed $t$ test, $n = 9$ mice of each genotype and age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>9 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3.83 ± 0.27</td>
<td>2.91 ± 0.10*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.72 ± 0.05</td>
<td>1.03 ± 0.15</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.42 ± 0.77</td>
<td>10.69 ± 0.82</td>
</tr>
</tbody>
</table>

Table 3.3 Blood plasma cholesterol, triglyceride and glucose levels (in mmol/L) in male wild-type (WT) and P2X7 knockout (KO) mice.
Table 3.4 Blood plasma cholesterol, triglyceride and glucose levels (in mmol/L) in female wild-type (WT) and P2X7 knockout (KO) mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>9 months</th>
<th></th>
<th>12 months</th>
<th></th>
</tr>
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<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.32 ± 0.17</td>
<td>2.33 ± 0.09</td>
<td>2.23 ± 0.21</td>
<td>2.12 ± 0.11</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.81 ± 0.06</td>
<td>0.74 ± 0.16</td>
<td>0.60 ± 0.09</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.33 ± 0.62</td>
<td>10.57 ± 0.35</td>
<td>12.56 ± 0.48</td>
<td>12.91 ± 1.17</td>
</tr>
</tbody>
</table>

Glucose, triglyceride, and total cholesterol (mmol/L) were measured in blood plasma samples obtained by cardiac puncture. No significant differences were observed between KO and WT. Data are means ± SEM, n ≥ 7 mice per group.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Liver</td>
<td>3.93 ± 0.47</td>
<td>3.74 ± 0.16</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3.36 ± 0.05</td>
<td>3.46 ± 0.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.70 ± 0.13</td>
<td>4.26 ± 0.34</td>
</tr>
<tr>
<td>Heart</td>
<td>1.73 ± 0.03</td>
<td>1.64 ± 0.02*</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.43 ± 0.17</td>
<td>6.25 ± 0.20</td>
</tr>
<tr>
<td>Salivary Glands</td>
<td>4.71 ± 0.22</td>
<td>4.35 ± 0.15</td>
</tr>
</tbody>
</table>

Data are means ± SEM in mg per gram of tissue.
Data are based on 6 animals per group.
*indicates significant difference from WT ($p < 0.05$), based on unpaired two-tailed $t$ test.
3.5 Discussion

The P2X7 receptor serves a number of important functions in multiple tissues throughout the body. Previous *in vitro* studies have shown that P2X7 suppresses expression of adipocyte differentiation markers (Panupinthu et al., 2008) and acts through phospholipases and sphingomyelinases to generate bioactive lipid signalling molecules (Costa-Junior et al., 2011). Here, we report abnormal adipocyte and lipid accumulation in mice lacking functional P2X7, pointing to a generalized role for P2X7 in regulating lipid storage and metabolism *in vivo*.

3.5.1 Loss of P2X7 receptor function in mice imparts changes in whole-body composition due to adipocyte hyperplasia

We used the Pfizer KO mouse in which there is global disruption of P2X7 function (Masin et al., 2012; Solle et al., 2001). This mouse model has been studied extensively; however, most studies have focused on the phenotype of younger animals. It has previously been reported that the Pfizer KO mouse displays no gross phenotypic differences at early ages (Ke et al., 2003; Sun et al., 2012; Taylor et al., 2009). In the present study, we allowed these mice to age, which revealed an unexpected increase in body mass due to greater lipid accumulation in older KO mice. Previous characterization of the femurs and tibias of older Pfizer KO mice revealed diminished periosteal bone formation and excessive trabecular bone resorption (Ke et al., 2003). In the present study, we did not detect any difference in skeletal mass at the whole-body level, perhaps indicating regional effects of P2X7 disruption.

We observed that fat pad mass was greater in KO than in WT mice at 9 months of
age. An increase in adipose tissue mass can result from greater adipocyte size (hypertrophy), greater adipocyte cell number (hyperplasia), or both (Bjorntorp, 1991). Previous studies have shown that both visceral and subcutaneous adipocytes express P2X7 receptors (Madec et al., 2011; Volonte et al., 2006). Adipocyte size and density were similar in WT and KO, indicating that the increase in mass was due to adipocyte hyperplasia. Adipocyte number is determined both by the recruitment and differentiation of stem cells into mature adipocytes and by adipocyte apoptosis (Drolet et al., 2008). Therefore, our results suggest that loss of P2X7 may decrease apoptosis of mature adipocytes or increase adipogenesis from mesenchymal stem cells.

Mesenchymal stem cells are widely distributed in connective tissues such as bone marrow, as well as in the perivascular niche (Bouacida et al., 2012). These cells are capable of differentiating into multiple lineages including fibroblasts, chondrocytes, osteoblasts and adipocytes. Moreover, human mesenchymal stem cells have been shown to express several P2 receptors including P2X7 by both RT-PCR and immunoblotting (Zippel et al., 2012). In addition, we have shown previously that cultures of bone cells from Pfizer P2X7 KO mice display greater expression of adipogenic markers compared to wild-type cells (Panupinthu et al., 2008). Thus, P2X7 may suppress adipocyte differentiation from mesenchymal stem cells. In this regard, when mesenchymal stem cells are induced to differentiate into adipocytes, exogenous ATP reduces the formation of lipid droplets and suppresses adipogenic differentiation (Zippel et al., 2012). Taken together, these in vitro results support our in vivo findings of greater numbers of adipocytes in P2X7 KO mice.
3.5.2 Mice with loss of P2X7 receptor function exhibit ectopic deposition of adipocytes and lipid droplets

In the present study, the observed distribution of adipocytes in adipose tissues and ectopically in perivascular regions of the KO mice corresponds to locations where mesenchymal stem cells are known to reside. Previous studies have shown that mesenchymal stem cells exist in both lacrimal glands (You et al., 2011) and pancreas, specifically in ductal regions (Rovira et al., 2010). Thus, it is possible that loss of P2X7 enhances the differentiation of mesenchymal progenitor cells towards the adipocyte lineage, resulting in increased adipocyte numbers in periductal regions of the pancreas and lacrimal glands. In future studies, lineage tracing could be used to determine the commitment of mesenchymal progenitors to adipocytes, shedding light on the role of P2X7 in adipogenesis.

Recent work has shown that P2X7 regulates cellular metabolism. Grol and coworkers showed that activation of P2X7 leads to a dramatic, glucose-dependent increase in metabolic acid production by osteoblasts (Grol et al., 2012). In addition, work from another lab has shown that heterologous expression of P2X7 in vitro leads to upregulation of glycolytic enzymes and an increase in lactate production (Amoroso et al., 2012). Thus, overall energy expenditure may be less in mice lacking functional P2X7 receptors, resulting in increased accumulation of fat as the animals age. It is also possible that P2X7 receptors have direct effects on lipid metabolism. In this regard, several studies have shown that P2X7 couples to activation of phospholipases in various cell types (Costa-Junior et al., 2011; Le Stunff et al., 2004; Panupin thu et al., 2008; Panupin thu et al., 2007). Thus, loss of P2X7 function in cells that normally express this receptor may
disrupt lipid metabolism leading to the eventual accumulation of lipid droplets in the cytosol.

Novak and coworkers have shown previously that Pfizer KO mice display abnormal exocrine secretion from the pancreas and lacrimal glands (Novak et al., 2010). Pancreatic secretion was significantly reduced in KO mice, whereas tear production was increased. Interestingly, the secretory phenotype was affected by the sex of the animal, with males more dependent on P2X7 expression, as observed for ectopic lipid deposition in the present study and skeletal remodeling in a previous study (Ke et al., 2003). This sexual dimorphism may reflect interactions between estrogen and P2X7 signaling. In this regard, it has been shown previously that 17β-estradiol inhibits P2X7-mediated currents through a non-genomic mechanism (Cario-Toumaniantz et al., 1998).

In the present study, accumulation of lipid droplets was also observed in the renal tubular epithelium. Previous reports have shown that, in healthy mice, P2X7 is expressed in the renal collecting duct, but not in the glomeruli or other tubules (Hillman et al., 2002). The function of P2X7 in collecting duct remains poorly understood. A previous study reported no abnormalities in renal morphology or histology in a different P2X7 KO mouse model (6- to 8-week-old female mice) (Taylor et al., 2009). In the present study, lipid droplets were observed only in older male mice, perhaps explaining the lack of phenotype in the earlier study.

In our study, no histological differences were observed in liver, heart, skeletal muscle or Harderian glands. Although it has been reported that P2X7 is expressed in hepatocytes, and cardiac and skeletal myocytes (Emmett et al., 2008; Volonte et al., 2006; Young et al., 2012), there was no apparent accumulation of lipid in these cells.
Therefore, absence of P2X7 alone is not sufficient to trigger lipid abnormalities in all tissues.

### 3.5.3 Metabolic dysregulation in P2X7 KO mice — a potential role for P2X7 in metabolic syndrome

Analysis of blood plasma revealed significantly reduced levels of total cholesterol in the male KO mice in older age groups. In contrast to humans, mice carry cholesterol mainly in the form of high-density lipoprotein (HDL) (Daugherty, 2002; Getz and Reardon, 2012; Zadelaar et al., 2007). On the other hand, fasting blood glucose concentrations were not significantly different between WT and KO mice of all age groups and sexes (although glucose levels were noted to be relatively high in both WT and KO mice). Therefore, it was unlikely that the heavier KO males were diabetic. Furthermore, assessment of food intake in our study revealed no significant differences between WT and KO mice at any age. Additionally, others have reported no differences in spontaneous locomotor activity of P2X7 WT and KO mice (Basso, 2009). Therefore, the changes in adiposity in older males are likely due to metabolic dysregulation. The present study reveals that P2X7 KO mice exhibit some of the characteristics of metabolic syndrome, including increased central adiposity, reduced levels of HDL and the deposition of ectopic lipids in lean tissues.

