DEVELOPMENTAL ORIGINS OF CARDIOVASCULAR DISEASE IN YUCATAN MINIATURE SWINE

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Abstract

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Epidemiological studies have consistently indicated that low birth weight is associated with increased risks of chronic diseases in adulthood. Due to limitations of human studies, various animal models are used to elucidate the mechanisms regulating developmental programming of chronic adult diseases. A large proportion (80-90%) of the human incidence of intrauterine growth restriction, resulting in low birth weight, is may be due to impaired nutrient perfusion through the placenta. Spontaneous low birth weight animal models also represent placental insufficiency and may be appropriate models for the human. The overall objective of this thesis was to determine whether the spontaneous naturally occurring low birth weight (i.e., runt) Yucatan miniature pig represents a suitable model for studying developmental origins of chronic adult diseases by investigating biological markers of obesity and cardiovascular diseases. The runts showed qualities similar to low birth weight infants, i.e., small size at birth, an increased rate of postnatal growth (catch-up growth), and organ and metabolic changes which led to the development of obesity and early indicators of cardiovascular diseases. Specifically, runts experienced catch-up growth (prior to sexual maturity at 7 mo old). The catch-up growth was partly due to increased feed intake, which was independent of the post-weaning diet provided, suggesting developmental programming of food intake regulation. Catch-up growth was also associated with increased adiposity in the runts. Furthermore, blood pressure was inversely related to birth weight, similar to findings in

epidemiological studies. The higher blood pressure in the runts was significantly correlated to lower nephron number. The results showed that low birth weight was associated with a dyslipidaemic plasma profile as indicated by the higher plasma triglyceride levels in the runts in both the fasted and postprandial states. Finally, a post-weaning dietary intake also plays an important role as a determinant of chronic disease outcomes; a post-weaning Western-style diet that was high in salt, fat and sugar exacerbate early programming of blood pressure and lipid profile in the runts. Overall, the Yucatan miniature swine has many attributes for a good animal model to explore mechanisms that contribute to the developmental origins of human adult chronic diseases.

Keywords: Birth weight, Blood pressure, Catch-up growth, Developmental programming, Lipids, Nephron, Obesity, Radio-telemetry, Yucatan miniature pig,

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List of Abbreviations

ADFI	Average daily feed intake
ANOVA	Analysis of variance
AUC	Area under the curve
Bl	Blood
BP	Blood pressure
CHD	Coronary heart disease
Chol	Cholesterol
CM	Chylomicron
CMF	Chylomicron-free fractions
CVD	Cardiovascular diseases
DAP	Diastolic arterial pressure
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediamine tetra-acetic acid
FOAD	Fetal origins of adult disease
GFR	Glomerular filtration rate
GLM	General linear model
H&E	Hematoxylin and eosin
HDL	High density lipoprotein
HR	Heart rate
HSFS	High-salt-fat-sugar
IGF	Insulin-like growth factor
IST	Insulin sensitivity test

IUGR	Intrauterine growth restriction (retardation)
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MAP	Mean arterial pressure
MTP	Microsomal triglyceride transfer protein
NaCl	Sodium Chloride (salt)
NIBP	Non-invasive blood pressure
NPY	Neuropeptide Y
PARs	Predictive adaptive responses
PP	Pulse pressure
PUN	Plasma urea nitrogen
RAS	Renin-angiotensin aldosterone system
SAP	Systolic arterial pressure
SEM	Standard error mean
SD	Standard deviation
SGA	Small-for-gestational age
TG	Triglyceride
VLDL	Very low density lipoprotein

CHAPTER 1. INTRODUCTION

Developmental origins of health and disease (DOHaD) is the study of the relationship between early (i.e., pre- and post-natal) nutritional insults and the risk for chronic diseases in adulthood. Terms such as 'fetal programming', 'fetal origins of adult disease', 'Barker's hypothesis', and 'thrifty phenotype hypothesis' are all related to DOHaD. The aim of this chapter is to provide an overview of the concepts of DOHaD, with some focus on developmental origins of cardiovascular diseases (CVD) including the roles of blood pressure and lipid metabolism. This review will also discuss animal models that are currently used to study the underlying mechanisms of DOHaD as well as the potential of the spontaneous naturally occurring low birth weight Yucatan miniature pig (i.e., runt) as a model animal to elucidate the mechanisms underlying DOHaD. The chapter will conclude with the overall hypotheses and objectives of this thesis.

1.1 Developmental origins of health and disease (DOHaD) - An overview

In the late 1980s David Barker and colleagues popularized the concept of 'fetal programming' in their attempt to explain the relationship between an individual's birth size and the subsequent risk for development of chronic diseases such as CVD and type 2 diabetes mellitus (Barker and Osmond 1986; Barker, Osmond et al. 1989; Barker, Osmond et al. 1989; Barker 1990). The fetal programming hypothesis, which is also known as 'fetal origins of adult disease' (FOAD) hypothesis, deals with the notion that various adult chronic diseases may have originated from 'programming' events that occurred due to a nutritionally poor environment *in utero*. The theory suggests that small

birth size, which is considered a proxy for intrauterine growth retardation or other consequences of it, is a reflection of permanent metabolic reprogramming due to fetal undernutrition during gestation (Fall 2003; Fall 2006; Fowden, Giussani et al. 2006). That is, maternal dietary insults, or other *in utero* insults, result in an inadequate supply of nutrients and/or oxygen to the fetus; consequently, the fetus adapts to these insults in a number of ways to prioritize the growth of immediately essential organs such as the brain, at the expense of other organs (and tissues) such as the liver, pancreas, heart and kidneys (the needs of these organs become more critical later in life). The fetal programming hypothesis proposes that although these organ adaptations are occurring in response to a transient phenomenon (i.e., fetal undernutrition, which only occurs at specific stages of fetal development), they become permanent or 'programmed' because they occur during critical periods of early development (Lucas 1991; Fall 2006), resulting in an increased and irreversible susceptibility to chronic diseases later in life.

Although the fetal programming/FOAD concept focuses on the intrauterine environment, more recently, researchers have started to realize that the early childhood environment is just as significant, or maybe even more so, for the actualization of chronic disease outcomes (Singhal and Lucas 2004; Gluckman, Hanson et al. 2005; Gluckman 2006; Symonds 2007)Hence, the developmental origin of health and disease (DOHaD) hypothesis was introduced in recognition of the importance of the postnatal nutritional influences on chronic disease outcomes. In other words, DOHaD is an expansion of the fetal programming/FOAD concept, acknowledging the importance of both the prenatal (i.e., the intrauterine environment) and postnatal environments, and the interaction

between these environments in the manifestation of various chronic disease outcomes (Gluckman, Hanson et al. 2005; Gluckman 2006).

1.2 Intrauterine growth restriction (IUGR), birth size and DOHaD

DOHaD grew as a research field based on human and animal studies that consistently demonstrated that smaller birth size is associated with increased risk of chronic disease onsets (see reviews by (McMillen and Robinson 2005; Yajnik and Deshmukh 2008). Birth size is usually used as a reflection of fetal growth and gestational maturation (i.e., gestational age), both of which are influenced by environmental and genetic factors (Pietilainen, Kaprio et al. 2002; Silventoinen, Pietilainen et al. 2007). In other words, small birth size is used as an expression of intrauterine growth restriction (IUGR). IUGR is defined in terms of the infant size at birth for a given gestational age, such as reduced weight, reduced length, and/or increased thinness. For both humans and animals, low birth weight is the most commonly used measure of IUGR (Buehler, Kleinman et al. 1987; Kramer, Olivier et al. 1990; WHO 1995; Styne 1998), followed by short stature (Paz, Seidman et al. 1993; Leger, Limoni et al. 1997). In humans, the clinical definition of IUGR is a birth weight below the 10^{th} percentile (or <2500 g) for a term baby ($\geq$ 37 completed weeks of gestation) (WHO 1995; WHO 1995; Styne 1998). Although the mechanisms are not fully understood, it is believed that most infants born with IUGR have experienced a reduction in the delivery of essential substrates (oxygen and nutrients) during fetal development due to maternal and/or placental insufficiencies (Owens, Falconer et al. 1986; McMillen and Robinson 2005). IUGR is associated with substantially increased risk of morbidity and mortality during infancy (reviewed in:

(Ashworth 1998; Greenwood and Bell 2003) as well as during adulthood via increased risk for adult onset chronic diseases – in other words, fetal programming (Barker, Godfrey et al. 1991). Worldwide, over 30 million infants are born each year at term with low birth weights (WHO 1995).

IUGR can also be considered a generic term used to describe babies who did not meet their genetically-determined birth weight potential due to abnormal environmental conditions, such as an adverse *in utero* environment; as such the association between birth size and IUGR can be viewed as a graded relationship. At one extreme there are small-for-gestational age (SGA) infants - the term most often used for clinically defined IUGR infants (i.e., < 10^{th} percentile for birth weight); however, not all adverse events that occur *in utero* will result in SGA; therefore, as an assessment parameter, birth size has limitations (Rasmussen 2001; Fall 2006; Gluckman 2006). Most epidemiological studies that assessed the relationship between birth weight and adult chronic disease outcomes did not include SGA (< 2500 g) infants; rather, the birth weights were in the normal range (2500 – 4000 g), suggesting that even moderately restricted fetal growth carries the risk for increased susceptibility to chronic diseases (reviewed in: (Rasmussen 2001; Fall 2006). Indeed, epidemiological studies by Barker and colleagues indicated that the effects of birth weight on chronic disease risk outcomes were linear and graded across the whole range of birth weights (Osmond, Barker et al. 1993). In summary, with respect to the DoHaD hypothesis, IUGR also refers to infants that are >10^{th} percentile for birth weight; but overall, IUGR neonates can be considered smaller than their genetic potential. As it may be misleading for researchers to assess intrauterine growth restriction based only on

birth size; many investigators consider birth weight in conjunction with rapid early postnatal growth rate. Accelerated early growth rate is related to the concept of compensatory growth (i.e., catch-up growth), which will be discussed later in this chapter.

1.3 Critical periods of programming - Biological frameworks of DOHaD

Several frameworks have been proposed to explain the biological association between birth weight and chronic disease risk outcomes. The two prominent frameworks are the 'thrifty phenotype' and the 'predictive adaptive responses' hypotheses. These two hypotheses are not mutually exclusive. The thrifty phenotype was proposed to explain the fetal programming/FOAD hypothesis, but as this hypothesis has expanded to become DOHaD hypothesis, the predictive adaptive responses hypothesis is a modification of the thrifty phenotype hypothesis that better explains the impact of the postnatal environment on chronic disease risk outcomes.

1.3.1 Thrifty phenotype hypothesis

An understanding of the thrifty phenotype hypothesis first requires defining the concept of 'programming', which is the mechanism by which a stimulus or insult at a critical period of development has lasting or lifelong effects (Lucas 1991; Roseboom, van der Meulen et al. 2001; de Rooij, Painter et al. 2007). The concept of programming is not novel in biology; however, what was significant about the application of this concept to FOAD (and later DOHaD) is the notion that events during early development might be linked to chronic diseases in adulthood (Lucas 1991; Barker, Martyn et al. 1993). The idea is that intrauterine nutritional insults that occur at critical or sensitive periods during

early development may result in permanent or long-term changes in the structure or function of the organism with adverse consequences later in life (Lucas 1991; Roseboom, van der Meulen et al. 2001; de Rooij, Painter et al. 2007).

The thrifty phenotype hypothesis, which focuses on survival through metabolic adaptations, is a variation on the thrifty genotype hypothesis (which deals with survival through genetic adaptations) (Hales and Barker 1992). The differences between the thrifty genotype and the thrifty phenotype are best explained by the concept of developmental plasticity (which is a more specific definition of programming in FOAD or DOHaD). Developmental plasticity is defined as a regulated phenomenon by which one genotype can give rise to a range of phenotypes, thereby allowing the developing fetus to respond to environmental cues by choosing a trajectory of development that often has adaptive advantages (Gluckman, Hanson et al. 2005). The processes of developmental plasticity involve the commitment of cells to specific lineages, tissue differentiation and growth, and are regulated, in part, by epigenetic changes in gene expression (Gluckman, Hanson et al. 2005). Therefore, the application of developmental plasticity to the thrifty phenotype hypothesis can be interpreted as follows: the thrifty phenotype hypothesis suggests that a nutritionally poor *in utero* environment will result in the fetus switching to an energy conservative metabolic mode in order to survive. This nutritionally poor fetal environment triggers altered adaptive metabolic responses in the fetus, which optimizes the growth of key organs such as the brain, at the expense of other organs (e.g., pancreas, liver and kidneys), leading to altered postnatal metabolism in the individual. The hypothesis proposed that these metabolic adaptations only became detrimental if nutrients

are more abundant in the postnatal environment than there were in the *in utero* environment (Lucas 1991; Roseboom, van der Meulen et al. 2001; Gluckman, Hanson et al. 2005).

Although the thrifty phenotype model is widely accepted, it has limitations. It does not readily explain the continuous relationship across birth sizes (as seen in many epidemiological data). In other words, it does not explain how programming could operate in a well-nourished fetus, i.e. over-exposure to specific nutrients during gestation, such as maternal consumption of a Western-style that is high in saturated fat (Gluckman, Hanson et al. 2005). Consequently, the predictive adaptive hypothesis was proposed as a modification to the thrifty phenotype hypothesis.

1.3.2 Predictive adaptive responses (PARs) hypothesis

Extending the thrifty phenotype hypothesis, the predictive adaptive responses' (PARs) hypothesis proposes that based on the fetal environment, the fetus makes adaptations *in utero* or during the developmental plastic (programming) phase (fetal and neonatal), in preparation for its predicted postnatal environment (post-plastic phase) (Gluckman, Hanson et al. 2005). The belief is that the fetus constantly interprets its environment created by the maternal environment and placental function. Based on those conditions, some fetal responses have immediate adaptive advantages; others reflect developmental disruption which resonates throughout life (e.g. reduced nephron numbers), and others have little immediate adaptive value, instead they are assumed to represent long-term advantages by establishing metabolic physiology appropriate for the predicted postnatal environment (Gluckman and Hanson 2004; Gluckman, Hanson et al. 2005). But, overall

the theory proposed that many of the *in utero* adaptive programming responses, which induce irreversible changes in structure and functions of the organism, were not made for immediate advantage for the fetus, but rather as adaptive advantages in expectation of the future postnatal environment (Gluckman, Hanson et al. 2005). If the fetus predicts a nutritionally poor postnatal environment, it chooses a developmental path appropriate for a lower postnatal nutritional range, than if it predicted an enriched postnatal environment (Gluckman and Hanson 2004; Gluckman, Hanson et al. 2005). These responses are said to be appropriate if the predicted and actual path match, in which case, the phenotype is 'normal', i.e., no increase of early onset of various chronic diseases. However, chronic diseases are observed when mismatch occurs between the predicted and actual postnatal environments (Gluckman and Hanson 2004; Gluckman, Hanson et al. 2005). Thus, the PARs model is said to explain both the transition from an adverse *in utero* environment to an enriched postnatal one which leads to chronic disease risk, and also the reverse situation when an enriched *in utero* environment is followed by poor postnatal environment; both transitions increase risk for later diseases (Eriksson, Forsen et al. 2003).

Furthermore, the underlying biological mechanisms of FOAD and DOHaD are said to be explainable within the context of the biological framework of PARs.

1.4 Biological mechanisms of intrauterine programming

The underlying biological mechanisms that contribute to FOAD and DOHaD are undoubtedly multi-factorial; but the current dogma suggests that these mechanisms are all

related to programming events during development. For instance, researchers have shown that programmed changes *in utero* induce altered pathogenic events such as reduced insulin sensitivity, reduced pancreatic beta cell mass, and reduced nephron numbers, all of which are associated with increased risk of various chronic diseases (McMillen and Robinson 2005; Fowden, Giussani et al. 2006; Yajnik and Deshmukh 2008). Thus, the current aim in DOHaD research is to elucidate the underlying mechanisms of these programmed changes. Over the last 10 years, a number of frameworks were proposed to explain the underlying biological mechanisms of the association between early nutritional perturbations and chronic disease risk outcomes. Overall, these mechanisms have been categorized into three general groups: 1) epigenetic changes in gene expression; 2) altered tissue differentiation; and 3) altered homeostatic metabolism and regulation. These groupings of mechanisms are believed to act in isolation or synergistically to affect the risk for various chronic disease outcomes (Fall 2006; Fowden, Giussani et al. 2006). For instance, Fowden and colleague (Fowden, Giussani et al. 2006) compiled a detailed review of research on intrauterine programming of physiological systems in rat models. Using the rat model, they illustrates that intrauterine programming of physiological systems occurs at the gene, cell, tissue, organ, and system levels, and causes permanent structural and functional changes, which can lead to overt disease. Similar levels of programming are believed to occur in other model systems (McMillen and Robinson 2005), also leading to overt disease.

1.4.1 Epigenetics

The studies conducted in this thesis did not address epigenetic mechanisms; however, epigenetics provides the most likely mechanism underlying early programming of adult diseases. In brief, epigenetic changes refers to permanently altered gene expression patterns during the development of the individual caused by environmental influences such as maternal and early postnatal diets (reviewed in: (Fowden, Giussani et al. 2006; Vickaryous 2006; Waterland 2006; Burdge, Hanson et al. 2007)). It is a leading mechanistic theory because: epigenetic patterns can be manipulated within a short window of time early in development (including both fetal and postnatal development); epigenetic profiles are conserved through cell division beyond this critical window; and epigenetic patterns are sensitive to dietary interventions during these critical windows. Epigenetic changes are also suggested to play a major underlying role in the commitment of cells to specific lineages, tissue differentiation and growth (Gluckman, Hanson et al. 2005; Fowden, Giussani et al. 2006).

1.4.2 Altered tissue differentiation and organ development

Researchers have suggested that altered tissue differentiation and organ development may be the result of: a biological trade-off to conserve resources in response to deprivation during a critical window of development; adaptive strategies for surviving the predicted poor postnatal conditions; or developmental disruption with no adaptive value (Gluckman, Hanson et al. 2005; Fall 2006; Godfrey 2006). Furthermore, the timing of the insult determines the tissue that may be subjected to altered tissue differentiation (Roseboom, van der Meulen et al. 2001; Fowden, Giussani et al. 2006). For example,

with organs such as the kidney, there is a broad window for *in utero* programming effects because the kidneys are not completely developed until late gestation (37 week gestation in humans). Therefore, *in utero* insults that occur throughout gestation, or in mid to late gestation alone, are believed to impact nephrogenesis, with increased risk for development of hypertension. Conversely, other tissues such as the pancreas, liver, muscle and vasculature are said to be targeted in early gestation, where as tissues like the brain are susceptible to programming throughout postnatal development (Fowden, Giussani et al. 2006; Godfrey 2006).

1.4.3 Altered homeostatic metabolism and regulation

One of the general theories of fetal programming is that the fetus adapts to an adverse *in utero* environment by making permanent readjustments to homeostatic systems in order to maximise its chances for survival (Lucas 1991). These adaptations may include resetting metabolic and endocrine systems which leads to a change of growth trajectory and increased risk of chronic onset of diseases later in life (Fowden, Giussani et al. 2006; Godfrey 2006). That is, programmed changes may lead to an adjusted homeostatic metabolism and regulation later in life. For instance, a fetus that has growth restriction with relative sparing of cranial growth (asymmetric fetal growth) may have an altered blood flow through the liver, possibly causing disruptions in cholesterol metabolism and coagulation, which may raise the risk of atherosclerosis and related cardiovascular disease in adult life (Haugen, Hanson et al. 2005). There are also developmental changes in the homeostatic set points of many hormones and alterations in tissues sensitive to these

hormones (Godfrey 2006). For instance, altered sympathetic activity interacts with obesity and appetite control via leptin and fat metabolism (Godfrey 2006).

1.5 Compensatory (catch-up) growth following IUGR

Worldwide (in both developed and developing countries), the incidence of obesity and type 2 diabetes is increasing and the individuals who appear to be at greatest risk are those who are born small following IUGR, have increased postnatal growth and grow up in a society which is increasingly characterized by low physical activity levels and diet high in calories (Law 2001). Most IUGR infants undergo a period of accelerated growth after birth termed catch-up growth (Prader, Tanner et al. 1963; Karlberg and Albertsson-Wikland 1995). Research in the field of DOHaD has led to a resurging interest in understanding the metabolic consequences of catch-up growth. Such interest stems from the emerging evidence that small birth weight infants that also experience accelerated postnatal growth are at even greater risk for the development of chronic diseases (e.g., (Ong, Ahmed et al. 2000; Eriksson, Forsen et al. 2001; Eriksson, Forsen et al. 2003). The concept of catch-up growth has been studied for years and is well documented in many species. Indeed, animal research indicates that catch-up growth increases short-term survival rates for most animals, allowing them to reach sexual maturity more quickly; however, in most species the long-term (i.e., post-reproductive age) consequences are poorly understood and with little research in this area (Metcalfe and Monaghan 2001).

