The Role of Maternal High Fat Diet in the Pathogenesis of Metabolic and Bone Disease in the Adult Offspring

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ABSTRACT

Chronic diseases such as osteoporosis, type 2 diabetes, and cardiovascular disease are diseases of long duration, slow progression, and are by far the leading cause of death worldwide. A growing body of evidence links adverse exposures in early development with an increased risk of chronic diseases in adult life. The studies presented in this dissertation sought to exploit this phenomenon to determine the extent to which gestational and lactational exposure to a high fat diet predisposes the offspring to certain diseases in later life and if the eating habits of adult offspring would be able to mitigate or exacerbate these conditions. In the study presented in Chapter III, dams fed an atherogenic high fat diet prior to conception and throughout gestation and lactation experienced excess hepatic lipid accumulation and poor birth outcome as characterized by smaller litter sizes and higher post-delivery mortality. In the offspring, gestational and lactational exposure to such a diet resulted in growth restriction and skeletal aberrations indicative of osteoporosis, despite being fed a standard rodent diet post-weaning. We propose that dietary-induced hyperlipidemia, along with pregnancy-associated factors, resulted in fatty liver and subsequently reduced litter sizes and increased early mortality, and that the skeletal aberrations seen in the mature offspring represent dietary-induced inhibition of osteogenesis in favor of adipogenesis. In the study presented in Chapter IV, early exposure to a high fat diet resulted in central obesity, elevated lipid levels, hyperglycemia, and additional markers used in the diagnosis of the metabolic

syndrome. Altering the diets of the mature offspring demonstrated that the eating habits of adulthood have the potential to mitigate or exacerbate certain metabolic parameters established earlier in life. Mechanisms contributing to the observed metabolic aberrations could include developmental plasticity and mismatch, catch-up growth, and altered programming of the appetite regulatory network. Collectively, this research suggests that early exposure to a fat-rich diet can lead to metabolic and skeletal aberrations in the adult offspring and adds support to the developmental origins of health and disease hypothesis by finding that adverse nutritional exposures in early life can play a role in the chronic diseases of adulthood.

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DEDICATION

I dedicate this work to my husband, Danny, who provides constant support and unconditional love. Danny, you and Dylan are my raison d'être.

ATTRIBUTION

Several colleagues aided in the preparation and completion of the research presented in Chapters III and IV of this dissertation. Their current affiliation and a brief description of their contributions are provided here.

CHAPTER III: Maternal Atherogenic High Fat Diet Results in Hepatic Lipidosis, Poor Birth Outcome, Growth Restriction, and Skeletal Aberrations in C57BL/6 Mice

Javiera Bahamonde, DVM, PhDc: Dr. Bahamonde is a candidate for the Doctor of Philosophy Degree in Biomedical and Veterinary Sciences at Virginia Tech. Dr. Bahamonde directly assisted in the research, including data collection and analysis, as well as contributed editorial comments for the chapter.

Megan E. Oest, PhD: Dr. Oest is an assistant professor at Upstate Medical University in Syracuse, NY. Dr. Oest performed the micro-computed tomographic imaging and analysis for trabecular organization of the left femur.

M. Renee Prater, DVM, MS, PhD: Dr. Prater is Associate Dean for Medical Education at Edward Via College of Osteopathic Medicine. Dr. Prater was the principle investigator for the project and was responsible for project organization, direction, and approval acquisitions. Dr. Prater also directly assisted in the research and contributed editorial comments for the chapter.

CHAPTER IV: Maternal Nutrition and Fetal Programming of the Metabolic Syndrome: Mitigating or Exacerbating the Effects through Altered Eating Habits in Adulthood

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Deborah J. Good, PhD: Dr. Good is an associate professor in the Department of Human Nutrition, Foods, and Exercise. Dr. Good was principle investigator for the project and was responsible for project organization, direction, approval acquisitions, and supply orders.

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LIST OF ABBREVIATIONS

AFLP	Acute Fatty Liver of Pregnancy
AgRP	Agouti-related Peptide
ANOVA	Analysis of Variance
aHFD	Atherogenic High Fat Diet
BMI	Body Mass Index
CVD	Cardiovascular Disease
CT	Computed Tomography
DOHaD	Developmental Origins of Health and Disease
ELISA	Enzyme-Linked Immunosorbent Assay
EC	Experimental Conditions
ECM	Extracellular Matrix
FFAs	Free Fatty Acids
GTT	Glucose Tolerance Test
H&E	Hematoxylin and Eosin
HDL	High Density Lipoprotein
HFD	High Fat Diet
HPA	Hypothalamic–Pituitary–Adrenal
IRS	Insulin Receptor Substrate
IL-6	Interleukin-6
IUGR	Intrauterine Growth Restriction
LDL	Low Density Lipoprotein
MSH	Melanocyte-stimulating Hormone
MSCs	Mesenchymal Stems Cells
micro-CT	Micro-Computed Tomography
NPY	Neuropeptide Y
NBF	Neutral Buffered Formalin
NO	Nitric Oxide
NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
NMR	Nuclear Magnetic Resonance
Pdx1	Pancreatic and duodenal homeobox 1
RER	Respiratory Exchange Ratio
REML	Restricted Maximum Likelihood
SE	Standard Error
RD	Standard Rodent Diet
TCA	Tricarboxylic Acid
TNF-α	Tumor Necrosis Factor-alpha
VLDL	Very Low Density Lipoproteins
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine
WHO	World Health Organization

CHAPTER I: Literature Review

Portions of this review were submitted to the *Journal of Pregnancy* for their special issue on “Pregnancy and Lifestyle: Short- and Long-Term Effects on Mother's and Her Children's Health".

Introduction

Chronic diseases, such as type 2 diabetes and cardiovascular disease, are diseases of long duration and generally slow progression and are by far the leading cause of mortality in the world, representing 63% of all deaths.[1] Mounting research suggests that the fetal environment may be a determining factor of such diseases developing in later life. The period from conception to birth is a time of rapid cellular proliferation, differentiation, and organization as well as functional maturation of tissues and organs. Important determinants of fetal growth include adequate nutrient supply and the proper hormonal milieu.[2] An adverse gestational environment, such as one lacking appropriate nutrition, can cause the developing fetus to make certain adaptive responses to ensure survival. If permanent, these programming changes may be the origins of a number of conditions in later life including metabolic syndrome, cardiovascular disease, and osteoporosis.[3-10] This phenomenon prompted the “fetal origins of adult disease” hypothesis, now often referred to as the “Developmental Origins of Health and Disease (DOHaD)” hypothesis, and was spearheaded by physician and researcher David Barker. Barker’s initial work focused on the association between lower birth weights and higher risk of coronary heart disease later in life.[6] Our

research has its roots in the DOHaD hypothesis and extends Barker's work by utilizing a mouse model to study the impact of a mother's poor diet, particularly one that is high in fat, on the maintenance of pregnancy and the immediate and long-term health of the progeny. This avenue of research carries the hope of chronic disease prevention at its earliest beginnings.

Developmental Origins of Health and Disease

In the late 1980s reports emerged on the relationship between poor nutrition in fetal and early life, small size at birth, and increased risk of chronic disease in adulthood.[11,12] These reports were based on a considerable body of epidemiological data that revealed an association between an adverse intrauterine environment, as determined primarily by low birth weight, and an increased risk of coronary heart disease later in life. Since that time, there has been an explosion of research into the fetal origins of disease relating not only to cardiovascular disease, but also to metabolic syndrome and osteoporosis. Evidence to support the DOHaD hypothesis in relating to these disease conditions is discussed under their respective sections in this review. It bears mentioning that although the vast majority of the literature concerning the DOHaD hypothesis is focused on the relationship between small birth size and increased incidence of disease in adult life, it is now recognized that higher incidences of disease occurs in both those born small and those born large, thus reflecting a U-shaped curve.[13,14] The putative concepts and mechanisms behind the DOHaD hypothesis are discussed below and, although separated into individual sections, should not be interpreted as mutually exclusive.

Developmental Plasticity

Developmental plasticity is the ability of an organism to change its phenotype in response to changes in the environment.[15] If this change or adaptation is permanent, it is considered a “programming” change and is associated with persistent effects in structure and/or function that endure throughout the life of the organism.[15,16] Living creatures are capable of developing in a variety of ways, forming characteristics that are well adapted to the environments in which they are likely to live.[17] In most cases these programming changes are beneficial for the health and survival of the organism. Barker uses the functional activity of the sweat glands as a simple example of plasticity and programming.[5] Humans have similar numbers of sweat glands at birth, but they are essentially nonfunctional. In the first few years of life a proportion of the glands become functional depending on the temperature to which the child is exposed. The hotter the conditions, the greater number of functional sweat glands. After a few years the process is complete and the number of sweat glands is fixed. Thereafter, the child who has experienced hot conditions will be better adapted to such conditions in later life, because they have more functioning sweat glands, and thus cool down faster.[5] For most organs and systems the critical period of plasticity occurs *in utero*.[5] Fetal development may involve a particular pattern that prepares the individual for the environment in which he or she is likely to live. However, the problem of “mismatch” occurs when individuals developmentally adapted to one environment are exposed to another.[17] Using the example of the functional activity of sweat glands, those born and raised in cool climates will have fewer functioning sweat glands and will experience a mismatch between adaptation and environment when relocated to a hot climate. Other examples of this mismatch phenomenon include people whose birth weights were

towards the lower end of normal and who subsequently grow up in affluent societies being at increased risk for heart disease, type 2 diabetes, and hypertension.[13,18]

Thrifty Phenotype Hypothesis

The “thrifty phenotype” hypothesis, put forth by Barker and Hales, proposes that poor fetal and early postnatal nutrition imposes mechanisms of nutritional thrift upon the growing individual. For example, they proposed that one of the major long-term consequences of inadequate nutrition *in utero* is impaired development of the endocrine pancreas and a greatly increased susceptibility to the development of type 2 diabetes in later life. The hypothesis is based on a study of 468 men, in which the percentage of those with impaired glucose tolerance or type 2 diabetes fell progressively with increasing birth weight and weight at 1 year.[19] Data from this research suggest that intrauterine undernutrition results in the growth of vital organs (e.g., brain) at the expense of other organs (i.e., endocrine pancreas leading to reduced β cell mass/islet function). Such adaptations may increase the chance of fetal survival by means of “brain sparing”, but result in difficulty in coping with nutritional abundance in later life. Another such example is the concept that the number of nephrons, the functional units of the kidney, may be programmed during gestation. In both animal and human studies maternal undernutrition, in the form of a protein-restricted diet, has been found to produce small offspring with reduced numbers of nephrons which leads to the later development of hypertension. This reduction in nephrons may reflect an adaptation that has the immediate advantage of energy and resource conservation, but no long-term advantage.[13,20]

Catch-up growth

"Catch-up growth" is where children return to their genetic trajectory for size after a period of growth delay or arrest. It may occur at any stage of growth, but it is most commonly observed in the first 1 to 2 years of life.[21] Studies have found that catch-up growth often results in overcompensation, whereby the organism exceeds normal weight and often has excessive fat deposition. This rapid and excessive growth has been associated with the development of adult obesity, insulin resistance, metabolic syndrome, and type 2 diabetes.[8,21,22] Cianfarani and colleagues speculate that the tremendous effort to recover lost growth shortly after birth involves the overactivation of insulin and insulin-like growth factors (IGF). Tissues exposed to low levels of insulin and IGF-1 during fetal life and then suddenly exposed to increased concentrations of the two hormones shortly after birth may counteract the actions of insulin by developing insulin resistance as a metabolic defense mechanism to protect the organism from hypoglycemia.[22] Interestingly, this novel implication of catch-up growth goes against the current medical practice of promoting rapid growth of small babies. It implies that rapidly enhancing infant growth rate by a nutrient enriched diet may cause harm over time and that encouraging slower growth rates may actually be beneficial.

Epigenetics

Epigenetics refers to all modifications to genes other than changes in the DNA sequence itself. Epigenetic changes include DNA methylation, which is associated with the silencing of genes, and histone modification, which is associated with transcription of genes.[15,23] Every cell in the body has the same genetic information; what makes cells, tissues, and organs different is that different sets of genes are turned on or expressed. An increasing body of evidence supports that environmentally-induced

epigenetic changes play a role in disease susceptibility. In addition, some of these changes seem to be passed on through subsequent generations. For example, in a simple, but groundbreaking experiment, fat yellow female agouti mice were provided a methyl-supplemented diet (including folate) two weeks prior to conception and throughout gestation and lactation.[24,25] Agouti mice are so named because they carry the agouti gene that, in addition to making them ravenous and yellow, renders them prone to type 2 diabetes, cancer, and other life-shortening diseases. In this study "hypermethylated" dams passed along the agouti gene to their offspring intact, yet demonstrated a shift in distribution towards having more offspring with the pseudoagouti phenotype. Pseudoagouti mice are darker/more brown, leaner, healthier, and longer lived than their yellow siblings.[24,26] Another example is reduced pancreatic and duodenal homeobox 1 (Pdx1), also known as insulin promoter factor 1, in a rodent model of intrauterine growth restriction (IUGR). Pdx1 is a transcription factor necessary for development and function of the insulin producing pancreatic β cell. In this study, IUGR led to permanently reduced expression of Pdx1 in β cells.[27] Reduced Pdx1 transcription was mediated through a cascade of epigenetic modifications (loss of upstream stimulatory factor-1 binding at the proximal promoter of Pdx1, recruitment of the histone deacetylase 1 and the corepressor Sin3A, and deacetylation of histones H3 and H4) culminating in the eventual silencing of Pdx1 and the development of diabetes in adulthood.[28] There is strong evidence that epigenetic changes are transgenerational. One example is the Dutch winter famine, in which women who were exposed to famine while in the womb later had offspring with birth weights lower than offspring of women not exposed to famine. This effect of *in utero* exposure to famine on birth weight in the

subsequent generation persisted after control for potential confounding and intervening variables.[29]

Reactive oxygen species

Excessive reactive oxygen species can cause modulation of gene expression and/or direct damage to cell membranes and other molecules at critical developmental windows. Many believe that oxidative stress is the primary link between adverse fetal growth and later elevated risks of the metabolic syndrome, type 2 diabetes, hypertension, and other disorders.[8] Smoking, hypertension/preeclampsia, inflammation/infection, obesity and malnutrition are common causes of preterm and/or low birth weight as well as known sources of oxidative stress. As an example, undernutrition and/or malnutrition often involve protein and micronutrient deficiencies. These nutritional deficiencies may impair antioxidant capacities because proteins provide amino acids needed for antioxidant synthesis, such as glutathione and albumin, and many micronutrients themselves, such as vitamins A, C and E, are antioxidants.[30] A study using cord blood to compare the status of oxidative stress between small for gestational age (SGA) infants born to undernourished mothers and appropriate for gestational age (AGA) infants born to healthy mothers found elevated oxidative stress, as determined by increased quantities of malondialdehyde (one of the major products of lipid peroxidation), reduced quantities of the antioxidant glutathione, and decreased activity of the antioxidants superoxide dismutase and catalase in the SGA infants as compared to the AGA controls.[31]

Overnutrition can also lead to an imbalance between oxidants and antioxidants. Using a rat model, maternal consumption of a “cafeteria diet” that had 42% of energy

from fat resulted in lower plasma total antioxidant status and catalase activity as well as higher plasma hydroperoxides (markers of lipid peroxidation) and carbonyl proteins (markers of protein oxidation) in both the dams and their offspring which were fed the control diet after they were weaned. Interestingly, this cafeteria diet did not affect maternal or offspring plasma levels of vitamins A, C, or E. In addition to the imbalance between oxidants/antioxidants favoring oxidative stress, dams and their offspring developed increased body weight, hyperglycemia, hyperinsulinemia, hyperleptinemia, and hyperlipidemia suggesting an unrelenting effect of maternal diet on metabolism and body habitus of maturing offspring.[32] Similarly, an experiment by our laboratory used C57BL/6 mice to examine the effects of early exposure to a high saturated fat diet and the potential role of antioxidants in ameliorating certain dietary-induced conditions. In this study, gestational and lactational exposure to a high saturated fat diet resulted in the development of key parameters of the metabolic syndrome including obesity and hypertension in the adult offspring, despite being fed a standard rodent diet post-weaning. These conditions were reduced by supplementing the pregnant dam with quercetin, a powerful antioxidant.[33]

Neuropeptides

The hypothalamus plays a critical role in the regulation of appetite and body composition by way of responding to cues from neuropeptides. A series of studies have explored the possibility that maternal nutrition during pregnancy may alter the level of energy intake in the offspring through inducing changes in the expression, localization, and action of specific neuropeptides. Central and peripheral neuropeptides function in

the regulation of appetite. Appetite stimulating neuropeptides include neuropeptide Y (NPY), agouti-related peptide (AgRP), and ghrelin. Conversely, appetite suppressing neuropeptides include cocaine and amphetamine related transcript (CART), melanocyte-stimulating hormone (MSH), serotonin, insulin, and leptin.[34] It bears mentioning that the overweight and obese tend to develop resistance to insulin and leptin; therefore these two neuropeptides are usually elevated in such groups. Studies in rats have found that exposure to overnutrition in the fetal or neonatal period can result in permanent changes in body fat mass and in the hypothalamic neuronal circuitry regulating appetite in the adult brain. Plagemann and colleagues investigated the effects of early postnatal overnutrition, as compared to normo- and undernutrition, on leptin and NPY in rats.[35] Those in the overnutrition group became overweight and developed hyperleptinemia and hyperinsulinemia while those in the undernutrition group had decreased leptin and insulin concentrations. In assessing NPY content, those rats that were in the undernutrition group had increased concentrations of NPY whereas the NPY content in the overnutrition group was not decreased or significantly different from the “normonutrition” rats. The authors concluded that these findings might indicate an acquired resistance of the hypothalamic NPY system to increased levels of insulin and/or leptin in early postnatally overfed rats. Interestingly and perhaps somewhat related to findings of the aforementioned study, many believe that the dominant role of leptin is not as an appetite suppressor in the prevention of obesity, a relatively new problem in human history, but as a signal to avoid starvation.[36] This alternate interpretation suggests that reduced levels of leptin confer an evolutionary protective mechanism in times of limited food supply as starvation decreases leptin levels leading to increased appetite. Another rat study looked at the effects of exposure to high

glucose milk from diabetic dams and found that offspring of control dams cross-fostered to diabetic dams developed early postnatal growth delay and showed structural and functional hypothalamic "malprogramming" as it relates to appetite stimulation and suppression.[37] Using immunocytochemical staining, exposure to milk from a diabetic dam resulted in an up-regulation of the appetite stimulants NPY and AgRP and a down-regulation of the appetite suppressant MSH. Morphometric analyses demonstrated increased total number of neurons in the paraventricular nucleus[37] which is involved in body weight control and the regulation of blood pressure[38,39]. These studies suggest early nutritional exposures may impact the development of the hypothalamic appetite regulatory system which is critically involved in the lifelong regulation of appetite, body composition, and metabolism.

Hypothalamic-Pituitary-Adrenal Axis

Experimental data in animals and observational data in humans have suggested that an alteration in the set point of the hypothalamic-pituitary-adrenal (HPA) axis is an important long-term change that occurs in association with reduced fetal growth. Pregnant animal models have shown that exposure to a variety of stressors, including nutrient restriction, results in the birth of offspring with elevated basal or stress-induced glucocorticoid secretion.[40,41] It is thought that maternal exposure to stressors during pregnancy subsequently leads to excessive fetal exposure to glucocorticoid hormone resulting in persistent alterations in HPA axis activity. In support of this hypothesis are studies conducted in rats in which fetoplacental exposure to maternally administered dexamethasone throughout gestation reduced birth weight and produced hypertensive adult offspring. In one such study, dexamethasone administration to pregnant rats on

days 15-20 of gestation resulted in offspring with reduced birth weight, elevated blood pressure, increased basal plasma corticosterone, lower mRNA expression of hippocampal neuronal glucocorticoid receptor, and decreased gene expression of hippocampal mineralocorticoid receptor.[42] In a study in pregnant sheep, severe brief undernutrition in late gestation altered the function of the HPA axis of adult offspring such that those exposed to gestational undernourishment for 10 days demonstrated altered steroid levels including an increased adrenocorticotropic hormone (ACTH) response as compared to offspring from dams fed *ad libitum* or offspring from dams undernourished for 20 days.[43] In another sheep study, dams were treated with dexamethasone over 2 days; treatment group 1 was treated during 22–29 days of pregnancy and treatment group 2 was treated during 59–66 days of pregnancy (term 145 days). Offspring from dams that had received dexamethasone during 22–29 days gestation, but not days 59–66 of gestation had elevated blood pressures. Such studies suggest that excess glucocorticoid exposure at certain developmental stages or "windows" programs higher blood pressure.[44]

Summary

In summary, the DOHaD hypothesis attempts to link an adverse intrauterine environment with a higher incidence of chronic disease in adult life. As mentioned in the introduction, chronic diseases such as type 2 diabetes and cardiovascular disease are by far the leading cause of mortality in the world. This avenue of research carries the hope that certain chronic diseases of adulthood may be prevented or mitigated by appropriate nutritional interventions in early life. The studies presented in this

dissertation were conducted to further establish a link between early development and adult health and to explore the mechanisms behind the DOHaD hypothesis.

Metabolic Syndrome

The metabolic syndrome has become a major public health challenge with an estimated 22% of US adults having this condition.[21] Although the disorder is defined in various ways, the ultimate importance of recognizing this syndrome is that it helps identify individuals at high risk for both type 2 diabetes and cardiovascular disease. The cause of the syndrome remains obscure but the pathophysiology seems to be largely attributable to insulin resistance, excessive flux of fatty acids, and a chronic proinflammatory state.[45] There is no specific treatment for metabolic syndrome. Therapeutics include lifestyle changes (e.g., weight reduction and increased physical activity) and pharmaceutical agents, but as with all diseases, prevention would be preferred. New theories have suggested that certain adverse exposures during the fetal/perinatal period could be contributing to the development of metabolic syndrome and thus this early life stage may offer an attractive point in the disease process for intervention strategies.

A consensus group for the International Diabetes Federation defines metabolic syndrome as central obesity, plus any two of the following: raised triglycerides, reduced HDL-cholesterol, raised fasting plasma glucose, and raised blood pressure.[46] The consensus group also recommends that additional criteria should be part of further research into the metabolic syndrome, including tomographic assessment of visceral adiposity and liver fat, biomarkers of adipose tissue (leptin, adiponectin), and glucose

tolerance testing. The defining parameters of the metabolic syndrome (i.e., obesity, dyslipidemia, insulin resistance/hyperglycemia, and hypertension) are discussed below.

Obesity

Surprisingly, the obesity component of the metabolic syndrome was omitted when the syndrome was first described in 1988.[47] Obesity can be measured by various methods and has been defined as a body mass index (BMI) of ≥ 30 kg/m^2 , percent body fat ≥ 25% in men and ≥ 35% in women, and a waist circumference of ≥ 102 cm in men and ≥ 88 cm in women.[48] Waist circumference is frequently used in clinical practice given its ease of assessment and that excess central (intra-abdominal) fat is most strongly associated with the metabolic syndrome as well as type 2 diabetes and cardiovascular disease.[49,50] Once regarded as merely a heat source and storage site for excess free fatty acids (FFAs), fat, primarily abdominal fat, is now recognized as a central player in the metabolic syndrome due to its ability to secrete FFAs, proinflammatory cytokines, and hormones such as leptin and adiponectin.[50-52]

Free fatty acids are produced when there is lipolysis of adipose tissue, and obese persons release increased amounts of FFAs into circulation.[53] Free fatty acids can be used as an energy source; however when the amount of FFAs exceeds tissue needs, they start to accumulate, specifically in muscle and liver leading to insulin resistance and, in the liver, also to fatty liver disease.[50] Adipose tissue can also synthesize and secrete the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Levels of these so-called “adipokines” are higher in the obese than the non-obese.[50] In the acute phase response, TNF-α and IL-6 are key inflammatory mediators that contribute to leukocyte activation, vascular changes, fever,

etc. In the low-grade chronic inflammatory condition of obesity, these cytokines may have similar functions which could be causing damage and contributing to additional maladies. Both adipokines can also stimulate lipolysis resulting in increased circulating FFAs which, as mentioned previously, contribute to insulin resistance.[54,55]

Adipose tissue is also an endocrine organ producing the hormones leptin and adiponectin. In 1994 the discovery of leptin, the so-called “satiety hormone”, was greeted with much excitement as many scientists believed that it could be the cure for obesity.[56] This excitement soon diminished when it was found that overweight and obese people developed leptin resistance and, as such, treatment with leptin had minimal to no effect on weight loss in these patients. Incidentally, hyperleptinemia is now recognized as a marker for the metabolic syndrome. Leptin exerts its effects on energy balance mainly by acting in the brain, specifically the leptin-sensitive neurons in the hypothalamic portion, where it inhibits appetite by counteracting the effects of the feeding stimulants NPY and anandamide as well as promoting the synthesis of the appetite suppressant MSH.[34] In 1995 and 1996, four different groups working independently discovered adiponectin.[57] Circulating levels of most adipocyte-derived hormones and cytokines, including leptin, TNF-α, and IL-6, are positively correlated to body adiposity. In contrast, circulating adiponectin levels are reduced in obese animals and humans, and increase after weight loss. One proposed explanation is that adiponectin is primarily produced by intra-abdominal fat stores and as these adipocytes become enlarged/triglyceride-filled, as occurs in overweight/obesity, they produce less adiponectin. Adiponectin has 2 receptors: AdipoR1 is mainly expressed in muscles and AdipoR2 is mainly expressed in liver.[57] Overall, adiponectin has anti-inflammatory, anti-atherosclerotic, and anti-diabetic effects. The administration of adiponectin has been

accompanied by lower blood glucose levels as well as increased insulin sensitivity, whereas reduced expression and lower circulating levels of adiponectin are associated with insulin resistance and development of type 2 diabetes. Hypoadiponectinemia is now recognized as an independent risk factor for metabolic syndrome and administration of adiponectin is a potential pharmaceutical treatment for obesity and type 2 diabetes.[58]

Dyslipidemia

Dyslipidemia is an abnormal amount of lipid in the blood. The combination of raised triglyceride levels, elevated low density lipoprotein (LDL) cholesterol particles, and low levels of high density lipoprotein (HDL) cholesterol has become referred to as the lipid triad or the atherogenic lipoprotein phenotype.[45,59] The function of lipoproteins is to transport lipids in the bloodstream. Lipoproteins are composed of an outer group of hydrophilic phospholipids and apolipoproteins (which function as structural components as well as ligands for surface receptors) and an inner core of triglyceride-fats and cholesterol.[60] There are 5 major groups of lipoproteins which, in order of size largest to smallest, are chylomicrons, very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, and HDL.[61] Much of the focus of atherosclerosis and heart disease studies has been on the role of LDL and its oxidized forms, but in fact LDL is a metabolic end product of triglycerides and is regulated by HDL cholesterol, so all lipids are involved in lesion development.[62]

A triglyceride level of < 150 mg/dL is considered normal. A triglyceride is composed of a glycerol backbone and 3 fatty acids. Exogenous dietary triglycerides enter the intestine where lipases and bile split them in a process called lipolysis so that

they can be absorbed by intestinal enterocytes. They are rebuilt and packaged with cholesterol to form chylomicrons which are transported in blood to various tissues which capture the chylomicron, releasing the triglycerides to be used as a source of energy. Fat and liver cells can synthesize and store triglycerides. The liver has the additional ability to use triglycerides to form VLDLs which are secreted into the bloodstream to deliver endogenous triglycerides to various tissues to be used as energy.[63] Although triglycerides, in their full form, are not a part of the atheromatous plaque, many prospective and case-controlled studies have shown that serum triglyceride levels are positively associated with the risk of coronary artery disease, even after adjusting for HDL-cholesterol and other risk factors.[64,65] Potential mechanisms include oxidative susceptibility of triglyceride-rich lipoproteins as well as partially hydrolyzed lipoproteins derived from chylomicrons and VLDL catabolism.[62,66]

Low density lipoprotein particles are often called the “bad cholesterol” lipoprotein because they collect cholesterol from the liver and deliver it to the cells of the body. Blood tests typically report LDL cholesterol, the amount of cholesterol contained in LDL, as a clinically useful estimate of the amount of low density lipoproteins present. A LDL cholesterol level of < 150 mg/dL is considered normal. Low density lipoprotein particles are formed as VLDLs lose triglyceride through the action of lipoprotein lipase and become smaller and denser, containing a higher proportion of cholesterol. Foods high in saturated and trans fats are known to contribute to elevated levels of LDL cholesterol.[67] Low density lipoproteins appear harmless until they are within the blood vessel wall and are oxidized by free radicals. Oxidized LDL contributes to atherosclerosis by 1) facilitating its uptake by macrophages via the scavenger receptors, 2) attracting

monocytes/macrophages to the subendothelium, and 3) stimulating the production of monocyte chemoattractant protein and other inflammatory cytokines.[68]

High density lipoprotein particles are often called the “good cholesterol” lipoprotein because they collect triglycerides and cholesterol from the blood and various tissues and bring it back to the liver for excretion or re-utilization, and thus protect against cardiovascular disease.[69] As with LDL, blood tests typically report HDL cholesterol, the amount of cholesterol contained in HDL, as a clinically useful estimate of the amount of high density lipoproteins present. A HDL cholesterol of ≥ 60 mg/dL is considered protective against heart disease.[70] High density lipoproteins are the smallest of the lipoproteins and are the densest because they contain the highest proportion of protein to cholesterol. Lipid-poor HDL is synthesized and secreted by the liver and intestine to collect triglycerides and cholesterol from blood and tissues and bring them back to the liver.[63] High-density lipoprotein cholesterol concentrations are inversely associated with coronary heart disease.[71]

Insulin Resistance

Insulin resistance is perhaps the most accepted and unifying component of the metabolic syndrome.[45] An insulin resistant individual can have normal, low, or high levels of insulin largely based on the duration and severity of overstimulation of pancreatic β cells.[72] The term “insulin resistance” is used to denote the lack of response to insulin’s efforts to remove glucose from the blood and utilize and store it in cells, specifically the target tissues of fat, muscle, and liver.