We found that loss of P2X7 had noticeable effects on body weight and adiposity only in older animals. These findings are consistent with other studies showing no differences in body weight between P2X7 KO and WT mice at younger ages, even when fed a high-fat diet (Glas et al., 2009; Sun et al., 2012). Aging itself is known to be
associated with metabolic dysfunction including alterations in glucose and fatty acid metabolism, and redox homeostasis (Houtkooper et al., 2011). Thus, it is possible that the additional metabolic impairment arising from loss of P2X7 results in a phenotype only when combined with age-related changes in metabolism. Alternatively, it is conceivable that loss of P2X7 affects younger mice, but results in such gradual lipid accumulation that it manifests only in older animals. Interestingly, total mass and fat pad mass were significantly greater in KO than in WT mice at 9 months of age, but there were no significant differences in 12-month-old animals. Thus, it is possible that loss of P2X7 function accelerates age-related changes in body composition.

In summary, we have shown that male mice lacking functional P2X7 receptors develop ectopic lipid accumulations as they age. Our study reveals that P2X7 receptors play a generalized role in regulating lipid storage and metabolism in vivo. Further research is required to elucidate the role of P2X7 in regulating the differentiation of adipocytes from mesenchymal stem cells. As well, our findings provide additional impetus to probe the emerging roles of P2X7 in the control of energy and lipid metabolism.
3.6 References


CHAPTER FOUR

LOW-MAGNITUDE, HIGH-FREQUENCY VIBRATION HAS NO EFFECT ON MURINE WHOLE-BODY COMPOSITION AND BONE MICROARCHITECTURE
4.1 Chapter Summary

Whole-body vibration (WBV) has been shown by some investigators to both promote bone formation and suppress adipogenesis, leading to changes in whole-body composition. The P2X7 receptor is a nonselective cation channel activated by the binding of extracellular ATP. P2X7 receptors are expressed by both osteoblasts and osteoclasts, and have been suggested to play a role in skeletal mechanotransduction. Our objective was to study the effects of WBV on whole-body composition and bone microarchitecture, and to assess whether these effects were mediated by the P2X7 receptor. Male 8-week-old wild-type (WT) and P2X7 loss-of-function (KO) mice were placed on a vibration or control (non-vibrated) platform for 15 min/day, 5 days/week for 18 weeks. The experimental group was subjected to low-magnitude, high-frequency WBV (45 Hz, 0.3 g maximum acceleration). Mice were scanned by micro-CT to assess whole-body composition prior to and during the experimental period. In contrast to some previous studies, we did not observe significant effects of vibration on whole-body mass, adipose, lean or skeletal tissue masses, bone mineral density, or bone mineral content in WT or KO mice. Ex vivo high-resolution scans of hind limbs revealed small but significant differences between genotypes in femoral mid-diaphyseal cortical area, and tibial distal-diaphyseal cortical area and thickness. In contrast, there were no significant effects of vibration on skeletal microarchitecture. Moreover, femoral and tibial lengths were unaffected by genotype or vibration. We conclude that, under the conditions studied, WBV does not induce changes in murine whole-body composition or bone microarchitecture either in the presence or absence of functional P2X7 receptors.
4.2 Introduction

Obesity and musculoskeletal diseases such as osteoporosis are some of the most prevalent diseases in our society. Though pharmacological agents are available to treat these conditions, they are expensive and vary in their efficacy and side effects. As such, development of cost-effective non-pharmacological approaches that are non-invasive is highly desirable. Whole-body vibration (WBV) platforms, a popular commercially available product, aim to meet this need. These devices are described as a non-invasive mechanical loading technology and a safer alternative than drug therapies, which some propose build bone (Chan et al., 2013; Rubin et al., 2001), increase muscle mass and strength (Milanese et al., 2013; Osawa et al., 2013), and reduce adiposity (Cristi-Montero et al., 2013; Vissers et al., 2010). However, while some studies support WBV as a potential therapeutic treatment, other studies do not replicate the benefits (Roelants et al., 2004; Tapp and Signorile, 2014; von Stengel et al., 2011). Therefore, whether whole-body vibration has beneficial effects on body composition is still controversial, providing the rationale for systematic investigation.

It is well established that mechanical loading is an anabolic stimulus in bone (Frost, 2001; Prisby et al., 2008; Robling et al., 2006). In 1892, Julius Wolff published his “Law of Transformation of Bone”, reporting that bone alters its inner architecture due to loading and, as a secondary effect, alters its shape (Wolff, 1986). Mechanically-induced osteogenesis results in improved bone strength, particularly in areas of bone subjected to increased loading (Frost, 2001). Mechanical stimuli are also necessary for the normal functioning of joints (Liu et al., 2001). The adaptive response of the skeleton is made possible by the ability of cells to sense and translate a mechanical stimulus into a
biochemical signal, a process known as mechanotransduction (Grol et al., 2009; Robling and Turner, 2009).

Low-magnitude, high-frequency vibration has previously been shown to increase bone formation (Rubin et al., 2001; Xie et al., 2008), reduce fat formation (Luu et al., 2009; Rubin et al., 2007), increase lean muscle tissue and strength (McKeehen et al., 2013; Mettlach et al., 2014; Xie et al., 2008) and promote anabolic response in intervertebral discs (Gusi et al., 2006; McCann et al., 2013). However, the cellular and molecular mechanisms underlying the response of tissues to mechanical stimuli remain largely unknown. Moreover, the actions of vibration are inconsistent, with some investigators reporting that vibration has minimal effects on musculoskeletal tissues (Christiansen et al., 2009; Lynch et al., 2010; Lynch et al., 2011; Manske et al., 2012).

Potential pathways mediating cellular responses to mechanical loading have been investigated. Perhaps the most widely endorsed view is that nucleotides are released from cells in response to mechanical stimulation (Bodin and Burnstock, 2001; Burnstock, 2007), and serve as mediators of the response of skeletal cells to mechanical loading (Dixon and Sims, 2000; Lenertz et al., 2011; Panupinthu et al., 2008; Robling et al., 2006; Robling and Turner, 2009). ATP is released by mechanical stress (Romanello et al., 2001) and binds to P2 receptors, resulting in bone remodelling (Buckley et al., 2003; Lenertz et al., 2011; Li, 2005).

P2 receptors are subdivided into two classes: P2X (ATP-gated nonselective cation channels) and P2Y (G protein-coupled receptors) (Burnstock, 2007). Osteoblasts and osteoclasts express multiple P2 receptor subtypes (Lenertz et al., 2011; Orriss et al., 2010). Seven subtypes of P2X receptors, P2X1-7, are known to exist. Of these, P2X7 is...
emerging as a key player in a number of physiological and pathological processes. An extended C-terminal tail confers unique properties to this receptor, such as the ability to form pores and induce plasma membrane blebbing (Burnstock, 2007; Garcia-Marcos et al., 2006; Grol et al., 2009; Panupinthu et al., 2008). Moreover, P2X7 receptors are implicated in bone remodeling (Gartland et al., 2012; Grol et al., 2013; Husted et al., 2013; Ke et al., 2003; Li, 2005; Wesselius et al., 2013), cellular metabolism and energy homeostasis (Amoroso et al., 2012; Grol et al., 2012), as well as adiposity and lipid accumulation (Chapter 3).

Mice that have been genetically modified to lack functional P2X7 receptors (KO) exhibit diminished periosteal bone formation and excessive trabecular bone resorption (Ke et al., 2003). Interestingly, these mice show an impaired skeletal response to axial loading of the ulna (Li, 2005). In comparison to cultures from wild-type (WT) mice, bone cells from P2X7 KO mice (which include mesenchymal progenitor cells) exhibit greater expression of adipogenic markers accompanied by suppression of osteogenic markers (Panupinthu et al., 2008). Therefore, P2X7 may be activated during mechanotransduction, directing differentiation towards the osteoblast lineage and away from the adipocyte lineage.

The objective of the present study was to explore the effects of WBV on murine body composition and bone microarchitecture, and the possible role of P2X7 in these processes. We used a custom-built platform for in vivo low-magnitude, high-frequency WBV of mice, as described previously (McCann et al., 2013). The vibration parameters used in the present study were similar to those used by other investigators who demonstrated anabolic effects on bone in mice (Xie et al., 2006; Xie et al., 2008).
Quantitative micro-CT imaging was used to assess murine whole-body composition and bone microarchitecture. We found no significant effects of vibration on whole-body composition parameters, including total body mass, and adipose, lean and skeletal tissue masses. Although genotype was found to influence skeletal microarchitecture, there were no significant effects of vibration. Together with the results of other recent studies, our findings highlight the need to re-evaluate current thinking on the effects of low-magnitude, high-frequency WBV on body composition.
4.3 Materials and Methods

4.3.1 Animals

The P2X7 KO mouse was obtained from Pfizer (Solle et al., 2001). A splice variant (with C-terminal truncation) is detectable in some tissues of the Pfizer KO mouse; however, it is inefficiently trafficked to the cell surface and displays greatly diminished receptor function (Masin et al., 2012) thereby justifying the use of this mouse as a loss-of-function model. Mice were maintained on a mixed genetic background (129/Ola × C57BL/6 × DBA/2) by crossbreeding of heterozygous mice. Genotypes were identified by PCR. Mice were individually housed in standard cages and maintained on a 12-hour light/dark cycle, with water and standard mouse chow (2018 Teklad Global 18% protein rodent diet, Harlan Laboratories, USA) available ad libitum. Mice were weighed immediately after scanning, and food was weighed weekly using a portable electronic scale (Acculab VICON VIC-511; Sartorius Group, Germany). Food intake was assessed throughout the study by subtracting the weight of food remaining after each week from the known amount of food provided. One outlier mouse was excluded from the food intake data due to abnormal grinding of food without ingestion. All aspects of this study were conducted in accordance with the policies and guidelines of the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario, London, ON, Canada. Data for the WT NV group in the present study also appear in Chapter 2 for Cohort 2 mice (ages 8 to 26 weeks) and in the whole-body composition and food consumption data in Chapter 3 for 8-week-old (2 month) and 26-week-old (6 month) WT mice.
4.3.2 Vibration platform and procedure

We developed a low-magnitude, high-frequency vibration platform designed specifically for small animal studies, as described previously (McCann et al., 2013). Briefly, vibrations were produced by an electromagnetic shaker regulated by an open-loop controller. To effectively reduce resonance frequencies, the platform itself was constructed using 12-mm thick aerospace-grade aluminum-core honeycomb, and was then mounted atop the electromagnetic shaker. This system allowed us to apply vertical sinusoidal vibrations with up to 1 g maximum acceleration, 10 to 90 Hz frequency range, and 0.01 to 0.33 mm peak-to-peak displacement. Acceleration was monitored using a precision accelerometer, and validated by high-speed imaging. The acceleration accuracy was confirmed to be within 4% and precision at 45 Hz was better than 0.70%.

