Development of Functional Interactions Among Cortical and Trabecular Traits During Growth of the Lumbar Vertebral Body

Melissa Ramcharan
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DEVELOPMENT OF FUNCTIONAL INTERACTIONS AMONG CORTICAL AND TRABECULAR TRAITS DURING GROWTH OF THE LUMBAR VERTEBRAL BODY

by

MELISSA A. RAMCHARAN

A dissertation submitted to the Graduate Faculty in Biomedical Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2015
This manuscript has been read and accepted for the Graduate Faculty in Biomedical Engineering in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

DEVELOPMENT OF FUNCTIONAL INTERACTIONS AMONG CORTICAL AND TRABECULAR TRAITS DURING GROWTH OF THE LUMBAR VERTEBRAL BODY

by

Melissa A. Ramcharan

Advisor: Dr. Karl J. Jepsen

Variation in bone traits that contribute to increased fracture risk in the elderly is mainly established in adulthood. Previous studies have shown that in adults, cortical and trabecular traits are functionally related. How variations in traits develop to establish mechanical function in adult bone is not well understood. In this study, we examined temporal changes in the development of cortical and trabecular traits during growth in mouse lumbar vertebral body structures that have a wide range of genetic variants. We determined a sequence of events among traits that would suggest how functional bone structures developed. Examining bones in A/J, C57/BL6 and C3H/HeJ inbred mouse strains during postnatal growth, we identified inter-strain variation in trabecular architectural traits as seen in adult strains were established by 1 week of age while inter-strain variation in cortical area largely occurred after 4 weeks of age. Across a panel of 20 AXB/BXA Recombinant Inbred mouse strains, we observed a similar sequence in trait development from 4 weeks of age to 16 weeks of age. In addition, the alignment of trabeculae was shown to be a primary variant relative to bone size at an early age. Vertebral bodies that tended to show a large increase in trabecular alignment from 4 weeks of age to 16 weeks of age tended to show a small increase in cortical area over time. However, load borne on the trabecular bone region from 4 weeks of age
despite trabecular alignment was important for mechanical stiffness and strength throughout growth. The interaction of anisotropy and bone size in conjunction with the interaction between load sharing and trabecular bone volume at an early age suggested predictive patterns in how traits changed over time relative to bone size. Together these results have great clinical significance because they provide a novel way of assessing mechanical function of the skeletal system by means of coordination of traits and benefit development of predictive models of fracture risk in humans. Understanding the interaction of corticocancellous traits during growth has important implications for genetic analyses and for interpreting the response of bone to genetic and environmental perturbations.
Dedicated in loving memory of

Phulchan Ramcharan
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# TABLE OF CONTENTS

Chapter 1: Introduction 1

Chapter 2: Variation in the development of cortical and trabecular traits during growth of the mouse lumbar vertebral body
- Introduction 27
- Materials and Methods 28
- Results 35
- Discussion 50
- Acknowledgements 59
- References 60

Chapter 3: Genetic randomization reveals novel interactions among cortical and trabecular traits during growth of the lumbar vertebral body
- Introduction 64
- Materials and Methods 66
- Results 71
- Discussion 89
- Acknowledgements 97
- References 98
- Appendix A 101

Chapter 4: The contribution of load sharing between cortical and trabecular bone to the development of a mechanically functional vertebral body
- Introduction 106
- Materials and Methods 108
- Results 118
- Discussion 140
- Acknowledgements 148
- References 149
- Appendix B 152

Chapter 5: General Conclusions and Future Directions 168
- General Conclusions 169
- Future Directions 175
- References 180

Bibliography 182
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 2.1</strong></td>
<td>Body weight and morphological traits for the lumbar vertebral body of three inbred mouse strains between postnatal 1 day and 105 days of age.</td>
<td>42</td>
</tr>
<tr>
<td><strong>Table 2.2</strong></td>
<td>Width, depth and length measures for lumbar vertebral body of three inbred mouse strains between postnatal 1 day and 105 days of age.</td>
<td>43</td>
</tr>
<tr>
<td><strong>Table 3.1</strong></td>
<td>Variation in vertebral body size, and morphology among RI mouse strains at 4 weeks of age.</td>
<td>72</td>
</tr>
<tr>
<td><strong>Table 3.2</strong></td>
<td>Multiple linear regression analysis of traits that contribute to the change in cortical traits.</td>
<td>86</td>
</tr>
<tr>
<td><strong>Table 3.3</strong></td>
<td>Multiple linear regression analysis of traits that contribute to the whole bone mechanical properties of the mouse lumbar vertebral body.</td>
<td>89</td>
</tr>
<tr>
<td><strong>Table 4.1</strong></td>
<td>Trabecular and cortical bone load fraction, mass fraction for a panel of 20 AXB/BXA RI mouse strains at 4 weeks of age along the length of the vertebral body.</td>
<td>123</td>
</tr>
<tr>
<td><strong>Table 4.2</strong></td>
<td>Trabecular and cortical bone load fraction, mass fraction along the length of the vertebral body for each of 20 AXB/BXA RI mouse strains at 4 weeks of age.</td>
<td>124</td>
</tr>
<tr>
<td><strong>Table 4.3</strong></td>
<td>Multiple Linear Regression analysis of traits that contribute to the change in cortical and trabecular traits.</td>
<td>133</td>
</tr>
<tr>
<td><strong>Table 4.4</strong></td>
<td>Multiple Linear Regression analysis of traits that contribute to whole bone mechanical properties of the lumbar vertebral body.</td>
<td>134</td>
</tr>
<tr>
<td><strong>Table 4.5</strong></td>
<td>Sorting of bone traits and change in traits from 4 weeks of age to 16 weeks of age based on RI mouse strains that have a low or high degree of anisotropy (DA) relative to bone size.</td>
<td>136</td>
</tr>
</tbody>
</table>
CHAPTER 1

Figure 1.1. The hierarchical structure of bone at various size scales for each tissue component. 4

Figure 1.2. Diagram of the shaft of long bone showing cortical bone, trabecular bone, and various channels. 5

Figure 1.3. Schematic of skeletal development in a long bone. 9

Figure 1.4. Image of a mid-sagittal section of the lumbar vertebral body indicating the cortical and trabecular bone regions. 10

CHAPTER 2

Figure 2.1. Representative 3D images indicating growth in width, depth and length of the lumbar vertebral body. 31

Figure 2.2. 2D cross-sectional images indicating analysis of cortical and trabecular traits in the mouse vertebral lumbar. 32

Figure 2.3. Representative images indicating endochondral ossification and periosteal apposition bone development processes in the lumbar vertebral body. 35

Figure 2.4. Mid-transverse and mid-coronal midsections of the lumbar vertebral body for AJ, B6, and C3H inbred mouse strains. 37

Figure 2.5. Total area, width, depth, and length values of the vertebral body in AJ, B6, and C3H mice. 41

Figure 2.6. Images of in vivo bone labeling of the L4 vertebral body and the mineral apposition rate for three inbred mouse strains. 46

Figure 2.7. Percentage of trabecular bone area and cortical area values in lumbar vertebral body in three inbred mouse strains. 47

Figure 2.8. Anisotropy values for AJ, B6, and C3H mice during postnatal growth from 1day of age to 105days of age. 49
CHAPTER 3

Figure 3.1. Average values of total cross-sectional area for AJ, B6, and 20 RI AXB/BXA RI mouse strains. 71

Figure 3.2. Average body weight and total area at 16 weeks of age relative to 4 weeks of age across a panel of RI mice. 74

Figure 3.3. Cortical and trabecular traits at 16 weeks of age relative to 4 weeks of age across a panel of RI mice. 76

Figure 3.4. Variation in total area, structural stiffness and maximum load to failure relative to body weight in RI mice at 16 weeks of age. 78

Figure 3.5. Partial regression analysis of whole bone mechanical properties relative to bone size at 16 weeks of age taking body weight at 16 weeks of age into consideration. 78

Figure 3.6. Variation in cortical and trabecular traits relative to body weight in RI mice at 4 weeks of age. 80

Figure 3.7. Variation in cortical and trabecular traits relative to body weight in RI mice at 16 weeks of age. 81

Figure 3.8. Partial regression analysis of cortical and trabecular traits relative to bone size at 4 weeks of age taking body weight at 4 weeks of age into consideration. 82

Figure 3.9. Partial regression analysis of cortical and trabecular traits relative to bone size at 16 weeks of age taking body weight at 16 weeks of age into consideration. 83

Figure 3.10. Partial regression analysis of the change in cortical and trabecular traits from 4 weeks of age to 16 weeks of age relative to bone size at 4 weeks of age taking body weight at 4 weeks of age into consideration. 85

Figure 3.11. Partial regression analysis of the change in cortical traits relative to the change in trabecular traits taking the change in body weight from 4 weeks to 16 weeks of age into consideration. 85

Figure 3.12. Partial regression analysis of stiffness and maximum load to failure relative to cortical and trabecular traits at 16 weeks of age taking body weight at 16 weeks of age into consideration. 88
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.1.</td>
<td>Schematics of finite element 3D mesh and position of endplate platens on a mouse lumbar vertebral body.</td>
<td>111</td>
</tr>
<tr>
<td>Figure 4.2.</td>
<td>Images indicating contact surfaces along vertebral body endplates used to apply boundary and loading conditions on a finite element model.</td>
<td>112</td>
</tr>
<tr>
<td>Figure 4.3.</td>
<td>Calibration of stress values from finite element analysis.</td>
<td>114</td>
</tr>
<tr>
<td>Figure 4.4.</td>
<td>Image indicating regions of interest along the length of the vertebral body for load fraction analysis.</td>
<td>114</td>
</tr>
<tr>
<td>Figure 4.5.</td>
<td>Images of transverse sections along the length of the lumbar vertebral body with a color gradient of stress distributions from finite element analysis.</td>
<td>119</td>
</tr>
<tr>
<td>Figure 4.6.</td>
<td>Variation of trabecular load fraction relative to position along the length of the vertebral body.</td>
<td>121</td>
</tr>
<tr>
<td>Figure 4.7.</td>
<td>Variation in load fraction relative to mass fraction along the length of the vertebral body in the trabecular and cortical region.</td>
<td>122</td>
</tr>
<tr>
<td>Figure 4.8.</td>
<td>Variation in trabecular load fraction at 4 weeks of age relative to body weight and total area at 4 weeks of age.</td>
<td>126</td>
</tr>
<tr>
<td>Figure 4.9.</td>
<td>Variation in trabecular load fraction at 4 weeks of age relative to body weight and total area at 16 weeks of age.</td>
<td>126</td>
</tr>
<tr>
<td>Figure 4.10.</td>
<td>Variation in trabecular load fraction at 4 weeks of age relative to cortical and trabecular traits at 4 weeks of age.</td>
<td>128</td>
</tr>
<tr>
<td>Figure 4.11.</td>
<td>Variation in trabecular and cortical traits at 16 weeks of age relative to trabecular load fraction at 4 weeks of age.</td>
<td>128</td>
</tr>
<tr>
<td>Figure 4.12.</td>
<td>Partial regression analysis of trabecular load fraction at 4 weeks of age relative to trabecular and cortical traits at 4 weeks of age.</td>
<td>130</td>
</tr>
<tr>
<td>Figure 4.13.</td>
<td>Partial regression analysis of the change in traits between 4 and 16 weeks of age relative to trabecular load fraction at 4 weeks of age.</td>
<td>131</td>
</tr>
<tr>
<td>Figure 4.14.</td>
<td>Variation in adult stiffness and maximum load to failure relative to trabecular load fraction at 4 weeks of age.</td>
<td>134</td>
</tr>
<tr>
<td>Figure 4.15.</td>
<td>Representative images of the development of mouse lumbar vertebral body structures from 4 to 16 weeks of age relative to variation in bone size and anisotropy at 4 weeks of age.</td>
<td>139</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

APPENDIX A – Additional linear regression analyses

Appendix A- Figure 3.1. Variation in cortical and trabecular traits relative to total area in RI mice at 4 weeks of age. 102

Appendix A- Figure 3.2. Variation in cortical and trabecular traits relative to total area in RI mice at 16 weeks of age. 102

Appendix A- Figure 3.3. Partial regression analysis of cortical and trabecular traits relative to BVTV at 4 weeks of age taking body weight at 4 weeks of age into consideration. 103

Appendix A- Figure 3.4. Partial regression analysis of cortical and trabecular traits relative to BVTV at 16 weeks of age taking body weight at 16 weeks of age into consideration. 103

Appendix A- Figure 3.5. Trabecular and cortical TMD at 16 weeks of age relative to TMD values at 4 weeks of age for each RI mouse strain. 104

APPENDIX B - Parametric studies to determine the reliability of the finite element analysis system

Appendix B- Figure 4.1. Validation of the number of samples used for finite element analysis. 154

Appendix B- Figure 4.2. Trabecular load fraction and mass fraction for samples with either a low or high BVTV value subjected to various threshold values. 157

Appendix B- Figure 4.3. Schematic drawings of various loading scenarios applied to FE models in a parametric study. 159

Appendix B- Figure 4.4. Derivation of a line equation used to calculate a load gradient that was applied to an FE model. 161

Appendix B- Figure 4.5. Trabecular load fraction values for bone samples with different external size subjected to various loading scenarios. 164

Appendix B- Figure 4.6. Mid-transverse sections of the lumbar vertebral body showing stress distributions, range of stress, and strain values under various applied force magnitudes. 166
Chapter 1

INTRODUCTION
The primary function of the skeletal system is to provide a structural framework for the body, protect vital organs, and enable body movement when bone is attached to other skeletal tissues such as tendons, ligaments, joints and skeletal muscle. These critical mechanical functions depend on bones being sufficiently stiff and strong to resist deformation and failure under physiological load bearing and movement. Bones must also be capable of enduring forces of about 2-3 times body weight during physical activity (Davy et al., 1988).

Osteoporosis is a major problem that compromises the mechanical function of bones and lead to skeletal fragility and a high incidence of bone fracture. This condition also known as “porous bone” is associated with a loss in bone mass and a decrease in bone strength that leads to fractures commonly in cortico-cancellous structures such as the hip, wrist and spine. In the United States, vertebral compression fractures due to osteoporosis occur in nearly 700,000 patients each year (American Academy of Orthopaedic Surgeons). Vertebral fracture cases occur nearly twice as many times as those in hips and wrists. It is predicted that by 2025, osteoporosis will be responsible for approximately 3 million fractures in patients over the age of 50 and $25.3 billion in cost each year (National Osteoporosis Foundation). Women are at higher risk of fracture mainly because they tend to have smaller and thinner bones than men. In addition, an increase in bone loss occurs in women reaching menopause due to a sharp decrease in estrogen, a hormone that protects bones. Efforts to improve diagnosis and early identification of patients at risk of fracturing will benefit treatments that can reduce vertebral fractures later in life.
Currently, areal bone mineral density (aBMD) measured by dual-energy X-ray absorptiometry (DEXA) is the most widely used index of bone strength. A low aBMD value is associated with high vertebral fracture risk (American Academy of Orthopaedic Surgeons). However, nearly 50% of patients that have a high aBMD according to diagnostic thresholds from the World Health Organization have experienced vertebral fractures (McDonnell et al. 2007; Schuit et al. 2004). The singular trait measure does not take into account the multiple structural and compositional skeletal features or traits within bone that contributes to bone strength and how these traits may vary among individuals. It is important to consider a range of variation in skeletal traits for a more specific diagnosis of those at higher risk of fracturing.

**Bone composition and structural organization**

The mechanical properties of bone depend on material properties, mass and microarchitecture of bone tissue (Jepsen 2009). Bone tissue is a composite of both organic and inorganic components that together provide a hard and rigid tissue that is also flexible and resilient under load (Figure 1.1). The organic extracellular matrix consists of mainly highly organized Type I collagen fibers which accounts for approximately 25% to 30% dry weight of bone. Inorganic mineral in the form of hydroxyapatite crystals are arranged along each collagen fiber so that they are known as mineralized collagen fibers. The mineral accounts for 65%-70% of the dry weight of bone and gives bone its solid consistency. In addition, 5% of the extracellular matrix consists of a gelatinous ground substance made up of non-collagenous glycoproteins and proteoglycans that lay between the layers of mineralized collagen fibers to bind them together. Water is also an important component which accounts for 25% of the
total weight of bone (Martin et al. 1998; Frankel et al. 2001). Water can be found in the organic matrix, around the collagen fibers and ground substance, surrounding the bone crystals as well as in canals and cellular regions and, aids in carrying nutrients to the bone tissue (Frankel et al. 2001).

Figure 1.1: The hierarchical structure of bone at various size scales for each tissue component (Rogel et al. 2008)

A whole bone structure is composed of two main types of bone tissue, cortical and trabecular bone which are made up of the same matrix elements, but are different in both microstructure and function. Cortical bone or compact bone forms the outer shell or cortex and has a dense structure. Cortical bone is organized in fundamental structural units known as osteons or the Haversian system (Figure 1.2). At the center of each osteon a small channel called a Haversian canal contains blood vessels and nerve fibers. The osteon consists of a series of layers or lamellae of mineralized matrix
organized in concentric rings surrounding the central canal. The intertwining pattern of mineralized collagen fibers in the lamellae contributes to the bones’ resistance to mechanical stress (Bilezikian et al. 2008). Along the boundaries of each lamella are small cavities or lacunae that contain a single osteocyte. Numerous canaliculi radiate from each lacunae connecting the lacunae across lamellae and reaching the Haversian canal to allow transport of nutrients from the blood vessels to osteocytes throughout the osteon. Each osteon is approximately 200 microns in diameter and is surrounded by ground substance that mainly consists of glycoaminoglycans which acts as a cement line binding adjacent osteons and interstitial lamellae that occupy the region between osteons (Martin et al. 1998). Volkmann’s canals transversely connect Haversian canals to the outer surfaces of the bone and also contain blood vessels and nerves.

Figure 1.2: Diagram of the shaft of long bone showing cortical bone, trabecular bone, and various channels (Prezbindowski 1983).
Trabecular or cancellous bone is located within the shell and is composed of a mesh-like structure of plates and struts known as trabeculae. Space between the cortical shell and trabeculae is filled with a highly vascular marrow tissue. Trabecular bone also has an arrangement of concentric layers of lamellae that contain lacunae that house osteocytes. However, trabeculae do not contain Haversian canals. Instead, osteocytes receive nutrients through canaliculi from blood vessels in the surrounding marrow tissue.

The primary structural difference between cortical and trabecular bone is porosity. Cortical bone has a porosity of approximately 5% to 10% of the volume mainly from the openings of Haversian and Volksmann canals. In the trabecular space, the porosity is 75% to 95% based on the total internal volume of the bone. The majority of the non-calcified volume is occupied by bone marrow, blood vessels, and connective tissue. Typically, the dense cortical bone is stronger than trabecular bone. In humans, the compressive ultimate stress of cortical bone is approximately 100 times greater than that of trabecular bone (Martin et al. 1998, p137, 166). However, the greater porosity in trabecular bone allows this bone type to withstand greater deformation compared to cortical bone (Carter and Hayes, 1976). The proportions of cortical and trabecular bone will vary depending on location and function of the bone. In long bone, trabecular bone is found within the ends (metaphysis and epiphysis) making up approximately 8% of the total bone mass (Jee 2001) and acts as energy absorbing regions when load is applied along joints. The shaft (diaphysis) is made up of only cortical bone to provide greater stiffness and rigidity under different modes of loading (compression, tension, bending, and torsion).
The human skeleton consists of two subdivisions - axial and appendicular bones that vary in anatomy and function. Axial bones along the central axis of the human body include flat bones of the skull, ossicles of the inner ear, and irregular bones of the spinal column and ribs. These bones are primarily important for central weight bearing, balance, and protection of inner organs and maintenance of posture. Appendicular bones are arranged symmetrically on either side of the body, which include long bones such as the arm and leg, short bones such as those in the wrist and ankles, as well as the shoulder and pelvic girdles that connect the upper arms and femur, respectfully to the axial skeleton. The functions of appendicular bones also include balance as well as stability, locomotion, and digital manipulation which are important for feeding and reproduction (http://www.differencebetween.com/difference-between-axial-and-vs-appendicular/).

Growth and development of bone

The development of a whole bone structure begins during the embryonic growth stage as mesenchymal condensations. These condensations form bone through either intramembranous or endochondral ossification processes. Intramembranous ossification is involved in the formation of flat bones of the skull. Here, an ossification center occurs within the embryonic tissue where mesenchymal cells cluster together, differentiate into osteoblasts, and secrete bone matrix. Some of the osteoblasts become trapped in the bone matrix and become osteocytes. Blood vessels grow into the area to supply the cells and matrix with oxygen and nutrients. These blood vessels are then surrounded by
the developing bone. The bone assumes a foundation of spongy bone remodeling creates compact bone and the finalized form of the bone.

Endochondral growth is the primary process of bone ossification in the human skeleton including limbs, hips and the spinal column. As illustrated in the growth of a long bone (Figure 1.3), cartilage cells differentiate into osteoblasts (bone forming cells) and secrete a bony collar around the diaphysis of the hyaline cartilage model. An ossification center (physis) is formed within a cartilage template or anlagen. There is also vascular penetration from the perichondral sleeve surrounding the anlagen which provides a source of blood for bone cell precursors. An orderly arrangement of chondrocytes adjacent to the physis allows the ossification center to expand in radial directions toward the periphery of the bone. During early postnatal development, the ossification center enlarges creating parallel growth plates along the superior and inferior ends for longitudinal growth. At these growth plate ends, rapidly maturing hypertrophic chondrocytes synthesize calcified matrix. Through cell signaling from bone lining cells, osteocytes (bone-forming cells), and marrow cells, the calcified matrix is resorbed by osteoclasts. Osteoblasts then form ossified tissue or trabeculae along the primary spongiosa. Shortly before or right after birth, a secondary ossification center appears in the epiphyses. The epiphysis undergoes ossification and bone is remodeled - marrow cavity expands, trabeculae extend into the secondary spongiosa (metaphysis), and compact bone is formed, until the bone is in its final form. Some hyaline cartilage remains to form the articular cartilage and the epiphyseal cartilage separating the epiphysis and the diaphysis.
In this study, we are focused on understanding how functional bone structures develop through the coordination between different features of cortical bone and trabecular architecture during growth in the lumbar vertebral body. The vertebral body is a cortico-cancellous structure that consists of trabecular bone throughout the centrum and surrounded by a cortical shell (Figure 1.4). Therefore, this structure does not have a diaphysis consisting of only cortical bone as seen in long bones. The primary ossification of the vertebral body was suggested to be is similar the epiphyseal ossification seen in long bones (Bogduk et al. 2005).

Figure 1.3: Schematic of the endochondral ossification process during skeletal development in long bone (https://thesebonesofmine.wordpress.com/category/taphonomy/).
In the spinal column, the vertebral body of each vertebra primarily bears compressive loads along with some anterior-posterior bending loads. Previous studies that used finite element analysis to simulate a functional adult lumbar vertebral body showed that load shared between the cortex and trabecular region varies along the length of the vertebral body, and cortical load fraction is inversely proportional to the modulus of the trabecular region (Cao et al. 2001). In humans, the average thickness of the cortical shell in the vertebral body is approximately 0.38mm, cortical mass fraction can range from 21% to 39% and the maximum load fraction can range from 38-54% along the length of the vertebral body (Eswaran et al. 2006). Cortical load fraction and proportional mass fraction was shown to be largest at the mid-transverse plane and lowest at the superior and inferior growth plate ends (Cao et al. 2001; Eswaran et al. 2006; Silva et al.1997). In addition, trabecular load fraction was shown to be inversely proportional to cortical load fraction (Webster et al. 2012). Differences in load fraction
and mass fraction along the vertebral body were consistent with the concept of Wolff’s Law of bone adaptation that indicated mechanical loading influenced the form and function of bone (Frost, 1994). Therefore, the geometry of both the cortical shell and trabecular bone throughout a bone structure is important for whole bone mechanical function.

**Functional adaptation**

Functional adaptation is an important developmental process that ensures bone is sufficiently stiff and strong with changes in applied load. It is well known that the bone architecture adapts to mechanical loads (Frost 1994; Cowin 1997, Odgaard et al. 1997). In adult bone, functional adaptation was observed as an increase in bone mass in the dominant arm of tennis players (Kannus et al. 1995), or bone loss in response to immobilization (Maeda et al. 1993; Rittweger et al. 2009; Sievanen 2010), or anti-gravity during space travel (Doty 2004). A strain-based feedback system enables that structures attain a form that sufficiently provides stiffness and strength to resist fracture (Rubin et al. 1985; Frost 1987; Turner et al. 1998). Previous studies have shown that applied load induced tissue-level strains that affected the development of the trabecular architecture, peak bone mass, bone shape, and matrix architecture (Olson et al. 1958; Cheverud 1996, Moro et al. 1996; Sumner et al. 1996; van der Meulen et al. 1996; Ruff et al. 2006). In addition, bone cells influenced by mechanical stimuli were suggested to regulate local formation and functional adaptation of trabecular architecture (Roux 1881; Mullender et al. 1995). However, very little is known about how functional adaptation occurs during growth and among the cortical and trabecular regions of bone. A previous study showed that during growth of the femoral mid-shaft in mice, a
coordination of bone surface expansion, marrow expansion and matrix mineralization of the cortex was important to maintain function over time with an increase in weight-bearing load (Jepsen et al. 2009). In more complex cortico-cancellous structures such as the lumbar vertebral body, it is not known how both cortical and trabecular traits coordinate during growth to achieve whole bone function.