Although the primary role of insulin is to maintain normal blood glucose levels, insulin has wide-ranging physiological processes including lipid metabolism. Insulin is a

critical regulator of virtually all aspects of adipocyte biology. The intimate relationship between insulin and adipose tissue is disrupted in an insulin resistant individual and is illustrated by the self-perpetuating state of insulin resistance.[73] Insulin promotes adipocyte triglyceride stores by promoting the differentiation of preadipocytes to adipocytes, stimulating glucose transport and triglyceride synthesis (lipogenesis), and inhibiting lipolysis. Insulin also increases uptake of FFAs derived from circulating lipoproteins by stimulating lipoprotein lipase activity in adipose tissue.[73] In relating insulin's role in adipocyte metabolism, insufficient insulin output leads to increased lipolysis, elevated FFAs, and reduced uptake of circulating FFAs. Excessive FFAs strongly contribute to insulin resistance by way of decreasing glucose utilization in muscle and stimulating hepatic glucose production.[74]

Obesity can also cause elevations in FFAs as well as the proinflammatory cytokines TNF-α and IL-6 which can directly interfere with insulin signaling. Normal insulin signaling involves a cascade of events initiated by insulin binding to its cell surface receptor, followed by receptor autophosphorylation and activation of receptor tyrosine kinases, resulting in stimulatory *tyrosine phosphorylation* of IRS. Binding of IRS to the regulatory subunit of phosphoinositide 3-kinase (PI3K) results in *activation of PI3K* which is necessary for insulin action on glucose transport.[75,76] Free fatty acids, TNF-α, and IL-6 can lead to inhibitory *serine phosphorylation* of IRS-1 and the *activation of c-Jun N-terminal kinase (JNK)* resulting in the prevention of insulin signaling.[54,55]

The effect of insulin resistance is impaired glucose tolerance which is the inability to properly metabolize glucose resulting in hyperglycemia. According to criteria of the American Diabetes Association and the World Health Organization (WHO), impaired

glucose tolerance is defined as either a fasting plasma glucose level ≥ 100 mg/dL to < 126 mg/dL or a two-hour glucose level of ≥ 140 to < 200 mg/dL following an oral glucose load of typically 75 g of glucose (i.e., a glucose tolerance test).[77,78] The ultimate importance of insulin resistance and impaired glucose tolerance is that they are strong indicators of type 2 diabetes. The WHO defines diabetes as a fasting plasma glucose of ≥ 126 mg/dL or a two-hour glucose level ≥ 200 mg/dL following a glucose load.[77] Diabetes is a serious, potentially fatal, disease that is the leading cause of kidney failure, non-traumatic low limb amputations, and new cases of blindness in US adults. Adults with diabetes are at 2 to 4 times higher risk for stroke and have heart disease death rates about 2 to 4 times higher than adults without diabetes.[79]

Hypertension

Normal blood pressure is less than 120 over 80 (120/80 mmHg). Though hypertension remains somewhat mysterious in that its etiology in most cases is undetermined, it has been linked to certain risk factors including insulin resistance and endothelial dysfunction. Insulin resistance and hypertension are interrelated by several different mechanisms. For example, insulin has been found to have vasodilatory effects. Several reports have demonstrated that circulating insulin concentrations increase blood flow to skeletal muscle, and thus insulin and glucose delivery.[80,81] The mechanism by which insulin causes vasodilaton in skeletal muscle vasculature is unknown, but insulin has been demonstrated to modulate the synthesis and release of endothelium-derived nitric oxide (NO), which is a powerful vasodilator.[82] Insulin also has sodium regulatory effects in the kidney. In multiple studies, increased reabsorption of renal sodium is consistently and independently associated with the metabolic pattern of

insulin resistance, suggesting that renal sodium handling is involved in the hypertension component of the metabolic syndrome.[83,84] Also hypertension is known to contribute to endothelial damage and vascular disease. The vascular endothelial lining has important homeostatic functions for the regulation of vascular tone and structure. Under normal conditions, endothelial cells are able to synthesize and secrete a large spectrum of vaso-protective substances, the most characterized of which is NO, which functions in vasodilation and prevention of leukocyte and platelet adhesion.[85] In disease conditions the endothelium undergoes functional and structural alterations and loses its protective capabilities, thus leading to vasoconstriction, adhesion of leukocytes and platelets, and release of substances and mediators that further damage the vessel wall.[85,86] A large body of evidence demonstrates that the presence of endothelial dysfunction is a hallmark of the hypertensive patient[87,88], but the designation of endothelial dysfunction as a pathogenic mechanism for hypertension is questionable as it may be more of a consequence rather than a cause of the disease process.[89]

Discussion and Summary

Both epidemiological and experimental studies have revealed that perinatal factors play a role in the origin of the metabolic syndrome. Epidemiological evidence includes the Rancho Bernardo Study which examined the association between low birth weight and metabolic syndrome by measuring metabolic and anthropometric variables in 303 postmenopausal Caucasian women aged 50-84 years and comparing them to documented birth weights. Metabolic syndrome, defined in this study as the simultaneous presence of hypertension, dyslipidemia, and abnormal glucose tolerance, was present in 7.9% of these women. Compared with women in the highest birth

weights (8.1-13.0 lb, mean 9.4 lb), those in the lowest birth weights (2.5-6.8 lb, mean 5.5 lb) exhibited an increased prevalence (12.0 vs. 4.3%) and 2.41 times the risk of developing metabolic syndrome.[90] Women with a heavy birth weight had an increased risk of adult obesity; however women with a low birth weight who became adults in the highest tier of BMI (> 25.2kg/m^2) or waist circumference (> 80.7 cm) had the highest prevalence of the metabolic syndrome at 30%.[90] A study of adult offspring from women with diet-treated gestational diabetes (GDM) or type 1 diabetes found that the risk of overweight was doubled in the offspring of women with GDM or type 1 diabetes (compared with offspring from a background population), moreover, the risk of metabolic syndrome was 4- and 2.5-fold increased, respectively. [91] Offspring risk of the metabolic syndrome increased significantly with increasing maternal fasting blood glucose as well as 2 hour blood glucose following an oral glucose load (i.e., glucose tolerance test). Based on these findings, the authors concluded that intrauterine exposure to hyperglycemia, in addition to other factors, contributes to the pathogenesis of overweight and the metabolic syndrome.[91]

Using C57BL/6 mice, our laboratory studied the effects of gestational and lactational exposure to a high saturated fat diet and found that such exposure resulted in the development of obesity, hyperglycemia, insulin resistance, and hypertension in the adult offspring.[33] The study presented in Chapter IV of this dissertation sought to further validate the findings from this pilot study and to determine if the dietary habits of the adult offspring could exacerbate or mitigate certain parameters of the metabolic syndrome.

Hepatic Lipidosis

Metabolic hepatic disease is generally manifested as vacuolar change (lipid and/or glycogen type) and hepatic lipidosis, also known as fatty liver disease, is the most common condition of the liver seen at postmortem examination.[92,93] In human medicine, the term steatosis is used more commonly than lipidosis and alcoholism is a common cause; however fatty liver can occur in the absence of excess alcohol consumption and is termed nonalcoholic fatty liver disease (NAFLD). The pathogenesis of NAFLD remains unclear. It is proposed that an aberration in fatty acid and triglyceride metabolism leads to excess hepatic lipid accumulation.[94] Normally, fatty acids enter the liver where they are either utilized for energy in the hepatic mitochondria or converted to triglycerides that are further processed into lipoproteins which are actively secreted into the plasma as VLDL. Any disturbance in energy, supply of structural components to form lipoproteins, or physical disruption of the organelles involved in synthesis, assembly, and secretion of lipoproteins has the potential to render the liver incapable of ridding itself of fatty acids resulting in accumulation of triglyceride globules in the hepatocyte cytoplasm.[92]

The amount of fat in the liver depends on the balance between the processes of delivery, utilization, and removal. Hyperlipidemia is a known risk factor for fatty infiltration of the liver.[95-97] In a study demonstrating the association between hyperlipidemic states and fatty liver, ultrasound findings of 95 adult human patients referred to a clinic for management of hyperlipidemia (hypercholesterolemia, hypertriglyceridemia, or mixed hyperlipidemia) revealed that 47 patients (50%) had diffuse fatty liver.[98] The majority of patients with hypercholesterolemia had normal ultrasounds, whereas severe hypertriglyceridemia and mixed hyperlipidemia were frequently associated with radiologic evidence of fatty liver. Using a model that

incorporated known risk factors for fatty liver (e.g., , triglycerides, diabetes, obesity, age, etc.), diabetes was the only risk factor other than hypertriglyceridemia that was significantly associated with fatty infiltration.[98]

Pregnancy state can be a contributing factor to hepatic lipidosis. In veterinary medicine, fatty liver disease is frequently encountered in cows and other ruminant species and is attributed to negative energy balance due to insufficient feed intake to meet the nutritional demands for maintenance of late pregnancy and early lactation.[99] Commonly referred to as fat cow syndrome, fatty liver disease is typically seen in cows that were overfed in the dry (non-lactating) period resulting in overly fat cows at calving.[99] The pathogenesis follows that negative energy balance results in mobilization of the body's fat reserves in an effort to rebalance the deficit resulting in the release of FFAs into the blood which subsequently accumulate in the liver. Cows which calve in fat to very fat body condition are more likely to develop fatty liver likely because their feed intake is depressed so as to not match the high energy requirements that arise during late pregnancy/early lactation.

Humans also experience a pregnancy-associated fatty liver disease termed acute fatty liver of pregnancy (AFLP).[100] This is a rare disease of unknown etiology that occurs during the third trimester. In many cases it is believed to be caused by mitochondrial dysfunction resulting in abnormal β-oxidation of fatty acids. Beta-oxidation of fatty acids is the process by which fatty acids are broken down in the mitochondria to be used in the tricarboxylic acid (TCA) cycle for energy and, interestingly, it has been found to be decreased in pregnant mice.[101] This finding led researchers to suspect that the effects of pregnancy on hepatic fatty acid oxidation may be due to the female sex

hormones estrogen and progesterone. To test their hypotheses, estradiol and progesterone were administered to non-pregnant mice, this resulted in a decrease in β-oxidation of fatty acids and in the activity of the TCA cycle, as well as ultrastructural lesions of mitochondria and decreased recovery of mitochondrial proteins.[102]

No matter the ultimate cause of fatty liver, when occurring in pregnancy, it can be associated with maternal and fetal mortality. Using data from the human condition AFLP, as recently as the 1980s AFLP carried a mortality rate for both mother and fetus of about 85%.[103] A more recent report indicates that death, due to the disease and its associated and complicating conditions, has decreased dramatically, but is still substantial with a fetal/perinatal mortality rate of 9-23% and a maternal mortality rate of 7-18%.[104] In a clinicopathologic study of 35 women with AFLP, 22 of the cases were diagnosed at autopsy, and of these, 12 had stillbirths, 8 had live births, and the status of 2 of the offspring was not established.[105] Delivery of the fetus, regardless of gestational age, is the primary treatment for AFLP once the diagnosis has been made. Infants that survive post-delivery face the potentially fatal complications from premature delivery, fetal distress, asphyxia, and hypoxia.[106] The study presented in Chapter III proposes that dietary-induced hyperlipidemia, along with pregnancy-associated factors, resulted in fatty liver in the dams and, subsequently, reduced litter sizes and increased early mortality in the offspring.

Cardiovascular Disease

The WHO lists cardiovascular disease (CVD) as the number one cause of death globally. This is a group of diseases that occurs almost equally in men and women and that took the lives of an estimated 17.3 million people in 2008. Behavioral risk factors,

mainly an unhealthy diet and physical inactivity, are largely responsible, but there are also a number of underlying determinants.[107] Maternal-fetal health is becoming increasingly recognized as potentially playing a role in the development of CVD.

Cardiovascular disease is actually a group of diseases that involve the heart or blood vessels. Although the causes of CVD are diverse, the chronic inflammatory process of atherosclerosis is the most common.[108] Various methods are employed to diagnose CVD including electrocardiogram, echocardiogram, and cardiac catheterization with coronary angiography[109]; however frequently the diagnosis is made at postmortem examination. In veterinary medicine atherosclerosis is relatively rare. It becomes important in laboratory animal medicine where animals serve as models for human disease.[92] In humans, atherosclerosis and its complications of stenosis, thrombosis with infarction (e.g., heart attack and stroke), and peripheral vascular disease are major causes of morbidity and mortality, particularly in industrialized societies. The essential lesion is the atheromatous plaque which has 3 principal components: 1) cells, including smooth muscle cells, macrophages, and T cells, 2) extracellular matrix (ECM), including collagen, elastic fibers, and proteoglycans, and 3) intra- and extracellular lipid.[92,110] Called the "response-to-injury hypothesis", atherosclerosis is viewed as a chronic inflammatory and healing response of the arterial wall to endothelial injury. The pathogenesis of atherosclerosis is as follows: endothelial "injury" (caused by hyperlipidemia, hypertension, diabetes, smoking, toxins, immune reactions, etc.)→ increased vascular permeability→ monocyte adhesion, platelet adhesion, and accumulation of lipoproteins (mainly LDL and its oxidized forms) +/- calcium in the vessel walls→ monocyte adherence to the endothelium, migration into the tunica intima of the vessel wall, and transformation into macrophages and foam

cells (macrophages or smooth muscle cells that contain lipid)→ factor release from activated platelets, macrophages, and vascular wall cells induce smooth muscle recruitment, proliferation, and ECM production→ arterial wall thickening/atherosclerosis.[92,110]

Barker's fetal origins hypothesis was based on the discovery of a link between lower birth weights and a higher risk of CVD later in life[6] and much of the focus of this area of study has been on maternal-fetal undernutrition as the cause. The association between birth weight and heart disease came to light by way of conducting a search of the archives and records offices of Britain which led to the discovery of three important groups- Hertfordshire, Preston, and Sheffield. Sixteen thousand men and women born in Hertfordshire, England between 1911 and 1930 were traced from birth to the present. The results indicated a correlation between birth weight of 5.5 pounds or less and increased risk for death due to CVD.[111] The study in Sheffield supported the findings of the Hertfordshire Study in that those who were small at birth because they failed to grow, rather than because they were born early, were at increased risk of CVD.[111] Critics of Barker's early work argued that Barker failed to take into account confounding variables, particularly that those born into poor socioeconomic circumstances often remain in those circumstances into adulthood.[112] Rich-Edwards and colleagues analyzed data from the Nurses' Health Study in the United States to specifically address the association between birth weight and adult CVD while controlling for potential confounders such as socioeconomic group and adult lifestyle.[113] In this study, there were 1,216 cases of non-fatal CVD, including myocardial infarction and stroke. Among women who were singletons and had been born full term, the relative risks adjusted for several risk factors (e.g., cigarette smoking, alcohol use, and socioeconomic status)

decreased steadily as weight increased such that the relative risk for CVD was 1.49 for birth weight < 5 lb and 0.68 for birth weight > 10 lb.[113]

As mentioned previously, the majority of studies on the relationship between maternal nutrition, birth weight, and CVD have focused on maternal-fetal undernutrition.[114] However, with the current problem of nutritional excesses leading to obesity and obesity-related conditions, which include CVD, it would seem more pertinent to focus on maternal-fetal overnutrition, specifically fats. The study presented in Chapter III employed a diet rich in unhealthy fats and cholesterol to induce lesions of CVD in the pregnant dam and to determine if such lesions would also develop in their offspring.

Osteoporosis

Osteoporosis is a skeletal disorder characterized by low bone mass and deterioration of the micro-architecture producing increased bone fragility and susceptibility to fracture.[115] The disease is most commonly diagnosed via bone mineral density (BMD) test in which a dual-energy x-ray absorptiometry (DEXA) scan is performed and results are reported as a “T score” and “Z score”. The T score compares the patient’s bone density with that of healthy young women and the Z score compares the patient’s bone density with that of other people of the same age, gender, and race. In either score, a negative number means that bones are thinner than the standard. The more negative the number, the higher the risk of a bone fracture.[116] Osteoporosis causes 8.9 million fractures each year, of which more than 4.5 million occur in the Americas and Europe. The lifetime risk for such a fracture is estimated to be in the order of 30 to 40% in developed countries. This disease can deprive people of their mobility

leaving those affected bedridden and facing serious complications.[116] The disease commonly occurs with aging and accelerates at menopause, but can also develop secondary to certain medical conditions and medications that disrupt normal bone remodeling. In menopause, it is the loss of ovarian hormone production that confers the major portion of risk to women. Although the precise mechanisms are still not understood, steroids, especially estrogen, regulate the bone-remodeling process. Estrogen deficiency causes an increased rate of skeletal remodeling with the rate of bone resorption exceeding the rate of bone formation.[117] Although generally considered a disease of old age, the health of the skeleton is a reflection of everything that has happened to it from its uterine existence to the time of senility. Achieving a high adult peak bone mass is protective against late-life fragility fractures; therefore there is strong interest in discovering all factors contributing to optimal peak bone health.[118] Osteoporosis is a disease with a multifactorial etiology and new theories suggest that adverse environmental exposures in early life can impair skeletal development and contribute to permanent bone lesions.

A growing body of evidence, including data produced by our laboratory, supports the intrauterine and postnatal environment as a large determinant of adult bone health including bone mass, density, and architecture. Cooper and colleagues performed an longitudinal epidemiological study, in which 153 women born during 1968-1969 were traced and studied in 1990, aiming to identify the relationships between childhood growth, lifestyle, and peak bone mass.[119] Data from their growth in childhood were compared to current bone mineral measurements and there was a statistically significant positive correlation between body weight at 1 year of age and bone mineral

content of the lumbar spine and femoral neck. The authors concluded that infant growth determines the size of the skeletal envelope/framework and its trajectory is established by 1 year of age.[119] Our laboratory has performed experimental studies using a mouse model to evaluate the impact of maternal high saturated fat diet on bone health of the offspring. In one such project published in 2009, bone health was examined by means of micro-computed tomography. The offspring exposed to a high saturated fat diet *in utero* and until the age of weaning exhibited lesions reminiscent of osteoporosis as characterized by lower average bone mineral density at 6 months of age and dysregulation of distal femoral trabecular architecture at 12 months of age.[33] Similar research published in 2010 and conducted by the Bone and Joint Research Group at the University of Southampton School of Medicine found that 7 month old offspring from dams fed a high fat diet showed increased adiposity in addition to altered trabecular structure in the femur as compared to offspring of dams fed a standard rodent diet. Both studies suggested altered osteogenic programming during the period of developmental plasticity as a possible part of the disease pathogenesis.[33,120] Our laboratory also proposed and tested elevated oxidative stress as a possible mechanism contributing to the skeletal aberrations, and demonstrated that maternal antioxidant supplementation significantly protected against diet-induced reduction of trabecular connectivity density in the adult offspring.[33] This is yet one proposed mechanism and, given the lack of an accepted complete disease pathogenesis linking maternal high fat diet and the development of osteoporosis in the adult offspring, it is clear that more research is needed. In Chapter III of this dissertation, morphologic analysis of the skeleton was performed to determine the effects of gestational and lactational exposure to an atherogenic high fat diet on bone.

Hypotheses

The research presented in this dissertation was conducted to more fully understand the role and effects of maternal diet before, during, and after pregnancy on lifelong health of the offspring as well as how eating habits of the adult offspring mitigate or exacerbate disease conditions initiated in early life. Our overall hypotheses were: 1) a high fat diet will negatively impact maternal health and birth outcome and 2) offspring born to mothers consuming such a diet will show morphologic and biochemical alterations in metabolic and bone health. With this more complete understanding of the contribution of maternal diet to birth outcome and lifelong health of the offspring comes the hope to reverse this advancing trend of metabolic and bone disease through improved prenatal care, education, and dietary interventions.

CHAPTER II: Materials, Equipment, and Diagnostic Techniques Utilized

A mouse model of human disease was utilized in this dissertation. All experiments were approved by the Virginia Tech Animal Care and Use Committee.

Mice: Generally speaking, the use of mice in research provides numerous advantages over the use of larger mammals. Mice require less space, housing costs are less, and restraint and handling are relatively easy. Yet the more important reason mice were used for this research was that they have particular attributes relating to the pathophysiology and expected outcomes of the studies presented in this dissertation. In general, mice are commonly used in reproductive and obstetric studies because of the strong similarities between human and mouse placentas.[121-123] Both species have hemochorial placentation in which maternal blood is in direct contact with fetal trophoblast. This region is the site of feto-maternal exchange and is called the villous tree in humans and the labyrinth in mice. In both species this exchange site is involved in the transport of gases, nutrients, and wastes between maternal and fetal circulations throughout the majority of pregnancy, thus is critically important in shaping fetal growth and development.[122] In addition to the aforementioned advantages of mice in research, the C57BL/6 strain of mice in particular has many qualities that make them ideal for this avenue of research. The key reasons the C57BL/6 strain was utilized are: 1) isogenicity, 2) susceptibility to diet-induced atherosclerosis, and 3) propensity to develop osteoporosis. Firstly, the C57BL/6 strain is an inbred mouse strain and as such is considered isogenic. Inbred strains are produced using at least 20 consecutive generations of sister x brother or parent x offspring matings. In other words they have

had at least 20 generations of inbreeding, making inbred strains as genetically alike as possible, similar to identical twins, being homozygous at virtually all of their loci.[124] The benefit of using isogenic mice is that the outcomes of certain experimental conditions (e.g., diet) are not influenced by genetic variation. Secondly, no known inbred mouse strain spontaneously develops atherosclerosis, but the C57BL/6 strain is considered the most susceptible to diet-induced atherosclerosis.[125-127] Lastly, aging C57BL/6 mice are known to develop osteoporosis having low cortical bone density[128] as well as a decline in trabecular bone volume and deterioration of trabecular bone architecture[129].

Diets: All diets were in pellet form and were formulated to meet or exceed the nutritional requirements of mice. For each study, mice in control groups were fed a standard rodent diet (Harlan Teklad Global Diet 2018, Madison, WI). In Chapter III, mice in the experimental group were fed an atherogenic high fat diet (Harlan Teklad Global Diet TD.10121, Madison, WI) and in Chapter IV, mice in the experimental group were fed a high fat diet (Harlan Teklad Global Diet TD.06414, Madison, WI). Experimental diets were formulated to reflect the macronutrient content of a fatty fast food diet with total energy from fats within the upper limit of intake estimates for people, and although high, such intake is not unrealistic and was chosen to permit detection of effects. Studies evaluating diet-induced obesity and obesity-related diseases in the rodent model typically require a prolonged course of treatment with a diet that has 40-60% of energy derived from fat.[130,131] Along similar lines, although the C57BL/6 strain is considered the most susceptible to diet-induced atherosclerosis, lesion development necessitates the use of modified diets that promote hyperlipidemia.[126] Diets used most commonly are enriched in saturated fat, cholesterol, and cholate. In the present study,

diets rich in fat and/or fats and cholesterol were used to address the aforementioned challenges of studying obesity and atherosclerosis in rodents.