Eight-week-old male WT and P2X7 KO mice were separated into either a sham (non-vibrated, NV) or experimental (vibrated, VIB) groups. Mice in the experimental groups were placed in cages affixed to the vibration platform and subjected to whole-body vibration at 45 Hz, 74 µm peak-to-peak amplitude, and 0.3 g maximum acceleration for 15 min/day, 5 days/week over an 18-week period. These vibration parameters were selected as they have been reported in some studies to induce anabolic skeletal effects in mice (Prisby et al., 2008; Xie et al., 2006; Xie et al., 2008). Mice in the sham groups were placed in cages on a control platform for the same duration of time, in close proximity to the actual vibration platform while it was in operation for the corresponding experimental group. All mice were housed individually in custom plastic cages during experimentation. The plastic cages were affixed to the vibration platform to reduce
resonance frequencies. Cages did not contain bedding so as to avoid dampening the vibration.

4.3.3 Whole-body in vivo micro-CT image acquisition

Longitudinal in vivo micro-CT imaging was used to quantify whole-body composition (including skeletal, adipose and lean tissue masses) prior to (at week 0), during (at 3, 6, 9, 12 and 15 weeks) and immediately following the experimental period (at 18 weeks). The duration of the study was determined based on the results of previous studies, which showed skeletal effects of WBV within 3 weeks and effects on adipose tissue distribution within 15 weeks (Rubin et al., 2007; Xie et al., 2006; Xie et al., 2008). Mice were anesthetized with isoflurane (Forane, catalog # CA2L9100, Baxter Corporation, Mississauga, Canada) and imaged using an eXplore Locus Ultra micro-CT scanner (GE Healthcare Biosciences, London, ON, Canada). Scans were analysed as described previously (Chapter 2). Briefly, a calibrating phantom composed of air, water and cortical bone-mimicking epoxy with a mineral value of 1100 mg/cm³ (SB3; Gammex, Middleton, WI, USA) (White, 1978) was scanned together with the animals. One thousand projection images were obtained over a single 16-second rotation (80 kVp, 55 mA tube current, 16 ms exposure). While still anesthetized, immediately after scanning, mice were weighed gravimetrically using a portable electronic precision scale (Acculab VICON VIC-511; Sartorius Group, Germany).

Data sets were reconstructed into 3D volumes from the X-ray projection data with nominal isotropic voxel spacing of 154 µm using a cone-beam filtered backprojection algorithm. The reconstructed data were linearly rescaled into Hounsfield units (HU), with
the voxel gray-level of air being -1000 HU and water 0 HU.

### 4.3.4 Whole-body composition analysis

Using MicroView software (GE Healthcare Biosciences), three signal-intensity thresholds (-200, -30 and 190 HU) were used to classify each voxel as adipose (-200 to -31 HU), lean (-30 to 189 HU), or skeletal tissue (≥190 HU). In-house-designed software was used to calculate tissue masses from assumed tissue densities of 0.95 (adipose), 1.05 (lean), and 1.92 (skeletal) g/cm$^3$ as listed by the International Commission on Radiation Units and Measurements (ICRU, 1989). The sum of all tissue masses yielded a CT-derived estimate of whole-body mass, which corresponded well to the gravimetric mass of each mouse (within 3%). In addition, the software was used to calculate bone mineral density (BMD; mg HA/cm$^3$) and bone mineral content (BMC; mg HA). Briefly, BMD was computed as the ratio of the average HU value of the skeletal region of interest (ROI) to the measured HU value of the SB3 calibrator, multiplied by the known hydroxyapatite (HA)-equivalent density of the SB3 (1100 mg/cm$^3$). Thereafter, the program automatically computed BMC as the product of the BMD and the total volume of the skeletal ROI used in the BMD calculation.

### 4.3.5 High-resolution micro-CT image acquisition

Mouse hind limbs were isolated post-mortem, fixed in 10% neutral buffered formalin, and imaged using an eXplore Locus RS micro-CT scanner (GE Healthcare Biosciences, London, ON, Canada) to characterize trabecular and cortical bone properties. The calibrating phantom was scanned together with specimens. The scanning protocol was as
follows: 900 X-ray projection images were acquired at an angular increment of 0.4 degrees over a single 2.75-hour gantry rotation (80 kVp, 450 mA tube current, 4.5 s exposure, 2 frames per view angle averaged). Using a filtered backprojection algorithm, the data sets were reconstructed into 3D volumes with an isotropic voxel spacing of 20 µm, and then linearly rescaled into HU using the internal air and water calibration standards.

4.3.6 High-resolution micro-CT analysis

Reconstructed images were cropped and analysed using MicroView software. Briefly, femur and tibia were spline cropped and reoriented to the axes. Selected ROIs were then assessed using the Bone Analysis Tool in MicroView. Consistently sized box ROIs were placed in the mid-diaphysis of the femur and tibia, and distal tibia for cortical analyses, avoiding the presence of trabeculae. BMD and cortical analyses (including slice data and radial thickness) were selected and reported using user-defined threshold values. Trabecular analyses were performed on cylindrical ROIs from the distal femur and proximal tibia, landmarked by the distal-most aspect of the epiphyseal line, and contained within the marrow space ensuring that no cortical bone was included. BMD, structure model index (SMI), direct measures, anisotropy and stereology analyses were selected, using the same threshold values as above. Images of isosurfaced bones were obtained using the snapshot option within MicroView with the highest surface quality and no decimation.
4.3.7 **Hind limb long-bone analyses**

Bone lengths were measured using the line measurement tool in MicroView. Femurs were measured from the base of the lateral femoral condyle to the tip of the greater trochanter. Tibial lengths were measured from the base of the medial malleolus to the tip of the intercondylar eminence.

4.3.8 **Statistical analyses**

Data are presented as means ± standard error of the mean (SEM). Differences among three or more groups were evaluated by two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons test. Differences were accepted as statistically significant at $p < 0.05$. All $n$ values represent the number of mice used in each group.
4.4 Results

4.4.1 Evaluation of the effect of vibration on murine whole-body composition

We first investigated the effects of low-magnitude, high-frequency vibration on murine body composition and the possible role of P2X7. Eight-week-old male WT and P2X7 KO littermate mice were separated into age-matched control (non-vibrated, NV) and experimental (vibrated, Vib) groups. Experimental groups were subjected to WBV at 45 Hz and 74 μm peak-to-peak amplitude (giving maximum acceleration of 0.3 g) for 15 min/day, 5 days/week over a period of 18 weeks. To assess the systemic effects of WBV, in vivo micro-CT was used to quantitatively monitor whole-body composition longitudinally at 3-week intervals. Each voxel was assigned as adipose, lean or skeletal tissue based on threshold values using an in-house-designed program. The sum of all tissue masses yielded a CT-derived estimate of whole-body mass (Figure 4.1 A). When the change in total body mass (in g) was evaluated over the 18-week period, we did not observe any significant differences between NV and Vib or between WT and KO mice. We then calculated changes in total body mass as percentages of baseline values at week 0 (Figure 4.2 A). Again, no significant effects of vibration or genotype were observed.

WBV of mice has been shown by some authors to enhance trabecular bone volume fraction, inhibit trabecular bone resorption, increase the rate of bone formation on the endocortical surface, and increase cortical and bone marrow areas in the proximal tibia (Xie et al., 2006; Xie et al., 2008). However, over the 18-week period of our study, we found no significant effect of vibration or genotype on whole-body BMD, BMC or skeletal tissue mass, both when data were analysed as absolute values (Figure 4.1 B-D) or
Figure 4.1 Vibration does not significantly alter whole-body composition of WT or P2X7 KO mice.

WT and P2X7 KO mice were subjected to vibration (Vib) or sham control (non-vibrated, NV). Mice were anesthetized and scanned by micro-CT at baseline (week 0, 8 weeks of age) and at 3-week intervals for 18 weeks (26 weeks of age). An in-house-designed program was used to assign each voxel as adipose, lean or skeletal tissue, based on threshold values. Tissue masses were then calculated from assumed tissue densities of 0.95 (adipose), 1.05 (lean), and 1.92 (skeletal) g/cm³. Data are changes in indicated whole-body composition parameters from baseline (week 0) to week 18, expressed in absolute units. There were no significant effects of vibration on whole-body composition parameters. Additionally, there were no significant effects of genotype on whole-body composition. Data are means ± SEM (n = 6-8 mice per group), where p > 0.05 by two-way ANOVA.
Figure 4.2 Effects of vibration on whole-body composition of WT or P2X7 KO mice expressed as relative changes.

