MATERNAL PROTEIN RESTRICTION IN MICROSWINE: PROGRAMMING OF POSTNATAL GROWTH, BODY COMPOSITION AND ADIPOSE TISSUE STRUCTURE AND FUNCTION

By

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LIST OF ABBREVIATIONS

AGA	Appropriate for Gestational Age
AgRP	Agouti-Related Peptide
AMPK	5'-cyclic Adenosine Monophosphate (AMP) Activated Protein Kinase
BMI	Body Mass Index
CAD	Coronary Artery Disease
CRP	C-Reactive Protein
DEXA	Dual-energy X-ray Absorptiometry
DIO	Diet-induced Obesity
DOHAD	Developmental Origins of Health and Disease
FL	Feed Limitation
GC	Glucocorticoid(s)
GD	Gestation Day
GH	Growth Hormone
GLUT4	Glucose Transporter 4
GR-α	Glucocorticoid Receptor-α
HPA	Hypothalamic-Pituitary-Adrenal
11βHSD1	11-beta Hydroxysteroid Dehydrogenase type I
11βHSD2	11-beta Hydroxysteroid Dehydrogenase type II
IAT	Intra-abdominal Adipose Tissue
IGF	Insulin-like Growth Factor
LPL	Lipoprotein lipase
LPO	Low Protein Offspring
MCP-1	Monocyte Chemoattractant Protein-1
MNR	Maternal Nutrient Restriction
MPR	Maternal Protein Restriction
NPO	Normal Protein Offspring
NPY	Neuropeptide Y
RAS	Renin-Angiotensin System
PPAR	Peroxisome Proliferator-Activated Receptor
POMC	Pro-opiomelanocortin
SAT	Subcutaneous Adipose Tissue
SGA	Small for Gestational Age
TNF-α	Tumor Necrosis Factor-α
WAT	White Adipose Tissue

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ABSTRACT

In low birth weight babies, accelerated childhood growth amplifies risk of adult cardiovascular and metabolic diseases, but roles of rapid growth *per se* vs obesity typically associated with accelerated growth are unknown. In microswine offspring exposed to maternal protein restriction (MPR) in late gestation plus early lactation, we examined the hypotheses that: 1) MPR would lead to accelerated growth; 2) accelerated growth would be associated with development of obesity; 3) altered adipocyte size and function would be observed concurrently with obesity; 4) these effects would be related to altered hypothalamic-pituitary-adrenal (HPA) axis and/or tissue-level glucocorticoid (GC) function; and 5) these effects are programmed indirectly as consequences of accelerated growth. Compared to Normal Protein Offspring (NPO) controls, Low Protein Offspring (LPO) have reduced weight at birth and at 2 wks but similar weights as juveniles. In LPO, growth rates (weight and length gain) are reduced from 2-5 wks but increased from 6-12 wks vs NPO. Also over 6-12 wks, feed intake in LPO was higher by ~16% and feed utilization efficiency was increased. Percent body fat and lean mass were reduced in LPO at 6 wks. By 11wks, % body fat and lean mass in LPO were not different, reflecting significantly increased accrual rates for both fat and lean tissue. Post-weaning Feed Limitation (FL) applied in a subset of offspring (preliminary data), slowed growth rate in both sexes but prevented the increased rate of fat mass accrual only in females. HPA axis and local cortisol production and activity were not affected by MPR or by post-weaning FL except for a transient decrease in plasma cortisol at 2 wks. In juveniles, fasting plasma glucose was increased in LPO males but decreased in LPO

females compared to sex-matched controls; FL did not affect plasma glucose, suggesting that fasting plasma glucose is programmed directly by MPR. Fasting plasma glucose also appears to be regulated independently of adipose tissue dysfunction. In females, FL after MPR causes altered LPL transcription in a manner suggesting potential for increased lipid deposition in Intra-abdominal (IAT) vs. Subcutaneous (SAT) Adipose Tissue. Adipocyte size in IAT, while not significantly different, tended to be decreased in LPO vs NPO. FL reduced adipocyte size similarly in all groups. Adiponectin mRNA was decreased in both fat depots in LPO vs NPO. FL restored adiponectin mRNA levels to normal in IAT, but not in SAT, suggesting that impaired adiponectin transcription is a secondary effect of accelerated growth in IAT, but is programmed directly by MPR in SAT. TNF-α mRNA was not altered by MPR in either depot and was not changed by FL. Adiponectin mRNA expression was reduced in SAT vs IAT. In summary, perinatal MPR programs accelerated overall body growth and adipose tissue accrual, accompanied by reduced adiponectin expression without obesity and without adipocyte hypertrophy. Accelerated growth is accompanied by hyperphagia and increased feed utilization efficiency; these data do not preclude that hyperphagia may be secondary to growth acceleration mechanisms. Neither HPA axis activation nor local GC production were involved in accelerated growth or adipose tissue function. Some programmed MPR effects are direct, while others are programmed indirectly as a consequence of accelerated growth. Reduced adiponectin transcription may represent one important link between early development and later risk of disease.

Chapter 1: Introduction

Low birth weight in humans is associated with increased risk of developing coronary artery disease (CAD) and/or metabolic syndrome, a cluster of diseases including insulin resistance, type II diabetes mellitus, hypertension, dyslipidemia, and obesity. In animals, these outcomes have been generated by a variety of experimental interventions, including maternal nutrient restriction (MNR), maternal protein restriction (MPR), maternal high fat diet, prenatal glucocorticoid administration, and placental insufficiency. Accelerated postnatal growth in humans born small is associated with exacerbated risk of disease, but whether accelerated growth is developmentally programmed and how it contributes to disease risk remain unanswered.

Using a microswine perinatal MPR model, five hypotheses related to accelerated growth were addressed. First, because accelerated postnatal growth has not been documented previously in a large animal nutrient-restriction model, and in order to discern whether accelerated growth is a programmed feature of MPR or merely coincidental in some individuals, it was hypothesized that MPR in microswine would result in reduced birth weight followed by rapid postnatal growth, and that this would be accompanied by hyperphagia. Second, also as described in rodent models, but again not documented in a large animal nutrient-restriction model, it was hypothesized that accelerated growth would be accomplished by preferential deposition of fat, leading to development of obesity by the point of weight equality.

The third hypothesis addressed was that MPR in microswine would result in altered adipose tissue structure and function typical of those observed in diet-induced and genetic

models of obesity. In human and rodent obesity, increased adipocyte size has been documented, and in both human and animal studies, increased adipocyte size has been associated with altered gene expression, metabolic perturbations, and increased inflammation in adipose tissue. A few rodent MPR studies have examined adipocyte size, but no large animal models with substantial subcutaneous adipose tissue similar to that observed in humans have been examined. Adipocyte size and potential dysfunction (increased circulating leptin, decreased adiponectin transcription, increased inflammation as indexed by TNF-α transcription) were thus examined in both intra-abdominal and subcutaneous adipose tissue.

The fourth hypothesis relates to a potential mechanism whereby accelerated growth with increased fat deposition could occur. Glucocorticoids (GC: cortisol in man and in swine, corticosterone in rodents) have been implicated as causative factors in the "programming" of increased disease risk. Prenatal GC administration mimics the phenotypes observed following MPR. Also, in placentas exposed to MPR, decreased deactivation of maternal GC has been observed, leading to the concept that increased fetal exposure to GC may be the mechanism by which MPR exerts its effects. In fetal sheep exposed to MNR, hypothalamic-pituitary-adrenal (HPA)-axis activation and increases in indices of GC activity in tissues, including in perirenal adipose tissue, are observed. Additionally, increased GC levels at either systemic or adipose tissue level are known to cause increased obesity in adults, especially in the intra-abdominal compartment. It was thus hypothesized that GC would be increased in fetal microswine Low Protein Offspring (LPO), and that there would be programmed long-lasting increases in tissue indices of

GC activity, including 11β hydroxysteroid dehydrogenase type 1 (11βHSD1), the enzyme which reactivates cortisone to cortisol within tissues, and glucocorticoid receptor (GR)-α.

Last, because accelerated growth is often observed following low birth weight, it is difficult to determine whether detrimental effects later in life are programmed indirectly as a consequence of accelerated postnatal growth, or by some other mechanism resulting from MPR. In order to separate these effects, a post-weaning Feed Limitation (FL) protocol was applied to a subset of offspring. Based upon the prediction that the observed effects are due to accelerated postnatal growth, and not to programming by maternal diet, it was hypothesized that prevention of accelerated growth via post-weaning feed limitation would prevent the development of obesity, adipose tissue cellular hypertrophy and dysfunction, and adipose tissue GC activation.

Review of the literature

Human Epidemiology of Developmental Origins of Health and Disease (DOHAD)

The first associations of birth weight and adult disease were described by David JP Barker in the 1980s. Using the careful recordings of a British midwife in the early 1900s, Barker and colleagues were able to show that risk of death from ischemic heart disease in adulthood was inversely related to birth weight in men[1]. Based on those data, Barker proposed a hypothesis suggesting that adverse events or conditions experienced *in utero* could cause long lasting, or "programmed", changes in structure and/or function, resulting in increased risk of later disease. For example, administration of dexamethasone to pregnant rats results in changes in both structure and function: adult offspring have reduced nephron number, salt retention and high blood pressure despite normal kidney size[2].

Since that time, Barker and colleagues extended these observations to cardiovascular disease in women[3,4]; associations have been made between low birth weight and increased risk of many diseases and conditions; and observations have been extended to multiple countries and population groups. Barker and colleagues have now published relationships between low birth weight and incidence of: CAD[5,6]; hypertension[4,7,8 9 10,11,12,13,14]; dyslipidemia[4,15]; glucose tolerance[16,17,18,4]; insulin sensitivity[19,20]; type II diabetes[20]; central adiposity[21,22]; body composition and grip strength[23]; end-stage renal disease[24] and ultimately of metabolic syndrome, defined as the presence of type II diabetes, hypertension, and hyperlipidemia in the same individual[25].

To reconcile how low birth weight could be related to later metabolic syndrome, the "thrifty phenotype" hypothesis was proposed. It states that when nutritional resources are scarce during gestation, the fetus may alter gene expression patterns and/or structural development to encourage gain and conservation of energy as fat, ensuring the individual will have the best chance of surviving famine postnatally. In the context of a postnatal environment which matches the prenatal environment, the outcome is a healthy adult. However, in much of Western society and increasingly in the developing world, nutritional resources are more abundant later in life than they were during gestation. On the background of a thrifty phenotype, this causes an increased propensity for obesity, glucose intolerance, and dyslipidemia, culminating in increased risk of death from ischemic heart disease.

Animal Models of DOHAD

Animal models have been generated to study the mechanisms by which early life events may be linked to later disease. Maternal nutrient restriction (MNR, which refers to an equal reduction in all dietary components and an overall caloric deficit) and maternal protein restriction (MPR, which refers to a diet deficient in protein, but with additional carbohydrate, such that the feed as offered is calorically equivalent to the control diet) are two basic paradigms which have been studied in detail. MNR has been applied to sheep, rodents, and baboons, while MPR has, until now, been studied primarily in rodents with few studies performed in swine.

MATERNAL NUTRIENT RESTRICTION

Sheep

In sheep, MNR has been applied in varying timing schemes and severity. The three time periods studied most intensively are the early-to-midgestation period (~d28-d80; term at ~d145), which is the period of greatest placental growth in the sheep; the late gestation period (varies, but generally d110-term); and the periconceptional period (~60d prior to mating until 7d post-mating). Most studies use a 30-50% reduction in calculated feed requirements.

Studies of early-to-midgestation MNR in sheep report many alterations during fetal development, including placental enlargement at term[26,27] following an initial reduction in placental weight during the time of MNR[28]; altered HPA axis function[29-32]; altered GC metabolism in tissues[33,34]; altered growth hormone (GH) and insulin-like growth factor (IGF) status[35-38]; altered glucose levels and glucose metabolism[27,39]; altered blood pressure and cardiovascular structure and function[30,37,39-42]; altered adipose tissue mass and function[31,34]; altered amino acid metabolism and transport[43]; reduced/altered skeletal muscle development[44,45]; and altered renin-angiotensin system (RAS) development[41]. Severe (50%) reduction in maternal feed intake causes vascular dysfunction in the late gestation fetus, while mild (15%) reduction in maternal feed does not[46]. At birth, early-to-midgestation MNR results in no difference in body weight or length[47], but causes increased adiposity and altered adipose tissue gene expression[48] and altered GC and RAS development in tissues, including perirenal adipose tissue[49]. Some effects of early-to-midgestation MNR have been shown to persist into the late juvenile or early adult

periods, or to cause new effects to become evident. GC metabolic indices were shown to be altered in tissues, including adipose tissue, up to 6 mo after birth (juvenile)[34,50]; increased blood pressure, low nephron number, and altered RAS expression was observed at 245d of age (adult)[51]; a different study showed decreased resting BP with reduced nephron number at 6 mo (juvenile)[52]; altered skeletal muscle development in the neonatal period[45] or in 5 to 9-mo-old offspring[47,53,54]; altered GH-IGF axis in 6-mo-old juvenile[38] and 3-yr-old adult offspring[55]; age-dependent alterations in glucose tolerance and insulin sensitivity[47]; and increased adiposity at 4 mo and 9 mo[47].

Periconceptional MNR in sheep (applied ~60d prior to mating and up to 7 d afterward) results in altered HPA activity later in gestation in twin, but not singleton, fetuses[56]; this effect was only visible in juveniles during corticotrophin releasing hormone and/or vasopressin administration, and was sex-dependent or apparent only in males[57,58]. MNR applied only during the first month of gestation causes different prenatal and postnatal growth rates based on number and sex of offspring[46,59].

MNR applied during late gestation in the sheep shows some similar effects as early-to-midgestation MNR, but some important differences exist. Like early-to-midgestation MNR, late gestation MNR results in altered HPA axis activation[60], altered adipose tissue function[61] and altered adipose tissue GC metabolic indices[50]. Unlike MNR applied in early-to-midgestation or throughout gestation[62], MNR applied only in late gestation results in reduced fat deposition by term[63], and there were no significant effects on the

GH-IGF axis[64] unless the feed limitation was severe (75% reduction in maternal feed intake), in which case fetal GH was high and IGF-I was low[65].

Primates

The baboon is another model used to examine the effects of MNR in early-to-midgestation (0.16 to 0.50 of gestation). In this model, MNR resulted in altered renal development without a change in nephron number[66]. Postnatal effects have not yet been published using this model, and adipose tissue biology has not been reported.

Rodents

Rodent models have also been used to study effects of MNR. In rats, MNR was first applied as a 70% reduction in caloric intake (i.e., very severe: maternal caloric intake was only 30% of controls). In this first series of studies, Vickers, Breier, and colleagues showed increased appetite, blood pressure, fasting plasma insulin and leptin, and fat mass without complete catch-up in body weight in adult male[67] and female[68] offspring. In females at least, these effects are largely remedied by administration of IGF-I to the offspring in adulthood[68] or by leptin administration to offspring in the neonatal period[69]. The same model programs sedentary behavior in both male and female adult offspring[70], leptin resistance in adult female offspring[71], and seems to involve altered development of appetite-regulatory neural circuitry[72]. By comparing this model of obesity to diet-induced obesity (DIO), these authors concluded that the etiology of prenatally programmed obesity is fundamentally different from that of adult-onset DIO[73].

MNR carried out with less severe caloric restriction (50% reduction in maternal feed) caused increased susceptibility to negative effects of high-fat/high-calorie feeding[74]; cardiac and/or vascular dysfunction in some[75-78], but not all studies[79,]; altered leptin levels at birth and 9 mo[80]; altered adrenal development with increased circulating catecholamines[81]; and altered pancreatic development[82].

In the mouse, MNR during the last week of gestation causes low birth weight, increased growth rate from birth to two weeks and again from 3-5 wks, and glucose intolerance and obesity at 6 mo[83]. Post-weaning feed restriction prevented catch-up growth, glucose intolerance and obesity[83]. Mild MNR prior to and throughout gestation in guinea pigs caused altered placental IGF system expression, and is associated with altered placental development[84].

MATERNAL PROTEIN RESTRICTION

Rodents

Another general model used to study the developmental origins of health and disease is maternal protein restriction (MPR). Most studies to date have been performed in rodents, and studies have been performed by applying MPR throughout gestation, throughout both gestation and lactation, or for one week of the rodent three-week gestational period. MPR throughout gestation causes low weight at birth which in at least one study persists to weaning, but catch-up growth can be encouraged by administration of GH or IGF-I in the neonatal period[85]. Lifespan was shown to be reduced in rats[86] and mice[87] exposed to MPR but an older study in rats showed no effect of severe or moderate MPR on

lifespan[88]. MPR in rats has been shown to cause systolic hypertension in offspring in both a sex- and timing-dependent manner[89], and these effects may be dependent upon neonatal suppression of intrarenal RAS and perhaps reduced nephron number[90,91]. An additional explanation for the mechanism of increased blood pressure is increased fetal exposure to GC via reduced inactivation of maternal GC in the placenta[92]. Other effects observed in MPR-exposed rat offspring include impaired recovery following myocardial infarction[93]; altered DNA methylation of specific genes (transmitted to the subsequent generation)[94]; impaired glucose tolerance, insulin sensitivity and pancreatic alterations[95-97]; altered appetite and/or food choice[98,99,100]; and vascular dysfunction[101]. In the mouse, MPR caused low weight at 10d of age, impaired glucose clearance and vascular dysfunction[102].

MPR in the rat during both gestation and lactation leads to asymmetric growth restriction in the offspring[97]; reduced nephron number [91]; increased cardiac fibrosis and capillarization[103]; altered glucose metabolism, blood lipids, leptin levels and appetite[95-97,98,99]; and visceral adiposity with altered gene expression in the adipose tissue[104]. MPR in gestation and lactation in the mouse results in reduced nephron number[105].

Several studies in rats have compared the effects of MPR during specific periods of gestation to those effects observed when MPR is applied throughout gestation, and have also examined the effect of sex of the offspring. MPR during any single week of gestation causes increased feed intake and altered macronutrient choice (i.e., high-fat preference) in females, but not males[99], while MPR throughout gestation causes high-fat

preference in offspring of both sexes[100]. The greatest effect of MPR on offspring hypertension is observed when MPR is applied throughout pregnancy, as compared to effects observed when MPR is applied in early, mid, or late gestation[89]. The same study showed different effects on RAS function based on timing of MPR: late gestation MPR caused increased plasma renin activity in offspring, while early- or mid-gestation MPR caused a reduction in plasma angiotensin II levels at weaning[89]. Effects of MPR on pancreatic development and function were strongest in female offspring when MPR was applied during mid-gestation; but effects were strongest in male offspring when MPR was applied during late gestation; weaker effects were seen during other periods or when MPR was applied throughout gestation[95].

Direct comparison of MNR and MPR in rats showed that MPR offspring do not catch up in body weight, while MNR offspring did catch up at 140d; feed intake was low in early postnatal life but normalized later in both groups; serum leptin was low at 12d but high at 21d in both groups[106].

Swine

Severe MPR in early pregnancy in pigs caused no alteration in fetal growth by GD 44, but by birth, weight was reduced, and liver and muscle weight were reduced; similar effects were observed by MPR in late pregnancy but effects were greatest when MPR was applied throughout pregnancy[107]. The same severe MPR also caused low insulin levels throughout life[108] and elevated GH at least through 12 wks of age[109].

Taken together, it is clear that maternal malnutrition (either protein deficiency or caloric deficiency) leads to cardiovascular and metabolic dysfunction across species, with specific effects differing based on type and timing of exposure, species, sex and age of the offspring, and perhaps other, as yet undefined factors. It is also clear that the use of a variety of models will allow these factors and their effects to be better defined. The Bagby laboratory has developed the microswine MPR model for use in studying cardiovascular and metabolic effects of developmental programming. This dissertation is focused on metabolic effects, including growth, body composition, and adipose tissue structure and function.

Developmental Origins of Obesity

Adipose Tissue Biology

Until recently, adipose tissue was thought of as merely a storage depot for energy. However, since the discovery of leptin in the 1980s, it has become clear that adipose tissue functions as an endocrine tissue. It has also become clear that intra-abdominal and subcutaneous adipose tissue depots function differently, with intra-abdominal (visceral) adipose tissue being more highly associated with adverse cardiovascular outcomes.

Obesity, defined in humans as a body mass index (BMI) greater than 30 kg/m^2, is associated with increased production and secretion of leptin, and decreased production and secretion of adiponectin. Both leptin and adiponectin are cytokines produced almost exclusively by adipocytes, and both have effects on the brain and peripheral tissues. Leptin, the product of the *ob* gene, together with its receptor, *ObRb*, act as the body's

adipostat, sending signals to the hypothalamus regarding status of energy stores. Binding of leptin to the ObRb (leptin receptor type b) causes decreased orexigenic Neuropeptide Y (NPY)/ Agouti-Related Peptide (AgRP) neuron activity and increased anorexigenic Pro-opiomelanocortin (POMC) neuron activity[110], leading to decreased appetite as part of a negative feedback mechanism maintaining energy stores at a constant level. However, during obesity leptin resistance develops by an undefined mechanism, disrupting the normal negative feedback and allowing continued storage of lipid in adipose tissue. Leptin also regulates cellular metabolism via AMP-activated protein kinase (AMPK) in peripheral tissues. Leptin prevents ectopic fat deposition in peripheral tissue such as pancreas[111]; this probably occurs through activation of AMPK. In mouse myoblast culture, leptin directly activates AMPK and stimulates β-oxidation[112]. Leptin administration activated AMPK in skeletal muscle of lean mice, but not in mice with diet-induced obesity[113].

Adiponectin was first described in 1996, and is the product of the adipose most abundant gene transcript-1 (*ApM-1*)[114]. It was independently discovered simultaneously by four different groups, and therefore is also known as AdipoQ[115], ACRP30[116], and gelatin-binding protein-28[117]. Adiponectin is positively associated with insulin sensitivity[118,119,120], negatively associated with inflammation[121, 122], and is paradoxically decreased as body fat stores rise[123]. Adiponectin is thus thought to be the key mediator of obesity-associated insulin resistance and type II diabetes. Adiponectin effects reduced gluconeogenesis in the liver[124] and increased β-oxidation in skeletal muscle[125,126,127] thereby causing increased insulin sensitivity in both major insulin target organs. These

effects appear to be mediated via AMP-activated protein kinase (AMPK)[126,127] and peroxisome-proliferator activated receptor (PPAR)-α[128,129]. Two receptors for adiponectin have been cloned, AdipoR1 and AdipoR2[130], and the presence of these receptors have been confirmed in swine[131]. Based on single and double adiponectin receptor knockout studies in mice, adiponectin binding to AdipoR1 seems to signal via AMPK and leads to reduced gluconeogenesis and increased β-oxidation, while adiponectin binding to AdipoR2 appears to signal through PPAR-α, and increases β-oxidation without affecting gluconeogenesis[129].

In adiponectin-deficient mice, C-reactive protein (CRP) levels are increased in white adipose tissue (WAT)[132] and tumor necrosis factor (TNF)-α levels are increased both in WAT and in plasma[133] compared to wild-type mice. There are many studies reporting negative correlations between adiponectin and tissue or circulating levels of inflammatory markers in humans and rodents, but no studies in humans that have shown a relationship between plasma adiponectin and plasma TNF-α, an inflammatory molecule known to negatively regulate adiponectin transcription in adipose tissue (see Review[121]). Overall, there is a great deal of evidence suggesting that adiponectin acts as an anti-inflammatory agent, but that it is itself negatively regulated by inflammatory molecules. TNF-α is known to be produced by adipose tissue and related to percent body fat[134], but based on the plasma levels in arterial vs venous blood across a SAT bed, it does not seem to be released into the circulation by adipose tissue, and thus adipose-derived TNF-α may act in autocrine or paracrine manners, rather than as an endocrine signal[135].

In human and mouse studies, increased adipocyte size has been observed in diet-induced obesity (DIO) and genetic models of obesity, and this adipocyte hypertrophy is associated with altered gene transcription: larger adipocytes secrete more leptin[136,137] and correlate with lower plasma adiponectin levels[122] (though one study shows increased adiponectin secretion with increasing cell size)[137]. In addition, increased mediators of inflammation such as TNF-α[122,138] and monocyte chemoattractant protein (MCP)-1, together with increased numbers of macrophages in adipose tissue are observed in obese mice[139,140] and humans[139] and are also associated with increased adipocyte size[139].