Sets of traits contribute to bone function

Within a whole bone structure, the combination of multiple morphological and tissue quality traits from both cortical and trabecular bone contribute to bone function. Bone traits can vary among individuals based on their genetic background. Morphological traits include measures of bone size and distribution of bone tissue. In long bones, bone size can range from slender (narrow cross-sectional area relative to length) to robust (wide cross-sectional area relative to length). In the lumbar vertebral body, bone size is measured by the total cross sectional area which can range from narrow to wide in diameter. A bone with a narrow diameter tends to have a low stiffness compared to a wider bone. A small increase in bone diameter translates to an exponential increase in bone stiffness and strength (Jepsen 2009). Tissue-level mechanical properties are based on micro-structural and compositional traits. Micro-structure refers to traits such as porosity, lamellar thickness, trabecular volume fraction and trabecular number and thickness. Compositional traits include water, mineral, collagen and proteoglycan content. Assessing mechanical properties of compositional traits involves invasive techniques. Bone morphology has been shown to be a predictor of tissue fragility and fracture risk using non-invasive imaging techniques (Tommasini et al. 2005).
Previous studies have shown that variation in bone traits including bone size that contributed to increased fracture risk in the elderly was mainly established by adulthood (Duan et al. 2003). In addition, variation in the vertebral body and femoral bone size at approximately 16 years of age in males and females was established during early puberty at approximately 12 years of age (Loro et al., 2000). Narrow bones are commonly associated with fracture risk because loss of bone mass with age may reduce the cortical thickness leading to greater loss of stiffness and strength (Tommasini et al. 2005; Tommasini et al. 2008; Tommasini, et al. 2007; Jepsen et al. 2007; Giladi et al. 1987). Other studies have shown that adults acquired specific sets of cortical and trabecular traits relative to bone size to create mechanically functional structures. In human proximal femurs (Zebaze et al. 2007) and mouse vertebral bodies (Tommasini et al. 2005; Tommasini et al. 2009), wide bones have a thin cortical shell and low trabecular volume whereas narrow bones have a thick cortical shell and high trabecular volume. Therefore, not all narrow bones are at risk of fracture. Healthy mouse and human skeletons showed narrow bones with a set of traits that are different from wide bones but designed to be functional under physiological loads (Zebaze et al. 2007; Tommasini et al. 2009). Altered proportions of cortical and trabecular traits relative to bone size due to genetics, aging or disease may compromise function and lead to bone fragility and fracture (Bell et al., 1999; Duan et al., 2003; Jepsen et al., 2009; Szulc et al. 2006). For example, patients with either wide or narrow femoral necks developed fractures when the cortical shell thickness was reduced (Duan et al., 2003). However, it remains unclear how sets of traits interact for function and may also contribute to fracture susceptibility later in life.
To better understand functional adaptation of a complex, and highly adaptive system such as bone, it is important to understand the relationship among physical bone traits during growth. The goal of this dissertation is to understand how cortical and trabecular morphological traits interact during growth to establish mechanical function as shown in adulthood. A better understanding of how traits interact to develop functional structures will provide a basis to detect alterations in development that may suggest the onset of fracture risk. Our working hypothesis of how functional bone structures are established is based on the concept that there is a coordination of sets of traits relative to variation in external bone size when load is applied to the bone system (Waddington 1942). We are focused on understanding the coordination of cortical and trabecular traits and whether the coordination is the same across bone structures with natural variation in bone size. To further simplify our understanding of the complex adaptive nature of bone, we adopted a working model which postulates that early variation in one trait leads to subsequent adaptive changes in other traits (Jepsen 2009). Few studies have addressed the interaction of cortical and trabecular bone using this working model. In the lumbar vertebral body of adult mice, multivariate analysis was used to show that interactions among cortical and trabecular traits were important for whole bone mechanical function (Tommasini et al. 2009). A computer simulation of bone adaptation during growth along the metaphysis of long bones showed that under applied load, trabeculae near the periphery of the growth plate tended to become compact and form cortical bone (Tanck et al., 2006). In addition, parametric studies on a simulated model of an adult lumbar vertebral body showed that the magnitude of load borne by the cortical shell was sensitive to the changes in the degree of trabecular
anisotropy (Silva et al. 1997). Studies on the development of cortical and trabecular bone examined age-related changes of individual cortical and trabecular traits in mouse lumbar vertebral bodies from a young adult at 4-6 weeks of age (Glatt et al. 2007; Buie et al. 2008) to aged mice at 20 months of age (Glatt et al., 2007). From 8 weeks of age, there appeared to be a pattern where a large decrease in bone volume fraction and trabecular thickness throughout aging coincided with a small increase in total cross-sectional area and cortical thickness (Glatt et al., 2007). In addition, isolated trabecular samples from porcine lumbar vertebral bodies examined from 6 weeks of age to 230 weeks of age showed that a decrease in bone volume fraction and an increase in trabecular alignment was a mechanical adaptation of the trabecular region to produce efficient architecture (Tanck et al. 2001). Therefore, how cortical and trabecular traits interact during growth to establish whole bone mechanical function is not fully understood. For this thesis, our global hypothesis of how traits functionally interact is that variation in trabecular traits lead to subsequent adaptive changes in cortical traits.

To test this hypothesis, inbred mouse models were used to study relationships among cortical and trabecular traits in the lumbar vertebral body. Mice are powerful tools that have been used to characterize genes and biological processes in many complex diseases including heart disease, cancer and diabetes (Nadeau et al. 2003; Nadeau et al. 1998). Here, inbred mouse strains were used to better understand the genetic basis of establishing skeletal function. Advantages for using mice are that each mouse strain is homozygous within a strain, and has a different set of phenotypic characteristics from other strains (Rosen et al. 2001). There are hundreds of strains available which can be used to examine genetic and phenotypic variability in different
biological systems (The Jackson Laboratory, Bar Harbor, ME). In addition, mice have short generation times of approximately 10 weeks until sexual maturity, short breeding durations of twenty-one days to produce a litter of about 4-7 pups, and mice live to about 2-3 years of age. These attributes are useful for studying complex trait development during growth (Price et al. 2005), puberty, and aging (Glatt et al. 2007; Buie et al. 2008) in both cross-sectional and longitudinal studies. Mouse bone is small and does not have an extensive osteon microstructure like human bone, however mineralization as a function of variation in bone size is similar between mice and humans in adults (Zebaze et al. 2007; Tommasini et al. 2009). Biological concepts involving functional adaptation of bone traits sets from the mouse femur (Jepsen et al., 2007) were translational to the human skeleton (Tommasini et al. 2009; Tommasini et al. 2007; Jepsen et al. 2007). Therefore, the network of trait interactions required for mechanical function is suggested to be similar between mice and humans in long bone. Further examining other skeletal sites such as the mouse lumbar vertebral body will improve our understanding of biological processes among trait interactions that occur in a site of high fracture risk in humans. The mouse vertebral body has proportionally fewer trabeculae compared to the human vertebral body. However, age-related changes in mouse trabecular bone and associated changes in the cortex are consistent with those shown in the human skeleton (Glatt et al., 2007; Khosla et al., 2006). In addition, recent studies showed that the mouse spine is mainly loaded by axial compression, similar to humans (Smit, 2002) even though mice ambulate as a quadruped.
Recombinant inbred mouse strains as a model to study the genetic basis of skeletal function

Recombinant Inbred (RI) mouse strains have been used to determine the tendency of various traits to correlate to better understand function in different biological systems including heart (Llamas et al. 2007), musculature (Lionikas et al., 2010), and bone (Bailey 1986; Jepsen et al. 2010; Tommasini et al. 2009; Sanger et al. 2011) to name a few. A panel of RI mouse strains is an animal model where two parental inbred strains of known divergent genetic or phenotypic characteristics are used to generate multiple new mouse strains that each have a genome consisting of a unique randomization of parental alleles. To create an RI panel of mice, parental strains are crossed, and first generation progeny are intercrossed to produce large numbers of second generation animals. Random mating of brother and sister are selected as founders for each RI strain. Interbreeding within each RI strain occurs for approximately 20 additional generations to create a genome within each RI strain that is 100% homozygous (Rosen et al. 2001; Silver 1995). In this study, A/J (A) crossed with the C57/BL6 (B) mouse strain was used because they are known to show large variability in bone size, bone area, failure load as well as other heritable traits that contribute to both strength and fragility in the femur (Price et al., 2005) and vertebral body (Tommasini et al. 2005). As a result, the panel of AXB/BXA RI mouse strains represented a wide range of bone phenotypes that are natural perturbations of parent strains with non-pathological variation in sets of bone traits. These attributes of each RI strain leads us to assume that biological controls that regulate trait co-variation was important to build each mechanically functional bone structure. Previous work from our lab used this panel
of RI mice to determine functional interactions among cortical traits at the mid-diaphysis of mouse femurs in adult RI mice (Jepsen et al., 2007) and during growth (Jepsen et al., 2009), as well as to predict functional interactions among bone traits in the adult lumbar vertebral body (Tommasini et al. 2009). Further utilizing this panel of RI mice to determine the tendency of cortical and trabecular traits to correlate during growth will provide insight into biological processes to develop functional bone structures across a diverse population that is similar to the variation in trait sets seen among humans (Zebaze et al. 2007; Marshall et al. 1992; Nesbitt et al. 1984).

Summary

To understand how cortical and trabecular traits functionally interact in the mouse lumbar vertebral body, we determined the sequence of events of when traits develop during growth. When significant trait interactions arose provided insight into which traits were considered variant and which traits may show an adaptive response. First (Chapter 2), we examined temporal changes in cortical and trabecular morphology during postnatal growth for A/J, C57/BL6 and C3H/H3J inbred mouse strains to identify when patterns of inter-strain variation of traits arose that was similar to the variation seen in adulthood (Tommasini et al. 2005). The timing of inter-strain variation in traits suggested phenotypic development that was important for function in each strain with a different genetic background. Second (Chapter 3), based on the temporal patterns of traits shown across three inbred mouse strains, we further analyzed relationships among cortical and trabecular traits during growth across a panel of AXB/BXA RI mouse strains. Determining the coordination of traits across a population with a wide range of genetic variants provided insight into a global biological process of how functional
structures develop. In addition, determining how cortical and trabecular morphological traits developed relative to variation in bone size provided a better understanding of functional interactions among traits for individuals. Lastly (Chapter 4), we investigated how the interactions among cortical and trabecular morphological traits during growth across the panel AXB/BXA RI mouse strains was explained by load sharing between the different bone regions from an early age. This finding provided a better understanding of the coordination of traits over time under the influence of applied load, therefore, extending the concept of Wolff’s Law and leading to suggestive functional adaption processes for whole bone vertebral bodies with different bone sizes.

This study will benefit efforts to develop predictive models of adult trait variation and provide a basis to further understand bone adaptation processes leading to skeletal fragility. In addition, this body of work emphasized the importance of analyzing variation of sets of traits over time which has important implications for genetic analyses and interpreting changes during bone development due to genetic or environmental perturbations.
References


among morphologic and tissue-quality traits that contribute to bone strength and fragility. *Mammalian Genome*, 18(6-7), 492–507.


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Chapter 2

VARIATION IN THE DEVELOPMENT OF CORTICAL AND TRABECULAR TRAITS
DURING GROWTH OF THE MOUSE LUMBAR VERTEBRAL BODY
Introduction

Variation in bone traits in the elderly is largely established by adulthood (Duan et al. 2001). In clinically relevant structures like the vertebral body, previous studies have shown that cortical and trabecular traits are functionally related in young adults (Zebaze et al. 2007; Tommasini et al. 2009). Individuals achieve mechanical function by acquiring a specific set of cortical and trabecular traits that are predictable based on the natural variation in bone size (Zebaze et al. 2007). The variation in bone size ranges from narrow to wide. To achieve mechanical function, slender bones have traits such as a small diameter, thick cortical shell and high trabecular volume. In contrast, wide bones have a large diameter, thin cortical shell and lower trabecular volume. Particular combinations of traits allow for flexibility in the way mechanical function can be achieved across a population. The interactions among cortical and trabecular traits throughout the vertebral body are important for load bearing. Tanck et al suggested that trabeculae in the peripheral metaphysis of porcine long bones merged to form the cortex during growth (Tanck et al. 2006). Tommasini et al suggested a mechanism in the adult mouse vertebral body where genotypic variation in cortical bone traits leads to an adaptive response of trabecular bone (Tommasini et al. 2009). Therefore, it is unclear how functional interactions among these traits arise during growth.

To better understand the complex adaptive nature of bone, we adopted a working model which postulates that variation in one trait leads to adaptive changes in other traits during growth (Tommasini et al. 2009; Jepsen 2011). Herein, we examined the temporal sequence of postnatal development in three mouse strains - A/J (AJ), C57BL/6J (B6) and C3H/HeJ (C3H) to provide insight into underlying biological
processes in establishing functional bone structures. Each strain has a distinct adult phenotype. AJ mice exhibit a narrow bone with low cortical area and high trabecular volume. B6 mice have a wide bone with greater trabecular volume compared to AJ, and C3H has an intermediate vertebral body size with a thick cortex and low trabecular bone mass (Tommasini et al. 2005; Turner et al. 2001). The goal of this study was to determine when inter-strain variation arises in cortical and trabecular traits during postnatal growth in AJ, B6 and C3H mice. Growth patterns among bone traits during growth are expected to reveal how cortical and trabecular traits co-develop for each strain and whether this pattern is similar across strains. Understanding the ontogenic origins of these functional interactions will benefit efforts to identify developmental anomalies that affect the proportion of cortical and trabecular tissue for a particular bone size and lead to fracture risk later in life.

**Materials and Methods**

**Animals**

Female (n=6/strain) and male (n=3/strain) A/J (AJ), C57BL/6J (B6) and C3H/HeJ (C3H) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at 6-8 weeks of age and used to establish breeding colonies under standard environmental conditions. The Institutional Animal Care and Use Committee approved the handling and treatment of mice. Mice were fed a standard rodent chow (Purina Rodent Chow 5001) and water ad libitum, subjected to a 12-hour light/dark cycle, and housed at a maximum of 5 mice per cage in the same room. This mouse model was previously used by our lab to determine how genetic variability in adult bone traits correlated to
differences in mechanical properties (Tommasini et al. 2005). To examine the temporal changes in bone trait development in the lumbar vertebral body, L3 to L4 vertebrae were harvested from female pups at seven ages: 1, 4, 7, 10, and 14 days of age (n=10/age/strain) and 28, and 105 days (n=6/age/strain). These ages were similar to previous work examining variation in the development of femoral morphology during growth (Price et al. 2005). Mice 1 day to 10 days of age were decapitated at sacrifice. Those older than 10 days of age with better developed respiratory systems were euthanized using carbon dioxide asphyxiation. Lumbar vertebrae were placed in 10% neutral buffered formalin fixative then prepared for histological examination of bone morphology.

Histomorphometry

**Bone Morphology** – Histological preparation in plastic resin allowed for accurate assessment of morphological traits particularly in bone structures at early postnatal ages with low mineral content. Vertebrae were fixed with 10% neutral buffered formalin for 72 hours at room temperature, washed with deionized water, then dehydrated with ethyl glycol methylether for 48 hours and acetonitrile for 24 hours. Samples were cleared with methyl salicylate for 24 hours and infiltrated with a series of methylmethacrylate solutions. After the methylmethacrylate polymerized at 37-degrees Celsius for 48 hours, a low-speed diamond-coated wafering saw (Buehler, Lake Bluff, IL, USA) was used to cut the L3 and L4 vertebral bodies along separate orthogonal planes. L3 vertebrae were cut along the mid-transverse plane and L4 vertebrae were cut along the mid-coronal plane. 300 micron thick sections from either plane were
affixed to glass slides and polished to a reflective surface finish. The autofluorescence of bone and surrounding tissue was viewed at 100x magnification using a fluorescence microscope (Zeiss Axioplan, Zeiss, Thornwood, NY, USA) configured with Apitome image enhancement. Images were captured with a CCD camera and Axiovision Imaging Software (version 4.8).

*Bone Morphology and Micro-architectural Measurements*

ImageJ Software (version 1.47i) was used to analyze morphological traits in transverse and coronal orthogonal planes. In the transverse plane, within the cross-sectional total area (Tt.Ar) of the bone we measured the vertebral body width in the lateral direction and depth along the anterior-posterior direction (Figure 2.1). In the coronal plane, vertebral length was measured in the cranial-caudal direction.
Figure 2.1: Representative 3D images indicating directional expansion of the lumbar vertebral body during whole bone growth. Expansion in width and depth was measured along the transverse direction (left), and length was measured along the coronal direction (right).

At the mid-transverse plane, cortical traits were also measured including, cortical area (Ct.Ar) where Ct.Ar = total area - marrow area (Ma.Ar) (Figure 2.2A), and relative cortical area (RCA) where, RCA = cortical area/ total area. In the mid-coronal plane, trabecular bone traits were examined by removing the auto-fluorescence of marrow tissue using Adobe Photoshop Elements 4.0 software. The images were then smoothed with a median filter algorithm, thresholded, and binarized to quantify the fraction of bone tissue from surrounding surfaces (ImageJ Software). Trabecular measures included the percentage of trabecular bone area (%Tb.Ar) and trabecular thickness (Tb.Th) as seen in Figure2.2B, as well as the trabecular degree of anisotropy (DA) in the direction of...
axial compression (cranial-caudal) within the secondary spongiosa. The %Tb.Ar measured in 2D sections was equivalent to the percentage of trabecular bone volume/total bone volume (BV/TV) derived from 3D volumes (Parfitt et al. 1983). Traits were measured for 3 sections per bone which were then averaged for each sample.

Figure 2.2. 2D cross-sectional images indicating analysis of cortical and trabecular traits in the mouse vertebral lumbar. In the (A) mid-transverse plane, the outermost outline indicated total area (Tt.Ar). Cortical area (Ct.Ar) was calculated by total area minus marrow area (Ma.Ar). In the (B) mid-coronal plane, the outline indicates the area used to determine the percentage of trabecular bone area (%Tb.Ar). The average width of individual trabecular throughout the vertebral body was used to measure the average trabecular thickness (Tb.Th).

Trabecular anisotropy was estimated with the mean intercept length (MIL) method (Odgaard 1997; Harrigan et al. 1984) from a stack of ten consecutive 2D images from the mid-coronal region of each bone sample. A region of interest between the cranial and caudal growth plates was selected. BoneJ software (Doube et al. 2010) was used to compute a MIL fabric tensor by fitting an ellipsoid to a resulting plot of points based on MIL data. DA was calculated as 1- [length of the shortest axis]/ [length of the longest axis] of the ellipsoid. The value of DA ranged from 0 (isotropic) to 1 (anisotropic). The region of interest to measure anisotropy in the mid-coronal plane was consistent for the
three mouse strains at each age. Means and standard deviations were calculated for each strain at each age.

**In vivo Bone Labeling**

Additional female (n=5/strain) AJ, B6 and C3H mice were purchased from Jackson Laboratory at 3-3.5 weeks of age. To determine the mineral apposition rate (MAR) of bone growth in the cortical shell and the direction of cortical drift patterns, mice were injected with three different fluorescent bone labels. After a three day acclimation period, mice received a series of intraperitoneal injections of fluorescent bone labels (Sigma-Aldrich, St. Louis, MO, USA) over the course of 1 week. Day 1: 90mg/kg Xylenol Orange, Day 3: 10mg/kg Calcein Green and, Day 5: 20mg/kg Alizarin Red. Mice were sacrificed on Day 7 (4.5weeks old) by carbon dioxide asphyxiation. Body weights were recorded prior to initial injection and at the time of sacrifice to ensure injections had negligible effects on normal systemic behavior. L4 vertebrae were prepared for plastic embedding as described earlier. 200μm thick sections were cut in the transverse direction along the mid-transverse plane and approximately 20% above the growth plate at the caudal end where the vertebral body extends beyond the transverse processes. Sections were affixed to glass slides and, polished to a reflective surface. Bone labels within polished sections were then viewed using FIT-C and Texas Red filters using a fluorescence microscope configured with Apitome image enhancement (Zeiss Axioplan, Zeiss, Thornwood, NY, USA). An overlay of the fluorescent image and brightfield image was used to identify the proximity of bone labels relative to the size and shape of the cortical or trabecular bone.
To quantify the cortical mineral apposition rate in sections along the mid-transverse plane and the caudal end, the distance between consecutive bone labels (orange to green, green to red) were measured along the perimeter of the bone using ImageJ software. Labeling with no distance between the lines was classified as a point of mineralization with no apposition. MAR was calculated by dividing the distance between two bone label lines by the inter-labeling periods in days. Within a strain, MAR values were compared between the mid-transverse plane and caudal end sections. Values were also compared among strains at similar locations.

Statistical Analysis

All results were expressed as mean ± standard deviation. Differences in morphological trait and mineral apposition rate values among the three mouse strains at each age were determined using one-way ANOVA with Tukey’s posthoc tests and Student t-tests. Differences were considered statistically significant at p <0.05 (GraphPad Software 5.0, San Diego, CA).
Results

*Qualitative morphological changes of the lumbar vertebral body during growth*

Examining postnatal growth in the mouse lumbar vertebral body starting at 1 day of age, we quantified morphological changes in cortical and trabecular traits into adulthood at 105 days of age. In addition, we observed bone developmental processes ([Figure 2.3](#fig2.3)). Representative images in transverse plane show vertebral body expansion in the lateral direction (width) through endochondral ossification toward the pedicles, and in the anterior-posterior direction (depth) through periosteal apposition ([Figure 2.3A](#fig2.3A)). In coronal sections, expansion in the cranial-caudal direction (length) occurred through endochondral ossification ([Figure 2.3B](#fig2.3B)).

![Figure 2.3](#fig2.3)

*Figure 2.3.* Representative images indicating growth in (A) cross-sectional total area along the mid-transverse plane was attributed to expansion in width from lateral endochondral ossification, and expansion in depth in the anterior to posterior direction from periosteal appositional growth. (B) Along the mid-coronal plane showed growth in length from cranial-caudal endochondral ossification.
Overall morphological development of the vertebral body during growth was not qualitatively different among AJ (Figure 2.4A), B6 (Figure 2.4B) and C3H (Figure 2.4C) inbred mouse strains. At one day of age, the primary ossification center exhibited a cuboidal appearance and lacked a distinct cortex; however, mesh-like trabecular architecture was clearly visible. Between 4 days of age and 7 days of age, there seemed to be less trabecular bone and there was a thinner peripheral growth region (as a result of endochondral ossification). At 7 days of age, the trabecular region appeared to be organized along the cranial-caudal direction. By 14 days of age, a well-defined cortical shell was evident and the trabecular region showed an increase in anisotropy in the direction of axial compression. Structurally, the 14 day old lumbar vertebral body was consistent with the adult structure. In particular, cranial-caudal growth plates continued to expand the bone structure in length while lateral ends were joined to transverse processes. After 14 days of age, lateral endochondral ossification was not seen.
Figure 2.4. Auto-fluorescent microscopy images of the lumbar vertebral body in female (A) AJ, (B) B6 and (C) C3H mouse strains. Transverse and coronal midsections from each strain were examined for morphological changes in cortical and trabecular traits at 1, 4, 7, 14, 28 and 105 days during postnatal development. Scale bar: 1mm.
Variation in total area was mainly established through endochondral ossification

Variation in total cross-sectional area was observed among the inbred mouse strains as early as 1 day of age (Figure 2.5A). Each strain showed progressive increases in total cross-sectional area (Tt.Ar) and length with growth (Table 2.1). Over time, Tt.Ar for AJ mice was significantly smaller than B6 or C3H mice. B6 had a larger total area that C3H from 10 days of age to 28 days of age. By adulthood at 105 days of age, C3H had a larger Tt.Ar value compared to AJ or B6 mouse strains (Figure 2.5A). Inter-strain variation in length was observed at 1 day of age (Figure 2.5D). In addition, AJ mice tended to have a smaller length compared to B6 and C3H mice over time. However, there was not a consistent difference in length values among all three strains during most of growth. To understand how variation in a simple trait such as Tt.Ar was established, we analyzed the pattern of inter-strain variation in lateral (width) and anterior-posterior (depth) growth at particular ages. Throughout growth, AJ mice consistently had a smaller measure in width compared to B6 or C3H mice except at 28 days when there was no difference among all strains (Figure 2.5B). There was not a consistent difference in depth among the three strains at any particular age (Figure 2.5C). However, by 1 day of age the depth of the vertebral body was 56%, 43% and 47% of adult values in AJ, B6 and C3H mice, respectfully. Over 83% of adult values in depth were seen by 28 days of age for the three strains. The trend in inter-strain variation in width among the three mouse strains was similar to the variation in total area. The coinciding pattern in width and total area suggested that variation in cross sectional bone size was mainly attributed to expansion in width through a lateral endochondral ossification process.
Figure 2.5: (A) Total area, (B) width, (C) depth, and (D) length of the vertebral body in AJ (△), B6 (●) and C3H (□) mice during postnatal growth from 1 day of age to 105 days of age. Overall significant differences among strains for each measure at a given age from an ANOVA are indicated by * (p<0.05). The “#” symbol represented significant inter-strain variation (Tukey’s posthoc tests) as seen in adulthood at 105 days of age. Differences among individual strains analyzed from posthoc tests are shown in Table 2.1 and Table 2.2.
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<td>0.063 ± 0.005a</td>
<td>0.115 ± 0.018a</td>
<td>21.2 ± 2.1abc</td>
<td>0.024 ± 0.002c</td>
<td>0.72 ± 0.09c</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>4.9 ± 0.9</td>
<td>0.83 ± 0.05abc</td>
<td>0.103 ± 0.008abc</td>
<td>0.124 ± 0.013a</td>
<td>24.2 ± 2.7abc</td>
<td>0.025 ± 0.002ac</td>
<td>0.81 ± 0.03c</td>
</tr>
<tr>
<td></td>
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<td>10</td>
<td>7.2 ± 0.7</td>
<td>0.90 ± 0.05abc</td>
<td>0.123 ± 0.010a</td>
<td>0.136 ± 0.011a</td>
<td>29.0 ± 2.3abc</td>
<td>0.034 ± 0.002ab</td>
<td>0.78 ± 0.03b</td>
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<td>0.231 ± 0.016abc</td>
<td>0.181 ± 0.012c</td>
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<td>0.307 ± 0.011abc</td>
<td>0.211 ± 0.007c</td>
<td>33.9 ± 1.7abc</td>
<td>0.043 ± 0.002c</td>
<td>0.80 ± 0.04c</td>
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<tr>
<td>C3H</td>
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<td>1.5 ± 0.2</td>
<td>0.28 ± 0.02ab</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>20.7 ± 2.1b</td>
<td>0.013 ± 0.001ab</td>
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<tr>
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<td>4</td>
<td>10</td>
<td>2.6 ± 0.9</td>
<td>0.43 ± 0.04ab</td>
<td>0.026 ± 0.001</td>
<td>0.061 ± 0.006a</td>
<td>22.3 ± 1.8ab</td>
<td>0.023 ± 0.002ab</td>
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<td>10</td>
<td>3.4 ± 0.7</td>
<td>0.51 ± 0.04ab</td>
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<td>0.123 ± 0.011a</td>
<td>21.2 ± 1.2ab</td>
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<td>5.4 ± 0.8</td>
<td>0.61 ± 0.03ab</td>
<td>0.082 ± 0.005ab</td>
<td>0.135 ± 0.009</td>
<td>16.5 ± 1.5ab</td>
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<td>14</td>
<td>10</td>
<td>6.9 ± 0.6</td>
<td>0.79 ± 0.04ab</td>
<td>0.116 ± 0.008ab</td>
<td>0.147 ± 0.012</td>
<td>17.7 ± 1.8ab</td>
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<td>0.86 ± 0.05b</td>
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<td>11.9 ± 2.8</td>
<td>0.98 ± 0.02ab</td>
<td>0.188 ± 0.007ab</td>
<td>0.192 ± 0.008</td>
<td>20.8 ± 1.0ab</td>
<td>0.038 ± 0.001ab</td>
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<td>1.51 ± 0.06ab</td>
<td>0.385 ± 0.037ab</td>
<td>0.255 ± 0.025ab</td>
<td>23.6 ± 0.9ab</td>
<td>0.048 ± 0.002ab</td>
<td>0.92 ± 0.04ab</td>
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</table>

