EFFECT OF PERINATAL HIGH FAT DIET ON STRESS RESPONSIVITY, MOTIVATION, AND THE INDUCTION OF METABOLIC SYNDROME IN OFFSPRING USING A BORDERLINE HYPERTENSIVE RODENT MODEL

By

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To my parents, Nilima and Bhaskar.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BHR	Borderline Hypertensive Rat
BMI	Body Mass Index
BMR	Basal Metabolic Rate
BP	Blood Pressure
DBP	Diastolic Blood Pressure
FR	Fixed Ratio
FOAD	Fetal Origins of Adult Disease
HPA	Hypothalamic-Pituitary Adrenocortical Axis
PD	Postnatal Day
PR	Progressive Ratio
MAP	Mean Arterial Pressure
NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel III
SBP	Systolic Blood Pressure
SD	Sprague-Dawley
SE	Standard Error of the Mean
SES	Socioeconomic Status
SHR	Spontaneously Hypertensive Rat
TEF	Thermic Effect of Food
VPR	Volume Pressure Recording
WHO	World Health Organization

Abstract of Dissertation Presented to the Graduate School
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Animal models are especially useful tools with which to study the effects of maternal obesity on the development and progression of disease in the offspring.

We were interested in studying the programming effects of maternal obesity on the development of metabolic syndrome in offspring, using a borderline hypertensive rodent (BHR) model. To the best of our knowledge there have been no studies on metabolism and food intake in this strain. Wistar females were maintained on either a high fat (60% calories from fat) or control (10% calories from fat) diets for 6-8 weeks, at which point they were mated with male spontaneously hypertensive rats to generate borderline hypertensive offspring. As we were interested in studying the separate effects of a prenatal or a postnatal hypercaloric environment, we cross-fostered all litters such that they were either placed with a dam in the same or the opposite dietary condition as their gestational dam. The offspring generated from these matings and fosterings were at weaning (Postnatal Day 21; PD21) separated based on sex. Half of them were fed a rotating junk food diet, while the other half were fed a standard chow diet. The rotating junk food diet consisted of 2 day presentations of either cookie dough, peanut butter and chow (1:1), Vienna sausages, processed cheese product, condensed milk and chow (1:1), or

D12492 (lard based diet from Research diets®), alternating each with 2 days of chow in order to ensure normal growth and development in the junk food cohort.

We examined for differences in bodyweight and total body adiposity in the Wistar dams at the time when the litters were weaned. Food intake was monitored every day for the first week on the experimental diets, and on a weekly basis thereafter. Body weight was assessed on the first day of the diet, 6 weeks after being on the special diets, the day before parturition, the day of parturition and the day of weaning. The litters were weighed and culled to a total of 10 pups (6 males and 4 females). Litter weights were assessed on PD0, 5, 10, 15 and 21. The Wistar dams were sacrificed when their litters were weaned (PD 21) and fat pads were harvested in order to assess differences in adiposity as a function of the different diets the dams had been maintained on. The Wistar dams maintained on the high fat diet were initially hyperphagic compared with dams fed the low fat control diet. Within 2 weeks the high fat fed dams had reduced their daily intake by weight so that their caloric intake was no longer distinguishable from that of the dams fed the low fat control diet. Complementing their intake patterns, dams in the 2 dietary groups showed no differences in body weights at any time during the course of the experiment. There were also no differences in pup weight as a function of the dam's diet, but by PD10 those pups that were being suckled by dams fed the high fat diet were heavier than those that were being suckled by dams fed the low fat control diet. This difference became more prominent by PD 21.

We assessed the effects of a psychosocial stressor on food intake, adiposity and blood pressure in the male offspring. These male rats underwent 6 days of acclimation to having their blood pressure measured via a tail cuff method. After the 6^{th} day, half the males were placed in a social defeat situation in which they were placed in a larger male rat's cage for a total of 10 minutes each day for 6 days. The rats' blood pressures were assessed both prior to and after the

social defeat sessions in order to examine for stress-induced changes in blood pressure. On the last day of social defeat, no blood pressure readings were taken and 20 minutes after the end of the social defeat session the rats were sacrificed. Blood and organs were harvested and frozen at -60º C for future analyses. The rats that had been subjected to social defeat had significantly elevated serum concentrations of corticosterone, but there were no differences in blood pressure either as a function of the rats' dietary histories, or as a function of stress exposure. The rats fed the junk food diet had heavier fat pads than the chow-fed controls and had reduced levels of non-fasting serum insulin. Rats fed the junk food also had higher levels of corticosterone compared with the chow-fed rats in both the stressed and non-stressed groups.

After 6 months, we assessed the long-term effects of the junk food diet on the development of obesity, hypertension, hyperleptinemia, and hyperinsulinemia on the female rats generated from the above mentioned matings. Post-weaning (PD21) these rats were maintained on either the rotating junk food diet described above or standard chow alone. Blood pressures were measured indirectly using the tail cuff method. At about 7 months of age blood was harvested by tail nick following an 18 hour fast. At about 7.5 months of age, without prior fasting, the same rat was sacrificed and organs, fat pads and blood were harvested. The remaining siblings of each pair of rats were then placed on an FR1 schedule of reinforcement, and then on a PR schedule of reinforcement in order to assess their motivation to obtain a food reward. The results showed that rats fed the junk food diet had heavier fat pads, and were hyperleptinemic and hyperinsulinemic compared with their chow-fed counterparts. Surprisingly, those rats gestated in dams fed the low fat control diet had higher blood pressures than those that had been gestated in dams fed the high fat diet. An effect of the post-weaning diet was evident in motivation to obtain food, with the chow-fed controls obtaining greater numbers of food rewards than rats fed the junk food fed. An

effect of the gestational and lactational environments was evident in the leptin levels such that those rats that had either been gestated in or suckled by a dam fed the high fat diet had higher levels of leptin compared with those from a mother fed the control diet.

In summary, these experiments have for the first time defined some early life programming effects as a result of high fat exposure of the mothers in BHR. In particular, changes in energy-sensing homeostatic systems were identified that could have detrimental health effects later in life. Further studies will be needed to more fully examine sex differences suggested in our results, as well as the generality of this result to other genetic backgrounds.

CHAPTER 1
INTRODUCTION

The United States today is facing an unprecedented obesity epidemic with more than 62% of its population being classified as either overweight or obese. While more prevalent in developed nations, widespread obesity is also seen in developing nations at the wealthier levels of society. Experiments involving manipulations of the *in utero* environment are abundant in the literature, and all point to the same conclusion - perturbations experienced prenatally significantly impact fetal development, ultimately altering adult regulatory mechanisms, a phenomenon known as fetal programming.

Today being either overweight, or morbidly obese is medically recognized as being a metabolically altered state that increase a given individual's proclivity towards developing a whole host of other potentially life-threatening conditions; diabetes, hypertension, and cardiovascular disease, to name a few. In line with this notion, the altered hormonal milieu of the mother's uterus, within which the fetus develops, is likely to have long-term postnatal effects on the physiology of the fetus. The precise mechanisms responsible for inducing these metabolic changes are unknown. But with the proportion of obese and overweight individuals in the population growing, it is important to elucidate the precise metabolic and behavioral repercussions the offspring of these obese mothers will have to contend with in their lifetime.

Sociological Significance of Obesity Research

With more than 61% of the American population being classified as overweight (US Surgeon General) and over one billion people worldwide being classified as overweight (WHO), the obesity epidemic has most certainly become a global crisis. The difference between an obese and an overweight individual is based on their Body Mass Index (BMI). This is calculated by

dividing body weight (in kilograms) by height (in meters) squared. Overweight is defined as a BMI between 25-29.9, whereas obesity is defined as a BMI greater than 30.

The obesity epidemic affects more than just those burdened with the disease; the economic toll borne by society at large is enormous. Just in the US, the economic impact on health-insurance is estimated at a staggering 75 billion US dollars in 2003; more than half of which was financed by Medicare and Medicaid (Finkelstein et al., 2004), and thus ultimately the US tax-payer at the rate of $175 per obese person.

Socioeconomic and ethnographic studies reveal that in developed nations like the United States, sex, race and socio-economic status interact to influence the proclivity of a given demographic towards becoming obese or overweight (Paeratakul et al., 2002) with Hispanic-American and African-American populations being particularly vulnerable (Perry et al., 2004). When it comes to being obese and overweight, a cross-cultural double standard is evident; to be a woman and obese is a far greater transgression than to be a man and obese. Consequently, the socioeconomic repercussions of being obese are more severe for women, than for men (Gortmaker et al., 1993). In the United States, the highest rates of obesity and overweight are negatively correlated with socioeconomic status (SES) and educational level (Goldblatt et al., 1965; Drewnowski, 2004). Both Drewnowski (2004) and Turrell (2004) assert that one's SES impacts what food-type are within one's economic reach, with calorically dense foods (high in fats and sugars) being cheaper options than foods high in nutritional value (fruits and green leafy vegetables). Perhaps most alarming are the statistics seen in the youth of America. According to the American Obesity Association, today's youth are the most inactive in the history of the nation; consequently over 30% of children (aged 6-11) and over 30% of adolescents (aged 12-19) are considered overweight or obese.

The state of being either obese or overweight, is not an isolated condition; they are almost always shadowed by myriad other problems, including hypertension, heart disease, type II diabetes, and psychological problems specifically relating to body image and self-esteem issues. The extent of chronic health problems experienced by obese individuals exceeds those associated with smoking or problem drinking (Sturm, 2002).

Historically, obesity has been considered a problem more to do with a lack of will power, rather than a genuine metabolic disorder. With the media bombarding consumers with images relating thinness to beauty, anti-fat attitudes are disturbingly commonplace, and obese and overweight people continue to be stigmatized. Longitudinal studies and self-report data indicate that obese and over-weight individuals suffer discrimination in situations ranging the gamut from applying for jobs to visiting the doctor's office (Puhl and Brownell, 2001).

This rapid rise in obesity cannot be explained by one's genetic predisposition alone, but must be the consequence of the interaction of genes with the sedentary lifestyle maintained by a majority of people today. In humankind's evolutionary past, before the agricultural revolution, the environment did not allow for over-consumption. Not only was food scarce, but obtaining it was energetically expensive. Thus it was advantageous to not only have a low basal metabolic rate, but also to have a preference for high fat food. In terms of caloric gain, one gram of fat provides more than twice the calories obtained from one gram of carbohydrate. Over the last several centuries, humans have not undergone any dramatic changes in their physiology; however their environment has changed dramatically. With the advent of agriculture and the domestication of livestock, food has become a reliable/storable commodity. In this post-modernization era, increasingly greater numbers of people maintain a sedentary lifestyle, thus exacerbating this growing trend towards overweight and obesity.

With increasing numbers of women being classified as obese and overweight, it follows that more and more mothers will be overweight or obese during their pregnancies. Thus it is of critical importance to develop a more sophisticated understanding of how this altered prenatal environment is affecting not only the physiology, but the dietary choices of children born to these women.

Etiology of Obesity

Non-genetic forms of obesity are fundamentally the result of an imbalance between energy intake and energy expenditure, and the cause of this imbalance is mediated by the combination of dietary choices, sedentary versus active lifestyle choices, and how these factors further modulate the individual's metabolism.

Energy Intake, Expenditure and Metabolism

The preference for high fat foods appears to be a universal mammalian trait, and choice studies in humans (Nysenbaum and Smart, 1982) and rodents (Lucas and Sclafani, 1996, Imaizumi et al., 2001) suggest a preference for higher fat options. Rodent studies have shown that rats will typically overeat and become overweight when given diet high in fat or sugar (Eckel and Moore, 2004) and this hyperphagia is further increased when fats and sugars are provided together (Sclafani, 1993). From an evolutionary standpoint, in an environment where food was a scarce commodity, it stands to reason that genes modulating preferences for calorically dense foods would be selected for. In the 21st century however, food scarcity is not a problem in the developed world, nor is it is a problem for the economically advantaged sections of society in the developing world. While undernutrition is no longer a major problem, malnutrition broadly defined as "a pathological state resulting from inadequate nutrition, including undernutrition (protein-energy malnutrition) due to insufficient intake of energy and other nutrients; overnutrition (overweight and obesity) due to excessive consumption of energy

and other nutrients; deficiency diseases due to insufficient intake of one or more specific nutrients such as vitamins or minerals" (Ge and Chang, 2001) continues to plague us today, primarily in the form of overnutrition. Overnutrition typically results from consuming an unbalanced diet; one that disproportionately consists of consumption of nutrient-poor energy dense foods like candy, cakes, savory snacks and nutrient-poor energy dense beverages (carbonated beverages, juices etc) . An unbalanced diet may be defined as one that is disproportionately high in fat, deficient in vitamins and minerals and typically low in fiber. The incidence of metabolic syndrome associated pathologies (obesity, diabetes, hypertension *etc*) is greater in people that consume such diets (Kant, 2000; Gray et al., 2004).

When considering the caloric composition of a given individual's diet, the jury is still out on whether a calorie is a calorie, or whether a fat calorie is distinct from a carbohydrate calorie which is distinct from a protein calorie. The basis for fad diets such as the Atkins Diet is that the body processes calories from different macronutrients differently. Work by Lewis et al. (1973) investigating weight-loss in men maintained on isocaloric high-fat or high-carbohydrate diets showed equivalent losses. Another study by Brown et al. (2000) found no differences in body-weight or fat mass after maintaining 2 groups of cyclists on either a high fat diet or a high-carbohydrate diet for 3 months. These data suggest that the macronutrient source of the calorie is not the critical factor, but rather it is the overall caloric intake, and also whether this is commensurate with energy expenditure.

The incidence of obesity in human populations often correlates positively with dietary fat (Gray and Popkin, 1998; Macdiarmid et al., 1998), and reductions in the intake of dietary fat produce weight loss (Astrup et al., 1999; Swinburn et al., 2001). However these associations are far from perfect and consumption of a high fat diet does not necessarily guarantee a high BMI

(Blundell and Macdiarmid, 1997). The probable reason for this is that diet is only one part of the equation; the other part is energy expenditure. Also, most of the information on energy intake is based on self-report data which is known to be an unreliable measure, and work by Heitmann and Lissner (1995) has shown the rates of under-reporting energy intake are higher in people with higher BMIs. Nonetheless, physical activity, or the lack thereof, is an important contributing factor to this fast growing epidemic. Total daily energy expenditure consists of basal metabolic rate , the thermic effect of food, and activity-associated energy expenditure (Novak and Levine, 2007). The basal metabolic rate (BMR) which is responsible for nearly 60% of an individual's daily caloric expenditure is typically based on lean body mass (Ravussin et al., 1986). As measures of physical activity correlate inversely with fat mass (Westerterp and Goran, 1997), physically active people typically have less fat mass, and proportionally greater lean mass, and consequently a higher BMR. Obese and over-weight people are also less likely to participate in voluntary physical activity, thus their activity-associated energy expenditure is also typically lower, than that of non-obese individuals. The thermic effect of food (TEF) is the energy expenditure that occurs during the consumption, digestion and absorption of food, and typically accounts for less than 10% of the total daily energy expenditure. Although it has been suggested that TEF is reduced in obese individuals, there is no current consensus in the literature as to whether this is the case or not (for further review see Granata and Brandon, 2002).

Finally the genetic contribution in the development of obesity cannot be denied. Epidemiological and laboratory work reveals that genetic factors play a significant role in food choice and level of physical activity (Loos et al., 2005; Tung et al., 2007; Bouchard et al., 1990). The observation that mice genetically susceptible to obesity become more obese on low fat diets than their wild-type litter mates provides further support for the idea that dietary fat is not

necessary for the expression of an obese phenotype (Genuth, 1976). The interplay of an obesogenic environment on a given genotype is what ultimately produces an obese phenotype.

Metabolic Syndrome

The term Syndrome X was first introduced by Gerald Reaven in 1988 to describe a cluster of symptoms that are typically associated with insulin resistance (Reaven, 1988). Since that time, Syndrome X has come to be better known as Metabolic Syndrome X, or simply Metabolic Syndrome. There was originally some debate as to whether obesity (particularly visceral and abdominal obesity) should be considered one of the central tenets of the Metabolic Syndrome, with Reaven (1993) arguing against and Björntorp (1991) arguing for its importance. Today it is accepted that "abdominal obesity, atherogenic dyslipidemia (elevated triglyceride, small LDL particles, low HDL cholesterol), raised blood pressure, insulin resistance (with or without glucose intolerance), and pro-inflammatory states" are the physiological abnormalities associated with Metabolic Syndrome (National Cholesterol Education Program Adult Treatment Panel III: NCEP-ATP III). The cut-off criteria for diagnosing Metabolic Syndrome (as proposed by the NCEP-ATP III) are presented in Table 1-1. When 3 of these risk factors present together, the individual is diagnosed with having Metabolic Syndrome.

Table 1-1. The NCEP-ATP III cut-off criterion for diagnosing Metabolic Syndrome.

Abdominal Obesity	
Men	>102 cm (40 in)
Women	>88 cm (35 in)
Triglycerides	≥ 150 mg/dL
HDL Cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
Blood Pressure	≥130/≥85 mm Hg
Fasting Glucose	≥110 mg/dL

(From Reaven, 2002)

Stress and Obesity

In day to day life, people use the term "stress" to refer to both the cause (i.e. the stressor) and its effects (i.e. their response to the stressor). While there is no universally accepted definition for stress, broadly speaking it may be defined as the physiological response to a change, which may be real or perceived, in an organism's environment (Herman and Cullinan, 1997). These changes in the organism's environment may be referred to as the stressors, and these can further be divided into physiological and processive/emotional stressors. Examples of physiological stressors include starvation, hemorrhaging or prolonged cold exposure. Processive or emotional stressors do not pose an immediate organic threat; examples include job stress, marital strife, and caring for an ailing family member *etc.* (Herman and Cullinan, 1997). It is also important to consider the differences between acute and chronic forms of stressors. Acute stressors are typically of a short duration and once passed, the organism is not subjected to any prolonged effects. In contrast, a chronic stressor that sustains an elevated glucocorticoid profile may have pathological physiological consequences (Dallman et al., 2006).

The hypothalamic-pituitary-adrenocortical (HPA) axis is central in the body's response to stress; specialized cells in the paraventricular nucleus release corticotrophin-releasing hormone and other peptides (e.g. arginine vasopressin) into the hypophysial-portal system of the anterior pituitary, which in turn releases adrenocorticotropic hormone into the circulation, which stimulates the release of cortisol (corticosterone in rodents) from the adrenal glands (for review see Herman et al., 2003). Short-term activation of the HPA axis in response to an immediate or perceived threat is indeed adaptive; however chronic activation of the system is associated with a variety of pathophysiological conditions, and today chronic stress has been identified as a risk factor for the metabolic syndrome cluster of chronic diseases (obesity, diabetes, and hypertension) (Van Itallie, 2002).

While the effects of stress on energy balance have been investigated extensively, no clear conclusions can be drawn from the literature because it is fraught with controversy. There are studies demonstrating anabolic effects of chronic stress or chronic glucocorticoid administration on body weight and adiposity (Michel et al., 2003; Zakrzewska et al., 1999). There is an equally compelling body of literature asserting the catabolic effects of chronic stress on the same parameters (Krahn et al., 1990; ; Harris et al., 1998). Other studies report that elevations in circulating corticosterone levelspromote hypertrophy of visceral fat depots and stimulate hyperphagia (Dallman et al., 2003; Pecoraro et al., 2004), but others report decreases in body fat content (Michel et al., 2005) and reduced food intake (Harris et al., 1998) following exposure to chronic stress.

The current state of the literature strongly suggests that stress has a mediating influence on the development of obesity and the metabolic syndrome; however differences in animal models, forms of stressors (acute/chronic, physiological/processive) and types of diets used (to assess hyperphagia) are probable explanations for the great variability seen in this literature.

Given that in the 21st century, modern humans are subject to a large variety of processive stressors, and live in an obesogenic environment with easy access to a large variety of palatable foods, a relevant animal model to study the effects of stress-induced obesity would be one which employs a stressor that is processive in nature, while providing the animal with a large variety of palatable foods, in order to best emulate the present human condition.

Fetal Origins of Adult Disease (FOAD): The Impact of the Prenatal Environment on Health in Adulthood

The prenatal environment is particularly sensitive to physical and chemical insult, and perturbations at critical periods of development can have devastating effects on the fetus. A horrifying example is the Thalidomide disaster of the 1960s in which nearly 1 in every 3 women

who took the medication had children born with limb abnormalities, a condition called phocomelia (McBride, 1961). Since the late 1980s epidemiological studies have been accumulating, supporting the theory that poor maternal nutrition has long-term consequences on adult health of the offspring. In the first wave of these studies, it was demonstrated that men in the Hertfordshire area of the UK who had lower birth weights, had a higher tendency to develop type 2 diabetes and impaired glucose tolerance in adulthood (Hales et al.,1991). The children born to women that had been pregnant during some part of the Dutch Hunger Winter (1944-45) had higher rates of obesity in later life (Ravelli et al., 1976; Ravelli et al., 1999). This long-lasting effect of a suboptimal prenatal/perinatal environment has been termed 'developmental programming' and may be defined as "an adverse stimulus or environmental insult during critical periods of development (that) can reprogram normal physiological responses and give rise to metabolic and hormonal disorders later in life (Barker, 2002)". There is a vast literature supporting the assertion that the prenatal environment is highly sensitive to both physical and chemical insult (for reviews see Yajnik, 2000; Hales and Barker, 2001).

However, today with the increase in the number of obese and overweight people world-wide, overnutrition, rather than undernutrition is the primary public health concern. Investigations into the programming effects of maternal obesity on the health of future generations are therefore an increasingly important area of research.

Programming Effects of Maternal Obesity

Human Studies

The metabolic and behavioral consequences of gestational overnutrition will have a significant impact on health and economic conditions of future generations. Epidemiological work has shown that there has been a 20% increase in mean maternal weight in the United States as recorded at the first prenatal visit (Lu et al., 2001). It is already well documented that women

with a BMI ranging between 25.1 - 30 kg/m^2 tend to give birth to offspring that are large for their gestational age (LGA) (Ehrenberg et al., 2004), and these children are at a greater risk of becoming obese (Guo et al., 2002) and developing type 2 diabetes in adulthood (Hampton 2004).