WT and P2X7 KO mice were subjected to vibration (Vib) or sham control (non-vibrated, NV). Mice were anesthetized and scanned by micro-CT at baseline (week 0, 8 weeks of age) and at 3-week intervals for 18 weeks (26 weeks of age). Images of mice were obtained and analysed as described in Figure 4.1. Data are changes in indicated whole-body composition parameters from baseline (week 0) to week 18, expressed as a percentage of the baseline values. There were no significant effects of vibration on whole-body composition parameters. Additionally, there were no significant effects of genotype on whole-body composition. Data are means ± SEM (n = 6-8 mice per group), where p > 0.05 by two-way ANOVA.
as percent changes from baseline values (Figure 4.2 B-D). Previous studies have also shown that P2X7 KO mice display differences compared to WT in histomorphometric parameters of individual bones (Ke et al., 2003). However, differences were not apparent in the present study at the whole-body level, consistent with regional effects of P2X7 disruption.

Some investigators have reported that WBV reduces adipose tissue volume (Rubin et al., 2007) and enhances muscle accretion (Xie et al., 2008). However, we did not find any significant effect of vibration or genotype (Figure 4.1 E, F, 4.2 E, F). The absence of effect of genotype on adiposity in the 6-month-old mice used for this study is consistent with our findings in Chapter 3, which demonstrate an effect of P2X7 only at 9 months of age.

Some previous studies have suggested that vibration can induce skeletal changes in as little as 3 weeks (Xie et al., 2006) and adipose tissue changes in 12-15 weeks (Rubin et al., 2007). Though gravimetric body mass (Figure 4.3 A) corresponded well to the CT-derived total body mass (within 3%), no effects of vibration or genotype were found on body mass or skeletal tissue mass (Figure 4.3 B). Similarly, there were no significant effects of vibration or genotype on adipose or lean tissue masses at any time point (Figure 4.3 C, D).\(^1\) Taken together, these data reveal that, under the conditions studied, WBV does not induce changes in murine whole-body composition.

As an additional measure, we assessed whether offsetting effects of vibration on

\(^1\) In contrast to the results of the Bonferroni test, two-way ANOVA indicated an overall significant effect of vibration on adipose and lean tissues mass in WT mice. However, the cohort of WT mice that were vibrated began at time 0 with a greater percentage of adipose tissue mass (and less lean tissue mass) than the non-vibrated cohort, accounting for this apparent effect of vibration.
Figure 4.3 Time course of changes in whole-body composition of WT and P2X7 KO mice.

WT and P2X7 KO mice were subjected to vibration (Vib) or sham control (non-vibrated, NV), scanned, and analysed as described in the legend to Figure 1. A Data indicate the gravimetric mass of mice over the 18-week experimental period. B-D Skeletal, adipose and lean tissue mass are expressed as a percentage of CT-derived total body mass at each time. No significant differences were seen among WT NV, WT Vib, KO NV and KO Vib at any time point throughout the course of the experimental regimen. Data are means ± SEM (n = 6-8 mice per group).
food intake might have masked possible effects of vibration on whole-body composition. Food consumption was measured by weighing food once weekly, then calculating mean food consumption per day over the experimental period for each group (Figure 4.4). No significant effects of vibration or genotype were observed in eating behaviour. Thus, diet did not appear to have any confounding effect on our assessment of whole-body composition.

4.4.2 Evaluation of the effect of vibration on bone microarchitecture

Other studies have shown that the effects of mechanical loading are localized to loaded bones (Robling et al., 2006; Sugiyama et al., 2010; Turner, 1999) and, within those bones, site-specific effects are observed (Fritton et al., 2005). Therefore, we assessed the local effects of vibration on microarchitectural parameters in the femur and tibia. High-resolution (20 \( \mu \text{m} \)) ex vivo micro-CT scans of hind-limbs were performed at the end of the experimental period. Visual assessment of representative images at the gross level did not reveal any marked differences in shape or architecture (Figure 4.5). Moreover, no significant effects of vibration or genotype were observed on long bone lengths (Figure 4.6).

We then evaluated specific regions of interest in the femur and tibia. Others have reported an effect of vibration on cortical bone parameters in the femoral diaphysis (Vanleene and Shefelbine, 2013). In the present study, an ROI containing only cortical bone of the mid-diaphysis was isolated, and we used the Bone Analysis Tool in MicroView to assess cortical parameters. Our data did not reveal significant effects of vibration on BMD, BMC, BV/TV, Ct.Th, Ma.Ar or Ct.Ar (Figure 4.7). However, we did observe a small but significant effect of genotype on Ct.Ar (Figure 4.7 F).
Figure 4.4 Food consumption by mice.

Food consumption was measured by weighing food once weekly over the course of the experimental period. No significant differences were observed among groups. Data are the mean consumption per day over the 18-week experimental period and are expressed as means ± SEM ($n \geq 6$ mice per group), $p > 0.05$, determined by two-way ANOVA.
Figure 4.5 Representative high-resolution micro-CT images of femur and tibia.

Immediately following the 18-week experimental period, mice were sacrificed and left hind-limbs were isolated from mice for *ex vivo* scanning. Using the eXplore Locus RS micro-CT scanner, images were reconstructed with nominal isotropic voxel spacing of 20 µm and rescaled into Hounsfield units. MicroView software was used to crop femur and tibia from the whole hind-limb volume. Each column shows images from a single representative mouse in each of the study groups. The first row shows coronal sections of the femur that are 3D surface-rendered to illustrate trabeculae and cortical bone. The second row shows surface-rendered femurs from the posterior perspective. The third row shows surface-rendered tibias from the medial perspective. The fourth row shows a sagittal view of the cropped proximal tibia to illustrate trabeculae. Images are representative of left femur and tibia from 6 mice in each group.
Figure 4.6 Lengths of femurs and tibiae at the completion of the 18-week experimental period.

Left tibiae and femurs were measured using the line measurement tool in MicroView from base of the lateral femoral condyle to tip of the greater trochanter (for femurs, A) and base of the medial malleolus to tip of the intercondylar eminence (for tibiae, B). There were no significant differences between genotypes or vibrated (Vib) and non-vibrated (NV) groups. Data are means ± SEM (n = 6 mice per group), p > 0.05 determined by two-way ANOVA.
Figure 4.7 Analysis of mid-diaphyseal cortical region of the femur.

Using MicroView software, left femurs were cropped from the hind-limb images and axes were reoriented. A region of interest (ROI) that encompassed the mid-diaphysis in all three spatial directions was cropped from each of the volumes. The ROI included only cortical bone. The cropped ROI was then used to determine the bone mineral density (BMD, mg HA/cm³), bone mineral content (BMC, mg HA), bone volume fraction (BV/TV), cortical mean thickness (Ct.Th, mm), cortical marrow area (Ma.Ar, mm²) and cortical area (Ct.Ar, mm²) with the Bone Analysis Tool in MicroView. There were no significant differences between vibrated (Vib) and non-vibrated (NV) groups. There was a significant effect of genotype on Ct.Ar. Data are means ± SEM (n = 6 mice per group), * indicates p < 0.05 by two-way ANOVA with a Bonferroni post hoc test.
Thereafter, we assessed the effects of vibration on the distal femoral trabeculae (Figure 4.8), as some other investigators have shown responses to WBV in this region (Judex et al., 2002; Tezval et al., 2011). A cylindrical ROI was placed in the marrow cavity of the distal femur that encompassed only trabecular bone immediately above the growth plate. BMD, BMC, BV/TV, Tb.N, Tb.Th and Tb.Sp were assessed. No significant effects of vibration or genotype were observed.

We also evaluated cortical and trabecular regions of the tibia. Others have reported an effect of WBV on cortical bone parameters in tibia (Vanleene and Shefelbine, 2013; Xie et al., 2006). We first examined cortical bone of the mid-diaphysis (Figure 4.9).

Again, no significant effects of vibration or genotype were observed. Next, we assessed the distal-diaphyseal cortical region of the tibia (Figure 4.10). Our data did not reveal significant effects of vibration; however, we did observe a significant effect of genotype on Ct.Th (Figure 4.10 D). As well, two-way ANOVA revealed an overall significant effect of genotype on Ct.Ar (Figure 4.10 F), with significantly smaller cortical area in P2X7 KO compared to WT mice. Finally, we assessed trabecular bone in the proximal tibia (Figure 4.11). No significant effects of vibration or genotype were observed in this region.

Taken together, these data indicate that, under the conditions studied, WBV does not induce changes in the microarchitecture of the murine femur and tibia.
Figure 4.8 Analysis of distal femoral trabeculae.

A cylindrical region of interest (ROI) that encompassed the perimeter of the marrow cavity in all three spatial directions was cropped from each of the volumes. The ROI began at the proximal end of the growth plate in the distal metaphysis and extended proximally while excluding the surrounding cortical bone. The cropped ROI was then used to determine the BMD (mg HA/cm$^3$), BMC (mg HA), BV/TV, trabecular number (Tb.N), trabecular thickness (Tb.Th, mm) and trabecular spacing (Tb.Sp, mm) with the Bone Analysis Tool in MicroView. A - F) There were no significant differences between genotypes or between vibrated (Vib) and non-vibrated (NV) groups. Data are means ± SEM ($n = 6$ mice per group), where $p > 0.05$ determined by two-way ANOVA.
Figure 4.9 Analysis of mid-diaphyseal cortical region of the tibia.

A region of interest (ROI) that encompassed the mid-diaphysis in all three spatial directions was cropped from each of the volumes. The ROI included only cortical bone. The cropped ROI was then used to determine the BMD (mg HA/cm³), BMC (mg HA), BV/TV, Ct.Th (mm), Ma.Ar (mm²) and Ct.Ar (mm²) with the Bone Analysis Tool in MicroView. There were no significant differences between genotypes or between vibrated (Vib) and non-vibrated (NV) groups. Data are means ± SEM (n = 6 mice per group), where p > 0.05 determined by two-way ANOVA.
Figure 4.10 Analysis of distal-diaphyseal cortical region of the tibia.