Comparing traits among strains in the same age groups using one-way ANOVA:

a significantly different from AJ, Tukey’s posthoc test p<0.05
b significantly different from B6, Tukey’s posthoc test p<0.05
c significantly different from C3H, Tukey’s posthoc test p<0.05

**Table 2.1:** Body weight and morphological traits for the lumbar vertebral body of three inbred mouse strains between postnatal 1 day and 105 days of age.
### Table 2.2: Width, depth and length measures for the lumbar vertebral body of three inbred mouse strains between postnatal 1 day and 105 days of age.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (days)</th>
<th>N</th>
<th>Width (mm)</th>
<th>Depth (mm)</th>
<th>Length (mm)</th>
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</thead>
<tbody>
<tr>
<td>AJ</td>
<td>1</td>
<td>10</td>
<td>0.41 ± 0.03&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.51 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34 ± 0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>0.58 ± 0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.58 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55 ± 0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
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<td>10</td>
<td>0.73 ± 0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.63 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.75 ± 0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
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<td>10</td>
<td>0.96 ± 0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.69 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>10</td>
<td>0.99 ± 0.03&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.70 ± 0.03&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.16 ± 0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
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<td>1.31 ± 0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>1.86 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>6</td>
<td>1.65 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.91 ± 0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.69 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>B6</td>
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<td>10</td>
<td>0.52 ± 0.03&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.48 ± 0.03&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.44 ± 0.02&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>0.71 ± 0.07&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>0.66 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10</td>
<td>0.90 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.67 ± 0.03&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.90 ± 0.06&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>1.16 ± 0.06</td>
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<td>1.33 ± 0.07&lt;sup&gt;a,c&lt;/sup&gt;</td>
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<td>1.39 ± 0.04&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.92 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.15 ± 0.07&lt;sup&gt;a,c&lt;/sup&gt;</td>
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<tr>
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<td>105</td>
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<td>1.70 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.11 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.76 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C3H</td>
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<td>10</td>
<td>0.66 ± 0.03&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.50 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
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<td>0.80 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.65 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.70 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.64 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>10</td>
<td>1.12 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.18 ± 0.06</td>
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<td>14</td>
<td>10</td>
<td>1.22 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.44 ± 0.10&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>28</td>
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<td>1.47 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.99 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.95 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>6</td>
<td>1.91 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.07 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.03 ± 0.08&lt;sup&gt;a,b&lt;/sup&gt;</td>
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</tbody>
</table>

Comparing traits among strains in the same age groups using one-way ANOVA:

- <sup>a</sup> significantly different from AJ, Tukey's post hoc test p<0.05
- <sup>b</sup> significantly different from B6, Tukey's post hoc test p<0.05
- <sup>c</sup> significantly different from C3H, Tukey's post hoc test p<0.05
To understand how variations in cross sectional bone size among the three strains occurred through cellular coordination, we examined a series of in vivo fluorescent bone labels to determine cortical drift patterns as well as the mineral apposition rate (MAR) at different cross-sections along the length of the vertebral body. All three mouse strains examined at 4.5 weeks of age showed cortical drift patterns of periosteal apposition along the anterior surface and endosteal apposition along the posterior surface at both the mid-transverse and caudal end sections (Figure 2.6A). Bone labels also showed clear distinctions of the cortical and trabecular bone including regions where trabeculae were adjoined to the cortical shell.

At 4 weeks of age, B6 mice had a larger Tt.Ar at 1.23 ± 0.07mm² while C3H mice had a value of 0.98 ± 0.02mm² (p=0.23), and AJ mice had a smaller Tt.Ar of 0.67 ± 0.04mm² (p<0.05). In addition, in the mid-transverse plane of the vertebral body, width and depth measures were not different among the three mouse strains at this age (Table 2.2). Within a strain, the mineral apposition rate at 4.5 weeks of age in each of the mid-transverse or caudal end section of the mouse lumbar vertebral body was not different at the lateral surfaces and the posterior surface. Therefore, for each cross-section, the average MAR value was examined. In the mid-transverse section, AJ mice which had a small Tt.Ar, had a MAR value of 3.25 ± 0.69µm/day which was similar to the value for C3H mice at 3.02 ± 0.49µm/day but greater than the MAR for B6 mice at 2.57 ± 0.87µm/day (p=0.03) (Figure 2.6C). In the caudal end sections, the vertebral body was not adjoined to transverse processes (Figure 2.6B). In these sections, little to no label along the anterior surface was observed for all strains, which indicated high bone resorption activity in this region. An average MAR value at the caudal end for AJ
mice was 2.60 ± 1.12µm/day which was not different from C3H with a value of 2.76 ± 0.62µm/day (p=0.63). B6 mice with a MAR value of 1.91 ± 0.64µm/day was lower than that of C3H (p=0.0014), which tended to be lower than AJ mice (p=0.06). There were no differences in MAR values between the mid-transverse and caudal end sections for AJ mice (p=0.08) or C3H mice (p=0.25). However, at this snapshot in time, B6 mice tended to show a greater MAR value in the mid-transverse section compare to the caudal end (p=0.03).
Figure 2.6: Fluorescent microscopy images of in vivo bone labeling of the L4 vertebral body in a 4.5 week old AJ mouse. Sections were taken at the (A) mid-transverse plane and (B) a transverse slice approximately 20% above the growth plate at the caudal end. In vivo bone labels were administered through interperitoneal injections. Xylenol Orange applied 7 days pre-sacrifice followed by Calcein Green at 5 days and finally Alizarin Red at 3 days pre-sacrifice. Bone drift patterns seen in these images are representative for all three strains. Cortical drift occurred in the anterior direction (closed arrows) along the anterior (window a) and posterior (window b) surfaces at either location within the vertebral body. In addition, we observed cortical drift expansion in the lateral directions (window c) at the inferior end. Mineral apposition was also seen along trabeculae (open arrow). Scale bar: 1mm. (C) Cortical mineral apposition rate (MAR) was plotted for both mid-transverse and inferior end sections for each AJ, B6 and C3H mouse strains. Bars represent mean ± SD. * represents significant difference at p<0.05, assessed by unpaired t-test.
Growth patterns in establishing variation in trabecular and cortical traits

The development of external bone size was associated with the development of specific sets of cortical and trabecular traits. Along with Tt.Ar, all strains showed a progressive increase in cortical area, relative cortical area and trabecular thickness during postnatal growth (Table 2.1). However, for each strain, between 1 day of age and 4 days of age a gradual increase in the percentage of trabecular bone area (%Tb.Ar) was observed. AJ mice showed the greatest change in %Tb.Ar with a 50% increase during this age interval (p<0.0001) (Figure 2.7A). For A/J and C3H mice, the %Tb.Ar was 94% of adult values by 4 days of age, and 78% of adult values for B6. Between 4 days of age and 7 days of age each strain underwent a significant decrease in %Tb.Ar (p<0.0001 within each strain). After 7 days of age, a progressive increase in % Tb.Ar where over 80% of the adult trait was achieved by 28 days of age. The variation in %Tb.Ar among the three

Figure 2.7: Lumbar vertebral body (A) % trabecular bone area (%Tb.Ar) and (B) cortical area in three inbred mouse strains – AJ (△), B6(●) and C3H(□) during growth from 1 day of age to 105 days of age. Overall significant differences among strains for each measure at a given age from an ANOVA are indicated by * (p<0.05). Differences among individual strains analyzed from posthoc tests are shown in Table 2.1. The “#” symbol represented significant inter-strain variation as seen in adulthood at 105 days of age.
strains seen at 15 weeks of age was retained at all postnatal ages beginning at 7 days of age (p<0.05 across all strains). In contrast, cortical area values continued to change over this time period. After 7 days of age, AJ mice consistently had a smaller cortical area value compared to B6 and C3H mice (Figure 2.7B, Table 2.1). Cortical area between B6 and C3H mice were not different until 28 days of age at which point B6 had a significantly greater cortical area at a value of 0.231mm² ± 0.016mm² (p<0.0001) compared to other strains. The B6 cortical area value was 45% and 31% larger than AJ and C3H, respectfully. By 15 weeks of age, C3H mice had the largest cortical area among the three strains at a value of 0.385mm² ± 0.037mm² as a result of a 135% increase between 4 weeks and 15 weeks of age. Differences in morphological bone traits occurred while there were nearly identical age-related increases in body weight among all the strains (Table 2.1).

Within a strain, degree of anisotropy (DA) was 87%, 91% and 90% of adult values by 7 days of age for AJ, B6 and C3H, respectfully (Figure 2.8). AJ mice appeared to show a spike in trabecular alignment at 14 days of age which was significantly greater than the adult value (p=0.0002). Among the three strains, AJ and B6 mice did not show a difference in anisotropy between strains at 15 weeks of age (p=0.31) while C3H has a significantly greater trabecular alignment compared to AJ mice (p=0.0003) or B6 mice (p=0.002). This pattern was not different from that seen at 7 days of age. Between 4 days of age and 7 days of age, all three strains showed an increase in anisotropy coincided with a large decrease in the %Tb.Ar. This age interval appeared to be a critical age interval when variation in trabecular traits arose while the
trabecular architecture underwent highly adaptive changes to increase alignment in the axial direction to support physiological load.

Figure 2.8: Trabecular anisotropy values in the direction of axial compression for AJ (∆), B6(●) and C3H(□) mice during postnatal growth from 1 day of age to 105 days of age. Data represented as mean ± SD. Overall significant differences among strains for each measure at a given age from an ANOVA are indicated by * (p<0.05). The "#" symbol represented significant inter-strain variation. Differences among individual strains analyzed from Tukey's posthoc tests are shown in Table 2.1.
Discussion

Analyzing the temporal changes in bone traits during growth of the mouse lumbar vertebral body, the data showed that for three inbred mouse strains, variation in trabecular traits developed prior to the variation in cortical traits. The inter-strain variation in trabecular %Tb.Ar at 15 weeks of age occurred as early as 7 days of age. %Tb.Ar was highest in B6 mice followed by AJ then C3H mice while. The variation in %Tb.Ar among AJ, B6, and C3H mouse strains was also retained from 7 days of age into adulthood. In contrast, variation in cortical area was highly variable throughout growth with C3H showing a 135% increase in Ct.Ar between 4 and 15 weeks of age. Based on our adopted paradigm of how traits interact to establish functional bone structures, the data suggested that early variation in trabecular traits leads to an adaptive response in cortical traits to establish whole bone mechanical function. Determining the sequence of when variations in traits first appeared is an important first step toward understanding how traits functionally interact during early postnatal growth in mice. Prior to the rise of inter-strain variation in %Tb.Ar at 7 days of age, between 4 days of age and 7 days of age, a decrease in %Tb.Ar coincided with an increase in trabecular anisotropy. This pattern was similar to previous studies examining the development of trabecular microarchitecture in 6 week-old to 230 week-old porcine lumbar vertebral bodies (Tanck et al. 2001). We identified a narrow window from 1 day of age to 7 days of age during early postnatal growth in mice when inter-strain variations in multiple traits such as width, length, total area, trabecular thickness, and % Tb.Ar were similar to those seen as the adult mouse strains. This finding was important to
provide new insight into when phenotypic variation in traits were established to build functional bone structures for a given genotype.

Examining the coordination of cortical and trabecular bone traits in mice with different genetic backgrounds is an advance in understanding how bone function is achieved. Previous studies that analyzed developmental stages to determine the origin of variation in traits as seen in adults primarily focused on anatomical development. Variation in adult long bone length among LG/J and SM/J recombinant inbred mouse strains was shown to be a result of differences in growth rate as early as 3 weeks of age to 5 weeks of age (Sanger et al. 2011). The data further showed that between 4 days of age and 7 days of age a decrease in %Tb.Ar in the lumbar vertebral body coincided with an increase in Ct.Ar. This coordination among traits was similar to the development of changes among traits that occurred during trait maturation in adult mouse vertebral bodies (Glatt et al. 2007). Although analytical methods and age groups were different, this previous study and our current study both showed a compensating nature between cortical and trabecular bone during development. A pattern of trabecular traits followed by cortical traits at an early stage in development was similar to a study examining bone maturation in the L5 lumbar vertebral body of inbred mouse strains including B6 and C3H mice (Buie et al. 2008). Trabecular BV/TV, Tb.Th, TbN and TbSp values did not differ between 12 weeks of age to 32 weeks of age within a strain. Maturity in cortical thickness was achieved later in life at 17 weeks of age. In other studies, anatomical development of cortical and trabecular bone was primarily focused on the metaphyseal region of long bones along the periphery of the growth plate (Wang et al. 2011; Cadet et al. 2003; Tanck et al. 2006). Metaphyseal trabecular bone adaptation to mechanical
load played an important role in endosteal expansion and cortical elongation of long bone in adult rabbits (Cadet et al. 2003) and during growth in pigs (Tanck et al. 2006). However, heterogeneous distribution of trabecular and cortical bone in long bone makes it difficult to determine how endochondral ossification processes at the proximal and distal ends and periosteal apposition at the diaphyseal region of the bone may influence the trabecular and/or cortical adaptive response. Examining the coordination of cortical and trabecular trait development that occurred throughout the lumbar vertebral body provided insight into interactions of traits for whole bone function.

Bone traits examined as early as 1 day of age in inbred mouse strains was an advance in understanding age-related developmental patterns during postnatal growth. Previous studies on maturation examined individual traits in the lumbar vertebral body from a young adult mouse at 4-6 weeks of age (Glatt et al. 2007; Buie et al. 2008) to aged mice at 20 months of age (Glatt et al. 2007). The data further showed that inter-strain variation in both cortical and trabecular morphological traits as seen in adulthood emerged prior to 4 weeks of age. In addition to the inter-strain variation in % Tb.Ar and increase in trabecular anisotropy as early as 7 days of age, we also observed that the variation in Tb.Th was apparent at 1 day of age and remained consistent during the early developmental stage from 1 day of age to 7 days of age. These patterns among multiple trabecular traits during growth suggested an interdependent connection that influenced the phenotypic variation in trabecular area fraction among the different mouse strains. We speculate that the interaction of trabecular traits at an early age provided an internal structural integrity and established mechanical function during early postnatal development. In C3H mice, a low %Tb.Ar coincided with highly aligned trabeculae in
the direction of axial compression mainly near the inferior and superior growth plates, which later coincided with a rapid increase in cortical area after 4 weeks of age. In a previous study, finite element analysis on a geometric representation of a lumbar vertebral body showed how load was shared between the cortex and trabecular network (Silva et al. 1997). Overall shell force was largest for reduced trabecular alignment in the axial compressive direction. In an actual vertebral body, the mid-section of the vertebral body has high cortical area, and low trabecular anisotropy. Together, our biological data along with the previous computer simulation findings, suggested that the trabecular architectural arrangement influenced mechanical adaptation of the cortical shell. We speculate that increased external load and body mass with growth leads to more efficient arrangement of the trabecular architecture to allow mid-sectional cortical development during whole bone expansion. Therefore, we hypothesize that load sharing between cortical and trabecular bone is based on trabecular traits leading to cortical trait adaptation during growth.

Identifying mineral apposition rates along the mouse vertebral body advanced our understanding of the coordination of bone formation and resorption at different regions of the bone given natural variation in bone size and across three inbred mouse strains. In a previous study of 14-day-old mouse femora in AJ, B6 and C3H mouse strains, it was shown that variation in osteoclast number, spatial distribution and in vitro differentiation activity among the three strains (Gerstenfeld et al. 2010). However, how relative activities of osteoblasts and osteoclasts coordinate or what controls bone cell activity to establish trait phenotypes are not known. In this study, at 4 weeks of age, AJ mice showed a smaller total cross-sectional area and cortical area compared to C3H
mice (Table 2.1). However, the two strains had a similar mineral apposition rate in the cortical region at both the mid-transverse plane and the caudal end (Figure 2.6). This suggested that at this age, osteoblasts are forming and mineralizing bone at a similar rate for these two strains. At this particular age, B6 mice showed a lower MAR in the mid-transverse section compared to AJ, a lower MAR in the caudal section. This suggested that the B6 mouse strain developed most of the total cross-sectional area by 4.5 weeks of age therefore did not have a high MAR compared to other strains. In addition, differences in the rate of mineral apposition along the vertebral body were observed in B6 mice, which suggested that the B6 mouse may be growing differently that the other strains.

There may be differences in load sharing between cortical and trabecular bone at the two sections within the B6 mouse bone that would influence the rate of mineralization. Determining MAR at 7 days of age would provide insight into cellular activity at that rise of inter-strain variation in trabecular traits and AJ and C3H mice showed differences in width. We speculate that by 7 days of age, genetic variation in osteoblast and osteoclast activity among the different strains fundamentally forms the external bone size. In addition, the rapid decrease in % Tb.Ar and increase in trabecular alignment between 4 days of age and 7 days of age suggested the onset of establishing specific sets of trabecular and cortical traits among the individual mouse strains. The data suggested that between 4 days of age and 7 days of age, excessive resorption activity from osteoclasts may lead to the rapid clearing of trabecular bone tissue and an increase in anisotropy to establish mechanical function in the trabecular region and lead an adaptive response in the cortical shell.
Determining developmental patterns across multiple bone traits will benefit non-invasive predictions of phenotypic changes and interpret results due to genetic or environmental perturbations for different genetic backgrounds. For example, a C3H mouse with a relatively thin trabeculae and relatively high % Tb.Ar by 7 days of age is unlike the known adult phenotype. It would be imperative to see what other adaptive changes in the bone structure may arise particularly in the cortical shell and determine if the structure would be susceptible to fracture later in life. In the case of transgenic mice, genetic mutations that affect bone quality may be analyzed among trabecular traits earlier than 7 days of age to determine alterations that may affect functionality. However, after 7 days of age, alterations to trabecular trait development may lead to compensating changes in cortical traits therefore, there would not be a noticeable difference in whole bone mechanical properties because of functional adaptation of bone. Previous studies using a panel of AXB/BXA Recombinant Inbred mouse strains have found quantitative trait loci (QTLs) that regulate the interaction among cortical traits in the mouse femur for mechanical function during growth (Jepsen et al. 2010). B6 and C3H mice have been studied to identify genes that may contribute to differences in bone mass (Turner et al. 2000). Our data analysis can provide better insight into the genetic basis of differences in bone mass by identifying genes associated with early growth in mice between 1 day of age and 7 days of age. These genes may regulate trabecular and cortical trait interaction in the vertebral body and contribute to phenotypic differences in bone mass. Targeted genetic regions can be used to manipulate phenotypic development in bone structures that show early signs of compromised function.
Early developmental patterns of genetic variation among bone traits coincide with mouse behavioral changes during growth. Early behavioral development of mice for locomotion indicate that as early as 2 days of age, mice have increased strength for mobility through limb reflexes to orient the body and seek nourishment from the mother (Fox 1965). Strength for mobility increases with age, therefore, we speculate that this early loading activity along with genetic factors contributed to the individual adaptive rate of change in %Tb.Ar and anisotropy seen between 1 day of age and 4 days of age for each mouse strain. By 7 days of age, when a rise in inter-strain variation in %Tb.Ar was observed, mice tended to show the ability to right themselves to normal stance position on all four paws and, crawl in a straight line. Between 9 days of age to 15 days of age, when inter-strain variation in cortical traits continues to develop, mice have locomotion movements similar to an adult (Fox 1965). This suggested that these early stages of behavioral development including an increase in body size and weight with age, provide necessary loading forces on the skeletal system that coincide with establishing phenotypic variation in both trabecular traits and cortical traits. This also advocates the importance of studying both genetics and environmental factors to understand functional adaptation (Tommasini et al. 2009; Jepsen et al. 2009; Cheverud 1982).

Importantly, our findings do not exclude whether trabecular traits continue to adapt (or co-adapt) with subsequent changes in the cortical shell. Both trabecular and cortical traits are genetically regulated and sets of traits are developmentally and functionally interdependent to establish a particular phenotype (Cheverud 1982). However, the outcomes of this study do not distinguish whether the coordination among
cortical and trabecular traits occurred simultaneously or serially. We could not establish a causal nature of how traits interact. Mapping the temporal sequence in which trabecular and cortical traits develop during postnatal growth, simply established that early variation in trabecular traits may be a principal event contributing to the characteristic phenotype of these three inbred mouse strains.

Examining morphological bone trait measures from histological sections along mid-orthogonal planes represented values throughout the bone structure. However, this posed as a limitation to understand whether there were spatial differences in trait development and interactions that would occur in a three dimensional space. Micro-computed tomography (microCT) techniques have been used to quantify morphological and tissue quality traits in three dimensional bone volumes of the mouse vertebral body as early as 4 weeks of age (Glatt et al. 2007). However, the resolution of the x-ray technology in a microCT makes it difficult to detect low mineralization density in bone structures younger than 4 weeks of age. A high resolution imaging system such as a nanoCT may allow quantification of architectural and compositional bone traits including changes in 3D anisotropy and tissue mineral density in the cortical and trabecular bone over time.

Female AJ, B6 and C3H mouse strains were used as a model to represent genetic variation in bone size and corresponding sets of traits that occurs in nature. All three strains showed a similar pattern in trait development where trabecular trait variation preceded cortical trait variation. However, to better understand whether this pattern is a global phenomenon of how bone traits interact, it is essential to examine patterns among traits across more diverse populations. We can examine trait
development in multiple inbred mouse strains or use recombinant inbred (RI) mouse strains. RI mouse strains are a powerful tool based on genetic randomization of parent strain genomes to create non-pathological trait variation used to measure the tendency for different traits to cosegregate or correlate (Tommasini et al. 2009; Nadeau et al. 2003; Bailey 1981). AXB/BXA RI mouse strains (parental strains, AJ and B6 inbred mouse strains) have been studied to understand the phenotypic integration of cortical and trabecular traits in adults (Tommasini et al. 2009). Using this panel of mice, we can further understand how cortical and trabecular traits interact during growth in order to understand how functional structures with a wide range of genetic variants develop. Males generally have wider bones than females (Duan et al. 2001; Bhola et al. 2011). Determining gender-specific growth patterns in the development of phenotypic traits in trabecular and cortical bone would provide insight into whether there are dimorphic trait development processes. Additionally, RI strains can be used to determine quantitative trait loci (QTLs) that may regulate cortical and trabecular traits and help explain how genetic variants are associated with bone strength and fragility.

In conclusion, examining the temporal changes in both trabecular and cortical morphological traits in the lumbar vertebral body from 1 day of age to adulthood, we were able to identify when inter-strain variations in traits occurred among the three strains similar to the variation seen in adulthood. Among three different mouse strains, there was a similar pattern in the co-development of bone traits to establish functional lumbar vertebral bodies. We determined that variation in trabecular traits preceded the development of variation in cortical traits. According to our adopted working model of how functional bone structures develop, our results suggest variation in cortical traits
arise from an adaptive response to early genetic variation in trabecular traits. Using inbred mice with different genetic backgrounds provided a model to determine biological processes during growth that are important in establishing mechanical function given natural variation in traits. This is beneficial for interpreting adaptive changes in bone due to genetic or environment perturbations.

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References


Chapter 3

GENETIC RANDOMIZATION REVEALS NOVEL INTERACTIONS AMONG CORTICAL AND TRABECULAR TRAITS DURING GROWTH OF THE LUMBAR VERTEBRAL BODY
Introduction

Adult cortico-cancellous structures achieve mechanical function by acquiring specific sets of cortical and trabecular traits that are predictable based on the natural variation in bone size (Zebaze et al. 2007; Tommasini et al. 2009). Understanding how specific sets of traits co-develop relative to bone size during growth is important to identify individuals that may be at risk of compromised bone function early in life, and benefit efforts to personalize treatments to prevent fracture. Both genetic and environmental factors contribute to variation in bone mass and multiple skeletal traits (Rosen et al. 2001). Therefore, bone undergoes complex adaptive mechanisms during growth to maintain stiffness and strength with changes in physiological load (Ruff et al. 2006). A previous study showed a functional adaption process in the trabecular architecture of porcine lumbar vertebral bodies where trabecular BV/TV was inversely correlated with trabecular anisotropy with weight bearing-load during growth (Tanck et al. 2001). Other studies showed coordinated changes involving morphological and compositional traits in the cortical shell were important to establish function during growth of long bones with a wide range of external bone sizes (Jepsen et al. 2009; Jepsen et al. 2010; Price et al. 2005). Few studies have identified developmental patterns among cortical and trabecular traits in whole bone structure. During bone maturation in the mouse lumbar vertebral body from a young adult at 4-6 weeks of age (Buie et al. 2008; Glatt et al. 2007) to aging at 20 months of age (Glatt et al. 2007), one observation was that a decrease in trabecular bone volume fraction coincided with an increase in cortical thickness (Buie et al. 2008; Glatt et al. 2007) and an increase in external cross sectional area (Glatt et al. 2007). However, these studies did not apply a
systematic approach to determine how cortical and trabecular traits interacted to achieve mechanical function during growth and relative to natural variation in bone size. To better understand the complex adaptive nature of bone, we adopted a working model, which postulates that early variation in one trait leads to subsequent adaptive changes in other traits during growth (Tommasini et al. 2009; Jepsen 2011). The objective of this study is to understand how cortical and trabecular traits functionally interact during growth in whole bone vertebral bodies across a population that has a wide range of genetic variants including external bone sizes. In our previous work, we identified a temporal sequence in the co-development of cortical and trabecular traits during postnatal growth in the lumbar vertebral body of AJ, B6 and C3H inbred mouse strains (Chapter 2). We found that variation in trabecular BV/TV across the three strains as seen in adulthood was already present by 1 week of age whereas variation in cortical area was observed after 4 weeks of age. Based on this observation, we hypothesized that early variation in trabecular architectural traits leads to adaptive changes in cortical area during growth in the mouse lumbar vertebral body.