Though the mechanisms of catch-up growth are not fully understood, catch-up growth can be defined as a period of accelerated growth, in either absolute or fractional terms,

following a period of nutritional restriction. Specifically, catch-up growth has been defined as 'a growth velocity in terms of height and/or length above the statistical limits of normality for age and/or maturity during a defined period of time following a transient period of growth inhibition (Prader, Tanner et al. 1963), with the hope of helping the individual return to his/her pre-determined growth trajectory. There is a narrow window of time during which nutrient efficiency facilitates accelerated growth until the individual has caught up to a normal growth curve (Colle, Schiff et al. 1976). However, this window of nutrient efficiency is not well defined; consequently, excessive nutrients that are supplied beyond this window can result in obesity.

Low birth weight *per se* does not cause catch-up growth, rather catch-up growth occurs as a result of a period of nutritional insult or illness; therefore even infants within the normal range of birth weights can undergo catch-up growth (Rasmussen 2001). During this period, nutrient efficiency (utilization) is typically increased allowing for rapid growth. However, this increased efficiency is transient and once over, growth continues along a trajectory appropriate to age and size. If the animal continues to receive abundant nutrients once the period of compensatory growth is complete, the excess nutrients will be catabolised and deposited as fat. In humans, catch-up growth is commonly evident in infants or children born following a transient period of growth inhibition (i.e., IUGR) (Prader, Tanner et al. 1963). Catch-up growth following IUGR occurs predominantly during the first year of life. Studies have shown that catch-up growth in terms of weight occurs in the majority (85%) of IUGR infants, begins as early as 2 weeks and is largely completed by 5 months of age, whereas catch-up growth in terms of height begins as

early as 3 months of age (Albertsson-Wikland, Wennergren et al. 1993) and the majority caught-up completely in terms of height and weight by 5 to 12 months of age (Albertsson-Wikland, Wennergren et al. 1993; Karlberg and Albertsson-Wikland 1995). However, some infants (~15%) do not catch-up and have a reduced final adult stature, while those who do catch-up may have increased adiposity as measured by skin fold thickness and waist circumference (Ong, Ahmed et al. 2000). Hence, an important aspect for catch-up growth is the actual composition of body growth.

The process of catch-up growth can be categorized as being disadvantageous for two reasons: 1) altered body composition (i.e., increased adiposity); and 2) excess demand on tissues which are incapable of compensatory hyperplasia (e.g., pancreas and the kidneys) (Fall 2006). Thus, it is believed that prevention of catch-up growth during early neonatal life (e.g. during the first 6 months of life) could potentially reduce the incidence of many of the observed later metabolic disturbances (Ong, Ahmed et al. 2000; Ekelund, Ong et al. 2007). For instance, studies have shown that children who showed catch-up growth in terms of weight and length between zero and two years of age are fatter and have more central fat distribution at five years (i.e., greater body mass index, percentage body fat and waist circumference) than those children that did not catch-up (Ong, Ahmed et al. 2000). Catch-up growth and increased adiposity have been found to be most evident in developing countries that are increasingly adopting a high fat Western diet (Law 2001).

1.6 Developmental origins of cardiovascular disease - an example of DOHaD

CVD is the leading cause of death in developed countries; accounting for >35% of all deaths in the United States, and >25% in Canada (StatsCanada 2007; AHA 2008; AHA 2008). The main forms of CVD are hypertension, coronary heart disease (myocardial infarction and angina pectoris), stroke and heart failure. Some major CVD risk factors include insulin resistance, elevated blood pressure, type 2 diabetes and obesity, which have all been shown to have significant inverse relationships with birth size (McMillen and Robinson 2005; Yajnik and Deshmukh 2008). In comparison to the aforementioned risk factors, dyslipidaemic plasma lipid profiles (such as high plasma cholesterol and triglyceride levels), which are also established risk factors for CVD, show some association with birth size; however, the results are less consistent (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004). This discussion about the developmental origins of CVD will focus on early developmental programming effects on blood pressure (BP) and lipid metabolism.

1.6.1 Blood pressure and developmental origins of cardiovascular disease

Hypertension represents the number one contributor to CVD morbidity and mortality (AHA 2008; AHA 2008; WHO 2008) and is therefore potentially the greatest health concern in adult populations (Armitage, Khan et al. 2004). As such, a majority of investigations in the field of DOHaD are focused on trying to elucidate the underlying mechanisms related to the association between BP and birth weight. Indeed, Barker was the first to report a significant inverse relationship between birth size and BP (Barker,

Osmond et al. 1989); since then these findings have been confirmed through numerous epidemiological and animal studies (see reviews by (McMillen and Robinson 2005; Alexander 2006; Yajnik and Deshmukh 2008). Reduced nephrogenesis during early development is currently being investigated as the major underlying mechanism responsible for the inverse association between birth size and hypertension later in life.

1.6.1.1 Blood pressure and the kidneys – fetal programming of nephrogenesis

The aetiology of hypertension is multi-factorial. The kidneys are major players because they are long-term regulators of systemic blood pressure, with the nephron as the primary functioning unit of the kidney. Studies have shown that biological programming of the kidneys involves alterations at all levels, from the gene to the physiological system (McMillen and Robinson 2005; Fowden, Giussani et al. 2006; Yajnik and Deshmukh 2008), but for the purpose of this thesis, the discussion will be focused at the organ level.

Various studies have demonstrated that alterations in kidney development during gestation (i.e., impaired nephrogenesis caused by programming events due to nutritionally poor environment) result in an increased susceptibility to the development of hypertension later in life (reviewed in: (McMillen and Robinson 2005; Alexander 2006; Yajnik and Deshmukh 2008). Some animal models of maternal protein (or other nutrient) deficiency or glucocorticoid exposure generally results in a reduction in glomerular (and nephron) numbers in the offspring, with resulting increase in BP later in life. Defining how nephrons may be programmed *in utero* requires an understanding of what determines the normal development of nephrons during fetal development. In human fetuses, the number of nephrons has been shown to be reduced by ~35% in infants with a birth weight

below the 10th percentile (Hughson, Farris et al. 2003) and studies in IUGR animal models have shown similar decreases (McMillen and Robinson 2005; Alexander 2006; Yajnik and Deshmukh 2008). The decrease in nephron numbers is believed to be due to changes in the expression of genes that have been identified to be critical for normal branching morphogenesis in the kidney (Fowden, Giussani et al. 2006; Moritz 2006).

1.6.1.2 Measurement of blood pressure

In the field of DOHaD research, the non-invasive cuff technique is the most routine method to measure BP and has been used in many animal models; however, recently, these measurements have been the subject of much criticism, primarily because they create an artificially high BP due to the stress from the measurement method (Tonkiss, Trzcinska et al. 1998). There are three common methods for measuring BP in animal studies (Van Vliet, Chafe et al. 2000; Kurtz, Griffin et al. 2005; Pickering, Hall et al. 2005): 1) Many studies use the indirect cuff plethysmography method, which involves restraint. Only systolic pressure can be reliably measured and the method gives a single point measurement. 2) An in-dwelling carotid or iliac artery catheter allows continuous measurements of diastolic, systolic and pulse pressures to be taken with minimal disturbance, though the animal is continuously attached to a cable is inherently stressful. The catheter is implanted under general anaesthesia, and the time taken to recover is not clearly established. 3) More recently, it has been possible to use radio-telemetry methods which use an in-dwelling catheter in the aorta coupled to an intraperitoneal transmitter. This method has the benefit of a stress-free environment and the opportunity for 24-h monitoring of diastolic, systolic and pulse pressures (Van Vliet, Chafe et al. 2000).

1.6.2 Coronary heart disease and DOHaD – the role of lipid metabolism

A dyslipidaemic plasma lipid profile, such as high blood cholesterol level, is an established hallmark of cardiovascular and cerebrovascular diseases such as coronary heart disease (CHD) and strokes; with epidemiological and animal studies showing that low birth weight is associated with dyslipidaemic profiles (Barker and Osmond 1986; Barker, Gluckman et al. 1993; Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004; McMillen and Robinson 2005; Poston 2006). Specifically, a large number of studies have reported inverse relationships between birth size and components of the serum lipid profile (i.e., total plasma cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, apolioprotein A or triglycerides) later in life. However, such reported relationships have been found to be variable (see reviews: (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004; Poston 2006). For instance, in the Bogalusa Heart Study, children ages 7-11 showed an association between low birth weight and elevated serum triglyceride, but not total cholesterol (Donker, Labarthe et al. 1997). A large birth cohort study found that lower birth weight in men was associated with higher adult total cholesterol levels, but no association was observed in women (Davies, Smith et al. 2004). Recently, two large systematic reviews concluded that birth weight was only consistently associated with triglyceride levels, showing either an inverse or U-shaped relationship (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004).

Furthermore, some studies demonstrated that fetal undernutrition can permanently change lipid metabolism, with alterations in the liver as the underlying mechanism (Ozanne 2001; Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004; McMillen and Robinson

2005; Poston 2006).The liver is one of the key organs that are believed to be most likely to be impaired by fetal growth retardation (Barker, Martyn et al. 1993; Desai, Crowther et al. 1996). For instance, Barker and colleagues (Barker, Gluckman et al. 1993) found that smaller abdominal circumference at birth was associated with higher serum cholesterol levels in adult life. They suggested that abdominal circumference at birth was a reflection of liver size, and since cholesterol metabolism is regulated by the liver, impaired liver growth in uterus re-sets cholesterol concentrations towards a more atherogenic profile (Barker, Gluckman et al. 1993). The rationale for this hypothesis stems from the knowledge that in most species the liver is the hub for lipid metabolic processes such as fatty acid synthesis and lipid circulation through lipoprotein synthesis (Nguyen, Leray et al. 2008); that is, the liver plays a key role in blood lipid homeostasis, therefore altered liver growth during gestation may permanently reprogram lipid metabolism. Indeed, alterations in lipid metabolism have been found to be associated with alterations in the structure of the liver (Desai, Byrne et al. 1997; Roberts, Nava et al. 1999; Ozanne 2001; Souza-Mello, Mandarim-de-Lacerda et al. 2007). For instance, when compared to normal birth weight rats, low birth weight rats (offspring from maternal protein restriction) were shown to have reduction in heptaocyte number with steatosis and elevated plasma lipid profile and with significant enhancement of hepatic steatosis at 6-month old when they were fed a post-weaned high fat diet (Souza-Mello, Mandarim-de-Lacerda et al. 2007).

1.6.3 Impact of a post-weaning Western-style diet on early programming of CVD

Some studies have shown that early growth restriction and/or postnatal catch-up growth creates a disposition to chronic diseases, which at a later age manifest as overt diseases only if certain conditions are met. For instance, a study of arteriosclerosis in the United States and South America showed that in both regions, the development of coronary arteriosclerosis began very early, developing slowly and continuously up to about 20 years of age. However, while in South America this development continued to be slow and never reached a level resulting in a high incidence of coronary heart disease, in the US, the development of arteriosclerosis was accelerated and the incidence of coronary heart disease increased significantly with age (Leon 1998). The explanation for the decreased incidence of overt diseases in developing countries has been that while the predisposing factors are programmed and present, the conditions needed to express the disease at a later age were not widely prevalent in these countries. However, with the increased Westernization of developing countries, the prevalence of exacerbating conditions, such as high energy intake from fat and a sedentary lifestyle, is rapidly increasing in populations of developing countries and leading to much higher rates of disease morbidity and mortality. In other words, it is not the disease per se, but the susceptibility to disease, that is being programmed, and environmental factors that exceed an individual's programmed limits will ultimately lead to overt disease. For example, a hypercaloric postnatal diet exacerbates the adverse effects of fetal undernutrition in relation to hypertension, obesity, hyperleptinemia (Vickers, Breier et al. 2000; Vickers,

Krechowec et al. 2007). Therefore, adaptations that lead to the development of hypertension and CVD are initiated during fetal life and are progressively amplified after birth and throughout adult life. The interaction between fetal programming and the postnatal environment has been recognized in epidemiological studies, with evidence that postnatal diet can indeed amplify detrimental programming effects (Barker, Osmond et al. 1989); also see reviews:.

The typical Western diet is high in levels of saturated fat, simple sugars and salt, and low in vitamins and minerals (due in part to dilution by calorie-rich, low nutrient dense foods) and accounts for over 30% of the world's heart attacks (AHA 2008; WHO 2008). In Western societies, the dietary intake of salt is particularly high and salt sensitivity may be an important contributor to the prevalence of essential hypertension. Moreover, there might be different mechanisms involved in controlling acute (i.e., days to weeks) versus chronic (i.e., months to years) salt sensitivity. Acute salt sensitivity is somewhat reversible, while chronic/progressive salt hypertension is not fully reversible (Van Vliet and Montani 2008).

1.7 Animal models to study DOHaD

Although there is substantial epidemiological evidence for DOHaD, there are of course many limitations; including obtaining tissue samples, invasive procedure and other ethical issues, with the use of clinical studies to elucidate the mechanisms regulating DOHaD. Thus, animal models provide more rapid and controlled conditions to study many aspects of DOHaD. Various animals models that have been developed to study the phenomenon

of DOHaD, including, the rat, mouse, guinea pig, sheep, non-human primate and swine (see reviews: (Bertram and Hanson 2001; Armitage, Khan et al. 2004; Armitage, Taylor et al. 2005; Langley-Evans, Bellinger et al. 2005; McMillen and Robinson 2005).

Although the mechanisms for FOAD or DOHaD are not completely understood, it is thought that fetal undernutrition plays a dominant role. Fetal undernutrition could in turn be caused by maternal undernutrition or by impairment of uteroplacental blood flow or nutrient exchange in an otherwise well-nourished mother (Bertram and Hanson 2001; Armitage, Khan et al. 2004; Armitage, Taylor et al. 2005; Langley-Evans, Bellinger et al. 2005; McMillen and Robinson 2005). In most species, including humans, the major cause of IUGR is placental insufficiency due to reduced supply of oxygen and nutrients (Bertram and Hanson, 2001; McMillen and Robinson, 2005). To date, many animal models of placental restriction (fetal undernutrition) have been studied, including dietary (maternal protein, global food or specific nutrient restrictions), surgical (placental embolization, caruncleclectomy and intrauterine artery ligation), pharmacological, genetic and natural (restriction due to litter size) interventions, and these models have been studied in many different animal models including rat, sheep, and pig (e.g., (Owens, Falconer et al. 1987; Ritacco, Radecki et al. 1997; Jansson and Lambert 1999; Langley-Evans 2000). However, most of these models have been criticized because they may use maternal insults that, although effective in programming the offspring, are more severe insults (e.g., protein restriction from normal 18% to 8% or even 5%) than would normally be encountered by humans in Western society. Alternatively, swine and other animals with a bicornuate uterus have natural variations in fetal growth occurring at least partially

because of the somewhat complicated architecture of the vascular supply to the uterus and placenta (Swindle 1994). These models of placental insufficiency are said to be more appropriate, because a large proportion (80-90%) of the human incidence of intrauterine growth retardation is thought to be due to impaired nutrient perfusion through the placenta, which leads to a reduced offspring birth weight as well as altered organ development such as reduced nephron number and impaired renal function (Bauer, Walter et al. 2002; Bauer, Walter et al. 2003; Woods 2006).

The naturally occurring runt piglet model has been argued to represent a suitable model for IUGR newborn infants (Ritacco, Radecki et al. 1997; Bauer, Walter et al. 2002; Bauer, Walter et al. 2003). IUGR occurs spontaneously in a variety of mammalian species, including human, swine, rabbit, sheep and guinea pig, and is said to exhibit quite similar signs of adaptive programming with catch-up growth characteristics as shown in humans (Bauer, Walter et al. 2003).

Some animal researchers have suggested that uterine capacity is the primary determinant of runting. That is, the uterus of the pig is limited in the number of piglets it can support during pregnancy, termed 'uterine capacity'. Uterine capacity has been traditionally defined as the ability of the uterus to provide the necessary nutrients to maintain fetuses until farrowing (Vallet and Freking 2007). One of the consequences of limitations in uterine capacity in piglets born alive is runting. "The term 'uterine capacity' focuses attention on the uterus, but in fact uterine capacity is a combination of the ability of the uterus to provide nutrients, the ability of the placenta to transfer nutrients to the fetus, and the ability of the fetus to efficiently use those nutrients for growth and

development" (Vallet and Freking 2007). Furthermore, the term uterine capacity is also a deceptive terminology, because some researchers have shown that limited uterine space is not a primary determinant of fetal growth. In fact, inadequately grown pig fetuses can statistically be identified as early as 30 days into the 114 days gestation period in sows (Ashworth, Finch et al. 2001). In summary, runting in swine seems to be primarily based on placental insufficiency rather than uterine capacity *per se.* The naturally occurring Yucatan miniature runt piglet model used in this thesis represents an appropriate model of placental insufficiency in humans and can be used to investigate mechanisms of DOHaD as represented in the epidemiological literature.

The majority of the animal research in the field of DOHaD is conducted in rodent models. The popularity of rodent models is attributed to several factors, including the short gestation and lifespan periods of these models, as well as the fact that they are widely accepted models for studying many facets of human metabolism and physiology. However, one of the key disadvantages is that rodents are altricial animals; that is, they experience significant organ maturation during weaning (unlike humans) which makes it difficult to extrapolate to the human, especially when trying to delineate the role of fetal versus postnatal nutrition. The sheep model represents over 10% of the research in this field. Sheep are used primarily to study *in utero* fetal physiological changes. This is feasible in this model because it is relatively easy to catheterize fetal sheep because of the large uterus with only singleton or twin fetuses, allowing for surgical manipulation models such as placental ligation, placental embolisation, and carunclectomy (review in: (Bertram and Hanson 2001; McMillen and Robinson 2005). Swine have been used in

biomedical research because of the similarity in terms of physiology, metabolism and nutritional requirements to humans. Furthermore, like humans, they are precocial and experience most of their organ development during gestation.

In conclusion, primarily because of physiological, metabolic and nutritional similarities, along with the moderate placental insufficiency model via spontaneous IUGR (i.e., runt), the swine is an appropriate model to study many aspects of FOAD and DOHaD, and, further investigation that utilizes the swine model is required.

1.7.1 Swine model of IUGR with catch-up growth

There is limited research on swine as a model of fetal programming or DOHaD but the available research with a domestic swine model (Poore, Forhead et al. 2002; Poore and Fowden 2002; Poore and Fowden 2003; Poore and Fowden 2004; Poore and Fowden 2004) shows that early catch-up growth contributes to later chronic disease onset. In these studies the researchers found that in comparison to larger littermates, the runt piglets had a faster rate of growth from 3 to 12 months of age with impairment in glucose tolerance (Poore and Fowden 2002; Poore and Fowden 2004) and increased fat depth (Poore and Fowden 2004) at 12 months of age. Recently, it was shown that Yucatan miniature runt piglets undergo compensatory growth in terms of fractional, but not absolute growth rates for weight by the third week of life (McKnight 2008). Others have also reported this form of catch-up growth in other runt pig models (Ritacco, Radecki et al. 1997; Schoknecht, Ebner et al. 1997); however, these researchers did not examine long-term consequences of this early catch-up growth.

1.8 Thesis rationale and objectives

1.8.1 Hypotheses

1.8.1.1 General hypothesis

Naturally occurring IUGR Yucatan miniature pigs (i.e., runts) will display some biological markers of cardiovascular disease in contrast to their larger weight siblings. This difference will be exacerbated by a nutritionally poor post-weaning Western-style diet.

1.8.1.2 Specific hypotheses

Runt pigs will: 1) experience a period of accelerated postnatal growth rate; 2) have elevated blood pressure; 3) display components of altered lipid metabolism, such as elevated plasma triglyceride and cholesterol concentrations; 4) undergo altered tissue and metabolic differentiation *in utero,* which will contribute to development of CVD markers later in life.

1.8.2 Overall objectives

To determine the effects of intrauterine growth restriction in Yucatan miniature pigs on postnatal growth and development of biomarkers of cardiovascular disease from birth to early adult life. Specifically, the overall project objectives were:

1. To validate the Yucatan miniature pig as a model for studying DOHaD, specifically CVD in terms of BP and CHD.

2. To examine plausible biological mechanisms which contribute to programming of BP and CHD in this model.
3. To examine the effects of a post-weaning Western-style diet on fetal/ early programmed BP and CHD.

