Effect of a high fat maternal diet on body composition and bone development in male offspring

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ABSTRACT

Direct high fat (HF) feeding has adverse effects on body composition and bone development in rodents. However, it is unclear whether maternal HF feeding has similar effects in male rat offspring. The objectives of this thesis were to determine if maternal HF feeding altered body composition, plasma hormones, bone development, and bone fatty acid composition in male offspring at weaning and 3 months of age. Maternal HF feeding increased bone mass and altered femur fatty acid composition at weaning, without differences in fat mass, lean mass, plasma hormones, or bone mass (femur or lumbar vertebrae). However, early differences did not persist at 3 months of age or contribute to lower bone strength – following consumption of a control diet post-weaning. These findings suggest that maternal HF feeding can alter body composition and bone development in weanling male offspring, without long-lasting effects if a healthy control diet is consumed post-weaning.

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ABBREVIATIONS

DNA – deoxyribonucleic acid

BMC – bone mineral content

BMD – bone mineral density

DEXA – dual energy x-ray absorptiometry

IL-6 – interleukin-6

TNF-α – tumor necrosis factor-α

CHO – carbohydrates

CON – control

HF – high fat

RUNX2 – runt related transcription factor-2

RANKL – receptor activator of nuclear factor kappa-β ligand

pQCT – peripheral quantitative computed tomography

Micro-CT – micro-computed tomography

SFAs – saturated fatty acids

MUFAs – monounsaturated fatty acids

PUFAs – polyunsaturated fatty acids

RTP – retroperitoneal

OM – omental

EPI – epididymal

PND – postnatal day

LV1-3 – lumbar vertebrae 1-3

ROI – region of interest

LV3 – lumbar vertebra 3

RPM – revolutions per minute

ANOVA – analysis of variance

SEM – standard error of the mean

BD – below detection

FFQ – food frequency questionnaire

GPAQ – global physical activity questionnaire

CHAPTER 1.0: GENERAL INTRODUCTION

1.1: Nutritional programming

Nutritional programming refers to the phenomenon in which nutrients consumed *in utero* and/or during suckling have long-lasting effects on the function or metabolism of a tissue [1]. Conceptually, the term nutritional programming originated from observations that under-nutrition during pregnancy, as a result of the Dutch Famine (1944), was associated with altered offspring health at adulthood [2]. For example, individuals born to mothers exposed to famine during late gestation demonstrated higher rates of coronary artery disease and diabetes in adulthood compared to offspring not exposed to famine in utero [2].

Due to the ethical considerations underlying the study of nutritional programming in humans (i.e., randomizing mothers to unhealthy diets – either under-nutrition or over-nutrition), rodent models have proven useful to understand this phenomenon. Although there are examples of nutritional programming in both male and female offspring, this thesis focuses specifically on the response of male offspring to nutritional programming. In this regard, maternal under-nutrition (via protein restriction) has been shown to alter body composition and bone development in male rodent offspring [3-5]. For instance, maternal consumption of a low protein diet (9% energy from protein) resulted in lower fat mass in male Wistar rat offspring at 36 weeks of age; compared to offspring from dams fed a control diet [3]. It has also been shown that male offspring from dams fed similar low protein diets had higher markers of bone turnover (i.e., osteocalcin) at 4 weeks of age [4], and lower bone strength [4] and bone mineral content [5] at 12 and 75 weeks of age, respectively – following the consumption of a control diet post-weaning.

Together, these studies show that male offspring have been responsive to maternal protein restriction, as differences in body composition and bone development have been sustained at adulthood. Hence, males may be 'programmed' by maternal nutrient supply in utero and during suckling. However, little information is available regarding whether maternal over-nutrition, in the form of a high fat diet, programs body composition and bone development in male offspring.

1.2: Potential mechanisms of nutritional programming

Although the exact mechanisms underlying nutritional programming are unclear, epigenetics may play a role [6]. Epigenetic mechanisms involve changes in gene expression (i.e., phenotype), without changes in DNA sequencing (i.e., genotype) [7]. In regard to nutritional programming studies, two potential epigenetic mechanisms include DNA methylation [8] and histone modification (i.e., acetylation and methylation) [9].

1.2.1. Altered DNA methylation status

In utero exposure to nutritional alterations (e.g., protein restriction) may alter DNA methylation status and resultant gene expression in the offspring [6]. DNA methylation involves the addition of a methyl group – particularly to gene sequences containing high numbers of cytosine-guanine nucleotides (i.e., CpG islands) near the promoter region of a DNA strand [10]. In order for methylation to occur, methyl groups must be donated by nutrients consumed such as folate or methionine (an essential amino acid). In this regard, an enzyme known as 'methyltransferase' receives the methyl group from respective donors and adds them to CpG islands within a DNA sequence. Subsequently, gene transcription can be prevented in two ways: 1) methylation near the promoter region of a DNA strand (i.e., critical for transcription initiation) prevents RNA

polymerase (an enzyme that facilitates gene transcription) from binding to the promoter region, thus, inhibiting gene transcription; or 2) ‘methyl-CpG binding proteins’ translocate to the gene and tighten the coiling of the DNA strands, thus, inhibiting DNA separation and preventing gene transcription [11,12]. In contrast, the removal of methyl groups can up-regulate gene expression in an opposing fashion by loosening the DNA coiling to improve transcription.

Few studies have investigated whether maternal high fat feeding affects DNA methylation status (of any gene). It has been demonstrated that maternal high fat feeding may alter DNA methylation status of genes involved in determining food preferences of male offspring. In turn, this may perpetuate adverse body composition long-term. For instance, offspring from dams fed a high fat diet showed that genes regulating food palatability (i.e., dopamine and opiod receptors) were hypomethylated compared to offspring from dams fed control diet [13]. Therefore, it was suggested that maternal high fat feeding ‘programmed’ offspring to consume greater levels of fat, which may contribute to greater weight gain and adverse body composition (i.e., greater fat mass).

1.2.2. Histone acetylation and methylation

DNA sequences are surrounded by histone proteins that can regulate gene expression. In this regard, histone proteins are susceptible to modifications involving acetylation status (i.e., addition or removal of an acetyl group; CH_3-CO) and methylation [14]. Changes in acetylation status are facilitated by the enzymes ‘histone acetyltransferase’ (addition of an acetyl group) or ‘histone deacetylase’ (removal of an acetyl group), corresponding to loosening (increasing transcription) and tightening (reducing transcription) of DNA coiling, respectively. In contrast, histone methylation

modifications tighten (addition of a methyl group; reduced transcription) and loosen (removal of a methyl group; increased transcription) DNA strands [14].

In regard to maternal high fat feeding, research pertaining to histone modifications has focussed on liver glucose metabolism. As such, it has been shown that maternal high fat feeding (45% energy from fat; gestation only) resulted in histone modification (unspecified type) of an enzyme regulating glucose production in the liver (i.e., phosphoenolpyruvate carboxykinase), leading to excess glucose accumulation in the blood [15]. Thus, suggesting that maternal high fat feeding may exert programming effects through epigenetic mechanisms. However, the exact genes modified *in utero*, as well as the mechanisms underlying high fat feeding and gene modification, have yet to be fully elucidated in response to a maternal high fat diet - specifically in regard to fat, lean, and bone development.

CHAPTER 2.0: EFFECT OF HIGH FAT FEEDING – BODY COMPOSITION

2.1: Background information & methods used in previous rodent studies

Body composition refers to three separate components: fat mass (adipose tissue), lean mass (skeletal muscle), and bone mass (bone mineral content, BMC; or bone mineral density, BMD) [16] – each contributing to overall body weight. Body composition and body weight may be influenced in utero by maternal diet, but are also modulated across the lifespan depending on genetics, nutrition, and physical activity [17]. Since one modifiable factor includes nutrition, interventions such as direct and/or maternal high fat feeding may modulate changes in body composition and body weight.

One of the most cited non-invasive technologies used to assess body composition is dual energy x-ray absorptiometry (DEXA) [16,18], however, the 'gold standard' for body composition measurement in rodents is through chemical analysis (i.e., ashing) [19]. Although these two methods can accurately assess total body composition (including fat mass), they do not distinguish between visceral (surrounding organs) and subcutaneous (beneath the skin) fat [20]. This identification may be important because visceral fat has been linked with greater adipokine (i.e., leptin) [21] and pro-inflammatory cytokine (i.e., interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)) [22] release than subcutaneous fat [23]. Ultimately, these may contribute to a chronic state of low-grade inflammation that can result in damage of tissues such as bone [24-26]. Thus, fat pad weight (e.g., retroperitoneal; behind the kidneys) measurements are useful in identifying regions of visceral adiposity [27,28], either alone or combined with DEXA measurements (i.e., total fat mass).

2.2: Effect of direct high fat feeding in rodent models

Many studies have demonstrated that high fat feeding adversely affected body composition in male or female rats. These findings have predominately shown that high fat feeding resulted in elevated whole body fat mass [18], and lower lean [28] and bone mass [28,29], despite differences in study design (e.g., fat amount/type, duration of feeding) and methods used to assess fat mass. For example, Franco et al. (2012) demonstrated that high fat feeding (female Wistar rats; 41% energy from fat (lard); 8 weeks) resulted in higher fat mass (27% relative to body weight; DEXA) without differences in body weight compared to rats fed a control diet. However, food intake and measurements of lean and bone mass were not reported. Therefore, the extent to which higher dietary intake of fat affected whole body lean or bone mass was unknown. In contrast, Lac et al. (2008) reported that high fat feeding (male Wistar rats; 39% energy from fat (vegetable oil; unknown source); 10 weeks) led to lower lean (10% less) and bone mass (BMC, 15% less; BMD, 5% less); without differences in fat mass (DEXA and perirenal fat pad weight; compared to rats fed a control diet). However, these results were reported in absolute values, which did not account for lower body weights in the high fat fed rats [28]. Therefore, it was unclear whether 10 weeks of high fat feeding altered body composition in addition to body weight. In contrast, a more recent study reported body composition (including bone mass; DEXA) relative to body weight [29]. As such, higher fat mass (~12% greater) and lower bone mass (BMC & BMD) was shown in rats fed a similar percentage of dietary fat as the aforementioned study (male Wistar rat; 40% energy from fat (beef tallow); 8 weeks) [29]. This finding supports the contention that high fat feeding may adversely affect body composition, resulting in higher fat mass and

lower lean and bone mass. Although, these studies did not adjust the level of protein, vitamins (e.g., vitamin D), and minerals (e.g., calcium) for the higher energy content provided by the high fat diets. Since these nutrients are all important in supporting body growth, the accumulation of fat, lean, and bone mass may have been the result of differences in both macronutrient and micronutrient composition – rather than fat content alone. For example, lower protein intake relative to the high fat diet may have resulted in a lower accumulation of lean and bone mass, along with higher fat mass. Ultimately, it is unclear whether altered body composition reported in previous studies were due to high fat feeding alone, or due to the combination of excess fat and unadjusted levels of protein, vitamins, and minerals in the high fat diet (that resulted in lower levels of these nutrients per kg of diet when compared to control diet).

In summary, it has consistently been shown that direct high fat feeding resulted in altered body composition in rats, especially when diets began during early developmental windows of life (i.e., shortly after weaning) and were continued for 8-10 weeks (Table 1). However, whether maternal high fat feeding alters body composition in male offspring at similar stages of life is not well characterized.

Table 1. Direct High Fat Feeding Studies: Body Composition in Rodents

Study	Rodent Model	CON Diet (% energy)	HF Diet (% energy)	Feeding Duration	Method Used	Results (HF vs. CON)
Franco et al., 2012	Wistar rat (female)	**9% Fat (lard)** 68% CHO 23% Protein	**41% Fat (lard)** 45% CHO 14% Protein	8 weeks	DEXA	↑ Fat mass
Lac et al., 2008	Wistar rat (male)	**8% Fat (unknown source)** 72% CHO 20% Protein	**39% Fat (vegetable oil**) 41% CHO 20% Protein	10 weeks	DEXA Fat pad weights	↔ Fat mass ↓ Lean mass ↓ Bone mass
Macri et al., 2012	Wistar rat (male)	**16% Fat (soybean oil)** 66% CHO 18% Protein	**40% Fat (beef tallow)** 44% CHO 16% Protein	8 weeks	DEXA	↑ Fat mass ↓ Bone mass

CHO, carbohydrates; CON, control; HF, high fat; DEXA, dual energy x-ray absorptiometry. ↑, higher; ↓, lower; ↔, no difference.

2.3: Effect of maternal high fat feeding on male rodent offspring

There are inconsistent findings regarding maternal high fat feeding and male offspring body composition, particularly whole body fat mass (as lean and bone mass are not usually reported). As such, studies have shown higher [18,30], lower [31], and no difference [32] in male offspring fat mass following maternal high fat feeding (compared to offspring of dams fed a control diet; 6-16 weeks prior to breeding and throughout pregnancy and lactation) (Table 2). These divergent findings may be related to differences in study design. For example, maternal high fat feeding studies that focus on offspring body composition have varied in terms of rodent model selected (i.e., rats versus mice), and age at which offspring have been studied (i.e., weaning versus 3-6 months of age). Franco et al. (2012) found that maternal high fat feeding (Wistar rats; 41% energy from fat (lard + soybean oil)) resulted in higher fat mass (53% greater) in

male offspring at weaning, without differences in body weight. However, whether a 'programming' effect occurred is unclear as rats were not studied beyond weaning. Rather, offspring could have been studied to an older age (e.g., 3-6 months of age) to identify if effects demonstrated shortly after suckling (thus consumption of mother's diet) were sustained following consumption of a control diet post-weaning. In contrast, Howie et al. (2009) reported that maternal high fat feeding (Wistar rats; 45% energy from fat (lard + soybean oil)) led to greater fat mass (~17% higher) in male offspring at 20 weeks of age. Although these authors isolated potential 'programming' effects by studying offspring after they had surpassed periods of their most rapid development (i.e., 10 weeks of age [33]), these differences pertained to a main effect of maternal diet (thus combining offspring control and high fat dietary conditions). Therefore, the results may be attributed to offspring diet and not maternal high fat feeding *per se*. Moreover, whether differences were evident to the same extent (or potentially greater) at weaning is unknown. Therefore, it is unclear when differences in body composition first became evident prior to 5 months of age. This information could provide insight on the time period in which interventions should occur to avoid adverse body composition at adulthood. In addition, Howie et al. (2009) found that maternal high fat feeding throughout pregnancy and lactation alone showed similar elevations in fat mass (~41% higher) of male offspring. This finding suggested that maternal high fat feeding had the strongest influence on offspring body composition between conception and the end of lactation [30].