To test this hypothesis, we determined relationships among cortical or trabecular traits during growth using a panel of AXB/BXA recombinant inbred (RI) mouse strains. Each strain is made up of a unique pattern of genetic randomization of the A/J (AJ) and C57BL/6 (B6) parental genomes (Nadeau et al. 2003). Adult AJ mice are known to have a characteristic set of traits that include a narrow bone size with a small cortical area and high trabecular volume. B6 mice are known to have a wide bone size with a greater trabecular volume compared to AJ mice. Non-pathological variation in cortical and trabecular sets of traits within each RI strain allows us to examine mechanically
functional structures and determine patterns of trait interactions during growth. We measured the tendency for different traits to co-vary or correlate across the panel of RI mouse strains (Bailey 1971; Bailey 1986). In addition, variation in bone size and corresponding sets of traits that we observed in adult RI mouse strains represents the phenotypic variation that we see in humans (Zebaze et al. 2007; Tommasini et al. 2009).

**Materials and Methods**

Recombinant Inbred mouse strains

Female AXB/BXA RI mouse strains were derived from A/J (AJ) and C57BL/6J (B6) progenitor strains. Female samples have been used in our previous studies (Tommasini et al. 2009; Jepsen et al. 2009; Jepsen et al. 2010; Jepsen et al. 2007). Therefore, our findings can be compared to skeletal trait development in different bones and at different ages. Within each RI strain, natural perturbation of inherited A/J and B6 alleles through meiosis created various combinations of cortical and trabecular traits to build mechanically functional bones in slightly different ways. Traits were examined across the panel of RI mice as a powerful experimental model to quantify functional relationships among traits (Li et al. 2006; Nadeau et al. 2003).

AJ, B6, and 20 AXB/BXA RI strains were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and examined at 4 weeks of age (n=8-10/strain) and 16 weeks of age (n=9-10/strain). These ages were chosen based on prior work that showed variation in mouse femoral cortical traits across three inbred mouse strains was achieved by 4
weeks and continued to steadily grow until 16 weeks of age when bone was skeletally mature (Price et al. 2005). Our previous work also reported that the same three inbred mouse strains showed variation in both trabecular and cortical traits as seen in the adult lumbar vertebral body between 4 weeks of age and 16 weeks of age (Chapter 2). We examined cortical and trabecular traits in the L4 vertebral body at both ages to provide insight into whole bone structural development. In addition, the L4 vertebral body was previously examined at 16 weeks of age to determine functional interactions between cortical and trabecular traits in skeletally mature bones (Tommasini et al. 2009). 4 week old mice were retrieved from storage at -60 degrees-C. The L4 vertebrae were harvested then placed in phosphate buffered saline solution and stored at -40 deg-C.

Physical bone traits

L4 vertebrae samples were scanned using an eXplore Locus SP PreClinical Specimen MicoComputed Tomography (microCT) system (TriFoil Imaging, Chatsworth, CA, USA). For scanning, the L4 vertebrae samples were placed in an air-tight chamber filled with phosphate buffered saline solution. 16 week-old samples were scanned at a 16-micron voxel size, and the 4-week-old samples were scanned at an 8.7-micron voxel size. Each scan contained a calibration phantom containing air, water and hydroxyapatite crystals (SB3; Gamex RMI, Middleton, WI, USA), which was used to adjust mineral density measurements because of variability in X-ray attenuation inherent to each scan (Jepsen et al. 2007). MicroView Advanced Bone Analysis software (v 1.2.1 GE Healthcare) was used to reconstruct a 3D rendition of the whole bone, isolate the vertebral body of each sample and manually segment cortical and trabecular regions as previously described (Tommasini et al. 2009). The volume of
interest consisted of trabecular bone in the secondary spongiosa and the surrounding cortical bone. Cortical and trabecular volumes were thresholded separately to differentiate calcified from non-calcified voxels (Otsu 1979). Total bone volume (Tt.V) was a measure of the total bone volume plus the marrow volume. Cortical traits measured included cortical area (Ct.Ar) and cortical thickness (Ct.Th), average total cross-sectional area (Tt.Ar) along the length of the vertebral body region of interest, and relative cortical area (RCA) - calculated as Ct.Ar/Tt.Ar. Trabecular traits measured included trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular spacing (Tb.Sp), and structural degree of anisotropy (DA) in the cranial-caudal direction.

In the MicroView analytical software, DA was calculated using the mean intercept length (MIL) method. This method measured the intersections of a test grid with the trabecular structure and calculated the fabric ellipsoid (3D ellipse) (Odgaard 1997; Harrigan et al. 1984). Trabecular structures with no preferred orientation had a spherical ellipsoid, whereas structures with more alignment in one direction had the major axis of the ellipse aligned in that direction. An eigen analysis of the second rank tensor provides the length of the axes of the ellipsoid and their corresponding directions (a1, a2, a3). The degree of anisotropy is then defined as the ratio of the maximum length axis/length of the minimum length axis. In the vertebral body, the maximum length was in the cranial-caudal direction (a1) and the minimum length was in the lateral direction (a3). Therefore, the degree of anisotropy was calculated as the ratio of a1/a3. The values of trabecular anisotropy ranged from 1 (isotropic) to infinity (anisotropic). Higher
DA values indicated greater alignment of the trabecular architecture in the cranial-caudal direction.

Trait analyses for 16 week old RI strains were repeated to ensure the regions of interest were consistent for samples at both 4 weeks of age and 16 weeks of age. This was important to provide a more precise understanding of the changes in traits that occurred within a particular space and over time.

**Adult whole bone mechanical properties**

Stiffness (S) and maximum load to failure (F) were previously reported for 16 week old RI mice strains (Tommasini et al. 2009). In brief, the vertebral body region of whole L4 vertebrae (n=9-10/strain) were compressed at a cross-head speed of 0.05mm/s using a servohydraulic material testing machine (Instron model 8872; Instron Corp, Canton, MA, USA). The endplates of the vertebral bodies were minimally shaved flat and the samples were aligned and secured in a holding device within the Instron machine. A 3-mm diameter platen was positioned above the cranial end to apply a uniaxial displacement while a platen positioned against the caudal end remained stationary. Stiffness and failure load were determined from deformation output measurements reported on LabView i6 (National Instruments).

**Interaction of cortical and trabecular traits for skeletal function**

Multiple cortical and trabecular traits in the lumbar vertebral body were obtained from the microCT bone analysis. However, this study focused on five morphological traits that represented features of the whole bone structure and microarchitecture – total area, cortical area, relative cortical area, bone volume fraction, and anisotropy.
Previously acquired trait values and stiffness and failure load values at 16 weeks of age for each RI strain (Tommasini et al. 2009) were used to generate new associations between traits and variation in bone size. These values were also used in combination with 4 week old data to better understand the development of traits during growth. To test for functional interactions, we conducted a series of bivariate regression analyses to identify correlations between cortical and trabecular traits at 4 weeks and 16 weeks of age and the percentage change that occurred between 4 and 16 weeks of age. We also conducted multiple regression analyses to test whether there were multiple trabecular traits that could explain the change in cortical traits over time and to determine which traits contributed to whole bone mechanical properties. Correlations were considered statistically significant at a p<0.05 (GraphPad Software 5.0, San Diego, CA).
Results

*Variation in physical bone traits among RI mouse strains as early as 4 weeks of age*

Randomization of the A/J and B6 genomes resulted in a wide variation in vertebral size and cortical and trabecular morphological features among the panel of RI mouse strains at 4 weeks of age (Table 3.1). At this early age, the total-cross sectional area of the vertebral body in A/J mice was smaller than B6 mice (p=0.04) at 1.13 ± 0.03mm² and 1.17 ± 0.06mm², respectfully. Mean values of total area for each strain showed that the RI vertebral bodies ranged from smaller to larger in size compared to the parental strains (ANOVA, p<0.0001, Figure 3.1). Average body weight and architectural features of each strain also varied within and beyond the values of A/J and B6 progenitor strains (ANOVA, p<0.0001).

![Figure 3.1](image-url) **Figure 3.1.** Mean values of total cross-sectional area of the lumbar vertebral body for A/J, B6, and 20 AXB/BXA RI mouse strains. Data represented as mean ± SD for n=8-10 samples per strain.
Table 3.1: Variation in vertebral body size, and morphology among 20 AXB/BXA RI strains at 4 weeks of age. Data shown as mean ± standard deviation (italics).
Development of individual traits: 16 week old trait values relative to 4 week old trait values

To understand overall development of traits across the population of RI mouse strains, we compared trait values at 16 weeks of age to 4 weeks of age. Across the panel of RI mice, body weight at 16 weeks of age was positively correlated with body weight at 4 weeks of age ($R^2=0.81$, $p<0.0001$, Figure 3.2A). The slope of the regression line was 1.7, which indicated a uniform amount of growth in body size across the RI panel. The average percentage change in bodyweight for RI mice over time was approximately 53% ± 9%. RI mice with a large body size at 4 weeks of age grew proportionally more than RI mice with a small body size. The position of the cluster of points above the 1:1 trend line suggests that an increase in body weight from 4 weeks of age to 16 weeks of age, which was expected with overall body growth during this time period.

Total cross sectional area of lumbar vertebral bodies at 16 weeks of age was also positively correlated with total area at 4 weeks of age ($R^2=0.70$, $p<0.0001$, Figure 3.2B). The slope of the regression line was 0.96, which indicated a uniform amount of growth in total area across the RI panel from 4 weeks of age to 16 weeks of age, which was approximately 18 ± 6%. The AXB4 strain appeared to have a very small total area of 0.88 mm$^2$ ± 0.04 mm$^2$ at 4 weeks of age and 1.04 mm$^2$ ± 0.06 mm$^2$ at 16 weeks of age compared to other mouse strains. However, the average body weight for this strain was also below the overall average at both 4 weeks of age and 16 weeks of age indicating that the AXB4 strain was simply small for size. The position of the cluster of points above the 1:1 trend line indicated an increase in total area from 4 weeks of age to 16
weeks of age, which was expected with growth and expansion of the whole bone with increasing body size over time.

Figure 3.2: Comparison of average (A) body weight and (B) total area values at 16 weeks of age to 4 weeks of age for each of 20 RI mouse strains showed that the majority of these traits were established by the later time point.

Cortical area, trabecular BV/TV, relative cortical area (RCA) and the Degree of Anisotropy (DA) values at 4 weeks of age correlated positively to trait values at 16 weeks of age (Ct.Ar, $R^2=0.40$; BV/TV, $R^2=0.58$; RCA, $R^2=0.20$; DA, $R^2=0.32$, for all $p\leq0.05$, Figure 3.3). We observed that each trait appeared to develop at different rates and mature at different ages. For cortical area (Figure 3.3A) and trabecular BV/TV (Figure 3.3B), the slope of the regression line was 1.0 and 1.3, respectfully. This indicated a uniform amount of growth in cortical area or BV/TV across the RI panel. Mice that tended to have a small cortical area or BV/TV at 4 weeks of age tended to have small trait values at 16 weeks of age. Data points for cortical area above the 1:1 trend line indicated that there was an increase in cortical area values by 16 weeks of age. This finding was expected and coincided with the expansion of the total cross-
sectional area. The position of points close to the 1:1 trend line indicated that BV/TV tended to not be different at 4 weeks and 16 weeks across the RI strains. However, mice that tended to have a high BV/TV value (above 0.22) at 4 weeks of age tended to show a further increase in BV/TV with growth. For RCA (Figure 3.3C) and trabecular anisotropy (Figure 3.3D), the slope of the regression line was 0.42 and 0.50, respectfully, indicating that RCA and the alignment of the trabecular architecture developed differently across the RI strains. Mice that tended to have a low RCA at 4 weeks of age (less than 0.21) tended to have an increase in RCA with growth whereas mice that initially had a high RCA value tended to retain their RCA values. Aside from three strains that appeared to retain a low RCA, the data suggested that out of 17 mouse strains, those that tended to have a low RCA at 4 weeks of age underwent a greater change over time. For most strains, the anisotropy or alignment of the trabeculae was observed by 4 weeks of age. In addition, mice that tended to have highly aligned trabecular architecture at 4 weeks of age tended to have a reduced alignment by 16 weeks of age. This suggested a change in the alignment of trabecular architecture over time that was important to maintain function in these structures.
Figure 3.3: Comparison of cortical and trabecular trait values at 16 weeks of age to those at 4 weeks of age for each of 20 RI mouse strains. (A) Cortical area was mainly established by 16 weeks of age. (B) Most mouse strains appeared to establish BV/TV at 4 weeks of age. (C) Relative cortical area appeared to have different developmental patterns relative to 4 week old values, and the (D) degree of anisotropy appeared to mostly be established at the early age.
**Functional equivalence relative to bone size**

In adult RI mouse strains at 16 weeks of age, total area, stiffness, and whole bone failure load (a measure of bone strength) correlated positively with body size (BW versus Tt.Ar, $R^2=0.60$; BW versus Stiffness, $R^2=0.41$, BW versus Maximum load to failure, $R^2=0.58$, $p\leq0.002$, **Figure 3.4**). To better understand co-variation of mechanical properties and total cross sectional area of the vertebral body, we conducted partial correlation regressions taking into consideration the effects of body weight at 16 weeks of age. In **Figure 3.5A**, residuals of stiffness calculated from the Stiffness-BW regression (**Figure 3.4B**) was plotted against residuals of total area calculated from the TtAr-BW regression (**Figure 3.4A**). Therefore, negative residuals referred to RI strains that had a small value for size and positive residuals referred to strains that had a large value for size. Mice that tended to have a small total area for size had a stiffness value that was not different from mice that tended to have a larger total area for size ($R^2=0.04$, $p=0.43$). Failure load was also not significantly correlated with total area ($R^2=0.08$, $p=0.24$, **Figure 3.5B**). This suggested that mouse lumbar vertebral bodies were functionally equivalent relative to bone size. We further determined how cortical and trabecular traits developed and led to bones maintaining similar levels of function.
Figure 3.4: Variation in (A) total cross-sectional area, (B) structural stiffness, and (C) maximum load to failure as a function of body weight at 16 weeks of age. Total area and whole bone mechanical properties positively correlated with body weight.

Figure 3.5: Whole bone mechanical properties relative to total cross-sectional area of lumbar vertebral bodies at 16 weeks of age. Partial regression analyses were conducted to take the effects of body weight into consideration showing that (A) whole bone stiffness and (B) maximum load to failure were not different relative to variation in total area.
Development of morphological traits relative to body weight

Plotting cortical and trabecular traits against overall body weight at both 4 weeks of age and 16 weeks of age, we expected that bone traits would have significant positive correlations with body weight at both ages. This would indicate bone growth with weight-bearing load as early as 4 weeks of age and with increased weight during growth to 16 weeks of age. At 4 weeks of age, total area of the vertebral body was highly correlated with body size ($R^2=0.69$, $p<0.0001$, Figure 3.6A), similar to what we observed in the 16 week old mice (Figure 3.4A). Cortical area was also highly correlated with body size ($R^2=0.61$, $p<0.0001$, Figure 3.6B). However, RCA (Figure 3.6C), BV/TV (Figure 3.6D) and the degree of anisotropy (Figure 3.6E) showed a weak relationship with body weight (BW vs RCA, $R^2=0.08$, $p=0.22$; BW vs BV/TV, $R^2=0.11$, $p=0.16$; BW vs DA, $R^2=0.09$, $p=0.20$). At 16 weeks of age, cortical area appeared to have a higher correlation to body size compared to 4 weeks of age (BW versus Ct.Ar, $R^2=0.81$, $p<0.0001$, Figure 3.7A). At this later time point, BV/TV continued to have a weak relationship with body size (BW versus BV/TV, $R^2=0.19$, $p=0.06$, Figure 3.7B) as well as RCA (Figure 3.7C) and the degree of anisotropy (Figure 3.7D) (BW vs RCA, $R^2=0.14$, $p=0.10$; BW vs DA, $R^2=0.08$, $p=0.84$).
Figure 3.6: Variation of (A) total cross-sectional area, (B) cortical area, (C) RCA, (D) trabecular BV/TV, and (E) trabecular anisotropy as a function of body weight at 4 weeks of age.
Figure 3.7: Variation of (A) cortical area, (B) trabecular BV/TV, (C) RCA, (D) trabecular anisotropy as a function of body weight at 16 weeks of age.
Development of morphological traits relative to bone size

To determine how cortical and trabecular traits co-varied with bone size at both 4 weeks of age and 16 weeks of age, we conducted partial correlation regression analyses taking the effects of body weight into consideration. At 4 weeks of age, cortical area showed a weak relationship with total area ($R^2=0.14$, slope=0.07, and $p=0.11$, Figure 3.8A). BV/TV was not correlated with total area across the RI panel ($R^2=0.001$, slope= -0.01, and $p=0.92$, Figure 3.8B). In the unadjusted data, we observed a large amount of variability in BV/TV relative to total area across the RI panel (Appendix A-Figure 3.1B). However, mice with bones that tended to be small for size tended to have a large RCA ($R^2=0.37$, slope= -0.13, and $p=0.005$, Figure 3.8C) and high degree of anisotropy ($R^2=0.33$, slope= -0.89, and $p=0.008$, Figure 3.8D) at this early age.

Figure 3.8: Partial regression analyses conducted to take the effects of body weight at 4 weeks of age into consideration showed that, (A) cortical area and (B) BV/TV was not correlated with total area however (C) RCA and (D) trabecular anisotropy had weak yet significant correlations with total area at this early time point.
At 16 weeks of age, the relationships among cortical or trabecular traits relative to total area were similar to those observed at 4 weeks of age, but with some subtle differences. At this later time point, cortical area showed a stronger relationship to bone size ($R^2=0.27$, slope= 0.09, and $p=0.02$, Figure 3.9A) and anisotropy showed a weaker relationship to bone size ($R^2=0.18$, slope= -0.46, and $p=0.06$, Figure 3.9D) compared to the earlier time point.BV/TV continued to not be different relative to bone size ($R^2=0.05$, slope= -0.12, and $p=0.36$, Figure 3.9B). In addition, the unadjusted data showed that BV/TV was not correlated with total area at 16 weeks of age ($R^2=0.04$, $p=0.37$), whereas BV/TV values were widely variable relative to total area (Appendix A-Figure 3.2B). RCA continued to be negatively correlated with bone size ($R^2=0.44$, slope= -0.10, and $p=0.001$, Figure 3.9C).

Figure 3.9: Partial regression analyses conducted to take the effects of body weight at 16 weeks of age into consideration showing that, (A) Cortical Area has a weak yet significant correlation with total area (B) BV/TV was not different, (C) RCA was correlated with total area and (D) Anisotropy has a low correlation with total area in adult mice.
In the unadjusted data, cortical area was highly correlated with total area at both 4 weeks of age (Appendix A-Figure 3.1A) and 16 weeks of age (Appendix A-Figure 3.2A), which was expected. However, the significance was improved for RCA and Anisotropy when accounting for the effects of body weight (Appendix A-Figure 3.1C-D, 3.2C-D).

To understand whether the initial variation in bone size at 4 weeks of age had an effect on how traits developed from 4 weeks of age to 16 weeks of age, we plotted the percent change in cortical or trabecular traits relative to total area at 4 weeks of age taking into account the effects of body weight at 4 weeks of age. The percent change in cortical area, BV/TV, RCA and anisotropy tended to not be different relative to total area at 4 weeks of age (Figure 3.10). Therefore, the data suggested that cortical and trabecular traits grew uniformly across the panel of RI mouse strains. To better understand the co-variation of cortical and trabecular traits during growth, the percent change in cortical traits between 4 weeks of age and 16 weeks of age was plotted against the percent change in trabecular traits. A correlation analysis was conducted among all variables. From the analysis, we found two significant correlations. Mice that tended to show an increase in BV/TV from 4 weeks of age to 16 weeks of age tended to show an increase in RCA regardless of bone size ($R^2=0.31$, $p=0.01$, Figure 3.11A). Mice that tended to show an increase in anisotropy (trabecular architecture became more aligned over time), tended to show a small increase in cortical area ($R^2=0.22$, $p=0.04$, Figure 3.11B).
Figure 3.10: Partial regression analyses to take the effects of body weight at 4 weeks of age into consideration showing that the percent changes from 4 weeks of age to 16 weeks of age in (A) Cortical Area, (B) BV/TV, (C) RCA and, (D) anisotropy were not different relative to variation in total area at 4 weeks of age.

Figure 3.11: Partial regression analyses to take the effects of the percent change in body weight from 4 weeks of age to 16 weeks of age into consideration showing that (A) the percent change in RCA was positively correlated with the percent change in BV/TV and (B) the percent change in cortical area was negatively correlated with the percent change in anisotropy.
We conducted multiple linear regressions to assess the relative contributions of trabecular traits and body size to the change in cortical traits from 4 weeks to 16 weeks of age (Table 3.2). Using best subsets regression analyses, the data showed that 67.4% of variation in the change of cortical area across the RI panel was explained by the change in anisotropy, the change in body weight, and BV/TV at 4 weeks of age ($R^2=0.67$, $p=0.001$). Of these traits, the change in anisotropy and change in body weight over time were significant contributors. 47.8% of the change RCA was explained by the change in body weight, the change in BV/TV and trabecular anisotropy at 4 weeks of age ($R^2=0.48$, $p=0.004$). Significant contributors to the change in RCA included the change in body weight and the change in BV/TV over time. This indicated that the development of the cortical shell was predicted by change in weight-bearing load and trabecular architectural traits during growth.

<table>
<thead>
<tr>
<th>Equation</th>
<th>$R^2$-adj</th>
<th>$p$</th>
</tr>
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<tbody>
<tr>
<td>%Δ Cortical Area = -0.31 - 0.80 %Δ DA + 0.72 %Δ BW + 0.69 BV/TV</td>
<td>67.4%</td>
<td>0.001</td>
</tr>
<tr>
<td>%Δ RCA = -0.31 + 0.26 %Δ BW + 0.22 %Δ BV/TV + 0.12 DA</td>
<td>47.8%</td>
<td>0.004</td>
</tr>
</tbody>
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*Bold font indicates traits making significant contributions to the variation in %change in cortical trait ($p<0.05$).

BW = Body Weight; BV/TV = Trabecular bone volume fraction; DA = Degree of Anisotropy

**Table 3.2**: Multiple Linear Regression analysis of traits that contribute to the change in cortical traits.
Interaction among traits that contribute to whole bone stiffness and strength

We further investigated cortical or trabecular traits that contribute to the structural stiffness and strength of the vertebral body. We conducted partial correlation regressions of stiffness and failure load relative to cortical and trabecular traits at 16 weeks of age taking the effects of body weight into consideration. BV/TV was the only trait that was significantly correlated with stiffness (Figure 3.12B). Mice that tended to have a low BV/TV for size had a low stiffness value ($R^2=0.35$, $p=0.006$). Failure load was positively correlated with BV/TV ($R^2=0.65$, $p<0.0001$, Figure 3.12F) and RCA ($R^2=0.27$, $p=0.02$, Figure 3.12G). Using best subsets regression analyses, multiple regression equations showed that nearly 60% of variation in stiffness was explained by trabecular BV/TV, trabecular anisotropy and body weight in the adult structure ($R^2=58.7\%$, $p=0.001$, Table 3.3). Of these traits, BV/TV was the predominant contributor followed by body weight. 90.1% of the variation in the failure load was explained by RCA, BV/TV and body weight at 16 weeks of age, which were all significant contributors to the variation in failure load. In addition, 60.7% of the variation in adult stiffness was shown to be explained by the change in BV/TV from 4 weeks of age to 16 weeks of age and the body weight at 4 weeks of age ($R^2=60.7\%$, $p=0.001$). 91.2% of the variation in adult failure load was explained by the adult RCA values as well as BV/TV and body weight at 4 weeks of age and the change in BV/TV and change in anisotropy over time ($R^2=91.2\%$, $p=0.001$). This indicated that early trabecular traits were predictors of adult mechanical properties.
Figure 3.12: Partial regression analyses conducted to take the effects of body weight at 16 weeks of age into consideration showing that stiffness (A-D) was significantly correlated with (B) BV/TV and had weak relationships with (A) Ct.Ar, (C) RCA, and (D) trabecular anisotropy. Maximum load to failure (E-H) was significantly correlated with (F) BV/TV and (G) RCA and had weak relationships with (E) Ct.Ar and (H) anisotropy.
**Table 3.3**: Multiple Linear Regression analysis of traits that contribute to the whole bone mechanical properties of the lumbar vertebral body.