1.8.3 Significance of project

This project will determine the suitability of the naturally occurring runt Yucatan miniature pig as a valid model to study aspects of developmental origins of adult disease. Specifically, this project will study whether the runt Yucatan miniature pig, in comparison to its siblings, will develop biomarkers of cardiovascular disease later in life. Furthermore, we will examine some of the underlying mechanisms for the development of these biomarkers in the runt. This will provide some evidence to support the Yucatan miniature swine as an animal model to aid in unravelling the mechanistic basis of developmental origins of adult disease, and therefore, provide a model that can subsequently be used to test alternative therapies to overcome the adverse consequences of early programming.

1.9 Proposed studies

1.9.1 Characterizing blood pressure in Yucatan miniature swine

The inverse association between birth size and BP is the most widely observed phenomenon in the field of fetal programming and DOHaD. BP measurement by non-invasive cuff technique is the most widely used method in BP research. However,

recently, in the field of blood pressure research, there have been criticisms of BP measurements by cuff methods due to the stress created by restraining the animal (Tonkiss, Trzcinska et al. 1998). More researchers are switching to the radio-telemetry (invasive) technique, the gold-standard for BP measurements in animals (Van Vliet, Chafe et al. 2000). However, this technique is still fairly novel for large animals such as swine; thus we wanted to first characterize BP in Yucatan miniature swine by radio-telemetered 24-h BP measurements in conscious, freely moving pigs.

1.9.2 Developmental programming of CVD in Yucatan miniature swine

A comprehensive literature search found only one other major research group that uses swine (domestic) as a model to study DOHaD (Poore, Forhead et al. 2002; Poore and Fowden 2002; Poore and Fowden 2003; Poore and Fowden 2004; Poore and Fowden 2004). Here, we aim to validate the naturally occurring IUGR (i.e., runt) Yucatan miniature pig as another swine model for studying DOHaD, specifically CVD in terms of BP and CHD.

1.9.3 Effects of post-weaning Western-style diet on developmental programming of CVD in Yucatan miniature swine

The prevalence of CVD in industrialized societies has always been due in part to nutritionally poor diet choices throughout life. Recently epidemiological and animal studies have reported an acceleration of overt CVD outcomes due to an additive effect of pre-and post-natal diets in low birth weight offspring. The suggestion is that a postnatal

nutritionally poor diet might be needed for the manifestation of early markers of diseases attributed to DOHaD. In this thesis we wanted to investigate whether early (post-weaning) exposure to a Western-style diet that was high in salt and caloric content would exacerbate CVD onset in our runt Yucatan miniature pig model.

CHAPTER 2. MATERIALS AND METHODS

2.1 Animals

The Institutional Animal Care Committee at Memorial University of Newfoundland approved all animal procedures and protocols used in this project, and all animals were treated in accordance with guidelines set by the Canadian Council of Animal Care.

This project consisted of two main studies, which are described in details in chapters 4 through 7 (study 1: **Chapter 4** and **5**; study 2: **Chapter 6** and **7**). There were twelve pigs in each of the main studies, and a third blood pressure validation study (**Chapter 3**) which used a total of twelve pigs, thus, a total of thirty-six Yucatan miniature pigs were used in this project (**Figure 2.1**). All pigs were obtained from Memorial University of Newfoundland swineherd. Sows were allowed to farrow naturally at term (114 ± 3 d) and then 1 day postpartum, each piglet in the litter was weighed. Defining the IUGR pig in the litter was done based on similar procedures used by Ritacco (Ritacco, Radecki et al. 1997) for domestic pigs. For this thesis, a runt piglet was defined as a piglet weighing less than 900 g, with a same-sex littermate weighing at least 300 g more than the runt (note: this larger littermate is referred to throughout the thesis as a large littermate). There was only one runt per litter. A third littermate, that was similar in weight to the large littermate, was also selected for the project; however, unlike the runt and large littermate, this piglet (sow-fed control) was left with the sow until weaning at 4 wk old. During this 4 week period the third littermate received *ad libitum* nutrition from the sow. On average there were two piglets left on the sow. Within each litter, we attempted to have the sow-

fed control the same sex as the runt and large littermates; however, this was not possible in two of the litters that were used in the standard pig diet study (i.e., the control diet study). That is, the sex distribution of the litter was such that there was only one male or one female born to the litter, therefore, we always ensure to sex-match the runt and large littermate per litter; however, the third littermate was not necessarily the same sex. However, overall there were equal numbers of males and females for each study.

2.2 Study protocols: Diets and housing

2.2.1 Neonatal phase: Milk feeding and housing

After allowing for colostrums intake, the runt and the large littermate were taken from the sow at 3 d old and placed on reconstituted sow milk replacer (Piglet-Gro, Grober Nutrition, Cambridge, Ontario, Canada) until they were weaned from milk replacer 4 wk later (i.e., 31-32 d old). Sow milk replacer contained a composition of (units/L) of 60 g protein, 63 g fat, 72 g lactose, and 4.6 MJ of energy, and a nutrient profile similar to that of sow milk (Table 2.1)(GroberNutrition 2004). During the milk feeding phase, the runt and large littermate from the same litter were housed together but were fed separately, thus individual intakes were measured and recorded daily. The sow milk replacer was rehydrated in pre-boiled water and bowl-fed to the runts and large littermates *ad libitum*, eight to ten times daily, at 2-3-h intervals. Piglets were monitored during feedings to ensure that the milk was being consumed, and to minimize spillage. Each housing pen was provided with thick rubber mats and dried straw for bedding, which was changed routinely. The pig room was temperature controlled (25^0C), however, the pens that were

housing piglets were also provided with infrared heat lamps for supplementary heat. The pigs were provided with squeaky and chewy toys to enjoy the environment. The sow-fed piglet was left with the sow until weaning at 31-32 d old.

2.2.2 Post-weaning phase: diet feeding and housing

2.2.2.1 Control diet – Standard pig grower diet

At 4 wk old, all 3 pigs from the same litter (runt, large littermate and sow-fed littermate) were weaned from milk replacer or the sow onto grower diet (Eastern Farmers Co-op, St. John's, NL - providing 12% of their caloric intake from fat, 67% from carbohydrate and 21% from protein) (Table 2.2). From 1 mo old to 5 d before surgery (i.e. ~ 9 mos old) the triplet was group-housed in the same pen, with the exception of the two groups of mixed sex (i.e., sow-fed pig of different sex than the runt and large littermate siblings), where the sow-fed pig was housed with another pig of the same sex and similar age. Throughout the entire study, each pig was fed individually for 5 h (1200 – 1700h) *ad libitum* daily and intakes recorded. All pigs had 24-h *ad libitum* water access and were maintained on a 12-h day-night cycle (0700 – 1900h). A total of 18 pigs (6 sets of littermates: 6 runts, 6 large littermates and 6 sow-fed littermates) were placed on the standard pig grower diet.

2.2.2.2 Western-style diet

As partly illustrated in Figure 2.1, after the 4 weeks of the milk feeding phase, another six groups of triplets (runt, large littermate and sow-fed littermate) followed the same protocol as outlined above for pigs fed the regular diet, except these pigs were fed a high-salt-fat-sugar (HSFS) diet (Table 2.2) from weaning. This diet was formulated to

represent a North American-style diet, with the added sugars, sodium and fat (saturated and trans) levels based on the 95th percentile for American young adult males (20-39 yrs old) (Kant 2003; CDC 2004; Romieu, Mannino et al. 2004). This HSFS diet was made by the addition of salt (40g/kg; Windsor, Clarkson, ON, Canada), melted hydrogenated margarine (50 g/kg; Central Dairies, St. John's, NL, Canada),melted lard (150 g/kg; Loblaw Inc., Toronto, ON, Canada) and granulated sugar (100 g/kg; Lantic, Montreal, QC, Canada) to ground standard pig diet (Eastern Farmers Co-op, St. John's, NL, Canada), thus providing 50% of caloric content from fat, 40% from carbohydrate and 10% from protein. The HSFS diet was prepared in 150 kg and 300 kg batches and frozen at -20^{0}C in 10-20 kg aliquots until used; the daily aliquots were stored at 4^{0}C. We ensured that the mineral and vitamin contents were adequate for the predicted growth rate given the lower protein content of this diet (NRC 1998).

The HSFS diet was fed from weaning until ~12 mo of age; these pigs were held longer for several reasons: 1) because the protein-to-energy ratio of this diet was lower, pigs on this diet grew slower so more time was allowed to try and match the final body weights with the pigs in the control diet study group; 2) more time was allowed for the HSFS diet to exert its metabolic effects; and 3) because of housing limitations for surgically altered pigs ,which did not allow for complete synchronization between groups. Therefore, we ensured each diet group was controlled within each diet, but not between the diets.

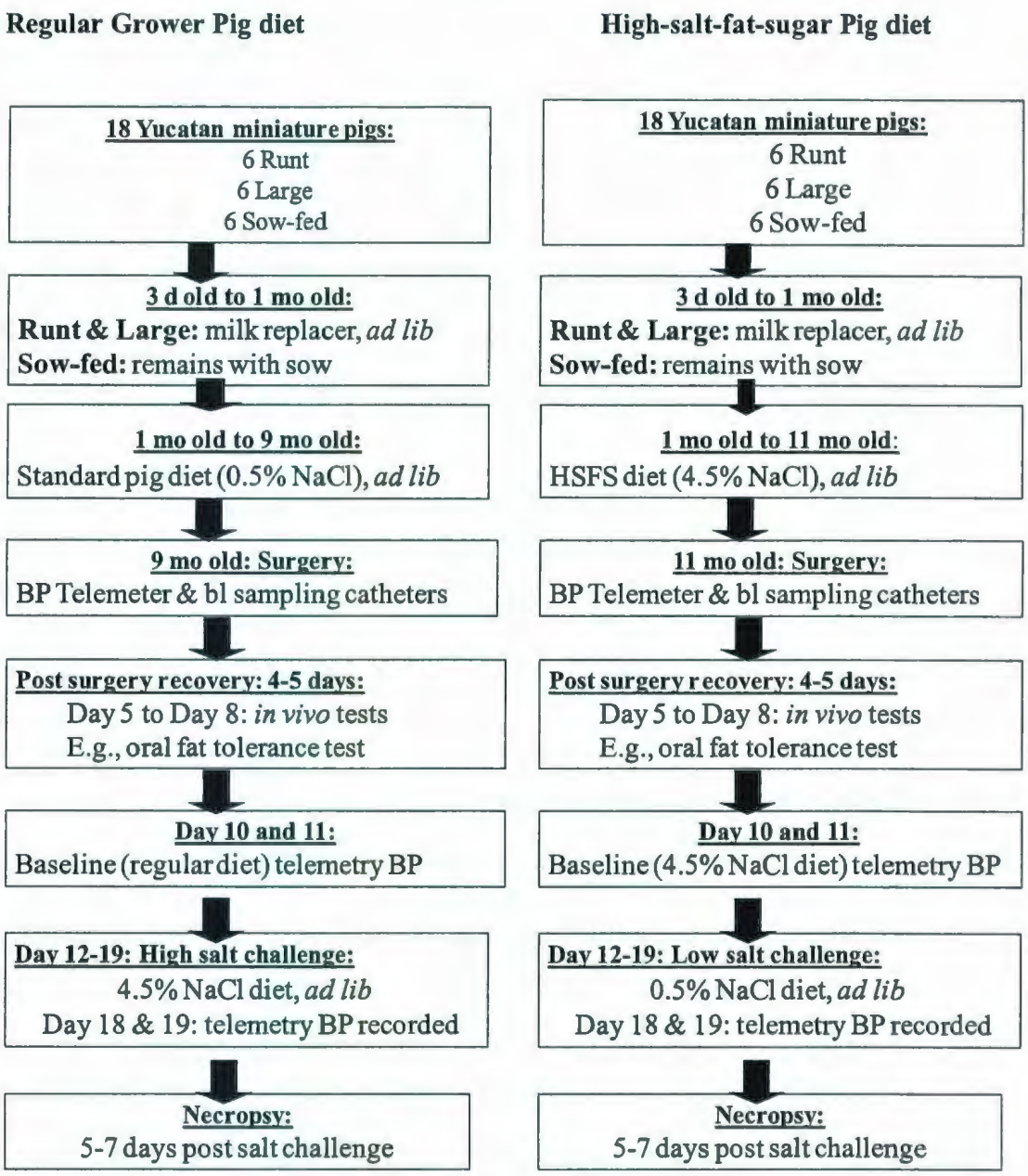

Figure 2.1: An overview of the study design for the runts, large littermates and sow-fed littermates on the regular grower pig diet and those on the high-salt-fat-sugar diet.

BP = blood pressure; bl = blood; Large refers to the large littermate; HSFS = High-salt-fat-sugar. **In vivo tests:** [Day 5 Intravenous glucose tolerance test; Day 6 Insulin sensitivity test]; Day 8 Oral fat tolerance test. **Note:** Day 5 and 6 tests are not part of this thesis.

Table 2.1 Guaranteed nutrient levels[1] and main ingredients[2] in the milk replacer fed to the runts and large littermates.

Nutrient	Guaranteed nutrient levels[1]
Crude Protein	23% (Minimum)
Crude Fat	17% (Minimum)
Crude Fibre	0.15% (Maximum)
Ash	8% (Maximum)
Sodium	0.8% (Actual)
Calcium	0.75% (Actual)
Phosphorus	0.75% (Actual)
Zinc	105 mg/kg (Actual)
Copper	125 mg/kg (Actual)
Vitamin A	40,000IU/kg (Minimum)
Vitamin D3	4,000 IU/kg (Minimum)
Vitamin E	150 IU/kg (Minimum)

[1]Grober Nutrition, Cambridge, Ontario, website: http://www.grobernutrition.com/speciality-milk-replacers/piglet/piglet-gro-2/.

[2]**Ingredients**: Whey protein concentrate, dried whey, animal fats, coconut oil, lecithin, starch, L-lysine, DL-methionine, dicalcium phosphate, calcium carbonate, vitamins and minerals. Information on the exact inclusions of ingredient levels were not available.

Table 2.2 Composition of the regular grower diet[1] and high-salt-fat-sugar (HSFS) diet[2] fed to the Yucatan miniature pigs during the regular diet-feeding phase.

	Regular diet (Low salt)	Regular diet (High salt)	HSFS diet (High salt)	HFS diet (Low salt)
Energy distribution (% total energy)				
Carbohydrates	67	67	40	40
Complex	67	67	30	30
Sugar	-	-	10	10
Fat	12	12	50	50
Saturated fatty acid	1	1	16	16
Protein	21	21	10	10
Nutrients (g/kg DM[3])				
Wheat shorts	400.5	400.5	264.3	264.3
Canola	49.0	49.0	32.3	32.3
Meat meal	19.0	19.0	12.5	12.5
Limestone	13.0	13.0	8.6	8.6
Corn gluten feed	40.0	40.0	26.4	26.4
Ground barley	297.0	297.0	196.0	196.0
Oats	175.0	175.0	115.5	115.5
Vitamin mix	0.8	0.8	0.5	0.5
Mineral mix	1.0	1.0	0.7	0.7
Lard[€]	-	-	150.0	150.0
Margarine[£]	-	-	50.0	50.0
Sugar[¥]	-	-	100.0	100.0
Sodium chloride*	4.7	40.0	40.0	4.7

[1]Standard diet = standard pig grower diet, manufactured by Eastern Farmers Co-op, St. John's, NL, Canada. [2]HSFS diet = High-salt-fat-sugar diet. HSFS diet was made by the addition of salt* (40g/kg; Windsor, Clarkson, ON), hydrogenated margarine£ (50 g/kg; Central Dairies, St. John's, NL), lard€ (150 g/kg; Loblaw Inc., Toronto, ON) and granulated sugar¥ (100 g/kg; Lantic, Montreal, QC) to ground standard pig diet (Eastern Farmers Co-op, St. John's, NL). [3]DM = dry matter basis.

2.3 Study protocols: Procedures & Techniques

2.3.1 Swine body measurements

Routine growth measurements were taken from all pigs from birth to 9 or 11 mo of age for regular or HSFS treatments, respectively. Body weight, snout to rump length, crown to rump length, and abdominal circumference were measured 1-2 times weekly during the milk feeding phase (i.e., 0-4 wk old). During the regular diet feeding phase (i.e., 1-9 or 1-11 mo old) these measurements were made twice monthly. The pigs were weighed in a tared container, using appropriate electronic scales accurate to the nearest gram (Sartorius GmbH, Gottingen, Germany). Crown-rump length was measured from the mid-point between the ears, along the neck, down the back and to the first joint in the tail using a tailor's tape measure. Abdominal circumference was measured around the largest section of the trunk using a tailor's tape measure.

2.3.2 Blood sampling

From 1 to 3 mo old, about 1-2 times monthly, 10 mL of blood was collected in EDTA tubes (K_2EDTA BD Vacutainer®; BD, Franklin Lakes, NJ) from each pig via jugular venipuncture while the pig was restrained in the supine position in a V-trough. From 3 mo old to the end of the study, 20 mL of blood was collected from each animal in EDTA tubes 1-2 times monthly. Blood samples were immediately centrifuged for 10 min at 4000 x g at 4^0C, with the plasma collected and stored at -20^0C for later analyses.

2.3.3 Non-invasive blood pressure measurement:

From ages 4 to 8 mo-old, using the blood pressure (BP) cuff technique, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR) were measured once every two weeks in pigs on the regular diet. Specifically, pigs were restrained in the supine position in a V-trough on the floor. In this position, the hind leg was in-line with the heart level. The appropriate size cuff (Surgivet V6004 NIBP monitor, Waukesha, WI) was placed on the lower part of the left hind leg (the lower part of the leg is more cylindrical, which was more suitable for cuff function than the upper part of the leg). Consecutive haemodynamic measurements were recorded for 30 min using Surgivet V6004 NIBP monitor (Waukesha, WI), with approximately 35-45 haemodynamic recordings during this period. On average, the pigs were more relaxed after 10 min in this position (Figure 2.2) and data were reproducible between pigs (Figure 2.3). Measurements were taken at the same time of day for each pig and on the same day of the week. Specifically, measurements were taken in the afternoon from 1300h-1800h; during this time of the day, the animals were in the fed state and were less stressed during the procedure as measured by heart rate (data not shown).

2.3.4 Surgical techniques – Telemetry and blood sampling catheters

2.3.4.1 Blood pressure telemetry implantation

At 9 mo old (i.e., regular diet group) or 11 mo old (i.e., HSFS diet group), the pigs were transported from the general animal housing facility (i.e., Memorial University Vivarium) to the Memorial University Health Sciences Centre (HSC) animal housing facility where

they remained for the rest of the study. There, the animals underwent surgery for implantation of the telemeter and blood sampling catheters. The pigs were taken to the HSC at least 2 days before the surgery day and fasted overnight prior to surgery. Anaesthesia was induced with ketamine (Ketalean, Bimeda-MTC, Cambridge, Canada; 22 mg/kg *i.m.*) and xylazine (Rompum, Bayer, Toronto, Canada; 2 mg/kg *i.m.*) and was maintained with 1.0- 1.5% halothane and a 3:2 mixture of oxygen and nitrous oxide via intubation and ventilation. The body of the BP telemeter (TA11PA-D70; Data Sciences International, St. Paul, MN) was implanted in a subcutaneous pouch over the inner thigh of the left hind-limb with the telemeter catheter inserted 10 cm into the femoral artery with the catheter tip close to the aorta.

2.3.4.2 Blood sampling catheter implantation

During the telemeter implantation, two blood sampling catheters (0.04 in. ID x 0.70 in. OD, Tygon® tubing, Norton Performance Plastics, Akron, OH) were also inserted in the left femoral vein and advanced to the inferior vena cava; the tips were staggered by ~15 cm. The catheters were tunnelled under the skin and exteriorized between the shoulder blades. To reduce risk of infection, pigs were given 0.07 mL/kg *i.v.* trimethoprim (40 mg/mL)/ sulfadoxine (20 mg/mL) (Borgal, Intervet Canada Ltd., Whitby, ON) during surgery and the first 2 d post-surgery. To alleviate pain, animals were given 300 μg buprenorphrine hydrochloride (Temgesic, Schering-Plough Ltd., Hertfordshire, UK) immediately after surgery and 1 d post-surgery. Patency of the catheters in the pigs was maintained by flushing with 5 mL of 0.2% heparinized saline .Body temperature was

measured daily with a digital ear thermometer. If any animal presented with a temperature $> 40^0$C, then antibiotics were provided to the animal.