Micro-Computed Tomography (Figure 1): For the study presented in Chapter III, morphologic assessment of bone was achieved through whole body micro-computed tomography (micro-CT) scans (MicroXCT-400, Xradia, Pleasanton, CA) as well as scans of individual femurs (VivaCT 40, Scanco Medical, Bassersdorf, Switzerland). Image data for the femurs were further manipulated using medical imaging software (OsiriX, version 2.6, Medical Imaging Software, Los Angeles, CA) to determine bone volume, bone density, connectivity, trabecular number, trabecular thickness, and trabecular spacing. The femurs were also submitted for routine histologic processing, after undergoing decalcification, and the slides examined via light microscopy (see following section). Rodents serve as important models of human development and disease. The use of advanced imaging is vital in understanding these models. The introduction of computed tomography (CT) in the early 1970s revolutionized medical radiology.[132] For the first time, clinicians were able to obtain high-quality tomographic (cross-sectional) images of the whole body, including bone and internal structures. Computed tomography uses X-rays to produce “slices” of the body which can then be reconstructed into a three-dimensional image without destroying the original model.[132] With clinical CT scanners the voxel resolution is ~ 1 mm^3.[133] With micro-CT, the machine is much smaller in design compared to the human version, thus more ideal for small animals, and the voxel resolution is in the micrometer range (10^{-3} mm^3 or 10 µm)[133], making it eminently suited for imaging smaller sized rodent anatomical structures.[134] In the micro-CT setup, the object of interest is placed in the center of the

machine and a gantry, carrying the X-ray tube and detector, is rotated around it. In the X-ray tube, an electron beam is produced on the tip of a hairpin tungsten filament and focused by several magnetic lenses onto a focal spot of 1-10 µm on a transmission target.[134] The detector system records the ionizing radiation and transforms it into an electrical signal that is then amplified and converted to a digital form.[135] Given that this imaging modality is nondestructive, it allows visualization of tissue architecture without compromising the ability to pursue subsequent histological analysis.[136]

Histopathology: For the study presented in Chapter III, morphologic assessment of tissues was performed by histologic examination. Tissues collected from dams and offspring included those routinely taken at necropsy such as heart and liver as well as tissues of special interest to include femur. All tissues were fixed and stored in 10**%** neutral buffered formalin. Routine histologic processing, embedding, sectioning, and staining were performed by the Histology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). The resulting slides, containing 4 µm thick hematoxylin and eosin stained sections, were evaluated by light microscopy. Prior to histologic processing, femurs had the additional treatment of decalcification in which they were placed in decalcifying solution (Enhanced Decalcification Formulation, StatLab Medical Products Catalog #SL85-32, McKinney, TX) for 48 hours. To assess lipid content in the liver of a dam exhibiting signs of neurologic disease, a portion was frozen and submitted to the VMRCVM Histology Laboratory for 5 µm thick cryosectioning and special staining with Oil Red O. Histopathology is considered the gold standard in the diagnosis of many diseases including fatty liver disease.[137]

Nuclear Magnetic Resonance (Figure 2): Total body weight is a rather crude assessment of fatness in that it fails to distinguish between fat and lean mass. In the study presented in Chapter IV, nuclear magnetic resonance (NMR) (Bruker LF90 NMR Analyzer, Billerica, MA) was used to measure fat, lean tissue, and fluid in order to provide a more comprehensive evaluation of body composition. Nuclear magnetic resonance is a physical phenomenon in which nuclei of atoms have magnetic properties. The nuclei of different atoms absorb unique frequencies of electromagnetic radiation depending on their environment, thus by placing a sample in a strong magnetic field and observing the frequencies absorbed and later emitted again when the magnetic field is removed, it is possible to learn much about the sample's composition.[138] For the Bruker LF90 NMR analyzer, mice were individually placed in a restraining chamber. The restraining chamber was placed into the electromagnetic apparatus for approximately 1 to 3 minutes while the analyzer took measurements, which were performed in triplicate. The use of the Bruker LF90 NMR analyzer reflects an alternative approach to whole body micro-CT scans, which is how our laboratory attempted to gather information about body composition in past projects. Although micro-CT provides excellent images, particularly of bone, it poses numerous challenges such as the prolonged time animals and/or samples are required to stay in the machine and the inadequacy of soft tissue imaging and assessment.

Metabolic System (Figure 3): For the study presented in Chapter IV, a metabolic system [PhenoMaster/LabMaster, Technical Scientific Equipment (TSE) Systems, Chesterfield, MO] was employed to simultaneously measure energy expenditure,

physical activity, and water intake over a 48 hour period.[139] For this test, mice were maintained in their home cage which limited stress thereby allowing an accurate reflection of typical metabolic performance. The home cage was outfitted with monitoring devices and the data generated were recorded and analyzed by integrated hardware and software programs. Indirect calorimetry, which calculates energy produced by measuring O_2 consumption and CO_2 production, was used to determine the respiratory exchange ratio and energy expenditure. As an index of physical activity, infrared light-beam frames surrounding the home cage measured activity in three dimensions (X-Y-Z axis). Water intake was measured by high-precision weighing sensors attached on top of the cage lids.[139] The rational for employing this system in the present study was to provide a more complete assessment of the metabolic syndrome which is often correlated to fuel source type (carbohydrates or fats), energy intake and expenditure, and physical activity.[45,140]

Glucose Tolerance Test: In the study presented in Chapter IV, blood glucose levels in mg/dL were determined by performing a glucose tolerance test (GTT). Mice underwent an overnight, 6-12 hour, fast. The following morning, mice were placed into a restraining device that allows easy access to the tail. Blood from the lateral tail vein was taken via insertion and removal of a 25G needle to produce a drop of blood which was then placed on a hand-held glucometer (FreeStyle Lite glucose meter, Abbott Diabetes Care Inc., Alameda, CA). Tail blood was taken at time 0 (fasted measurement), and then a glucose load equaling 2g/kg was injected intraperitoneally. Tail blood glucose levels were obtained at 15, 30, 60, 90 and 120 minutes post-injection. Fasting glucose levels

and the GTT provide a measure of glucose homeostasis, which is lost in the pre-diabetic and diabetic state. The use of the GTT reflects an alternative approach to single non-fasted glucose measurements performed in past projects.

Enzyme-Linked Immunosorbent Assays: In the study presented in Chapter IV, enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum insulin (Catalog# 80-INSMSU-E01, ALPCO Diagnostics, Salem, NH) and leptin (Catalog #90030, Crystal Chem Inc., Downers Grove, IL) concentrations in ng/mL. Blood was collected after a 6-12 hour fast and at two time points, 7 and 18 weeks of age. Blood collected at the 7 week age mark was attained via the lateral saphenous vein and blood collected at the 18 week age mark was attained via cardiac puncture (see Chapter IV for description of blood collection procedures). Blood was allowed to clot for 30 minutes to 1 hour at room temperature, centrifuged for 20 minutes, and then the serum collected. Serum aliquots of 20 µl were stored at -80^{o}C until the time of performing the ELISA. An immunoassay is a technique for measuring the presence of a substance using an immunological reaction and is almost exclusively used to describe tests that exploit the reaction between antibody and antigen.[141] When an antibody combines with an antigen it forms an immune complex. For both insulin and leptin, the kits come with an antibody-coated plate and are considered a sandwich type immunoassay. The steps for this type of ELISA are: 1) the plate is coated with capture antibody, 2) samples (standards, controls, and samples/unknowns) are added and any antigen (e.g., insulin) present binds to capture antibody, 3) detecting antibody is added and binds to antigen, 4) enzyme-linked secondary antibody is added and binds to detecting antibody, 5)

chemical substrate is added and is converted by the enzyme into a color, and 6) absorbency of the sample is measured to determine quantity of antigen.[141] The ELISA kits were completed per manufacturer's instructions with standards, samples, and controls run in duplicate. Once completed, the absorbance was measured by a spectrophotometer (SpectraMax 250 and SoftMax Pro software, Molecular Devices, Sunnyvale, CA) at 450 nm. For both the insulin and leptin ELISA kits, the intensity of the color generated was directly proportional to the amount of the respective hormone in the sample. Results were determined by taking the mean absorbance of the sample and comparing it to the reference curve using a 4 parameter logistic curve fit equation. Knowing the levels of insulin and leptin are helpful in the diagnosis of the metabolic syndrome. Insulin resistance is a major component of the metabolic syndrome and is an early indicator of type 2 diabetes. Patients with insulin resistance typically have hyperinsulinemia together with normoglycemia or hyperglycemia.[142] Leptin is an adipose-derived hormone that plays a key role in regulating energy intake and expenditure. People who are overweight or obese tend to develop a resistance to leptin, thus have hyperleptinemia.

Lipid Panel: Serum aliquots of 300 to 500 µl were submitted to the VMRCVM Clinical Pathology Laboratory for determination of serum triglyceride and cholesterol levels using their Olympus AU480 Chemical Analyzer. For this analysis, most samples were submitted within hours of blood collection; however for samples attained over the weekend when the Clinical Pathology Laboratory was closed, the serum was stored at 2-8^{o}C and submitted within 48 hours. Serum lipid levels were compared to published clinical chemistry reference ranges for adult female C57BL/6 mice[143] as well as the

control group for the experiment. Blood and serum collection procedures were the same as those described in the previous section on the use of ELISA for serum insulin and leptin concentrations for offspring at 18 weeks of age. The justification for performing this test is that hyperlipidemia is a component of the metabolic syndrome and a risk factor for cardiovascular disease.

Figures

Fig. 1. Micro-computed tomography for the morphologic assessment of bone. The object of interest is placed in the center of the machine and a gantry, carrying the X-ray tube and detector, is rotated around it. X-rays produce cross-sectional images, or “slices”, which can be reconstructed into a three-dimensional image. These micro-CT units are located in the Institute for Critical Technology and Applied Science (ICTAS) Center for Biomedical Imaging at VA Tech.

Fig. 2. Nuclear magnetic resonance for the evaluation of body composition. Animals are placed into a restraining chamber which is then inserted into the electromagnetic apparatus for approximately 1 to 3 minutes while the analyzer measures fat, lean tissue, and fluid in grams and percent. This analyzer is located in the Integrated Life Sciences Building (ILSB) at VA Tech.

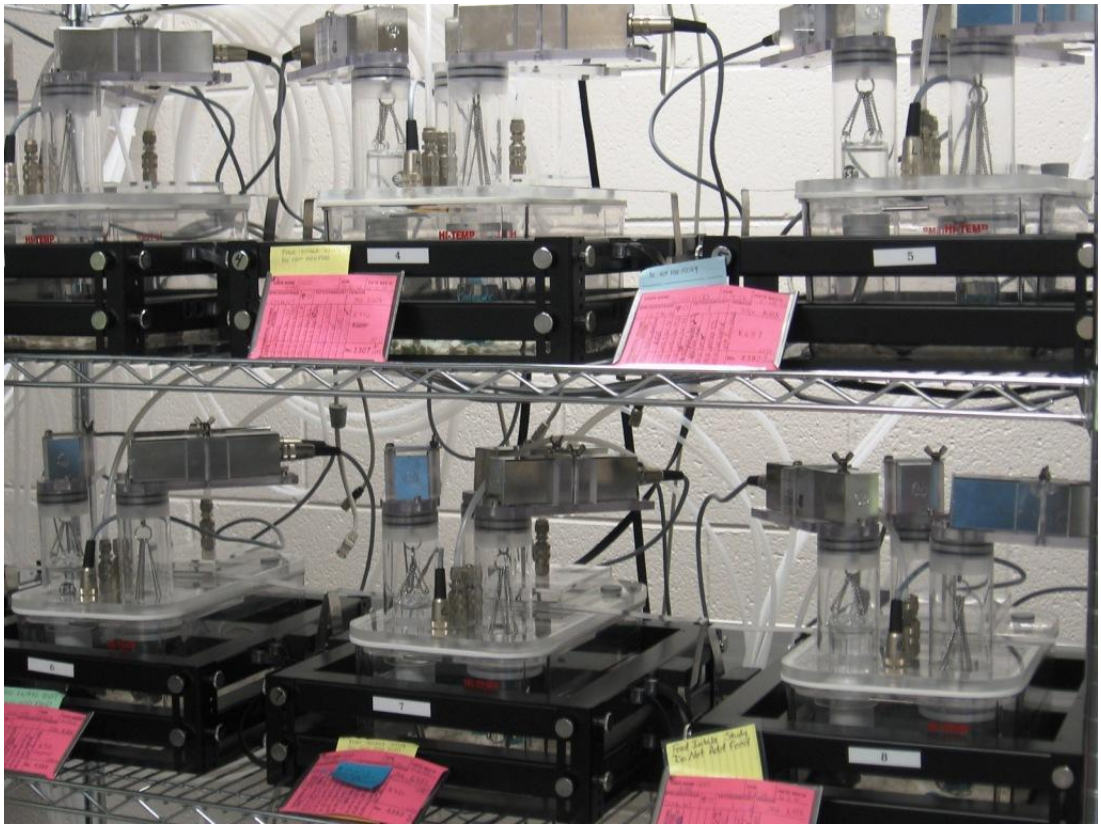

Fig. 3. Metabolic monitoring system to assess metabolic performance. Animals are maintained in their home cage which is outfitted with various monitoring devices that generate data on energy expenditure, physical activity, and water intake. This system is located in the Integrated Life Sciences Building (ILSB) at VA Tech.

CHAPTER III: Maternal Atherogenic High Fat Diet Results in Hepatic Lipidosis, Poor Birth Outcome, Growth Restriction, and Skeletal Aberrations in C57BL/6 Mice

Running Title: aHFD Morbidity and Mortality

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Abstract

The Western diet is high in unhealthy fats and cholesterol. Mounting research suggests that women of childbearing age consuming such a diet may be placing their unborn children at increased risk for certain chronic diseases in later life; however mechanistic explanations are not presently understood. In this study, the impact of maternal consumption of an atherogenic high fat diet (aHFD) on pregnancy and development of offspring into adulthood was determined. Female C57BL/6 mice were separated into two groups: standard rodent diet (RD) or aHFD. Diets were maintained for 4 weeks prior to breeding and throughout gestation and lactation. Pups were weaned at postnatal day (PND) 21 and placed on RD for the duration of the project. As compared to the RD dams, the aHFD dams experienced excess hepatic lipid accumulation and poor birth outcome as characterized by lower litter sizes and higher mortality. Offspring exposed to aHFD had smaller body weights and crown-to-rump lengths than age-matched RD controls. Micro-computed tomography (micro-CT) of aHFD-exposed offspring revealed skeletal aberrations including shorter bone lengths in whole body scans of PND21 progeny and irregularities of distal femoral trabecular architecture in detailed micro-CT analysis of PND42 progeny. Histologically, PND42 aHFD offspring had higher numbers of adipocytes in the distal femur compared to their RD counterparts. In this study, exposure to a diet rich in fats and cholesterol resulted in fatty liver in the dams and early mortality, growth restriction, and lesions mimicking human osteoporosis in the offspring. We propose that dietary-induced hyperlipidemia, along with pregnancy-associated factors, resulted in fatty liver and subsequently reduced litter sizes and increased early

mortality, and that the skeletal aberrations seen in the mature offspring represent dietary-induced inhibition of osteogenesis in favor of adipogenesis.

KEYWORDS: nutrition, saturated fat, trans fat, cholesterol, pregnancy, lactation, growth restriction, micro-computed tomography, osteoporosis

Introduction

The Western diet is characterized by high intakes of fatty meats, dairy products, and refined vegetable oils.[144] This diet is epitomized on fast food menus across the United States in such items as burgers, fried chicken, french fries, chips, cookies, and pastries, which are high in saturated fat and cholesterol, and often contain trans fat.[145,146] It is generally well-accepted knowledge that unhealthy fats and cholesterol contribute to cardiovascular disease, obesity, and obesity-related disorders; however the effects on pregnancy, fetal development, and lifelong health of the offspring are still largely unknown.

The Developmental Origins of Health and Disease (DOHaD), a hypothesis put forward by physician and researcher David Barker, proposes that various adult diseases can be traced back to alterations that occurred in the womb.[3,5] The DoHaD hypothesis emerged from a considerable body of epidemiological data that revealed an association between an adverse intrauterine environment, as determined primarily by low birth weight, and an increased risk of coronary heart disease later in life.[6] Such associations are also being made between the fetal environment and the development of osteoporosis.[147] Our research has its roots in the DOHaD hypothesis and extends Barker's work by utilizing a mouse model to study the impact of a mother's poor diet,

particularly one that is high in fat, on the maintenance of pregnancy and the immediate and long-term health of the progeny.

In the present study, the effects of chronic consumption of an unhealthy fast food diet, including components that contribute to atherosclerosis, on pregnancy and development of the offspring were examined, particularly looking for evidence of alterations in vasculature and bone morphology. The C57BL/6 strain was utilized in this research because of three important attributes: 1) isogenicity[124], 2) susceptibility to diet-induced atherosclerosis[125-127], and 3) propensity to develop osteoporosis[128,129]. Findings of this research suggest that exposure to a diet rich in fats and cholesterol leads to fatty liver disease in the dams and early mortality, growth restriction, and skeletal aberrations in the offspring. It is notable that no appreciable atherosclerotic lesions were identified in the dams or offspring. We propose that dietary-induced hyperlipidemia, along with pregnancy-associated factors, resulted in fatty liver and, subsequently, reduced litter sizes and increased early mortality, and that the skeletal aberrations seen in the mature offspring represent dietary-induced inhibition osteogenesis in favor of adipogenesis.

Materials and Methods

Animals and Diets

The study was approved by the Virginia Tech Animal Care and Use Committee. Four-week-old male and female C57BL/6 mice (Harlan Laboratories, Dublin, VA) were acclimated for 2 weeks at 22.0 ± 1^{o}C, 40-60% humidity, and 12/12-hour light/dark cycle with 4 mice per cage. During acclimation, mice were provided standard rodent diet (RD) (Harlan Teklad Global Diet 2018, Madison, WI) and water *ad libitum*. Following

acclimation, the breeder males were continued on RD for the duration of the project and the females were arbitrarily assigned to one of two dietary groups: RD (*n* = 18) (Table 1) or atherogenic high fat diet (aHFD) (Harlan Teklad Global Diet TD.10121, Madison, WI) (*n* = 16) (Table 2). Dietary treatments were continued for 4 weeks prior to breeding. During this time female mice were housed together with a maximum of 4 mice per cage and food consumption and body weight were measured weekly. For breeding, females were placed overnight with males (2:1 ratio) and checked the following morning for the presence of a vaginal plug, which indicated a successful mating event. Plug-positive females were designated as Gestation Day (GD) 0 and were then singly housed while remaining on their assigned diet throughout gestation and lactation. At the time of delivery, typically at gestation day 19, pup number was determined by gentle, non-disruptive, inspection of the cage and pup age was designated as postnatal day (PND) 1. At PND21, pups were weaned and provided a standard rodent diet for the duration of the project. Dams and offspring were euthanized via carbon dioxide gas inhalation at designated time points for tissue collection and analysis. In struggling with small litter sizes and high post-delivery mortality experienced by the aHFD dams, three of the aHFD breeder females were re-bred to increase the number of offspring available for evaluation. Taking into account the dams euthanized at GD14 and the multiparous aHFD dams, the *n* for litters was 16 for the RD group and 17 for the aHFD.

Measurements and Assessments

Dams: During the 4 week period of dietary treatment prior to breeding mice were housed together with a maximum of 4 per cage and food consumption and body weight were measured weekly. At gestation day 14, two mice were sacrificed for blood and

tissue collection. Blood was collected via perimortem cardiac venipuncture and then pooled, providing one sample per group. Blood was allowed to clot for 30 minutes to 1 hour at room temperature, centrifuged for 20 minutes, and then the serum collected. Serum aliquots of 300 to 500 µl were submitted to the Clinical Pathology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) for determination of triglyceride and cholesterol levels using their Olympus AU480 Chemical Analyzer. Lipid levels for aHFD dams were compared to published clinical chemistry reference ranges for adult female C57BL/6 mice[143] as well as the RD controls. Tissues collected included heart with roots of the great vessels and liver; tissues were fixed and stored in 10% neutral buffered formalin (NBF) and submitted for routine histologic processing by the VMRCVM Histology Laboratory. Four micron thick, hematoxylin and eosin (H&E) stained sections were examined by light microscopy. One aHFD dam experienced a decline in health characterized by weight loss, repetitive flipping, and self-induced trauma to the perianal region within 1 week of delivering two dead pups. This mouse was subsequently euthanized and the liver and brain collected for microscopic evaluation. The liver was fixed and processed the same as the livers from dams at gestation day 14 described above; however a portion was frozen and submitted to the VMRCVM Histology Laboratory for 5 µm thick cryosectioning and Oil Red O staining for lipid. The brain was cut into multiple cross sections and those containing diencephalon and metencephalon were submitted for routine histologic processing and subsequent microscopic evaluation of 4 µm thick H&E stained slides.

Birth Outcome and Litter Size: Birth outcome was assessed by determining litter size and mortality rate. Litter size was established by counting the number of pups delivered

at PND1. Offspring mortality rate was calculated as number of pups that died at any point post-delivery by number of pups delivered.

Offspring: The body weights of progeny were measured weekly, starting at GD14 and continuing until PND35, using an electronic scale. Crown-to-rump lengths were measured at GD14 and PND1, 21, and 42 using the Fisherbrand Traceable Digital Caliper (Fisher Scientific, Pittsburgh, PA). Whole body micro-computed tomography (micro-CT) (MicroXCT-400, Xradia, Pleasanton, CA) was performed on PND21 pups. The left femurs of PND42 offspring were isolated and examined by micro-CT (VivaCT 40, Scanco Medical, Bassersdorf, Switzerland) for trabecular organization; skeletal image data were archived and manipulated using OsiriX, a DICOM-compatible freeware program (OsiriX, version 2.6, Medical Imaging Software, Los Angeles, CA). Heart with roots of the great vessels, thoracic aorta, liver, and right hindlimb of PND42 offspring were collected, fixed in 10% NBF, submitted for routine histologic processing at VMRCVM Histology Laboratory, and 4 µm thick H&E stained slides evaluated by light microscopy. The right femurs were isolated and then had the additional treatment of decalcification in which they were placed in decalcifying solution (EDF, StatLab Medical Products Catalog #SL85-32, McKinney, TX) for 48 hours prior to histologic processing. Adipocyte number was determined by taking the average number of adipocytes out of three high power fields at a magnification objective of 40.

Statistical Analysis

One-way analysis of variance (ANOVA), restricted maximum likelihood (REML) method, Student's t-test, and Tukey-Kramer test (JMP 9.0, SAS Institute Inc., Cary, NC) were

used to establish differences between groups. Specifically, ANOVA and Student's t-test were used for the analysis of maternal food consumption, litter size, post-delivery mortality rate, and bone marrow adipocyte numbers in the offspring. For maternal body weight and offspring body measurements, REML method in addition to Tukey-Kramer test and Student's t-test were used. Data were determined to be statistically significant when $p < 0.05$.

Results

Maternal Food Consumption and Body Weights

In the 4 week period of dietary treatment prior to breeding in which food and body weights were measured weekly, there were no statistically significant differences in food consumption or body weight between the two groups.

Maternal Cholesterol and Triglyceride Levels

The pooled sample from aHFD dams demonstrated hypercholesterolemia at 166 mg/dL (76.1–107.9 mg/dL) and hypertriglyceridemia at 255 mg/dL (65.8–90.2 mg/dL); the sample from the RD dams had the respective levels of 97 mg/dL and 125 mg/dL.

Pathology of Dams

Tissues from RD and aHFD dams, to include heart with roots of the great vessels, and liver were evaluated via light microscopy. Neither the RD nor the aHFD dams had gross or microscopic cardiac lesions and of particular interest, no atherosclerotic lesions were identified. Livers of aHFD dams were grossly pale. Microscopically, these livers

exhibited moderate diffuse micro- and macrovesicular hepatic lipidosis (Figure 1B). Also present were rare foci of inflammation composed mainly of neutrophils and a few Kupffer cells containing small amounts of finely granular yellow pigment (lipofuscin). No such lesions were observed in RD controls (Figure 1A). No appreciable fibrosis was identified in the aHFD or RD dams on routine H&E stain or on a special stain for collagen fibers (i.e., Masson's trichrome). An Oil Red O stain performed on the liver of the aHFD dam exhibiting weight loss and neurologic disease confirmed lipid accumulation (Figure 2) with multiple groups of hepatocytes having mild to moderate lipid accumulation; however no brain lesions, particularly no lesions of hepatic encephalopathy (i.e., spongiform change with Alzheimer type II astrocytosis), were identified.

Birth Outcome: Litter Size and Mortality Rate

As compared to the RD group, the aHFD group had lower mean litter sizes, $p < 0.05$ (Figure 3) and experienced higher post-delivery mortality, $p < 0.01$ (Figure 4). The vast majority of deaths occurred at the time of birth (stillbirths) and in the early postnatal period.

Offspring Body Measurements

Atherogenic, high fat diet pups had overall lower body weights than age-matched RD controls, $p < 0.01$ (Figure 5) as well as shorter crown-to-rump lengths, $p < 0.05$ (Figure 6).

MicroCT Evaluation of Offspring

Whole body scans of a female PND21 pup per group demonstrated shorter bone lengths in the aHFD-exposed offspring (Figure 7) as compared to the age and sex-matched RD control; bones of the appendicular and axial skeleton were measured. Detailed analysis of the left femur from PND42 pups, one male and female per group, revealed striking skeletal aberrations in aHFD offspring as compared to RD controls. Similar to human osteoporotic lesions, aHFD-exposed offspring demonstrated reduced bone density, trabecular connectivity, and trabecular number with the female more severely affected than the male and having increased trabecular spacing (Table 3 and Figure 8). Notably, these changes occurred in the absence of changes in bone volume.

Pathology of Offspring

The right distal femur of PND42 offspring demonstrated higher numbers of adipocytes in aHFD offspring as compared to RD controls ($p < 0.05$) (Figure 9). No gross or microscopic lesions were identified in the heart, thoracic aorta, and liver of either group.

Discussion

Studies in humans and experimental animals addressing the “fetal origins of adult disease” have established a connection between an adverse intrauterine environment and the predisposition to chronic disease in adulthood; however few studies have directly assessed the relationship between maternal nutritional excesses and cardiovascular and skeletal phenotype of the offspring. In the present study, habitual consumption of a diet rich in unhealthy fats and cholesterol prior to conception

and throughout gestation and lactation in C57BL/6 mice resulted in hepatic lipidosis in the dams and early mortality, growth restriction, and skeletal aberrations in the offspring. It is notable that no appreciable atherosclerotic lesions were identified in the aHFD dams or offspring. We propose that dietary-induced hyperlipidemia, along with pregnancy-associated factors, resulted in fatty liver and, subsequently, reduced litter sizes and increased early mortality, and that the skeletal aberrations seen in the mature offspring represent dietary-induced inhibition osteogenesis in favor of adipogenesis.