In DIO and genetic models of obesity, dysfunction can occur at several steps, all contributing to the development of metabolic syndrome. In normally functioning adipocytes, leptin inhibits feed intake, and adiponectin feeds back on adipocytes to reduce fat storage; both of these effects act to maintain adipocytes at a normal size. Leptin and adiponectin both act on peripheral target tissues to prevent ectopic fat deposition via increased β-oxidation and reduced fatty acid import, thus maintaining insulin sensitivity (liver, skeletal muscle) and glucose tolerance (pancreas). Adiponectin further acts on the liver to reduce gluconeogenesis, and acts on the vasculature to prevent atherosclerosis and thrombosis. In metabolic syndrome, leptin resistance occurs in both central and peripheral tissues. This prevents the negative feedback inhibition of feed intake, allowing adipocytes to enlarge unchecked; and this allows ectopic fat deposition via reduced β-oxidation and increased fatty acid import into target tissues, resulting in insulin resistance and impaired glucose tolerance. As adipocytes enlarge, adiponectin production falls; this reduces the negative feedback inhibition of fat storage in

adipocytes, also allowing adipocytes to enlarge unchecked. This also causes ectopic fat deposition in target tissues, and causes increased gluconeogenesis, atherosclerosis, and thrombosis, all leading to metabolic and cardiovascular disease (see **Fig.1-1**).

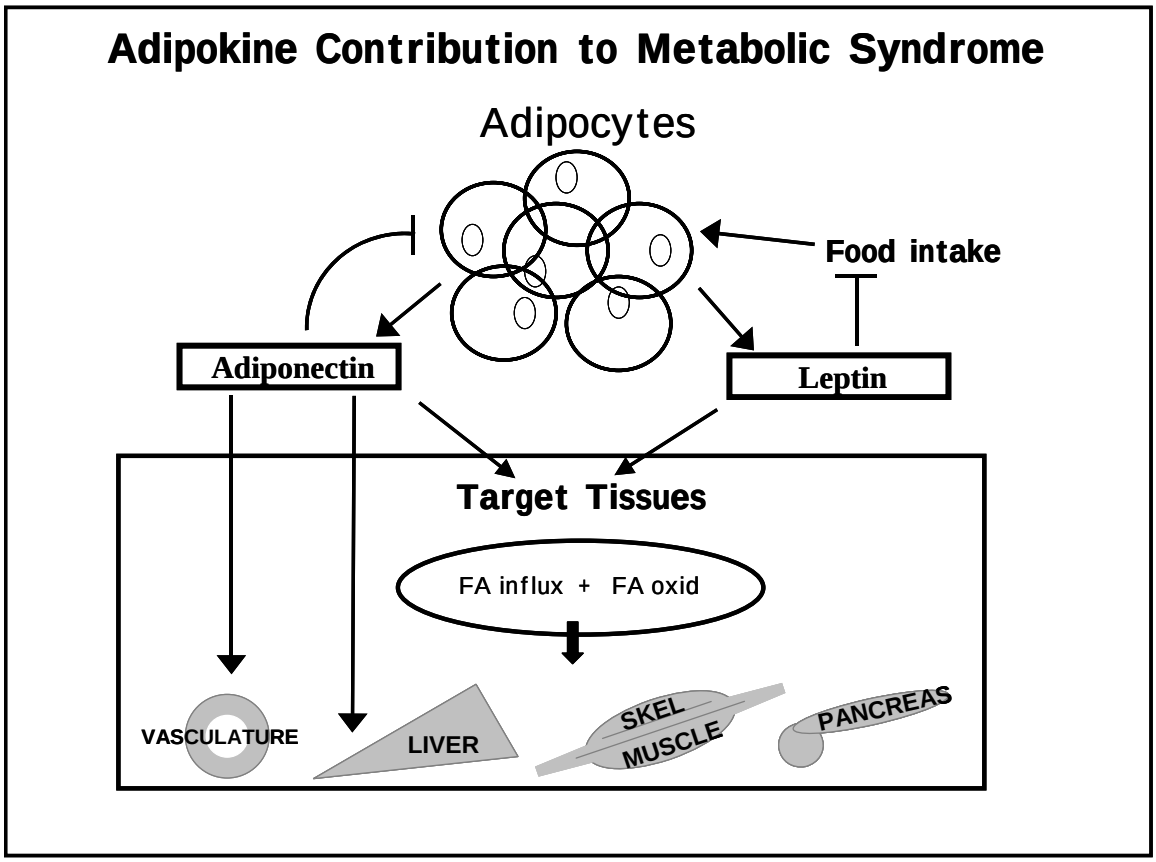

FIGURE 1-1: Actions of Adipokines Leading to Metabolic Syndrome. Leptin and adiponectin, both produced by adipocytes, prevent ectopic fat deposition, and maintain insulin sensitivity, glucose tolerance, and healthy vasculature. In metabolic syndrome, leptin resistance and reduced adiponectin production leads to enlarged adipocytes, ectopic fat deposition, insulin resistance, impaired glucose tolerance, atherosclerosis and thrombosis, all leading to metabolic and cardiovascular disease.

Developmental Programming of Adipocyte Function

While there have been many epidemiological studies connecting birth weight to later obesity, very few studies have addressed developmental origins of adipocyte function. Babies born small-for-gestational age (SGA) have high serum leptin levels during the first 3 years of life and these children lose the normal regulation of leptin by BMI and sex

observed in appropriate-for-gestational age (AGA) children[141]. However, young adults born SGA have low serum leptin levels despite moderately increased percent body fat[142]. In contrast, the reduction in serum adiponectin levels observed in SGA children[143] does persist at least into early adulthood, and the normal relationship between adiponectin and insulin resistance is disrupted such that high adiponectin levels were not protective against insulin resistance[144]. No published studies of humans born SGA have examined adipose tissue inflammation.

Using a rat MPR model, Ozanne and others showed decreased adipocyte size in male MPR offspring epididymal fat depots, in association with increased basal but impaired response to insulin in the same tissue[145]. Bieswal et al showed similar results in perirenal adipose tissue, though adipocyte hypertrophy in MPR offspring fed a hypercaloric diet resulted in cells the same size as controls fed the same diet (both groups hypertrophied), indicating a larger effect on adipocyte size in MPR offspring in response to high calorie feeding[146]. In that study, adiponectin mRNA levels were not affected by MPR, but adiponectin mRNA levels were increased in offspring by MNR (global caloric restriction)[146]. A similar rat MPR model with visceral adiposity but no differences in overall body weight or adipocyte size was used in gene microarray analysis of adult visceral adipose tissue. The authors found a variety of genes were upregulated by MPR, including fatty acid synthase, the insulin-sensitive glucose transporter GLUT4, and leptin. Surprisingly, 48 genes involved in inflammation were down-regulated, and two of three inflammation-related genes which were upregulated are negative regulators of

inflammation[104], supporting the notion that programmed obesity is fundamentally different than adult diet-induced obesity.

No developmental programming studies have yet studied adipocyte biology in an animal model which carries a significant proportion of its fat in the subcutaneous depot, as humans do. This dissertation is the first description of such work.

Glucocorticoids as Mediators of Developmental Origins of Disease

Glucocorticoids (GC) have been implicated in both rodent and sheep studies as potential mediators of effects of MPR or MNR. It was noted first that outcomes in offspring following MPR were similar to those in offspring whose dams were given synthetic GC capable of crossing the placenta[147]. Subsequently, many studies have shown altered GC status, either at a systemic or local level, in offspring following MPR and MNR. For example, alterations in indices of GC activity have been implicated in programming of hypertension in rodents by MPR throughout pregnancy[148,149].

In species which are relatively mature at birth (e.g., sheep), fetal plasma GC concentrations are higher than maternal GC concentrations throughout gestation, then peak just prior to term (see Review[150]). In species relatively less mature at birth (e.g., rodents), fetal plasma GC concentrations are lower than maternal levels, but also rise prior to birth[150]. In both animals and humans, excess prenatal GC exposure causes reduced birth weight and altered placental development, and programs hypertension in adults (see Review[151]).

In human studies, adults born small had elevated 9 a.m. plasma cortisol concentrations[152,153]. Cortisol concentrations were reported to be increased in low birth weight neonatal pigs[154]. This effect seems not to persist, as a separate study showed no differences in plasma cortisol concentrations in juvenile low birth weight pigs[155].

Tissue GC levels are regulated by the balance of two enzymes acting in opposite directions to activate or deactivate GC. 11βhydroxysteroid dehydrogenase (11βHSD) Type 1 converts inactive cortisone (human, pig) or deoxycorticosterone (rodent) to the active cortisol (human, pig) or corticosterone (rodent). 11βHSD Type 2 performs the reverse reaction, inactivating GC in tissues. In normal placenta, 11βHSD2 is thought to result in reduced transfer of maternal GC to the offspring, protecting the fetus from potentially damaging GC[151]. In swine, like humans, placental 11βHSD2 activity increases toward term[156], whereas in rats and mice, placental 11βHSD2 decreases toward term[157]. This species difference has not been well studied, but may be due to the relative immaturity of the rodent offspring at birth. Placental 11βHSD2 in rats is reduced by MPR applied throughout gestation, and this is associated with hypertension in adult offspring[147].

In rats, MPR throughout pregnancy results in tissue-specific increased expression of GR and decreased expression of 11βHSD2 in adulthood[158]. In sheep, MNR during early-to-mid gestation results in altered tissue-specific expression of GR and 11βHSD1 in neonates[49], effects which persist to 6 mo[50]. Studies have not yet been performed in

human low birth weight populations. No studies in late gestation MPR or MNR models have been reported; the present work is the first to examine local expression of GR and 11βHSD1 in offspring following late gestation MPR.

Studies have been done in human populations in order to examine the role of GC in acquired obesity. GC are well-known to cause obesity, specifically in the visceral/central compartment as is observed in Cushing's disease. Sex-specific activity of 11βHSD1 was observed in human subcutaneous adipose tissue, with women having higher activity than men; 11βHSD1 activity was shown to correlate closely with its mRNA expression[159]. In rats, MPR throughout gestation causes increased expression of 11βHSD1 in male fetal liver, but no change in expression in female liver[160].

GC effects are mediated by the glucocorticoid receptor, GR, a nuclear receptor which binds cortisol (corticosterone in rodents), translocates to the nucleus, and initiates transcription of numerous tissue-specific genes. Two splice variants of GR, each with multiple translational isoforms have been reported (see Review[161]) but it seems that the GR-α isoform is the transcriptionally active form. GR-α levels are increased in visceral fat compared to the subcutaneous depot in both humans[162] and rodents[163]. Lipoprotein lipase (LPL), a well-described target of GR-α in adipose tissue, is a vital step in storing fat. MPR-programmed or sex-based differences in LPL or its upstream regulators might contribute to increased adiposity but have not yet been studied.

Maternal Protein Restriction in Microswine: A Novel Model for Study of DOHAD

A new swine model for the study of the developmental origins of health and disease has been generated in the laboratory of Susan P. Bagby, MD, my advisor. Swine are especially suited for developmental research due to their remarkable similarity to humans with respect to cardiovascular, renal, digestive and immunological anatomy and physiology. In addition, their extended gestation make the pig an attractive model for gestational manipulations, including dietary restriction. For studies of appetite in offspring, pelleted swine diets are more easily manipulated compared to sheep diet, which is composed of both pellets and hay, and swine do not have the potentially confounding factors of ruminant digestion. Moreover, like humans, but unlike sheep and primates, swine are omnivorous. Of particular importance to this dissertation, swine are very similar to humans in their distribution of body fat; neither rodents nor non-human primates have large amounts of body fat distributed in the subcutaneous depot, as swine and humans do.

Results in Microswine Model Independent of Dissertation Studies

Microswine sows subjected to Low Protein (LP) diet during the last quarter of gestation plus the first two weeks following parturition bear offspring with low birth weights and an asymmetric form of growth restriction which, by the end of MPR (2 wks), reduces body weight more than length and reduces heart and adrenal weight less than body weight (see **Appendix Table A-3**).

Juvenile (3-5 mo old, prepubertal) microswine Low Protein Offspring (LPO) exhibit normal arterial blood pressure, as assessed by continuous telemetric monitoring in unrestrained pigs over 48 hrs. However, in response to the stress of sling restraint, pressure increases more in LPO as compared to NPO. In parallel, LPO show increased *ex vivo* mesenteric vascular reactivity to KCl (increased sensitivity and maximum response) and to norepinephrine (increased sensitivity without altered maximum response). Moreover, mesenteric vascular reactivity to angiotensin (Ang) II is increased in LPO, a response due almost entirely to enhanced signaling via the NADPH-oxidase dependent/EGF receptor transactivation pathway. No alterations in circulating Renin-Angiotensin System (RAS) components have been observed at any age. However, the intrarenal RAS shows age-dependent alterations. Increased intrarenal angiotensin-generating components during MPR (in fetal and neonatal LPO) successfully maintain normal intrarenal AngII levels in LPO, while increased AT1 ± AT2 receptor densities appear additionally necessary to sustain normal renal vascular AngII responses in near-terms but convey increased AngII vascular responses in neonates. In contrast, in juvenile LPO renal tissue, AngII generating components, AngII tissue levels, and angiotensin effectors (i.e., angiotensin AT1 receptor) are unchanged. Thus, the normal AngII levels in both circulating and renal tissue sites encounter vascular AT1 receptors with enhanced vasoconstrictive signaling. Studies are underway to determine whether the enhanced AT1R-NADPH Oxidase-EGFR transactivation pathway reflects programmed abnormalities in one or more of the signaling components, as well as to assess whether food limitation/prevention of accelerated growth modifies the vascular reactivity to AngII.

Chapter 2: Methods and Materials

Animal Care and Use

Time-mated microswine sows were obtained from Sinclair Research Laboratories (formerly Charles River Laboratories) approximately two-thirds into gestation. Sows were housed at the OHSU Department of Comparative Medicine under approved Institutional Animal Care and Use Committee protocol A439. Sows were maintained on a 12hr:12hr light:dark cycle in a temperature-controlled room. At least two sows were studied at a time, and cages were placed in the same room to allow pigs to have social contact with each other. Eight juvenile litters were studied [2 Normal Protein (NP), 6 Low Protein (LP)]. An additional four litters (2 NP, 2LP) were studied at 2 wks after parturition ("neonatal"); six additional litters (3 NP, 3 LP) were studied at 113d gestation ("near-term fetal") with all sows randomized to NP or LP diet as for juvenile litters. On gestation day 84 (approximately three-quarters into gestation; term is 115 days), sows were randomized to NP (containing 14% protein) or LP (containing 1% protein) diet (see **Table 2-1** at end of this chapter for dietary composition). Previous studies in the laboratory tested 0.5% low protein diet, which resulted in increased loss of offspring; and 3% protein, which did not produce any significant reduction in body weight of offspring at birth. 1% protein diet produces a significant reduction in body weight of offspring (approximately 14% lower weight) without affecting the number of piglets born live per litter, or the postnatal survival of piglets. Sows were maintained on the assigned diet throughout the remainder of gestation, and for two weeks after delivery. The duration of protein restriction was designed to approximate the last trimester of human gestation in terms of renal and adipose tissue development. In pigs, nephrogenesis continues for two weeks postnatally, while in humans, nephrogenesis is complete before birth. Also,

piglets are born without any significant fat depots, while human babies are born with a relatively large amount of fat. Because of the lack of body fat, piglets are unable to maintain body temperature. Therefore, two days prior to expected due date, ceramic heaters were placed outside the sows' pens to provide an external heat source; these heaters remained available until weaning.

Beginning at 3 weeks, piglet chow (Lab Mini-pig Chow: Starter (5080), PMI Nutrition International, LLC Brentwood, MO; see **Table 2-2** at end of this chapter) was made available to piglets, and piglets were acclimatized to the post-weaning feed measurement procedure by placing them in individual cages for one hour twice per day with piglet chow. At 4 wks, piglets were permanently placed in individual cages to prevent interference with feed intake based on social hierarchy. In the last four litters studied, piglets were randomized at 4 wks to either *ad libitum* (AL) or feed limited (FL) post-weaning diet.

Weight and Length Measurements

Piglets had weights and lengths measured daily for the first 4 weeks, then 3-5 d/wk thereafter until harvest. Weights were measured on a baby scale to the nearest 10g until they became too large for the baby scale (~11wks of age, or 10-13 kg). After this point, piglets were weighed on a livestock scale. Lengths were measured from crown (between ears) to rump (base of tail) along the curve of the spine using a flexible tape measure to the nearest half-cm with the head raised to approximately horizontal to the ground.

Feed Intake Measurements

Feed intake was measured from wks 6-8 (one NPO litter and one LPO litter) or from wk 4 to at least wk 13 or until harvest (remaining litters). Feed dishes were placed inside a half kennel secured to the walls of the pen using clips or zip ties. Pre-measured feed was placed in the feed dish, and piglets were allowed to eat for 45-60min. Remaining feed was collected and measured. Feed consumed was calculated by subtracting remainder weight from starting weight. If significant spillage occurred, data were not measured. For AL offspring, piglets were offered more feed than they could eat. For FL offspring, enough feed was offered to maintain piglets on a particular growth trajectory. Each piglet's growth trajectory was projected by calculating the percent reduction in weight at 2 wks (nadir weight) compared to the average sex-matched control weight at 2 wks (for an example, see **Fig. 5-1**). Each individual was fed enough to keep the percent reduction in weight steady throughout the duration of the study. To begin, piglets were offered 25g/kg body weight, an amount determined by initial studies to be the average amount consumed by NPO. Growth was analyzed weekly, and feed offerings were adjusted as needed. In addition, feed offerings were calculated every day weights were measured, and weekend feedings were projected using the previous two weeks' weights.

Body Composition Measurements

At 6 (NPO:39.1 ± 0.3 d; LPO: 39 ± 0.5 d) and 11 wks (NPO: 80 ± 1.9 d; LPO: 82.8 ± 0.9d), piglets were subjected to isoflurane anesthesia, then transported to the dual energy X-ray absorptiometry (DEXA) scanner (Hologic QDR-4500W) and placed in a prone position on the table with a sheet of plastic under the animal. As the scan progressed, the

table moved back and forth. During this movement, a veterinary technician adjusted the anesthesia tube as needed to prevent snags and to keep the tube within the head region of the scan. The scanner was equipped with pediatric software (Experimental Pediatric Whole Body v8.26 & v12.3) used to analyze scans. Scans were analyzed by region: trunk, right leg, and left leg. The head region, also containing the forelimbs due to anatomical constraints, was excluded from analysis because of the high lipid content of the brain. The software reported fat, lean, and bone mineral content for each region, and summed the regions to yield whole-body totals. For bone mineral content and density data, see the **Appendix Table A-2**.

Plasma and Tissue Collection

For collection of fetal plasma and tissue, sows were placed under isoflurane anesthesia on gestation day 113 (term is 115d). Near-term fetal piglets were delivered by Caesarean section one at a time, and blood and tissues were quickly dissected. Blood was collected by cardiac puncture in EDTA; skeletal muscle from the hind limb was snap-frozen in liquid nitrogen and stored at -80ºC until use. Fetal piglets lack sufficient body fat for collection. Blood was centrifuged immediately at 4ºC and plasma was stored at -80ºC.

For neonatal plasma and tissue, 2 wk old [NPO: 13.3 ± 2.4 (mean ± SEM) days; LPO: 12.5 ± 2.0 days] piglets were anesthetized with isoflurane to maintain perfusion of tissues during harvest. Blood was collected by cardiac puncture in sodium heparin and centrifuged; plasma was stored at -80ºC.

At 14-24 wks, blood was collected from juvenile offspring in sodium heparin from awake pigs via an indwelling inferior vena caval catheter placed under isoflurane anesthesia the day prior. Pigs were first placed in a sling and allowed to acclimate before collection of blood. Blood was centrifuged immediately and stored at -80ºC. Five to eight days later, pigs were anesthetized with isoflurane for tissue harvest. Gastrocnemius muscle, intra-abdominal adipose tissue (IAT), and subcutaneous adipose tissue (SAT) samples were collected, snap-frozen in liquid nitrogen, and stored at -80ºC.

24-hr Urine Collections

At 11-12 wks, juvenile piglets were placed in metabolic cages for 48 hrs. Two consecutive 24-hr urine collections were stored separately at -20°C until use. Total "urine" volume was recorded (urine + spilled drinking water; all calculations were based on total volume of fluid collected). The second day's collection was used for cortisol assays.

RNA Extraction and Real-Time PCR

Snap-frozen tissue samples were weighed, minced, and homogenized in TRIzol (Invitrogen Corp., Carlsbad, CA) using a Tissuemiser rotor-stator homogenizer (Fisher Scientific, Waltham, MA). After an initial spin to clear lipid and cell debris, total RNA was extracted according to manufacturer instructions. RNA concentration was quantified by optical density (Absorbance at 260nm). 1μg RNA was treated with DNase I (Invitrogen), then reverse transcribed using random hexamer primers (Operon Biotechnologies, Huntsville, AL) and SuperScript II reverse transcriptase (Invitrogen).

Gene-specific primers to swine lipoprotein lipase [LPL, an adipose tissue target of glucocorticoid receptor (GR)-α, used here as an index of GR-α activity], adiponectin, tumor necrosis factor (TNF) -α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s rRNA were designed using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by Operon Biotechnologies; primers for GR-α and 11β-hydroxysteroid dehydrogenase (HSD)-1 were adapted for swine from human sequences reported by Wake, et al[159]; see **Table 4-1** for all primer sequences, amplicon lengths and gene accession numbers. Resulting cDNA was used in real-time PCR using the SYBR Green method. Triplicate reactions of 5.0 μL sample (diluted 1:20) or standard (pooled mix of samples, diluted 1:5, 1:20, and 1:80), 0.75 μL of each primer (forward and reverse, 5 μM each), 12.5 μL 2X SYBR Green PCR Master Mix (Applied Biosystems), and 6.0μL nuclease-free water were performed in 96-well plates. Each plate contained the transcript-of-interest and 18s rRNA (or GAPDH for juvenile gastrocnemius) as an endogenous control for each sample; each transcript had its own standard curve generated from pooled sample cDNA. Amplification reactions were run on an ABI Prism 7500 (Applied Biosystems). Cycling conditions were as follows: 50ºC for 2 min, 95ºC for 10 min, then 40 cycles of 90ºC for 15 sec and 60ºC for 60 sec. Fluorescence was detected during the annealing phase of each cycle. Baseline values were auto-subtracted and thresholds were assigned automatically by the instrument software. Relative gene expression was calculated by the standard curve method, and normalized to endogenous controls. Neither of the endogenous controls' (18s rRNA or GAPDH) abundance was affected by maternal diet. Values represent a fold-change compared to the pooled sample control. Ratios were Ln transformed prior to statistical analysis.

Adipocyte Size Measurements

Formalin-fixed paraffin-embedded IAT slides were cut to 5 μm thickness. For each animal, two slides were cut from each of two blocks for each adipose tissue depot. Slides were stained with hematoxylin to visualize plasma membranes. One image from the center of each quadrant was captured for every section analyzed. Images were taken using a CCD camera attached to a Leica microscope using a 20X objective. Images were analyzed using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Prior to image analysis, images were prepared using a macro which flattened background, attempted to close any breaks in cell membranes, and adjusted contrast to reduce bias in analysis; further adjustments to each image were made if needed. Cross-sectional area was obtained for every cell in the image, excluding those cells touching the border of the image as well as any objects smaller than 150 μm^2. Data from 4 quadrants were averaged to generate mean cell size for each slide; coefficient of variation (CV) between slides within subjects was 10.8%.

Cortisol and Leptin Radioimmunoassay

To assay cortisol concentrations in plasma and urine, the Coat-A-Count Cortisol RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) was used. Cortisol was extracted from urine samples with dichloromethane prior to assay. Urine extract or plasma was pipetted into antibody-coated tubes. ^{125}I-Cortisol was added to the tubes, incubated 45 min at 37°C and decanted thoroughly. Tubes were counted in a gamma counter, and results were analyzed according to kit instructions. For urine samples, concentrations were multiplied by 24-hr volume to yield an excretion rate in nmol per

day. 24-hr urine values were reported both in absolute excretion rates and as normalized to lean body mass measured by DEXA scan at 11 wks (~one week prior to urine collection). Within-assay coefficient of variation (CV) was 2.45% and between-assay CV was 4.24%.

Plasma leptin concentrations were assayed using the Multi-Species Leptin RIA kit (Millipore, Inc, Billerica, MA, formerly Linco Research, St. Charles, MO) according to kit instructions. Because standards were human leptin and because the antibody was raised against human leptin (cross-reactivity with porcine leptin is ~67% as reported in kit instructions), results are reported as ng/mL Human Equivalent (HE). Intra-assay CV was 7.3%.

TABLE 2-1. Maternal Diet Composition

Ingredient	Low Protein Diet (1.0% Protein)	Normal Protein Diet (14% Protein)
	g/kg diet	g/kg diet
Soybean Meal (47.5% protein)	18.3	301.1
Alfalfa Meal (15% protein)	10.0	10.0
Corn Starch	679.2	466.4
Glu monohydrate	230.0	160.0
Soybean Oil	30.0	30.0
Limestone (grd)	10.0	10.0
Dicalcium PO_4	15.0	15.0
Salt	5.0	5.0
Vitamin/Min supplement	2.5	2.5

Normal- (14%) or low- (1%) protein diets were fed to microswine sows during the last 1/4 of gestation plus the first two weeks of lactation. Diets were isocaloric, with the reduction in calories in the low-protein diet made up by adding carbohydrate (both starch and glucose).