Additional problems found in the offspring of obese mothers in human studies include neural tube defects (Shaw et al., 1996) and renal anomalies (Honein et al., 2003). Obese women are often insulin-resistant, and when they become pregnant they are more likely than non-obese women to develop gestational diabetes (for review see Catalano, 2007). Furthermore, there is an extensive body of work suggesting that prenatal exposure to a hyperinsulinemic environment may predispose the developing fetus to obesity and diabetes in adulthood (for reviews see Fernandez-Twinn and Ozanne, 2006; Devaskar and Thamotharan, 2007; Plagemann, 2008). While there has not been a lot of work in humans studying the programming effects of maternal obesity on the development of cardiovasculature in the offspring, work by Napoli et al., (1997) has shown increased fat deposition in fetal arteries as a result of maternal hypercholesterolemia (for review see Palinski et al., 2007). Obese and overweight women have also been found to be less inclined to breastfeed their babies (Kugyelka et al., 2004); a consequence of this is that these formula-fed babies have lower levels of circulating leptin than their breast-fed counterparts (Savino et al., 2004). Alternatively breast-fed babies of mothers with gestational diabetes are likelier to develop glucose intolerance and become obese later in life than babies of normoglycemic mothers (Plagemann and Harder, 2005). Issues like this illustrate that it is as yet mechanistically unclear how a hypercaloric prenatal and/or perinatal environment programs the development of metabolic syndrome-associated pathologies in the offspring.

Animal Models of Maternal Obesity

While the great majority of studies investigating the effects of developmental programming have involved investigations into the effects of maternal undernutrition, the

increase in obesity world-wide makes it important that we begin to shift our focus to the programming effects of maternal overnutrition on the development of disease in adulthood. The majority of animal studies that have investigated the effects of maternal obesity or perinatal overnutrition have been conducted on rodents and sheep.

Ovine models

Sheep, with a gestation period of ~5 months, are a useful model in which to study the effect of programming because much of their hypothalamic development occurs prenatally, as is the case in humans (for review see McMillen et al.,2005; Mühlhäusler et al., 2004). However, to date, there are a very limited number of studies that have investigated the programming effects of maternal obesity/overnourishment on changes in sensitivity of hypothalamic systems to metabolic signals (such as leptin and insulin) in the offspring. Ovine studies have found increases in fetal weight and/or fetal adiposity as a result of maternal overnutrition (Mühlhäusler et al., 2003) and maternal hyperglycemia (Devaskar et al., 2002). Maternal overnutrition during the last trimester in sheep resulted in increased POMC mRNA expression in the arcuate nucleus of the offspring (Mühlhäusler et al., 2006) suggesting programming of the central networks that regulate appetite and energy balance. Other work that has been conducted on gestation in adolescent sheep (to compare with pregnancy during adolescence in human females) suggests that maternal overnourishment during gestation results in greater rates of maternal growth, increased maternal adiposity and reduced birth weight of the resulting lambs (Wallace et al., 2006). Finally, work by Mühlhäusler et al. (2008) reported an association between low birth weight and greater weight gains in adulthood in lambs. Collectively, these studies clearly demonstrate that as in the case of the human and rodent condition, perturbations in the ovine prenatal environment have permanent programming effects on the adult phenotype.

Rodent models

With their short gestation time (21 days), rats and mice are ideal animals to study the programming effects of maternal obesity. Additionally, rats and mice are useful species in which to tease out differences in programming that may occur prenatally (i.e. *in utero*) versus those that occur postnatally, particularly during the suckling period.

Early overnourishment, often induced by reducing litter size, has been shown to have programming effects on the offspring. Work by Morris et al., (2005) has shown that reducing litter size produced greater adiposity, hyperleptinemia and increased body weight in adulthood. Supporting this, work by Plagemann's group has also shown increased body weight, adiposity, hyperleptinemia and hyperinsulinemia as a result of postnatal overnutrition (Plagemann, 1999). Hypotheses that have been proposed to explain the association between early life nutrition and the development of obesity-related pathology in adulthood include perturbations in the development of hypothalamic circuitry (Davidowa et al., 2003; Plagemann, et al., 2000) and reduced insulin (Davidowa and Plagemann, 2007) and leptin (Davidowa and Plagemann, 2000; Férézou-Viala et al., 2007) signaling. Work by Taylor's group, also investigating the effects of maternal and postnatal overnutrition, has shown the development of hypertension, dyslipidemia, insulin resistance and hyperglycemia in the programmed offspring (Khan et al., 2003; Khan et al., 2005; Taylor et al., 2005).

Published studies on the effects of maternal obesity on the development of obesity in the offspring need to be examined carefully, however, because many of them did not implement cross-fostering procedures. This omission makes it impossible to assess unequivocally whether the differences observed in adulthood are a function of either prenatal or postnatal programming. For example, Levin and Govek (1998) demonstrated maternal obesity promoted obesity in adult offspring regardless of whether the offspring were maintained on a high fat or control diet but

they did not employ cross-fostering. Samuelsson et al., (2008) showed the induction of hyperphagia, hypertension, and obesity in male and female offspring gestated in dams fed a high fat/high energy diet, but again they did not cross-foster. So, while these results are intriguing, they do not resolve the problem. In contrast, Shankar et al., (2008) appropriately cross-fostered all offspring to dams fed a control diet in order to isolate the effects of gestational obesity on development. They reported that the male offspring gestated in the high fat dams were more susceptible to the obesogenic effects of a high fat post-weaning diet compared with offspring gestated in dams fed a control diet. Bayol et al., (2007) implemented a cross-fostering regimen, similar to that to be used in the present experiments, and reported increased preference for junk foods (high in sugars, fats and salt) in male and female offspring that had been gestated and suckled by high fat fed dams as compared with those offspring that had been on standard chow either during gestation or lactation alone. However, that study did not standardize litter size which again makes the results hard to interpret. It is also important, when considering these experiments, to know whether the dams were diabetic during gestation or not. Typically gestational diabetes induces macrosomia (Khan, 2007), or an increase in litter size (Holemans et al., 2004), which increases the likelihood for impaired glucose handling in adulthood (Van Assche et al., 2001).

The precise biological mechanisms that link early nutrition and development of obesity and related pathology in adult life are still unclear and require further investigation at this time. It is important that we take into account differences in programming that may be occurring prenatally versus those that may be occurring postnatally, while designing our experiments.

CHAPTER 2
EFFECT OF DIETARY FAT ON PRENATAL AND EARLY POSTNATAL PARAMETERS IN MOTHERS AND OFFSPRING

Introduction

The steadily increasing prevalence of obesity among women is a serious public health concern today. According to the 1999-2002 National Health and Nutrition Examination Survey, more than 50% of non-pregnant women of child-bearing age (20-39 years of age) were overweight or obese (BMI of 25-29.9 kg/m^2), 29% were obese (BMI $\geq$ 30 kg/m^2) and 5.6% were extremely obese (BMI of $\geq$ 40 kg/m^2) (Hedley et al., 2004). Even more concerning are the data on adolescent girls; more than 30% of girls between the ages of 12-19 were either at risk of being overweight or were overweight (defined as a BMI for age $\geq 85^{th}$ percentile) (Hedley et al., 2004). These girls are the mothers of tomorrow and if present trends continue, more and more pregnant women will be either overweight or obese during their pregnancy.

It is well-documented that obesity can cause complications during pregnancy. Obese women are likelier to develop gestational diabetes (Solomon et al., 1997), are at an increased risk for preterm delivery (<33 weeks) (Bhattacharya et al., 2007), and are more likely to have reduced success with breast-feeding (Hilson et al., 1997). There is also an increased risk of congenital abnormalities in the offspring of obese women (Naeye, 1990, Honien et al., 2003), but the mechanisms by which this may be occurring are poorly understood. Possible explanations include decreased serum folic acid levels (Mojtabai, 2004), increased incidence of gestational diabetes and reduced effectiveness of ultransonography equipment to identify congenital abnormalities early during gestation (Hendler et al., 2004). Furthermore, women that are obese prior to conception, are at an increased risk of undergoing a cesarean delivery, as compared to non-obese women (Crane et al., 1997); this is likely a consequence of slower progression of labor (Vahratian et al., 2004) and/or fetal macrosomia (Sheiner et al., 2004). Not only as a

consequence of the higher rate of cesarean sections, but also as a consequence of increased antenatal and postnatal care (for both mother and infant), health care costs are much greater for over-weight and obese women. A study by Galtier-Dereure et al. (2000) reported that health-care costs are increased by between 5-16 times (depending on degree of obesity), as compared with health-care costs of normal weight women. Thus the increase in obesity rates in women of child-bearing age is a significant public health concern not only from the perspective of the mother and her child's health, but the increasing costs and additional treatment often required by obese pregnant women.

The over-arching theme of these experiments was to study the effect of maternal obesity on the offspring, so it was of initial importance to examine whether our dietary manipulations were inducing obesity in the dams prior to conception. The objective of the present experiment was to examine food intake and body weight gain in the Wistar female rats maintained on a high fat diet (60% calories from fat) compared with Wistar females maintained on a control diet (10% calories from fat). In addition, litter weights were monitored at birth and through weaning to examine possible differences as a result of maternal dietary condition either during gestation or lactation, or both.

Materials and Methods

Animals and Housing Environment

Primiparous Wistar rats (Harlan Laboratories, Room 212A, Indianapolis, IN) weighing 290-320 g at the beginning of the study were housed individually in polycarbonate cages with stainless steel wire mesh lids in a controlled environment (21-24°C, 45-55% relative humidity, 12:12 cycle, with lights off 10:00-22:00 h). These females were maintained on one of two semi-purified pelleted diets (purchased from Research Diets, New Brunswick, NJ) for 6-8 weeks prior to mating. The high fat diet (cat no. D12492) contained 34.9% fat by weight which is

approximately 60% calories from fat and had a caloric density of 5.24 kcal/gram. The control diet (cat no. D01060501) contained 4.3% fat by weight which is approximately 10% calories from fat and had a caloric density of 3.85 kcal/gram. The other constituents were matched between the diets (Table 2-1). When initially placed on these diets, the rats' food intake and body weight were monitored every day for the first 6 days, and then every 5 days through week 6. Prior to the start of the experiments, animals were handled frequently, in order to minimize stress during the experiments. All experiments were conducted during the early part of the 12 h dark period. All experiments were conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals, and were approved by the UF Animal Care and Use Committee.

Table 2-1. Composition of the high fat and control diets given to the dams. Both diets were purchased from Research Diets, New Brunswick, NJ.

Product #	High-Fat Diet (D12492)		Control Diet (D01060501)	
	gm%	kcal%	gm%	kcal%
Protein	26.2	20	19.2	20
Carbohydrate	26.3	20	67.3	70
Fat	34.9	60	4.3	10
Ingredient	gm	kcal	gm	kcal
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	0	0	575	2300
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275.2	0	0
Cellulose (BW200)	50	0	50	0
Soybean Oil (EFA)	25	225	25	225
Lard	245	2205	20	180
Mineral Mix (S10026)	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin Mix (V10001)	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	773.85	4057	1055.05	4057

Synchronous Mating and Cross-fostering Procedures

The primiparous Wistar dams were mated with proven-breeder SHR males (Harlan Labaratories, Room 202A, Indianapolis, Indiana) after being maintained on the above mentioned diets for 6-8 weeks. The dams continued to be weighed through gestation, at parturition, and weaning. At parturition, the litters were weighed and culled to 10 pups (approximately 6 males and 4 females). All litters were cross-fostered such that they were housed with a dam either in the same or opposite dietary condition as their gestational mother. Cross-fostering thus produced 4 dietary groups (Table 2-2). Litters were weighed at PD0, 5, 15 and 21. The litters were weaned at PD 21 and littermates were separated based on sex. The dams were sacrificed at weaning, and their fat pads (subcutaneous, visceral, periovarian and perirenal) were dissected and weighed.

Table 2-2. Maternal diet and cross-fostering procedures.

Diet During Week 1 (adaptation)	Diet During Week 2-Week 8 and through Gestation	Litter Type Received at Cross-Fostering
Chow	High-Fat Diet (H)	High-Fat Litter (HH)
		Control Litter (HL)
	Control Diet (L)	High-Fat Litter (LH)
		Control Litter (LL)

Data Analysis

One-way ANOVAs and post-hoc Tukey tests (where appropriate) were used to examine for differences in maternal caloric intake, maternal body weights, maternal fat pads, mean litter

weights and general litter statistics (i.e. number of males, females and total number of pups per litter). Significance levels were set at $p<0.05$.

Results

Caloric intake was significantly greater in the high fat fed dams during the first week of being introduced to the special diets (Figure 2-1) [$F(1,399)=224.03$; $p<0.001$], after which the difference in the intake of the two diets was no longer significant ($p>0.05$).

Mean body weight of the dams (Figure 2-2) did not differ significantly as a function of diet ($p>0.05$). However, their total fat pad mass observed in the dams at the time the pups were weaned (Figure 2-3) was found to be significantly heavier in the high fat dams compared with their control diet counterparts regardless of the litter type they had suckled during the lactation period [$F(3,35)= 17.982$; $p<0.00001$].

There were no significant differences in general litter statistics (total litter size, male:female ratio) between the high fat and control-diet litters (Figure 2-4) ($p>0.05$). Mean litter weights did not differ significantly between the 4 groups (HH, HL, LH and LL) at either PD0 or PD5 (Figure 2-5) ($p>0.05$). By PD10, LH litters were significantly heavier than HL and LL litters [$F(3,33)=3.198$; $p<0.05$]. At PD15, while a similar trend was apparent, the differences were not significant ($p>0.05$). At PD21, the litters that had had a high fat dam during the lactation period (HH and LH litters) were significantly heavier than those that had had a control-diet dam during the lactation period (HL and LL litters) [$F(3,33)=11.604$), $p<0.001$].

Discussion

These experiments characterized the effects of manipulating fat content in the maternal diet (60% vs. 10%) on intrauterine and early postnatal life in terms of growth parameters, as well as differences in maternal white adipose tissue weight.

The results indicate that when first exposed to the experimental diets, the high fat dams initially consumed almost twice the number of calories of their control group, but that after a week on the diets the intakes of the two groups were comparable. This pattern of initially elevated intake upon exposure to a high fat or palatable diet followed by a return to normal intake over the next several weeks has been reported by others (Beck and Richy, 2008). In contrast, Ribot et al. (2008) reported that female rats maintained on a cafeteria-style diet consumed significantly more than chow-fed controls over a 10 day period. However within this 10 day time period, the elevated intake declined from 5-fold to 3-fold. It is possible that had they followed the rats' intakes beyond 10 days they too would have seen a complete normalization of intake. The critical difference between studies that show normalization of intake (such as the present study) and those that do not is most likely due to variety in the cafeteria paradigm.

Given that the caloric intake of the high fat dams was elevated only transiently over that of the control-diet dams, it is not surprising that there were no differences in body-weight between the 2 groups either after 6 weeks on their respective diets before mating, or at parturition or weaning. Consistent with this observation, Johnson et al. (2007) maintained two groups of 3 month old female SD rats on either a high-fat or low-fat diet (diets slightly different from those used in the present experiment, but also from the same commercial supplier) and at the end of 6 weeks the groups were not significantly different from one another. Other studies have reported significant differences in body weight between low and high fat fed rats despite a lack of difference in caloric intake (Torre-Villalvazo et al., 2008; Férézou-Viala et al., 2007). It is possible that there are factors such as age, strain, or the particular diet that influence this result. However, despite no difference in body weight, our data do suggest a change in body energy partitioning in the two dietary groups. Examination of the total fat pad mass

(subcutaneous+perirenal+ovarian) revealed that the dams on the high fat diet had significantly heavier fat pads than the control diet dams, regardless of the litter type they had nursed. These findings are in concordance with other work that has investigated the impact of high fat versus high carbohydrate diets on the development of obesity and metabolic syndrome in rats. The control diet used in the present study had a greater proportion of calories from carbohydrates as compared to the high fat diet (70% vs. 20%). Boozer et al. (1995) found that after 6 weeks on either a low fat (12%), 24% fat, 36% fat or 48% fat, male SD rats did not show any differences in body weight or interscapular brown fat mass. However total body fat of the 48% fat group was elevated above the control group.

At PD0, there were no differences in mean pup weight as a result of the different gestational environments; this is consistent with previous reports by Holemans et al. (2004) and Bayol et al. (2005) who also manipulated the maternal diet during gestation and lactation. There were also no differences in litter size or male:female ratio as a consequence of being gestated in a high-fat versus control-diet dam. By PD10 those pups that had been gestated in a control-diet dam, but cross-fostered to a high fat dam (LH) were significantly heavier than the other groups of pups (HH, HL and LL). At PD21, those pups that had been nursed by high fat dams (HH and LH) were significantly heavier than those that had been nursed by the control-diet dams (HL and LL). It is of consequence to note that the pups' weights began to diverge as a function of maternal diet as early as PD10; that is prior to the age at which the pups begin to consume solid food. Possible explanations for the greater weight gain in the litters suckled by the high fat dams are either a higher fat content in the milk, or an overall increase in milk production (Del Prado et al., 1997). It has been shown that the fat content of the milk supply is related to the fat content of the maternal diet (Trottier et al., 1998; Averette et al., 1999). The increased fat content of the

milk of the high fat dams could have possibly made the milk more palatable to the pups, resulting in longer or more frequent suckling bouts, which would also be an explanation for the greater weight gain in the HH and LH litters.

In conclusion, while the maternal diet did not have an effect on maternal caloric intake or bodyweight, the high-fat fed dams did have increased fat mass as measured at weaning. In addition to this, the litters suckled by the high-fat fed dams were heavier than the litters suckled by the control-diet dams.

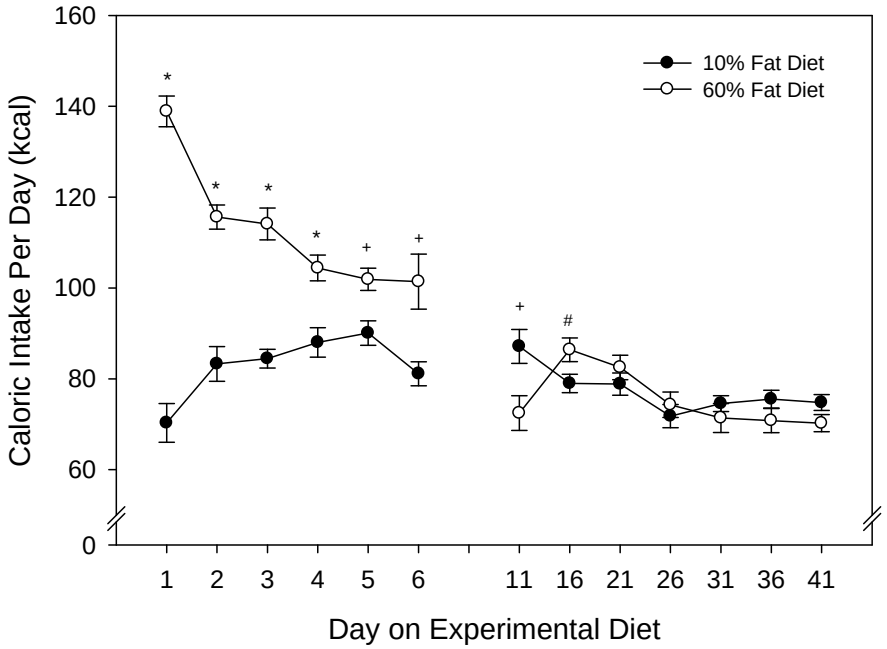

Figure 2-1. Mean (±SE) caloric intake per day. Rats placed on the high fat diet ate significantly more than the control diet rats until about Day 16, after which there was no differences in their total daily caloric intake(* $p<0.001$, + $p<0.01$, # $p<0.05$).

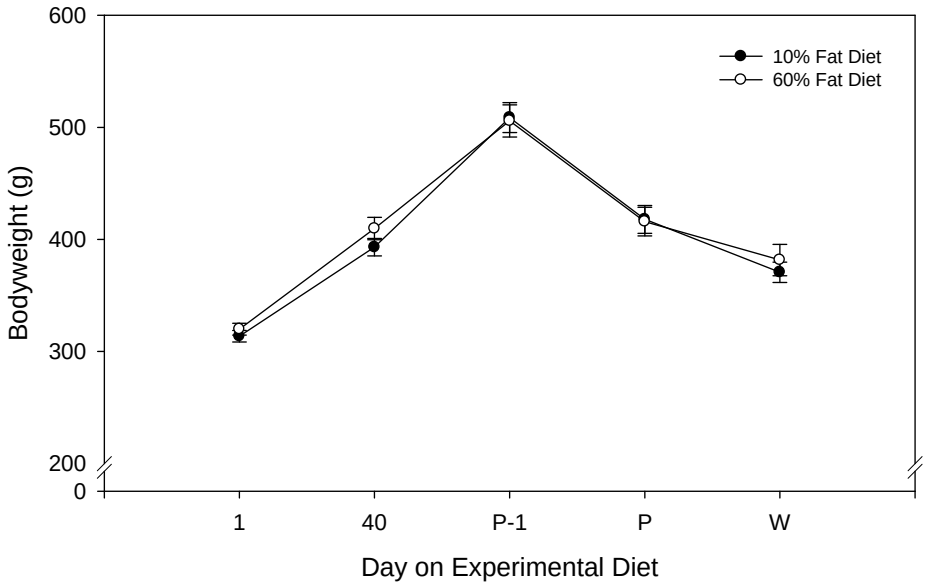

Figure 2-2. Mean (± SE) maternal bodyweights on days 1 and 40 of the experimental diets, the day before parturition, the day of parturition, and the day of weaning. Regardless of diet type, there were no significant differences in body weight between the 2 dietary groups (Ps>.05).