**Discussion**

The objective of this study was to test the hypothesis that early variation in trabecular architectural traits lead to adaptive changes in cortical area during growth of the mouse lumbar vertebral body given a wide range of genetic variants. Correlation analyses across a panel of AXB/BXA RI mouse strains showed trabecular architectural traits were established prior to cortical traits during growth. Both trabecular BV/TV and anisotropy values were observed by 4 weeks of age. Cortical area and total area values were greater at 16 weeks of age indicating increased development compared to trabecular traits. The temporal changes in cortical and trabecular traits in a panel of RI mouse strains were similar to our previous finding that showed variation in trabecular traits as seen in adulthood, occurred early in development compared to cortical traits in three inbred mouse strains. At 4 weeks of age, there was a significant difference in trabecular anisotropy relative to bone size taking body weight into consideration (**Figure 3.8D**). Mice that tended to have a small total cross sectional area had a greater trabecular alignment than mice that has a larger total area. A significant difference in
cortical area relative to bone size was observed at 16 weeks of age (Figure 3.9A). In addition, we determined that bone structures that tended to show an increase in trabecular alignment from 4 weeks of age to 16 weeks of age tended to show a small increase in cortical area over time (Figure 3.11B). This relationship between trabecular anisotropy and cortical area traits during growth is an advance from a previous study that used a finite element simulation of the lumbar vertebral body to determine that the load borne to the cortical shell was largest when there was a low degree of trabecular anisotropy (Silva et al. 1997). Here, our analysis provided further insight into the inverse relationship between the cortex and the trabecular architecture as a biological adaptation process that is important to build functional structures. We also found that structures that tended to show an increase in BV/TV over time tended to show an increase in RCA (Figure 3.11A). This finding appeared to be different from what was previously predicted as the interaction between BV/TV and RCA to achieve whole bone stiffness in adult AXB/BXA RI strains (Tommasini et al. 2009). However, analysis of the change in BV/TV and RCA traits during growth provided insight into an additional cortical-trabecular trait interaction taking place that is important for achieving mechanical function.

Determining interactions among cortical and trabecular traits relative to bone size was an advance in understanding how function is achieved in bone structures. Tommasini et al utilized 16 week old AXB/BXA RI mouse strains to determine that there were functional interactions among cortical and trabecular traits across the panel of adult mouse lumbar vertebral body (Tommasini et al. 2009). In this study, we analyzed data from the adult mice in a different way to better understand co-variation of cortical
and trabecular traits relative to variation in bone size and taking into consideration the effects of body weight. Bone morphology is a primary determinant of the development of bone stiffness and strength during growth (Sumner et al. 1996), therefore, we focused our analyses on whole bone structural traits. Figure 3.4A and 3.6A showed a small variation in 16 week and 4 week old Tt.Ar-BW residuals, respectfully. However, we were able to show significant associations between cortical and trabecular traits using this analytical model. Therefore, assessing how the skeletal system coordinated traits to establish mechanical homeostasis relative to natural variation among traits appeared to be an important experimental paradigm. The data showed that the adult lumbar vertebral bodies across the panel of RI strains tended to not be different in stiffness and strength values relative to bone size (Figure 3.5). Examining the co-variation of cortical and trabecular structural traits relative to bone size, we were able to provide a better understanding of how mice with a small bone cross-sectional area for body size can support the same load as mice with a large cross-sectional area. Trends in cortical or trabecular trait values relative to bone size were not different at 4 weeks of age and 16 weeks of age (Figure 3.8 and 3.9). This suggested that cortical and trabecular sets of traits relative to body size were mainly present by 4 weeks of age and later underwent subtle changes. This finding was consistent with our study on the temporal changes of traits in inbred mouse strains that showed inter-strain variation in both cortical and trabecular traits in AJ and B6 mice as seen in adulthood were observed as early as 2 weeks of age (Chapter 2). In addition, the percentage of change in bone traits from 4 weeks of age to 16 weeks of age relative to body size tended to not be different, which suggested that traits developed uniformly across the RI mouse panel from 4 weeks of
age to 16 weeks of age (Figure 3.10). This also suggests that initial variation in bone size may be due to early differences in bone formation and resorption activity among individuals to adjust cortical and trabecular morphological traits and intrinsic properties to provide sufficient mechanical properties for a given bone size. However, the cellular processes that regulate the co-variation among cortical and trabecular trait sets remain to be fully understood.

An unexpected finding was that the trabecular bone fraction did not correlate with bone size or body weight. Plots of the unadjusted data showed a large variation in BV/TV values relative to bodyweight or total area at both 4 weeks of age and 16 weeks of age (Figure 3.6D and 3.7B; Appendix A-Figure 3.1B and 3.2B). This indicated that BV/TV or the change in BV/TV over time tended to not be influenced by weight-bearing load. In addition, we did not observe significant interactions between BV/TV and cortical area, relative cortical area, or anisotropy traits at either 4 weeks or 16 weeks of age (Appendix A-Figure 3.3 and 3.4). Though BV/TV did not correlate with bone size or other structural traits at individual ages, BV/TV was the only bone trait in our analysis that was significantly correlated with whole bone stiffness after adjusting for body size (Figure 3.12). Tommasini et al used multivariate analysis to show that both BV/TV and total tissue mineral density explained 66% adult stiffness in AXB/BXA RI mouse strains (Tommasini et al. 2009). In adult human vertebral bodies, trabecular micro architectural traits including BV/TV was shown to strongly correlate with whole bone stiffness and load to failure but not anisotropy or cortical thickness (Roux et al. 2010). This suggested that the trabecular bone volume fraction including BMD appear to be associated with the load bearing capacity of the bone while the cortical shell was important for flexibility.
and energy absorption. This further supported that trait interactions were complex, and the integration of multiple morphological and tissue quality traits is important to understand whole bone function. We can speculate that the mass and composition of the trabecular bone volume may be designed from an early age as a “shock absorber” to resist deformation under increasing weight-bearing load with growth and externally applied load to provide a structural foundation from which the whole bone develops. During growth, the data showed that mice that tended to have an increase in BV/TV from 4 week of age to 16 weeks of age tended to have an increase in relative cortical area (Figure 3.11A). We also showed that adult bone strength was mainly explained by the adult RCA value followed by the adult BV/TV value (Table 3.3), indicating that the interaction of both the cortical and trabecular structure is important for whole bone function. Adult strength could also be explained by a combination of adult RCA values, 4 week old BV/TV values, a change in anisotropy and a change in BV/TV during growth and body weight at 4 weeks of age. The coordination of multiple bone traits further supports that trabecular traits from an early age are predictors of the mechanical properties that are achieved in adulthood.

Correlation analyses showed differences in growth patterns between trabecular architectural traits and the development of cortical traits relative to variation in bone size in the mouse lumbar vertebral body. However, many of the simple linear relationships between cortical or trabecular traits and total area showed weak correlations where $R^2$ values were less than 0.40, yet p values were less than 0.05 indicating significant relationships. One explanation is that variation in trabecular anisotropy at 4 weeks of age or cortical area at 16 weeks of age depended on important interactions between
anisotropy and BV/TV than just the total cross sectional area of bone. For example, tissue mineral density in combination with morphological traits may be important in structural differences relative to differences in bone size. Another explanation is that there was a limitation in the number of RI strains used in this study. 20 RI strains were used to draw conclusions on bone development mainly based on a few data points that represented the extreme cases of bones that tended to be narrow or wide for size. Data points near the center of partial regression plots indicated an interaction of traits that each had a high correlation to bodyweight in the unadjusted data plots. We expect that analyzing a greater number of strains would provide a stronger correlation of points since there would be a better probability of acquiring samples that could be considered narrow and wide for body size. However, the relationships that we have identified in how traits interact as early as 4 weeks of age and into adulthood is not expected to change.

A second limitation of the methods was the ages used to determine growth patterns among traits. We analyzed traits in AXB/BXA RI mice starting at 4 weeks of age based on the availability of samples. Our previous study on the development of trait sets in AJ, B6 and C3H mice showed that variation in cortical and trabecular traits for AJ and B6 mice as seen in adulthood was observed as early as 2 weeks of age (Chapter 2). By 4 weeks of age, female AJ and B6 mice achieved 85% of the adult trait values in BV/TV, anisotropy and RCA. These strains also achieved over 50% and over 75% of the adult cortical area and total area trait values, respectfully. This may explain the subtle differences in the development of bone traits that we see in the female AXB/BXA RI mice specimens between 4 weeks of age and 16 weeks of age. Examining trait
development from 2 weeks of age in the RI panel, we would expect to see variation in trabecular architectural traits relative to bone size and greater amounts of change in the cortex relative to bone size from this early age into adulthood. This would provide further evidence that early variation in trabecular traits leads to the changes in cortical morphological traits and would suggest allelic variation in trabecular traits influences processes of development of the whole bone structure before puberty in mice.

Mice and humans have similar developmental patterns in their biological systems (Rossant et al. 2002). Biological concepts involving functional adaption of bone trait sets from the mouse femur (Jepsen et al. 2007) have been translated to the human skeleton (Tommasini et al. 2007; Tommasini et al. 2005). Therefore, the temporal patterns of trait development and interactions in mice provided a reliable model of what may occur in humans. In addition, females represent the gender group that is highly susceptible to osteoporotic fracture risk (Melton et al. 1992; Vondracek 2010; “National Osteoporosis Foundation” 2014). Therefore, studying functional development of bones in females may help early diagnosis and treatments that can reduce fracture risk in this population.

Previous studies have shown that mineralization along with trabecular bone volume fraction are important contributors to adult vertebral body stiffness and strength (Tommasini et al. 2009). Including tissue quality traits such as tissue mineral density would be important to add to the complexity of understanding how traits interact to build mechanically functional structures and make data interpretation more difficult. Bone samples at 4 weeks of age and 16 weeks of age were scanned in a microCT system using different resolutions; therefore, the calibration of the mineral content may have
been affected due to partial volume effects. Our data showed a weak correlation between TMD values at 4 weeks of age and at 16 weeks of age in either trabecular or cortical bone regions (Tb.TMD: $R^2=0.04$, $p=0.42$; Ct.TMD: $R^2=0.04$, $p=0.40$, Appendix A-Figure 3.5). However, RI mice tended to show an increase in TMD values by 16 weeks of age similar to a previous study (Buie et al. 2008). In addition, across the RI panel, the average trabecular TMD at different ages during growth tended to be lower than average cortical TMD values as expected. Proper validation studies of the tissue mineral density components in the trabecular and cortical regions compared to the software output were not performed in this study. Therefore, interpreting changes in TMD over time or the interactions between TMD and cortical and trabecular morphological traits may be inaccurate. In addition, an advanced model of higher resolution and calibrated systems may be able to determine spatial differences in TMD within the cortical or trabecular region along the length of the vertebral structure as well as over time.

Investigating the developmental patterns among bone traits in other cortico-cancellous structures would be important to determine whether there are common biological processes in achieving mechanical function. The proximal femur has a notable dual-loading system of tension and compression regions compared to the lumbar vertebral body, which is mainly under compressive forces. We expect that a structure such as the proximal femur would rely on similar trait interactions as seen in the lumbar vertebral body. However, the nature of relationships may vary depending on applied loads, size and shape of the skeletal structure.
In conclusion, this study provided evidence that cortical and trabecular traits develop differently relative to bone size. However, we did reveal associations among traits that suggested how cortical and trabecular traits interact to develop functional bone structures. The data showed that BV/TV positively correlated with adult mechanical properties. In addition, anisotropy was positively correlated with bone size at 4 weeks of age, which suggested that trabecular architectural traits contributed to mechanical function of the whole bone vertebral body from an early age. Total cross-sectional area and cortical area showed a high positive correlation to body weight but not BV/TV, which suggested that cortical traits continued to increase with increased weight-bearing load due to a possible stress-shielding affect to the changes in the trabecular architecture. However, the amount of change in the cortical traits depended on the amount of change in bone volume fraction and trabecular alignment of the over time. This study emphasized the importance of analyzing the developmental patterns of multiple traits to explain how function is achieved. This is clinically significant because understanding trait co-development patterns relative to external bone size and the interaction of particular cortical and trabecular traits during growth to provide a more individualized assessment of bone function and early age prediction of fracture susceptibility. There are also important implications for genetic analyses and interpreting bone function during growth in response to genetic or environmental perturbations.

Acknowledgements

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References


Appendix A

ADDITIONAL LINEAR REGRESSION ANALYSES
Appendix A-Figure 3.1: Variation of (A) cortical area, (B) trabecular BV/TV, (C) RCA, (D) and trabecular anisotropy as a function of total cross-sectional area at 4 weeks of age.

Appendix A-Figure 3.2: Variation of (A) cortical area, (B) trabecular BV/TV, (C) RCA, (D) and trabecular anisotropy as a function of total cross-sectional area at 16 weeks of age.
Appendix A-Figure 3.3: Partial regression analyses to take the effects of body weight at 4 weeks of age into consideration showing that the (A) cortical area, (B) total area, (C) RCA and, (D) trabecular degree of anisotropy were not different relative to variation in BV/TV at 4 weeks of age.

Appendix A-Figure 3.4: Partial regression analyses to take the effects of body weight at 16 weeks of age into consideration showing that the (A) cortical area, (B) total area, (C) RCA and, (D) trabecular degree of anisotropy were not different relative to variation in BV/TV at 16 weeks of age.
Appendix A-Figure 3.5: Comparison of TMD values at 16 weeks of age to 4 weeks of age for each of 20 RI mouse strains. In the (A) trabecular region, and (B) cortical region, TMD values were greater at 16 weeks of age. Average trabecular TMD values across the RI panel at 4 weeks of age was $498 \pm 39$ mg/cc, and at 16 weeks of age was $661 \pm 55$ mg/cc. Average cortical TMD values at 4 weeks of age was $631 \pm 44$ mg/cc, and at 16 weeks of age was $838 \pm 48$ mg/cc.
Chapter 4

THE CONTRIBUTION OF LOAD SHARING BETWEEN CORTICAL AND TRABECULAR BONE TO THE DEVELOPMENT OF A MECHANICALLY FUNCTIONAL VERTEBRAL BODY
Introduction

In adult bone, external loading conditions are thought to influence adaptation of bone mass and architecture (Fritton et al. 2008; Frost 1994; Rubin et al. 1994; Rubin et al. 1985). How load is shared between the cortical shell and the trabecular region is important for whole bone mechanical function. In corticocancellous structures such as the lumbar vertebral body, load is shared between an outer cortical shell and trabecular centrum. In the adult human lumbar vertebral body, finite element models were used to show that under applied load, the maximum cortical load fraction was at the mid-transverse section whereas maximum trabecular load fraction was located near the endplates (Eswaran et al. 2006; Cao et al. 2001; Silva et al. 1997). In addition, cortical load fraction was shown to be proportional to cortical mass fraction at different regions along the length of the vertebral body (Eswaran et al. 2006; Cao et al. 2001). Therefore, variation in load transferred between the outer cortical shell and the internal trabecular region throughout the vertebral body is necessary to provide stiffness and strength in each vertebral segment within the spinal column (Silva et al. 1997). Few studies have associated applied load to changes in the bone morphology to better understand bone adaptation in the vertebral body. Webster et al showed load-induced increases in both cortical area and trabecular bone volume in adult B6 mouse caudal vertebral body (Webster et al. 2012). Smit et al showed a coordination of low bone volume fraction and high trabecular anisotropy in trabecular biopsies of an adult human lumbar vertebral body. This coordination of trabecular architectural traits was consistent with concepts from Wolffs’ Law of bone adaptation under regions of higher compressive load (Smit et al. 1997). In addition, Silva et al used finite element models of an adult lumbar vertebral body to show an inverse relationship between trabecular anisotropy and the load borne
by the cortical shell (Silva et al. 1997). However, it is unclear how load shared in the vertebral body influences the interaction of cortical and trabecular sets of traits.

We hypothesize that load borne by the trabecular region leads to changes in cortical area. Concepts of Wolffs’ Law indicate that trabecular alignment along principal stresses is closely related to mechanical function (Frost 1994; Smit et al. 1997). In addition, our previous study suggested that early variation in trabecular alignment may lead to an adaptive response in cortical area later during growth of the mouse lumbar vertebral body (Chapter 3). Therefore, we cannot assume that cortical or trabecular bone mass is a direct measure of how load is shared within the vertebral body. To test our hypothesis, we determined load sharing in 4 week old vertebral body samples across a panel of AXB/BXA RI mouse strains using finite element analysis. We examined mice at both 4 weeks of age and 16 weeks of age to provide a better understanding of how mechanically functional structures develop through a sequence of events during growth.

Determining relationships among load sharing at an early age, the change in trabecular architectural traits as well as the change in cortical traits from 4 weeks of age to 16 weeks of age, led to a better understanding of patterns in the development of sets of traits relative to load sharing. Therefore, our findings provided insight into a biomechanical coordination of traits to develop functional structures. RI mouse strains are a powerful tool to study the tendency of traits to co-segregate or correlate after genetic randomization of the parental genomes (Bailey 1986; Nadeau et al. 2003; Tommasini et al. 2009). In addition, a homozygous genotype within a strain allowed us to examine changes in traits between different ages in a cross-sectional study.
Material and Methods

Bone Samples

Female AXB/BXA Recombinant Inbred (RI) mouse strains were examined in this study as previously described in Chapter 3. In brief, the L4 vertebrae was harvested from 20 RI mouse strains at 4 weeks of age (n=6/strain) and 16 weeks of age (n=9-10/strain). The vertebral body from each mouse sample was examined for cortical and trabecular traits. Traits in samples at 16 weeks of age were previously examined by Tommasini et al. (Tommasini et al. 2009). However, bone traits in the adult bone were reanalyzed to ensure consistency in the methods in which values were acquired for the different ages.

Physical bone traits

L4 vertebrae samples were scanned using an eXplore Locus SP PreClinical Specimen MicroComputed Tomography (microCT) system (TriFoil Imaging, Chatsworth, CA, USA) as previously described in Chapter 3. In brief, L4 vertebrae were placed in a chamber filled with phosphate buffered saline solution. 16 week-old samples were scanned at a 16-micron voxel size, and the 4-week-old samples were scanned at an 8.7-micron voxel size. Each scan contained a calibration phantom containing air, water and hydroxyapatite (SB3; Gamex RMI, Middleton, WI, USA) to adjust mineral density measurements because of variability in X-ray attenuation inherent to each scan (Jepsen et al. 2007). MicroView Advanced Bone Analysis software (v 1.2.1 GE Healthcare) was used to reconstruct a 3D rendition of the whole bone and manually define cortical and trabecular regions of the vertebral body, as previously described (Tommasini et al.
The volume of interest consisted of trabecular bone in the secondary spongiosa and the surrounding cortical bone. Cortical and trabecular volumes were thresholded separately to differentiate calcified from non-calcified voxels (Otsu 1979). Multiple cortical and trabecular traits were obtained through the MicroView analysis software. However, for this study, we examined five morphological traits at both 4 weeks of age and 16 weeks of age to focus on variation in cortical and trabecular architectural traits across the RI mouse strains during growth. Cortical traits measured included Cortical Area (Ct.Ar), Total Area (Tt.Ar) and relative cortical area (RCA) - calculated as Ct.Ar/Tt.Ar. Trabecular traits measured included trabecular bone volume fraction (BV/TV), and trabecular degree of anisotropy (DA) in the cranial-caudal direction of compressive load. Higher DA values indicated greater alignment of the trabecular architecture in the cranial-caudal direction.

**Adult whole bone mechanical properties**

Stiffness (S) and maximum load to failure (F) for 16 week old RI mice strains were previously reported (Tommasini et al. 2009).
**Finite Element Model**

To better understand load sharing in vertebral body samples with different bone sizes and sets of cortical and trabecular traits, microCT-based finite element models were generated from L4 vertebral body samples of RI mouse strains at 4 weeks of age (n=6/strain). Examining how load was shared in the vertebral body from 4 weeks of age, we can determine relationships between load borne in the trabecular region of the bone at an early age and bone traits during growth to better understand how adult mechanical function was achieved given variation in bone size.

microCT reconstructed images of L4 vertebrae were cropped to isolate the vertebral body region (excluding transverse and spinal processes). Vertebral body images were imported into Simpleware (ScanIP v.5.0, Exeter, United Kingdom), thresholded based on Hounsfield units provided by Microview for the vertebral body region, and filtered (median) to remove noise (Figure 4.1A). To apply boundary and loading conditions to the external surfaces of the vertebral body, solid elliptical platens were created using AutoCAD (version 2002) to fit within the surface area of the endplates. The surface area of the platens simulated the region of load transferred from adjacent intervertebral discs. Using Simpleware (Scan+CAD v.1.4), platens were positioned parallel to one another to simulate a uniform axial compression scenario (Figure 4.1B, 4.1C). Boolean operations were used to remove portions of the platens that internally extended beyond the bone surface. In addition, a rectangular cuboid 1mm x 0.05mm x 2mm was generated to represent a 1mm scalebar that spanned along the length of the vertebral body. This scalebar was later used as a calibration tool to ensure that 2D transverse images extracted along the length of the vertebral body each had the
same scale during load fraction analysis. In addition, the scalebar was used to
determine the area of a pixel in each 2D transverse image (Equation 1). The calibrated
pixel size per image was then used to further calculate cortical and trabecular load
fraction values (See Material and Methods – Determining load fraction).

**Figure 4.1.** Finite element model of a mouse lumbar vertebral body. (A) 3D mesh, (B) bone mesh with
parallel elliptical platens applied to the external surfaces of the vertebral endplates, and (C) sagittal view
with added scalebar.

*FE Model - Material properties*

Both the cortical shell and trabecular region were assigned a Youngs’ Modulus, $E = 20\text{GPa}$ and a Poisson ration of 0.3 (Eswaran et al. 2006; Somerville et al. 2004; Webster et al. 2008). Female mouse bone matrix was shown to have a Young’s modulus ranging from $18\text{GPa}$ to $20\text{GPa}$ from 1 month of age to 6 months of age (Somerville et al. 2004). Although the cortical shell has a higher density of bone tissue
than the trabecular region, the cortical shell is often described as condensed trabeculae
(Roy et al. 1999; Mosekilde 1993; Silva et al. 1994). Assigning the same hard tissue
properties for the whole bone structure allowed us to focus on load sharing based on
variation in architecture among samples.
**Finite Element Analysis**

Each finite element mesh of the vertebral body was approximately 7 million elements consisting of 90% tetrahedron and 10% hexahedron and an average of 2.5 million nodes. A linear elastic finite element analysis was performed on each FE model (Abaqus v.6.10-2, SIMULIA, Dassault Systemes, Walthan, MA, USA). The area of the elliptical platens represented a contact area along the bone surface to apply boundary and loading conditions (**Figure 4.2**). The cranial end was held stationary (**Figure 4.2A**) as a uniform axial compressive force of 1N was applied upward at the inferior end of the vertebral body (Webster et al. 2012) (**Figure 4.2B**). The high resolution micro-CT based analysis was run on a Dell Precision T7500 Workstation (2 x 2.8GHz Quad-Core Intel Xeon processors). A stress distribution profile was generated throughout each bone sample. Longitudinal stress (S33) and strain (E33) values were acquired in the direction

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**Figure 4.2.** Images from the FEA software showing elliptical areas on the FEA model represent regions along the bone surface where boundary and loading conditions were applied. A) At the cranial end, a stationary boundary condition was applied indicated by circular nodules. B) At the inferior end a compressive load was applied indicated by upward arrows. C) A diagram summarizing the boundary and loading conditions.
parallel to the applied axial compressive load. Across the panel of RI mice, stress values ranged from 10 to -60 MPa. Stress intervals within this range differed among bone samples depending on bone size and geometry. Strain values were monitored to ensure they were in the physiological range (300 to -1000 microstrain) for each sample.

*Calibration of stress values*

To determine actual stress concentration values in a bone structure, the gradient of stress from the FE analysis was calibrated to grayscale values. An FE model was created in Simpleware (ScanCAD) consisting of 10 cylindrical posts measuring 10mm in diameter and 100 mm in height. As an example, for a sample that had a range of stress from 5 to -40 MPa, we conducted a calibration FE analysis where different stress magnitudes ranging from 5 to -40 MPa were applied to the bottom surface of a cylinder (compressive) as top surfaces was held stationary. A grayscale image of the mid-transverse section of cylinders was imported into ImageJ (v. 1.47v) to determine grayscale intensity values ranging from 0(black) to 255 (white) that corresponded to the different applied stress magnitudes (*Figure 4.3A*). Later, an output report from the FE analysis was examined to determine actual stress values in the S33 direction at the mid-transverse section of each cylinder. The actual stress values were plotted against the grayscale intensity values ($R^2=1$, *Figure 4.3B*). From this calibration curve, we obtained an equation of the line that was used to determine stress values for different grayscale intensities within the bone sample. A different stress range in the FE analysis of a sample required a new calibration curve and equation of a line.
Figure 4.3. Calibration of stress values from finite element analysis. A) Mid-transverse sections of calibration cylinders indicating a set stress applied to each cylinder ranging from 5 to -40 MPa. Each applied stress had a corresponding grayscale value. B) Actual stress values were plotted against grayscale intensity values to generate an equation of a line used to for stress analysis in bone samples.

**Determining load fraction**

To determine cortical and trabecular mass fraction and load fraction values along the length of the vertebral body, transverse cross-sections from the FE analysis were isolated from 20%-80% of the total length of each sample as indicated in Figure 4.4. The region of interest for the total length (0% to 100%) of the vertebral body was measured at a mid-sagittal section from the start of the secondary spongiosa along the anterior surface at both the cranial and caudal end (Bab et al. 2007). The average length of the vertebral body samples...
across the RI panel was $1.4 \pm 0.11\text{mm}$ with an 8% variation among all samples. We interpolated the distances of sections from 20% to 80% starting from the caudal end.

Representative images of transverse cross-sections from 20% to 80% along the length of the vertebral body are shown in **Figure 4.5**. Sections were converted to grayscale and imported into ImageJ software. For each section, a histogram was generated indicating the number of pixels for each grayscale intensity value within the total cross-sectional area, the cortical area and the trabecular area. Grayscale values that equaled 0 or 255 (typically accounting for less than 8 pixels) were discarded since they represented extreme stress concentrations outside of the indicated stress range.