A region of interest (ROI) that encompassed the distal-diaphysis in all three spatial directions was cropped from each of the volumes. The ROI included only cortical bone. The cropped ROI was then used to determine the BMD (mg HA/cm$^3$), BMC (mg HA), BV/TV, Ct.Th (mm), Ma.Ar (mm$^2$) and Ct.Ar (mm$^2$) with the Bone Analysis Tool in MicroView. There were no significant differences between vibrated (Vib) and non-vibrated (NV) groups. There was a significant effect of genotype on Ct.Th and Ct.Ar. Data are means ± SEM ($n = 6$ mice per group), * indicates $p < 0.05$ by two-way ANOVA with a Bonferroni post hoc test. The effect of genotype on Ct.Ar was significant based on the results of two-way ANOVA ($p < 0.05$).
Figure 4.11 Analysis of proximal tibial trabeculae.

A cylindrical region of interest (ROI) that encompassed the perimeter of the marrow cavity in all three spatial directions was cropped from each of the volumes. The ROI was selected starting at the end of the growth plate in the proximal metaphysis and extended distally while excluding the surrounding cortical bone. A-F) The cropped ROI was then used to determine the BMD (mg HA/cm$^3$), BMC (mg HA), BV/TV, Tb.N, Tb.Th (mm) and Tb.Sp (mm) with the Bone Analysis Tool in MicroView. There were no significant differences between genotypes or between vibrated (Vib) and non-vibrated (NV) groups. Data are means ± SEM ($n = 6$ mice per group), where $p > 0.05$ determined by two-way ANOVA.
4.5 Discussion

Whole-body vibration has recently gained popularity due to studies indicating that it may be a non-pharmacological approach to building bone (Gilsanz et al., 2006; Xie et al., 2008), increasing muscle strength (Tapp and Signorile, 2014; Verschueren et al., 2004; Xie et al., 2008) and reducing fat tissue mass (Milanese et al., 2013; Rubin et al., 2007; Vissers et al., 2010; Xie et al., 2008). WBV is appealing due to its ease of use, minimal time requirements, and accessibility for those who are unable to do high-impact exercises (such as infirmed, injured, elderly or obese individuals) (Chan et al., 2013; Tapp and Signorile, 2014). However, the effects of WBV are controversial and poorly understood.

The aim of the present study was to assess the effects of WBV on murine body composition and bone microarchitecture, and the possible role of P2X7 in these processes. We analysed whole-body composition using in vivo quantitative micro-CT. Unexpectedly, our analysis did not reveal significant changes in body composition in response to vibration.

Some previous studies showed anabolic skeletal responses to WBV using the same vibration parameters (Xie et al., 2006; Xie et al., 2008). However, consistent with our findings, recent studies from a number of other groups have not demonstrated anabolic effects of vibration on skeletal tissues (Lynch et al., 2011; Manske et al., 2012; van der Jagt et al., 2012; Wenger et al., 2010). In addition, we did not see a significant effect of vibration on adiposity. Our findings, though in contrast to those of Rubin and coworkers, who observed a ~28% decrease in adipose tissue volume in the torso after 12 weeks measured using CT (Rubin et al., 2007), are consistent with a more recent report showing fat pad masses are unaffected by WBV (Lynch et al., 2010). It should be noted
that the study by Rubin and colleagues used a vibration protocol of 90 Hz with a
displacement of ~12 \( \mu \text{m} \) (0.2 g maximum acceleration) for 15 min/day, 5 days/week over
the course of 15 weeks (Rubin et al., 2007). In contrast, our study used 45 Hz and ~74
\( \mu \text{m} \) peak-to-peak amplitude (0.3 g maximum acceleration) for 15 min/day, 5 days/week
over the course of 18 weeks. It is possible that these small differences in vibration
protocols may have impacted the effects of vibration on adipose tissues. Unfortunately,
some investigators do not include specific information on vibration parameters or how
parameters were validated (Lorenzen et al., 2009). This may be one reason for
inconsistencies in the literature.

It is also possible that responses to vibrational stimuli are dependent on genetic
differences among strains of mice (Judex et al., 2002; Prisby et al., 2008). For example,
the study by Rubin et al. showed effects of WBV on mice of male C57BL/6 strain (Rubin
et al., 2007), while other studies from this group showed enhanced cortical bone
properties in female mice on a BALB/cByJ background (Xie et al., 2008). Reports from
another author do not show anabolic skeletal effects of WBV in male BALB/c mice
(Lynch et al., 2011), and another group shows no response in several skeletal sites and
some response in other sites of male C57BL/6 mice (Wenger et al., 2010). Additionally,
we noted large ranges in sample sizes of animal populations used in vibration studies,
from as little as 3 to as many as 20 animals per treatment group (Mettlach et al., 2014;
Rubin et al., 2007). In our study, a sample size calculation was performed with a two-
side alpha of 0.05 and power of 80%. We used the variability of gravimetric mass (SD =
1.3 g), BMD (SD = 8.67 mg HA/cm\(^3\)), BMC (SD = 41.1 mg HA), lean tissue (SD = 1.0
g), and skeletal tissue (SD = 0.17 g) in WT non-vibrated mice. We determined that
sample sizes of $n = 5, 3, 6, 6$ and $3$ mice, respectively, were required to detect a difference of $\geq 10\%$ between treatment groups. Our study used a minimum of $6$ animals per group; thus ensuring that sample sizes were sufficient to detect effect sizes of $10\%$ or less. Taken together, the differing strains, sexes and sample sizes used in other studies likely contribute to the inconsistent body of literature on the effects of WBV treatment.

Because we failed to detect changes in whole-body composition induced by vibration, we proceeded to use high-resolution micro-CT to determine whether vibration leads to localized skeletal changes in hind-limb long bones. This decision was based on observations in the literature that suggest that the effects of mechanical stimuli are exhibited solely in loaded bones (Robling et al., 2006; Sugiyama et al., 2010; Turner, 1999). In this regard, some studies show favourable effects of vibration of BV/TV in trabeculae, as well as greater cortical bone area and marrow area in the proximal tibia (Xie et al., 2008). However, we saw no differences in tibial trabecular or cortical bone structure; consistent with that reported by Lynch and colleagues (Lynch et al., 2010). On the other hand, that bone lengths did not change significantly with vibration in this study is consistent with the findings of most other groups (Xie et al., 2006; Xie et al., 2008). Bone lengths also did not differ significantly with genotype; a finding consistent with previously reported phenotype for P2X7 KO mice (Ke et al., 2003). Additionally, it is possible that the scan resolution could have affected the outcomes in this study. Although we scanned with $20\, \mu m$ voxel size; however, the width of mouse trabeculae fall within $30 – 50\, \mu m$ (Bouxsein et al., 2010). Thus, it is possible that even higher scan resolutions ($< 20\, \mu m$) could have produced different results, albeit at the expense of markedly increased scan times and impaired signal-to-noise ratios.
With regards to the P2X7 KO mouse model, the current study also did not observe any effects of genotype at the whole body level for mice between 2 and 6 months of age. This is in keeping with our findings in Chapter 3 that show only at older ages (9 months) do KO mice exhibit greater adiposity than WT mice. Additionally, Ke and coworkers showed reduced total bone content and smaller bone diameters in femurs of KO mice compared to WT mice, differences which were subtle at 2 months of age and more pronounced at 9 months (Ke et al., 2003; Li, 2005). Thus, the P2X7 KO phenotype manifests with age and as only young animals were included in the present study, only subtle effects of genotype were observed.

The possible mechanism through which vibration affects skeletal tissues is unclear, as is the reason for conflicting reports as to the in vivo effects of WBV. In vitro studies reveal that mechanical stimulation increases mesenchymal stem cell (MSC) proliferation and differentiation (Luu et al., 2009). Moreover, vibration is reported to maintain the osteogenic potential of bone marrow (Ozcivici et al., 2010). Others have shown that mechanical strain enhances osteogenesis while suppressing adipogenesis in MSC cultures, even under conditions favoring adipogenesis (Sen et al., 2008). However these findings are controversial, as other studies have shown that vibration reduces the osteogenic potential of MSCs, diminishing osterix expression and inhibiting matrix mineralization (Lau et al., 2011). Therefore, further investigation of mechanotransduction in response to vibrational stimuli is required using both in vitro and in vivo models.

Skeletal aging may affect how bone senses WBV, as the sensitivity of bone to mechanical signals appears to decrease with age (Prisby et al., 2008; Robling et al., 2006; Rubin et al., 1992). Using a tibial compression loading model, Willie and coworkers
showed anabolic responses in young mice at 10 weeks of age, but not in older mice at 26 weeks of age (Willie et al., 2013). Some have observed anabolic effects of WBV on bone in mice at 7, but not 22 months of age (Lynch et al., 2010), whereas others show effects of vibration on mice at 18 months of age (Wenger et al., 2010). Our animals ranged from 8 to 26 weeks of age, which may have affected our outcomes. Given that some studies have observed anabolic responses in older mice, future studies examining WBV in this KO mouse model may yield different results at an older age when the phenotype is more pronounced. Additionally, future work could also assess the acute, rather than chronic, effects of vibration in both younger and older mice.

Some research has shown that extending the duration of skeletal loading does not result in proportional increases in bone mass, since the response fades as bone cells become desensitized to mechanical loading over time (Robling et al., 2006). Whether the existence of such a period of desensitization applies to WBV is still controversial, as some investigators have shown that a rest period during vibration fails to its potentiate cellular effects (Xie et al., 2006), whereas others have shown that it can inhibit adipogenesis (Sen et al., 2011) while enhancing osteogenic responses (Gross et al., 2004; LaMothe and Zernicke, 2004). Our experimental procedure entailed one bout of WBV per day for 5 days/week over the course of 18 weeks. It is possible that multiple bouts of vibration over a shorter time period might be more effective.