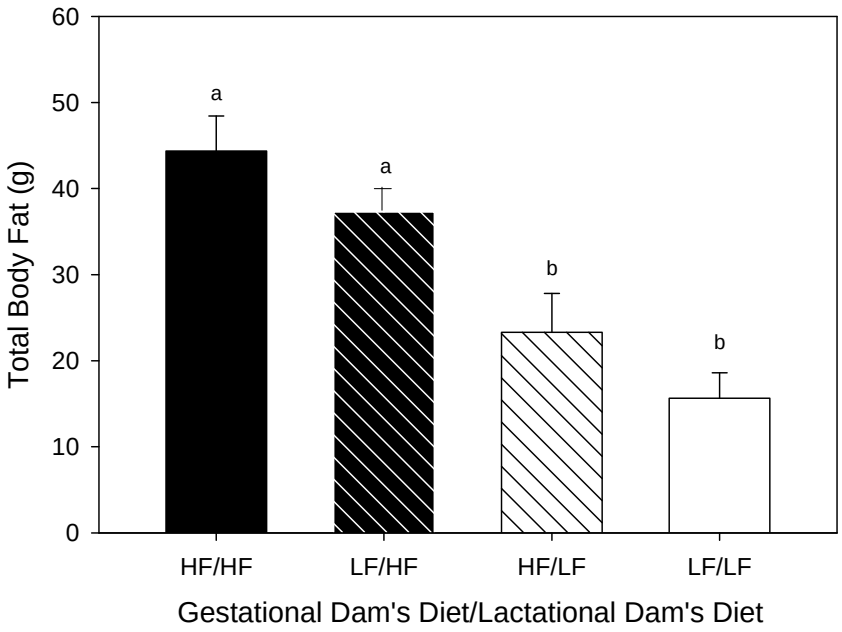

Figure 2-3. Mean (± SE) total body fat (subcutaneous + visceral + periovarian + perirenal fat pads). The dams that were maintained on the high fat diet had significantly heavier fat mass compared to the dams maintained on the control diet ($p<0.001$). There was no effect of litter type that the dam had suckled during the lactation period. Bars marked with different letters are significantly different from each other ($p<0.05$).

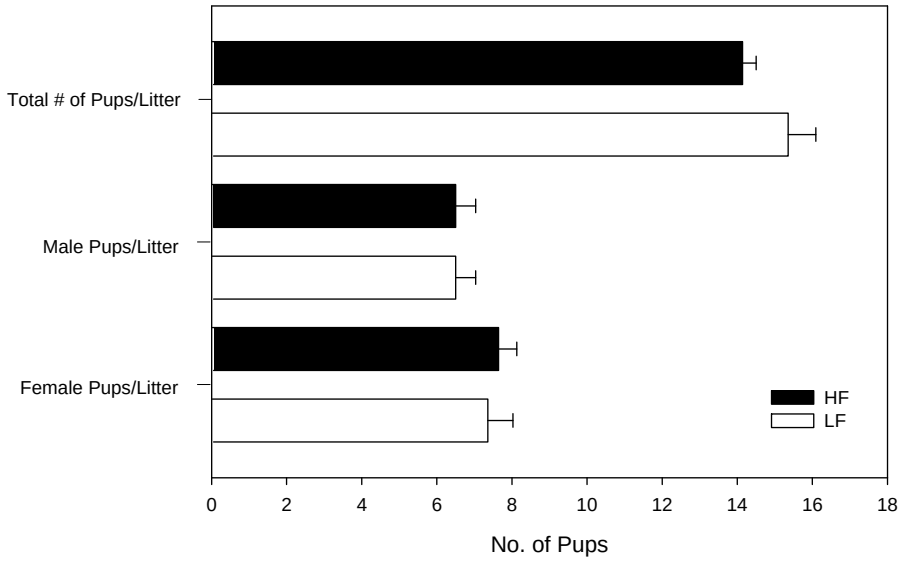

Figure 2-4. Mean (± SE) number of pups per litter. There was no effect of maternal diet (HF vs. LF) on litter size, or sex ratio in the litters (Ps>.05).

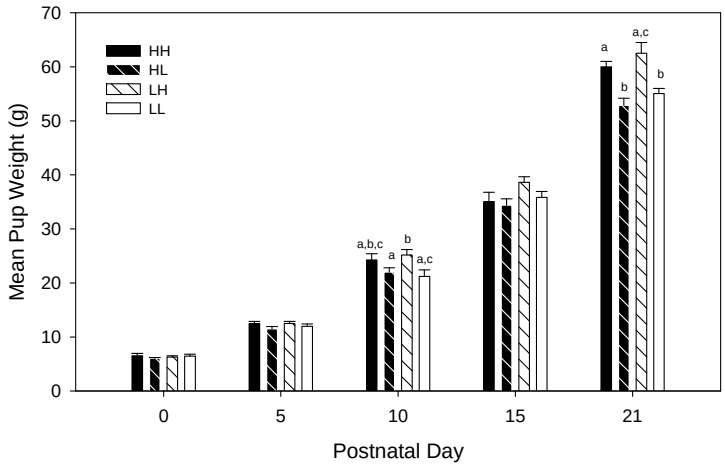

Figure 2-5. Mean (±SE) pups weights at approximately 5 day intervals from birth (PD0) through weaning (PD21). While there were no group differences seen at PD0 or PD5, from PD10 onwards the LH pups (gestated in control diet dams, fostered to high-fat dams) were significantly heavier than HL (gestated in high-fat dams, fostered to control dams) or LL (gestated and fostered with control diet dams) groups. Bars with different letters are significantly different from each other (Ps<0.05).

CHAPTER 3
EFFECT OF HIGH-FAT DIET ON PHYSIOLOGIC RESPONSES TO SOCIAL DEFEAT STRESS IN BORDERLINE HYPERTENSIVE RATS.

Introduction

In the previous chapter we compared the efficacy of high- and low-fat diets to induce obesity in the Wistar dams. At weaning, the dams on the high fat diet had significantly heavier fat pad mass relative to those on the control diet. There were no effects of maternal diet on litter size, litter weight and sex ratio at PD0, but by PD21 those litters that had been suckled by a high fat dam were significantly heavier than those that had been suckled by a control diet dam, regardless of the dietary condition of their gestational dam.

Given the rise in obesity in developing countries and the attendant health risks it is important to develop a greater understanding about how stress and obesity interact and potentially exacerbate pathological conditions associated with the metabolic syndrome. It is well established that stressors, be they systemic (hemorrhage, cold-exposure etc.) or processive (emotional, fiscal etc.) in nature, activate the hypothalamic-pituitary axis (HPA) to initiate an array of adaptive counter-responses. While short-term activation of the HPA axis in response to an immediate or perceived threat is indeed adaptive, chronic activation of the system is associated with a variety of pathophysiological conditions ranging from dampened immune response (Webster Marketon and Glaser, 2008), increased risk of heart disease (Otsuka, 2007), and increased susceptibility to depression and other mood disorders (Gold and Chrousos, 2002; Pariante 2003). There is also a growing body of literature suggesting that high levels of dietary fat may itself be a stressor, increasing HPA activity (Hüllsman 1978; Pascoe et al., 1991, Tannenbaum et al., 1997; Kamara et al., 1998). Conversely, there is a considerable literature asserting that high fat feeding might ameliorate the behavioral and neurophysiological effects of stress (Prasad and Prasad, 1996; Pecoraro et al., 2004; Dallman et al., 2005).

There have been numerous investigations into the relationship between stress and obesity (Contreras et al., 1991; Rosmond et al., 1998; Steptoe et al., 1999; Dallman et al., 2003). This relationship appears to be bi-directional, but it remains unclear as to whether stress stimulates or attenuates food intake. Although it has been suggested that people use food as a coping mechanism (McCann et al., 1990; Michaud et al., 1990; Markus et al., 2000), the effect of stress on food intake has by no means been clearly defined. There are studies showing both increases (Wallach et al.. 1977; Rowland and Antelman, 1976; Pecoraro et al.. 2004) and decreases (Marti et al,. 1994; Harris et al., 1998) in consumption following exposure to a variety of stressors and diets. Investigations into macronutrient selection following glucocorticoid administration have also yielded varying results; adrenalectomized rats have shown increases in fat intake (Bligh et al., 1993) as well as carbohydrate intake (Kumar and Leibowitz, 1988) following corticosterone administration. Stress has been associated with increased visceral adiposity in obese humans (Randrianjohany et al., 1993; Gluck et al., 2004). Complementing this work, there is evidence that corticosterone, the major stress hormone, plays an important role in energy balance. High-fat feeding in humans has been shown to be followed by elevations in cortisol levels (O'Connell et al., 1973). Furthermore, Castonguay et al. (1986) reported reduced adiposity, smaller meal size and meal frequency (Freedman et al., 1985) following adrenalectomy in Zucker rats and reinstatement of obesity following glucocorticoid administration to these rats.

Based on the literature it is evident that the jury is still out on how precisely a high fat diet modulates HPA axis activity, and vice-a-versa. The goal of the present set of experiments was to develop a model that could be used to further investigate the pathophysiological interaction of obesity and stress. In order to do this, we investigated differences in stress responsivity as a function of diet in the male BHR offspring. We selected social defeat stress as it

has been demonstrated to produce a reliable stress response in rats; one to which rats do not readily habituate (Tornatzky and Miczek, 1993) and is an ecologically valid model, as it is best suited to mimic the processive stressors that increasing numbers of people are contending with today.

We hypothesized that those rats that had been weaned onto high fat diets would show greater stress responsivity than those that had been weaned onto low-fat control diets. More specifically, we hypothesized that those rats that had been gestated in a high fat dam, suckled by a high fat dam and weaned onto a high fat diet (HHH condition) would be most sensitive to stress (as measured by serum corticosterone concentrations, and blood pressure readings) and those that had been in the opposite condition (LLL) would be most resistant to its effects.

Materials and Methods

Animals and Housing Environment

This study used the male offspring (4 per litter) generated from the matings described in the previous chapter. To summarize that design, there were four litter types at weaning (PD21): HH, HL, LH and LL with 5 litters of each (20 litters in total). The litters were separated based on sex and diet type and housed (n=2/3 pups per cage) in polycarbonate cages with stainless steel wire mesh lids in a controlled environment (21-24°C, 45-55% relative humidity, 12:12 cycle, with lights on 10:00 pm and off at 10:00 am). Half the rat pups in each litter were placed on a rotating high fat diet (details provided in the following section), and the other half were placed on standard Purina chow diet. This resulted in 8 groups based on their gestational, lactational and post-weaning history: HHH, HHL, HLH, HLL, LHH, LHL, LLH, LLL (see Table 3-1).

At PD45 4 males from each litter (80 males in total) were moved to another vivarium that was maintained at a similar temperature and relative humidity as the original room, but had a

normal light cycle (lights on at 8 am and off at 8 pm). The rats were also housed singly for the remainder of the study.

Table 3-1. Outline of experimental design showing assignment of offspring from different dietary protocols to stress and control groups.

Litter Condition at Weaning (PD21)	Diet From PD21-PD61	Test Condition During Social Defeat Stress
HH (5 Litters; n=20 ♂rats)	Rotating High-fat Diet 5 Pairs of Rats; n=10 rats. Condition: HHH	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
	Standard Chow Diet 5 Pairs of Rats; n=10 rats. Condition: HHL	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
HL (5 Litters; n=20 ♂rats)	Rotating High-fat Diet 5 Pairs of Rats; n=10 rats. Condition: HLH	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
	Standard Chow Diet 5 Pairs of Rats; n=10 rats. Condition: HLL	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
LH (5 Litters; n=20 ♂rats)	Rotating High-fat Diet 5 Pairs of Rats; n=10 rats. Condition: LHH	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
	Standard Chow Diet 5 Pairs of Rats; n=10 rats. Condition: LHL	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
LL (5 Litters; n=20 ♂rats)	Rotating High-fat Diet 5 Pairs of Rats; n=10 rats. Condition: LLH	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)
	Standard Chow Diet 5 Pairs of Rats; n=10 rats. Condition: LLL	Per pair (5 pairs) 1 intruder rat (n=5 rats) 1 control rat (n=5 rats)

Post-weaning Diets

The rotating junk-food diet comprised of a presentation of one of six high fat foods: cookie-dough (4.98 kcal/g, made from flour, sugar, shortening, and vanilla essence); peanut-butter/chow (4.78 kcal/g, 50% powdered Purina 5001+50% smooth peanut butter); Vienna sausages (2.83 kcal/g), processed cheese product (2.85 kcal/g), condensed-milk/chow (3.32 kcal/g, 50% powdered Purina 5001+50% sweetened condensed milk) and the high fat semi-synthetic diet D12492 (5.24 kcal/g). With the exception of the standard chow and D12492 (Table

2-1), all of these ingredients were generic brands from a local supermarket. Each of these diets was presented for two days separated by two days of standard chow (Purina 5001; 3.34 kcal/g). The rationale for these chow periods was because the protein:calorie ratio of the junk foods could be lower than needed to sustain optimal growth, so chow (>25% protein:calorie ratio) periods should ensure that protein availability was not a limiting factor. This diet regimen produced 8 groups: maternal dam high fat diet, foster dam high fat diet, post-weaning junk-food diet (HHH), HHL, HLH, HLL, LLL, LLH, LHH, and LHL (Table 3-1). Food intake and body weights were monitored every two days from PD 21 through PD 61.

Surgical Procedures

Each rat undergoing surgery (n=10) was anesthetized with ketamine-xylazine (ketamine, 100mg/kg + xylazine, 5mg/kg), administered by the intraperitoneal route. These rats were also given a subcutaneous injection of ketorolac (2mg/kg) analgesic at the time of the anesthesia. Surgical level of anesthesia was determined by a firm paw pinch. Once anesthetized, the rats were shaved immediately above their scrotal sac. The shaved area was scrubbed with Betadine followed by 70% ethanol; this was repeated three times. A 1 cm ventral midline incision was made with a scalpel and the vas deferens was located and grasped with forceps. Using a microcautery tool, a 0.5 cm section of both the left and right ducts was removed. The abdominal wall was then sutured with absorbable 4-0 monofilament nylon non-wicking suture (Ethilon, Ethicon Inc.), and the external incision was closed up with stainless steel wound clips (9mm, World Precision Instruments Inc.). These clips were removed a week following the surgery. After the surgery, the rats were given a subcutaneous injection of 0.9% NaCl (1 ml) and then placed in a recovery chamber with a heating pad. The rats were returned to their home cage when fully ambulatory.

Social Defeat Stress Paradigm

The vasectomized Long-Evans (LE) rats (n=9) were double-housed with female LE (n=9) rats for 5 weeks prior to the start of the social defeat sessions. These males will henceforth be referred to as the *residents.* Of the 4 males from each litter that were used in this experiment, 2 had been placed on the rotating high fat diet, and 2 on the standard chow diet as described previously. In each case, 1 male was placed in the social stress condition, while the other male served as its unstressed control. This is further described in Table 3-1. The rats that were tested in the social defeat session will be referred to as the *intruders*.

On the day of a social defeat session, the co-habiting female LE rat was removed from a given resident's cage 10 minutes prior to the start of the defeat session. The intruder rat was then placed in the resident's cage for up to 5 minutes or 3 defeats which were defined as the resident pinning the intruder on his back for a minimum of 2 seconds. At this point the intruder was quickly removed from the cage, and placed in a small double-wire mesh protective cage and returned to the resident's cage for another 5 minutes. This procedure was repeated for 6 days and, to avoid habituation, the intruder rat was placed with a different resident rat on each occasion. .

Blood Pressure Measurements

Blood pressure was measured using a Volume Pressure Recording (VPR) system (CODA 6+, Kent Scientific, Torrington, CT). The principle of the VPR method is similar to tail cuff inflation, however it uses two tail cuffs: the occlusion cuff (O-cuff) constricts the tail artery, while the VPR cuff then measures the change in tail-artery volume when blood flow is restored as the O-cuff deflates. These tests were performed in a room maintained at approximately 31°C. The warmer room temperature ensured an adequate blood flow through the tail and improved the signal at the transducer. Rats were habituated to the restraint tubes for 6 days (PD51-56) during

which time 4 sets (5 cycles in each) of blood pressure measurements were taken over approximately 20 minutes per session. The social defeat sessions began on PD 57 at which point blood pressure measures were obtained both before and after the social defeat taking 3 sets of 5 cycles each time (i.e. each session consisted of a total of 15 cycles which took approximately 15 minutes to run). Average systolic, diastolic, and mean arterial pressures were computed over the last 10 cycles and these averages were used for statistical analysis.

Physiological Measures

Organs (brain, heart, kidneys, pancreas, thymus, adrenals and spleen) were harvested and weighed at PD 62. Fat pads (visceral, perirenal and epididymal pads combined, and subcutaneous fat pads) were also harvested and weighed. Non-fasting blood was collected by decapitating the rat 20 minutes after the end of the last (6^{th}) social defeat session. After coagulation, blood was centrifuged at 3000 rpm for 20 minutes and the plasma was collected and stored at -60ºC until later analyses. Corticosterone, insulin and leptin concentrations were measured in plasma using commercially available RIA kits (Rat Corticosterone: PITKRC-2; DPC® Los Angeles, CA, Rat Leptin kit: RL-83K and Rat Insulin kit: RL-RI-13K; Linco®, St. Charles, MO). The manufacturer's protocol was followed and the assay tubes were counted for 1 min using a Beckman 8000 gamma detector. The concentrations of the hormones in the samples were read from a standard curve constructed using standards supplied in the kits. Each sample was run in duplicate and the average value used for calculation.

Data Analysis

Three-way ANOVAs were conducted to examine for significant differences in body weight, organ weight, blood pressure and fat pad mass of the rats as a function of the rats' dietary (gestational, lactational and post-weaning diet) history and their exposure to stress. There was a strong positive correlation between subcutaneous fat pad mass and the visceral + epididymal +

perirenal fat pad mass (Pearson's r=0.836, $p<0.01$), so the ANOVAS for the fat pads were conducted using the total fat pad mass. Similarly ANOVAs were also conducted to examine for significant differences as a result of stress and dietary history in serum corticosterone, leptin and insulin levels. Subsequent t-tests were conducted as necessary in order to assess effects of gestational, lactational or post-weaning diet with stress. Significance levels were set at $p<0.05$.

Results

From PD23-47 there were no significant differences in caloric intake of the rats based on their gestational, lactational or dietary histories (Figure 3-1) ($p>0.05$). Similarly from PD49-61, caloric intake did not differ either as a function of stress or dietary history (Figure 3-3) ($p>0.05$). However both during and prior to stress exposure, the rats on the high fat post-weaning diet consumed the greater proportion of their calories from the high fat diets, and compensated by a reduction in chow intake (Figure 3-2). While there were no significant differences in body weight (Table 3-2) ($p>0.05$), there was an overall effect of diet on total fat pad mass [$F(7, 79) = 16.541$] (Figure 3-5); specifically the rats maintained on the post-weaning high fat diets had significantly heavier fat pads than those maintained on the standard chow diet [$F(1, 79) = 60.868$] (Figure 3-6). There were no significant differences in organ weights (brain, heart, or kidneys) as a function of the rats' dietary histories (Table 3-3) ($p>0.05$). There were no differences in spleen or thymus weight as a result of stress exposure (Figure 3-7) ($p>0.05$). However the adrenal glands of the intruder rats (which had been subjected to 6 days of social defeat) were significantly heavier than those of the control rats [$F(1, 79) = 8.105$] (Figure 3-7). There was a main effect of post-weaning diet on thymus gland weight [$F(1, 79) = 4.195$], such that those rats on the high fat post-weaning diet had heavier thymus glands as compared to those on the standard chow diet.

Mean arterial blood pressure (MAP) readings did not increase following the social defeat sessions nor did they differ significantly as a function of the rats' dietary histories (Figure 3-8) ($p>0.05$). Similarly, the systolic and diastolic pressures did not differ between groups (data not shown). When the rats were compared based on gestational history, a significant interaction was noted between gestational history and stress exposure [$F(1,159)=4.456$, $p<0.05$].

The rats exposed to social defeat stress had significantly elevated levels of non-fasting serum corticosterone (Figure 3-9) [$F(1, 78)=115.256$, $p<0.00001$]. An overall effect of post-weaning diet was seen such that the rats maintained on the high fat post-weaning diet had significantly higher levels of serum corticosterone [$F(1,78)=4.319$, $p<0.05$] (Figure 3-10). There was no significant interaction between stress and post-weaning diet however.

Non-fasting serum leptin levels were significantly higher in the rats as a function of diet history [$F(7,74)=5.576$), $p<0.0001$] and stress exposure [$F(1,74)=4.662$, $p<0.05$] (Figure 3-11). This diet effect was derived from the lactational dams' diet (Figure 3-12), such that those rats that had been with a high fat dam during lactation had significantly higher leptin levels than those that had been with a low fat dam during lactation [$F(1,74)=36.572$, $p<0.00001$].

Non-fasting serum insulin levels were significantly lower in the stressed rats [$F(1,79)=5.965$, $p<0.05$], and an effect of diet was also observed [$F(7,79)=3.330$, $p<0.01$] (Figure 3-13); those rats maintained on the high fat post-weaning diet had significantly higher insulin levels than the chow-fed rats [$F(1,79)=8.827$, $p<0.01$] (Figure 3-14).

Discussion

These experiments characterized differences in stress responsivity of male BHR offspring as a function of different gestational, lactational and post-weaning dietary environments.

It has been previously reported that borderline hypertensive rats are susceptible to environmentally-induced hypertension (Lawler et al., 1981; Sanders and Johnson, 1989; Fisher

and Tucker, 1991), but the present set of experiments did not find any differences in blood pressure as a function of stress. While the present experiment employed a psychosocial form of stress, the stressors used in the above mentioned studies included electric shock, elevated sodium intake and air-jet noise. Gelsema et al., (1994) reported that social stress (created by colony housing designed to increase aggressive/competitive interactions between male BHRs, and later changing the composition of the groups, thus preventing the establishment of a dominance hierarchy) stimulated an increase in aggressive interactions and subsequent increase in adrenal weight; however it did not induce hypertension in the rats.

We found no differences in blood pressure as a function of their gestational, lactational or post-weaning diets. While there are a number of studies demonstrating programming effects of maternal diet (Langley-Evans, 1997; Samuelsson et al., 2008) or high fat post-weaning diet (Velkoska et al., 2005; Souzo-Mello et al., 2007) on the development of hypertension in the offspring, there are an equally impressive number of studies that report no effects of prenatal and postnatal dietary environments on the development of hypertension (Zimanyi et al., 2002; Leary et al., 2005; Woods et al., 2005). Differences in diets, strain of rat, sex, method of assessing blood pressure and age at which blood pressure is measured are likely explanations for these differences. What is peculiar in the present set of experiments is that regardless of the rats' experimental or dietary condition, their blood pressures were atypically high, with average SBP readings of 190 mmHg and higher, DBP readings of 158 mmHg, and MAP readings of 170 mmHg and higher. Other studies conducted using male BHRs typically report MAP readings around 130-140 mmHg (Sanders and Lawler, 1992). We discuss below some possible reasons for the discrepancy, although we find no compelling arguments at this time.