To determine load fraction and mass fraction in each of the total area, cortical area and trabecular area region in each transverse section, the following equations were applied:

The length of a pixel was determined by the reciprocal of the number of pixels along the 1mm scalebar, therefore,

\[
\text{The area of a pixel} = \left(\text{length of 1 pixel}\right)^2 \text{mm}^2
\]  
**Equation 1**

\[
\text{The area of a grayscale intensity \(m^2\)} = \left(\text{number of pixels per grayscale intensity}\right) \times \text{Equation 1}
\]  
**Equation 2**

The stress value of particular grayscale intensity was calculated from the equation of line from the calibration curve:

\[
\text{Stress value (MPa)} = m(\text{grayscale intensity}) + b
\]  
**Equation 3**

Based on the equation, Force (N) = Stress (MPa) \times Area (mm$^2$),  
**Equation 4**
The load for a grayscale intensity = Equation 3 x Equation 2 \[ \text{Equation 5} \]

Load for a particular region was equal to the sum of the load for each grayscale intensity value and the area was equal to the sum of the areas for each grayscale intensity value. The trabecular load fraction was the ratio of the load in the trabecular region to the load in the total section. Trabecular mass fraction was the ratio of area in the trabecular region to the area of the total section. The maximum trabecular load fraction was equivalent to the minimum cortical load fraction (cortical load fraction=1-trabecular load fraction). An average trabecular load fraction for each RI mouse strain (n=6/strain) was reported for each position from 20% to 80% along the length of the vertebral body. The average of trabecular load fraction values from 20%-80% for each strain was used to examine relationships between trabecular load fraction and bone traits at 4 weeks of age and 16 weeks of age.

Parametric analyses were conducted to determine the reliability of the FEA model to provide consistent results on how load was shared between the cortical and trabecular region in the vertebral body (Appendix B). We analyzed variations in threshold values, position of the external surfaces when applying boundary and loading conditions and applied load. How load was shared between bone types in in each sample was not experimentally validated as shown in previous studies (Webster et al. 2012; Webster et al. 2008). This procedure utilized microFE models, which was impractical for this study based on available laboratory resources. However, these analyses showed that load sharing in vertebral bodies was invariant to the parameters chosen in our finite element model. Therefore, we generated a working model to analyze load sharing in different samples. In addition, the parameters used in this study
were based on optimal and physiologically relevant conditions that were used in previous literature (Eswaran et al. 2006; Webster et al. 2012; Somerville et al. 2004; Yang et al. 2012).

**Statistical Analysis**

Average trabecular and cortical load fraction and mass fraction across the panel of RI mouse strains and within each RI strain was represented as mean ± one standard deviation. In linear and multiple regression analyses, significant correlations were considered at p<0.05 (GraphPad Software, 5.0, San Diego, CA). To understand how load sharing and bone traits at an early age contributed to changes in traits over time, a sorting analysis of traits was conducted across the RI panel. Residuals from the Tt.Ar-BW regression was sorted from negative to positive indicating total area values that were small to large for body size, respectfully. Strains were then segregated into tertiles, where each tertile consisted of 6-7 mouse strains. Within each tertile, the data was sorted by the degree of trabecular anisotropy at 4 weeks of age, then subdivided into groups that showed a low or high anisotropy (n=3 to 4 strains/ anisotropy subgroup). Within each tertile, a Students't-test was used to compare average trait values from RI mice with low anisotropy to trait values from RI mice with high anisotropy. Significance was indicated by ** for p<0.05, or * for p<0.08 (Microsoft Excel 2010).
Results

Stress and area distributions along the length of the vertebral body

Representative images from FEA show variation in cross-sectional area, proportions of cortical and trabecular bone and stress distributions at different locations along the length of the mouse vertebral body (Figure 4.5). Toward the caudal end at the 20%-30% positions, there was a higher trabecular area compared to cortical in the cross section slice. At the 50% to 80% positions, cross-sections showed a thicker cortex and less trabecular bone. Low compressive stress (positive value) was indicated by a red color at the top of the stress gradient, yellow or green at the center of the stress gradient indicated moderate compressive stress concentrations (negative value), followed by blue at the bottom of the stress gradient indicated high compressive stress concentrations. In these representative images, the different positions throughout the bone structure show an orange to green color gradient indicating moderate stress levels, which was expected since bone architecture is built to minimize high stress concentrations. Lower compressive stress was observed near the posterior end of the vertebral body compared to more moderate stress values near the anterior end.
Figure 4.5. (Above) Coronal view of a mouse lumbar vertebral body with stress distributions from finite element analysis. (Below) Visualization of transverse sections from 20% to 80% along the length of the vertebral body showing distribution of stress at each position.
Variation in microarchitectural load fraction along the length of the vertebral body

The amount of load taken by the trabecular region depended on the axial distance from the endplates (Figure 4.6). At each position along the length of the vertebral body, the trabecular load fraction value was inversely proportional to the cortical load fraction value. Across the panel of 20 RI mouse strains, the maximum trabecular load fraction occurred at the caudal end of the vertebral body (position at 20% of the vertebral body length) and ranged in value from 0.71 to 0.52 with an average value of 0.61 ± 0.05 (Table 4.1). The maximum cortical load fraction was near the cranial end (position at 70%) and ranged in value from 0.81 to 0.60 with an average value of 0.72 ± 0.05. Near the caudal end at the 20% and 30% positions, the coefficient of variation was 9% and 10%, respectfully, for both trabecular and cortical load fraction across the RI panel. The coefficient of variation for mass fraction in the different bone regions at the caudal end was approximately 10%. At the mid-section and cranial end (positions from 50% to 80%), trabecular load fraction showed a coefficient of variation of 16% to 20% with a 12% to 16% coefficient of variation in trabecular mass fraction. The coefficient of variation for cortical load fraction and mass fraction in the midsection to cranial end was approximately 10% across the RI panel.
Figure 4.6. Variation of trabecular load fraction across transverse slices 20% (caudal) to 80% (cranial) along the length of the lumbar vertebral body for 20 RI strains.

Within a RI strain, average load fraction values from 20% to 80% along the length of the vertebral body positively correlated with the average mass fraction values ($R^2=0.91$, p<0.0001, Figure 4.7). This was expected where regions with a small area would bear a lower load than regions with a larger area. The average trabecular load fraction for the 20 RI mouse strains was $0.39 \pm 0.05$ where the average coefficient of variation across the RI panel was $31 \pm 7\%$ along the length of the vertebral body (Table 4.2). The average trabecular mass fraction was $0.43 \pm 0.04$ where the average coefficient of variation across the RI panel was $10 \pm 4\%$. Cortical values were inversely proportional to trabecular values so that the average cortical load fraction for the 20 RI mouse strains was $0.61 \pm 0.05$ where the average coefficient of variation across the RI panel was $20 \pm 3\%$, and the average cortical mass fraction was $0.57 \pm 0.04$ where the average coefficient of variation across the RI panel was $7 \pm 2\%$. 
Figure 4.7. Variation of load fraction relative to mass fraction along the length of the vertebral body in the (A) trabecular and (B) cortical region. Each point represents the average value of transverse sections from 20% to 80% for each RI strain (n=6/strain).
Table 4.1. Average trabecular and cortical bone load fraction, mass fraction, and coefficient of variation values across a panel of 20 AXB/BXA RI mouse strains at 4 weeks of age at different positions along the length of the vertebral body (20% to 80%).

<table>
<thead>
<tr>
<th>Position along length of bone (%)</th>
<th>Number of RI Strains</th>
<th>Trabecular Load Fraction</th>
<th>COV (%)</th>
<th>Trabecular Mass Fraction</th>
<th>COV (%)</th>
<th>Cortical Load Fraction</th>
<th>COV (%)</th>
<th>Cortical Mass Fraction</th>
<th>COV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (caudal)</td>
<td>20</td>
<td>0.61 ± 0.05</td>
<td>9%</td>
<td>0.49 ± 0.04</td>
<td>9%</td>
<td>0.39 ± 0.05</td>
<td>14%</td>
<td>0.51 ± 0.04</td>
<td>9%</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>0.49 ± 0.05</td>
<td>10%</td>
<td>0.44 ± 0.04</td>
<td>9%</td>
<td>0.51 ± 0.05</td>
<td>10%</td>
<td>0.56 ± 0.04</td>
<td>7%</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>0.42 ± 0.05</td>
<td>13%</td>
<td>0.46 ± 0.05</td>
<td>10%</td>
<td>0.58 ± 0.05</td>
<td>9%</td>
<td>0.54 ± 0.05</td>
<td>9%</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>0.35 ± 0.06</td>
<td>16%</td>
<td>0.43 ± 0.05</td>
<td>12%</td>
<td>0.65 ± 0.06</td>
<td>9%</td>
<td>0.57 ± 0.05</td>
<td>9%</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
<td>0.30 ± 0.06</td>
<td>20%</td>
<td>0.40 ± 0.06</td>
<td>16%</td>
<td>0.70 ± 0.06</td>
<td>9%</td>
<td>0.60 ± 0.06</td>
<td>11%</td>
</tr>
<tr>
<td>70</td>
<td>20</td>
<td>0.28 ± 0.06</td>
<td>19%</td>
<td>0.38 ± 0.05</td>
<td>13%</td>
<td>0.72 ± 0.06</td>
<td>8%</td>
<td>0.62 ± 0.05</td>
<td>8%</td>
</tr>
<tr>
<td>80 (cranial)</td>
<td>20</td>
<td>0.30 ± 0.05</td>
<td>16%</td>
<td>0.41 ± 0.05</td>
<td>13%</td>
<td>0.70 ± 0.05</td>
<td>7%</td>
<td>0.59 ± 0.05</td>
<td>9%</td>
</tr>
<tr>
<td>RI strain</td>
<td>N</td>
<td>Trabecular Load Fraction</td>
<td>COV (%)</td>
<td>Trabecular Mass Fraction</td>
<td>COV (%)</td>
<td>Cortical Load Fraction</td>
<td>COV (%)</td>
<td>Cortical Mass Fraction</td>
<td>COV (%)</td>
</tr>
<tr>
<td>-----------</td>
<td>---</td>
<td>--------------------------</td>
<td>---------</td>
<td>--------------------------</td>
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<td>-----------------------</td>
<td>---------</td>
</tr>
<tr>
<td>AXB1</td>
<td>6</td>
<td>0.32 ± 0.13</td>
<td>42%</td>
<td>0.35 ± 0.04</td>
<td>11%</td>
<td>0.68 ± 0.13</td>
<td>20%</td>
<td>0.65 ± 0.04</td>
<td>6%</td>
</tr>
<tr>
<td>AXB2</td>
<td>6</td>
<td>0.40 ± 0.11</td>
<td>28%</td>
<td>0.43 ± 0.03</td>
<td>7%</td>
<td>0.60 ± 0.11</td>
<td>19%</td>
<td>0.57 ± 0.03</td>
<td>5%</td>
</tr>
<tr>
<td>AXB4</td>
<td>6</td>
<td>0.34 ± 0.12</td>
<td>36%</td>
<td>0.37 ± 0.03</td>
<td>10%</td>
<td>0.66 ± 0.12</td>
<td>19%</td>
<td>0.63 ± 0.03</td>
<td>5%</td>
</tr>
<tr>
<td>AXB5</td>
<td>6</td>
<td>0.33 ± 0.14</td>
<td>42%</td>
<td>0.37 ± 0.06</td>
<td>17%</td>
<td>0.67 ± 0.14</td>
<td>20%</td>
<td>0.63 ± 0.06</td>
<td>10%</td>
</tr>
<tr>
<td>AXB6</td>
<td>6</td>
<td>0.45 ± 0.15</td>
<td>33%</td>
<td>0.46 ± 0.06</td>
<td>13%</td>
<td>0.55 ± 0.15</td>
<td>27%</td>
<td>0.54 ± 0.06</td>
<td>11%</td>
</tr>
<tr>
<td>AXB8</td>
<td>6</td>
<td>0.32 ± 0.11</td>
<td>36%</td>
<td>0.36 ± 0.04</td>
<td>11%</td>
<td>0.68 ± 0.11</td>
<td>17%</td>
<td>0.64 ± 0.04</td>
<td>6%</td>
</tr>
<tr>
<td>AXB10</td>
<td>6</td>
<td>0.41 ± 0.12</td>
<td>30%</td>
<td>0.45 ± 0.02</td>
<td>5%</td>
<td>0.59 ± 0.12</td>
<td>21%</td>
<td>0.55 ± 0.02</td>
<td>4%</td>
</tr>
<tr>
<td>AXB12</td>
<td>6</td>
<td>0.47 ± 0.08</td>
<td>18%</td>
<td>0.49 ± 0.02</td>
<td>5%</td>
<td>0.53 ± 0.08</td>
<td>16%</td>
<td>0.51 ± 0.02</td>
<td>5%</td>
</tr>
<tr>
<td>AXB13</td>
<td>6</td>
<td>0.40 ± 0.11</td>
<td>26%</td>
<td>0.43 ± 0.05</td>
<td>11%</td>
<td>0.60 ± 0.11</td>
<td>18%</td>
<td>0.57 ± 0.05</td>
<td>9%</td>
</tr>
<tr>
<td>AXB15</td>
<td>6</td>
<td>0.42 ± 0.09</td>
<td>22%</td>
<td>0.45 ± 0.04</td>
<td>9%</td>
<td>0.58 ± 0.09</td>
<td>16%</td>
<td>0.55 ± 0.04</td>
<td>8%</td>
</tr>
<tr>
<td>AXB18</td>
<td>6</td>
<td>0.39 ± 0.11</td>
<td>27%</td>
<td>0.44 ± 0.04</td>
<td>8%</td>
<td>0.61 ± 0.11</td>
<td>17%</td>
<td>0.56 ± 0.04</td>
<td>6%</td>
</tr>
<tr>
<td>AXB19</td>
<td>6</td>
<td>0.35 ± 0.12</td>
<td>34%</td>
<td>0.41 ± 0.06</td>
<td>15%</td>
<td>0.65 ± 0.12</td>
<td>18%</td>
<td>0.59 ± 0.06</td>
<td>10%</td>
</tr>
<tr>
<td>AXB20</td>
<td>6</td>
<td>0.40 ± 0.11</td>
<td>28%</td>
<td>0.44 ± 0.03</td>
<td>6%</td>
<td>0.60 ± 0.11</td>
<td>18%</td>
<td>0.56 ± 0.03</td>
<td>5%</td>
</tr>
<tr>
<td>AXB23</td>
<td>6</td>
<td>0.38 ± 0.16</td>
<td>41%</td>
<td>0.40 ± 0.08</td>
<td>19%</td>
<td>0.62 ± 0.16</td>
<td>25%</td>
<td>0.60 ± 0.08</td>
<td>13%</td>
</tr>
<tr>
<td>AXB24</td>
<td>6</td>
<td>0.48 ± 0.13</td>
<td>27%</td>
<td>0.48 ± 0.04</td>
<td>8%</td>
<td>0.52 ± 0.13</td>
<td>24%</td>
<td>0.52 ± 0.04</td>
<td>7%</td>
</tr>
<tr>
<td>BXA7</td>
<td>6</td>
<td>0.36 ± 0.14</td>
<td>39%</td>
<td>0.40 ± 0.05</td>
<td>12%</td>
<td>0.64 ± 0.14</td>
<td>22%</td>
<td>0.60 ± 0.05</td>
<td>8%</td>
</tr>
<tr>
<td>BXA14</td>
<td>6</td>
<td>0.46 ± 0.13</td>
<td>28%</td>
<td>0.49 ± 0.04</td>
<td>9%</td>
<td>0.54 ± 0.13</td>
<td>23%</td>
<td>0.51 ± 0.04</td>
<td>9%</td>
</tr>
<tr>
<td>BXA17</td>
<td>6</td>
<td>0.44 ± 0.13</td>
<td>29%</td>
<td>0.49 ± 0.04</td>
<td>8%</td>
<td>0.56 ± 0.13</td>
<td>22%</td>
<td>0.51 ± 0.04</td>
<td>7%</td>
</tr>
<tr>
<td>BXA25</td>
<td>6</td>
<td>0.43 ± 0.12</td>
<td>31%</td>
<td>0.45 ± 0.05</td>
<td>11%</td>
<td>0.57 ± 0.13</td>
<td>24%</td>
<td>0.55 ± 0.05</td>
<td>9%</td>
</tr>
<tr>
<td>BXA26</td>
<td>6</td>
<td>0.38 ± 0.12</td>
<td>31%</td>
<td>0.44 ± 0.04</td>
<td>9%</td>
<td>0.62 ± 0.12</td>
<td>20%</td>
<td>0.56 ± 0.04</td>
<td>7%</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>0.39 ± 0.05</td>
<td>31%±7%</td>
<td>0.43 ± 0.04</td>
<td>10%±4%</td>
<td>0.61 ± 0.05</td>
<td>20%±3%</td>
<td>0.57 ± 0.04</td>
<td>7%±2%</td>
</tr>
</tbody>
</table>

Table 4.2. Trabecular and cortical load fraction, mass fraction and coefficient of variation values along the length of the vertebral body (average of cross-sectional positions from 20% to 80%) for 20 RI strains at 4 weeks of age. Data shown as mean ± standard deviation.
Load sharing in the vertebral body versus body size and bone size

At 4 weeks of age, load borne by the trabecular region weakly correlated with body size and bone size (BW versus Tb.LF, $R^2=0.08$, $p=0.24$; Tt.Ar versus Tb.LF, $R^2=0.07$, $p=0.25$, Figure 4.8). There was a trend where mice that tended to have a low trabecular load fraction in the vertebral body tended to have a low body weight and small external bone size. At 16 weeks of age, there appeared to be a slight improvement to the correlation between trabecular load fraction from 4 weeks of age and either body weight and total cross sectional area values across the RI panel (Tb.LF versus BW, $R^2=0.14$, $p=0.11$; Tb.LF versus TtAr, $R^2=0.16$, $p=0.08$, Figure 4.9). Thus, body size and bone size were weak predictors of load sharing.
Figure 4.8. Variation in trabecular load fraction relative to (A) body weight and (B) total cross-sectional area at 4 weeks of age.

Figure 4.9. Variation in (A) body weight and (B) total cross-sectional area at 16 weeks of age as a function of trabecular load fraction at 4 weeks of age.
The interaction of load shared in the vertebral body from an early age and bone traits during growth

To understand the relationship between load shared in the vertebral body and micro-architectural traits across the RI panel at 4 weeks of age, we plotted trabecular load fraction values relative to cortical and trabecular traits. We assumed at an early age, morphological traits would influence how load was distributed between cortical and trabecular bone. We expected that the load borne by the trabecular region would be negatively correlated with cortical area and positively correlated with trabecular bone volume fraction. At 4 weeks of age, load borne by the trabecular bone along the length of the vertebral body was not correlated with cortical area ($R^2=0.001$, $p=0.88$, Figure 4.10A). However there was a strong positive correlation between trabecular load fraction and trabecular bone volume fraction (BV/TV) ($R^2=0.75$, $p<0.0001$, Figure 4.10B). RCA (Figure 4.10C) and Anisotropy (DA) (Figure 4.10D) showed a weak negative relationship with trabecular load fraction (RCA versus Tb.LF, $R^2=0.15$, $p=0.09$; DA versus Tb.LF, $R^2=0.09$, $p=0.21$). At 16 weeks of age, the trabecular load fraction from 4 weeks of age continued to have a strong positive relationship with adult BV/TV values ($R^2=0.60$, $p<0.0001$, Figure 4.11B). There was a trend where adult cortical area values was positively correlated with the trabecular load fraction from 4 weeks of age (Tb.LF versus Ct.Ar, $R^2=0.09$, $p=0.20$, Figure 4.11A). Adult RCA (Figure 4.11C) and anisotropy values (Figure 4.11D) did not appear to be different relative to the load borne by the trabecular region at an early age (Tb.LF versus RCA, $R^2=0.01$, $p=0.62$; Tb.LF versus DA, $R^2=0.03$, $p=0.49$).
Figure 4.10. Variation in trabecular load fraction at 4 weeks of age as a function of (A) cortical area (B) trabecular BV/TV, (C) relative cortical area, and (D) anisotropy at 4 weeks of age.

Figure 4.11. Variation in (A) cortical area (B) trabecular BV/TV, (C) relative cortical area, and (D) anisotropy at 16 weeks of age as a function of trabecular load fraction at 4 weeks of age.
Based on the positive trend that we observed in Figure 4.8A between load fraction and body weight at 4 weeks of age, we conducted partial correlation regression analyses taking into consideration the effects of bodyweight to identify how the load borne by the trabecular region co-varied with bone traits at this early age. The significance for correlations between Ct.Ar and Tb.LF or DA and Tb.LF was improved compared to the unadjusted data, which indicated that these bone traits may have been influenced by body weight. Results continued to show a strong relationship between BV/TV and trabecular load fraction at 4 weeks of age (Figure 4.12B). RI mice that tended to have a low BV/TV tended to have a low load borne by the trabecular region (or a high load borne by the cortical region) ($R^2=0.73$, $p<0.0001$). There were weak relationships between trabecular load fraction and trabecular anisotropy ($R^2=0.14$, $p=0.10$, Figure 4.12D), cortical area ($R^2=0.10$, $p=0.16$, Figure 4.12A), and RCA ($R^2=0.12$, $p=0.14$, Figure 4.12C).
Figure 4.12. Partial regression analyses conducted to take the effects of body weight at 4 weeks of age into consideration showed that trabecular load fraction at 4 weeks of age had a weak relationship with (A) cortical area; (C) RCA, and (D) trabecular anisotropy yet, was positively correlated with 4 week old (B) trabecular BV/TV values.
To further understand whether load shared within the vertebral body at an early age affected how traits developed from 4 weeks of age to 16 weeks of age, we plotted the percent change in bone traits relative to the load borne by the trabecular region taking into account the effects of body weight at 4 weeks of age. RI mice that tended to show a low trabecular load fraction (or high cortical load fraction) at 4 weeks of age, tended to show an increase in BV/TV (positive change) over time ($R^2=0.19$, $p=0.05$, Figure 4.13A). The simple linear regressions showed that the percentage change in trabecular anisotropy, cortical area, RCA or total area tended to not be different relative to the load borne by the trabecular region at 4 weeks of age ($\%\Delta DA$, $R^2=0.02$, $p=0.51$; $\%\Delta$ Ct.Ar, $R^2=0.10$, $p=0.17$; $\%\Delta$RCA, $R^2=0.02$, $p=0.59$; $\%\Delta$TtAr, $R^2=0.09$, $p=0.20$, Figure 4.13B-E).

**Figure 4.13.** Partial regression analyses conducted to take the effects of body weight at 4 weeks of age into consideration showed that the percent changes from 4 weeks of age to 16 weeks of age in (A) BV/TV had a positive correlation with trabecular load fraction at 4 weeks of age. The change in (B) Anisotropy, (C) cortical area, (D) RCA, and (E) total cross-sectional area over time were not different relative to the trabecular load fraction at an early age.
Multiple linear regressions showed that load sharing from an early age contributed to the change in cortical and trabecular traits from 4 weeks of age to 16 weeks of age (Table 4.3). Using best subsets regression analysis, the data showed that 58.5% of the variation in the change in BV/TV in RI mouse strains was explained by the change in anisotropy, Tb.LF at 4 weeks of age, the change in RCA, the change in Ct.Ar and the change in body weight ($R^2=0.59$, $p=0.003$). Of these traits, the change in anisotropy, RCA and Ct.Ar and Tb.LF were significant contributors. 43.3% of the variation in the change in anisotropy over time was explained by Tb.LF, the change in Ct.Ar, the change in body weight and the change in BV/TV over time ($R^2=0.43$, $p=0.012$). Of these traits, Tb.LF, and the change in Ct.Ar and BV/TV were significant contributors where Tb.LF was a primary contributor to the variation in change in anisotropy across the RI panel. Approximately 65% of the variation in the change in cortical area was explained by the change in anisotropy, the change in body weight, Tb.LF, and the change in BV/TV over time ($R^2=0.65$, $p=0.001$). Of these traits, the change in anisotropy and the change in bodyweight were significant contributors not Tb.LF at 4 weeks of age. 52.1% of the variation in the change in RCA was explained by the change in BV/TV, Tb.LF and body weight at 4 weeks of age ($R^2=0.52$, $p=0.002$). The variation in the change in RCA was primarily influenced by the change in BV/TV followed by body weight at an early age. Therefore, trabecular load fraction was not a significant contributor to the change in cortical traits over time.
**Equation#**

<table>
<thead>
<tr>
<th>Equation</th>
<th>R^2-adj</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Δ BV/TV = -0.82 - 2.33 %Δ DA + 1.73 Tb.LF4 + 1.52 %Δ RCA -1.35 %Δ Ct.Ar + 0.47 %Δ BW</td>
<td>58.5%</td>
<td>0.003</td>
</tr>
<tr>
<td>%Δ DA = -0.21 + 0.40 Tb.LF4 - 0.28 %Δ Ct.Ar + 0.14 %Δ BW - 0.13 %ΔBV/TV</td>
<td>43.3%</td>
<td>0.012</td>
</tr>
<tr>
<td>%Δ RCA = -0.22 + 0.27 %ΔBV/TV - 0.18 Tb.LF4 + 0.03 BW</td>
<td>52.1%</td>
<td>0.002</td>
</tr>
<tr>
<td>%Δ Cortical Area = -0.32 - 1.07 %Δ DA + 0.58 %Δ BW + 0.42 Tb.LF4 + 0.27 %ΔBV/TV</td>
<td>64.8%</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Bold font indicates traits making significant contributions to the variation in % change in bone trait (p<0.05).*

BW=Body Weight; BV/TV = Trabecular bone volume fraction; DA = Degree of Anisotropy; Ct.Ar = Cortical Area; RCA = Relative Cortical Area; Tb.LF = Trabecular Load Fraction

**Table 4.3.** Multiple Linear Regression analysis of traits that contribute to the change in cortical and trabecular traits.