2.3.5 In vivo tests

The 5 h daily feeding regime was re-established the day immediately following surgery; on average, by the third day post-surgery, food intake was similar to pre-surgery levels. Animals were allowed to recover for 4-5 days after surgery before any testing was started. The studies in this thesis were only part of a larger series of studies conducted with these pigs. As part of the overall project (which includes this thesis and two other Masters theses), the pigs underwent additional *in vivo* tests, including the intravenous glucose tolerance and insulin sensitivity tests (IST) to assess parameters of type 2 diabetes mellitus – this work is not part of my thesis. Two days after the IST an oral fat tolerance test was performed in each animal after an overnight (i.e., 12-14 h) fast. Two days after the fat test, telemeter blood pressure recording began. The fat tolerance test and blood pressure measurements are part of this thesis and are discussed in more detailed in later chapters.

2.3.6 Telemetry system

The telemeter (TA11PA-D70) monitored the arterial BP and heart rate (HR) of the animal and transmitted these signals in the form of radio frequency to receivers located within the perimeter of the animal housing pen. Specifically, the BP telemeter signal was picked up using a network of six receivers (RLA1020, Data Sciences International, St. Paul, MN) positioned three on either side of the holding pen (1.9 m L x 1.2 m W x 2.0 m H).

The strongest signal was selected by a consolidation matrix (RMX10, Data Sciences International, St. Paul, MN) and passed to an analog adapter (R11CPA, Data Sciences International, St. Paul, MN), which provided a calibrated voltage output after correcting for atmospheric pressure using an ambient pressure monitor (APR-1, Data Sciences International, St. Paul, MN). The calibrated signal was then recorded using a computerized data acquisition system and processed as previously described (Montani, Mizelle et al. 1995). Overall, the telemeters were pre-calibrated (relative to a vacuum) by the manufacturer (Data Sciences International, St. Paul, MN) with additional pressure calibrations of the telemeters assessed by the researchers before implantation and after removal; corrections were made to the data for any deviation from the original factory calibration (Van Vliet, Chafe et al. 2000). The BP signal was sampled for 10 s at 30-s intervals, and the levels of systolic (SAP), diastolic (DAP), and mean (MAP) arterial pressure and heart rate over this sample period were computed and stored for offline analysis. Locomotor activity data were derived from the activity signal reported by the Data Sciences International telemetry equipment and were expressed in arbitrary units, with the value 0 corresponding with inactivity.

2.4 Post-mortem

On average, about 32 d after surgery pigs were anaesthetized with 105 mg/kg sodium pentobarbital (Euthanyl, Biomeda-MTC Cambridge, ON) and ventilated and maintained with 0.5 – 1 % halothane gas mixed with oxygen via intubation. The animals died of exsanguination caused by removal of the liver. The following organs and tissues were removed, weighed, and samples stored in 5% formalin and/or snap-frozen in liquid

nitrogen and stored at -80^{0}C for later analyses: pancreas, liver, kidneys, spleen, visceral fat, small and large intestines, stomach, muscle, lung, heart, entire aorta, back-fat, brain, and vertebra (L2) and femur. The carcass and visceral organs were frozen separately and stored at -20^{0}C for later analyses. As soon as possible after necropsy, the whole empty carcass and the visceral organs were separately ground by extrusion through a series of grinding plates. The carcass was ground one time through a grinding plate with 12.5 mm diameter holes, three times through a series of grinding plates with diameters 6 to 3 mm; the visceral organs were ground three times through grinding plates range from 6 to 2 mm diameters. Subsamples of the ground tissues were stored at -20°C for determination of lipid content.

2.5 Statistical analyses

Data are expressed as mean ± standard deviation (SD), unless stated otherwise. Statistical analyses varied depending on the parameters that were analyzed – specific statistical analyses are indicated in the appropriate chapters of this thesis. After assessing the initial outcomes, the data were packaged appropriately. For example, because no differences were observed between sow-fed and large littermates, we decided to use the sow-fed groups as reference data culminating in **Chapter 3** and runt-large littermate comparisons were made throughout the other chapters (**Chapters 4, 5, 6, 7**). This separation improved statistical analyses by allowing paired t-tests between runt and large littermates, which were all sex-matched.

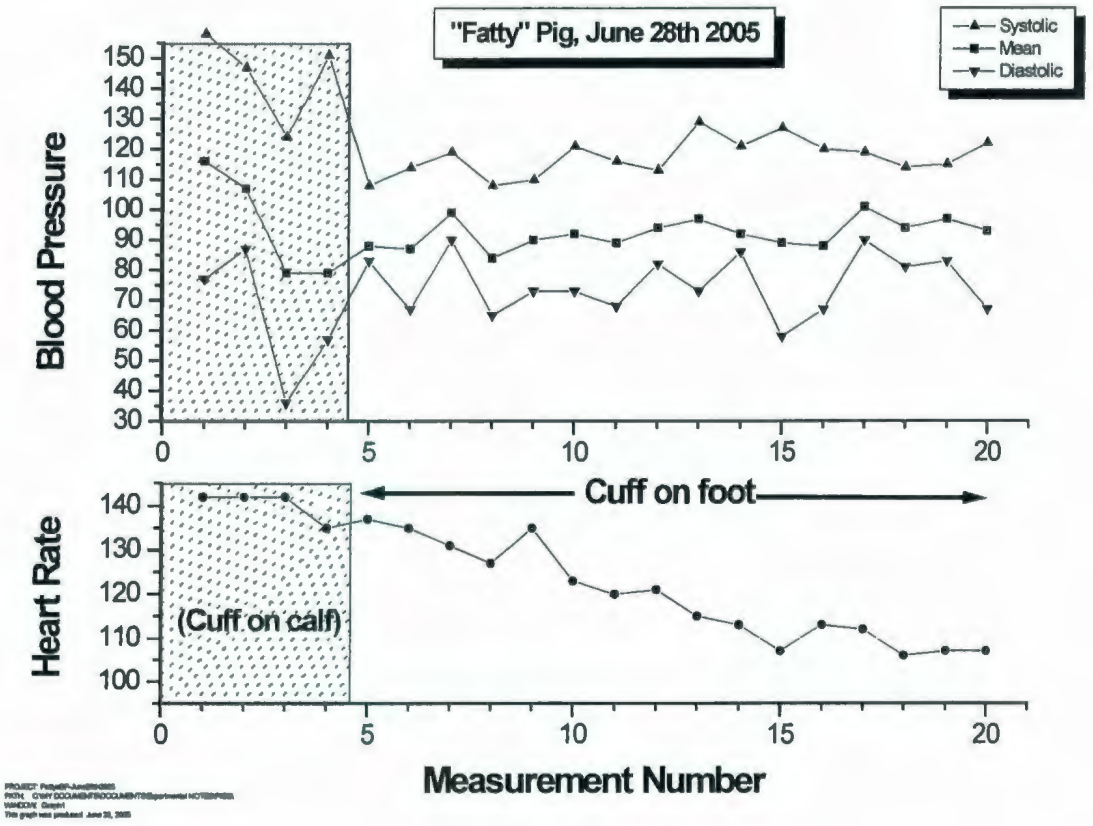

Figure 2.2: Representative recordings of systolic, mean and diastolic blood pressure and heart rate by foot cuff measurements (i.e., upper hind leg – shown in the shaded region and lower hind leg- un-shaded) in an individual Yucatan miniature swine on standard pig diet.

Values were plotted at 1 min intervals for a 30-min period. Heart rate = HR (beats/min); diastolic, mean and systolic blood pressure = (mmHg).

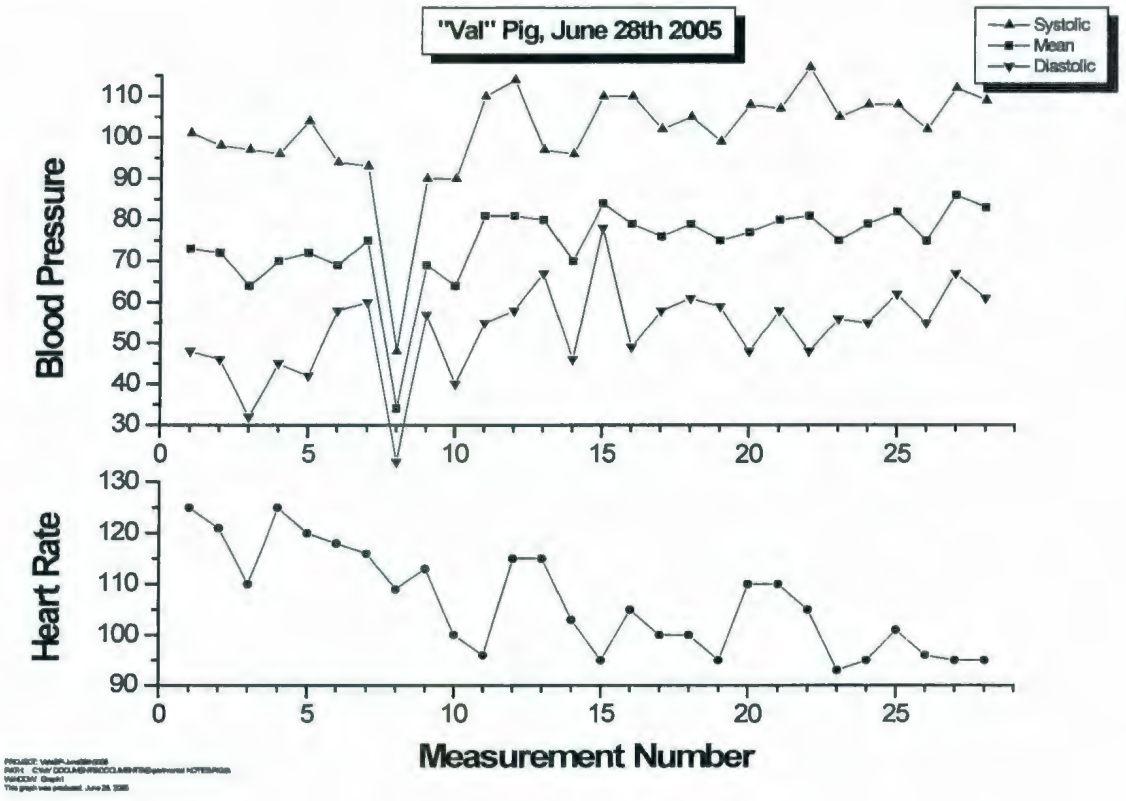

Figure 2.3: Representative recordings of systolic, mean and diastolic blood pressure and heart rate by foot cuff measurements in an individual Yucatan miniature swine on standard pig diet.

Values were plotted at 1 min intervals for a 30-min period. Heart rate = HR (beats/min); diastolic, mean and systolic blood pressure = (mmHg).

Characterising Blood Pressure in Yucatan Pigs

CHAPTER 3 Absence of Blood Pressure Dipping in Yucatan Miniature Swine

CHAPTER 3. TECHNIQUE DEVELOPMENT: ABSENCE OF BLOOD PRESSURE DIPPING IN YUCATAN MINIATURE SWINE

3.1 ABSTRACT

Objective: A fall or "dipping" in blood pressure (BP) during the inactive phase of the 24-h cycle is common in mammals, and a non-dipping BP profile is regarded as an independent risk factor for cardiovascular events and target organ damage. The purpose of this study was to investigate the dipping phenomenon in Yucatan miniature swine.

Methods: BP telemeters were implanted via the femoral artery to assess the 24-h profile of blood pressure, heart rate (HR) and locomotor activity in unrestrained, 9-month-old Yucatan miniature swine of either sex maintained on regular diet (0.5% NaCl, N = 6). We repeated our analysis in the same pigs after a 7-d salt challenge (4.5% NaCl diet), and a separate group of pigs maintained on a diet high in salt, sugar and fat (4.5% NaCl, N = 6).

Results: In pigs maintained on regular feed, HR and indices of locomotor activity showed pronounced diurnal variation, both being significantly ($P<0.05$) greater in the light than dark phases of the 24-h cycle (HR: 90 ± 4 vs. 82 ± 6 beats/min; Activity index: 2.6 ± 1.6 vs. 0.5 ± 0.4). In contrast, BP indices showed little if any diurnal variation. BP dipping was clearly absent, with the mean BP level actually tending to be slightly higher during the dark phase of the 24-h cycle than during the light phase (mean: 113 ± 4 vs. 110

± 7 mmHg). Subsequent analysis revealed that during both the light and dark phases, episodes of locomotor activity were associated with increased HR but not increased BP. In fact, BP was significantly reduced during periods of locomotor activity during the light phase (mean: 112 ± 6 vs. 104 ± 10 mmHg, $P<0.05$). After a 7-d salt challenge, BP increased significantly from 110 ± 7 mmHg to 119 ± 8 mmHg indicating that the pigs were moderately sodium sensitive. However, BP dipping remained absent on the high salt diet. Finally, a group of pigs maintained on a high-salt-fat-sugar diet demonstrated similar patterns for BP, HR and activity, including the absence of BP dipping.

Conclusion: Our results demonstrate that in this swine model, irrespective of their experimental diet, the phenomenon of BP dipping is absent, despite the clear presence of diurnal variations of locomotor activity and HR. The absence of dipping may in part be due to an unusual response to exercise, as periods of locomotor activity were associated with slight reductions, not increases, in BP.

3.2 INTRODUCTION

A fall in blood pressure (BP) during the inactive phase of the 24-h daily cycle is common in mammals, having been observed not only in adult humans (Pickering 1990), dogs (Mishina, Watanabe et al. 1999), cats (Mishina, Watanabe et al. 2006), rats (van den Buuse 1994), mice (Van Vliet, Chafe et al. 2003), and rabbits (Antic, Van Vliet et al. 2001), but even in fetal sheep (Brace and Moore 1991). In healthy human subjects, BP falls by 10% or more during sleep, and this has been defined as "dipping" of BP (Birkenhager and van den Meiracker 2007). In both human and animal studies, a "non-dipping" BP profile has been identified as an independent risk factor for the development of cardiovascular events and target organ damage, including an increased risk of left ventricular hypertrophy, carotid artery plaques, stroke, cognitive impairment, nephropathy and retinopathy (Roman, Pickering et al. 1997; Mancia and Parati 2003; Verdecchia and Angeli 2005; Izzedine, Launay-Vacher et al. 2006). These findings have led to an interest in chronotherapies for the management of high BP. However, a complete understanding of the significance of daily BP variations will require considerable additional research, including the use of appropriate animal models to investigate mechanisms contributing to the non-dipping BP phenomenon.

Swine are widely used in cardiovascular research. However, despite the growing use of sophisticated methods such as radio-telemetry for the continuous monitoring of BP in unrestrained animals, to our knowledge, a comprehensive description of the 24h BP profile in swine has not been reported. Therefore, the initial objective of this study was to

characterize the 24h profile of BP and activity in healthy Yucatan miniature swine under normal conditions. During this process we found that these pigs demonstrated a complete absence of BP dipping despite clear evidence of diurnal variations in locomotor activity and heart rate. Therefore, we conducted additional analyses of the impact of locomotor activity on BP and HR, and confirmed our main observations in swine fed a high salt diet for 7 days and in an additional set of pigs fed a high-salt-fat-sugar diet for 11 months.

3.3 MATERIALS AND METHODS

3.3.1 Animals and study protocol:

The Institutional Animal Care Committee at Memorial University of Newfoundland approved all procedures and protocols used in the study, and all animals were treated in accordance with guidelines set by the Canadian Council of Animal Care. Twelve Yucatan miniature pigs (6 females and 6 non-castrated males) from the Memorial University swineherd were studied. At 4 weeks old, the pigs were weaned from the sow and half of the pigs (3 females and 3 males) were fed a standard pig grower diet (Eastern Farmers Co-op, St. John's, NL - providing 12% of their caloric intake from fat, 67% from carbohydrate and 21% from protein – see **Chapter 2, Table 2.1** for the diet composition), while group-housed with two of their siblings of the same sex (these siblings were not used in the present study – see **Chapter 2 , Figure 2.1** for project outline). However, throughout the study, each pig was fed separately for 5 h *ad libitum* daily and intakes were recorded. All animals had 24-h *ad libitum* water access and were maintained on a 12-h day-night cycle (0700 – 1900h). At ~8.5-9 mo old (mean 8.7 mo

old), with average weights of 69 ± 6 kg, the animals underwent surgery for implantation of a telemeter and blood sampling catheters. The second group of six pigs (3 females and 3 males) followed the same protocol as outlined above, except that these pigs were fed a high-salt-fat-sugar (HSFS) diet immediately at weaning and underwent surgery at ~10.5-11 mo old (mean 10.9 mo old). This HSFS diet was prepared by the addition of salt (40g/kg), hydrogenated margarine (50 g/kg; Central Dairies, St. John's, NL), lard (150 g/kg; Loblaw Inc., Toronto, ON) and granulated sugar (100 g/kg; Lantic, Montreal, QC) to ground pig diet, thus, providing 50% of caloric intake from fat, 40% from carbohydrate and 10% from protein (see **Chapter 2, Table 2.1** for the diet composition).

3.3.2 Telemetry and catheter implantation

Anaesthesia was induced with ketamine (Ketalean, Bimeda-MTC, Cambridge, Canada; 22 mg/kg *i.m.*) and xylazine (Rompum, Bayer, Toronto, Canada; 2 mg/kg *i.m.*) and was maintained with 1.0- 1.5% halothane and a 3:2 mixture of oxygen and nitrous oxide. The body of the BP telemeter (TA11PA-D70; Data Sciences International, St. Paul, MN) was implanted in a subcutaneous pouch over the inner thigh of the left hindlimb with the telemeter catheter inserted 10 cm into the femoral artery. Because this investigation was part of a larger series of studies, two blood sampling catheters (Tygon® tubing, Norton Performance Plastics, Akron, OH) were also inserted in the left femoral vein and advanced to the inferior vena cava; the catheters were tunnelled under the skin and exteriorized between the shoulder blades. To reduce risk of infection, pigs were given 0.07 mL/kg *i.v.* trimethoprim (40 mg/mL)/ sulfadoxine (20 mg/mL) (Borgal, Intervet Canada Ltd., Whitby, ON) during surgery and the first 2 days post surgery. To alleviate

pain, animals were given 300 μg buprenorphrine hydrochloride (Temgesic, Schering-Plough Ltd., Hertfordshire, UK) immediately after surgery and the next day.

3.3.3 Telemetry system

The BP telemeter signal was received by a network of six receivers (RLA1020, Data Sciences International, St. Paul, MN) positioned three on either side of the holding pen (1.9 m L x 1.2 m W x 2.0 m H). The strongest signal was selected by a consolidation matrix (RMX10, Data Sciences International, St. Paul, MN) and passed to an analog adapter (R11CPA, Data Sciences International, St. Paul, MN), which provided a calibrated voltage output after correcting for atmospheric pressure using an ambient pressure monitor (APR-1, Data Sciences International, St. Paul, MN). The calibrated signal was then recorded using a computerized data acquisition system and processed as previously described (Montani, Mizelle et al. 1995). Pressure calibrations of the telemeters were assessed before implantation and after removal, and corrections were made to the data for any deviation from the original factory calibration (Van Vliet, Chafe et al. 2000). The BP signal was sampled for 10 s at 30-s intervals, and the levels of systolic (SAP), diastolic (DAP), and mean (MAP) arterial BP and heart rate (HR) over this sample period were computed and stored for offline analysis.

3.3.4 Locomotor activity

Locomotor activity (i.e. body movement) was obtained by the telemetry system by monitoring changes in the received signal strength, which occur upon movement of the animal within the pen. Changes in signal strength of more than a predetermined amount

generated a digital pulse, which was counted by the data acquisition software system. One digital pulse was generated each time the signal level changes. It is important to note that in this protocol, the transmitter had to move, implying that simple head movement or body twitch by the animal would not be registered as activity. Specifically, because of the location of the implantation of the telemeter in the left thigh flank, to generate activity signal the animal had to stand up and walk. Also, the detection ability of the receivers allowed for collection of radio-frequency signals in the enclosed pen without any signal loss by environmental factors or position of the animal. Thus, locomotor activity data were derived from the activity signal reported by the telemetry equipment and were expressed in arbitrary units, (a.u.) representing the number of events, with the value 0 corresponding with inactivity.