TABLE 2-2: Dietary Composition of Piglet Growth Chow

Ingredient	Percentage of feed (wt/wt)
Crude Protein	20.0%
Lysine (min)	.065%
Crude fat (min)	4.00%
Calcium (min)	0.65%
Calcium (max)	1.15%
Phosphorus (min)	0.5%
Salt (NaCl) (min)	0.5%
Salt (NaCl) (max)	1.00%
Zinc (min)	100 ppm
Selenium	0.3 ppm
Ash (max)	6.50%

Piglets were fed Lab Mini-pig Chow: Starter (5080) (PMI Nutrition International, LLC Brentwood, MO.)

Chapter 3: Late-Gestation/Early Lactation Maternal Protein Restriction in Microswine Programs Excess Feed Intake and Accelerated Growth Without Prepubertal Obesity

ABSTRACT

In low birth weight babies, accelerated childhood growth amplifies risk of adult cardiovascular disease, but roles of rapid growth *per se* vs obesity potentially associated with accelerated growth are unknown. In microswine offspring exposed to maternal protein restriction (MPR) in late gestation plus early lactation, we examined growth, feed intake, body composition, and indices of glucocorticoid (GC) production over 0-12wks. At 2 wks, Low Protein Offspring (LPO) were asymmetrically growth restricted compared to Normal Protein Offspring (NPO), with weight deficit (28%) exceeding length deficit (9%) and with heart and adrenals exhibiting relative protection vs kidney and liver. LPO grew slowly vs controls from 2-5wks, then from 6-12wks showed accelerated growth of weight and length, together with increased feed intake and efficiency of gain. Lean and fat mass, both low in LPO at 6wks, were similar in LPO and NPO at 11wks, reflecting in LPO proportional accrual of - but greater fold-increases in - lean and fat mass over 6-11wks. Plasma cortisol was low in neonatal LPO but normal in fetal and juvenile LPO; 24-hr cortisol production was not altered in juvenile LPO. In juveniles, fasting plasma glucose was increased in male LPO but decreased in female LPO. Hyperphagia and accelerated growth following MPR do not yield obesity prior to puberty. Cortisol deficit in early postnatal life may contribute to altered hypothalamic neural circuits and increased appetite.

Introduction

In human cohorts from both developed and developing countries, low birth weight is associated with increased abdominal obesity[4] and cardiovascular disease[4,164,165,166] in adulthood. In children of low birth weight, accelerated growth between 4 and 12 years of age further increases risk for development of hypertension, type 2 diabetes, and central obesity in later life[10,167]. Whether accelerated growth per se mediates disease risk, or whether it acts through altered body composition, *i.e.*, obesity or lean mass excess, is unresolved.

In rats, severe maternal undernutrition (70% reduction in global caloric intake) throughout gestation led to low birth weight, followed by increased appetite, central obesity, and hypertension in the adult offspring; these postnatal effects were exacerbated by feeding a high-fat diet postweaning[67]. While in that model, offspring remained small throughout life[68], low birth weight in a mouse model of more moderate maternal nutrient restriction was followed by accelerated weight gain during suckling so that body weights had normalized by weaning[83]. The effects of maternal diet on offspring growth in rodents are thus inconsistent. However, because rodents have a very short period of time between weaning and puberty, it is difficult to assess the period equivalent to human childhood; use of a model with an extended juvenile period is optimal for study of postnatal growth patterns.

In humans, prenatal and early postnatal growth patterns have been associated with the development of obesity. Several studies have shown an inverse[21,168] or U-shaped[166]

relationship between birth weight and later BMI. Men exposed early in gestation to maternal nutritional deprivation during the Dutch Winter Hunger had an increased risk of obesity during adulthood[169,170]. Additionally, rapid growth during infancy[171] or childhood[172,173] has been associated with development of increased adiposity.

Glucocorticoids (GC) are well-known to affect body composition in adults, with cortisol excess leading to increased visceral adiposity. Low birth weight has been associated with increased fasting plasma cortisol[153,152] and increased 24-hr cortisol excretion and plasma cortisol following ACTH administration[174], all evidence of hypothalamic-pituitary-adrenal (HPA) axis activation. Animal studies have suggested that the circulating and tissue-level GC system is altered by maternal nutrient restriction[158,49,50] or maternal protein restriction (MPR)[92,148]. Such programmed changes in GC activity may thus lead to increased total or central adiposity later in life.

The present study was designed to characterize the postnatal patterns of growth and changes in body composition in prepubertal offspring exposed to MPR. We chose microswine, a large and long-lived omnivore, for its remarkable similarity to humans in cardiac, vascular, digestive, adipose and renal anatomy and physiology[175]. The longevity of swine permits a more precise definition of critical windows of development, while the extended period of time between weaning and puberty allows modeling of human childhood. We hypothesized that, following MPR, microswine offspring would subsequently exhibit increased appetite, accelerated increase in body mass, and increased plasma cortisol, collectively leading to excess fat deposition.

Materials and Methods

Experimental Design and Animal Care

Time-bred Yucatan microswine sows were obtained commercially (Charles Rivers Laboratories, later Sinclair Research Labs) and maintained in the OHSU Animal Care Facility until study under OHSU Institutional Animal Care and Use Committee approved protocol (#A439). Sows were randomly assigned to either normal (14%) or low (1%) protein diets (for dietary composition, see **Chapter 2: Methods and Materials, Table 2-1**) for the last fourth of gestation (beginning Gestation Day 85 of 115) plus two weeks post delivery. Sows were meal-fed twice per day. Since swine are protein-efficient and therefore not growth restricted by 3% maternal protein diet, but showed excess mortality with 0.5% MPR (SP Bagby, unpublished observations), the 1% protein diet was selected. There was no difference in numbers of piglets born in Low Protein (LP) vs Normal Protein (NP) litters (6.2 vs 5.9, n= 9 LP litters and 7 NP litters, p=.59). Male:female offspring ratio at birth was approximately 50:50, and not affected by maternal diet. The window of undernutrition was chosen to approximate, from a developmental perspective, the last 1/3 of human gestation, a period linked with developmental hypertension in other animal models. At 2 wks postnatally, all sows were returned to a normal diet (Purina Mills Lab Porcine Diet Grower). Sows and offspring were housed in a facility with 12:12-hr light-dark cycle. Offspring were weaned at 4 wks to juvenile piglet diet (Purina Mills) and were meal-fed twice per day (see Feed Intake methods, below).

Plasma and Urine Collection

Near-term fetal piglets were delivered by Caesarean section under isoflurane anesthesia

at gestation day 113 (gestation length = 115d); blood was collected by cardiac puncture in EDTA, and organs were rapidly removed for weighing and processing. 2-wk old neonatal piglets under isoflurane anesthesia underwent midline thoraco-abdominal incisions for organ harvest, and blood was collected by cardiac puncture in sodium heparin. Blood was centrifuged immediately at 4ºC and plasma was stored at -80ºC. In litters assigned to study as juveniles, piglets were placed in metabolic cages for 48 hrs at age 11-12 wks. Two consecutive 24-hr urine collections were stored separately at -20°C until use. Total "urine" volume was recorded (urine + spilled drinking water; all calculations were based on total volume of fluid collected and reported values were total nmol excretion per day, not nM). The second day's collection was used for cortisol assays. Blood was collected from awake pigs via an indwelling inferior vena caval catheter placed under isoflurane anesthesia the day prior to blood collection. Plasma glucose was analyzed by the OHSU Clinical Laboratory.

Body Size Measurements

Weight and crown-to-rump length (measured from base of tail to crown along the curve of the back using a flexible tape measure to the nearest half-cm) were measured 5-7 days/wk for the first 6 wks, then 3-5 days/wk thereafter. Low-Protein Offspring (LPO) weights were converted to z-scores [standard deviation scores, normalized to the average Normal-Protein Offspring (NPO) weight at each time point] and plotted against age to quantify initial reduction in body weight (z-score at day 0), reduction in body weight at day 14 (end of protein restriction period), and to visualize differences in rate of growth. Body length and body mass index [BMI: weight (kg)/length $(m)^2$] were similarly plotted.

Weight, length and BMI were also analyzed by linear regression (Proc Mixed procedure, SAS software) to determine rate of growth (slope of regression line).

Feed Intake

After weaning at 4 wks, piglets were housed separately to facilitate feed measurement and avoid confounding by social hierarchy or aggressive behaviors. Adjacent chain-link cages were used, allowing piglets to see and hear each other, and have physical contact. From completion of weaning at 4 wks of age through the end of the study, piglets were individually fed two meals per day, each in excess of spontaneous intake. For piglet growth chow composition, see **Chapter 2: Methods and Materials, Table 2-2**. Feed intake was measured by subtraction for both meals each day, 5 d/wk during wks 7-9 for the first set of litters, and 3 d/wk during wks 6-14 for subsequent litters. Pre-measured feed (more than the piglet could eat) was placed in feed bins constructed to minimize spillage; residual feed was removed after one hour (based on observation that after ~45 min pigs were no longer interested in feeding) and weighed to nearest g. On rare occasions when spillage did occur, data were not collected. Feed intake (g) per meal was normalized to current body weight; weekly per-meal averages were determined for each piglet and results were analyzed according to weeks of age. To calculate feed utilization efficiency, weekly weight gain was divided by weekly feed intake. Weekly weight gain was determined by subtracting weight on a given day from weight 7 days later; if data were missing, values were interpolated using linear regression equation obtained from data 7 calendar days prior to and following the missing data point. Weekly feed intake was calculated by multiplying weekly per-meal average by 14 meals per week.

Body Composition

At 6 wk (when weights were significantly reduced in LPO and NPO) and 11 wk (when weights were statistically similar between LPO and NPO), body composition was assessed by dual-energy x-ray absorptiometry (DEXA). Piglets were anesthetized by inhaled isoflurane and placed prone on the DEXA scanner (Hologic QDR-4500W). Fat mass and lean mass were measured by region – trunk, right leg, and left leg. The head region was excluded from calculations because of the high lipid content of the brain; forelimbs were excluded due to anatomic constraints imposed by use of pediatric software (Experimental Pediatric Whole Body v8.26 & v12.3). Lean mass was normalized to length as an index of thinness because length is less affected than weight at birth and 2 wk time points, suggesting it is a more appropriate index of the size of the piglet than weight.

Cortisol RIA

To assay cortisol concentrations in plasma and urine, the Coat-A-Count Cortisol RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) was used. Cortisol was extracted from urine samples with dichloromethane prior to assay. Urine extract or plasma was pipetted into antibody-coated tubes. ^{125}I-Cortisol was added to the tubes, incubated 45 min at 37°C and decanted thoroughly. Tubes were counted in a gamma counter, and results were analyzed according to kit instructions. For urine samples, concentrations were multiplied by 24-hr volume to yield an excretion rate in nmol per day, then normalized to lean body mass. Within-assay coefficient of variability was 2.45% and between-assay coefficient of variability was 4.24%.

Statistical Methods

Growth rate (weight, length, and BMI) was analyzed using SAS version 9.1 (SAS Institute, Inc., Cary, NC). Multivariate linear regression models using Proc Mixed procedure in SAS were generated to determine slope (rate of growth) of 2-5 and 6-12 wk periods. Z-scores were calculated as (individual value – NPO mean)/NPO standard deviation. Feed intake and feed utilization efficiency were analyzed by two-way Analysis of Variance (ANOVA) using Maternal Diet and Time or Sex and Time as variables; all body composition, plasma glucose and GC data were analyzed by two-way ANOVA using Maternal Diet and Sex as variables. ANOVA analyses were performed using GraphPad Prism v4.00 software (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± SEM. For all statistical tests, a P value <.05 was considered significant.

Results

Growth Patterns

Female LPO were reduced in body weight by 2.0 standard deviations (SD) at birth, while male LPO were reduced by 1.3 SD at birth (**Fig 3-1A**). Postnatal growth patterns in LPO compared to NPO controls were multiphasic; no sex differences were observed. Thus, from birth to 2 wk, as predicted during ongoing MPR, LPO grew slowly compared to controls, achieving a body weight of 3.7 SD (female LPO) or 3.3 SD (male LPO) below NPO at 2 wk of age. After this time point, once LP sows were returned to normal feed, LPO gained weight more quickly than controls from 2 wk to weaning at 4 wk. In the two weeks after weaning, however, LPO unexpectedly experienced a transient cessation of

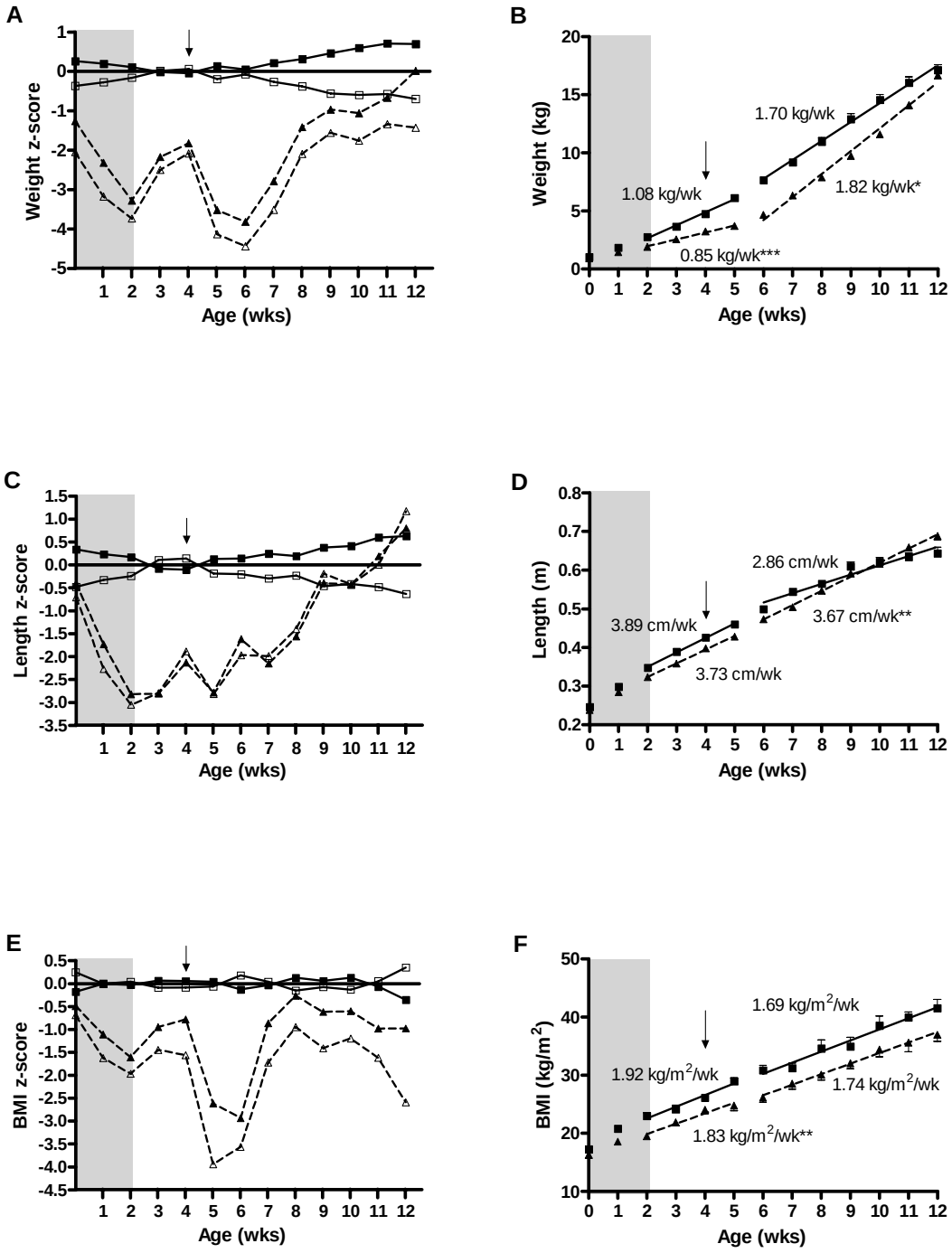

FIGURE 3-1: Growth Rates for Weight, Length and BMI. Weekly values converted to z-scores (standard deviation scores) are shown for weight (A), length (C), and BMI (E). ■, male NPO; □, female NPO; ▲, male LPO; Δ, female LPO. Linear regressions from 2-5 wks and 6-12 wks are shown for weight (B), length (D), and BMI (F). Points shown are weekly mean ± SEM condensed from several data points for each animal: squares, NPO; triangles, LPO. Shaded regions represent postnatal continuation of maternal protein restriction; arrows mark age at weaning. * LPO rate > NPO rate, p<.003; ** LPO rate > NPO rate, p<.001; *** LPO rate < NPO rate, p<.0001.

weight gain, whereas NPO merely slowed weight gain. In the 2-5 week period overall, LPO remained smaller and gained weight more slowly ($p<.0001$). From 6 to 12 wk, LPO underwent a period of significantly accelerated weight gain ($p=.003$)(**Fig 3-1B**).

Linear growth, expressed as crown-to-rump length, was also multiphasic in LPO compared to NPO and also without sex differences. Lengths in LPO were not significantly reduced at birth; however linear growth was slow from 0-2 wks, so that length at 2 wks was reduced by 2.9 SD (female LPO) or 3.0 SD (male LPO) (**Fig 3-1C**). Linear growth rates were similar between LPO and NPO from 2-5 wk. From 6-12 wk, linear growth was accelerated ($p=.0005$; **Fig 3-1D**) such that lengths at 12 wk were not significantly different between LPO and NPO.

BMI (weight (kg)/[length (m)]^2) followed trends similar to those observed for weight. At birth, BMI in LPO was not significantly reduced, but by 2 wk, BMI in LPO was 1.6 SD (female LPO) or 2.0 SD (male LPO) lower than NPO controls (**Fig 3-1E**). From 2-5 wk, BMI increased more slowly in LPO than in NPO ($p=.0003$). From 6-12 wk, BMI increased at a statistically similar rate in LPO compared to NPO (**Fig 3-1F**), however, this analysis masks a rapid increase in BMI over 5-8wks in LPO (**Fig 3-1E**), followed by a leveling off of BMI increase due to continued rapid increase in length with concurrent normalization of rate of weight gain.

Feed Intake

LPO consistently consumed more feed than NPO (LPO: 33.7 ± 0.6 g/kg/meal; NPO: 29.0

± 1.3 g/kg/meal; p < .0001; **Fig 3-2A**) and showed a slower decline with age vs NPO (p=.03). Sex did not affect feed intake in either NPO or LPO. Feed utilization efficiency (conversion of ingested feed to tissue accumulation) was increased in LPO (0.393 ± 0.04 g weight gain/g feed consumed) vs NPO (0.359 ± 0.04; p = .005; **Fig 3-2B**). Feed utilization efficiency decreased over time in both LPO and NPO (p < .0001), indicating an age-related decrease in feed utilization efficiency. However, the decline in feed utilization efficiency with age was nearly significantly slower in LPO (time x diet interaction p = .059). Sex did not affect feed utilization efficiency.

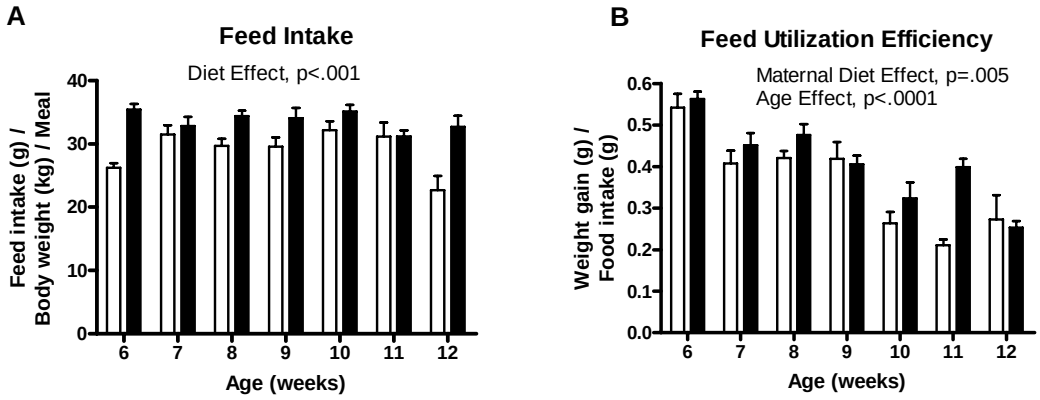

FIGURE 3-2: Feed Intake and Feed Utilization Efficiency. Feed intake (A) and feed utilization efficiency (B) are shown as weekly means ± SEM for LPO and NPO of ages 6-12 weeks. White bars, NPO; black bars, LPO. A) Average feed intake in LPO (n=9-15) vs. NPO (n=6-11) B) Feed utilization efficiency (g weight gain / g feed intake) in LPO (n=6-15) vs. NPO (n=3-11).

Body Composition: Adiposity

Body composition data are presented in **Table 3-1**. At 6 wks, when weights were still significantly low in LPO, percent body fat in LPO was decreased compared to NPO

(p=.003), and females had higher percent body fat than males (p=.012). Body fat mass:lean mass ratios in LPO were also decreased compared to NPO (p=.003), and females had higher fat mass:lean mass ratios compared to males (p=.011). Truncal adiposity, calculated as truncal fat mass divided by whole-body fat mass, showed a strong trend toward decrease in 6-wk-old LPO (p = .06), and was not different in males vs females.

In contrast, at 11 wks, when weights in LPO were statistically similar to controls, there were no differences in whole-body or truncal adiposity between LPO and NPO. Female offspring had higher percent body fat and whole-body fat:lean ratios than males (p=.003). There were no differences in whole-body fat:lean ratios based on either maternal diet or sex.

Body Composition: Lean Mass

At 6 wks, normalized lean mass (lean mass:length ratio, an index of thinness) was reduced in LPO compared to NPO (p=.0007). By 11 wk, there was no significant effect of maternal diet on normalized lean mass; however, male offspring had increased normalized lean mass as compared to females (p=.003).

Accrual of Fat and Lean Mass

The fold-increase in fat mass from 6 to 11 wk (grams fat at 11wks/grams fat at 6 wks) (**Fig. 3-3A; Table 3-1**) was greater in LPO than NPO (p=.003). Fold-increase in lean mass from 6 to 11 wk (grams lean at 11wks/grams lean at 6 wks) (**Fig 3-3B**) was also

greater in LPO than NPO (p=.002). The ratio of increase in fat to increase in lean was not different between LPO and NPO, indicating a balanced accrual of fat vs lean mass during the accelerated growth period (**Fig 3-3C**). Females accrued relatively more fat than lean as compared to males (p=.004; **Fig 3-3C**).

FIGURE 3-3: Increase in Fat and Lean Mass Over Weeks 6 to 11. The fold-increases in fat and lean mass over weeks 6 to 11 are shown as mean ± SEM. White bars, NPO (n=9: 4 male, 5 female); black bars, LPO (n=12: 7 male, 5 female). A) Fold increase in fat mass B) Fold increase in lean mass C) Ratio of the increase in fat mass to the increase in lean mass. See Table 3 for statistical summary of body composition.

Plasma Cortisol and Urinary Cortisol Excretion

Plasma cortisol levels were not different between LPO and NPO at near-term fetal or juvenile ages (**Table 3-2**). In 2wk neonatal LPO, plasma cortisol was lower than NPO (LPO: 502 ± 22.7, n=7; NPO: 605 ± 31.9, n=9, $p<.05$) with no sex differences observed. In juvenile offspring, 24-hr urinary cortisol excretion was similar in LPO and NPO (**Table 3-2**). Normalizing 24-hr cortisol excretion to lean body mass at 11wks of age did not affect the results (**Table 3-2**). A positive correlation was observed between 24-hr cortisol excretion and % body fat at 11 wks ($r^2 = .20$, $p<.05$), which remained significant after adjusting for lean body mass ($r^2 = .21$, $p<.04$). No correlation existed between plasma cortisol and % body fat.

Plasma Glucose

In juveniles, fasting plasma glucose was increased in male LPO (111.3 ± 5.7mg/dL, n=8) and decreased in female LPO (93.8 ± 7.0mg/dL, n=5) compared to sex-matched controls (male NPO: 85.2 ± 6.0 mg/dL, n=5; female NPO: 111.3 ± 5.7mg/dL, n=6; interaction, p<.02; **Fig. 3-4**). No significant differences were observed in near-term fetal offspring (NPO: 55.4 ± 8.2mg/dL, n=17; LPO: 59.4 ± 9.1 mg/dL, n=18) or neonatal offspring (NPO: 158.1 ± 4.8mg/dL, n=9; LPO: 163.8 ± 10.1 mg/dL, n=8).