In summary, our data are consistent with a number of other studies reporting little-to-no improvement of skeletal parameters in response to WBV (Castillo et al., 2006; Lynch et al., 2010; Lynch et al., 2011; Manske et al., 2012). Together with the results of
these studies, our findings highlight the need to re-evaluate current thinking on the effects of low-magnitude, high-frequency WBV on body composition \textit{in vivo}. 
4.6 References


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CHAPTER FIVE

GENERAL DISCUSSION
5.1 Summary and Conclusions

This thesis characterized a non-invasive method to monitor body composition during growth and aging in mice, and also evaluated the effects of P2X7 and mechanical vibration on body composition. We demonstrated that whole-body composition analysis using micro-CT could be performed rapidly and accurately in mice. We then applied this methodology to study the effects of P2X7 and whole-body vibration. Using wild-type (WT) and P2X7 knockout (KO; P2rx7−/−) mice, we found that P2X7 plays a previously unknown role in the regulation of adiposity and metabolism. Next, we evaluated the effects of whole-body vibration on WT and KO mice. We found that, under the conditions used, low-magnitude, high-frequency vibration does not induce changes in body composition or bone microarchitecture either in the presence or absence of P2X7 receptors. The specific objectives, results and conclusions of each data chapter are summarized in greater detail below.

5.1.1 Quantitative Micro-Computed Tomography for Assessment of Age-Dependent Changes in Murine Whole-Body Composition: Objective, Summary and Conclusions

Chapter 2 – Specific Objective – To characterize changes in whole-body composition of mice during growth and aging using a novel, high-throughput method for analysis of micro-CT images.

Chapter 2 – Summary and Conclusions

1. Cohorts of mice at several ages were scanned using micro-CT to assess whole-body composition, which included assessment of total, adipose, lean and skeletal tissues. The resulting images provided a useful means for
visually determining changes in the distribution of tissues during growth and aging in mice.

2. Quantification of the micro-CT data using whole-body composition analysis revealed distinct age-related changes in tissue masses. Specifically, total body mass, bone mineral content (BMC), lean and skeletal tissue masses exhibited biphasic changes consisting of rapid increases up to 11 weeks of age, followed by a slow increase up to 52 weeks of age. Bone mineral density (BMD) rapidly increased up to 11 weeks of age then plateaued, while adipose tissue exhibited a continuous increase with age.

3. Variability within our cohorts of mice at each age was assessed. Individual mouse whole-body composition data and coefficients of variation were computed. The coefficients of variation were within 10% of the mean, with the exception of adipose tissue, which ranged between 16-33% and became more variable with age.

4. We describe a method to reproducibly measure tibia and femur lengths using anatomical landmarks and a line measurement tool in MicroView software. Tibia and femur both rapidly increased in length prior to 11 weeks of age, then exhibited a slow, continuous increase up to 52 weeks of age.

5. Using regions of interest in the femoral mid-diaphysis, we quantified maximum BMD values. The maximum BMD values show a biphasic curve, rapidly increasing to 11 weeks of age then exhibiting much slower increases with age.

6. To assess reproducibility, we examined two separate cohorts of mice at 8
weeks of age at different times, using the same scanner and protocol. Similar values were obtained for the two cohorts of mice.

7. We compared values for total body mass, obtained gravimetrically and CT-derived, at each age. There was excellent agreement, with CT-derived masses slightly (< 3%) less than gravimetrically determined masses.

8. A higher-resolution scanner (speCZT) was used to image younger mice, and data were compared to those obtained from our lower-resolution micro-CT scanner (Ultra). There were slight but statistically significant differences in the volumetric measures of total mass, BMD, BMC, and adipose, lean and skeletal tissue mass.

9. The present study is the first to report CT-derived, whole-skeleton data for growing and aging mice. Our micro-CT methodology offers the ability to accurately and precisely quantify adipose and lean tissue volumes of the mouse in addition to measuring skeletal tissue volume, providing normative comparisons for researchers using mouse models.

5.1.2 Loss of P2X7 Nucleotide Receptor Function Leads to Abnormal Fat Distribution in Mice: Objective, Summary and Conclusions

Chapter 3 – Specific Objective – To assess the role of P2X7 in adipocyte distribution and lipid accumulation in vivo.

Chapter 3 – Summary and Conclusions

1. We studied mice at 2, 6, 9 and 12 months of age. By allowing mice to age, we revealed an unexpected increase in body mass due to lipid accumulation in
male KO mice at 9 months of age compared to WT controls. This effect was sex-dependent, as no differences were noted for female mice at any age.

2. Quantitative micro-CT whole-body composition analysis confirmed greater total body mass in KO mice at 9 months of age, and revealed significantly greater adipose tissue mass in these mice compared to WT controls.

3. We observed significantly greater epididymal fat pad mass was in KO than in WT male mice at 9 months of age, with no significant difference observed at 12 months of age.

4. Histological assessments of epididymal fat pads in 9- and 12-month-old male mice revealed similar adipocyte morphology, adipocyte size and adipocyte density in WT and KO mice. Thus, the greater fat pad mass was attributed to adipocyte hyperplasia, rather than hypertrophy.

5. Lipid staining revealed significantly greater lipid accumulation in the following male KO tissues compared to WT controls: lipid droplets in kidney at 9 and 12 months of age, adipocytes in the pancreas at 12 months of age, and both lipid droplets and adipocytes in the extraorbital lacrimal glands at 12 months of age.

6. Lipid accumulations were not observed in the following tissues in KO compared to WT: the submandibular salivary glands, heart, spleen, liver, Harderian glands and skeletal muscle. However, at 12 months of age the spleen had significantly greater mass in male KO mice compared to WT controls. H&E of spleen revealed greater basophilic staining coupled with megakaryocyte hyperplasia.
7. No significant differences in average daily food consumption were seen between male WT and KO mice at any age, suggesting that the phenotype may be due to metabolic differences, which we assessed with blood plasma analysis. Male KO mice had significantly reduced plasma cholesterol levels at both 9 and 12 months of age. No significant differences in plasma triglyceride or glucose levels were seen at either age for WT and KO mice.

8. We report abnormal adipocyte and lipid accumulation in mice lacking functional P2X7 receptors, revealing a novel role for P2X7 in regulating lipid storage, deposition and metabolism in vivo.

5.1.3 Low-Magnitude, High-Frequency Vibration has No Effect on Murine Whole-Body Composition and Bone Microarchitecture: Objective, Summary and Conclusions

Chapter 4 – Specific Objective – To investigate the effects of whole-body vibration on body composition and bone microarchitecture, and the possible role of P2X7 in these processes.

Chapter 4 – Summary and Conclusions

1. We assessed the systemic effects of WBV in vivo using micro-CT to quantify whole-body composition of WT and KO mice, which were separated into either non-vibrated or vibrated groups throughout the experimental period.

2. No effects of vibration or genotype were found in body mass, BMD, BMC, skeletal tissue mass, adipose or lean tissue masses at any time point. In contrast to the findings of some other investigators, our data reveal that under
the conditions studied, WBV does not induce changes in murine whole-body composition.

3. No significant effects of vibration or genotype were observed on food intake. Thus, diet did not appear to have any confounding effect on our assessment of whole-body composition.

4. High-resolution (20 µm) ex vivo micro-CT scans of hind-limbs were performed at the end of the experimental period. Visual assessment of representative images did not reveal any marked differences in shape or architecture. No significant effects of vibration or genotype were observed on long bone lengths.

5. WBV does not induce changes in cortical properties of the mid-diaphyseal femur, the mid-diaphyseal or distal diaphyseal tibiae. However, there was a small but significant effect of genotype in Ct.Ar of mid-diaphyseal cortical femur, as well as Ct.Ar and Ct.Th distal-diaphyseal cortical tibiae.

6. WBV does not induce changes in trabecular properties of the distal femur and proximal tibia.

7. Overall, we did not observe an effect of vibration using our study parameters; therefore, we are unable to draw conclusions about the role of P2X7 in mediating the biological effects of WBV.

8. Together with the results of other recent studies, our findings highlight the need to re-evaluate current thinking on the effects of low-magnitude, high-frequency WBV on whole-body composition in vivo and bone microarchitecture.
5.2 Limitations of the Research and Future Research Directions

Genetically modified mouse models — All studies in this thesis relied on mouse models, and as such inherent issues arise. The wild-type mice from the study in Chapter 2 were used in the whole-body composition assessments to validate the methodology and provide normative values for other researchers. The mice used for studies in this thesis established a role for P2X7 in lipid distribution and metabolism; whereas, we were unable to establish whether or not P2X7 might play a role in mediating the effects of whole body vibration reported by others. The P2X7 KO mouse was obtained from Pfizer (Solle et al., 2001). Although initial studies showed an absence of P2X7 protein in these mice, recent studies have shown a splice variant (with C-terminal truncation) is detectable in some tissues of the Pfizer KO mouse (Masin et al., 2012). This group demonstrated that this splice variant, which is present in the salivary glands and spleen, is inefficiently trafficked to the cell surface, resulting in greatly diminished receptor function (Masin et al., 2012). Thus, while not a complete KO, there is global loss of P2X7 function in the Pfizer KO mouse model.