In this study hepatic lipidosis was identified by gross and microscopic examination of the liver of aHFD female mice at day 14 of a 19 day gestation period as well as a peripartum female demonstrating neurologic disease. Metabolic hepatic disease is generally manifested as vacuolar change (lipid and/or glycogen type) with fatty liver disease being now recognized as the most common liver disease of the 21st century.[93] In human medicine, the term steatosis is used more commonly used than lipidosis and alcoholism is often the cause; however fatty liver can occur in the absence of excess alcohol consumption and is thus termed nonalcoholic fatty liver disease (NAFLD). The pathogenesis of NAFLD remains unclear. It is proposed that an aberration in fatty acid and triglyceride metabolism leads to excess hepatic lipid accumulation.[94] Normally, fatty acids enter the liver where they are either utilized for energy in the hepatic mitochondria or converted to triglycerides that are further processed into lipoproteins which are actively secreted into the plasma as VLDL.[85] Any disturbance in energy, supply of structural components to form lipoproteins, or physical disruption of the organelles involved in synthesis, assembly, and secretion of lipoproteins has the potential to render the liver incapable of ridding itself of fatty acids resulting in accumulation of triglyceride globules in the hepatocyte cytoplasm and fatty

liver. Most individuals with NAFLD are asymptomatic.[148] When present, symptoms can include fatigue, upper abdominal pain, bruising, jaundice, and hepatic encephalopathy.[148,149]

The amount of fat in the liver depends on the balance between the processes of delivery, utilization, and removal. One proposed mechanism for the excess hepatic lipid in our mice is dietary-induced hyperlipidemia resulting in increased fatty acid delivery, overwhelming the ability of the liver to utilize and remove this fuel source. Hyperlipidemia is a known risk factor for fatty infiltration of the liver.[95-97] In this study, aHFD dams at gestation day 14 were hyperlipidemic, having elevated levels of both cholesterol and triglyceride. In a study demonstrating the association between hyperlipidemic states and fatty liver, ultrasound findings of 95 adult human patients referred to a clinic for management of hyperlipidemia (hypercholesterolemia, hypertriglyceridemia, or mixed hyperlipidemia) revealed that 47 patients (50%) had diffuse fatty liver.[98] As researchers have worked to develop animal models of NAFLD, a handful of studies have been conducted to induce fatty liver disease in rodents through dietary means and have found that rodents fed a high fat diet can develop NAFLD as well as its progressed form, nonalcoholic steatohepatitis (NASH).[150,151] One such study using C57BL/6 mice found that those on a high fat diet (rich in nonsaturated fats and fructose) or a “fast food” diet (relatively rich in saturated fats and cholesterol and fructose) developed hepatic steatosis; those in the fast food group also demonstrated disease progression to steatohepatitis having inflammation and fibrosis.[151] Similarly, a study conducted on obese *fa/fa* Zucker rats and their lean, Fa/?, littermates found that steatohepatitis developed in the *fa/fa* rats fed a high fat diet as did elevation in cytokine production and oxidative stress.[150]

In the present study, in addition to a dietary-induced hyperlipidemic state promoting excess fats being delivered to the liver, we suspect that pregnancy state was also a large contributing factor. In veterinary medicine, fatty liver disease is frequently encountered in cows and other ruminant species and is attributed to negative energy balance due to insufficient feed intake to meet the nutritional demands for maintenance of late pregnancy and early lactation. Commonly referred to as fat cow syndrome, fatty liver disease is often seen in cows that were overfed in the dry (non-lactating) period resulting in overly fat cows at calving.[99] The pathogenesis follows that negative energy balance results in mobilization of the body's fat reserves in an effort to rebalance the deficit resulting in the release of free fatty acids into the blood which subsequently accumulate in the liver. Cows which calve in fat to very fat body condition are more likely to develop fatty liver likely because their feed intake is depressed so as to not match the high energy requirements that arise during late pregnancy/early lactation. Humans also experience a pregnancy-associated fatty liver disease termed acute fatty liver of pregnancy (AFLP).[100] This is a rare disease of unknown etiology that occurs during the third trimester. In many cases it is believed to be caused by mitochondrial dysfunction resulting in abnormal β-oxidation of fatty acids. Beta-oxidation of fatty acids is the process by which fatty acids are broken down in the mitochondria to be used in the tricarboxylic acid (TCA) cycle for energy and, interestingly, it has been found to be decreased in pregnant mice.[101] This finding led researchers to suspect that the effects of pregnancy on hepatic fatty acid oxidation may be due to the female sex hormones estrogen and progesterone. To test their hypotheses, estradiol and progesterone were administered to non-pregnant mice which resulted in a decrease in β-oxidation of fatty acids and in the activity of the TCA cycle, as well as ultrastructural lesions of

mitochondria and decreased recovery of mitochondrial proteins.[102] The implications to this project are that the fatty liver identified in our mice may be attributed to pregnancy in two ways: 1) negative energy balance leading to increased mobilization and delivery of lipids to the liver and 2) decreased ability to utilize these lipids due to a decrease in mitochondrial β-oxidation of fatty acids.

In this project we did not experience spontaneous death occurring in our dams, but we did experience reduced litter size and increased early mortality in the offspring, mainly stillbirths and deaths in the early postnatal period. Fatty liver development in pregnancy is frequently associated with maternal and fetal mortality. Using published reports on the human condition of AFLP, maternal death is often the result of liver failure, bleeding, and/or sepsis.[152-154] The etiology of fetal mortality is not well established, but is often attributed to preterm delivery, fetal distress, and asphyxia.[152-154] In the 1980s AFLP carried a mortality rate for both mother and fetus of about 85%.[103] A more recent report indicates that death, due to the disease and its associated and complicating conditions, has decreased dramatically, but is still substantial with a fetal/perinatal mortality rate of 9-23% and a maternal mortality rate of 7-18%.[104] In a clinicopathologic study of 35 women with AFLP, 22 of the cases were diagnosed at autopsy, and of these, 12 had stillbirths, 8 had live births, and the status of 2 of the offspring was not established.[105] Delivery of the fetus, regardless of gestational age, is the primary treatment for AFLP once the diagnosis has been made. Infants that survive post-delivery face complications from premature delivery, fetal distress, asphyxia, and hypoxia.[106]

In this project, aHFD-exposed offspring had skeletal aberrations including shorter bones in both the axial and appendicular skeleton as well as substandard trabecular architecture and increased adiposity in the femur. Our proposed pathogenesis for this observation follows that diet-induced increases in bioactive oxidized lipids inhibit osteoblastic differentiation in favor of adipogenic differentiation during a critical period of bone growth, thus altering osteogenic programming and predisposing the animal to osteoporosis. The trabecular lesions identified by microCT are similar to a study conducted in our laboratory and published in 2009, in which the offspring exposed to a high saturated fat diet *in utero* and until weaning exhibited lesions reminiscent of osteoporosis, having lower average bone mineral density at 6 months of age and dysregulation of distal femoral trabecular architecture at 12 months of age.[33] Parallel research published in 2010 and conducted by the Bone and Joint Research Group at the University of Southampton School of Medicine found that 7 month old offspring from dams fed a high fat diet showed increased adiposity in addition to altered trabecular structure in the femur as compared to offspring of dams fed a standard rodent diet. Both studies suggested altered osteogenic programming during the period of developmental plasticity as a possible part of the disease pathogenesis.[33,120] Our laboratory also postulated and tested elevated oxidative stress as a mechanism contributing to the skeletal aberrations, and demonstrated that maternal antioxidant supplementation significantly protected against high fat diet-induced reduction of trabecular connectivity density in the adult offspring.[33]

In the current project we take our previously proposed mechanism of elevated oxidative stress causing damage to bone, and refine it to incorporate the inhibitory effects of bioactive oxidized lipids on osteogenesis. In osteoporotic bone there are fewer

osteogenic cells and more adipocytes in the marrow. Since both osteoblasts and adipocytes arise from multipotent mesenchymal stem cells (MSCs) in the bone marrow, it has been suggested that the increased number of adipocytes is due to a greater propensity for adipogenesis versus osteogenesis.[155] Parhami and colleagues have performed *in vitro*, *ex vivo*, and *in vivo* studies demonstrating that atherogenic oxidized lipids inhibit osteoblastic differentiation by directing marrow MSCs to undergo adipogenic instead of osteogenic differentiation. With their *in vivo* work, they were able to show that minimally oxidized low density lipoprotein (MM-LDL) caused reduction of osteoblastic markers in marrow MSCs including reduced alkaline phosphatase (ALP) activity, and that treatment of MSCs with MM-LDL along with a selective peroxisome proliferator-activated receptor y (PPARy) agonist, resulted in accelerated adipocyte formation, producing positive Oil Red O staining as early as 8 days after treatment, at which time cultures treated with a PPARγ agonist alone contained only sparse positive-staining cells and untreated cells had none.[156] *Ex vivo* and *in vivo* projects were conducted with C57BL/6 mice that were fed either a standard rodent diet or an atherogenic high fat diet containing added cholesterol and sodium cholate. In these projects the marrow MSCs collected from animals fed the atherogenic high fat diet failed to undergo osteogenic differentiation and mice on the atherogenic high fat diet had significantly lower mineral content and density, and reduced osteocalcin expression.[156,157] Interestingly, although we did not identify atherosclerosis or any cardiac disease in our animals, their research puts forward the “lipid hypothesis of osteoporosis” which postulates that lipids involved in causing cardiovascular disease also contribute to osteoporosis.[156]

In summary, our results indicate that habitual consumption of a diet rich in fats and cholesterol prior to conception and throughout gestation and lactation in C57BL/6 mice leads to hepatic lipidosis in the dams and early mortality, growth restriction and skeletal aberrations in the offspring. The suspected etiopathogenesis of the maternal hepatic lipidosis includes dietary-induced hyperlipidemia and pregnancy-associated factors, specifically an imbalance between energy supply and demand, and possibly, a decreased ability of the liver to utilize fatty acids for energy. The reduced litter size and increased post-delivery mortality experienced in the aHFD group is suspected to be due to complications arising from maternal development of fatty liver, as this condition in pregnant women is frequently associated with fetal and perinatal mortality. The proposed pathogenesis of the skeletal aberrations follows that exposure to an atherogenic high fat diet during the critical periods of *in utero* and postnatal growth results in increased bioactive oxidized lipids which inhibit osteogenic differentiation in favor of adipogenic differentiation resulting in permanent skeletal compromise and osteoporosis. Atherosclerosis was not detected, which could be the result of the fairly short duration of exposure of both the dams and offspring and/or that the lesion of atherosclerosis in this susceptible inbred strain is often small and restricted in location to the aortic root[126]. To conclude, this study emphasizes the importance of maternal diet to birth outcome and lifelong health of the offspring and serves as an example of how the foundation of certain chronic diseases such as osteoporosis may actually be laid down *in utero*.

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and dedication of veterinary student Rachael Edwards and osteopathic medical students Lisa Mitchell, Megan Friedman, and Eric Jenkie.

Tables and Figures

Table 1. Standard Rodent Diet (RD) Composition

RD	Kcal (%)
Protein	24
Carbohydrate	58
Fat	18
Fat Composition (%)	
Saturated	16
Monounsaturated	23
Polyunsaturated	60

Table 2. Atherogenic High Fat Diet (aHFD) Composition

aHFD	**Kcal (%)**
Protein	18.40
Carbohydrate	21.30
Fat	60.30
Fat Composition (%)	
Saturated	39
Trans	15
Monounsaturated	35
Polyunsaturated	11
Cholesterol	1.25
Sodium Cholate	0.50

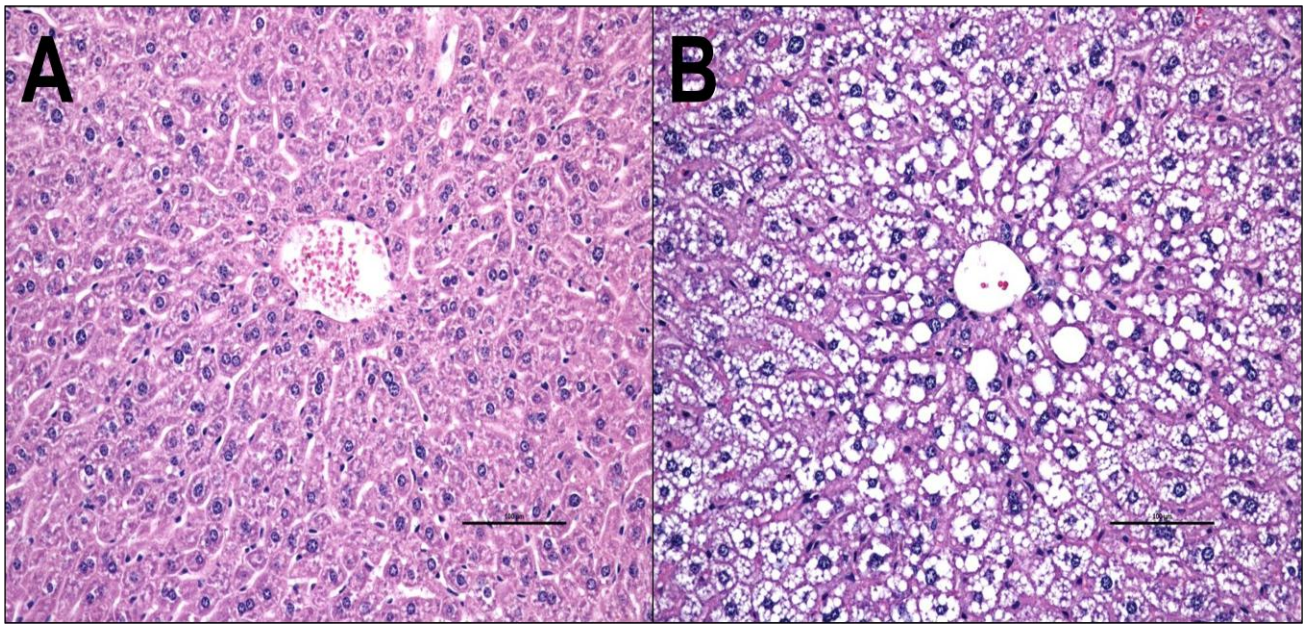

Fig. 1. Liver from Rodent Diet (RD) and Atherogenic High Fat Diet (aHFD) Dams at Gestation Day 14. Note the moderate diffuse micro- and macrovesicular hepatic lipidosis in the aHFD dams (B) as compared to the RD dams (A). Hematoxylin and eosin stain, 20x magnification, scale bars = 100 µm.

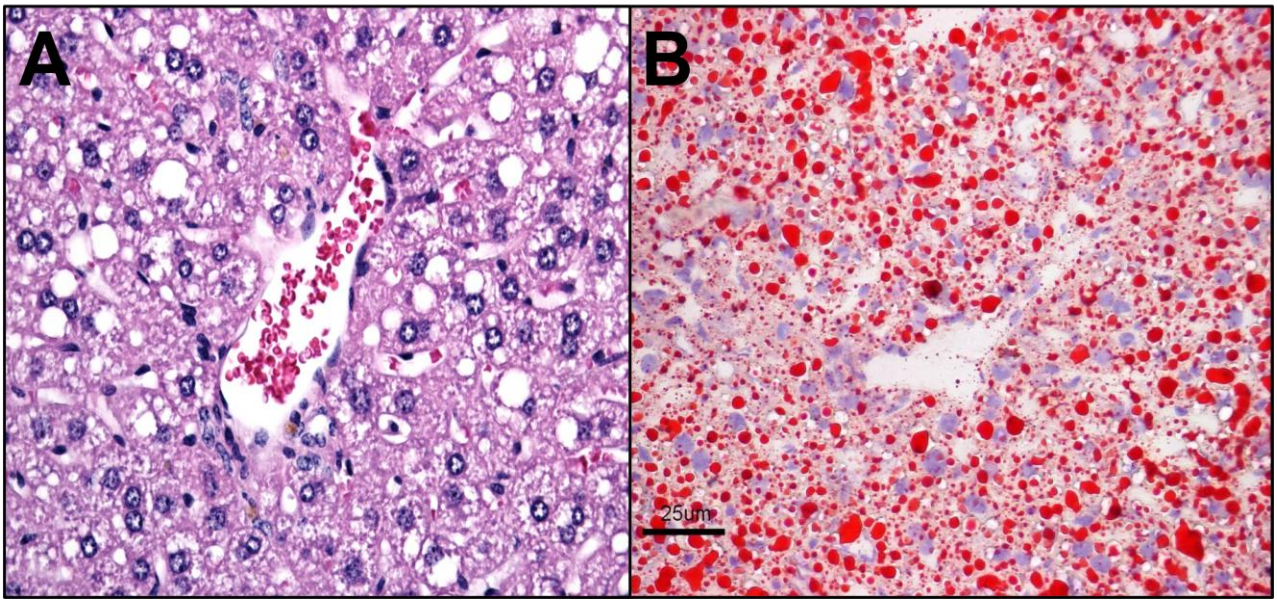

Fig. 2. Liver from a Postpartum Atherogenic High Fat Diet (aHFD) Dam that was Demonstrating Neurologic Signs. Note the accumulation of lipid seen in both the routine hematoxylin and eosin stain (A) and the Oil Red O stain for lipid (B). 40x magnification, scale bar = 25 µm.

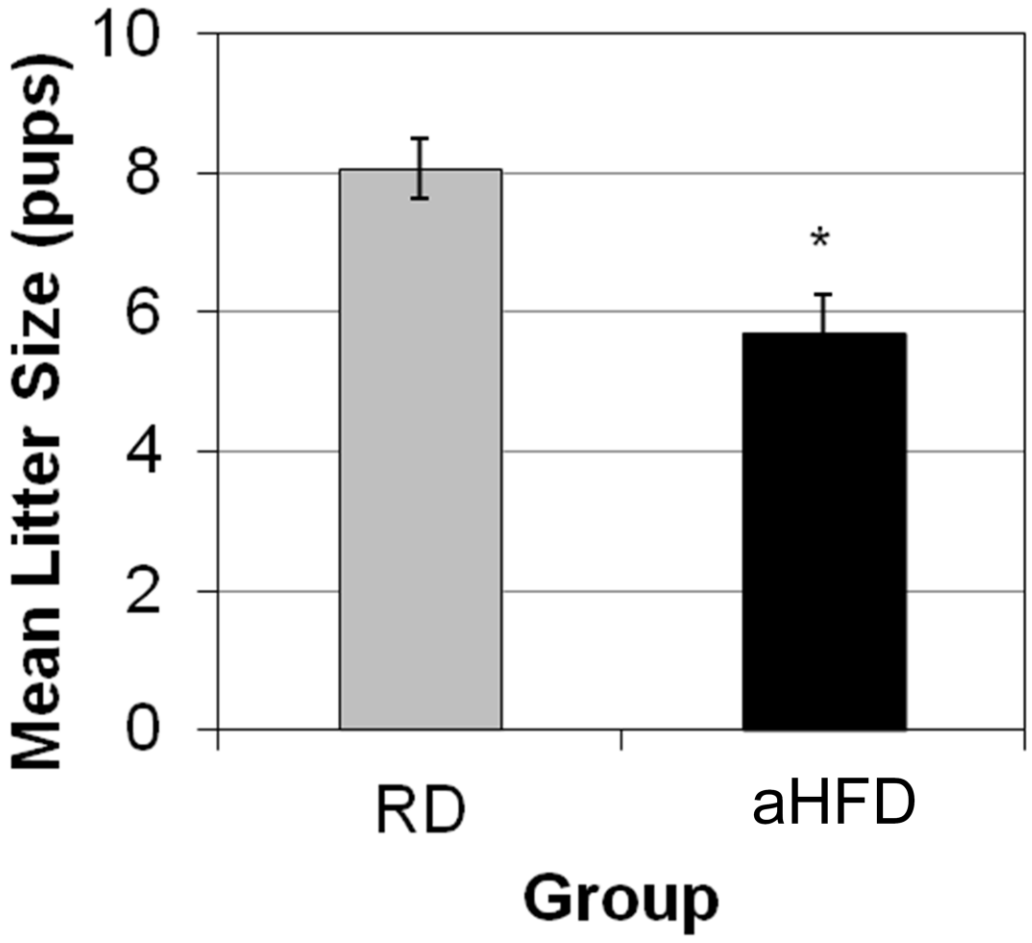

Fig. 3. Litter Size in Rodent Diet (RD) and Atherogenic High Fat Diet (aHFD). Data are mean ± SE; *n* = 16 for RD and *n* = 17 for aHFD; *$p < 0.05$.

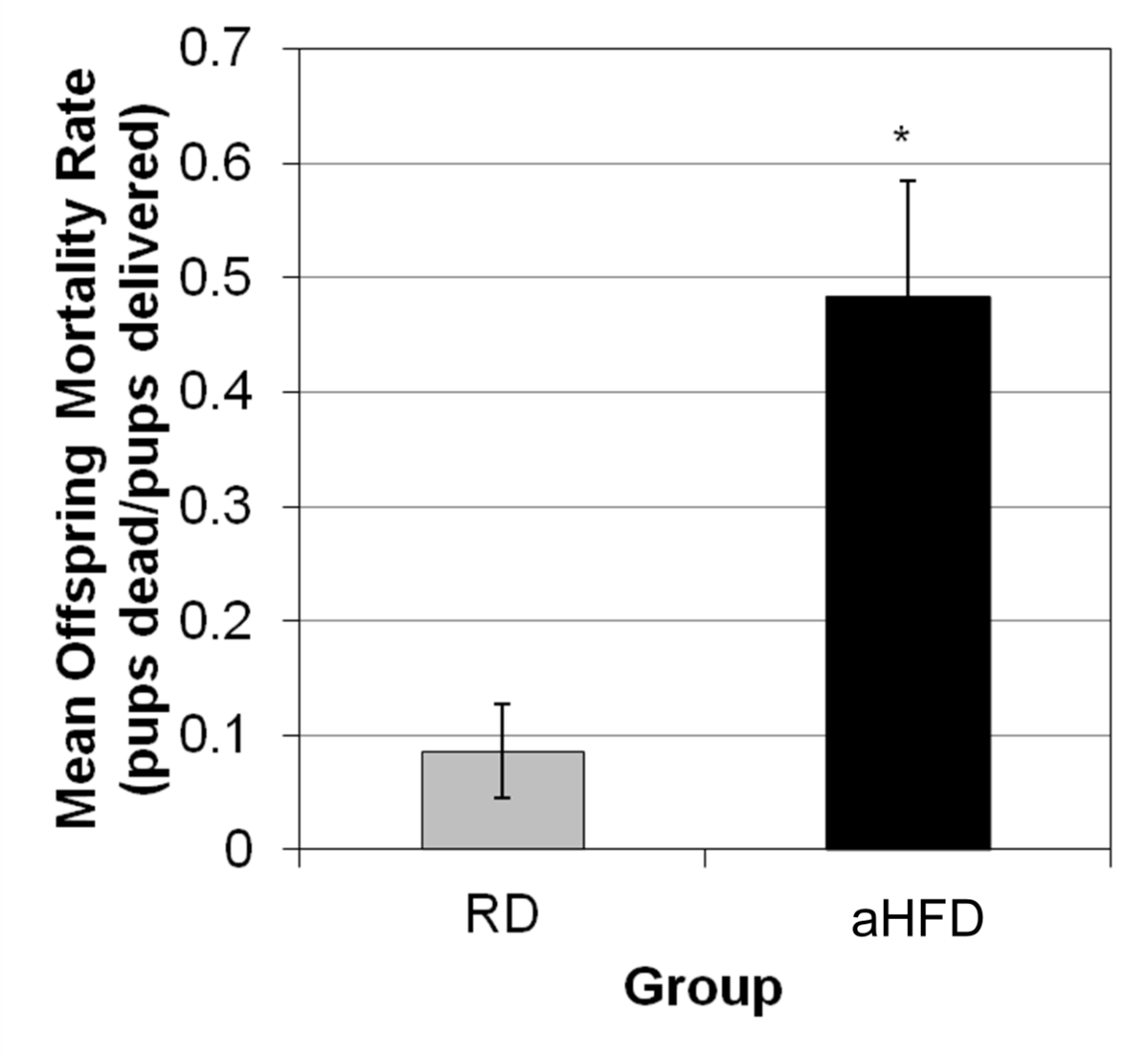

Fig. 4. Mortality Rate of Offspring for Rodent Diet (RD) and Atherogenic High Fat Diet (aHFD). Data are mean ± SE; n = 16 for RD and n = 17 for aHFD; *$p < 0.01$.

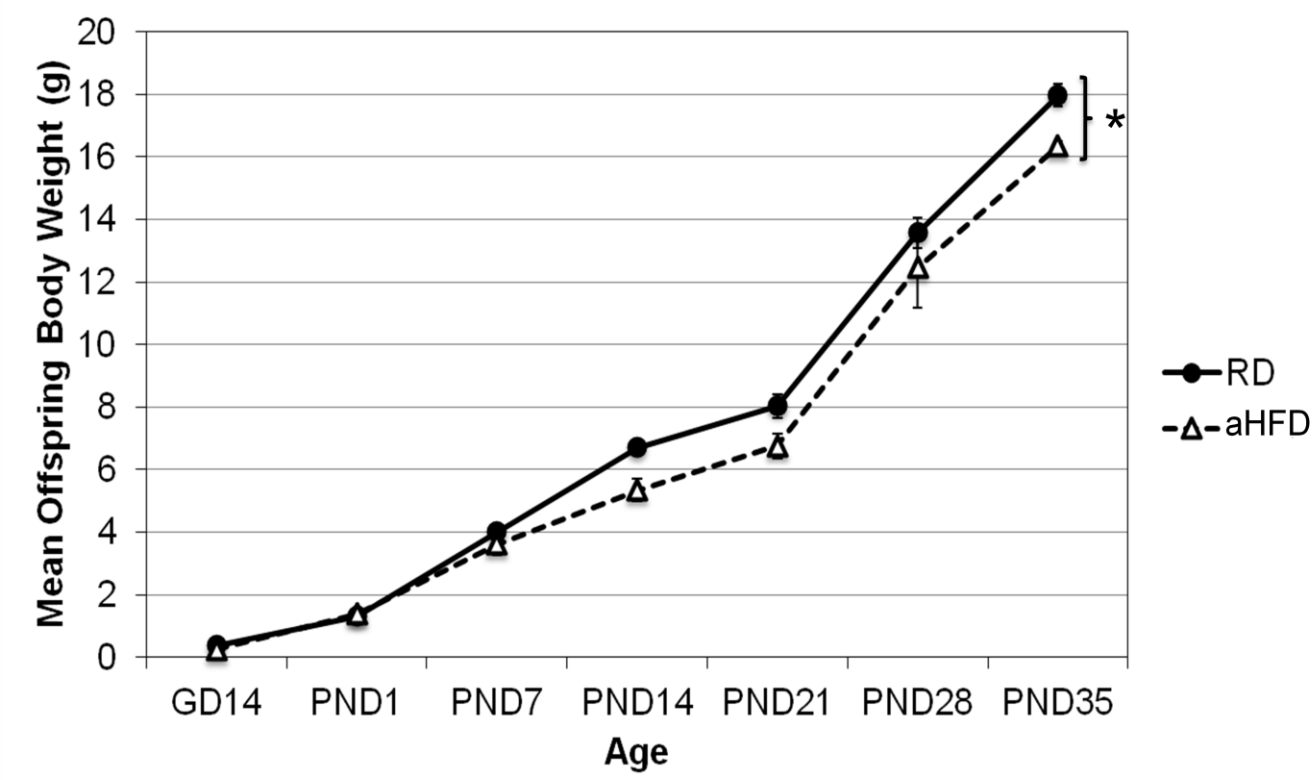

Fig. 5. Body Weights of Offspring Over Time for Rodent Diet (RD) and Atherogenic High Fat Diet (aHFD). Data are mean ± SE; sample size varied within dietary groups and ages with body weight *n* ranging from 1 to 4 for GD14 and PND1, *n* = 7 for PND7 through 21, and *n* = 3 for PND28 and 35; *$p < 0.01$. GD = gestation day, PND = postnatal day.

