HIGH-FAT DIET AND RAT BONE

THE EFFECT OF A HIGH-FAT DIET ON BONE STRAIN IN ADULT RAT FEMURS

By CHERYL DRUCHOK, H.B.Sc.Kin.

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AUTHOR: Cheryl Druchok, H.B.Sc.Kin. (McMaster University)

SUPERVISOR: Gregory R. Wohl, Ph.D., P.Eng.

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ABSTRACT

A high-fat diet can adversely affect bone mechanical properties, but it is unknown how these changes affect bone adaptation. Bone adaptation occurs in response to strain-related mechanisms, and strain in the bone is affected by the size and mechanical properties of the bone. The purpose of this study was to compare the strain during loading in femurs from rats fed a high-fat (HF) or normal control (NC) diet. At 3 weeks of age, male and female Wistar rats were randomly assigned to receive a NC (NC–17% fat; N=8 per gender) or HF diet (HF–41% fat; N=8 per gender) until termination (39 weeks of age). Right femurs were loaded *ex vivo* in 3-point bending to physiologic levels and mechanical strain was measured. The mechanical properties of the left femurs were determined by 3-point bend tests to failure. The dietary effects were limited in both genders. Femoral cross-sectional area properties (bone area, moment of inertia), determined from microCT scans, were significantly greater in HF femurs vs. NC for males and females. Elastic modulus was calculated from strain and deformation data and no dietary effects were seen in either gender. At the applied loads, despite significantly larger cross-sectional area properties in the HF femurs, there was no significant difference in strain between HF and NC femurs for either gender. As a comparison, an expected HF femur strain was calculated using the average elastic modulus of the NC femurs and the HF femur cross-sectional properties. A significant difference was found between the expected HF strain and the measured NC strain. Together, these findings suggest that the greater cross-sectional area properties are a compensatory mechanism for deficient material properties in the HF femurs. Adaptive modeling during growth appears to take place to target a predetermined level of strain to preserve bone structural integrity.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	per cent
°C	degrees Celsius
δ	deformation or displacement
θ	angle
γ	shear strain
ε	strain (mm/mm)
με	microstrain (10^{-6} mm/mm)
μm	micrometres
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BMU	basic metabolic unit
BW	body weight
cm	centimetres
CO_2	carbon dioxide
cos	cosine
DEXA	dual-energy x-ray absorptiometry
DVRT	differential variable reluctance transducer
E	elastic modulus
ECM	extracellular matrix

EDNP	energy dense nutrient poor
EMG	electromyography
F	applied load
F-HF	female high-fat
F-NC	female normal control
FFM	fat free mass
FM	fat mass
g	grams
GPa	gigapascal
HF	high-fat
HF-NR	high-fat non-responders
HF-Pred E=NC	predicted high-fat strain using normal control elastic modulus
HFS	high-fat sucrose
Hz	Hertz
IGF-1	insulin-like growth factor 1
IL-1β	interleukin-1 beta
IL-6	interleukin-6
I_{xx}	second moment of area
kg	kilogram
l	distance between supports
L	litres
M-HF	male high-fat

M-NC	male normal control
mg	milligram
microCT	micro-computed topography
min	minute
mJ	milliJoules
mL	millilitre
mm	millimetres
N	Newton
n-3	omega-3 fatty acid
n-6	omega-6 fatty acid
NC	normal control
PTH	parathyroid hormone
ROS	reactive oxygen species
SEM	scanning electron microscope
sin	sine
STD	standard deviation
TNF-α	tumor necrosis factor-alpha
TRAP	tartrate-resistant acid phosphatase
XSA	cross-sectional area
Y_{post}	distance from centroid to posterior endosteum

CHAPTER ONE

INTRODUCTION

1.1 Introduction

The average age of the North American population has increased and is projected to increase dramatically through 2025 [1]; with this aging population, osteoporosis has become a significant public health concern and cost to the health care system. Osteoporosis is a disease of bone fragility that affects a significant portion of the aging North American population. Approximately one in two women and one in four men over the age of fifty will have an osteoporosis-related fracture in their lifetime [1]. It has been suggested that the rate of fractures related to bone fragility is disproportionately high for the rate of aging [1, 2] and therefore factors other than age must be taken into consideration. Among the factors that contribute to bone health (*i.e.*, genetics and hormones), nutrition, including dietary fat, can influence bone adaptation and may contribute to an increased risk of osteoporosis-related fractures [3].

Obesity has become a major public health concern mostly due to the "North American" or "Western" diet which is characteristically high in fat and refined sugars [4]. In the past, obesity has been thought to be protective for bone health. There is a well established positive correlation between body weight, which increases bone loading, and bone mineral density (BMD) [5]. However, accumulating recent data is now suggesting that increased fat mass (FM) is detrimental to bone [3]. Obesity has been shown to alter bone structural and material properties and, as a result, may be a contributing factor to the

increased fracture risk seen in the aging population. Preventing fracture is key to reducing consequential morbidity, mortality, and health care costs. The potential of obesity to exacerbate fracture risk warrants a closer look at obesity and its effects on bone. A better understanding of the consequences of obesity on bone is needed — not only the consideration of how obesity affects bone structural and material properties, but also how these changes then affect the ability of bone to adapt to various loading conditions.

The ability of bone to adapt to changes in the loading environment is not only important but also necessary in order to minimize the consequences of increased loads on the bone. Altering the ability of bone to adapt to its environment could potentially result in compromised bone properties leading to fracture and physical disability. Bone adaptation is thought to occur in response to strain-related mechanisms, and strain in the bone is affected by both structural and material properties [6]. Because obesity has been shown to adversely affect material properties and alter structural properties, the strain experienced by bones from obese animals at a given load may differ from the strain in bones from non-obese animals. If the strain levels in the bones are different then the ways in which bone adapts to environmental stimuli will also be different; different rates and types of bone formation could occur, ultimately affecting how the bone stands up to continued loading. It is important to understand how bone adaptation is affected by obesity so that interventions can take place before bone health has been compromised.

The overall goal of this thesis project was to study the effects of obesity on the ability of bone to adapt to mechanical stimuli through the examination of bone structural and material properties and bone strain. The hypothesis was that male and female rats fed

a lifelong high-fat diet, designed to induce obesity, would exhibit decrements in bone mechanical properties, thus altering bone strain during mechanical loading and consequently affecting bone adaptation.

To accomplish this goal the specific aims were:

1. Use a rat forelimb compression model to examine the effects of diet on lamellar bone formation induced by mechanical loading.
2. Measure and compare femoral strain during 3-point bending and compression in high-fat and normal control rats.
3. Compare the results from the two methods of mechanical testing.
4. Examine the structural and material properties of femurs from obese rats.

The effects of bone adaptation in response to loading can be examined at four different levels: 1) organ, 2) tissue, 3) cellular and 4) molecular [7]. At the organ level, structural parameters such as force, displacement, stiffness, and failure load can act as factors governing bone adaptation [7]. At the tissue level the factors that influence bone adaptation are determined by material parameters like stress and strain [7]. At the cellular level factors such as fluid flow, cell shape changes, cell-matrix interactions, oxygen and nutrient supply and electrical potentials control adaptation [7]. At the molecular level factors that influence adaptation are cytoskeletal changes, stretch activated ion channels, cell-surface integrins, cytokines and cell receptors [7]. For the purpose of this thesis, the

main focus was on organ (structural) and tissue (material) levels with some discussion on the cellular level. The characterization of structural and material properties, behavior and adaptation in high-fat bones will provide a basis for subsequent studies to examine bone adaptation at the cellular and molecular levels.

1.2 Bone Function and Composition

Bones are the primary structural elements in the body, protecting vital organs and providing a rigid framework for movement and locomotion. In addition, the skeleton participates in mineral homeostasis and is a primary site of hematopoiesis. Overall health requires the maintenance of all of these functions, thus emphasizing the importance of optimizing bone health.

Bone tissue consists of two components: cells and extracellular matrix (ECM). The ECM itself is composed of four components: hydroxyapatite mineral, collagen, water, and small amounts of proteoglycans and noncollagenous proteins (Table 1.1).

Mineral in bone consists mostly of hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$ and gives the bone rigidity and compressive strength. Bone mineral is impure containing many structural substitutions (*e.g.*, carbonate, fluoride, citrate) that are governed by the composition of body fluids and in turn affect the solubility of the bone mineral [8]. Collagen is a structural protein and the predominant type found in bone is type I. Collagen gives bone flexibility and tensile strength. Some of the water in the calcified bone matrix is free and some is bound to other molecules [8]. The mineralization of osteoid, the organic portion of extracellular bone, displaces part of its water [8].

Therefore, the water content of new bone tissue changes as it mineralizes. Ground substance of bone consists of proteoglycans. The specific roll of these in bone is unclear. They may function to control the location or rate of mineralization in bone through their calcium-binding properties [8]. Non-collagenous proteins include a number of molecules whose functions are also unclear. The most abundant non-collagenous protein is osteocalcin which is secreted by osteoblasts and is important in the mineralization of new bone [8].

Table 1.1. Composition of the bone extracellular matrix (ECM) [8].

Component	**Mass (%)**
Hydroxyapatite	65
Collagen (mostly type I)	23
Non-collagenous proteins and proteoglycans	2
Water	10

1.3 Cortical Bone Structure

There are two types of bone present in the human body: cortical and trabecular. For the purpose of this research only cortical bone was examined in the rat femur. Cortical bone is the dense bone that lines the outer surface of most bones and is found in the shaft of long bones such as the femur (Figure 1.1). It has a porosity of 5-10% [9]. The microarchitecture and composition of cortical bone determines its mechanical properties and, due to its structure, it is stronger and heavier than trabecular bone. Cortical bone contains two major types of bone tissue: lamellar and woven bone. Lamellar bone forms in response to anabolic stimuli and is a slow-forming but very strong, dense bone. It is highly organized consisting of parallel layers or lamellae in which the collagen fibres run

parallel in each lamella and change orientation by 90° between layers. Lamellar bone has fewer cells compared to woven bone. Woven bone is poorly organized and a weaker bone compared to lamellar bone but it forms rapidly enabling it to provide support to damaged bone quickly. The collagen fibres and mineral crystals appear to be randomly arranged but it may be more mineralized than lamellar bone helping to compensate for its poor organization. Woven bone has a low mineral density and high cellularity.

Cortical bone can be characterized as primary or secondary bone. Primary bone is tissue laid down *de novo* on an existing bone surface during growth. It is composed of lamellae that are parallel to the bone surface. Blood vessels are interspersed throughout the lamellar structure and are surrounded by several circular lamellae, forming a primary osteon with a primary Haversian canal at its centre (Figure 1.2). Haversian canals are approximately aligned with the long axis of the bone and also contain nerves. Volkmann's canals are short transverse canals connecting Haversian canals to each other and to the outer surface of the bone. These canals also contain blood vessels and nerves. Secondary bone results from the resorption of existing bone and the replacement by new lamellar bone. This process is known as remodeling. In cortical bone secondary tissue consists of cylindrical structures known as secondary osteons in which cylindrical lamellae surround a central Haversian canal (Figure 1.3). Cement lines form the boundary between the osteon and the surrounding bone and are scalloped in secondary osteons. In adult humans, most compact bone is composed entirely of secondary bone. Rats do not typically undergo true cortical bone remodeling [10] and as such the cortical bone exhibits only a few secondary osteons [11].

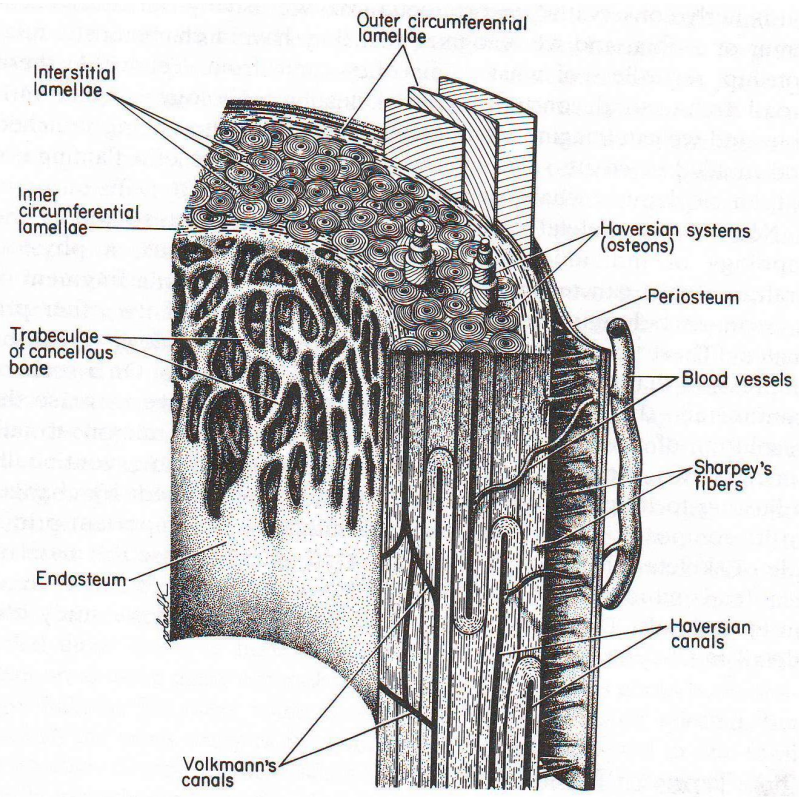

Figure 1.1 Schematic of the internal structure of a long bone [9].

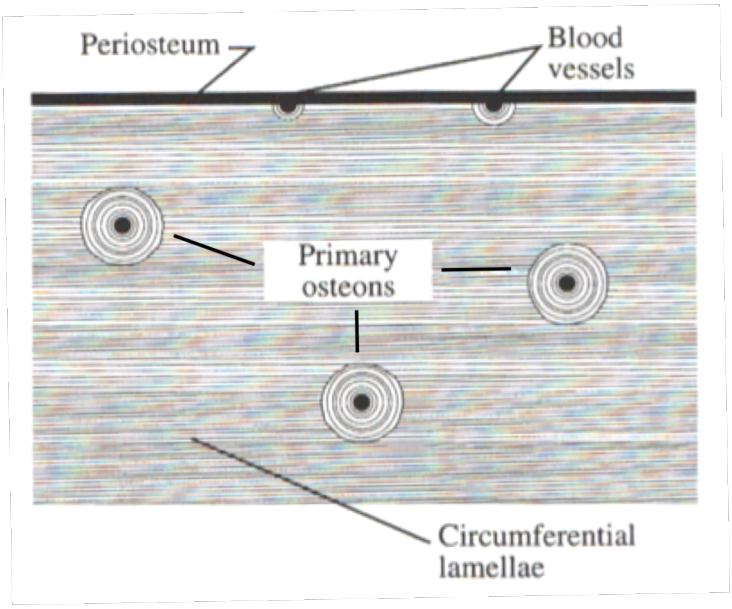

Figure 1.2. Schematic of primary lamellar bone structure [9]. Primary osteons, concentric lamellae surrounding a blood vessel, are shown.

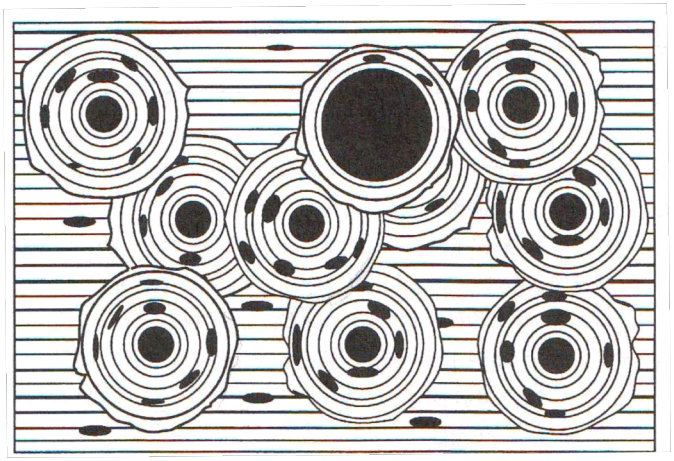

Figure 1.3. Schematic of secondary osteons [9]. Their characteristic scalloped cement lines are shown.

1.4 Bone Cells

Formation and maintenance of the ECM is carried out by the cells that make up a small percentage of the bone volume. There are four types of bone cells.

Osteoclasts are the cells that resorb bone. They are multinucleated because they are formed by the fusion of cells called monocytes originating in the hematopoietic portion of the bone marrow [12]. Osteoclasts erode their way through bone by first demineralizing the adjacent bone with acids and then dissolving its collagen with enzymes.

Osteoblasts are mononuclear cuboidal cells that produce osteoid, the organic portion of the bone matrix [12]. Osteoid contains collagen and non-collagenous proteins, proteoglycans, and water [8]. Osteoblasts are differentiated from mesenchymal cells and it appears that differentiation may be triggered by mechanical stress to the bone [12]. Osteoblasts have cell processes that extend into the newly formed bone connecting them to osteocytes within the calcified matrix.

Osteocytes are former osteoblasts that have become embedded in the bone matrix as it is being secreted. They sit in cavities called lacunae and communicate with each other and with osteoblasts via processes passing through tunnels called canaliculi [12]. Processes from adjoining cells are connected by gap junctions. The osteocytes are found throughout the entire skeleton and this is one reason that they are thought to be important in transporting mineral into and out of the bone and in sensing mechanical stress.

Bone lining cells are like osteocytes, quiescent osteoblasts. These are the osteoblasts that escaped being buried in newly formed bone and remained on the surface when bone formation ceased [8]. Lining cells cover more than 90% of the surfaces of adult bone and they contain gap junction connections to osteocytes and osteoblasts to maintain communication [12]. Like osteocytes they are thought to be responsible for the transfer of mineral into and out of bone and for sensing mechanical strain [8]. They are also believed to initiate bone remodeling in response to various chemical and mechanical stimuli [12].

1.5 Osteocytes

Osteocytes are thought to be the main mechanosensing structure of bone because they are the most numerous bone cells and are spaced regularly throughout the mineralized matrix (Figure 1.4). They are connected with each other via long, slender cell processes and have the ability to communicate with other bone cells through their extensive network of processes connected at gap junctions, ensuring the entire bone is communicating [13]. Mature osteocytes reside within the lacuna-canalicular network of

bone with the cell bodies residing in the lacunae and the long, slender processes radiating out in all directions but with the highest density perpendicular to the bone surfaces [13]. The processes pass through the bone matrix via small canals, the canaliculi, connecting the osteocytes to each other and to other bone cells [13]. Osteocytes must remain in contact with other cells and with the bone surface to ensure they have access to oxygen and nutrients. Osteocyte viability may play a significant role in the maintenance of bone homeostasis and integrity. Osteocyte apoptosis can occur due to a number of different factors including: microdamage, estrogen deprivation, elevated cytokines, osteoporosis, and aging [13]. Obesity and its associated effects (including microdamage and elevated cytokines) could potentially affect osteocytes and therefore mechanotransduction.

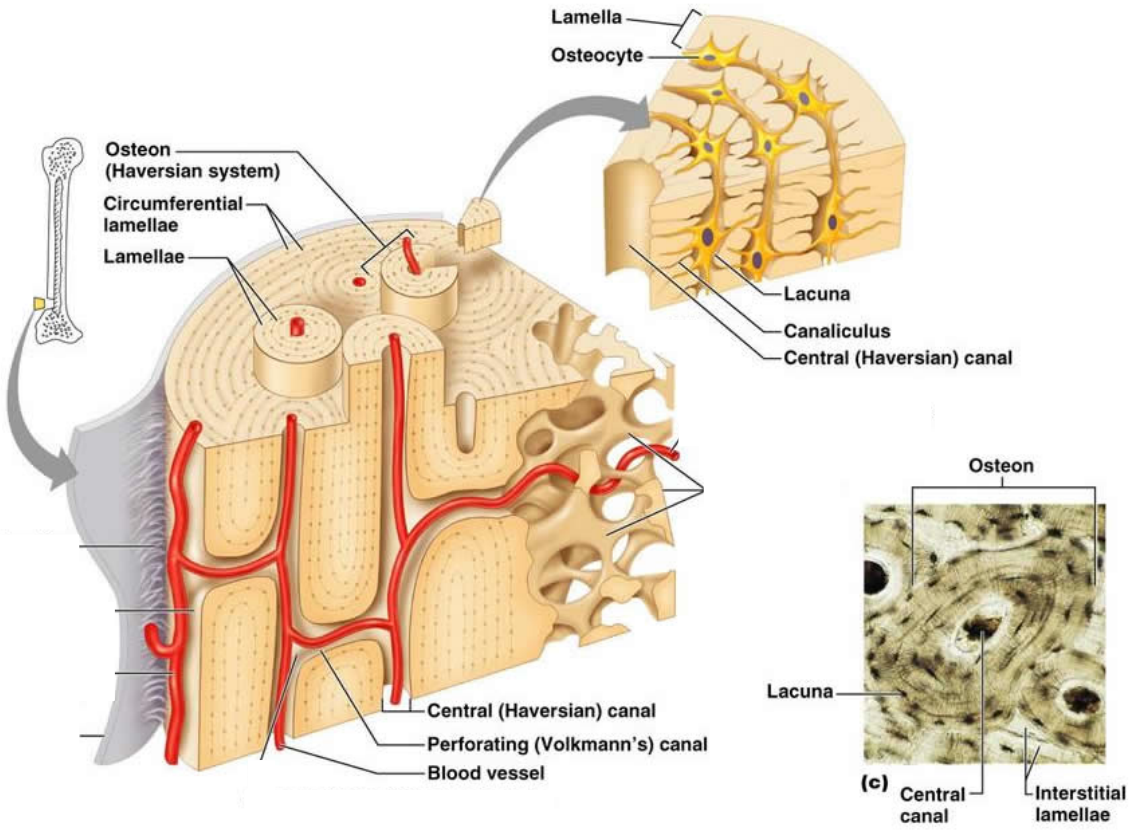

Figure 1.4. Schematic of the osteocyte lacuna-canalicular network [14].

1.6 Mechanotransduction

The currently favoured model of mechanotransduction involves osteocytes and their lacuna-canalicular network [13]. It is presumed that mechanical loads applied to bone are transduced through the skeleton and received by a cellular network [13]. The mechanical signal is then detected by certain receptor cells leading to the generation of a secondary signal aimed at target cells that modulate actual bone formation and resorption [13]. The mechanisms of mechanotransduction, mechanosensitivity, and response signaling in bone are not completely understood. It is thought that bone strain causes fluid flow in the lacuna-canalicular network which creates shear stress that stimulates the osteocytes, the receptor cells, which then activate the osteoblasts and osteoclasts, the effector cells, to maintain bone homeostasis [15].

It was first believed that loading would strain the cells directly, but the degree to which bone cells need to be stretched to elicit an osteogenic response is greater than the surrounding mineralized tissue can bear [16]. It has not yet been established how the loading of intact bone is transduced into a signal for the osteocytes. The application of force to bone during movement results in several potential cell stimuli including: changes in hydrostatic pressure, direct cell strain, fluid flow, and electric potentials resulting from electrokinetic effects accompanying fluid flow [15]. Evidence has been increasing steadily for fluid shear stresses caused by the flow of canalicular interstitial fluid as the likely stress-derived factor that informs the osteocytes about the level of bone loading, as it can generate an osteogenic response [15].

The magnitude of fluid flow through the lacuna-canalicular network is directly related to the amount of bone strain [13] and could be the means by which mechanical information is transmitted. The flow of fluid through the bone canaliculi has two effects: a mechanical one, derived from the fluid shear stress, and an electrokinetic one, derived from strain-generated potentials [13]. Either of the two, or both in combination, might activate osteocytes, although cell culture experiments suggest that cells are more sensitive to fluid forces than they are to electric potential [17]. Strain-generated potentials may modulate the movement of ions such as calcium whereas shear stress might cause movement of cell attachments [15].

Experimental studies show that osteocytes are sensitive to stress applied to intact tissue and loading can produce rapid changes in their metabolic activity [15]. *In vitro* osteocytes are quite sensitive to fluid shear stress and their response is stronger than osteoblasts and osteoprogenitor cells [15]. Both of these findings suggest that they may indeed function as mechanosensors in bone. As the fluid flows over the membranes of the osteocyte and its processes (the mechanosensitive part of the osteocyte has not yet been determined) and interacts with the proteoglycan matrix, the resulting shear stresses on the cell cause the release of intracellular calcium ions [13]. It is thought that the release of these calcium ions leads to propagation of signals across gap junctions from cell to cell activating many downstream signaling cascades. However, many steps in the mechanosignalling cascade are still unknown.