*Load sharing at an early age was predictive of adult mechanical properties*

The load borne by the trabecular region of the vertebral body at 4 weeks of age was positively correlated with adult stiffness (R^2=0.21, p=0.04, **Figure 4.14A**) and maximum load to failure (R^2=0.29, p=0.01, **Figure 4.14B**). We conducted multiple linear regressions to assess the relative contribution of trabecular load fraction and bone traits to adult mechanical properties (**Table 4.4**). A best subsets analysis showed that approximately 65% of the variation in adult bone stiffness was explained by the change in BV/TV, Tb.LF at 4 weeks of age and body weight at 4 weeks of age (R^2=0.649, p=0.001). Of these traits, the change in BV/TV and the body weight at an early age were significant contributors where the change in BV/TV accounted for the majority of the variation in adult stiffness. 85.2% of the variation in maximum load to failure of the whole vertebral body was explained by RCA at 16 weeks of age, Tb.LF, the change in
BV/TV, body weight at 4 weeks of age, and the change in anisotropy over time ($R^2=0.852$, $p=0.001$). Of these traits, adult RCA, Tb.LF, the change in BV/TV and body weight at 4 weeks of age were significant contributors. This indicated that load borne by the trabecular bone at an early age was a predictor of the adult mechanical strength of whole bone structure.

Figure 4.14. Variation in (A) stiffness and (B) maximum load to failure values in 16 week old mice relative to trabecular load fraction at 4 weeks of age.

<table>
<thead>
<tr>
<th>Equation#</th>
<th>$R^2$-adj</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiffness = -64.3 + 107 %Δ BV/TV + 56.8 Tb.LF$_4$ + 12.56 BW$_4$</td>
<td>64.9%</td>
<td>0.001</td>
</tr>
<tr>
<td>Max Load = -52.7 + 154 RCA$_{16}$ + 43.2 Tb.LF$_4$ + 13 %Δ BV/TV + 1.7 BW$_4$ - 22.6 %Δ DA</td>
<td>85.2%</td>
<td>0.001</td>
</tr>
</tbody>
</table>

#Bold font indicates traits making significant contributions to the variation in stiffness or maximum load to failure ($p<0.05$).

BW=Body Weight; BV/TV = Trabecular bone volume fraction; DA = Degree of Anisotropy; RCA= Relative Cortical Area; Tb.LF=Trabecular Load Fraction

Table 4.4: Multiple Linear Regression analysis of traits that contribute to whole bone mechanical properties of the lumbar vertebral body.
Understanding how load sharing and bone traits at an early age contribute to growth patterns relative to bone size

Load fraction was weakly correlated with body size or bone size. Therefore, further data interpretation was needed to understand how load shared in the vertebral body at an early age would explain functional interactions among cortical and trabecular traits during growth relative to bone size. Our previous study showed that at 4 weeks of age, there was a significant negative correlation between trabecular anisotropy and total cross sectional area of the vertebral body across a panel of AXB/BXA RI mouse strains (Chapter 3, Figure 3.7D). RI mice that tended to have a small cross-sectional bone size tended to show a greater trabecular alignment than mice with a larger bone size. Therefore, we conducted a sorting analysis of traits from the panel of 20 RI mouse strains based on residuals from the Tt.Ar-BW regression, which was then separated into tertiles of total areas that were small, medium, and large for body size. Within each tertile, traits were further sorted based on trabecular anisotropy at 4 weeks of age. Table 4.5 is a summary of average trait values of strains that showed a small bone for size or larger bone for size and was further subdivided into groups of either a low or high degree of anisotropy. It is important to note that in examining patterns relative to bone size, it was difficult to test for significance with 3-4 samples per subdivision of anisotropy. Therefore, interpretation of patterns among traits during growth was primarily based on observed differences of trait values.
<table>
<thead>
<tr>
<th>Trait</th>
<th>Small bone for size (Small Tt.Ar₄-BW₄ residual tertile)</th>
<th>Large bone for size (Large Tt.Ar₄-BW₄ residual tertile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low DA₄</td>
<td>High DA₄</td>
</tr>
<tr>
<td>DA₄</td>
<td>1.987 ± 0.061 **</td>
<td>2.081 ± 0.027</td>
</tr>
<tr>
<td>BV/TV₄</td>
<td>0.228 ± 0.026</td>
<td>0.216 ± 0.040</td>
</tr>
<tr>
<td>Ct.Ar₄ (mm²)</td>
<td>0.216 ± 0.002</td>
<td>0.229 ± 0.013</td>
</tr>
<tr>
<td>Tt.Ar₄ (mm²)</td>
<td>1.01 ± 0.12</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>RCA₄</td>
<td>0.22 ± 0.03</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>BW₄ (g)</td>
<td>12.3 ± 1.5</td>
<td>13.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>%ΔDA 0.053 ± 0.033</td>
<td>-0.027 ± 0.038</td>
</tr>
<tr>
<td></td>
<td>%ΔBV/TV 0.027 ± 0.118</td>
<td>-0.107 ± 0.182</td>
</tr>
<tr>
<td></td>
<td>%ΔCt.Ar 0.252 ± 0.123</td>
<td>0.227 ± 0.121</td>
</tr>
<tr>
<td></td>
<td>%ΔTt.Ar 0.210 ± 0.047</td>
<td>0.198 ± 0.069</td>
</tr>
<tr>
<td></td>
<td>%ΔRCA 0.037 ± 0.092</td>
<td>0.022 ± 0.046</td>
</tr>
<tr>
<td></td>
<td>%ΔBW 0.593 ± 0.092</td>
<td>0.499 ± 0.060</td>
</tr>
<tr>
<td>Tb.LF₄</td>
<td>0.39 ± 0.05</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>Ct.LF₄</td>
<td>0.61 ± 0.05</td>
<td>0.61 ± 0.08</td>
</tr>
<tr>
<td>Stiffness₁₆ (N/mm)</td>
<td>111.5 ± 40.2</td>
<td>119.3 ± 39.8</td>
</tr>
<tr>
<td>Max. Load₁₆ (N)</td>
<td>20.7 ± 5.8</td>
<td>20.1 ± 8.1</td>
</tr>
<tr>
<td>Tb TMD₄ (mg/cc)</td>
<td>506 ± 35</td>
<td>518 ± 38</td>
</tr>
<tr>
<td>Tb TMD₁₆ (mg/cc)</td>
<td>664 ± 54</td>
<td>668 ± 61</td>
</tr>
<tr>
<td>Ct TMD₄ (mg/cc)</td>
<td>632 ± 31</td>
<td>640 ± 47</td>
</tr>
<tr>
<td>Ct TMD₁₆ (mg/cc)</td>
<td>830 ± 36</td>
<td>836 ± 33</td>
</tr>
</tbody>
</table>

**Table 4.5.** Variation in architecture, composition, body size, and load fraction at 4 weeks of age as well as the percentage change in traits from 4 weeks of age to 16 weeks of age, and adult whole bone stiffness strength and composition for RI mouse strains that have a low or high degree of anisotropy (DA) relative to bone size. Students’ t-tests were performed between samples that had a low DA value and high DA value for a given total area tertile. Significant difference indicated by **: p<0.05, *: p<0.08.
We identified patterns among traits relative to bone size and differences in anisotropy to understand how bone structures developed between 4 weeks of age and 16 weeks of age. Mice that tended to have a small bone for size had an average total area of 1.03 ± 0.08 mm² at 4 weeks of age whereas those that tended to have a large bone for size had an average total area of 1.18 ± 0.04 mm² (p < 0.001). Cortical area for mice that tended to have a small bone size was significantly smaller than mice that tended to have a large bone for size (p < 0.05). RCA, and BV/TV values at 4 weeks of age were not different relative to bone size (p > 0.10) however, we observed trends in these traits at an early age and trends in the percentage change in traits over time. As illustrated in Figure 4.15, an inverse relationship between BV/TV and anisotropy was seen across the different bone sizes. Variation in bone size showed different patterns in the percentage of change in cortical and trabecular traits over time. The expected positive relationship between BV/TV and trabecular load fraction was mainly seen among mice that tended to show a larger bone for size. RI mice that tended to have a large bone for size and high BV/TV tended to show a trabecular load fraction of 0.42 ± 0.04, which was greater than RI mice that tended to have a low BVTV with a trabecular load fraction value of 0.37 ± 0.02 (p = 0.075). RI mice that tended to show a small bone size, and high trabecular anisotropy at 4 weeks of age, tended to show a low BV/TV at this early age. However, these RI mice tended to show an average trabecular load fraction value of 0.39 that was not different (p = 0.920) based on anisotropy or BV/TV values.

Across the RI panel, between 4 weeks of age and 16 weeks of age, mice tended to show a decrease in trabecular bone volume fraction and alignment over time. RI mice
also tended to show an increase in cortical area and total area over time. However, the amount of change in bone traits was different based on the early coordination of trabecular architectural traits. RI mice that tended to show low trabecular alignment and high trabecular bone volume fraction from an early age, tended to show a small decrease in bone volume fraction and a large increase in cortical area and total area over time. However, mice that tended to have a small bone for size and low anisotropy at 4 weeks of age tended to show greater decrease in trabecular alignment and greater increase in RCA over time compared to mice that were large for size and had low trabecular anisotropy at an early age.
Figure 4.15. Representative images of the development of mouse lumbar vertebral bodies from 4 weeks of age to 16 weeks of age relative to variation in bone size and anisotropy at 4 weeks of age. Percentage change in cortical and trabecular traits over time indicate different trait development patterns for each scenario.
**Discussion**

The objective of this study was to determine how load sharing in the lumbar vertebral body at an early age could explain functional interactions among cortical and trabecular traits during growth. A correlation analysis across a panel of AXB/BXA RI mice showed a strong relationship between load fraction at 4 weeks of age and trabecular bone volume fraction during growth. Surprisingly, load fraction was not correlated to cortical area or total area, which suggested that early variation in trabecular BV/TV was important for whole bone stiffness and strength throughout growth. The correlation between load sharing and BV/TV at an early age was not consistent with variation in bone size. RI mice that tended to show a large bone for size, tended to follow the pattern of a positive correlation between trabecular load fraction and BV/TV. However, RI mice that tended to show a small bone for size, tended to show a similar trabecular load fraction value with variation in BV/TV. This discrepancy suggested that load sharing was important for structural mechanical function, but could not directly explain how sets of traits develop. In our previous study, a correlation analysis among cortical and trabecular traits across a panel of AXB/BXA RI mice showed that at 4 weeks of age, trabecular alignment negatively correlated with total area taking body weight into consideration (Chapter 3). This finding suggested that early variation in trabecular alignment relative to bone size was important to maintain function at an early age. Further analysis of trait patterns across the RI panel showed that an inverse coordination between anisotropy and BV/TV at 4 weeks of age was consistent among bones with different external sizes. RI mice that tended to show a low anisotropy and high BV/TV at this early age, tended to show a developmental pattern of
a small decrease in BV/TV, and large increase in cortical area and total area over time (Table 4.5). This coordination of traits suggested that the amount of change in the cortical shell during growth depended on interactions among trabecular traits at an early age. Therefore, the data provided insight into the developmental patterns of traits in different bone structures, which extended the concept of Wolff’s law toward a better understanding of how bone architecture changes relative to loading and morphological features from an early age. This study advanced our understanding of how mechanical loading and genetics influence the development of functional structures.

Determining patterns among traits during growth relative to an inverse relationship between BV/TV and trabecular anisotropy was an advance in understanding how functional vertebral body structures develop. A previous study showed the inverse relationship between BV/TV and trabecular alignment occurred throughout the trabecular region of an adult human lumbar vertebral body (Smit et al. 1997). Regions under high compressive load showed greater alignment of trabecular and low bone volume fraction, whereas regions with multiple load conditions (therefore, a lower compressive load) showed low trabecular alignment and higher bone volume fraction. The data was consistent with the concept of Wolffs’ Law that there was a balance between form and function at different locations in the lumbar vertebral body. In this study, we further showed that the inverse coordination between BV/TV and trabecular alignment relative to bone size at 4 weeks of age tended to influence how bones developed into adulthood. RI mice that tended to show a pattern of low trabecular alignment and high BV/TV at an early age, tended to show a small decrease in BV/TV over time. In addition, these mice tended to show a large increase in cortical area and
total area. The small decrease in BV/TV suggested that stresses within the trabecular region continued to be distributed in different directions and there was a small decrease to achieve a mechanical efficient architecture as the whole bone structure underwent elongation and expansion during growth. We can speculate that the large increase in cortical area was due to the high amount of load borne by the cortical shell compared to the trabecular region in these samples. The large amount of trabecular bone that was not highly aligned in the cranial-caudal direction, allowed for greater load transfer to the cortical shell.

Determining a strong relationship between load fraction and BV/TV at 4 weeks of age was an advance in understanding how the vertebral body architecture was related to mechanical function during growth. We expected that both BV/TV and the alignment of the trabecular architecture would be significantly correlated with trabecular load fraction based on the concept that bone material was arranged to optimally bear applied load (Ruff et al. 2006). The data showed that at 4 weeks of age, trabecular load fraction was positively correlated with BV/TV and weakly correlated with trabecular anisotropy. We observed a trend that trabecular load fraction was negatively correlated with anisotropy. The weak relationship between trabecular load fraction and anisotropy suggested that there may be interactions with other traits to improve this correlation at 4 weeks of age. Previous studies have shown that mineralization along with trabecular bone volume fraction were important contributors to adult vertebral body stiffness and strength (Tommasini et al. 2009). Determining whether there were interactions between anisotropy and tissue mineral density at an early age, would add to the complexity of understanding how traits interact to build mechanically functional structures. Average
tissue mineral density values of the trabecular and cortical region of the vertebral body samples relative to bone size are listed in Table 4.5. This data was used only as a reference of tissue mineral density values at different ages. Proper validation studies such as determining bone ash content, or utilizing Raman spectroscopy to detect mineralization in the trabecular and cortical regions, were not performed in this study to compare to the Microview software output. However, the data suggested a trend where mice that tended to show a high alignment of the trabecular architecture at 4 weeks of age tended to show greater values in trabecular tissue mineral density at 4 weeks of age and 16 weeks of age. We can speculate that examining a complex interaction between anisotropy, tissue mineral density, and BV/TV over time would improve our understanding of how mechanically functional structures were built early in life and throughout growth.

Relationships between load sharing and bone traits between 4 weeks of age and 16 weeks of age across a panel of RI mouse strains advanced our understanding how bone structures achieve function. Previous studies used finite element analysis to examine load sharing between cortical and trabecular bone along the length of the vertebral body in adult human samples (Eswaran et al. 2006), and simulate models of human bone (Cao et al. 2001). The maximum cortical shell load fraction was observed at the mid-transverse cross-section, and the maximum trabecular load fraction occurred at the endplates. In this study, the distribution of load along the length of the vertebral body in twenty RI mouse strains at 4 weeks of age was consistent with that seen in the adult human samples. This finding indicated that at an early age in mice, we were able to determine similarities in form and function of the vertebral body across different
strains and species given variation in bone size and sets of cortical and trabecular traits, as well as discriminations in loading conditions between a biped and quadruped animal. The data also showed that the average load fraction in either region of the bone was not different relative to variation in bone size or body size across the RI panel (Figure 4.8-4.9). This finding suggested that variation in sets of multiple cortical and trabecular traits regulated how load was shared within the vertebral body to create structures of similar function. Similar load sharing patterns in vertebral bodies across the RI panel suggested that load sharing was important for geometric design of the whole bone structure, which follow the concepts of Wolff’s Law that bone is able to adapt to mechanical loads.

Genetic and environmental factors were important to build a vertebral body that showed similar form and function across the RI panel. However, to understand how sets of traits develop during growth, this study suggests there are other genetic influences, or cues within individual structures.

Patterns of cortical and trabecular development in the vertebral body during growth reveal complex interactions among traits that may advance our understanding of the development of different bones in the skeletal system. Previous studies on bone mechanical adaptation of cortical bone at the diaphysis of long bone showed a coordination of outer-surface expansion, marrow expansion and matrix mineralization influenced the development of functional cortical structures during growth from 1 day of age to 16 weeks of age in AXB/BXA RI mice (Jepsen et al. 2009). Here, our data suggested that in a cortico-cancellous structure of the same RI panel, a complex interaction between trabecular architectural traits is important for the co-variation in cortical and trabecular traits during growth. Along the mid-diaphysis of metacarpals in
children, there were also differences in periosteal and endosteal expansion during growth relative to variation in bone size (Bhola et al. 2011). In this study, the change in cortical area and total area of the vertebral body cortex in mice from 4 weeks of age to 16 weeks of age appeared to show a similar coordination in the expansion of cortical morphological traits as seen along the diaphysis of long bone. In long bones, trabecular bone is localized to the distal and proximal ends unlike the internal architecture of trabecular bone seen throughout the vertebral body. Studies have shown that trabecular regions at the proximal end of rodent tibiae bones have a greater response to change in applied load (Fritton et al. 2005; Fritton et al. 2008) or, gonadal removal (Fritton et al. 2008; Waarsing et al. 2004) compared to the diaphyseal cortical bone. Therefore, it appears that the trabecular bone in the lumbar vertebral body and at the proximal ends of long bone show similarities in the adaptive response to applied load though the distribution of bone types differs among bones based on function within the skeletal system. In addition, previous studies have shown evidence of anatomical development near the growth plate of long bones where metaphyseal trabecular bone coalesce to form cortical bone (Cadet et al. 2003). This developmental process was suggested to be regulated by load transfer from the trabeculae to the peripheral cortex (Tanck et al. 2006). A future study may be able to determine whether load bearing in the trabecular region of long bones is associated with the co-variation of metaphyseal trabecular traits at an early age to influence the co-variation of cortical traits seen in the diaphysis. This finding would enhance our understanding of biological mechanisms of how whole bone functional structures develop within the skeletal system.
There were several limitations to this study. One limitation was that patterns among traits were based on trends and observational differences in values because only seven samples were grouped for different bone sizes, which were further subdivided based on differences in trabecular anisotropy at 4 weeks of age. We expect that increasing the number of RI mouse strains would improve the statistical significance in differences of trait values at 4 weeks of age and the amount of change in traits between 4 weeks of age and 16 weeks of age given variation in bone size. It would be important to test whether these patterns would change. Mechanical testing on 4 week samples was not performed to determine stiffness and maximum load to failure measure at this early age. It would be interesting to determine whether stiffness and strength correlated with BV/TV at a young age however, this finding would not alter the outcomes that we have shown in the relationships between how load was shared and development of traits over time. A second limitation was that Young’s modulus and Poisson ratio were taken from a previous study that investigated material properties for female mice tibiae as early as 1 month of age (Somerville et al. 2004). Previous studies used similar material property values for both cortical and trabecular regions in finite element analyses of mouse vertebral body samples (Eswaran et al. 2006; Webster et al. 2012). Therefore, we maintained consistency in values to focus on architectural development. A third limitation was that load sharing and trait analyses were performed in mice between 4 weeks of age and 16 weeks of age, which represented a later stage of growth. We previously showed that inter-strain variation in the percentage of trabecular area fraction was established at one week of age in three inbred mouse strains, which preceded the observed inter-strain variation in cortical area (Chapter 2).
Determining whether BV/TV influenced patterns of change in traits at an earlier stage of growth into adulthood, would provide further insight into the importance of trabecular architecture early in development. Examining traits at earlier ages would also imply a time period of interest for genetic analyses to better predict the development of whole bone structures.

Importantly, determining patterns in the change in traits over time provided insight into a bone adaptation process during growth. However, our data does not indicate a causal behavior in bone adaptation. To better understand adaptation we would need to perform a direct perturbation on trabecular traits to better understand the relationship with the change in cortical traits.

In conclusion, load sharing from an early age did help explain how cortical and trabecular bone co-varied during growth for overall structural conformation which was consistent with concepts of Wolff’s law. Here, we determined that load borne to the trabecular region strongly correlated to BV/TV, and previously we showed that trabecular anisotropy varied relative to bone size at an early age. Together, these findings gave rise to complex interactions in the trabecular architecture relative to bone size at an early age that explained developmental patterns in sets of traits during growth. Across the panel of RI mice and variation in bone size, a low trabecular BV/TV and high trabecular alignment at an early age lead to a greater decrease in BV/TV, as well as a small increase in cortical area and total area between 4 weeks of age and 16 weeks of age. This was in line with mechanical adaptation principles of organizing bone and utilizing minimal mass to achieve efficiency for load bearing. The amount of change in the alignment of the trabecular architecture over time was associated with an
inverse relationship between anisotropy and BV/TV relative to bone size at an early age. Across a population of mice with a wide range on genetic variance, we revealed common patterns in trait development where trabecular architectural traits arise as key components in determining how whole bone structures develop. Identifying these patterns during growth has important implications for early age prediction of structural development including detection of anomalies in patterns of trait development. In addition, genetic analyses and perturbation studies may be focused on how variation among trabecular traits affects whole bone growth.

**Acknowledgements**

We thank the National Institute of Health (AR056639) for support of this research.
References


Appendix B

PARAMETRIC STUDIES TO DETERMINE THE RELIABILITY OF THE FINITE ELEMENT ANALYSIS SYSTEM
1) Justification for number of samples used for FE Analysis

Twenty AXB/BXA female RI mouse strains at 4 weeks of age were used to analyze load sharing between cortical and trabecular bone in the L4 lumbar vertebral body. This animal model was also used to analyze the interaction of cortical and trabecular traits during growth (n=8-10/strain) [Chapter 3]. In this study, subsets of 6 samples per RI mouse strain were used for both finite element and trait analysis due to limitations in the availability of finite element analysis software. Cortical and trabecular traits from this sample subset strongly correlated with average trait values from the complete data set (Tt.Ar, \( R^2 = 0.96, p < 0.0001 \); Ct.Ar, \( R^2 = 0.89, p < 0.0001 \); RCA, \( R^2 = 0.92, p < 0.0001 \); BV/TV, \( R^2 = 0.97, p < 0.0001 \); Trabecular Anisotropy, \( R^2 = 0.88, p < 0.0001 \), Appendix B-Figure 4.1). Therefore, the sample sets used were representative of the full data set.
Appendix B-Figure 4.1. Validation of number of samples used for finite element analysis. (A) Total cross sectional area, (B) Cortical area, (C) RCA, (D) BV/TV, and (E) Anisotropy trait values for RI mouse samples used for FEA strongly correlated with traits across the entire RI panel. Significance indicated by p<0.05.
2) Parametric analyses

We conducted several parametric studies to determine the reliability of the FEA system in calculating cortical and trabecular load fractions in vertebral body samples with different external bone sizes and microarchitectural geometry. These analyses were important to validate how load was shared in vertebral bodies was invariant to parameters chosen for the finite element model. We evaluated variation in threshold values of the bone sample, size and position of the endplate surfaces used to apply boundary and loading conditions, and force applied to the vertebral body.

Variation in threshold values

Autothreshold values of trabecular and cortical regions from Microview software was important to analyze microCT scans for trabecular traits including BV/TV, trabecular thickness, number and spacing and cortical traits including total area and cortical area. For microCT based-finite element modeling, the threshold value from Microview was not always consistent with the value interpreted in Simpleware. Threshold values in Simpleware were shown to differ from the auto-threshold values acquired from Microview by 0.4% to 4%. Whether the variation in threshold values between the different software would affect further analysis of relationships between trait analysis and load fraction values in the same bone samples was not clear. To determine whether variation in threshold values would affect structural mass fraction and load fraction for trabecular and cortical bone, we created FE models based on the approximated autothreshold value from Microview, and thresholds that were 10% above and 10% below this given value. We expected that a ± 10% variation in threshold values in Simpleware would not affect the load fraction values within bone samples, therefore the
actual variation in threshold between different software would provide comparable data for trait and load sharing analyses.

Methods. RI mouse strain samples were chosen based on divergent variation in trabecular volume fraction (BV/TV). Previous studies showed that the trabecular region was highly sensitive to changes in loading conditions compared to the cortical region (Fritton et al. 2008; Kaneps et al. 1997; Globus et al. 1986). A change in threshold indicated a change in bone mass, which we expect would mainly influence the trabecular region. The average BV/TV value for all RI strains used for FEA analysis was 0.22 ± 0.02. A sample from the AXB8 strain showed a small BV/TV value at 0.17, and a sample from the AXB6 strain showed a large BV/TV value of 0.25. In addition, the AXB8 sample showed a small Ct.Ar (0.21 mm\(^2\)) and small Tt.Ar (1.02 mm\(^2\)). A large Ct.Ar (0.25 mm\(^2\)) and a large Tt.Ar (1.20 mm\(^2\)) was observed for the AXB6 sample.

In Microview, the vertebral body was cropped from extended processes and an autothreshold value was recorded for the entire sample. Three finite element models were created per sample using thresholds based on the 1) estimated Microview value, 2) 10% below this value, and 3) 10% above this value. In each model, material properties were set to be the same for both the cortical and trabecular region. The boundary and loading surfaces were in a parallel position, and a 1N load was applied along the caudal endplate whereas the cranial endplate remained stationary. In the finite element analysis, transverse sections with stress distributions were isolated from 20%, 50% and 80% along the length of the vertebral body as previously described (Chapter 4, Material and Methods). The trabecular load fraction was calculated for each section. Average trabecular load fraction values for the three sections were calculated
for each threshold condition. One sample was examined with either a low or high BV/TV value. Therefore, for each sample, the percent (%) difference in load fraction values from ±10% of the Microview threshold value was compared to the estimated Microview threshold value. A difference equal to or less than 10% was considered not different.

Results. Trabecular load fraction values were not sensitive to variations in threshold that were ±10% from the estimated threshold value for each bone sample (Appendix B-Figure 4.2A). The sample with a low BV/TV showed that the average trabecular load fraction for the estimated threshold was 0.46 ± 0.14. Threshold values that were 10% above and below the given value showed a trabecular load fraction values of 0.43 ± 0.17 and 0.42 ± 0.17, which was a difference of 5.6% and 8.4%, respectfully. A threshold value 10% above the given value tended to show a decrease in the overall bone mass, whereas a threshold value 10% below the given value tended to show an increase in the overall bone mass. However, there was no difference in the average trabecular mass fraction for the selected sections. The difference in threshold value 10% above and 10% below the given threshold value was 10%, and 2.9% respectfully (Given threshold, 0.44 ± 0.04; -10%, 0.43 ± 0.06; +10%, 0.40 ± 0.06,
Appendix B-Figure 4.2B). For a sample with a high BV/TV, the given threshold condition showed an average trabecular load fraction of 0.50 ± 0.20 (Appendix B-Figure 4.2A). This trabecular load fraction value tended to be higher than the value for the low BV/TV sample but, they were not significantly different. Threshold values 10% above or below the given value for the high BV/TV sample showed a difference in trabecular load fraction of 5.9% and 0.6%, respectfully. Therefore, there was not a difference in the trabecular load fraction (-10%, 0.52 ± 0.20; +10%, 0.50 ± 0.26). In addition, there were not significant differences in the average trabecular mass fraction for the different threshold conditions. The % difference in mass fraction from a threshold value 10% above and 10% below the estimated threshold value was 1.3% and 8.6% (Given threshold, 0.45 ± 0.12; -10%, 0.49 ± 0.12; +10%, 0.44 ± 0.13, Appendix B-Figure 4.2B).