Although the previously mentioned studies reported greater fat mass following maternal high fat feeding, this finding was not consistently reported in the literature. Elahi et al. (2009) showed that maternal high fat feeding (C57BL6 mice; 39% energy

from fat (lard)) did not lead to differences in fat mass (relative to body weight) of male offspring at 36 weeks of age (Table 2). In contrast to previous studies, these findings may be due to the fact that the levels of protein, vitamins, and minerals between the control and high fat diet were matched to account for the higher energy content of the high fat diet. As such, it is unclear whether discrepancies in previous literature are actually due, in part, to lower levels of these nutrients rather than high fat exposure *per se*. Furthermore, it is unknown whether differences were evident earlier in life (e.g., weaning or 3 months of age). In this regard, maternal high fat feeding could have mediated changes in younger offspring that did not translate 'permanently' into late adulthood. Therefore, consuming a control diet post-weaning for 33 weeks could have attenuated differences established at younger ages. In contrast, another study in the same mouse strain reported lower fat mass (5% less relative to body weight) in male offspring at 14 weeks of age following maternal high fat feeding (C57BL6J mice; 45% energy from fat (lard)). Similar to Elahi et al. (2009), it is unknown whether these differences were present earlier in life. Since soft tissue acquisition and bone accretion is greatest during rapid periods of growth (i.e., first 5 weeks of age [34]), it would be expected that the largest differences in body composition might be observed at this stage of the life cycle.

There is limited information regarding the effects of maternal high fat feeding and whole body bone mass in male offspring. However, two of the previously discussed studies reported this parameter in their body composition analysis. For instance, Devlin et al. (2013) and Howie et al. (2009) found no differences and higher whole body BMC in male offspring from dams fed a high fat diet (versus control), respectively. However, the significance in the latter study pertained to a main effect of maternal diet – which

combined post-weaning control and high fat diet interventions. Therefore, whether maternal high fat feeding alone followed by a control diet post-weaning can modulate male offspring bone mass warrants further investigation.

Overall, there is limited information regarding maternal high fat feeding on all three tissues underlying body composition – fat, lean, and bone mass – at both an early (weaning) and adult (3 months of age) life stage (Table 2). It is important to study both time points in order to discern when differences first become evident. For instance, if differences exist as early as weaning, it may be important to study whether these differences are sustained following control diet consumption post-weaning (thus studying 'long-lasting' effects of maternal diet). In contrast, differences may not be evident at weaning but emerge later on in life. Moreover, as previously mentioned, these studies vary in regard to rodent model (i.e. whether rats or mice are studied), thus, different species may respond differently to high fat feeding. For example, the previously discussed studies have used either Wistar rats or C57BL6 mice; each yielding different results on male offspring body composition in response to maternal high fat feeding. As such, the Wistar rat studies reported higher fat mass following maternal high fat feeding, however, C57BL6 mouse studies showed no difference [32] or lower [31] fat mass. One potential contributor to these discrepancies between species may be the age at which offspring were studied. As such, a given 'age' may represent different stages of life for each species, which can potentially be due to distinctive growth rates for rats and mice. For example, male Wistar rats tend to grow in size and weight rapidly until about 10 weeks of age [33], while C57BL6 male mice tend to grow rapidly until ~5 weeks of age [34]. Therefore, selected time points represent different periods of maturity for rats and

mice, thus, the impact of maternal high fat feeding on offspring body composition may depend on which stage of the life cycle is studied.

Table 2. Maternal High Fat Feeding Studies: Body Composition in Male Offspring

Study	Model	Maternal CON Diet (% Energy)	Maternal HF Diet (% Energy)	Duration of feeding	Offspring Diet	Age of Offspring Studied	Method	Offspring Results
Franco et al., 2012	Wistar rat	**9% Fat (lard)** 68% CHO 23% Protein	**41% Fat (lard)** 45% CHO 14% Protein	8 wk prior to breeding + PL	N/A	Weaning	DEXA	↑ Fat mass (vs. maternal CON)
Howie et al., 2009	Wistar rat	**18% Fat (lard)** 58% CHO 24% Protein	**45% Fat (lard)** 35% CHO 20% Protein	16 wk prior to breeding + PL	CON or HF	20 weeks	DEXA	↑ Fat and bone mass (offspring consuming HF vs. CON)
Devlin et al., 2013	C57BL6 mice	**18% Fat** 58% CHO 24% Protein	**45% Fat (lard)** 36% CHO 19% Protein	6 wk prior to breeding + PL	CON	14 weeks	DEXA	↓ Fat mass ↔ Bone mass (vs. maternal CON)
Elahi et al., 2009	C57BL6 mice	**14% Fat (lard)** 60% CHO 26% Protein	**39% Fat (lard)** 35% CHO 26% Protein	6 wk prior to breeding + PL	CON or HF	36 weeks	Fat pad weight	↔ Fat mass (vs. maternal CON)

CHO, carbohydrates; CON, control; HF, high fat; wk, weeks; PL, pregnancy and lactation; N/A, not available due to offspring studied at weaning; ↑, higher; ↓, lower; ↔, no difference.

CHAPTER 3.0: EFFECT OF HIGH FAT FEEDING – BONE DEVELOPMENT

3.1: Background information

3.1.1. Composition of femur and vertebrae

Chronic high fat feeding has been shown to alter bone composition [35-40]. Briefly described, bone is composed predominately of type 1 collagen and calcium hydroxyapatite [41]. Bone can be classified into cortical (external surface of bone) or trabecular (deep to cortical bone) compartments, with proportions varying depending on the site considered within the femur (e.g., femur neck versus midpoint) or vertebrae. For example, cortical bone is predominately found at the femur midpoint and external surface of vertebrae. In contrast, trabecular bone is extensively found at the femur neck and in the centre of vertebrae [41]. Higher levels of trabecular bone may result in greater pro-inflammatory cytokine secretion in response to dietary invention (e.g., high fat feeding), and thus, may be implicated in bone remodelling/turnover (discussed in a later section).

Although little information is available regarding fatty acid storage in bone, bone is highly responsive to dietary lipid consumption [37,39,40,42]. As such, bone lipid composition has been shown to reflect the fat composition of the diet consumed [37,40]. However, the importance of altered bone fatty acid composition is not established, and whether a high saturated fat diet translates into functional differences in bone strength and/or structure is unclear [37].

3.1.2. Femur and vertebral formation and growth

Bone formation (i.e., ossification) is initiated in utero, and growth progresses until peak bone mass (i.e., maximal mineralization) is achieved. Therefore, nutritional manipulation during pregnancy and/or postnatal dietary consumption may influence the

maximal bone mass attained. In this regard, endochondral ossification proceeds to develop bone from a hyaline cartilage model. The outer cartilaginous surface (i.e., perichondrium) produces chondrocytes that thicken the existing cartilage model [43]. Subsequently, the perichondrium produces osteoblasts that migrate towards the centre of the bone, creating a bony 'collar' around the cartilage model [43,44] and facilitating bone elongation. As such, cells closest to the centre of the bone proliferate and differentiate into pre-hypertrophic chondrocytes (no cartilage secretion), which eventually undergo hypertrophy and secrete extracellular cartilaginous matrix [45]. The extracellular matrix becomes calcified and limits nutrient availability to the chondrocytes, thus, inducing apoptosis [43]. Subsequently, the necrotic tissue is invaded by blood vessels – delivering osteoblasts and osteoclasts to the center of the bone [45]. Consequentially, the osteoclasts remove the cartilage matrix while the osteoblasts utilize existing cartilage as a blueprint for bone deposition [45].

3.1.3. Bone remodelling

The consumption of a high fat diet may affect bone remodelling (i.e., removal and replacement of bone), an ongoing process facilitated by osteoblasts, osteoclasts, and osteocytes. For instance, bone remodelling is initiated when mesenchymal stem cells (originating in the bone marrow) differentiate into osteoblasts via the influence of runt-related transcription factor 2 (RUNX2) [46]. Subsequently, mature osteoblasts deposit primary bone matrix (i.e., osteoid; osteocalcin and type 1 collagen) and secrete receptor activator of nuclear factor kappa-β ligand (RANKL), which is pertinent to activating osteoclasts upon binding to RANK (RANKL receptor on the osteoclast cell surface) [46]. Once activated, osteoclasts resorb bone tissue, which is later replaced with osteoid

deposition by osteoblasts. However, osteoblasts occasionally become enclosed within the matrix they deposited and differentiate into osteocytes. These cells potentially sense bone deformation, and thus, signal the initiation of bone remodelling [43] (Figure 1).

The process of bone remodelling continues throughout life, although, the level of deposition versus resorption varies across the lifespan. In this regard, growing bones have greater levels of bone deposition by osteoblasts, whereas bone resorption dominates in mature bone at later stages of life (e.g., elderly) [43,44]. If bone resorption is greater than bone deposition, bone loss will occur and increase the risk for fragility fracture [44].

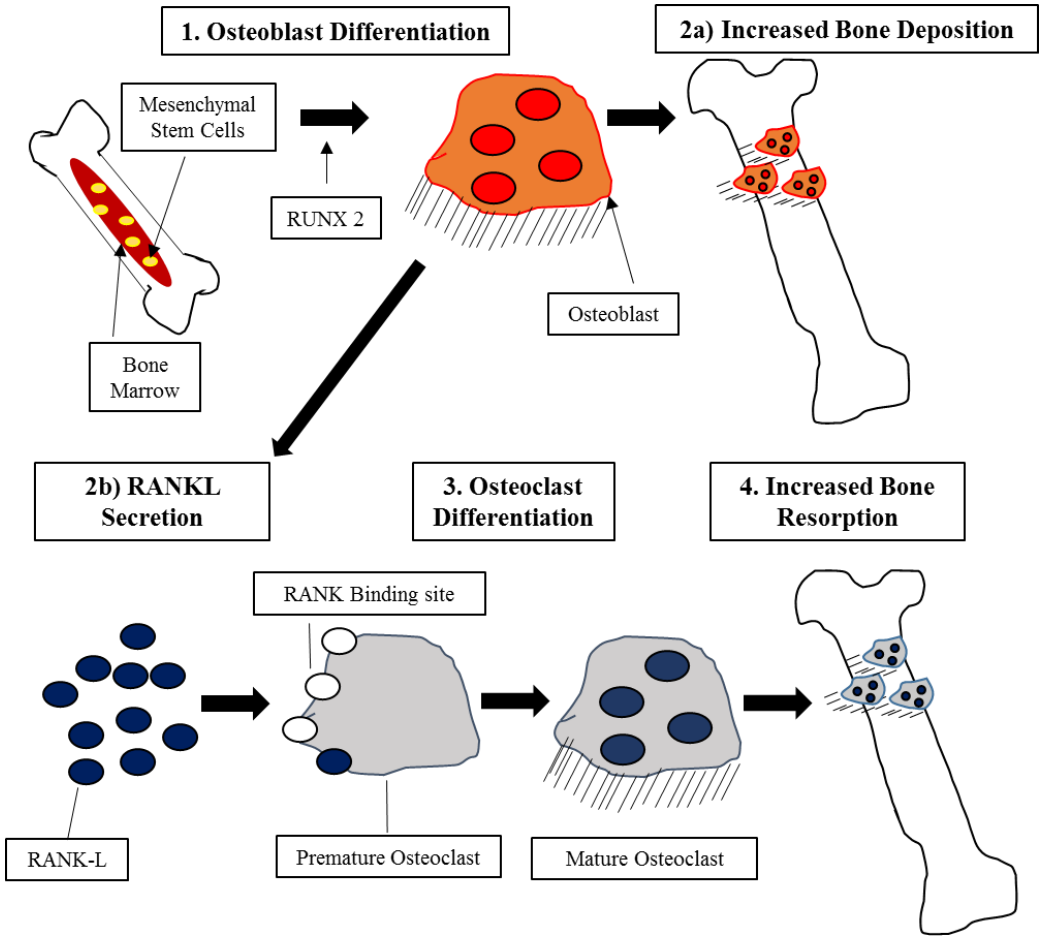

Figure 1. Bone Remodelling. 1. Mesenchymal stem cells within bone marrow differentiate into osteoblasts via the RUNX2 transcription factor. 2a) Osteoblasts facilitate bone deposition. 2b) Osteoblasts secrete RANKL that binds to RANK receptors on premature osteoclasts. 3. Osteoclasts are activated by RANKL-RANK interactions and differentiate into mature osteoclasts. 4. Active osteoclasts resorb bone. Step 1 repeats after step 4. ***Miotto 2014 (unpublished)***

3.1.4. Potential mechanisms of high fat feeding on bone remodelling

As previously discussed, chronic high fat feeding is associated with increased fat mass and resultant adipokine (i.e., leptin) and pro-inflammatory cytokine (e.g., TNF-α and IL-6) release. As a result, bone turnover may be affected (Figure 2). For instance, at the tissue level, leptin may promote osteoblast differentiation while inhibiting osteoclast differentiation (i.e., secreting osteoprotegerin to divert RANKL from binding to RANK and activating osteoclasts) [47]. Thus, leptin may exert positive effects on bone by improving bone mass long-term. In contrast, TNF-α and IL-6 may up-regulate RANKL expression (via osteoblast release), and thus, stimulate osteoclast differentiation to enhance bone resorption [22]. Theoretically, this may lead to lower bone mass and altered bone structure, which may contribute to lower bone strength. However, the net result of the actions of leptin and pro-inflammatory cytokines on bone is not well understood.