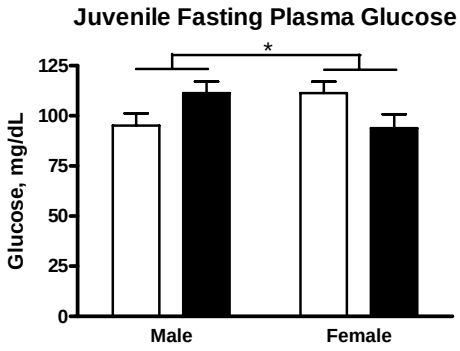

FIGURE 3-4: Fasting Plasma Glucose in Juvenile Offspring of Protein-Restricted Sows. Fasting plasma glucose concentrations in juvenile (3-5 mo) offspring are shown as mean ± SEM. White bars, NPO (n=11; 5 male, 6 female); black bars, LPO (n=13; 8 male, 5 female). *Interaction between maternal diet and sex, p<.02.

Discussion

In those born small, altered growth patterns in childhood exacerbate risk of developing cardiovascular and metabolic diseases in adulthood. It is unknown whether alterations in body composition potentially associated with accelerated growth are required to convey this increased risk of disease. In this study, microswine sows were placed on either normal or low protein diet for the last quarter of gestation plus the first two weeks postnatally. This protocol was designed to encompass developmental processes in the late-maturing piglet that occur before birth in the human. MPR in microswine causes reduced birth weight in the offspring with nadir weight occurring at 14 days, the end of

the protein restriction period. We describe four major findings in LPO following restoration of normal nutrition at two wks of age: 1) A biphasic post-nadir growth pattern, with slow growth persisting over 2-5 wks despite return of sows to normal diet at 2 wks, followed by marked acceleration of weight and linear growth rates post-weaning, over 6-12 wks; 2) sustained 16% increase in feed intake (g/current wt/meal) over 6-12 wks with increased feed utilization efficiency over the same period; 3) fasting plasma glucose alterations in a sex-dependent manner suggesting the development of abnormal glucose homeostasis in prepubertal male LPO; and 4) increased rates of fat and lean mass accumulation – but in balanced proportions compared to controls – yielding normal body composition at 11 wks. Thus, isocaloric MPR in late fetal/early neonatal development programs hyperphagia, accelerated growth, altered glucose homeostasis and enhanced metabolic efficiency; these abnormalities pertain in the absence of prepubertal obesity. In keeping with normal distribution of lean and fat tissues at 11 wks, we also found no persisting abnormalities in plasma cortisol or 24-hr cortisol excretion in juvenile LPO, though plasma cortisol was reduced in early postnatal life during active MPR.

Growth Patterns

Prior large-animal studies of MNR effects on postnatal growth have focused on early-gestational nutrient restriction;[176,177,178,179,180,47] to our knowledge, ours are the first to examine effects of a late-gestational nutritional intervention on postnatal growth patterns. Following MPR in microswine, abnormal postnatal growth in LPO occurs in distinct phases. Body weight, length, and BMI are reduced in LPO in a pattern consistent with asymmetric intrauterine growth restriction (IUGR), both at birth and at the end of the

protein restriction period at 2 wks. Following the sows' return to normal diet, LPO catch up at varying rates in each of these parameters such that, at 11 wk – while still prepubertal – LPO weight, length, and BMI are similar to NPO controls. The maintenance of normal linear growth from 2-5 wks while weight gain is slow, indicating that length is unaffected by immediate post-weaning factors limiting weight gain, and suggests a different mechanism may drive linear growth as opposed to mass accrual. The post-weaning slowing of weight gain may be due to impaired absorption of solids based on immaturity of the gastrointestinal system or to specific defects of the gut, both described in piglets following IUGR[181,182]. Our findings are similar to those described by Poore and Fowden in naturally-occurring low birth weight pigs[183]. During suckling, these low birth weight pigs showed increased fractional growth rate vs high birth weight littermates[183]. After weaning (1-3 months), the low birth weight pigs had a decreased fractional growth rate; from 3-12 months, fractional growth rate was again increased[183]. Because these data were based on only four weight measurements (at birth and at 1, 3, and 12 months), it is possible that nuances in growth rate, such as the adverse response to weaning observed in the present study, were missed. The high fractional growth rate reported over 3-12 months by Poore and Fowden[183] suggests that the accelerated growth observed in the present study in prepubertal LPO may persist into adulthood. While our data do not distinguish whether the increased feed utilization efficiency observed in LPO from 6-12 wks is a result of increased absorption in the GI tract and/or due to metabolic

alteration allowing increased growth, persistence of increased feed utilization efficiency could contribute to an increased rate of growth or obesity in adulthood.

Accelerated postnatal growth, when it follows fetal growth restriction, is a potentiating factor in the development of human disease, including hypertension[10,167], diabetes and obesity[184]. A recent report from a Finnish birth cohort showed two patterns of early growth which were associated with hypertension. In one pattern, low birth weight and continued small size through four years of age was followed by accelerated growth to yield normal weight and BMI at 11 years of age; this was followed by earlier-onset and more severe hypertension together with obesity and insulin resistance in adulthood[184]. Microswine LPO follow this pattern, and demonstrate, by the prospective study design, what human birth cohorts cannot: that MPR programs not only the slow, but also the accelerated component of this postnatal growth pattern.

The postnatal growth pattern we report here is distinct from those previously published in animal models in that accelerated growth with full weight catch-up is delayed until after weaning despite normalized nutrition, more closely modeling the growth pattern leading to a more severe hypertension in the Helsinki birth cohort[184]. In contrast to human studies and the present report, while MNR or MPR in rats resulted in reduced birth weight of the offspring, offspring never reached the same adult weight as control animals[67,100]. In other studies in rats, MPR during pregnancy and/or lactation had different growth patterns in the offspring depending upon the timing of the MPR and the sex of the offspring[98]. In mice, caloric restriction in the last third of pregnancy resulted in low birth weight in offspring, followed by accelerated growth during the first week after birth so that weights were not different by weaning at 3 wks of age[83]. As mentioned

above, gastrointestinal immaturity and/or dysfunction has been described in IUGR piglets and may contribute to the observed delay in onset of accelerated growth in the microswine MPR model; it is unknown whether similar factors may delay onset of growth acceleration in babies born small. Irrespective of the reason for delayed growth acceleration, the growth pattern in microswine LPO more closely models human growth patterns leading to adult disease than do other previously described models.

Programming of Appetite

The present study in microswine is the first large animal model to show that a specific nutritional intervention – *i.e.* isocaloric MPR - can result in hyperphagia. Increased appetite accompanies the accelerated growth phase over 6-12 wk. Both male and female LPO exhibit a programmed increase in appetite compared to NPO, eating ≅ 16% more feed per kg current body weight at all measured time points. Persistence of increased appetite (>12 wks) beyond the point of weight equality (11 wks) suggests the potential for lifelong energy imbalance, as observed in spontaneous low birth weight pigs[185], but it is unknown how long the increased appetite persists in LPO. Some rodent studies support this pattern, reporting increased appetite after MPR[100] or severe MNR[67]. Alternatively, it is possible that factors mediating the programming of accelerated growth also drive the increase in appetite. In spontaneously low birth weight lambs, elevated appetite from birth decreases to match that of high birth weight lambs at 60-80 days of age[186], suggesting that accelerated growth may drive hyperphagia. If this is the case in our model, we would predict that the hyperphagia would normalize as growth rates return to normal. Studies which showed no effect of MPR on appetite[99] may have assessed feed

intake after catch-up growth was complete, and thus after appetite normalized. Extension of appetite and growth measurements from 12 wks into adulthood will be required to distinguish these possibilities in the microswine model.

Body Composition

Microswine LPO at 6 wk have both reduced fat mass and reduced lean mass compared to NPO; fat mass is decreased to a greater degree than lean mass, suggesting that lean tissue is relatively protected in LPO. By 11 wk, these deficits were eliminated through accelerated growth of both fat and lean tissue in a balanced fashion. The absence of differences in body composition just prior to puberty in LPO is consistent with findings of still-normal BMI in low birth weight children who became hypertensive and obese as adults[184]. If the programmed changes in appetite and/or enhanced feed utilization efficiency observed in these prepubertal LPO persist into adulthood, then LPO (especially females) may still develop excessive amounts of fat with aging. Though the lack of obesity observed in this study may seem to contradict studies showing obesity in animals exposed to maternal nutrient or protein restriction[170,187,188,100,47], spontaneously low birth weight pigs developed increased back fat by 12 mo, but not by 3 mo[185]. These findings, together with the propensity of our female LPO toward increased % body fat at 11 wks, suggest the potential for overt obesity with aging in the microswine model.

Glucocorticoids

Normal body composition in juvenile LPO is concordant with our finding of normal cortisol plasma levels and 24-hr urinary excretion rates, the latter approximating daily

cortisol production, in juveniles. GC are well-known to cause increased obesity specifically in the visceral/central compartment. Alterations in indices of GC activity have been implicated in programming of hypertension in rodents by MPR throughout pregnancy[148,149]. The normal plasma cortisol observed in juvenile offspring is in agreement with a previous report that juvenile low birth weight pigs did not have altered cortisol plasma concentrations[155]. However, our findings stand in contrast to a study showing increased cortisol in low birth weight neonatal pigs[154] and to studies of humans which showed elevated morning plasma cortisol in adults born small[152,153]. Our data do not exclude the possibility that the normal circadian trough in plasma cortisol levels may be disrupted; however, the present data do not support inappropriate HPA activation.

In parallel studies by our group, juvenile LPO were found to exhibit enhanced *ex vivo* mesenteric vascular reactivity to pressors despite normal telemetric blood pressures under basal conditions (48 hrs unrestrained). (See **Introduction.**) Our present findings thus also suggest that neither obesity nor elevated cortisol is necessary for development of the vascular dysfunction identified in prepubertal LPO.

Fasting Plasma Glucose

Fasting plasma glucose concentrations were altered in a sex-dependent manner in juvenile LPO. Male LPO had increased glucose concentrations, suggesting the development of impaired glucose homeostasis at a prepubertal stage in the absence of obesity. Female LPO had decreased glucose concentrations, suggesting improved glucose handling under basal conditions in spite of a suggestion of increased adiposity in

some individuals (2 juveniles female LPO had % body fat > 2 SD above female NPO mean). Adult female rat offspring exposed to MNR exhibited increased glucose disposal in vivo due to enhanced GLUT4 cell-surface expression; however offspring had impaired responses to exogenous insulin stimulation[189]. While the absolute values of the changes were small, the fact that these animals are still prepubertal suggests that the relatively small effects observed here may translate into large effects in adults.

Limitations of the Study

The microswine MPR model has distinct advantages, including remarkable similarity to human anatomy and physiology, and the extended period of time between weaning and puberty, allowing for modeling of human childhood. However, the model as applied in this study cannot differentiate between effects of poor nutrition during gestation vs. during early lactation. Altered milk production has been documented in a rat uteroplacental insufficiency model[190]. Similarly, MPR results in changes in maternal body composition and physiology which likely persist throughout the lactation period, potentially extending the period of nutrient deficit or altering hormonal milieu beyond the intended window. Cross-fostering piglets onto Normal Protein sows at birth and at 2 wks of age differentiate pre- vs postnatal effects. It would also serve to distinguish the effects of the active protein restriction period vs lingering effects during later lactation, as in studies performed by Thamotharan, *et al*[189] and Di Nicolantonio, *et al*[191]. Additionally, the nature of isocaloric dietary intervention is that reduction of one macronutrient requires the increase of one or more other macronutrients. Moreover, the protein-to-carbohydrate ratio may be more important to fetal growth than either element alone[192].

Last, our use of a soy-based diet (both as protein and fat sources) vs the typical casein-based diet may have reduced some adverse effects. Studies in adult rodents have shown soy-based diets result in lower plasma and liver lipids[193,194], better preserved insulin action[194], less promotion of oxidative stress[195], reduced hepatic steatosis and adipocyte hypertrophy[196], reduced adiposity[197], renal protection by restoration of nitric oxide production[198], and reduced the development of metabolic syndrome in obese[199] and lean[200] rats; however, one study in mice showed worsening of cardiomyopathy with soy vs casein[201]. The multiple effects observed on metabolism suggest that the effects observed in the present study may be understated compared to what might have been observed had the sows been fed a casein diet.

In summary, MPR in microswine, applied in a period designed to encompass in the piglet developmental processes occurring before birth in the human, generates growth patterns which mimic those described in human cohorts among young subjects destined to develop hypertension, insulin resistance, and central obesity as adults[184]. MPR results in low birth and nadir weights, continued slow growth over 2-5 weeks, followed by accelerated growth over 6-12 wk. The latter is accompanied by programmed increase in appetite and efficiency of tissue gain. Unexpectedly, the excessive tissue accrual in LPO was normally proportioned in terms of fat vs. lean, differing from NPO primarily in representing greater fold-increases over initial mass. Concomitantly, cortisol levels and excretion were normal in juveniles. Accordingly, the mesenteric vascular hyperreactivity and altered BP regulation, reported separately in these normotensive juvenile LPO (SP

Bagby, unpublished observations; **Introduction**), do not depend on obesity; nor do they depend on excess GC production or circulating levels.

The dissociation of prepubertal growth acceleration in swine LPO from obesity and hypercortisolemia suggests that later abnormalities which impact energy metabolism - operative during or beyond puberty – may be required to generate adult obesity following early asymmetric growth restriction. How MPR, accelerated growth and/or hyperphagia contribute to metabolic and vascular abnormalities will be discussed in Chapters 4 and 5.

TABLE 3-1: Body Composition in Juvenile Offspring of Protein Restricted Sows[1]

	NPO			LPO		
	Male	Female	All	Male	Female	All
6 weeks						
% Body Fat	13.7 ± 0.8[c] n=7	16.4 ± 0.8[c] n=5	14.8 ± 0.7 n=12	11.8 ± 0.7[c] n=10	13.2 ± 0.5[c] n=6	12.3 ± 0.5[a] n=16
Truncal Adiposity[2]	0.868 ± 0.010 n=7	0.873 ± 0.024 n=5	0.870 ± 0.011 n=12	0.845 ± 0.008 n=10	0.843 ± 0.015 n=6	0.844 ± 0.007 n=16
Fat:Lean[3]	0.162 ± 0.011[d] n=7	0.201 ± 0.012[d] n=5	0.178 ± 0.010[a] n=12	0.137 ± 0.009[d] n=10	0.149 ± 0.009[d] n=6	0.144 ± 0.007[a] n=16
Lean Mass[4]	87.4 ± 3.4 n=7	81.7 ± 4.4 n=5	85.0 ± 2.7[b] n=12	67.8 ± 2.23 n=10	68.5 ± 6.9 n=6	68.1± 2.8[b] n=16
11 weeks						
% Body Fat	22.2 ± 1.0[d] n=4	25.0 ± 0.5[d] n=5	23.8 ± 0.7 n=9	21.6 ± 1.1[d] n=8	26.3 ± 1.2[d] n=6	23.6 ± 1.0 n=14
Truncal Adiposity[2]	0.901 ± 0.006 n=4	0.906 ± 0.005 n=5	0.904 ± 0.004 n=9	0.905 ± 0.004 n=8	0.900 ± 0.006 n=6	0.903 ± 0.003 n=14
Fat:Lean[3]	0.291 ± 0.016[d] n=4	0.341 ± 0.009[d] n=5	0.319 ± 0.012 n=9	0.283 ± 0.018[d] n=8	0.366 ± 0.023[d] n=6	0.318 ± 0.018 n=14
Lean Mass[4]	159.1 ± 11.1[d] n=4	137.3 ± 4.8[d] n=5	147.0 ± 5.6 n=9	147.4 ± 3.9[d] n=8	125.7 ± 7.2[d] n=6	138.1 ± 4.4 n=14
Change from 6-11wks						
Fold-increase in Fat[5]	5.1 ± 1.5 n=4	4.0 ± 0.3 n=5	4.5 ± 0.7 n=9	6.8 ± 0.6 n=8	7.7 ± 0.5 n=6	7.2 ± 0.4[a] n=14
Fold-increase in Lean[5]	2.5 ± 0.24 n=4	2.3 ± 0.14 n=5	2.4 ± 0.11 n=9	3.2 ± 2.4 n=8	3.2 ± 0.28 n=6	3.2 ± 0.13[a] n=14
Incr. Fat: Incr. Lean[6]	0.373 ± 0.024[d] n=4	0.455 ± 0.031[d] n=5	0.419 ± 0.024 n=9	0.341 ± 0.027[d] n=7	0.501 ± 0.052[d] n=5	0.408 ± 0.035 n=12

[1]Data are presented as mean ± SEM, and were analyzed by two-way ANOVA using maternal diet and sex as factors. [a]p<.005 LPO vs NPO; [b]p<.001 LPO vs NPO; [c]p<.05 male vs female; [d]p<.005 male vs female.

[2]Truncal adiposity is calculated as fat (g) in trunk region divided by fat (g) in whole body.

[3]Fat:Lean ratio is fat mass (g) in whole body divided by lean mass (g) in whole body.

[4]Lean mass is normalized to crown-to-rump length (in cm).

[5]Fold-increases in fat or lean are calculated as grams of tissue at 11wks divided by grams of tissue at 6wks.

[6]Increase in fat : increase in lean is (grams fat at 11wks - grams fat at 6 wks) / (grams lean at 11 wks - grams lean at 6 wks)

TABLE 3-2: Plasma Cortisol and Cortisol Excretion in Fetal, Neonatal and Juvenile Offspring of Protein Restricted Sows[1]

	NPO			LPO		
	Male	Female	All	Male	Female	All
Near-Term Fetal						
Harvest Cortisol[2], nM	386 ± 46 n=11	373 ± 72 n=6	381 ± 38 n=17	324 ± 71 n=7	291 ± 46 n=12	303 ± 38 n=19
2 Wk Neonatal						
Harvest Cortisol[2], nM	656 ± 38 n=5	540 ± 35 n=4	604 ± 32 n=9	601± 128 n=4	519 ± 49 n=3	501 ± 23* n=7
3-5 Mo Juvenile						
Awake Restraint Cortisol, nM	429 ± 101 n=5	471 ± 155 n=5	450 ± 88 n=10	338 ± 54 n=10	489 ± 66 n=7	400 ± 44 n=17
24-hr Cortisol Excretion, nmol/d	68.6±11.8 n=5[3]	61.7 ± 9.5 n=4	65.5 ± 7.4 n=9[3]	52.0 ± 6.7 n=10[3]	56.6±14.3 n=7	53.9±6.83 n=17[3]
24-hr Cortisol Excretion, nmol/d normalized to lean mass	4.90±0.01 n=3[3]	7.39±1.25 n=4	6.32±0.84 n=7[3]	5.03±0.74 n=8[3]	6.30±1.48 n=6	5.57±0.75 n=14[3]

[1]Data are presented as mean ± SEM. Data were analyzed by two-way ANOVA using maternal diet and sex as factors. *p<.05 vs NPO. Abbreviations: Normal Protein Offspring, NPO; Low Protein Offspring, LPO.

[2]Harvest cortisol measurements were obtained from blood drawn from the heart under isoflurane anesthesia.

[3]Some animals did not undergo DEXA body composition analysis; thus no lean mass data are available to normalize cortisol data.

Chapter 4: Altered Adipocyte Function without Adipocyte Hypertrophy in Juvenile Microswine Offspring Following Maternal Protein Restriction

ABSTRACT

Microswine offspring exposed to late gestation/early lactation maternal protein restriction (MPR) have low birth weight and low birth weight:length ratios, followed by relatively accelerated post-weaning growth rate. We hypothesized that rapid accrual of adipose tissue in Low Protein Offspring (LPO) may have adverse metabolic consequences even without development of classically defined obesity. In juvenile offspring, adiponectin, tumor necrosis factor (TNF)-α and glucocorticoid-related mRNA levels were assessed in intra-abdominal (IAT) and subcutaneous (SAT) adipose tissue depots by real-time PCR. Adipocyte size was assessed in juveniles by histological methods with image analysis. Plasma leptin levels were measured in near-term fetal, neonatal, and juvenile offspring by radioimmunoassay. Adipocyte size in juveniles increased with age over 3-5 mo; after adjustment for age, adipocyte size was not significantly affected by maternal diet, though adipocyte sizes tended to be smaller in LPO compared to Normal Protein Offspring controls. In LPO, adiponectin mRNA was reduced in both sexes and in both adipose tissue depots. In contrast, in LPO, TNF-α mRNA was not affected in either depot. Overall, adiponectin mRNA was lower in SAT vs IAT. Adipose tissue glucocorticoid indices were not altered by MPR, but females had lower 11βHSD1 mRNA compared to males in both adipose tissue depots. Plasma leptin concentrations were decreased in LPO at 2wks but were normal by 3-5 mo, suggesting a delayed maturational increase in leptin levels during a potentially critical window of development for neural circuits regulating appetite. Thus, rapid accrual of adipose tissue during post-weaning development following MPR is accompanied by altered adipose tissue function without adipocyte hypertrophy or obesity in a sex-dependent manner in juvenile microswine offspring.

Introduction

Low birth weight increases risk of developing cardiovascular and metabolic disease in humans and animal models. Accelerated postnatal growth in humans is associated with exacerbated risk, but it is unknown how accelerated growth is related to the increase in risk of cardiovascular disease. We have previously shown that maternal protein restriction (MPR) during late gestation/early lactation programs both low birth weight and accelerated post-weaning growth in microswine offspring (Chapter 3) but without generating obesity in prepubertal juveniles. Fat mass is accrued at accelerated rates in low protein offspring (LPO) vs normal protein offspring (NPO) controls; the mechanism and implications of the accelerated fat accrual have not yet been studied.

Adipocyte size across the range of normal and obese BMIs has been associated with infiltration of macrophages and expression of inflammatory molecules, and it has been suggested that reductions in adiponectin and increased local chronic inflammation in adipose tissue comprise the link between obesity and cardiovascular and metabolic disease. Accelerated fat accrual observed previously in microswine (Chapter 3) may lead to altered adipocyte size and/or function. We therefore hypothesized that late gestation/early lactation MPR, accompanied by accelerated post-weaning growth, causes increased adipocyte size with concomitant decrease in adiponectin and increase in tumor necrosis factor (TNF)-α, a mediator of inflammation.

Human acquired obesity has been associated with increased mRNA levels and activity of 11β hydroxysteroid dehydrogenase type 1 (11βHSD1), the enzyme which converts

inactive cortisone to active cortisol[159]. In support of the concept that local reactivation of cortisol can influence whole-body physiology, transgenic mice overexpressing 11βHSD1 only in adipose tissue develop visceral obesity, accompanied by insulin resistance and hyperlipidemia[202]. In neonatal sheep exposed to maternal nutrient restriction in early to mid gestation, tissue-level increases in glucocorticoid receptor (GR) and 11βHSD1 have been reported in perirenal adipose tissue. However, neonatal microswine offspring under ongoing MPR have reduced plasma cortisol levels (see **Chapter 3**). A rational physiological adaptation to explain these observations might be increased tissue-level production or sensitivity to cortisol. If this increased production or sensitivity were to persist through life, it would contribute to increased risk of disease in vulnerable individuals – those who were born with low birth weight and undergo subsequent accelerated growth during childhood. We therefore also hypothesized that local production of - or response to - glucocorticoids (GC) in juvenile offspring contributes to the increased rate of fat mass accrual. The two hypotheses were addressed using a microswine maternal protein restriction (MPR) model previously used to demonstrate accelerated growth with increased rate of fat accrual during the juvenile period.

Methods

Experimental Design and Animal Care

Time-bred Yucatan microswine sows were obtained commercially (Charles Rivers Laboratories, later Sinclair Research labs) and maintained in the OHSU Department of Comparative Medicine until study under approved OHSU Institutional Animal Care and Use Committee protocol (A439). Sows were randomly assigned to either normal (14%)

or low (1%) protein diet for the last quarter of gestation (starting Gest Day 85 of 115) and for two weeks postnatally as described in Chapters 2 and 3. This period of undernutrition was chosen to approximate, from a developmental perspective, the last third of human gestation, a period linked to developmental adult hypertension in other models. Sows were returned to normal diet (Purina Mills Lab Porcine Diet Grower) at 2 wks postnatally. Sows and offspring were housed under 12:12hr light-dark cycle. From two days prior to expected due date until weaning, ceramic heaters were placed just outside the sows' pens to provide extra heat for microswine offspring, which are born with essentially no body fat, and hence are unable to maintain body temperature without an external heat source. Offspring were weaned at 3-4 wks to juvenile piglet diet (Purina Mills, see **Chapter 2**) and were meal-fed twice per day. These juvenile offspring are the same animals described previously (**Chapter 3**). Two other separate groups of animals were sacrificed at day 113 of gestation (term 115d; near-term fetal) and at 2 wks after birth (neonatal).

