DEVELOPMENTAL ORIGINS OF ADULT DISEASE IN THE YUCATAN MINIATURE PIG: THE EFFECTS OF PRE-NATAL GROWTH ON GLUCOSE AND METHYL METABOLISM

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DEVELOPMENTAL ORIGINS OF ADULT DISEASE IN THE YUCATAN MINIATURE PIG:

The effects of pre-natal growth on glucose and methyl metabolism

By

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Abstract

The effects of birth weight variations on growth and glucose metabolism later in life were investigated in Yucatan miniature pigs. Pigs were fed a high salt, high sugar, and high fat diet for ~12 months. Glucose metabolism *in vivo* was studied using intravenous glucose tolerance tests (IVGTT) and insulin sensitivity tests (IST). Glucose tolerance or insulin sensitivity was not affected by birth weight but was significantly affected by gender. Female pigs had reduced glucose tolerance and insulin sensitivity compared to male pigs. The female pigs also had higher visceral fat concentrations than male. Increased visceral fat was detrimental to glucose metabolism in all the pigs. The hepatic gene expression of cystathionase (CTH) was lower in runt piglets but betaine-homocysteine methyltransferase (BHMT) expression and hepatic global DNA methylation were not different. Thus the role of methyl metabolism in fetal programming of adult disease remains unclear.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ATP	Adenosine triphosphate
AUC	Area under the curve
BHMT	Betaine homocysteine methyltransferase
BMI	Body mass index
CβS	Cystathionine beta synthase
CγL	Cystathionine gamma lyase
CTH	Cystathionase
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DPM	Disintegrations per minute
FE	Feed efficiency
FFA	Free fatty acid
FGR	Fractional growth rate
GHL	Glucose half-life
GLM	General linear model
GR	Growth rate
IGF	Insulin like growth factor
ISGHL	Insulin stimulated glucose half-life
IST	Insulin sensitivity test
IVGTT	Intravenous glucose tolerance test
MAT	Methionine adenosyltransferase

MS	Methionine synthase
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PIC	Pancreatic insulin concentration
RIA	Radioimmunoassay
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SGA	Small for gestational age
TGR	Total growth rate
TTRB	Time to return to baseline

Chapter 1 Introduction and literature review

1.1 Overview

The work in this thesis is a continuation of two other studies completed previously in our laboratory. The first study assessed the development of diabetes and impaired glucose metabolism as it related to birth weight and early post-natal diet in Yucatan miniature pigs (McKnight, 2008). Those pigs were fed a normal pig feed after weaning and raised for an average of 9.7 months. The second study measured the activities of sulfur amino acid enzymes in newborn Yucatan miniature piglets (Brophy, 2006). Differences between runt and large piglets were observed for betaine homocysteine methyltransferase (BHMT) and cystathionase (CTH) activities. In this thesis research, Yucatan miniature pigs were raised for ~12 months on a "cafeteria" style diet designed to mimic poor dietary practices in developed countries. The long grow-out period and poor diet were used to further stress the glucose metabolism beyond what was achieved in the previous study and to determine if diabetes would develop. The expression of the BHMT and the CTH, as well as global DNA methylation status, was measured in samples from the previous piglet study.

1.2 Developmental origins of adult disease

The concept of "fetal origins" of adult disease was first hypothesized by David Barker and colleagues at the University of Southampton (Barker, 1995). They hypothesized that factors *in utero* influence the development of disease later in life. This hypothesis was based on the positive relationship observed in England and Wales between cardiovascular and stroke death in the early 1970's and neonatal mortality some fifty years earlier (Barker & Osmond, 1986). High infant mortality was taken to reflect

poor nutrition across the whole study population, and this was linked to high cardiovascular and stroke death later in life in adults who survived. From this relationship they concluded that poor health of mothers was important in stroke risk of the children later in life. Low birth weight was then shown to increase the risk of cardiovascular disease (Osmond, et al., 1993). This, however, was not the first time such relationships had been demonstrated. As early as 1934, Kermack and colleagues showed a relationship between adult death rates and childhood living conditions (Kermack et al., 1934). Forsdahl (1977) showed a relationship between infant mortality and surviving adult cardiovascular disease. Many studies have since demonstrated links between pre-natal growth rate (which determines birth weight) and adult diseases, including hypertension, insulin resistance and type 2 diabetes (McMillen & Robinson 2005).

Some of these observations led to Hales and Barker's "thrifty phenotype" hypothesis (Hales & Barker, 2001), which contrasted Neel's "thrifty genotype" (Neel, 1962). The "thrifty genotype" hypothesis argued that genes selected for during periods of evolution when food was scarce were the cause of insulin resistance, obesity and type 2 diabetes in environments where nutrients were plentiful. Alternately, the "thrifty phenotype" hypothesised that poor fetal nutrition "set in train mechanisms of nutritional thrift" (Hales & Barker, 2001) as a developmental adaption to the predicted post-natal environment. This thrift, which is intended to increase post-natal survival, allows for the development of key organ systems, at the expense of others. However, if the adaptations are inappropriate for the post-natal (nutritional) environment, disease risk is increased. The term "programming" was used to refer to the relationship between early infant feeds and diseases later in life (Lucas, 1991). It has since been used to refer to the

consequences of early life on long term health. Studies also show that early nutrition and post-natal growth rates relate to disease in adult life, changing the "fetal origins" to "developmental origins" which extends beyond birth (McMillen & Robinson, 2005).

The term "developmental plasticity" is now preferred to "programming". The formal definition of developmental plasticity is "the ability of a single genotype to produce more than one alternative form of structure, physiological state or behaviour in response to environmental conditions" (Barker, 2004a). This refers to the range of development options that can arise from the same genetic or mechanical constraints. The underlying mechanisms that are thought to bring about the changes that occur in developmental plasticity are epigenetic changes, such as altered DNA methylation (Waterland & Garza, 1999, Waterland & Jirtle, 2004). This is supported by data that show that manipulation of methyl donors and folate in maternal animal diets can perturb DNA methylation in the offspring (Wu et al., 2004).

1.3 Pre- and post-natal growth and adult disease

Birth weight is a product of fetal growth. Fetal growth is dependent upon maternal circulation which delivers nutrients to the fetus. This delivery is affected by the mother's nutrient intake, metabolism and blood flow as well as the size of the placenta (Jansson & Powell, 2007). If any of these factors were altered to decrease the nutrient supply to the fetus, then lowered birth weight could result.

As mentioned previously, numerous epidemiological studies show that reduced fetal growth and the resulting low birth weight is related to the development of diseases later in life. These diseases include hypertension, cardiovascular disease, stroke, insulin resistance and type 2 diabetes (McMillen & Robinson, 2005).

As mentioned above, however, fetal growth may not be the only factor in the development of diseases later in life; growth rates after birth are involved as well. Feeding a low caloric diet to babies born pre-term reduced their early growth, and may have a positive effect on insulin resistance during adolescence (Singhal et al., 2003). This suggests that early post-natal growth can have an impact on the development of diseases later in life. Risk of adult disease increases as birth weight decreases; however, this risk has also been shown to increase with rapid weight gain following birth (Barker, 2004b). In a large cohort of men and women from Helsinki, those with low birth weight and low weight at one year of age also had an increased risk of type 2 diabetes (Eriksson et al., 2002). Individuals who had low weight at birth and at one year of age, and who experienced rapid weight gain following one year of age, had the highest risk of developing diabetes. In both men and women, disease risk was more related to rate of weight gain, rather than body size at any age. Another study found that low growth in the first 6 months of life, and a rapid increase in body mass index (BMI) between 2- 11 years of age, was related to the development of impaired glucose tolerance (IGT) (Eriksson et al., 2006).

Children born with higher or lower than average birth weight often experience "catch-down" or "catch-up" growth, which is defined as significant centile crossing on infant growth charts, in a negative or positive direction, respectively. In developed countries with well-nourished populations, approximately 25% of children experience catch-down growth, and ~30% catch-up growth (Ong et al., 2000). Catch-up and catch-down growth is thought to return infants to their genetic size potential; however, in nutrient-rich environments, catch-up growth can cause infants to overshoot their optimal

weights and result in higher BMI, fat mass, and increased central adiposity in childhood (Ong et al., 2000). Rapid catch-up growth has also been shown to strongly predict childhood insulin resistance and obesity, particularly increased visceral (central) obesity (Ong & Loos, 2006). Rapid post-natal growth following pre-natal growth restriction, and its resulting physiological effects such as reduced cell number (i.e. glomeruli in the kidney, or beta cell mass in the pancreas), may result in demands that exceed the capacity of those systems. This may explain the increased risk of hypertension and type 2 diabetes later in life (Barker, 2004a; McMillen & Robinson, 2005).

In animal models, if an animal is deprived of nutrients *in utero* or in early life, and is then put in a nutrient-rich environment, accelerated growth occurs. This animal equivalent of "catch-up" growth is called "compensatory growth", which is defined as a period of accelerated weight gain and growth following a period of nutritional restriction (pre-natal or post-natal) (Hornick et al., 2000). This accelerated growth period is marked by an increased nutrient efficiency. This compensatory growth period does not continue indefinitely; initially it is characterized by increased lean tissue mass, which eventually turns to increased fat deposition, especially with high feed intake, and finally a return to normal growth rate (Hornick et al., 2000).

The mechanisms suggested for the effects of compensatory growth include permanent hormonal and physiological changes, including insulin resistance and structural changes in developing organs, as well as reduced cell number in specific organs. Changes to the timing and form of metabolic differentiation and rate of telomere shortening are also thought to be involved (McMillen & Robinson, 2005). Similar to the

changes that occur due to reduced pre-natal growth, the changes brought about by compensatory growth are thought to be caused by epigenetic changes (Park, 2005).

The costs of reduced pre-natal growth and the costs of rapid compensatory growth are difficult to separate, as low birth weight is related to rapid weight gain in early childhood (Barker, 2004b). Therefore, it is often beneficial to investigate both pre- and post-natal nutrition and growth together.

1.4 Diabetes and insulin resistance

Proper glucose metabolism, or the ability to maintain glucose homeostasis, is of utmost importance in the maintenance of health. An impaired ability to maintain glucose homeostasis can result in blood glucose levels that are too high or too low, which has been linked to numerous negative health outcomes (Hornick & Aron, 2008). Glucose tolerance is the ability of the muscle, adipose and liver tissue to remove glucose from the blood and maintain glucose homeostasis. The most important factor in glucose tolerance is the body's ability to respond to insulin, which is referred to as insulin sensitivity. If normal amounts of insulin fail to produce the expected results in the liver, muscle and fat cells, a person is said to be insulin resistant (Lann & Leroith, 2007). Decreased response to insulin by liver cells leads to reduced hepatic uptake and disposal of glucose from the blood. The liver stores glucose as glycogen for release when blood glucose levels are low. Insulin resistance in the liver can also fail to halt hepatic glucose output while blood glucose levels are high, leading to even higher blood glucose levels (Mlinar et al., 2007). Insulin resistance in muscle cells leads to reduced uptake of glucose by muscle, which is the primary means of removing excess glucose from the blood following a meal, resulting in hyperglycaemia (Mlinar et al., 2007). Muscle cell insulin resistance has also been

linked to increased obesity (Guilherme et al. 2008). Insulin resistance in fat cells results in decreased uptake of free fatty acids (FFA) from the blood via a decrease in adipose lipoprotein lipase activity, and increased hydrolysis of stored triglycerides (Goldberg, 2000). Hepatic insulin resistance can also lead to decreased FFA oxidation and increased triglyceride synthesis (Lann & Leroith, 2007). Insulin resistance is a contributing factor in the dyslipidemia associated with type 2 diabetes and the increased risk of cardiovascular complications. In general, decreased lean body mass and increased fat mass, especially visceral fat, increases insulin resistance. Low physical activity levels, which often accompany obesity and high visceral fat mass, can also lead to insulin resistance (Rizzo et al., 2008). Insulin resistance has also been associated with certain medications, such as corticosteroids, anti-retrovirals, and some antipsychotics (Calmy et al., 2007; Tschoner et al., 2007) as well as high carbohydrate diets, especially those high in fructose (Kahn, 2003).

The body first attempts to compensate for decreased insulin sensitivity, or insulin resistance, by releasing larger amounts of insulin from the pancreas to maintain blood glucose homeostasis. This results in increased fasting plasma insulin levels, and normal glucose levels (Kahn, 1998). Therefore, insulin resistance can go unnoticed as blood glucose levels can remain normal. However, high circulating levels of insulin can lead to dyslipidemia and thereby increase the risk of cardiovascular disease. Often, insulin resistance will progress into type 2 diabetes (Cali & Caprio, 2008). This occurs when cells become so resistant to insulin that increased insulin levels fail to keep blood glucose under control, and/or the pancreatic beta cells lose their ability to respond to

hyperglycaemic stimuli and stop releasing insulin, leading to persistent hyperglycaemia and eventual destruction of beta cells (Cali & Caprio, 2008; Kahn, 2003).

The development of type 2 diabetes is often gradual. Starting with normal glucose levels and hyperinsulinemia, progressing to mildly elevated glucose levels and hyperinsulinemia, and finally hyperglycemia, with or without high insulin levels (Cali & Caprio, 2008). An oral glucose tolerance test is a diagnostic test used to assess glucose metabolism and diagnose diabetes and insulin resistance. It consists of a standardized dose of 1.75 grams of glucose per kg of body weight (to a maximum of 75 g) given to a fasted individual; a typical protocol involves sampling blood before the glucose is administered, and 2 hours after glucose administration. Two hours post test, glucose levels should be below 7.8 mM. Glucose levels two hours post test between 7.9 mM and 11.0 mM are indicative of impaired glucose tolerance. A fasting glucose concentration between 6.2-7.8 mM is considered impaired fasting glucose (WHO, 2006). The diagnostic criteria for overt type 2 diabetes include a fasting blood glucose $\geq$ 7.0 mM, or a two-hour post test blood glucose value $\geq$ 11.1 mM according to the World Health Organization (WHO, 2006).

An abundance of epidemiological data link low birth weight and compensatory growth to impaired glucose tolerance, insulin resistance, and the development of type 2 diabetes (Eriksson et al., 2006; McMillen & Robinson, 2005). Animal studies of developmental plasticity that induce diabetes primarily use rodent models. This is due to the rodent's shorter lifespan, well established research protocols, and their limited space and animal care requirements. Reduced fetal growth can be induced in rodents by low maternal protein or energy diets, or maternal exposure to glucocorticoids. Chemical

induction of diabetes in rodent mothers will also result in reduced fetal growth (Vuguin, 2007). This growth restriction leads to permanent structural and functional changes in the organs and tissues of the offspring. These changes, such as reduced beta cell mass or insulin secretion, and decreased liver, skeletal, and adipose tissue insulin sensitivity, lead to obesity and with time, to the development of type 2 diabetes (Martin-Gronert & Ozanne, 2007; Waterland & Garza, 1999).

Although not used as extensively as the rodent models, sheep and pigs are used to study fetal programming and its effects on glucose metabolism. Pre-natal growth can be restricted in fetal lambs by surgical removal of endometrial caruncles in the ewes, which causes reduced placental size and fetal growth restriction. Size at birth of male lambs has a negative relationship with glucose tolerance at one year of age. In female lambs, the surgical placental restriction was associated with increased insulin sensitivity, demonstrating a gender difference in response to the reduced fetal growth (Owens et al, 2007). Poore and Fowden (2004a) demonstrated a relationship between pre-natal growth and glucose tolerance in pigs. Low birth weight pigs had higher glucose area-under the curve values than high birth weight pigs at 12 months of age. Poore and Fowden also showed that early catch up growth in pigs was associated with insulin resistance at 12 months of age across all birth weights; with no difference in insulin resistance between high and low birth weight piglets (Poore & Fowden, 2004b).

1.5 Developmental origins of obesity

The marked increase in the rate of adult and childhood obesity in developed countries is well documented, and of epidemic proportion (James, 2008). More than 50% of adults in the United States are overweight, with a BMI of 25 or higher (Flegal et al.,

2002). Epidemiological data have linked both reduced pre-natal growth (children who are born small for gestational age (SGA)) and rapid catch-up growth, with increased risk of obesity and a predisposition toward increased fat mass, particularly visceral fat (Druet & Ong, 2008). In a longitudinal cohort of children born SGA had gained more total and abdominal fat by 4 years of age than the normal birth weight control group. At 6 years of age, the SGA children had 50% more visceral fat than the controls (Ibanez et al., 2008a). A larger study found higher fasting insulin levels and visceral fat masses in SGA children than controls at 6 years of age. In the latter study fasting insulin concentration was strongly predictive of visceral fat mass (Ibanez et al., 2008b). The amount of visceral fat was also associated with catch-up growth in both of these studies. In animal models of compensatory growth, rapid catch-up growth often results in obesity and/or increased visceral fat later in life (Zhan et al., 2006). This obesity may be a result of leptin resistance, leading to inappropriate appetite regulation, or insulin resistance (McMillen & Robinson, 2005). Obesity, particularly high visceral adiposity is a risk factor for insulin resistance and development of type 2 diabetes (Rader, 2007). In this relationship between insulin resistance and obesity, it is difficult to define causation, as insulin resistance has been shown to increase the risk of obesity (Guilherme et al., 2008)

1.6 Early nutrition and DNA methylation

Epigenetic changes may be an underlying mechanism in the plasticity associated with early development (Waterland and Garza, 1999, Waterland and Jirtle, 2004). As plasticity associated with early development does not change an organism's DNA, but can produce substantial physiological changes, it must be mediated by differential expression of the DNA. DNA modification plays a central role in the regulation of a

gene's expression, and could have the potential to bring about changes observed in developmental plasticity.

DNA methylation is chemical modification of DNA involving the addition of a methyl group to a nucleotide. The most common site of methylation in all vertebrates is the 5 position of a cytosine's pyrimidine ring. DNA methylation is considered an epigenetic mechanism, as DNA methylation can alter gene expression without changing the sequence of DNA (Waterland & Michels, 2007). Epigenetic mechanisms, of which DNA methylation is the most well known, can regulate gene expression and are therefore critical during development and throughout life (Van den Veyver, 2002). In mammals, DNA methylation typically occurs on cytosines next to a guanine residue (CpG, p denoting a phosphate group). In mammals, approximately 70% of CpGs are typically methylated, however clusters of hypomethylated (approximately 30% methylation) CpGs, called "CpG islands" are often found in the 5'regulatory region of many genes (Rees, 2002; Waterland & Jirtle, 2004). These islands are thought to be one of the ways by which DNA methylation regulates gene expression. Typically, hypermethylation of these islands has a silencing effect on its corresponding gene, as demonstrated by hypermethylation of CpG islands in tumour suppressor genes which are often involved in different forms of cancer (Van den Veyver, 2002). The method by which methylation reduces gene expression is likely by blocking transcription factors, by recruiting proteins that interfere with transcription, or by modifying chromatin structure itself or recruiting proteins that do so (Van den Veyver, 2002).

The DNA methylation patterns are set early in development and vary between cells of different tissue types. These patterns are then replicated in progeny cells and are

maintained throughout life. In early human development, DNA methylation is set up by DNA-methyltransferase enzymes (DNMT), of which DNMT 1, 3a, and 3b are essential for embryonic development. DNMT 3a and 3b are *de novo* methyltransferases that establish the methylation patterns and DNMT 1 is primarily a maintenance methyltransferase, which preferentially methylates hemi-methylated DNA (Van den Veyver, 2002).