Our mice were maintained on a mixed genetic background (129/Ola × C57BL/6 × DBA/2) by crossbreeding of heterozygous mice. The maintenance of the mixed background, rather than backcrossing onto a pure C57BL/6 strain, enabled comparisons between our data and the results of other studies that characterized the KO mouse (Ke et al., 2003; Labasi et al., 2002; Li, 2005; Panupinthu et al., 2008; Panupinthu et al., 2007; Solle et al., 2001). It should also be noted that a naturally occurring polymorphism (P451L) with some loss-of-function occurs in the C-terminus of P2X7, and is found in several commonly used mouse strains, including C57BL/6 and DBA/2 (Adriouch et al.,
2002). Given that the mixed genetic background our mice contains both of these strains, it is possible that the differences observed between WT and KO mice could be underestimated. Future studies should consider backcrossing the KO mouse onto a pure C57BL/6 strain and re-characterizing the skeletal phenotype, which may differ from the current KO mouse phenotype due to reduced genetic variability. Additionally, it may be of interest to cross this mouse onto a different background with higher BMD values, such as BALB/c or C3H strains (Judex et al., 2002), to determine if P2X7 loss-of-function produces greater severity of osteopenia in these animals. On the other hand, these strains with higher BMD values have been shown to be less responsive to the osteogenic effects of mechanical loading, when compared to strains with intrinsically lower BMD such as C57BL/6 (Judex et al., 2002). Thus, crossing onto a strain with higher BMD would perhaps make these animals more suitable for studies not involving mechanotransduction. Moreover, the generation of tissue-specific P2X7 knockouts would help clarify the specific role of P2X7 in, for example, adipocytes and osteoblasts.

*Micro-CT-derived data —* In this thesis, whole-body composition data are an integral part of each study. As such, it was imperative that we confirmed the validity and reliability of our technique, as reported in Chapter 2. Although we conducted a rigorous study to validate our protocol, some limitations exist. Though micro-CT is the gold standard technique for measuring radio-opaque tissues, repeated acquisition of *in vivo* data from living mice is a recently developed method. Studies that have used this technology have not performed repeated measurements *in vivo* in a longitudinal manner. As such, we had little literature to compare with as most studies use pQCT and DXA for
live or post-mortem quantification of whole-body composition. Moreover, whole-body composition data acquired from micro-CT scans as described in Chapter 2 is a novel technique. Thus, the numerical values attained using pQCT, DXA and micro-CT imaging modalities are not comparable. As *in vivo* micro-CT continues to gain popularity, future studies should consider using our threshold-based whole-body composition methodology for rapid image analysis in longitudinal studies of mice.

It also should be noted that micro-CT resolutions for *in vivo* and *ex vivo* scanning differ markedly, and as such the data attained differ as well. *In vivo* micro-CT is appropriate for whole-body composition analyses, but when assessing microarchitectural properties of bone, the resolution is simply not sufficient due to the small size of mouse bones. We chose to use an *ex vivo* high-resolution scanning protocol at 20 µm voxel spacing to assess femoral and tibial microarchitecture, as trabeculae are approximately 30-50 µm in width (Bouxsein et al., 2010). Therefore, assessments of mouse bone microarchitecture must be performed at a scan resolution of \( \leq 20 \) µm as post-mortem specimen scans. We used 2.75-hour, 20 µm resolution scans. Although higher resolutions are possible with our micro-CT scanners (~6 µm), the cost is greater and, as spatial resolution is increased, scan time and image noise increase significantly. Though 20 µm voxel spacing appeared to be sufficient for our purposes, the possibility remains that additional information would have been obtained with higher-resolution scanning. Future work should attempt to optimize higher-resolution scanning protocols.
Whole-body vibration — Little is known about the WBV parameters required to elicit changes in whole-body composition. Some previous studies have shown that low-magnitude, high-frequency vibration is anabolic to bone using a protocol of 45 Hz, 0.3 g acceleration for 3-6 weeks (Xie et al., 2006; Xie et al., 2008), while studies using 90 Hz, 0.2 g acceleration had extended experimental periods of up to 15 weeks observed reduced adiposity (Rubin et al., 2007). We chose our WBV parameters based on studies showing the anabolic effect on bone (45 Hz, 0.3 g), and extended the time course of the experimental period (up to 18 weeks) to assess effects on adiposity. As we did not observe these effects in our mouse model, the possibility arises that differing WBV protocols have distinct consequences. Thus, it is plausible that altering the treatment regimen (time-course or vibration frequency and/or acceleration) could produce tissue-specific effects. This possibility should be explored in future work. Moreover, the effects of acute vibration on gene expression levels should be assessed using real-time PCR. Only recently have studies begun to incorporate this approach, and these studies will be critical for defining tissue-specific protocols for in vivo vibration.

Quantification in Chapter 4 was heavily reliant on micro-CT data. Future work should include histomorphometric assessments in calcified and decalcified long bones from this study, to determine the effects of vibration at the cellular level. This includes assessment of bone lining cells, osteoclasts, quantification of marrow adiposity, and evaluation of the knee joint to determine if WBV affects these tissues. Our preliminary data from collaborative studies indicate that WBV may have a detrimental effect on articular cartilage of the knee, inducing osteoarthritis-like changes in some mice within 2 weeks of vibration treatment (unpublished). Thus, histological assessment of these tissues
may add valuable insights into tissue-specific effects of WBV.

The use of other mouse models with skeletal defects that include compromised bone quality, as well as mouse models of metabolic dysfunction and excess adipose deposition, should be considered for future work. These studies would provide insight as to whether mechanical stimulation has any therapeutic effects in a range of skeletal and metabolic disease models. Additionally, unknown signalling pathways that play a role in transducing WBV may be identified.

It remains a possibility that the mixed genetic background of our animal model could have influenced the response to vibration treatment, as different mouse strains (with dissimilar bone mineral densities) respond differentially to vibration treatment (Judex et al., 2002). Moreover, humans have a great deal of genetic variability, which affects bone mineral density and may also result in differential responses to vibration treatment. Therefore, studying WBV in genetically-modified mice may be useful for determining signalling pathways involved in mediating skeletal mechanotransduction, but may not be directly applicable to clinical WBV due to the inherent genetic variability in humans.

We studied a chronic model of WBV, anticipating greater bone accrual and reduced fat tissue with a prolonged exposure to vibration. However, the longevity of this treatment may be limitation. While some studies have shown anabolic responses in bone with prolonged exposure to WBV (Xie et al., 2008), others have noted that bone can become desensitized to repeated exposures of mechanical loading over time (Robling, 2006; Willie et al., 2013). Similar observations have been made in humans during exercise activities such as walking or running, where remodelling slows as the bone
adapts to repeated mechanical stimulation of similar intensities. Interestingly, it is believed that this adaptation occurs during a defined refractory period, after which bone is capable of anabolic responses to mechanical stimuli once more. The presence of such a refractory period suggests that multiple bouts of vibration separated by a time interval may be necessary to induce effects on whole-body composition (Robling, 2006; Sen et al., 2011).

Another future goal would be to identify potential signalling pathways mediating mechanotransduction _in vivo_. To do so, the use of _in vitro_ approaches will be critical in determining the underlying signalling mechanisms. Only recently have studies begun to reveal that mechanical stimulation can influence mesenchymal stem cell (MSC) proliferation and differentiation (Luu et al., 2009). Furthermore, vibration has been shown to maintain the osteogenic potential of bone marrow stromal cells (Ozcivici et al., 2010), while discouraging commitment to the adipocyte lineage (Sen et al., 2011). At the same time, others have shown that osteoblast commitment is reduced and matrix mineralization inhibited with vibration _in vitro_ (Lau et al., 2011). Given the controversial nature of these findings, future studies should further investigate these effects.

Additionally, the specific signalling pathways that regulate the commitment of mesenchymal stem cells to either the osteoblast or adipocyte lineage in response to vibration should be explored.

We have specifically designed, built and tested two vibration platforms to explore the effects of vibration _in vitro_. The first of these platforms is designed to image cell cultures using microscopy during treatment in individual cell culture dishes (Holdsworth et al., 2012), while the other is a modification of the _in vivo_ whole-body vibration...
platform with custom 6-well cell culture plate inserts. These systems may allow us in future studies to rigorously determine the key players in mechanotransduction at the cellular level, and unveil the signalling pathways mediating the effects of vibration.
5.3 Contributions of the Research to the Current State of Knowledge

Novel imaging approaches — Medical imaging is a large and diverse field that incorporates several different imaging modalities, providing insights into disease by revealing internal structures in a non-invasive manner. The imaging modality used in each this thesis was micro-CT, the gold standard for quantification of radio-opaque tissues (Donnelly, 2011; MacNeil and Boyd, 2007). New micro-CT machines have the capacity for short scan times and low radiation dosages, which has opened up the possibility of repeated in vivo scanning of mice in longitudinal studies (Glatt et al., 2007; Granton et al., 2010; Judex et al., 2003).

Prior to this thesis, there had been no reports of repeated in vivo scanning of mice over a long time course. Our study reported in Chapter 2 is the first characterization of changes in whole-body composition during growth and aging of mice using micro-CT and whole skeleton data. This method offers the ability to quantify adipose and lean tissue components of the mouse in addition to measuring the skeletal tissues. The methodology described in Chapter 2 facilitates analysis of large data sets. Moreover, our study was the first to scan mice as young as 2 weeks of age in vivo; other studies have only used post-mortem scans for young age groups (Guldberg et al., 2004; Oest et al., 2008). Furthermore, our study provided many novel methods, including a precise method to measure the length of long bones using anatomical markers. We also described a method for determining maximum bone mineral density, which reflects mineralization better than mean BMD. Our data showed marked differences in whole-body composition values during the period of rapid growth between 2 and 5 weeks of age, highlighting the importance of collecting data at precise ages when comparing groups within this range.
Moreover, our findings provide critical reference data for other investigators using mice as disease models. Therefore, Chapter 2 makes significant contributions to the field of micro-CT imaging of mouse models, as others can use this methodology for characterizing their mouse models in an efficient, non-invasive, and high-throughput manner.