One possible explanation for these unexpectedly high readings is that the restraint- and thermal stress associated with indirect tail-cuff plethysmography may have confounded the effects (if any) of social-stress induced hypertension. It is important to note that the equipment was calibrated to ensure that the readings were accurate. However, it is also of value to note that the rats had received 6 days of adaptation to the restraint and warming associated with this procedure and it is well-established that rats will habituate to repeated restraint stress (Melia et al., 1994; Girotti et al., 2006). Furthermore, Lawler et al. (1981) used tail-cuff plethysmography to measure blood pressures in male BHRs and reported significant differences in SBP as a result of an electric-shock exposure. In contrast, Gelsema et al. (1994) used telemetry and direct carotid artery cannulation to measure blood pressure in male BHRs and, similar to the present results, found no induction of hypertension as a result of psychosocial stress. To our knowledge, this is the first use of the VPR method in assessing blood pressures in BHRs, and while VPR has been validated in a number of other studies (Aukes et al., 2007; Euser and Cipolla 2007; Starr et al., 2008), it is possible that this technique may be inappropriate for male BHRs.

Rats subjected to social defeat stress had significantly elevated levels of serum corticosterone on day 6 compared to the non-stressed control rats. Elevations in circulating corticosterone levels as a result of stress have been reported by numerous studies (for review see Dallman et al., 2004) and our data demonstrate that our rats had not habituated to the repeated social defeats by day 6. Importantly, a significant effect of post-weaning diet was found, such that the rats maintained on the junk-food diet had higher serum corticosterone levels compared to those on standard chow; this was seen for both the stressed and non-stressed groups, indicating that basal corticosterone levels were higher in the junk-food fed rats compared to those maintained on standard chow. The stressed rats were also hyperleptinemic. It has been

established that glucocorticoids increase *ob* gene mRNA expression and leptin production (Slieker et al., 1996; Devos et al., 1995). Thus the elevated corticosterone levels in the stressed rats may have been driving the elevated leptin levels observed in the stressed rats. It is also known that leptin attenuates elevations in plasma corticosterone and adrenocorticotrophic hormone induced by restraint-stress (Heiman et al., 1997). It has also been suggested that dietary fat is a stressor (Hüllsman 1978; Pascoe et al., 1991; Tannenbaum et al., 1997; Kamara et al., 1998), which may be an alternative explanation for the higher corticosterone levels observed in the junk-food fed rats (as compared with the rats maintained on standard chow alone).

Despite differences in total body adiposity as a function of post-weaning diet, the junk-food fed rats did not have elevated serum leptin compared with the rats maintained on the standard chow diet. Instead an effect of the lactational dam's diet was observed, such that those rats that had been gestated in the high fat dams had higher serum leptin levels compared with those rats that had been gestated in the control diet dams. Although serum leptin levels were not assessed in the dams it is likely that the high-fat fed dams were hyperleptinemic as compared with the control diet dams, since fat pad mass has been shown to correlate with circulating leptin (for review see Friedman and Halaas, 1998). Furthermore it has been shown that not only do leptin levels in milk correlate with maternal BMI and serum leptin levels, (Casabiell et al., 1997; Houseknecht et al., 1997) but also that leptin transfer occurs from dam to pup during lactation (Casabiell et al., 1997). Though we are unable to propose a mechanism at this time, increased leptin transfer via maternal milk in the pups suckled by the high-fat fed dams may have induced a resistance to leptin's effects centrally, which in turn may explain their elevated leptin levels in adulthood.

Serum insulin levels were lower in the stressed rats than in the non-stressed controls. This is consistent with a number of other studies that report decreases in insulin levels following exposure to a variety of stressors (forced swim, intermittent noise, varying forms of restraint stress) (Armario et al., 1985; Zardooz et al., 2006). The rats in the present study were decapitated in the early part of their light cycle, so it is likely that they were still digesting food they had eaten towards the end of their dark cycle. This would typically stimulate increases in circulating insulin levels, however in the case of the stressed rats, activation of the sympathetic nervous system would have had the dual effect of reducing gastric motility and inhibiting insulin release which may explain the reduced insulin levels observed in the stressed rats relative to their non-stressed controls. Rats that had been maintained on the junk-food diet post-weaning had lower serum insulin levels than those that had been placed on the standard chow diet. It is known that high fat diets induce decreases in pancreatic insulin production (Sako and Grill, 1990; Zhou and Grill, 1994) so the reduced levels of serum insulin in the junk-food fed rats may be reflecting a diet-induced reduction in production. An alternative explanation is that although the rats on the junk-food diet had only a moderately elevated caloric intake relative to the chow-fed rats, they did consume more of their calories from the junk-food diets, some of which had a higher protein content than standard chow (particularly the peanut butter+chow and sausage diets). This greater protein intake may have improved their insulin sensitivity, as high-protein intake has been associated with decreases in blood glucose and improved insulin sensitivity (Demigné et al., 1985; Karabatas et al.,1992; Gannon and Nuttall, 2004).

Complementing their serum corticosterone profile, the rats exposed to 6 days of social defeat stress had heavier adrenal glands compared to the non-stressed control rats. While no differences in spleen weight were seen, the stressed rats did exhibit a trend towards thymus

involution, although this was not statistically significant. Adrenal gland enlargement and thymus involution are consistent with other reports examining physiological responses to chronic stress (Seyle, 1936; Schmidt et al., 1992; Aguilera et al., 1996; Kubera et al., 1998). Interestingly, those rats on the junk-food post-weaning diet had heavier thymus glands than those on the standard chow diet. With the progression of age in humans, it is known that there is increasing fat accumulation within the thymus gland (Kendall, 1984) so it is possible that the junk-food-fed rats had greater fat accumulation within their thymus gland, which would explain their heavier thymus weights.

Neither prior to nor after the introduction of the social defeat stress (at PD57) were any differences in caloric intake noted, either as a function of dietary history or exposure to stress. While there are a number of studies reporting stress-induced hyperphagia (Rowland and Antelman, 1976; Bell et al., 2002; Pecoraro et al., 2004), it is important to consider the type of diets they provided. Specifically all these studies reporting stress-induced hyperphagia provided the rats with a high-carbohydrate option (typically either some concentration of sucrose or sweetened condensed milk). The present experiment provided a rotating junk food diet; however the social defeat stress began on PD57, at which point all the rats were on standard chow. On PD59 the junk-food diet rats were given processed cheese product, and then on PD61 all the rats were placed back on standard chow. So at no point during the social defeat stress did the junk-food diet group have access to a sweet palatable food option. Supporting our results, studies that report either no differences in food intake as a result of stress (Legendre and Harris, 2006) or even an inhibition of intake following stress (Harris et al., 1998; Bates et al., 2008) provided either a high fat diet or standard chow, rather than a sweet palatable option. Work by Sucheki et al. (2003) and Uhlrich-Lai et al. (2007) report that rats given saccharin solutions had reductions

in HPA axis responses following stress exposure. Thus it is possible that sweet foods may specifically be reducing HPA activity, which may explain the stress-induced hyperphagia seen in studies where a sweet palatable food is provided.

It is noteworthy that the rats on the junk-food diet consumed a greater portion of their calories from the high fat dietary options available to them, and compensated to some extent by reducing their chow intake. Possible reasons for the rats' apparent failure to completely compensate for the higher caloric density of the high fat foods is likely due to a preference for those foods, based on their orosensory and postingestive properties (Reed et al.,1990; Lucas et al., 1998). As expected based on the similar caloric intake in the experimental groups, there were no group differences in bodyweight. However like their dams (Chapter 2), despite the absence of differences in body weight, significant differences in adiposity were found, not only as a function of the post-weaning diet but also as a function of the gestational and lactational dams' diets. Specifically those rats that had either been gestated in a high-fat fed dam, or suckled by a high-fat-fed dam or weaned onto a high fat diet had greater total body adiposity compared to their low-fat counterparts. The effect of the post-weaning diet is readily apparent as the junk-food diet rats were consuming more calories than those fed standard chow. The increased adiposity seen in the offspring that were gestated in the high fat dams points towards a programming effect of the gestational environment. The increased adiposity in the offspring suckled by the high fat dams complements their high plasma leptin levels, both compared with offspring of dams fed the control diet. Hypothalamic development is incomplete at parturition in rats, and a postnatal leptin surge is thought to influence the development or sensitivity of hypothalamic circuitry relating to energy regulation (Bouret et al., 2004). Thus, it is possible that the relative size of the leptin surge differs between pups nursed by a high fat compared with a control diet dam, and the net

result of that may be greater adiposity in adulthood. However, the mechanism(s) by which this could occur, for example leptin in milk, as well as the direction of the diet-related change, are unclear at this time.

There were no differences in total body adiposity between the stressed and non-stressed rats in the present study. While the present study did not discretely measure visceral fat pads, it is known that elevations in glucocorticoids have been associated with increased visceral adiposity in humans (Randrianjohany et al., 1993, Gluck et al., 2004) and glucocorticoid administration promotes obesity in rats (Zakrzewska et al., 1999). This may suggest that a more prolonged stress exposure may be necessary before the adipogenic effects of elevated glucocorticoids become apparent.

In conclusion, while the social defeat stress produced marked activation of the HPA axis, there were no effects on blood pressure measured using the VPR method. It is important to note that as the rats' blood pressures were so tremendously elevated, it is feasible that were there an effect of the stress may have been masked. Overall, stress exposure did increase serum leptin and decrease serum insulin levels. Additionally, rats maintained on the junk-food diet had higher basal and stress-induced corticosterone levels relative to the chow-fed controls. In the human population stress-induced hypertension typically occurs in genetically susceptible individuals (Light et al., 1999; Saab et al., 2001), so while studying social stress in a genetically predisposed animal model (BHR) makes theoretical sense, the data suggest otherwise. Given the obesogenic and stressful environment of the 21st century, it is important to create an animal model that will allow us to better study the development of stress-induced hypertension and its associated pathological effects.

Table 3-2. Mean (± SE) body weight of rats at PD21, 45, 57 and 61. There were no significant differences in body weight either as a result of the rats' dietary histories or as a result of their exposure to the social defeat stress.

BODY WEIGHTS (g)	HHH	HHL	HLH	HLL	LHH	LHL	LLH	LLL
PD 21 BW (Weaning)								
Stress	63.2 ± 4.1	61.9 ± 3.4	54.1 ± 2.7	53.2 ± 2.8	64.1 ± 3.5	65.5 ± 3.5	53.5 ± 5.6	56.8 ± 3.2
Control	62.0 ± 3.4	61.5 ± 2.5	52.9 ± 2.4	53.5 ± 2.9	61.9 ± 1.9	65.9 ± 3.3	57.3 ± 4.6	58.0 ± 3.4
PD 45 BW (End of 1st Diet Cycle)								
Stress	219.6 ± 8.5	223.6 ± 10.0	224.4 ± 4.9	229.4 ± 5.7	229.6 ± 5.9	235.1 ± 7.9	206.3 ± 12.9	222.8 ± 8.9
Control	216.6 ± 7.8	220.2 ± 8.1	215.4 ± 5.3	222 ± 6.8	229.6 ± 7.8	239.2 ± 5.8	219.4 ± 11.7	217.1 ± 9.6
PD 57 BW (Day 1 of Social Defeat Stress)								
Stress	304.8 ± 11.0	307.6 ± 10.9	315.8 ± 7.1	320.4 ± 5.1	314.6 ± 6.6	322.0 ± 4.5	281.6 ± 14.7	307.4 ± 10.3
Control	307.0 ± 12.2	301.2 ± 8.2	300.8 ± 5.7	306.2 ± 6.6	315.8 ± 8.4	325.0 ± 7.1	302.8 ± 12.3	299.0 ± 10.8
PD 61 BW (Day 5 of Social Defeat Stress)								
Stress	313.2 ± 9.9	319.0 ± 10.7	320.6 ± 5.7	324.4 ± 5.6	320.9 ± 6.5	326.4 ± 7.3	286.2 ± 14.0	308.8 ± 13.2
Control	316.6 ± 9.0	308.4 ± 7.9	305.6 ± 4.5	313.4 ± 7.0	322.8 ± 8.3	328.2 ± 7.9	307.1 ± 13.6	306.4 ± 11.0

Table 3-3. Mean (± SE) organ weights harvested on PD 62. There were no significant differences in organ weights either as a result of the rats' dietary histories or as a result of their exposure to the social defeat stress.

ORGAN WEIGHTS (g)	HHH	HHL	HLH	HLL	LHH	LHL	LLH	LLL
				BRAIN				
Stress	2.04 ± 0.02	2.03 ± 0.03	2.05 ± 0.02	2.07 ± 0.02	1.98 ± 0.08	2.09 ± 0.02	2.05 ± 0.05	2.10 ± 0.03
Control	2.04 ± 0.01	2.02 ± 0.04	2.04 ± 0.03	2.06 ± 0.02	2.09 ± 0.01	2.08 ± 0.03	2.10 ± 0.04	2.07 ± 0.05
				HEART				
Stress	1.24 ± 0.05	1.16 ± 0.02	1.23 ± 0.03	1.20 ± 0.02	1.23 ± 0.02	1.20 ± 0.02	1.13 ± 0.03	1.18 ± 0.06
Control	1.24 ± 0.03	1.15 ± 0.04	1.17 ± 0.02	1.18 ± 0.02	1.29 ± 0.03	1.26 ± 0.05	1.20 ± 0.03	1.16 ± 0.03
				KIDNEYS				
Stress	1.32 ± 0.05	1.35 ± 0.05	1.36 ± 0.04	1.30 ± 0.03	1.32 ± 0.06	1.39 ± 0.03	1.32 ± 0.09	1.32 ± 0.06
Control	1.32 ± 0.02	1.28 ± 0.06	1.29 ± 0.03	1.29 ± 0.04	1.37 ± 0.05	1.39 ± 0.04	1.31 ± 0.07	1.29 ± 0.06

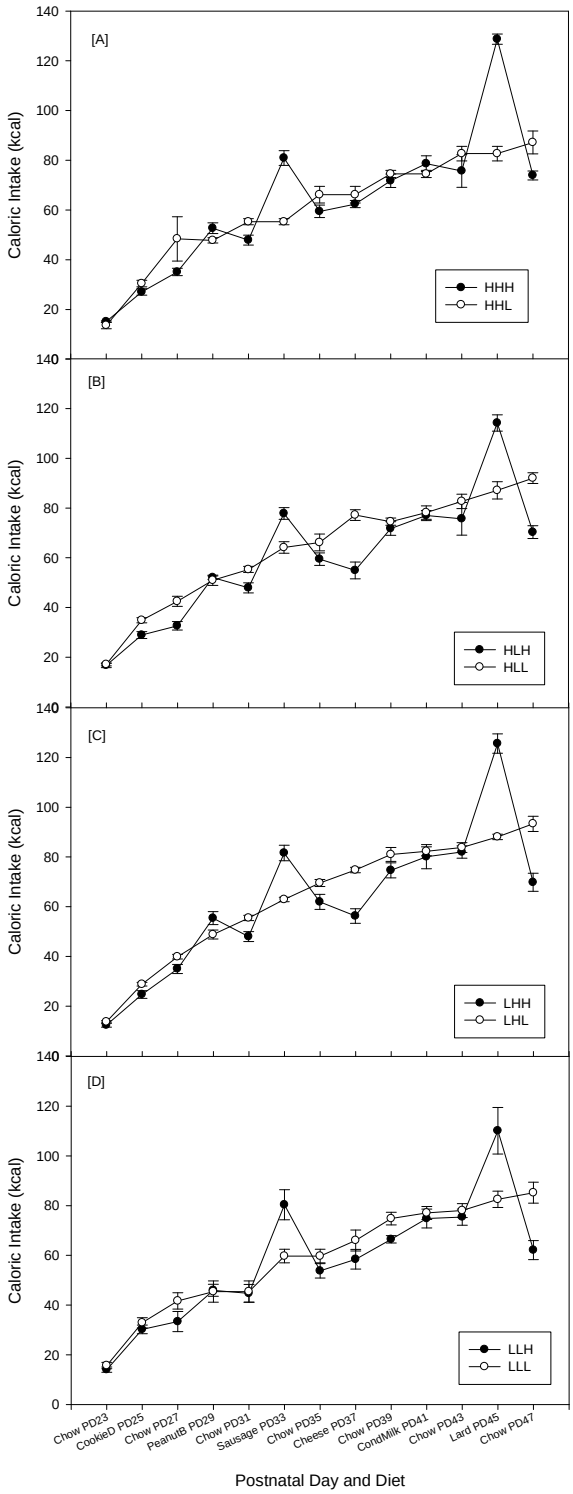

Figure 3-1.Mean (±SE) caloric intake every 2 days from PD23-47. The rats on the high fat diets (•) consumed more calories from the high fat options, particularly sausage and lard. Panel A: HHH vs. HHL, Panel B: HLH vs. HLL, Panel C: LHH vs. LHL, Panel D: LLH vs. LLL

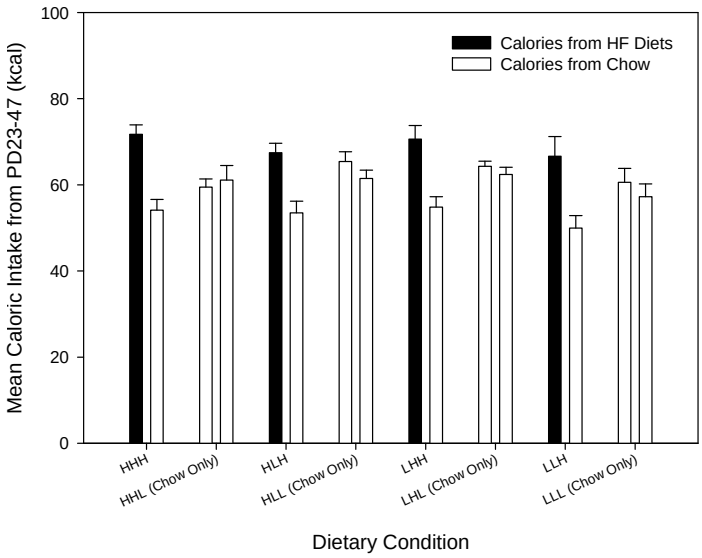

Figure 3-2. Overall Mean (±SE) caloric intake from PD23-47. There were no differences in mean caloric intake between the high fat fed and chow fed groups. However the high-fat fed rats consumed more of their calories from the high fat diets (black bars) and compensated by reducing their chow intake (white bars).

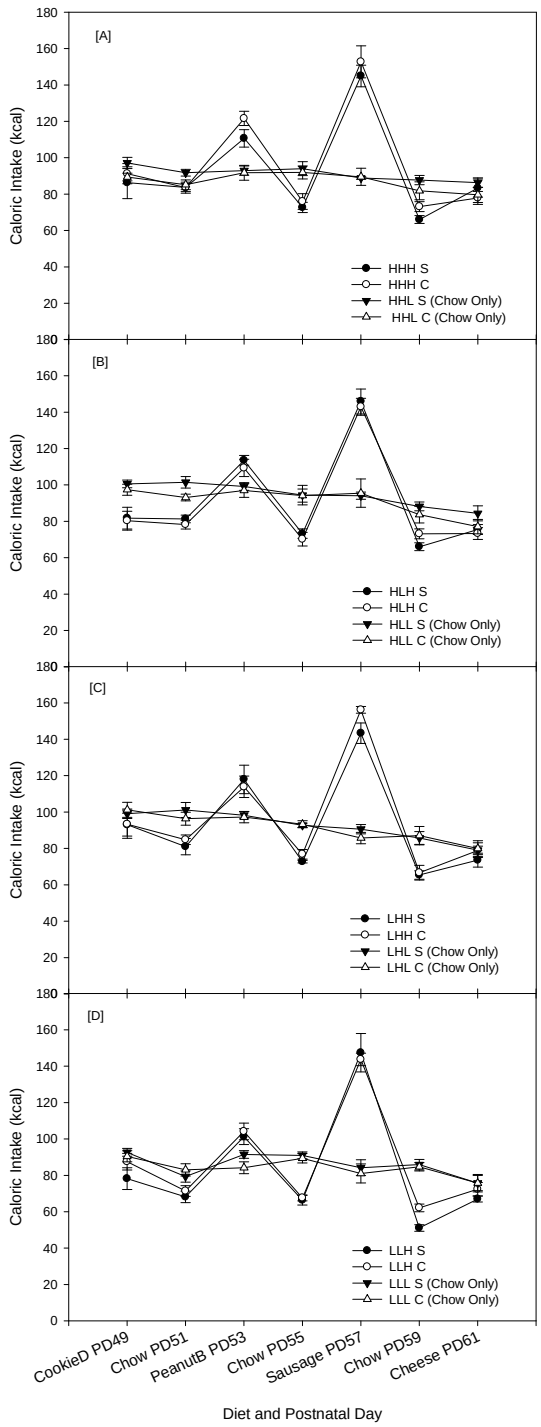

Figure 3-3. Mean (±SE) caloric intake every 2 days from PD47-61 There were no differences in caloric intake as a result of stress exposure. The rats fed high fat diets (•◦) reflect the same trend seen in Figure 3-1: they consume a greater proportion of their calories from the high fat diets, specifically peanut butter and sausage in this case. Panel A: HHH vs. HHL, Panel B: HLH vs. HLL, Panel C: LHH vs. LHL, Panel D: LLH vs. LLL. S = Stress, C= Control.

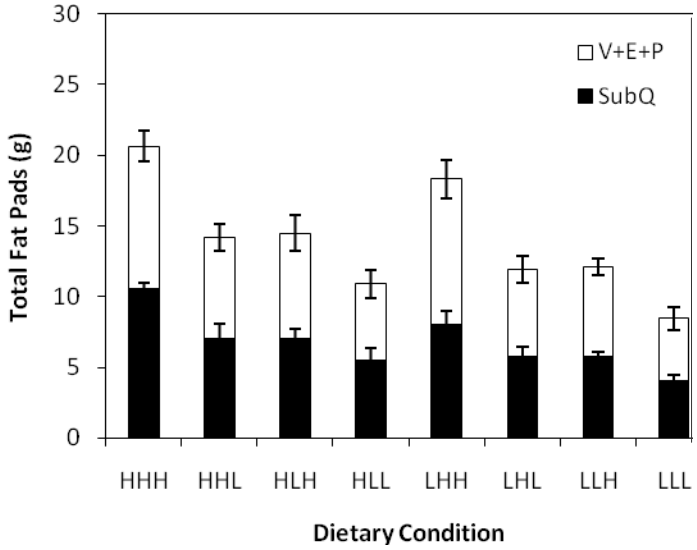

Figure 3-4. Mean (±SE) fat pad mass. V+E+P = Visceral+Epididymal and Perirenal fat pads, SubQ = Subcutaneous fat pads. A main effect of gestational diet, lactational diet and post-weaning diet was found (Ps<0.001). An interaction between lactation and post-weaning diet was also seen (p<0.05).