Early nutrition has been shown to have an impact on DNA methylation. Manipulation (over or under supply) of the single carbon donors, such as methionine or choline, or critical co-factors such as folic acid or vitamin B_{12}, has led to changes in DNA methylation. Analysis of DNA from fetal livers of dams fed varying levels of protein shows decreased global DNA methylation and proportionally reduced birth weights. The reduction in DNA methylation was thought to be in response to lowered amounts of methionine in the diets. (Rees et al., 2000). The *yellow agouti* mouse has a genetic mutation that produces yellow hair pigmentation. Oversupply of methyl donors in *yellow agouti* mice dam diet by supplementation with folic acid, vitamin B_{12}, choline, and betaine has been shown to increase DNA methylation and suppress the expression of the yellow pigment in mice pups, returning the mice to a typical grey coat colour (Waterland & Jirtle, 2003). The $Axin^{Fu}$ mouse, which develops a kinky tail due to a genetic mutation, can also have expression of kinky tails suppressed by methyl oversupply in dam diets (Waterland et al., 2006). The *yellow agouti* mouse and $Axin^{Fu}$ mouse are both examples of phenotypic changes mediated by DNA methylation changes resulting from methyl oversupply. The fetal nutrient environment is important in the establishment of DNA methylation, and changes in the nutrient environment can affect global DNA methylation.

Alterations in DNA methylation can have profound effects on gene expression, some resulting in significant phenotypic changes. DNA methylation is thought to be the primary epigenetic mechanism behind the developmental origins of adult disease. Changes in gene expression brought on by developmental factors such as pre- or post-natal diet, and compensatory growth are thought to be mediated by changes in DNA methylation (Waterland &Michels, 2007).

1.7 Methyl supply and the sulfur amino acid cycle

The methyl groups required for DNA methylation come from S-adenosyl methionine (SAM), which is formed directly from the amino acid methionine. The enzyme methionine adenosyltransferase (MAT) attaches an adenosyl group from ATP to the sulfur atom of methionine to form SAM. When a SAM-dependent methyltransferase, such as the DNA methyltransferases (DNMTs), requires a methyl group, SAM is used as the methyl group donor and S-adenosyl homocysteine (SAH) is formed. SAH is then hydrolyzed by S-adenosylhomocysteine hydrolase (SAHH), to form homocysteine and adenosine (Brosnan et al., 2007; Van den Veyver, 2002). This process is called the transmethylation pathway.

Homocysteine is the branch point in the sulfur amino acid cycle. Homocysteine can be remethylated to methionine, or catabolised via the transsulfuration pathway to cysteine. The remethylation of homocysteine occurs via two separate enzymes: betaine-homocysteine methyltransferase (BHMT), which converts a betaine and homocysteine to dimethylglycine and methionine, or methionine synthase (MS), which transfers a methyl group from 5-methyl tetrahydrofolate to homocysteine to form methionine and tetrahydrofolate. The catabolism of homocysteine to cysteine is a two-step process. In the

first step, homocysteine is condensed with serine to form cystathionine, catalyzed by cystathionine β-synthase (CβS). In the second step cystathionine is broken down to cysteine and α-ketobutyrate by the enzyme cystathionase (CTH or cystathionine γ-lyase, CγL) (Brosnan et al 2007; Stipanuk, 2004).

Lack of methyl donors such as folate (actually an essential carrier of serine-derived methyl groups), betaine, or choline (which can be converted to betaine), or essential cofactors in the sulfur amino acid cycle, such as zinc or vitamin B_{12}, can result in an accumulation of homocysteine and reduced SAM levels. Due to the fact that SAH hydrolysis is a reversible reaction, as homocysteine increases so will SAH. This increase in SAH will decrease the SAM/SAH ratio. The SAM/SAH ratio is called the methylation index because SAH is inhibitory to most methyltransferases, and in general, as the SAM/SAH ratio decreases so too does methylation (Van den Veyver, 2002).

Some of the effects of reduced pre-natal growth may be related to sulfur amino acid metabolism. Some animal models of developmental plasticity involve feeding reduced protein diets to mothers to reduce offspring birth weight, this leads to an increased risk of many adult diseases, especially hypertension and reduced glucose tolerance (Ozanne, 2001). These models led to the hypothesis that a lack of amino acids was perhaps the cause of reduced fetal growth, and its later consequences. However, human trials have shown that protein supplementation of mothers did not improve fetal growth (Rees, 2002).

1.8 Swine models

Pigs are litter-bearing animals which show natural variation of birth weights within a litter. The variation in fetal growth has been attributed to variation in nutrient

supply (via umbilical blood supply). The fetuses that have reduced blood supply, and therefore fewer nutrients, are born as runts or are spontaneous aborted prior to birth (Bertram & Hanson, 2001). These runts, when compared to their larger, genetically similar littermates, make excellent animal models to investigate developmental plasticity due to reduced intrauterine growth (Poore & Fowden, 2002).

Runt piglets are known to experience compensatory growth, although some may never reach the weight of their larger littermates. This compensatory growth is thought to occur early in life during the suckling phase (Ritacco et al., 1997). Low birth weight piglets are less glucose tolerant later in life than their larger littermates, and in these piglets, rapid compensatory growth is associated with insulin resistance (Poore & Fowden, 2002).

Miniature pigs, such as the Yucatan, are an ideal animal model for use in the study of human health. Miniature pigs are precocial, meaning they are relatively mature at birth, unlike rodents, which are altricial (born before they are completely developed) (Vuguin, 2007). Pigs are phenotypically similar to humans, having a similar cardiovascular system, metabolism, lipoprotein profile, and size. Miniature pigs' omnivorous diets, eating habits and nutrient requirements are also very similar to those of humans (Bellinger et al., 2006). Pigs, unlike humans, are litter-bearing animals; however this is a benefit in studying developmental plasticity. As previously discussed, the natural variation in blood supply to pig fetuses results in the development of runts, thereby providing an excellent model to study differences in pre-natal growth (Poore and Fowden, 2004b).

1.9 Previous studies

In a previous study in our laboratory, the effect of pre-natal and post-natal growth, as well as early post-natal diet, on the development of diabetes was investigated in Yucatan miniature pigs (McKnight, 2008). Post-weaning, the pigs were fed a normal pig feed diet until approximately 10 months of age. No overt type 2 diabetes developed in any of the pigs. Neither pre-natal growth nor post-natal diet affected glucose tolerance or insulin sensitivity. Males, however, were more glucose tolerant than females. Males also had less visceral fat. Visceral fat percentage showed a negative relationship with glucose tolerance which explained the sex differences. Compensatory growth was observed during the milk feeding phase, with runts experiencing accelerated growth compared to their large littermates. This growth, however, was not related to insulin sensitivity or glucose tolerance. The pigs were fed a nutritionally balanced pig feed used in commercial pig farming, which, combined with the young age of these pigs (approximately 10 months, equivalent to young adults in humans), may have protected them from developing more profound symptoms of insulin resistance or diabetes. The logical progression of this study is to investigate the effects of poor dietary practices and a longer feeding phase on the symptoms of insulin resistance and diabetes, and this will be done in this thesis.

In an earlier study in our laboratory the activity of sulfur amino acid enzymes was measured in runt and large piglets 3-5 days old (Brophy, 2006). Runt piglets were found to have lower activity of betaine homocysteine methyltransferase (BHMT), and cystathionase (CTH) than their larger siblings. The activities of these sulfur amino acid enzymes are both related to the available methyl supply. BHMT is the key enzyme in one

of the two pathways that remethylate homocysteine to methionine, CTH is the second enzyme on the transsulfuration pathway that converts homocysteine to cysteine. Both of the enzymes are important in reducing the amount of homocysteine, and thereby increasing the SAM/SAH ratio. The next step is to investigate whether the gene expression levels of the enzymes are also different, to determine if the differences in activity are due to pre- or post-translational regulation.

This SAM/SAH ratio, as described previously, is called the methylation index, which has been linked to the activity of methylating enzymes. One of the hypothesized mechanisms in fetal programming is altered gene expression due to changes in DNA methylation. This altered DNA methylation could be a generalized effect, or targeted to specific genes or gene families. As differences in BHMT and CTH gene expression or activities could alter methyl supply, these differences between the runt and large piglets will be investigated in this thesis.

Chapter 2: Glucose metabolism study

2.1 Objective

Pre-natal growth and compensatory growth after birth have been shown to increase the risk of developing diseases later in life. Insulin resistance, and impaired glucose tolerance (precursors of overt type 2 diabetes) in particular, are linked to low pre-natal growth, and rapid post-natal compensatory growth. As outlined previously, a study in Yucatan miniature pigs looking at the development of type 2 diabetes as it related to developmental plasticity observed no development of overt type 2 diabetes, nor an effect of pre-natal or post-natal growth or post-natal diet on later insulin sensitivity or glucose tolerance (McKnight, 2008). In that study, however, the pigs were fed a balanced normal pig feed, and were raised until approximately 8 months of age. This healthy diet may have protected the pigs from developing type 2 diabetes, as it was low in fat and simple carbohydrates. Although the development of type 2 diabetes in humans is increasingly being observed at earlier ages, 8 months of age in a pig is equivalent to early adulthood in humans. Because the risk of developing type 2 diabetes increases with age, it is possible that these pigs were simply too young to exhibit the possible consequences of reduced pre-natal growth and compensatory growth. The current study will use the same basic design as the previous study: 18 Yucatan miniature pigs, comprising 6 triplets (3 all male, 3 all female), each containing a runt (<800g) and large littermate (>1100g) fed formula for the first month of age, and a sow-fed littermate (>1000g) left with the sow for the first month of age. At 1 month of age the triplets will be housed together and fed a 'cafeteria' diet modeled towards a high intake of sugar, salt, saturated fat, trans fat and total fat.

This style of diet is thought to be diabetogenic and promotes obesity. The pigs will also be raised for a longer period of approximately 12 months. After which time a glucose tolerance test (IVGTT) and insulin sensitivity test (IST) will be performed. The main objectives of this study are to: 1) determine the effects of pre-natal growth on glucose metabolism and the development of type 2 diabetes; 2) determine the effect of early post-natal diet on glucose metabolism and the development of type 2 diabetes; 3) determine the effects of post-natal growth on glucose metabolism and the development of type 2 diabetes.

2.2 Hypothesis

Runt piglets will have reduced glucose tolerance and increased insulin resistance compared to their larger littermates. The sow-fed piglets will have better glucose tolerance and reduced insulin resistance compared to the larger littermates due to the health benefits of sow's milk over formula. Rapid early growth will show a negative effect on glucose metabolism and insulin sensitivity. The high fat, high salt, high sugar diet fed to the pigs will induce obesity and be detrimental to the glucose metabolism of the pigs. The insulin sensitivity and glucose tolerance of the pigs will deteriorate with increased obesity.

2.3 Methods

2.3.1 Animals, housing, feeding and suometric measures

Eighteen Yucatan miniature pigs (9 male, 9 female) were obtained and housed at the at the Vivarium animal care facility at Memorial University. The 18 pigs consisted of 6 triplets from individual litters; triplets were used in an attempt to control for genetic variability, age and birth conditions. Each triplet contained a runt (R) (<800g birth weight), a large (L) (>1100g), and a sow–fed (SF) littermate (>1000g) of the same sex. The runts and large piglets were taken from the sow at 3 days of age and fed milk replacer (Piglet-Gro, Grober Nutrition, Cambridge Ontario) for 28 days on the schedule outlined in Table 2.3.1.1. Sow-fed piglets were left with the sow until 31 days of age. At 31 days of age the triplets were housed together, but separated and individually fed *ad libitum* 'cafeteria' diets for 5 hours daily. The 'cafeteria' diet was made from commercial pig grower chow from Eastern Co-op feeds (Table 2.3.1.2). The commercial pelleted diet was milled and mixed with melted lard (NoName, Loblaw Companies Limited, Brampton, ON) and hydrogenated margarine (Central Dairies, St. John's, NL), sugar (Lantic, natural granulated, Lantic Sugar Limited, Montreal, QC), and salt (Windsor, free running iodized, The Canadian Salt Company Limited, Pointe-Claire, QC). The cafeteria diet ingredients are shown in Table 2.3.1.3. The cafeteria diet composition calculated as a percent of total calories is outlined in Table 2.3.1.2. The composition of this diet is similar to other diets fed to miniature pigs to study atherosclerosis and the development of diabetes (Dixon et al. 1999; Otis et al. 2003). The diet is also within the range of intakes of total and saturated fat observed in humans (Food and Nutrition Board, 2005). This diet

was stored frozen as granular feed and fed by bowl to the pigs *ad libitum* for five hours daily (1200-1700 h).

Feed intake was monitored by subtracting feed remaining after 5 hours from starting feed amount. Feed intake during the milk feeding phase, from 8-28 days, was measured daily and summed to calculate feed intake for a given period. During 'cafeteria' diet feeding, feed intake was measured on weekdays; weekly feed intakes were calculated for each animal by determining the average daily feed intake during the 5-day week and multiplying by 7.

Body weight and abdominal circumference were measured bi-weekly. Body weight was measured using a digital scale (XI-120K, Denver Instruments, Denver, CO). Abdominal circumference was measured by a tape measure at the largest point of a pig's abdomen. Blood sampling by jugular puncture was also performed bi-weekly with pigs restrained in a supine position in a steel V-trough. Blood samples were centrifuged for 10 minutes at 4000 x g at 4°C. The plasma was obtained and stored at -20 C for later analysis of plasma glucose. In accordance with the Canadian Council of Animal Care guidelines, this study was approved by the Institutional Animal Care Committee of Memorial University.

Table 2.3.1.1 Milk replacer feeding schedule

Week 1	Week 2	Week 3	Week 4
6:00am 7:30am 9:00 am 10:30am 12:00pm 1:30pm 3:00pm 4:30pm 6:00pm 7:30pm 11:00pm	6:00am 7:30am 9:00 am 10:30am 12:00pm 1:30pm 3:00pm 4:30pm 6:00pm 7:30pm	6:00am 7:30am 9:00 am 10:30am 12:00pm 1:30pm 3:00pm 4:30pm	6:00am 7:30am 9:00 am 10:30am 12:00pm 1:30pm 3:00pm 4:30pm
	Expand pen size	Separate piglets for 0.5-1.0 hours, twice daily.	Last 2 days: begin introducing cafeteria diet.

Table 2.3.1.2 Diet compositions

As % of total calories		
	Pig grower feed	Cafeteria diet
Carbohydrate	60.10	40.32
- Sugar	0.0	10.38
Crude fat	11.64	50.02
- Saturated fat	0.30	16.28
- Trans fat	0.0	2.30
Crude protein	21.26	10.57
Sodium	0 (0.22% by weight)	0 (1.93% by weight)

Table 2.3.1.3 Cafeteria diet components by weight

Ingredient	Weight added (g) to make 1 kg of diet
Co-op pig grower feed	660
Lard	150
Margarine	50
Salt	40
Sugar	100

2.3.2 Growth measures

Total growth rate (TGR) was measured by subtracting weight at birth, from weight at 10 months and dividing by days of age. Growth rates (GR) for 8-14, 15-21, 22-28, 8-28, 30-120, 120-210, and 210-300 days were calculated by subtracting starting weight from final weight of each period, and dividing by days in the period. Fractional growth rate was calculated by dividing the GR by the pig's starting weight. The growth phases were selected as pre-sexual maturity (30-120 days), sexual maturation (120-210 days), and post sexual maturation (210-300 days) (Figure 2.3.2.1). Sexual maturation in Yucatan miniature pigs occurs at approximately 4-6 months of age or between 20-30 kg of weight (Smith and Swindle, 2006). The sexual maturation phase defined in this study was deliberately larger than these literature values to ensure all pigs reached sexual maturation during this time period. Feed efficiency (FE) was calculated by dividing the kg of body weight gained in a growth phase by the kg of feed intake over the same time period.

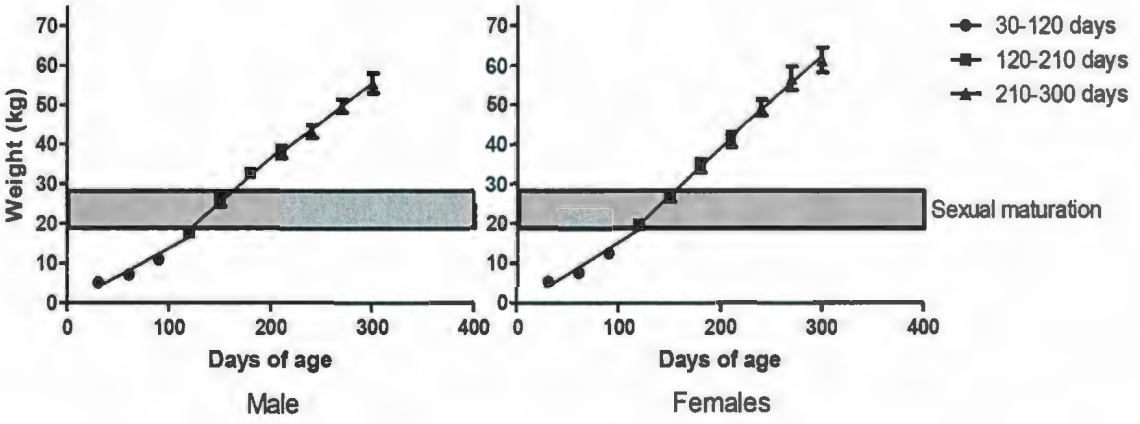


Figure 2.3.2.1 Growth phases and sexual maturation in Yucatan miniature pigs.

2.3.3 Surgical procedures

At approximately 10 months of age (Table 2.3.3.1 for exact ages at surgery and necropsy) the triplets were transported to the Health Sciences Centre, and housed there for at least 24 h before surgery. As only two surgeries could be completed each day, two pigs from each triplet had surgery on the same day, with the third undergoing surgery the following day. All pigs were fasted (24h) the day before surgery. Pigs were anaesthetized with ketamine hydrochloride (0.25 mL/kg, Ketalean, Bimeda-MTC Cambridge ON) and xylazine (0.02 mL/kg, Rompum, Bayer Toronto ON), and maintained with 0.5 – 1.5 % halothane gas and 3/2 oxygen/nitrous oxide. A small incision was made on the proximal end of the medial anterior surface of the left leg, and the femoral vein was isolated. Tygon catheters, each 2.4 metres in length, with inner and outer diameters of 0.040 and 0.070 mm (Norton Performance Plastics, Akron Ohio), respectively, were inserted into the femoral vein and advanced to the inferior vena cava. The catheters were tunneled under the skin and out through a small incision made between the scapulae. As part of another study, a radiotelemeter catheter was also inserted in the femoral artery and the telemeter body was implanted between the anterior thigh and peritoneum. The telemeters were used to measure blood pressure and physical activity. The pigs were administered intravenous antibiotics (Borgal: Trimethoprim 40 mg/ml, Sulfadoxine 20 mg/ml, Intervet Canada Ltd. Whitby, ON) immediately after surgery and for three days post- surgery. Buprenorphine hydrochloride 300 μg (Temgesic, Schering-Plough Ltd., Hertfordshire, UK) was administered at the end of surgery and 24 hours after surgery to minimize post-surgical pain.

Table 2.3.3.1 Age of pigs at surgery and necropsy.

Litter ID #	Age at surgery (days)	Age at necropsy (days)
6	310-311	338-339
7	338-339	365-366
9	273-274	301-302
11	321-322	349-350
12	342-343	370-370
13	358-359	386-387
Average	~324	~352

2.3.4 Study design

Animals were allowed to recover from surgery (4-5 days) before *in vivo* testing was started. During the recovery period, the animals' catheters were flushed daily with 5 mL of heparinized saline (0.2%). Body temperature was measured daily using a digital ear thermometer to monitor post-surgical infection. Animals with a temperature greater than 40°C were given antibiotics (Borgal, as above). The surgical incisions and catheter sites were treated with topical antibiotic cream (Hibitane, Ayerst, Guelph, ON, or Polysporin, Pfizer, Toronto, ON) to prevent infection. The feeding schedule was re-established on the day following surgery.