**Implications for metabolic disease** — The P2X7 receptor has been linked to a role in directing osteoblast and adipocyte commitment from mesenchymal progenitors, as cultured cells from KO mice show greater adipogenic markers accompanied by reduced osteogenic markers (Panupinutha et al., 2008). Additionally, P2X7 activation has been shown to enhance cellular energy metabolism, suggesting a role for this receptor in regulating energy homeostasis (Amoroso et al., 2012; Grol et al., 2012). P2X7 receptors are present in mouse adipocytes (Sun et al., 2012) and human adipocytes (Madec et al., 2011), but little is known about their role in these tissues. In Chapter 3, we reported that loss of P2X7 function leads to increased adiposity and lipid accumulation in older male mice. P2X7 KO mice were found to have increased body and epididymal fat pad weights, coupled with reduced total plasma cholesterol levels compared to WT mice at 9 months of age. Additionally, we showed that male mice lacking functional P2X7 receptors develop ectopic lipid accumulations as they age. Our study revealed previously unrecognized roles for the P2X7 receptor in adipogenesis, lipid storage and metabolism *in vivo*. This provides the impetus to further elucidate the roles of P2X7 in the control of energy and lipid metabolism. Moreover, these findings highlight the need for research to probe the role of P2X7 in regulating the differentiation of adipocytes and in bone-fat
crosstalk.

In terms of clinical implications, patients with metabolic syndrome show enhanced expression of P2X7 in adipocytes (Madec et al., 2011). Obese patients have elevated P2X7 expression levels, while patients with type II diabetes mellitus show reduced levels of P2X7 expression (Glas et al., 2009). Taken together with our results, this suggests a link between P2X7 and metabolic syndrome. Moreover, increased marrow fat in humans is associated with aging as well as chronic diseases such as diabetes mellitus and osteoporosis (Gourine et al., 2009). Thus, our study in mice provides further insights into the role of P2X7 in adipose tissues and lipid metabolism, which may be clinically relevant in humans with chronic metabolic diseases.

**P2 receptors and mechanotransduction in bone** — Our lab has previously proposed that mechanotransduction in bone is mediated by nucleotide release and subsequent activation of P2 receptors in bone cells (Dixon and Sims, 2000). The P2X7 receptor has demonstrated roles in bone remodelling (Grol et al., 2009; Grol et al., 2013; Panupinuth et al., 2008), cellular metabolism and energy homeostasis (Amoroso et al., 2012; Grol et al., 2012), and adiposity (Chapter 3). Moreover, the P2rx7 KO mouse exhibits an osteopenic phenotype (Ke et al., 2003) and a reduced osteogenic response to axial compressive loading (Li, 2005), implicating P2X7 in mechanotransduction. In Chapter 4, we assessed the effects of WBV on wild-type and P2rx7 KO mice, and anticipated observing changes whole-body composition in response to vibration (at least in wild-type mice). However, our data revealed no significant effects of WBV at the whole-body and microarchitectural level using our vibration protocol.
Future studies could investigate the effects of different WBV protocols, varying frequency, acceleration, duration and/or intervals. WBV remains a promising avenue for further research as a potential approach for non-invasive, non-pharmacologic treatment of diseases such as osteoporosis.

*Anabolic treatment for osteoporosis* — A long-term goal is to identify signalling pathways underlying mechanotransduction in skeletal tissues. As musculoskeletal diseases are extremely prevalent, and the burden becomes of greater concern with an aging population, the need for clinical interventions is urgent (Woolf and Pfleger, 2003). Currently, there are no non-pharmacological interventions that are effective in countering the destruction of bone in osteoporotic patients. The limited therapeutic interventions available for treatment of osteoporosis are only marginally successful and primarily anti-resorptive in their properties (Akesson, 2003). Presently, one anabolic (bone building) pharmacological treatment is available (teriparatide, a parathyroid hormone peptide). However, due to the extremely high cost of this medication, this treatment is currently limited to patients with severe osteoporosis (Maeda and Lazaretti-Castro, 2014). Thus, it is not difficult to comprehend the allure of WBV, a non-drug-based (and thus has no drug interactions, a common problem among elderly patients taking several medications), non-invasive, relatively affordable and simple-to-use therapy for a common, debilitating disease. Therefore, we suggest that future studies should continue to investigate the role of WBV, and to determine the therapeutic conditions required to mitigate bone loss in osteoporosis.
5.4 References


APPENDIX A

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APPENDIX B

Ethics Approval of Animal Use
AUP Number: 2008-043-06
AUP Title: Role of cytosolic calcium in the regulation of osteoclasts and bone resorption// Ion Transport and Signalling in Skeletal Cells: P2 Nucleotide Receptor Function in Bone

Yearly Renewal Date: 09/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-043-06 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
CURRICULUM VITAE
Kim L. Beaucage

PERSONAL DATA
Date of Birth: April 8\textsuperscript{th}, 1985
Citizenship: Canadian

EDUCATION
Spring 2010 – Summer 2018
Dental Clinician-Scientist (DCS) Program, Candidate
Doctor of Dental Surgery, Class of 2018
Schulich School of Medicine and Dentistry
University of Western Ontario, London, ON
Supervisor: Dr. S. Jeffrey Dixon

Fall 2008 – Summer 2014
Doctor of Philosophy in Physiology, specialization in Musculoskeletal Health Research
Department of Physiology and Pharmacology
University of Western Ontario, London, ON
Supervisor: Dr. S. Jeffrey Dixon
Co-supervisor: Dr. David W. Holdsworth
Thesis Title: Roles of P2X7 Receptors in Adipose and Skeletal Tissues of Mice

Fall 2004 – Spring 2008
Bachelor of Sciences
Honours Science, Biology Minor
University of Waterloo, Waterloo, ON
(Conferred in June 2008)

PUBLICATIONS


**TEACHING EXPERIENCE**

Fall 2011 – Spring 2013  
Teaching Assistant  
Department of Physiology and Pharmacology 4980e: Physiology/Pharmacology/Psychology Honours Thesis  
University of Western Ontario, London, ON

Fall 2010 – Spring 2011  
Tutorial Teaching Assistant  
Department of Physiology and Pharmacology 1021: Human Physiology  
University of Western Ontario, London, ON

Fall 2009 – Spring 2010  
Laboratory Teaching Assistant  
Department of Physiology and Pharmacology 3130y: Physiology Laboratory  
University of Western Ontario, London, ON

Fall 2008 – Spring 2009  
Laboratory Teaching Assistant  
Department of Biology 1222/12223: General Biology/Introductory Biology  
University of Western Ontario, London, ON
ACADEMIC SERVICE ACTIVITIES
(SELECTED)

Fall 2010 –
Summer 2013
Graduate Studies Committee Student Representative
The Department of Physiology and Pharmacology
University of Western Ontario, London, ON

Fall 2009 –
Summer 2013
Physiology and Pharmacology Graduate Student Council Member
The Department of Physiology and Pharmacology
University of Western Ontario, London, ON

Winter 2010 –
Summer 2012
Program Operations Committee Trainee Representative and Social Committee Member
Joint Motion Program (JuMP), a CIHR training program in musculoskeletal health research
University of Western Ontario, London, ON

Winter 2010 –
Summer 2012
Research Committee Physiology Student Representative
The Department of Physiology and Pharmacology
University of Western Ontario, London, ON

Winter 2009 –
Summer 2012
Let’s Talk Science Physiology and Pharmacology Department Student Representative
The Department of Physiology and Pharmacology
University of Western Ontario, London, ON

May 2012
Trainee Workshop Organizer: “People and Project Management” and “Non-Academic Job Search and Interviewing”
Joint Motion Program (JuMP) Annual Retreat
University of Western Ontario, London, ON

May 2010
Organizer and Workshop Leader: “Scientific Collaboration”
Canadian Arthritis Network Training Day Committee
Canadian Arthritis Network/The Arthritis Society Conference, Gâtineau, QC
AWARDS AND SCHOLARSHIPS
(SELECTED)

Fall 2010 –
Joint Motion Program (JuMP) Doctoral Trainee Scholarship (Value: $13,500 each year for 3 years; joint funding) – Accepted
University of Western Ontario, London, ON

Fall 2010 –
Canadian Arthritis Network (CAN) Doctoral Trainee Scholarship (Value: $10,500 each year for 2 years; joint funding) – Accepted
CAN/The Arthritis Society, Toronto, ON

Fall 2009 –
Joint Motion Program (JuMP) Master’s Trainee Scholarship (Value: $13,500 each year for 1 year; joint funding) – Accepted
University of Western Ontario, London, ON

Fall 2009 –
Canadian Arthritis Network (CAN) Master’s Trainee Scholarship (Value: $9,000 each year for 1 year; joint funding) – Accepted
CAN/The Arthritis Society, Toronto, ON

Fall 2008 –
Schulich Graduate Scholarship (Value: ~$8,000/year, covers cost of tuition each year for 5 years) – Accepted
Schulich School of Medicine & Dentistry
University of Western Ontario, London, ON

Fall 2008
Schulich Graduate Entrance Scholarship (Value: $5,000) – Accepted
Schulich School of Medicine & Dentistry
University of Western Ontario, London, ON

June 2008
University of Waterloo Dean’s Honours List for the Faculty of Science
Description: Meritorious award for achieving greater than an 80% average in each term for the Honours Science program
Awarded: 5 consecutive terms
University of Waterloo, Waterloo, ON
CONFERENCE ABSTRACTS
(SELECTED FIRST AUTHOR SUBMISSIONS)

*Title*: “Effects of whole-body vibration on bone and body composition in mice” 

Canadian Connective Tissue Conference, Toronto, ON, Canada. June 2012. 
*Title*: “Effects of whole-body vibration on bone and body composition in mice” 

*Title*: “Absence of P2X7 nucleotide receptors leads to abnormal fat distribution in mice” 

Canadian Connective Tissue Conference, Montreal, QC, Canada. May 2011. 
*Title*: “Absence of P2X7 nucleotide receptors leads to abnormal fat distribution in mice” 

Canadian Arthritis Network Annual Scientific Meeting, Gatineau, QC, Canada. November 2010. 
*Title*: “The role of P2X7 nucleotide receptors in mediating mechanically-induced osteogenesis” 

*Title*: “The role of P2X7 nucleotide receptors in mediating mechanically-induced osteogenesis” 