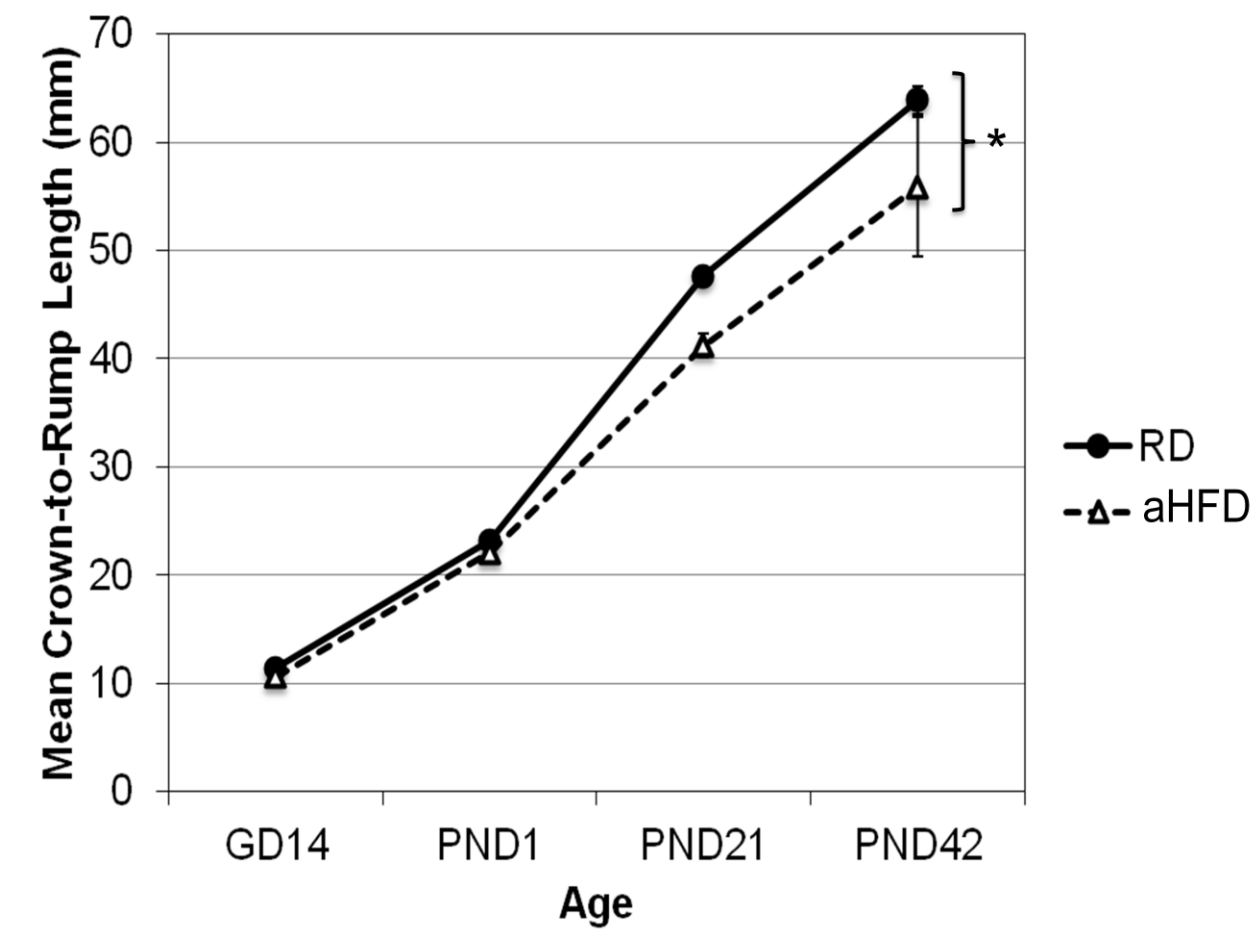

Fig. 6. Crown-To-Rump Length of Offspring Over Time for Rodent Diet (RD) and Atherogenic High Fat Diet (aHFD). Data are mean ± SE; sample size varied within dietary groups with *n* varying from 3 to 4 for all groups and ages; *$p < 0.05$. GD = gestation day, PND = postnatal day.

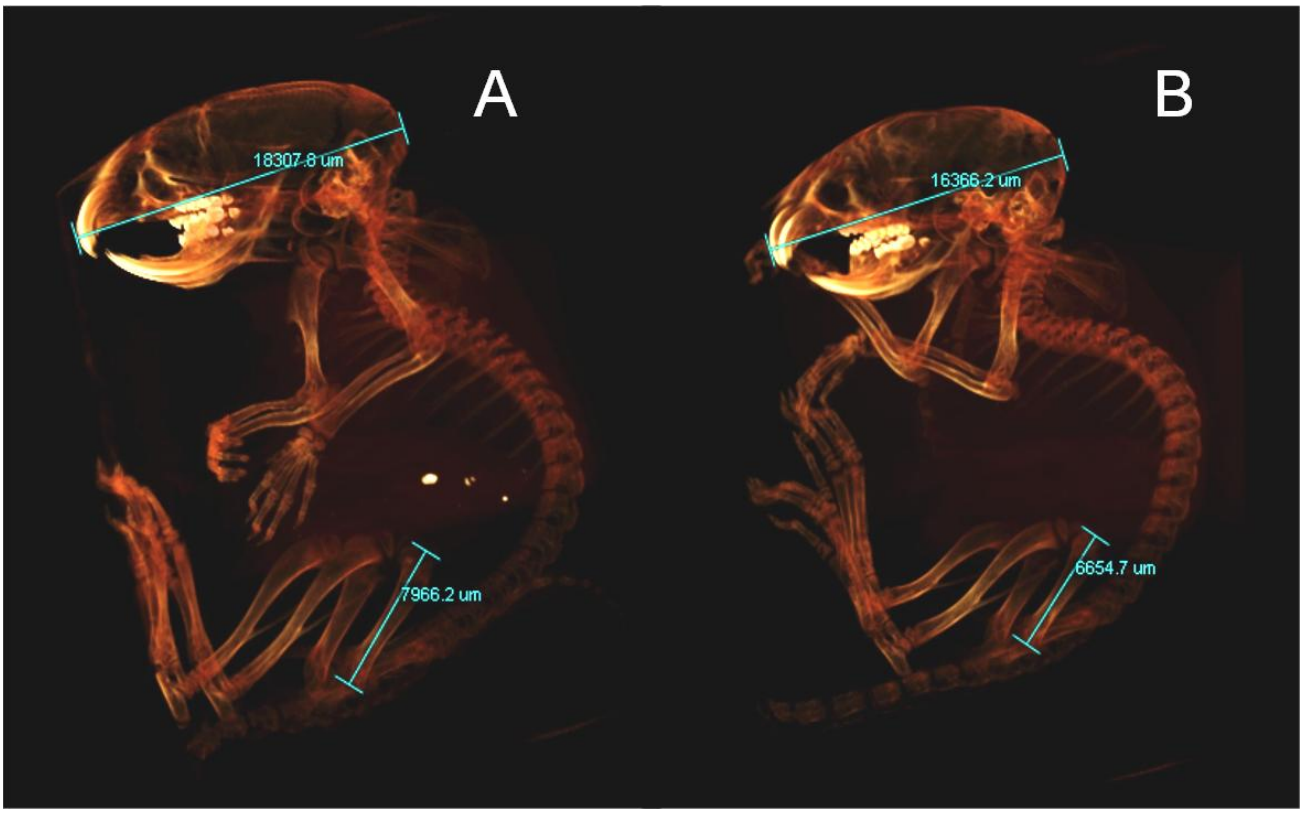

Fig. 7. Whole Body Micro-Computed Tomographic Image of Postnatal Day 21 Offspring. Note the shorter cranial and femoral bone lengths in the pup exposed to atherogenic high fat diet (B) as compared to an age- and sex-matched rodent diet control (A).

Table 3. Bone Microarchitecture Measurements of Postnatal Day 42 Offspring

Femurs	Bone volume (mm^3)	Bone density ($mgHA/cm^3$)	Connectivity ($1/mm^3$)	Trabecular number (1/mm)	Trabecular thickness (mm)	Trabecular spacing (mm)
RD, M	7.84	745.3	146.3	5.15	0.11	0.26
RD, F	7.11	736.4	111.09	4.31	0.11	0.21
aHFD, M	7.59	706.4	95.71	4.65	0.10	0.23
aHFD, F	7.72	703.5	63.73	3.88	0.12	0.30

RD = Rodent Diet, aHFD = Atherogenic High Fat Diet, M = Male, F = Female

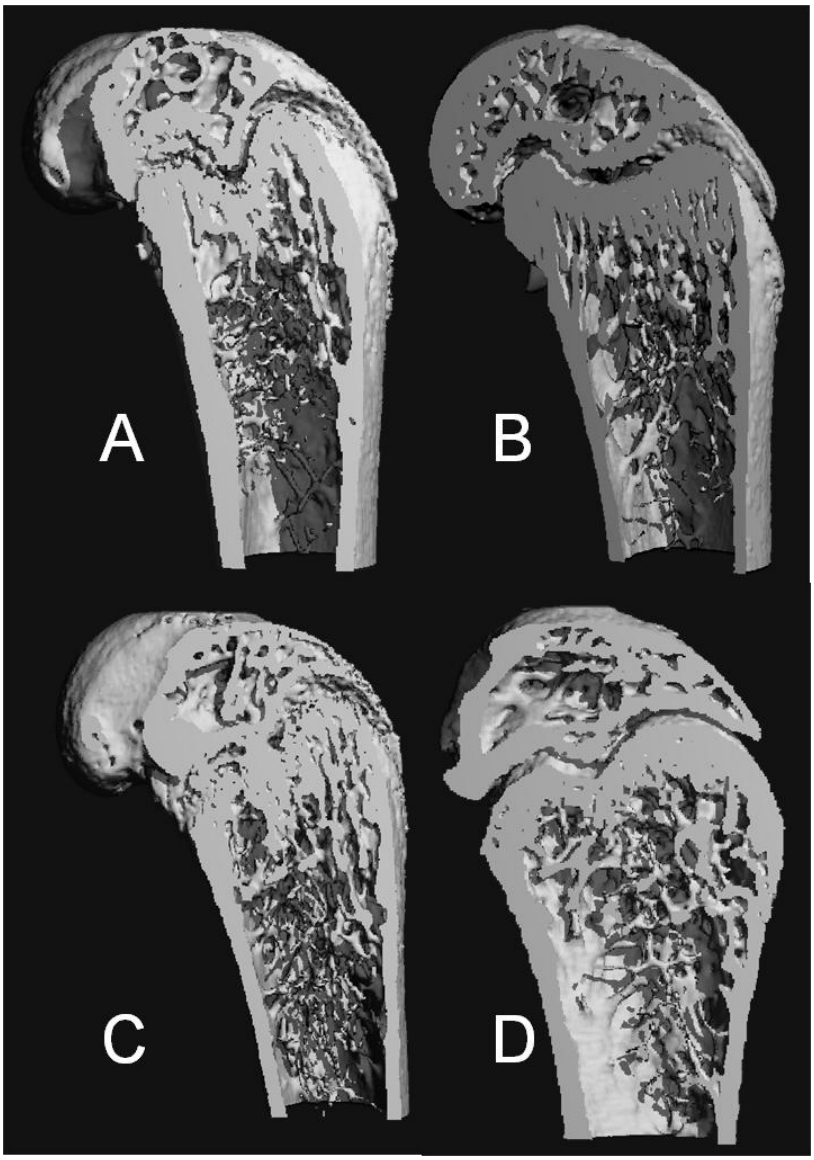

Fig. 8. Micro-Computed Tomographic Image of Femoral Trabecular Microarchitecture in Postnatal Day 42 Offspring. The atherogenic high fat diet offspring (B female and D male) demonstrated lesions similar to human osteoporosis including reduced bone density, trabecular connectivity, and trabecular number with the female more severely affected than the male and having increased trabecular spacing as compared to age and sex-matched rodent diet controls (A female and C male).

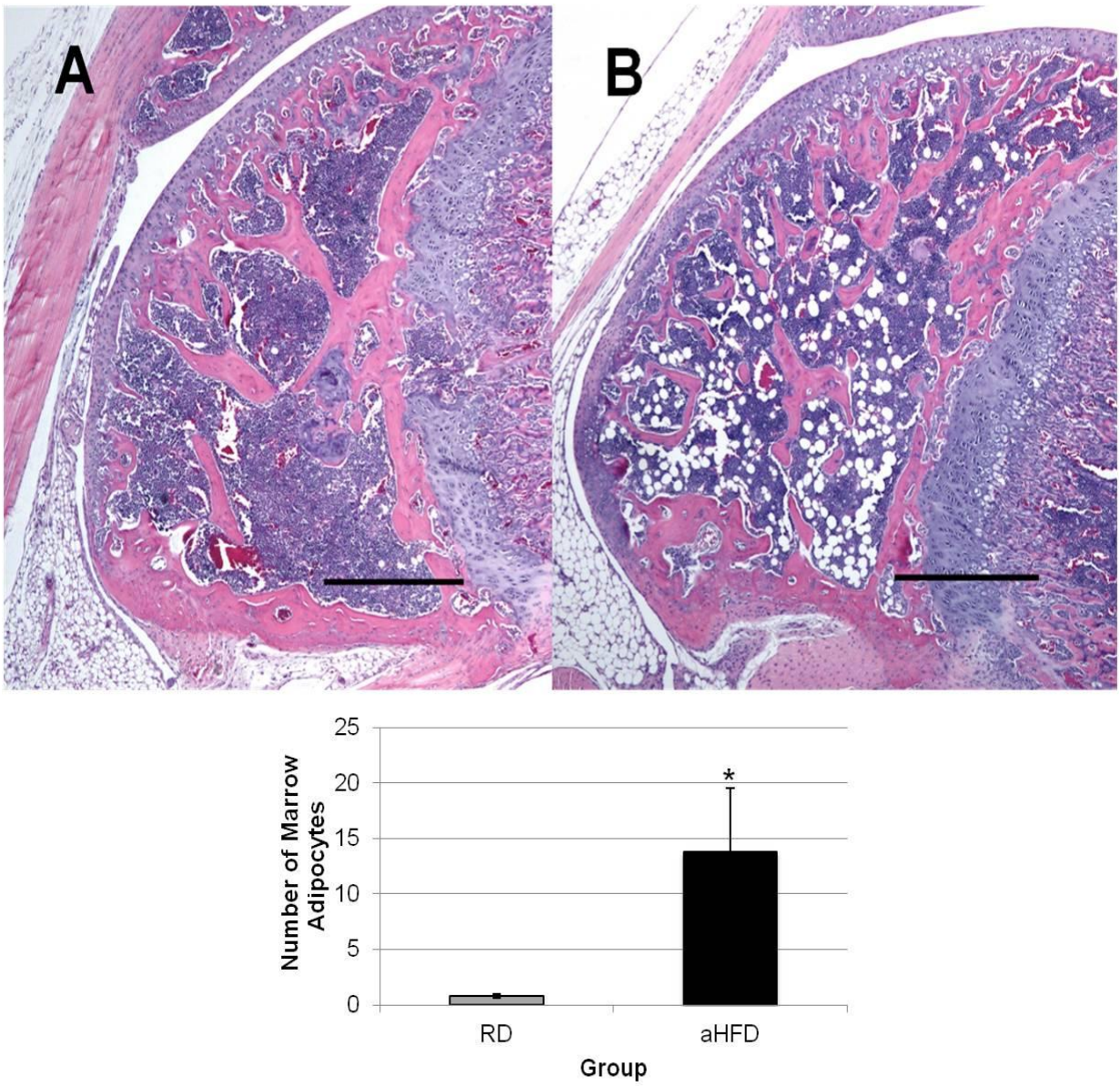

Fig. 9. Marrow Adipocytes in the Right Distal Femur of Postnatal Day 42 Pups. Note the higher number of adipocytes in the atherogenic high fat diet (aHFD) group (B) as compared to the standard rodent diet (RD) controls (A). Hematoxylin and eosin stain, 4x magnification, scale bars= 500 µm. Adipocyte number was determined by taking the average number of adipocytes out of three high power fields at a magnification objective of 40. Data are mean ± SE; n = 6 for RD and n = 5 for aHFD; *$p < 0.05$.

CHAPTER IV: Maternal Nutrition and Fetal Programming of the Metabolic Syndrome: Mitigating or Exacerbating the Effects through Altered Eating Habits in Adulthood

Running Title: Nutrition and Metabolic Syndrome

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Abstract

The *in utero* environment may determine, in part, lifelong susceptibility to chronic diseases of adulthood. Fetal undernutrition has been studied extensively and its role in elevating risk of metabolic and cardiovascular disease is well characterized. However, the contribution of exposure to nutrient excesses, specifically fats, to adult-onset metabolic disorders has only recently been recognized. To investigate a link between early exposure to a fat-rich diet and the development of metabolic syndrome, dams and female offspring were exposed to particular dietary treatments and anthropometric and metabolic parameters were evaluated. Dams were fed a standard rodent diet (RD) or a high fat diet (HFD) prior to conception and throughout gestation and lactation. At weaning, female offspring were continued on the diet of their dam until 7 weeks of age at which time they were assigned to one of six experimental conditions (ECs) that allowed them to continue on the diet of their dam, be placed on the opposing diet, or be given a choice between the two diets; ECs were maintained until 18 weeks of age. Results demonstrate that early exposure to a high fat diet induces central obesity, elevated lipid levels, hyperglycemia, and additional markers used in the diagnosis of the metabolic syndrome. Altering the diets of the mature offspring demonstrated that the eating habits of adulthood have the potential to mitigate or exacerbate certain metabolic parameters established earlier in life. Mechanisms contributing to the observed metabolic aberrations could include developmental plasticity and mismatch, catch-up growth, and altered programming of the appetite regulatory network.

KEYWORDS: nutrition, fetal programming, obesity, insulin resistance, hyperlipidemia, metabolic syndrome

Introduction

Chronic diseases, such as type 2 diabetes and heart disease, are diseases of long duration, generally slow progression, and are by far the leading cause of mortality in the world, representing 63% of all deaths.[1] Mounting research suggests that the *in utero* and postnatal environment may determine, in part, lifelong susceptibility to such diseases. The period from conception to weaning is a time of rapid cellular proliferation, differentiation, and organization as well as functional maturation and growth of tissues and organs. Important determinants of fetal growth include adequate nutrient supply and the proper hormonal milieu.[2] An adverse gestational environment, such as one lacking appropriate nutrition, can cause the developing fetus to make certain adaptive responses. If permanent, these programming changes may be the origins of a number of conditions in later life including the metabolic syndrome.[9] This phenomenon prompted the fetal origins of adult disease hypothesis, now often referred to as the Developmental Origins of Health and Disease (DOHaD) hypothesis, and was spearheaded by physician and researcher David Barker. Barker's work and others, focused on the associations between fetal undernutrition, low birth weights, and increased risk of coronary heart disease later in life.[6] Critics of the DOHaD hypothesis argue that the research fails to take into account confounding variables, particularly the impact of the choices and circumstances of adult life.[112] Furthermore, given the current problem of nutritional excesses with more than one-third (35.7%) of US adults being obese[158], it seems more pertinent to study the effects of maternal-fetal overnutrition on long-term health of the offspring.

The metabolic syndrome (central obesity, hyperlipidemia, hyperglycemia, and hypertension) has become a major public health challenge with an estimated 22% of US

adults having this condition.[21] Although the disorder is defined in various ways, the ultimate importance of recognizing this syndrome is that it helps identify individuals at high risk for both type 2 diabetes and cardiovascular disease. The cause of the syndrome remains obscure but the pathophysiology seems to be largely attributable to insulin resistance, excessive flux of fatty acids, and a chronic proinflammatory state.[45] There is no specific treatment for metabolic syndrome. Therapeutics include lifestyle changes (e.g., weight reduction and increased physical activity) and pharmaceutical agents, but as with all diseases, prevention is preferred. New theories have suggested that certain adverse exposures during the fetal/perinatal period could be contributing to the development of metabolic syndrome and thus this early life stage may offer an attractive point in the disease process for intervention strategies.

In a pilot study, we found that gestational and lactational exposure to a high fat diet resulted in the adult offspring developing obesity, hyperglycemia, insulin resistance, and hypertension.[33] The present study sought to further validate the findings of metabolic disturbance seen in offspring of dams fed a high fat diet and to determine if the diet consumed by the adult offspring would influence certain parameters of the metabolic syndrome. As an additional component and as a precursor to a follow-up project, we investigated the influence of early nutritional exposures on the food selection choices of adulthood by providing mice with a choice between diets. With this body of research the etiopathogenesis of such chronic diseases as the metabolic syndrome, type 2 diabetes, and cardiovascular disease may become more completely understood leading to early and effective prevention strategies.

Materials and Methods

Animals and Diets

The study was approved by the Virginia Tech Animal Care and Use Committee. Mice used in this protocol were identified by a unique four digit ear tag (National Band and Tag Company, Newport, KY). Four-week-old male and female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were acclimated for 1 week at 22.0 ± 1^{o}C, 40-60% humidity, and 12/12-hour light/dark cycle with 4 mice per cage. During acclimation, mice were provided standard rodent diet [(RD) Harlan Teklad Global Diet 2018, Madison, WI] and water *ad libitum*.

Following acclimation, the breeder males were continued on RD for the duration of the project and the females were arbitrarily assigned to one of two dietary groups: RD (*n* = 13) (Table 1) or high fat diet [(HFD) Harlan Teklad Global Diet TD.06414, Madison, WI) (*n* = 17) (Table 2). Both diets were formulated to meet or exceed the nutritional requirements of mice. Dietary treatments were continued for 5 weeks prior to breeding to establish chronic consumption of the specific diet. During this time female mice were housed together with a maximum of 4 mice per cage.

For breeding, females were placed overnight with males in a 2:1 ratio and checked the following morning for the presence of a vaginal plug, which indicated a successful mating event. Plug-positive females were designated as gestation day 0 and were then singly housed while remaining on their assigned diet throughout gestation and lactation. At the time of delivery, typically at gestation day 19, pup number was determined by gentle, non-disruptive, inspection of the cage and pup age was designated as postnatal day 1. At postnatal day 25, pups were weaned and female offspring were kept in the study and continued on the diet of their dam until 7 weeks of age. At 7 weeks of age, offspring were assigned, via stratified random sampling, to one

of six experimental conditions (ECs) (Figure 1) that allowed them to continue on the diet of their dam, be placed on the opposing diet, or be given a choice between the two diets. For the choice conditions (EC3 and EC6) approximately equal amounts RD and HFD were placed in the same style top-feeder used by the all the mice in the experiment and the two diets were separated by a metal plate. The sample size for each EC was as follows: EC1) n = 11, EC2) n = 11, EC3) n = 11, EC4) n = 8, EC5) n = 9, and EC6) n = 9. Experimental conditions were continued until 18 weeks of age.

Measurements and Assessments

Body Weight, Food Consumption, and Body Composition of Dams and Offspring: Body weight and food consumption were measured using an electronic scale (Catalog# 80104002, OHAUS Scout Pro, Parsippany, NJ). Body composition was determined via nuclear magnetic resonance (NMR) (Bruker LF90 NMR Analyzer, Billerica, MA). For the dams, body weight and food consumption were measured weekly during the 5 weeks of dietary treatment prior to breeding. Body composition was determined at three time points: 1) before starting the diet, 2) after one month on the diet, and 3) at weaning. For the offspring, body weight was measured weekly from 1 to 18 weeks of age. The sex of the pups was not determined until 3 weeks of age and only females were kept in the study; therefore body weights for week 1 and 2 include males while weeks 3 through 18 reflect female offspring only. Food consumption was measured weekly from 7 to 18 weeks of age (i.e., once they entered their EC). Although recorded, data on body weight and food consumption at 8 and 9 weeks of age were not analyzed due to potential irregularities incurred as the mice adjusted to the diet of their EC, which may or may not have been different from their previous diet. Body composition was determined at 7 and

18 weeks of age. The amount of intra-abdominal fat was subjectively assessed immediately following euthanasia at 18 weeks of age.

Litter Size, Offspring Mortality Rate, and Number of Male and Female Offspring: Litter size was established by counting the number of pups delivered. Offspring mortality rate was calculated as number of pups that died at any point post-delivery by number of pups delivered. The sex of the pups was determined by 3 weeks of age and the number of males and females in each litter was documented.

Glucose Tolerance Test of Offspring at 7 and 18 Weeks of Age: Blood glucose levels in mg/dL were determined by performing a glucose tolerance test (GTT). Mice underwent an overnight, 6-12 hour, fast. The following morning, mice were placed into a retraining device that allows easy access to the tail. Blood from the lateral tail vein was taken via insertion and removal of a 25G needle to produce a drop of blood which was then placed on a hand-held glucometer (FreeStyle Lite glucose meter, Abbott Diabetes Care Inc., Alameda, CA). After determining fasted blood glucose levels (time 0), a glucose load equaling 2g/kg was injected intraperitoneally and tail blood glucose was obtained at 15, 30, 60, 90 and 120 minutes post-glucose load. Blood glucose levels were compared among the treatment groups including RD controls as well as evaluated based on published glucose reference ranges for adult female C57BL/6 mice (114 – 154.7 mg/dL)[143] and levels used by the American Diabetes Association (ADA) and the World Health Organization (WHO) for the establishment of imparied glucose tolerance. The ADA and the WHO define impaired glucose tolerance as either a fasting plasma glucose level ≥ 100 mg/dL to < 126 mg/dL or a two-hour glucose level of ≥ 140 to < 200

mg/dL post-glucose load. Levels greater than the high reference ranges for impaired glucose tolerance are used to define type 2 diabetes.[77] Glucose levels in plasma are generally higher than glucose measurements in whole blood. A constant factor of 1.11 was used to convert concentration in whole blood to the equivalent concentration in the plasma.[159]

Serum Insulin and Leptin Levels of Offspring at 7 and 18 Weeks of Age: Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum insulin (Catalog# 80-INSMSU-E01, ALPCO Diagnostics, Salem, NH) and leptin (Catalog #90030, Crystal Chem Inc., Downers Grove, IL) concentrations in ng/mL. At both ages, blood was collected after a 6-12 hour fast. Blood collected at 7 weeks of age was attained via the lateral saphenous vein. The mouse was placed in a restraining device that allows easy access to the hindlimbs, the fur covering the lateral saphenous vein was shaved to allow visualization of the vein, a 27G needle was used to puncture the vein, and approximately 200 µl of blood was collected in Microvette® CB 300 capillary tubes (Catalog #NC9059691, Fisher Scientific, Pittsburgh, PA). Blood collected at 18 weeks of age was attained via cardiac puncture. The mouse was placed in an induction chamber containing oxygen (1 to 2 % flow rate) and isoflurane (1.5-3.5% flow rate) until the stage of surgical anesthesia was reached. The mouse was then moved to a table, placed in dorsal recumbency, and maintained on nose cone anesthesia. A 1 mL syringe with a 27G, ½ inch needle was introduced at the lower part of the sternum, just lateral to the xiphoid process, and 700 to 900 µl of blood was collected. While under nose cone anesthesia, mice were euthanized via cervical dislocation and death was confirmed by

thoracotomy. For both ages, blood was allowed to clot for 30 minutes to 1 hour at room temperature, centrifuged for 20 minutes, and then the serum collected. Serum aliquots of 20 µl were stored at -80^{o}C until the time of performing the ELISAs.

Serum Lipid Levels of Offspring at 18 Weeks of Age: Serum aliquots of 300 to 500 µl from each mouse were submitted to the Clinical Pathology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) for determination of triglyceride and cholesterol levels using their Olympus AU480 Chemical Analyzer. For this analysis, most samples were submitted within hours of blood collection; however samples attained over the weekend, when the Clinical Pathology Laboratory was closed, were stored in 2-8^{o}C and submitted within 48 hours. Lipid levels were compared among the various treatment groups including the RD controls as well as evaluated based on published reference ranges for adult female C57BL/6 mice for cholesterol (76.1–107.9 mg/dL) and triglycerides (65.8–90.2 mg/dL)[143]. Blood and serum collection procedures were the same as those described in the previous section on serum insulin and leptin levels for offspring at 18 weeks of age.