Five days following surgery, an intravenous glucose tolerance test (IVGTT) was conducted following an overnight fast. Animals with a fever were not tested until their body temperatures and feed intakes returned to normal and they had no longer received antibiotics in the previous 24 hours. Prior to the test, two fasting blood samples were taken 5 minutes apart and blood glucose was measured in a drop of blood using an Ascensia Contour blood glucometer (Bayer, Toronto, ON) to establish baseline values. An intravenous bolus of 0.5 g/kg body weight of 50% glucose solution was then administered. Blood was sampled every 5 minutes and blood glucose levels were checked every 2.5 minutes using a blood drop drawn from a venous catheter (following the removal of the void volume of the catheter). The test was completed when blood glucose concentrations returned to the baseline value range; the number of blood samples varied among pigs from 6-13. Pigs were not restrained during blood sampling, and all blood samples (4 mL vials) were taken from an intravenous catheter, to minimize stress, and stored immediately on ice in an EDTA treated blood tube. Blood samples were

centrifuged for 10 minutes at 4000xg at 4°C. The plasma was stored at -20°C for later analysis of plasma glucose, C-peptide and insulin concentrations.

The next day, an insulin sensitivity test (IST) was conducted following an overnight fast. A blood sample was taken to establish baseline values of glucose 10 minutes prior to the test. After 5 minutes, a 4 μg/kg intravenous dose of somatostatin was administered to inhibit endogenous pancreatic hormone secretion. At time zero, a 0.05 g/kg bolus of 50% glucose solution was administered intravenously. Blood samples were taken every 2.5 minutes, and blood glucose was measured using an Ascensia Contour glucometer. When blood glucose reached a plateau, 0.5 U/kg of body weight bolus of Humulin R insulin (Eli Lilly, Toronto, ON) was given intravenously. Blood samples were taken every 5 minutes, and blood glucose was tested every 2.5 minutes (as above) until blood glucose returned to baseline values. Blood samples were treated as above. Methods for the IVGTT and IST were adapted from Otis and colleagues (2003).

2.3.5 Necropsy

Pigs were anaesthetized with sodium pentobarbital (30 mg/kg, IV), intubated and maintained by 0.5-1.5% halothane gas mixed with oxygen. Two pigs from each litter were killed on the first day, with the third killed on the following day. Organs (lung, heart, stomach, small intestine, large intestine, pancreas, kidney, liver, spleen) were removed and samples were immediately frozen in liquid nitrogen for later analyses or immersed in formalin for histological analysis. Carcass and remaining visceral organs were taken and stored at -20°C for later compositional analyses.

2.3.6 Biochemical analyses

Plasma glucose and insulin levels were measured in all IVGTT and IST blood samples. To assess whether endogenous pancreatic hormone secretions were suppressed by somatostatin, C-peptide levels were measured in plasma samples. Plasma glucose values were measured on a Rapid Lab 865 analyzer (Bayer Diagnostics, Toronto, ON) using a glucose oxidase electrode. Plasma insulin levels were measured using a radioimmunoassay (RIA) kit for porcine insulin (Linco Research, St. Charles Missouri). Briefly, a known quantity of radiolabelled (^{125}I) porcine insulin competes with an unknown amount of unlabeled porcine insulin for binding with a known quantity of antibodies specific for porcine insulin. Once the amounts of labelled and unlabeled porcine insulin are equilibrated, the concentration of bound labelled insulin can be measured and used to calculate the amount of unlabeled insulin present. A standard curve is constructed using known concentrations of labelled and unlabeled porcine insulin. Radioactivity was measured using a gamma counter (Wallac 1480 Wizard, Perkin Elmer, Waltham, MA), and plasma insulin concentrations were determined using the standard curve. Plasma C-peptide concentrations are also measured using a C-peptide RIA kit (Linco Research, St. Charles Missouri) using the same principles. Pancreatic insulin concentration (PIC) was measured by homogenizing pancreas samples in acid alcohol, incubating overnight at 4°C, centrifugation, and assaying supernatant for insulin using a RIA kit, as described above (Modified from Srinivasan et al., 2005). All insulin RIA measurements were performed in duplicate, duplicates which differed by >10 % CV were repeated. The intra-assay CV for the insulin RIA was 8.09%, the inter-assay CV was 7.10%.

2.3.7 Visceral carcass analysis

Intra-peritoneal tissues (small and large intestines, mesentery, stomach, spleen, pancreas, liver) taken at necropsy were homogenized using a meat grinder with a final die size of 1/8" (Model # 4146, Hobart, Troy, OH). Fat was extracted using chloroform: methanol extraction technique in duplicate samples (~10g) from each pig (Folch et al., 1957). The lipid-containing phase was evaporated to yield the dry weight of lipids in each sample, which was expressed as a percentage of original sample's mass. The average of each duplicate was used to calculate the visceral fat percentage.

2.3.8 Calculations

Area under the curve (AUC) for the IVGTT was calculated using GraphPad Prism 5 (GraphPad Software Inc.). Glucose or insulin concentration was plotted against time in minutes and AUC was calculated using the software's AUC function. The baseline for the AUC function was set as the average of the first two values, and peaks that were less than 10% of the overall height of the curve were ignored, only positive peaks were used. The insulin-stimulated glucose half-life (ISGHL in minutes) was calculated using the formula: ISGHL = Ln (2)/K (the half-life equation for a first order equation). K, the rate constant, was calculated by plotting the natural logarithm of plasma glucose concentration vs. time, and taking the slope after insulin administration (Poore and Fowden, 2002). The glucose half–life (GHL in minutes) was calculated from the IVGTT (as described above for the ISGHL), starting after the administration of glucose.

2.3.9 Statistics

All statistical analyses were performed using Minitab Software version 15.1. (Minitab inc. State College, PA). The effects of treatment and gender, as well as possible

interactions were investigated using general linear model (GLM). Because many of the variables studied were related to visceral fat percentage, pancreatic insulin concentration or birth weight, these outcomes were included as covariates, if linear regression of covariate and response variable yielded a $p < 0.05$, and the addition of the covariate increased the adjusted R-squared value of the GLM. Differences among individual means identified by GLM were compared by Tukey's post hoc test. Pearson correlation and linear regression analysis was performed to assess relationships between variables. For statistical tests, data were considered to be significantly different if $p < 0.05$. A statistical trend was defined as between p values ≥ 0.05 and ≤ 0.10. All data are expressed as mean ± standard deviation.

2.4 Results

2.4.1 Fasting plasma analyses

Fasting plasma glucose concentrations did not differ by age (Figure 2.4.1.1), and did not differ among runt, large and sow fed pigs at ~11 months of age (Table 2.4.1.1). The slopes of plasma glucose over time did not differ significantly from zero or between groups (Figure 2.4.1.1). Fasting plasma insulin and plasma glucose:insulin ratio did not differ between groups at ~11 months of age. Gender significantly affected fasting plasma glucose:insulin ratio, with males having higher ratios than females ($p=0.034$) (Figure 2.4.1.2).

2.4.2 Intravenous glucose tolerance test (IVGTT)

Time to return to baseline of glucose (TTRB), glucose area under the curve (gAUC), peak glucose, insulin area under the curve (iAUC), peak insulin, ratio of insulin AUC to glucose AUC (iAUC:gAUC), and glucose half life (GHL) were determined from

a graph of glucose and insulin values over time for each pig (Figure 2.4.2.1) (Table 2.4.2.1). There were no variables that differed among runt, large or sow-fed groups, but gender significantly affected peak glucose, peak insulin and iAUC/gAUC. Females had a higher peak glucose ($p=0.008$), peak insulin ($p=0.005$), and iAUC/gAUC ($p=0.046$) than males (Table 2.4.2.2). Visceral fat content was a significant covariate of gAUC, iAUC and GH; as visceral fat content increased, so did gAUC ($p<0.001$), iAUC ($p=0.008$) and GHL ($p=0.039$). Pancreatic Insulin Concentration (PIC) also significantly affected GHL ($p=0.041$); as PIC increased, so did GHL.

There were no significant correlations between variables from the IVGTT and fasting glucose or insulin for all pigs.

Table 2.4.1.1 Average fasting glucose, insulin and glucose:insulin ratio at ~11 months of age.

	Runts	Large	Sow-fed
Plasma Glucose (mmol/L)	5.9 ± 0.6	5.9 ± 0.2	6.0 ± 0.7
Plasma Insulin (μU/mL)	13.82 ± 6.45	12.71 ± 4.73	17.03 ± 4.44
Glucose:insulin ratio	0.51 ± 0.23	0.53 ± 0.20	0.36 ± 0.07

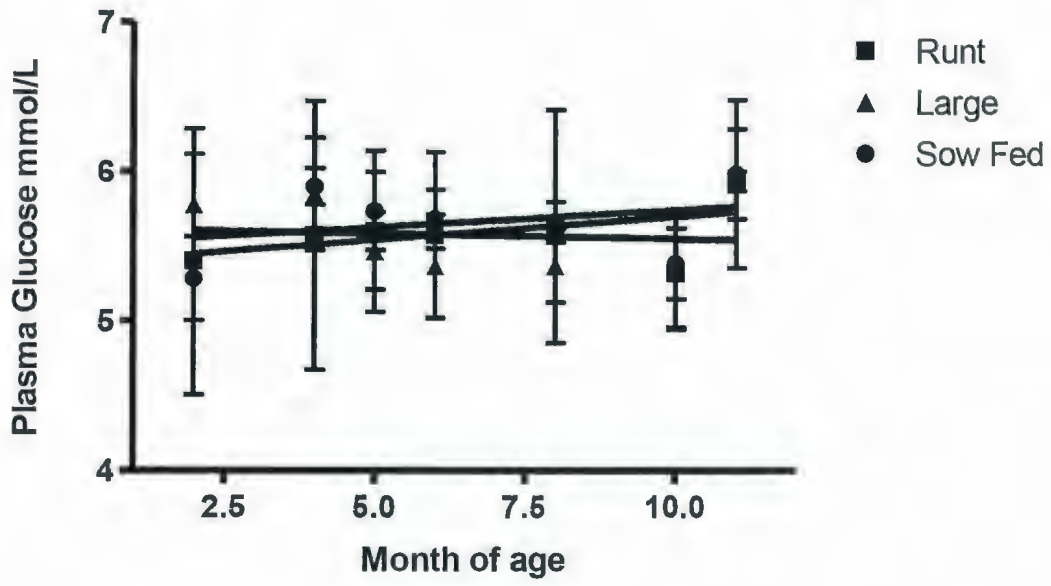


Figure 2.4.1.1 Plasma glucose over time.

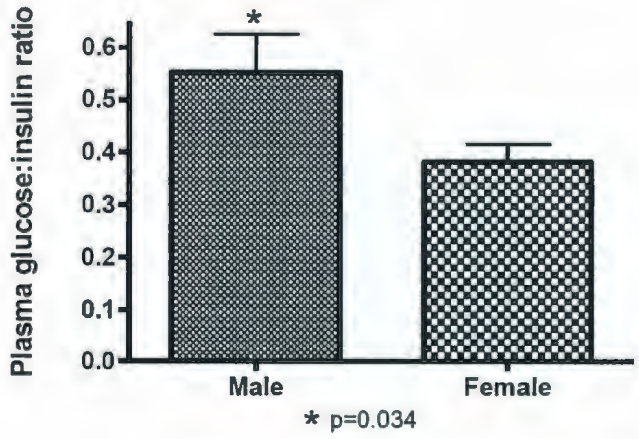


Figure 2.4.1.2 Fasting plasma glucose:insulin ratio by gender.

2.4.3 Insulin sensitivity test (IST)

The insulin stimulated glucose half-life (ISGHL) was used to assess insulin sensitivity. A lower half-life indicates greater insulin sensitivity. There was no difference among runts, large or sow-fed pigs in ISGHL (Figure 2.4.3.1). Visceral fat content and PIC were both significant covariates of the ISGHL; ISGHL increased with visceral fat content ($p=0.014$) and PIC ($p=0.004$). ISGHL correlated positively with the glucose half-life (GHL) calculated from the IVGTT ($r=0.56$, $p=0.019$). There were no significant correlations between variables from the IST and fasting glucose or insulin for all pigs.

2.4.4 Pancreatic insulin concentration (PIC)

PIC did not among between runts, large or sow-fed pigs. Gender significantly affected PIC (Figure 2.4.4.1) with females having a higher pancreatic insulin concentration than males ($p=0.013$) (Figure 2.4.4.2). PIC was positively correlated with GHL ($r=0.52$, $p=0.024$), and ISGHL ($r=0.70$, $p=0.002$) and showed a trend towards increased gAUC ($r=0.46$, $p=0.055$). Abdominal circumference at 3 months of age showed a positive trend with PIC ($r=0.45$, $p=0.058$).

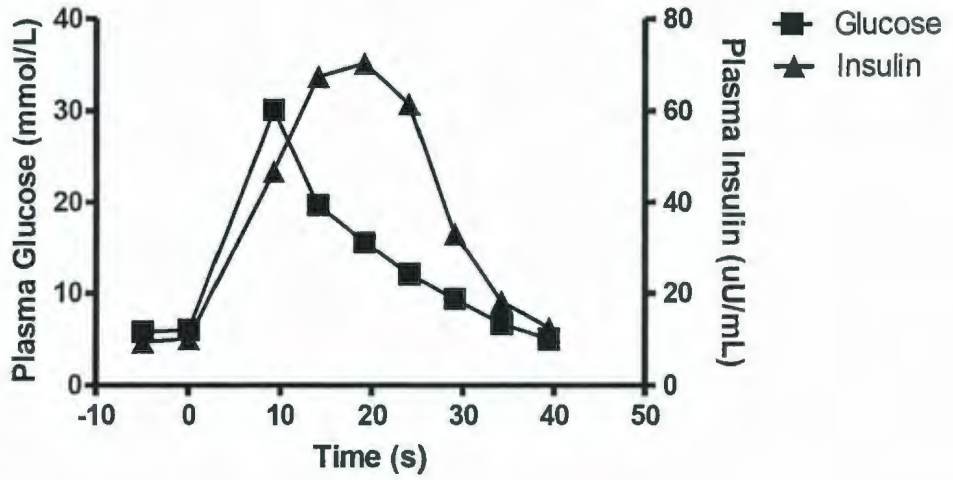


Figure 2.4.2.1 Glucose and insulin AUC from IVGTT of L9 pig.

Table 2.4.2.1 Variables from IVGTT at ~11 months of age.

	Runt	Large	Sow-fed
TTRB (min)	39.5 ± 13.2	50.6 ± 18.5	42.3 ± 15.8
gAUC (mmol·min·L^{-1})	473.6 ± 168.5	505.5 ± 116.45	481.67 ± 187.40
iAUC (μU ·min·L^{-1})	3451 ± 1388	4277 ± 2186	3706 ± 3180
Peak Glucose (mmol/L)	31.0 ± 5.0	29.35 ± 2.63	31.10 ± 4.89
Peak Insulin (μU/mL)	139.3 ± 65.3	158.09 ± 87.78	123.66 ± 72.09
iAUC/gAUC (μU/mmol)	7.52 ± 2.71	8.09 ± 3.25	7.12 ± 3.72
GHL	17.0 ± 6.3	21.71 ± 9.09	16.88 ± 6.04

Table 2.4.2.2 Gender differences in measures of glucose tolerance derived from IVGTT at ~11 months of age.

	Male	Female	p-value
gAUC (mmol·min·L^{-1})	394.5± 108.6	579.3± 132.6	*
iAUC (μU ·min·L^{-1})	2453 ± 1287	5170 ± 2230	*
Peak Glucose (mmol/L)	28.0 ± 3.9	33.0 ± 2.7	0.008
Peak Insulin (μU/mL)	96.2 ± 34.7	184.5 ± 74.7	0.005
iAUC/gAUC (μU/mmol)	6.15 ± 2.27	9.00 ± 3.24	0.046

*No effect of gender due to significant covariate visceral fat content.

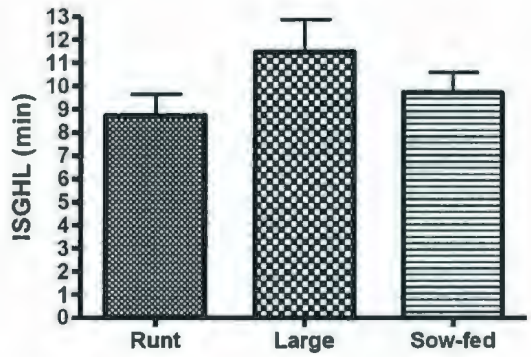


Figure 2.4.3.1 Insulin stimulated glucose half-life of runts, large and sow-fed pigs at ~11 months of age (n=6 for each group).

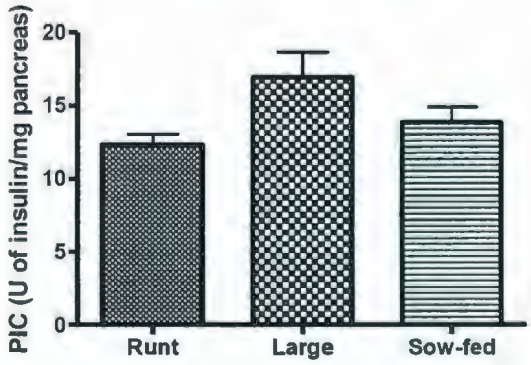


Figure 2.4.4.1 Pancreatic insulin concentration of runts, large and sow-fed pigs at ~11 months of age (n=6 for each group).

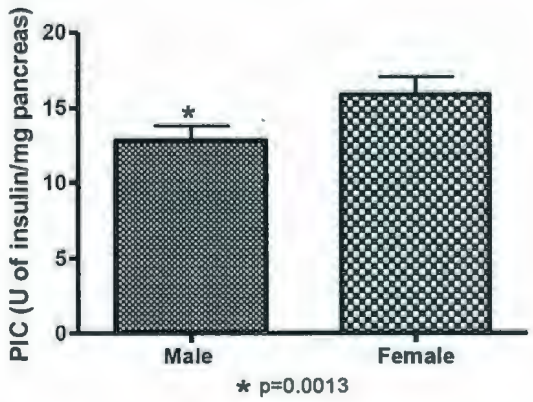


Figure 2.4.4.2 Pancreatic insulin content by gender at ~11 months of age (n=9 for each gender).

2.4.5 Visceral fat

There was no difference among runts, large or sow-fed pigs in visceral fat percentage (Figure 2.4.5.1). Gender significantly affected visceral fat percentage, with females having higher visceral fat than males ($p=0.001$) (Figure 2.4.5.2). Visceral fat percentage correlated positively with abdominal circumference at 1 ($r=0.54$, $p=0.025$) and 3 ($r=0.65$, $p=0.004$) months of age, and showed a positive trend at ~11 months of age ($r=0.45$, $p=0.061$). Visceral fat also tended to correlate positively with PIC ($r=0.44$, $p=0.067$)

2.4.6 Correlations

In order to determine the relationship between visceral fat percentage and pancreatic insulin concentration on the test outcomes, correlations were performed for all the variables measured in the IVGTT and IST (Table 2.4.6.1).

2.4.7 Birth weight and weight at 10 months

Birth weight differed among runt, large and sow-fed groups (Table 2.4.7.1) ($p<0.001$) but did not differ by gender. Weight at 10 months of age did not differ among runt, large or sow-fed group, or by gender, however visceral fat content was a significant covariate of weight at 10 months ($p=0.048$). Birth weight did not significantly correlate with any of the measures of glucose metabolism. Weight at 10 months correlated positively with gAUC ($r=0.51$, $p=0.029$), negatively with plasma glucose:insulin ratio ($r=-0.507$, $p=0.032$) and showed a positive trend with fasting insulin ($r=0.43$, $p=0.076$), iAUC ($r=0.45$, $p=0.063$) and peak glucose during the IVGTT ($r=0.41$, $p=0.093$).