In healthy adequately adapted bone, strains resulting from physiological loads are quite small. Quantitative studies of the strain in bones of many animals found normal

locomotor strains *in vivo* ranging from about 500–2000 microstrain (με) and maximal strains no higher than 2000-3000 με [18]. However, *in vitro* studies of strained bone cells have shown that much higher strains, in the order of 7000-10000 με, are required to obtain a cellular response but the average strain of the bone matrix cannot exceed approximately 7000 με or the bone will be permanently damaged [13]. In intact bone, 1500 με was sufficient to activate adaptive bone formation *in vivo* [15]. If bone strain is involved in bone cell mechanosensing, then bone tissue seems to possess the ability to amplify small strains experienced by the bone into larger ones that can be detected by osteocytes.

Overall, it appears that strain is the stimulus for bone adaptation, osteocytes are indeed the mechanosensors and osteoblasts and osteoclasts work together to adjust the bone accordingly. In healthy bone, normal physiological loading is needed to maintain the movement of nutrients and waste to osteocytes and to provide them with enough fluid shear stress to meet a threshold level of strain [15]. Under such conditions bone cells, osteoblasts and osteoclasts, would not be recruited. If the level of loading were increased so would the fluid shear stress and thus, the osteocytes would call upon the osteoblasts or may inhibit osteoclasts. The production of new bone would then reestablish the strain threshold, stopping the stimulation of osteocytes. If the bone becomes underloaded, then fluid shear stress would decrease and so would the transportation of nutrients and wastes and the osteocytes would become inactivated or may even die. This inactivation may be the trigger for the recruitment of osteoclasts or the inhibition of osteoclast suppression or both [15].

1.7 Cortical Bone Mechanical Properties

It is important to distinguish between the mechanical behavior of a bone as a structure (structural behaviour) and the mechanical behaviour of the bone tissue itself (material behaviour). The material behaviour of a specimen is independent of its geometry, size and shape, and it reflects the intrinsic properties of the material itself.

To determine the mechanical properties of a bone as a structure, typically a load-deformation test is conducted. In the case of this thesis project, both three-point bending and compression tests were used. When a load is applied to the bone the bone will deform. That is, there will be a change in length in the bone being tested and the results can be portrayed in a load deformation curve (*e.g.*, Figure 1.5). Such a mechanical test evaluates the bone as a structure and the shape of the load-deformation curve is dependent on the size and shape of the bone and also on the properties of bone tissue itself. Generally there is a linear region of the curve referred to as the elastic region — within this region the bone will return to its original size and shape when it is unloaded. The slope of the elastic region is the bone structural stiffness. Beyond the linear region, where the slope of the curve drastically decreases, is the plastic region. If a bone is loaded into the plastic region it will experience permanent deformation, and if the bone is unloaded in this region it will not return to its original shape. The point where the two regions meet and the curve changes shape is referred to as the yield point. The point where the curve stops and drops off is the ultimate failure point or the point where the bone fractures. The failure or maximum load is usually considered the strength of the bone. The energy

required to cause failure of the bone is defined as the area under the load-deformation curve to the failure point and is termed the work to failure.

The mechanical properties of a structure depend on both its geometry and the properties of the material that composes the structure. To determine material properties, the size and shape of the structure must be taken into account. The material properties are usually determined by calculations that use a combination of the measured bone structural properties and the bone geometric properties. Normalizing the load and deformation to the size of the structure results in stress and strain respectively. Stress is defined as load per unit area. Strain is defined as the fractional change in dimension of a loaded structure. For example, when a femur is compressed, the load is divided by the cross-sectional area (XSA) of the femur to obtain the stress, and the deformation is divided by the original length of the femur to obtain the strain. A load-deformation curve can be converted to a stress-strain curve by changing load to stress and deformation to strain. The stress-strain curve is analogous to the load-deformation curve but reflects the material behaviour of the bone tissue.

The shape of the stress-strain curve is similar to that of the load-deformation curve as shown in Figure 1.5. Similar to the load-deformation curve, there is a linear region – the elastic region, and a nonlinear region – the plastic region. At physiologic levels of stress, there is a linear relationship between the applied stress and the resultant strain. The slope of the linear region is the modulus of elasticity or Young's modulus. The elastic modulus is a useful determinant of bone material properties. Typical values of the elastic modulus in human femora are shown in Table 1.2. Similar to the load-deformation curve,

in the elastic region, the material bonds will stretch or bend but they will not be permanently rearranged. In the plastic region, the material will undergo permanent deformation. The strength of the bone material is determined by calculating the maximum stress at the point of failure. Integration of the curve, giving the area under the curve, is a measure of the strain energy or toughness of the material.

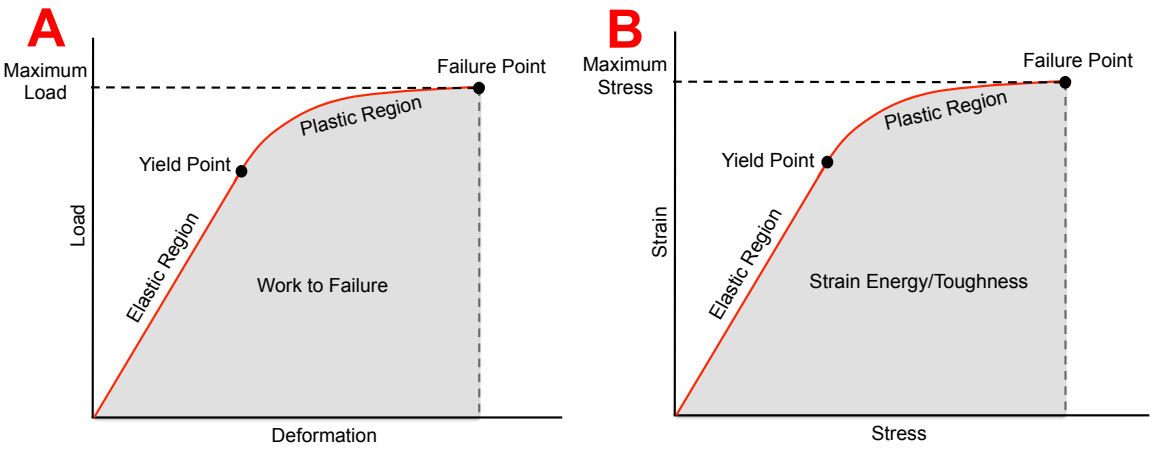

Figure 1.5. Typical load-deformation curve (A) and stress-strain curve (B) of a bone under compression.

Table 1.2. Elastic modulus values of the human femur in various loading directions [19].

Modulus (GPa)	Value
Longitudinal	17.0
Transverse	11.5
Shear	3.3

Bone material properties are determined by two different factors: 1) the calcification of the solid bone matrix, 2) the composition and spatial arrangement of crystals, collagen fibers, lamellae, and 3) the density of microdamage inside the calcified tissue. More than 80% of the variation in the elastic modulus in cortical bone can be

explained by the matrix mineralization and bone porosity [20]. The major determinant of the elastic modulus is related to the mineral phase whereas the yield strength is determined both by the mineral composition and by the integrity of the collagenous matrix [20]. The elastic behavior of bones is mainly affected by the collagen quality and mineralization, while bone properties under plastic conditions depend on microstructural factors and microdamage that affect the ability of the tissue to resist fracture [20]. The ability to incur microdamage is a protective mechanism against complete fracture, but microcracks compromise bone strength as they accumulate.

The mechanical behaviour of bone varies according to the direction and rate of loading. Bones have different mechanical properties in different loading directions, a property known as anisotropy. The anisotropic behaviour of bone reflects its function as a load bearing structure because it is generally stiffest and strongest in the primary loading direction. Cortical bone is stronger along the long axis of the bone than the transverse axis and is stronger in compression than in tension. The stiffness and strength of cortical bone also depend on the rate at which the bone is loaded or strained. Bone is a viscoelastic material, one that undergoes material flow under sustained stress and exhibits different mechanical properties under different loading rates [21].

1.8 Bone Adaptation

Bone is a dynamic tissue that can adapt to mechanical loading with changes in shape and structure to achieve a better balance between stress and load. Julius Wolff is credited with first proposing that mechanical stress is responsible for determining the

architecture of bone and that bone tissue is able to adapt its mass and three-dimensional structure to mechanical usage to obtain a higher efficiency of load bearing [9]. Bone is able to optimize itself to provide the best support for daily loading. If loading patterns change then bone can adapt accordingly. The ability of bone to adapt is key to maintaining structural stability.

One key concept regarding the adaptation of bone to mechanical loading is the idea that this occurs via a self-regulating biologic mechanism. Claude Bernard first introduced the idea of homeostasis to physiology, the concept that physiologic systems self-regulate to keep a critical internal variable constant [9]. Frost [22] was the first to describe the mechanostat theory stating that bones adapt in response to changes in the mechanical environment so that they can remain mechanically competent. As Bernard had done previously, Frost [22] suggested that bone has a homeostatic regulatory mechanism that can sense changes in mechanical demands and alter its structure accordingly to meet these new demands. It is thought that below a certain threshold of use, bone will be resorbed because it is not necessary, and above a certain threshold, where mechanical demands are higher than typical, bone formation will occur to increase the strength of the bone to withstand the new loading. That is, bone has a mechanostat that can regulate bone adaptation based upon the loading environment. The bone homeostatic control system or the mechanostat has a stimulus – strain or a strain-related characteristics, a sensory mechanism – osteocytes, and an effector mechanism that can bring the system back to homeostasis – modeling and remodeling [22].

The internal variable that bone tries to keep constant, as Bernard described it, is strain. Bone strain is defined as the fractional change in dimension of a loaded bone and therefore results in changes in the bone environment. Because of this, it is strongly believed that the level of strain experienced by the bone provides the threshold or stimulus for bone adaptation. Rubin and Lanyon [18] found that measured bone strains during physiological activities were nearly equivalent on different bones and in different animals. When a mature musculoskeletal system is functioning at a high physiologic level, peak periosteal strains usually lie between 2000 and 3000 με regardless of the bone or animal [18]. This finding supports the notion that the skeletons of all animals are adapted to control the magnitude of strain.

Bone modeling and remodeling are the mechanisms that bones use to adapt to mechanical strain. Bone modeling is the process by which bone is shaped to maximize functional capacity. It is the process by which bone is formed by osteoblasts without prior bone resorption and occurs on the surfaces of bone [12]. This process is vigorous through growth and produces changes in bone size and shape. It slows during aging but continues to maintain optimal bone shape. Bone modeling can be induced in mature bone with a change in the mechanical environment. In animals, bone modeling can be induced experimentally through *in vivo* external loading of a bone. This loading can produce lamellar bone formation on the periosteal surface.

Bone can also be remodeled. This process involves the resorption of existing bone by osteoclasts and subsequent new bone formation by osteoblasts in the same location. Together, the osteoclasts and osteoblasts form the basic metabolic unit (BMU) that

reconstructs the internal bone structure [23]. Bone remodeling works to maintain or alter mineral balance, adapt bone structure to meet changes in mechanical loading, or to repair microdamage caused by excessive loading and fatigue damage [23].

Bone adaptation requires a balance between bone formation and resorption to optimize bone structure. Changes in the body that can affect osteoblasts and osteoclasts such as obesity, can compromise bone adaptation and, in turn, affect the integrity of bone.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diet

Obesity, although once considered a state of overnutrition, has now been recognized as a risk factor for several nutrient deficiencies, including lower levels of antioxidants and certain fat-soluble vitamins. The causes of the nutrient deficiencies are not fully understood but it is most likely the result of eating energy dense nutrient poor (EDNP) foods. Positive associations between body mass index (BMI) and consumption of EDNP foods have been found [24, 25]. EDNP foods provide approximately 27% of the total daily energy in the North American diet, with one-third of the population consuming an average of 45% of energy from EDNP foods [26]. EDNP foods tend to substitute for, rather than supplement, the more nutrient-dense foods in the North American diet. This pattern leads to: 1) high energy intake, 2) marginal micronutrient intake, 3) poor compliance with current nutrient- and food group–related dietary recommendations, and

4) low serum concentrations of vitamins [26]. Kant et al. [26] found that the odds of consuming less than the standard intake of most micronutrients and the odds of having low serum vitamin concentrations rose with increasing EDNP food intake. As well, EDNP food consumption was inversely related to the odds of consuming 30% of energy from fat and obtaining less than 10% of energy from saturated fat [26].

An EDNP diet would be considered what Fung et al. [27] refers to as a "Western" pattern diet. It is characterized by a higher intake of red and processed meat, high-fat dairy products, and high-fat foods such as French fries, eggs, refined grains, sugar-containing beverages, sweets, desserts, beer, and liquor [27]. As intake of nutrient-poor food increases, the intake of unprocessed, nutrient-dense foods decreases proportionately. Although risk factors of this diet do include increased fat consumption, they also include increased sugar consumption and decreased consumption of micronutrients key to bone health. There are a number of dietary factors associated with obesity that may contribute to the changes seen in the bone mechanical properties of obese individuals.

Hypothetically, overweight and obese people may be disadvantaged nutritionally in two ways. Firstly, although their diets are high in energy, intakes of micronutrients may be low as a consequence of poor dietary choices [28]. However, micronutrient intakes have been shown to be strongly influenced by energy intakes [29]. Secondly, overweight and obese people may have higher requirements of key nutrients because obesity affects the absorption, storage, oxidation and excretion of nutrients [30-32].

Many studies have investigated associations between micronutrient status and markers of adiposity, generally finding a low micronutrient status in overweight or obese

populations for one or more nutrients [33]. Research on micronutrient status is often carried out on patients awaiting bariatric surgery. Surveys in patient groups found inadequate levels of nutrients, such as iron and vitamins A, B6, B12, C, D and E [34-37]. Surveys in general populations also show associations between markers of adiposity and a lower micronutrient status [28].

A large portion of the population consistently consumes fast food. In fact over one-third of North Americans report having eaten fast food on any given day, while 7% eat fast food daily [38]. Nutritional analysis shows fast food to be high in fat, saturated fat, energy density, fructose and glycaemic index, yet poor in fibre, vitamins A and C and calcium [39]. Over the past 20 years, sugared beverage intake has increased 70% in children aged 2–18 and 83% in young adults aged 19–36 [40]. The Canadian Community Health Survey found that one-quarter of all Canadians reported consuming fast food on the day before their interview including one third of adolescents (aged 14-18) [41].

2.1.1 Vitamin D

Vitamin D inadequacy constitutes a largely unrecognized epidemic in many populations worldwide. More than one-third of the US population exhibits vitamin D deficiency [42]. It has been reported in healthy children, young adults, middle-aged and elderly adults [42]. Typically, the prevalence of low 25-hydroxy vitamin D 25(OH)D levels is approximately 36% in otherwise healthy young adults aged 18 to 29 years [42].

Vitamin D plays a central role in calcium and phosphorus homeostasis and skeletal health. Chronic vitamin D inadequacy in adults can result in secondary hyperparathyroidism, increased bone turnover, enhanced bone loss, and increased risk of

fragility fracture [42]. The increase in parathyroid hormone (PTH) mediated osteoclastogenesis results in increased numbers and activity of osteoclasts [42]. Osteoclasts resorb bone, releasing calcium and phosphorus. The result is increased skeletal porosity, defective bone mineralization, decreased BMD, osteoporosis, and increased risk of fragility fracture [42]. Secondary hyperparathyroidism can also affect phosphorus levels causing poor calcium-phosphorus ratios resulting in poor bone mineralization, making the skeleton less rigid [42].

The most important source of vitamin D is the sun and, to date, there is no evidence of reduced sun exposure in obese groups [43]. The second most important source of vitamin D is food, and it has been speculated that malnutrition in the obese group could be a reason for their vitamin D depletion [44]. It may not be a lack of sun exposure or a lack of consumption but rather vitamin D sequestration by adipose tissue [44] that leads to vitamin D deficiency in obese individuals. A study by Wortsman et al. [45] found that conversion of the vitamin D precursor was similar in obese and lean subjects, but that the resulting increase in serum vitamin D in the obese group was 57% lower than that measured in the lean group. The authors proposed that vitamin D production in response to sunlight was inefficient in obese people caused by deposition of vitamin D in subcutaneous adipose tissue. As well, oral supplementation in the obese group resulted in a lower serum vitamin D level compared to the effect seen in the lean group [45]. Other studies have confirmed differences in vitamin D and calcium metabolism between obese and lean subjects [46]. This has led to suggestions that obese people may have a higher requirement for vitamin D [31].

Several studies have reported differences in circulating levels of 25(OH)D between lean and overweight populations. In these studies a lower vitamin D status was found in participants with a higher BMI [44], abdominal obesity [47, 48] and greater body fatness [49]. As well, recent studies have shown that body mass index and body fat are inversely related to serum 25(OH)D levels and directly related to PTH levels, which is likely due to vitamin D sequestration in body fat compartments [42]. Abnormalities in calcium metabolism in the morbidly obese have been demonstrated with vitamin D deficiency as the common denominator [50]. Several investigators have reported vitamin D deficiency and raised PTH levels in obese populations, some in more than 50% of the study population [36, 50-53]. Debate continues on whether the low levels of circulating nutrients in obese populations reflect true unavailability or increased adipose storage, both risk factors for deficiency.

2.2 Effects of Obesity on Bone

2.2.1 Systemic Effects

Obesity can affect bone both systemically and locally. Systemically, obesity can affect bone metabolism in the following ways:

- It has been established that obesity is associated with low-grade chronic inflammation and elevated production of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) [54-57]. These cytokines are released from adipocytes and have been shown to increase osteoclast activity [54-56].

- Adipocytes and osteoblasts are derived from a common multipotential mesenchymal stem cell, and these stem cells exhibit an equivalent tendency for differentiation into adipocytes or osteoblasts [55, 56] (Figure 2.1). Numerous interacting pathways determine the balance of the differentiation and a diet high in fat may increase bone marrow adipogenesis while inhibiting osteoblastogenesis [54, 55].
- A diet high in fat can interfere with intestinal calcium absorption and utilization [55, 58]. In particular, saturated fatty acids form insoluble soap complexes with calcium in the intestine and therefore the calcium cannot be absorbed [57-60].
- The levels of several hormones are altered by obesity, many of which can impact bone. Insulin is a potential regulator of bone growth, since osteoblasts have insulin receptors as well as insulin-like growth factor 1 (IGF-1) receptors, which can also mediate the effects of insulin [5]. Insulin can stimulate osteoblast proliferation and in clinical studies, circulating insulin levels are related to bone density [5]. As well, IGF-1 concentrations increase with weight and it has been suggested that IGF-I acts to increase bone size [61]. Hyperinsulinemia can cause androgen and estrogen overproduction increasing free concentrations of sex hormones which can reduce osteoclast activity and have positive effects on osteoblasts [5]. Adipocyte-derived hormones such as leptin and adiponectin also play a role in bone's response to obesity by a variety of mechanisms [61].
    - Leptin is secreted primarily by adipocytes, and whether it is beneficial or antagonistic to bone quality is under debate [54, 55]. Although, there is some consensus that increased leptin production is associated with higher bone

mineral density and bone size [61]. Leptin affects bone both locally and centrally. Locally, osteoblasts express leptin receptors and leptin increases osteoblast proliferation and differentiation and also inhibits osteoclastogenesis [5]. Centrally, leptin enhances sympathetic activity, which in turn promotes bone resorption through the inhibition of beta-adrenergic receptors expressed by osteoblasts [62]. The local and central effects are in opposition with respect to bone health, but since leptin originates primarily in adipocytes, in most situations the local effects will dominate [5, 63].

- Adiponectin is the most abundant protein secreted from adipose tissue and its concentration in circulation is much higher than that of other hormones and cytokines [64]. Unlike other adipokines, serum adiponectin levels are decreased in obesity [65]. Studies report negative correlations between circulating adiponectin and bone mass [65]. Adiponectin is expressed on osteoblasts and increases osteoblast proliferation, differentiation and mineralization suggesting a positive effect of adiponectin on bone [5, 65]. Adiponectin has also been shown to inhibit osteoclastogenesis and osteoclast activity by modulating osteoblasts resulting in reduced bone resorption and increased bone mass [5, 65].

- High levels of dietary fat have been reported to induce oxidative stress, overproduce reactive oxygen species, and decrease antioxidative enzyme activity, all of which may contribute to osteoclastogenesis and bone resorption and to increased osteoblast apoptosis and decreased bone formation [66].

- A high sucrose diet can also affect bone as it can produce hyperinsulinemia that can then induce hypercalciuria by inhibition of renal resorption of calcium, which can potentially influence bone mineralization [57-59].

Overall, systemically, the effects of obesity on bone are both positive, with increases in bone size and bone formation and negative, with potential compromises in mineralization and bone resorption.

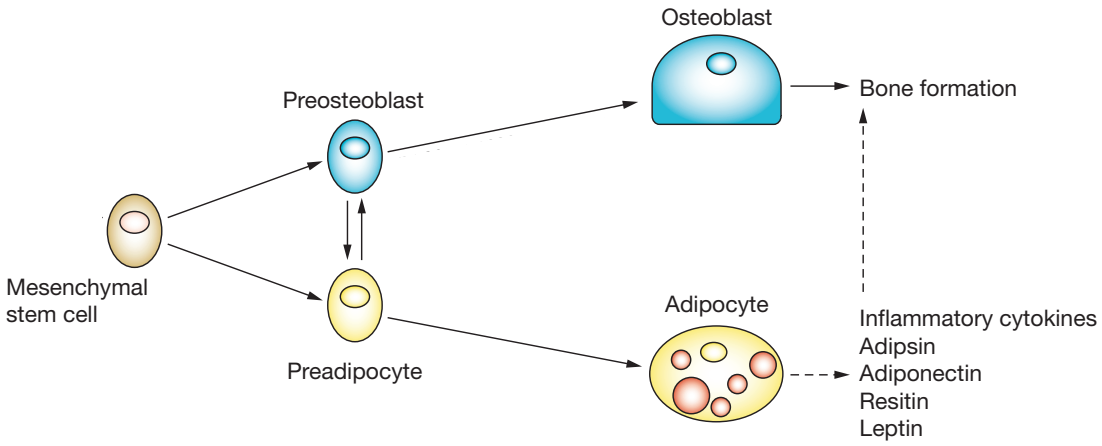

Figure 2.1. Schematic of the lineage allocation of mesenchymal stem cells [62]. The solid arrows represent confirmed networks for regulation and the dashed arrows represent potential regulatory pathways.

2.2.2 Local Effects

Evidence suggests that the strain responsible for bone adaptation is sensed by osteocytes through the production of fluid shear stress but the mechanisms behind the origin of the strain are debatable. It is strongly believed that muscular forces exerted upon bone mainly determine bone morphology, structure and strength [67-69]. Muscles put the largest loads on bones, even on weight bearing bones [9, 70, 71]. Therefore, bone would adapt a weight bearing bone's strength more to muscle strength than to body weight or

other sources of bone loads [72, 73]. It is the muscle forces that cause the strains on the bones and, in turn, control bone adaptation. Thus with reduced muscle mass and strength, strains exerted on bone are weaker, and the bones are not stimulated sufficiently. However, reaction forces or gravitational forces can also cause bone loading. The local effects of obesity on bone therefore include muscular forces and may include, for weight bearing sites, body mass effects. Muscular forces and gravitational forces are not mutually exclusive in weight bearing animals. Mechanical loading as a result of body mass occurs at a frequency of 1 to 2 Hz whereas the frequency of muscle contraction ranges from 15 to 60 Hz [74]. It has been shown that these higher frequencies are significantly related to bone adaptation responses [74]. Muscle forces are present, significant, and capable of eliciting adaptive responses but whether they are the primary stimuli or not has not been well established.

In younger adults, muscle mass is generally increased in obesity, which is assumed to be an anatomical response to the stress imposed by increased body weight. As mentioned previously, epidemiological data show that higher body weight or BMI is positively correlated with bone mass, and weight loss may actually cause bone loss [75-77]. It has been shown that changes in body weight generally involve proportional changes in fat mass (FM) and fat-free mass (FFM) with, on average, 30% of any change in weight, gain or loss, comprised of FFM, mainly muscle [78]. Therefore obesity not only involves an increase in fat mass, it is also accompanied by an increase in FFM so it may be the greater muscular forces as a result of the increased FFM, the overall increase in body mass that is causing changes in the bone structure or it may be a combination of

both. Resolving whether changes in bone size, mass and density seen with obesity are mainly the result of increased FFM or overall body mass is difficult.

Though the mechanisms by which lean mass influences bone mass are not clear, the most accepted explanation is that increasing muscular loading can induce periosteal apposition, and that bone formation can also be stimulated directly by mechanical strain via mechanoreceptors in osteocytes [79-81].

2.2.3 Demonstrated Effects of Obesity on Bone Properties

Obesity has been shown to affect a number of bone properties including structural, morphological, material, and microarchitectural, but many of the results are inconsistent.