3.3.5 Experiment protocols

3.3.5.1 Experiment 1:

An initial group of 6 pigs was housed individually and allowed at least 8 d of recovery following surgery before BP recordings were made on the standard pig diet. There were at least 48 h continuous BP recordings at 30 s intervals, while on the standard pig diet (0.5% NaCl). Pigs were maintained on a 12-h light-dark cycle (light from 0700 to 1900 h). The recording room had a radio turned on at a low volume to reduce the impact of environmental noise. SAP, DAP, MAP, pulse pressure (PP), HR and locomotor activity were recorded in each animal.

3.3.5.2 Experiment 2:

In response to the findings from Experiment 1, the same pigs were placed on a high salt (4.5% NaCl) diet (regular diet was ground, moistened, supplemented with NaCl and then re-dried) for 7 d, with haemodynamic data collected during the last 48 h. At the end of the salt-challenge recording period, the pigs were placed back onto the regular diet for 5-7 d and then killed using an intravenous infusion of sodium pentobarbital.

3.3.5.3 Experiment 3 and 4:

Experiment 3: The protocol in experiment 1 was repeated in a second group of pigs that had been fed the HSFS diet from weaning (i.e., 1 mo of age). After recording haemodynamic and activity measurements on the HSFS diet (containing 4.5% NaCl), pigs were challenged with a lower salt (0.5% NaCl) version of the HSFS diet for 7 d with BP recorded during the last 48 h. **Experiment 4:** Similarly to experiment 2 above, the pigs were placed back on the HSFS (4.5% NaCl) diet for 5-7 d, and then killed using an intravenous infusion of sodium pentobarbital.

3.3.6 Data analysis

A modified version of the Microsoft Excel 2000® template (HdStats, see www.med.mun.ca/Medicine/Faculty/Van-Vliet,-Bruce.aspx for link to download) was used for routine analysis of 24-h telemetry data sets. In addition to calculating means of the 12 h daytime and 12 h nighttime periods, we computed several indices of locomotor activity. These included: 1) the mean value of the raw activity signal (arbitrary units), 2) the mean value of activity for values of activity > 0 (arbitrary units; an index of the

intensity of activity, when active), 3) the mean of the logarithm of activity values >0 (similar to #2 above, but log transformed to normalize the otherwise highly skewed distribution of values), and 4) inactive time, calculated as the percentage of raw activity values =0. Furthermore, we computed the mean values for BP and HR associated with sustained periods of activity and inactivity. For these calculations, we only included data for which the activity signal was >0, for at least three consecutive samples (sustained activity), or = 0 for at least three consecutive samples (sustained inactivity).

3.3.7 Statistical analysis

Values are expressed as mean ± SD, unless otherwise stated. Statistical comparisons were made by paired t-tests using Prism 4 (GraphPad Software Inc., CA). A value of $P \leq 0.05$ was considered significant.

3.4 RESULTS

3.4.1 Circadian variation in blood pressure, heart rate, and locomotor activity

As illustrated in Figure 3.1 and Figure 3.2 Yucatan miniature pigs exhibited a prominent circadian variation in both HR and locomotor activity, both variables being significantly lower during the dark phase of the 24-h period (Table 3.1). HR and activity tended to rise and fall in parallel, and were highly correlated; for instance, using the same data as shown in Figure 3.2, correlation of 24-h HR data to activity levels indicated R = 0.70 with

P<0.0001, when the pigs were fed regular diet (0.5% NaCl). In contrast, dipping of BP was entirely absent with BP values actually tending to be slightly increased during the dark phase of the 24-h cycle (**Table 3.1, Figure 3.3**). Although correlations of BP with HR were not significant, (R = 0.07, P = 0.62), BP was found to be significantly inversely correlated with activity level (R = -0.41, P = 0.003). Therefore, we subsequently explored the relationship between BP and activity in greater detail (see below).

The pigs appeared to be moderately salt sensitive as indicated by an 8 - 9% increase in BP when the salt content of the regular diet was increased from 0.5% to 4.5% (P < 0.05, **Table 3.1**). However, the high salt regular diet did not appear to affect either the pattern or time course of diurnal variation of BP or HR variation, and BP dipping remained absent (**Table 3.1**).

In order to verify the absence of dipping in other conditions (i.e., unhealthy subjects), we repeated these analyses in an additional group of pigs fed a HSFS diet since weaning (**Table 3.2**). Consistent with our earlier findings, dipping of BP was also absent in pigs on the HSFS diet, and remained absent (P = 0.88) when switched to a lower salt version of the HSFS diet (**Table 3.2**). As with the previous set of pigs, the absence of dipping occurred despite quite clear and significant dark-light differences in HR and locomotor activity (**Table 3.2**).

3.4.2 Effects of activity on blood pressure and heart rate

To help clarify the causes underlying the absence of dipping of BP in Yucatan miniature swine, we assessed the levels of BP and HR associated with sustained periods of activity

(raw activity >0) and inactivity (raw activity =0). As shown in Table 3.3 periods of locomotor activity were associated with significantly elevated HR, as expected. However, BP did not increase during periods of activity, instead, it decreased significantly. This fall in BP with activity was particularly pronounced and significant for all three BP indices (systolic, mean, and diastolic) during the light phase of the day (**Table 3.3**).

Table 3.1. Basic haemodynamic and activity data for Yucatan miniature pigs on normal salt (0.5%) and high salt (4.5%) versions of the regular grower pig diet.

Period	Variable	Regular diet (Normal salt)	Regular diet (High salt)	P value
Light period	SAP (mm Hg)	130.3 ± 8.4	141.9 ± 9.4	0.014
	MAP (mm Hg)	109.9 ± 6.9	118.9 ± 8.0	0.027
	DAP (mm Hg)	91.3 ± 6.8	99.1 ± 7.9	0.029
	PP (mm Hg)	39.0 ± 7.1	42.8 ± 8.9	ns (0.118)
	HR (beats min^{-1})	89.5 ± 3.7	84.4 ± 8.9	ns (0.250)
	Inactive time (%)	70.9 ± 9.9	69.1 ± 6.0	ns (0.716)
	Mean activity (a.u.)	2.6 ± 1.6	3.0 ± 1.3	ns (0.611)
	Mean (activity >0) (a.u.)	23.6 ± 7.1	25.2 ± 8.3	ns (0.693)
	Mean log (activity > 0)	1.27 ± 0.10	1.26 ± 0.14	ns (0.892)

Dark period	SAP (mm Hg)	134.1 ± 7.3	146.3 ± 8.6	<0.0001
	MAP (mm Hg)	112.7 ± 3.8	122.6 ± 4.0	<0.0001
	DAP (mm Hg)	93.5 ± 3.2	101.6 ± 3.8	0.001
	PP (mm Hg)	40.6 ± 7.8	44.6 ± 9.4	ns (0.125)
	HR (beats min^{-1})	82.3 ± 9.1	75.7 ± 5.6	ns (0.143)
	Inactive time (%)	93.5 ± 5.4	94.2 ± 2.2	ns (0.659)
	Mean activity (a.u.)	0.5 ± 0.4	0.5 ± 0.2	ns (0.887)
	Mean (activity >0) (a.u.)	17.1 ± 7.9	20.5 ± 7.6	ns (0.512)
	Mean log (activity > 0)	1.17 ± 0.21	1.25 ± 0.13	ns (0.473)
Dark-light difference	SAP (mm Hg)	3.8 ± 6.8	4.4 ± 5.9	ns (0.813)
	MAP (mm Hg)	2.8 ± 5.6	3.7 ± 4.8	ns (0.739)
	DAP (mm Hg)	2.1 ± 6.1	2.5 ± 4.6	ns (0.889)
	PP (mm Hg)	1.6 ± 1.5	1.9 ± 3.5	ns (0.860)
	HR (beats min^{-1})	-7.2 ± 6.7*	-8.7 ± 5.0*	ns (0.672)

a.u., arbitrary units; DAP: diastolic pressure; HR: heart rate; MAP: mean arterial pressure; PP: pulse pressure; SAP: systolic arterial pressure. Each value represents the mean ± SD in n = 6 pigs. Significant difference between dietary treatments were assessed by paired t-test, $P<0.05$, ns = not significant. *Within diet, the dark-light difference for HR was found to be significant (P = 0.05).

Table 3.2. Basic haemodynamic and activity data for Yucatan miniature pigs on High-Salt-Fat-Sugar (HSFS) diet: normal salt (0.5%) and high salt (4.5%) versions.

Period	Variable	HSFS diet Normal salt	HSFS diet High salt	P value
Light period	SAP (mm Hg)	146.3 ± 9.7	157.0 ± 17.0	ns (0.055)
	MAP (mm Hg)	116.3 ± 8.7	126.8 ± 16.1	0.036
	DAP (mm Hg)	91.7 ± 8.3	100.5 ± 15.4	ns (0.058)
	PP (mm Hg)	54.7 ± 5.0	56.5 ± 4.0	ns (0.446)
	HR (beats min^{-1})	91.2 ± 6.4	94.7 ± 3.3	ns (0.126)
	Inactive time (%)	71.4 ± 7.1	67.8 ± 9.2	ns (0.433)
	Mean activity (a.u.)	2.5 ± 0.7	3.2 ± 2.0	ns (0.369)
	Mean (activity >0) (a.u.)	23.5 ± 5.6	25.3 ± 4.9	ns (0.545)
	Mean log (activity > 0)	1.25 ± 0.08	1.29 ± 0.07	ns (0.295)
Dark period	SAP (mm Hg)	146.2 ± 6.7	154.8 ± 11.3	0.048
	MAP (mm Hg)	116.0 ± 7.4	123.9 ± 10.9	ns (0.067)
	DAP (mm Hg)	91.6 ± 8.6	97.3 ± 11.5	ns (0.163)
	PP (mm Hg)	54.7 ± 6.3	57.5 ± 4.5	ns (0.078)
	HR (beats min^{-1})	86.3 ± 9.8	86.3 ± 5.3	ns (0.998)
	Inactive time (%)	92.6 ± 4.9	91.1 ± 4.7	ns (0.667)
	Mean activity (a.u.)	0.6 ± 0.6	0.9 ± 0.6	ns (0.546)

	Mean (activity >0) (a.u.)	16.4 ± 6.7	20.2 ± 9.2	ns (0.392)
	Mean log (activity > 0)	1.15 ± 0.17	1.21 ± 0.14	ns (0.475)
Dark-light difference	SAP (mm Hg)	-0.1 ± 5.3	-2.2 ± 10.0	ns (0.605)
	MAP (mm Hg)	-0.3 ± 4.4	-2.9 ± 9.2	ns (0.488)
	DAP (mm Hg)	-0.1 ± 4.4	-3.2 ± 8.0	ns (0.362)
	PP (mm Hg)	0.0 ± 4.1	1.0 ± 2.6	ns (0.531)
	HR (beats min^{-1})	-4.9 ± 5.7	-8.4 ± 3.7	ns (0.335)

a.u., arbitrary units; DAP: diastolic pressure; HR: heart rate; MAP: mean arterial pressure; PP: pulse pressure; SAP: systolic arterial pressure. Each value represents the mean ± SD in n = 6 pigs. Significant difference between dietary treatments were assessed by paired t-test, $P<0.05$, ns = not significant. *Within diet, the dark-light difference for HR was found to be significant ($P = 0.003$) on the 4.5% NaCl version of the HSFS diet, but only a trend ($P= 0.09$) towards significant on the 0.5% NaCl version of the diet.

Table 3.3 Basic haemodynamic data for Yucatan miniature pigs during activity or inactivity on regular grower pig diet (0.5% salt)

Period	Variable	Activity = 0	Activity > 0	P value
Light period	SAP (mm Hg)	132.6 ± 7.4	125.5 ± 10.5	0.005
	MAP (mm Hg)	111.9 ± 6.4	103.9 ± 9.5	0.031
	DAP (mm Hg)	93.0 ± 6.7	86.6 ± 7.2	0.021
	PP (mm Hg)	39.6 ± 7.2	38.8 ± 5.0	ns (0.608)
	HR (beats min^{-1})	84.5 ± 3.5	115.7 ± 14.6	0.002
Dark period	SAP (mm Hg)	134.4 ± 7.8	127.0 ± 5.0	0.037
	MAP (mm Hg)	112.8 ± 4.1	109.3 ± 4.2	ns (0.221)
	DAP (mm Hg)	93.4 ± 3.3	92.7 ± 7.6	ns (0.852)
	PP (mm Hg)	41.0 ± 7.9	34.2 ± 10.4	0.030
	HR (beats min^{-1})	80.2 ± 6.6	117.4 ± 19.2	0.001

Inactivity and activity were measured as sustained in/activity; DAP: diastolic pressure; HR: heart rate; MAP: mean arterial pressure; PP: pulse pressure; SAP: systolic arterial pressure. Each value represents the mean ± SD in n= 6 pigs. Significant difference between dietary treatments were assessed by paired t-test, $P<0.05$, ns = not significant.

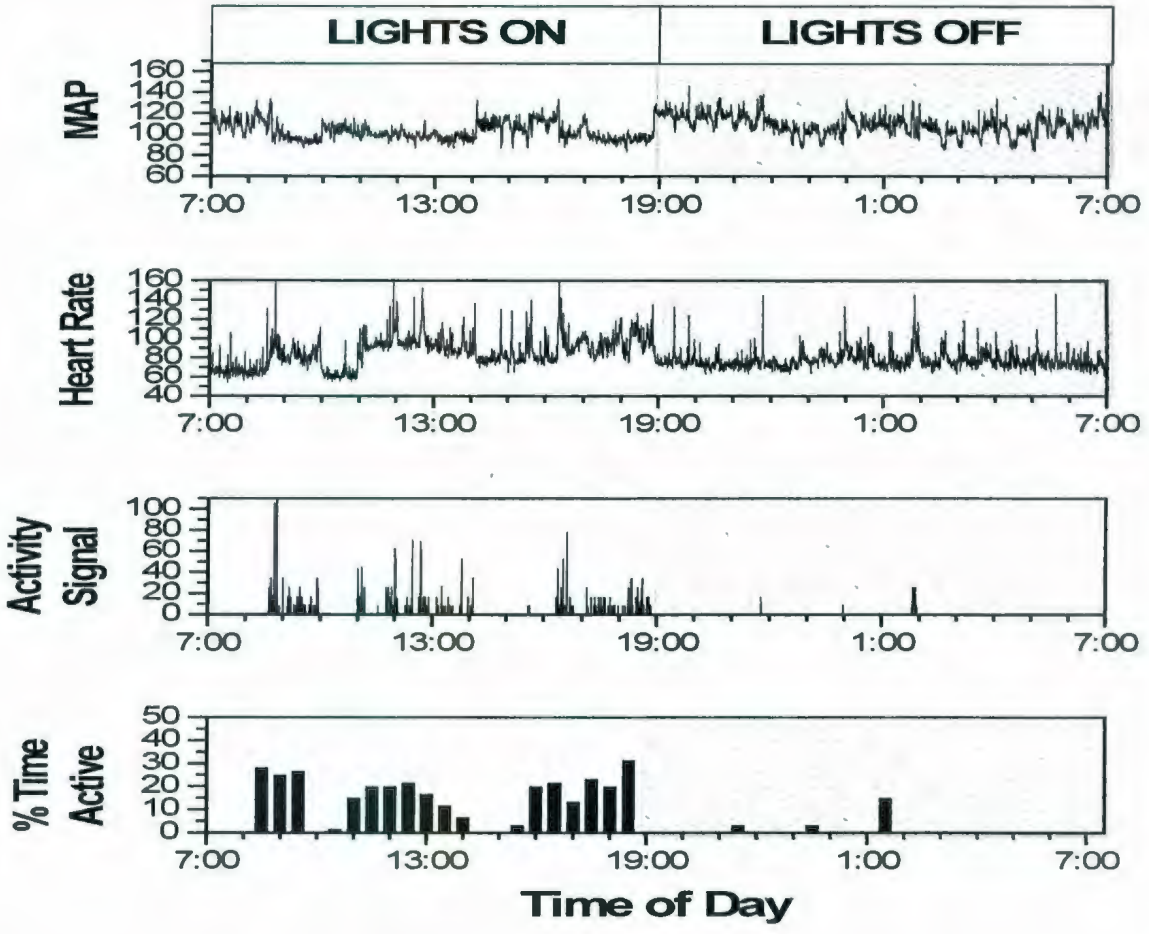

Figure 3.1. Representative 24-h recordings of MAP, HR, and locomotor activity in an individual Yucatan miniature swine on standard pig diet.

Day represented by the un-shaded section of graphs, where light came on at 0700 h and off at 1900 h. Night represented by the shaded section of graphs, from 1900 h to 0700 h. Activity signal (arbitrary unit); Heart rate = HR (beats/min); MAP = Mean arterial pressure (mmHg); % Time Active = percent time spent active. This animal

represents the median level of 24 h MAP, HR, activity and time active for animals on the standard pig diet.

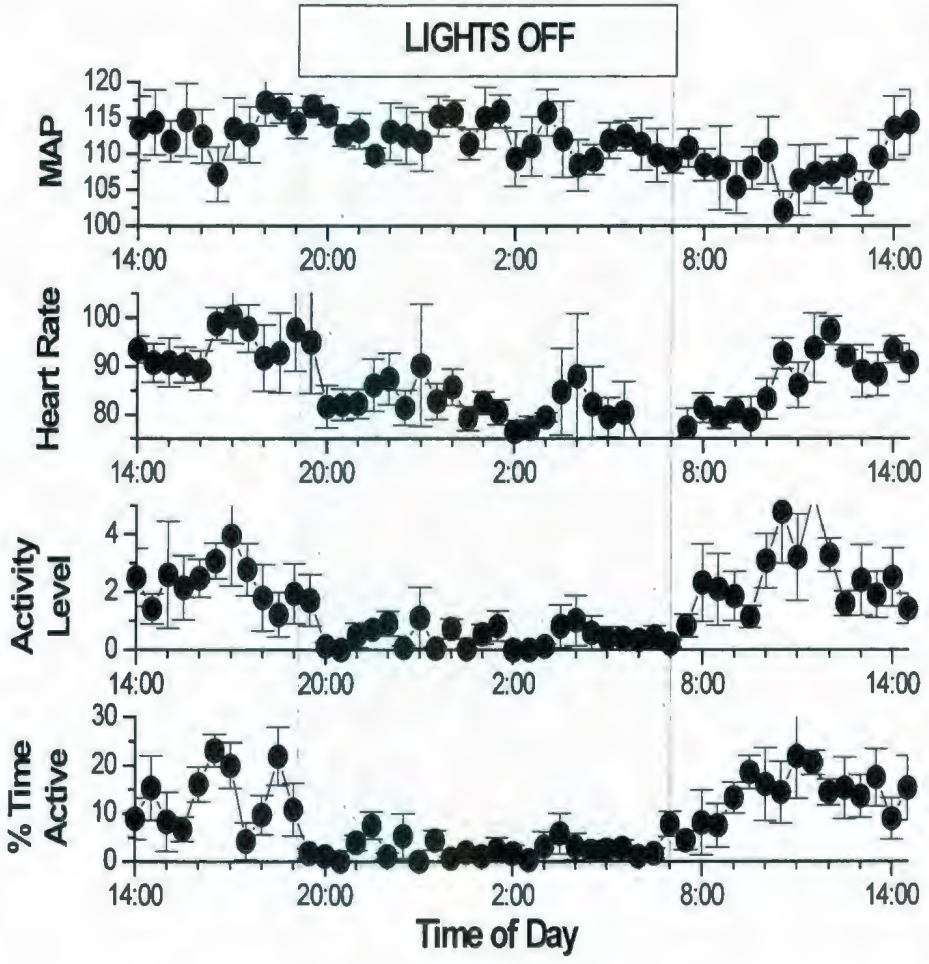

Figure 3.2. Mean 24-h variations in MAP, HR and locomotor activity in pigs on standard pig diet.

Data were plotted over a 24-h period at half-hourly intervals. Activity level (arbitrary unit); Heart rate = HR (beats/min); MAP = Mean arterial pressure (mmHg); % Time Active = percent time spent active. Mean values for the group are indicated by black

filled circles. Day represented by the un-shaded section of graphs, where light came on at 0700 h and off at 1900 h. Night represented by the shaded section of graphs, from 1900 h to 0700 h. Regular = standard grower pig diet (0.5% salt). N = 6 pigs per diet treatment. Error bars represent SEM.