In addition to hormonal influences on bone, high fat feeding may directly reduce intestinal calcium absorption [48] by forming soaps with calcium and being excreted in feces [49]. Therefore, lower calcium availability may compromise bone mineralization (i.e., lower bone mass) and increase the risk of a fragility fracture. Overall, high fat feeding may influence bone remodelling through changes in adipokine, cytokine, and intestinal calcium absorption that are related to elevated fat mass. However, whether maternal high fat feeding can affect offspring bone growth and development is not known.

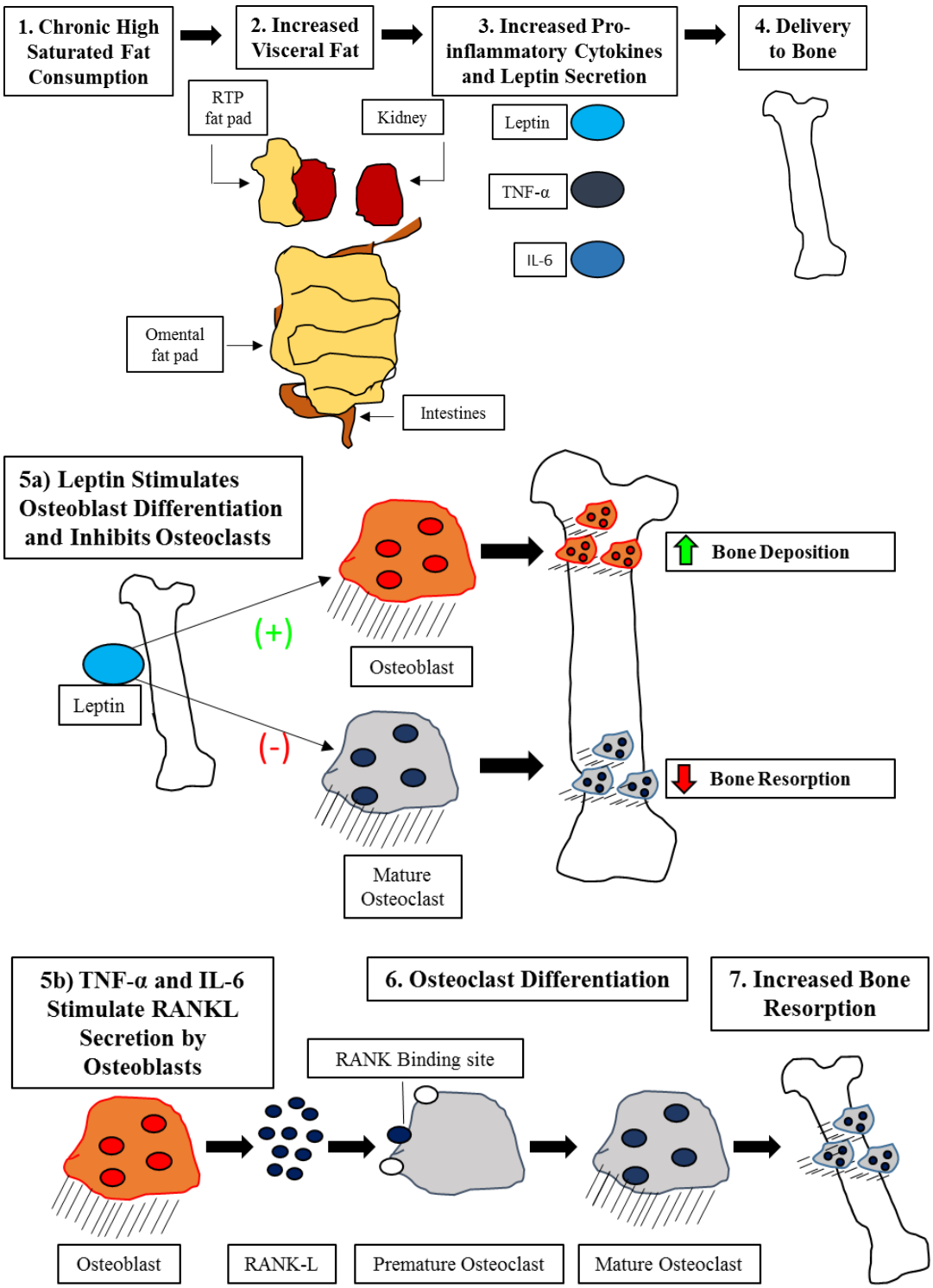

Figure 2. Mechanism of Leptin and Pro-inflammatory Markers on Bone Remodelling. 1. Chronic high fat consumption may result in: 2. Increased visceral fat mass. 3. Increased visceral fat mass can lead to elevated leptin and pro-inflammatory cytokine secretion into the circulation. 4. Circulating leptin and pro-inflammatory cytokines are delivered to bone. 5a). Leptin promotes osteoblast differentiation and efficiency, resulting in elevated bone deposition. 5b) TNF-α and IL-6 promote RANKL secretion from osteoblasts. 6. RANKL binds to RANK receptors on premature osteoclasts, resulting in osteoclast differentiation. 7. Active osteoclasts resorb bone. ***Miotto 2014 (unpublished)***

3.2: Bone terminology and methods used in previous rodent studies

Bone is often described in terms of bone quantity, quality, and strength. All of these properties can be altered by high fat feeding. Bone quantity refers to the amount of bone mineral acquired [50], and can be described as either BMC (measured as a total amount; g or mg) or BMD (expressed as the quantity of mineral per area of site measured; mg/mm^2). In previous studies, bone quantity of excised femurs and vertebrae were commonly assessed using DEXA ('gold standard technique') [51], providing a two-dimensional image of the excised bone. However, DEXA cannot distinguish between cortical and trabecular bone [52]. This is important because there may be different responses to dietary interventions, depending on the type of bone (e.g., trabecular bone may respond faster than cortical bone due to higher turnover rate) [40]. Thus, studying more than one skeletal site, that differ in cortical and trabecular content, provides a more comprehensive understanding of high fat feeding on bone composition (e.g., the femur midpoint is predominately cortical bone, femur neck contains both trabecular and cortical bone, and lumbar vertebrae contain mostly trabecular bone). In contrast, peripheral quantitative computed tomography (pQCT) uses 3-dimensional imaging at the periphery of bone to track changes in the quality of cortical and trabecular bone [52]. This method has also been used to measure BMD in rat bones after high fat feeding [35].

Bone quality refers to the structural integrity of the bone [50,53] and can be measured using micro-computed tomography (μCT). A μCT creates 3-dimensional images of bone, which may provide information regarding bone volume, density, or microarchitecture [54]. A few specific examples of the outcomes obtained by using μCT are trabecular number, thickness, and separation [55].

Collectively, bone quantity and quality contribute to bone strength – ultimately assessed by 'peak load'. Peak load is the maximum force a bone can withstand before fracturing, and has been considered the most clinically relevant outcome when assessing risk of fracture in humans [56]. To measure bone strength, materials testing systems can be used. As such, each bone is placed below a descending cross-head that applies incremental levels of force (measured in Newtons) on the bone (at a constant speed) until a fracture occurs [37].

Following high fat feeding, some (but not all) studies have assessed bone lipid content. Previous studies have identified the total fatty acid content of bone (bone marrow removed) or adipocyte number and/or diameter in the bone marrow itself. As such, total fatty acid content in a bone sample can be measured by gas chromatography (GC) to identify the amount and type of saturates, monounsaturates, or polyunsaturates incorporated into bone [37,39]. The exact location and function of these fatty acids in bone is unclear and requires further investigation. In contrast, adipocyte number and diameter have been measured using histological methodologies [31,57].

3.3: Effect of direct high fat feeding in rodent models

Previous research has shown that direct high fat feeding altered bone mass (BMC and/or BMD) [35-37], structure [38], strength [37,58], and fatty acid composition [37,39,40] in excised bones. However, there is limited information regarding all 4 outcomes together, especially in different bones (e.g., femur; lumbar vertebra). For instance, it has been shown that high fat consumption resulted in lower femur BMC after 13 (63% energy from fat (lard)) [36] and 28 weeks of feeding (35% energy from fat (cocoa butter)) [35]. Moreover, the latter study also reported lower bone mass (BMC &

BMD) in the lumbar vertebrae after 28 weeks of feeding [35], whereas the former study did not measure vertebral bone mass. It is currently unknown if high fat feeding also affects femur (midpoint/neck) and vertebral structure and/or strength. It is important to also consider these outcomes to determine whether differences in mineralization parallel changes in bone structure, which may ultimately contribute to bone strength. In regard to bone structure, high fat feeding (male C57BL6J mice; 45% energy intake from fat (lard)) resulted in lower tibial bone volume and higher trabecular separation after 14 weeks of feeding [38]. These results suggest a poorer bone structure, which may contribute to lower bone strength, however, bone strength was not directly measured. In contrast, it has been shown that high fat diets abundant in saturated fat (38% coconut oil) did not lead to differences in femur bone mass or strength. However, peak load was higher in the n-3 and n-6 polyunsaturated fat groups compared to controls, thus, different sources of fat may have varied effects on bone outcomes (Table 3). Whether alternative sources of high saturated fat (i.e., lard) can affect bone parameters is unknown. There may be differences in the fatty acid composition of lard and coconut oil that contribute to bone strength; as the fatty acid composition of the bones reflected the sources of fat that were consumed [37].

Collectively, previous research has shown that direct high fat feeding led to lower bone mass, poorer bone structure, potentially lower bone strength (although not often studied), and altered fatty acid composition in excised bones. This has been shown at different skeletal sites (e.g., tibia, femur, vertebrae), however, has yet to be studied together at multiple skeletal sites containing varied proportions of cortical and trabecular bone (i.e., femur **and** lumbar vertebrae) within the same study. More importantly, less

information is available regarding *maternal* high fat feeding and male offspring bone mass, structure, strength, and fatty acid composition.

Table 3. Direct High Fat Feeding Studies: Bone Outcomes in Rodents

Study	Model	CON Diet (% Energy)	HF Diet (% Energy)	Feeding duration	Outcomes measured	Method	Results (HF versus CON)
Cao et al., 2009	C57BL6 mice (male)	**10% Fat** 70% CHO 20% Protein	**45% Fat (lard)** 35% CHO 20% Protein	14 wk	Bone structure (tibia)	µCT	↓ Bone volume ↑ Trabecular separation
Xiao et al., 2011	C57BL6 mice (male)	**28% Fat** 69% CHO 3% Protein	**63% Fat (lard)** 35% CHO 2% Protein	13 wk	Bone mass (femur)	Ashing	↓ BMC
Parhami et al., 2001	C57BL6 mice (male)	**18% Fat** 59% CHO 23% Protein	**37% Fat (cocoa butter)** 42% CHO 21% Protein	16 and 28 wk	Bone mass (femur & vertebra)	pQCT	↓ Femur BMC/BMD (16 & 28 weeks) ↓Vertebrae BMD (28 weeks)
Lau et al., 2010	Sprague Dawley rats (male)	**14% Fat** 65% CHO 21% Protein	**38% Fat (coconut oil)** 42% CHO 20% Protein	~8 wk	Bone mass	DEXA	↔ BMD
					Bone strength (femur)	3 pt. bending	↔ Peak load (high saturated fat)
					FA comp. (femur)	GC	Reflected level of SFAs & PUFAs consumed

CHO, carbohydrates; HF, high fat; wk, weeks; FA comp., fatty acid composition; GC, gas chromatography; SFAs, saturated fatty acids; PUFAs, polyunsaturated fatty acids; µCT, micro-computed tomography; ↑, higher; ↓, lower; ↔, no difference.

3.4: Effect of maternal high fat feeding on male rodent offspring

Although direct high fat feeding has been shown to lower bone mass, reduce the structural integrity of bone, and alter fatty acid composition, the potential influence of maternal high fat feeding on bone development in male offspring is not well characterized. The literature pertaining to this topic involves two studies, each sharing similar study designs (i.e., C57BL6J mice; ~41% energy intake from fat; offspring

studied after maturity has been reached) (Table 4). For instance, Lanham et al. (2010) maintained female mice on a high fat diet (41% energy from lard/soybean oil) for 7 weeks prior to breeding, pregnancy, and lactation. At weaning, male offspring were fed either a high fat or control diet. There were 3 overall dietary conditions: 1) maternal control diet – offspring control diet post-weaning; 2) maternal high fat diet – offspring high fat diet post-weaning; or 3) maternal control diet – offspring high fat diet post-weaning. At 30 weeks of age (~8 months old; late adulthood), male offspring that consumed a high fat diet after weaning had greater femoral BMD than offspring consuming control diet. Since this finding involved a main effect including multiple conditions (i.e., maternal control diet – offspring high fat diet post-weaning; maternal high fat diet – offspring high fat diet post-weaning), offspring dietary consumption may have been a stronger contributor to BMD than maternal diet. However, this cannot be concluded because none of the offspring exposed to a maternal high fat diet received a control diet post-weaning. Moreover, offspring that consumed a high fat diet (regardless of maternal diet) demonstrated greater peak load at the femur midpoint than offspring consuming control diet [57]. However, these mice weighed more than the control mice at 30 weeks of age. Therefore, differences in bone strength may be attributed to mechanical loading (due to greater body weight) and/or larger bone size instead of maternal diet. Whether similar findings would be evident in regard to femoral neck and vertebral bone strength is unknown. It is important to identify these sites because they contain greater levels of trabecular bone than the femur midpoint (highly cortical), and as such, may respond differently to maternal high fat feeding (which may yield differences in bone strength).