- *Geometry*

Several murine studies have shown altered bone geometry caused by obesity. Increased second moment of area [59, 61, 82], and bone cross sectional area have been found in obese animals [58, 61, 83]. No changes [54, 55, 58] as well as reductions in cross sectional area have also been found [3, 84]. These different findings could be the result of different diet durations and/or the different bones examined.

- *Bone Volume and Mass*

Significant decreases in bone volume in animals fed a high-fat diet have been found [54, 55, 60, 85]. A high-fat diet has also shown to decrease femoral bone mass despite a significant increase in body weight [4, 55].

- *Mechanical and Material Tests*

The maximum load has been shown to be greater [59, 83] in animals fed a high-fat diet compared to controls but it has also been shown to be lower [3, 4, 57, 58, 61]. The energy to failure has also been inconsistent, with some studies showing lower [57] and others no change [58]. No differences have been found between high-fat diet animals and controls with respect to maximum stress and stiffness [83] but a lower stiffness [4, 61] has been shown in high-fat animals as well. The elastic or Young's modulus has shown to be unchanged in some studies [4, 83] and significantly lower in rats fed a high-fat diet in others [4, 58].

- *BMD*

The effects of a high-fat diet on bone mineral density (BMD) are inconsistent. No differences in BMD of cortical bone between obese animals and controls [54, 55, 61] have been found, indicating that high-fat diet may not impair mineralization. However, others have found that the BMD indicated a substantial bone loss in obese animals compared to controls [56, 64, 85]. Li et al. [58] found both results depending upon the bone examined. An increase in BMD has also been reported [61].

- *Bone Mineral Content (BMC)*

Similar to BMD the effects of a high-fat diet on BMC are unclear. Both increases [59, 61] and decreases [4, 64, 66] in BMC with a high-fat diet have been found. The increase in BMC was said to be consistent with the increase seen in bone size [61].

- *Cortical Bone Microarchitecture*

In general, trabecular bone has been said to be more responsive than cortical bone to diet or drug treatments or aging because trabecular bone is more actively remodeled than cortical bone due to the larger surface to volume ratio [54]. Ionova-Martin et al. [61] used SEM to show that both the orientation of osteocyte lacunae and the lamellar structure are less ordered and aligned in mice fed a high-fat diet compared to controls. In the control mice, the bone was well aligned and organized; the high-fat diet mice displayed poorer mineral organization [61]. As well, a diet high in fat has been shown to cause a significant accumulation of adipocytes in the bone [56], compromising the bone material.

Overall the effects of obesity on bone are inconsistent, possibly due to the different bone sites examined and the length and composition of the diets. However, many studies do indicate adverse effects of obesity on bone. Osteoporosis and fractures remain a large health issue and if obesity and its complications can potentially affect bone and increase the risk of low-energy fractures, then obesity must be considered to be a significant factor in bone health.

2.3 Muscle and Bone

A role for muscle in the determination of bone properties can be seen in weight – bearing and non weight-bearing bones. Daly et al. [86] measured muscle and bone size in the playing and non playing arm of tennis players and found increased muscle and bone mass in the playing arm compared to the non playing arm. Studies looking at the lower

limb or bones that experience both muscular and ground reaction forces have shown a primary role for muscle force in bone mass regulation. Lu et al. [87] used force transducers placed in the shaft of prostheses and a force platform to show that more than 70% of the forces generated within the femur during a normal gait cycle were found to result from muscle forces (which were also monitored via EMG), leaving less than 30% derived from body weight. However, during more strenuous exercise, when bone formation would be stimulated, the proportion of forces in the long bones resulting from muscle contraction might change. Other studies using animal models strengthen the role of muscles in osteogenesis, even in the absence of loading from ground reaction forces. Umemura et al. [88, 89] trained rats to jump up to an elevated platform, using muscular contraction of their hindlimbs, and then catch the edge of the platform using their front paws to avoid the effects of the ground reaction forces on the bones. The results from these studies showed significant increases in bone weight, mechanical properties, and structural properties [88, 89]. These studies, therefore, suggest that loading achieved by muscles only can cause a significant osteogenic response.

2.3.1 Fat and Muscle Effects on Bone

Recent reports suggest that fat mass negatively impacts bone mass when the confounding effects of weight and mechanical loading are controlled [90-92]. However, it has been shown that obesity can be protective against osteoporosis and fractures [93, 94], and that fat mass is the main determinant of a higher BMD [95]. Gnudi et al. [77] found that the relationship between BMC and BMD measurements and body composition is

different between women with or without osteoporosis. In osteoporotic women, FFM and FM were shown to be significantly associated with BMC and BMD, but in women without osteoporosis only lean mass was significantly associated with BMC and BMD [77]. However, in osteoporotic postmenopausal women, FFM has been demonstrated as the major component of body composition associated with BMC and BMD measurements, and FM has shown no association with BMC or BMD measurements [96]. It may be site specific, as muscle mass has been shown to be the only predictor of lumbar vertebrae BMD, whereas both fat and lean body mass determined femoral neck BMD [69]. The specificity of the site may play a role because trabecular bone is more metabolically active and is therefore subject to more accelerated remodeling when compared to cortical bone. Any adverse effects on cortical bone are offset more slowly and to a lesser extent than within trabecular bone. As well, the femur undergoes extensive mechanical loading from the increased body and fat mass whereas the lumbar spine does not undergo loading to the same extent.

In a number of studies of women with osteoporosis, a correlation between FFM and BMD have been reported. In some, it has been considerably weaker than the association between FM and BMD [95, 97], in others it has been greater [77]. Support for the positive association between muscle mass and bone has been demonstrated in young women as well. Low muscle mass was identified as a risk factor for low proximal femur BMD, whereas higher fat was only protective when associated with substantial muscle mass [98]. Teasing out the effects of body mass, fat mass, and lean mass on bone is very difficult and it remains to be seen which plays a larger role. However, if some studies

show that obesity, and specifically fat mass, do not provide protection for bone and rather muscle mass does, then interventions targeting physical activity would be ideal.

2.3.2 Sarcopenic Obesity

Examining sarcopenic obesity may help to tease out the effects of muscle vs. body mass on bone properties. Sarcopenic obesity describes individuals who are sarcopenic, have a low muscle mass, but who are obese. Therefore these individuals exhibit increased total body mass, through increased fat mass, but are lacking muscle mass, so, if muscle were the main determinant of bone mass, then these individuals would show decreased bone mass despite their increased overall mass.

Osteoporosis has been shown to be more prevalent in sarcopenic and sarcopenic obese individuals than normal lean and obese groups [99]. Despite no difference in BMI or percent body fat, osteoporosis was more than 3 times more prevalent in women who were sarcopenic obese compared to nonsarcopenic obese [100]. Osteoporosis was also 2.5 times more prevalent in sarcopenic obese women compared to sarcopenic nonobese women, despite the fact that the obese women had a greater BMI and greater percent body fat [100]. Lima et al. [101] found significantly lower trochanter and whole body BMD in sarcopenic individuals when compared with nonsarcopenic individuals independent of age, percent body fat, weight, BMI, and physical activity level [101]. These results suggest that an increase in body mass or fat mass cannot exert great enough loads upon bones to compensate for decreased muscular force needed to sustain BMD and may actually hinder the bone.

However, Aubertin-Leheudre et al. [102] found that sarcopenia did not influence hip or spine BMD, two main sites of osteoporosis and fractures, in obese sarcopenic postmenopausal women. They showed that when absolute fat mass is controlled for, muscle mass has no impact on BMD [102]. This suggests that obesity may exert a protective effect on BMD and fracture risk in sarcopenic postmenopausal women. The large mass of adipose tissue may provide an increased level of circulating estrogen, which, in turn, may help prevent BMD loss [103] or it may be that the increased bone loading caused by body weight does, indeed, affect BMD.

In summary, sarcopenic obesity studies to date do not appear to help decipher the role of muscle, fat and overall body weight on bone. This remains an area of much needed research.

Many factors associated with obesity can influence bone geometric, structural and material properties and, in turn, can affect the ability of bone to adapt to mechanical stimuli. The overall goal of this thesis project was to study the effects of obesity on the ability of bone to adapt to mechanical stimuli through the examination of bone structural and material properties and bone strain. The hypothesis was that male and female rats fed a lifelong high-fat diet, designed to induce obesity, exhibit decrements in bone mechanical properties, thus altering bone strain during mechanical loading and consequently affecting bone adaptation.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animals

The Animal Research Ethics Board at McMaster University approved all animal experiments. At weaning, 3 weeks of age, 38 male and female Wistar rats were randomly assigned to receive a normal control diet (NC) or high-fat diet (HF) (Table 3.1). A portion of the rats were assigned to the diets according to their litter. Four rats from each litter were divided up with one male and one female placed on the NC diet and one male and one female placed on the HF diet. The number of rats per group is not equivalent because litter sizes were not equal among the dams and the litters did not have equal sex ratios. It was decided *a priori* to keep 6 males and 6 females per mother but that was not always possible and some pups were sacrificed at earlier ages for examination, resulting in unequal group numbers (Table 3.2).

The animals were placed on this diet until termination at 39 weeks of age. The rats were housed one per cage and given food and water *ad libitum*. Upon termination, the rats were weighed and the mass of the gonadal, mesenteric and perirenal fat pads were measured. The carcasses were frozen until dissection.

Table 3.1. Distribution of experimental groups.

Diet	Male	Female
Normal Control (NC - 17% fat)	8	11
High Fat (HF - 41% fat)	10	9

Table 3.2. Litter pairings of NC and HF male and female rats.

Litter Number	# of M-NC	# of M-HF	# of F-NC	# of F-HF	Total Number
1	1	1	1	1	4
2	-	-	-	1	1
3	1	1	-	1	3
4	1	1	1	1	4
5	1	1	1	1	4
6	-	2	3	-	5
7	1	1	1	1	4
8	1	1	2	1	5
9	1	1	1	1	4
10	1	1	1	1	4
Sum	**8**	**10**	**11**	**9**	**38**

M-NC, male NC; M-HF, male HF; F-NC, female NC; F-HF, female HF

3.2 Diets

The NC diet is the Harlan 8640 diet in which 17% of the calories came from fat (Appendix I). The HF diet is the Research Diets RD Western Diet (D12079B) in which 41% of the calories are from fat (Table 3.3, Appendix II). The primary fat in the HF diet is milk fat. It makes up 39% of the 41% of calories from fat. Milk fat contains a variety of fatty acids but saturated fats compose over 50% of the total fat found in milk (Table 3.4).

Table 3.3. Macronutrient and micronutrient breakdown of the NC and HF diets and the average Canadian diet.

Macronutrient (% of calories)	Normal Control Diet (NC)	High Fat Diet (HF)	Rat Recommended	Average Canadian Diet*
Protein	29	17		17
Carbohydrate	54	43		51
Fat	17	41		32
Saturated Fat (% of total fat)	0.9	~50		10
Sugars	~5-8% of mass	>35% of mass		
Micronutrient				
Calcium	11 g/100g	9.2 g/1000g	≥2.5 g/kg [104]	<adequate
Vitamin D	3 IU/g	1000 IU/kg	1000IU/kg [105]	<adequate

* Statistics Canada [41]

Table 3.4. Common fatty acids in milk fat [106].

Common Name	Carbon Number	Type	Typical Composition (% w/w)
Butyric	4:0	short chain, saturated	3.9
Caproic	6:0	short chain, saturated	2.5
Caprylic	8:0	short chain, saturated	1.5
Capric	10:0	medium chain, saturated	3.2
Lauric	12:0	medium chain, saturated	3.6
Myristic	14:0	long chain, saturated	11.1
Palmitic	16:0	long chain, saturated	27.9
Stearic	18:0	long chain, saturated	12.2
Oleic	18:1	long chain, unsaturated	21.1
Linoleic	18:2	long chain, unsaturated	1.4
Linolenic	18:3	long chain, unsaturated	1.0
Others			10.6

3.3 Bone Adaptation – Lamellar Bone Formation

Mechanical loading was performed on a total of 20 of NC and HF male and female Wistar rats to investigate the effects of diet on mechanical induced bone

adaptation. The mechanical loading was performed using a forelimb compression model and a protocol intended to induce lamellar bone formation. The protocol was a total of ten days and sacrifice of the rats occurred on day 9, at 39 weeks of age. The experimental group breakdown is shown in Table 3.5.

Table 3.5. Breakdown of experimental groups for the bone adaptation loading protocol.

Diet	Male	Female
Normal Control (NC - 17% fat)	4	7
High Fat (HF - 41% fat)	5	4

3.3.1 Forelimb Model

Torrance et al. [107] were the first to use the rat forelimb compression model, which has benefits not offered by previous loading methods. The forelimb model is noninvasive and the loading contact points are at the wrist and elbow, both of which are away from the midpoint, the expected site of the highest strain and therefore greatest bone formation. This ensures that any bone apposition that occurs in this area is from the strain produced from the bending of the ulna due to the applied load and not from contact with the loading points themselves.

All loading was performed using an ADMET 5100 series (ADMET, Norwood, MA) material testing system (Figure 3.1). Custom cups were designed and manufactured to hold the carpus (wrist) and olecranon (elbow) during loading, similar to Figure 3.2. The z-axis was aligned with the long axis of the forelimb. Translation of the elbow was constrained in the x, y, and z directions and translation of the wrist was constrained in the x and y directions. The loading actuator was attached to the upper cup housing the wrist

and applied a downward displacement in the z-direction. Both the wrist and elbow were allowed to rotate freely in their cups; therefore, no external moments were applied on either end of the forelimb. The rat was placed on a custom designed table while the loading was performed. The height of the bottom cup (holding the elbow) was adjusted such that the elbow was not being pulled up or down but rather sat naturally as it would when the rat was lying on its back.

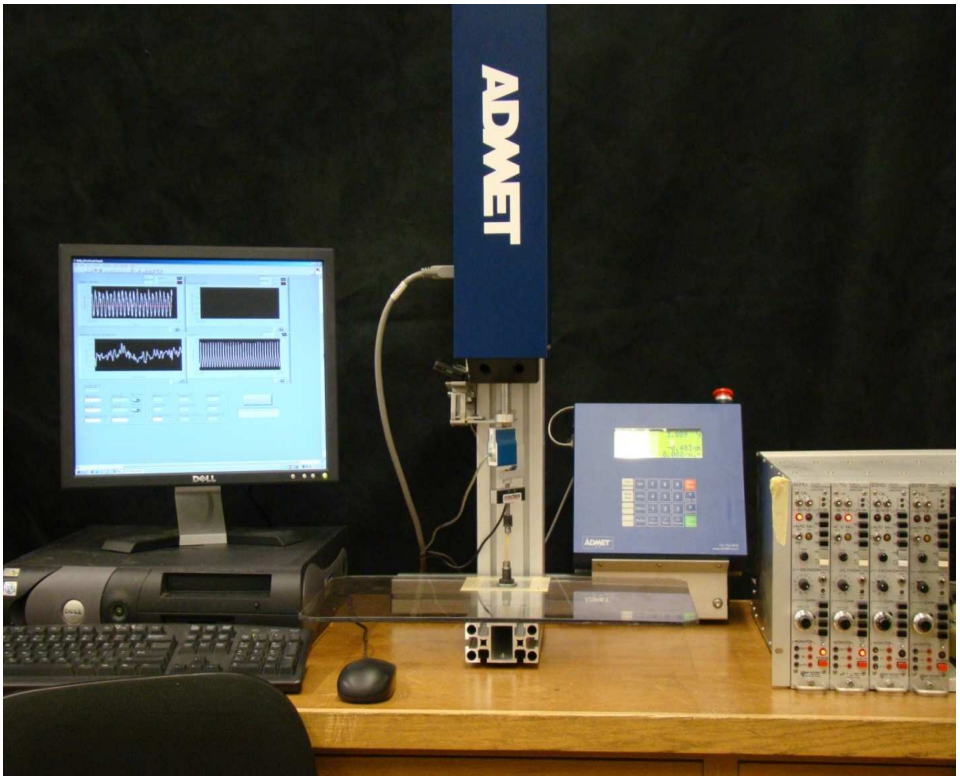

Figure 3.1. Material testing system setup.

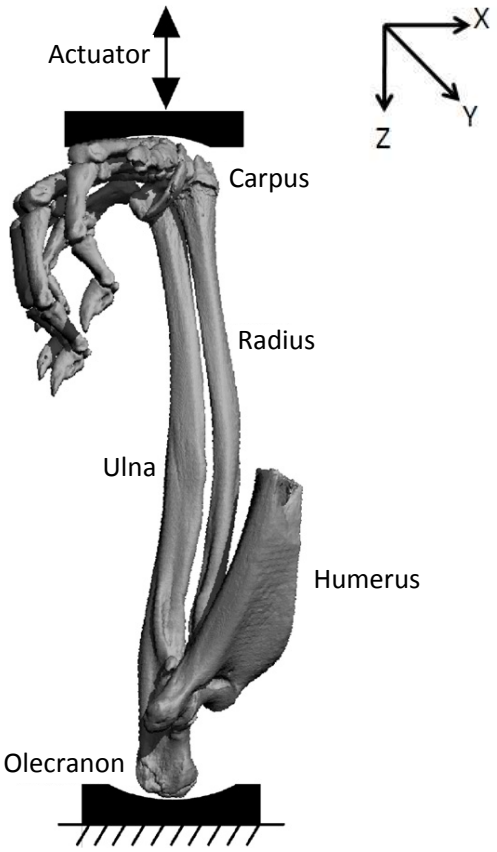

Figure 3.2. Schematic of the rat forelimb compression model [108].

3.3.2 Loading Protocol

Based on results from a previous study [109], a five-day forelimb loading protocol was chosen to induce lamellar bone formation in the ulna. The rats were anesthetized using 4-5% Isofluorane at 1 L/min. Cyclic loading was applied in a triangular waveform at a frequency of approximately 2 Hz with a 9.5 second delay at the end of each cycle, making a total loading cycle period of approximately 10 seconds. Fifty cycles were performed each day on the right forelimb of each rat to a target load of approximately 15 N, as established from a previous study [109]. The left forelimb acted as the non-

loaded control. The target load was approximate because the ADMET 5100 series material testing system had significant overshoot. For example, the target load was 15 N. To achieve this load at a frequency of 2 Hz, an input load of 8.5 N was used. The forelimb was unloaded to a load of approximately 2 N. The displacement rate was 14100 N/min. Gender also caused changes in the peak and minimum load with the females experiencing a smaller peak load at the 8.5 N setting than the males, possibly because of less stiffness or more compliance in their forelimbs (Table 3.6). Although the input parameters remained constant throughout the loading, response of the system did show some variability. Notably, changes in the stiffness of the forelimb throughout a given loading bout resulted in slight variations of frequency and peak load.

Table 3.6. The average peak load experienced by male and female rats during the forelimb loading protocol. The ADMET input load was 8.5 N and the actual target load was 15 N.

	NC-Male	HF-Male	NC-Female	HF-Female
Average Peak Load (N)	15.2	15.3	14.3	14.5

3.3.3 Fluorochrome Labels

Fluorochrome labeling is a widely used standard technique in skeletal research, which is simple and efficient for the investigation of the dynamics of bone formation [110]. In animal research this technique can be used for the measurement of bone formation and formation rate. The fluorochrome labels have a high affinity for calcium and therefore bind readily to it [110]. The labels only bind to calcium inside living cells, which are forming the new bone. When they are bound to calcium ions, they are incorporated at sites of mineralization in the form of hydroxyapatite crystals. The two

labels used during this study were alizarin complexone and calcein green. Alizarin and calcein have different excitation and emission wavelengths. Alizarin fluoresces red and calcein fluoresces green. In the first 24-36 hours after administration, the label will bind with calcium and the unincorporated label will be rapidly excreted by the kidneys [110]. This allows the visualization of the lamellar bone apposition rate. The bone apposition rate can be determined from the distance between labels in histological sections and the time between label administrations.

To evaluate lamellar bone formation, the fluorochrome labels calcein and alizarin complexone were injected into the rats on days when bone formation was known to occur (day 2 and 7) (Figure 3.3). Calcein (Alfa Aesar, Lancaster, UK) salt was diluted into Millipore water at a concentration of 5 mg/mL. Alizarin complexone (Alfa Aesar, Lancaster, UK) salt was diluted into Millipore water at a concentration of 10 mg/mL. One molar hydrochloric acid and sodium hydroxide solutions were used for pH balancing (pH between 7.2-7.4). The solutions were administered through subcutaneous injections in the abdomen. Calcein doses were 20 mg/kg body mass (4 mL/kg) and alizarin doses were 30 mg/kg body mass (3 mL/kg).

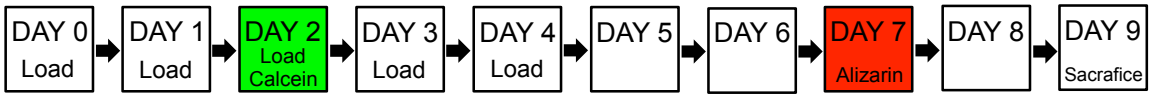

Figure 3.3. The forelimb loading and fluorochrome label injection timeline. The rats were loaded for five consecutive days, day 0 to day 4. On day 2 they were injected with Calcein and on day 7 they were injected with Alizarin. On day 9 the rats were sacrificed.

3.3.4 Sacrificing

The rats were sacrificed nine days after loading began, at 39 weeks of age. The rats were sacrificed through CO_2 asphyxiation. A clean animal housing was charged with CO_2 gas for one minute prior to asphyxiation. The rats were then placed in the charged CO_2 housing for at least five minutes. Rat carcasses were then stored in a freezer (-20°C).

3.3.5 Dissection

Both loaded and non-loaded control ulnas were harvested for histology after freezing. Minimal amounts of soft tissue were removed from the ulnas, leaving the muscle on the bone as not to disrupt the periosteum on the bone. After removal, bones were stored in a 70% ethanol solution at 3°C until the start of the dehydrating procedures.

3.3.6 Bone Sample Preparation for Histological Assessments

For histological evaluation, the ulnas were embedded in a plastic resin to ensure integrity when cutting them into thin sections (120 μm) for examination and analysis. After harvesting, forelimbs were placed in 15 mL Falcon tubes and dehydrated in increasingly concentrated ethanol solutions (70%, 95%, and 100%) stored at 3ºC in the refrigerator (Appendix III). They were then placed in 16 mL glass vials with xylenes to remove the fat, also stored at 3ºC. In the same vials, the forelimbs then underwent infiltration with methyl methacrylate, dibutyl phthalate and benzoyl peroxide solutions. These solutions were stored at 3ºC as well to prevent the resin from hardening. The final

embedding required the forelimbs to be placed in 4 mL glass vials and for the solution to be warmed to 37ºC to achieve a hard resin that could be sectioned properly.

3.3.7 Sectioning

After three days of curing, the resin had hardened and the embedded forelimbs were removed from the glass vials by breaking the glass off around them. The lengths of the forelimbs were then measured and the midpoint was marked. The area of interest, where the most bone formation was expected to occur, was approximately 1 mm distal to the midpoint [109]. The sample was placed with the distal end in the cutting clamp and an Isomet low speed diamond wafer saw (Buehler, Lake Bluff, IL) was used to cut the sample at the midpoint. A second cut was made 500 μm distal to the midpoint to achieve a section thickness of approximately 120 μm (the blade has a thickness of 380 μm). The section was then fixed to a microscope slide using Permount (Fisher Scientific, Toronto, Canada) and coverslipped. The samples were left to dry for twenty-four hours.

3.3.8 Imaging and Analysis

Upon drying, a Nikon DS-Fi microscope (Nikon, Tokyo, Japan) was used to image the slides and images were captured using a RETIGA 2000R camera (QImaging, Vancouver, BC) and accompanying NIS-Elements AR software package. Twelve bit 1650 x 2150 pixel images were obtained with an individual picture resolution of 1.68 μm/pixel. From the captured images, the total bone perimeter and the perimeter of the calcein and alizarin labels were determined using ImageJ (http://rsbweb.nih.gov/ij/).

3.4 Measurement of Physiological Mechanical Strains in the Right Femur

The ability to measure strain is extremely beneficial, not only for determining the mechanical properties of bone but also for quantifying the mechanical input to bone. A strain gauge is used to measure the deformation of the material it is attached to, in this case bone. *In vivo* strain gauge recordings provide a direct measure of bone deformations caused by all the parts of the musculoskeletal system not just estimates from ground reaction forces and analytical models. *In vivo* strain gauge implantation of a rat is difficult because of their small size, but measuring bone strain during physiological conditions offers more relevant data. Very few studies have reported *in vivo* strain gauge data from the rat. However, they can help provide a link between the mechanical responses of bone and the metabolic processes that are occurring within the bone. Combining the two, the factors that govern changes in bone metabolism can be established.