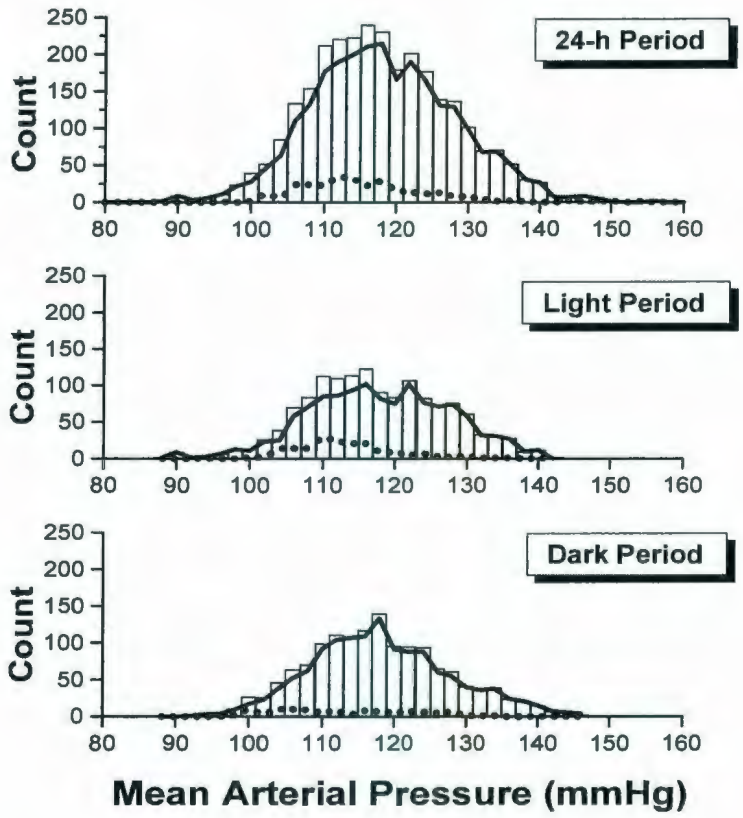

Figure 3.3 Representative distributions of the mean arterial pressure in an individual Yucatan miniature swine on standard pig diet.

The histogram shows the total number of samples occurring within 2 mm Hg bins during the 24 h (top) or 12 h (middle and bottom) periods. The dotted and continuous lines indicate the number of samples recorded with or without concurrent locomotor activity, respectively. Light period = daytime from 0700 h to 1900 h. Dark period = nighttime from 1900 h to 0700 h. This animal represents the median level of 24 h MAP on the standard pig diet.

3.5 DISCUSSION

The results demonstrate that nocturnal dipping of BP is absent in Yucatan miniature swine. This is in contrast to the findings from a number of other mammalian species (van den Buuse 1994; Mishina, Watanabe et al. 1999; Antic, Van Vliet et al. 2001; Van Vliet, Chafe et al. 2003; Mishina, Watanabe et al. 2006). In these pigs, though not significant, the BP values tended to be slightly higher during the nighttime period in comparison to the day period. More importantly, the absence of BP dipping occurred despite the presence of clear diurnal variation in heart rate and locomotor activity. Thus, Yucatan miniature swine represents one of a very short list of animal models in which a selective non-dipping of BP has been clearly documented. Other similar models include the transgenic hypertensive rat (TGR[mREN-2]27) (Lemmer, Mattes et al. 1993; Witte and Lemmer 1999; Lemmer, Witte et al. 2003)and the Fischer 344 normotensive rat strain (Basset, Laude et al. 2004). Together, these examples may represent a resource for investigating mechanisms underlying non-dipping of BP and for testing potential therapeutics to normalize the diurnal variation of BP.

This absence of the circadian pattern of BP without a disturbance in circadian pattern of HR clearly illustrates that the circadian regulation of BP differs from that of HR. The cause for this dissociation in regulation of BP and HR is still not fully understood. Environmental factors such as physical activity as well as sympathetic nervous system activity are thought to be important factors influencing circadian BP variations (Teerlink and Clozel 1993; van den Buuse 1994; Makino, Hayashi et al. 1997; Pickering and Kario 2001). For example, physical inactivity has been proposed as a factor to explain non-

dipping nocturnal BP (Kario, Schwartz et al. 1999; O'Shea and Murphy 2000). In our study, locomotor activity patterns displayed the expected circadian rhythm; the highest frequency and intensity of activity was observed during the daytime when the lights were on and the animals were feeding (Figure 3.1), and activity was synchronized with circadian pattern of HR. In other words, there was a noticeable dipping of activity, which could be matched to dipping of HR, but not BP (Figure 3.2). As evident in Figure 3.1 and summarized in Table 3.2, periods of physical activity in Yucatan swine were associated with decreases in BP values, in contrast with the increase in BP that has generally been associated with periods of activity in humans and other animal models (Mishina, Watanabe et al. 1999; Van Vliet, Chafe et al. 2003; Cavelaars, Tulen et al. 2004; Mishina, Watanabe et al. 2006). Thus, it would appear that a lack of BP dipping in this model is associated with, and may be partly due to, an unusual BP response to periods of locomotor activity. The pigs on the regular diet spent on average less than 15% (i.e., 13.7 ± 5.8%) of their time engaged in any locomotor activity. Thus, this low activity could contribute to the lack of diurnal dipping BP; however, if the pigs were more active, based on the observed significant inverse relationship between these parameters, we would predict that the daytime BP would be even lower overall, demonstrating a reversed circadian rhythm compared to most species. However, additional studies are required to fully test this hypothesis because this study only measured spontaneous activity, that is, the animals were not subjected to any test related to maximal physical activity capacity.

Heightened activation of sympathetic activity is known to be associated with elevation of BP, and some researchers have suggested that a non-dipping BP profile could be due to a

failure to withdraw sympathetic tone during nighttime (Hojo, Noma et al. 1997; Ragot, Herpin et al. 1999; Sherwood, Steffen et al. 2002; Kanbay, Turgut et al. 2008; Okamoto, Gamboa et al. 2009). Although our study was not designed to assess this possibility, our results suggest that the situation may be somewhat different in Yucatan miniature swine. Our data show that the BP in the miniature swine failed to rise during periods of locomotor activity, despite clear increases in heart rate. Thus, this raises the possibility that the lack of BP dipping in this animal model could be due to a lack of an elevation of BP during daytime periods of arousal and activity, rather than the absence of a nocturnal fall in BP.

There is also evidence in humans and animals suggesting that issues related to salt and water balance may contribute to a non-dipping BP profile. Salt sensitivity BP has been found to be more prevalent in non-dippers (Uzu, Ishikawa et al. 1997; Uzu, Fujii et al. 1999; Sachdeva and Weder 2006) and in some subjects, the presence or absence of BP dipping has been shown to be related to the time course of nocturnal fluid volume changes (Uzu and Kimura 2000; Fukuda, Mizuno et al. 2008). In the present study, animals had a non-dipping BP profile on a regular salt diet and we demonstrated that acute high salt exposure significantly increased BP, indicating a moderate degree of salt sensitivity in Yucatan miniature swine (Table 3.1). However, changes in the level of salt intake did not influence the pattern (Table 3.1 and Table 3.3) or time course (data not shown) of diurnal variation of BP in Yucatan swine, similar to findings in the spontaneously hypertensive rat (Calhoun, Zhu et al. 1994; Basset, Laude et al. 2004). Thus, as suggested by those researchers (Calhoun, Zhu et al. 1994; Basset, Laude et al.

2004), the preservation of BP and HR pattern during regular and high dietary salt ingestion suggests that dietary salt supplementation alone does not seem to disrupt the neural mechanisms underlying the circadian rhythm of BP and HR.

In other animal models, feeding a standard diet and then switching to a high-fat diet typically alters the circadian rhythms of both BP and HR from dipping to non-dipping profiles (Antic, Van Vliet et al. 2001; Carroll, Thaden et al. 2005). In this swine model, we observed the same patterns of non-dipping BP profiles and dipping HR profiles, regardless of whether the animals were on regular or high-fat diets. Thus, this observation would suggest that the fat content of the diet is not the initiating factor for dipping BP, nor did it appear to worsen the non-dipping profile. Notably, when pigs were fed HSFS diets and then switched to a low salt version of the same diet, most haemodynamic parameters tended to decrease ($P<0.10$) in both daytime and nighttime periods. Therefore, these findings further support our conclusion that Yucatan miniature swine are moderately salt sensitive but they lack BP dipping regardless of whether they are on a standard or high salt diet.

In conclusion, the data in this study show that in this Yucatan miniature swine model, there is a blunting of circadian BP rhythm without disturbances of circadian HR and locomotor activity rhythms, suggesting different regulatory mechanisms. While the underlying mechanisms for the absence of BP dipping in this model are unknown, the absence of dipping is associated with a lack of increase in BP during periods of activity, which may indicate an abnormal cardiovascular or autonomic reactivity to periods of activity and sympathetic arousal. This swine model represents one of a small number of

animal models which may be useful for evaluating the mechanisms and consequences of a non-dipping BP profile. .

Developmental Origins of Cardiovascular Disease in Yucatan Miniature Pigs

CHAPTER 4 *Reduced Nephron Numbers in the Low Birth Weight Yucatan Miniature Pig is Associated with Elevated Blood Pressure in Adulthood*

CHAPTER 5 *Low Birth Weight Yucatan Miniature Swine that Experience Early Catch-up Growth have Impaired Lipid Metabolism as Adults*

CHAPTER 4. REDUCED NEPHRON NUMBERS IN THE LOW BIRTH WEIGHT YUCATAN MINIATURE PIG IS ASSOCIATED WITH ELEVATED BLOOD PRESSURE IN ADULTHOOD

4.1 ABSTRACT

Background and Objectives: Epidemiological and animal studies have reported higher blood pressure (BP) during adolescence and adulthood in individuals of smaller birth weight. Reduced nephron number due to impaired nephrogenesis is one of the most studied theories to explain early programming of later BP. The objectives of this study were to evaluate the effects of birth weight on nephron number and BP parameters in the Yucatan miniature pig. Additionally, we investigated whether birth weight was related to acute salt sensitivity of BP in this swine model.

Methods: Each runt piglet (<850 g) (n=6) was paired with the largest same sex littermate (>1100 g) (n=6) from the same litter and taken from the 6 sows at 3 d of age. After 4 weeks of *ad libitum* milk replacer, pigs were fed standard diet *ad libitum* for 5 h/d. Throughout the study, body weights and feed intake were recorded, and monthly fasted blood samples were taken to measure plasma creatinine and urea nitrogen concentrations. From 4 to 8 mo of age, BP was measured by the non-invasive blood pressure (NIBP) technique using an automated oscillometric device (Surgivet V6004) via a cuff affixed to the pig's hind lower hind leg while restrained in a V-trough. At 9 mo of

age, BP radio-telemeters were implanted via the femoral artery to measure continuous BP, heart rate and locomotor activity in conscious, unrestrained pigs when fed standard diet (0.5% NaCl) followed by measurements after a 7-d salt challenge (4.5% NaCl). At 10 mos old, kidneys were removed and nephron numbers and average glomerular size were determined using unbiased stereological techniques.

Results: Prior to sexual maturity (i.e., 0-4 mo), the runts had greater ($P < 0.05$) feed intake-per body weight and experienced significant catch-up growth. From 4 to 8 mo of age, NIBP measurements remained more elevated in the runts relative to the large littermates, with a steady increase in BP in all pigs with age. Using telemetry, runts had higher diastolic BP, as young adults (10 mo old) compared to their larger siblings (SAP: 140.8 ± 7.8 vs. 134.2 ± 5.3, $P=0.08$; DAP: 93.8 ± 5.5 vs. 90.0 ± 8.7, $P = 0.05$, respectively). Increased BP was partly due to fewer nephrons per kidney in runts compared to their larger siblings ($296{,}084 \pm 70{,}218$ vs. $518{,}330 \pm 166{,}812$, $P < 0.05$); but hyperfiltration of nephrons was not evident, as there was no enlargement of glomeruli in the runts ($P > 0.05$). An acute high salt (4.5% NaCl) intake led to higher ($P < 0.05$) BP values in both groups; however, runts did not show enhanced salt sensitivity relative to the large littermates ($P > 0.05$).

Conclusion: Naturally occurring small birth weight Yucatan miniature swine have elevated BP later in life, which was strongly correlated to their nephron number. However, glomeruli morphology and salt sensitivity was not altered by birth weight.

4.2 INTRODUCTION

Within the last two decades, epidemiological and animal studies have demonstrated the impact on long-term health resulting from fetal adaptation to a poor intrauterine environment (Barker, Osmond et al. 1989; Hales and Barker 1992; Alexander 2003; McMillen and Robinson 2005). In terms of hypertension, studies have reported an inverse relationship between fetal growth and BP in adulthood, and this concept has been documented in induced (Dodic, May et al. 1998; Brawley, Itoh et al. 2003) and naturally occurring (Poore, Forhead et al. 2002; Woods and Weeks 2004) animal models of intrauterine growth retardation (IUGR). Although, the fetal/early programming mechanisms resulting in the development of hypertension in later life have not been fully elucidated, the kidneys likely play a crucial role (Hughson, Farris et al. 2003; Adair and Dahly 2005; McMillen and Robinson 2005; Bagby 2007; Schreuder and Nauta 2007). Evidence suggests that a nutritionally poor intrauterine environment leads to impaired nephrogenesis, resulting in a reduction in the number of glomeruli (i.e., reduced nephron endowment), which in part may lead to various alterations in renal functions, with subsequent development of hypertension later in life (Hughson, Farris et al. 2003; Vehaskari and Woods 2005; Schreuder and Nauta 2007; Vehaskari 2007).

There are various proposed mechanisms relating congenital low nephron numbers in low birth weight offspring to altered renal development and elevated BP later in life (Brenner and Chertow 1994; Adair and Dahly 2005; Bagby 2007; Schreuder and Nauta 2007). For instance, Brenner's hypothesis (Brenner, Garcia et al. 1988; Brenner and Chertow 1994; Mackenzie, Lawler et al. 1996) suggests that reduced renal mass (i.e., reduced nephron

number) leads to decreased filtration of salt (NaCl), resulting in increased extracellular volume, and consequently increased blood volume and systemic BP. Moreover, Brenner's hypothesis proposes that over time, reduced nephron number (i.e., reduced glomeruli) will also contribute to elevated BP through renal damage. Reduced nephron endowment initially leads to glomerular hyperfiltration, in order to maintain adequate renal clearance; but with age, this hyperfiltration leads to subsequent glomerular enlargement, resulting in glomerular injury and systemic hypertension with further reduction in nephron numbers. The salt retention ("tubular theory") and the hyperfiltration ("glomerular theory") events may not be mutually exclusive, thus both could contribute to the development of elevated BP in the low birth weight offspring later in life.

In addition to the fetal environment, postnatal factors, such as diet and early growth rate (i.e., accelerated catch-up growth following low birth weight), are believed to be just as important for the manifestation of adult hypertension in low birth weight offspring (Huxley, Shiell et al. 2000; Law, Shiell et al. 2002; Morris, Velkoska et al. 2005; Bagby 2007; Lurbe, Garcia-Vicent et al. 2007; Singhal, Cole et al. 2007). A reduced nephron number, however, may not be enough to confer hypertension later in life. Possibly, the programming of a postnatal increase in appetite leads to accelerated postnatal growth, resulting in excess body mass which becomes superimposed on reduced nephron number and creates an imbalance between excretory load (body mass) and excretory capacity (nephron number); these two factors may therefore be required for the actualization of adult hypertension in the low birth weight offspring (Bagby 2007; Griffin, Kramer et al. 2008).

The main objectives of the present study were to determine whether naturally occurring IUGR Yucatan miniature pigs had increased BP values later in life compared to their normal same-sex littermates, and, if so, to examine the possible role of the kidneys in the development of the elevated BP. We also investigated whether the responsiveness to an acute high salt challenge is affected by IUGR.

4.3 MATERIALS AND METHODS

All animal procedures were approved by Memorial University of Newfoundland's Institutional Animal Care Committee, and carried out in accordance with guidelines set by the Canadian Council of Animal Care.

4.3.1 Animals and study protocol:

Six pairs of runt and large littermates (3 female and 3 male pairs – see **Chapter 2, Figure 2.1**) of Yucatan miniature pigs from six sows from the Memorial University of Newfoundland swineherd were studied (average litter size was 7 ± 1). One day after a sow gave birth, the entire litter was weighed. The mean birth weight of all piglets born in the 6 litters used in this study was 0.92 ± 0.08 kg (average body weight per litter ranged from 0.83 to 1.03 kg). Within each litter, a runt piglet was defined as a piglet weighing less than 850 g. A same sex larger littermate weighing at least 300 g more than the runt was chosen as a littermate control. The runt and the larger same sex littermate were taken from the sow at 3-d-old. The pair was housed together, but individually provided with rehydrated sow milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) eight to ten times daily *ad libitum*, with all intakes recorded. The piglets were housed in pens

containing straw bedding and infrared heat lamps. Pigs were weaned at 4 wk old and siblings remained group-housed throughout the trial; however, each pig was fed separately for 5 h (1200-1700 h) during which time the diet was freely available. Daily feed intakes were recorded. The pigs were fed standard pelleted grower pig diet (Eastern Farmers Co-op, St. John's, NL, Canada) containing typical levels of protein (21%), and NaCl (0.5%). All animals had 24 h *ad libitum* water access and were maintained on a 12 h day-night cycle (lights on 0700 – 1900 h).

4.3.2 Swine body measurements:

Serial growth measurements were taken throughout the study. During the milk-feeding phase (birth to 1-mo-old), body weight, snout to rump length, and abdominal circumference were measured at least twice weekly; thereafter, until the end of the study, these measurements were made bimonthly.

4.3.3 Biochemical analyses:

From 1 to 9 mo of age, monthly blood samples were collected from all animals using jugular venipuncture technique with pigs supine in a V-trough. Blood samples (5 to 10 mL) were collected in EDTA tubes (BD; Franklin Lakes, NJ, US) and immediately centrifuged at 3, 000 x g for 15 min at 4°C for separation of plasma, which was stored at – 20°C until later analyses for plasma creatinine and plasma urea nitrogen (PUN) using enzymatic assays (Bioassay Systems, Hayward, CA, US).

4.3.4 Part 1: Blood Pressure measurement by non-invasive blood pressure (NIBP) cuff technique

From 4 to 8 mo old, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR) were measured once every two weeks in pigs using a leg cuff technique (Surgivet V6004 NIBP monitor, Waukesha, WI, US) while the pigs were restrained in the supine position in a V-trough; cuffs were placed on the upper part of the hind leg. Continuous haemodynamic measurements were recorded for 30 min, with approximately 35-45 readings recorded during this period; measurements were taken at the same time of day for each pig on the same day of the week.

4.3.5 Part 2: Radio-telemetric measurement of haemodynamics and locomotor activity:

At 9 mo old, with an average weight of 69 ± 7 kg, the animals underwent surgery, as previously described (**Chapter 3**), for implantation of a radio-telemeter (TA11PA-D70; Data Sciences International, St. Paul, MN, US) in the femoral artery of the back left leg, and because this study was part of a larger series of studies, of blood sampling catheters in the femoral vein. After surgery, each animal was housed individually. The telemetry system (Data Sciences International; St. Paul, MN, US) was set up to monitor haemodynamics and spontaneous locomotor activity in the pigs, as previously described (**Chapter 3**). Individual haemodynamics and activity data were exported from the data acquisition software and transferred to Microsoft Excel 2000® spreadsheet program for calculations (**Chapter 3**). All measured parameters (BP, HR and activity) were sampled every 30 s and data analysed over 24-h and 12-h periods (Van Vliet, Chafe et al. 2003).

4.4 Experimental protocols for radio-telemetry blood pressure assessment:

4.4.1.1 Part A: Baseline 24-h haemodynamics:

Pigs were allowed at least 8 d of recovery from surgery followed by at least 48 h of continuous BP recordings at 30-s intervals, while on the standard pig diet (0.5% NaCl). Pigs were maintained on a 12-h light-dark cycle (lights on 0700 to 1900 h). The recording room had a radio turned on at a low volume to reduce the impact of environmental noise. SAP, DAP, MAP, pulse pressure (PP), HR and locomotor activity were recorded in each animal.

4.4.1.2 Part B: Salt (NaCl) challenge study:

After the 48-h baseline BP recordings, the pigs were given a high salt (4.5% NaCl) version of the standard pig diet (**Chapter 3**) for 7 d, with continuous BP recordings for the last 48 h on this diet. At the end of the salt challenge the pigs' diet reverted to the standard pig diet (0.5% NaCl) until necropsy.