In addition to bone strength, Lanham et al. (2010) also reported that mice fed a high fat diet post-weaning had greater bone marrow adipocyte content in the femur than mice consuming a control diet (as anticipated) [57]. This finding demonstrated that direct consumption of a high fat diet altered bone marrow composition, without compromising bone strength. However, the extent to which maternal high fat feeding altered the level of fatty acids stored within the cortical and trabecular compartments of bone is unknown. This information could be more functionally relevant if this study included a dietary condition that isolated maternal high fat feeding on offspring bone development (i.e., maternal high fat diet – offspring control diet post-weaning). As such, this would help determine if maternal diet permanently affected bone fatty acid composition with or without changes in bone strength.

Similar to Lanham et al. (2010), Devlin et al. (2013) provided mice a high fat diet (C57BL6J; 45% energy from lard; 6 weeks prior to breeding) throughout pregnancy and lactation. However, these authors weaned all offspring onto the same control diet (2 conditions overall: maternal high fat diet or maternal control diet), thus, isolating the effects of maternal high fat feeding. All offspring were studied at either 14 weeks (~3.5 months old) or 26 weeks (~6 months) of age, slightly earlier than Lanham et al. (2010). The results demonstrated that femurs from offspring of dams fed a high fat diet had greater trabecular bone volume/total volume (38%) and cortical bone area (6%) at 14 but not 26 weeks of age (compared to offspring from dams fed a control diet) – without differences in peak load at either age. These findings suggested that maternal high fat feeding improved bone structure without translating into functional differences in bone strength at 14 weeks of age. Furthermore, consuming a control diet for 23 weeks may

have countered earlier differences in bone structure, as these effects were no longer evident at late adulthood (i.e., 6 months of age; similar to Lanham et al. 2010) [31]. Although no differences were found at the femur midpoint, inclusion of mechanical testing of the femur neck and lumbar vertebrae could have provided information regarding trabecular characteristics in response to maternal high fat feeding. In regard to bone histology, no differences were found in bone marrow adipocyte diameter at 14 or 26 weeks of age. Total fatty acid composition was not measured. Such analyses may have provided information regarding whether fatty acids in offspring bone reflected maternal diet at 14 weeks of age (in addition to structural differences evident), and whether these fatty acids remained stored in bone to a similar extent following prolonged consumption of a control diet until 26 weeks of age.

In consideration of these two studies (Table 4), the former demonstrated that direct consumption of a high fat diet post-weaning may have been more critical for bone development and strength acquisition than maternal diet. This is because all offspring consuming a high fat diet demonstrated similar results in bone mass and strength, regardless of maternal diet. In contrast, Devlin et al. (2013) isolated the impact of maternal high fat feeding on bone development by weaning all offspring onto a control diet. The results suggested that maternal diet affected offspring bone development at young adulthood (14 weeks), but these effects diminished by late adulthood (26 weeks), after prolonged control diet consumption. However, it is unknown whether maternal high fat feeding alters bone development at earlier stages of life (i.e., at weaning) when bone growth is rapid – compared to measurements at young adulthood (after the consumption of a control diet; growth rate has slowed). It is important to identify bone outcomes at

both ages because it verifies if: a) the maternal diet was strong enough to elicit changes in offspring development at some point in time; b) if earlier differences in offspring persist despite a switch in diet consumed post-weaning; or c) if differences do not become evident until later stages of life. Furthermore, neither study addressed if maternal high fat feeding contributed to differences in bone mass, structure, and strength at other skeletal sites such as the lumbar vertebrae. It may be important to highlight different bones since *direct* high fat consumption has demonstrated results that are skeletal site specific (i.e., femur responded sooner than vertebrae) [35].

Table 4. Maternal High Fat Feeding Studies: Bone Outcomes in Male Offspring

Study	Model	Maternal CON Diet (% Energy)	Maternal HF Diet (% Energy)	Feeding Duration	Offspring Diet	Age of Offspring Studied	Bone Outcomes (Femur)	Method	Results (Maternal: HF vs. CON)
Lanham et al., 2008	C57BL 6J mice	**5% Fat (lard + soybean oil)** 49% CHO 21% Protein	**41% Fat (lard + soybean oil)** 37% CHO 22% Protein	7 wk before breeding, PL	CON or HF	30 wk	BMC, BMD	µCT	↑BMD*
							Strength	3 pt B (F. MP)	↑ Peak load*
							Bone marrow lipid content - adipocyte diameter	Histology	↑ Diameter *
Devlin et al., 2013	C57BL 6J mice	**18% Fat (unknown source)** 58% CHO 24% Protein	**45% Fat (lard + soybean oil)** 36% CHO 19% Protein	6 wk before breeding, PL	CON	14 or 26 wk	Structure	µCT	↑**
							Strength	3 pt.B (F. MP)	↔
							Lipids – adipocyte # and diameter	Histology	↔

CHO, carbohydrate; CON, control; HF, high fat; wk, weeks; PL, pregnancy + lactation; BMC, bone mineral content, BMD, bone mineral density; µCT, micro-computed tomography; pt.B, point bending; F. MP, femur midpoint. * represents a main effect of maternal HF versus CON conditions (therefore offspring dietary groups are combined); **bone volume, cortical content, moment of inertia; ↑, higher; ↓, lower; ↔, no difference between maternal dietary groups.

CHAPTER 4.0: THESIS

4.1: Statement of the problem

There is limited information regarding the effects of maternal high saturated fat feeding on male offspring body composition and bone development. It is important to study both under the same experimental conditions, in order to clarify whether these parameters parallel one another in response to maternal high fat feeding. If so, this may reveal relationships between fat, lean, and bone mass on specific aspects of bone health that may be secondary to the maternal high fat diet itself. Moreover, it is unclear whether maternal high fat feeding exerts different effects on male offspring at earlier (weaning; rapid growth and development) versus later (young adulthood; slower growth and development) stages of life, following the consumption of a control diet post-weaning. This is important because it will demonstrate whether effects at adulthood can be attributed to maternal diet or the offspring's diet post-weaning. For example, if differences in body composition and/or bone health are evident at earlier but not later stages of life, this may suggest that the most recent diet consumed (e.g., via suckling) is more critical for these parameters. Furthermore, it is unclear whether high fat feeding when adjusted for protein, vitamins, and minerals (on a basis of energy per kg of diet) impacts body composition and bone health in the dams, as well as male offspring. In this regard, findings from previous literature may not have provided enough nutrients to support optimal growth and development. As a result, decrements in protein, vitamins, and minerals may play a role in the differences noted in fat mass, lean mass, and bone mass among previous studies.

No study has reported measurements of excised bone mass, structure, and strength in both the femur and lumbar vertebrae in response to maternal high fat feeding. These measurements will highlight whether bone mass, structure, and strength change proportionately to one another (irrespective of inherent differences in composition (i.e., varied proportions of cortical and trabecular bone)) in response to maternal high fat feeding, and if the results are skeletal site specific. Moreover, it is currently unknown whether maternal high fat feeding alters offspring bone fatty acid composition. This measurement may provide valuable insight as to whether offspring bone fatty acid composition can be implicated in bone development due to maternal diet.

4.2: Objectives

The primary objective of this study was to determine if maternal high fat feeding exerted long-term effects on body composition and bone development in male offspring. The specific objectives were to determine if male offspring (at weaning and 3 months of age) exposed to maternal high fat feeding (in utero and lactation) led to differences in:

1. Body composition (fat, lean, and bone mass) – including fat pad weight (retroperitoneal, RTP; omental, OM; and epididymal, EPI).
2. Mineral mass (BMC and BMD), structure, and bone strength (at 3 months of age only) of excised femurs and lumbar vertebrae.
3. Femur fatty acid composition.
4. Plasma leptin, TNF-α, and IL-6 concentrations.

Although the focus of this thesis pertains to the effects of maternal high fat feeding on male offspring body composition and bone development, it was important to measure specific outcomes in dams as their status could influence outcomes in their male

offspring. Thus, the secondary objective was to determine whether dams consuming a high fat diet (versus control) led to differences in:

1. Fat pad weight.
2. Mineral mass (BMC and BMD), structure, and bone strength of excised femurs and lumbar vertebrae.
3. Femur fatty acid composition.
4. Plasma leptin, TNF-α, and IL-6 concentrations.

4.3: Hypotheses

In male offspring at weaning and 3 months of age, maternal high fat feeding would result in:

1. Altered body composition, reflected by higher fat mass (including fat pad weight) and lower lean and bone mass.
2. Lower bone mineral mass, altered bone structure, and lower bone strength (at 3 months of age only) in excised femur and lumbar vertebrae.
3. Higher saturated fatty acid content and lower unsaturated fatty acid content in femur that reflects maternal diet.
4. Higher plasma leptin, TNF-α, and IL-6 concentrations.

In dams at 5 months of age, high fat feeding would result in:

1. Altered body composition, reflected by higher fat pad weights.
2. Lower bone mineral mass, altered bone structure, and lower bone strength in excised femur and lumbar vertebrae.
3. Higher saturated fatty acid content and lower unsaturated fatty acid content in femur that reflects the diet consumed.

4. Higher plasma leptin, TNF-α, and IL-6 concentrations.

CHAPTER 5.0: STUDY DESIGN AND METHODOLOGY

5.1. Study design

All experimental procedures complied with the Canadian Council on Animal Care [59] and were approved by the Brock University Animal Care Committee. Female Wistar rats (n = 18, Charles River Laboratories, Canada) were obtained at weaning (~ 28 days old) and were housed two per cage with a 12:12 hour light/dark cycle. Rats were randomly assigned to receive one of two diets: control (CON; AIN93G diet, 7% soybean oil by weight) or high fat (HF; modified AIN93G diet, 20% lard by weight), *ad libitum* for 10 weeks. The purpose of altering the amount and source of fat in the diets was to mimic traditionally considered 'healthy' (i.e., CON, containing higher polyunsaturated fat content) and 'unhealthy' (i.e., HF, contains higher saturated fat) fat diets, respectively. The energy content of the CON diet was from 18.8% protein, 63.9% carbohydrate, and 17.2% fat (TD.94045; Harlan Teklad, Mississauga, ON). The energy content of the HF diet was from 19.1% protein, 39.9% carbohydrate, and 41.0% fat (TD.02016; Harlan Teklad, Mississauga, ON). The fatty acid composition of each diet was obtained using gas chromatography (Table 5). The amount of protein, vitamins, and minerals in the HF diet was increased by a factor of 1.2 compared to the CON diet to account for the 1.2 fold higher energy content (3.8 kcal/g CON diet versus 4.4 kcal/g HF diet). This adjustment was done to ensure that any observed effects in the offspring could be attributed to the higher level of fat in the diet, and not to lower levels of other nutrients on an energy basis (Kcal/g of diet). Body weights were recorded weekly (Acculab, VI-1200 electronic scale).

After 10 weeks of feeding, females were bred with male Wistar rats (n = 6) of similar age. Each male rat was bred with the same number of females from each intervention, ensuring equal paternal influence among groups. Nine females from each diet group became pregnant during the 1 week breeding period. Female rats were fed their respective diets throughout pregnancy and lactation. Offspring were weaned at postnatal day (PND) 19. Dams were fasted overnight and were euthanized the following morning by an overdose of sodium pentobarbital (12 mg/100 g body weight) and blood and bones (femurs and lumbar vertebrae 1-3; LV1-3) were collected (to obtain the same measurements as the offspring). Two males per litter were euthanized at weaning – 1 male was measured for body composition while the other male was used for blood and bone collection. Additionally, 2 males per litter were weaned onto CON diet and studied until 3 months of age (housed 2 per cage). Body weights of offspring were recorded weekly from weaning through to 3 months of age. It is important to note that for every outcome, only 1 male per litter was reported to ensure that the results were not biased towards significance due to over-representation of each litter (i.e., "litter effect") [60]. At 3 months of age, similar to weaning, 2 male offspring per litter were euthanized with an overdose of sodium pentobarbital after an overnight fast. One rat was used for body composition assessment while the other rat was used for blood and bone collection. All blood samples and excised bones were stored at -80ºC until analysis. It is important to note that the same measurements for female offspring were conducted by another graduate student. These results were published with the present thesis findings [61].

Table 5. Fatty Acid Composition of Treatment Diets.