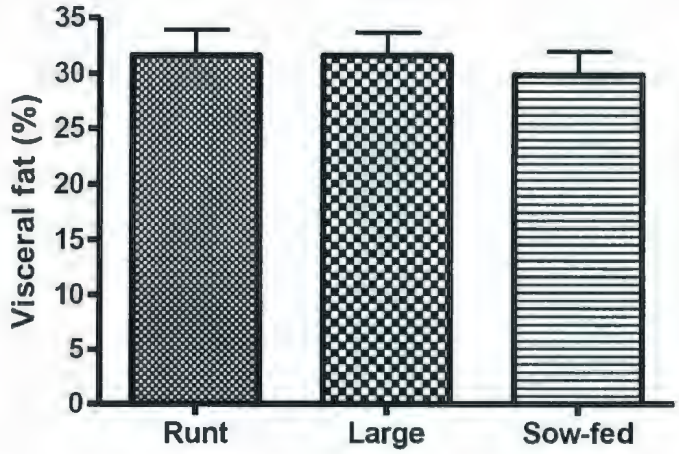


Figure 2.4.5.1 Visceral fat percentage of runts, large and sow-fed pigs at ~12 months of age (n=6 for each group).

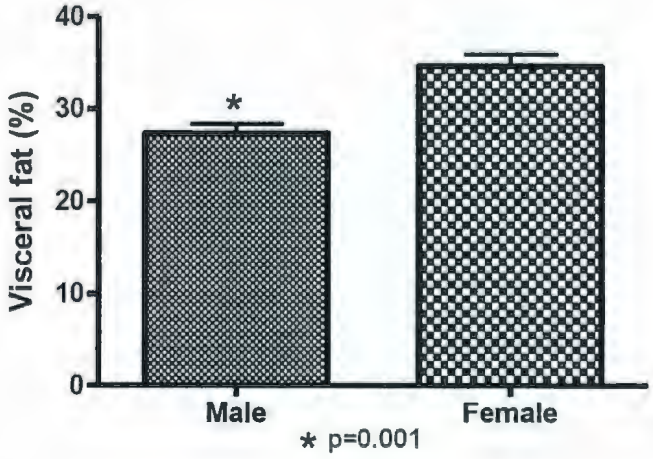


Figure 2.4.5.2 Visceral fat percentage by gender (n=9 for each gender).

Table 2.4.6.1 Measures of glucose tolerance from the IVGTT and IST correlated to visceral fat percentage and PIC.

	Visceral Fat	PIC
gAUC	r=0.807 p=<0.001*	r=0.459 p=0.055
iAUC	r=0.659 p=0.003*	r=0.306 p=0.217
iAUC: gAUC	r=0.354 p=0.150	r=0.071 p=0.778
Peak Glucose	r=0.461 p=0.054	r=0.075 p=0.767
Peak Insulin	r=0.413 p=0.088	r=0.207 p=0.409
GHL (min)	r=0.456 p=0.057	r=0.529 p=0.024*
ISGHL (min)	r=0.620 p=0.008*	r=0.700 p=0.002*
TTRB (min)	r=0.431 p=0.074	r=-0.043 p=0.866

*p<0.05

Table 2.4.7.1 Birth weight and weight at 10 months.

	Runts		Large		Sow Fed	
Litter ID #	Birth Weight (kg)	Weight at 10 Months (kg)	Birth Weight (kg)	Weight at 10 Months (kg)	Birth Weight (kg)	Weight at 10 Months (kg)
6	0.751	44.10	1.133	60.16	1.038	61.62
7	0.869	59.46	1.251	65.12	1.029	57.34
8	0.754	45.30	1.057	48.78	0.978	56.84
11	0.848	56.28	1.119	50.96	1.094	65.50
12	0.618	61.16	1.061	51.56	0.856	69.04
13	0.804	59.44	1.243	76.96	1.001	80.66
Mean	0.774	54.29	1.144	58.92	0.999	65.17
St.Dev	0.090	7.60	0.085	10.82	0.080	8.93

2.4.8 Abdominal circumference

Abdominal circumference at 1 month did not differ among runt, large or sow-fed groups, or gender (Table 2.4.8.1), but did correlate positively with gAUC (r=0.717, p=0.001), iAUC (r=0.504, p=0.033) (Table 2.4.8.2), final weight (r=0.483, p=0.042), visceral fat content at ~12 months of age (r=0.526, p=0.025) and abdominal circumference at 3 months(r=0.690, p=0.002). Abdominal circumference at 1 month also showed a positive trend with abdominal circumference at 10 months (r=0.459, p=0.056). Abdominal circumference at 3 months did not differ among runts, large or sow-fed groups; however an effect of gender was observed, with female pigs having a significantly larger abdominal circumference than males (p=0.023). Abdominal circumference at 3 months also correlated positively with gAUC (r=0.730, p=0.001), iAUC (r=0.576, p=0.012), peak glucose (r=0.534, p=0.022), weight at 10 months (r=0.507, p=0.032), abdominal circumference at 10 months (r=0.547, p=0.019), visceral fat content at ~12 months (r=0.649, p=0.004), and showed a positive trend with PIC (r=0.454, p=0.054) and ISGHL (r=0.451, p=0.069). At 10 months of age a significant interaction of sex and group was observed in abdominal circumference (p=0.040), with female sow-fed pigs having a larger abdominal circumference than male sow-fed pigs. Abdominal circumference at 10 months correlated positively with gAUC (r=0.615, p=0.007), iAUC (r=0.576, p=0.012), peak glucose (r=0.525, p=0.025), weight at 10 months (r=0.694, p=0.001), and showed a positive trend towards visceral fat content (r=0.450, p=0.061).

Table 2.4.8.1 Average abdominal circumferences (cm) at 1, 3 and 10 months of runt, large and sow-fed pigs (n=6 for each group).

Age (months)	Runt	Large	Sow-fed
1	42.8 ± 1.9	42.0 ± 2.9	41.2 ± 4.3
3	58.7 ± 4.4	59.3 ± 1.9	58.8 ± 5.4
10	94.6 ± 6.0	99.2 ± 5.2	100.7 ± 12.0

Table 2.4.8.2 Abdominal circumference at 1, 3 and 10 months correlated to measures of glucose tolerance and insulin sensitivity.

	Abdominal circ at 1 month	Abdominal circ. at 3 month	Abdominal circ. at 10 month
gAUC	r=0.717 p=0.001 *	r=0.730 p=0.001 *	r=0.615 p=0.007 *
iAUC	r=0.504 p=0.033 *	r=0.576 p=0.012 *	r=0.576 p=0.012 *
iAUC: gAUC	r=0.206 p=0.412	r=0.249 p=0.32	r=0.326 p=0.187
Peak Glucose	r=0.305 p=0.22	r=0.534 p=0.022 *	r=0.525 p=0.029 *
Peak Insulin	r=0.104 p=0.681	r=0.353 p=0.151	r=0.336 p=0.172
GHL (min)	r=0.389 p=0.110	r=0.297 p=0.232	r=0.226 p=0.367
ISGHL (min)	r=0.238 p=0.357	r=0.451 p=0.069	r=0.316 p=0.217
TTRB (min)	r=0.259 p=0.299	r=0.307 p=0.215	r=0.071 p=0.780

*p<0.05

2.4.9 Total growth rate (TGR)

TGR (1-300 days) did not differ among runt, large and sow-fed pigs or by sex (Figure 2.4.9.1). TGR correlated negatively with plasma glucose:insulin ratio($r=-0.519$, $p=0.027$), and positively with gAUC ($r=0.514$, $p=0.029$). TGR showed a positive trend towards iAUC ($r=0.452$, $p=0.060$) and fasting plasma insulin ($r=0.449$, $p=0.062$). TGR also showed a positive trend with visceral fat percent ($r=0.442$, $p=0.081$).

2.4.10 Growth rates

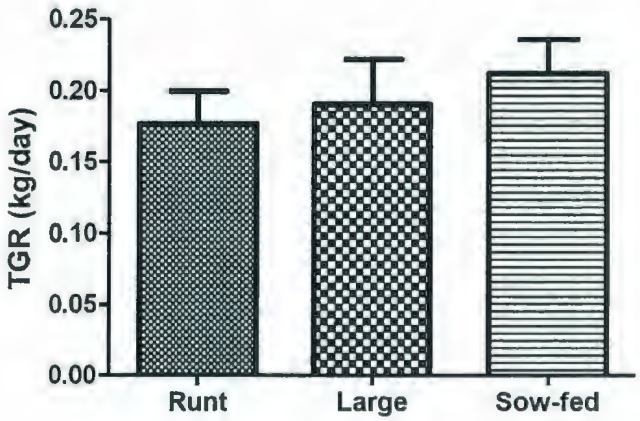
Growth rates for the milk feeding phase, 8-14, 14-21, 21-28, and 8-28 days all differed among runt, large and sow-fed piglets ($p=0.003$, $p<0.001$, $p=0.017$, and $p=0.001$ respectively). The large and sow-fed piglets had higher growth rates than the runts. No significant difference in growth rates was observed between large and sow-fed pigs. Birth weight also correlated positively with growth rates for each time period (Table 2.4.10.1). Growth rates for 8-28, and 22-28 days also correlated positively with TGR ($r=0.527$, $p=0.025$ and $r=0.473$, $p=0.047$). Growth rates in the milk feeding phase did not correlate with any measure of glucose metabolism or suometric measurement. Growth rates for 30-120, 120-210 and 210-300 days did not differ among runt, large or sow-fed pigs or by gender (Figures 2.4.10.2 and 2.4.10.3). GR at 30-120 days shows a positive trend with birth weight ($r=0.448$, $p=0.063$). GR at 120-210 days correlated negatively with feed efficiency at 30-120 days ($r=-0.474$, $p=0.047$) and positively with FE at 120-210 days($r=0.479$, $p=0.044$). GR at 210-300 days also correlated positively with FE at 210-300 days($r=0.778$, $p<0.001$). GR at 30-120 days did not correlate with FE at 30-120days. GR at 120-210 days and 210-300 days correlated positively with final weight ($r=0.757$,

$p<0.001$, and $r=0.766$, $p<0.001$). GR at 120-210 days also correlated positively with abdominal circumference at 10 months ($r=0.751$, $p<0.001$).

GR at 30-120 days correlated negatively with plasma glucose:insulin ratio ($r=-0.478$, $p=0.045$) and fasting glucose ($r=-0.581$, $p=0.011$). GR at 30-120 days correlated positively with iAUC ($r=0.482$, $p=0.043$). GR 120-210 days showed a positive trend towards iAUC ($r=0.416$, $p=0.086$). GR at 210-300 days showed a positive trend with fasting insulin ($r=0.444$, $p=0.065$).

2.4.11 Fractional growth rate

Fractional growth rate at 120-210 days differed among runt, large and sow-fed ($p=0.044$) (Figure 2.4.11.1) with runts having a higher fractional growth rate than large pigs ($p=0.048$). FGR at 210-300 days did not differ among runts, large or sow-fed pigs. FGR at 30-120 days correlated negatively with fasting glucose ($r=-0.600$, $p=0.008$) and showed a positive trend with abdominal circumference at 3 months ($r=0.465$, $p=0.052$).



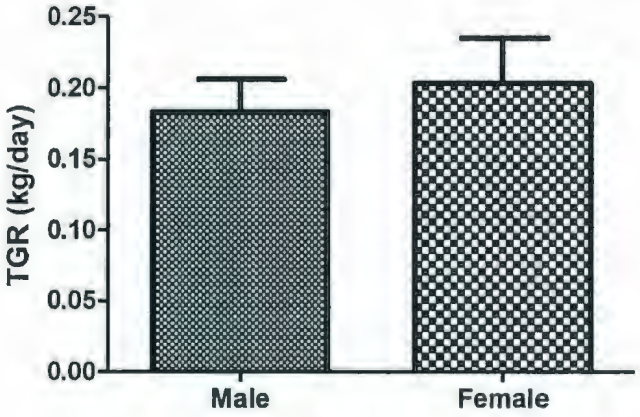


Figure 2.4.9.1 Total growth rate of runt, large and sow-fed pig and by gender.

Table 2.4.10.1 Growth rates correlated to birth weight during milk feeding.

	8-14 Days	14-21 Days	21-28 Days	8-28 Days
Birth weight	r=0.686, p=0.002	r=0.807, p<0.001	r=0.506, p=0.032	r=0.700, p=0.001

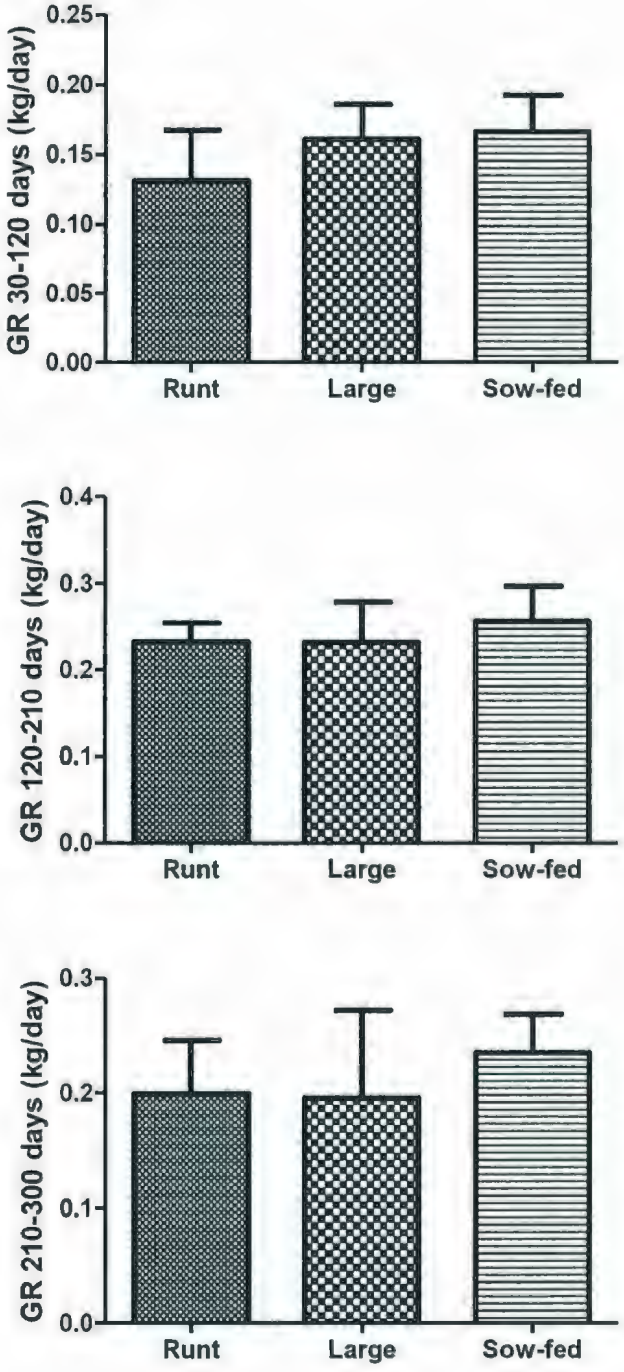


Figure 2.4.10.1 Growth rates of runt, large and sow-fed pigs.

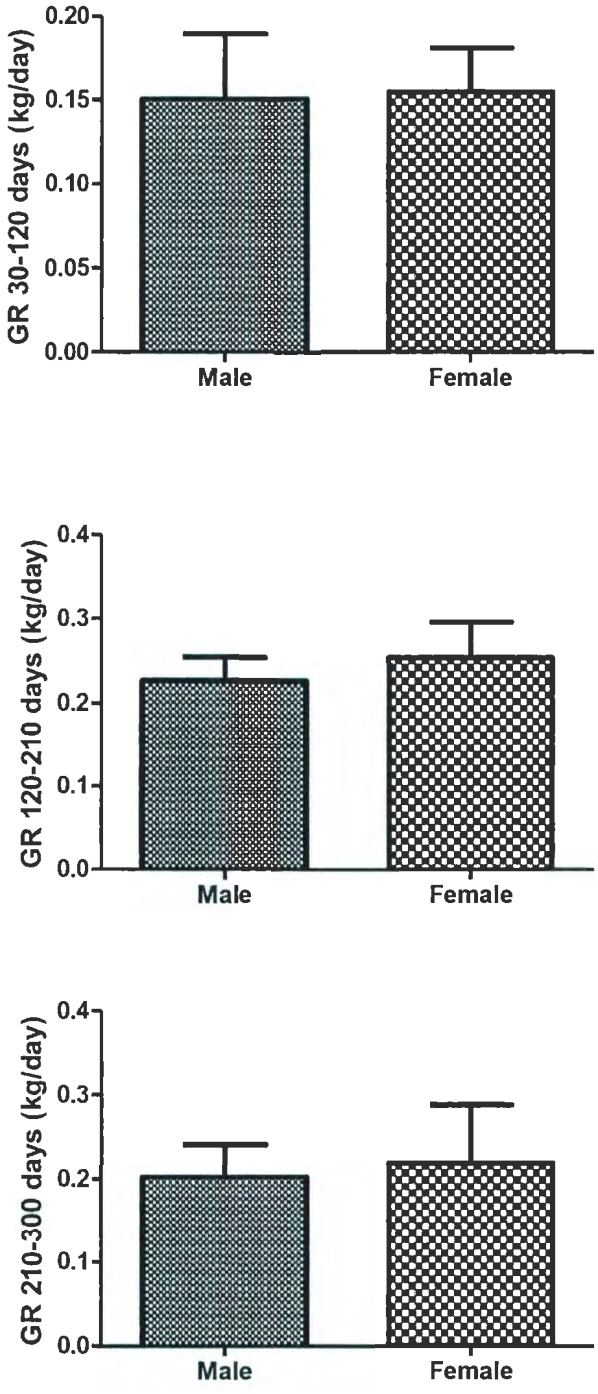


Figure 2.4.10.2 Growth rates by gender.

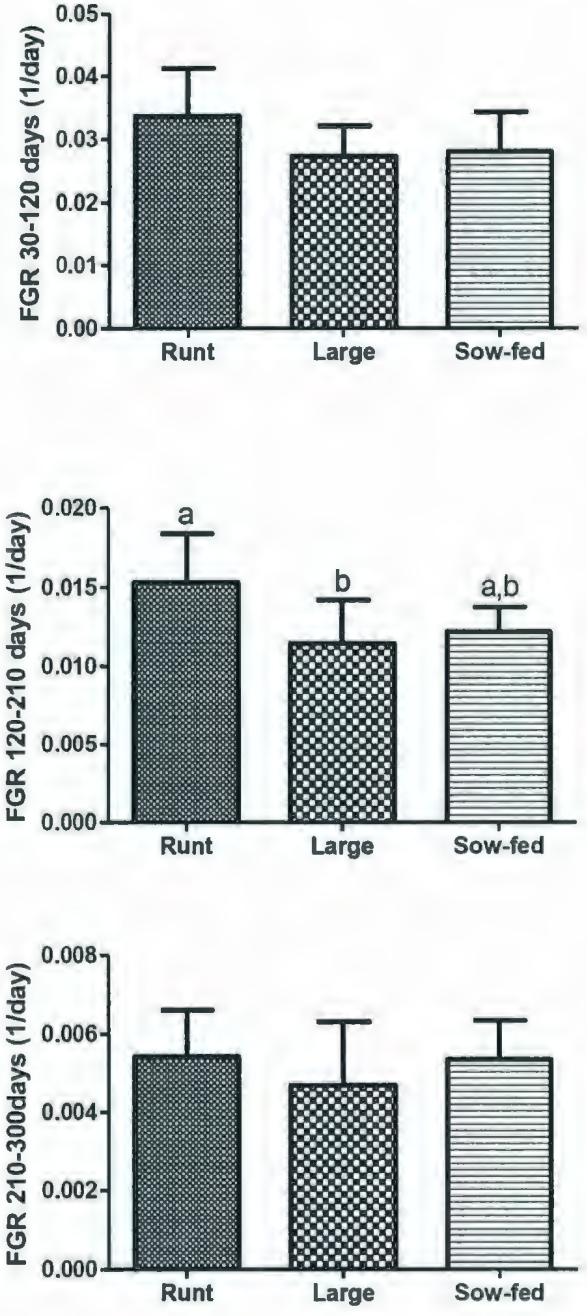


Figure 2.4.11.1 Fractional growth rates by runt, large and sow-fed. Groups with differing superscripts are significantly different ($p<0.05$).