Plasma and Tissue Harvest

At 14-22wk (i.e., 3-5 mo), juvenile offspring were placed under isoflurane anesthesia. Plasma was immediately collected from the jugular vein. Subcutaneous adipose tissue (SAT) from the ventral abdomen, intra-abdominal adipose tissue (IAT) from the lateral abdominal wall, and gastrocnemius muscle were quickly harvested, snap-frozen in liquid nitrogen, and stored at -80ºC until use. Separate aliquots of adipose tissues were fixed in formalin, then paraffin embedded. In a separate group of animals (n= 9 NPO, 7 LPO; 2 litters in each group), 2-wk-old offspring were placed under isoflurane anesthesia, and

gastrocnemius muscle was collected as for juvenile tissues. At 2 wks, microswine have too little body fat for analysis. Plasma was collected by cardiac puncture.

Real-time PCR

Snap-frozen tissue samples were weighed, minced, and homogenized in TRIzol (Invitrogen Corp., Carlsbad, CA) using a Tissuemiser rotor-stator homogenizer (Fisher Scientific, Waltham, MA). After an initial spin to clear lipid and cell debris, total RNA was extracted according to manufacturer instructions. RNA concentration was quantified by optical density at A_{260}. 1μg RNA was treated with DNase I (Invitrogen), then reversed transcribed using random hexamer primers (Operon Biotechnologies, Huntsville, AL) and SuperScript II reverse transcriptase (Invitrogen). Gene-specific primers to swine lipoprotein lipase (LPL, an adipose tissue target of GR, used here as an index of GR-α activity), adiponectin, tumor necrosis factor (TNF)- α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s rRNA were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primers for GR-α and 11βHSD1 were adapted for swine, based on those used in human studies by Wake, et al[159]. All primers were synthesized by Operon Biotechnologies; see **Table 4-1** for sequences. Resulting cDNA was used in real-time PCR using the SYBR Green method. Triplicate reactions of 5.0 μL sample (diluted 1:20) or standard (pooled mix of samples, diluted 1:5, 1:20, and 1:80), 0.75 μL of each primer (forward and reverse, 5 μM each), 12.5 μL 2X SYBR Green PCR Master Mix (Applied Biosystems), and 6.0μL nuclease-free water were performed in 96-well plates. Each plate contained the transcript-of-interest and 18s rRNA (or GAPDH for juvenile gastrocnemius) as an endogenous control for each

sample; each transcript had its own standard curve generated from pooled sample cDNA. Amplification reactions were run on an ABI Prism 7500 (Applied Biosystems). Cycling conditions were as follows: 50ºC for 2 min, 95ºC for 10 min, then 40 cycles of 90ºC for 15 sec and 60ºC for 60 sec. Fluorescence was detected during the annealing phase of each cycle. Baseline values were auto-subtracted and thresholds were assigned automatically by the instrument software. Relative gene expression was calculated by the standard curve method, and normalized to endogenous controls. Neither of the endogenous controls' (18s rRNA or GAPDH) abundance was affected by maternal diet. Values represent a fold-change compared to the pooled sample control.

Adipocyte Size Measurements

Formalin-fixed paraffin embedded IAT blocks were cut in 5 μm sections, then stained with hematoxylin. All analysis procedures were performed by an individual blinded to the maternal diet and sex of the animal. Digital images were captured from four quadrants per section using a Leica microscope with a 20X objective and CCD camera. Images were analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) software. Cell area (μm^2) estimates were obtained for each quadrant field analyzed. Two sections were averaged from each block; two blocks were counted per animal. Cell areas were averaged across blocks to obtain a single average for each animal. **Fig. 4-1** shows slides from two individuals, and displays the wide range of cell sizes observed between and within animals. Image-Pro Plus automatically highlighted cell membranes and measured the area of each cell, minimizing observer bias. Cells touching the border were not measured.

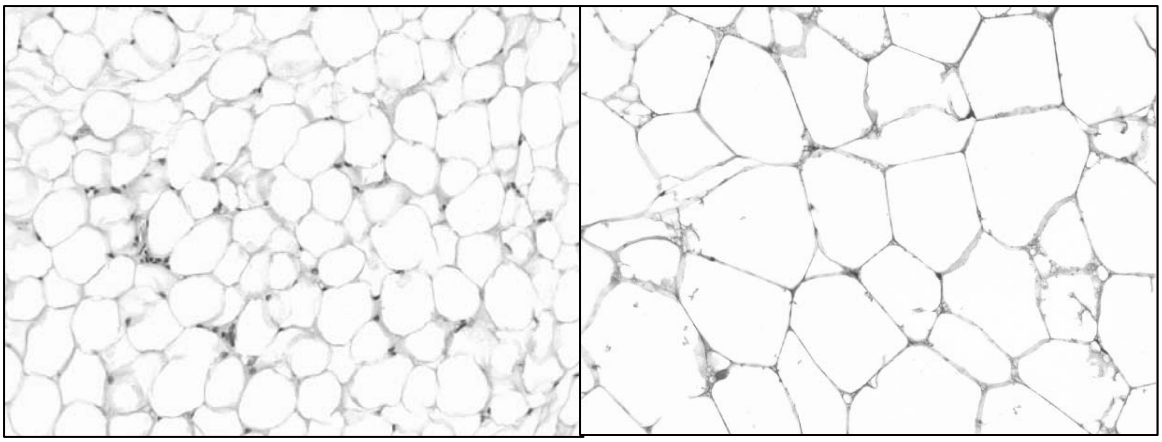

FIGURE 4-1: Intra-Abdominal Adipose Tissue Representative Slides. Panels are from two different individuals, and illustrate the wide range of cell sizes observed both between and within individuals. Computer-based image analysis was performed to remove observer bias.

Leptin Assay

Fetal, neonatal and juvenile EDTA plasma samples were analyzed using the Multi-Species Leptin Radioimmunoassay Kit (Millipore, Inc., Billerica, MA; formerly Linco Research, St. Charles, MO) according to the kit instructions. Plasma leptin concentrations are reported in Human Equivalents (HE) because human leptin was used as standard and the antibody was raised against human leptin. Intra-assay coefficient of variation was 7.3%; all measurements were performed in a single assay.

Statistical Methods

For all real-time PCR assays, results (**Tables 4-2 and 4-3**) are expressed as a ratio of gene-of-interest to endogenous control (either 18S rRNA or GAPDH). These ratios were Ln transformed then analyzed by two-way Analysis of Variance (ANOVA) using maternal diet and sex as factors. For adiponectin and TNF-α, three-way ANOVA was performed using maternal diet, sex, and tissue depot as factors. Plasma leptin concentrations were also analyzed by two-way ANOVA using maternal diet and sex as

factors. Because sex had no effect in fetal and neonatal animals, sexes were subsequently pooled in these groups then analyzed using age and maternal diet as factors in two-way ANOVA. Adipocyte size (area in μm_2) measurements were analyzed by pooling cell areas across all fields analyzed to obtain one mean value for each animal. Using age as a covariate in a General Linear Model univariate analysis, values were analyzed using maternal diet and sex as factors (SPSS 14.0 for Windows, SPSS Inc., Chicago, IL).

Results

Tissue Glucocorticoid Indices

In juvenile IAT and SAT, there were no differences in 11βHSD1, GR-α or LPL mRNA due to maternal diet. Females had decreased 11βHSD1 mRNA levels compared to males in both IAT (p=.002) and SAT (p=.02) (**Table 4-2**).

In skeletal muscle (gastrocnemius), MPR had no effect on GR-α or 11βHSD1 mRNA in both neonatal and juvenile offspring. However, neonatal female offspring had higher skeletal muscle GR-α mRNA levels than neonatal males (p=.02) (**Table 4-2**). This sex difference was not maintained in juvenile offspring. 11βHSD1 mRNA was not different between sexes at either age in muscle.

Adipocyte Size and Function

Plasma leptin concentrations are shown in **Fig. 4-2**. In near-term fetal offspring, plasma leptin concentrations were not different between LPO (0.95 ± 0.01ng/mL Human Equivalent (HE), n=13) and NPO (0.94 ± 0.01 ng/mL HE, n= 14). At 2 wks, plasma

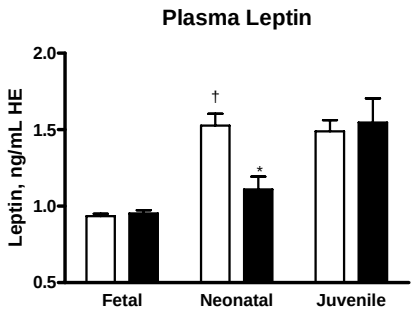

FIGURE 4-2: Plasma Leptin Concentrations in Fetal, Neonatal and Juvenile Offspring. Plasma leptin concentrations (mean ± SEM in ng/mL Human Equivalent, HE) are shown for near-term fetal, 2-wk neonatal, and 3-5 mo juvenile offspring. White bars, NPO; black bars, LPO. *LPO vs NPO within age group, p=.004 by unpaired t-test. †NPO neonate vs NPO fetus, p<.001 by Bonferroni post-test after two-way ANOVA.

leptin concentrations were reduced in LPO vs NPO (LPO: 1.10 ± 0.03 ng/mL HE, n=8; NPO: 1.53 ± 0.03 ng/mL HE, n=9; p=.004). In juvenile offspring, plasma leptin concentrations were not affected by MPR. In NPO, the plasma leptin concentrations were significantly higher in neonates than in fetal offspring, while in LPO, fetal and neonatal levels were similar, indicating a delayed maturational increase in leptin levels in LPO. Female juvenile offspring had increased plasma leptin concentrations compared to males (female: 1.79 ± 0.18 ng/mL HE, n=12; male: 1.31 ± 0.06 ng/mL HE, n=15; p=.01).

In juvenile IAT, linear regression showed that adipocyte size increased with age across the 3-5 mo juvenile period (r^2=.63, p<.0001; **Fig 4-3A)**. After adjusting for age, adipocyte size was not significantly altered by MPR, but tended to be reduced in LPO of both sexes (male NPO: 3224 ± 439; male LPO: 2546 ± 307; female NPO: 3711 ± 397; female LPO: 3350 ± 369, p=.14). Cell size also tended to be increased in females vs males (p=.11)(**Fig. 4-3B)**.

Adiponectin mRNA was decreased in both adipose tissue depots in LPO vs NPO (p=.01) (**Fig. 4-4**; all adipokine PCR data presented in **Table 4-3**). Adiponectin mRNA was higher in IAT than SAT (p<.0001). TNF-α mRNA levels tended to be decreased in LPO

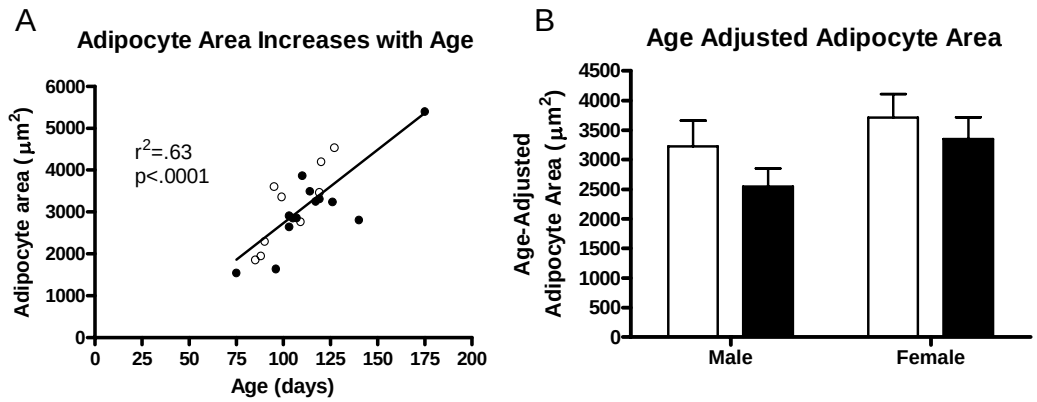

FIGURE 4-3: Adipocyte Size in Juvenile Offspring of Protein-Restricted Sows. A) Adipocyte size (mean area) vs age (in days) is shown for 3-5 mo juvenile offspring. ○, NPO. ●, LPO B) Adipocyte size (mean ± SEM in μm^2) is shown for 3-5 mo juvenile offspring. White bars, NPO (n=9; 4 male, 5 female); black bars, LPO (n=14; 8 male, 6 female).

vs NPO (p=.06), with values in IAT similar to those in SAT. There was a marginally significant interaction between maternal diet and sex with female LPO showing decreased TNF-α mRNA but no difference or elevated TNF-α mRNA in male LPO compared to sex-matched controls (p=.07).

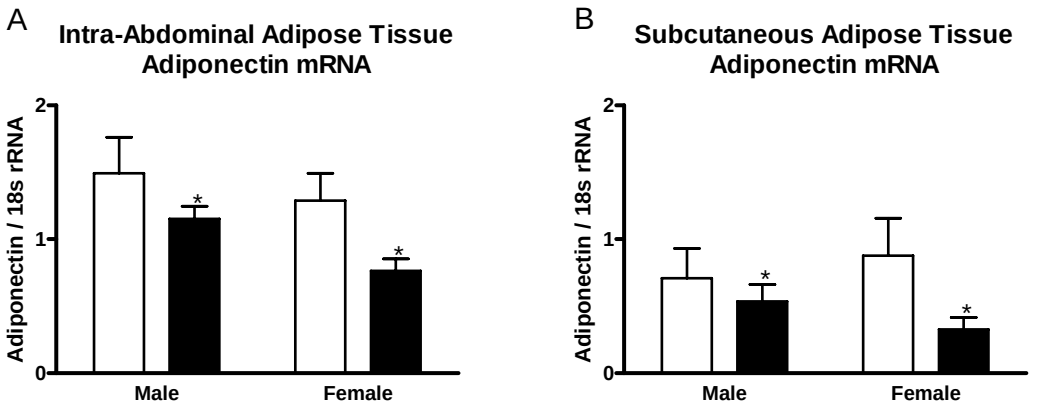

FIGURE 4-4: Adipose Tissue Adiponectin mRNA expression in Juvenile Offspring Following Maternal Protein Restriction. Adiponectin mRNA levels, normalized to 18s rRNA, are shown as mean ± SEM. White bars, NPO (n=10; 5 male, 5 female); black bars, LPO (n=16; 9 male, 7 female). A significant depot effect was observed, with subcutaneous adipose tissue expressing less adiponectin mRNA compared to intra-abdominal adipose tissue (p<.0001). *Maternal Diet, p<.02.

Adipocyte size did not correlate with serum leptin or IAT adiponectin or TNF-α mRNAs, either prior to or after age adjustment of cell sizes.

Discussion

In prepubertal juvenile offspring following MPR, we report unaltered adipocyte size and reduced adiponectin mRNA in IAT and SAT of both sexes. We also report a marginally significant (p=.06) reduction in TNF-α mRNA in both fat depots. These effects are observed in concert with an accelerated fat mass accrual and, at 11 wks, a normal body fat mass (see **Chapter 3**) and a normal plasma leptin concentration in juvenile LPO. In addition, in juvenile offspring we report a decrease in 11βHSD1 mRNA in both SAT and IAT in females as compared to males, and significant age effect in adipocyte size. This is the first study of adipocyte size in both male and female offspring in a developmental programming model, and the first study to report adiponectin mRNA levels in either sex in such a model. Additionally, this is the first report of adipocyte size and function in a large animal programming model wherein a large proportion of body fat is stored in the SAT depot, similar to the pattern observed in humans.

Adipocyte Size

We previously reported that MPR programs low birth weight and accelerated post-weaning growth, accompanied by increased rates of both fat and lean accrual without altered body composition at the point of weight equality. In the present report, adipocyte sizes in LPO were unaltered, although they tended to be smaller, compared to sex-matched controls. This finding clearly refutes our hypothesis that rapid fat mass accrual

generates increased adipocyte size. The lack of adipocyte hypertrophy is in agreement with two published reports in rat MPR models: Ozanne et al, reported smaller adipocytes in male rat offspring following MPR[203], while Guan et al, reported, in a similar rat MPR model with visceral obesity in male adult offspring, no difference in adipocyte size[104]. A reduction in adipocyte size, together with normal quantities of body fat, suggest hypercellularity of adipose tissue, which may lead to increased risk of developing obesity later in life.

In these juvenile microswine, adipocyte size increased with age. This is in agreement with a study in human children and adolescents showing that both adipocyte size and number increase throughout development[204]; in that study, adipocyte size and number were increased in obese vs lean, and appeared to track over time, so that lean children with small adipocytes early on still had relatively small adipocytes as young adults[204]. This suggests that cell size hypertrophy is unlikely to develop in microswine LPO as they age.

Increased adipocyte size has been observed in DIO and genetic obesity, and is associated with increased production of the leptin and reduced production of adiponectin. In contrast, in rodent MPR models with adult obesity, reduced or unaltered adipocyte size has been observed in male offspring; female offspring have not been studied[145,104]. However, neither of these studies reported adiponectin or TNF-α levels, which have been shown to be inversely (for adiponectin) or directly (for TNF-α) related to adipocyte size and to whole-body fat mass.

Adiponectin

Adiponectin mRNA levels are reduced in LPO in both sexes and in both fat depots. This reduction is observed in the absence of both obesity and adipocyte hypertrophy. The possibility that adiponectin protein levels or high-molecular weight:total adiponectin ratios may be increased is not ruled out by our study, but the results reported here seem contrary to the typically-reported inverse relationship between adiponectin and adipocyte size. The reduction in adiponectin mRNA, if found to be concordant with activity, would imply that LPO have a greater risk of developing insulin resistance over time, and would suggest that risk of obesity is also enhanced via impaired adiponectin-dependent β-oxidation. In light of the multiple actions of adiponectin on fat deposition in adipocytes, fat oxidation in non-adipose tissue, insulin sensitivity, and atherogenic disease, it is possible that the reduction in adiponectin transcription is a major contributor to the increased risk in adults born small.

Leptin

Leptin, a cytokine produced almost exclusively by adipocytes, is secreted into the circulation and acts to regulate appetite and body fat on a long-term basis. Plasma levels of leptin generally increase with increasing body fat mass. Microswine LPO plasma leptin levels were low at 2 wks, the end of the protein restriction period when body weights in LPO are significantly reduced compared to NPO (see **Chapter 3**). Leptin levels are normal in juvenile LPO, when body composition has also normalized (see **Chapter 3**). This pattern reflects a delayed maturational increase in leptin levels over fetal, neonatal, and juvenile ages. The increased leptin in juvenile females over males,

along with the greater accrual of body fat in females by 11wks (**Chapter 3**), suggests that the leptin-body fat relationship is maintained normally in LPO.

While the pattern of leptin levels in LPO vs NPO reflects the pattern of body weight, and presumably of body fat, low circulating leptin observed in LPO during the neonatal period may affect development of the appetite-regulating neural circuitry. In rodent models, low leptin levels during the early postnatal period caused altered neuronal development in the paraventricular nucleus of the hypothalamus, the brain center largely responsible for controlling appetite[205]. Aberrant neuronal development due to low leptin levels in microswine LPO may contribute to the increased appetite observed from 6-12 wks in these same offspring (see **Chapter 3**).

TNF-α

TNF-α is produced by both adipocytes and macrophages in adipose tissue, and it is thought to be an initiator of the inflammatory response, attracting more inflammatory cells to the adipose tissue. It is also involved in the development of insulin resistance in genetic or diet-induced obesity. In all published reports except one (involving antiretroviral drug in adipocyte cell culture), adiponectin and TNF-α levels (both protein and mRNA) are inversely related, with low circulating adiponectin levels and increased TNF-α levels being associated with insulin resistance. In contrast, in our microswine offspring, adiponectin is reduced while TNF-α is maintained at normal or marginally reduced levels, but certainly is not increased. The reason for the discrepancy between our data and those reported in the literature are unknown; however, a rat MPR model with

adult visceral obesity showed reduced transcription of many inflammatory genes by microarray of white adipose tissue[104]. It is thus possible that obesity and adipocyte dysfunction observed following developmental programming are fundamentally different from that observed in diet-induced or genetic forms of obesity.

The current report demonstrates that MPR and/or the consequent post-weaning growth acceleration result in potentially adverse metabolic consequences without adipocyte hypertrophy and without the development of obesity. This is in agreement with human epidemiological studies which show that the children who go on to develop both obesity and metabolic disease (Type II diabetes or impaired glucose tolerance) as adults are those who were both born small and also grew rapidly to catch up to normal body weight by age 12 yrs. Importantly, juvenile obesity was not required for increased risk of adult disease[206].

A large body of data suggests that altered GC production and/or activity are related to increased risk of cardiovascular disease and obesity in offspring following MPR and MNR(see Review[151]). Also, it is well-known that excess cortisol, either in circulation or at a local level, causes increased fat accrual, especially in the visceral compartment. In neonatal microswine LPO there is a decrease in plasma cortisol levels that does not persist (**Chapter 3**). This led to the hypothesis that the reduction in plasma cortisol could cause a persisting increase in tissue indices of cortisol activity, which would achieve a normal cortisol response at 2 wks (adaptive), but an abnormally increased cortisol response later on. However, in our microswine, we observed no significant differences in

indices of tissue-level GC activity at neonatal or juvenile ages. On the other hand, a trend toward a decrease in GR-α mRNA abundance in SAT in LPO, without a corresponding decrease in GR-α mRNA in IAT increases the ratio of abdominal-to-subcutaneous GR-α in LPO (50% higher than NPO). This may shift lipid deposition from the subcutaneous toward the abdominal compartment, a pattern associated with adverse metabolic and cardiovascular consequences. We previously showed in these same LPO that truncal fat deposition (assessed by dual energy X-ray absorptiometry, DEXA, scan) was low at 6 wks ($p=.06$) but normal at 11 wks in LPO, reflecting a more rapid accumulation of central fat (**Chapter 3**). While DEXA cannot distinguish truncal subcutaneous from truncal intra-abdominal adipose tissue, this GR-α imbalance creates the potential for development of abdominal obesity over time.

The present study reports a decrease in 11βHSD1 mRNA in female IAT and SAT compared to males. While this difference was not observed in human preadipocytes in culture[207], whole-body 11βHSD1 activity is reduced in women compared to men[208], and 11βHSD1 mRNA in abdominal subcutaneous adipose tissue is lower in women than in men (Jonathan Purnell, personal communication).

Findings from Chapters 3 and 4 are summarized in **Fig. 4-5**. MPR programs asymmetric growth restriction with reduced plasma leptin concentration in neonates. These offspring, once weaned to ad lib diet, exhibit hyperphagia and increased feed utilization efficiency, characteristic of the Thrifty Phenotype. Though accelerated growth and rapid fat accrual occur over 6-12 wks, obesity and adipocyte hypertrophy do not develop. In spite of

normal adipocyte size and body fat mass, adiponectin transcription is reduced in both IAT and SAT. The reduction in adiponectin transcription may be responsible for the altered glucose homeostasis observed in juvenile offspring. The results presented in this chapter support the concepts 1) that nutritional programming of adipose tissue function is distinct from diet-induced or genetic forms of obesity; 2) that reduced adiponectin may be the mechanism through which accelerated growth conveys adverse metabolic effects independently of total body fat and independently of adipocyte size; and 3) that adiponectin itself is regulated by fat-independent mechanisms.

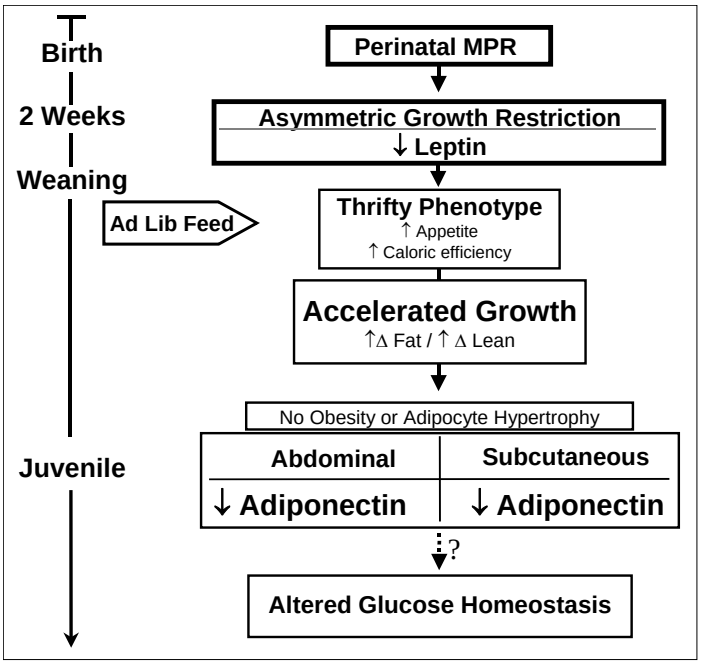

FIGURE 4-5: Effects of Perinatal Maternal Protein Restriction in Offspring. MPR results in thrifty phenotype and accelerated accrual of fat without obesity or adipocyte hypertrophy. Despite normal fat mass and adipocyte size, adiponectin transcription is reduced, and may lead to the altered glucose homeostasis observed in juvenile offspring.