Fatty acid	CON	HF
14:0	0.59 ± 0.01	2.31 ± 0.03
16:0	12.25 ± 0.10	25.79 ± 0.03
18:0	4.19 ± 0.02	11.95 ± 0.21
16:1	0.20 ± 0.01	2.75 ± 0.01
18:1	20.42 ± 0.36	36.34 ± 0.17
18:3*n*3	8.46 ± 0.06	0.82 ± 0.01
18:2*n*6	51.91 ± 0.31	16.38 ± 0.29
Total SFAs	18.52 ± 0.10	41.67 ± 0.12
Total MUFAs	21.01 ± 0.36	40.10 ± 0.21
*n*3 PUFAs	8.46 ± 0.06	1.10 ± 0.01
*n*6 PUFAs	52.00 ± 0.30	17.14 ± 0.29

Values are expressed as percent mole fraction of total fatty acids. Percent mole fraction of fatty acids below 1% for both diets are not shown; CON, AIN93G diet; HF, AIN93G with 20% lard by weight; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

5.2: Body composition analysis

Body composition (lean, fat and bone mass) was determined using dual-energy x-ray absorptiometry (DEXA; pSabre, Orthometrix) and specialized software (Host Software version 3.9.4; Scanner Software version 1.2.0) in offspring at weaning and 3 months of age. All rats were placed on the scanning field in a prone position (nose facing left side of scanning field) prior to analysis. In weanling rats, the region of interest (ROI) surrounded the abdominal region (4 cm in width, 3.5 cm in height), with the lower boundary immediately above the femur heads [62] and scan parameters of 10 mm/sec (speed) and a resolution of 0.2 x 0.2 mm. To analyze 3 month old offspring, the ROI was 10 cm in width and 7 cm in height, fixed in the abdominal region, again with the lower boundary immediately above the femur heads [62] and scan parameters of 10 mm/sec (speed) and a resolution of 0.5 x 1.0 mm. Percent lean, fat and bone mass were calculated by dividing lean, fat or bone mass in grams by the total mass of the ROI and multiplying by 100. Additionally, fat pad weights were obtained and adjusted for body weight by dividing

mass (g) by total body weight (g) and multiplying by 100 (i.e., % retroperitoneal, % omental, and % epididymal).

5.3: Bone mineral and strength of excised femurs and lumbar vertebrae

Femur and LV1-3 BMC (mg) and BMD (mg/mm^2) of dams and offspring at weaning and 3 months of age were measured using DEXA (pSabre, Orthometrix) and specialized software (host software version 3.9.4; scanner software version 1.2.0) [37]. Scans of femurs and LV1-3 were conducted using the following parameters: speed of 2 mm/sec, resolution of 0.1 X 0.1 mm (weanlings); or speed of 10 mm/sec, resolution of 0.2 X 0.2 mm (dams, 3 month old offspring).

To measure bone strength, peak load was measured at the femur midpoint, femur neck, and 3rd lumbar vertebrae (LV3) in dams and 3 month old male offspring using a materials testing system (Model 4442, Instron), and specialized software (Series IX Automated Materials Tester, version 8.1 5.00). A cross-head was lowered perpendicularly to the sample at a constant speed of 2 mm/min, until peak load was achieved (i.e., fracture).

5.4: Femur fatty acid analysis

Femurs obtained from dams and offspring were sliced in half using a band saw. Bone marrow was extracted and fragments of bone were wrapped in aluminum foil and placed in liquid nitrogen. The bones were hammered and then pulverized using a mortar and pestle under liquid nitrogen as previously described [40]. The resultant powder was added into 15 mL glass screw cap Kimax tubes and total lipids were extracted as per Sacco et al. (2009) [63]. Extracted lipids were dried under nitrogen, weighed, and reconstituted in 2 mL of chloroform to form the lipid stock.

Total lipid from each sample was saponified and methylated as previously described [40]. Briefly, 0.0625 mg of lipid from each lipid stock was saponified and methylated at 100ºC using 0.5 M of potassium hydroxide and 6% H_2SO_4-MeOH, respectively. The resulting fatty acid methyl esters were separated on a UFM-RTX WAX analytical column (Thermo Electron Corp., Milan, Italy) using gas chromatography (Trace GC Ultra, Thermo Electron Corp, Milan, Italy) fitted with a fast flame ionization detector, a split-splitless injector, and Triplus AS autosampler [64]. Fatty acids were identified by comparison of retention times with those of a known standard (Supelco 37 component FAME mix, Supelco, Bellefonte, PA) and absolute amounts of individual fatty acids were calculated with the aid of an internal standard, tridecanoic acid (13:0), added to the samples prior to methylation. Prior analysis determined no detectable endogenous 13:0 in the samples (data not shown).

5.5: Plasma leptin, TNF-α, and IL-6 concentrations

Blood was collected in EDTA tubes and centrifuged at 3000 RPM for 10 minutes at 4ºC to obtain plasma from dams and offspring (weaning and 3 months of age). Plasma was analyzed using Magpix technology with xPONENT software (Luminex Corporation, Austin, Texas). Plasma from dams and all male offspring were measured for leptin, IL-6 and TNF-α using a Rat Metabolic Magnetic Bead Panel (Cat. #RMHMAG-84K; Millipore Corporation, MA).

5.6: Statistical analyses

Statistical analyses were performed using SigmaStat (version 3.5, Systat). For dams, independent samples t-tests were used for body weight (at breeding and 6 months of age), plasma hormones, bone mineral, bone strength, and bone lipid profile. For male

offspring, repeated measures 2-way ANOVA followed by a Student-Newman Keul's post-hoc analysis was used to analyze changes in body weight. The two factors used were age (1 month, 2 months, and 3 months of age) and maternal diet (CON, HF). Male offspring body composition (lean, fat and bone mass expressed as % of ROI), plasma hormones (leptin, IL-6, TNF-α), bone mineral of excised femurs and lumbar vertebrae, and femur lipid profile were conducted using 2-way ANOVA. The two factors used were age (weaning and 3 months of age) and maternal diet (CON, HF). Independent samples t-tests were also used to analyze bone strength of offspring at 3 months of age; bones at weaning age were too small for the strength analyses. Data are expressed as mean ± standard error of the mean (SEM). Significance was determined as $P \leq 0.05$.

CHAPTER 6.0: RESULTS

6.1: Effect of maternal high fat feeding on male offspring

6.1.1. Body weight and body composition

At 3 months of age, male offspring of dams fed HF diet had significantly greater body weight than offspring of dams fed CON diet (Figure 3). In regard to body composition, there were significant main effects of age and maternal diet for % lean mass and % fat mass (Table 6). As such, offspring at weaning had greater % lean mass and lower % fat mass than at 3 months of age, regardless of maternal diet. Also, offspring of dams fed HF diet had lower % lean mass and higher % fat mass than those of dams fed CON diet, regardless of age. There were significant interactions of age and maternal diet for % bone mass (Figure 4). Weanling offspring from dams fed HF diet had greater % bone mass than weanling offspring from dams fed CON diet. Additionally, weanling offspring from dams fed HF diet had greater % bone mass than at 3 months of age (Table 6; Figure 4).

For % fat pad weight (i.e., % RTP, % OM, and % EPI), there were main effects of age (Table 6). As such, 3 month old male offspring demonstrated greater % RTP, % OM, and % EPI fat pad weight than weanling offspring, regardless of maternal diet.

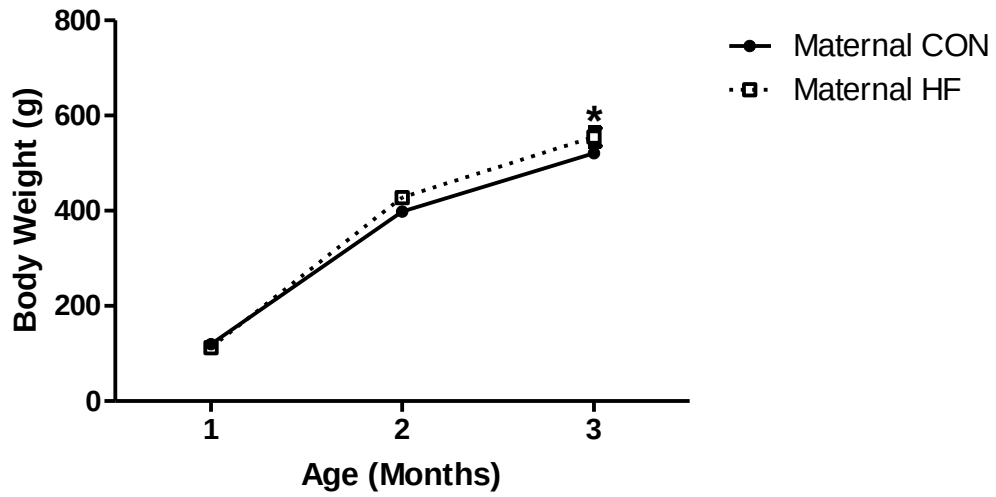

Figure 3. Male Offspring Body Weight at 1, 2, and 3 Months of Age.

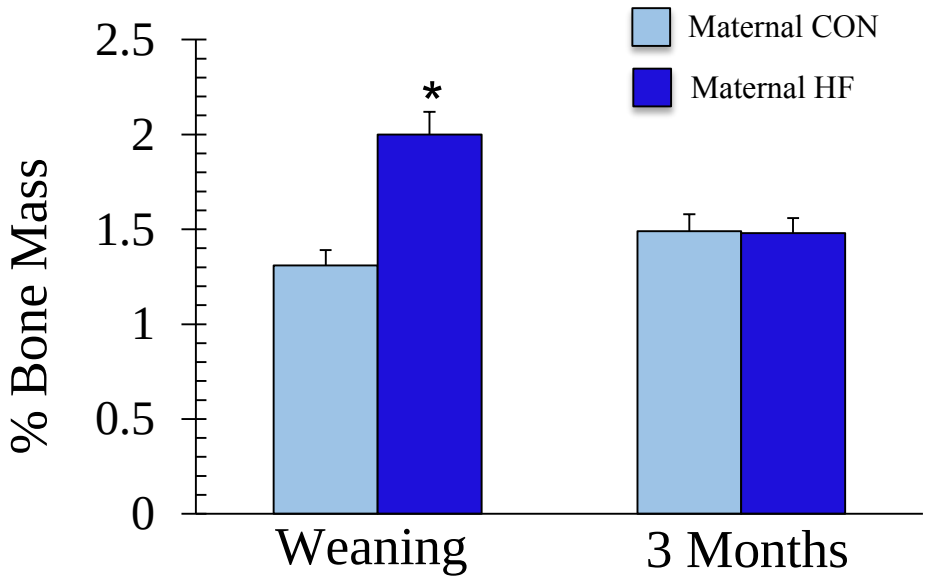

Figure 4. Male Offspring % Bone Mass at Weaning and 3 Months of Age.

6.1.2. Bone mineral and bone strength of femurs and lumbar vertebrae

There were significant main effects of age for BMC and BMD at all bone sites. As such, 3 month old offspring had greater BMC and BMD in the whole femur, 1/3 proximal femur, and LV1-3 than offspring at weaning, regardless of maternal diet. There were also significant main effects of maternal diet for whole femur BMC, 1/3 proximal femur BMD, and LV1-3 BMD. As such, offspring from dams fed HF diet had greater whole femur BMC, 1/3 proximal femur BMD, and LV1-3 BMD than offspring from dams fed CON diet, regardless of age (Table 6).

Peak load did not differ between males from dams fed HF or CON diet at the femur midpoint, femur neck, or LV3 at 3 months of age (Table 6).

6.1.3. Plasma leptin, TNF-α, and IL-6 concentrations

There were no differences in plasma leptin, TNF-α, or IL-6 between groups (Table 6).

Table 6. Male Offspring Body Composition, Plasma Hormones, and Bone Outcomes.

Outcomes	Weaning		3 Months		*P* Values		
	Maternal CON	Maternal HF	Maternal CON	Maternal HF	Age x Diet	Age	Diet
Body Composition							
% Lean mass	85.3 ± 2.0	68.5 ± 2.6	64.9 ± 4.5	62.9 ± 4.2	0.063	0.002	0.020
% Fat mass	13.4 ± 1.9	29.5 ± 2.5	33.6 ± 4.4	35.6 ± 4.1	0.070	0.001	0.022
% Bone mass	1.31 ± 0.08	2.00 ± 0.12*	1.49 ± 0.09	1.48 ± 0.08†	<0.001	0.083	0.001
% OM fat pad weight	0.88 ± 0.10	0.93 ± 0.07	2.12 ± 0.13	2.22 ± 0.17	0.404	<0.001	0.315
% RTP fat pad weight	0.29 ± 0.04	0.51 ± 0.03	3.36 ± 0.33	3.55 ± 0.24	0.920	<0.001	0.320
% EPI fat pad weight	0.40 ± 0.05	0.48 ± 0.06	2.36 ± 0.17	2.56 ± 0.10	0.567	<0.001	0.200
Plasma Hormones							
Leptin (pg/ml)	6255 ± 520	7367 ± 902	5535 ± 916	6796 ± 1126	0.934	0.475	0.193
TNF-α (pg/ml)	11.58 ± 1.03	13.29 ± 1.62	11.51 ± 2.92	8.74 ± 0.99	0.275	0.437	0.738
IL-6 (pg/ml)	179 ± 28	230 ± 50	217 ± 34	188 ± 24	0.264	0.954	0.759
Bone Mineral							
Whole femur							
BMC (mg)	18.8 ± 1.2	25.3 ± 2.5	499.0 ± 7.3	524.0 ± 8.2	0.121	<0.001	0.011
BMD (mg/mm^2)	0.35 ± 0.01	0.42 ± 0.03	2.11 ± 0.03	2.13 ± 0.01	0.261	<0.001	0.068
1/3 Proximal femur							
BMC (mg)	5.3 ± 0.4	7.0 ± 0.7	172.2 ± 2.9	179.8 ± 3.5	0.227	<0.001	0.060
BMD (mg/mm^2)	0.36 ± 0.01	0.42 ± 0.03	2.18 ± 0.02	2.20 ± 0.01	0.383	<0.001	0.039
LV1-3							
BMC (mg)	10.3 ± 1.1	13.8 ± 1.5	377.9 ± 10.1	394.5 ± 18.4	0.540	<0.001	0.350
BMD (mg/mm^2)	0.31 ± 0.02	0.36 ± 0.03	2.07 ± 0.02	2.14 ± 0.04	0.571	<0.001	0.029
Bone Strength							
Femur midpoint							
Peak load (N)	NM	NM	172.2 ± 9.6	174.0 ± 5.3			0.872
Femur neck							
Peak load (N)	NM	NM	110.8 ± 11.2	127.3 ± 8.5			0.258
LV3							
Peak load (N)	NM	NM	347.1 ± 42.6	444.9 ± 33.0			0.098

Data is expressed as mean ± SEM ((n = 9 per group); except for measures of body composition at weaning (n = 7/group) and LV3 peak load (n = 4 or 5/group as vertebral strength exceeded the load cell capacity of 475 N)). *Values are significantly different from maternal CON at weaning. †Values are significantly different from maternal HF at weaning. NM, not measured due to small bone size of weanlings; OM, omental; RTP, retroperitoneal; EPI, epididymal.