2.4.12 Feed efficiency

Feed efficiency at 8-14, 14-21, 21-28, 30-120, 120-210, and 210-300 days did not differ among runts, large or sow-fed pigs. Feed efficiency approached significant difference between genders for 8-28 days ($p=0.067$) and 30-120 days ($p=0.060$) and was significantly different by gender at 120-210 days ($p<0.001$). FE from 210-300 days did not differ by gender (Figure 2.4.12.1). FE from 8-14 days correlated negatively with PIC ($r=-0.604$, $p=0.038$). FE from 22-28 days correlated negatively with abdominal circumference at 10 months ($r=-0.619$, $p=0.032$) and positively with plasma glucose:insulin ratio ($r=0.646$, $p=0.023$). FE from 8-28 days correlated positively with plasma glucose:insulin ratio ($r=0.637$, $p=0.026$) and negatively with abdominal circumference at 3 months and PIC ($r=-0.740$, $p=0.006$ and $r=-0.607$, $p=0.036$). FE from 30-120 days correlated negatively with GR from 120-210 days ($r=-0.474$, $p=0.047$), FE from 210-300 days ($r=-0.492$, $p=0.038$), visceral fat content ($r=-0.506$, $p=0.032$) and abdominal circumference at 10 months of age ($r=-0.481$, $p=0.043$). FE at 30-120 days also showed a negative trend towards iAUC ($r=-0.416$, $p=0.086$), weight at 10 months of age ($r=-0.460$, $p=0.055$) and FE from 120-210 days ($r=-0.397$, $p=0.100$). Unexpectedly FE from 30-120 days did not correlate with GR from 30-120 ($r=0.078$, $p=0.758$), unlike the 120-210 and 210-300 day periods. FE from 120-210 days showed at positive correlation with gAUC ($r=0.468$, $p=0.050$), visceral fat content ($r=0.597$, $p=0.009$), abdominal circumference at 10 months ($r=0.596$, $p=0.009$) and GR from 120-210 days ($r=0.479$, $p=0.044$). FE from 120-210 days also showed a positive trend towards peak glucose ($r=0.430$, $p=0.075$), and ISGHL ($r=0.414$, $p=0.098$). FE at 210-300 days correlated positively with GR from 210-300 days ($r=0.778$, $p<0001$).

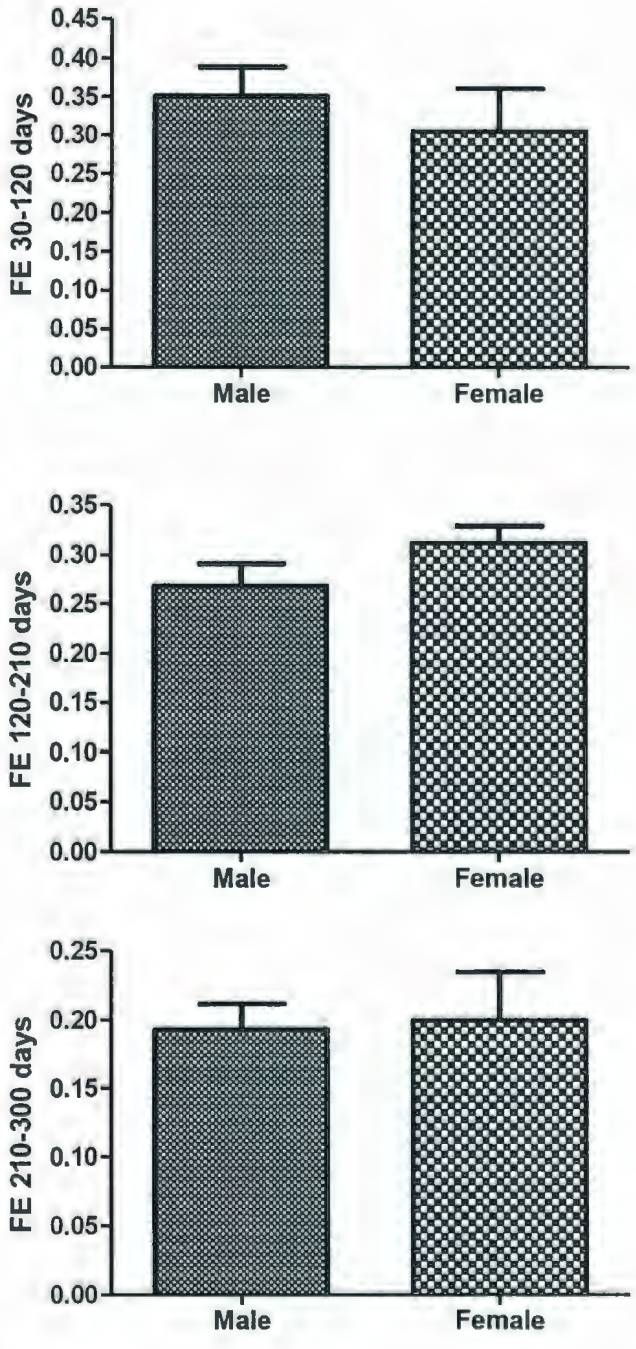


Figure 2.4.12.1 Feed efficiencies by gender.

2.5 Discussion

Analysis of fasting glucose and insulin levels is the first step in assessing glucose metabolism. High insulin levels and low glucose to insulin ratios suggest insulin resistance. High fasting glucose levels suggest the development of type 2 diabetes. Fasting plasma glucose values in our pigs were measured on a monthly basis throughout the study to identify possible trends in blood glucose concentrations over time, and to look for differences in blood glucose among runt, large and sow-fed groups. Normal fasting serum glucose values for healthy, sexually mature Yucatan miniature pigs were determined by Radin and colleagues (1986). They found a range of 2.40-7.40 mmol/L (μ = 3.71 mmol/L) and no difference between genders at 80 weeks of age. A fasting blood glucose concentration range of 3.0-5.0 mmol/L has been reported as normal in other more recent studies utilizing Yucatan miniature pigs (Larsen et al., 2001; Otis et al., 2003; Xi et al., 2004). In our pigs, mean fasting blood glucose values did not change over the course of the study (Figure 2.4.1.1). These values were on the higher end of recently published normal values for Yucatan miniature pigs, but well within the range set out by Radin and not indicative of overt diabetes. At approximately 11 months of age, the pigs had a mean fasting glucose concentration of 5.94 ± 0.51 mmol/L and a mean plasma insulin level of 14.52 ± 5.31 μU/mL. Fasting plasma insulin levels of 5-16 μU/mL have been reported in recent studies using healthy, sexually mature Yucatan miniature pigs (Larsen et al., 2001; Otis et al., 2003; Xi et al., 2004). The plasma glucose to insulin ratio was 0.47 ± 0.19 in our pigs, which was similar to healthy pigs used in other studies (Larsen et al., 2001; Otis et al., 2003; Xi et al., 2004). The glucose and insulin values did not differ significantly among the runt, large and sow-fed piglets. This suggests that the pre-natal growth rate

(runt vs. large) or post-natal diet (large vs. sow-fed) did not have an effect on the fasting plasma values of glucose or insulin.

From a fetal programming perspective, higher insulin levels and therefore a lower glucose to insulin ratio might be expected in the runt piglets, suggesting the development of insulin resistance. From an early nutrition perspective, sow's milk should be better suited for piglets' optimum growth and development compared to cow's milk-based formula. As the large pigs were formula-fed, we hypothesized that large pigs would have decreased glucose tolerance compared to the sow-fed controls, similar to epidemiological evidence in formula-fed humans (Owen et al., 2006). However, this was not the case. Birth weight across all the pigs showed no relationship with fasting glucose or insulin values. However, when plasma insulin levels were compared by gender, female pigs had higher insulin levels than males, and females had lower fasting glucose to insulin ratios. These data suggest that the females were more insulin resistant than the males. Although both males' and females' average fasting plasma insulin levels were within the normal range from the literature, the females were on the upper boundary of normal with a mean of 16.32 ± 4.18 μU/mL. These findings are similar to recent findings in Goettingen miniature pigs, in which higher plasma insulin levels were found in females with no gender difference in plasma glucose levels (Christoffersen et al., 2007). These findings are also in agreement with results from human studies (Williams et al., 2003). In addition, a Spanish study found fasting insulin levels to be higher in obese pre-menopausal women compared with obese men of similar age (Garaulet et al., 2000). This Spanish study

showed a positive correlation between central obesity and insulin levels for both men and women.

Although monitoring fasting plasma glucose and insulin concentrations are convenient and affordable, fasting plasma values do not provide a complete assessment of glucose metabolism. Fasting plasma values cannot demonstrate how the body responds to the stimulus of glucose to maintain normal blood glucose. *In vivo* analysis of glucose metabolism is the most appropriate way to measure glucose tolerance and insulin sensitivity. In humans, an oral glucose tolerance test (OGTT) is used to access *in vivo* glucose metabolism. This test can be modified to yield far more information on glucose tolerance by including blood sampling every five to ten minutes post glucose dose, and measuring plasma glucose and insulin levels. This allows the calculation of a glucose and insulin area under the curves, peaks, and times to return to baseline. Unfortunately, we were unable to perform OGTT on our pigs, as it was impossible to administer an oral dose of glucose to the pigs without stressing them. The administration of oral glucose was attempted in some pigs in a previous study, but this administration of glucose led to extreme agitation in the pigs, causing them to become stressed (McKnight, 2008). The oral administration of the appropriate amount of glucose to each pig was also difficult to ascertain and it was impossible to verify if all the glucose had been completely ingested. If pigs are stressed during an OGTT, then the measured values would be unusable as stress hormones, such as glucocorticoids, cause blood glucose concentrations to increase. We employed a modification of the OGTT and administered the glucose dose intravenously. This intravenous glucose tolerance test (IVGTT) allowed us to test the

pig's glucose tolerance without stressing the pig. The bolus of glucose administered intravenously to the pigs did not cause any noticeable agitation, and it was easy to administer the correct dosage of glucose to each pig.

The results from a IVGTT differ from the results of an OGTT. In a IVGTT the glucose bypasses the gastrointestinal tract and is injected directly in the blood, this removes the variation in intestinal glucose absorption and the gastrointestinal glucoregulatory hormones known as incretins. Incretins can control the amount of insulin released after a meal and vary the gastric emptying rate, as well as inhibit glucagon release (Drucker and Nauck, 2006). If it were possible to administer a stress free OGTT along with the IVGTT more information could have been gleaned on this important gastrointestinal component of glucose metabolism. The IVGTT measures the circulatory component of glucose metabolism in response to a rise in blood glucose by monitoring plasma glucose and insulin levels. Plasma glucose levels rise rapidly after the administration of glucose, and then begin to decrease as the glucose is removed from the blood by the liver and muscles in response to insulin produced by the pancreas. Predictably the levels of insulin also increase following the administration of glucose, and begin to decrease as the blood glucose decreases. The plasma glucose and insulin levels were plotted against time to yield glucose (gAUC) and insulin (iAUC) areas under the curve, peak glucose and insulin values, as well as the time to return to baseline for glucose (TTRB), glucose half life (GHL) which was calculated from the rate of glucose clearance, and the ratio of iAUC to gAUC (iAUC:gAUC). An animal with reduced glucose tolerance would have higher plasma glucose concentrations, and the glucose level

would take longer to return to baseline. This elevated glucose concentration would be due to a decreased clearance of glucose from the blood, likely due to a decreased action of insulin. Glucose intolerant animals would therefore have higher gAUCs, higher peak glucose values, a longer TTRBs and a longer GHL, suggesting a slower clearance of glucose from the blood. Insulin resistant animals would require more insulin to return their plasma glucose levels to normal due to their reduced insulin sensitivities. Therefore insulin resistant animals would have a higher iAUC, peak insulin levels, and iAUC:gAUC ratios.

Similar plasma glucose curves were observed in all pigs in response to glucose administration, and glucose concentrations in all pigs returned to fasting levels by 90 minutes. The gAUC, peak glucose, and TTRB for our pigs did not differ among runt, large or sow-fed pigs. Jonsson and colleagues (2006) studied crossbred Landrace x Yorkshire x Hampshire pigs on a cereal-based or cereal-free (Paleolithic) diet, at 17 months of age. The dose of glucose used in the IVGTT was 0.5 g/kg body weight, as in our study, but the average gAUC was 1076 ± 113 and 1199 ± 212 $mmol \cdot min^{-1} \cdot L^{-1}$ for each diet respectively. Poore and Fowden (2002) on the other hand also used a dose of 0.5g/kg body weight of glucose in their IVGTT in purebred large white breed domestic pigs that were fed a standard pig diet for 12 months, and found average gAUC values for low birth weight and high birth weight pigs of approximately 280 and 200 $mmol \cdot min^{-1} \cdot L^{-1}$, respectively. In Poore and Fowden's study, birth weight negatively correlated with gAUC across both low and high birth weight groups. The average gAUC in our pigs was 487 ± 151 $mmol \cdot min^{-1} \cdot L^{-1}$. These values were much higher than those of Poore and Fowden,

but far lower than those of Jonssen and colleagues. It must be noted that the protocol by Jonssen and colleagues had the pigs restrained in a supine position, although they did not mention the use of sedatives or anesthesia, which have been shown to affect glucose metabolism. Poore and Fowden's techniques most closely resemble ours. It is challenging to compare gAUC values from one study to another, as differing methods will affect the results. The amount of glucose administered and how the area under the curve is calculated will yield vastly different results. The age and diet of pigs in a study may also make comparisons between studies difficult. These differing findings may also be due to differences in glucose metabolism between strains of pigs.

Our findings suggest that neither pre-natal growth nor post-natal diet affected the glucose tolerance of these pigs. As low birth weight has been linked to impaired glucose tolerance, it was expected that the runt pig would be less glucose tolerant than the large pigs. However, birth weight across all pigs did not correlate with any of the measures of the IVGTT, unlike the data from Poore and Fowden. There were no differences in any of the IVGTT measures between the large and sow-fed pigs, suggesting that early post-natal diet did not affect glucose tolerance at ~11 months of age.

We did find an effect of gender in our pigs. Female pigs had higher gAUC values, with an average of 579 ± 133 $mmol \cdot min^{-1} \cdot L^{-1}$ versus males with an average of 395 ± 109 $mmol \cdot min^{-1} \cdot L^{-1}$. This was congruent with our fasting plasma values, which suggested that female pigs were more insulin resistant than males. This gender difference has not been reported by other studies doing similar tests in pigs (Jonsson et al., 2006; Poore & Fowden, 2004a). The experimental groups of pigs used in Poore and Fowden's study

were not balanced for gender. Indeed, the low birth weight group in their study had more female than male pigs, while the high birth weight group had an equal number of male and female pigs. The gender effect observed in our pigs on gAUC, disappeared when visceral fat content was added to the statistical model as a covariate. Visceral fat content was a significant covariate, suggesting that the difference between genders in gAUC was explained by the difference in visceral fat content, with females averaging 34.7 ± 3.9% fat and males 27.4 ± 3.0% fat. Visceral fat also correlated positively with gAUC, suggesting that as visceral fat content increased, glucose tolerance decreased. This relationship between visceral fat and glucose tolerance has been documented in humans (Attalah et al., 2006; Weiss et al., 2003).

A gender effect was also observed for peak glucose concentrations during the IVGTT (females 33.0 ± 2.7 mmol/L, males 28.0 ± 3.9 mmol/L). This could be due to the dosage regimen used in our study. The bolus of glucose was standardized to total body weight, not lean mass, which resulted in higher peak glucose levels in the more obese females. This phenomenon was also noted by Dyson and colleagues (2006), who found higher peak glucose levels in obese Ossabaw pigs than normal weight Ossabaw pigs, in spite of no difference in glucose tolerance between the two groups. It is likely that our more obese females received a higher dose of glucose relative to their metabolic lean mass.

TTRB did not differ by gender, but visceral fat content was a significant covariate, which showed a positive relationship with TTRB. In other words, as visceral fat increased, glucose tolerance decreased, and the time required for glucose values to

return to normal increased. GHL was not significantly different among runt, large or sow-fed pigs, or between genders. The average glucose half-life of our pigs was 18.5 ± 7.3 minutes which is higher than the values reported by Poore and Fowden (2002) of ~12 minutes for low birth weight pigs and ~9 minutes for high birth weight pigs at 12 months of age on a normal pig feed diet. Visceral fat content was a significant covariate in the analysis of GHL, and as visceral fat increased so did GHL. Pancreatic insulin concentration (PIC) was also a significant covariate of GHL; pigs with higher PIC had a longer GHL. This relationship between higher insulin concentration in the pancreas and longer GHL suggests that insulin levels in the pancreas are elevated in pigs with slower glucose clearance, perhaps as a result of insulin resistance, which causes hyperinsulinemia to maintain normal glucose levels. Pancreatic insulin concentrations may be elevated to compensate for higher insulin requirements.

Insulin AUC, peak insulin and iAUC to gAUC ratio from the IVGTT did not differ between runt, large or sow-fed groups which suggests that pre-natal growth or post-natal diet did not affect insulin sensitivity in our pigs at ~11 months of age. Although we hypothesized an increased iAUC or iAUC to gAUC ratio in runt pigs, which would have been suggestive of the development of insulin resistance, no relationship between insulin levels and birth weight was observed in our pigs. In contrast, Poore and Fowden (2004a) found increased iAUC in low compared to high birth weight pigs. iAUC values are affected by the same factors which affect gAUC, making it difficult to compare values from different studies. Poore and Fowden had average iAUC values of approximately 1500 $\mu U \cdot min^{-1} \cdot L^{-1}$ for low birth weight pigs, and 1200 $\mu U \cdot min^{-1} \cdot L^{-1}$ for high birth weight

pigs. Our results yielded an average iAUC of 3811 ± 2252 $\mu U \cdot min^{-1} \cdot L^{-1}$, almost identical to those reported by Dyson and colleagues (2006) in obese female Ossabaw pigs who had an average of 3811 $\mu U \cdot min^{-1} \cdot L^{-1}$, but much higher than those of Poore and Fowden (2004a). The results, once again, showed a strong effect of gender, females having an average iAUC of 5170 ± 2229 $\mu U \cdot min^{-1} \cdot L^{-1}$, twice as high as the males, with an average of 2452 ± 1287 $\mu U \cdot min^{-1} \cdot L^{-1}$. This large difference between genders, coupled with two female pigs with iAUCs in excess of 7000 $\mu U \cdot min^{-1} \cdot L^{-1}$ suggested that the females were insulin resistant compared to the males. The effect of gender on iAUC lost statistical significance when visceral fat content was added to the statistical model as a covariate. Visceral fat was a significant covariate of iAUC; similarly to gAUC, iAUC demonstrated a positive relationship with visceral fat content. This suggests the differences in iAUC between the genders may be related to the differences in visceral fat content. There was also a gender difference observed in peak insulin and iAUC to gAUC ratio, with females having higher peak insulin levels, and higher iAUC to gAUC ratios than the males. This suggests that the female pigs were more insulin resistant than male pigs, and required more insulin to return their plasma glucose levels to normal. Visceral fat content correlated positively with iAUC. This suggests that across both genders as visceral fat content increases, so does insulin resistance. This is consistent with many findings in humans and other animal models (Frayn et al., 2000; Zhao et al., 2007).