4.4.2 Necropsy:

On average, 5 d after the salt challenge BP recording period, all pigs were anaesthetized with 105 mg/kg sodium pentobarbital (Euthanyl, Biomeda-MTC Cambridge, ON, Canada) and ventilated and maintained with 0.5 – 1 % halothane gas mixed with oxygen. The animals died of exsanguination caused by removal of the liver. The heart was removed and weighed, and relative cardiac mass was assessed by calculation of the ratio of the cardiac weight to the whole-body weight. The heart was also dissected into left and

right ventricles and each ventricle weighed. Both kidneys were removed and weighed, and the right kidney was immediately frozen with liquid nitrogen and stored at –80°C for later analyses, while the left kidney was removed and immediately perfused-fixed with 10% buffered formalin, then immersion-fixed in more formalin until histology was performed for nephron measurements, as described below. Backfat thickness was measured on the carcass at the midline of the back, caudal to the last rib.

4.4.3 Kidney stereology and histology:

Kidney glomeruli were counted using unbiased stereological procedures, similar to the method used by Johnson et al. (Johnson, Wreford et al. 2000). Briefly, the fixed left kidney was cut along the frontal plane into two approximately equal halves, and one half randomly chosen for further analysis. Using a razor blade slicing device, the sampled kidney half was sliced perpendicular to the blade into 5 mm slices then every 4^{th} slice was taken for further analysis with the first slice chosen at random from the interval 1 to 4. Each of the chosen slices was cut into 5 mm strips and the strips were rotated and cut into small blocks so they were all approximately the same size. For each slice, every 5^{th} block was sampled from the total number of blocks, with the first block chosen at random from the interval 1 to 5. All sampled blocks were paraffin embedded, then using a 20-μm cutting thickness, the 24^{th} and 25^{th} sections of each block were sampled and stained with hematoxylin and eosin (H&E) for unbiased stereological counting of glomeruli numbers (Q) using the optical fractionator program (Stereo Investigator v.4.36, MicroBrightField, Inc., Williston, VT, US). Total nephron numbers per kidney (N_{kid}) were estimated using the following equation: $N_{kid} = f1 \times 5 \times 24 \times (P_s/2P_F) \times Q$. Where, f1 is the reciprocal of

the first sampling fraction (i.e., the weight of the sampled slices divided by the weight of the whole kidney); 5 was the inverse of the second sampling fraction (1/5 of the blocks); 24 was the inverse of the section sampling fraction (1/24 of the sections); $P_s/2P_F$ was the fraction of the section area used for glomerular counting. The nucleator program (Stereo Investigator v.4.36) was used to measure each counted glomerulus identified via optical fractionator, thus allowing for determination of average glomerular surface area and volume per kidney.

4.4.4 Blood pressure data analyses:

Cuff measurements: All cuff measurements were collected for 30 continuous min, with the last 20 min used to calculate the mean BP values (see **Chapter 2** for details).

Telemetry: A modified version of the Microsoft Excel 2000® template (HdStats, see www.med.mun.ca/Medicine/Faculty/Van-Vliet,-Bruce.aspx for link to download) was used for routine analysis of 24-h telemetry data sets (see **Chapter 3** for more detail).

4.4.5 Statistical analyses:

All values are expressed as mean ± SD. Initially all means between groups (i.e., runts versus large littermate) were analyzed using two-way ANOVA (Prism 4, GraphPad Software Inc., CA, US), where the factors were treatment, sex and the interaction between them. Then using Pearson's correlations we found that haemodynamic values were significantly affected by various measured physiological parameters, thus the haemodynamic data were reassessed by general linear model (GLM) (Minitab v.15.1, Minitab Inc., State College, PA, US). The GLM analysis model included treatment, sex

and the interaction between the two, as well as covariates including birth weight, current weight, kidney weight (and nephron/kidney), ventricle weights, visceral fat, subcutaneous fat measurement, fractional growth rates and activity level. When significant interaction was found between treatments, pair-wise comparisons were made using Tukey's *post hoc* test. Responses to the salt challenge were compared using the paired t-test.

For non-haemodynamic parameters, all means were initially analyzed using 2-way ANOVA; if no sex effect was found we reduced the model to a paired-t-test. For all statistical analyses, a value of $P < 0.05$ was considered significant.

4.5 RESULTS

4.5.1 Effect of birth weight on growth and feed intake:

In this study, birth weights of runts averaged 34 ± 5% lower than those of their larger littermates (Table 4.1), and the weight difference remained significant until sexual maturity, which occur between ages 4 to 7 mo in miniature pigs (Smith and Swindle 2006; Nunoya, Shibuya et al. 2007). For instance, weight at 1 mo of age (i.e., weaning) was significantly correlated with birth weight ($R = 0.80$, $P = 0.002$). However, by 7 mo of age, body weight was no longer significantly correlated to birth weight ($R = 0.41$, $P = 0.19$). Indeed, as highlighted in Table 4.1, in terms of fractional growth rates, the runts experienced significant catch-up growth before sexual maturity. From 1 to 4 mo of age, the average relative daily feed intake was significantly ($P < 0.05$) greater for the runts. However, runts and large littermates were similarly efficient in utilizing feed intake for body weight gain throughout the study. At the end of the study at 10 mo of age,

measurement of backfat thickness showed that the runts had more subcutaneous fat compared to the large littermates (0.90 ± 0.19 vs. 0.82 ± 0.17 mm backfat/kg bodyweight, P = 0.04). Thus, these data indicate that catch-up growth in the runts was due to greater relative feed intake resulting in increased obesity.

4.5.2 Haemodynamic recordings by NIBP:

From 4 to 8 mo old, BP measurements by the NIBP cuff technique indicated more elevated BP in the runts relative to the large littermates, and this difference reached significance at 6-8 mo old (Figure 4.1). Also, there was a noticeable steady increase in BP in all pigs as they age (Figure 4.1).

4.5.3 Basal haemodynamic recordings by radio-telemeter:

As illustrated in Table 4.2, at 9-10 mo of age the runts had a significant (P <0.05) elevation in diastolic BP levels compared to their larger littermates. Indeed, the BP values were more elevated in the runts whether we assess 24-h values (Table 4.2) or when the data were separated into day and night periods (Figure 4.2). Furthermore, there tended to be inverse relationships between birth weight and BP (Figure 4.3). We also found that runts had significantly fewer nephrons than large littermates (P <0.05, Table 4.3), and that nephron numbers were positively correlated (P <0.05) (Figure 4.3) to birth weight and inversely correlated (P <0.05) to BP (Figure 4.4). In addition, there were no significant differences in HR values between runts and large littermates (Table 4.2), nor was HR correlated to birth weight (P = 0.81).

Absolute and relative heart weights were not different between groups (Table 4.3), nor were there any significant correlations with birth weight (R = 0.37, P = 0.23).

In this swine model we found that sex, birth weight, current weight, ventricle mass (left and right, and the ratio of the two), subcutaneous fat, visceral fat, kidney size and activity level all significantly associated to BP; more importantly, when these parameters were included as covariates there were even greater significant differences in all BP parameters between runts and large littermates (Table 4.2).

4.5.4 Locomotor activity and haemodynamics:

In most animals, an increase in activity level acts on the nervous system, resulting in elevation of arterial pressures. However, as previously reported (**Chapter 3**), we observed the opposite relationship between activity and BP in this animal model. That is, activity was shown to decrease BP while increasing HR and an inactive state had the reverse effects (Table 4.2). Overall, one of the main points illustrated in Table 4.2 regarding BP and fetal/early programming is that whether active or inactive, BP parameters remained higher in the runts compared to their large littermates. Because the animals were predominantly inactive, with more than 80% of the time spent resting, the difference in BP values between runts and large littermates only reached significance (i.e., DAP, P <0.05) during the inactivity period (Table 4.2).

4.5.5 Comparison of NIBP and radio-telemetry BP:

We found no significant correlations between NIBP at 8 mo old and baseline radio-telemetry BP measurements at ~ 9 mo old for SAP (P = 0.77), DAP (P = 0.63) or MAP (P

= 0.66). Interestingly, there was a significant inverse relationship between HR measurement by cuff technique and HR measurements by radio-telemetry (R = -0.70, P = 0.01).

4.5.6 Nephron number and renal function:

There was no significant difference in the absolute or relative weights of kidneys between groups (Table 4.3); however, birth weight tended to correlate with kidney weight (R = 0.51, P = 0.09). Runts had significantly fewer glomeruli (and therefore nephrons) in the kidneys compared to their littermates (Table 4.3); however, there was no significant correlation between nephron number and kidney size (R = 0.36, P = 0.25). Furthermore, there were no significant differences in average individual glomerular size or volume, or in plasma creatinine and PUN concentrations between the groups.

4.5.7 Salt challenge:

In both runts and large littermates, there were significant ($P < 0.05$) increases in most BP parameters (Figure 4.5, Column A) in response to the 7-d salt (NaCl) challenge, where the dietary salt content increased from 0.5% to 4.5%. These data indicated acute salt sensitivity in this Yucatan miniature swine model. But because there was noticeable salt sensitivity in both runts and large littermates, and the degree of salt sensitivity was similar between the groups (Figure 4.5, Column B), salt sensitivity itself was not subject to fetal/early programming in this swine model. The high salt diet increased BP values in both groups to very similar final values, which suggested greater changes in BP in the large littermates, but this increase was not significant. Furthermore, there were no

significant correlations between salt sensitivity (i.e., changes in DAP) and any of the following parameters: 1) birth weight ($R = 0.20$, $P = 0.54$), 2) nephron numbers ($R = 0.38$, $P = 0.22$), and 3) glomerular area ($R = -0.02$, $P = 0.95$).

Table 4.1. Growth and feed intake characteristics in runt and large littermate Yucatan miniature pigs.

	Runt	Large	P-value	Runt/ Large
		Body Weight (kg)		
Birth	0.73 ± 0.11	1.11 ± 0.13	<0.0001	0.66
1 mo old (weaning)	4.60 ± 0.53	6.04 ± 1.03	0.007	0.77
4 mo old	29.54 ± 2.69	34.19 ± 2.50	0.013	0.87
7 mo old	55.70 ± 5.37	60.93 ± 3.58	0.113	0.92
10 mo old	69.42 ± 7.58	74.48 ± 6.19	0.181	0.94
		Fractional Growth Rate (g• kg body wt^{-1}• day^{-1})		
Birth to 1 mo old	146.3 ± 27.9	116.5 ± 8.6	0.025	1.25
1 to 4 mo old	56.9 ± 6.9	46.2 ± 4.7	0.015	1.24
4 to 7 mo old	10.4 ± 2.2	8.5 ± 3.2	0.260	1.22
7 to 10 mo old	3.1 ± 0.6	2.8 ± 1.0	0.531	1.11
		Average Daily Feed Intake (kg body wt $^{-1}$)		
Birth to 1 mo old (mL)	370.2 ± 40.7	364.1 ± 37.3	0.808	1.02
1 to 4 mo old (g)	50.9 ± 1.9	49.1 ± 3.0	0.035	1.04
4 to 7 mo old (g)	30.0 ± 1.3	28.4 ± 3.5	0.185	1.07
7 to 10 mo old (g)	21.2 ± 2.7	21.4 ± 0.9	0.817	0.11
		Feed Efficiency (body wt gain (g)• feed intake (kg) $^{-1}$)		
Birth to 1 mo old	146.3 ± 21.7	140.0 ± 16.5	0.586	1.04
1 to 4 mo old	401.7 ± 40.6	389.8 ± 41.0	0.584	1.03
4 to 7 mo old	271.1 ± 54.8	266.4 ± 34.3	0.870	1.02
7 to 10 mo old	165.2 ± 16.1	150.6 ± 52.8	0.525	1.11

Each value represents the mean ± SD in n = 6 pigs. Significant differences ($P < 0.05$) between dietary treatments were assessed by GLM.

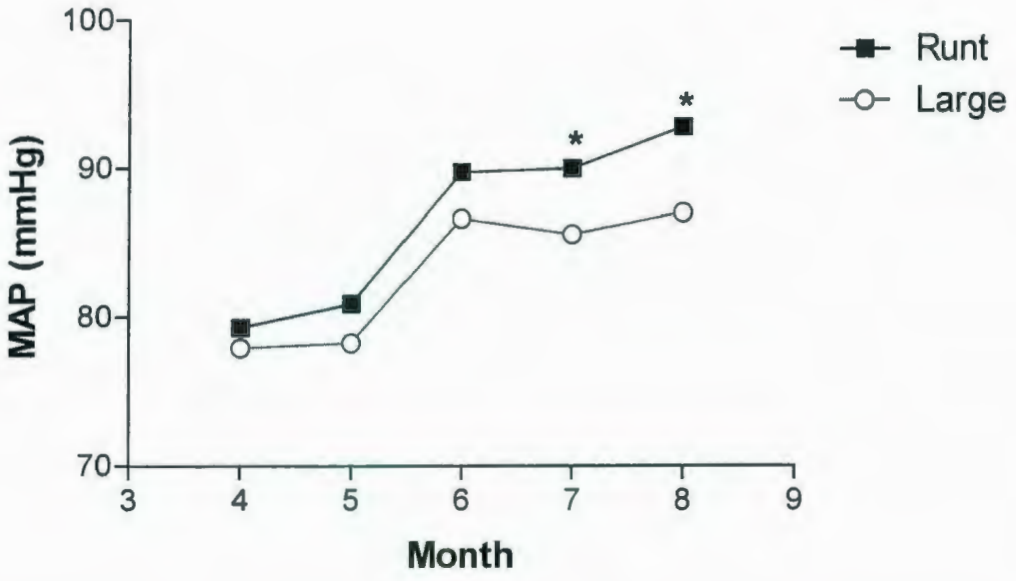

Figure 4.1: Mean blood pressure (MAP) by NIBP foot cuff measurements in Yucatan miniature pigs on standard (0.5% NaCl) pig diet from 4 to 8 mo old.

Each symbol is represented by mean in n = 6 pigs. $^{*}P < 0.05$ is significant as assessed by paired t-test

Table 4.2. Summary of basic haemodynamic and activity parameters for Yucatan miniature pigs on standard (0.5% NaCl) diet.

Period	Variable	Runt	Large	P value	P-value#
24 h	SAP (mm Hg)	140.8 ± 7.8	134.2 ± 5.3	ns (0.084)	0.004
	MAP (mm Hg)	115.7 ± 7.2	110.6 ± 8.0	ns (0.058)	0.021
	DAP (mm Hg)	93.8 ± 5.5	90.0 ± 8.7	0.017	<0.001
	PP (mm Hg)	47.0 ± 6.0	44.2 ± 4.3	ns (0.345)	0.042
	HR (beats min^{-1})	87.8 ± 5.9	87.7 ± 9.1	ns (0.967)	
	Activity				
	Mean activity (a.u.)	1.9 ± 0.9	1.5 ± 0.4	ns (0.394)	
	Active time (%)	10.1 ± 3.4	8.0 ± 1.6	ns (0.236)	
Activity = 0	SAP (mm Hg)	142.4 ± 8.4	135.1 ± 4.8	ns (0.081)	
	MAP (mm Hg)	116.9 ± 7.7	111.3 ± 7.4	ns (0.065)	
	DAP (mm Hg)	94.7 ± 5.7	90.5 ± 8.1	0.029	
	PP (mm Hg)	47.7 ± 6.1	44.6 ± 4.3	ns (0.318)	
	HR (beats min^{-1})	82.6 ± 6.2	84.2 ± 8.5	ns (0.651)	

Activity > 0	SAP (mm Hg)	135.1 ± 9.2	131.4 ± 11.5	ns (0.409)
	MAP (mm Hg)	111.8 ± 8.5	109.1 ± 12.9	ns (0.409)
	DAP (mm Hg)	90.7 ± 7.5	88.6 ± 13.4	ns (0.382)
	PP (mm Hg)	44.4 ± 6.0	42.8 ± 3.5	ns (0.559)
	HR (beats min^{-1})	117.0 ± 8.8	119.4 ± 16.7	ns (0.660)
Change with inactivity (Inactive – Active)	SAP (mm Hg)	7.3 ± 5.1	3.7 ± 7.1	ns (0.237)
	MAP (mm Hg)	5.1 ± 5.0	2.2 ± 5.9	ns (0.292)
	DAP (mm Hg)	4.0 ± 5.3	1.9 ± 5.6	ns (0.437)
	PP (mm Hg)	3.3 ± 1.9	1.8 ± 1.9	ns (0.153)
	HR (beats min^{-1})	-34.3 ± 8.6	-35.2 ± 10.0	ns (0.865)

SAP: systolic arterial pressure; MAP: mean arterial pressure; DAP: diastolic pressure; HR: heart rate; PP: pulse pressure; a.u., arbitrary units. Inactivity and activity were measured as sustained in/activity (see Methods). Each value represents the mean ± SD in n = 6 pigs. Significant differences between dietary treatments were assessed by GLM with $P < 0.05$ as significant; ns = not significant.

P-value# - indicates significant difference when sex, viscera fat, subcutaneous fat, and activity level are included as covariates in the statistical analysis.

Table 4.3. Renal and cardiac parameters in runt and large littermate Yucatan miniature swine at the end of the study at 10 months old.

	Runt	Large	P-value
Renal Parameters			
Total kidney wt (kg)	0.206 ± 0.043	0.231 ± 0.075	ns (0.077)
Total kidney/body wt ratio (%)	0.297 ± 0.046	0.307 ± 0.082	ns (0.539)
Nephron number per kidney	296,084 ± 70,218	518,330 ± 166,812	0.012
Average glomerular surface area (μm^2)	20,717 ± 2,987	20,220 ± 4,591	ns (0.808)
Average glomerular volume ($\mu m^3 \times 10^6$)	2.52 ± 0.53	2.43 ± 0.82	ns (0.814)
Total glomerular volume ($\mu m^3 \times 10^6$)	73.34 ± 16.64	93.44 ± 49.88	ns (0.178)
Plasma urinary nitrogen (PUN) (mmol/L)	3.24 ± 0.41	3.29 ± 0.83	ns (0.894)
Plasma creatinine (μmol/L)	86.0 ± 13.7	100.6 ± 13.7	ns (0.122)
Cardiac Parameters			
Heart wt (kg)	0.176 ± 0.035	0.183 ± 0.047	ns (0.471)
Heart/body wt (%)	0.253 ± 0.039	0.244 ± 0.049	ns (0.645)
Left ventricle (kg)	0.130 ± 0.026	0.137 ± 0.032	ns (0.416)
Right ventricle (kg)	0.045 ± 0.009	0.047 ± 0.015	ns (0.660)
Right/left ventricle	0.346 ± 0.010	0.336 ± 0.032	ns (0.389)

Each value represents the mean ± SD in n = 6 pigs. Significant differences between dietary treatments were assessed by GLM with $P < 0.05$ as significant; ns = not significant.

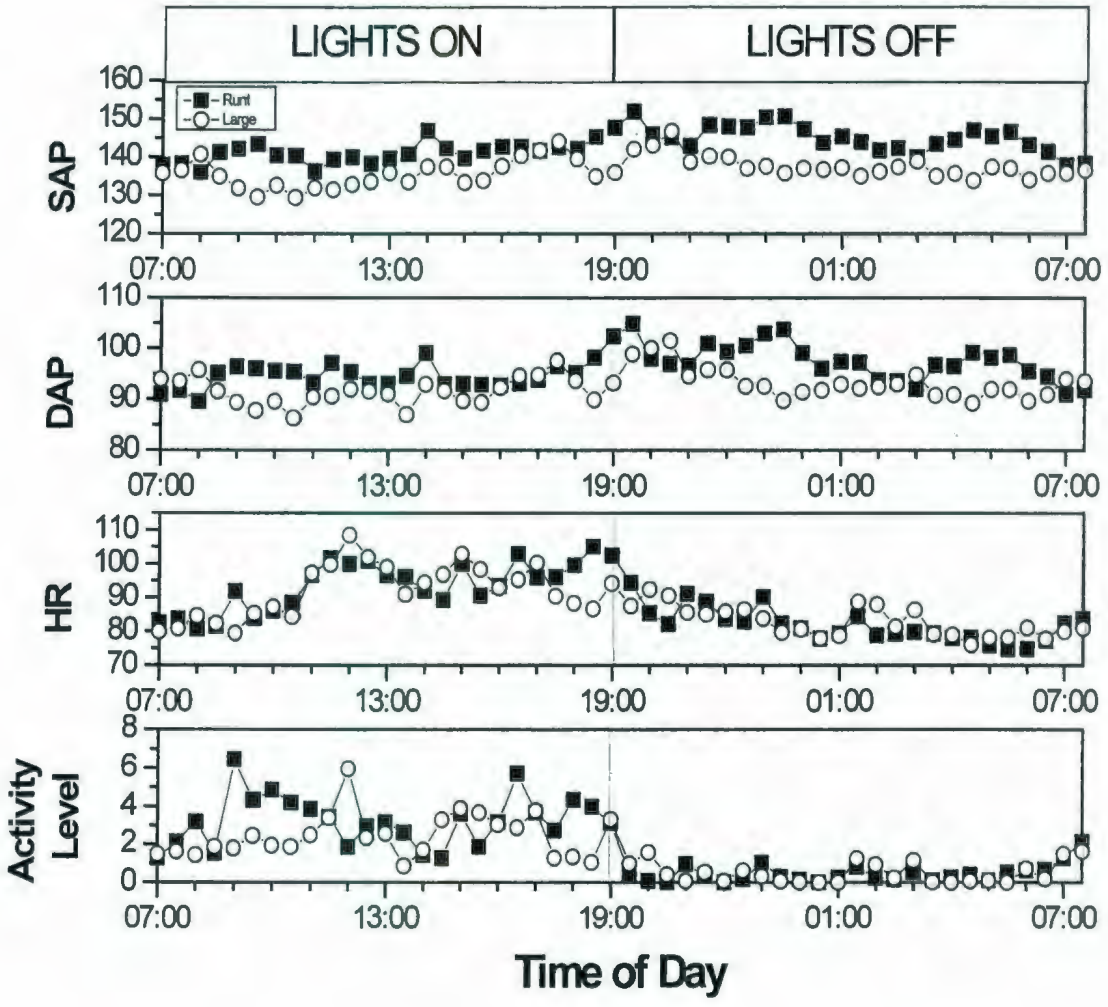

Figure 4.2: Diurnal patterns in SAP, DAP, HR, and locomotor activity in runt and large Yucatan miniature swine on the standard pig diet (0.5% NaCl).