6.1.4. Femur fatty acid composition

In regard to lipid composition, femurs from offspring were responsive to maternal diet at weaning but not 3 months of age. There was a significant main effect of age for SFAs between groups, whereby offspring at weaning had greater SFAs than at 3 months of age, regardless of maternal diet. There were significant interactions for age and maternal

diet regarding percent mole fractions of total MUFAs, PUFAs, and *n*6 PUFAs (Table 7). Weanling offspring from dams fed HF diet had greater MUFAs and lower total PUFAs and *n*6 PUFAs than weanling offspring of dams fed CON diet. In regard to offspring from dams fed HF diet, weanling offspring had greater MUFAs and lower total PUFAs and *n*6 PUFAs than at 3 months of age. With respect to offspring from dams fed CON diet, weanling offspring had lower MUFAs than at 3 months of age (Table 7; Figure 5). There were no differences in fatty acid composition between groups at 3 months of age (Table 7; Figure 6).

Table 7. Male Offspring Femur Fatty Acid Composition.

	Weaning		**3 Months**		***P* Values**		
Fatty Acid	**Maternal CON**	**Maternal HF**	**Maternal CON**	**Maternal HF**	**Age x Diet**	**Age**	**Diet**
14:0	6.02 ± 0.32	3.05 ± 0.28*	1.92 ± 0.06*	2.18 ± 0.11†	<0.001	<0.001	<0.001
16:0	25.00 ± 0.45	22.49 ± 1.59	26.66 ± 1.17	28.21 ± 0.85	0.077	0.002	0.666
18:0	11.61 ± 1.47	15.26 ± 1.54	9.39 ± 1.39	8.19 ± 2.33	0.166	0.011	0.479
16:1	3.03 ± 0.22	2.78 ± 0.17	6.03 ± 0.31	6.05 ± 0.40	0.633	<0.001	0.675
18:1	17.31 ± 0.68	24.94 ± 1.95*	19.28 ± 1.41	18.69 ± 1.07†	0.006	0.131	0.016
18:3*n*3	1.66 ± 0.47	0.33 ± 0.12*	1.68 ± 0.07	1.64 ± 0.13†	0.022	0.018	0.015
18:2*n*6	16.16 ± 0.67	8.23 ± 0.65*	18.22 ± 0.94	18.21 ± 1.41†	<0.001	<0.001	<0.001
20:2*n*6	1.16 ± 0.41	2.33 ± 0.96	1.04 ± 0.26	1.35 ± 0.43	0.486	0.366	0.228
20:4*n*6	4.79 ± 1.19	4.25 ± 0.88	5.65 ± 1.45	5.95 ± 1.37	0.734	0.304	0.926
Total SFAs	48.93 ± 1.31	45.44 ± 1.61	40.56 ± 0.98	41.12 ± 1.80	0.178	<0.001	0.326
Total MUFAs	23.61 ± 1.37	35.53 ± 1.77*	28.88 ± 1.32*	27.41 ± 1.19†	<0.001	0.337	0.001
Total PUFAs	27.46 ± 1.68	19.03 ± 0.85*	30.56 ± 1.56	31.47 ± 1.51†	0.003	<0.001	0.013
*n*3 PUFAs	8.76 ± 1.18	7.34 ± 0.49	10.2 ± 1.02	10.31 ± 0.61	0.390	0.018	0.460
*n*6 PUFAs	18.7 ± 0.68	11.69 ± 1.13*	20.36 ± 0.90	21.16 ± 1.06†	<0.001	<0.001	0.003

Data is expressed as mean ± SEM (n = 8 or 9 per group) for percent mole fraction of femur total fatty acids. Percent mole fraction of fatty acids below 1% are not shown.*Values are significantly different from maternal CON at weaning. †Values are significantly different from maternal HF at weaning.

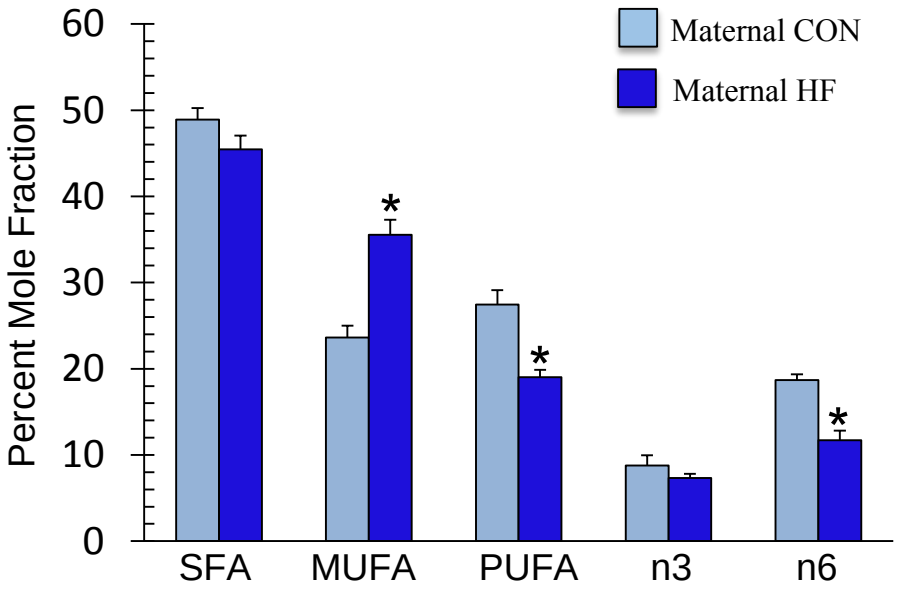

Figure 5. Male Offspring Femur Fatty Acid Composition at Weaning. Percent mole fraction of major fatty acid subclasses extracted from whole femur of weanling male offspring from dams fed a control or high fat diet. Values are expressed as mean ± SEM; * denotes significance from control; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n3, n3 PUFAs; n6, n6 PUFAs.

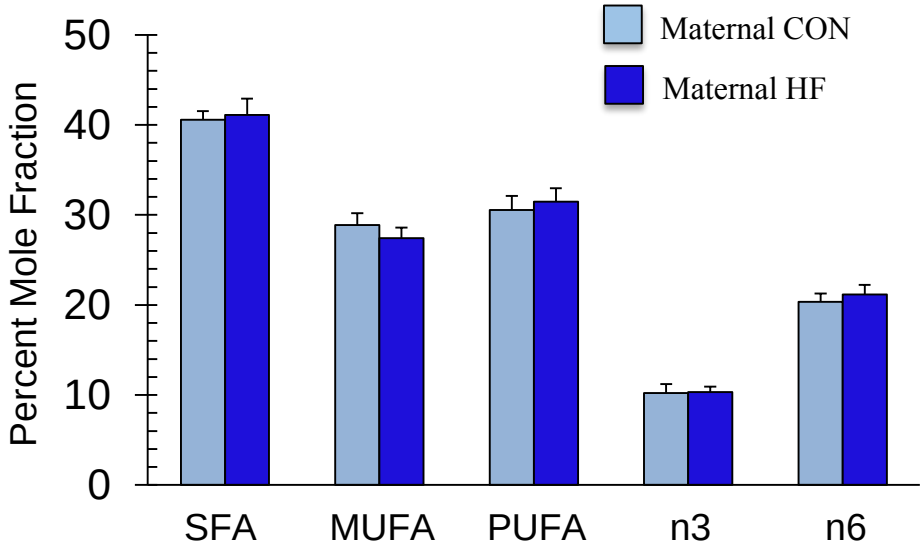

Figure 6. Male Offspring Femur Fatty Acid Composition at 3 Months of Age. Percent mole fraction of major fatty acid subclasses extracted from whole femur of 3 month old male offspring from dams fed a control or high fat diet. Values are expressed as mean ± SEM. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n3, n3 PUFAs; n6, n6 PUFAs.

6.2: Effect of high fat feeding in dams

6.2.1. Body weight and fat pad weight

There were no differences in body weight at time of breeding, however, final body weight of dams fed HF diet were significantly higher than dams fed CON diet at the end of lactation (Table 8). Dams fed HF diet had significantly higher energy intake than dams fed CON diet for 8 out of the 10 weeks that food intake was measured, from the time of arrival through to the start of breeding. It was not possible to accurately measure food intake of dams during breeding due to the presence of the male, or during pregnancy and lactation, as we tried to minimize cage disturbances.

6.2.2. Litter characteristics

There were no differences in litter size (n = 14-15), the ratio of females to males in a litter (females, n = 7; males, n = 7), or litter weight at PND 3 between groups (CON, 133.42 ± 3.69; HF, 136.13 ± 9.66). However, at PND 12, offspring in litters from dams fed HF diet weighed significantly more than offspring from dams fed CON diet (438 ± 14 g and 386 ± 6 g, respectively).

6.2.3. Bone mineral and bone strength of femurs and lumbar vertebrae

There were no differences in BMC or BMD of whole femurs, at the 1/3 proximal site of femurs, or LV1-3 between groups. There were also no differences in peak load at the femur midpoint, femur neck, or LV3 between groups (Table 8).

Table 8. Body Weight, Plasma Hormones, and Bone Outcomes of Dams.

Outcome	Treatment		*P* Value
	CON	HF	Diet
Body Weight			
Time of breeding (g)	330 ± 9	343 ± 12	0.412
Final body weight (g)	324 ± 5	350 ± 10	0.028
Body Composition			
% OM fat pad mass	1.33 ± 0.12	1.56 ± 0.11	0.179
% RTP fat pad mass	1.52 ± 0.13	1.75 ± 0.13	0.244
% PM fat pad mass	1.86 ± 0.15	2.27 ± 0.23	0.150
Plasma Hormones			
Leptin (pg/ml)	1357.7 ± 158.5	1936.8 ± 331.8	0.135
IL-6 (pg/ml)	227.7 ± 63.1	506.3 ± 122.4	0.130
TNF-α (pg/ml)	BD	BD	
Bone Mineral			
Whole femur			
BMC (mg)	355.9 ± 15.4	367.0 ± 13.4	0.584
BMD (mg/mm^2)	1.70 ± 0.05	1.76 ± 0.04	0.379
1/3 Proximal femur			
BMC (mg)	122.0 ± 6.2	123.9 ± 4.6	0.800
BMD (mg/mm^2)	1.62 ± 0.05	1.69 ± 0.05	0.332
LV1-3			
BMC (mg)	244.5 ± 15.6	249.5 ± 11.5	0.792
BMD (mg/mm^2)	1.45 ± 0.05	1.48 ± 0.04	0.625
Bone Strength			
Femur midpoint			
Peak load (N)	125.4 ± 9.1	138.3 ± 7.1	0.948
Femur neck			
Peak load (N)	82.9 ± 17.4	62.3 ± 8.2	0.300
LV3			
Peak load (N)	188.2 ± 18.4	220.4 ± 17.2	0.219

Data is expressed as mean ± SEM (n = 9 per group). BD, values below the level of detection, no comparison made.

6.2.4. Plasma leptin, TNF-α, and IL-6 concentrations

There were no differences in plasma concentrations of leptin, and IL-6 between dams fed HF versus CON diet (Table 8). No comparison was made for plasma TNF-α as some rats had levels below the level of detection for the assay. However, more dams fed HF diet (n = 5) had detectable levels than dams fed CON diet (n = 2).

6.2.5. Femur fatty acid composition

Dams that consumed HF diet had higher percent mole fraction of MUFAs, as well as lower *n*3 and *n*6 PUFAs compared to dams fed CON diet. There were no differences in percent mole fraction of SFAs between dams fed CON and HF diet (Table 9).

Table 9. Femur Fatty Acid Composition of Dams.

	Treatment		*P* Value
Fatty acid	**CON**	**HF**	**Diet**
14:0	1.66 ± 0.10	1.62 ± 0.04	0.750
16:0	24.97 ± 0.89	25.07 ± 0.34	0.917
18:0	7.94 ± 0.26	9.53 ± 0.27	<0.001
16:1	4.47 ± 0.25	3.88 ± 0.11	0.068
18:1	25.52 ± 0.92	38.31 ± 0.55	<0.001
18:3*n*3	1.85 ± 0.09	0.39 ± 0.02	<0.001
20:3*n*3	3.42 ± 0.23	3.35 ± 0.42	0.884
18:2*n*6	25.09 ± 0.71	12.84 ± 0.15	<0.001
Total SFAs	36.02 ± 1.26	37.76 ± 0.57	0.251
Total MUFAs	31.10 ± 0.85	43.58 ± 0.61	<0.001
Total PUFAs	32.89 ± 0.71	18.66 ± 0.70	<0.001
*n*3 PUFAs	6.42 ± 0.21	4.22 ± 0.46	<0.001
*n*6 PUFAs	26.47 ± 0.69	14.44 ± 0.27	<0.001

Data is expressed as mean ± SEM (n = 7 or 8 per group) for percent mole fraction of femur total fatty acids. Percent mole fraction of fatty acids below 1% are not shown.