TABLE 4-1: Real-time PCR Primer Sequences

Transcript/ Accession #	Forward (5'-3')	Reverse (5'-3')	Amplicon Length
18s rRNA	CAGCAGCCGCGGTAATTC	ACGAGCTTTTTAACTGCAGCAA	64
AF179868			
GAPDH	AGAACGGGAAGCTTGTCATC	TCTCATACTTCTCATGGTTC	237
U48832			
GR-α*	CATTGTCAAGAGGGAAGGAAACTC	GATTTTCAACCACATCATGCATGGA	95
NM001008481			
11βHSD1*	GAATATTCAGTGACCAAGGTCAA	TAATTTCCAGGGCGCATTCTT	139
NM214248			
LPL	GCTCCAAGCCGCCTTTC	GACCCTCTGGTGAATGTGTGTAAG	68
NM214286			
Adiponectin	GGCCGTGATGGCAGAGAT	CCCTTAGGACCAGTAAGACCTGTATC	81
AY672882			
TNF-α	CTCTGGCCCAAGGACTCAGA	GTGGGCGACGGGCTTAT	60
X54859			

Sequences for real-time PCR primers. Primers were designed using Primer Express software (Applied Biosystems) and synthesized by Operon Biotechnologies

*Primer sequences for GR-α and 11βHSD1 were adapted for the pig from the human primers published by Wake et al[159].

TABLE 4-2: Adipose and Skeletal Muscle Tissue Indices of Glucocorticoid Activity in Fetal, Neonatal and Juvenile Offspring of Protein Restricted Sows [1]

	NPO			LPO		
	Male	Female	All	Male	Female	All
2 Wk Neonatal						
Muscle GR-α mRNA[2]	0.95 ± 0.07* n=5	1.51 ± 0.35* n=4	1.23 ± 0.28 n=9	0.99 ± 0.12* n=5	1.37 ± 0.05* n=3	1.18 ± 0.19 n=8
Muscle 11βHSD1 mRNA[2]	0.86 ± 0.08 n=5	1.36 ± 0.42 n=4	1.11 ± 0.25 n=9	1.06 ± 0.08 n=5	1.26 ± 0.14 n=3	1.16 ± 0.10 n=8
3-5 Mo Juvenile						
Abd Adipose GR-α mRNA[2]	1.34 ± 0.16 n=5	1.19 ± 0.10 n=5	1.27 ± 0.07 n=1 0	1.14 ± 0.07 n=9	1.35 ± 0.24 n=7	1.24 ± 0.11 n=16
Ab Adipose 11βHSD1 mRNA[2]	1.36 ± 0.12** n=5	0.78±0.06** n=5	1.07 ± 0.29 n=10	1.47±0.19** n=9	0.90±0.22** n=7	1.19 ± 0.28 n=16
Abd Adipose LPL mRNA[2]	1.01 ± 0.19 n=5	0.89 ± 0.08 n=5	0.95 ± 0.06 n=10	0.76 ± 0.08 n=8	1.13 ± 0.16 n=7	0.94 ± 0.19 n=15
SC Adipose GR-α mRNA[2]	0.71 ± 0.14 n=5	0.67 ± 0.20 n=5	0.69±0.02 n=10	0.51 ± 0.07 n=9	0.42 ± 0.08 n=7	0.47±0.04 n=16
SC Adipose 11βHSD1 mRNA[2]	1.27±0.24* n=5	0.65±0.15* n=5	0.96 ± 0.31 n=10	0.99±0.15* n=9	0.60±0.11* n=7	0.80 ± 0.20 n=16
SC Adipose LPL mRNA[2]	0.63 ± 0.13 n=5	0.87 ± 0.27 n=5	0.75 ± 0.12 n=10	0.48 ± 0.08 n=9	0.55 ± 0.16 n=7	0.51 ± 0.04 n=16
Muscle GR-α mRNA[2]	1.31 ± 0.45 n=4	0.95 ± 0.08 n=5	1.13 ± 0.18 n=9	1.17 ± 0.11 n=8	1.25 ± 0.15 n=5	1.21 ± 0.04 n=13
Muscle 11βHSD1 mRNA[2]	1.20 ± 0.49 n=4	1.22 ± 0.47 n=5	1.21 ± 0.01 n=9	1.63 ± 0.56 n=8	1.14 ± 0.13 n=5	1.39 ± 0.25 n=13

[1]Data are presented as mean ± SEM. Data were analyzed by two-way ANOVA with maternal diet and sex as factors. * Male vs female, p=.02 **Male vs female p =.002 . Abbreviations: Normal Protein Offspring, NPO; Low Protein Offspring, LPO; Glucocorticoid Receptor alpha, GR-α; 11β-Hydroxysteroid Dehydrogenase type I, 11βHSD1; Lipoprotein Lipase, LPL.

[2]Juvenile adipose tissue and neonatal muscle mRNA values are ratios of transcript-of-interest to 18S rRNA; juvenile muscle mRNA values are ratios of transcript-of-interest to GAPDH.

TABLE 4-3: Adipose Tissue Cytokine mRNA in Juvenile Offspring of Protein Restricted Sows[1]

	NPO			LPO		
	Male	Female	All	Male	Female	All
Abdominal						
Adiponectin† mRNA	1.49 ± 0.27 n=5	1.29 ± 0.21 n=5	1.39 0.16* n=10	1.15 ± 0.09 n=9	0.76 ± 0.09 n=7	0.98 ±0.08* n=16
TNF-α mRNA	1.28 ± 0.87 n=5	2.62± 1.10 n=5	1.95 ± 0.70 n=10	3.02 ± 0.97 n=9	0.68± 0.23 n=7	2.00 ± 0.62 n=16
Subcutaneous						
Adiponectin† mRNA	0.71 ± 0.22 n=5	0.88 ± 0.28 n=5	0.79 ± 0.17 n=10	0.54 ± 0.13 n=9	0.33 ± 0.09 n=7	0.44 ± 0.08 n=16
TNF-α mRNA	1.50 ± 0.51 n=5	1.88 ± 0.94 n=5	1.69 ± .51* n=10	1.26 ± 0.59 n=9	0.37 ± 0.21 n=7	0.87 ±0.35* n=16

[1]Data are presented as mean ± SEM of ratios of transcript-of-interest to 18S rRNA. Data were Ln transformed then analyzed by two-way ANOVA with maternal diet and sex as factors. *NPO vs LPO, $p<.03$; †Intra-abdominal adipose tissue > subcutaneous adipose tissue, $p<.0001$.

Abbreviations: Normal Protein Offspring, NPO; Low Protein Offspring, LPO; Tumor Necrosis Factor-α, TNF-α

Chapter 5: Post-Weaning Feed Limitation Prevents Accelerated Growth and Adipocyte Dysfunction in Juvenile Microswine Offspring Following Maternal Protein Restriction

ABSTRACT

Low birth weight and accelerated postnatal growth are associated with increased risk of cardiovascular and metabolic diseases later in life. Late-gestation/early lactation maternal protein restriction (MPR) in microswine causes low birth weight, accelerated post-weaning growth, altered plasma glucose and reduced adiponectin transcription in juveniles without adipocyte hypertrophy or obesity. It is unknown whether the altered adipocyte function and plasma glucose levels are programmed by MPR directly during nutrient restriction, or indirectly via accelerated growth. Microswine offspring exposed to MPR were randomized to either *ad libitum* diet or Feed Limitation (FL) from weaning onward. FL was designed to maintain body weight percent reduction at the level observed at 2 wks, thereby preventing accelerated growth in Low Protein Offspring (LPO). Growth rates were reduced in FL vs ad lib offspring. Lean mass was reduced in both male and female LPO, but % body fat was reduced only in females. Post-weaning FL reduced intra-abdominal (IAT) adipocyte size and TNF-α mRNA similarly in LPO and NPO. The reduced adiponectin mRNA in LPO was abolished by post-weaning FL in IAT but not subcutaneous adipose tissue (SAT). Adiponectin and TNF-α mRNAs were reduced in SAT vs IAT. Plasma glucose alterations were not affected by post-weaning FL. Thus, IAT adiponectin mRNA levels following MPR are determined by post-weaning growth rate. In contrast, plasma glucose levels may be programmed by MPR independently of both accelerated growth and of adipocyte size and function. LPL transcription is unexpectedly altered in LPO females in a way that may cause shunting of fat deposition to the IAT depot. Reduced adiponectin transcription may be the link between early growth restriction and later risk of metabolic and cardiovascular disease.

Introduction

Low birth weight causes increased vulnerability to development of cardiovascular and metabolic diseases later in life. Accelerated postnatal growth, characterized by upward crossing of weight centiles, exacerbates risk. Previously we showed that late-gestation plus early lactation maternal protein restriction (MPR) in microswine causes low birth weight and accelerated post-weaning growth in offspring. This was associated with reduced adiponectin transcription in adipose tissue and altered plasma glucose levels suggestive of insulin resistance (in males but not females) in juvenile offspring.

While epidemiological studies have been instrumental in showing the patterns of growth associated with particular diseases in adulthood, it is unknown whether increased risk is programmed directly by the factors influencing prenatal and postnatal growth, or indirectly via accelerated growth. Using the same microswine MPR model as previously described, we assessed whether post-weaning Feed Limitation would restore adipocyte function and glucose homeostasis to control levels. The results presented here are preliminary pending verification by addition of more animals.

Methods and Materials

Animal Care and Use

Time-bred Yucatan microswine were obtained from Sinclair Laboratories (formerly Charles River Laboratories) and maintained in the OHSU Department of Comparative Medicine until study under approved OHSU Institutional Animal Care and Use Committee protocol (A439). Sows were randomly assigned to either Normal Protein

(14%, NP) or Low Protein (1%, LP) diet for the last quarter of gestation (day 85 of gestation of 115) plus the first two wks postnatally as described in **Chapter 2: Methods and Materials**. Sows were returned to normal diet (Purina Mills Lab Porcine Diet Grower) at 2 wks after delivery. Sows and offspring were housed under 12hr:12hr light:dark cycle at constant temperature, and ceramic heaters were available from two days prior to expected due date through weaning for piglets. At 4 wks of age, piglets were weaned to *ad libitum* or Feed Limited (FL) diets (n=13; 3 male NPO-FL, 3 female NPO-FL, 4 male LPO-FL, 3 female LPO-FL; 1 NPO-FL litter, 3 LPO-FL litters). Ad lib animals included in this report are identical to those studied previously. Because of the low numbers and low litter numbers included in the FL groups, these data are considered to be preliminary, and conclusions will be verified following study of additional litters.

Feed Limitation

As reported in Chapter 3, body weight reduction in Low Protein Offspring (LPO) was greater at 2 wks than at birth. The weight deficit for each animal was therefore calculated as a percentage of the sex-matched Normal Protein Offspring (NPO) average reported in Chapter 3 at 2 wks of age. FL offspring were offered a specific amount of feed designed to keep offspring weights reduced to the same degree throughout postnatal development as calculated at 2 wks for each individual. Because LPO were previously shown (**Chapter 3**) to eat ~16% more feed than NPO, feed was initially offered at 25g/kg/meal (two meals per day), approximately the same amount consumed by NPO offspring. This was adjusted as necessary every 2-3 days, never falling below 20g/kg, to keep FL

offspring on the projected growth trajectory (see **Fig. 5-1** for example). Actual feed intake was recorded by measuring the amount of feed remaining (if any) after each meal.

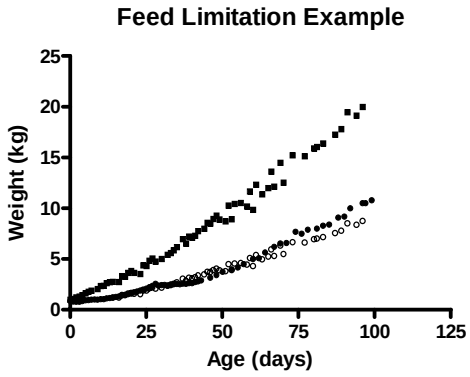

FIGURE 5-1: Feed Limitation Example. To calculate each individual's targeted growth rate, weights at 2 wks were converted to percentage reduction compared to the average sex-matched control. In this example, this LPF piglet weight was 56.2% of NPF mean on postnatal day 14. Therefore, from the day of weaning (d28) onward, a weight trajectory for this animal was plotted by calculating 56.2% of NPF mean for each data point available. Feed was offered at 25 g/kg initially; weights were measured ≥3 d/wk and feed was adjusted as necessary, between 20-30 g/kg, to maintain weights on the projected growth trajectory.■, Sex-matched NPO-AdLib Means; ○, Projected Weights; ●, Actual Weights

Body Composition

Body composition was measured by dual energy X-ray absorptiometry (DEXA) scan at 6 and 11 wks of age as described in Chapter 3. Regional analysis of fat and lean mass was performed using pediatric software.

Tissue, Plasma and Urine Collection

24-hr urine collections were made at 12 wks of age as described in Chapter 3. Tissues and plasma were collected in 3-5 mo-old juvenile offspring as described in Chapter 4. Fasting plasma glucose was measured by the OHSU Clinical Laboratory.

Adipocyte Size

Formalin-fixed paraffin embedded intra-abdominal adipose tissue slides were analyzed by non-biased image analysis software as described in Chapter 4.

Real-Time PCR

Total RNA was extracted from intra-abdominal (IAT) and subcutaneous (SAT) adipose tissue and gastrocnemius skeletal muscle using TRIzol (Invitrogen Corp, Carlsbad, CA), then was reverse transcribed as described in Chapter 4. cDNA was analyzed by real-time PCR using the SYBR Green method using primers for adiponectin, TNF-α, glucococorticoid receptor (GR)-α, 11βHSD1, lipoprotein lipase (LPL), glyceraldehyde phosphate dehydrogenase (GAPDH), and 18s rRNA as described in Chapter 4.

Cortisol and Leptin RIA

Commercially available RIA kits for cortisol (Coat-A-Count Cortisol RIA kit, Siemens Medical Solutions Diagnostics, Los Angeles, CA) and leptin (Millipore, Inc., Billerica, MA; formerly Linco Research, St. Charles, MO) were used as described in Chapters 3 (cortisol) and 4 (leptin) to analyze plasma levels.

Statistical Methods

Growth rates were analyzed by collapsing daily weights into weekly values, then using linear regression to determine rate of weight accrual over 6-12 weeks (GraphPad Prism). Body composition, circulating and tissue GC-related parameters and plasma leptin data were analyzed using GraphPad Prism. Preliminary analysis was done to determine whether sex differences existed for each parameter. If sex differences were observed, sexes were analyzed separately, but if not, sexes were pooled for analysis; data were analyzed by two-way ANOVA using maternal diet and post-weaning diet. Adipocyte size and function (adiponectin and TNF-α mRNA) data were analyzed using SPSS

software. Using General Linear Modeling Univariate Analysis, it was first determined whether age was a significant covariate. For adipocyte size, age was used as a covariate, and maternal diet, post-weaning diet, and sex were used as factors in analysis. For adiponectin and TNF-α mRNA, age did not significantly affect expression, so maternal diet, post-weaning diet, sex, and adipose depot were used as factors.

Results

It is important to note that results from the FL series is still preliminary, based on one litter of NPO-FL (n=6) and 3 litters of LPO-FR (n=7).

Growth rates

Weights from 6-12 wks were condensed into weekly values for each diet group (NPO-AdLib, LPO-AdLib, NPO-FL, LPO-FL), then analyzed by linear regression to obtain growth rates (**Fig. 5-2)**. Rates of weight gain were 1.63 ± 0.05 kg/wk for NPO, 1.98 ± 0.09 kg/wk for LPO, 1.38 ± 0.04 kg/wk for NPO-FL, and 1.49 ± 0.11 kg/wk for LPO-FL. Weight gain was significantly increased in LPO-AdLib vs NPO-AdLib ($p<.01$ by Bonferroni post-test after two-way ANOVA of slope of weight gain), but LPO-FL was not different compared to NPO-FL. Rates of weight gain also were not different between NPO-AdLib and NPO-FL, although small numbers limit this comparison.

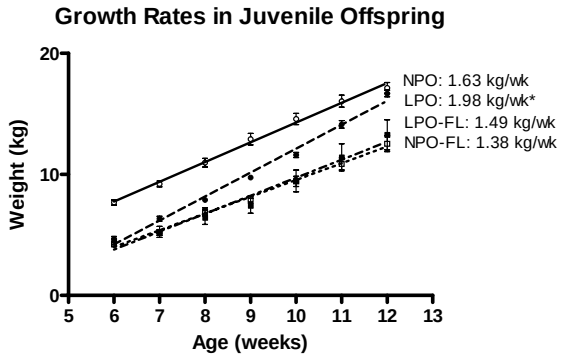

FIGURE 5-2: Growth Rates for Offspring of Protein-Restricted Sows with or without Post-Weaning Feed Limitation. Weekly values shown are mean ± SEM condensed from several data points for each animal: ○, NPO-AdLib; ●, LPO-AdLib; □, NPO-FL; ■, LPO-FL. Linear regression from 6-12 wks is shown for each group. *Growth rate > NPO growth rate, $p<.001$.

Feed intake

Feed intake results are shown in **Fig. 5-3**. Feed intake was adjusted on an individual basis to maintain a projected growth pattern. However, at times some animals did not eat all feed offered. Feed intake patterns show a "ramp up" period following weaning in all groups, achieving an average level from weeks 6-12 of 29.0 ± 1.3 g/kg body weight/meal in NPO-AdLib, 33.7 ± 6 g/kg/meal in LPO-AdLib, 25.7 ± 0.3 g/kg/meal in NPO-FL, and 26.5 ± 0.7 g/kg/meal in LPO-FL. After this time, both NPO-FL and LPO-FL had a voluntary decrease in feed intake over time (i.e., offspring did not eat all the feed offered); low n in groups after week 12 preclude formal analysis. Feed utilization efficiency decreased over 6-12 wks in all groups.

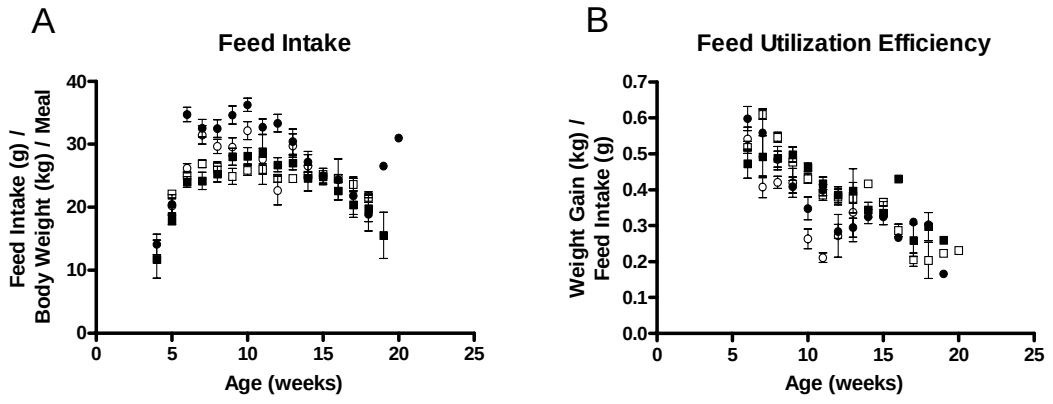

FIGURE 5-3: Feed Intake and Feed Utilization Efficiency in Offspring of Protein-Restricted Sows with or without Post-Weaning Feed Limitation. Feed intake (A) and feed utilization efficiency (B) are shown as mean ± SEM for NPO-AdLib (○), LPO-AdLib (●), NPO-FL (□), and LPO-FL (■) over 5-20 weeks. A) Average feed intake in NPO-AdLib (n=3-11), LPO-AdLib (n=3-18), NPO-FL (n=3-7), LPO-FL (n=4-8). B) Feed utilization efficiency in NPO (n=3-11), LPO (n=3-18), NPO-FL (n=4-6), LPO-FL (n=2-8).

Body composition

At 6 wks (two weeks after weaning), post-weaning FL did not affect percent body fat or the ratio of fat mass to lean mass. Truncal adiposity was altered in males: a significant

interaction between maternal diet and postnatal diet was observed, with male NPO-FL having less and LPO-FL having more of their fat distributed in the trunk region compared to AdLib maternal diet matched controls (p=.001, **Fig. 5-4A**; see **Appendix, Table A-1** for a complete statistical summary of body composition parameters in all offspring). Females had no significant changes in truncal adiposity. Normalized lean mass at 6 wks was unaffected by sex, so sexes were pooled for analysis. Post-weaning FL caused a significant reduction in normalized lean mass (p<.0001), and a significant interaction between maternal diet and post-weaning FL was observed (p=.009), indicating the reduction in lean mass was larger in NPO-FL compared to LPO-FL due to the trend in LPO-Ad Lib toward lower lean mass compared to NPO-Ad Lib (**Fig. 5-4B**).

By 11wks, post-weaning FL in females caused large reductions in % body fat (**Fig. 5-5A**), ratio of fat mass to lean mass (**Fig. 5-5B**), and fold-increase in fat mass over 6-11

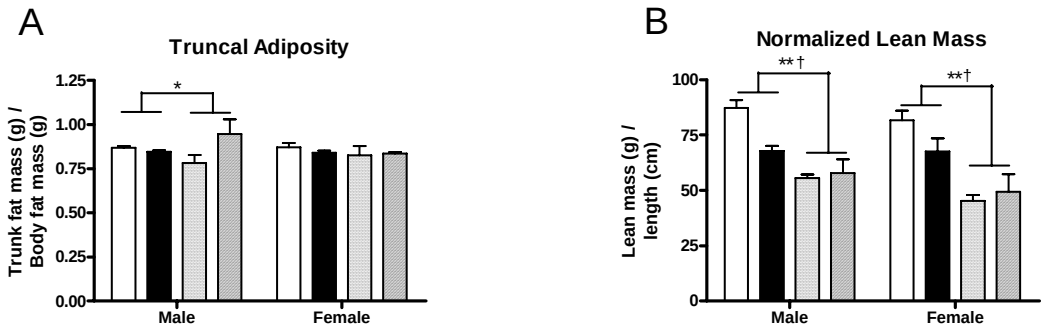

FIGURE 5-4: Body Composition at 6 Weeks in Offspring of Protein Restricted Sows with or without Post-Weaning Feed Limitation. Truncal adiposity and normalized lean mass in male and female offspring age 6 wks are shown as mean ± SEM. White bars, NPO-AdLib (n=12: 7 male, 5 female); black bars, LPO-AdLib (n=14: 8 male, 6 female); dotted bars, NPO-FL (n=7: 3 male, 4 female); hatched bars, LPO-FL (n=5: 3 male, 2 female). A) Truncal adiposity (g fat mass in trunk / g whole-body fat mass) B) Normalized lean mass (g lean mass / cm length). *Interaction between Maternal Diet and Postnatal Diet, p<.001; **Postnatal Diet, p<.0001; †Interaction between Maternal Diet and Postnatal diet, p=.009. See **Appendix: Table A-1** for statistical summary of body composition.

wks compared to Ad Lib offspring (**Fig. 5-6A;** p<.0001 for each measure), but truncal adiposity was not affected by FL. In males, no measure of adiposity was altered by FL, and the fold-increase in fat mass over 6-11 wks was not different in FL vs Ad Lib offspring. Normalized lean mass was reduced by FL in both males (p=.0003) and females (p=.0009; **Fig. 5-5C**). Fold-increase in lean mass over 6-11 wks was not affected by sex, so males and females were pooled for analysis. There was a significant interaction (p=.004) between maternal diet and post-weaning diet, with fold-increases in lean mass being higher in both NPO-FL and LPO-FL compared to NPO-Ad Lib, but similar compared to LPO-Ad Lib (**Fig. 5-6B**). The ratio of the increase in fat to the increase in lean was reduced by FL in females but not changed in males, indicating a relative protection of lean mass at the expense of fat accrual in females (**Fig. 5-6C**).