Metabolic Performance of Offspring at 18 Weeks of Age: A metabolic system [PhenoMaster/LabMaster, Technical Scientific Equipment (TSE) Systems, Chesterfield, MO] was employed to simultaneously measure respiratory exchange ratio, energy expenditure, and physical activity over a 48 hour period.[139] For this test, mice were maintained in their home cage, which limited stress thereby allowing an accurate reflection of typical metabolic performance. The home cage was outfitted with monitoring devices and the data generated were recorded and analyzed by integrated

hardware and software programs. Indirect calorimetry, which calculates energy produced by measuring O_2 consumption and CO_2 production, was used to determine the respiratory exchange ratio and energy expenditure. As an index of physical activity, infrared light-beam frames surrounding the home cage measured activity in three dimensions (X-Y-Z axis).[139]

Statistical Analysis

One-way analysis of variance (ANOVA), restricted maximum likelihood (REML) method, Student's t-test, and Tukey-Kramer test (JMP 9.0, SAS Institute Inc., Cary, NC) were used to establish differences between groups. Data sets were analyzed via determining differences due to age, time point, dam's diet, offspring's diet, and/or EC, which is a reflection of the diet of the dam and of the offspring, as deemed appropriate for each endpoint. Data were determined to be statistically significant when $p < 0.05$.

Results

Body Weight, Body Composition, Food Consumption, and Caloric Intake of Dams:

Body weights between the RD and HFD groups did not differ significantly at any time during the 5 weeks of dietary treatment prior to breeding; however HFD dams did have an increasingly significant higher body fat percentage when measured after one month of dietary treatment ($p < 0.01$) and at weaning ($p < 0.0001$) (Figure 2). Of note is the striking amount of body fat detected in HFD dams at the time of weaning. No significant difference in percent body fat was present when measured prior to the start of dietary treatments. HFD dams consumed significantly *less* food, averaging 2.17

grams/mouse/day, than their RD counterparts that averaged 2.76 grams/mouse/day ($p < 0.0001$), but had a significantly *higher* caloric intake with an average of 11.05 kcal/mouse/day as compared to 8.55 kcal/mouse/day for RD dams ($p < 0.001$) (Figure 3).

Litter Size, Offspring Mortality Rate, and Number of Male and Female Offspring: In comparing RD and HFD litters, there were no statistical differences in litter size, offspring mortality rate, or in the number of males and females in each litter with both groups having predominantly (60%) males.

Body Weight, Body Composition, Food Consumption, and Caloric Intake of Offspring:

In the initial few weeks of life HFD offspring weighed *less* than their RD counterparts, reaching significance at 2 weeks of age ($p < 0.05$); however at 5, 6, and 7 weeks of age they weighed significantly *more* ($p < 0.05$ for each time point) (Figure 4). No significant differences in body weight were present between the ages of 10 and 14 weeks. From 15 to18 weeks of age, offspring on Choice weighed significantly more than those on RD ($p < 0.05$ for weeks 15, 16, and 18, and $p < 0.01$ for week 17) (Figure 5). No significant differences in body weight were present between offspring on RD and HFD or HFD and Choice.

Body composition was assessed at 7 and 18 weeks of age (Figure 6). At 7 weeks of age, when the offspring remained on the diet of their dam, the percent body fat was

significantly higher in the HFD offspring as compared to their RD counterparts ($p < 0.0001$). At 18 weeks of age, when the offspring had been in their EC for eleven weeks, those on HFD or Choice had a higher percent body fat than those on RD ($p < 0.05$ between RD and HFD and $p < 0.01$ between RD and Choice). There was no significant difference in percent body fat between HFD and Choice. Although it did not reach statistical significance, based on the diet of the dam, body composition analysis at 18 weeks of age demonstrated that offspring from HFD dams had higher percent body fat than those from RD dams. Another observation was that based on the EC those offspring that were continued on the HFD of their dam into adulthood (EC5 and 7 due to their preference for HFD) had a higher percent body fat than the other ECs. The subjective assessment of intra-abdominal fat supported the quantitative assessment of percent body fat. On postmortem examination, the mice on HFD or Choice had larger amounts of adipose tissue, particularly within the central abdominal region, than those on RD. The amount of fat observed was highest for those in the Choice conditions (Figure 7).

Analysis of week 10 through 16 food consumption and caloric intake data demonstrated significant differences based on diet of the dam, diet of the offspring, and the EC. On the basis of *diet of the dam*, offspring born from HFD dams consumed more grams of food overall ($p < 0.01$), regardless of their current diet, and had a significantly higher caloric intake ($p < 0.05$) than offspring from RD dams. On the basis of *diet of the offspring*, those on RD consumed significantly *more* food than those on HFD ($p < 0.0001$) and significantly more than those on Choice ($p < 0.0001$). No significant difference in food consumption was present between HFD and Choice. Even though offspring on RD consumed more food than those on HFD and Choice, they had a

significantly *lower* caloric intake ($p < 0.0001$ for comparisons between RD and HFD and RD and Choice). There was no significant difference in caloric intake between HFD and Choice. On the basis of *EC*, food consumption was significantly *higher* for those in EC1 and EC4 with EC4 consuming the most food and both EC1 and EC4 consuming more than all other ECs (overall $p < 0.0001$) (Figure 8A). Conversely, caloric intake was *lower* for EC1 and EC4 with EC1 being the lowest and both EC1 and EC4 being significantly lower than all other ECs ($p < 0.0001$ between EC1 and 2, 3, 5, and 6 as well as between EC4 and 5; $p < 0.001$ between EC4 and 2 and 3; $p < 0.01$ between EC1 and 4; $p < 0.05$ between EC4 and 6) (Figure 8B). Of important note is that mice from EC4, those born from HFD dams and later changed to RD in adulthood, consumed the most food of all the ECs and had higher caloric intake than EC1, those born from RD dams and continued on RD in adulthood.

Analysis of food consumption and caloric intake also demonstrated an overwhelming preference for HFD by those mice in the Choice conditions. For both EC3 and EC6, the mice preferred to eat the HFD almost at the exclusion of the RD with the HFD composing 96% of food consumed and 97% of caloric intake.

Glucose Tolerance Test of Offspring at 7 and 18 Weeks of Age: Glucose tolerance testing performed at 7 weeks of age demonstrated no differences in blood glucose levels between RD and HFD offspring at time 0 (fasted blood glucose) or at 120 minutes post-glucose load. At all other time points HFD offspring did have significantly higher blood glucose levels: 15 ($p < 0.05$), 30, 60, and 90 ($p < 0.01$ for each time point) as compared to their RD counterparts (Figure 9A). At 18 weeks of age GTT was again

performed. At time 0, there were no significant differences in blood glucose levels based on diet of the dam, diet of the offspring, or the EC. At all other time points there were significant differences based on the diet of the offspring with offspring on HFD or Choice having higher glucose levels than those on RD. Offspring on HFD had significantly higher blood glucose levels at time 15 ($p < 0.01$), 30 ($p < 0.01$), 60 ($p < 0.0001$), 90 ($p < 0.001$), and 120 ($p < 0.01$) minutes post-glucose load. Offspring on Choice had significantly higher blood glucose levels at time 30 ($p < 0.0001$), 60 ($p < 0.001$), and 120 ($p < 0.05$) minutes post-glucose load (Figure 9B). Of note is that although at 7 weeks of age HFD offspring returned to essentially the same glucose level as RD offspring at the 120 minute mark, at 18 weeks of age glucose levels of offspring on HFD or Choice remained significantly higher than those on RD at this time point. Also, for the offspring on HFD or Choice, glucose levels at the 120 minute mark were well within the range of impaired glucose tolerance 2 hours post-glucose load (≥140 to < 200 mg/dL) set by the ADA and WHO. Using the constant factor of 1.11 to convert whole blood values to plasma values, the average plasma equivalent values in mg/dL for the offspring were 126 for RD, 163 for HFD, and 155 for Choice.

Serum Insulin and Leptin Levels of Offspring at 7 and 18 Weeks of Age:

Insulin levels at 7 weeks of age were significantly higher for RD offspring as compared to their HFD counterparts ($p < 0.05$). At 18 weeks of age there were no significant differences in insulin levels due to either diet of the dam, diet of the offspring, or the EC. Leptin levels at 7 weeks of age were significantly higher for the HFD offspring as compared to their RD counterparts ($p < 0.0001$) (Figure 10C). At 18 weeks of age a significant difference in leptin levels was present based on the diet of the offspring with

those on HFD ($p < 0.05$) or Choice ($p < 0.01$) having higher leptin levels as compared to those on RD (Figure 10D). Leptin is produced by adipose tissue and leptin levels are generally higher in animals that have more body fat. Hyperleptinemia is now recognized as an additional marker of the metabolic syndrome.[160] This relationship between the amount of adipose tissue and serum leptin levels is supported by our data on body fat percentage (Figure 10A and 10B) and serum leptin levels (Figure 10C and 10D). Regression analysis demonstrated a significant linear association between body fat percentage and serum leptin levels ($p < 0.0001$, RSquare = 0.67) (Figure 10E).

Serum Lipid Levels of Offspring at 18 Weeks of Age: Offspring on HFD or Choice had significantly higher serum cholesterol and triglyceride levels as compared to their RD counterparts (mean cholesterol values in mg/dL = 76 for RD, 94 for HFD, and 101 for Choice; mean triglyceride values in mg/dL = 44 for RD, 54 for HFD, and 56 for Choice; $p < 0.05$ and for each comparison to RD with the exception of $p < 0.01$ between cholesterol levels of RD and Choice) (Figure 11A and 11B). No significant differences in serum lipids were present between HFD and Choice. In comparing groups based on their EC, EC3 had the highest cholesterol levels of all the ECs, EC3 was significantly higher than EC1 ($p < 0.05$) and EC4 ($p < 0.01$), and EC2 was significantly higher than EC4 ($p < 0.05$) (Figure 11C). A similar trend occurred with triglyceride levels (Figure 11D). Of important note is that EC2 and EC3, offspring born from dams consuming RD and later changed to a fat-rich diet in adulthood, demonstrated rather high serum cholesterol and triglyceride levels as compared to the other ECs.

Metabolic Performance of Offspring at 18 Weeks of Age: The respiratory exchange ratio (RER) is indicative of the fuel source used. A value of 0.70 indicates that fat is the predominant fuel source, a value of 0.85 indicates that a mix of fat and carbohydrates are being used for fuel, and a value of 1.00 or above indicates that carbohydrates are the predominant fuel source. Fittingly, offspring on RD had a significantly higher RER as compared to offspring on HFD or Choice with a mean value of 0.88 as compared to mean values of 0.76 and 0.77 for HFD and Choice, respectively ($p < 0.0001$ for each comparison with RD) (Figure 12). No significant differences in RER were detected via analysis by diet of the dam or by the EC. On the basis of diet of the dam, activity level during both the day and night was significantly higher for offspring from HFD dams as compared to offspring from RD dams ($p < 0.05$) (Figure 13). For night activity level, mice in EC7 were significantly more active than those in EC3 ($p < 0.05$); no other significant differences in activity level were present based on ECs. No significant differences in activity level were detected via analysis by diet of the offspring. No significant differences in energy expenditure per fat-free mass (EE/FFM) were detected via analysis by diet of the dam, diet of the offspring, or by EC.

Discussion

The DOHaD hypothesis proposes that environmental factors, particularly nutrition, can act in early life to program the risks for chronic diseases in adult life. The present study supports this hypothesis. The putative concepts and mechanisms of the DOHaD hypothesis that are in concurrence with our results are discussed below. These

include developmental plasticity and programming, mismatch, catch-up growth, and altered programming of the appetite regulatory network.

Collectively the data clearly establish that exposure to a diet rich in fat is capable of inducing central obesity, elevated lipid levels, and hyperglycemia as well other useful markers for the diagnosis of the metabolic syndrome. It also establishes that the time of exposure (i.e., early in life, late in life, or throughout life) can influence the impact of such a diet on metabolic health. In this study early exposures were those that occurred from the age of conception up until the beginning of sexual maturity at 7 weeks (analogous to human adolescence) while late exposures were those that occurred from 7 weeks of age up until the age of mature adulthood at 18 weeks (analogous to humans at 20-30 years of age).[161] A common finding of the study is that mice, dams and offspring included, on RD ate *more* and a had *lower* caloric intake as compared to mice on HFD, while the reverse, mice on HFD ate *less* and had a *higher* caloric intake, is also true. This finding can be explained by a robust homeostatic ability of rodents to maintain body weight via regulation of food intake and energy expenditure.[162] Although those on HFD ate less, they still ingested more calories because the HFD is more calorie-dense than the RD. Incidentally, although aberrations to metabolic health observed in our mice were quite profound, they may actually be blunted as compared to what could occur in humans that lack this homeostatic drive to maintain standardized body habitus. Also, mice in the Choice condition overwhelmingly preferred to eat the HFD over the RD, thus the Choice diet produced similar effects on metabolic parameters as the HFD. Nonetheless, the Choice diet generally resulted in more profound perturbations in metabolic parameters than the HFD including higher body weight and percent body fat. This suggests that consumption of a fat-rich diet combined

with carbohydrates (i.e., RD), even in relatively small amounts, can have greater consequences to health than if consuming solely a fat-rich diet.

The results on lipid levels serve as a good example of how adult eating habits can exacerbate certain parameters of the metabolic syndrome. In comparing cholesterol and triglyceride levels based on experimental condition, EC2 and EC3, offspring with early nutritional exposure to a low fat diet (i.e., RD) and later exposed to a high fat diet (i.e., HFD or Choice) in adulthood, demonstrated higher serum cholesterol and triglyceride levels as compared to other ECs. This finding suggests that, at least for the metabolic parameter of serum lipids, a "mismatch" occurred in those offspring developmentally adapted to a low fat diet and later exposed to a high fat diet. The implications of this finding could suggest that benefits of exposure to a healthy diet throughout childhood can be negated if an unhealthy diet is consumed as an adult. Such an individual may face greater health challenges than even those consuming an unhealthy diet throughout life. This mismatch phenomenon is commonly addressed in discussions on the DOHaD hypothesis and generally includes the additional considerations of developmental plasticity, predictive adaptive responses, and changes in programming. Developmental plasticity is the ability of an organism to change its phenotype in response to changes in the environment.[15] If this change or adaptation is permanent, it is considered a programming change and is associated with persistent effects in structure and/or function.[15,16] In most cases these programming changes are beneficial for the health and survival of the organism. However, the problem of mismatch occurs when individuals developmentally adapted to one environment are exposed to another.[17] Nature has many examples of adaptive programming occurring during critical periods of developmental plasticity. Gluckman and Hanson use the

example of the meadow vole (*Microtus pennsylvanicus*) to illustrate these concepts.[13] Meadow moles born in autumn have a thicker hair coat than those born in the spring.[163] This occurs as a response of the fetus to maternally-derived signals for day length.[164] A thick coat confers no real advantage in the perinatal period, but reflects an adaptive response for survival in the cold environment that was predicted while in the womb.[13] But if, for example, a near-term dam pregnant with pups that are prepared for spring, thus do not have a thick haircoat, was transported to an area that is cold, then those offspring would face the problem of mismatch and even death being improperly prepared to survive in this unpredicted environment. Other examples of mismatch include people whose birth weights were towards the lower end of normal and who subsequently grow up in affluent societies being at increased risk for heart disease, type 2 diabetes, and hypertension.[13,18]

The analyses of body weight and body composition of offspring from 1 to 7 weeks of age demonstrate how early exposure to high fat diet can increase the risk of the metabolic syndrome. From 1 to 4 weeks of age, pups born from HFD dams were *smaller/weighed less* than those born from RD dams, reaching significance at 2 weeks of age. However, this situation was reversed by 5 to 7 weeks of age such that HFD offspring were *larger/weighed more* than their RD counterparts. This finding suggests that the HFD pups underwent a period of “catch-up growth”. Catch-up growth is the term used to describe the phenomenon in which children return to their genetic trajectory for size after a period of growth delay or arrest. It may occur at any stage of growth, but it is most commonly observed in the first 1 to 2 years of life.[21] Studies have found that catch-up growth often results in overcompensation, whereby the organism meets, then exceeds normal weight and frequently has excessive fat deposition. Indeed, when body

composition was assessed at 7 weeks of age, those exposed to HFD had significantly higher percent body fat than those exposed to RD. This rapid and excessive catch-up growth has been associated with the development of adult obesity, insulin resistance, metabolic syndrome, and type 2 diabetes.[8,21,22]

Results from week 10 through 16 food consumption and caloric intake data illustrate how perinatal exposure to a high fat diet can increase the risk of metabolic syndrome in the offspring and how adult eating habits can exacerbate certain parameters of the metabolic syndrome. On the basis of *diet of the dam*, overall offspring born from HFD dams consumed more grams of food, regardless of their current diet, and had a significantly higher caloric intake than offspring from RD dams. On the basis of *EC*, food consumption was significantly *higher* for those in EC1 and EC4 with EC4 consuming the most food and both EC1 and EC4 consuming more than all other ECs. Conversely, caloric intake was *lower* for EC1 and EC4 with EC1 being the lowest and both EC1 and EC4 being significantly lower than all other ECs. What is important to focus on is that EC4, mice born from HFD dams and later changed to RD in adulthood, consumed the most food of all the ECs and had higher caloric intake than EC1, those born from RD dams and were continued on RD in adulthood. Collectively the findings based on diet of the dam and the EC suggest that early nutritional exposures may have altered the programming for appetite regulation. A number of studies have explored the possibility that nutrition in early life can alter the level of energy intake of the offspring through inducing changes in the action, expression, and localization of specific neuropeptides. Appetite stimulating neuropeptides include neuropeptide Y (NPY) and agouti-related peptide (AgRP) while appetite suppressing neuropeptides include melanocyte-stimulating hormone (MSH) and serotonin. Studies in rats exposed to

hyperglycemia and/or hyperinsulinemia in the fetal or neonatal period provide an example of how early nutritional exposures may lead to an altered programming in appetite. These exposed rats developed permanent changes in body fat mass and in the hypothalamic neuronal circuitry regulating appetite in the adult brain. One such study used rats to look at the effects of exposure to milk from diabetic dams and found that offspring of control dams cross-fostered to diabetic dams developed early postnatal growth delay and showed structural and functional hypothalamic “malprogramming” as it relates to appetite stimulation and suppression.[37] Using immunocytochemical staining, exposure to the higher glucose milk from diabetic dams resulted in an up-regulation of the appetite stimulants NPY and AgRP and a down-regulation of the appetite suppressant MSH. Morphometric analyses demonstrated increased total number of neurons in the paraventricular nucleus[37] which is involved in body weight control and the regulation of blood pressure[38,39]. Such studies suggest that postnatal nutrition may impact the development of the hypothalamic-neuropeptide appetite regulatory system which is critically involved in lifelong regulation of appetite, body composition, and metabolism.

In conclusion, our results demonstrate that exposure to high fat diet in early life is capable of inducing central obesity, elevated lipid levels, and hyperglycemia as well other markers used for the diagnosis of the metabolic syndrome. By altering the diets of the mature offspring we demonstrated that the eating habits of adulthood have the potential to mitigate or exacerbate certain metabolic parameters established earlier in life. Mechanisms contributing to the observed metabolic aberrations could include developmental plasticity and mismatch, catch-up growth, and altered programming of the appetite regulatory network. The implications of this study are far reaching, carrying

importance to not only professionals in the field of pediatrics, preventative medicine, and others, but also to the general public. Given the knowledge that rodents prefer the high fat diet, future studies will explore the impact of policy-based interventions, such as the “junk food tax”, in controlling obesity. Such studies would make use of operant conditioning chambers and shaping to determine if rodents are willing to “pay a higher price” (i.e., press on a lever more times) for the preferred high fat food. In this way, our research team may begin to understand the importance of “price” on our food choices and consumption.

ACKNOWLEDGEMENTS: We would like to thank The Institute for Society, Culture, and Environment (ISCE) of Virginia Tech for their financial support of this project. We would also like to acknowledge the hard work and dedication of undergraduate research assistants Grayson Cobb, Jacklyn Daly, Tamima Hossain, and Michael Talanian.

Tables and Figures

Table 1. Standard Rodent Diet (RD) Composition

RD	Kcal (%)
Protein	24
Carbohydrate	58
Fat	18
Fat Composition (%)	
Saturated	16
Monounsaturated	23
Polyunsaturated	60

Table 2. High Fat Diet (HFD) Composition

HFD	Kcal (%)
Protein	18.4
Carbohydrate	21.3
Fat	60.3
Fat Composition (%)	
Saturated	37
Monounsaturated	47
Polyunsaturated	16

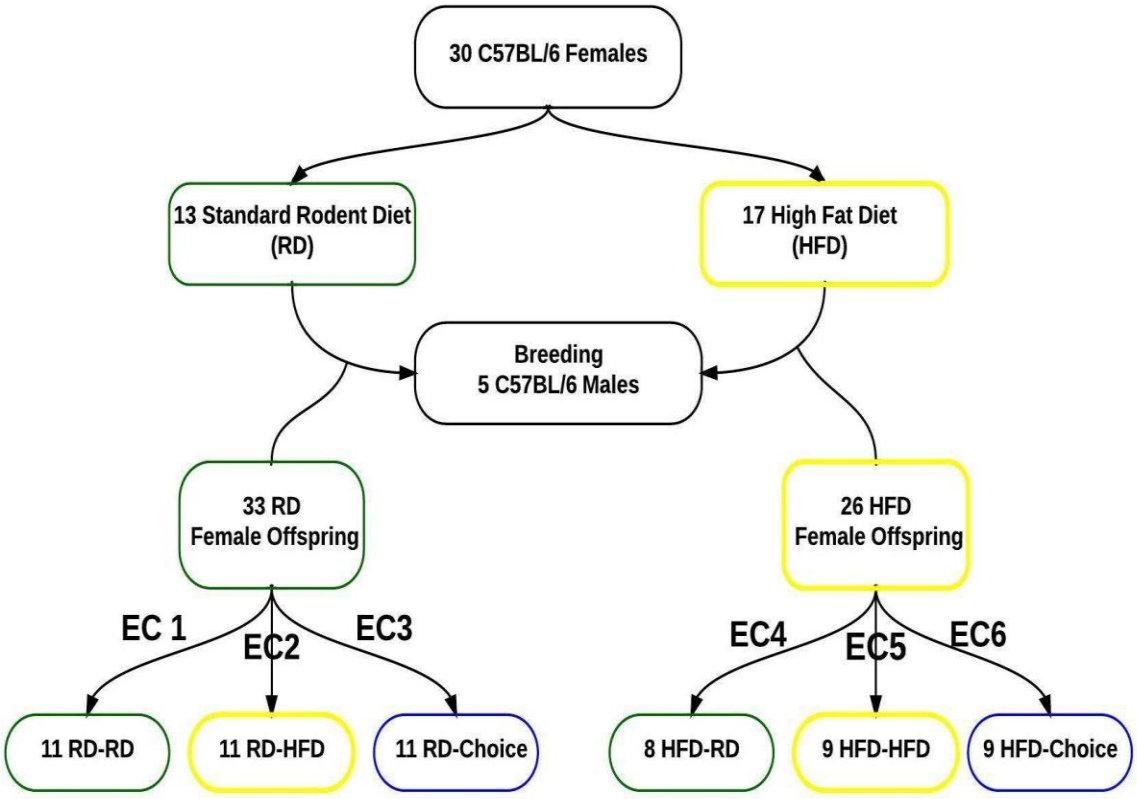

Fig. 1. Experimental Design. 30 C57BL/6 females were randomly assigned to either a standard rodent diet (RD) or a high fat diet (HFD). Dietary treatments were continued 5 weeks prior to breeding and throughout gestation and lactation. At weaning, female offspring were continued on the diets of their dam until 7 weeks of age at which time they were assigned, via stratified random sampling, to one of six experimental conditions (EC) that allowed them to continue on the diet of their dam, be placed on the opposing diet, or be given a choice between the two diets; EC were maintained until 18 weeks of age.

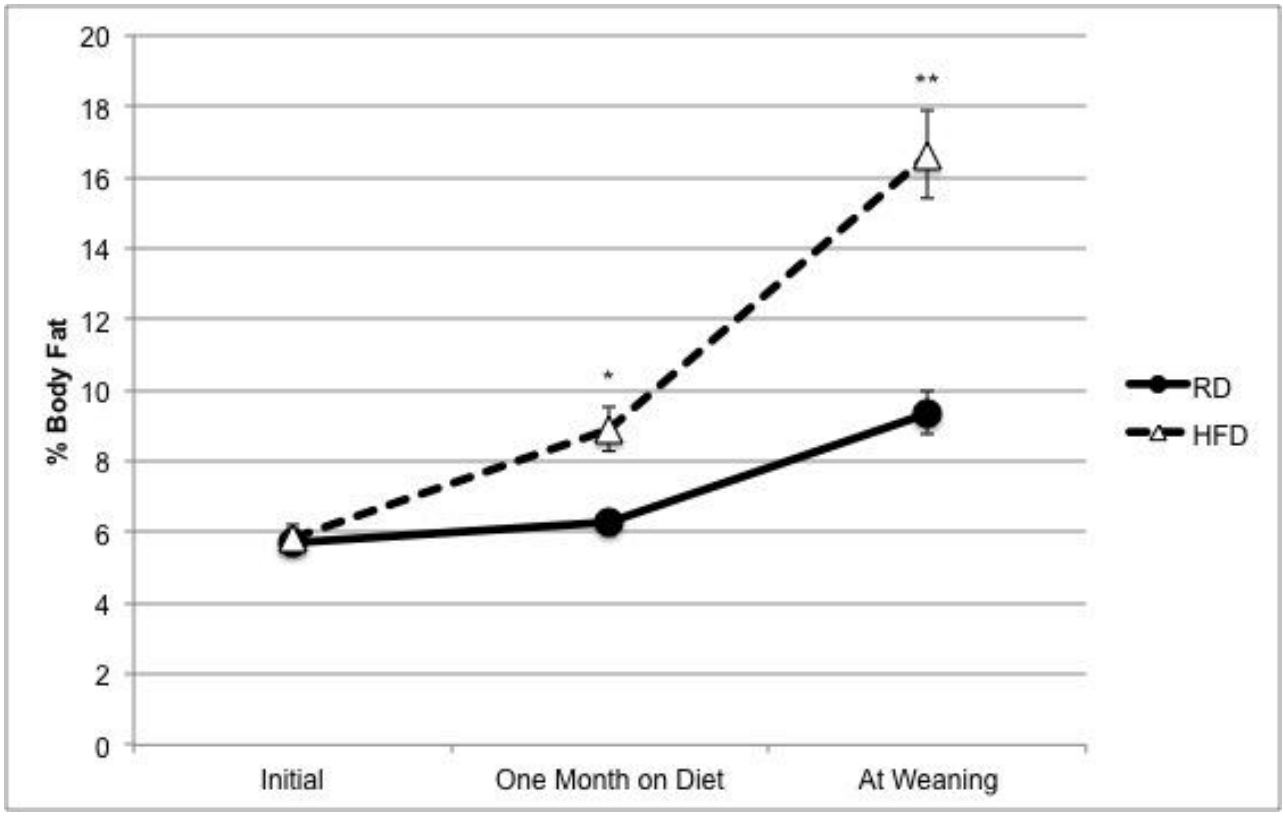

Fig. 2. Percent Body Fat of Dams. As compared to dams consuming a standard rodent diet (RD), those consuming a high fat diet (HFD) had a significantly higher percent body fat when measured after one month of dietary treatment and at weaning. No significant difference in percent body fat was present when measured prior to the start of dietary treatments. The body weights between the RD and HFD groups did not differ significantly at any time during the 5 weeks of dietary treatment prior to breeding. Data are mean ± SE; n = 13 for RD and n = 17 for HFD at the initial and one month time points; n = 12 for RD and n = 13 for HFD at weaning; *$p < 0.01$ and **$p < 0.0001$.