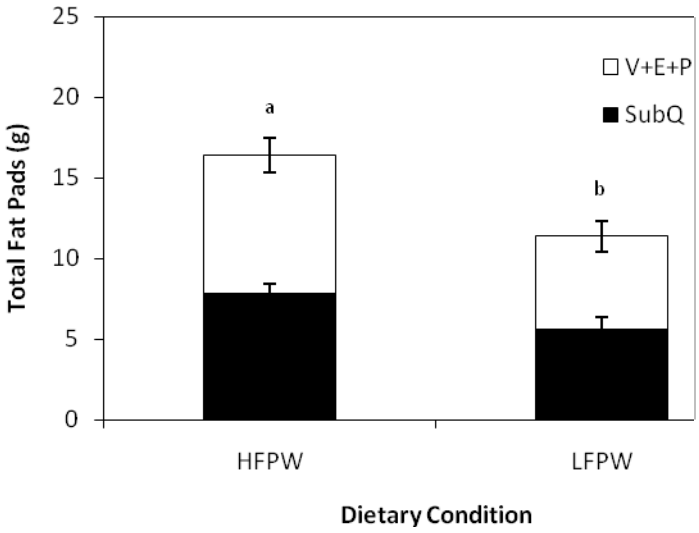

Figure 3-5. Mean (±SE) fat pad mass as a function of post-weaning diet. V+E+P = Visceral +Epididymal+ Perirenal fat pads, SubQ = Subcutaneous fat pads. Rats placed on the high fat post-weaning diet (HFPW) had significantly heavier visceral, epididymal, perirenal and subcutaneous fat pads than those maintained on the standard chow diet (LFPW) ($p<0.0001$). Bars denoted with different letters are significantly different ($p<0.0001$).

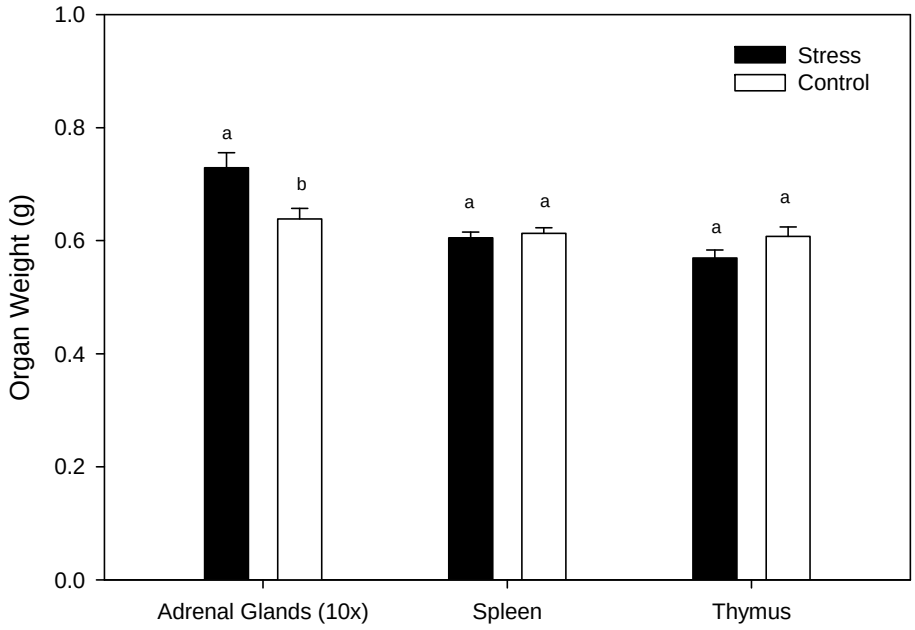

Figure 3-6. Mean (±SE) organ weights. The stressed rats had heavier adrenal glands ($p<0.01$) than the control rats. There were no differences in spleen weight; the thymus glands of the control rats were non-significantly heavier than the stressed rats, ($p=0.085$). Bars denoted with different letters are significantly different ($p<0.01$).

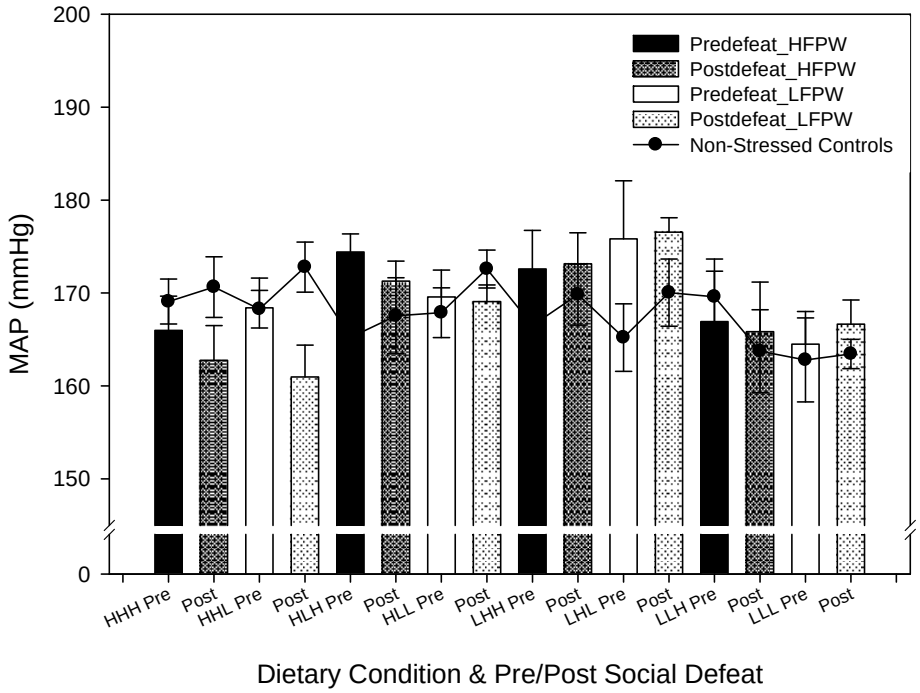

Figure 3-7. Average mean arterial blood pressure (MAP) (±SE). There were no significant differences in MAP, as a function of stress exposure or diet history ($p>.05$).

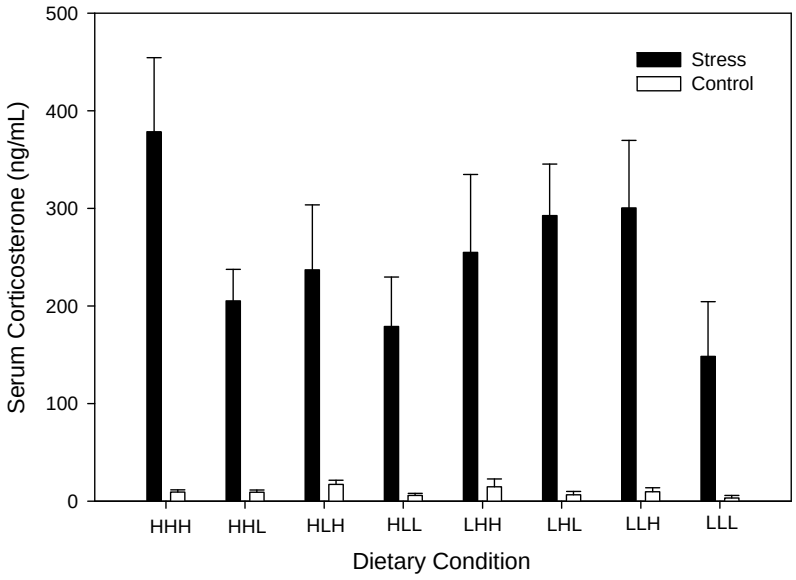

Figure 3-8. Mean (±SE) non-fasting serum corticosterone concentrations. The rats that were decapitated 20 minutes after the social defeat exposure had elevated serum corticosterone levels compared with the non-stressed control rats ($p<0.001$).

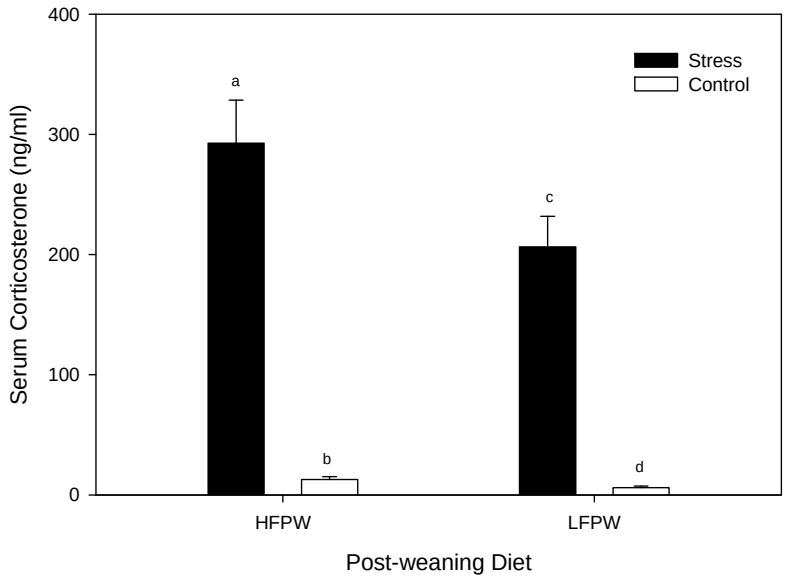

Figure 3-9. Mean (±SE) non-fasting serum corticosterone concentrations as a function of post-weaning diet. The rats on the high fat post-weaning diets (HFPW) had higher corticosterone levels than those maintained on the standard chow diet (LFPW) ($p<0.05$); this trend was seen in the stress ($p=0.057$) and control groups respectively ($p=0.022$). Bars denoted with different letters are significantly different ($ps<0.05$).

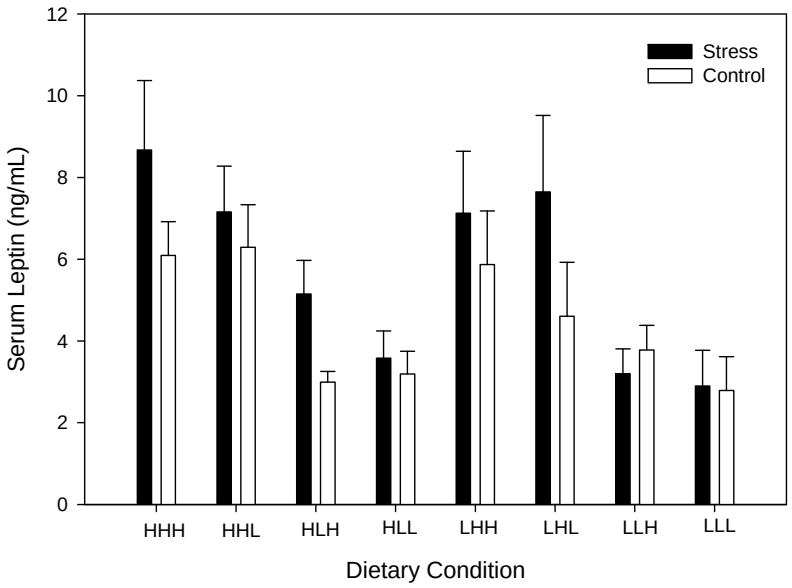

Figure 3-10. Mean (± SE) non-fasting serum leptin concentrations. An overall effect of stress was observed such that the stressed rats had higher leptin levels than unstressed controls ($p=0.035$). An overall effect of diet was also found ($p<0.0001$).

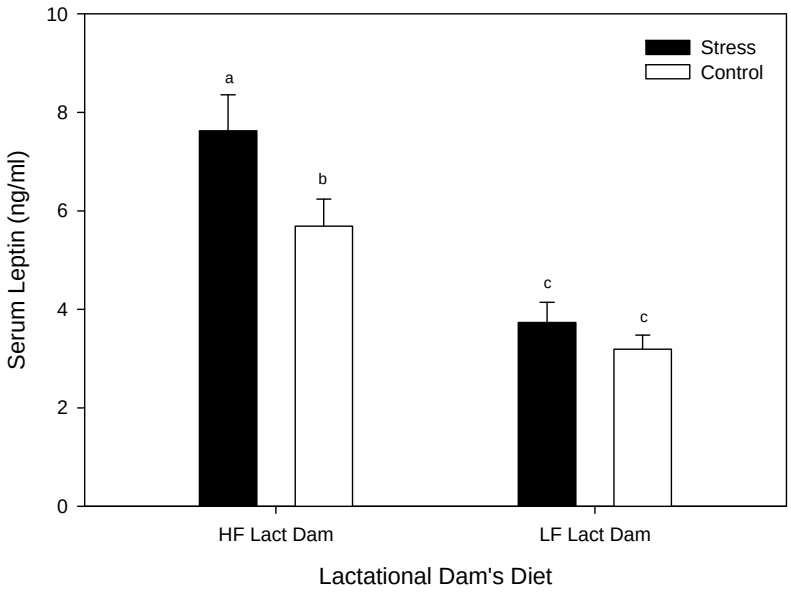

Figure 3-11. Mean (± SE) non-fasting serum leptin concentrations as a function of the lactational dam's diet. Those rats that were weaned by a high fat dam had significantly higher serum leptin levels than those that were weaned by the control diet (LF) dams. An effect of stress was observed only between the rats that were weaned by a high fat dam ($p=0.042$). Bars denoted with different letters are significantly different from each other ($ps<0.05$).

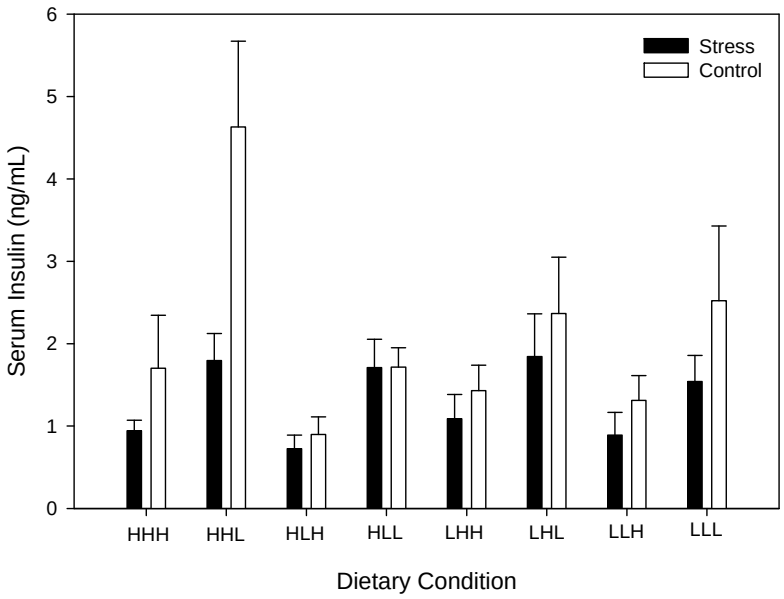

Figure 3-12. Mean (±SE) non-fasting serum insulin concentrations. An effect of stress was observed such that the stressed rats had lower insulin levels than unstressed controls ($p=0.006$). An overall effect of diet was also found ($p<0.001$).

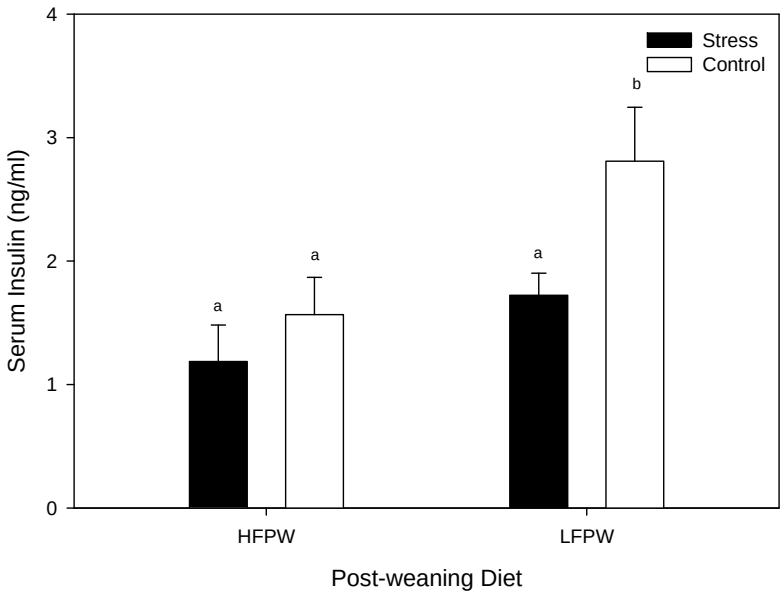

Figure 3-13. Mean (±SE) non-fasting serum insulin concentrations as a function of the post-weaning diet. The control rats had higher insulin levels than the stressed rats, although this difference was significant only for those rats on the standard chow (LFPW) diet ($p=0.027$). Bars denoted with different letters are significantly different ($Ps<0.05$).

CHAPTER 4
EFFECT OF MATERNAL DIET ON FEEDING BEHAVIORS AND METABOLIC PARAMETERS IN BORDERLINE HYPERTENSIVE RATS.

Introduction

There is a comprehensive body of research investigating the effects of maternal under-nutrition on the development of disease in adulthood. Both laboratory studies (Langley-Evans 2001; Vickers et al., 2005) and epidemiological work (Barker et al., 1997; Roseboom et al., 2001) have shown lasting effects of maternal undernutrition on the development of obesity and the metabolic syndrome in the affected offspring. The mechanism by which this fetal programming may be occurring is unclear at this time, but it is likely that gene expression is altered as a result of the inadequate nutritional environment, which in turn may alter organ development and have lasting effects on the physiology and behavior of the 'programmed' offspring.

Today however, maternal under-nutrition is globally no longer the primary concern; the World Health Organization asserts that obesity is now overtaking under-nutrition and infectious disease in terms of major public health concerns (WHO Tech. Rep. Series, 2000). While the adverse effects of obesity on the mother have been extensively studied (gestational diabetes, preeclampsia, prolonged delivery, delayed wound healing post-delivery *etc.*), the effects on fetal development and long-term health of the offspring remain unclear. What is known is that babies born to obese women have higher rates of congenital abnormalities (Naeye, 1990), that they tend to be large-for-gestational-age (LGA) and are at a greater risk of developing metabolic syndrome later in childhood (Boney et al., 2005). The importance of the postnatal environment cannot be overlooked, and it is clear that a hypercaloric postnatal environment combined with a sedentary lifestyle are both significant contributing factors in the development of the obesity epidemic.

It is presently unknown whether the increased rates of obesity in children are a consequence of the *in utero* environment, the immediate postnatal environment (e.g. breast versus formula feeding), behaviors learned from parents (i.e. poor diet choices) or a combination of these factors. It is therefore critical to develop a better understanding of precisely how maternal obesity impacts the developing offspring, both during gestation and post-parturition.

The present set of experiments examines, in female borderline hypertensive rats (BHR), whether exposure to a high-fat (60% fat) versus a control (10% fat) diet during either gestation or lactation has long term (i.e. programming) consequences for food intake and metabolism in the offspring. In chapter 2, I described the characteristics of exposure to high or low fat diet in the mothers during this period, as well as the birth characteristics of the offspring. Briefly, BHR offspring, produced by mating Wistar females with SHR males, were used because they may be genetically predisposed to hypertension. Furthermore to the best of our knowledge there have been no studies investigating the effects of diet-induced obesity in this strain.

Obesity is typically caused by an excess of food consumption relative to energy expenditure (Gray et al., 2004). Consumption of calorically dense high fat diets often produces hyperphagia and/or obesity in rodents (Warwick and Synowski, 1999) and so, after weaning the offspring in this experiment, they were fed either a standard and monotonous food (Purina 5001) only or were fed a changing regimen of high fat foods that might typify high fat foods eaten by humans: we termed this our junk food diet. To measure behavioral parameters, we recorded bi-daily food intake throughout the study. In addition, toward the end of the study we assessed food motivation using standard fixed (FR) and progressive ratio (PR) operant schedules of reinforcement for food pellets. We also measured blood pressure, fasting and non-fasting serum leptin levels and fasting serum insulin levels in order to examine for hypertension,

hyperinsulinemia and hyperleptinemia as these conditions typically accompany the development of obesity and the metabolic syndrome.

We hypothesized that those rats gestated in a high-fat dam, cross-fostered to a high-fat dam and weaned onto the junk-food diets (HHH rats) would be most susceptible to developing the metabolic syndrome (as indicated by the development of hypertension, greater overall adiposity, elevated serum insulin and leptin levels) relative to rats in the control condition of having been gestated in and fostered to the control diet dams, and subsequently weaned onto a standard chow diet (LLL rats). As hypothalamic circuitry is immature at birth in rats (Bouret et al., 2004), we also hypothesized that it was likely that the diet of the foster dam would in some way impact food intake patterns in the offspring as well.

Materials and Methods

Animals and Housing Environment

This study used the female offspring (4 per litter) generated from the matings described in chapter 2. To summarize that design, there were four litter types at weaning (PD21): HH, HL, LH and LL with 6 litters of each (24 litters in total). The litters were separated based on sex and diet type and housed (n=2 pups per cage) in polycarbonate cages with stainless steel wire mesh lids in a controlled environment (21-24°C, 45-55% relative humidity, 12:12 cycle, with lights off at 10 am and on at 10 pm). Half the rat pups in each litter were placed on a rotating high-fat diet (details provided in the following section), and the other half were placed on standard Purina 5001 chow diet. This resulted in 8 groups based on their gestational, lactational and post-weaning history: HHH, HHL, HLH, HLL, LHH, LHL, LLH, LLL (see Table 4-1).

At PD45 rats for this experiment were moved to another vivarium that was maintained at a similar temperature and relative humidity as the original room, but had a normal light cycle (lights on at 8 am and off at 8 pm). This resulted in 2 rats per cage per condition.

Table 4-1. Outline of experimental design showing dietary conditions of different litter types.