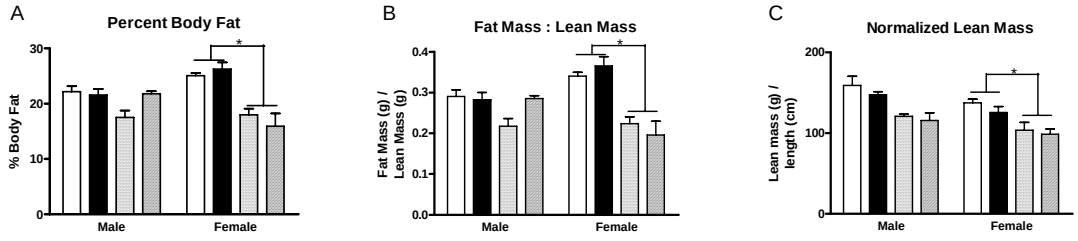

FIGURE 5-5: Body Composition at 11 Weeks in Offspring of Protein Restricted Sows with or without Post-Weaning Feed Limitation. Percent body fat, ratio of fat to lean, and normalized lean mass in male and female offspring age 11 wks are shown as mean ± SEM. White bars, NPO-AdLib (n=9: 4 male, 5 female); black bars, LPO-AdLib (n=14: 8 male, 6 female); dotted bars, NPO-FL (n=6: 3 male, 3 female); hatched bars, LPO-FL (n=8: 4 male, 4 female). A) Percent body fat (g fat mass / g total body mass) B) Ratio of fat mass : lean mass (g fat mass / g lean mass) C) Normalized lean mass (g lean mass / cm length). * Postnatal Diet, p<.005; **Interaction between Maternal Diet and Postnatal Diet, p<.01. See **Appendix: Table A-1** for statistical summary of body composition.

Glucocorticoids

Plasma cortisol levels were not significantly altered by post-weaning feed limitation. 24-hr urinary cortisol excretion, either absolute value or normalized to lean body mass, was also not affected by post-weaning FL. Positive correlations between % body fat and 24-

hr urinary cortisol (both absolute values and normalized to lean body mass) as reported in Chapter 3 for Ad Lib offspring were completely abrogated by post-weaning FL.

Glucocorticoid-related mRNA data are presented in **Table 5-1**. Post-weaning FL did not affect mRNA levels of GR-α or 11βHSD1 in skeletal muscle, IAT or SAT in male or female offspring. In females, LPL mRNA was increased in LPO-FL compared to NPO in IAT ($p<.05$), but the same transcript was decreased in LPO-FL compared to NPO in SAT ($p<.05$).

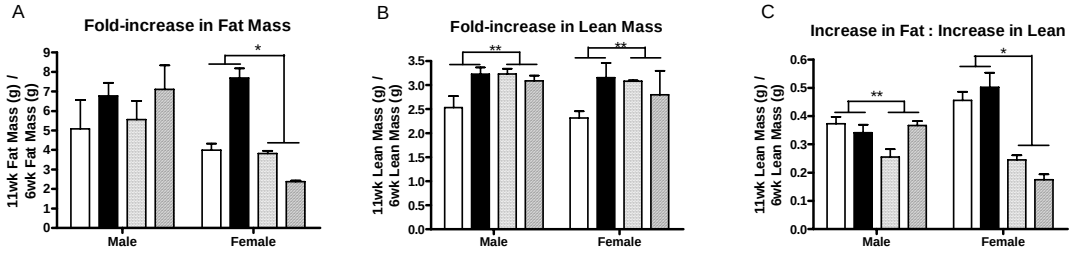

FIGURE 5-6: Fold-increases in Fat and Lean Mass in Offspring of Protein Restricted Sows with or without Post-Weaning Feed Limitation. The fold-increases in fat and lean mass over weeks 6 to 11 are shown as mean ± SEM. White bars, NPO-AdLib (n=9: 4 male, 5 female); black bars, LPO-AdLib (n=12: 7 male, 5 female); dotted bars, NPO-FL (n=6; 3 male, 3 female); hatched bars, LPO-FL (n=5; 3 male, 2 female). A) Fold increase in fat mass B) Fold increase in lean mass C) Ratio of the increase in fat mass to the increase in lean mass. ** Interaction between Maternal Diet and Postnatal Diet, p=.004. See Appendix Table A-1 for statistical summary of body composition.

Adipose Tissue Size and Function

Adipocyte size in IAT was significantly correlated with age ($p<.0001$). After adjusting for age, adipocyte size was reduced by post-weaning FL (age-adjusted means ± SEM: AL, 3646 ± 177; FL, 2012 ± 236; $p<.0001$; **Fig 5-7**), and females had larger adipocytes than males (age-adjusted means ± SEM: Male, 2446 ± 185; Female, 3211 ± 187; p=.007). The effect of FL to reduce adipocyte size was slightly smaller in LPO than in NPO (age-

adjusted means ± SEM: NPO-AdLib, 3843 ± 272; NPO-FL, 1718 ± 320; LPO-AdLib, 3448 ± 208; LPO-FL, 2306 ± 306; p=.07).

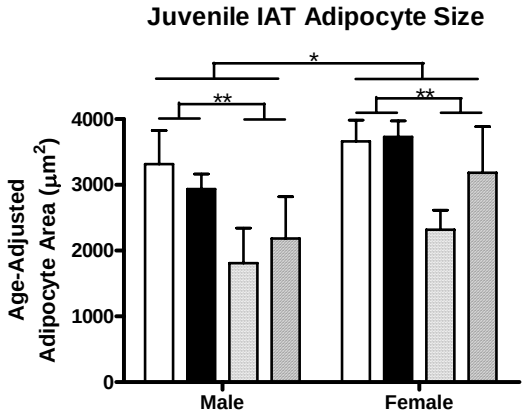

FIGURE 5-7: Age-adjusted Intra-Abdominal Adipocyte Sizes. Adipocyte size (mean ± SEM in μm^2) is shown for 3-5 mo juvenile offspring. White bars, NPO-AdLib (n=9; 4 male, 5 female); black bars, LPO-AdLib (n=15; 7 male, 6 female); dotted bars, NPO-FL (n=6; 3 male, 3 female); hatched bars, LPO-FL (n=7; 4 male, 3 female). *Male vs Female, p=.007; **AdLib vs FL, p<.0001

Plasma leptin levels in male LPO were marginally increased by post-weaning FL (Male LPO-AdLib, 1.25 ± 0.05; Male LPO-FL, 1.95 ± 0.55; p<.06; **Fig. 5-8**). Plasma leptin levels in NPO and female LPO were not affected by post-weaning FL.

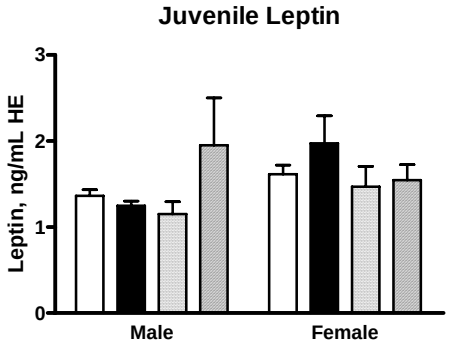

FIGURE 5-8: Juvenile Plasma Leptin in Ad Lib and Feed Limited Offspring. Plasma leptin levels (in ng/mL Human Equivalents, HE) are shown as mean ± SEM. White bars, NPO-AdLib (n=9; 4 male, 5 female); black bars, LPO-AdLib (n=17; 10 male, 7 female); dotted bars, NPO-FL (n=6; 3 male, 3 female); hatched bars, LPO-FL (n=7; 4 male, 3 female).

Adiponectin and TNF-α mRNA values are presented in **Table 5-2**. In juvenile IAT, adiponectin mRNA deficit observed in LPO was restored by post-weaning FL to levels

no longer different from controls (interaction between maternal diet and postnatal diet, p=.015; **Fig. 5-9**). In contrast, in juvenile SAT, adiponectin mRNA deficit observed in LPO was not improved by post-weaning FL. TNF-α mRNA levels were marginally decreased by post-weaning FL (p=.08).

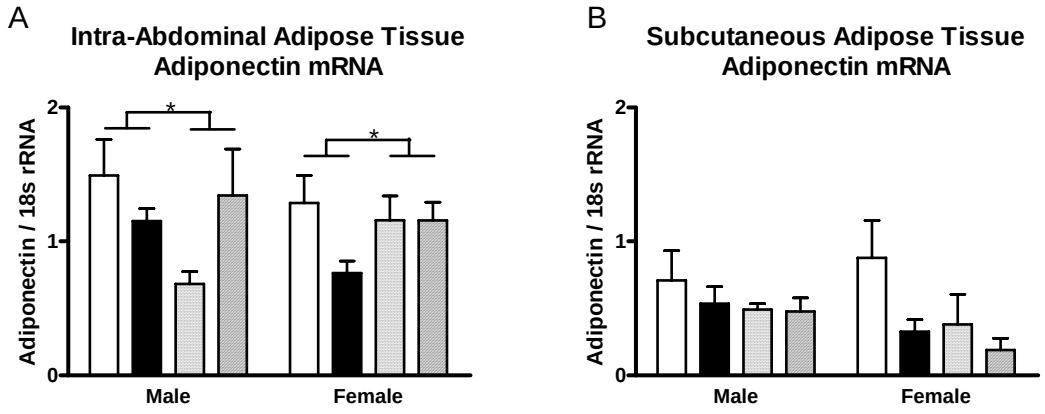

FIGURE 5-9: Adipose Tissue Adiponectin mRNA Expression in Juvenile Ad Lib and Feed Limited Offspring. Adiponectin mRNA levels, normalized to 18s rRNA, are shown as mean ± SEM. White bars, NPO-AdLib (n=10; 5 male, 5 female); black bars, LPO-AdLib (n=16; 9 male, 7 female); dotted bars, NPO-FL (n=6; 3 male, 3 female); hatched bars, LPO-FL (n=7; 4 male, 3 female). A significant depot effect was observed, with subcutaneous adipose tissue expressing less adiponectin mRNA compared to intra-abdominal adipose tissue (p<.0001). *Maternal Diet by Post-Weaning Diet Interaction, p<.02.

Adipocyte size correlated with plasma leptin concentrations both prior to and after age adjustment of cell sizes (r^2=.14, p<.03 for both correlations). Adipocyte size did not correlate with either adiponectin or TNF-α mRNA in IAT, either prior to or after age adjustment of cell sizes.

Fasting Plasma Glucose

Fasting plasma glucose (measured only in 4 LPO-FL offspring, and no NPO-FL offspring) appeared to be unaffected by post-weaning FL compared to sex-matched Ad Lib LPO.

Discussion

Individuals born with low birth weight who subsequently undergo accelerated growth during childhood are at increased risk for the development of metabolic and cardiovascular disease. However, it is unknown whether these risks are programmed directly by prenatal and early postnatal environment, or indirectly via accelerated growth often associated with adverse early environments. We therefore used a microswine model to address whether post-weaning feed limitation would correct adipose tissue dysfunction and glucose homeostasis abnormalities previously observed in low birth weight offspring following MPR.

As discussed in Chapter 4, juvenile LPO do not have adipocyte hypertrophy, and even tend to have smaller adipocytes compared to controls, disproving our hypothesis that accelerated growth leads to adipocyte hypertrophy. The overall reduction in adipocyte size by sustained post-weaning FL clearly shows that caloric restriction and/or growth rate reduction can influence adipocyte size. Moreover, effects of FL on both adipocyte size and adiponectin transcription in LPO (but not NPO) may indicate an altered adipocyte "setpoint" in LPO: i.e., normal/small size in LPO-AdLib is metabolically equivalent to hypertrophied adipocyte in NPO-AdLib and responds favorably to size reduction by post-weaning FL.

Adiponectin transcription is restored to normal levels by post-weaning FL in IAT, but not changed by FL in SAT. This suggests that MPR programs adiponectin transcription indirectly via accelerated growth in IAT, but by other mechanisms in SAT. Adiponectin

has effects on multiple systems, including reducing fat storage in both adipose and non-adipose (hepatic, muscle) tissues; sensitizing target organs to insulin; and reducing arterial plaque formation and embolization. Because of the pleiotropic actions of adiponectin, the reduction in IAT adiponectin mRNA via accelerated growth may be an important mechanism in the programming of metabolic syndrome in those born small.

TNF-α is marginally reduced by post-weaning FL (p=.08). The simplest explanation is that FL *per se* causes this reduction. In support of this concept, TNF-α transcription and secretion has previously been shown to be reduced in rat lung in response to allergen during moderate (25%) caloric restriction[209].

Plasma leptin concentrations in LPO-FL are increased in a marginally significant manner (p=.06) in males, and remain at normal levels in females. Leptin normally is highly correlated with body fat. However, in these offspring, male LPO-FL have normal % body fat, and female LPO-FL have dramatically reduced % body fat. This suggests that leptin may be inappropriately regulated in both male and female LPO-FL. The cause of "abnormally high" leptin in these chronic mildly feed-limited animals is unknown, as the typical response to acute feed limitation is a reduction in plasma leptin levels, and no effect is observed during chronic caloric restriction in humans[210]. In spite of the slight relative increase in leptin concentrations compared to body fat, leptin concentrations correlated positively with cell size, as would be expected. This is different from what was reported in Chapter 4 (no correlation) most likely because adding the FL offspring

increased both the number of individuals included in the analysis and the range of adipocyte size (FL had lower adipocyte sizes).

Based on our preliminary data, fasting plasma glucose levels are not normalized by post-weaning FL. This indicates that fasting plasma glucose, which is an indicator of glucose homeostasis, is programmed by MPR independently of post-weaning accelerated growth. Glucose levels also do not correlate with either adiponectin or TNF-α mRNA levels, both of which have been implicated in the obesity-related development of insulin resistance. Thus, the impaired fasting glucose observed in male LPO does not seem to be influenced by adipose tissue, and may be related to the function of other organs such as pancreas, liver and/or skeletal muscle. Interestingly, liver weights (normalized to body weight) are elevated in juvenile microswine LPO (see **Appendix, Table A-3**). Whether liver weights are increased due to steatosis, increased parenchyma, or for other reasons is unknown but may indicate functional changes relevant to glucose homeostasis.

Circulating and tissue-level GC metabolism does not appear to be affected by post-weaning FL. Although LPL was used as an index of GR activity, its transcription is regulated by several factors. Thus, the increase in LPL transcription in IAT and concomitant reduction in LPL transcription in SAT in female LPO-FL may, instead, reflect, not GC activation, but an alteration in other fat metabolism transcription factors, such as sterol regulatory element binding protein (SREBP)-1c, CCAAT enhancer binding protein (c/EBP)-β, c/EBP-δ, PPAR-α, or PPAR-γ. Each of these has been shown to regulate LPL transcription as well as having multiple other effects on fat metabolism and

adipocyte differentiation (see Review[211]). Further studies examining the roles of these transcription factors in MPR offspring may be useful to determine the etiology of adipose tissue dysfunction in the developmental programming of metabolic diseases.

The conclusions drawn from the FL experiment are suggestive, yet incomplete for two reasons. First, the numbers of animals and number of litters represented is small (only 1 litter, 6 animals for NPO-FL, and 3 litters, 7-8 animals for LPO-FL). Second, challenges with sow feeding occurred during the gestation and lactation periods of the only NPO-FL litter, and one of the LPO-FL litters, including 3 LPO-FL animals. As such, the sows likely consumed fewer calories in addition to being protein-deficient, and offspring may have exhibited different effects than would have been observed had sows been consuming a truly isocaloric diet. While this may in time yield important clues as to the mechanisms of programming, at this point, it represents a confounding factor in the analysis of the NPO-FL group in particular. Despite this limitation, comparison of the LPO-FL groups to NPO and LPO Ad Lib groups yields valuable preliminary information.

In conclusion, while this report is preliminary due to low numbers, the data presented here comprise the first evidence separating the indirect effects of MPR as a consequence of accelerated growth from other effects of MPR on adipose tissue function. **Fig. 5-10** summarizes the findings in both ad lib and feed limited offspring. Feed limitation prevents accelerated growth in both sexes, while preventing the rapid accrual of fat mass only in females. However, in both sexes, adiponectin transcription is restored to normal levels in IAT, but not SAT, suggesting that MPR regulates adiponectin transcription in

each fat depot in a distinct manner: in IAT as a consequence of accelerated growth, and in SAT via a separate adaptation resulting from perinatal MPR. Similarly, the altered glucose homeostasis observed in juvenile offspring does not result from accelerated growth, and does not appear to be dependent upon adipose tissue function, but is a function of some other early adaptation resulting from perinatal MPR. These results underscore the concept that adipose tissue function in offspring exposed to MPR is regulated in a manner distinct from that observed in diet-induced or genetic forms of obesity. While adiponectin transcription is only restored to normal in IAT, it is the IAT depot which is more strongly linked to cardiovascular and metabolic disease. Therefore, the reduction in adiponectin transcription may be an important mechanism in programming of metabolic and cardiovascular risk in individuals born small.

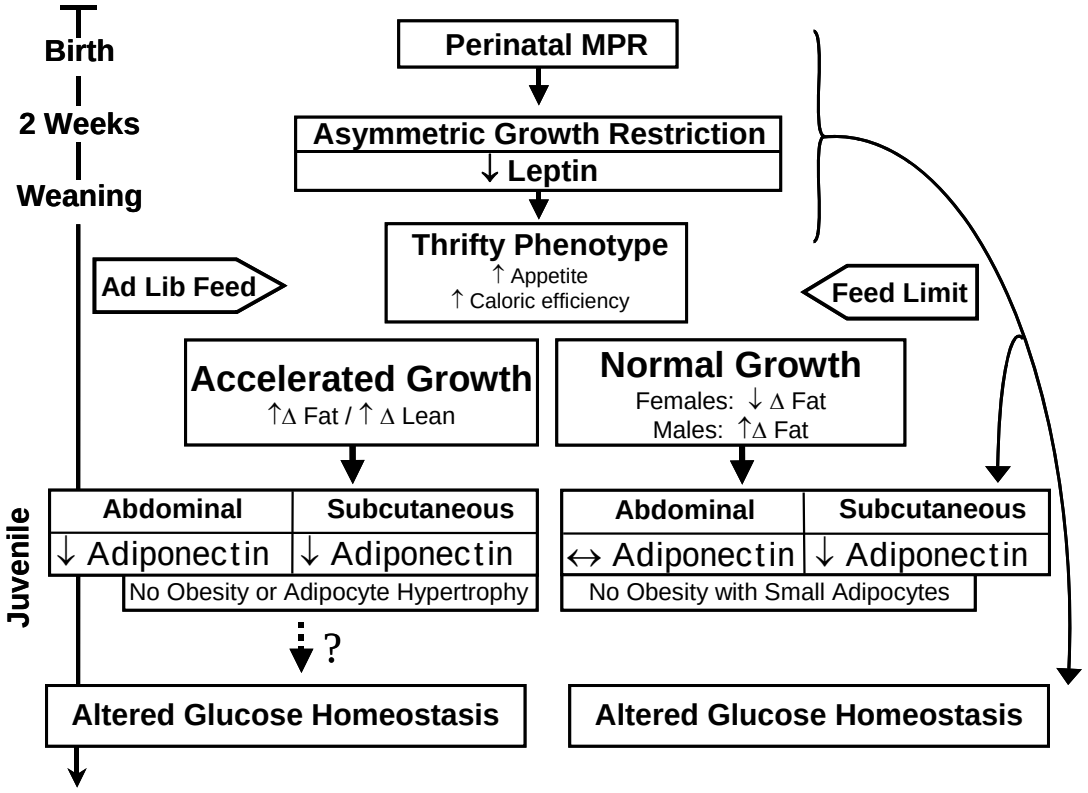

FIGURE 5-10: Effects of Perinatal MPR in Ad Lib and Feed Limited Offspring. Adiponectin transcription is reduced in both fat depots by MPR, but is restored to normal in IAT by post-weaning feed limitation, indicating IAT adiponectin transcription is dependent upon post-weaning accelerated growth. SAT adiponectin and glucose homeostasis are altered as a result of a different adaptation resulting from perinatal MPR.

TABLE 5-1: Adipose Tissue Indices of Glucocorticoid Activity in Juvenile Offspring of Protein Restricted Sows with Post-Weaning Feed Limitation [1]

	MALES				FEMALES			
	Ad Libitum		Feed Limited		Ad Libitum		Feed Limited	
	NPO	LPO	NPO	LPO	NPO	LPO	NPO	LPO
Abdominal Adipose								
GR-α mRNA[2]	1.34±0.16*†¶ n=5	1.14±0.07*†¶ n=9	0.56±0.31*†¶ n=3	1.32±0.22*†¶ n=4	1.19±0.10 n=5	1.35±0.24 n=7	2.16±0.69 n=3	1.91±0.72 n=3
11βHSD1 mRNA[2]	1.36±0.12** n=5	1.47 ± 0.19** n=9	1.13 ± 0.55 n=3	1.67 ± 0.42 n=4	0.78 ± 0.06** n=5	0.90 ± 0.22** n=7	1.07 ± 0.14 n=3	1.18 ± 0.30 n=3
LPL mRNA[2]	1.01 ± 0.19 n=5	0.76 ± 0.08 n=8	0.65 ± 0.26 n=3	0.82 ± 0.11 n=4	0.89±0.08 n=5	1.13±0.16 n=7	1.14±0.17 n=3	1.65±0.48 n=3
Subcutaneous Adipose								
GR-α mRNA[2]	0.71 ± 0.14 n=5	0.51 ± 0.07 n=9	0.64 ± 0.11 n=3	0.62 ± 0.17 n=3	0.67±0.20 n=5	0.42±0.08 n=7	0.60±0.09 n=4	0.37±0.08 n=3
11βHSD1 mRNA[2]	1.27 ± 0.24‡ n=5	0.99 ± 0.15‡ n=9	1.77 ± 0.38 n=3	0.87 ± 0.09 n=3	0.65±0.15‡ n=5	0.60±0.11‡ n=7	1.20±0.11 n=4	0.78±0.31 n=3
LPL mRNA[2]	0.63 ± 0.13 n=5	0.48 ± 0.08 n=9	0.47 ± 0.14 n=3	0.36 ± 0.25 n=3	0.87 ± 0.27 n=5	0.55 ± 0.16 n=7	0.56±0.09 n=4	0.34±0.10 n=3
Gastrocnemius Muscle								
GR-α mRNA[2]	1.31 ± 0.45 n=4	1.17 ± 0.11 n=8	No data	1.65 ± 0.57 n=3	0.95 ± 0.08 n=5	1.25 ± 0.15 n=5	No data	1.34±0.18 n=2
11βHSD1 mRNA[2]	1.20 ± 0.49 n=4	1.63 ± 0.56 n=8	No data	1.82 ± 0.52 n=3	1.22 ± 0.47 n=5	1.14 ± 0.13 n=5	No data	0.65±0.07 n=2

Data are presented as means ± SEM of ratios of gene-of-interest to 18s rRNA. Values were Ln transformed then analyzed by two-way ANOVA using either Maternal Diet and Sex (Ad Lib only) or Maternal Diet and Postnatal Diet (sexes analyzed separately) as factors. Maternal Diet by Postnatal Diet 2-way ANOVA: *Maternal Diet, $p<.03$; †Postnatal Diet, $p<.02$; ¶Maternal x Postnatal Diet Interaction, $p<.005$. Ad Lib Only Maternal Diet by Sex 2-way ANOVA: **Maternal Diet, $p<.005$; ‡ Sex, $p<.05$.

TABLE 5-2: Adipose Tissue Cytokine mRNA in Juvenile Offspring of Protein Restricted Sows with Post-Weaning Feed Limitation[1]

	MALES				FEMALES			
	Ad Libitum		Feed Limited		Ad Libitum		Feed Limited	
	NPO	LPO	NPO	LPO	NPO	LPO	NPO	LPO
Abdominal Adipose								
Adiponectin* mRNA	1.49±0.27†‡ n=5	1.15±0.09†‡ n=9	0.68±0.09†‡ n=3	1.34±0.35†‡ n=4	1.29±0.2†‡ n=5	0.76±0.09†‡ n=7	1.16±0.18†‡ n=3	1.16±0.14†‡ n=3
TNF-α** mRNA	1.28 ± 0.87 n=5	3.02 ± 0.97 n=9	0.49 ± 0.31 n=3	1.19 ± 0.59 n=4	2.62± 1.10 n=5	0.68± 0.23 n=7	0.58±0.19 n=3	0.31±0.09 n=3
Subcutaneous Adipose								
Adiponectin* mRNA	0.71 ± 0.22 n=5	0.54 ± 0.13 n=9	0.49 ± 0.04 n=3	0.48 ± 0.10 n=4	0.88 ± 0.28 n=5	0.33 ± 0.09 n=7	0.38 ± 0.22 n=3	0.19 ± 0.09 n=3
TNF-α** mRNA	1.50 ± 0.51 n=5	1.26 ± 0.59 n=9	0.32±0.09 n=3	0.14±0.05 n=4	1.88 ± 0.94 n=5	0.37 ± 0.21 n=7	0.36 ± 0.15 n=3	0.19 ± 0.02 n=3

[1]Data are presented as means ± SEM of ratios of gene-of-interest to 18s rRNA. Values were Ln transformed then analyzed by General Linear Model Univariate Analysis (SPSS) using Maternal Diet, Postnatal Diet, Sex and Adipose Tissue Depot as factors: *Intra-Abdominal Adipose Tissue>Subcutaneous Adipose Tissue, p<.0001; **Intra-Abdominal Adipose Tissue>Subcutaneous Adipose Tissue, p<.05. †Maternal Diet by Post-weaning Diet Interaction, p=.015; ‡Sex by Post-weaning Diet Interaction, p<.02. Abbreviations: NPO, Normal Protein Offspring; LPO, Low Protein Offspring; TNF- α, Tumor Necrosis Factor-α.