CHAPTER 7.0: DISCUSSION

7.1: Effect of maternal high fat feeding on male offspring

7.1.1. Body composition and body weight

Using a Wistar rat model, maternal HF feeding did not have long-lasting effects on male offspring body composition (i.e., % fat, % lean, % bone mass; and % fat pad weight) at 3 months of age. However, weanling offspring from mothers fed a HF diet *appeared* to be affected by elevated fat exposure. Although statistical significance was not evident in regard to body weight (potentially due to an underpowered sample size (i.e., n = 9 per group; power = 0.60 versus a sufficient 0.80)), offspring from dams fed HF diet had observably and palpably greater adiposity compared to offspring from dams fed CON diet. Although unknown in this study, offspring from dams fed HF diet may have consumed more milk and thus had a higher energy intake during suckling [65,66]. For example, it has been shown that neonates exposed to higher fat contents in milk consumed the milk more rapidly and consistently within a predetermined length of time (versus lower fat contents) [65]. This finding suggested that the higher fat content in milk may not have induced earlier onset of satiety, rather, it perpetuated offspring desire to consume more milk. Therefore, higher energy intake through elevated fat content in maternal diet could explain why offspring from dams fed a HF diet appeared to have greater adiposity than offspring from dams fed CON diet in the current study.

Although % fat and % lean mass were not significantly different between groups, weanling offspring from dams fed HF diet had higher % bone mass than weanling offspring from dams fed CON diet. This finding may be attributed to potentially higher intakes of all nutrients including vitamin and mineral contents (e.g., calcium) of the

maternal HF diet during suckling, thus, promoting bone growth. Although direct measurement of dam's milk was not obtained, previous work has shown that milk from lactating mothers reflected the composition of diet consumed [18,27,66]. Therefore, it can be speculated that these early differences in body composition may be due to HF exposure during suckling, thus, promoting greater weight gain (based on observation) from birth to weaning age. Furthermore, if there was higher energy consumption during this period, offspring from dams fed HF diet would then be consuming relatively higher levels of protein, calcium, and vitamin D (because absolute levels of these components increase proportionately to energy content). Thus, this may potentiate bone growth in offspring from dams fed HF diet by higher energy and nutrient delivery to support growth and bone mineralization.

At 3 months of age, there were no differences in body composition between offspring of mothers fed a HF or CON diet. This may be due to the consumption of a CON diet from weaning onwards, which may have altered the trajectory of body growth after weaning (especially since weanling offspring of mothers fed a HF diet appeared to be set on a trajectory to be heavier and have higher fat mass). However, offspring from dams fed a HF diet weighed more than offspring from dams fed CON diet at 3 months of age, irrespective of food intake. If these offspring were studied beyond 3 months of age, there might have been larger discrepancies in body weight, which may have corresponded to elevations in % fat mass. As such, this may have supported a nutritional programming effect due to maternal diet alone, as all offspring were weaned onto CON diet under the same environmental conditions (i.e., cage mate, body weight measurements, enrichment, etc.).

7.1.2. Bone mineral and bone strength of femurs and lumbar vertebrae

There were no interactive effects of maternal diet and offspring age on whole femur, 1/3rd proximal femur, and LV 1-3 bone mass (BMC and BMD). Therefore, any impact shown from maternal HF feeding was not dependent on the age of offspring studied for these outcomes, or vice versa. In contrast, main effects of maternal diet and offspring age were evident. In regard to whole femur BMC (mg), 1/3rd proximal femur BMD (mg/mm^2), and LV1-3 BMD (mg/mm^2), offspring from dams fed a HF diet had greater mineralization than offspring from dams fed CON diet. Since this is the first study to measure femur (whole and 1/3rd proximal) and lumbar vertebrae bone mass together, it is difficult to compare these results to other nutritional programming studies. However, a potential contributor to these findings may be related to vitamin and mineral adjustments in the HF diet. As such, perhaps these findings suggest that maternal HF feeding was not deleterious for bone health when other nutrients (i.e., calcium, vitamin D) are adequate within maternal diet (to support optimal bone development).

Similar to previous research [31], no differences in bone strength were found at any skeletal site (i.e., femur neck, femur midpoint, LV3) in 3 month old offspring. Overall, these findings suggest that slight differences in bone mineralization following a maternal HF diet (statistical main effect only) did not translate into functional differences in bone strength long-term. This is apparent at skeletal sites that contain more cortical (i.e., femur midpoint) or trabecular (i.e., femur neck and LV3) bone. However, bone strength measurements were not obtained from weanling offspring. This was due to the small size of the bones, as well as low bone mineral content (i.e., ~5-25mg and 172-524mg at weaning and 3 months of age, respectively) that may not be accurately assessed

using the materials testing system. If bone strength measurements were possible, it would be predicted that maternal HF feeding would result in comparable levels of bone strength among groups, as differences in bone mineralization were not detected (i.e., bone mineralization may be a surrogate estimate of bone strength [67]).

7.1.3. Bone structure

Due to non-significant differences in bone mineral and strength at 3 months of age, structural measurements were not conducted. This decision was consistent with previous research that has found comparable results between interventions (via DEXA and materials testing system, respectively) [68]; as changes in bone mass and/or bone structure would be expected to precede changes in bone strength.

7.1.4. Plasma leptin, TNF-α, and IL-6 concentrations

The results demonstrated that maternal HF feeding did not lead to differences in offspring plasma leptin, TNF-α or IL-6 concentrations at weaning or 3 months of age. Considering that the secretion of these adipokines and pro-inflammatory cytokines often correspond to the level of fat accumulated [18], this finding is fitting since there were no differences in % fat mass between maternal HF and CON groups at either age. Based on observation (albeit non-significant), weanling offspring from dams fed HF diet had higher levels of all plasma hormones measured compared to offspring from dams fed CON diet. Therefore, if the rats were maintained on a HF diet (via suckling or directly) longer, there may have been differences in plasma concentrations (i.e., leptin, IL-6, and TNF-α) mediated through elevated % fat mass. Thus, elevated concentrations may affect bone development by up-regulating osteoclastogenic activity either directly (i.e., TNF-α and IL-6 interactions with RANK receptors) [22] or indirectly (i.e., leptin potentiating IL-

6 and RANKL release from osteoblasts, thus, emphasizing osteoclast activation)[47]; ultimately facilitating greater bone resorption. However, it is difficult to draw this conclusion because this study was the first to analyze pro-inflammatory markers in a nutritional programming model, particularly in response to maternal HF feeding.

7.1.5. Femur fatty acid composition

This study was the first to examine whether maternal HF feeding altered offspring bone fatty acid composition. At weaning, femur bone fatty acid composition reflected that of maternal diet and femur composition of the dams. Moreover, the results show that after consuming a CON diet post-weaning, during a rapid period of growth, the fatty acid composition of femurs obtained from 3 month old male offspring reflected the fatty acid content of the CON diet. Thus, maternal diet alone did not permanently alter offspring bone fatty acid composition. Rather, the bone fatty acid composition reflected the diet consumed, in agreement with previous studies that have manipulated the fatty acid composition of diets fed to older rats [37,40]. Although the role of fatty acids in bone has not been established, the result in which fatty acid composition corresponded to the most recent diet consumed suggests that some form of fatty acid turnover may have occurred, which could be related to fuel utilization to support rapid growth (i.e., first 10 weeks of life). Moreover, although bone strength measurements were not obtained at weaning, early life changes in fatty acid composition did not compromise bone strength at 3 months of age. Therefore, these findings suggest that the trajectory for development can be altered according to changes in dietary consumption.

7.2: Effect of high fat feeding on dams

7.2.1. Body weight and fat pad weight

At the time of breeding (i.e., after 10 weeks of feeding), no differences in dam body weight were evident. In this Wistar rat model, 10 weeks of HF feeding may not have been long enough to elicit differences in body weight. Moreover, due to high variability of diets used in previous studies, it is difficult to compare this finding with other research. There is a possibility that the HF diet administered (relative to the AIN93G control diet) was not 'unhealthy' enough to elicit differences in body weight at breeding. Thus, body growth may have behaved similarly between groups throughout the 10 weeks of feeding. In contrast, other studies have shown differences in % fat mass (using DEXA) without changes in body weight [18]. Therefore, there is a possibility that dams fed a HF diet in this study had gained higher total % fat mass than dams fed CON diet at breeding, however, these measurements were not obtained. Although, the finding that % retroperitoneal, % omental, and % parametrial fat pad weights (% relative to total body weight) were comparable between conditions suggest that this was not the case (i.e., HF feeding may not have altered body composition).

During pregnancy, whether HF feeding perpetuated weight gain was unclear. Body weight and food intake measurements were not obtained to reduce cage disturbances. However, dams that consumed a HF diet weighed more at the time offspring were weaned (i.e., PND 19). This was found despite similarities in litter size and male:female ratio. Thus, perhaps there was a threshold duration of HF feeding (i.e., > 10 weeks) or an increased appetite (thus higher energy intake) for the HF diet during pregnancy and/or lactation that contributed to this finding. In support of this effect, it has

been previously shown that rats consumed more fatty foods during pregnancy and lactation when provided a choice [69]. Therefore, dams from this study potentially weighed more at the end of lactation because of higher fat consumption (thus elevated energy intakes).

7.2.2. Litter characteristics

The results demonstrated that 10 weeks of HF feeding did not alter litter size, litter weight at PND 3 (potentially due to similar body growth in utero), or male:female ratio. These findings are consistent with previous research [27]. Moreover, maternal HF feeding did not appear to affect maternal nurturing behaviour once offspring were born; as all offspring survived the lactation period (i.e., no cannibalism among groups and adequate nourishment for all pups to support body growth). The finding that offspring from dams fed a HF diet weighed more than offspring from dams fed CON diet at PND 12 suggests either: 1) higher fat content via milk transfer during suckling promoted greater weight gain post-natally (without differences in energy intake between groups); or 2) offspring had greater palatability for the HF diet and consumed more milk (thus greater energy intake; 4.4kcal/gram (HF) versus 3.8kcal/gram (CON)); resulting in greater weight gain. Although unknown in this study (i.e., milk composition, appetite regulation markers (e.g., leptin), or energy intake measurements), previous work has demonstrated that newborn pups exposed to a maternal HF diet consumed more milk (and higher fat) than offspring from dams fed CON [27]. Therefore, it is possible that offspring from the current study developed a greater appetite for fat, resulting in higher energy intake and body weight gain by PND 12.

7.2.3. Bone mineral and bone strength of femurs and lumbar vertebrae

In contrast to previous studies [35,36], 10 weeks of HF feeding did not adversely affect bone mass (i.e., represented by lower BMC and BMD) at the femur (whole and 1/3 proximal) or lumbar vertebrae; a skeletal site that has not been previously studied. A potential reason for these conflicting results may be due to the adjustment of protein, vitamins, and minerals in the HF diet in the current study. For instance, dietary protein consumption is important for extracellular bone matrix synthesis [70] as well as osteoblast differentiation and function. Therefore, previous work demonstrating compromised bone mass may be due, at least in part, by dietary protein deficiencies and resultant reductions in osteoblast bone deposition; rather than HF consumption *per se*. Furthermore, other potential nutrients not adjusted for in previous literature include calcium and vitamin D [18,36,38]. Therefore, in addition to reduced osteoblast viability via inadequate protein consumption, rodents from these studies may have had lower calcium availability for mineralization, thus, lower bone mass than controls. Therefore, the current study may be one of the first studies [58] to isolate the impact of HF feeding on bone health by minimizing potential decrements in other nutrients. In addition to potential dietary contributions on the bone mineral results, the finding that leptin, TNF-α, and IL-6 did not differ between dams fed HF or CON diet support these results. As such, circulating hormones may not have altered the ratio of bone deposition (via greater leptin availability) to bone resorption (greater TNF-α and IL-6 availability) (see Figure 2).

In support of the contention that changes in bone mass may precede changes in bone strength [71], there were no differences in bone strength at the femur (midpoint or neck) or lumbar vertebrae between groups. This finding was similar to Lau et al. (2010)

[37], suggesting that long-term high saturated fat consumption (regardless of source; coconut oil versus lard) may not lead to compromised bone strength.

7.2.4. Femur fatty acid composition

The results support previous evidence that bone fatty acid composition reflects the diet consumed [37,72,73]. Similar to Lau and colleagues (2010) [37], the results show that although differences in bone fatty acid composition were evident, this did not correspond to differences in bone strength. Therefore, supporting the contention that bone fatty acid composition and bone strength respond independently of one another to high fat consumption.

7.2.5. Plasma leptin, TNF-α, and IL-6 concentrations

Leptin

There were no differences in plasma leptin concentrations between dams consuming HF or CON diet. This finding is in contrast to previous work [74-77]. However, previous studies have reported higher % fat mass in addition to elevated leptin concentrations (thus elevated leptin release from adipose tissue). Therefore, the current finding that HF feeding did not alter % fat pad weight (OM, PM, and RTP) may potentially explain, at least in part, the non-significant leptin concentrations between groups. Moreover, since previous work has predominately fed rats diets containing higher % of energy from fat (i.e., 46-58%) for longer durations (6-10 months), this may have corresponded to the higher % fat and leptin concentrations evident in the literature [74-77].