An insulin sensitivity test (IST) differs from an IVGTT in its precision. An IVGTT looks at glucose tolerance, which can be affected by numerous factors including the speed at which the pancreas begins to release insulin following an elevation of

glucose, the amount of insulin released, and how effective the insulin is at lowering blood glucose (the insulin sensitivity). An IST looks only at the insulin sensitivity. Using somatostatin to inhibit endogenous secretion of insulin and glucagon, a standardized by weight dose of insulin was administered following a bolus of glucose (Otis et al., 2003). The rate at which the glucose is cleared from the blood following the insulin administration was calculated. The bolus of glucose was first administered to the pigs to elevate their blood glucose. This was done because the pigs were not overtly diabetic and did not have elevated fasting glucose levels. Blood glucose was initially raised to minimize the chance of severe hypoglycemia (Otis et al., 2003). The pre-treatment with somatostatin inhibits release of endogenous glucoregulatory hormones so that the rate of glucose clearance in response to a standardized amount of insulin can be determined. This rate, the insulin stimulated glucose half-life (ISGHL), is directly related to insulin sensitivity. As insulin sensitivity decreases and a subject becomes more insulin resistant, the ISGHL increases. The rate of glucose clearance is sometimes just given as the rate constant, or it can be converted to half-life using the formula ISGHL = Ln (2)/K, where K is the rate constant. To compare our results to those from the literature, this formula was used to convert rate constant to half-life.

The ISGHL in our pigs did not significantly differ among runts, large or sow-fed groups. This finding suggests that pre-natal growth and post-natal diet did not affect insulin sensitivity at ~11 months of age. It was hypothesized that at ~11 months of age the runt piglets would have a longer ISGHL than their large littermates; however this result was not found. In large white pigs, Poore and Fowden (2004b found no difference in

insulin sensitivity between low and high birth weights. The average ISGHL of our pigs was 9.98 ± 2.79 min. This is longer than the average of approximately 7.5 and 6.3 minutes reported by Otis and colleagues (2003). These average ISGHLs were from young (35-45 kg body weight at start of trial) adult male Yucatan miniature pigs fed a high fat diet, and a normal balanced diet, for 20 weeks respectively. This suggests that all of our pigs may have had reduced insulin sensitivity. Visceral fat was found to be a significant covariate of ISGHL. Insulin resistance measured by the IST increased with visceral fat content across all the pigs. PIC was also a significant covariate of ISGHL. Insulin resistance measured by the IST increased in a similar fashion as insulin concentration of the pancreas across all pig. This relationship between PIC and ISGHL is likely due to compensatory hyperinsulinemia, which requires more insulin production, and therefore higher levels of available insulin in the pancreas, to compensate for insulin resistance and maintain normal glucose levels. The relationship among visceral fat, PIC, and ISGHL was also observed in the GHL from the IVGTT. This would be expected, as the GHL from the IVGTT is largely determined by insulin sensitivity. ISGHL correlated significantly with GHL in our pigs.

Pancreatic insulin concentration (PIC) increases during the development of insulin resistance (Asghar et al., 2006; Srinivasan et al., 2005). The increased PIC coincides with increased insulin secretory response to hyperglycemia or insulin secretagogues such as arginine and leucine in rats fed a high carbohydrate formula (Srinivasan et al., 2005). Increased PIC is also related to increased insulin secretory response to glucose that worsens over time in the muscle IGF-I receptor (IGF-IR)-lysine-arginine (MKR) mouse

model of type 2 diabetes which expresses dominant-negative mutant IGF-IRs in skeletal muscle and is diabetic with insulin resistance in muscle, liver, and adipose tissue (Asghar et al., 2006). PIC in our pigs did not differ among runt, large and sow-fed groups. Had reduced pre-natal growth rates in our pigs caused insulin resistance one might have expected higher levels of PIC in the runt pigs. Had a post-natal diet of formula, rather than sow's milk caused more insulin resistance it might be expected that PIC would be elevated in the large pigs compared to the sow-fed. Neither of these results, however, were observed. PIC correlated positively with GHL, and ISGHL, as mentioned previously. The positive trend of PIC with gAUC, suggests a decrease in glucose tolerance with increasing PIC. This is consistent with increased PIC indicating decreased insulin sensitivity. A positive trend between PIC and visceral fat was also observed, suggesting a possible relationship between increased visceral fat and increased pancreatic insulin concentration, both of which show positive relationships with insulin resistance. This may be the first time that PIC was analyzed in pigs as it relates to glucose metabolism.

Gender affected PIC, with females having higher PIC than males. . This difference in PIC between males and females is likely related to differences in visceral fat content between genders. Visceral fat content was strongly related to glucose metabolism in our pigs. This PIC difference was expected given the results from the fasting plasma analysis, IVGTT and IST, which showed that female pigs were less glucose tolerant and more insulin resistance than males. Reduced pre-natal growth followed by *ad-libitum* feeding has been shown to induce obesity in some animal models (McMillen & Robinson, 2005).

Therefore it was expected that runt pigs might have higher visceral fat content, a measure of central obesity, than large pigs. If formula feeding were related to the development of obesity in our pigs, then large pigs would be expected to have higher visceral fat content than the sow-fed pigs. The visceral fat content in our pigs did not differ between runt, large or sow-fed pigs. This suggests that pre-natal growth or post-natal diet did not affect the development of central obesity in our pigs. There was a gender difference in the visceral fat content in our pigs. Female pigs had higher visceral fat than males. Visceral fat content correlated positively with gAUC, GHL, iAUC and ISGHL, suggesting reduced glucose tolerance and insulin sensitivity with increasing visceral fat. The link between visceral fat and glucose metabolism has been clearly established in humans (Kobayashi et al., 2001; Mittra et al., 2008). However, whether impaired glucose metabolism causes visceral adiposity or visceral adiposity causes impaired glucose metabolism has not been established. Both visceral adiposity and impaired glucose metabolism may result from some common pathology and may have synergistic effects on each other. The visceral fat content in this study was measured as a percentage, and absolute visceral fat mass could not be calculated due to an unrecoverable loss of data. Most human studies report visceral fat as a total mass and percentage fat calculated from an MRI, or as a percentage using DEXA scan. Ideally the percentage of visceral fat and the total visceral fat mass would allow for a better analysis of visceral fat's relationship with glucose metabolism and growth. Nevertheless, very strong relationships between the percentage of visceral fat used in this study and the measures of glucose metabolism and abdominal circumference were observed.

The early increase in abdominal circumference was predictive of visceral fat later in life in our pigs. Abdominal circumference as early as 1 month of age correlated significantly with visceral fat content at ~12 months. Abdominal circumference at 3 months of age correlated even more strongly with visceral fat at ~12 months. Visceral fat content at ~12 months of age showed a positive trend with abdominal circumference at 10 months of age. Abdominal circumference was also predictive of decreased glucose tolerance and insulin resistance. Abdominal circumference at 1 month, 3 months and 10 months correlated positively with gAUC and iAUC from the IVGTT at ~11 months of age. This suggests that development of obesity, reduced glucose tolerance, and insulin resistance may begin early in life. However, this development was not related to pre-natal growth or early post-natal diet. Abdominal circumference at 1, 3 and 10 months also correlated positively with weight at 10 months of the pigs.

Rapid growth in early life has been associated with negative health outcomes, including insulin resistance and impaired glucose tolerance (McMillen & Robinson, 2005). Growth rates and feed efficiencies were analyzed over numerous time periods and related to the outcomes of the IVGTT and IST, as well as PIC and visceral fat content. Growth rates in the milk feeding phase (8-14, 14-21, 21-28, and 8-28 days) were significantly lower in runts compared to large and sow-fed pigs. Moreover, growth in each phase of milk feeding was positively correlated with birth weight. This finding does not demonstrate compensatory growth in the milk feeding phase, as lower birth weight animals had lower growth rates than larger animals. This positive relationship between birth weight and early growth was also observed by Poore and Fowden (2004b). The rates

of growth during the milk feeding phase did not correlate with any of the measures of diabetes, fat or suometric measurements.

Total growth rate (TGR) (1-300 days) and growth during "cafeteria diet" feeding phase were calculated. The diet feeding phase was divided into three phases: the pre-sexual maturation phase (30-120 days), sexual maturation (120-210 days), and post-sexual maturation (210-300 days). TGR did not differ significantly among runt, large and sow-fed pigs in our study. This finding suggests a period of compensatory growth. The growth rates in the milk feeding phase were all positively related to birth weight and differed significantly between runt piglets and their large and sow-fed siblings. However, this significant difference disappeared over the life of the pigs suggesting that runt piglets made up for their reduced growth rate early in life. These findings also suggest that pre-natal growth or pre-weaning diet did not affect the overall lifelong growth of the pigs. TGR was significantly correlated with fasting glucose:insulin ratio (negatively) and gAUC (positively), and showed a positive trend with iAUC. This suggests that higher overall growth, which may involve obesity, is related to insulin resistance and reduced glucose tolerance. This finding was further supported by positive trends between TGR and visceral fat content. When growth rates during the different maturation phases were analyzed, no difference in any phase was observed among runt, large and sow-fed pigs or by gender. Pre-sexual maturation (30-120 days) growth rate correlated negatively with fasting glucose:insulin ratio and positively with iAUC. This suggests that accelerated early growth, from 30-120 days, may be linked to insulin resistance later in life. This pre-sexual maturation period may be a critical growth stage in which rapid growth rate may

lead to insulin resistance later in life, similar to findings in humans (Eriksson et al., 2006; Ong & Loos, 2006). Growth rates during sexual maturation correlated positively with weight and abdominal circumference at 10 months, and showed a positive trend with iAUC. Fractional growth rates (FGR) were also calculated for the different maturation phases during "cafeteria diet" feeding. The FGR during sexual maturation (120-220 days) was significantly higher in runts than both large and sow-fed pigs. The FGR for pre and post sexual maturation did not differ among runt, large and sow-fed pigs. These findings suggest that the runt piglets experienced greater growth during sexual maturation than the large and sow-fed pigs proportional to their starting weight. The runt piglets must undergo some compensatory growth during sexual maturation. However, fractional growth rates were not related to any measure of glucose metabolism.

Feed efficiencies (FE) for each growth phase (milk feeding and cafeteria diet) were calculated as compensatory growth in animals is often a result of increased feed efficiency. No significant difference in FE for any growth rate was observed between runt, large or sow-fed pigs. This is consistent with the lack of difference observed in the growth rates, but not with the difference in fractional growth rate during sexual maturation. Interestingly there were significant differences in feed efficiency by gender. FE from 120-210 days was significantly different, with females having the higher feed efficiency during sexual maturation. Feed efficiencies did show an interesting overall relationship with glucose metabolism. High early FE (i.e. before 120 days) may be beneficial to glucose metabolism at 10 months of age. This was demonstrated by FE from 8-28 days correlating positively with fasting glucose:insulin ratio, negatively with

abdominal circumference at 3 months, and PIC. FE from 8-28 days also showed a negative trend with gAUC. This positive relationship with glucose metabolism and health continued with high feed efficiency in the pre-sexual maturation (30-120 day) phase. FE from 30-120 days correlated negatively with gAUC and abdominal circumference at 10 months of age. A negative trend between iAUC and final weight was also observed with FE from 30-120 days. These findings suggest that high feed efficiencies before sexual maturation is protective against the development of insulin resistance, reduced glucose tolerance and obesity.

In contrast, the opposite relationship between FE and glucose metabolism was observed during sexual maturation. High FE during sexual maturation (120-210 days) had a negative relationship with glucose metabolism and health. FE from 120-210 days correlated positively with gAUC, visceral fat content, and abdominal circumference. FE from 120-210 days also showed a positive trend with ISGHL. These findings suggest that elevated FE during sexual maturation may lead to increased central obesity, and reduced glucose tolerance in our pigs, perhaps caused by insulin resistance. Interestingly, unlike the 120-210, and 210-300 day periods, FE from the 30-120 day period did not correlate positively with its corresponding growth rate. Furthermore, FE from 30-120 days correlated negatively with growth rate in the 120-210 day period and showed a negative trend with FE from 120-210. This would suggest that high feed efficiency during the pre-sexual maturation phase was related to reduced growth rate and feed efficiency during sexual maturation.

When feed efficiency and growth findings are analyzed together, it would seem that low feed efficiency and high growth before sexual maturation negatively impacts glucose metabolism. Furthermore, high feed efficiency during sexual maturation, which was related to a high rate of growth during sexual maturation, leads to increased development of central obesity, and reduced glucose tolerance.

Summary

Effects of pre-natal growth on glucose metabolism and the development of type 2 diabetes.

In order to investigate the effect of pre-natal growth on glucose tolerance and insulin sensitivity, comparisons between runt and large pigs were made. Runt pigs were expected to be more susceptible to insulin resistance and have reduced glucose tolerance compared to their large littermates, as has been demonstrated in other models (Bertram & Hanson, 2001; Poore & Fowden, 2002, 2004b; Vuguin, 2007). However there was no significant difference in any measurements of glucose tolerance or insulin sensitivity between runts and larger littermates at 10 months of age. There was also no significant difference in pancreatic insulin concentration, visceral fat content, or abdominal circumference between the runt and large pigs. Also, there were no significant correlations between birth weight and any measurement of type 2 diabetes.

Only one other study examined the relationship among birth weight, growth and the effect of diabetes and pigs (Poore and Fowden, 2002).Our findings are contrary to findings by Poore and Fowden, in large white breed of domestic pigs which showed that

low birth weight pigs had lower glucose tolerance compared to higher birth weight pigs.The lack of difference between low and high birth weight groups in our study could be due to the powerful effect of gender, attributed to differing visceral fat contents, observed in our pigs. Female pigs had far lower glucose tolerance and insulin sensitivity than the males in our study. Our experimental groups were balanced for gender; whereas Poore and Fowden's experimental groups were not, having more females than males in the lower birth weight group.

The lack of overt symptoms of diabetes in our pigs is likely a result of their relatively young age. Although they were fed a high fat, high sugar, high salt, nutrient dilute diet, the pigs were still in young adulthood (relative to humans) and likely unaffected by the age related decline in glucose tolerance observed in humans (Rosenthal et al., 1982; Rowe et al., 1983) and pigs (Larsen et al., 2001). This young age was likely protective against developing overt diabetes. In future studies using Yucatan miniature pigs the effect of pre-natal growth on glucose metabolism should be investigated in mid- to late adulthood.

Effect of early post-natal diet on glucose metabolism and the development of type 2 diabetes.

Early post-natal growth and development is influenced primarily by early nutrition. In humans, breast-feeding is protective against the chronic adult diseases such as insulin resistance and obesity (Ravelli et al., 2000; Weyermann et al., 2006). In order to investigate the effect of post-natal diet on glucose tolerance and insulin sensitivity,

comparisons between large and sow-fed pigs were made. It was expected that the large pigs that were formula fed may be more susceptible to insulin resistance and impaired glucose tolerance than the sow-fed pigs. However there was no significant difference in any measurements of glucose tolerance or insulin sensitivity between runts and larger littermates at 10 months of age. There were also no significant differences in pancreatic insulin concentration, visceral fat content, abdominal circumference, growth rates or feed efficiencies between the large and sow-fed pigs.

These findings are not consistent with findings in humans. Ravelli and colleagues (2000) found increased 120 minute (post glucose challenge) glucose concentration and elevated fasting insulin levels in adults who were formula fed as infants compared to those breast fed as infants. The age of participants in this study was 48-53 years, which is much older than the relative age of the pigs in our study. Again, this relatively young age of our pigs may have protected them from formula feeding associated metabolic consequences. The impact of formula feeding may not develop until later in life. In future studies using Yucatan miniature pigs, the effect of post-natal diet on glucose metabolism should be investigated in mid-to late adulthood.

Effects of post-natal growth on glucose metabolism and the development of type 2 diabetes.

Post-natal growth has been shown to impact the development of diseases later in life (Eriksson et al., 1999, 2006; McMillen & Robinson, 2005). Epidemiological data from Helsinki, Finland found that low birth weight infants who experienced rapid growth

after one year of age had the highest risk of developing type 2 diabetes (Eriksson et al., 2004). The disease risk was related to the rate of growth rather than actual size at any time period (Eriksson et al., 1999). These findings suggest that rapid post-natal growth increases the risk of developing impairments in glucose metabolism. It was expected that a negative relationship between growth rate and glucose tolerance and/or insulin sensitivity might be observed in our pigs. As low birth weight children and animals often experience compensatory or "catch up" growth, runt pigs in this study were thought to have the highest risk of elevated post-natal growth rates and the negative impact on associated glucose metabolism.

Growth during the milk feeding phase (i.e. 8-14, 14-21, 21-28, 8-28 days) differed significantly among runt pigs and the large and sow-fed pigs. Growth rates during milk feeding were positively related to birth weight, with the runt piglets having a lower rate of growth than the large and sow-fed pigs. The growth rates during the milk feeding phase did not correlate with any measure of glucose metabolism or suometric measurement later in life. This was contrary to finding by Poore and Fowden (2002) who found that gAUC was positively correlated with growth from birth to one month of age in their pigs.

Growth during the "cafeteria" diet feeding phase (i.e. 30-120, 120-210, 210-300, 0-300 days) did not differ among runt, large or sow-fed pigs. These findings are contrary to studies in humans that have shown higher rates of early growth (1-3 month) in breast-fed infants, but lower later (4-12 month) and overall growth rates in the first year of life than formula fed infants (Agostoni et al., 1999; Dewey et al., 1992; Zeigler, 2006). This pattern was not observed between large and sow-fed pigs in our study.

An interesting relationship between growth and later glucose metabolism was observed across all groups of pigs. High rates of growth before and during sexual maturations were related to decreased glucose tolerance and insulin resistance, as well as increased abdominal circumference and weight at 10 months. These findings were similar to findings by Eriksson et al. (2004) who found rapid growth during childhood was related to impaired glucose tolerance and increased risk of type 2 diabetes later in life. There was however no significant relationship between birth weight and growth rates during the cafeteria diet phase in our pigs.

Feed efficiencies during pre-sexual maturation, and sexual maturation also showed a relationship with growth and the development of impaired glucose tolerance, insulin resistance and obesity. High feed efficiency during sexual maturation was positively related to impaired glucose tolerance, increased visceral fat, abdominal circumference, and growth. These findings suggest that rapid growth during sexual maturation may lead to central obesity and impaired glucose metabolism. Conversely, high feed efficiency before sexual maturation may be protective against the development of insulin resistance, reduced glucose tolerance and obesity. High feed efficiency before sexual maturation was also related to reduced growth and feed efficiency during sexual maturation. Interestingly, feed efficiency before sexual maturation was not positively related to growth at the same time, in contrast to the sexual maturation phase. This would suggest that inefficient rapid growth before sexual maturation may lead to metabolic consequences later in life. Perhaps inefficient growth before sexual maturation results in physiological changes that affect body composition and increase central obesity. These

changes in turn may lead to metabolic problems later in life. This hypothesis is supported by our data, in which reduced feed efficiency before sexual maturation correlated with increased visceral fat and large abdominal circumference at 10 months of age.