Ex vivo strain gauging is a much easier procedure that can still provide insight into what the bone is experiencing during mechanical loading. The drawback of this technique is that the loading conditions are not truly physiological. There is a lack of muscle and soft tissue support and the physiological direction of loading cannot be replicated outside the body easily. Despite its drawbacks, *ex vivo* strain gauging can provide a basis for further testing.

3.4.1 Cross Sectional Area Properties

Cross-sectional area properties of the femurs were measured from micro-computed tomography (microCT) scans. The right femurs were excised and cleaned of

soft tissue and were wrapped in saline soaked gauze and frozen. The femurs were removed from the freezer two hours prior to microCT scanning. To examine the structural properties of the femurs, the bones were scanned using a GE Medical Systems MicroCT eXplore RS80 (GE, Toronto ON) at an isometric resolution of 45 μm. Three dimensional data sets were loaded into Microview (GE Healthcare, Waukesha, WI) to visualize the data. Cross sectional area properties for each femur were determined at the midpoint using ImageJ. After thresholding, images were saved as .txt files containing a matrix of ones and zeros representing pixels containing bone and pixels not containing bone. The .txt image files were then loaded into Excel (Microsoft, Redmond, WA) and a custom worksheet was used to determine cross-sectional area and second moment of area. Second moment of area values were calculated by determining which row of the matrix represented the geometric centroid and summing the square of the distances from this row of all pixels not in this row. Following scanning, the femurs were wrapped in saline soaked gauze and re-frozen.

3.4.2 Strain Gauging

The ex vivo femoral longitudinal strains were measured using rosette strain gauges. The femurs were removed from the freezer two hours prior to performing strain gauge attachment and mechanical testing. Rosette strain gauges (FRA-1-11-015LE, Tokyo Sokki Kenkyujo Co. Ltd., Tokyo, Japan) were attached at the femur mid-diaphysis on the posterior surface. The midpoint of the femoral diaphysis was estimated to be half the distance between the superior surface of the femoral condyles and the middle of the

lesser trochanter (Figure 3.4). To attach the rosette strain gauge the posterior surface at the midpoint of the bone was dried, dehydrated and cleaned using acetone. A drop of cyanoacrylate (Tokyo Sokki Kenkyujo Co. Ltd., Tokyo, Japan) was applied to the bone at the midpoint upon which the strain gauge was placed with gauge "1" aligned with the long axis of the femur. The gauge was held firmly in place for five minutes and afterward was coated with clear lacquer (clear nail polish). Upon drying, the femurs were wrapped in saline soaked gauze to keep them hydrated before mechanical testing.

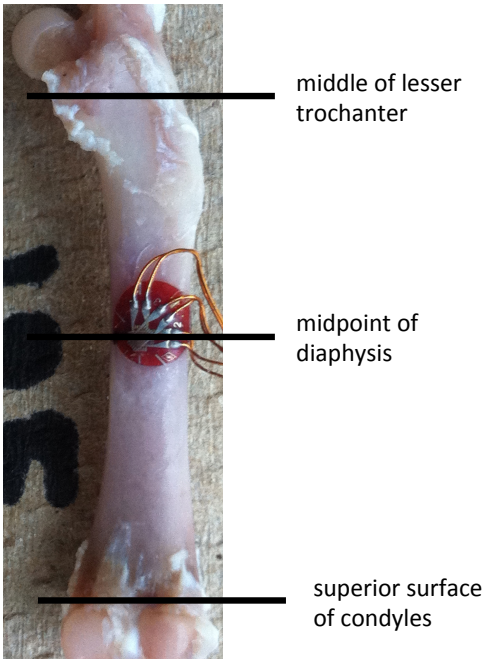

Figure 3.4. Strain gauge placement on the posterior surface of the femur. The strain gauge was placed at the midpoint of the diaphysis; determined as halfway between the middle of the lesser trochanter and the superior surface of the condyles.

3.4.3 Three-Point Bend Sub-Failure Tests

One of the main ways to examine bone structure is by performing mechanical testing. For whole bone testing, three-point bending has become the standard test for

determining bone mechanical properties associated with changes in the structure and material caused by various interventions. There are a number of long bones that can be tested using three-point bending including the femur, tibia, humerus, radius and third metatarsal [111]. Selection of an appropriate bone depends upon biological factors, the composition of the bone, the rate of bone turnover, and the site of interest and mechanical factors that will reduce errors and help to satisfy assumptions in various mechanical testing theories.

Custom designed and manufactured fixtures (Figure 3.5) were created for three-point bending using an ADMET 5100 series material testing system. Supports were manufactured from aluminum and capped with stainless steel dowels (2 mm diameter, Spaenaur, Kitchener, ON). An identical dowel pin was also fixed to the anvil. Femurs were removed from the freezer two hours prior to testing, and stored in saline soaked gauze. The posterior surface of each femur (where the strain gauge was attached) was placed on the two lower supports that were separated by a distance of 16 mm. The loading anvil made contact with the mid-point of the femur (opposite the location of the rosette strain gauge), causing the femur to bend in the anterior-posterior plane. Femurs were loaded to a 2 N preload and then were loaded cyclically for 30 cycles at a rate of 10 mm/min to loads of 10, 20 and 30 N. For loads of 40-100 N the femurs were loaded for 10 cycles. Force, displacement, and strain were recorded for each test (NI USB-6009 data acquisition card, National Instruments, Toronto, ON) using a custom data acquisition program (LabView 8.2, National Instruments, Toronto, ON). Load data were measured using an Interface SML-25, 25 lb load cell (Interface, Scottsdale, AZ) attached in series

with Interface SM-50, 50 lb load cell which provided feedback to the controller. Displacement was monitored using a differential variable reluctance transducer (DVRT) (M-DVRT-9, Microstrain, Williston, VT) and a custom extensometer. The load cell, extensometer's signal and strain were amplified using a Vishay 2310 Signal Conditioning Amplifier (Vishay, Shelton, CT).

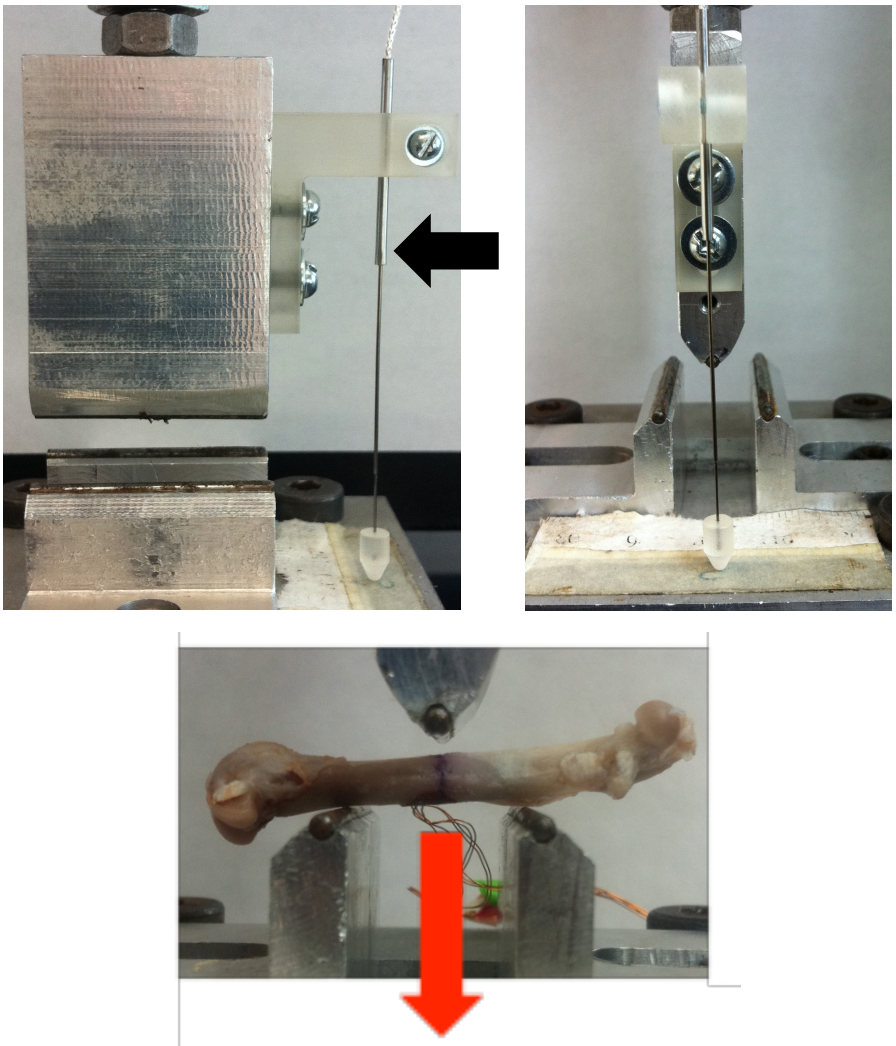

Figure 3.5. **Three-point bend test setup.** The two lower supports were 16 mm apart and the top loading anvil made contact with the midpoint of the bone. The DVRT is also shown (black arrow).

For loads of 10-30 N, 30 cycles were recorded. This was done to ensure the strain recorded from gauge "1" was within 5% of the calculated principal longitudinal strain. To collect the strain from each of the three gauges, a switch and balance unit (Automation Industries Inc., Phoenixville, PA) was used to switch between gauges every 3 cycles. This was done for a total of 30 cycles. The middle 9 cycles (3 cycles for each strain gauge) were used for analysis. The average peak force, displacement and strain of the 3 cycles for gauges 1,2 and 3 were taken (Figure 3.6). The principal strains were calculated for loads of 10, 20 and 30 N and the percent difference between the longitudinal principal strain and the strain recorded by gauge "1" was determined. For loads of 40-100 N only 10 loading cycles were performed. Strain was only recorded from gauge "1" because it was deemed as an acceptable representation of the longitudinal principal strain (<5% difference). The average peak force, displacement and strain of 3 cycles were used for analysis.

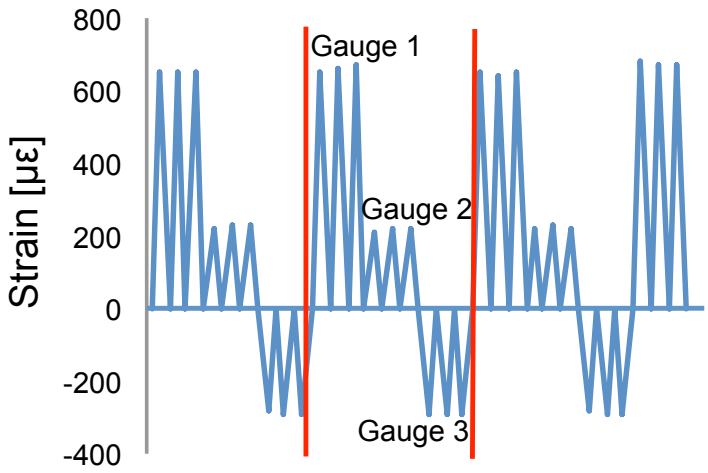

Figure 3.6. Representative graph of the strain recorded from the three gauges on a femur. The 9 cycles between the red lines (3 cycles/gauge) were the ones used for analysis.

The ADMET provided displacement data based on the position of the loading platen. However the bone displacement and testing machine displacement are never exactly equal. The ADMET itself has internal compliance, meaning there will always be some displacement in the machine and its components when mechanical tests are performed. To determine the internal compliance of the ADMET, the setup was loaded with no bone and the natural displacement of the apparatus was established through the range of loads. From this data, a load-displacement graph was constructed and the line of best fit was determined. The equation of this line was used to correct the recorded displacement values at a given load. The extensometer was attached to the ADMET and therefore was also affected by the compliance of the apparatus. The compliance tests were performed with the extensometer as well and the displacement values recorded by the extensometer were adjusted accordingly.

3.4.4 Principal Strain Calculations

The principal strains were determined using the following equation

$$\varepsilon_n = \varepsilon_x \cos^2\theta + \varepsilon_y \sin^2\theta + \gamma_{xy} \cos\theta \sin\theta$$

where:

ε_x = gauge 1 strain
ε_y = gauge 3 strain
γ_{xy} = shear strain
θ = angle of principal strain

The difference between the longitudinal principal strain and the measured longitudinal strain was calculated as follows:

$$\text{Percent Difference} = \frac{\text{(longitudinal principal strain – measured longitudinal strain)}}{\text{longitudinal principal strain}}$$

3.4.5 Material Property Calculations

Three-point bending tests determine the mechanical properties of the bone as a structure and these properties are then used to determine the mechanical properties of the bone as a material. To transition between the two, the Euler–Bernoulli beam theory, the gold standard to determine material properties of bones from mechanical testing, is used. However, along with this test come a few assumptions and limitations. The first assumption is that the length to width ratio of the beam is sufficient. For bone, it is suggested that the length to width ratio be at least 20:1 to guarantee shear displacements are insignificant [112]. It is also assumed that the cross-section of the beam is uniform throughout the long axis [113]. The last assumption is that the beam is a homogeneous, linear-elastic material [113]. These assumptions pose some issues when examining rat long bones because bones are not perfect beams and cannot be machined to meet these criteria. These limitations must be taken into consideration when examining the results of three-point bend tests of bones.

Using linear beam theory and the applied load and measured longitudinal strain, the elastic modulus of the femurs was determined (Figure 3.7).

$$\text{Elastic Modulus} = \text{stress/measured longitudinal strain}$$

where:

$$\text{Stress} = \frac{\text{applied load*support distance (0.016mm)*}Y_{post}}{4*I_{xx}}$$

Y_{post} = distance from centroid to posterior endosteum (Figure 3.8)

I_{xx} = second moment of area

The modulus was also determined from the displacement data measured by the DVRT.

$$\text{Elastic Modulus} = \frac{\text{applied load} * (\text{support distance } (0.016\text{mm}))^3}{48 * \text{beam deflection} * I_{xx}}$$

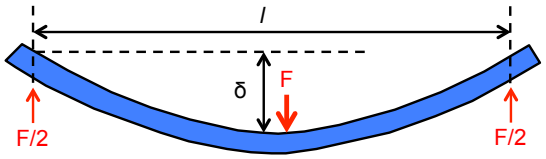

Figure 3.7. Schematic of a beam in 3-point bending. F is the applied load. *l* is the distance between supports. δ is the deformation or the distance the beam moves from the horizontal.

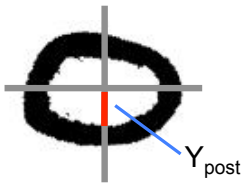

Figure 3.8. MicroCT cross-section of a femur at the mid-diaphysis. Y_{post} is the distance from the centre of the bone to the bottom (posterior) edge of the cortical bone.

3.4.6 Compression Tests

Compression tests of the strain gauged femurs were performed to load the bones in a more physiological type of loading and to also try to correct for some errors that can be caused by limitations of 3-point bending [113]. After the femurs were loaded in 3-point bending they were once again wrapped in saline soaked gauze and placed in the freezer. Approximately three months later the femurs were removed from the freezer and

left to thaw for two hours. Marks were then made 8 mm from the midpoint on either end of the femur to create a mid shaft segment length of 16 mm. A length of 8 mm was chosen because it was a distance that produced fairly uniform sized cross-sectional areas on either end of the femur. Longer lengths resulted in mismatched cross-sectional areas and smaller lengths would have made the loading too close to the strain gauges. An Isomet low speed diamond wafer saw (Buehler, Lake Bluff, IL) was used to cut off both ends of the femur to form the diaphyseal segments. The segments were then measured to make sure that each end was within ± 1 mm of the desired 8 mm from the midpoint. The length was approximate because the width of the saw blade made it difficult to get the cuts perfect as did the actual measuring and marking of the bone. However, the length was not an influential factor in determining material properties.

The femurs were placed in the ADMET material testing machine with the proximal end on the bottom loading platen. To ensure the long axis of the femur was aligned perpendicular to the loading platens, a wobble board type of device was constructed (Figure 3.9). This allowed the femur to self align when loading was performed to help prevent uneven loading. If the long axis of the bone is not aligned perpendicular to the loading actuator, bending of the bone can occur and thus the resultant strain may be inaccurate.

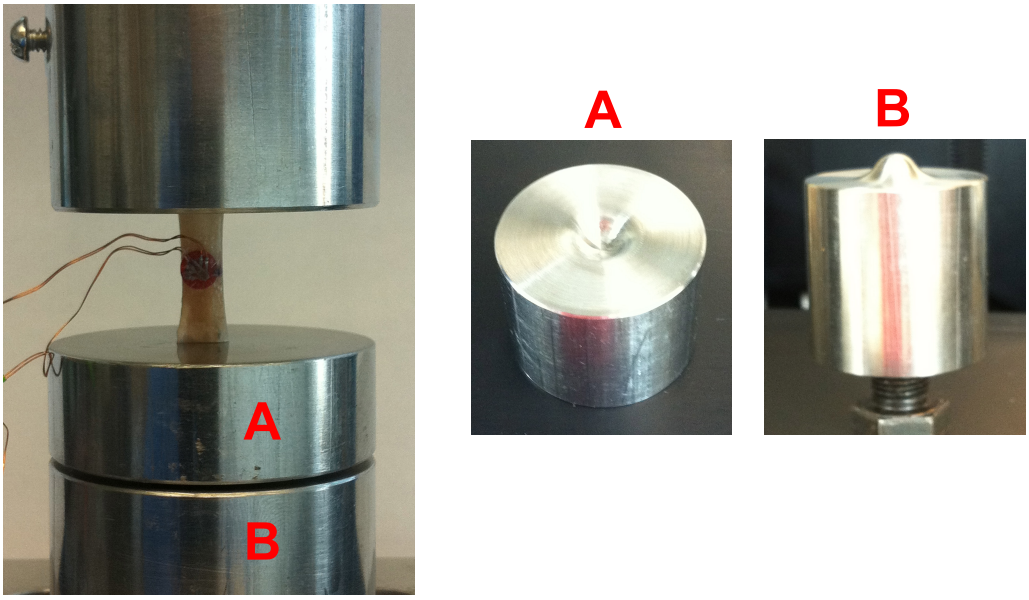

Figure 3.9. **Compression test setup.** The bottom platen is able to move (wobble), allowing the long axis of the bone to align perpendicularly to the platens.

The femurs were cyclically compressed at a rate of 10 mm/min to loads of 20, 40, 60, 80 and 100 N. Force, displacement and strain were recorded as was previously done with the 3-point bend tests. At 60 N, strain was recorded from each of the 3 gauges, as was previously done with the 3-point bend tests, but only 18 cycles were performed and the last 9 were used. The percent difference between the calculated longitudinal principal strain and measured longitudinal strain from gauge "1" was determined to be within 5% so only strain from gauge 1 was recorded for 20, 40, 80 and 100 N loads and only 10 cycles were performed for the remainder of the tests.

3.5 Left Femur Failure Tests

The left femurs from all 38 male and female Wistar rats, used for the strain gauging tests, were used to examine the failure properties of the NC and HF bones. The

left femurs were excised and cleaned of soft tissue and then wrapped in saline soaked gauze and placed in Falcon tubes for storage in the freezer. The femurs were removed from storage and were thawed two hours prior to mechanical testing.

The same three-point bending setup that was used for the strain gauged right femurs was used for the failure test of the left femurs. The supports were placed 16 mm apart and bending was performed in the anterior-posterior direction with the anvil making contact with the mid-point of the femur. A 5 N preload was set and the downward displacement of the loading anvil was set at a rate of 10 mm/min until fracture. Force, displacement, and time were recorded for each test, and stored temporarily in the ADMET controller. After each test, load-displacement data were transferred to the WinCOM software package (ADMET, Norwood, MA) where it was then imported into Excel (Microsoft, Redmond, WA) for analysis. The displacement was also measured using a DVRT (M-DVRT-9, Microstrain, Williston, VT). Load-displacement curves were constructed from the data collected from the ADMET and DVRT and then analyzed to determine the femoral mechanical properties (Figure 3.10). The cross-sectional area properties of the right femur were assumed to be equivalent to the left femur [114] and thus were used for the determination of the material properties.

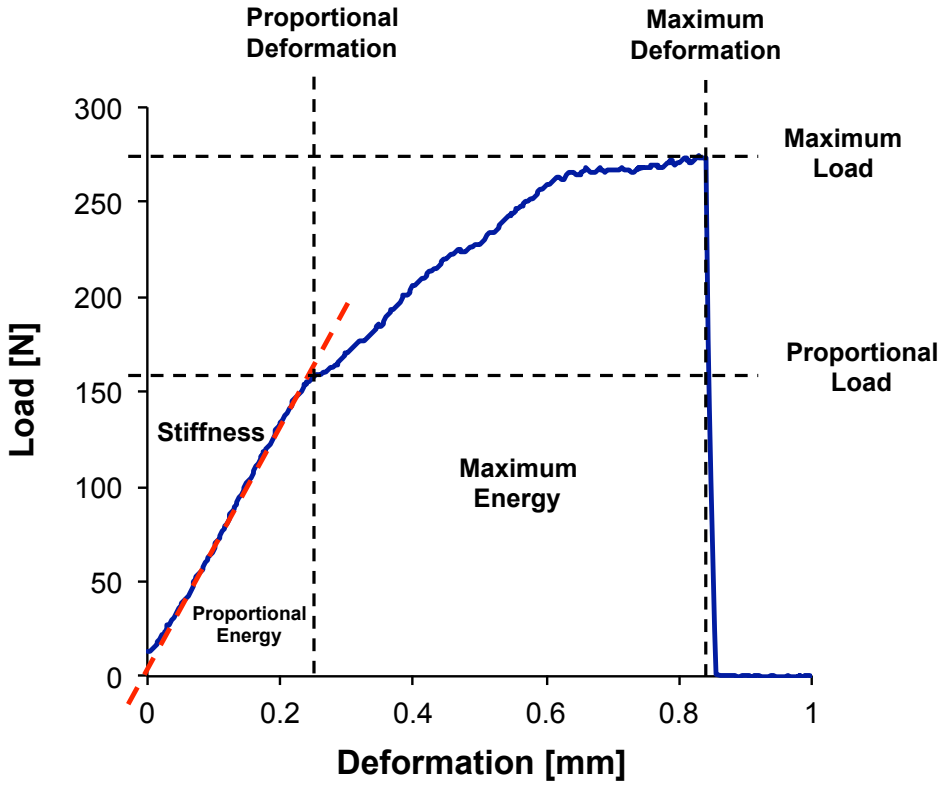

Figure 3.10. Representative load-deformation curve of a femur loaded in 3-point bending to failure. This curve was used to determine the mechanical properties of the femurs. The maximum load of the femur was the highest load the femur could withstand before failure; the highest point on the curve. The "proportional load" was the load at which the femur began to undergo plastic deformation; the end of the elastic or linear region of the curve (also known as the limit of the proportional load-deformation range). The proportional deformation was the deformation at the proportional load. The maximum deformation was the deformation at the maximum load. The stiffness was the slope of the curve in the elastic or linear region; it was calculated using a line of best fit, shown in red. The proportional energy was the area under the curve up the proportional load. The maximum energy was the area under the curve up to the maximum load.

3.5.1 Material Property Calculations

Using linear beam theory and the applied load and resultant femur deformation from the ADMET and the DVRT, the elastic modulus of the femurs was determined (Figure 3.6).

$$\text{Elastic Modulus} = \frac{\text{applied load} * (\text{support distance } (0.016\text{mm}))^3}{48 * \text{beam deflection} * I_{xx}}$$

where:

I_{xx} = second moment of area

3.6 Statistics

To determine diet effects, within a gender, and gender effects, within a diet group, on body composition and bone properties, t-tests (SPSS Inc., Chicago IL) were used. To examine overall diet and gender effects a two-way analysis of variance (SPSS Inc., Chicago IL) was used. A significance level of $p < 0.05$ was used for all statistical tests.

CHAPTER FOUR

RESULTS

4.1 Rat Body Composition and Exclusions

4.1.1 Body Composition

The average body mass of the HF rats, both males and females, was significantly greater than NC ($p < 0.05$) (Table 4.1, Figure 4.1). The males were significantly heavier than the females in both the HF and NC groups. The average mass of the three fat pads (gonadal, mesenteric and perirenal) was approximately two times greater in HF males than NC males and three times greater in HF females compared to NC females. The percent body fat was significantly greater in the HF rats compared to NC for both genders. It was similar between genders for both the NC and HF groups.

4.1.2 Exclusions

Two rats were excluded from all analyses. One sibling pair, a HF male and HF female, had body masses below the NC average body mass and as such were deemed to be non-responders to the diet and were consequently excluded from all analyses. In addition to not gaining mass, the bone properties (cross-sectional area and moment of inertia) of these two animals did not show any differences from the NC.