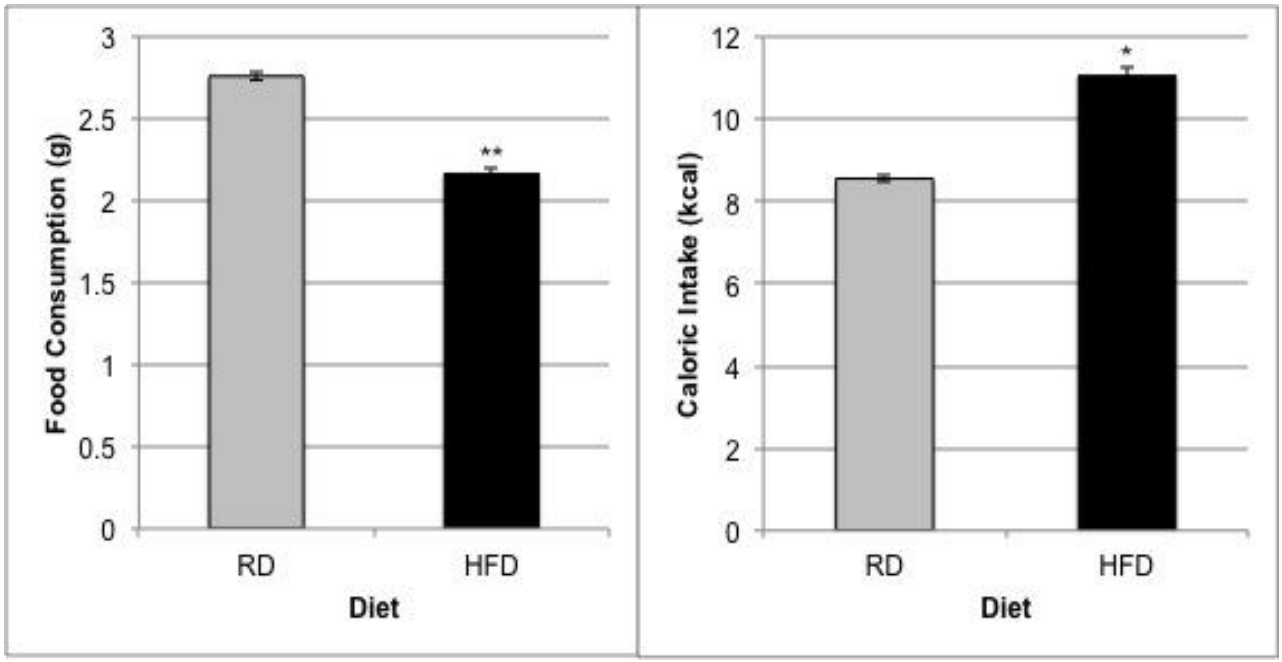

Fig. 3. Food Consumption and Caloric Intake of Dams. Those fed a high fat diet (HFD) consumed significantly *less* than their standard rodent diet (RD) counterparts, but had a significantly *higher* caloric intake. Food consumption and caloric intake were calculated as per mouse per day in grams and in kilocalories, respectively. Data are mean ± SE; $n = 3$ for RD and $n = 4$ for HFD; *$p < 0.001$ and **$p < 0.0001$.

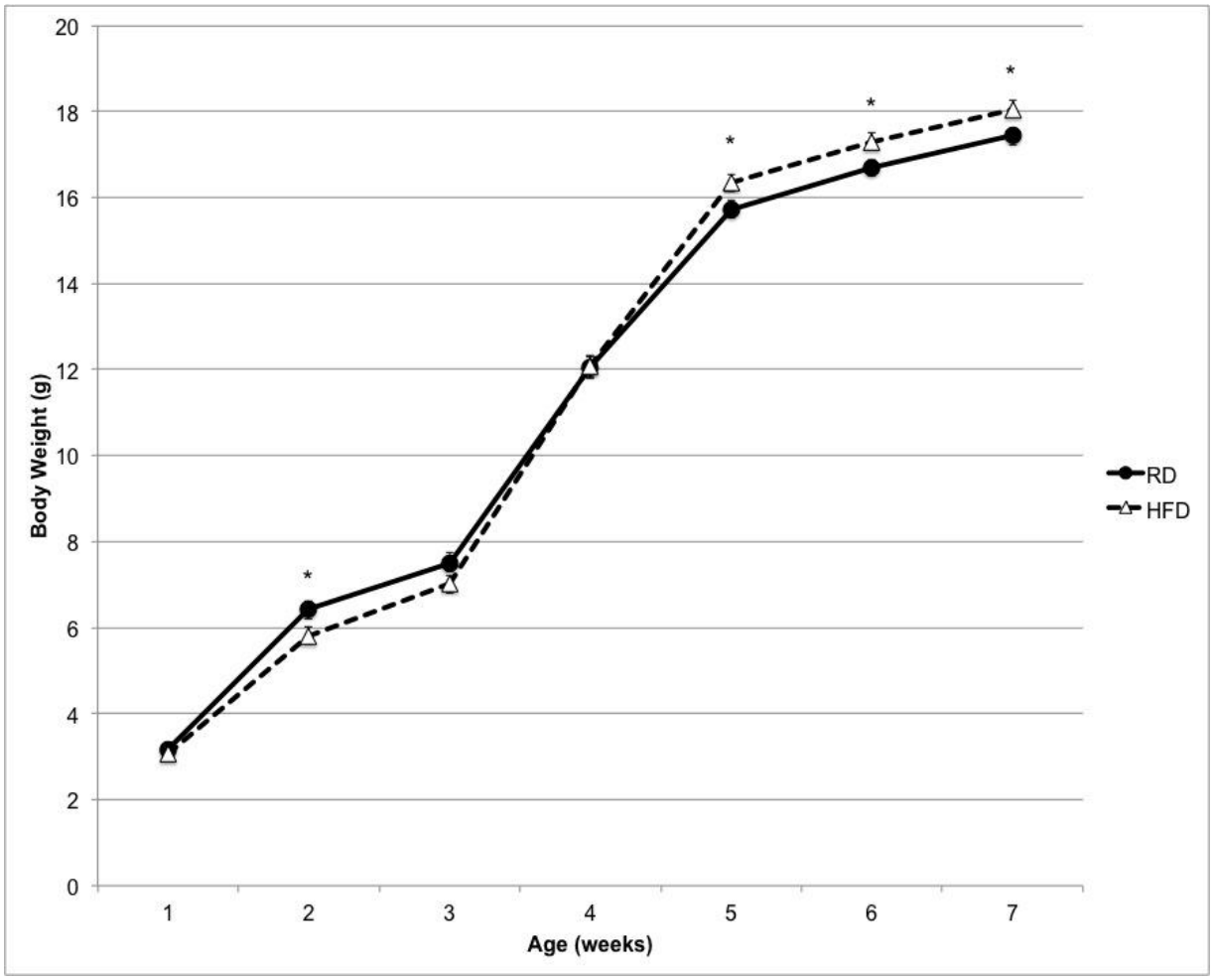

Fig. 4. Body Weight of Offspring from 1 to 7 Weeks of Age. In the initial few weeks of life HFD offspring weighed *less* than their RD counterparts, reaching significance at 2 weeks of age; however at 5, 6, and 7 weeks of age they weighed significantly *more*. Data are mean ± SE; n = 12 for RD and n = 14 for HFD for weeks 1 and 2; n = 33 for RD and n = 26 for HFD for weeks 3 through 7; *$p < 0.05$.

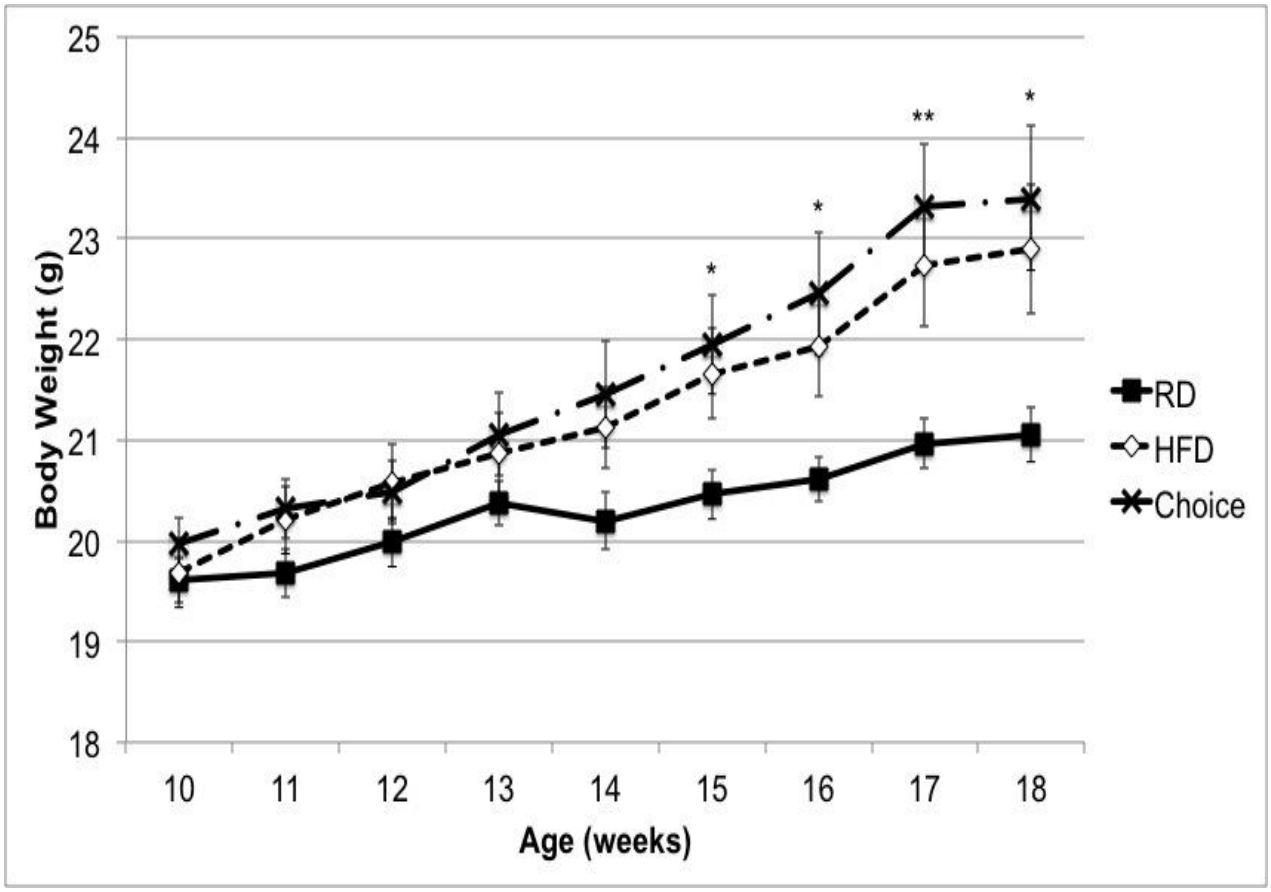

Fig. 5. Body Weight of Offspring from 10 to 18 Weeks of Age. No significant differences in body weight were present between the ages of 10 to 14 weeks. At 15 weeks of age and continuing until the termination of the project at 18 weeks of age, there was a significant difference in body weight based on the diet of the offspring with those on the Choice diet weighing significantly more than those on standard rodent (RD) diet. Data are mean ± SE; n = 19 for RD, n = 20 for high fat diet (HFD), and n = 20 for Choice for all weeks except week 14 in which n = 18, 19, and 19, respectively; *p < 0.05 and **p < 0.01.

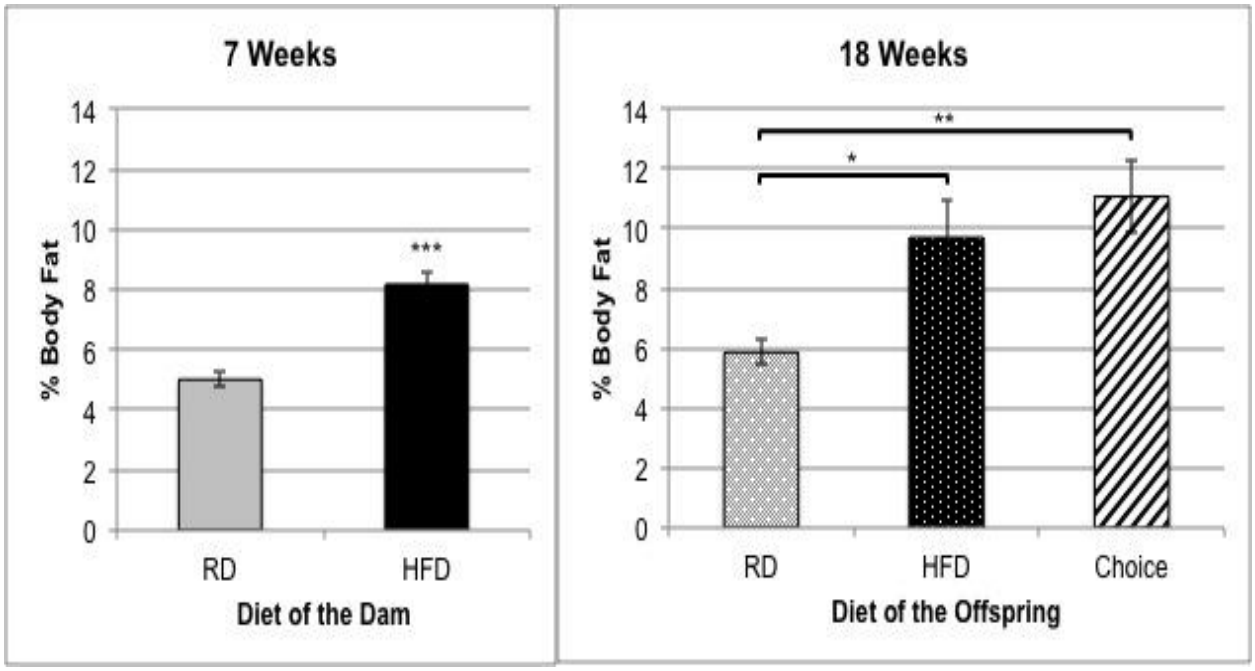

Fig. 6. Percent Body Fat of Offspring at 7 and 18 Weeks of Age. At 7 weeks of age the percent body fat was significantly higher in the high fat diet (HFD) offspring as compared to their RD counterparts. At 18 weeks of age offspring on HFD or Choice had higher percent body fat than those on standard rodent diet (RD). For the 7 weeks, data are mean ± SE; n = 33 for RD and n = 26 for HFD. For the 18 weeks, data are mean ± SE; n = 19 for RD, n = 20 for HFD, n = 20 for Choice; *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

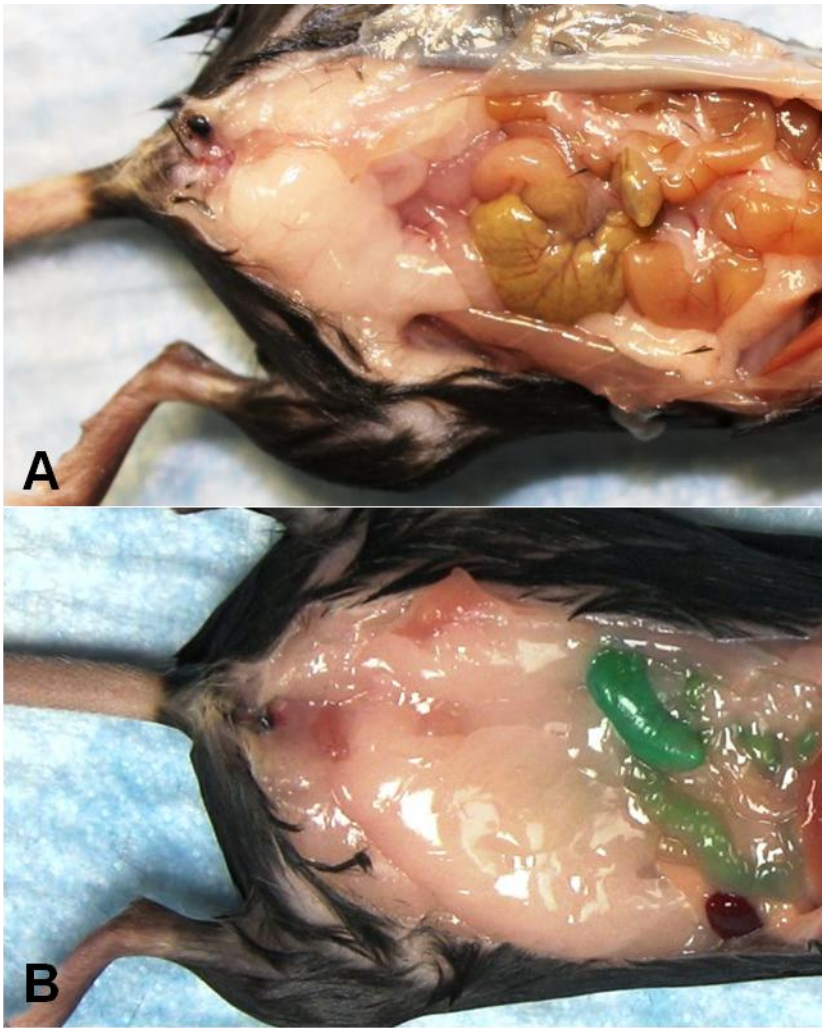

Fig. 7. Subjective Assessment of Intra-abdominal Fat of Offspring at 18 weeks of Age. On postmortem examination offspring on high fat diet (HFD) or Choice had larger amounts of adipose tissue, particularly within the central abdominal region, than those on standard rodent diet (RD). The amount of fat observed was highest for those in the Choice conditions. The animals pictured are representative of their group with (A) representing mice on RD from Experimental Condition (EC) 1 and (B) representing mice on Choice from EC3.

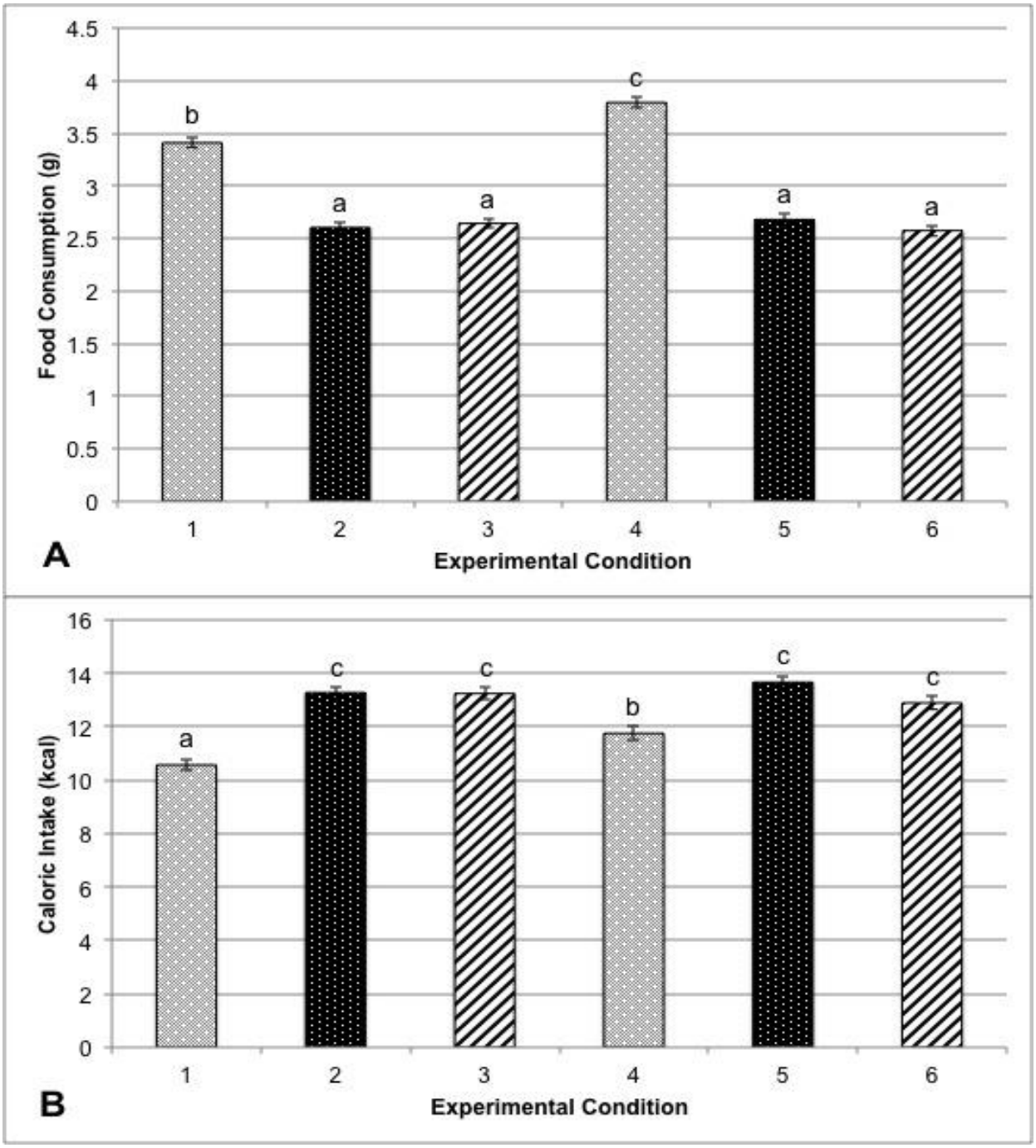

Fig. 8. Food Consumption and Caloric Intake of Offspring from 10 to 16 Weeks of Age. Food consumption was significantly higher for those in experimental condition (EC)1 and EC4 with EC4 consuming the most food and both EC1 and EC4 consuming more food than all other ECs (A). Conversely, caloric intake was lower for EC1 and EC4 with EC1 having the lowest caloric intake and both EC1 and EC4 being significantly

lower than all other ECs (B). For (A), data are mean ± SE; n = 11 for EC1, 2, and 3, n = 8 for EC4, and n = 9 for EC 5 and 6; overall $p < 0.0001$. For (B), data are mean ± SE; n = 11 for EC1, 2, and 3, n = 8 for EC4, and n = 9 for EC5 and 6; $p < 0.0001$ for comparisons between EC1 and 2, 3, 5, and 6 as well as for the comparison between EC4 and 5; $p < 0.001$ for comparisons between EC4 and 2 and 3; $p < 0.01$ between EC1 and 4; $p < 0.05$ between EC 4 and 6. Different letters represent differences between ECs.

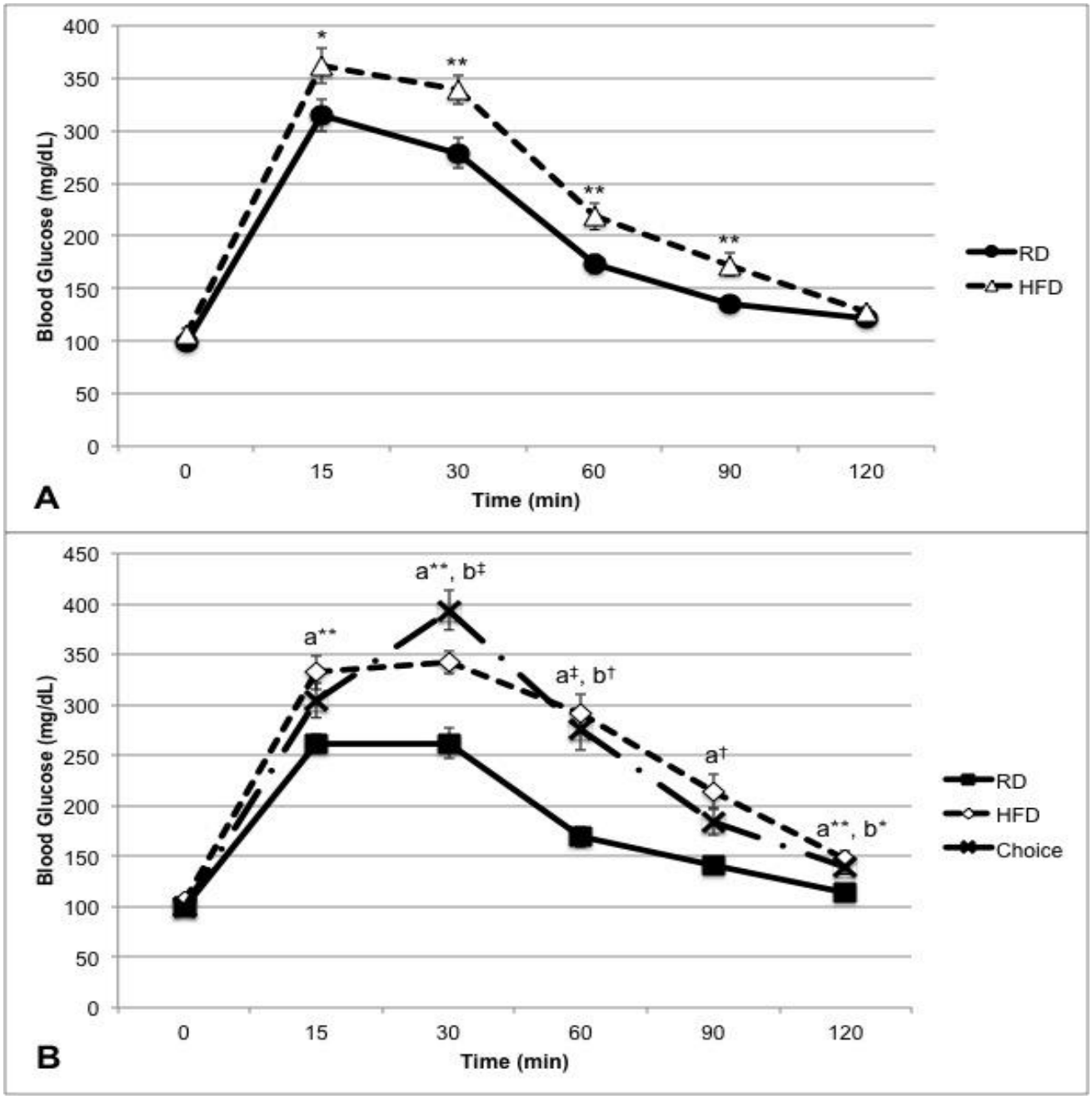

Fig. 9. Glucose Tolerance Test of Offspring at 7 and 18 Weeks of Age. At 7 weeks of age, high fat diet (HFD) offspring had significantly higher blood glucose levels at 15, 30, 60, and 90 minutes post-glucose load as compared to their standard rodent diet (RD) counterparts (A). At 18 weeks of age, there was a significant difference based on the diet of the offspring with HFD and Choice having higher glucose levels than RD (B). Offspring on HFD had significantly higher blood glucose levels at time 15, 30, 60, 90,

and 120 minutes post-glucose load. Offspring on Choice had significantly higher blood glucose levels at time 30, 60, and 120 minutes post-glucose load. For (A), data are mean ± SE; n = 33 for RD and n = 26 HFD. For (B), data are mean ± SE; n = 19 for RD, n = 20 for HFD, and n = 20 for Choice. Symbols for (A and B): a = HFD is higher than RD, b = Choice is higher than RD, * $p < 0.05$, ** $p < 0.01$, $^{\dagger} p < 0.001$, $^{\ddagger} p < 0.0001$.