Chapter 3: Methyl metabolism study

3.1 Objectives

Changes in sulfur amino acid metabolism have been suggested as a possible mechanism in developmental plasticity (Rees, 2002; McMillen & Robinson, 2005; Van den Veyver, 2002). A change in DNA methylation is one of the proposed methods by which gene expression could be altered during development. These changes in expression could be permanent, and result in physiological changes that mediate developmental plasticity. The activity of the enzymes in the sulfur amino acid cycle are possible contributing factors to DNA methylation status, as this cycle regulates the supply of methyl groups and the fate of homocysteine, which can have an inhibitory effect on all methylation if it is elevated. Enzyme activities of BHMT and CTH were observed in livers of newborn runt piglets compared to their larger littermates (Brophy, 2006). But it is unknown whether the changes in enzyme activities are due to pre- or post-translational mechanisms. The main objectives of this study were: 1) to determine if the hepatic expression of BHMT and CTH are affected by pre-natal growth; 2) to determine if the global DNA methylation in the liver of these piglets is affected by pre-natal growth; 3) to determine if DNA methylation is related to BHMT or CTH activity or expression.

3.2 Hypotheses

Runts will have reduced hepatic expression of BHMT and CTH compared to their larger littermates, which causes the lowered BHMT and CTH enzyme activity levels. The global hepatic DNA methylation of the runt piglets will be decreased compared to larger littermates due to decreased activity of enzymes responsible for disposing of homocysteine.

3.3 Methods

3.3.1 Animals and housing

Twelve Yucatan miniature piglets (5 male, 7 female) were obtained from the Vivarium at Memorial University. Six pairs of littermates, consisting of one normal-sized and one runt piglet, were removed from sows at the age of 3-5 days. Necropsies were performed the same day as removal from the sow. At the time of the necropsy the normal-sized littermates weighed 1.085 – 1.646 kg with a mean body weight of 1.362 ± 0.213 kg. Runt piglets weighed between 0.6912 – 1.0400 kg with a mean body weight of 0.849 ± 0.128 kg. Runts were at most 80 % of their littermate's body weight. Animal care and handling procedures were conducted in accordance with the guidelines of Memorial University of Newfoundland Animal Care Committee and the Canadian Council on Animal Care.

3.3.2 Necropsy

Necropsies were performed on each sibling pair on the same day. The piglets were anaesthetized with halothane (4% induction, 2% maintenance) delivered with oxygen by mask. Blood samples were procured by heart puncture; samples of liver were freeze-clamped and other tissue samples were weighed and sampled and then frozen using liquid nitrogen. Samples were stored at -70 °C until analyses.

3.3.3 Sulfur amino acid enzyme expression

Relative gene expression of betaine homocysteine methyl transferase (BHMT), and cystathionase (cystathionine gamma-lyase) (CTH) were measured using duplex real time reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was obtained from liver samples using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using 1 μg of total RNA with the QuantiTect reverse transcription kit (Qiagen, Valencia, CA) according to manufacturer's protocol, including a genomic DNA wipeout step.

PCR primers and probes for porcine BHMT, CTH and β-actin were designed using RealTimeDesign software (Biosearch Technologies, Novato, CA). Expression of β-actin was used as an endogenous reference gene to account for differences in sample loading and PCR efficiencies between reactions. Taqman probes for β-actin were labeled with reporter dye carboxyfluorescein (FAM) on the 5' end and black hole quencher (BHQ-1) on the 3'end. Taqman probes for BHMT and CTH were labeled with reporter dye PULSAR 650 on the 3' end and black hole quencher (BHQ-2) on the 5'end.

Amplification reactions contained 2 µl of cDNA, 10 µl of 2X x QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 2 µL of 10X β-actin primer/probe mix, 2 µL of 10X BHMT or CTH primer/probe mix (10x primer probe mixes made according to instruction in QuantiTect Multiplex PCR Kit). All reactions were performed in triplicate on a LightCycler 1.2 real-time PCR system (Roche, Missisauga, ON). The thermal cycling conditions were 10 min at 95 °C to activate HotStarTaq DNA polymerase, followed by 40 cycles of 94 °C for 45 sec, 56 °C for 45 sec, and 76 °C for 45 sec. A sample of the reverse transcriptase reaction without reverse transcriptase enzyme, and a sample of PCR reaction mixture without cDNA were used as negative controls.

Relative gene expression was calculated using the formula outlined by M. W. Pfaffl (2001). A PCR efficiency of 2.0 was used for all calculations. Gene expression was compared between runt and large piglets by a paired t-test, and genders were compared by unpaired t-test.

3.3.4 Cytosine extension assay

Hpa II is a methyl sensitive endonuclease which cuts at the sequence CCGG, if the middle CG is unmethylated, leaving an overhanging guanine nucleotide. This guanine can be paired with a cytosine using Taq polymerase, by using a radiolabelled cytosine in the nucleotide extension reaction; the amount of overhanging guanine residues, and thereby the amount of cleaved CCGG sequences in a DNA sample can be measured. As DNA methylation occurs primarily at CpG sites, and many of these CpG sites are found

in a CCGG sequence, an estimate of global methylation can be obtained. The method is outlined in detail by Pogribny and colleagues (1999).

Genomic DNA from liver samples was extracted using a classic chloroform phenol extraction. DNA concentration and purity was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Fifteen μg of DNA was added to 75 U (5-fold excess) of *Hpa* II (NEB, Ipswich, MA), in the *Hpa* II digestion buffer (NEB, Ipswich, MA), to make up 300 μl reactions. Negative controls containing 15 μg of genomic DNA in digestion buffer without the addition of endonuclease were used. Ten μL of each digestion reaction, including the negative controls were removed and added to I μL of plasmid (500 ng/μL in concentration, or equal to the amount of genomic DNA in the 10 μL samples). These reactions we carried out to assure that complete digestion occurred. All reactions were then incubated overnight at 37°C.

After 12-16 h, the 11 uL reactions were run on a 0.5% agarose gel, and stained with ethidium bromide and visualized under UV light. Completion of the digestion reaction was confirmed by the lack of intact plasmid. Endonuclease-negative samples showed intact plasmids. Any reactions which showed incomplete digestion were re-digested as previously described and checked again for complete digestion. When complete digestion was confirmed, the digested DNA samples and controls were precipitated by the addition of 2 volumes of 98% ethanol and 1/10 volume 3 M sodium acetate, followed by centrifugation at 13000 rpm at 4° C for 15 minutes. DNA samples were then dissolved in 30 μL DNase-free water, and DNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop products, Wilmington, DE).

For each *Hpa* II DNA digest, and the negative control, a single nucleotide extension reaction was set up using the following formula (Table 3.3.4.1).

Table 3.3.4.1 Single nucleotide extension reaction (25 μL).

2.0 μg digested DNA
2.5 μL 10 x Native Taq buffer (Invitrogen, Burlington, ON)
0.75 μL 50 mM MgCl
0.2 μL of Native Taq polymerase (Invitrogen, Burlington, ON)
0.2 μL [^{3}H] CTP (57.4 Ci/mmol) (Moravek Radiochemical, Brea, CA)

The extension reactions were incubated at 55°C for 1 h. 10 μL of each reaction digest was applied to 2 separate D-81 ion exchange filters, and each filter was washed 3 times in sodium phosphate buffer (pH=7.0). The filter papers were dried overnight at room temperature, and then prepared for scintillation counting by submersion in 10 mL of Scintiverse (Thermo Fisher Scientific, Waltham, MA) in a 20 mL scintillation vial. Radioactivity was measured for 5 min using a liquid scintillation counter for tritium. Duplicate samples had to have a coefficient of variation of less than 10%, or the single nucleotide extension and scintillation counting was performed again.

Using tritiated cytosine, the amount of methylation in a sample can be estimated by subtracting DPM of the control digest (background) from the *Hpa* II digest. The DPM of the samples were then expressed per 0.5 μg of digested DNA. As methylation in a DNA sample decreases the DPM/0.5 μg of digested DNA increases.

DNA methylation (DPM/0.5 μg DNA) was compared between runt and large piglets using a paired t-test, and genders were compared using an unpaired t-test.

3.3.5 Liver BHMT and CTH activity and plasma homocysteine, cysteine, and methionine

Liver BHMT and CTH enzyme activities as well as plasma homocysteine, cysteine, and methionine concentrations were assayed in these piglets by Julie Brophy, as part of an honours program. Her data will be included to allow for comparisons with the results of BHMT and CTH expression and DNA methylation.

3.4 Results

3.4.1 DNA methylation

Global DNA methylation was estimated by measuring the DPM/0.5 μg DNA in *Hpa* II digests. The DNA methylation did not differ between runt and large piglets (Figure 3.4.1.1) ($p=0.88$). The DPM/0.5 μg DNA of the runt piglets ranged from 3281 to 4502 with a mean of 3894 ± 469. The large piglets' DPM/0.5 μg DNA ranged from 3425 to 4178, with a mean of 3928 ± 314. The female piglets had a mean DPM/0.5 μg DNA of 3677 ± 375 and males with 3722 ± 316 ($p=0.12$) (Figure 3.4.1.2). DPM/0.5 μg DNA data in our pigs did not correlate with CTH or BHMT activity or expression. DPM/0.5 μg DNA data showed a negative trend with plasma homocysteine ($r=-0.549$, $p=0.065$) (Figure 3.4.1.3) and cysteine ($r=-0.499$, $p=0.099$) (Figure 3.4.1.4), suggesting a positive relationship between homocysteine and cysteine concentrations and DNA methylation.

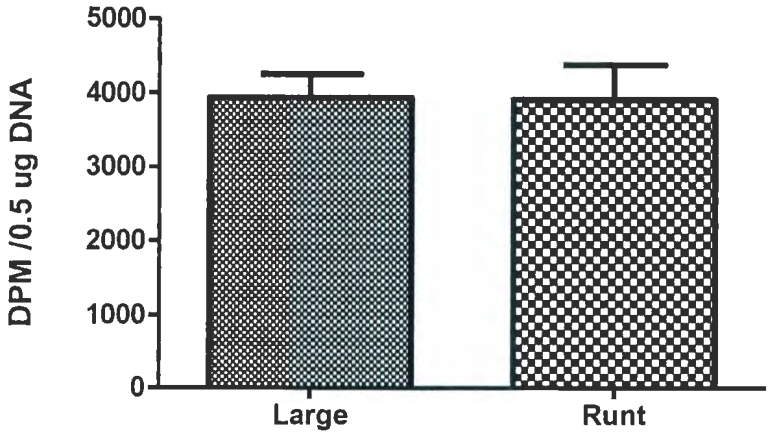


Figure 3.4.1.1 DNA methylation of runt and large piglets (n=6 for each group).

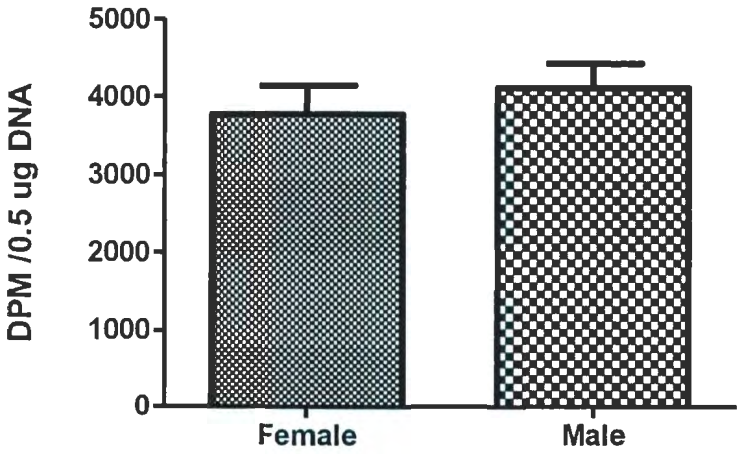


Figure 3.4.1.2 DNA methylation by genders (n=7 female, 5 male).

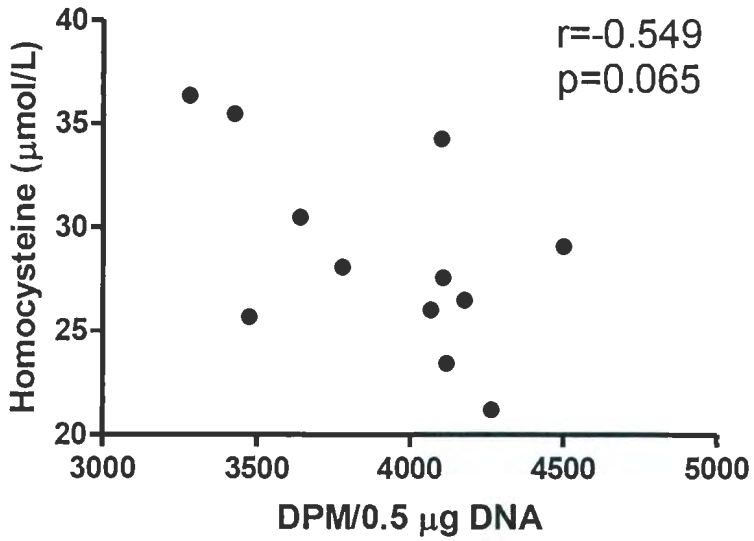


Figure 3.4.1.3 DNA methylation versus plasma homocysteine.

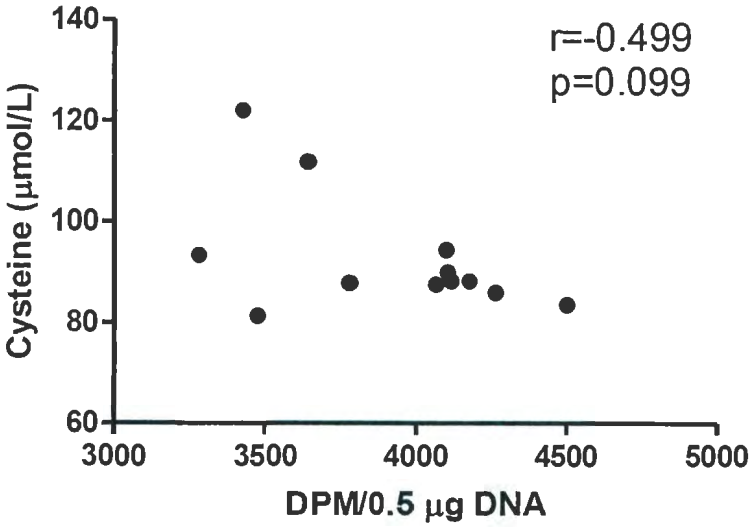


Figure 3.4.1.4 DNA methylation versus plasma cysteine.

3.4.2 BHMT expression

The expression of BHMT was normalized to β-actin expression in each sample and normalized to a calibrator sample to allow for direct comparison among all piglets, giving expression values in arbitrary units (AU). BHMT expression did not differ between runt and large piglets (p=0.96) (Figure 3.4.2.1). Runt piglets had a mean BHMT expression of 0.998 ± 0.24 AU while the large piglets had a mean BHMT expression of 0.991 ± 0.21 AU. Gender also did not affect the expression of BHMT (p=0.72) (Figure 3.4.2.2). BHMT expression did not correlate to BHMT activity, CTH activity or plasma homocysteine, cysteine, or methionine concentrations (data not shown).

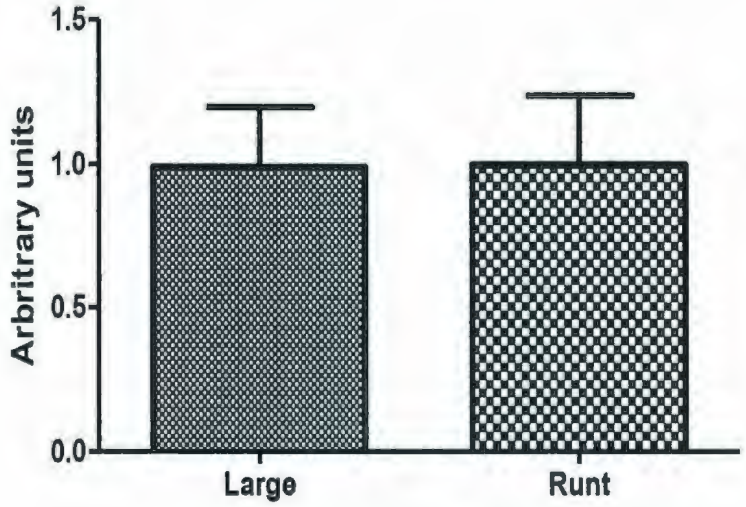


Figure 3.4.2.1 BHMT expression of runt and large piglets (n=6 for each group).

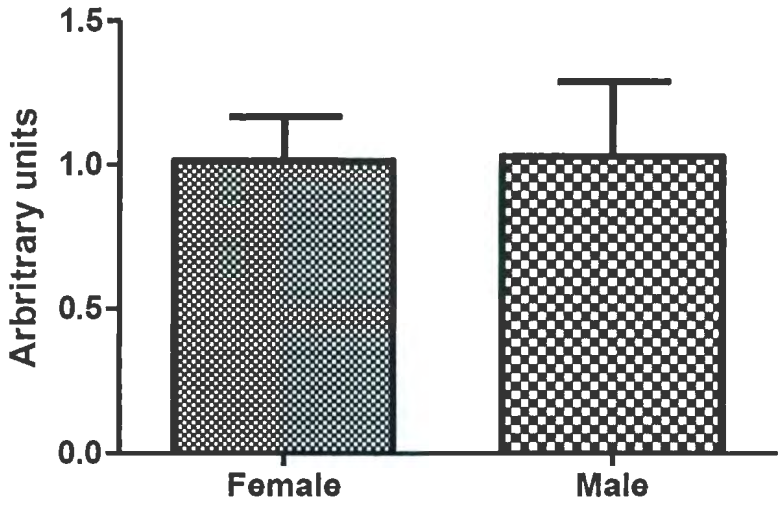


Figure 3.4.2.2 BHMT expression by gender (n=7 females, 5 males).

3.4.3 CTH expression

The expression of CTH was normalized as described for BHMT. The expression of CTH was significantly different between runt and large groups (p=0.01) (Figure 3.4.3.1). Large piglets had higher CTH expression than runts (1.24 ± 0.54 AU versus 0.85 ± 0.54 AU, respectively) (Figure 3.4.3.2). The expression of CTH was not affected by gender (Figure 3.4.3.2). CTH expression tended to correlate with CTH activity (p=0.16) (Figure 3.4.3.3). The removal of one runt and large sibling pair outlier (>2 SD greater than mean), allowed the CTH activity and expression to correlate significantly (p=0.05). Each large piglet had higher CTH expression than its sibling runt, a pattern that was also observed in the CTH activity data. CTH expression also significantly correlated with plasma homocysteine levels (r=0.703, p=0.007), but not plasma cysteine or methionine concentrations (data not shown).

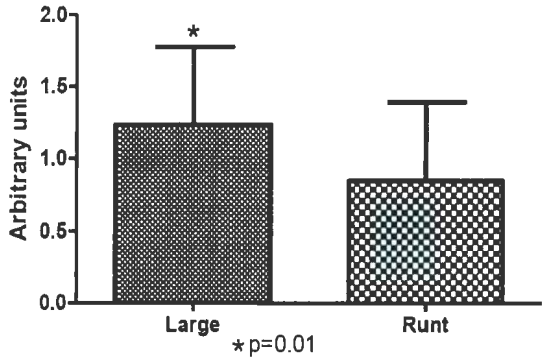


Figure 3.4.3.1 CTH expression of runt and large piglets.