Table 4.1. **Rat body composition.** Significant dietary differences within a gender are denoted by * $p < 0.05$. Significant between gender differences in HF rats is denoted by # $p < 0.05$ and in NC by ^ $p < 0.05$.

	Male		**Female**	
	NC	**HF**	**NC**	**HF**
Body mass (g)	660.7 ± 32.4	884.4 ± 92.1*	360.9 ± 29.1^	578.4 ± 57.3*#
Fat Pad Mass (g) ¶	43.4 ± 10.9	105.6 ± 28.4*	20.9 ± 5.2^	72.0 ± 13.1*#
% Body Fat ¶¶	6.5 ± 1.4	11.8 ± 2.3*	5.8 ± 1.0	12.4 ± 1.2*

¶ Sum of mesenteric, perirenal, and gonadal fat pads
¶¶ fat pad mass expressed as a percentage of body mass

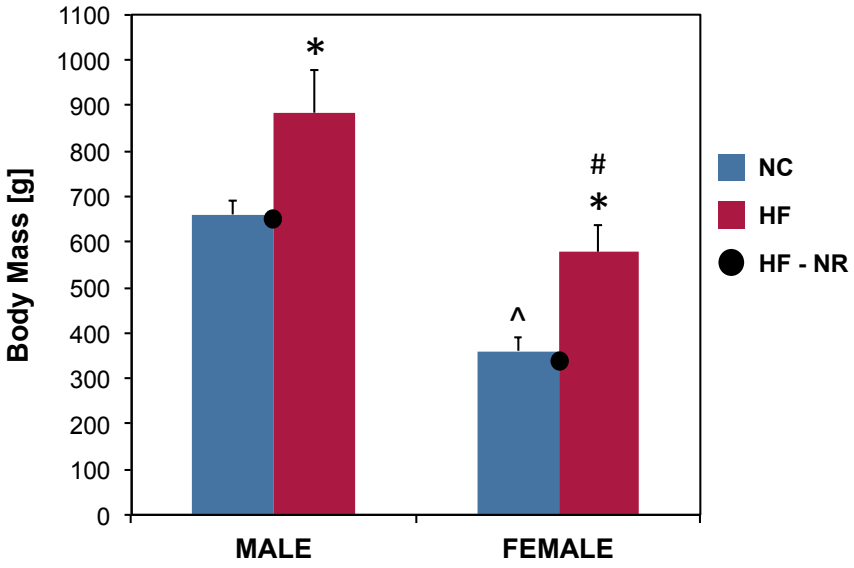

Figure 4.1. **Final rat body mass [g].** HF body mass was significantly greater than NC for both the males and females (* $p < 0.001$). The body mass of the male and female HF non-responder (HF – NR) rats is also shown. The male rats had significantly greater body masses than the females for both the HF (# $p < 0.001$) and NC groups (^ $p < 0.001$).

4.2 Lamellar Bone Formation

There were no differences in the bone, calcein or alizarin perimeter between the right (loaded) and left (non-loaded) ulnas of the male or female rats in both the NC and HF groups (Table 4.2, Figure 4.2). There was no difference between the HF and NC groups for males or females for the three variables analyzed. The NC male ulnas did have a significantly great ulnar perimeter than the NC females. There was no difference in ulnar perimeter between the HF males and females. No clear separation between the calcein and alizarin labels was found in the HF and NC ulnas, indicating that the protocol used did not actually produce lamellar bone formation, as was intended.

Table 4.2. **Bone, calcein and alizarin perimeters of the right and left ulnas.** There were no significant dietary effects found for either males or females. The only significant difference was between the ulnar perimeter of the NC males and females; the males had a larger perimeter (*$p < 0.05$).

Perimeter (cm)	Limb	Male		Female	
	Right	NC	HF	NC	HF
Bone		6.90 ± 0.20	6.89 ± 0.53	5.99 ± 0.48*	6.19 ± 0.33
Calcein		3.62 ± 1.48	3.08 ± 1.76	3.39 ± 1.25	3.06 ± 1.15
Alizarin		2.69 ± 1.85	2.89 ± 1.71	3.09 ± 1.25	2.73 ± 1.12
	Left				
Bone		6.90 ± 0.30	6.91 ± 0.27	5.85 ± 0.32*	6.17 ± 0.56
Calcein		3.78 ± 1.72	3.45 ± 1.02	2.93 ± 1.21	2.65 ± 1.53
Alizarin		3.48 ± 1.56	3.02 ±1.08	2.42 ± 1.03	2.37 ± 1.32

Values are means ± standard deviation (STD)

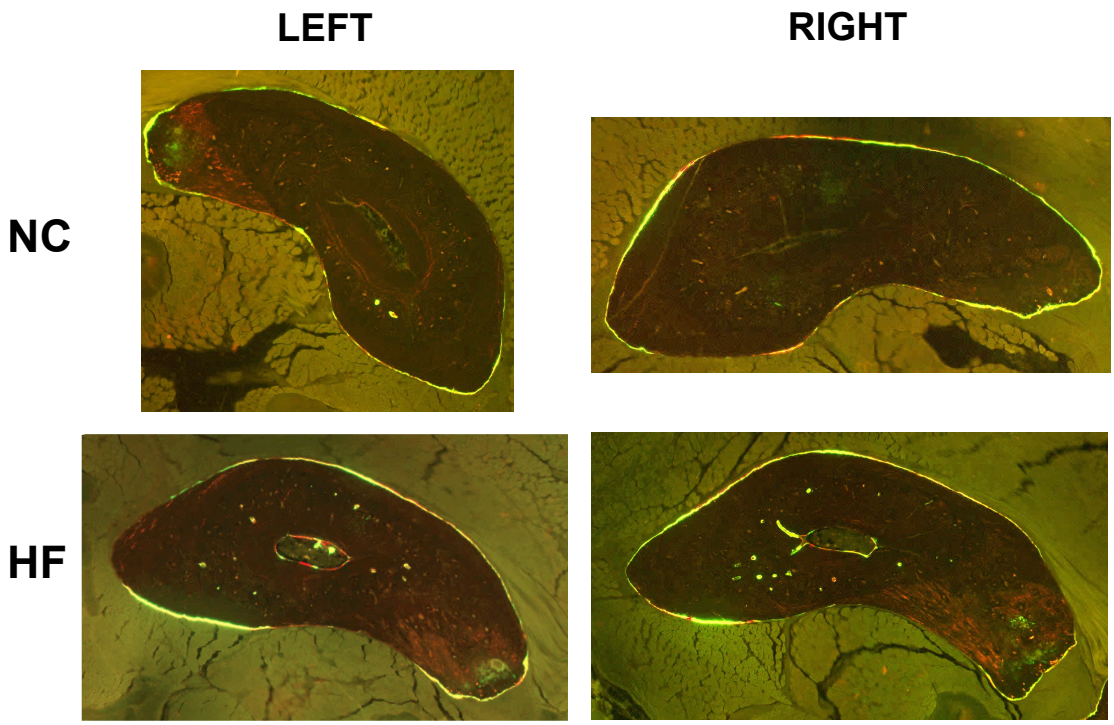

Figure 4.2. **Cross-sectional images of rat ulnas that underwent forelimb loading.** These are representative images of a NC and a HF female rat that had their right forelimbs loaded. The left forelimbs served as non-loaded controls. The 120 μm sections were taken at the mid-diaphysis of the ulna, the area of highest strain, and therefore the area of potentially greatest bone formation. The green and red colours surrounding the ulnas show the calcein and alizarin labels, respectively. The labels are present where new bone has formed.

4.3 Right Femur 3-Point Bend Sub-Failure Strain Tests

4.3.1 Geometric Properties

The femoral length was significantly longer in the HF femurs compared to the NC for males and females (Table 4.3). The males also had significantly longer femurs compared to the females for both HF and NC. The cross-sectional area (XSA) (Table 4.3, Figure 4.3, 4.4) and second moment of area (Table 4.3, Figure 4.5) were significantly greater in HF femurs compared to NC femurs in both males and females. As well, the male femurs had significantly greater XSA and second moment of area than the female femurs in both the HF and NC groups.

4.3.2 Mechanical Properties

At each of the applied loads there was no significant difference in measured longitudinal femoral strain between HF and NC femurs for males or females (Figure 4.6). Male femurs had significantly lower strains than female femurs at each load for both the HF and NC groups. Based on the significantly larger femoral XSA, the HF bones would be expected to experience less strain at a given load if the modulus was similar between the groups. However, there was no difference in strain between the NC and HF groups.

The elastic modulus was calculated using the applied load and the measured longitudinal strain. There was no statistical difference in the calculated elastic modulus between HF and NC femurs for males or females (Figure 4.7). The elastic modulus for the male femurs was significantly greater than the female femurs for NC but not for the HF rats.

The elastic modulus was also calculated using the femur deformation at sub-failure loads, 100 N for males and 60 N for females. The deformation was measured by a custom extensometer, the ADMET and a DVRT. As discussed in Methods Section 3.4.3, the extensometer and ADMET have an internal compliance associated with them, so the deformations were corrected based on this compliance. The ADMET deformation resulted in no significant differences in elastic modulus between the male or female NC and HF animals, although the NC femurs did tend to have a greater modulus (Appendix IV). There was also no significant difference between the male and female NC femurs or HF femurs. Using the extensometer deformation data, no significant differences were found between the elastic modulus of male or female NC and HF animals (Appendix IV). There was no significant difference between the male and female NC femurs. The HF female femurs did have a significantly greater modulus than the male HF femurs. The DVRT showed no significant modulus differences between the male or female NC and HF animals (Appendix IV). There was no also significant difference between the male and female NC femurs or HF femurs.

The extensometer and ADMET produced similar modulus values for each of the four groups with no significant differences between the male NC and HF or the female HF. However, for the female NC femurs there was a significant difference between the two methods of measuring deformation. The DVRT gave significantly greater modulus values for each of the experimental groups compared to the extensometer and ADMET.

4.3.3 Expected Strain in HF Femurs

The HF femurs had a significantly greater XSA than NC femurs. Based on linear beam theory, it would be expected that a greater cross-section would give rise to greater resistance to bending and the measured strain would be less. Despite the larger cross-section in HF femurs, a difference was not detected in measured strain between HF and NC femurs. The lack of difference in strain suggests that the HF bones are made of a more compliant material (so they bend more than expected). To test this, the average modulus of the NC femurs was used and an "expected" or "predicted" strain was calculated for the HF femurs (if they had the same modulus as the NC femurs). HF femur strain at 30N was predicted (HF-Pred E=NC) based on the average HF femur cross-sectional area properties and the average NC modulus. Using beam theory calculations, the predicted HF femur strain was significantly lower than the NC strain at 30N for males and females (Figure 4.8).

4.4 Right Femur Sub-Failure Compression Strain Tests

The compression tests did not yield valid results. A few femurs were loaded in compression and strains were recorded, but these strains differed greatly between trials for the same femur, at the same load. The repositioning of the femurs between the loading platens caused them to be loaded differently resulting in different strains for the same applied load (Figure 4.9). Due to these differing strains for the same bone it was decided that the compression test would not be carried out on all bones and the unusual results would not be analyzed.

Table 4.3. Right femur geometric and mechanical properties. The bone properties were determined from 3-point bend sub-failure tests. Significant dietary differences within a gender are denoted by * $p < 0.05$. Significant between gender differences in HF rats is denoted by # $p < 0.05$, in NC by ^ $p < 0.05$ and in HF-Pred E=NC by ≈ $p < 0.05$. Significant differences between NC and HF-Pred E=NC is denoted by † $p < 0.05$.

Property	Male		Female	
	NC	HF	NC	HF
Femur Length (mm)	43.3 ± 0.8	44.3 ± 0.9*	38.5 ± 0.7^	39.2 ± 0.5*#
XSA (mm^2)	9.39 ± 0.71	10.47 ± 0.93*	6.99 ± 0.49^	7.67 ± 0.84*#
Second Moment of Area (mm^4)	13.20 ± 2.29	16.82 ± 3.31*	7.36 ± 0.86^	8.84 ± 1.96*#
Strain at 30 N (με)	610.2 ± 65.2	554.4 ± 85.9	1122.2 ± 112.7^	1002.5 ± 160.2#
HF-Pred E=NC Strain at 30 N (με)		524.3 ± 86.9†		981.1 ± 180.5†≈
Elastic Modulus (GPa) (calculated using strain)	27.92 ± 2.93	26.45 ± 2.02	24.50 ± 3.13^	24.06 ± 2.95
Elastic Modulus (GPa) (calculated using deformation from Admet - corrected)	4.17 ± 0.87 (at 100 N)	3.46 ± 0.94 (at 100 N)	4.19 ± 0.45 (at 60 N)	3.70 ± 0.67 (at 60 N)

Values are means ± STD

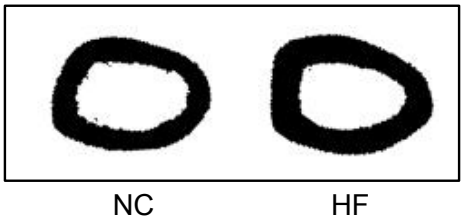

Figure 4.3. Representative microCT cross-section of a NC and HF femur at the mid-diaphysis.

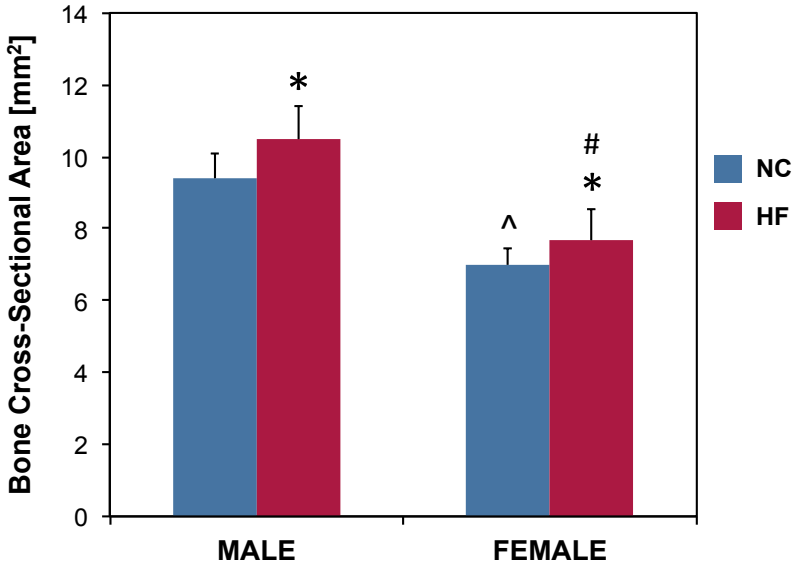

Figure 4.4. **Femoral cross-sectional area (XSA, mm^2) at the mid-diaphysis.** HF XSA was significantly larger than NC for both males (* $p = 0.020$) and females (* $p = 0.040$). The male femurs had significantly larger XSA compared to the females for both the HF (# $p < 0.001$) and NC (^ $p < 0.001$).

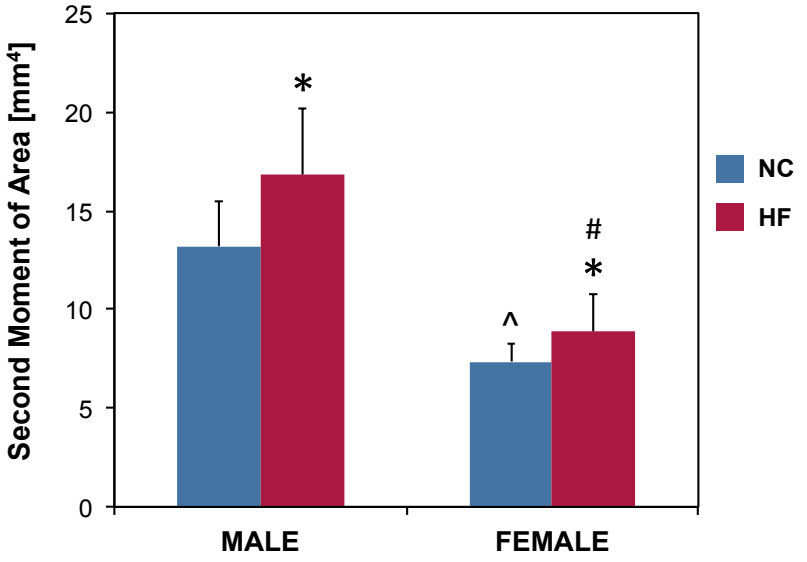

Figure 4.5. **Second moment of area [mm^4] of the femoral mid-diaphysis.** The second moment of area was significantly greater between the HF and NC males (* $p = 0.023$) and females (* $p = 0.039$). The males had significantly greater second moment of areas compared to the females for HF (# $p < 0.001$) and NC (^ $p < 0.001$).

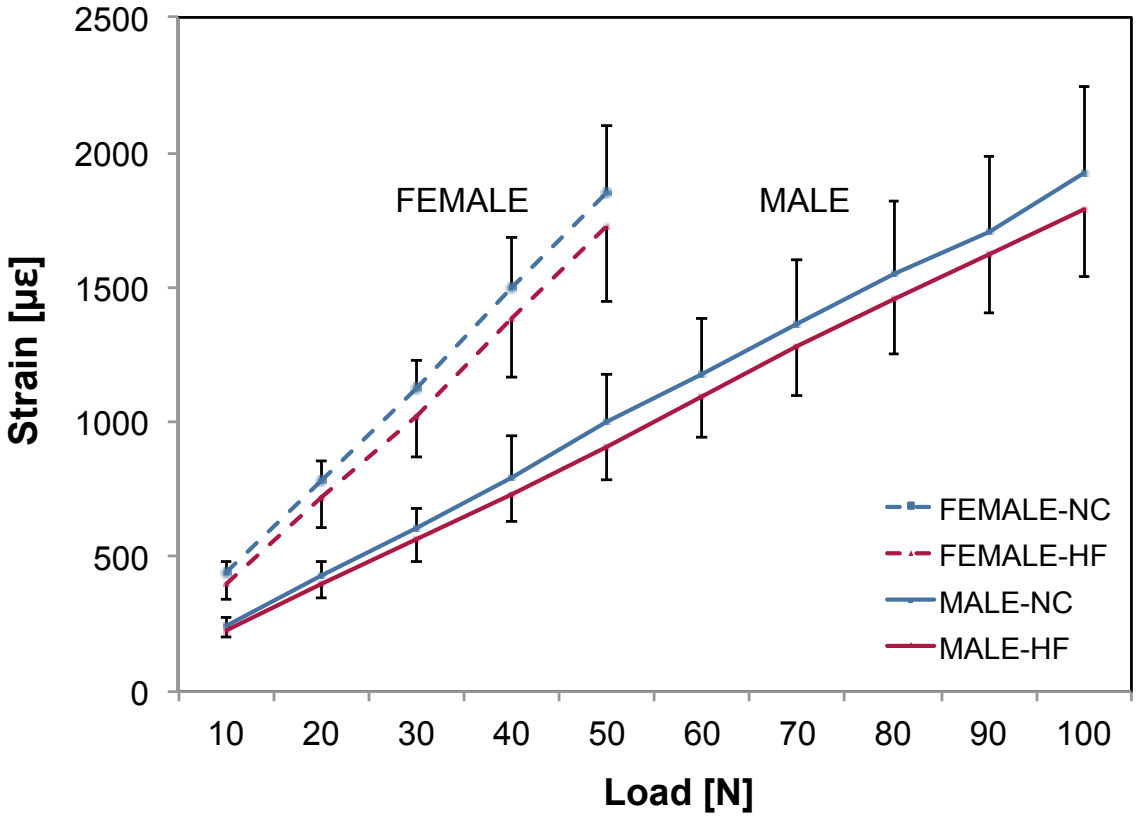

Figure 4.6. **Longitudinal femoral strain [με] at the applied sub-failure loads in 3-point bending.** Longitudinal strain was not different between HF and NC femurs for males or females. At each of the applied loads the strain in the male femurs was significantly less than in the female femurs for both HF and NC.

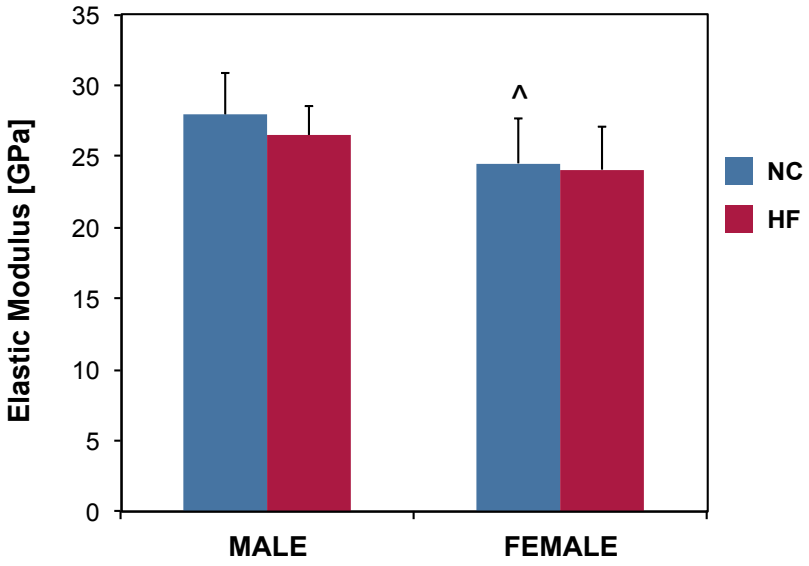

Figure 4.7. Femoral elastic modulus (E, GPa) calculated using measured longitudinal strain from 3-point bend tests. There was no significant difference in modulus between the HF and NC animals for either males (p = 0.264) or females (p =0.760). The modulus for HF male femurs was greater compared to HF female femurs but the difference was not significant (p = 0.079). The modulus for NC male femurs was significantly greater than NC female femurs (^ p = 0.027).

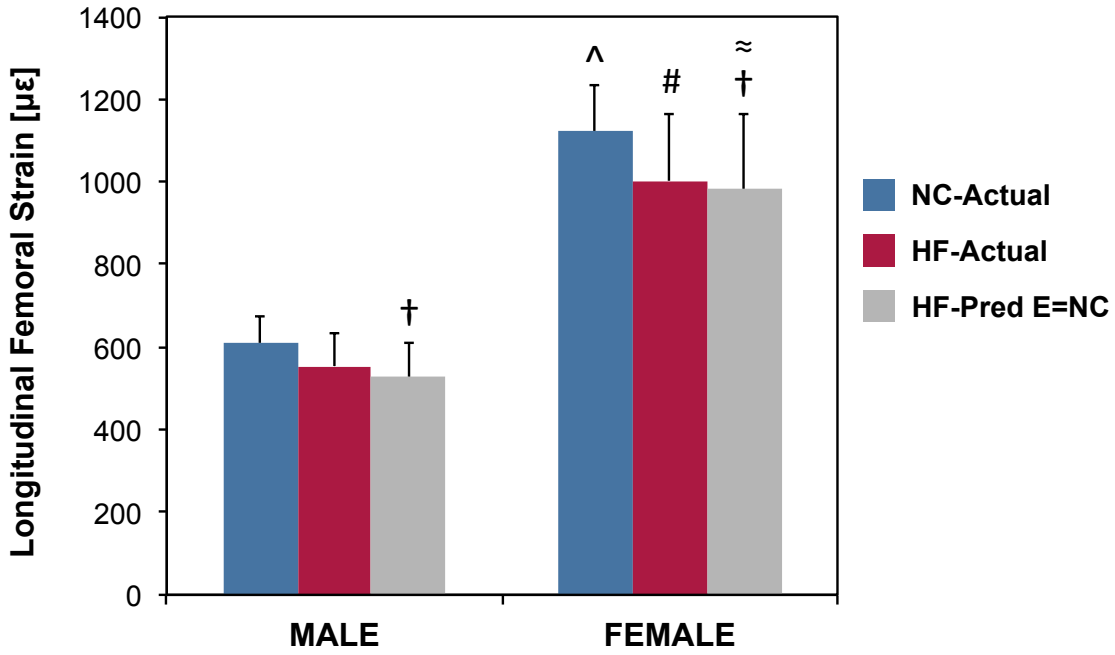

Figure 4.8. **Longitudinal femoral strain [με] at an applied load of 30 N in 3-point bending.** Femoral strain at 30 N did not differ between HF and NC for males ($p = 0.165$) or females ($p = 0.072$). The predicted HF femoral strain (HF-Pred E=NC), based on HF XSA properties and the NC modulus, was significantly less than the measured strain in NC males († $p = 0.042$) and females († $p = 0.050$). The strain in the NC, HF and HF-Pred E=NC male femurs was significantly less than in the NC (^ $p < 0.001$), HF (# $p < 0.001$) and HF-Pred E=NC (≈ $p < 0.001$) female femurs.