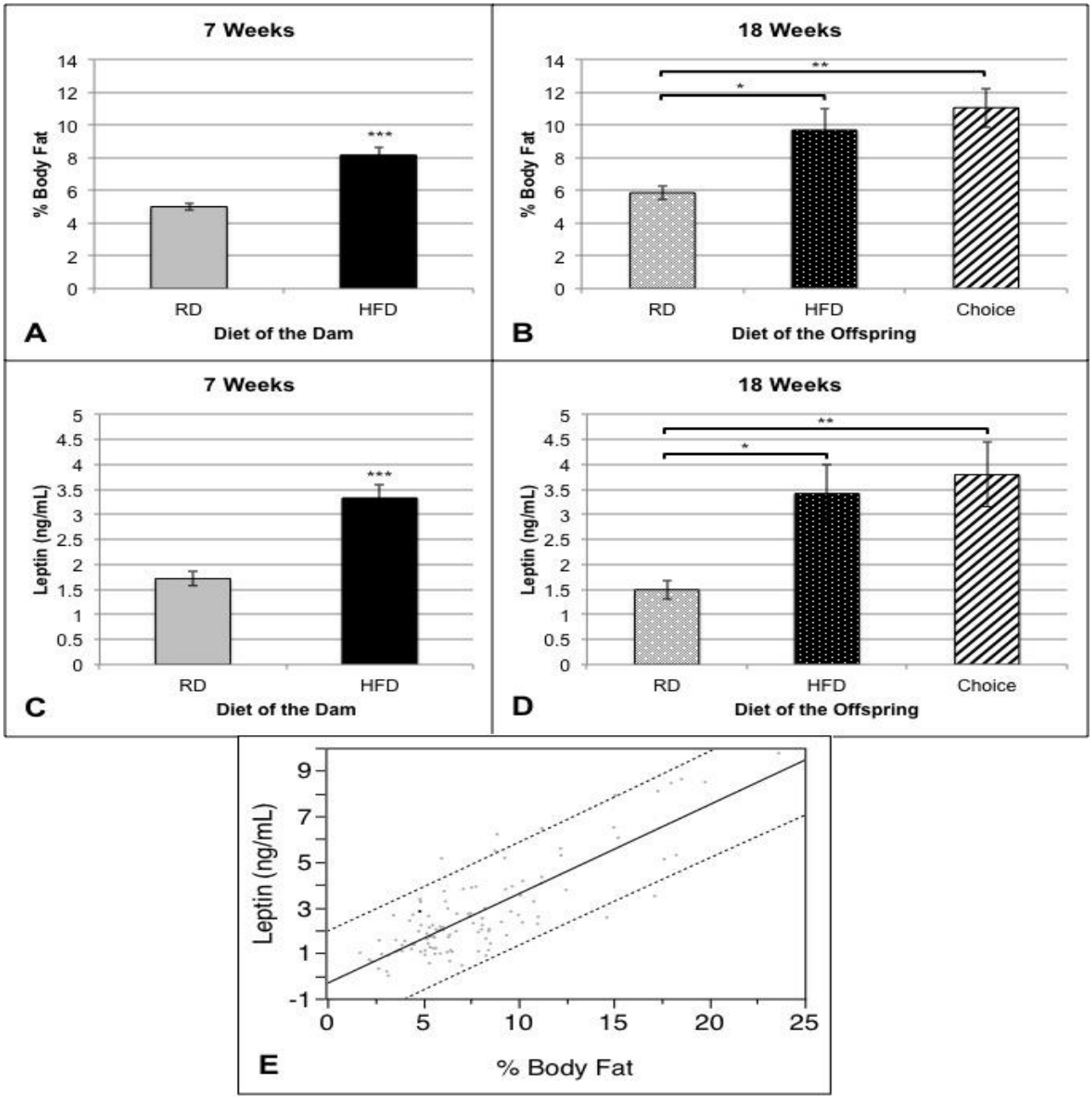

Fig. 10. Percent Body Fat and Serum Leptin Levels of Offspring at 7 and 18 Weeks of Age. At 7 weeks of age, percent body fat and serum leptin levels were significantly higher in the high fat diet (HFD) offspring as compared to their standard rodent diet (RD) counterparts (A and C). At 18 weeks of age, offspring on HFD or Choice had significantly higher percent body fat and serum leptin levels than those on RD (B and D). Regression analysis demonstrated a significant linear association between percent

body fat and serum leptin levels (E). For (A and C), data are mean ± SE; n = 33 for RD and n = 26 for HFD. For (B and D) data are mean ± SE; n = 19 for RD, n = 20 for HFD, n = 20 for Choice; *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. For (E) n = 118, RSquare = 0.67, and $p < 0.0001$.

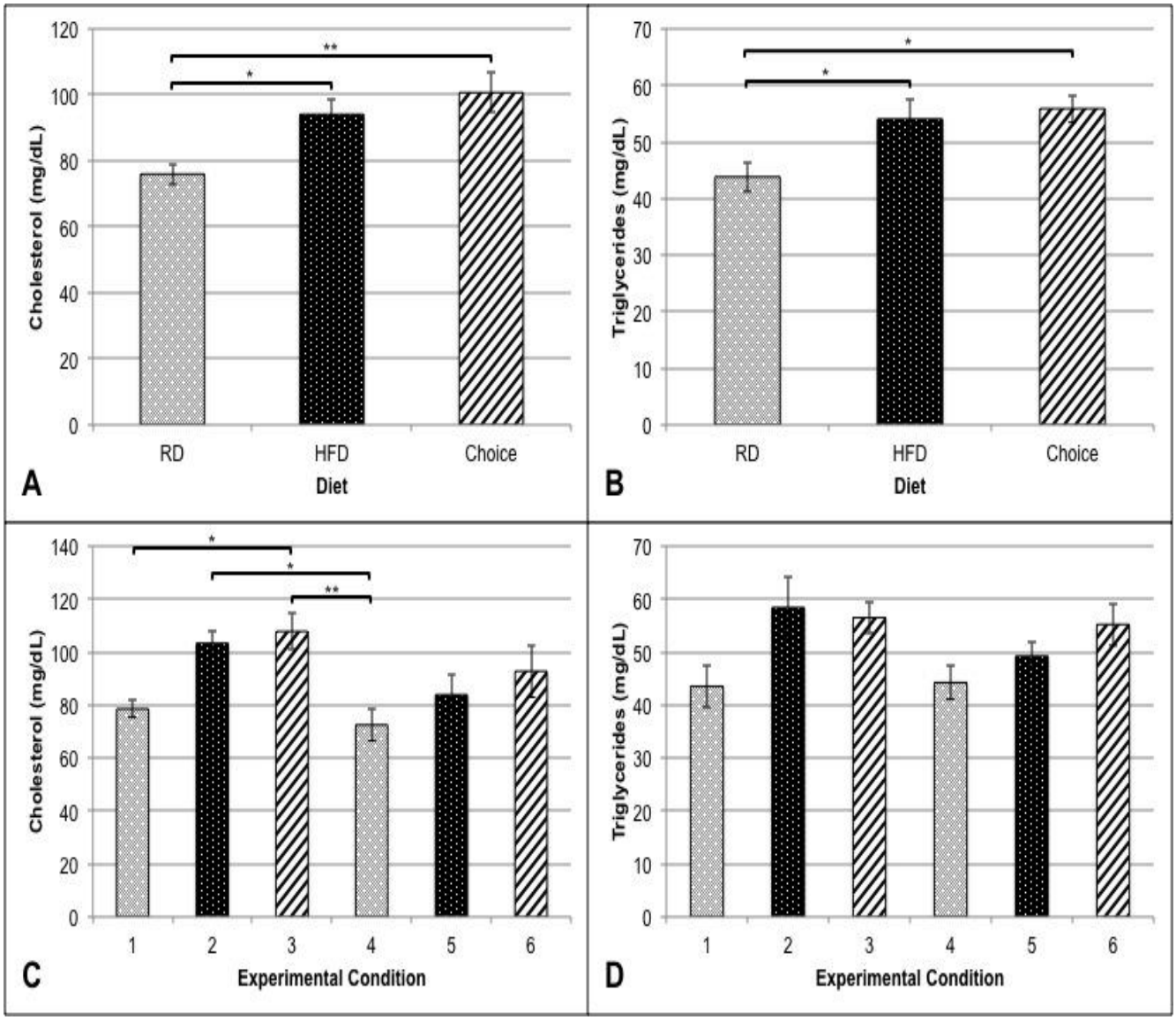

Fig. 11. Serum Lipid Levels of Offspring at 18 Weeks of Age. Offspring on high fat diet (HFD) or Choice had significantly higher cholesterol and triglyceride levels as compared to their standard rodent diet (RD) counterparts (A and B). In comparing cholesterol levels based on experimental condition (EC), EC3 had the highest cholesterol of all the ECs and was significantly higher than EC1 and EC4 (C). A similar trend occurred with triglyceride levels (D). For (A and B), data are mean ± SE; n = 19 for RD, HFD and Choice. For (C and D), data are mean ± SE; n = 11 for EC1, n =10 for EC2 and EC3, n = 8 for EC4, and n = 9 for EC 5 and EC6; *$p < 0.05$ and **$p < 0.01$.

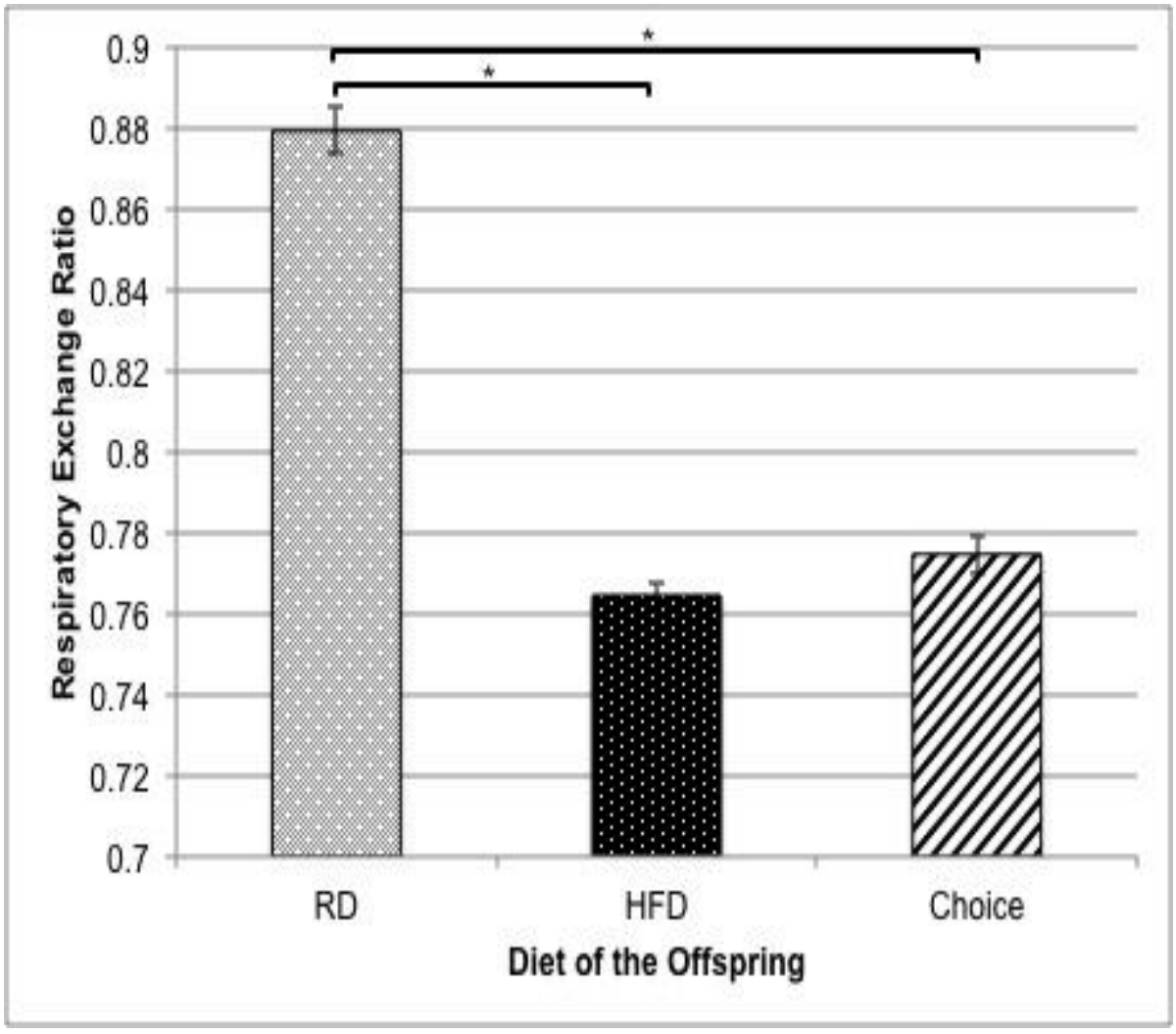

Fig. 12. Respiratory Exchange Ratio of Offspring at 18 Weeks of Age. The respiratory exchange ratio (RER) is indicative of the fuel source used. A value of 0.70 indicates that fat is the predominant fuel source, a value of 0.85 indicates that a mix of fat and carbohydrates are being used for fuel, and a value of 1.00 or above indicates that carbohydrates are the predominant fuel source. Offspring on standard rodent diet (RD) had a significantly higher RER, reflecting a mix of carbohydrates and fats being used for fuel, as compared to those on high fat diet (HFD) or Choice with both using fat as the predominant fuel source. Data are mean ± SE; n = 19 for RD, n = 20 for HFD and n = 20 for Choice; *$p < 0.0001$.

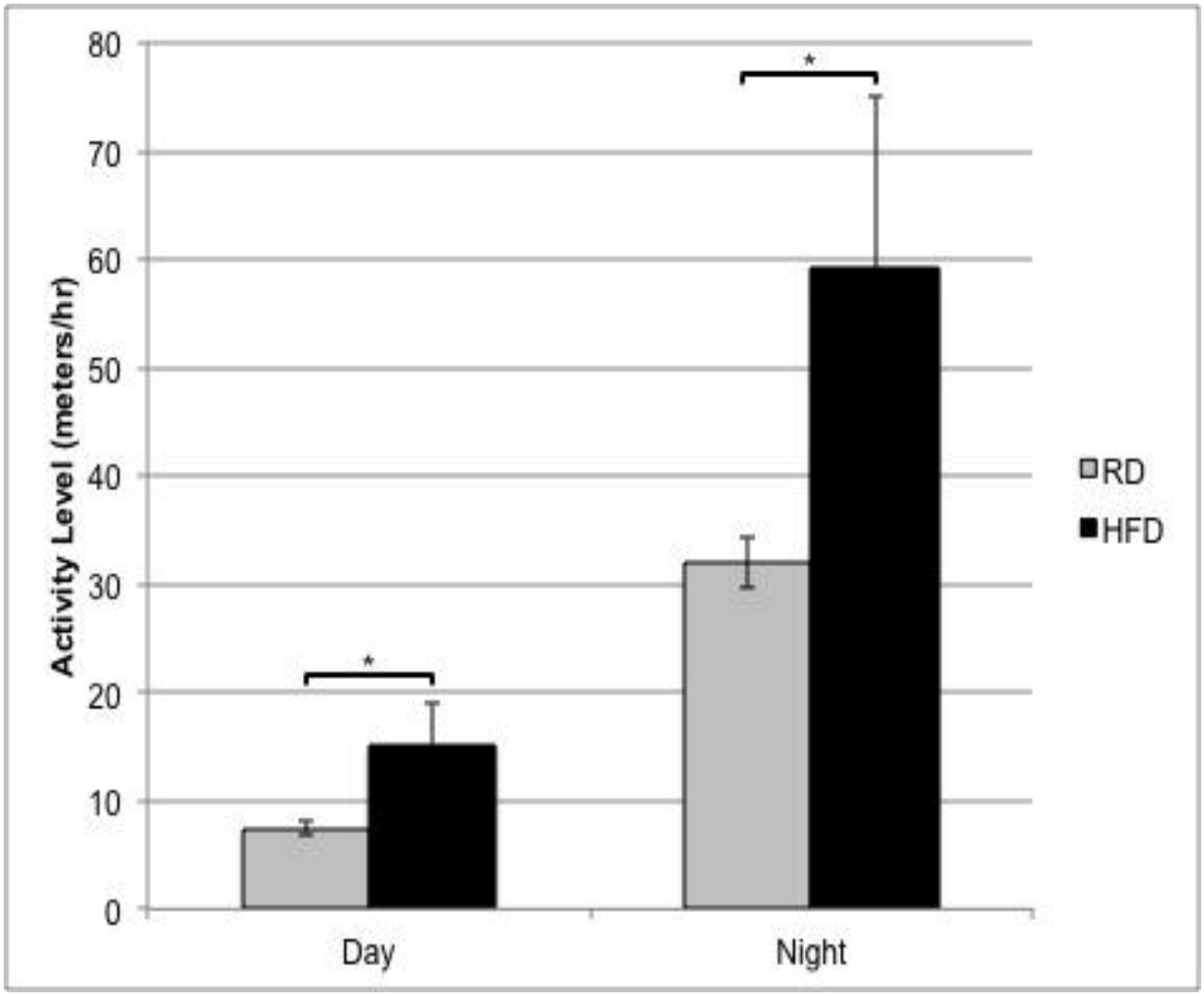

Fig. 13. Activity Level of Offspring at 18 Weeks of Age. Activity level during both the day and night differed significantly based on the diet of the dam with offspring from high fat diet (HFD) dams being more active than offspring from standard rodent diet (RD) dams. Data are mean ± SE; n = 27 for RD and n = 17 for HFD; *$p < 0.05$.

CHAPTER V: Summary, Conclusions, and Future Directions

Summary and Conclusions

The research presented in this dissertation was conducted to more fully understand the role and effects of early nutritional exposures, specifically to unhealthy fats, to lifelong health as well as how eating habits of the adult offspring mitigate or exacerbate disease conditions initiated in early life. Our overall hypotheses were that: 1) a high fat diet will negatively impact maternal health and birth outcome and 2) offspring born to mothers consuming such a diet will show morphologic and biochemical alterations in metabolic and bone health. With this more complete understanding of the contribution of maternal diet to birth outcome and lifelong health of the offspring comes the hope of chronic disease prevention through effective prenatal care, education, and dietary interventions.

In regard to our first hypothesis, it was demonstrated that chronic consumption of a high fat diet induced a number of untoward effects on both the dam and birth outcome. Research presented in Chapter III demonstrated that dams consuming an atherogenic high fat diet (aHFD) were hypercholesterolemic and hypertriglyceridemic and developed fatty liver, a serious and potentially fatal health condition. The suspected etiopathogenesis of the maternal hepatic lipidosis included dietary-induced hyperlipidemia and pregnancy-associated factors, specifically an imbalance between energy supply and demand, and possibly, a decreased ability of the liver to utilize fatty acids for energy. Dams from this group also experienced poor birth outcome as characterized by lower litter sizes and higher post-delivery mortality. As the development of fatty liver during pregnancy is known to carry high fetal and perinatal mortality, we postulated that the poor birth outcome arose out of complications from

maternal fatty liver. From Chapter IV, maternal consumption of a high fat diet (HFD) resulted in increasingly significant higher body fat percentage when measured at one month of dietary treatment and at weaning. Of note is the striking amount of body fat detected in HFD dams at the time of weaning. This overabundance of fat seen at the time of weaning carries strong implications to women's health particularly to mothers who face the challenge of shedding weight accumulated during pregnancy. Litter size and mortality rate were also evaluated for this study and no statistically significant differences were established between the HFD and RD groups. It bears mentioning that although we did not have significantly higher deaths in the HFD pups, these pups were small and unthrifty. This unthriftiness gave us so much concern that, for the well-being of the pups, we placed an amendment to our protocol that pushed the weaning date from 21 days of age to 25 days of age to allow the pups an extra few days in the care of their dam.

Regarding our second hypothesis, it was determined that early nutritional exposures to a high fat diet induced skeletal and metabolic aberrations in the maturing and mature offspring. Research presented in Chapter III demonstrated dramatic skeletal aberrations caused by gestational and lactational exposure to an aHFD. Offspring from aHFD dams had shorter bones in both the axial and appendicular skeleton, substandard trabecular architecture, and increased adiposity in the femur. Our proposed pathogenesis followed that diet-induced increases in bioactive oxidized lipids inhibit osteoblastic differentiation in favor of adipogenic differentiation during a critical period of bone growth, thus altering osteogenic programming and predisposing the animal to osteoporosis. In the study presented in Chapter IV, early exposure to a high fat diet resulted in central obesity, elevated lipid levels, hyperglycemia, and other markers used

in the diagnosis of the metabolic syndrome. Altering the diets of the mature offspring demonstrated that eating habits of adulthood have the potential to mitigate or exacerbate certain metabolic parameters established in the formative years. Suspected concepts and mechanisms involved in the observed metabolic aberrations are developmental plasticity and mismatch, catch-up growth, and altered programming of the appetite regulatory network.

In conclusion, the information presented in this body of work attempts to convey a relationship between perturbations in the early nutritional environment and the development of certain chronic diseases in adulthood, namely osteoporosis and the metabolic syndrome. The findings of our research are in accordance with, and thus support, the Developmental Origins of Health and Disease (DOHaD) hypothesis which proposes that adverse exposures, particularly in regard to nutrition, in the fetal/perinatal period can increase the risk for certain chronic diseases in adulthood. Concerns with the DOHaD hypothesis, which are shared by the author, are that the concepts and mechanisms behind this phenomenon are still rather speculative and that controlling for the numerous variables that could be involved in the association between exposures in early life and their effects on adult health is essentially impossible. In spite of these concerns and perhaps because of these concerns, it is clearly evident that more research is needed in this field.

Future Directions

The projects presented in this dissertation were developed to offer a more complete understanding of the role that maternal diet before, during, and after pregnancy plays on the lifelong health of the offspring as well as how the eating habits

of the adult offspring may mitigate or exacerbate disease conditions initiated in early life. In the future, we hope to expand upon our maternal high fat diet studies via two additions: 1) price controls in which mice are placed in operant conditioning chambers and are required to push on a lever to receive the food they want, having a higher "price" for the high fat food and 2) an *in vitro* model that would permit the study of how mesenchymal stem cells (MSCs), specifically pre-osteoblasts, respond to a lipid-rich environment. The first addition to our studies on high fat diet will assist in determining whether there is a cycle of intergenerational obesity and whether it can be broken by imposing higher prices on unhealthy food items. The second addition will provide a unique perspective on how poor diet choices effect development at the cellular level, specifically how multipotent MSCs differentiate into osteoblasts, adipocytes, etc. based on environmental cues.

As demonstrated in our research and many others, early exposure to poor nutrition poses a significant risk to birth outcome and is detrimental to lifelong health. However, the vast majority of such studies have been done in the absence of the influence of cost, a central element of the human environment. The "dollar menu" or "value menu" meals are quite popular for their low costs, but are frequently high in calories. One study that collected receipts from lunchtime customers at New York City fast-food chains found that such meals (purchases that included two or more items, at least one of which was a "dollar menu" or "value menu") accounted for up to 54% of receipts at one chain and that these purchases averaged over 800 calories across the three hamburger chains included in the study.[165] In response to the alarming incidence of obesity, governments around the world are actively seeking effective obesity prevention strategies.[166] Although policy-based interventions, such as imposing taxes

on soft drinks, snack foods, and/or fast foods, are likely to be key components of these strategies, few studies have been performed that examine the effectiveness of such imposed actions.[167] From the study presented in Chapter IV of this dissertation, it was determined that when given the choice, mice prefer a high fat diet over the conventional rodent diet. As a follow-up project and as a collaborative effort with Drs. Deborah Good and George Davis, who have performed several projects related to economic food pricing, mice would undergo a conditioning procedure referred to as a shaping to become accustomed to the levers and dispensers to receive food. Both a high fat and a standard rodent diet would be provided and mice would start off at a price of 1 lever press per pellet (both food types) and would be progressed over several days until they reach the price setting for the experiments. The aim of this project would be to determine if policy-based interventions, such as a "junk food tax", would indeed be effective in discouraging poor food choices and in lowering obesity prevalence through reduced consumption.

From past works completed in our laboratory[33] and in the present work described in Chapter III of this dissertation, it was discovered that adult mice born from mothers consuming a diet rich in fats throughout pregnancy and lactation developed lesions reminiscent of osteoporosis including increased adiposity in the marrow space. Our proposed pathogenesis for this observation follows that diet-induced increases in bioactive oxidized free fatty acids inhibit osteoblastic differentiation in favor of adipogenic differentiation during a critical period of bone growth, thus altering osteogenic programming and predisposing the animal to osteoporosis. However, this pathogenesis lacks specific molecular and cellular mechanisms by which this alteration in bone growth could be occurring. *In vivo* studies of growth and formation of bone

tissue have been addressed mainly by the utilization of advanced imaging and histopathology[168], as what was done in our research. Such studies provide an important foundation in the understanding of bone morphology, but are rather limited in their ability to address signaling and regulatory mechanisms. An *in vitro* study would be more ideal in addressing the molecular and cellular mechanisms that drive, and potentially derail, the development of pluripotent MSCs to preosteoblasts to osteoblasts. Using the results of the *in vivo* study discussed in Chapter III of this dissertation as a foundation, the molecular mechanisms by which fatty acids alter osteogenesis would be investigated through the use of murine mesenchymal stem cell line C3H10T1/2 and specialized media for osteogenic differentiation of MSCs that has been supplemented with fatty acids. The morphology of the resultant cells as well as a number of differentiation markers would be determined. Morphological assessment would include assessment of cell shape and any associated extracellular matrix as well as special stains for bone (alizarin red and von Kossa) and for fat (Oil Red O).[169] Markers of osteoblastic differentiation [e.g., runt-related transcription factor-2 (Runx-2), alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2), osteocalcin (OC), and osteopontin (OPN)] [169-171] and markers of adipogenic differentiation [e.g., peroxisome proliferation-activated receptor γ2 (PPARγ2) and leptin][169,172] would be evaluated. The aim of this project would be to determine, at the cellular level, how exposure to a high fat diet effects skeletal development.

In summary, results from the studies presented in this dissertation demonstrate that maternal consumption of a high fat diet is detrimental to both immediate birth outcome as well as the long-term health of the offspring specifically relating to the development of osteoporosis and metabolic syndrome, and that these detrimental

effects may persist no matter the dietary choices of the adult offspring. Future directions for this avenue of research include the addition of a price component to determine if implementing higher prices on unhealthy foods would be an effective anti-obesity strategy and the development of an *in vitro* model to study the effects of fatty acid exposure at the cellular level.

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