Chapter 6: Conclusions

This work describes a new model for study of developmental origins of adult metabolic and cardiovascular diseases. The use of microswine presents advantages compared to other commonly used models. First, for studies of postnatal accelerated growth, microswine are ideal because they have an extended period of time between weaning and puberty, unlike rodents which have only about one week between weaning and maturity. Thus, swine can be used to model human childhood more accurately than rodent models. Second, swine also - like humans but unlike rodents and non-human primates - have the capacity to deposit large amounts of fat in subcutaneous depots. For these reasons, microswine may provide a more faithful modeling of human MNR/MPR than do other species. To our knowledge, this study is the first report of adipose tissue function in a developmental programming model to consider both fat depots and both sexes, and the first in such a model to examine adiponectin, TNF-α, and LPL in postnatal adipose tissue.

In this study, five hypotheses were addressed. The first proposed that MPR in microswine would result in reduced birth weight followed by rapid postnatal growth, and that this would be accompanied by hyperphagia. In fact, we show in this report that MPR in swine leads to asymmetric growth restriction (i.e., weight reduced more than length) at birth and 2 wks of age, followed by accelerated growth from 6-12 wks; the latter was accompanied by hyperphagia and increased feed utilization efficiency. Use of the microswine MPR model supports the concept that the accelerated growth often observed in humans born small is not serendipitous, but rather is programmed by the same stimulus that leads to the stunted fetal growth. This could not be deduced from human

epidemiologic studies due to their retrospective nature. The current data do not distinguish whether hyperphagia is primary, and drives the accelerated growth, or whether accelerated growth is programmed, and drives hyperphagia secondarily. We have preliminary data suggesting that appetite normalizes after 12 wks; similar data exist in various sheep models. These data suggest that it may be that accelerated growth is programmed, and hyperphagia occurs to meet the demand for growth. This is an important point warranting further clarification through a series of feeding studies.

The second hypothesis posed that accelerated growth would be accomplished by preferential deposition of fat, leading to development of obesity by the point of weight equality. This hypothesis was not supported since % body fat and truncal fat were normal in LPO at 11 wks, the point of weight equality. These results run counter to the prevailing concept that accelerated growth obligates concomitantly increased fat deposition. We did, however, observe an increased rate of accrual of adipose tissue from 6-11 weeks without the development of obesity at the point of weight equality. Whether obesity develops at a later time has not been established and warrants further study. There are three reasons to believe that obesity may develop. First, accelerated fat deposition was observed in LPO from 6-11 weeks. If this were to continue beyond the period of rapid linear growth, obesity would ensue rapidly, probably by early adulthood. This is in fact observed in pigs naturally born small and followed to one year of age[185]. Second, 2 of the 5 female LPO-AdLib observed had a % body fat greater than 2 SD above the female NPO average by 11 wks of age. Finally, human epidemiological data show that, in those individuals born small who also undergo accelerated childhood

growth, the increased risk of both obesity and hypertension is not manifested until mid-adult life.

The third hypothesis addressed in this study predicted that MPR in microswine would result in altered adipose tissue structure and function typical of those observed in diet-induced obesity. While this hypothesis was based upon the ultimately incorrect prediction that LPO would be obese by 11 wks, adipocyte function was nonetheless altered by MPR in a depot-dependent manner without either obesity or adipocyte hypertrophy. This is one of the most striking findings of this work: aberrant adipose tissue function (reduced adiponectin transcription) typically observed in obesity in association with adipocyte hypertrophy, is already present in prepubertal, non-obese LPO with normal-to-small adipocyte size. Because of the pleiotropic effects of adiponectin, the reduction in adiponectin mRNA, if subsequently confirmed by protein level data, may at least partially explain why adults born with low birth weight are at increased risk for the development of metabolic syndrome[164] and cardiovascular disease[1].

The fourth hypothesis proposed that effects due to MPR would be mediated either by activation of the HPA axis or by increases in local adipose tissue GC generation or activity. GC, often thought to be mediators of developmental programming, seem not to be involved in mediating effects observed in the microswine MPR model. While altered circadian rhythmicity of cortisol production and local tissue GC metabolism in other organs were not ruled out, the bulk of the data shown here suggest that enhanced GC activity is not involved in the effects observed in this study.

The fifth hypothesis held that altered adipocyte size and function and altered fasting plasma glucose levels were programmed indirectly, as a consequence of accelerated post-weaning growth. The data presented here, while still preliminary, suggest that post-weaning feed limitation to that of normal levels prevents rapid growth in LPO of both sexes and prevents rapid adipose tissue accrual in females (not males). Feed limitation also restored adiponectin transcription in IAT, but not SAT. Thus, reduced adiponectin transcription is programmed in a depot-specific manner: it is programmed directly by MPR in SAT, but depends on post-weaning feed intake and/or growth rate in IAT. Fasting plasma glucose, which was altered in opposite directions in males vs females, was programmed directly by MPR in both sexes, in that post-weaning feed limitation did not alter the abnormalities.

In addition to addressing the five hypotheses, the results presented here yield several other important novel concepts related to adipose tissue function in nutritional programming. These data dissociate the relationship between adipocyte size, mass, and function typically reported in diet-induced and genetic obesity. Thus despite absence of obesity and clear absence of adipocyte hypertrophy, adiponectin mRNA levels were reduced. The reason for this discrepancy is unclear; however, it has been suggested that programmed obesity occurs via different mechanisms than adult diet-induced obesity[73]. The same could be true for adipocyte dysfunction, including adiponectin transcription. The only published gene microarray study in visceral adipose tissue in an MPR-induced visceral obesity model showed a different pattern of gene expression than that typically observed in either genetic or diet-induced models of obesity[104]. More detailed study is

needed to understand the relationship between adipocyte size and function in MPR models and to determine the potentially unique mechanisms underlying obesity and adipocyte dysfunction in developmental programming. The multiple functions of adiponectin (**Fig. 6-1**) impact every aspect of metabolic syndrome, and therefore, it is possible that the programmed reduction in adiponectin observed in this study may represent a primary link between early environmental factors, early growth, and the development of metabolic and cardiovascular disease later in life.

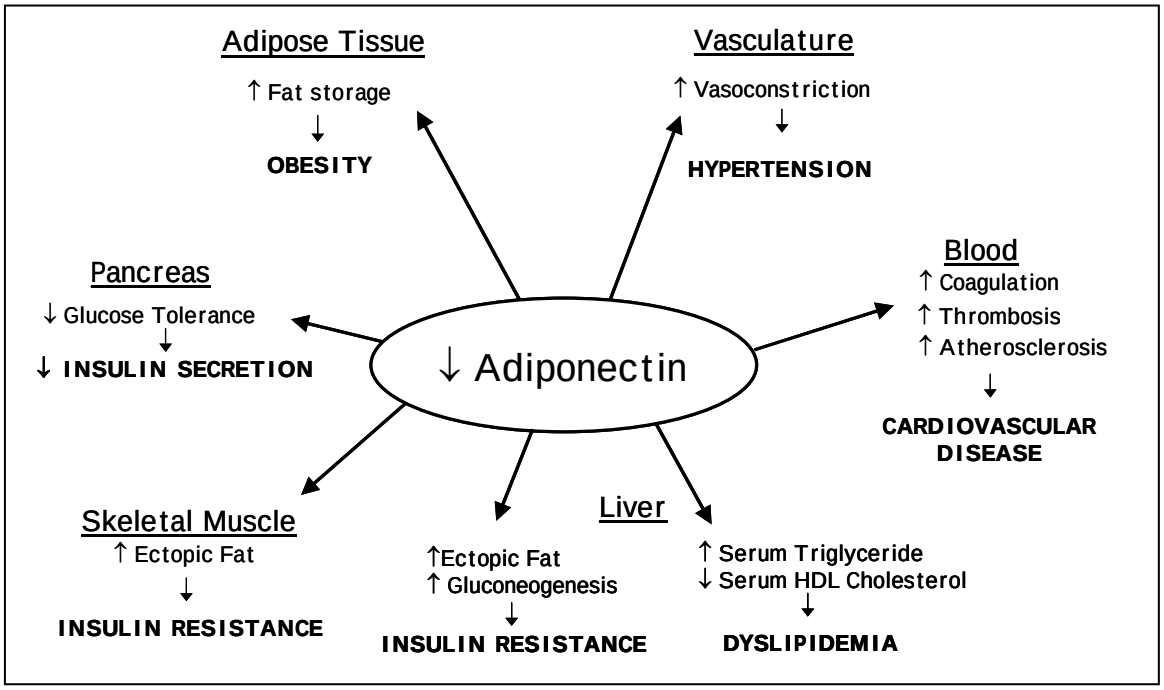

FIGURE 6-1: Multiple Functions of Adiponectin Impact Every Aspect of Metabolic Syndrome. Reductions in adiponectin lead to increased fat storage, reduced insulin secretion, insulin resistance, hypertension, and cardiovascular disease via effects on adipose tissue, pancreas, skeletal muscle, liver, vasculature, and blood. The pleiotropic effects of adiponectin support its role as a potential link between early development and later risk of disease.

This study also highlights the need for a variety of models in the DOHAD field, a surprisingly controversial topic. Contrary to what has been shown in sheep MNR and rodent MPR, altered GC status is not observed in microswine MPR offspring, either circulating or in adipose tissue, except for a transient reduction in cortisol in still-protein restricted neonates. This difference could be a result of the timing of MPR, age of offspring when studied, basal vs stimulated GC indices, or a species difference; further studies will be required to clarify this. An additional major difference is that microswine LPO show "catch-up" growth without the development of obesity, consistent with observations in children born small. The low birth weight followed by rapid post-weaning growth observed in the microswine MPR model more closely reflects patterns seen in some human populations, especially those who go on to develop cardiovascular and/or metabolic disorders; thus the microswine MPR model will be useful as a translational research tool in the DOHAD field.

As depicted in **Fig. 6-2**, perinatal MPR results in asymmetric growth restriction and reduced plasma leptin levels at two weeks of age. This leptin deficiency may program later hyperphagia, as observed from 6-12 wks, in conjunction with increased feed utilization efficiency and accelerated growth. This accelerated growth was in the form of rapid accrual of both fat and lean tissue, in balanced proportion. The rapid accrual of fat did not result in either obesity or adipocyte hypertrophy. In spite of the absence of these characteristics, adiponectin transcription was reduced in both intra-abdominal and subcutaneous adipose tissue depots. Based on similarities to human data, other models, and anecdotal evidence in the microswine MPR model, I predict that obesity will develop

in offspring exposed to perinatal MPR in early adulthood, with the potential to exacerbate adipocyte dysfunction. Post-weaning feed limitation prevented accelerated growth, and restored adiponectin transcription to normal in intra-abdominal adipose tissue, the depot more strongly linked to metabolic and cardiovascular disease. Because of the pleiotropic effects of adiponectin, it is possible that the reduction in adiponectin is the link between early development and later risk of disease. This research also suggests that postnatal intervention during childhood can reverse the alteration in adiponectin. While it may not be prudent to advise parents to limit growth of children, it is important proof of principle that environment during childhood can exacerbate or temper risk of disease. Such an intervention may prevent, or at least delay, the development of obesity and metabolic disease later in life.

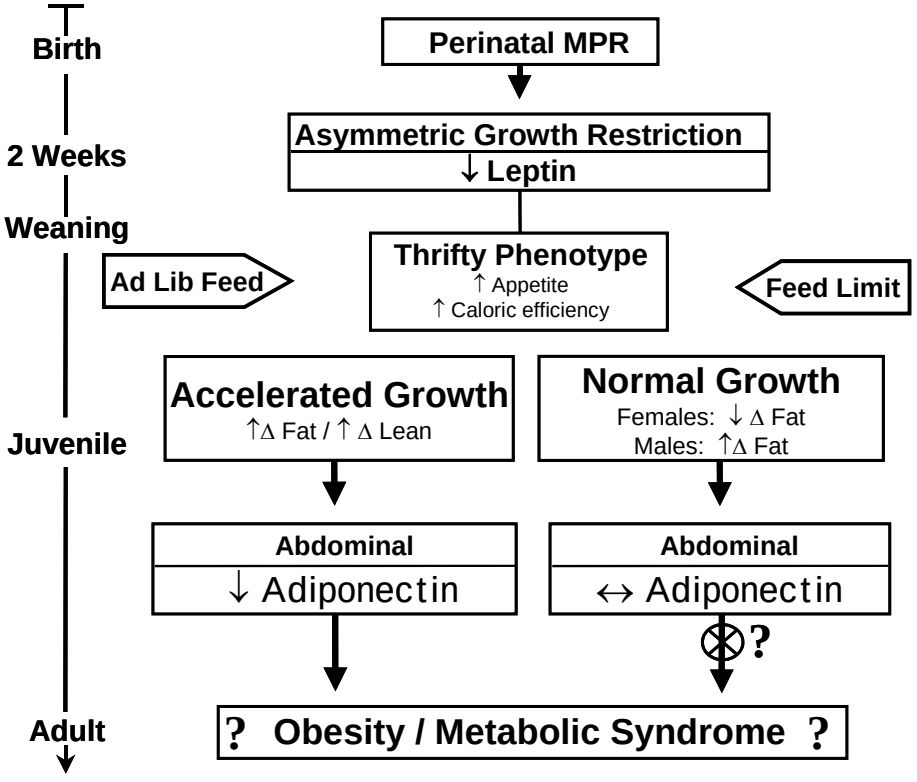

FIGURE 6-2: Programming of Metabolic Disease by Perinatal Maternal Protein Restriction. Perinatal MPR causes accelerated growth with rapid fat accrual. Adiponectin transcription is low, independent of usual regulators (diet-induced and genetic forms of obesity), reversible, and may cause the development of metabolic syndrome later in life.

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Appendix

TABLE A-1: Body Composition in Offspring Following Maternal Protein Restriction and/or Post-Weaning Feed Limitation

	MALES				FEMALES			
	Ad Libitum		Feed Limited		Ad Libitum		Feed Limited	
	NPO	LPO	NPO	LPO	NPO	LPO	NPO	LPO
6 Weeks								
Percent Body Fat	13.7 ± 0.8[ef] n=7	11.8 ± 0.7[ef] n=10	11.4 ± 1.1 n=3	12.4 ± 0.4 n=3	16.4 ± 0.8[ef] n=5	13.2 ± 0.5[ef] n=6	17.1 ± 2.2 n=4	17.9 ± 3.1 n=2
Truncal Adiposity	0.868 ± 0.010[bc] n=7	0.845 ± 0.008[bc] n=10	0.782±0.045[bc] n=3	0.992±0.098[bc] n=3	0.873 ± 0.024 n=5	0.843 ± 0.015 n=6	0.825 ± 0.052 n=4	0.836 ± 0.008 n=2
Fat:Lean	0.162±0.011[dg] n=7	0.137±0.009[dg] n=10	0.134 ± 0.015 n=3	0.144 ± 0.005 n=3	0.201±0.012[dg] n=5	0.149±0.009[dg] n=6	0.212 ± 0.033 n=4	0.220 ± 0.043 n=2
Lean Mass	87.4 ± 3.1[ace] n=7	67.8 ± 2.2[ace] n=10	55.7 ± 1.5[ac] n=3	54.6 ± 7.3[ac] n=3	68.6 ± 13.6[ace] n=5	68.5 ± 15.5[ace] n=6	45.2 ± 2.8[ac] n=4	49.4 ± 8.0[ac] n=2
11 Weeks								
Percent Body Fat	22.2 ± 1.0[d] n=4	21.6 ± 1.1[d] n=8	17.7 ± 1.8 n=3	21.8 ± 0.4 n=4	25.0 ± 0.5[ad] n=5	26.3 ± 1.2[ad] n=6	18.0 ± 1.1[a] n=3	15.9 ± 2.3[a] n=4
Truncal Adiposity	0.901±0.006 n=4	0.905±0.004 n=8	0.911 ± 0.008 n=3	0.914 ± 0.006 n=4	0.906±0.005 n=5	0.900±0.006 n=6	0.893 ± 0.014 n=3	0.876 ± 0.018 n=4
Fat:Lean	0.291±0.016g n=4	0.283±0.018g n=8	0.217 ± 0.019 n=3	0.286 ± 0.007 n=4	0.341±0.009[ag] n=5	0.366±0.023[ag] n=6	0.224 ± 0.017[a] n=3	0.196 ± 0.034[a] n=4
Lean Mass	159.1 ± 11.1[ag] n=4	147.4 ± 3.9[ag] n=8	121.0 ± 2.4[a] n=3	115.6 ± 9.5[a] n=4	137.3 ± 4.8[ag] n=5	125.7 ± 7.2[ag] n=6	103.7 ± 9.6[a] n=3	98.5 ± 6.7[a] n=4
Change from 6-11 Weeks								
Fold-increase in Fat Mass	5.1 ± 1.5[e] n=4	6.8 ± 0.6[e] n=8	5.6 ± 1.0 n=3	7.1 ± 1.2 n=4	4.0 ± 0.3[abce] n=5	7.7 ± 0.5[abce] n=6	3.8 ± 0.1[abc] n=3	2.4 ± 0.1[abc] n=2
Fold-increase in Lean Mass	2.5 ± 0.2[ce] n=4	3.2 ± 2.4[ce] n=8	3.2 ± 0.1[c] n=3	3.1 ± 0.1[c] n=3	2.3 ± 0.1[ce] n=5	3.2 ± 0.3[ce] n=6	3.1 ± 0.1[c] n=3	2.8 ± 0.5[c] n=2
Ratio Incr. Fat : Incr. Lean	0.373±0.024[cf] n=4	0.341±0.027[cf] n=7	0.255 ± 0.027[c] n=3	0.367 ± 0.015[c] n=3	0.455±0.031[af] n=5	0.501±0.052[af] n=5	0.245 ± 0.016[a] n=2	0.174 ± 0.020[a] n=2

Data are presented as means ± SEM and were analyzed by two-way ANOVA using either Maternal Diet and Sex (Ad Lib only) or Maternal Diet and Postnatal Diet (sexes analyzed separately) as factors. Maternal Diet by Postnatal Diet 2-way ANOVA: [a]Postnatal Diet, $p<.005$; [b]Maternal Diet, $p<.05$; [c]Maternal x Postnatal Diet Interaction, $p<.01$. Ad Lib Only Maternal Diet by Sex 2-way ANOVA: [d]Maternal Diet, $p<.05$; [e]Maternal Diet, $p<.005$; [f]Sex, $p<.05$; [g]Sex, $p<.005$.

TABLE A-2: Bone Mineral Content and Bone Mineral Density in Offspring of Protein Restricted Sows

	MALES				FEMALES			
	Ad Libitum		Feed Limited		Ad Libitum		Feed Limited	
	NPO	LPO	NPO	LPO	NPO	LPO	NPO	LPO
6 Weeks								
Bone Mineral Content	75.7 ± 4.7[fc] n=7	58.9 ± 2.9[fc] n=10	45.5 ± 7.1[c] n=3	38.4 ± 8.5[c] n=3	72.8 ± 6.4[fc] n=5	54.3 ± 4.5[fc] n=5	37.5 ± 5.6[c] n=4	39.5 ± 1.5[c] n=3
Bone Mineral Density	.432 ± .015[ac] n=7	.400 ± .007[ab] n=10	.373 ± .014[c] n=3	.363 ± .004[b] n=3	.423 ± .012[ac] n=5	.408 ± .008[ab] n=6	.362 ± .009[c] n=4	.393 ± .018[b] n=3
11 Weeks								
Bone Mineral Content	209.5 ± 30.2 n=4	208.9 ± 11.8[ce] n=8	181.5 ± 8.4 n=3	167.2 ± 23.9[ce] n=4	185.8 ± 8.6 n=5	190.0 ± 16.4[ce] n=6	147.3 ± 14.9 n=3	113.0 ± 10.5[ce] n=4
Bone Mineral Density	.530 ± .029 n=4	.529 ± .011[d] n=8	.513 ± .018 n=3	.529 ± .025[d] n=4	.532 ± .007 n=5	.540 ± .012[bd] n=6	.506 ± .009 n=3	.461± .020[bd] n=4
Fold Change from 6-11 Weeks								
Bone Mineral Content	2.59 ± 0.27[fc] n=4	3.81 ± 0.35[fd] n=7	4.18 ± 0.60[c] n=3	4.66 ± 0.39[d] n=3	2.62 ± 0.22[fc] n=5	3.69 ± 0.51[fd] n=4	3.55 ± 0.22[c] n=3	2.76 ± 0.37[d] n=3
Bone Mineral Density	1.22 ± 0.08[ab] n=4	1.34 ± 0.03[abde] n=7	1.38 ± 0.07 n=3	1.46 ± 0.08[de] n=3	1.26 ± 0.03[ab] n=5	1.35 ± 0.06[abde] n=5	1.37 ± 0.02 n=3	1.18 ± 0.08[de] n=3

Two-way ANOVA, maternal diet and sex (Ad Lib only): [a]Maternal Diet, $p<.05$; [f]Maternal Diet, $p<.01$. Two-way ANOVA, Postnatal Diet and Sex (NPO and LPO separate): [b]Postnatal Diet, $p<.05$; [c]Postnatal Diet $p<.01$; [d]Interaction (Postnatal Diet by Sex), $p<.05$; [e]Sex, $p<.05$

TABLE A-3: Body/Organ Size in Offspring of Protein Restricted Sows[1]

	Near-Term Fetus		2Wk Neonate		3-5Mo Juvenile	
	LPO	NPO	LPO	NPO	LPO	NPO
Age (Days)	GD 113	GD 113	12.5 ± 2.0	13.3 ± 2.4	113 ± 24	104 ± 15
Total n (male,female)	19 (7,12)	16 (10,6)	8 (5,3)	9 (5,4)	17 (8,9)	11 (5,6)
# Litters	3	3	2	2	5	2
BODY SIZE						
Body Wt (kg)	0.80 ± 0.10	0.87±0.09	2.01 ± 0.10¶	2.96 ± 0.50	25.1 ± 8.4	22.8 ± 5.2
Length (cm)	23.9 ± 1.4	24.1 ± 1.7	32.2 ± 1.3	33.8 ± 3.1	75.4 ± 8.8	72.6 ± 5.3
Wt:Lth Ratio (g/cm)	33.5 ± 3.4†	36.1 ± 3.9	59.3 ± 1.5¶	82.6± 3.3	30.2 ± 7.2	30.2 ± 4.8
BMI (kg/m^2)	14.03 ± 1.51	15.09 ± 2.34	19.6 ± 2.3¶	26.0 ± 3.4	41.4 ± 4.8	41.5 ± 4.9
KIDNEY						
TotKidWt(g)	4.97 ± 0.82	5.41 ± 1.21	13.5 ± 2.2¶	21.0 ± 3.5	160 ± 42‡	120 ± 38
AvgKid Lth(cm)	2.90 ± 0.24	3.05 ± 0.33	3.94 ± 0.23¶	4.57 ± 0.34	9.44 ± 0.78	8.67 ± 0.87
Avg Wt:Lth Ratio	0.85 ± 0.09	0.90 ± 0.12	1.72 ± 0.20¶	2.30 ± 0.23	8.41 ± 1.58‡	6.78 ± 1.44
TotKid:BWt Ratio	6.18 ± 0.56	6.18 ± 0.85	6.73 ± 1.13	7.10 ± 0.44	6.22 ± 1.00‡	5.45 ± 0.73
HEART						
Wt (g)	6.35 ± 0.87‡	7.19 ± 0.78	14.3 ± 0.9¶	19.2 ± 3.3	104 ± 21	97 ± 23
Ht:BWt Ratio	7.92 ± 0.36	8.28 ± 0.52	7.1 ± 0.5†	6.5 ± 1.0	4.10 ± 0.75	4.46 ± 0.43
ADRENAL						
Avg AdrWt (mg)	97 ± 19	95 ± 13	210 ± 20¶	260 ± 40	1233 ± 293	1172 ± 353
AvgAdr:BWt ratio	119 ± 22	113 ± 12	103 ± 11‡	85 ± 26	47 ± 9	53 ± 16
LIVER						
Wt (g)	26.1±4.4	28.2 ± 4.7	63 ± 10¶	100 ± 16	702 ± 205	527 ± 93
Liv:BWt Ratio	32.5±3.4	32.1 ± 3.0	31.1 ± 4.6	34.0 ± 2.4	28.0 ± 3.9	24.6 ± 2.8

[1]Data are presented as mean ± SEM, and were analyzed by two-way ANOVA using maternal diet and sex as factors. †$p<.05$ ‡$p<.02$ ¶$p<.002$