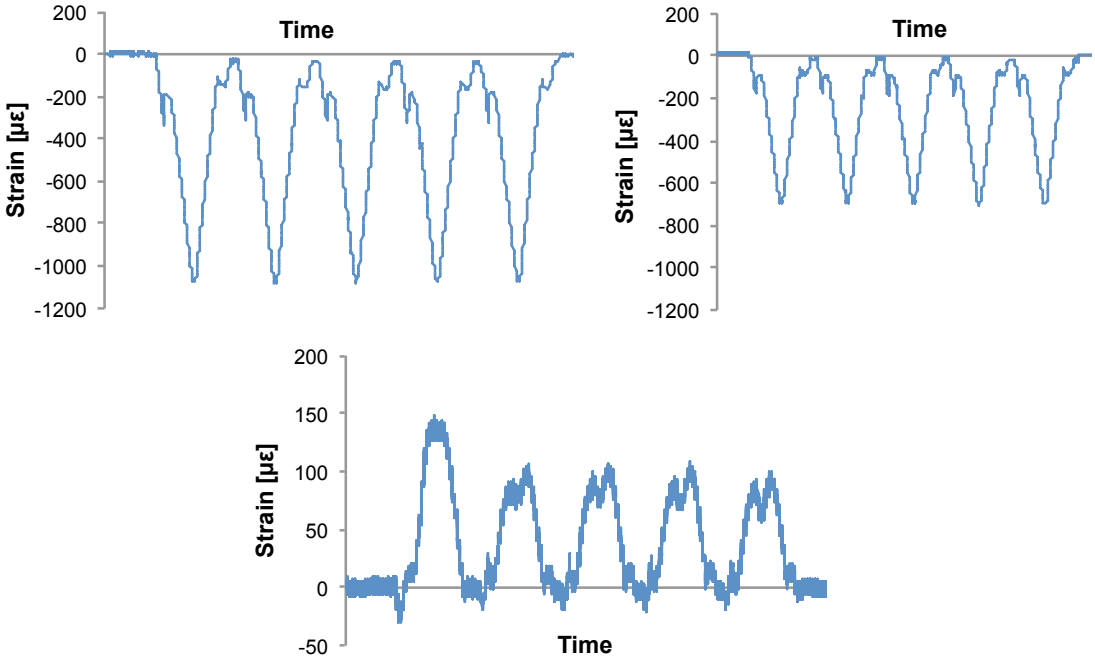

Figure 4.9. Longitudinal femoral strain [με] at an applied compressive load of 100 N. The measured strain was recorded over time for 5 compressive cycles. Each graph depicts the same femur loaded to 100 N, but each time the femur was repositioned between the loading platens.

4.5 Left Femur 3-Point Bend Failure Tests

4.5.1 Exclusions

One HF male (8U) was excluded from all analyses because it was a significant outlier for several mechanical properties.

4.5.2 Mechanical Properties

The maximum load of the left HF femurs was greater than the NC for both genders but the difference was not significant (Table 4.4, Figure 4.10). In addition, the males had significantly greater maximum loads compared to the females for both the HF

and NC rats. There was no difference in proportional load between HF and NC males (Table 4.4). The proportional load was significantly greater in the HF female femurs compared to the NC. Once again the proportional load was significantly greater in male femurs compared to the female for both HF and NC. There were no dietary effects on the maximum or proportional deformations (Table 4.4). There were no gender differences for the HF femurs. The male NC femurs had a significantly greater maximum deformation compared to females. There were no dietary effects on maximum and proportional energy (Table 4.4, Figure 4.11). The males had a significantly greater maximum load compared to the females both the HF and NC. Only the proportional energy was greater in the NC males compared to the females. There was no difference in proportional energy between males and females in HF femurs. The stiffness was greater in the HF femurs compared to the normal controls for both genders but the difference was only significant for the females (Table 4.4, Figure 4.12). The males did have greater stiffness than the females for both HF and NC. There were no dietary or gender effects on the maximum stress of the femurs but the HF femurs tended to experience less stress than the NC and the males tended to experience greater maximum stress than the females (Table 4.4, Figure 4.13). Lastly, the elastic modulus, calculated using deformation, was greater in the NC femurs compared to the HF for males and females but the difference was not significant (Table 4.4, Figure 4.14). There were no gender effects shown in the elastic modulus although the female femurs did tend to have greater modulus values compared to males for both HF and NC. There was no significant difference between the modulus values of the right

femurs, calculated using deformation at sub-failure loads, and the left femurs, calculated using deformation at failure loads, for any of the groups (Table 4.4, Appendix IV).

Table 4.4. **Mechanical properties of left femurs** (all deformations are corrected for internal compliance of the mechanical test system)**.** The bone properties were determined from 3-point bend failure tests. Significant dietary differences within genders are denoted by * $p < 0.05$. Significant between gender differences in HF rats is denoted by # $p < 0.05$ and in NC by ^ $p < 0.05$.

Property	Male		Female	
	NC	HF	NC	HF
Maximum Load (N)	256.57 ± 21.39	273.10 ± 30.31	189.17 ± 12.59^	203.79 ± 23.02#
Proportional Load (N)	156.14 ± 18.38	154.59 ± 20.59	111.67 ± 13.12^	124.14 ± 8.39*#
Max Deformation (mm)	0.665 ± 0.059	0.659 ± 0.184	0.585 ± 0.064^	0.579 ± 0.054
Prop Deformation (mm)	0.193 ± 0.019	0.171 ± 0.031	0.185 ± 0.029	0.187 ± 0.012
Max Energy (mJ)	118.82 ± 20.27	115.93 ± 32.32	75.20 ± 12.72^	80.72 ± 14.91#
Prop Energy (mJ)	16.33 ± 2.72	14.74 ± 4.35	11.57 ± 3.02^	12.73 ± 1.46
Stiffness (N/mm)	872.85 ± 112.20	990.79 ± 111.48	638.06 ± 67.50^	712.46 ± 53.71*#
Max Stress (N/mm^2)	174.39 ± 34.68	157.70 ± 41.66	159.45 ± 12.79	149.99 ± 18.76
Elastic Modulus (GPa) (calculated using deformation)	3.30 ± 0.77	2.82 ± 0.75	3.81 ± 0.50	3.53 ± 0.77

Values are means ± STD

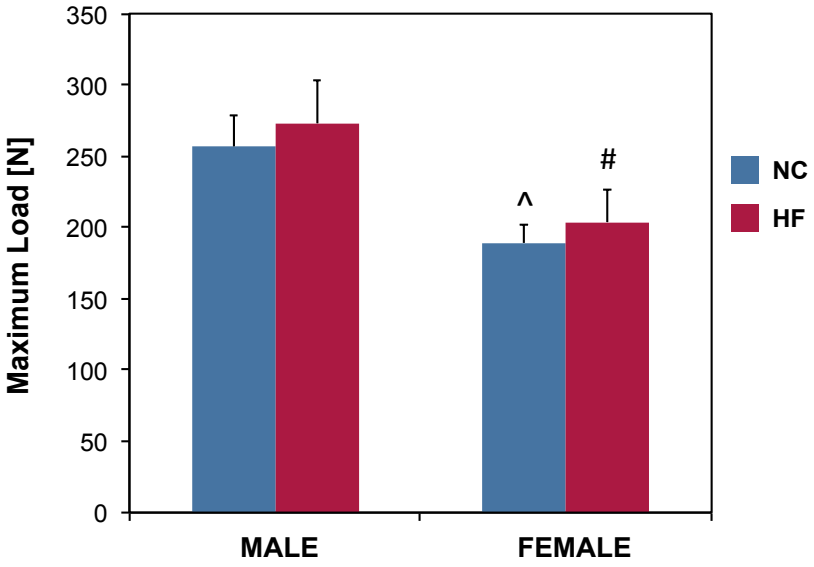

Figure 4.10. Maximum load [N] of the femurs in 3-point bending. The maximum load of the HF femurs did not differ from the NC for males ($p = 0.228$) or females ($p = 0.093$). The maximum load of the male femurs was significantly greater than the females for HF (# $p < 0.001$) and NC (^ $p < 0.001$).

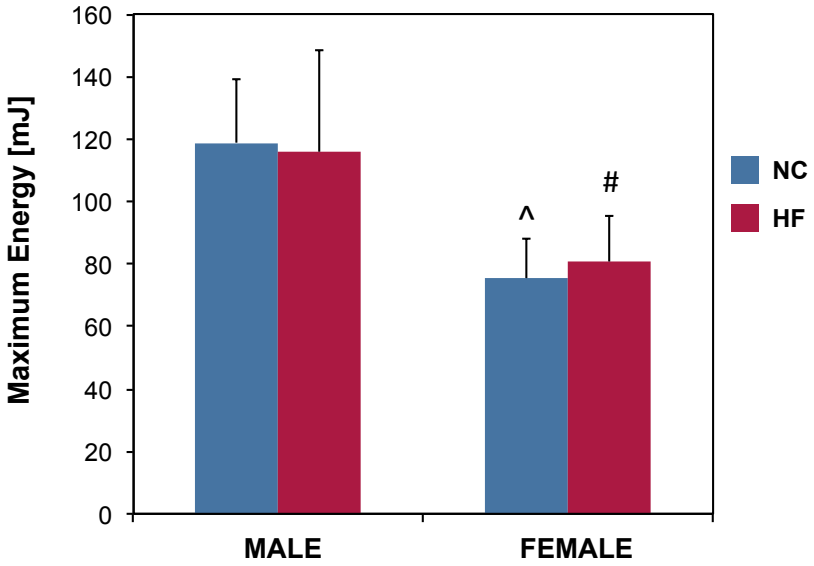

Figure 4.11. Maximum energy [mJ] of the femurs in 3-point bending. There was no significant difference in the maximum energy of the HF and NC femurs for either the males ($p = 0.833$) or females ($p = 0.396$). The HF (# $p = 0.014$) and NC (^ $p < 0.001$) male femurs did have significantly greater maximum energies compared to the females.

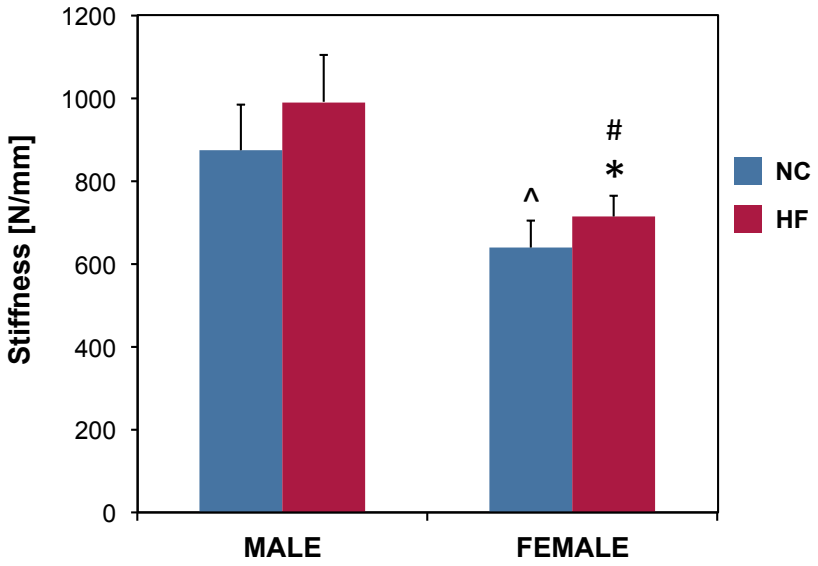

Figure 4.12. Femoral stiffness [N/mm] in 3-point bending. Stiffness of the male femurs was greater in the HF femurs compared to the NC but the difference was not significant ($p = 0.053$). The HF female femurs were significantly stiffer than the NC (* $p = 0.020$). The male femurs were significantly stiffer than the females for both the HF (# $p < 0.001$) and NC (^ $p < 0.001$).

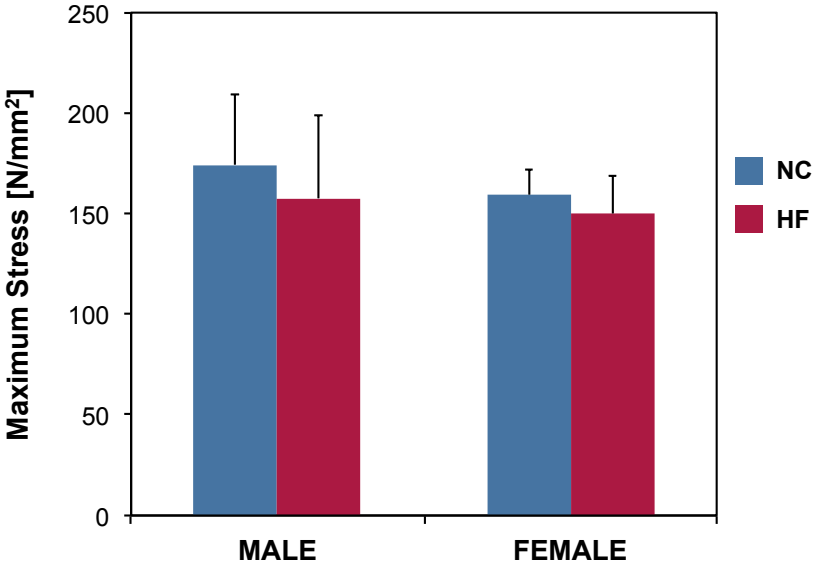

Figure 4.13. Maximum femoral stress [N/mm^2] in 3-point bending. Maximum stress was greater in the NC femurs compared to the HF femurs for males ($p = 0.399$) and females ($p = 0.207$) but the differences were not significant. There was no difference in stress between the male and female femurs for HF ($p = 0.640$) or NC ($p = 0.204$).

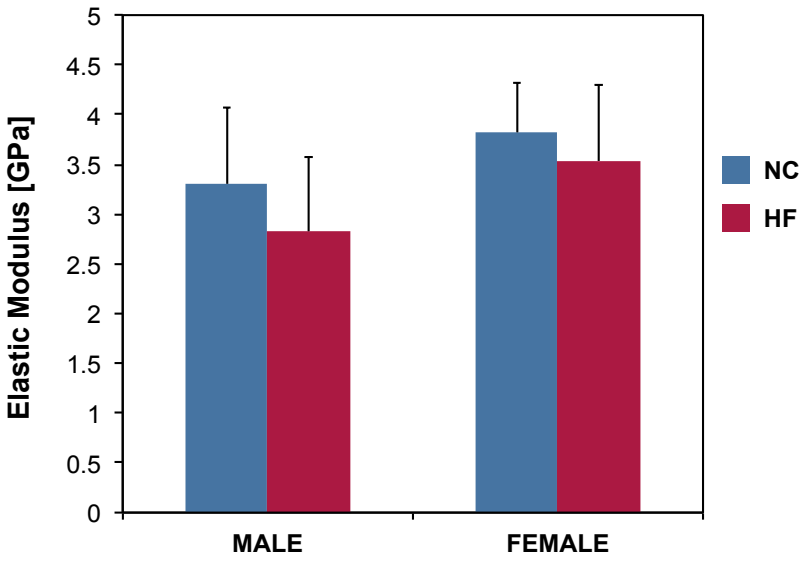

Figure 4.14. Femoral elastic modulus (E, GPa) calculated using maximum deformation from 3-point bend tests. The elastic modulus was greater in the NC femurs compared to the HF femurs for males ($p = 0.229$) and females ($p = 0.352$) but the differences were not significant. The females had greater modulus values compared to the males for the HF ($p = 0.082$) and NC ($p = 0.098$) but the differences were not significant.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The purpose of this project was to study the effects of obesity on the ability of bone to adapt to mechanical stimuli through the examination of bone structural and material properties and bone strain. The hypothesis was that male and female rats fed a lifelong high-fat diet, designed to induce obesity, would exhibit decrements in bone mechanical properties, thus altering bone strain during mechanical loading and consequently affecting bone adaptation. The findings did not support the hypothesis. No statistical differences in mechanical properties or strain were detected in the HF rats compared to the NC. However, femoral cross-sectional area properties (bone area and second moment of area) were increased in the HF rats.

Despite the significantly larger size of the HF femurs for both the males and females, there was only a tendency for them to have a greater maximum load, a structural property, as the difference was not significant compared to the NC femurs. In theory, with no difference in maximum load between the NC and HF femurs but significantly greater HF femoral cross-sectional area, the maximum stress, a material property, in the HF bones should be less. The stress tended to be less in the HF bones but the difference was not statistically significant. The stiffness, a structural property, tended to be greater in the HF male femurs than the NC male femurs, but again the difference was not statistically significant. The HF female femurs were significantly stiffer than NC. Once the stiffness

was normalized to the size of the bone and the material stiffness or the elastic modulus of the bone was examined, the HF femurs, for both genders, exhibited a lower modulus than the NC femurs, but the difference was not significant. There appears to be a tendency for the HF bones to have greater structural properties and diminished material properties compared to the NC bones. Based on the number of rats in the study the differences are not statistically significant, but there is a switch in these properties once the size of the bone is taken into account.

5.2 Dietary Effects on Bone

The HF diet in this thesis project was high in fat and sucrose but calcium and vitamin D replete (Table 3.3). However, vitamin D and calcium serum levels were not measured, and so whether the serum levels in the rats were adequate is unknown. Vitamin D may be sequestered by the large amount of adipose tissue found in the HF rats and thus they may not have access to a sufficient amount of available vitamin D. Both the high-fat and sucrose content in the HF diet are a realistic surrogate for what a significant segment of the North American population is consuming, those most at risk of obesity. Both the high-fat and sucrose levels could play a key role in the suspected compromised bone material properties seen in the HF rats.

The high saturated fat content of the HF diet may be contributing to the diminished material properties seen in the HF femurs. Atteh and Leeson [115] found that diets containing palmitic acid (saturated fat) led to low bone ash weight and bone calcium content and were associated with a reduction in bone magnesium content in broiler

chicks. The low bone mineral content was attributed to reduced mineral retention due to the formation of intestinal soaps [115]. An increased level of saturated fat in the diet impairs calcium absorption in the gut and intestine via formation of insoluble soaps [116-118]. Similar results were found in rats [3] and mice [57] fed diets high in saturated fats resulting in decrements in bone properties. The changes that were seen in the HF rat bones may be the result of the high saturated fat content of the diet causing the formation of intestinal soaps and thus adversely affecting both calcium absorption and retention and, in turn, altering mineral metabolism.

The high level of sucrose in the HF diet may have also adversely affected the HF bone. Diets high in sucrose can elevate blood insulin levels and induce hyperinsulinemia [119, 120] which in turn, produces hypercalciuria [121], as insulin can inhibit renal tubular resorption of calcium [122]. Calcium deficiencies resulting from high insulin levels can lead to poor bone mineralization [123], and can adversely influence bone mechanical properties [124]. Furthermore, high glucose levels can diminish renal reabsorption of magnesium [125], and magnesium depletion results in poor bone crystallization [126] and reduced bone turnover [127]. Insulin and glucose levels were not measured in the rats and if they were altered they could adversely affect the bone.

An increase in circulating pro-inflammatory cytokines, such as TNF-α, might also contribute to poor bone properties. In mice fed a high-fat sucrose diet (HFS), serum tartrate-resistant acid phosphatase (TRAP) (a marker of bone resorption) levels were significantly elevated relative to controls while serum osteocalcin (a marker of bone formation) levels were not different between cohorts [57]. This suggests that ingesting a

HFS diet disrupted the balance between bone formation and resorption, favouring resorption. Lorincz et al. [57] suggested that the increase in TRAP activity measured in the HFS mice was caused by pro-inflammatory cytokines such as TNF-α, which can affect bone resorption. Increases in TNF-α are associated with negative changes in bone metabolism [57]. Diets high in saturated fat can increase the release of TNF- α from adipose tissue [128] and expression increases with the level of obesity [129]. Increases in TRAP levels have been found in other animals fed a high-fat diet [54, 130], as well as decreases in osteocalcin [55, 130]. An increase in TNF-α was also shown in high-fat animals [130]. As the HF rats in the present study were obese and ingesting substantial amounts of saturated fat and sucrose, this group likely experienced elevated levels of proinflammatory cytokines, which may have contributed to increased porosity and thus reduced material properties. However, the HF femurs had greater cross-sectional area suggesting proinflammatory cytokines may not be increasing bone resorption.

An increase in the level of some hormones associated with a high-fat diet and obesity may have contributed to the suspected reduced material properties. An increase in IGF-1 and larger bone size has previously been associated with a high-fat diet in mice [61, 82]. Adipokine derived hormones, such as leptin, could also play a role in bone properties in HF rats. Leptin levels have been shown to increase in high-fat diet animals [55, 61, 82] but the results are equivocal, and the role of leptin remains unclear.

There are a number of different nutritional and hormonal factors that could or could not contribute to the effects of diet-induced obesity on bone properties. They work together and they work against each other so the overall outcomes and effects are

inconsistent. Studies show different effects with similar hormonal changes. Without further research into the individual mechanisms there is currently no easy or definitive way to conclude the cause of the changes in the bone properties.

5.3 Geometric Properties

MicroCT scans revealed that the HF femurs, from both males and females, had significantly greater cross-sectional areas and second moments of area than the NC femurs. Interestingly the femoral length of the HF rats was significantly greater than NC for both genders. Several studies report no change in length as a result of a high-fat diet [4, 55, 57, 61, 82], but none reported an increase. Increased second moment of area [59, 61, 82] and bone cross sectional area have been reported for obese animals [58, 61, 82]. The greater bone size seen in high-fat diet animals is generally explained by the resultant increase in body mass. However, muscle mass is also thought to play a possibly greater role than simply body mass in the determination of bone size.

Support for the influence of lean mass and not body or fat mass on greater bone size and strength, as seen in the HF animals, comes from a study by Wetzsteon et al. (2008) [131]. They found substantially lower bone strength relative to body weight and body fat values in overweight (BMI $\geq$ 85th percentile) compared to healthy weight (BMI $\leq$ 75th percentile) children and a 16 month change in bone strength that was significantly associated with a change in lean mass and not weight or fat mass. This suggests that bone is adapted to muscle mass and not fat mass or body weight. Other recent studies have also shown that bone mass and geometry are associated with lean mass but not fat mass in

children and adolescents [132, 133]. These results suggest that bone strength increases in response to greater muscle mass, but it may also increase to meet the added demands of extra body mass or to compensate for decrements in bone material properties, such as density, in obese individuals. Bone density may be decreased because of an accumulation of microdamage and the consequential bone turnover that may occur due to the increased loads on the bones in obese individuals [134]. This would then result in increased porosity in the cortical bone and thus, a lower apparent tissue density.

Increased muscle size and strength generates greater forces on the bone resulting in increased bone apposition on the periosteal surface, which adds bone away from the neutral axis, increasing bone area, and increasing the bone's resistance to bending demonstrated in enhanced bone strength. These findings are consistent with the mechanostat theory proposed by Frost, that suggests that bone adaptation is driven primarily by changes in mechanical load [135]. The highest loads on bone are generated by muscle forces, and not body weight or fat mass [136-138] so bone bending strength would be greater in the HF rats who theoretically have more muscle mass than NC rats because of their greater body mass. Although there was a tendency for the HF bones to exhibit greater strength, the difference was not significant. If the bones are significantly larger, as the microCT scans reveal, then their bending strength should be as well. Given this was not the case, something was apparently lacking in the bone below the structural level. As proposed above, the bone density may be lacking due to increased porosity and compensation for this increased porosity could have come from the addition of new bone to the existing bone, specifically the periosteal surface. The increase in bone area would

increase strength, and would keep strains within the target physiological levels. Based on the mechanostat theory and strain-dependent adaptation of bone, the ultimate goal of bone adaptation would be to keep the HF bone strain at its target or threshold level, to maintain bone metabolism. An increase in bone size is needed to do so.

The lack of change in bone cross-sectional area in the male and female HF rats that did not gain mass, implies one of three explanations: 1) that the increase in bone cross-sectional area is a result of the increased body mass imposing greater loads on the bone; 2) that the increase in bone size is required to compensate for the increase in body mass or 3) that fat mass is some how contributing to the greater bone size. Since an increase in fat mass is generally accompanied by an increase in muscle mass, the lack of increase in cross-sectional area may also be due to a lack of increase in muscle mass. In other studies, animals fed high-fat diets with resulting increased body mass either had no change in cortical bone area [3, 54, 55, 57], or had a significant increase in cortical bone area [61, 82]. In comparison, animals fed a high-fat diet that did not have greater body mass also had no change in bone mass [4, 58, 139]. Taken together, these findings suggest that an increase is body mass is necessary to cause an increase in bone size, but an increase in body mass is not sufficient to cause an increase in bone area. It does not appear that the high-fat diet itself is the cause of the increased bone size. Rather, an increased bone size is required to support the increase in body mass associated with the consumption of a high-fat diet.