Conclusion. Trabecular load fraction values were not affected by a ±10% deviation in threshold from the Microview value, which validated that smaller deviations in threshold between Simpleware and Microview would provide reliable load sharing data. The cortical load fraction was the inverse of the trabecular load fraction, therefore it was also not affected by the variation in threshold.

NOTE: Although we examined only two samples, samples with a low BV/TV and high BV/TV showed similar trabecular load fraction values. This analysis may indicate that the different bone structures were designed to bear load in a similar way.
2.1. Variation in boundary and loading surfaces

Platens were constructed and positioned at cranial and caudal endplate surfaces of 120 vertebral body structures to create an area where boundary and loading conditions were applied along the bone endplate. Given the wide range of variation in bone size across the panel of 20 RI mouse strains, the size and position of the platens may vary within a strain or across the different strains. It was unclear whether these variations would affect how load was shared between the cortical and trabecular region. To determine whether the size and position of the boundary and loading surfaces would affect load fraction values in cortical and trabecular bone, we selected bone samples that showed small, medium or large external bone size and subjected each sample to six different loading scenarios (Appendix B-Figure 4.3).

Appendix B-Figure 4.3. (Left) Schematic drawing of the position of the cranial and caudal platens at the endplates of a lumbar vertebral body. (Right) Schematic drawings of the different loading scenarios tested. Scenerios 1-5 represents size and position of the platen along the caudal endplate. 1) parallel to the cranial endplate platen, 2) not parallel to the cranial platen - positioned near the anterior side, 3) half the size of the full platen used in scenario 1 and positioned in the middle of the endplate, 4) parallel to the cranial endplate but the midsection was removed to create a “doughnut” shape, and 5) parallel to the cranial endplate with a load gradient applied along the surface – higher load at the anterior side. Scenario 6 represents a platen at the cranial end where loading was applied and was in parallel to the caudal platen.
In each scenario, we varied the size and/or position of the platens along the loaded surface. A stationary boundary condition was applied to the opposing end. The same material properties were set for both the cortical and trabecular region.

**Scenario 1:** Parallel platen surfaces – simulated a uniaxial compressive loading model, which was our primary model to use in this study. Elliptical platens were created to fit within the surface area of the cranial and caudal endplate. The caudal surface was larger than the cranial surface. The cranial platen was concentrically aligned to the caudal platen, and then both platens were translated to the endplates. The average area of the caudal platen for all strains was approximately 1 mm$^2$ ± 0.11 mm$^2$. Therefore, we set a compressive stress of 1MPa to create a 1N force along this surface area.

**Scenario 2:** Non-parallel surfaces – simulated uniaxial compressive loading but there was not a perfectly concentric alignment of platens as described for "parallel platen surfaces". In this scenario, the caudal platen was shifted toward the anterior size of the bone, whereas the cranial platen remained centered. This simulation was an extreme case that represented some samples that showed non-parallel alignment of opposing platens due to natural differences in size and shape of the endplates.

**Scenario 3:** Half-sized surface – uniaxial compressive load was concentrated at the center of the caudal endplate, which may be considered the nucleus pulposis region of an intervertebral disk. The platen surface area was half the original size; therefore, we set a compressive stress that was twice as large to generate a total force of 1N that was
similar to the other scenarios. The cranial platen remained large enough to fit the surface area of the endplate.

**Scenario 4**: Doughnut surface – The caudal platen was positioned in parallel to the cranial platen however, the center of the platen (one-half the area of the original platen) was removed. This region may be considered as the annulus fibrosis region of an intervertebral disk. The surface area of the platen was 75% of the solid platen, therefore we set a compressive stress of 1.33 MPa \(=1 \text{N} \div 0.75 \text{mm}^2\) to create a 1N applied load in the doughnut shaped surface.

**Scenario 5**: Load gradient – simulated a bending motion of the vertebral body. A linear variation of load was applied along the caudal platen surface in the anterior-posterior direction. To calculate the gradient, we specified a load into the finite element system in the form of a line equation similar to \(y=-2.5x+4.23\) (Appendix B-Figure 4.4). The line equation was based on the set positions of the platen in the anterior to posterior direction (x axis) and set magnitudes of load that was higher at the anterior side compared to the posterior side (y axis). Although a load gradient was applied to the sample, the average applied force was similar to other scenarios.

Appendix B-Figure 4.4. To calculate the load gradient, a line equation was generated.
**Scenario 6:** Cranial surface – platens were positioned in parallel, however the uniaxial compression was applied at the cranial surface. The average area of the cranial platen was 75% of the caudal platen (0.75 mm² ± 0.10 mm²), therefore we set a compressive stress of 1.33 N/mm² to create a 1N applied load.

**Methods.** RI mouse samples used for this analysis were chosen based on variation in external bone size but showed similar BV/TV values. The average total cross sectional area (Tt.Ar) for all RI mouse strains used for FEA analysis was 1.11 mm² ± 0.09 mm². Bone samples taken from the AXB8 strain showed a small Tt.Ar value at 1.02 mm², the AXB1 strain showed a medium Tt.Ar value at 1.10 mm², and the BXA7 strain showed a large Tt.Ar value of 1.22 mm². BV/TV values for each sample was 0.17, 0.17, and 1.93, respectfully. A finite element analysis was conducted on each sample for the six different loading scenarios. Transverse sections with stress distributions were isolated from 20%, 50% and 80% along the length of the vertebral body, and the average trabecular load fraction value of the three sections were calculated. One sample was examined that showed either a small, medium or large total cross sectional area. Therefore, for each bone sample, the % difference in the average trabecular load fraction from the different loading scenarios were compared to the parallel platen scenario. A % difference equal to or less than 10% was considered not different.

**Results.** A bone sample with a small Tt.Ar and platens positioned in parallel showed an average trabecular load fraction of 0.46±0.14. Compared to Scenarios 2, 4, 5, and 6, trabecular load fraction values were not significantly different (2: 0.45±0.12; 4: 0.46±0.08; 5: 0.46±0.10; 6: 0.47±0.07, **Appendix B-Figure 4.5**). The % difference for these scenerios was 2.3%, 0.7%, 0.3%, and 2.4%, respectfully. A bone sample with a
medium sized Tt.Ar and platens positioned in parallel appeared to have a low trabecular load fraction (0.37±0.16) compared to samples with a small or large Tt.Ar. However, there was not a significant different in load fraction value among the different samples. In addition, most of the loading scenarios with the medium Tt.Ar sample showed a % difference in trabecular load fraction from the parallel platen scenario that was less than 5% (2: 2.3%; 4: 0.4%; 5: 2.5%; 6: 3.2%) where the average values were 2: 0.38 ± 0.21; 4: 0.38 ± 0.13; 5: 0.38 ± 0.17, and 6: 0.39 ± 0.12. A bone sample with a large Tt.Ar and platens positioned in parallel, the average trabecular load fraction value was 0.50 ± 0.09. This value was not different from load fraction value from most of the other loading scenarios (2: 0.57 ± 0.10; 4: 0.58 ± 0.03; 5: 0.58 ± 0.06; 6: 0.60 ± 0.03) where the % difference from the parallel platens scenario was less than 10% (2: 2.6%; 4: 4.8%; 5: 4.8%; 6: 8.4%). In addition, there was a trend among the bone samples where a caudal platen that was half the size of the original (Scenario 3) tended to show higher trabecular load fraction values (Small TtAr 3:0.51±0.10; Medium TtAr 3: 0.42±0.14; Large TtAr 3: 0.66±0.06) and a % difference from the parallel platen scenario that was nearly 12% or greater (Small TtAr 3: 11.9%; Medium TtAr 3: 12.4%; Large TtAr 3: 18%).
Appendix B-Figure 4.5. Trabecular load fraction values for bone samples with a small, medium or, large total cross-sectional area that were subjected to various loading scenarios.

Conclusion. The data showed that the variation in size and position of platens at the vertebral body endplates did not affect the load fraction values throughout the bone structure except for the scenario where the caudal platen was half the size of the original. The large % difference in trabecular load fraction from this scenario may be due to a concentrated load in a small area directed at the center of the endplate and mainly the trabecular region. Fortunately, the half-sized plate was not used for this study. We preferred to position platens in parallel to simulate uniaxial compression. However, some samples may have a slightly non-parallel alignment. We validated that the unaligned platens would show load fraction values that were not different to the parallel platens.

Other loading scenarios represented possible physiological loading conditions but, were not used in this study. Previous studies have performed finite element analyses on human vertebral bodies using superior end loading (Eswaran et al. 2006; Yang et al. 2012; Unnikrishnan et al. 2013). Here, load applied to the cranial end or caudal end of
the mouse vertebral body did not affect load fraction values. We preferred to apply load at the caudal end because of the larger surface area and simple calculations in generating the applied force.

2.2. Variation in applied compressive force

To determine linearity of the finite element system, we applied different magnitudes of compressive force onto a bone sample and analyzed the stress distribution, and the range of stress and strain values, throughout a bone sample. We expected that the stress and strain values would vary in proportion to the magnitude of the applied load. However, we did not expect the stress distribution would change. Therefore, the fraction of load borne to different areas of the bone would not change.

Methods. In a finite element model, cranial and caudal platens were positioned in parallel at the endplates of a vertebral body sample. Material properties were the same for both the cortical and trabecular regions. Load was applied at the caudal endplate whereas the cranial endplate was held stationary. The contact area of the caudal platen was approximately 1 mm². We set compressive stress of 1 MPa, 2 MPa, or 5 MPa to create an applied force that was 1 N, 2 N or 5 N in magnitude. The stress distribution was analyzed at a transverse section 50% along the length of the vertebral body. Principal stress and strain values were analyzed for the whole bone sample.

Results. Stress distributions were insensitive to the magnitude of applied compressive force. The mid-transverse sections of a bone sample subjected to 1 N, 2 N or, 5 N of compressive load showed that there was no difference in the color pattern representing various stress concentrations (Appendix B-Figure 4.6). To maintain the
stress distribution pattern with increasing load, there was a proportional increase in the range of stress and strain values. A 1 N of applied force generated stress values that ranged from 5 to -40 MPa and strain values that ranged from 1000 to -1000 microstrain. A 2 N and 5 N applied force showed that the range of stress values and strain values increased linearly by two times and five times, respectfully.

Conclusion. The finite element system was linear. However, for our study we subjected bone samples to a 1N applied force since the range of stress and strain values were within the physiological range for bone (Homminga et al. 2004).

Appendix B-Figure 4.6. Mid-transverse sections of lumbar vertebral body sample showing stress gradients were not different with variation in applied compressive load of 1N, 2N or 5N. However, with increasing load there were linear increases in stress and strain range of values.
Appendix B - References


Chapter 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS
General Conclusions

The goal of this dissertation was to understand how cortical and trabecular traits interact to develop mechanically functional bone structures during mouse postnatal growth. Analyzing the coordination of bone traits will benefit predictive models of mechanical function, as well as the contribution to skeletal fragility. A systematic approach to better understand how traits functionally interact was to adopt a working model which postulated that early variation in one trait leads to subsequent adaptive changes in other traits during growth.

Our first objective was to identify patterns in morphological cortical and trabecular trait development during growth that would suggest variant or adaptive traits. Previous studies showed the maturation of individual traits in the mouse lumbar vertebral body (Buie et al. 2008; Glatt et al. 2007) or coinciding patterns in the development of trabecular bone volume fraction (BV/TV) and anisotropy during growth in the trabecular region of porcine lumbar vertebral body specimens (Tanck et al. 2001). We further examined temporal changes in cortical and trabecular traits during growth of the lumbar vertebral body among three inbred mouse strains to determine patterns of inter-strain variation of traits. Adult AJ, B6, and C3H mouse strains are known to have different genetic backgrounds and sets of cortical and trabecular traits that vary among the strains. Examining the inter-strain variation among cortical and trabecular traits during growth, we identified when variation in traits arose across the three mouse strains as seen in adulthood. This finding indicated phenotypic development that was important for function given a particular genotype. Temporal patterns during growth showed that inter-strain variation in the percentage of trabecular area was established at 1 week of
age and was retained into adulthood whereas variation in cortical area was established after 4 weeks of age (Chapter 2). This sequence of events in establishing phenotypic variation among cortical and trabecular traits suggested that the variation in the percentage of trabecular area lead to an adaptive response in cortical area. Therefore, we provided biological evidence of developmental patterns among traits that are important for establishing function. Morphological traits were measured in two-dimensional mid-sections of the lumber vertebral body and compared among three inbred mouse strains. We provided insight into how traits co-varied during growth and a narrow window in time when sets of traits are established for a given genotype. However, a three-dimensional analysis of the whole bone structure would advance our understanding of spatial differences in trait development throughout the vertebral body structure and over time. In addition, identifying developmental patterns in a population with greater genetic diversity would provide better evidence of whether the timing to establish variation in trabecular bone area was a global phenomenon. The timing to establish trait variations would benefit predictions of growth anomalies at an early age, and provide a time frame for genetic analysis and therapeutics to prevent fractures later in life.

Our second objective was to test the hypothesis that variation in trabecular architectural traits lead to adaptive changes in cortical area during growth given a wide range of genetic variants. This study provided insight into the coordination of cortical and trabecular traits throughout growth that was important to achieve mechanical function given a population of natural variation in bone size and sets of traits. We analyzed relationships among cortical and trabecular traits from 4 weeks of age to 16
weeks of age across a panel of twenty AXB/BXA Recombinant Inbred (RI) mouse strains. Trabecular BV/TV and anisotropy values were greater at 4 weeks of age whereas cortical area and total area continued to develop until skeletal maturity at 16 weeks of age across the RI panel. This finding suggested an adaptation process of the whole vertebral body system where a decrease in BV/TV and trabecular alignment efficiently adjusted the trabecular architecture and an increase in cortical traits was important for external expansion with increasing body size during growth. Across the RI panel, mice tended to develop cortical and trabecular traits uniformly over time relative to variation in bone size. However, we also showed that at 4 weeks of age, RI mice that tended to show a small bone size tended to show axially aligned trabecular architecture whereas RI mice with a larger bone size showed less trabecular alignment when taking body size into consideration. Cortical area showed a significant positive correlation with bone size at 16 weeks of age, which suggested that trabecular alignment relative to bone size was an important interaction for mechanical integrity of the bone structure at an early age. Analyzing interactions among micro-architectural traits, the data showed that an increase in trabecular alignment between 4 weeks of age and 16 weeks of age was associated with small increases in cortical area across the RI panel. This coordination of traits suggested interactions among cortical and trabecular traits as a biological process during growth where the amount of change in traits over time was important to build functional structures. This finding was an advance from previous studies that showed a consistent inverse relationship between the cortex and trabecular alignment in a computer simulation of the lumbar vertebral body (Silva et al. 2007), or predicted interactions using multivariate analysis in adult AXB/BXA RI mice.
Regression analyses suggested subtle differences in development of cortical and trabecular traits between 4 weeks of age to 16 weeks of age within each strain. Analyzing trait interactions as early as 2 week of age into adulthood in this panel of RI mice may show greater amounts of change in traits over time. This finding would further affirm that variation in trabecular architectural traits relative to bone size early in bone development occurred prior to variation in cortical traits. In addition, an analysis of morphological with compositional traits such as tissue mineral density would advance our understanding of the complexity of interactions among traits in developing mechanically functional structures.

Our final objective was to better understand how the functional interactions among cortical and trabecular morphological traits during growth was explained by load shared between the different regions of bone. Based on Wolffs’ Law, load bearing affects the form and function of bone (Frost 1994). This study provided a better understanding of how applied load at an early age contributed to the development of traits for mechanical function at different ages and during growth. We determined cortical and trabecular load fraction along the length of the lumbar vertebral body in twenty, 4 week old AXB/BXA RI mouse strains. We then examined how load borne by different regions of the bone was associated with variation in traits at 4 weeks of age, 16 weeks of age, and the change in bone traits over time. Positive correlations of BV/TV at 4 weeks and 16 weeks of age with trabecular load fraction and adult mechanical properties suggested that the early variation in trabecular bone volume fraction was important for whole bone stiffness and strength throughout growth. However, the interaction of BV/TV with load fraction relative to variation in bone size did not show a
consistent pattern across the panel of RI mouse strains. This finding suggested that load sharing between cortical and trabecular bone was not a direct predictor of how sets of traits developed in functional structures. Further analyses proposed that the interaction between trabecular alignment and BV/TV at an early age was important to understand developmental patterns among bone traits during growth. RI mice that tended to show either narrow or wide bones, as well as a high trabecular alignment and low BV/TV at 4 weeks of age, tended to show a large decrease in BV/TV, a small increase in cortical area, and a small increase in total area over time. Therefore, the data suggested that trabecular architectural traits were key variants that lead to adaptive changes in other traits relative to bone size. Identifying the coordination of trabecular traits early in life has important implications for diagnosing mechanical function of bone based on the coordination of trabecular traits relative to bone size at an early age, predicting patterns in bone development, and detecting traits that may lead to skeletal fragility. A larger cohort of RI mouse strains would provide a better statistical evaluation of the developmental patterns in traits relative to variation in trabecular architecture and bone size at an early age. In addition, examining developmental patterns at an early growth phase may show greater changes in traits over time. The patterns among traits may change with additional samples. However, we established an analytical approach to better understand how mechanical function is established during growth through interactions of cortical and trabecular traits. Examining the interaction among traits in different RI strains such as those derived from B6 and C3H progenitor strains, we would expect the sets of traits in each mouse strain would be different from
those shown in AXB/BXA RI mouse strains (Turner et al. 2001). We also expect there would be a similar biological sequence of events to develop functional structures.

In summary, this dissertation advances our knowledge of how function is achieved in mouse vertebral body structures through the interaction of cortical and trabecular traits during growth. The systems biology approach used to identify how sets of traits develop provided insight into biological mechanisms in establishing mechanical function. The timing of interactions during early growth provided important implications for interpreting bone function during growth in response to genetic and environmental perturbations. Temporal patterns in how cortical and trabecular morphological traits develop during growth, suggested that early variation in trabecular architectural traits contributed to structural development of the lumbar vertebral body. Based on the data, analyses targeting trabecular architectural development from an early age may benefit efforts to detect anomalies in this region that may be predictive of fracture risk later in life. Importantly, our findings did not establish a causal nature of how traits functionally interact during growth. To further examine causal behavior in a functional bone adaption process among traits, we speculate that specific genes that affect trabecular formation would have to be perturbed, and then the adaptive response in cortical traits would have to be analyzed during growth. In addition, perturbations applied after variation in traits has been established would be expected to have a much less effect on the change in traits over time. Genes that are specific to trabecular or cortical bone formation are not known.
Future Directions

The next step is to determine cellular mechanisms that lead to the rise in variation in trabecular bone volume fraction and anisotropy traits relative to bone size. Variation in bone morphological and tissue quality traits are fundamentally based on osteoclast and osteoblast activity regulated by both genetics and environmental changes in load. How bone cells coordinate will provide a better understanding of underlying biology to establish and maintain mechanically functional structures. Both cell types contribute to modeling and remodeling bone tissue to create mechanically functional structures (Gerstenfeld et al. 2010; Akhter et al. 2004). Previous studies showed that osteoclasts have a different spatial distribution and intrinsic activity with respect to genetic background, which was observed as early as 14 days of age in AJ, B6 and C3H femurs (Gerstenfeld et al. 2010). Temporal patterns during growth of the lumbar vertebral body among these three inbred mouse strains showed a rapid decrease in the % Tb.Ar between 4 days of age and 7 days of age occurred prior to the rise in inter-strain variation in this trabecular trait at 7 days of age (Chapter 2). In addition, by 7 days of age, there was an increase in trabecular alignment. Assessing both osteoclasts and osteoblasts activity along specific bone surfaces in the lumbar vertebral body among these three mouse strains will help to determine how cellular activity contributed to bone phenotypic variation during growth.

We hypothesize that 1) the rise in inter-strain variation in trabecular traits was due to excessive osteoclastic resorption activity within each strain, and 2) the amount of resorption varies among the mouse strains. These findings would suggest that osteoclasts play a primary role in establishing genotype-specific variation in bone traits.
as seen in adulthood. Histological analysis will provide insight into cellular and genetic mechanisms that guide the development of variation in trabecular traits. Histological staining for tartrate-resistant acid phosphatase (TRAP)-positive in osteoclasts is a reliable and established method to vividly detect resorption activity. However, immunohistochemical staining can also be used to detect Cathepsin K—a protein enzyme involved in bone resorption activity. Osteocalcin is a sensitive marker for bone turnover, however, osteocalcin also stained bone lining cells—an immature or quiescent osteoblasts (Chang et al. 2008) and the cytoplasm of chondrocytes (“RnD Systems, USA”). An alternative would be to stain for the PHEX/Phex (Phosphate-regulating gene with homologies to endopeptidases on the X chromosome) gene, which is a membrane glycoprotein expressed in fully differentiated osteoblasts (Alos et al. 2005). Dual immunohistochemical staining for osteoclasts and osteoblasts can be used to assess the simultaneous coordination of cellular activity at a particular location in the vertebral body. Further work can be directed toward understanding genetic regulation of other cell types during early bone development such as hypertrophic chondrocytes. We can determine the role these cells play in recruiting osteoblasts and osteoclasts, and directing the architectural structure of trabecular formations as a function of genetic variation in bone traits.

To determine how variation in bone traits is established through osteoclast and osteoblast activity, the temporal sequence of cell activity should be examined at three particular ages: 1) at 4 days of age—prior to the established variation of trabecular traits; 2) 7 days of age—when variation in trabecular traits is established, and 3) 14 days of age—when variation in both cortical and trabecular bone traits are established. The
regions of interest include the cranial and caudal growth plates where trabeculae are formed and mineralized trabecular struts within the primary spongiosa (Kronenberg 2003). The temporal distribution of cell activity at these specific regions will be critical to understand whether there is a pattern between osteoclastic activity and development of variation in adult trabecular traits. Excessive resorption activity at the growth plate would be associated with breakdown of calcified matrix synthesized by hypertrophic chondrocytes and apoptosis of these chondrocytes (Tschegg et al. 2012). Along the trabecular struts osteoclasts are recruited for remodeling of bone via signaling from apoptosis of osteocytes (Lorenzo et al. 2010). Therefore, the region of excessive resorption will have different biological implications as to why there is variation in osteoclasts activity among different mouse strains. Time-dependent changes can be assessed by plotting the amount of osteoclasts and osteoblasts on a specific bone surface as a function of age for each strain. The rate of change in cellular activity can be determined for each mouse strain by comparing cell numbers from the older mice to the mean value of activity in the younger mice. The difference in cell distribution among strains and the change in osteoclast activity with growth in a particular strain will provide insight into genetically regulated cell activity coordinated to establish variation in bone traits. The rate of change in depth of the vertebral body was not significantly different with time (Chapter 2). Cellular activity along the periosteal surface was suggested to mainly be associated with expansion and cortical drift to establish total cross sectional area and adaptive cortical thickness and areal traits relative to the variation in trabecular traits and increase in body mass. Therefore, we do not expect that osteoclastic and
osteoblastic activity would vary along the periosteal surface (anterior-posterior) of the vertebral body with growth for a particular strain.

Examining differences in the bone matrix among mouse strains would improve our understanding of how sets of traits are established. In human bone tissue, the extracellular matrix (ECM) consists of osteons made up of layers of lamellae which are composed of sub-structures containing mineral, collagen (types I, III, V, IX, and X), water, and other organic macromolecules (proteoglycans, glycoproteins, peptides, and lipids) (Figure 1.1). The organization of the matrix contributes to mechanical properties of the bone on the micro- and nano-structural level. Co-variation in ECM components in the cortical and trabecular region within a mouse strain would provide insight into the coordination of sets of traits. For example, a mouse strain that showed low mineralization or collagen content in the trabecular region, may show high mineralization in the cortical shell, which would influence the architecture of the whole bone to functionally bear load. We also speculate that during early development, temporal changes in bone matrix composition in the cortical and trabecular region among mouse strains would influence the rise in variation among bone traits. We hypothesized that inter-strain variation in trabecular bone fraction established by 7 days of age in AJ, B6, and C3H mice (Chapter 2) may be due to excessive resorption from osteoclasts. Further study may determine whether there is coinciding occurrences where genetic as well as mechanical influences on osteoblastic activity affect the structure and composition of the ECM on a nanoscale that in turn influence the trabecular architectural traits seen by 7 days of age.
Future studies can also determine whether temporal patterns of trait development differ between males and females. Previous studies have shown sexual dimorphism during long bone growth where males tend to show greater expansion of the periosteal surface along the cortex and greater lengthening of long bones during puberty compared to females (Seeman 2008). In addition, the maturation of individual bone traits in the lumbar vertebral body between male and female B6 mice showed that males tended to show a greater trabecular BV/TV after 2 months of age, and smaller cross-sectional area after 8 months of age compared to female mice (Glatt et al. 2007). However, it was not clear whether the temporal changes in establishing variation among cortical and trabecular traits in females are comparable to males. Using the mouse model of AJ, B6 and C3H mouse strains, we expect that males will show a larger total cross-sectional area than females at all ages. In addition, variation in trabecular BV/TV and anisotropy is expected to occur prior to variation in cortical area. Findings from Chapter 2 showed that variation in the percentage trabecular area among the three strains of mice occurred pre-puberty at 1 week of age, and mice showed variation in bone size as early as 1 day of age. Therefore, the timing of when variation in trabecular architectural traits is established early in bone development is expected to be similar to that shown in females. We also expect that interactions among traits during growth would be similar to what we have shown in females, which would suggest biological growth patterns relative to bone size that can be predicted across genders in a population.
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