Litter Condition at Weaning (PD21)	Diet From PD21-PD61
HH (6 Litters; n=24 ♀rats)	Rotating High-fat Diet n=12 rats Condition: HHH
	Standard Chow Diet n=12 rats Condition: HHL
HL (6 Litters; n=22 ♀rats)	Rotating High-fat Diet n=12 rats Condition: HLH
	Standard Chow Diet n=10 rats Condition: HLL
LH (6 Litters; n=24♀rats)	Rotating High-fat Diet n=12 rats Condition: LHH
	Standard Chow Diet n=12 rats Condition: LHL
LL (6 Litters; n=22 ♀rats)	Rotating High-fat Diet n=10 rats Condition: LLH
	Standard Chow Diet n=12 rats Condition: LLL

Post-weaning Diets

The rotating junk-food diet consisted of a presentation of one of six high fat foods: cookie-dough (4.98 kcal/g, made from flour, sugar, shortening, and vanilla essence); peanut-butter/chow (4.78 kcal/g, 50% powdered Purina 5001+50% smooth peanut butter); Vienna sausages (2.83 kcal/g), processed cheese product (2.85 kcal/g), condensed-milk/chow (3.32 kcal/g, 50% powdered Purina 5001+50% sweetened condensed milk) and the high fat semi-synthetic diet D12492 (5.24 kcal/g). With the exception of the standard chow and D12492, all of these

ingredients were generic brands from a local supermarket. Each of these diets was presented for two days separated by two days of standard chow (Purina 5001; 3.34 kcal/g). The rationale for these chow periods was because the protein:calorie ratio of the junk foods could be lower than needed to sustain optimal growth, so chow (>25% protein:calorie ratio) periods should ensure that protein availability was not a limiting factor. This diet regimen produced 8 groups: maternal dam high-fat diet, foster dam high-fat diet, post-weaning junk-food diet (HHH), HHL, HLH, HLL, LLL, LLH, LHH, and LHL (Table 4-1). Food intake and body weights were monitored every two days from PD 21 through PD189.

Blood Pressure Measurements

Blood pressure was measured using a Volume Pressure Recording (VPR) system (CODA 6+, Kent Scientific, Torrington, CT). The principle of the VPR method is similar to tail cuff inflation; however it uses two tail cuffs: the occlusion cuff (O-cuff) constricts the tail artery, while the VPR cuff then measures the change in tail-artery volume when blood flow is restored as the O-cuff deflates. These tests were performed in a room maintained at approximately 31°C. The warmer room temperature ensured an adequate blood flow through the tail and improved the signal at the transducer. Rats were habituated to restraint tubes for 4 days (PD165-168) during which time 4 sets (5 1-min inflation-deflation cycles in each) of blood pressure measurements were taken over approximately 20 minutes per session. The procedure was repeated for an additional 2 days (Day 5 and 6, PD169-170), and the average of the data from these 2 days were used for data analysis purposes.

Operant Procedures

All operant procedures were conducted in non-fasted rats. For this they were placed in operant chambers measuring 30x24x21cm with a steel rod floor (Med Associates, St. Albans, VT). One fixed lever protruded through one wall of the chamber, to the right of a recessed food

trough. A cue light was placed directly above the lever, and was illuminated for 1 second when the rats received a reward. The rats were first maintained on a fixed-ratio 1 (FR-1) schedule of reinforcement and then on a progressive ratio (PR) schedule of reinforcement. Rats were tested every second day for 48 days (first 11 sessions for FR1 and then 12 sessions for PR), to assess differences in motivation as a function of the different diets. Cue lights and pellet delivery were controlled by Med-PC software (Med Associates, St. Albans, VT) that also recorded the number of lever presses, rewards and session lengths. Body weights were measured on the first day of the first FR1 session and on the last day of the last PR session.

The rats received one session of training on the FR1 program. FR1 program lasted for a total of 60 minutes, and each time the rats successfully depressed the lever, they received a single pellet of similar composition to chow (45 mg pellet, Purina#1811155). On the day of their last FR1 session, they were placed on 2 consecutive PR training sessions. The PR schedule of reinforcement was set up such that the ratio requirement for successive rewards increased by a factor of 1.05 and was rounded to the nearest integer. The cumulative number of presses against pellets earned is illustrated in Figure 4-1. The program terminated whenever 15 minutes elapsed since the last pellet was received.

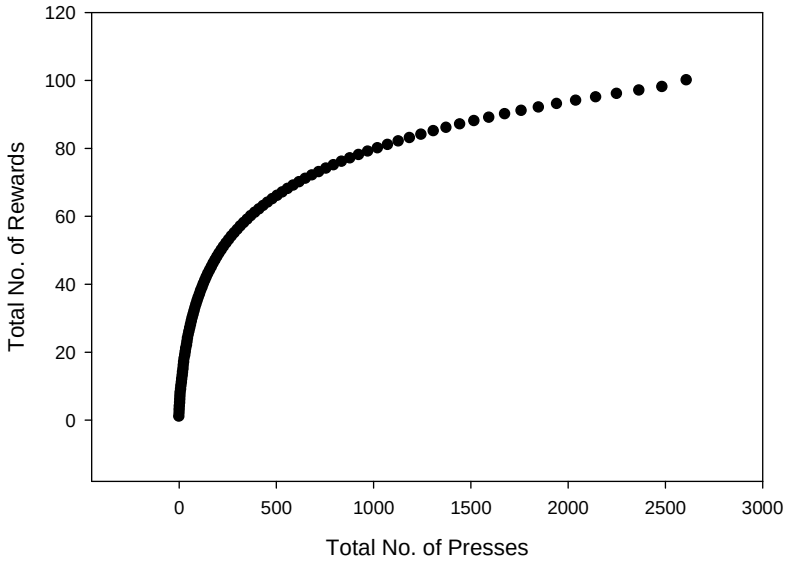

Figure 4-1. Cumulative number of presses versus pellets received in the PR schedule.

Physiological Measures

On PD200, fasting blood was collected after an 18-hour fast via heart puncture. On PD 224 the same rat from which the fasting blood had been collected was euthanized with sodium pentobarbitol, and non-fasted blood was collected from the aorta. At this time, organs (heart, kidneys, adrenals and spleen) and fat pads (visceral, perirenal and periovarian pads combined, and subcutaneous fat pads) were dissected out and weighed. Blood collected was allowed to coagulate after which it was centrifuged at 3000 rpm for 20 minutes and the serum was aspirated and stored at -60ºC until future analyses of insulin and leptin were performed. Commercially available RIA kits (Rat Leptin kit: RL-83K and Rat Insulin kit: RL-RI-13K; Linco, St. Charles, MO) were used for these assays; the manufacturer's protocol was followed and the assay tubes counted for 1 min using a Beckman 8000 gamma detector. The concentrations of the hormones in the samples were read from a standard curve constructed using standards supplied in the kits. Each sample was run in duplicate and the average value was taken for calculation.

Data Analysis

Three-way ANOVAs were conducted to examine for significant differences in body weight, organ weight, fat pad mass, serum leptin and insulin levels and blood pressure of the rats as a function of gestational, lactational and post-weaning environments. One way ANOVAs were conducted as necessary to examine for overall significance between the 8 dietary conditions, with post-hoc Tukey tests. For the PR1 and FR lever press studies, the total number of rewards earned was averaged across the total number of sessions for each animal. One-way and 3-way ANOVAs were then conducted on these means with post-hoc Tukey tests. Significance levels were set at $p<0.05$.

Results

The average daily caloric intakes from PD21-191 are shown in Figure 4-2. From PD21-191 there were no significant differences in caloric intake of the rats based on their gestational or lactational histories ($p>0.05$). There was a significant effect of post-weaning diet [$F(1,679) = 11.675$, $p<0.01$), such that the junk food-fed rats consumed approximately 10% more calories overall relative to the chow-fed controls. Junk food fed rats consumed a greater proportion of their calories from the junk foods, and had a corresponding reduction in chow intake (Figure 4-3). A comparison of the differences in mean overall intake as a function of the different diets provided to the junk-food rats is presented in Table 4-2. Table 4-3 shows the chow intake of the standard-chow control rats on the same days that the junk-food diet rats had their different diets. Clearly, these intakes did not differ between groups or across time.

Body-weights at PD21 were significantly heavier in rats suckled by high-fat dams as compared with the control-diet dams [$F(1,45)=15.177$, $p<0.0001$], but this difference was transient and disappeared after PD25. Post-weaning diet affected body weight of the offspring (Figure 4-4) with the junk food-fed groups weighing more than the chow-fed controls. This

effect first became evident at PD 45 [F (1, 45) =9.848, $p<0.01$) and, with the exception of PD49 ($p>0.05$), remained statistically significant through PD191 [F(1,45)=73.585, $p<0.00001$] . In addition to being heavier, those rats weaned onto the junk food diet were also longer, as measured from nose-to-anus at PD 224 [F(1,45)=23.094, $p<0.0001$] (Figure 4-5).

Complementing the body weight data, those rats that were weaned onto the junk-food diets had significantly heavier fat pads compared to their chow-fed counterparts; there were significant differences in subcutaneous fat pad mass [F(1,45)=58.979, $p<0.0001$], the combined mass of the visceral + periovarian + perirenal fat pads [F(1,45)=59.410, $p<0.0001$] , and the total fat pad mass [F (1, 45) =82.320, $p<0.0001$] (Figure 4-6). Interestingly an effect of the lactational dam's diet was also seen on the combined mass of the visceral + periovarian + perirenal fat pads [F (1, 45) =18.345, $p<0.0001$] and the total fat pad mass [F(1,45)=18.007, $p<0.0001$], such that the rats suckled by a high-fat dam had heavier fat pads as compared with those that had been suckled by a control diet dam (Figure 4-7). No effect of the gestational history was observed. A significant interaction was noted between post-weaning diet and the lactational dam's diet [F (1, 45) =5.114, $p<0.05$].

The heart [F (1, 45) =8.971, $p<0.01$] and kidney [F (1, 45) =19.141, $p<0.01$] weights of the junk food-fed rats were significantly heavier as compared with the chow-fed controls (Figure 4-8). There was a trend towards heavier adrenal glands in the junk food-fed rats, but this did not attain statistical significance ($p=0.060$). With regard to the gestational dams' diets, there was a trend for rats gestated in the high fat-fed dams to have reduced kidney weight, but this difference did not reach statistical significance ($p=0.075$). Organ weight data are presented in Table 4-4.

Mean arterial blood pressure (MAP) [F (1, 45) =13.467, $p<0.01$] and diastolic blood pressure (DBP) [F (1, 45) =9.178, $p<0.01$] readings differed as a function of the gestational

dams' diet (Figure 4-10 and 4-11). Rats gestated with control diet dams had consistently higher (by about 5 mm Hg) MAP and DBP than those gestated in high fat diet-fed dams. There was no effect on blood pressure of either lactational or post-weaning dietary histories.

Fasting (Figure 4-13) serum leptin (collected at PD200) levels were significantly higher in the rats as a function of post-weaning diet such that the junk-food fed rats had elevated serum leptin levels as compared to their chow-fed controls [$F(1,43)=46.107$, $p<0.0001$)]. Non-fasting serum leptin levels (collected at PD224) (Figure 4-14) differed significantly as a function of the gestational dams' diets [$F(1,40)=10.028$, $p<0.01$)], the lactational dams' diets [$F(1,40)=31.406$, $p<0.0001$)] as well as the post-weaning diets [$F(1,40)=86.835$, $p<0.0001$)]. Specifically, those rats that had either been gestated in or suckled by a high fat dam had significantly higher non-fasting serum leptin levels, and those rats that had been weaned onto the junk food diet also had significantly higher non-fasting serum leptin levels (Figure 4-15). Additionally significant interactions were observed for non-fasting leptin between the post-weaning and lactational diets [$F(1, 40)=22.964$, $p<0.0001$)], as well as the post-weaning and gestational diets [$F(1, 40)=9.993$, $p<0.01$)]. Fasted serum insulin levels collected at PD200 are shown in Figure 4-16. Junk food fed rats had higher insulin levels than chow-fed controls [$F(1, 44)=18.823$, $p<0.001$)], but there were no effects of gestational or lactational dams' diet ($p>0.05$).

The average performance across 11 sessions of FR1 and 12 sessions of PR are shown in Figure 4-17 and Figure 4-18. When comparing differences in FR1 performance, as a function of all the 8 diet groups a significant effect of diet was observed [$F(7,42)=4.166$, $p<0.01$]. Three-way ANOVA of the pellets consumed on the FR1 schedule revealed a significant effect of the post-weaning diet [$F(1,42)=16.737$, $p<0.0001$], such that the junk food-fed rats consumed fewer pellets (i.e. lever pressed fewer times) as compared with the chow-fed control rats.

When comparing differences in PR performance, as a function of all the 8 diet groups a significant effect of diet was observed [$F(7, 42) = 2.658$, $p<0.05$]. Three-way ANOVA of the pellets consumed on the PR schedule revealed a significant effect of the post-weaning diet [$F(1,42)=8.143$, $p<0.01$], such that the junk food-fed rats consumed fewer pellets (i.e. lever pressed fewer times) as compared with the chow-fed control rats.

Discussion

These experiments characterized the programming effects of different combinations of gestational, lactational and post-weaning environments on the development of metabolic syndrome in female borderline hypertensive rats.

There were no differences in caloric intake as a function of either the gestational or lactational dietary conditions. There is some controversy in the literature regarding programming of hyperphagia; while some studies have reported programmed hyperphagia as a consequence of maternal obesity (Bayol et al., 2007; Samuelsson et al., 2008), others have not found this effect (Shankar et al., 2008). In contrast, there seems to be more agreement among studies investigating the programming effects of maternal under-nutrition (Vickers et al., 2000; Desai et al., 2007) and/or protein deprivation (Bellinger et al., 2004); these all report the occurrence of hyperphagia in the programmed offspring. This may suggest differences in the programming of appetite regulatory mechanisms as a function of the *in utero* environment. Plausibly, if the developing fetus(es) were indeed gauging nutrient availability of the postnatal environment based on *in utero* cues, it stands to reason that a deprived maternal environment would induce programming which would reduce satiety thresholds, one outcome of which might be hyperphagia, while a hypercaloric maternal environment would likely have the opposite effect.

There was an effect of the post-weaning diet such that the junk food-fed groups were hyperphagic relative to the chow-fed rats; they ate more of their calories from the junk food

diets, and compensated by reducing their chow intake. While the reduced chow intake suggests that the rats were partially compensating for the junk food-induced hyperphagia, this compensation was imperfect; presumably the palatability, caloric density and post-ingestive cues provided by the junk food diets were responsible for their increased consumption (Reed et al., 1990; Lucas et al., 1998; Warwick et al., 2002). Complementing the hyperphagia seen in the rats fed the junk food diet, they were correspondingly heavier than their chow-fed controls after PD45. The difference in the body weights of the chow-fed versus junk food-fed rats increased with age. It is important to note that the junk food-fed rats were not only heavier, but also longer (as indicated by the nose-anus measurement obtained at PD224) than the chow-fed controls. This implies that the junk food-fed rats did not suffer any growth deficits as a result of the junk food diet, and in fact showed enhanced growth relative to the chow-fed rats.

Significant differences in adiposity were found, not only as a function of the post-weaning diet but also as a function of the lactational dams' diets. Specifically those rats that had either been suckled by a high fat-fed dam, or weaned onto a high fat diet had greater total body adiposity compared to their low fat counterparts. The effect of the post-weaning diet, which was also seen in the male offspring in chapter 3 is readily apparent as the junk food-fed rats were consuming more calories than those fed standard chow. The increased adiposity in the offspring suckled by the high fat dams was also seen in the male offspring described in chapter 3. This speaks to the importance of the suckling environment on the development of obesity. Work in humans (Plagemann and Harder, 2005) has shown that the breast-fed children of mothers that had gestational diabetes during lactation were at an increased risk of developing type II diabetes in adolescence. Furthermore, studies in rats have shown that maternal diet modulates both the fat content and the quantity of milk produced during suckling (Del Prado et al., 1997; Trottier et al.,

1998; Averette et al., 1999). The significance of the lactational period is further demonstrated by a multitude of studies, which overwhelmingly point to an increase in either adiposity, insulin resistance, hyperphagia, or hyperleptinemia as a result of either hypernutrition by reducing litter size (Oscai and McGarr, 1978; Plagemann et al., 1992; Velkoska et al., 2005) or by fostering pups to a dam fed a high fat diet as was done in the present experiment. How the suckling environment influences systems development is presently still unknown; possible explanations include an increase in preference for high fat food since lipid levels in milk correlate with dietary fat (Del Prado et al., 1997; Trottier et al., 1998; Averette et al., 1999), a programmed increase in intake due either to reduced litter size (Oscai and McGarr, 1978; Plagemann et al., 1992; Velkoska et al., 2005) or increased milk production in high-fat-fed dams(Del Prado et al., 1997), or a permanent change in neural orexigenic pathways due to inappropriate timing of the leptin surge (Bouret et al., 2004). The significant interaction that we observed between the lactational dam's diet and the post-weaning diet points to the idea that the high fat suckling environment may have induced changes in metabolic rate or energy efficiency, resulting in the increased adiposity. In addition to the elevated adiposity, gross heart and kidney weights of the junk food-fed rats were also greater than those of the control rats.

While there is considerable evidence indicating that factors such as maternal obesity (Samuelsson et al., 2008; Khan et al., 2003) and high-fat diets (Velkoska et al., 2005) increase the likelihood of developing hypertension, this was not the case in the present study. In striking contrast, it was those rats that had been gestated in the control diet fed (10% fat) dams that had elevated blood pressures, when compared with those that had been gestated in the high fat fed dams (60% fat). There is no clear explanation for these results. Young (2006, unpublished observations) reported increases in norepinephrine levels in offspring tissues (pancreata and

retroperitoneal fat pads) as a consequence of a high-carbohydrate diet (corn starch and sucrose) administered to the dam. The control diet on which the dams were maintained in the present experiment had approximately 60% of the calories from corn starch. This may suggest a possible programming effect of the high-carbohydrate diet on norepinephrine production in the offspring, thereby increasing susceptibility to hypertension in adulthood. While the following was not objectively evaluated, the control diet dams tended to be much more 'agitated' than high fat-fed dams when picked up for weighing or cage changes. Fetal stress and elevated prenatal glucocorticoid exposure has been linked to the development of hypertension (for review see Seckl, 2004), so while circulating glucocorticoid levels were not measured in the dams, it is possible that this might be another means by which the cardiovascular systems of the control-diet rats' offspring were programmed to increase susceptibility towards developing hypertension.

Consistent with increased adiposity in the junk food-fed rats, they were also hyperleptinemic and hyperinsulinemic compared with their chow-fed controls. The non-fasting serum leptin levels in the present study were considerably higher than those observed in the male rats in chapter 3. This is almost certainly a result of the greater adiposity and age of the female rats in the present study, as both of these factors have been found to be positively associated with increased levels of circulating leptin (for review see Friedman and Halaas, 1998). The non-fasting serum leptin levels differed not only as a function of the post-weaning diet, but also as a function of the gestational and lactational dams' diets. This was again similar to what was seen in the male rats in chapter 3, and points to the possibility that the prenatal and perinatal maternal environments program long-term changes in energy regulation and sensitivity to satiety signals. Given that caloric intake of the junk food-fed rats was higher than that of the standard chow-fed rats, and that there were no differences in intake as a function of the gestational or lactational

conditions, the hyperleptinemic condition of the rats that were either gestated in a high-fat dam, suckled by a high-fat dam or weaned onto the junk food diet may indicate reduced action of circulating leptin in the hypothalamus. Hyperinsulinemia was also noted in the fasted junk food-fed rats, which may suggest reduced insulin sensitivity that is typically associated with metabolic syndrome (Kahn et al., 2006).

Under a fixed-ratio (FR) schedule of responding, a fixed number of responses elicits a reward. Under a progressive ratio (PR) schedule of responding, the cost of the reward increases over time and the rat has to exert increasing effort for subsequent rewards. The break point is functionally the maximum amount of work that the rat is willing to exert for a given type of reward. The PR schedule of reinforcement was first used by Hodos (1961) to assess internal motivation states. The PR method of assessing motivational state is well validated and has been used for assessing incentive value of drug rewards (Arnold and Roberts, 1997) and food rewards (Hodos, 1961; Lowe et al., 2003).

Based on both the fixed-ratio (FR) and progressive ratio (PR) results, it is seen that junk food-fed rats had lower break points (i.e. were less inclined to work for the 45-mg pellets that were obtainable during these sessions) than standard chow- fed rats. It is important to note that the rats were in a non-deprived state during all the operant sessions. These data suggest that the incentive value of the reward is modulated by prior exposure to palatable foods; thus the incentive value of the 45-mg pellets was likely greater for the chow-fed than the junk food-fed rats. This phenomenon has been described as successive negative contrast by Crespi (1944). Successive negative contrast occurs when a rat is first given a stronger reward followed by a weaker reward (strength/weakness defined in terms of incentive value of the reward) and then

consumption of the weaker reward is significantly less than that seen in rats only exposed to the weaker reward.

In conclusion these data suggest that the maternal environment (both prenatal and perinatal) exerts programming effects on energy-related systems involving adipogenesis and sensitivity to insulin and leptin. It is important however to note that the post-weaning diet had significant effects on nearly all parameters measured: caloric intake, adiposity, gross organ weights, circulating serum and insulin levels, and motivation to obtain food. This would serve to emphasize the importance of the dietary choices made in adulthood, and suggest that those choices may well override programming effects of a sub-optimal maternal environment.

Table 4-2. Mean (± SE) calories consumed of the different diets from PD21-191 for the junk-food fed rats.

Mean Caloric Intake from PD21-191 (kcal) of Junk-food Fed Rats	HHH	HLH	LHH	LLH
Chow	39.7±2.7	42.7±2.4	41.4±2.8	42.1±2.7
Cookie Dough	73.4±3.3	70.2±2.6	77.4.8±3.9	76.8±3.6
Peanut Butter + Chow	72.8±5.6	70.8±3.6	67.0±4.2	77.4±5.0
Sausage	123.7±4.2	117.5±4.1	123.4.±5.5	120.4±4.7
Processed Cheese Product	62.5±3.1	57.4±2.3	60.8±3.7	63.4±3.3
Condensed Milk + Chow	62.3±2.2	60.1±2.3	59.9±2.9	61.4±4.2
D12492	106.2±3.8	101.5±3.8	110.4±4.0	109.0±4.7

Table 4-3. Mean (± SE) calories consumed of the different diets from PD21-191 for the standard chow-fed control rats.