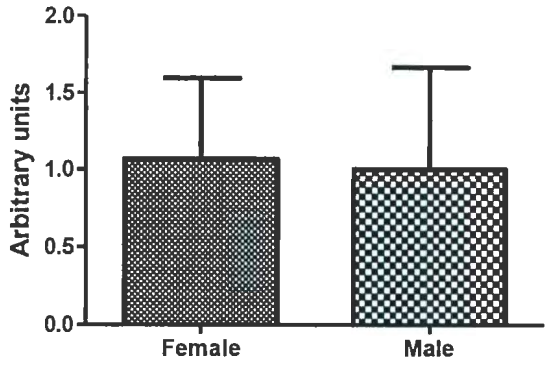


Figure 3.4.3.2 CTH expression by gender.

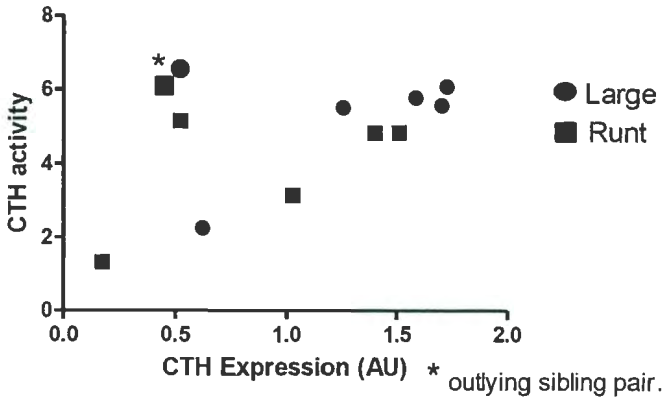


Figure 3.4.3.3 CTH expression as it relates to CTH activity.

3.4.4 BHMT and CTH activity and plasma cysteine and homocysteine.

BHMT and CTH activity, as well as plasma cysteine and homocysteine, were measured in our piglets by Julie Brophy, an honours student in our lab. As some of the results of this study related to these measures, the values will be included in tables for comparison purposes.

Table 3.4.4.1 BHMT and CTH activities.

	BHMT activity (nmol/min/mg)		CTH activity (nmol/min/mg)	
	Large	Runt	Large	Runt
Piglets 1,2	0.335	0.271	6.55	6.09
Piglets 3,4	0.443	0.260	5.78	4.82
Piglets 5,6	0.286	0.150	6.08	3.14
Piglets 7,8	0.299	0.316	5.52	5.15
Piglets 9,10	0.317	0.255	2.26	1.31
Piglets 11,12	0.401	0.229	5.57	4.84
Mean	**0.347**	**0.247***	**5.29**	**4.23****
St.Dev.	0.062	0.055	1.53	1.72

Runt is significantly different from large at *p=0.03 **p=0.04

Table 3.4.4.2 Plasma concentrations of cysteine and homocysteine.

	Cysteine (μmol/L)		Homocysteine (μmol/L)	
	Large	Runt	Large	Runt
Piglets 1,2	88.2	81.2	23.4	25.7
Piglets 3,4	111.8	93.3	30.5	36.3
Piglets 5,6	90.0	83.5	27.6	29.1
Piglets 7,8	122.0	87.6	35.5	26.0
Piglets 9,10	88.1	86.0	26.5	21.2
Piglets 11,12	94.4	87.9	34.3	28.1
Average	**99.1**	**86.6***	**29.6**	**27.7**
S.D.	14.4	4.2	4.7	5.0

Runt is significantly different from large at *p=0.05

3.5 Discussion

Changes in DNA methylation have been proposed as being one of the underlying mechanisms involved in the early origins of adult chronic diseases (McMillen & Robinson, 2005). Methyl supply has been shown to affect DNA methylation, which, in turn, can alter gene expression. This leads to the possibility that sulfur amino acid metabolism may be linked to the development of diseases later in life (Rees et. al, 2000, Rees, 2002). An honours student, who looked at the activity of sulfur amino acid enzyme in liver tissue, found significantly lower activity of BHMT and CTH in runt as compared to large piglets in our study. We hypothesized that lower activity of these homocysteine degradation enzymes in runt piglets may lead to the accumulation of homocysteine. Homocysteine has been shown to affect DNA methylation, and perhaps permanently alter gene expression and development, leading to the development of chronic diseases (Stipanuk, 2004). As methionine synthase (MS) activity did not differ between runt and large piglets, reduced BHMT activity could hypothetically lead to elevated homocysteine. However, it must be noted that the activity levels assayed represent the maximum activity capacity of each enzyme for each animal, not the *in vivo* activity levels of each enzyme. To determine whether the actual *in vivo* activity levels of sulfur amino acid enzymes are affected by developmental plasticity, the *in vivo* kinetics of the sulfur amino acid cycle would have to be investigated in runt and large piglets. Nevertheless, the fact that BHMT and CTH capacities were lower in runt piglets does presumably reflect a lower *in vivo* capability of removing homocysteine.

The plasma amino acid concentrations and homocysteine levels were also analyzed, but homocysteine ($p=0.23$) and methionine concentrations were not different ($p=0.86$) (data not included) (Brophy, 2006). Cysteine levels, however, were significantly lower in the runt piglets ($p=0.03$). Although homocysteine and methionine levels were not significantly different between runts and large piglets, the reduced cysteine levels suggest that runt piglets may have a lower rate of transsulfuration than the large piglets. As a follow up to these findings, the gene expression of BHMT and CTH were analyzed, and global DNA methylation was measured.

It was hypothesized that liver BHMT and CTH expression would show a similar pattern to their activities and that large piglets would have higher BHMT and CTH expression than runts. Indeed, CTH expression was significantly lower in runt versus large piglets, as hypothesized. Furthermore, consistent with the overall findings, CTH expression was higher in each large piglet compared to its runt sibling. The CTH expression and CTH activity correlation did not reach statistical significance, primarily because of one outlier pair of siblings that was >2 SD above the mean. The removal of this set of siblings from the correlation allowed CTH expression to have a significant positive correlation with CTH activity. These findings may suggest that the previously described difference in CTH activity between the runt and large piglets is due to regulation at the transcriptional level. As DNA methylation in the promoter regions of genes is often associated with the regulation of gene expression, it may be of interest to investigate the CTH gene-specific DNA methylation in these piglets. CTH is an essential enzyme in the removal of homocysteine and in the synthesis of cysteine from methionine.

Moreover, it has been suggested that this enzyme is rate-limiting for cysteine synthesis and its lower activity in neonates (particularly premature neonates) has led to the hypothesis that cysteine may be conditionally essential in early life because methionine cannot be transsulfurated adequately (Viña et al, 1995). It is possible that runt piglets have lower CTH similar to premature infants, and may not be able to meet their glutathione or cysteine requirements by transsulfuration. This could lead to increased oxidative stress in runt piglets due to reduced glutathione levels, as well as reduced overall growth due to insufficient availability of cysteine for protein synthesis. This reduction in protein synthesis and/or increase in oxidative stress could lead to increased risk of developing disease later in life, particularly atherosclerosis and cardiovascular disease in which oxidative stress has been implicated (Willcox et al., 2008). Furthermore, permanent changes in CTH expression via altered DNA methylation, which if maintained throughout life, may demonstrate one of the mechanisms behind developmental plasticity. If sulfur amino acid enzymes are permanently down regulated at a transcriptional level due to reduced fetal growth, then organisms with reduced fetal growth are more susceptible to elevated levels of homocysteine throughout life. Elevated homocysteine levels have been associated with numerous negative health outcomes, and these effects may be independent of the possible effect that elevated homocysteine concentrations can have on DNA methylation. This effect of elevated homocysteine on DNA methylation could alter the regulation of genes, possibly increasing the probability of disease (Rees et al., 2000; Stipanuk, 2004; Townsend et al., 2004).

CTH expression also correlated positively with plasma homocysteine levels. This observation was expected as the homocysteine level is directly related to S-adenosyl-homocysteine (AdoHcy) level, which is an activator of transsulfuration (Finkelstein, 2000). Surprisingly, this relationship was not observed for homocysteine and CTH activity. However, the removal of the one outlying sibling pair of piglets with elevated CTH activity showed a significant positive correlation between plasma homocysteine and CTH activity. S-adenosyl-methionine also activates transsulfuration; however, S-adenosyl-methionine concentration was not measured in our piglets and therefore could not be correlated with CTH expression or activity.

Unlike CTH expression, liver BHMT expression was not significantly different between runt and large piglets. This suggests that the differences in BHMT activity between runt and large piglets may be due to some type of post-translational mechanism. The type of post-translational mechanism that may be regulating BHMT activity merits further investigation. One possible mechanism of down regulation of BHMT activity in the runt piglets could be related to liver transglutaminases. Liver transglutaminases have been shown to intra- and inter-molecularly cross link BHMT subunits and thereby reduce BHMT activity in vitro (Ichikawa et al., 2004). Post-translational mechanisms of regulation may be more transient than regulation at an expression level and may be related to the availability of substrate. Although there was no significant difference in plasma homocysteine concentrations between runt and large piglets, we did not measure liver intracellular homocysteine or betaine levels. Perhaps the post-translational

regulation of hepatic BHMT activity was related to the availability of substrate in liver cells.

DNA methylation was measured by the cytosine extension assay. This assay measures the amount of tritiated cytosine incorporation into DNA following digestion by *Hpa* II, a methyl-sensitive restriction enzyme. There was no significant difference found in global DNA methylation between runt and large piglets. Furthermore, DNA methylation was not related to the activity or expression of either BHMT or CTH. Although not statistically significant, a trend was observed between DNA methylation and plasma concentrations of homocysteine and cysteine. Higher plasma homocysteine and cysteine levels tended to correspond to increased DNA methylation. This relationship is unexpected as homocysteine levels are related to S-adenosyl-homocysteine levels, and S-adenosyl-homocysteine is inhibitory of most methylation reactions and associated with reduced DNA methylation (Finkelstein, 2000; Rees, 2000; Van den Veyver, 2002). However, the ratio of S-adenosyl- methionine to S-adenosyl- homocysteine (the previously described methylation index) is more important to DNA methylation than the amounts of S-adenosyl-homocysteine, as S-adenosyl-homocysteine is a competitive inhibitor of methylation (Van den Veyver, 2002). The amount of S-adenosyl-methionine was not measured in our piglets, therefore the calculation of the SAM/SAH ratio was not possible.

Although global DNA methylation did not differ between runt and large piglets, it is possible that the gene-specific methylation of certain genes may be affected by developmental plasticity. Changes in cytosine methylation due to a global intervention,

such as a high methionine diet, may cause changes in methylation in a very specific fashion (McGowan et al. 2008). Such small changes in the important regulatory regions of genes may not be able to be detected on a global level, as variation in methylation of non-coding areas of DNA may be too great. The analysis of global DNA methylation is only a starting point, and can only detect differences across all coding and non-coding DNA methylation (Shen & Waterland, 2007). Where evidence suggests regulation by developmental programming, such as on CTH in the case of this study, DNA methylation should be investigated on a gene-specific level. The best method to investigate this specific methylation is through the use of bisulphite sequencing (Shen & Waterland, 2007). Bisulphite sequencing uses sodium bisulphite's transformation of unmethylated cytosine to uracil to induce cytosine (C) to thymine (T) transitions. DNA is modified by sodium bisulphite, and then regions of interest are amplified by PCR using primers that surround but do not overlap the CpG sites. The PCR fragments are then ligated into clones and amplified on selective media to produce individual colonies. The colonies are selected and their plasmid DNA is isolated and sequenced. Each colony's sequence represents the methylation of a single allele; by using many clones, the methylation of numerous CpG sites can be quantified. This method is the gold standard in gene-specific methylation analysis, and may be too time consuming to do on a large number of samples. Therefore, other methods such as bisulphite pyrosequencing, bisulphite PCR followed by matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS), or array based analysis of CpG methylation may be less precise but more useful.

Reduced pre-natal growth in Yucatan piglets may have profound effects on their metabolism of sulfur amino acids. However, this reduced growth and subsequent impairment of the transsulfuration pathway did not affect the overall global DNA methylation of the piglets.

Summary

Effects of pre-natal growth on gene expression of BHMT and CTH.

To investigate the effect of pre-natal growth on BHMT and CTH gene expression, comparisons between runt and large pigs were made. Runt piglets were expected to have reduced BHMT and CTH expression compared to their larger littermates, similar to the differences observed in BHMT and CTH activity. BHMT expression did not differ between runt and large piglets, suggesting that the differences in BHMT activity observed must be due to post-translation regulation. As hypothesized, CTH expression was significantly lower in runt piglets, suggesting that CTH is differentially regulated between runt and large pigs at pre-translational level. This effect of pre-natal growth on CTH expression merits further investigation to determine the mechanism and perhaps the cause of this differential expression, and its possible effects on health later in life.

Effect of pre-natal growth on global DNA methylation.

To investigate the possible effects of pre-natal growth on global DNA methylation, global methylation of the runt and large piglets was assessed using a cytosine extension assay. It was thought that because certain sulfur amino acid cycle enzymes had differing activity levels between runt and large piglets, this may have an

effect on the overall DNA methylation. No significant difference in DNA methylation was observed between runt and large piglets. Similar global methylation levels, however, do not preclude the possibility of individual differences in DNA methylation on a gene-specific level between runt and large piglets. As CTH activity and expression were shown to be affected by pre-natal growth, CTH is a possible candidate gene in which gene-specific methylation should be investigated.

Effect of BHMT and CTH activity or expression on DNA methylation.

As BHMT and CTH are enzymes involved in the control of methyl supply, it could be expected that variations in the activity of these enzymes would positively or negatively affect DNA methylation. BHMT and CTH activity and expression levels were correlated with DNA methylation to determine if any possible relationship between the variables existed. DNA methylation did not correlate with BHMT or CTH expression or activity. This may be due to the fact that the activity levels for each enzyme were measured in an in vitro assay, meaning that the activity measured was the maximum capacity of the enzymes and not the actual activity in vivo. The actual *in vivo* activity of the sulfur amino acid enzymes would be of great interest and perhaps better related to DNA methylation than the maximum capacities of the enzymes. The concentrations of liver SAH and SAM as well as the expression of DNA methylating enzymes would be of interest to investigate regarding their relationship with DNA methylation.

Chapter 4: Conclusions

Although numerous human epidemiological studies and some animal studies have demonstrated a negative relationship between fetal growth and impaired glucose metabolism later in life, the results of this study in Yucatan miniature pigs did not support those observations. This is not surprising as various swine models have failed to consistently demonstrate diet induced type 2 diabetes, the species as a whole may be resistant to such interventions (Bellinger et al.,2006) Glucose metabolism was assessed using an intravenous glucose tolerance test (IVGTT) and insulin sensitivity test (IST). The gold standard test for glucose metabolism is the hyperinsulinemic euglycemic clamp method, which measures the amount of glucose required to maintain a normal blood glucose level under hyperinsulinemic conditions. This clamp method, however, is not feasible on pigs of the age and size used in this study. The IVGTT and IST methods were sensitive enough to show gender dimorphisms in glucose metabolism in the pigs. The IVGTT and IST also revealed a clear relationship between visceral fat content and insulin sensitivity in the pigs which has also been observed in humans and other animal models. There was, however, no relationship observed between pre-natal growth or early post-natal diet and glucose metabolism, as measured by the IVGTT or IST. This finding is contrary to evidence from other studies in pigs and epidemiological data from humans. The pigs in this study may have been too young to show deteriorations in glucose metabolism caused by reduced pre-natal growth or post-natal diet. In future studies of this sort, the pigs should be tested at later ages to allow for the possible development of complications with glucose metabolism that could be linked to early life.

A negative relationship between visceral fat and glucose metabolism was clearly demonstrated by this study. Visceral fat tended to increase as insulin sensitivity and glucose tolerance decreased. Although, this relationship is well known, it is not known whether increased visceral fat causes insulin resistance, or vice versa. Other factors may be responsible for both the increases in visceral fat and insulin resistance. Even at the highest levels of visceral fat content, the plasma glucose levels of our pigs remained within normal ranges and no overt diabetes was observed. Plasma insulin levels were elevated, however, suggesting that the pigs were developing compensatory hyperinsulinemia.

The analysis of the pigs' growth rates and feed efficiencies throughout the study showed an interesting relationship between feed efficiency before and during sexual maturation and glucose metabolism and visceral adiposity. High feed efficiency before sexual maturation was related to lower feed efficiency during sexual maturation, lower visceral fat and improved glucose metabolism later in life. In contrast, high feed efficiency during sexual maturation was related to increased visceral adiposity. Because of these interesting relationships and the rapid growth and hormonal changes that occur during sexual maturation, the pre and peri sexual maturation phases are ideal developmental periods in which to start investigating the origins of obesity and impaired glucose metabolism.

Detailed analysis of metabolic hormones such as proinsulin, leptin and ghrelin in pigs should be investigated in future studies. Proinsulin could be used to access the proper production and packaging of insulin at a cellular level. High proinsulin levels suggest

improper insulin production and are related to the development of insulin resistance and the development of diabetes (Pfützner et al. 2004). Leptin and ghrelin levels in relation to food intake and weight gain could be used to determine if appetite dysregulation is related to the development of obesity. Leptin is a hormone produced by adipocytes and is responsible for signalling satiety to the brain. Ghrelin is a hormone produced by the cells in the stomach lining and epsilon cells in the pancreas; it is thought to be the counterpart to leptin and to stimulate appetite (Dezaki et al., 2008; Klok et al., 2007). Plasma levels of testosterone, estrogen, growth hormone and insulin-like growth factor 1 (IGF1) could also be analysed in future studies. All of these hormones play important roles in growth and sexual maturation and may be related to the development of obesity and diabetes (Regitz-Zagrosek et al., 2007). Finally plasma cortisol levels should be investigated in future studies. Cortisol can have profound effects on glucose and amino acid metabolism as well as fat deposition (Kaufman et al. 2007, Tomlinson & Stewart, 2007). Corticosteroid receptor levels could also be investigated, as an elevation or reduction in receptor number can change the responsiveness to cortisol.

Detailed analysis of gene expression of these aforementioned hormones and their receptors could also help in elucidating the development of visceral adiposity and impaired glucose metabolism. The expression of the genes in numerous tissue types, such as adipose, muscle and liver could be analyzed. This type of work would be best completed using gene chips, which allow for the expression analysis of numerous genes simultaneously; these chips are now being developed for pigs.

The analysis of CTH and BHMT gene expression in young piglets yielded mixed results. CTH expression was affected by pre-natal growth, similar to the CTH activity. BHMT expression was not affected by pre-natal growth. This was unexpected as BHMT activity measured in the same piglets was different between runts and large groups. This suggests that there may be differences in sulfur amino acid metabolism between runt and large piglets that are caused by pre and post translational regulation. The significance of these differences in sulfur amino acid metabolism merits further investigation, to determine how long these differences last during development and what the potential impact of these differences is to later health. The effect of high and very low methyl group consumption later in life on the runt piglets merits investigation. If the reduced capacity to remove homocysteine (via remethylation and transsulfuration) is lower in a runt piglet, then runts would be at an increased risk of developing hyperhomocysteinemia on a high methyl diet. If a runt piglet's capacity to remethylate homocysteine to methionine is permanently reduced, then a very low methyl diet may lead to a methionine deficiency.

The global DNA methylation in young piglets was not affected by pre-natal growth rate. We hypothesized that global methylation may be different between runt and large piglets due to differences in sulfur amino acid enzyme activities. The global DNA methylation did not differ between runt and large piglets and showed no relationship to CTH or BHMT activity. Future studies may want to look at gene specific DNA methylation differences between runt and large piglets, which may be disturbed, without significantly affecting the levels of global methylation.

The developmental origins of adult disease is a rapidly evolving field of research. Much of the research being performed uses smaller animal models. Large animal models such as the Yucatan miniature pig are an important step in bridging the gap between findings in small animals and their actual impact on human health.

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