TNF-α

TNF-α levels were below detectable limits of the Millipore assay. Previous work has also demonstrated difficulties obtaining TNF-α measurements using a different assay system (i.e., enzyme-linked immunosorbent assay) as well [78], however, HF consumption may still have altered tissue expression. For instance, previous work demonstrated that HF feeding increased TNF-α protein content in muscle and adipose tissue, despite non-detectable concentrations in blood [78]. As such, TNF-α may have produced autocrine inflammatory effects expressed by the tissue itself, independent of circulating pro-inflammatory markers. Moreover, another study demonstrated discrepancies between adipose tissue levels of TNF-α activities in different fat pads as a result of HF feeding [79]. As such, it was shown that HF feeding led to higher adipose derived TNF-α activity in RTP fat pads than epididymal or mesenteric (similar to omental) fat pads. Therefore, not only might differences be evident in tissue TNF-α activity, but there may also have been differences depending on fat pad type.

With respect to bone tissue in response to HF feeding, perhaps there was higher TNF-α protein concentrations and/or activity through elevated osteoblast release. As a result, greater osteoclastogenic activity may have occurred, thus, resulting in higher bone resorption (as long as this activity exceeds bone formation). However, since there were no differences in bone mass and strength in this study, it is expected that this did not play a role in response to HF feeding, or at least did not exceed bone depositing processes.

IL-6

Similar to previous work [21], there were no differences in IL-6 concentrations between dams fed a HF or CON diet. Although circulating levels of IL-6 may not reflect

tissue expression, this finding combined with comparable bone mineral and strength results suggest that HF feeding did not induce a state of bone inflammation (resulting in greater bone resorption due to elevated osteoclast expression/activity).

CHAPTER 8.0: CONCLUSIONS

Using a Wistar rat model, maternal HF feeding did not result in long-lasting effects on body composition and bone development in male offspring. However, exposure to a HF diet in utero and during suckling contributed to higher % bone mass and altered femur fatty acid composition in male offspring at weaning. Since these differences were not sustained at 3 months of age after consuming a control diet post-weaning, these findings suggest that components of body composition and bone health can be altered based on the most recent diet consumed.

CHAPTER 9.0: STRENGTHS AND LIMITATIONS

9.1: Study design

9.1.1. Strengths

- Used a Wistar rat model
    - Research has shown that developing Wistar rats resembles human body growth and metabolism (Charles River). This occurs at a much faster rate relative to humans, thus, provides information regarding maternal HF feeding in a succinct period of time.
    - Wistar rats have excellent breeding success and are tolerant of investigators handling their offspring during the suckling period (cannibalism is rare) [80]. All dams gave birth to at least 8 pups and all offspring survived. This allowed for evaluation of maternal HF feeding on outcomes such as litter size, male to female ratio, and changes in offspring body weight during the suckling period.
    - Bones extracted from rats resemble the structure and composition of human bone [81]. As such, the femur midpoint contains mostly cortical bone, femur neck contains both cortical and trabecular bone, and the lumbar vertebrae contains mostly trabecular bone in both rats and humans. Therefore, the sites chosen for bone strength measurements (i.e., femur midpoint, femur neck, and lumbar vertebrae) should be reflective of humans. However, it must be acknowledged that while closure of the epiphyseal plates occurs during puberty in humans [82] and mice [83], this does not happen in rats until a much later age. In this regard, a mouse model may have more closely reflected human bone growth than the rat model used.

- The level of protein, vitamins, and minerals were proportionate to the higher energy content of the HF diet (per kg of diet).
    - Therefore, differences between groups may be attributed to higher fat content in the diet and not deficiencies in other nutrients (particularly if there are no differences in energy intake). Many previous high fat feeding studies have not adjusted for these nutrients (i.e., protein, calcium, and vitamin D) in terms of energy per kg of diet [18,28,29,35,36,38]. Therefore, deleterious effects of HF feeding (either direct or maternal) on body composition (i.e., fat, lean, and bone mass) and bone outcomes (mass, structure, and strength) may have been due, at least in part, to inadequate protein, calcium, and vitamin D availability.
- Only 1 pup per litter was studied per outcome at weaning and 3 months of age.
    - Reducing 'litter effects' by maintaining original sample size (i.e., n = 9/group) at each time point. Since the dams were the population randomized to each treatment (i.e., CON or HF), it is expected that individual pups within each litter will be more similar to one another than pups from another litter. Therefore, pups within each litter are not necessarily 'independent' from one another. As such, studying more than 1 pup per litter over-represents the treatment applied to the dams. Ultimately, this may bias the results towards statistical significance by increasing the sample size and improving statistical power. Thus, studying one pup per litter removes potential bias on the results by appropriately representing each dam [60].
- Obtained measurements of body composition and bone health in dams as well as male offspring.

- Provided insight as to whether differences between offspring groups are reflected in body composition changes in the dams directly consuming a HF diet. This is important because it can help isolate whether maternal diet itself, or secondary changes due to the HF diet (i.e., elevated fat mass), can affect male offspring body composition and bone development.

9.1.2. Limitations

- Rats were housed 2 per cage.
    - Although a strength from a social and ethical perspective, housing multiple animals together results in less accurate food intake measurements. Therefore, it is unclear whether one cage mate consumed more or less than the other, and whether this contributed to differences in body composition at weaning.
- Unable to obtain mother's milk during suckling.
    - Although there is evidence that mother's milk reflects the diet consumed [18,84], no study has used the same HF and CON diets as this study. Therefore, it is inferred that weanling male offspring consumed higher fat content during suckling (due to maternal diet). However, they may have also consumed more milk, thus, it is difficult to tease out the exact effects of maternal high fat feeding on offspring development.
- Litters were not culled immediately after birth.
    - Therefore, milk availability may vary between litters depending on the number of pups suckling. For example, litters that are larger may have less suckling opportunities to support growth and development than smaller

litters. However, litters were not culled because it provided information regarding survival of the offspring between maternal CON and HF conditions.

- Complexity of human dietary behaviours
    - Rodents in the current study consumed the same level and composition of nutrients per gram of diet. Although an important strength from a study design perspective (thus able to isolate nutrients of interest for intervention sake), humans are more diverse in regard to food selection and nutrients consumed on a daily basis. Therefore, foods containing higher levels of fat may also contain higher levels of other nutrients such as sucrose and calcium (e.g., dairy products). In this regard, chronic high calorie and nutrient consumption may have varied effects on body composition and bone development in humans, which may result in different effects on offspring development from a maternal perspective.
- Complexity of human physical activity and exercise behaviours
    - Rodents from the current study may have been highly sedentary due to their consistent environment (i.e., caged, enrichment). Similar to the considerations noted pertaining to food intakes, this allows for isolation of the dietary intervention by limiting confounders such as highly variable energy expenditure. However, humans have much more autonomy in regard to physical activity (either voluntary or occupation related) and exercise behaviours [85]. Thus, humans may vary the type, frequency, amount, and duration of daily exercise that can implicate their body

composition and bone health long-term [85]. Moreover, changes in exercise behavior can also alter energy intake, thus, contribute to altered body composition and bone health [86]. Therefore, conclusions from the current study should focus on remotely sedentary populations consuming a HF diet.

9.2: Outcomes selected

9.2.1. Strengths

Out of the outcomes selected, this thesis studied:

- All components of body composition (i.e., fat, lean, and bone mass) at weaning and 3 months of age.
    - This is the first study to include all 3 aspects of body composition in dams, as well as male offspring at weaning and 3 months of age. Therefore, this study examined how maternal HF feeding can affect multiple tissues together, and whether early life differences are sustained following consumption of a control diet post-weaning. This is important because it can reveal how changes in fat, lean, and bone mass interact in response to maternal HF feeding.
- The femur and lumbar vertebrae together
    - Determined whether maternal HF feeding led to skeletal site specific changes in male offspring bone development. To date, no previous study has combined measurements for the femur and lumbar vertebrae. In this regard, bone mass and strength measurements at the femur midpoint, femur neck, and lumbar vertebrae provides insight on bones containing

various ratios of trabecular and cortical bone (femur midpoint, highly cortical; femur neck, both trabecular and cortical; lumbar vertebrae, highly trabecular) in response to maternal HF feeding.

- Plasma leptin and pro-inflammatory cytokines (TNF-α, IL-6).
    - Aimed to study potential mechanisms involving HF feeding (either direct of maternal exposure) that 'link' changes in body composition with changes in bone development. For example, if HF feeding (direct or maternal exposure) led to higher fat mass, lower bone mass, and lower bone strength in the rodents (dams and/or male offspring), elevated plasma pro-inflammatory cytokine (i.e., TNF-α and IL-6) concentrations may suggest an inflammatory state secondary to the HF diet itself (via release from adipose tissue). This could support previously proposed mechanisms of action regarding HF feeding, elevated fat mass, pro-inflammatory cytokine secretion, and altered bone remodeling (Figure 2). In contrast, if maternal HF feeding led to elevated fat mass, lower lean and bone mass without changes in plasma pro-inflammatory cytokines, this may suggest some other mechanism by which HF exposure acts on bone metabolism.

9.2.2. Limitations

- Measurements of bone strength were not feasible at weaning.
    - Therefore, it is unclear whether differences in femur fatty acid composition at weaning corresponded to differences in bone strength. It was not possible to obtain bone strength measurements at weaning because the bones were too small. Therefore, a materials testing system

would not accurately assess mechanical properties of the bones. If measurements were obtained and bone strength was different among groups, this would be a novel finding as bone strength was expected to be similar among groups because there were no differences in bone mass.

CHAPTER 10.0: IMPLICATIONS FOR HUMANS

As previously mentioned, rodent models have been used to mimic human development. Findings from the current maternal HF feeding study and previous literature allows for the following conclusions to be drawn:

1. Women consuming a HF diet throughout pregnancy and lactation may alter fat, lean, and bone mass development in male offspring during early life (i.e., during suckling). However, the magnitude and direction of alteration may depend on the amount and type of fat consumed, and whether protein, vitamins, and minerals are adequate within the diet. Ideally, elevated fat consumption (i.e., ~41% of energy intake) may not adversely impact offspring development, provided that protein, vitamins, and minerals are consumed proportionately to energy intake.
2. Early life perturbations in body composition (i.e., elevated fat mass) related to maternal diet may be lessened if the child is provided a well-balanced diet throughout childhood. Therefore, mother's diet throughout pregnancy and lactation may not be the only determinant of offspring body composition and bone health throughout life.

CHAPTER 11.0: FUTURE DIRECTIONS

11.1. Animal models

Future work should examine whether femur fatty acid composition from weanling male offspring corresponds with changes in mechanical properties. This may be examined using μCT, however, whether these measurements can be obtained from Wistar rats at weaning is unclear. Future bone fatty acid research may separate the phospholipid and triglyceride lipid components from femurs, in order to determine 'structure' versus 'storage' alterations that may correspond with changes in maternal diet. This may be important because changes in phospholipid composition could alter the function of cellular membranes [87], and thus, may affect signalling cascades that affect bone metabolism. In contrast, changes in lipid storage (i.e., triglycerides) might provide information as to whether or not bones store lipids for their own functional purpose (i.e., energy utilization).

11.2. Human models

Little information is available regarding how women consuming a HF diet throughout pregnancy and lactation impacts body composition and bone development of their children. Although a controlled study design that administers a HF diet to pregnant women is not feasible, food frequency questionnaires (FFQ), global physical activity questionnaires (GPAQ), and non-radiation based body composition techniques (i.e., Bod Pod if available or calipers) could be applied to observe potential associations between maternal diet, physical activity, and body composition on offspring fat, lean, and bone mass development. For instance, within each trimester (1^{st}, 2^{nd}, and 3^{rd}), after birth, and at the end of lactation, pregnant women could be assessed for:

1) Global food intakes - Completing a FFQ can provide insight into overall dietary consumption [88] prior to and throughout pregnancy and lactation. This information would allow the examiner to categorize subjects into various dietary groups (including high fat conditions).
2) Physical activity – Completing a GPAQ can identify typical activity levels that include occupational, leisure, and exercise behaviours [89]. Along with a FFQ, this information may provide insight into global energy intake and expenditure, respectively. Overall, this may allow for associations between maternal energy intake, energy output, and changes in body composition during pregnancy and lactation on offspring body composition and bone development.
3) Body weight and body composition measurements – Participants could be assessed for body weight and body composition once every trimester, after birth, and the end of lactation. These time points are important because they can demonstrate how the mother's body composition changes throughout pregnancy and lactation. This may allow relationships to be drawn between maternal diet, changes in maternal body composition, and offspring body composition development. This can be facilitated by an electronic scale (body weight) and either a bod pod (calculates body density [90], which can then be used to calculate fat, lean, and bone mass) or hand held calipers (estimates fat mass) [91].
4) Subsequently, the child born could be weighed and measured for body composition using calipers [91]. This could provide insight regarding the impact of maternal diet and body composition on *in utero* development. The offspring could also be tracked in a similar manner as the mothers throughout the first few

months or year of life. As such, information pertaining to offspring body composition development can be correlated to mother's diet and body composition. Moreover, longitudinal studies could also follow up with the offspring in a similar fashion as those in the Dutch famine cohort (i.e., FFQ), along with a GPAQ and body composition assessment [2]. This could provide insight on the lifestyle influences underlying offspring health at adulthood in addition to maternal diet.

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