Mean Caloric Intake from PD21-191 (kcal) of Std. Chow Fed Rats	HHL	HLL	LHL	LLL
Chow Intake (corresponding to Chow Intake of Junk-food rats)	55.6±2.6	56.2±2.1	57.0±2.7	59.0±2.6
Chow Intake (corresponding to Cookie Dough Intake of Junk-food rats)	52.4±1.9	52.4±1.7	53.6±2.1	53.6±2.6
Chow Intake (corresponding to PB+Chow Intake of Junk-food rats)	53.2±1.2	54.6±1.4	55.1±2.1	57.2±3.6
Chow Intake (corresponding to Sausage Intake of Junk-food rats)	55.3±1.6	56.1±2.3	56.3±2.4	57.5±3.7
Chow Intake (corresponding to Proc. Cheese Intake of Junk-food rats)	56.4±2.1	57.6±2.2	58.0±2.6	58.6±2.7
Chow Intake (corresponding to Cond.Milk+Chow Intake of Junk-food rats)	55.8±2.3	55.5±3.6	57.5±2.0	57.9±2.4
Chow Intake (corresponding to D12492 Intake of Junk-food rats)	55.2±3.5	57.0±2.2	57.6±2.2	55.4±5.8

Table 4-4. Mean (± SE) organ weights harvested on PD 224. There were significant differences in heart and kidney weights as a function of post-weaning diet ($p<0.01$). There were no significant differences as a function of gestational or lactational diets.

ORGAN WEIGHTS (g)	HHH	HHL	HLH	HLL	LHH	LHL	LLH	LLL
HEART	1.21 ± 0.01	1.15 ± 0.03	1.16 ± 0.03	1.10 ± 0.02	1.19 ± 0.04	1.15 ± 0.04	1.26 ± 0.08	1.06 ± 0.05
KIDNEYS	1.21 ± 0.04	1.02 ± 0.02	1.12 ± 0.02	1.06 ± 0.01	1.20 ± 0.05	1.13 ± 0.04	1.26 ± 0.07	1.02 ± 0.07
SPLEEN	0.61 ± 0.04	0.6 ± 0.02	0.50 ± 0.09	0.48 ± 0.11	0.59 ± 0.02	0.59 ± 0.01	0.63 ± 0.03	0.56 ± 0.02
ADRENALS	0.073 ± 0.007	0.064 ± 0.003	0.063 ± 0.003	0.062 ± 0.004	0.079 ± 0.007	0.062 ± 0.004	0.078 ± 0.013	0.068 ± 0.007

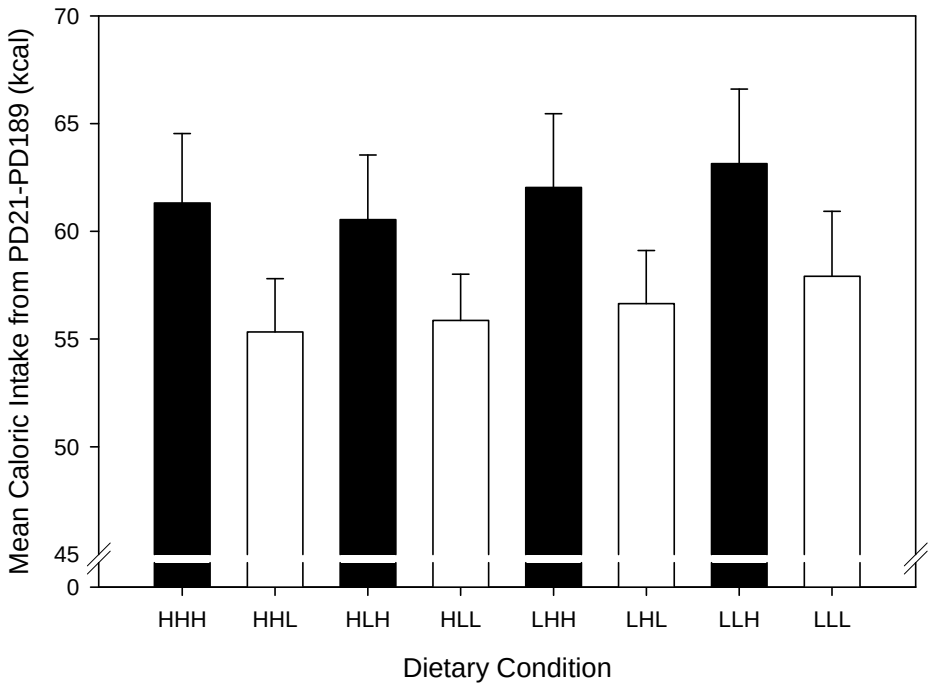

Figure 4-2. Total mean (±SE) caloric intake averaged from PD21-191. The rats on the junk-food post-weaning diets (dark bars) consumed more calories on average as compared to the chow-fed controls, regardless of their gestational or lactational histories ($p<0.01$ when comparing caloric intake based on post-weaning diet).

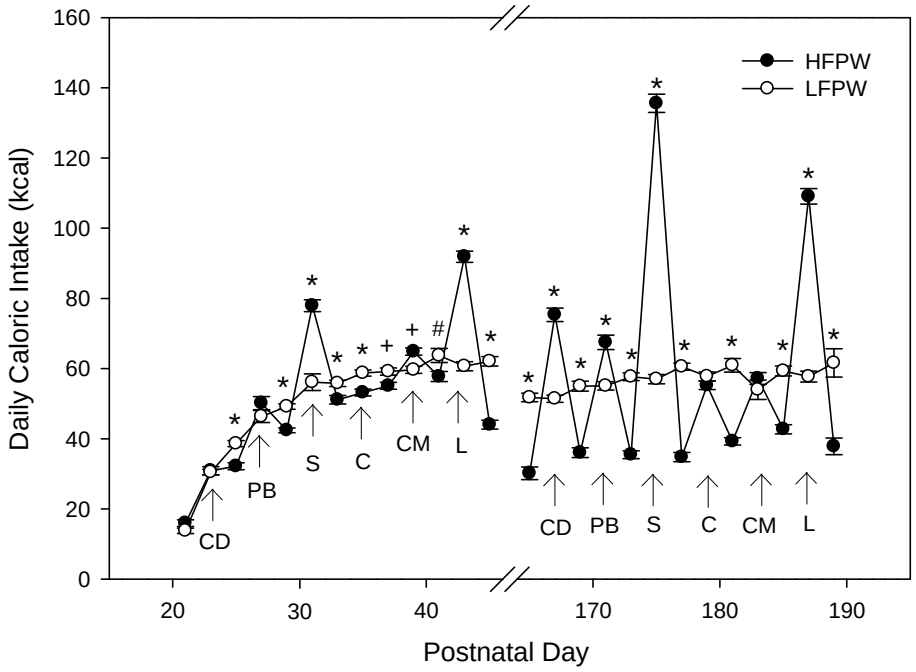

Figure 4-3. Mean (±SE) caloric intake from PD21-45, and then from PD165-189. The rats on the junk-food post-weaning diets (filled circles) consumed a larger proportion of their calories from the junk-food options, and compensated to an extent by reducing chow intake (* $p<0.001$, + $p<0.01$, # $p<0.05$). The intake between PD45-165 were very similar to that seen from PD165-189, thus are not shown for clarity. (CD=Cookie dough, PB=Peanut butter+Chow (1:1), S= Vienna sausage, C= Processed cheese product, CM= Condensed Milk+Chow(1:1), L=D12492).

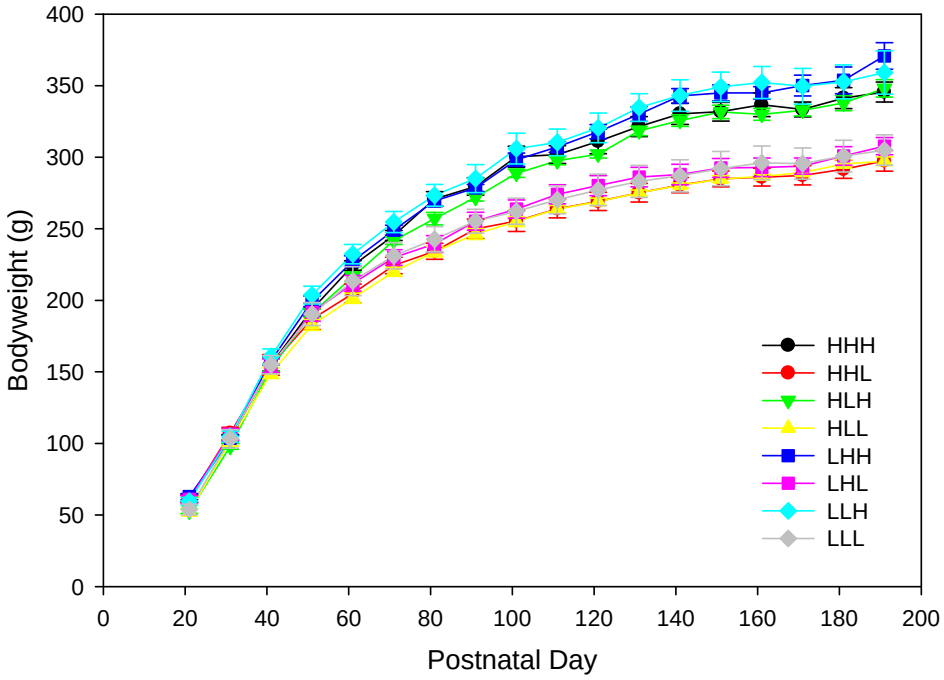

Figure 4-4. Mean (± SE) body weights every 10 days from PD21-191. The body weights began to diverge as a function of post-weaning diet from PD45 ($p<0.01$), and this difference increased with age ($p<0.00001$ at PD191).

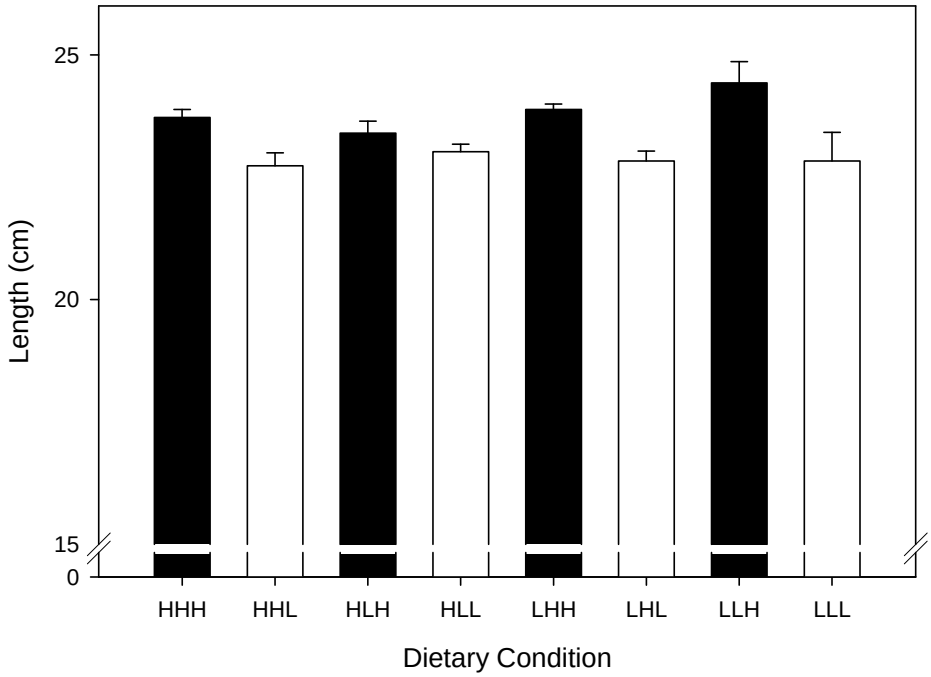

Figure 4-5. Mean (± SE) nose-to-anus lengths (PD224). The lengths of the rats differed as a function of post-weaning diet, such that the junk-food fed rats (dark bars) were longer than the standard-chow fed rats (white bars) ($p<0.001$).

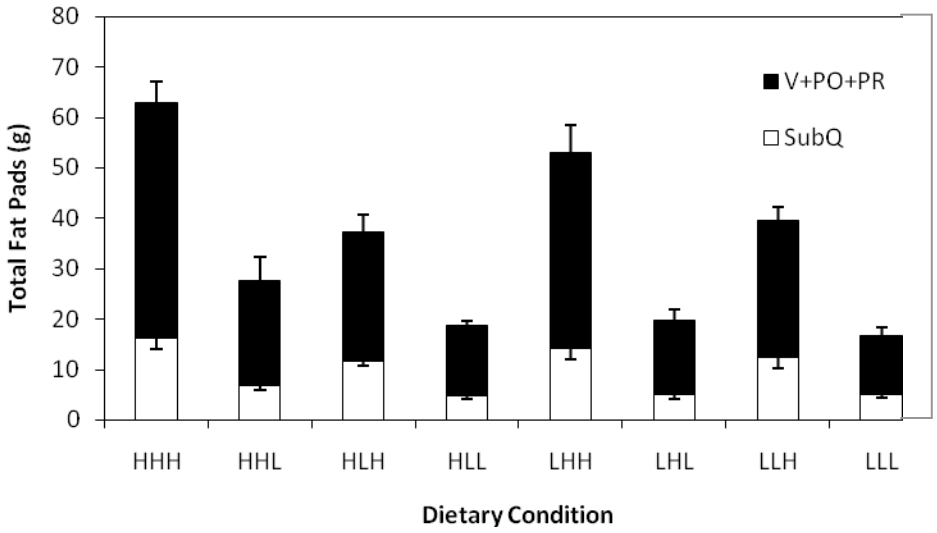

Figure 4-6. Mean (±SE) fat pad mass harvested (PD224). V+PO+PR = Visceral+Periovarian and Perirenal fat pads, SubQ = Subcutaneous fat pads. A main effect of post-weaning diet and lactational diet was observed (Ps<0.0001). An interaction between lactational and post-weaning diet was also seen ($p<0.05$).

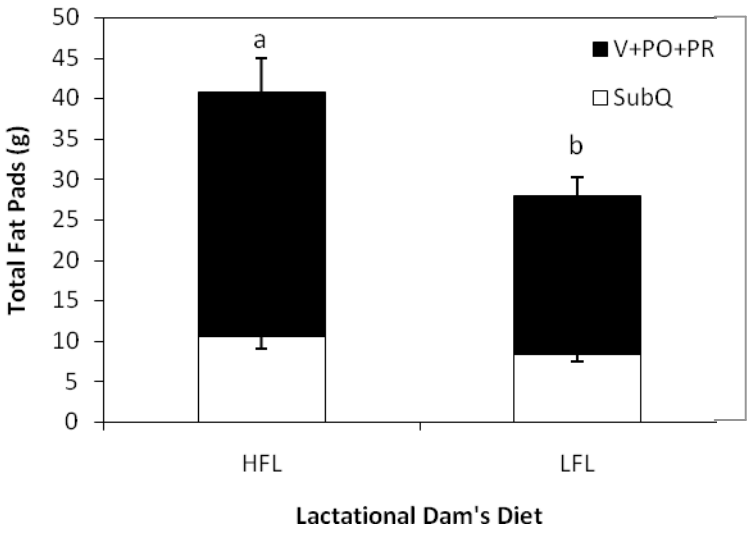

Figure 4-7. Mean (±SE) fat pad mass as a function of the lactational dam's diets (PD224). V+P+R = Visceral+Periovarian and Perirenal fat pads, SubQ = Subcutaneous fat pads. Total fat pad mass (VPR+SQ) was significantly greater in rats suckled by a high-fat dam as compared to those suckled by a control-diet dam ($p<0.0001$). VPR mass was also significantly greater in the rats suckled by a high-fat dam ($p<0.0001$). Bars denoted with different letters are significantly different ($Ps<0.0001$).

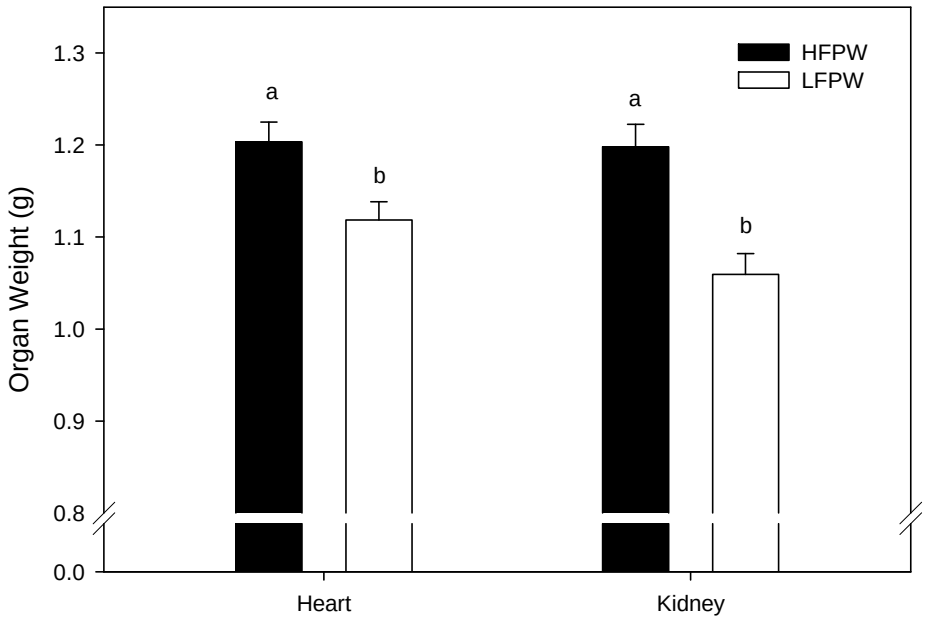

Figure 4-8. Mean (±SE) organ weights on PD224. Rats fed the post-weaning junk food diet had heavier heart and kidney weights as compared to the standard chow-fed control rats ($p<0.01$). Bars denoted with different letters are significantly different ($p<0.01$).

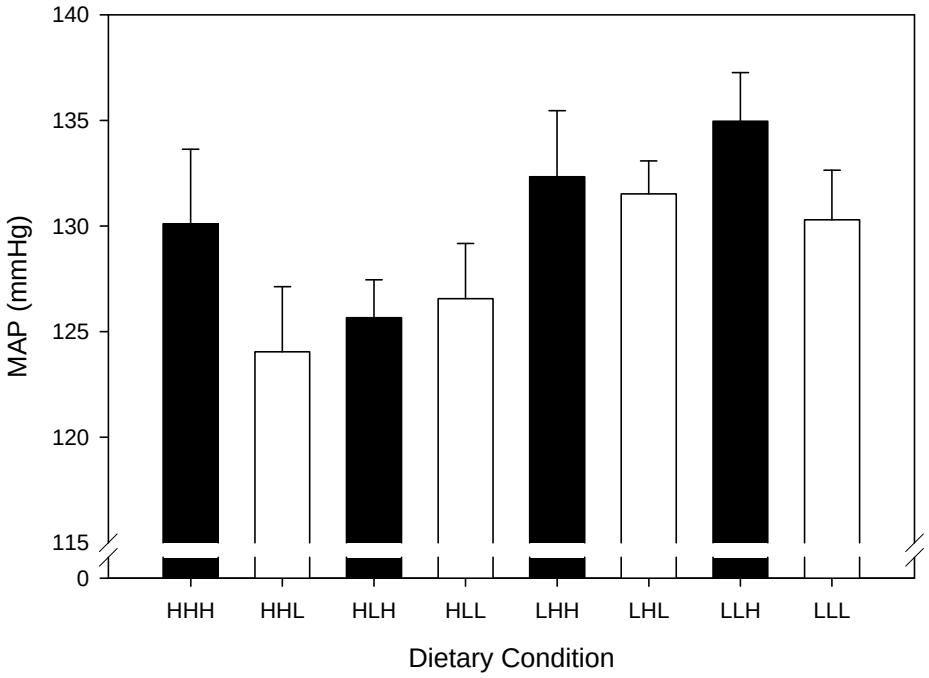

Figure 4-9. Mean (±SE) arterial blood pressure (MAP) on PD 170. There was a main effect of gestational diet on MAP such that those rats gestated in the control-diet dams had higher MAP as compared with those gestated in the high-fat dams ($p<0.01$).

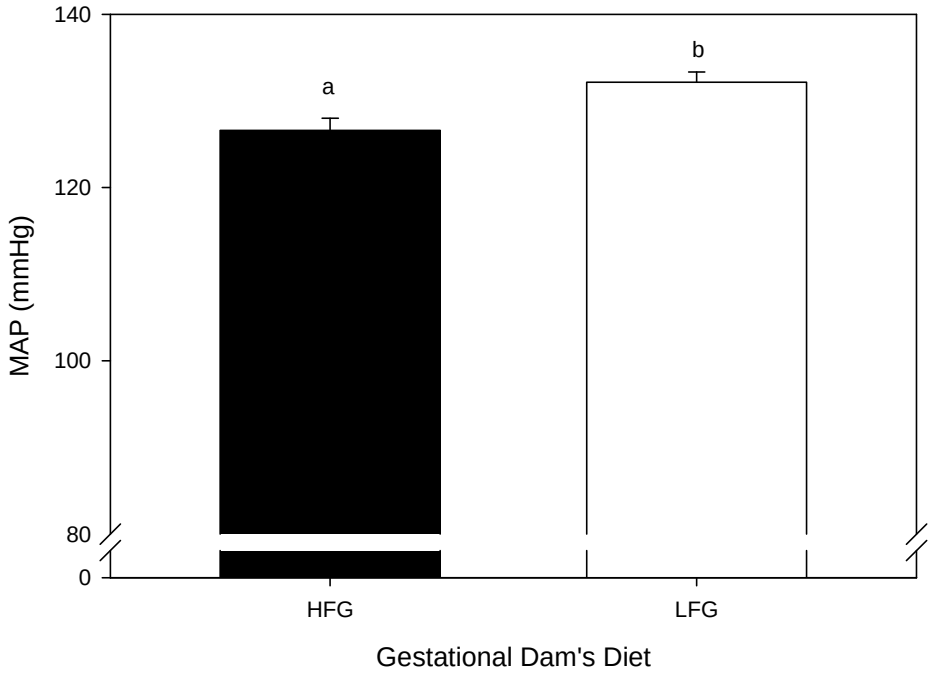

Figure 4-10. Mean (±SE) arterial blood pressure (MAP) on PD 170 as a function of the dams' gestational diets. There was a main effect of gestational diet on MAP such that those rats gestated in the control-diet dams had higher MAP as compared with those gestated in the high-fat dams ($p<0.01$). Bars denoted with different letters are significantly different ($p<0.01$).