A high-fat diet in mice resulted in an increase in lean mass and an increase in bone size [61]. However, a similar study showed no increase in lean mass from the high-fat

diet but there was an increase in bone size [82]. Both of these studies resulted in increased body mass suggesting that body mass is the contributing factor to the increased bone size and not muscle mass. Both studies, however, also found greater fat mass in the animals fed the high-fat diet so it may also be that the increased fat and its associated adipokines are causing the increase in bone size. This is only representative of two studies and therefore presents the need for further investigation into the main determinant of the increased bone size seen in obese animals.

The activity level of the rats could also be playing a role in the size of the bones, as more activity would mean more bone loading, both from increased muscular activity and body mass. If the HF rats had activity levels equal to the NC rats, the bones would experience more loading because of the increased body and muscle mass of the HF rats. Due to the large size of some of the HF male rats, it is highly possible that they did not experience as much physical activity as the smaller NC males or the females. If the HF rats are, indeed, less active but have greater muscle and body masses, and the NC rats are more active but have less muscle and body mass, then daily bone loading may be similar between them. However, since the activity levels of the rats were not monitored, the role of physical activity in bone loading and bone size in this study is unknown.

Determining the cause of the increased bone size is difficult. More work is needed in this area as support for the influence of muscle, fat and body mass on bone size can be found and in the end may involve a balance between them.

5.4 Femoral Material Properties

5.4.1 Elastic Modulus Calculations

One of the most commonly used methods for determining the mechanical properties of rat cortical bone is a three-point bending test of the rat femurs. To determine material properties of the bones from three-point bending tests, the Euler–Bernoulli beam theory is commonly used. There are, however, a few assumptions associated with this theory and some limitations when applying it to bone, particularly to a relatively short bone such as the rat femur. The first assumption is that the length-to-width ratio (aspect ratio) of the beam or in this case bone, should be at least twenty to obtain accurate results [112]. That is, the length of the bone should be twenty times the diameter. The aspect ratio for rat femurs is approximately four. This creates shear stresses, affecting results. Though it is possible to increase the aspect ratio, by moving the lower supports further apart, the femur shape changes quite dramatically towards the ends and the beam theory also assumes the cross-section of the bone is uniform throughout its length. The last assumption is that the structure being tested is made from a homogeneous, linear-elastic material. The material along the length of the femur transitions from cortical to trabecular bone. So, it is important to keep the loading support distance close enough to make sure the portion of the femur being tested has a relatively uniform cross-section and material composition. However, if the loading supports are too close together the local deformation that occurs at the contact points could influence results.

When loading a short bone in three-point bending, shear stresses result. The creation of shear stresses means that some of the elastic strain energy will be lost to or be

taken up by the shear that is created during the bending of such a short bone. This means that although no shear strain is actually being recorded at the surface of the bone, shear stresses are dominating the normal stresses and the deformation being measured is not the result of only normal stresses acting upon the bone but rather a result of both shear and normal stresses. To reduce the shear stresses, the loading support distance would need to be increased. If the support distance were to increase, so would the deformation measurements for two reasons: first, the normal stress would increase (the bending moment gets greater as the supports go wider) and second, the shear stress would take up relatively less of the elastic strain energy and therefore cause less deformation. The amount of shear stress does not decrease but rather its contribution to the stresses experienced by the bone decreases, and so, the normal stress now dominates and the deformation measured is a result of the normal stress. It has been estimated that shear stresses cause 15 to 20% of the measured deformation in bending tests of short whole rodent bones [112]. This causes the measured displacement to be greater than the actual bone deformation resulting in overestimation of strain and underestimation of the elastic modulus [112].

Using too small of a length-to-width ratio has been found to cause an underestimation of the elastic modulus, and, by increasing it, the error in the modulus can be reduced [140]. van Lenthe et al. [113] determined the effects of bone geometry and support width on elastic modulus as calculated from three-point bend tests using finite element analysis, a widely accepted method for the mechanical evaluation of bone samples. They found that tissue moduli are strongly underestimated and the extent of

underestimation depends on bone size and shape. Because the assumptions of beam theory include the shape of the bone, these findings do not seem unreasonable.

Elastic modulus values of rat femora vary throughout the literature (Table 5.1). The limitations associated with three-point bending tests and beam theory, the most commonly used method to evaluate the modulus of bone, may be the reasons for the varying numbers in the literature. Modulus values have been reported to be as low as 1 GPa [4, 141] and typically in the range of 3-9 GPa [4, 141-145] when using the resultant deformation from three-point bend tests to calculate the values. The values of modulus calculated based on deformation in the current study were in the same range (2.8-3.5 GPa). In comparison, Keller et al. [6, 146] used the strain measured from the anterior surface of femurs during cantilever bending to calculate modulus values of male Sprague Dawley rat femurs and found values between 22 and 40 GPa. In the current study, the modulus values calculated from strain measurements during three point bending were in the same range (25-30 GPa).

Modulus values, calculated using deformation from three-point bend tests, are very low, and when strain is used values are higher. The deformation used in the calculations is overestimated because it is the result of bending and shear, and therefore the modulus will be underestimated. The strain measured will be more accurate because there is no shear at the surface of the bone where the strain is being recorded. Therefore using measured bone strain, from three-point bend tests, appears to be the more accurate way to evaluate the modulus. The modulus values found for the HF and NC femurs from three-point bend tests seem to be reasonable and fall closely in line with what the

literature shows based on similar calculations.

Table 5.1. Rat femoral modulus values found in the literature. The values were calculated from either strain or deformation and in different rat strains and genders.

Author	Modulus Value (GPa)	Method of Calculation	Rat Strain	Gender
Keller et al. [6, 146]	22 - 40	strain	Sprague Dawley	male
Battraw et al. [114]	18.5 - 23	strain	Sprague Dawley	female
Ferretti et al. [141]	1 - 3	deformation	Wistar	male, female
Nakamura et al. [142]	2.7	deformation	Wistar	male
Fox et al. [143]	1.9	deformation	Sprague Dawley	female
Feldman et al. [144]	3	deformation	Sprague Dawley	female
Ward et al. [4]	3 - 9	deformation	Fisher 344	male, female
Hogan et al. [145]	6.5 - 9	deformation	Sprague Dawley	female
Currey et al. [147]	12 -18	deformation	Wistar	female

Other methods used to determine bone elastic modulus are nanoindentation and ultrasonic tests. Two studies using nanoindentation to examine the elastic modulus of rat femurs found values of approximately 19 GPa [148, 149]. Using ultrasound, Kohles et al. [150] found modulus values ranging between 22 and 26 GPa depending upon rat age and Vanleene et al. [151] reported modulus values between 8 and 20 GPa for male Wistar, rats depending upon age. These results are very close to the modulus value reported for the human femur in compression (17 GPa) [19]. Based on these findings, it seems as though there are more accurate methods to evaluate rat femora modulus values than three-point bend tests, but if that is the only available method, then calculating it using strain measurements is the more appropriate method.

Lastly, the elastic modulus calculated from three-point bend tests is not a test of the material in its intended loading direction and, as discussed above, is associated with many limitations and problems. Bone is an anisotropic material and therefore it exhibits different properties depending upon the direction of loading. The modulus calculated from compression tests, however, is a better indication of how the material behaves under physiological loading. Unfortunately because the compression testing was unable to be performed, the modulus of the femurs in compression could not be determined, leaving room for future investigation.

5.4.2 Material Properties

The modulus of elasticity is a material property of the bone and describes the stiffness of the bone material being tested. The modulus was calculated using the measured strain from the three-point bend tests and the femoral cross-sectional properties. It was also calculated using the deformation from the three-point bending at sub failure and failure loads and the cross-sectional properties. Though there are limitations with using such a calculation for determining absolute material properties, it is useful for determining the modulus for relative comparison between the NC and HF femurs. No differences were found between the NC and HF femur modulus values for the males or females for any of the three methods of calculating the modulus. However, the NC males did have a significantly greater modulus than the NC females when the modulus was calculated using strain. When the modulus was calculated using deformation, no gender differences were found. So, based on the modulus calculations, the NC and HF animals

have the same quality of bone material. The lack of change in the elastic modulus as a result of the diet is in agreement with another study examining the effects of a high-fat diet in rats [4]. There was no dietary effect on the modulus in the female rats, but a lower modulus was found in the high-fat males. Li et al. [58] also found no change in the elastic modulus of tibias from rats fed a high-fat-sucrose diet.

Based on the results of the strain measurements in which the NC and HF rats experienced the same level of strain at each of the applied loads, and the microCT scans revealed that the HF femurs had a significantly greater XSA compared to the NC femurs, the modulus of the HF femurs should, theoretically, be lower than the NC. There was, however, no statistical difference in moduli. The XSA of the femurs, based on microCT scans should be accurate, implying the calculated elastic modulus is not appropriate for the measured strain results. If the strains are the same but the femoral size is larger in the HF rats, then the bone material must be lacking something to cause equivalent strain. The calculated modulus values could very well be inaccurate due to the limitations of applying beam theory to bones, or it could be that the material differences are present but undetectable by mechanical testing and subsequent analysis.

If the bone material was the same (*i.e.*, same modulus) but the HF femurs had greater cross-sectional area, then the HF femurs should have had significantly reduced strains compared to NC femurs, but they did not. To investigate this further, the strain in the HF femurs was calculated for the 30 N load level using the average NC modulus and the HF cross-sectional area properties. The resulting "predicted" HF femur strain (HF-pred E=NC) was significantly less than the NC strain, suggesting that the HF femurs

were in fact different at the material level compared to the NC femurs. To determine the material differences further tests must be completed. These may include examining BMD or BMC or examining the microstructure of the cortical bone.

As mentioned earlier, more than 80% of the variation in the elastic modulus in cortical bone can be explained by matrix mineralization and porosity [20]. Poor matrix mineralization can result from the high saturated fat and sucrose levels in the diet, which interferes with calcium absorption and utilization. As well, the inflammatory state associated with obesity may increase bone resorption leading to increased bone porosity. Bone density may be reduced because of an accumulation of microdamage and the subsequent increase in bone turnover that may occur due to the increased loads on the bones in obese individuals [134]. This would then result in increased porosity and thus, a lower tissue density. There is a well established positive correlation between body weight, which increases bone loading, and areal BMD, as measured by DEXA in humans [5]. However, increases in BMD are not found in animals fed a high-fat diet despite increases in body mass compared to controls [3, 57, 61, 82]. Moreover, a decrease in bone mass has also been reported [55]. So despite increased bone loading, BMD may not be sufficient for the HF rats. In addition, Ionova-Martin et al. [82] used SEM to show that high-fat diet mice had reduced tissue organization, alignment of osteocyte lacunae and lamellae compared to controls. As well, a diet high in fat has been shown to cause a significant accumulation of adipocytes in the bone [56], potentially a sign that mesenchymal stem cells have differentiated in favour of adipocytes and not osteoblasts. This, in turn, could alter bone metabolism and affect bone properties. All of these bone changes could be

contributing to the suspected poor bone material properties in the HF rats.

5.5 Femoral Strain

By extrapolating the measured strain data for the HF and NC male and female femurs and using their respective maximum load data, the predicted failure strains were determined to be approximately 4800 με for the male femurs and 6800 με for the females. These values are in line with normal bone failure strains of 4500-7000 με [18] suggesting the strains being measured at the applied loads are realistic and the testing was appropriate.

It has been shown that it is not just bone geometry or bone material that determines bone strain, but rather strain is a function of both [6, 152]. Indrekvam et al. [153] found an increased load bearing capacity with age in adult rats, and despite increased mineralization and a lower rate of collagen synthesis contributing to stiffer bone with age, strains remained constant across the lifespan. This was likely due to the larger cross-sectional dimensions found in the older bones. Keller and Spengler [6] also found that although rat body weight and midshaft femoral geometry increased over 3-fold and 6-fold respectively from 6-18 weeks to over 18 weeks of age, respectively, the peak values of the *in vivo* strains remained unchanged. Similar results have also been found in growing chicks [154]. This seems to be the same phenomenon that is occurring in the HF bones. The HF diet femurs tended to be stronger and stiffer (both structural properties), likely due to their greater cross-sectional area, but they exhibited the same strain (non-significantly lower than NC) as the NC rats. The older rats also tended to have lower

strains than the younger ones [153]. These results from the aging rats and HF rats suggest the existence of a minimum effective strain. There seems to be a coordinated effort between both bone geometric and material properties to target this predetermined level of strain to keep bone structurally sound. Regardless of what is happening to the bone, whether it be growth, aging or a change in diet, as long as it is not too drastic, it appears that bone has the ability to adapt to the changes accordingly, in order to maintain a target level of strain. Controlling strain is important for the maintenance of bone because too much or too little can compromise the mechanical properties. Adaptive modeling during growth appears to take place to target this predetermined level of strain to preserve bone structural integrity.

One of the initial goals of this thesis project was to examine bone adaptation in response to mechanical loading. Bone adaptation is thought to occur in response to strain. The rat forelimb loading model is commonly used and involves compression of the forelimb along its longitudinal axis to a specified strain based on correlation with an externally applied load. A specific load, however, may result in different strains in the bone depending upon the size and material of the bone. To establish an appropriate model for producing lamellar bone, the strains in both the HF and NC bones needed to be the same. Based on the theory of strain-mediated bone adaptation [22], if the strains differ then the subsequent bone responses would also differ. Lamellar bone formation requires enough strain to produce bone but not so much as to damage the bone and invoke a reparative or woven bone response. Determining that the strains in the HF and NC femurs are equivalent provides support for similar strains in HF and NC ulnas. This suggests that

at a given load, the HF and NC forelimbs will experience the same strains and therefore the bone will receive the same signals. It is unknown whether or not the same strains, and theoretically the same signals, will induce the same amount and type of bone formation in the HF and NC bones. There are many factors involved with obesity that could affect the bone response to strain, and ultimately, bone adaptation.

The strain in rat femurs has been measured previously *in vivo*, during locomotion, but the number of studies is limited. Keller and Spengler [155] attached rosette strain gauges to the anterior-lateral surface of the femoral mid-diaphysis of male Sprague Dawley rats and recorded the strain as the rats walked on a wheel. They recorded axial strains between -260 and -280 με. The same procedure was performed again and average peak axial strains were between -260 and -330 με [6] and -400 με in another similar study [146]. Indrekvam et al. [153] used single strain gauges attached to the anterior surface of femurs of male Wistar rats to record peak strains between -180 and -390 με while walking. Wehner et al. [156] developed a numerical musculoskeletal inverse dynamics model of the rat's hindlimb to calculate musculoskeletal loads during gait. They calculated peak axial strains that were only 2 με (~1%) below the range of the *in vivo* strains measured by Keller and Spengler [6, 155]. Thus recorded *in vivo* strains are fairly consistent across studies.

However, it is difficult to compare the femoral strains measured in this thesis to the femoral strains reported in the literature because the strains were recorded under different loading conditions. A bone responds differently in three-point bending compared to compression. Cortical bone is strongest along its long axis under

compression. Using their numerical musculoskeletal model, Wehner et al. [156] showed that internal forces and moments varied along the femoral axis as well as over the gait cycle, but the greatest internal force in the femur acted in the longitudinal direction during gait. These maxima varied from 6.0 bodyweight (BW) in the proximal femur to 7.0 BW in the distal femur. Maximum shear forces were much lower in magnitude, 0.9 BW on the proximal femur and 0.6 BW on the distal femur.

In three-point bend tests the femur is loaded transversely, resulting in compression on the anterior surface and tension on the posterior surface; not in compression along its long axis, as is the case *in vivo.* Secondly, *in vivo* strains are a function of the whole musculoskeletal system as opposed to *ex vivo* where only the bone is being tested. The only literature that examined rat femoral strains *ex vivo* did not report their strain findings. *Ex vivo*, there is no muscle activity and the bone is being tested in a non-physiological loading manner. Although the femur does undergo some bending the greatest forces act compressively, not in the direction the strains were measured in this thesis. However, the actual strain values are not as critical as the overall general trends that show that a high-fat diet does not alter longitudinal bone mechanical strain during three-point bending.

Walking produces peak strains in the leg bones or hind limbs usually less than 1000 με in most animals [18] and, in the case of rats, less than 400 με. Humans can maintain normal bone with little activity, and the most intense exercise produces strains no more than 3000 με [18]. This suggests that the level of strain key to determining bone geometric and mechanical properties may not be the rare peak strains generated during

strenuous activity, but rather the consistent daily loading of low-magnitude, high-frequency strains [157]. If it is the daily loading of the bones that determines bone properties, then, in the case of the HF rats, the bone strains may have increased as they grew and gained mass because of gradually increased loading. This would, in turn, have caused bone apposition to occur with increasing bone size, as seen in the HF rats. The strains would then return to the target level until new loads were applied. The HF bones would have required the ability to adapt to mechanical loading in this case and therefore, decrements in bone adaptation may not be seen in the HF rats when they are mechanically loaded. However, the strains produced by mechanical loading are much greater than average daily strains and therefore the ability of the HF bones to adapt to these unusually high non-physiological strains may be altered.

5.6 Femoral Mechanical Properties

Three-point bend failure tests were performed on the left femurs to determine the mechanical properties of the femurs. The tests showed that the HF diet had little effect on the structural properties. The male and female HF animals had a tendency to have greater proportional and maximum loads and stiffness, but the only significant difference was greater stiffness in the HF female femurs compared to NC. No differences have been found before between high-fat diet animals and controls with respect to stiffness [83], but the diet only lasted four weeks. Since there was no difference in maximum load, the HF rats also tended to have lower maximum stresses than the NC, given their larger femoral size.

The male femurs, however, did exhibit higher maximum and proportional loads, maximum energy and stiffness compared to the females in both the NC and HF groups. This is expected given the significantly greater size of the male femurs compared to the female femurs. The male NC femurs also had significantly greater maximum deformation than the female NC femurs likely because of their greater size. As well, the proportional energy of the NC male femurs was significantly greater than the females. Given that the proportional deformation was not significantly different between genders, the reason for the difference in energy would lie in the significantly greater stiffness of the male femurs. The maximum stress did not differ between the male and female femurs. This is not surprising given that the male femurs were significantly larger and had greater maximum loads and the female femurs were smaller with lower loads. Stress is normalized load to the size of the bone so a large load normalized to a large bone and a small load normalized to a small bone could result in similar values.

The material properties of the left femurs were determined by applying beam theory to the data derived from the load-deformation curves of the three-point bend tests and the cross-sectional area properties of the right femurs. No significant differences in material properties, as determined by the elastic modulus, were found between the NC and HF femurs for the male or female animals. The HF femurs did have lower elastic moduli than the NC, but the differences were not significant.

Ionova-Martin et al. [61, 82] conducted two studies with a comparable diet regime to this thesis and found similar results. The lengths of the diets were 19 and 16 weeks, about half as long as the one examined in this study but lengthier than most reported in

the literature, and they began at four and three weeks of age, respectively. Their findings were similar to the current study with increases in bone size and second moment of area. They did, however, find significantly reduced material properties in the femurs as demonstrated by the decreased maximum and yield stress, and elastic modulus in the high-fat diet femurs. One study found no change in maximum load [61] whereas another found a decrease in the maximum load [82] in high-fat animals. Although significant differences were not seen in the material properties of the HF femurs in this thesis, trends toward diminished material properties were shown.

These thesis results show similar trends to other studies, regarding high-fat diet effects on bone, but lack statistically significant differences. Most studies have shown significant decrements in material properties with changes in bone size being inconsistently reported. Possible explanations as to why there was no effect on the material properties of the HF femurs in this study may include the length of the diet. Most studies implemented diets half the length of this thesis. It may be that bone possesses the ability to adapt to the poor long-term diet in order to minimize the effects on the bone. It is as though the bone is reaching a new steady state to maintain homeostasis. This may be what occurred in the HF bones in this project. The diet had been maintained for such a length of time that the bones are able to reach a new equilibrium. Interestingly, Zernicke et al. [3] found no change in the material properties of female rat femoral necks after a two year long high-fat-sucrose diet but, Hou et al. [139] found reduced material properties after only 10 weeks of the diet. These two studies suggest that the body is able to adapt to the diet to lessen the effects on the bone. It could also be that the diet is indeed

affecting the bone material, but in ways that are not detectable by mechanical testing and subsequent analysis.

It is difficult to compare these thesis findings to other studies examining the effects of a high-fat diet for several reasons. The first issue when comparing studies is the animals themselves. Some studies use rats, others mice and some even use poultry. The gender of the animals also varied between studies and this can play a role in diet effects as seen in this thesis and other studies. A second reason for the difficulties is the diet composition. Each study uses a different diet composition. Some diets are just high in fat whereas others are high in fat and sucrose. Many studies record and keep track of the dietary behaviours so as to have an idea of the number of ingested calories and the amount of fat the animals are consuming, but many others do not. This makes it difficult to compare studies because the animals are not consuming the same type or amount of food, which can greatly influence weight gain and, theoretically, effects on bone. The length of the diet is also a factor. Much of the literature regarding the effects of a high-fat diet on bone involves only short-term diet durations of 10-16 weeks. This thesis study placed animals on the high-fat diet for 36 weeks, three times as long as most other studies. The diet duration can play a large role in its effects on the body and bones. If the animals are placed on the diet for too short a period of time, the effects of the diet may not be detectable. If the animals are on the diet for an extended period of time, the effects may be minimized because of adjustment or normalization of the body in response to the diet (*e.g.*, current data and Zernicke et al. [3]). As well, if the diet is started at a young age then it could affect bone development whereas if it is started after skeletal maturity the

effects may be less pronounced [59]. Finally, the bone being examined in each study varied. Many studies looked at the vertebra and tibia and, as in this study the femur. The vertebrae are a non-weight bearing site whereas the femur and tibia are both weight bearing sites. The majority of the time a high-fat diet also caused an increase in body mass and therefore weight bearing sites may have experienced different changes in the bone environment in comparison to non weight bearing sites. As well, the type of bone under investigation, cortical or trabecular, varied between studies. Trabecular bone is more responsive to diet treatments than cortical bone because it is more metabolically active due to a greater surface to volume ratio than cortical bone allowing it to be remodeled more. All of the above factors not only make it difficult to compare studies but provide some explanations as to why there are so many inconsistencies between studies. The methodologies vary so much between studies that it is difficult to determine the cause(s) of bone changes.

5.7 Lamellar Bone Formation

No lamellar bone was formed in any of the ulnas as there were no distinct layers of bone formed or any distinct label separation seen. As a result, the label perimeters were determined. No differences were found in the calcein or alizarin perimeters between the right and left ulnas of the male and female rats in either the NC and HF groups. There were also no differences in the label perimeters between NC and HF ulnas for either gender. The reason for no bone formation is more than likely due to the lack of bone stimulation; that is to say that the loading protocol was insufficient to induce changes in

bone formation. It appears as though the loads applied did not stress the bone enough to cause sufficient strain to induce bone formation. It is also possible that the animals were too old, and the bones were less responsive to the prescribed loading protocol. Studies have shown that the strain threshold for osteogenesis is increased with age and the osteogenic response is suppressed [158-160].

Robling et al. [23] proposed that three factors that affect bone adaptation: a dynamic stimulus, duration of loading and accommodation to loading. The lamellar loading protocol applied here had a dynamic stimulus, which is the primary stimulus of bone adaptation [161, 162], a load applied at a frequency of 2 Hz. The minimum effective frequency was 0.5 Hz, and bone formation increased as loads were applied at higher frequencies up to approximately 10 Hz [163]. Experimental results demonstrated that the stimulus for bone formation was increased if either the magnitude or frequency of loading was increased [23]. The magnitude of the load used in this study may have been too low at only 15 N. It has been estimated that the rat forelimb normally withstands anywhere between 60-70% of body weight during normal locomotion [164] and the ulna itself carries about 65% of the total load [165]. Theoretically, given the average male body mass of the HF and NC rats was approximately 800 g, their body weight alone would cause a load of about 4.8-5.6 N on the forelimb and 3.4 N of that would be on the ulna. The forelimb compression model uses hyper-physiological loads in the range of 5-7 times the animal's body weight. So, loads of approximately 20 N could be applied to the male rats.