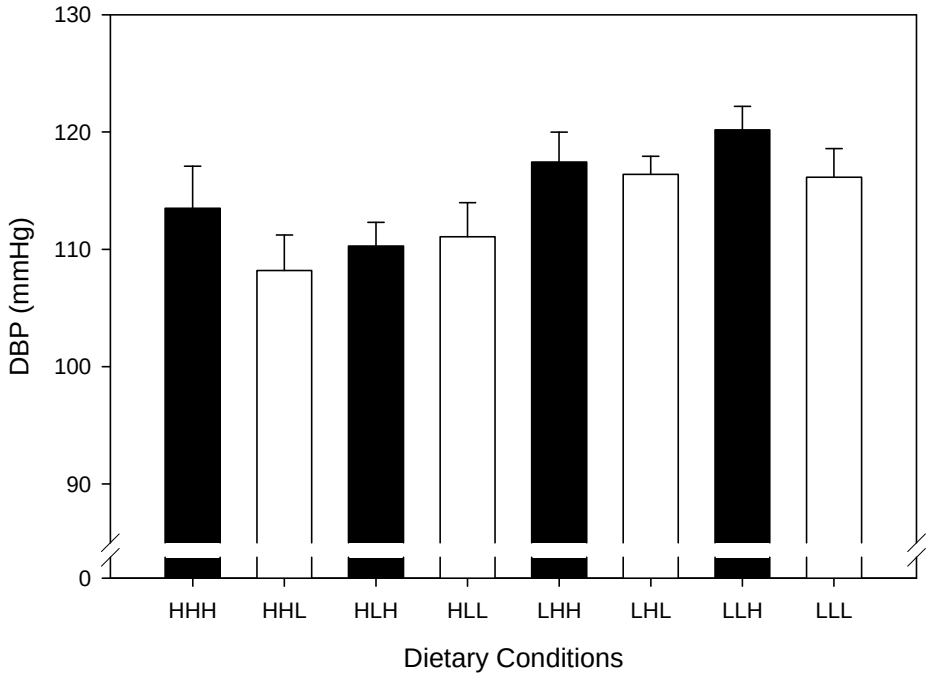

Figure 4-11. Mean (±SE) diastolic blood pressure (DBP) on PD170. There was a main effect of gestational diet on DBP such that those rats gestated in the control-diet dams had higher DBP as compared with those gestated in the high-fat dams ($p<0.01$). Bars denoted with different letters are significantly different ($p<0.01$).

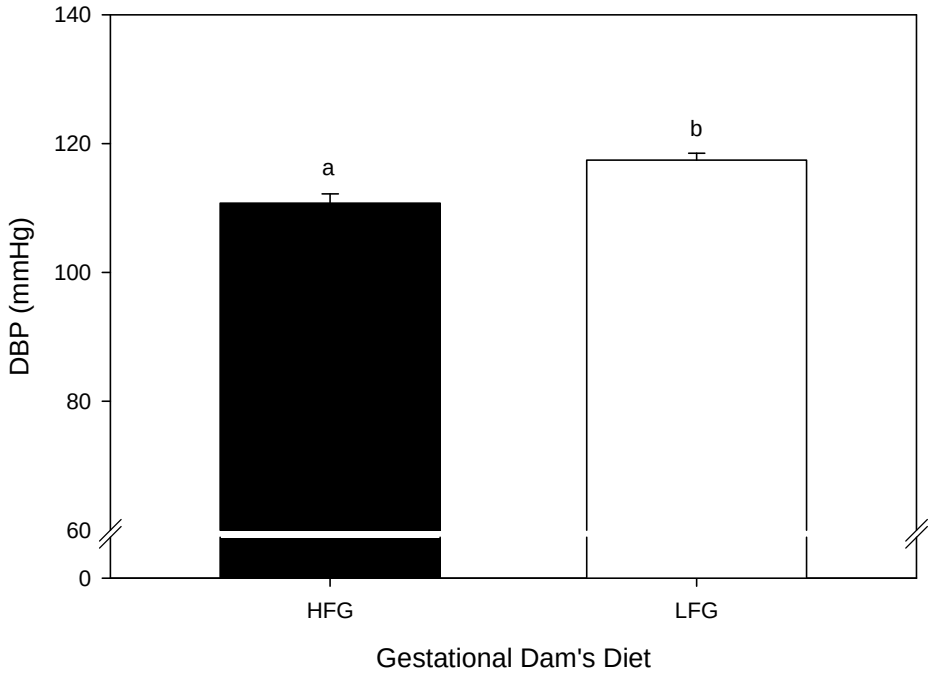

Figure 4-12. Mean (±SE) diastolic blood pressure (DBP) on PD 170 as a function of the dams' gestational diets. There was a main effect of gestational diet on MAP such that those rats gestated in the control-diet dams had higher MAP as compared with those gestated in the high-fat dams ($p<0.01$). Bars denoted with different letters are significantly different ($p<0.01$).

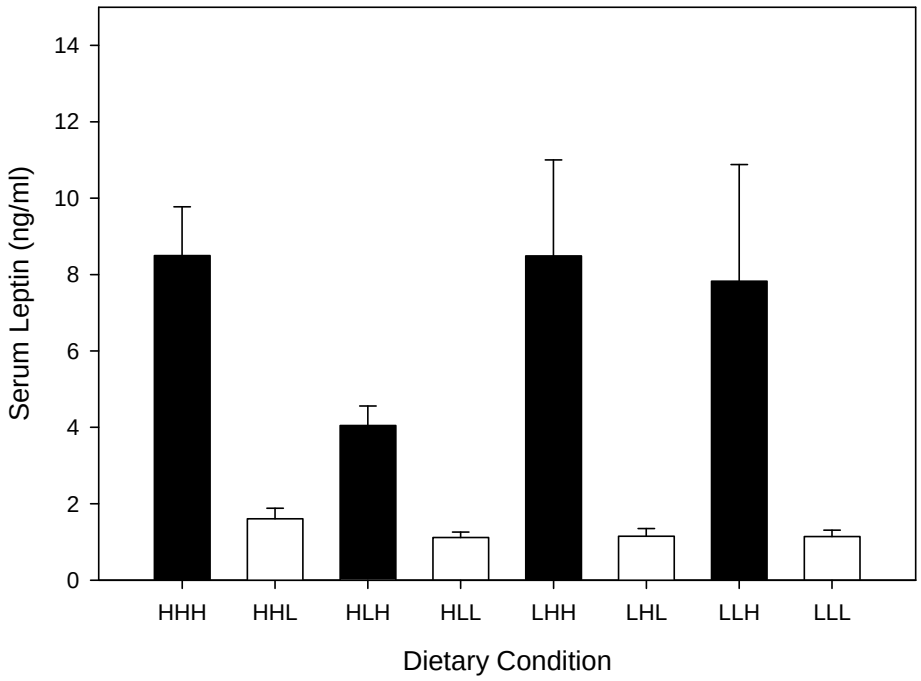

Figure 4-13. Mean (± SE) fasting (PD200) serum leptin. Junk-food fed rats had significantly higher fasting ($p<0.00001$) leptin levels as compared with the chow-fed control rats.

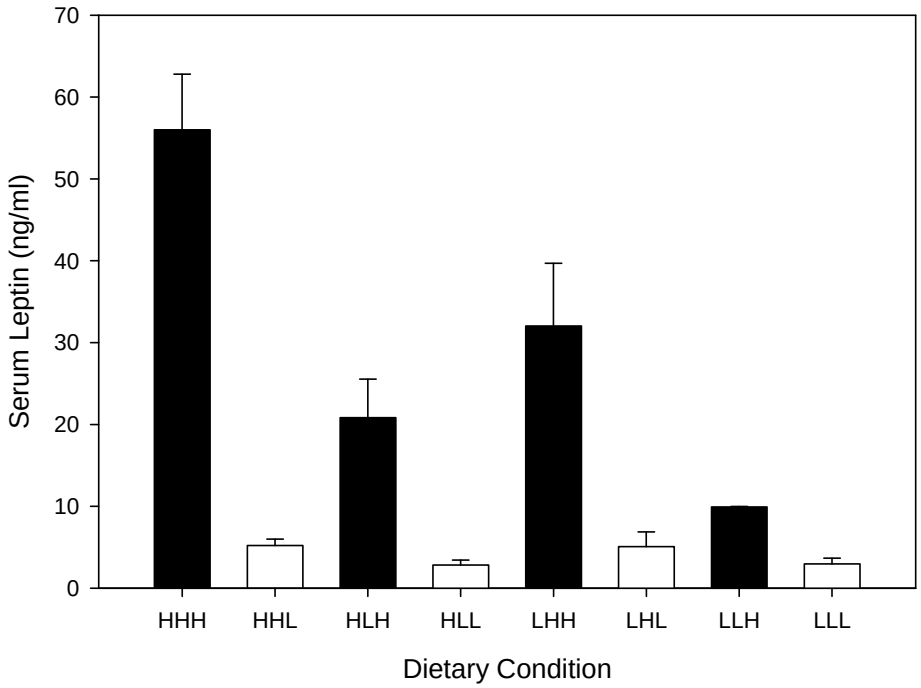

Figure 4-14. Mean (± SE) non-fasting (PD224) serum leptin. Junk-food fed rats had significantly higher non-fasting ($p<0.00001$) leptin levels as compared with the chow-fed control rats. Non-fasted serum leptin levels were also significantly higher in rats gestated in high-fat dams and those suckled by high-fat dams as compared with the control-diet dams ($Ps<0.0001$).

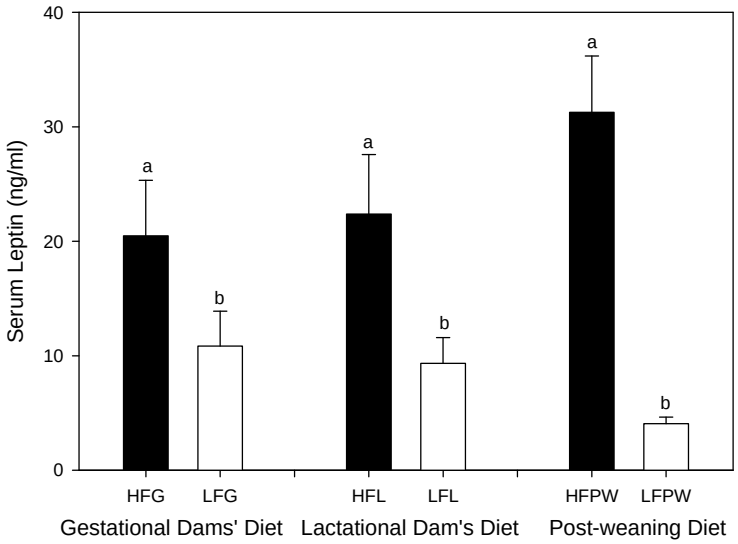

Figure 4-15. Mean (± SE) non-fasting serum leptin (PD224) as a function of the gestational ($p<0.01$), lactational ($p<0.0001$) and post-weaning ($p<0.0001$) diet history. Serum leptin levels were significantly higher in rats either gestated in or suckled by a high-fat dam, as well as in rats weaned onto the junk-food diet (dark bars). Bars denoted with different letters are significantly different ($Ps<0.0001$).

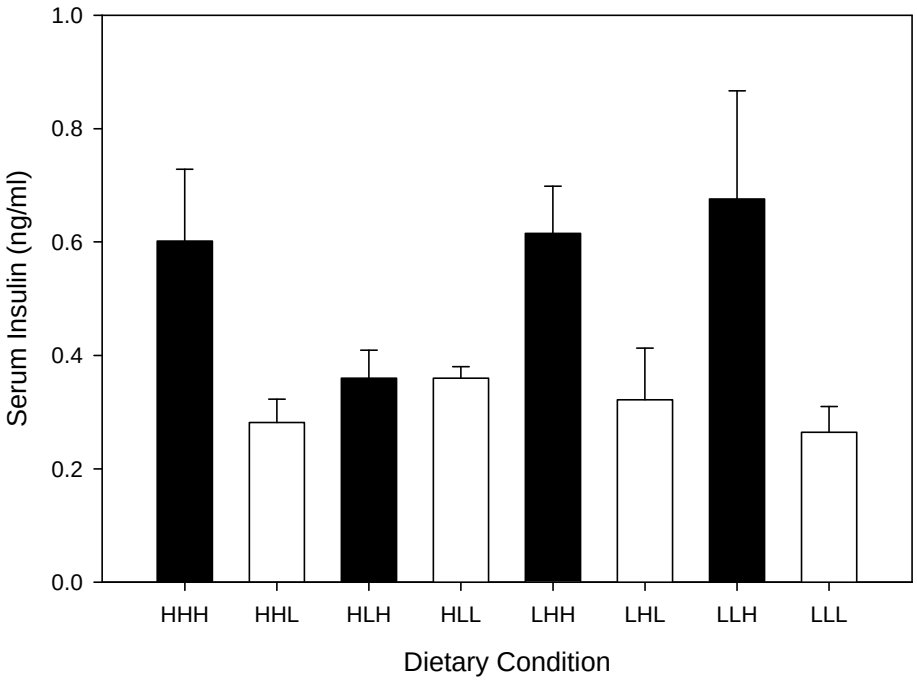

Figure 4-16. Mean (± SE) fasting serum insulin (PD200). A main effect of the post-weaning diet was observed, such that the junk-food fed rats (dark bars) had higher insulin levels than the standard-chow fed rats ($p<0.001$).

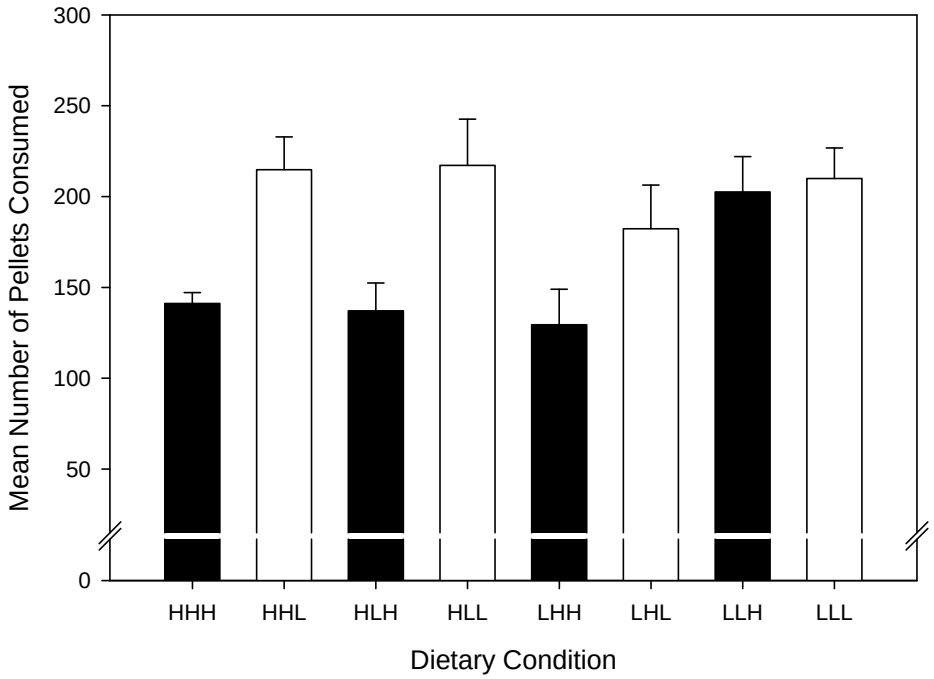

Figure 4-17. Mean (± SE) number of 45 mg pellets consumed across 11 FR1 sessions. Junk-food fed rats consumed fewer pellets (and pressed the lever fewer times) than the rats maintained on standard chow.

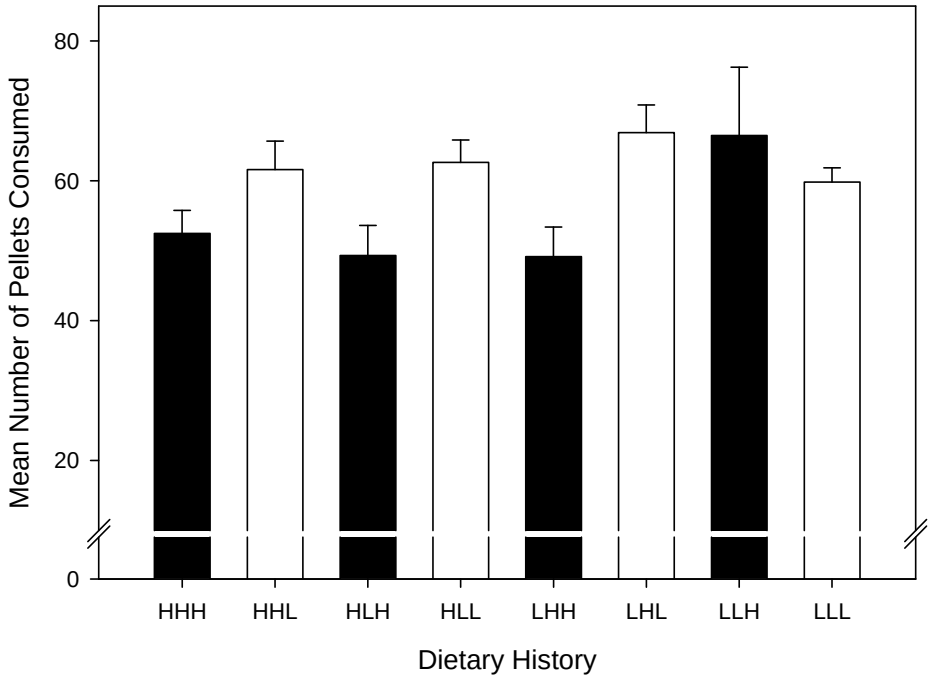

Figure 4-18. Mean (± SE) number of 45 mg pellets consumed across 12 PR sessions. Junk-food fed rats consumed fewer pellets (and pressed the lever fewer times) than the rats maintained on standard chow.

CHAPTER 5
GENERAL DISCUSSION

The experiments presented here aimed to assess whether a high fat maternal diet (and resulting maternal obesity) would impact development (i.e. program) of the offspring so as to induce long-term changes that might alter body-weight, adiposity, food intake, blood pressure and stress-responsivity in the offspring. In summary, the present series of experiments has demonstrated that the quality of the maternal diet during gestation impacts regulation of appetite and blood pressure in the offspring. The suckling environment was seen to be especially critical with the high fat maternal diet increasing predisposition towards greater adiposity and elevated serum insulin and leptin levels in adulthood. Most importantly, while there were effects of the gestational and lactational environments, the post-weaning diet on which the animals were maintained had even greater effects on measures such adiposity, body weight, insulin and leptin levels. The present results thus emphasize the importance of the diet quality in adulthood. Extrapolating these results to the human condition, the implications are quite obvious and allow us to make the hopeful observation that while a suboptimal prenatal or perinatal environment may not be ideal, our own adult lifestyle choices are likelier to have a greater impact on the development of obesity and metabolic syndrome, than are the lifestyle choices made by our mothers.

In humans, an obese maternal environment is typically a hyperenergetic one, and we had hoped to replicate this. However in the present series of experiments we ended up with a high fat exposure model, rather than hyperenergenetic one. The high fat dams compensated for the greater caloric density of their diet. Thus, the prenatal environments provided by the dams in the different dietary groups were isoenergetic; the difference was that the high fat dams obtained a greater portion (60%) of their calories from fat as compared with the control diet dams. While it

is indeed useful to separately study the effects of a high fat isoenergetic environment, it would be instructive to also study the effects of a hyperenergetic environment; with this in mind, were these experiments to be repeated I would like to supplement the high fat diet, with a sweet palatable food option such as condensed milk, which has been demonstrated in other studies to induce hyperphagia (Samuelsson et al., 2007). Furthermore it is of value to consider the fact that while obese women may be obtaining an adequate number of calories, they may still be malnourished (depending on what the source of their calories is). There is an extensive literature (for review see Gardner et al., 1998, Langley-Evans et al., 1998. Remacle et al., 2007) on the development of hypertension and obesity in animal models of protein malnutrition. Thus it is possible that an inadequate protein intake on the part of obese pregnant women may be a contributing factor in the development of metabolic syndrome in their offspring. In the present experiment the high-fat fed dams were not protein malnourished, so this would be another dietary intervention to consider were these experiments repeated.

While differences in adiposity were seen as a result of the lactational environment in the females, no such differences were observed in the male rats studied. This is most likely an effect of age (the females were nearly 8 months old, while the males were about 2.5 months of age). It would be valuable to examine the long term effects of the junk food diet in the males to better understand sex differences in the apparent programming effects of maternal obesity. Further, although males did not show differences in stress responsivity as a function of their gestational or lactational histories, it would be valuable to repeat these experiments in females. If such sex differences are robust, this would then suggest that gonadal steroids may interact during the programming phase, and this could be manipulated in a classic remove or replace type of perinatal hormonal experiment.

Finally given the significant effort and expense that goes into developmental models of maternal obesity such as the one used in the present set of experiments, in the future it would be prudent to set up a much wider set of collaborations with other laboratories so as to optimally utilize the 'programmed' offspring to answer as wide a variety of questions as possible. In particular it would have been useful to search for: changes in pancreatic insulin production, differences in central and peripheral sensitivity to leptin, differences in activity levels, differences in vascular reactivity etc.

In conclusion it is instructive to note that an obese maternal environment is most often accompanied by a host of physiological perturbations; elevated blood pressure, hyperglycemia, hyperinsulinemia, and hypertriglyceridemia to name a few. These physiological perturbations are also accompanied by behavioral perturbations in suckling and general quality of maternal care provided. So when laboratory studies of maternal obesity are conducted or epidemiological investigations of maternal obesity are conducted, one has to keep in mind that all these factors together provide a suboptimal environment. It is likely that the observed programming effects of maternal obesity are the result of the combined action of these physiological and behavioral perturbations. Because multiple factors are normally compounded in clinical populations, the isolation of the critical variables will require continued refinement of animal models such as used in the present work.

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BIOGRAPHICAL SKETCH

Anaya Mitra was born in Allahabad, India on May 12, 1980 to Bhaskar and Nilima Mitra. She completed her high school education in New Delhi, India, graduating from Sardar Patel Vidyalaya in 1998. She received her Bachelor of Arts in biology and psychology from Gustavus Adolphus College in 2003, after which she joined the Behavioral Neuroscience program at the University of Florida. She received her Master of Science in August 2005. In Fall 2006 she applied and was accepted into the College of Public Health and Health Professions to pursue a Master of Public Health (MPH) degree; also at the University of Florida. She has been interning at the Alachua County Public Health department since May 2007 promoting healthy body image, and smoking cessation in the Alachua county community. She graduated with an MPH in May 2008. She will graduate with her PhD in August 2008 after which she will be moving to the University of Minnesota for her post-doctoral work.