The loading may have been too long in this protocol without sufficient rest. It has

been shown that bone loses more than 95% of its mechanosensitivity after only 20 loading cycles [23]. It is thought that, if given time to rest, the cells may then be able to respond to the loading again. Resensitization has been shown to occur in seconds or hours depending on the nature of the loading stimulus. Time to rest is needed between loading bouts as well as between cycles. Experiments have shown that there is an osteogenic benefit of short-term (on the order of seconds) recovery periods in restoring sensitivity to loaded bone [166]. The number of loading cycles chosen was likely too low as well. Most literature cites over 100 cycles while this study only used 50. The number of cycles should be greater with appropriate rests inserted.

Lastly, the bone cells may have become accommodated to the loading routine. Bone adapts to mechanical loading because it senses a new loading state and therefore it must adjust accordingly. Bone cells must be stimulated above a certain threshold in order for a mechanical signal to cause a cellular response [22]. This threshold is a product of local loading history. Experiments show that the initial loading stimulus has the greatest influence on bone formation, not the long-term accumulation of loading [167]. Other experiments show that inserting a rest and stopping loading can reverse the effects of accommodation and restore mechanosensitivity [168]. The bones in this thesis were loaded for 5 consecutive days using the same loading protocol each day, likely resulting in accommodation. A better protocol would likely employ rest days between bouts of loading so the bone recognized each loading bout as new. Robling [169, 170] and Saxon [168] demonstrated that loading at 2 Hz with 360 cycles for at least 10 days of loading over at least two weeks with rest between loading bouts produced measurable lamellar

bone formation.

Mosley et al. [171] examined ulnar strains in male Sprague Dawley rats (approximately 300 g) during unrestricted locomotion (running) on a solid level surface and the peak compressive strains varied between animals from 700 to 1200 με. These strains would maintain bones but they would not be osteogenic, or more simply would not stimulate bone formation. Strains above these would be required to signal the bone of a new loading condition and then the bone would adjust accordingly by forming new bone to lessen the strains and get them back to the normal level. Hsieh et al. [79] assessed bone formation in relation to strain in female Sprague Dawley rats (approximately 300 g) using various loads and 360 cycles/day at 2 Hz for 10 days. They found that axial loading greater than 10 N was needed to induce new bone formation on the periosteal surface at the midshaft of the ulna, the point of greatest strain. The threshold strain for osteogenesis was 2284 με at the ulnar midshaft. A 15 N load produced 3000 με. In comparison to the loading protocol chosen for this thesis, a much larger number of cycles were performed each day and twice as many loading days were used. This may mean that the current loading protocol did not have enough loading cycles or days of loading especially since these rats were smaller than the NC and HF rats, on average. It is only speculation, but given the size of the rats used in this thesis, a 15 N load probably did not cause ulnar strains as high as 3000 με or even high enough to reach the threshold for osteogenesis shown in the Hsieh et al. [79] study, which may not necessarily be the threshold for osteogenesis in these HF and NC rats. Therefore the ulnar strains caused by the loading were probably too low to induce bone formation.

It has been recently demonstrated that a four week long protocol with loading performed three days per week to loads of 21 N at 2 Hz, for 20 cycles followed by 20 seconds of rest and then another 20 cycles, for a total of 300 cycles per day can produce lamellar bone in 20 week-old male Sprague Dawley rats (approximately 620 g) [172]. A protocol such as this addresses the three factors of bone adaptation, and incorporates the needed features of a complete protocol for inducing lamellar bone formation.

5.8 Exclusions/Non-responders

Genetics play a role in the susceptibility of an individual to obesity, but so do environmental factors. A genetic predisposition to be obesity-prone or resistant has been shown in animal models [173, 174]. Standard murine models for studying dietary obesity are different in their susceptibility to obesity, and Sprague-Dawley and Wistar rats can be easily categorized to prone and resistant phenotypes with *ad libitum* access to high-fat diets [175, 176].

Exposing some animals to a high-fat diet caused them to become obese (obesity prone), whereas exposing other animals to the same diet did not cause them to become obese (obesity resistant) [177, 178]. It was more of a continuum of response to the high-fat diet [179] but as with a continuum there will always be animals at the extremes. These different responses have been attributed to higher energy intakes in obesity-prone animals [180-183], but with similar intakes sometimes seen in prone and resistant animals, the suggested susceptibility in these animals was thought to come from the capability to store energy more efficiently [184, 185].

Other suggested mechanisms for the difference between the response of prone and resistant animals to high-fat diets are that prone animals have lower fat oxidation [179, 185, 186], increased lipoprotein lipase activity in their adipose tissue and no change in lipoprotein lipase activity of their muscles which favours fat storage [180, 186, 187]. However, Commerford et al. [183] showed that obesity prone male Wistar rats fed a high-fat diet did not have a greater capacity for lipogenesis or retention of dietary fat, and that a characteristic increase in energy intake, associated with obesity prone rats, appeared to be necessary and critical to accelerated weight and fat gain. Therefore, increased energy intake plays a central role in dietary induced obesity.

In this thesis project, one HF male and one HF female sibling pair were excluded from all analyses because they had body masses below the NC average body mass and, as such, were deemed to be non-responders to the diet. In addition to not gaining mass, the bone properties (cross-sectional area and moment of inertia) of these two animals did not show any differences from the average NC. These rats would be considered to be obesity resistant. The lack of weight gain as well as having about half the fat mass as the average HF rat suggests that these two rats were some how able to minimize the effects of the high-fat diet.

Several studies have shown that approximately half of the rats exposed to a high-fat diet will not become obese [83, 179, 188, 189]. Studies specifically examining a high-fat diet and bone also demonstrated a lack of body mass increase in animals fed a high-fat diet. Salem et al. [84], Hou et al. [139] and Li et al. [58] found no change in the body mass of female Sprague-Dawley rats placed on a high-fat-sucrose diet for 10 weeks. It

was suggested that the animals were able to adjust their caloric intake to maintain their body mass. Another study showed that along with no difference in body mass, the percent body fat of young female rats on a high-fat sucrose diet was not different from controls [120]. Ward et al. [4] also found no difference in body mass between their control and western-style diet male and female rats. The mean daily food intake of the male and female rats fed the western-style diet was significantly less than that of the control group, and despite a higher energy content in the western-style diet, energy intakes were also less among the male and female rats fed the western-style diet. This study supports the notion presented by Commerford et al. [183] that energy intake must be increased in order for a change in body mass to be seen.

Food and energy intake were not recorded for the NC or HF rats in this thesis project, so it cannot be discerned whether it was truly a lower energy intake that resulted in the two rat siblings being deemed non-responders (obesity resistant) or if genetics were a factor. The fact that the only two rats that were non-responders were siblings provides strong support for a genetic component in this thesis and obesity in general. Studies suggest that among different populations, the prevalence of obesity is largely determined by environmental factors. However, among individuals from the same population, living in a given environment, the variability in body size and body composition is mostly related to a genetically determined response to that environment [190]. Since the rats were provided with the same environment and were of the same strain, the non-responders likely did not gain weight because of their genetics.

With only 2 out of 19 rats not responding to the diet, a definitive conclusion cannot be drawn regarding the mechanisms behind the lack of increased body mass. The percentage of non-responders is well below the average reported by several studies and may be attributed to the rat strain, the diet or genetics. However, the limited number of non-responders or obesity resistant animals does provide some insight into how a high-fat diet can or cannot affect animals and bone.

5.9 Limitations

A limitation of this thesis was the sample size. Due to the litter pairings and the sacrifice of some rats before nine months, there were only approximately eight rats per group. Generally, for mechanical testing, a sample size of twelve is used. The power to detect change in the measured variables between diet groups is smaller when fewer animals are used. If the sample size had been increased, dietary induced effects on bone may have been seen. For example, a post-hoc analysis for sample size revealed that sixteen males per group and nine females per group were needed to have the power to detect a dietary difference in strain. This suggests that given an increased sample size, strain may have been statistically greater in HF rats. A post-hoc analysis for sample size also revealed that twenty-five males per group and 329 females per group were needed to have the power to detect a dietary difference in modulus calculated using strain and twenty-one males per group and thirty-five females per group were needed to have the power to detect a dietary difference in modulus calculated using deformation. The large estimated sample sizes needed to detect modulus differences may suggest that there is no

effect of diet on modulus. It may be that if a larger sample size were used, a significantly lower strain would have been detected in the HF femurs due to their greater cross-sectional area properties, and the modulus would not have been lower as is suspected. Retrospectively, the statistics can be examined but it does not change the results found.

One limitation that could play a significant role in the strain measurements obtained is the position of not only the strain gauge on the bone but also the positioning of the bone on the loading supports. The strain gauges were attached at the midpoint of each of the femurs and care was taken to ensure the location was the same on each femur in terms of positioning at the midpoint of the bone and at its centre. Upon completion of testing, the location of the gauges was compared across all femurs. The resulting measured strain values were also compared to gauge placement to ensure there were no significant outliers. Positioning of the bones on the supports was done the same way each time ensuring the bone was in the middle of the two lower supports and the loading anvil made contact with the midpoint of the bone. A 2 N preload was applied to all femurs to prevent them from twisting once loading began. During loading the femurs were visually examined to ensure no rotation was occurring, resulting in torsion as well as bending. However, this does not completely rule out the occurrence of rotation.

The compression tests required the femurs to be cut with a diamond wafer saw. Due to the inherent wobble in the saw blade, it was difficult to achieve perfectly square ends on the cut section. If the section is then loaded in compression, it is not just compression that is occurring but rather bending occurs and the strains recorded are therefore inaccurate. To help correct the uneven bone ends, a wobble board-type device

(Figure 3.6) was constructed. Because only one strain gauge was attached to the bone, it was not possible to determine if the bone was bending or not. However, upon visual inspection of the bone positioning upon the loading platens it appeared as though the wobble board adjusted the bone sections correctly.

In addition, the mid shafts of the femurs were not perfectly linear; they possessed a slight curvature, making it difficult to load them in pure compression. Because of the uneven ends and the curved femurs, the strain recordings differed between trials. Each time the same bone was placed into the loading apparatus different strains would result. In fact, some of the trials resulted in tensile strains as opposed to compressive strains (Figure 4.9). It was difficult to align the bone between the loading platens and this was shown in the strain outputs. Due to these inconsistencies it was decided that the compressions tests were providing invalid results and they were discontinued.

Femur deformation was recorded using three different methods, each with their own limitations. As discussed in the Methods Section 3.4.3, the ADMET and extensometer both had internal compliance that could have affected the displacement output. The DVRT had limitations as well. The DVRT had poor resolution at small displacements and it had inherent noise, making it difficult to gather reliable data at small displacements (low loads).

It seemed reasonable to use the ADMET displacements at the low loads (60 N for females and 100 N for males) and not the DVRT data. This is such because at the failure loads, the ADMET and DVRT displacements were very similar whereas at the low loads where the DVRT performed poorly, the displacement measurements between the two

methods were very different. Theoretically the DVRT should have produced reliable data at the higher loads where noise is less influential. Since the displacements matched at these higher loads, the ADMET appeared to be consistent and accurate throughout the loading range.

The modulus values calculated using the displacement data from the ADMET were similar at low and maximum loads implying that the displacements were consistent over the range of applied loads. In contrast, the DVRT displacement data resulted in very different modulus values depending upon the applied load. At the lower loads, where the DVRT was noisier and less accurate, the modulus values were very high. At the maximum loads, the modulus values calculated using the displacement data from the DVRT were similar to the ADMET modulus values at the maximum as well as the lower loads. The modulus values should not vary with load and therefore, the deformations measured by the ADMET were used for analysis.

5.10 Combining Diet and Gender to Increase Evaluation Power

The male and female and NC and HF animals were combined so the sample size could be approximately doubled, increasing the power to detect dietary and gender differences, respectively. In regards to diet differences, a two-way analysis of variance revealed similar findings as found when examining genders individually, but there were some additional statistical differences revealed. As with the genders separated, the HF rats had greater body and fat masses compared to NC, and the HF rats had larger bone architecture shown by greater femoral length, cross-sectional area and second moment of

area. The additional differences found with this analysis were that the maximum load and stiffness were significantly greater in the HF femurs. Stress and strain at 30 N in the HF femurs were also significantly reduced compared to NC. Although not significant, the maximum stress and modulus tended to be lower in the HF femurs.

The finding that the stress and strain were lower at a given load in the HF femurs suggests that bone may not be adapted to strain and that materially the properties may not be lacking in the HF femurs, as previously determined by looking within genders. The lower stresses and strains may have been a result of the increased HF femoral cross-sectional area. Combining the genders would result in sample sizes large enough to detect these dietary differences. The HF femurs were structurally stronger as demonstrated by the greater maximum load and still only showed a trend towards lower material properties.

In terms of gender differences, a two-way analysis of variance revealed similar findings as were found when gender was compared within diet groups. Greater body and fat masses, bone length, bone cross-sectional area, and second moment of area were shown in males compared to females. In agreement with the previous analyses, males had less stress and strain at 30 N. A significantly larger modulus at 30 N, calculated using strain, was found in the males but a significantly lower modulus, calculated using deformation, was also found. Structurally, as shown previously, the males had greater proportional and maximum loads, greater proportional and maximum energies and greater stiffness. Interestingly using this analysis, the males also had greater maximum deformation.

Surprisingly, depending on how the modulus was calculated determined whether males or females exhibited greater material properties. Because both results were shown in this analysis it is difficult to come to a definitive conclusion on which gender possesses greater femoral material properties. In addition, the first analysis between genders within a diet, revealed that males had a greater modulus calculated using strain for the NC diet and there was no difference in modulus calculated using deformation. Deciding on the most correct modulus values is difficult and therefore, whether they differ between genders will depend on which values are used and how the analysis is carried out.

5.11 Conclusions

This thesis studied the effects of a high-fat diet on bone properties in rats when compared to rats fed a NC diet; specifically bone strain for the purpose of investigating bone adaptation in animals consuming a high-fat det. The high-fat diet did not affect bone strain despite significantly larger bone cross-sectional area properties compared to NC rats. No differences in material properties were detected using 3-point bend tests. Most structural properties also appeared to be unaffected by the high-fat diet. If the HF femurs are not lacking structurally, tend to be stronger than NC femurs and are significantly larger, then the bone material must be lacking in some manner, because the strains were similar. The equivalent strains supports the idea that bone self regulates to control the strain environment and thus maintain its strength despite changes in the body that may be compromising optimal bone health.

The lack of difference in femoral strain between the HF and NC rats is suggestive of adaptive modeling. Compared to NC the HF rats had greater body mass and in theory, greater muscle mass, which is associated with an increase in body mass. The greater body as well as muscle mass increased bone loading and, in turn, increased strain on the bone. In agreement with Frost's theory [22], in which a minimum effective strain exists and strain is the main determinant of bone geometry, the HF rat femurs were larger than the NC. The strains surpassed the minimum effective strain because of the increased loading and bone formation was activated causing an increase in bone size. Once the bone size increased, the strains from loading decreased, back to the minimum effective strain or threshold level. In addition, it has been found that structural properties of rat bones are dependent on geometry to a greater extent than material [146] and in the case of the HF rats, they appear to be only relying on geometry, as their elastic modulus did not differ from the NC. The HF rats may not have been able to increase their material stiffness due to poor nutrition. The HF rats may have had a lack of calcium due to the formation of intestinal soaps or a lack of vitamin D due to deposition in adipose tissue. Both of which would compromise bone material properties. Bone appears to be able to adjust itself in accordance with strain to remain structurally competent.

5.12 Future Work

This thesis is the basis for further study into the effects of a high-fat diet on bone adaptation in rats. The results of this thesis suggest that loading the femurs of the HF and NC rats to the same degree induces the same amount of strain. This helps to establish a

loading protocol for the HF animals to determine the effects of a high-fat diet on bone adaptation due to mechanical loading. The next step in this research would be to strain gauge the ulna in NC and HF rats to determine if the bone strain at a given load, in this location, is also similar between groups just as it is in the femur. If so, then the same load can be applied to both groups during the bone formation loading protocol to induce the same strain and, in turn, to examine if the same bone response occurs.

Bone adaptation using the rat forelimb compression could be examined in rats of different developmental ages as well as in rats that experience different diet durations and compositions. Age is an important factor because poor diets are beginning at young ages [191], the time of critical bone development, and could impact the bone greatly as it develops. The length of diet may also play a critical role because the bone may be able to self adjust, as is thought to occur in this study, with an increase in bone size. The composition of the diet could also have an effect on bone adaptation and properties because the ratio of n-6 to n-3 fatty acids at the bone tissue level may alter the ability of bone to adapt to mechanical loading.

Prostaglandins are multifunctional regulators of bone metabolism that stimulate both bone resorption and formation. Fatty acids in the n-6 family are precursors to prostaglandin E1 and E2, both important modulators of bone adaptation. An imbalance of these fatty acids in bone tissue due to a diet high in saturated fats (typical of North American or Western diets) could impair the ability of bone to adapt to mechanical loading or damage. Thus, while examining bone adaptation, levels of prostaglandins should also be monitored. In addition to looking at bone adaptation on a whole bone

level, specifically looking at the effects of mechanical loading on osteoblasts in culture is also an area of further research.

The mechanical properties of bones from animals fed a high-fat diet could also be examined in various bones and at different ages. The literature is lacking a complete study. There are many pieces but as mentioned previously, it is difficult to compare them. In addition to mechanical testing, ideally bone mass and density would be determined as well as characterization of mineral organization and lamellar structure. This may help to determine just what the HF bones are missing. Hormone levels as well as bone formation and resorption markers would also be useful.

Lastly, the compression tests in the current study were not performed because of inconsistent findings, and establishing a consistent compression loading protocol would be helpful. The data gathered from this testing would be more physiologically relevant because of the orientation of the femurs and would have fewer limitations than 3-point bend tests. The new protocol would require a device that would align the femurs perpendicular to the loading platens to ensure the bones were only loaded in compression and the loading was consistent between trials and bones.

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APPENDIX I

Normal Control diet. Harlan Laboratories Inc. Teklad 22/5 Rodent Diet (8640).

Macronutrients	
Crude Protein	22%
Fat (ether extract)	5.5 %
Carbohydrate (available)	40.6 %
Crude Fiber	3.9 %
Neutral Detergent Fiber	12.8 %
Ash	8.1 %
Calories from Protein	29 kcal%
Calories from Fat	17 kcal%
Calories from Carbohydrate	54 kcal%
Minerals	
Calcium	1.1 %
Phosphorus	0.9 %
Non-Phytate Phosphorus	0.6 %
Sodium	0.4 %
Potassium	1.0 %
Chloride	0.7 %
Magnesium	0.2 %
Zinc	77 mg/kg
Manganese	102 mg/kg
Copper	24 mg/kg
Iodine	3 mg/kg
Iron	280 mg/kg
Selenium	0.27 mg/kg
Vitamins	
Vitamin A	15.8 IU/g
Vitamin D_3	3.0 IU/g
Vitamin E	150 IU/kg
Vitamin K_3 (menadione)	50 mg/kg
Vitamin B_1 (thiamin)	32 mg/kg
Vitamin B_2 (riboflavin)	9 mg/kg
Niacin (nicotinic acid)	66 mg/kg
Vitamin B_6 (pyridoxine)	14 mg/kg
Pantothenic Acid	23 mg/kg
Vitamin B_{12} (cyanocobalamin)	0.06 mg/kg
Biotin	0.41 mg/kg
Folate	3 mg/kg
Choline	2380 mg/kg

APPENDIX II

High Fat diet. Research Diets Inc. RD Western Diet (D12079B).

Macronutrient	**gm%**	**kcal%**
Protein	20	17
Carbohydrate	50	43
Fat	21	41
Ingredient		
Casein, 80 Mesh	195	780
L-Cystine	3	12
Corn Starch	50	200
Maltodextrin 10	100	400
Sucrose	341	1364
Cellulose	50	0
Milk Fat, Anhydrous	200	1800
Corn Oil	10	90
Mineral Mix S10001	35	0
Calcium Carbonate	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Cholesterol, USP	1.5	0
Ethoxyquin	0.04	0
Total	**1001.54**	**4686**

APPENDIX III

Dehydrating, infiltration and embedding procedure and schedule. This procedure prepares the bone samples for histology.

Day	Procedure	Solution	Time	# of Changes	Storage
1	dehydration	70% ethanol	1 hour	2	3ºC
		95% ethanol	1 hour	2	3ºC
		95% ethanol	overnight		3ºC
2	dehydration	100% ethanol	1 hour	3	3ºC
		xylenes	1 hour	2	3ºC
		xylenes	overnight		3ºC
3, 4	infiltration 1	85 ml methyl methacrylate 15 ml dibutyl phthalate	24 hours	daily	3ºC
5, 6	infiltration 2	85 ml methyl methacrylate 15 ml dibutyl phthalate 1 gram benzoyl peroxide	24 hours	daily	3ºC
7, 8	infiltration 3	85 ml methyl methacrylate 15 ml dibutyl phthalate 2 grams benzoyl peroxide	24 hours	daily	3ºC
9	embedding	85 ml methyl methacrylate 15 ml dibutyl phthalate 2 grams benzoyl peroxide	leave for 2-3 days		37ºC (water bath)

APPENDIX IV

Elastic modulus values of right femurs from sub-failure 3-point bend tests from the ADMET before correction, the extensometer and DVRT.

Property	Male		Female	
	NC	HF	NC	HF
Elastic Modulus (GPa) (calculated using deformation from extensometer)	2.75 ± 1.15 (at 30 N)	1.92 ± 0.63 (at 30 N)	3.54 ± 0.91 (at 30 N)	2.79 ± 0.70 (at 30 N)
Elastic Modulus (GPa) (calculated using deformation from extensometer-corrected)	9.79 ± 8.21 (at 30 N)	5.08 ± 3.09 (at 30 N)	9.01 ± 4.21 (at 30 N)	7.08 ± 4.19 (at 30 N)
Elastic Modulus (GPa) (calculated using deformation from Admet)	2.39 ± 0.46 (at 100 N)	1.94 ± 0.52 (at 100 N)	2.73 ± 0.29 (at 60 N)	2.39 ± 0.46 (at 60 N)
Elastic Modulus (GPa) (calculated using deformation from Admet - corrected)	4.17 ± 0.87 (at 100 N)	3.46 ± 0.94 (at 100 N)	4.19 ± 0.45 (at 60 N)	3.70 ± 0.67 (at 60 N)
Elastic Modulus (GPa) (calculated using deformation from DVRT)	14.17 ± 2.34 (at 100 N)	11.47 ± 2.71 (at 100 N)	14.31 ± 2.56 (at 60 N)	14.43 ± 3.38 (at 60 N)

APPENDIX V

Mechanical properties of the left femurs from failure 3-point bend tests from the ADMET before correction and the DVRT.

Property	Male		Female	
	NC	HF	NC	HF
Proportional Load (N) (ADMET)	156.02 ± 18.50	155.27 ± 20.57	112.41 ± 12.10	124.43 ± 8.46
Proportional Load (N) (DVRT)	152.41 ± 20.18	146.84 ± 20.01	108.71 ± 14.02	121.07 ± 7.20
Maximum Deformation (mm) (ADMET)	0.786 ± 0.061	0.791 ± 0.194	0.665 ± 0.074	0.674 ± 0.61
Maximum Deformation (mm) (DVRT)	0.692 ± 0.059	0.722 ± 0.181	0.595 ± 0.092	0.576 ± 0.074
Proportional Deformation (mm) (ADMET)	0.263 ± 0.023	0.246 ± 0.039	0.237 ± 0.031	0.244 ± 0.013
Proportional Deformation (mm) (DVRT)	0.223 ± 0.030	0.205 ± 0.029	0.199 ± 0.034	0.198 ± 0.018
Max Energy (mJ) (ADMET)	135.42 ± 22.27	134.80 ± 35.32	83.14 ± 14.07	91.25 ± 17.11
Max Energy (mJ) (DVRT)	118.20 ± 15.13	122.43 ± 31.09	74.76 ± 15.71	77.62 ± 15.46
Proportional Energy (mJ) (ADMET)	22.45 ± 4.04	21.19 ± 5.75	15.17 ± 3.26	16.77 ± 1.82
Proportional Energy (mJ) (DVRT)	18.33 ± 4.02	16.27 ± 4.17	12.18 ± 3.28	13.00 ± 1.53
Stiffness (N/mm) (ADMET)	605.39 ± 54.71	657.46 ± 50.26	474.59 ± 36.72	521.88 ± 28.60
Stiffness (N/mm) (DVRT)	682.28 ± 105.59	723.58 ± 66.49	536.24 ± 53.33	608.84 ± 54.37
Elastic Modulus (GPa) (calculated using deformation from ADMET)	2.79 ± 0.63	2.33 ± 0.59	3.36 ± 0.49	3.03 ± 0.66
Elastic Modulus (GPa) (calculated using deformation from DVRT)	3.16 ± 0.72	2.58 ± 0.74	3.78 ± 0.62	3.58 ± 0.90