Day is represented by the un-shaded section of the graphs, where lights came on at 0700 h and off at 1900 h. Night is represented by the shaded section of the graphs, from 1900 h to 0700 h. SAP = Systolic arterial pressure (mmHg); DAP = Diastolic arterial

pressure (mmHg); HR = Heart rate (beats/min); Activity (arbitrary units). Runts are represented by the solid squares; large littermates are represented by open circles. Each point in the graphs represents the mean of n = 6 pigs per treatment.

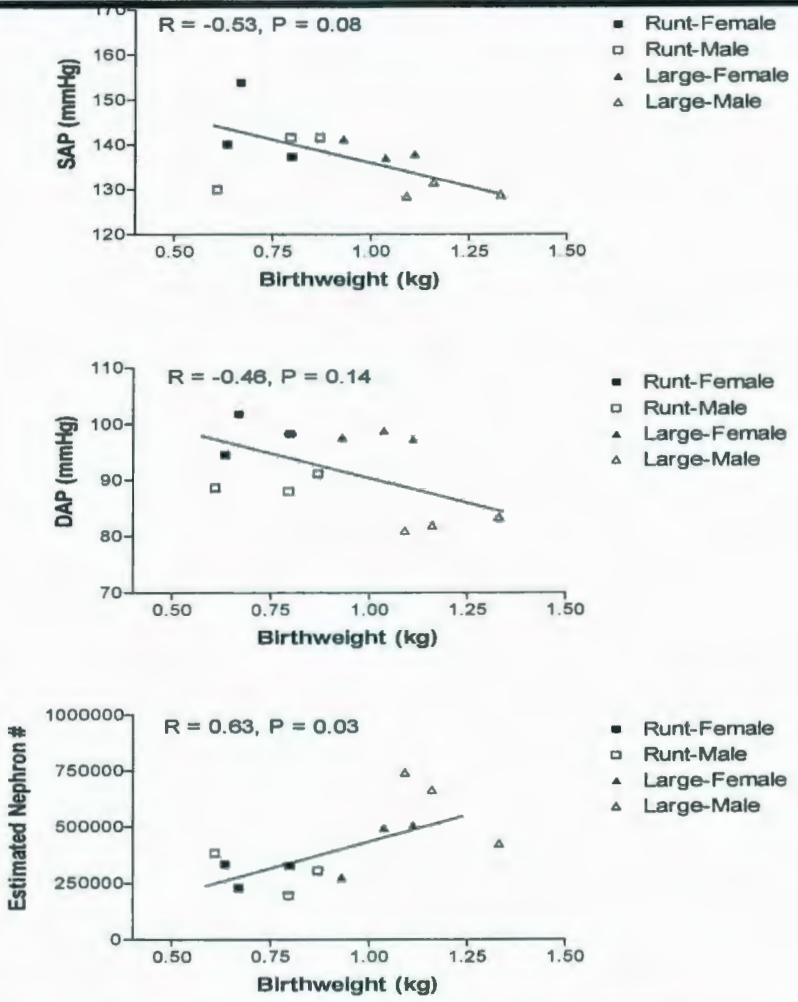

Figure 4.3: Relationship between birth weight and blood pressure or nephron numbers.

Blood pressure parameters were measured at about 9.5 mo of age in all pigs and correlated to birth weight. Kidneys were removed from the animals 2-3 weeks later and nephrons counted and correlated to birth weight. SAP = Systolic arterial pressure (mmHg); DAP = Diastolic arterial pressure (mmHg). Each symbol represents a pig.

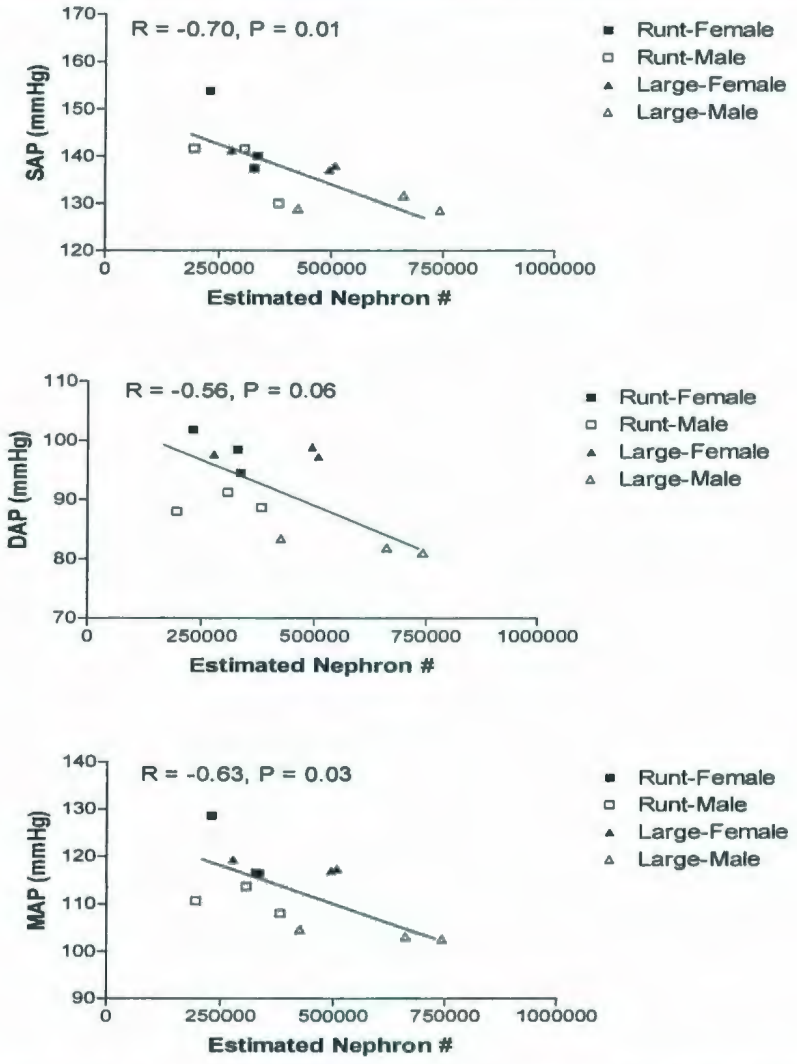

Figure 4.4: Relationship between nephron number and blood pressure.

Blood pressure parameters were measured at about 9.5 mo of age and kidneys were removed from the animals 2-3 weeks later and nephrons counted and correlated to blood pressure parameters. SAP = Systolic arterial pressure (mmHg); DAP = Diastolic arterial pressure (mmHg); MAP = Mean arterial pressure (mmHg). Each symbol represents the mean value from an individual pig.

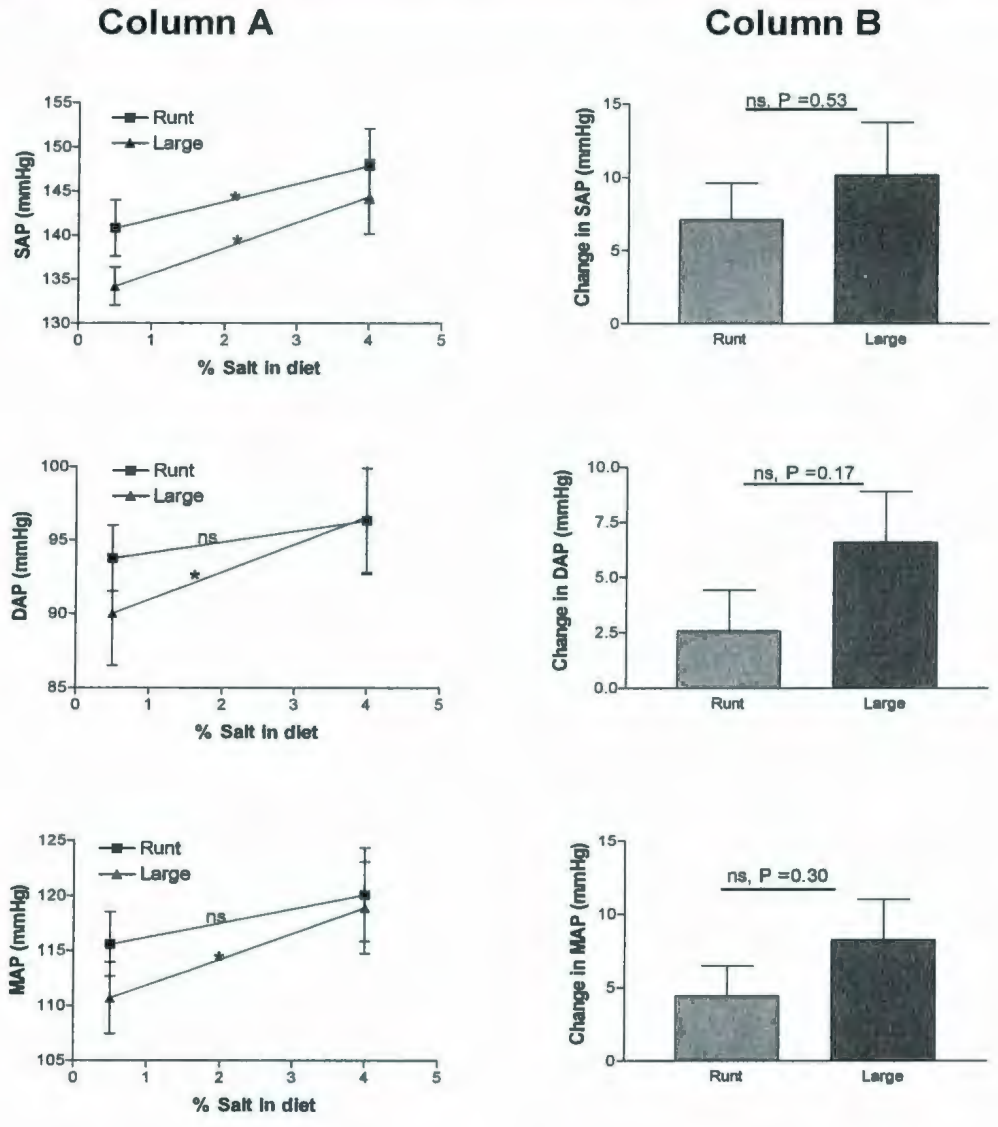

Figure 4.5: Effects of a high salt (4.5% NaCl) diet on blood pressure parameters in runts and large littermates.

Column A: On each graph, BP was initially measured in animals on the standard diet (0.5% NaCl), and then 7 d later on the high salt diet (4.5% NaCl). SAP = Systolic arterial pressure (mmHg); DAP = Diastolic arterial pressure (mmHg); MAP = Mean arterial pressure (mmHg). Each symbol represents the mean ± SD in n = 6 pigs. $^{*}P < 0.05$ is

significant- indicates a difference with change in salt intake within groups, ns = not significant ($P > 0.05$). **Column B:** Each graph shows the comparison of the differences between diets as the diet changed from 0.5% to 4.5% NaCl for the runts and the large littermates

4.6 DISCUSSION

The major findings of the present work are: 1) naturally occurring small birth weight Yucatan miniature pigs (runts) experience catch-up growth prior to sexual maturity; 2) these runts have elevated BP as adults compared to their larger birth weight siblings; 3) increased BP is associated with fewer glomeruli (i.e., reduced nephron number) per kidney; and 4) in spite of a lower number of nephrons, runts did not show enhanced salt sensitivity in response to an acute high salt (4.5%NaCl) intake.

4.6.1 Catch-up growth and blood pressure

Some research suggests that there are two parts to the relationship between fetal/early programming and BP regulation. The first is the adaptive mechanism associated with reduced placental blood flow during development of the fetus, which can lead to anatomical, physiological and metabolic alterations. For instance, reduced nephrogenesis has been shown to modify postnatal BP (Bagby 2007). Second, BP can be affected by postnatal factors such as catch-up growth (also known as compensatory growth), which is also associated with fetal growth restriction. Moreover, epidemiological studies have shown that those who are born small and experience catch-up growth are at an even more elevated risk for developing chronic diseases than those who are just born small (Huxley, Shiell et al. 2000; Adair and Dahly 2005; Singhal, Cole et al. 2007; Ben-Shlomo, McCarthy et al. 2008). Consistent with the epidemiological evidence, we observed that fetally growth restricted runts experienced a period of catch-up growth that started during the third week of the four-week milk-feeding phase (data not shown) and remained significant up to sexual maturity, which occurs in Yucatan miniature swine at 4 to 7 mo

old (Smith and Swindle 2006; Nunoya, Shibuya et al. 2007). Specifically, runts had a higher fractional growth rate from birth to 4 mo old, in comparison to their littermate controls ($P<0.05$); in other words, at a given body weight, runt pigs grew faster than their larger siblings. The increased weight gain in the runts was due to an increased food intake as indicated by the higher relative feed intake (Table 4.1). By the end of the study at 10 mos old, runts were more obese compared to the large littermates. Thus, in this swine model, catch-up growth in runts seems to occur because of an increase in food intake, as opposed to an increase in feed efficiency, resulting in increased obesity.

Although some epidemiological data (Law, Shiell et al. 2002; Franklin, Pio et al. 2005; Hardy, Sovio et al. 2006) suggest that postnatal growth rate (i.e., weight or body mass index) is more predictive of later hypertension, we were unable to detect significant relationships between fractional growth rates and BP values (e.g., DAP: $R = 0.40$, $P = 0.20$). However, we must caution against interpreting this as evidence that catch-up growth does not contribute to development of hypertension. We can conclude that runts, who do experience catch-up growth, will develop higher BP later in life. To test possible mechanisms, we also conducted multiple regression analyses and found that sex, birth weight, current weight, ventricle masses (and the ratio of the two), subcutaneous fat, visceral fat, kidney size and activity level all significantly associated with BP. Each of these covariates can independently increase the risk for hypertension, and when we included all these parameters as covariates, the relationships between BP and fetal growth restriction became even more significant (Table 4.2). Indeed, we also observed that there were significant correlations between subcutaneous fat and BP values (e.g. DAP: R =

0.88, P = 0.0001), and the runts had more subcutaneous fat compared to the large littermates ($P < 0.05$).

4.6.2 Kidneys and Blood Pressure

After over a decade, through epidemiological and animal studies, it is now accepted that adult hypertension can be programmed by prenatal and early postnatal environment (Adair and Dahly 2005; McMillen and Robinson 2005; Bagby 2007) Indeed, the results of this study are in agreement with this phenomenon. Yucatan miniature runt pigs had elevated BP as adults compared to similarly treated large littermates suggesting that early programming of BP occurs in this species. And similar to epidemiological data, BP in our pigs tended to correlate inversely with birth weight. Although the mechanisms underlying early programming of the development of hypertension in later life are not fully elucidated, the majority of experimental evidence suggests a crucial role for the kidney (Hughson, Farris et al. 2003; Adair and Dahly 2005; McMillen and Robinson 2005; Bagby 2007; Schreuder and Nauta 2007). For example, studies in the rat suggest that disruption of renal development during gestation results in hypertension in adulthood (Woods and Weeks 2004; Vehaskari and Woods 2005). Thus, part of the objectives of this study was to examine the role of the kidney in the development of elevated BP in this swine model. Several hypotheses have been proposed regarding early renal development and susceptibility to hypertension later in life (Kett and Bertram 2004; McMillen and Robinson 2005; Bagby 2007; Schreuder and Nauta 2007). In this study we attempted to explore two of these hypotheses, namely hyperfiltration in the kidney and impairment in the pressure-natriuresis system. In terms of hyperfiltration, the concept is that reduced

renal mass (or reduced nephron numbers) may promote increases in BP as a result of deterioration of renal structure and function. On the other hand, impairment in the pressure-natriuresis system suggests that reduced renal mass (or nephron numbers) may lead to increases in BP by promoting salt retention.

4.6.3 Kidney, Nephron and Blood Pressure

Human and other animal studies show that naturally occurring intrauterine growth restriction (IUGR) can result in decreases in total nephrons in the offspring (Bauer, Walter et al. 2000; Bauer, Walter et al. 2002; Hughson, Farris et al. 2003; Woods, Weeks et al. 2004). In this study, we found that runts had 43% fewer nephrons than the larger littermates, and nephron number was positively correlated to birth weight ($P<0.05$). Moreover, nephron number was inversely correlated with BP ($P<0.05$). But as others suggest, a reduction in nephron number is not enough to cause hypertension; the impact of reduced nephron numbers on renal functions (e.g., renal pressure) is important for the long-term consequences on systemic BP. For instance, this nephron reduction can lead to essential hypertension partially through hyperfiltration of the remaining nephrons (Brenner, Garcia et al. 1988; Brenner and Chertow 1994). Early programming can influence the kidney because nephrogenesis occurs during a critical window of development and impaired nephrogenesis during this period results in fewer nephrons. Outside of this critical window, it is impossible to compensate for this hyperfiltration to control BP by increasing nephron numbers. The hyperfiltration hypothesis (first proposed for kidney recipient studies), states that the low nephron number (i.e., due to reduce renal mass) will lead to glomerular hyperfiltration and elevated BP in the remaining nephrons

(i.e., glomerular hypertension). This hyperfiltration will eventually lead to subsequent glomerular enlargement in order to sustain adequate renal clearance, leading to glomerular injury, which results in proteinuria, glomerulosclerosis and systemic hypertension; the resulting vicious cycle leads to a further reduction in nephron numbers. Brenner and colleagues (Brenner, Garcia et al. 1988) proposed early on that this hypothesis may also apply to fetal programming of kidneys and may partially explain the systemic elevated BP observed in low birth weight offspring.

In this study we were not able to directly measure glomerular filtration rate (GFR); however, PUN and plasma creatinine were measured as indirect markers; impairment in GFR will result in elevated plasma creatinine and PUN. We measured monthly plasma creatinine and PUN concentrations throughout the study and found the levels remained relatively constant, and within normal values, with generally no differences between the groups. So, although runts had significantly fewer glomeruli, as reflected by nephron number, there was no indication of impaired renal function in either group. This would suggest that in the runts, each nephron did more work in order to maintain the same levels of plasma creatinine as observed in the large littermates (i.e., hyperfilitration). However, because we did not directly measure GFR and renal pressure, we can only speculate on this sequence of events.

According to the Brenner hypothesis, hyperfiltration to maintain GFR may eventually lead to glomerular damage and a further decline in nephron number. Prolonged hyperfiltration will eventually lead to glomerular enlargement in order to sustain adequate renal clearance. Thus, in this study, we evaluated the size of individual glomeruli to look

for evidence of glomerular enlargement in the runts. However, we found no differences in the average individual glomerular surface area or volume between groups. But interestingly, there were significant inverse relationships between BP and total glomerular volume (SAP: $R = -0.76$, $P < 0.001$, and DAP: $R = -0.82$, $P < 0.001$), suggesting that runts with higher BP still had smaller glomeruli. It is important to note that these pigs were still fairly young (early adulthood), with only modest elevations in BP in the runts (Table 4.2); so whatever hyperfiltration that might have been present in the runts did not appear to have progressed to the stage where it resulted in obvious enlargement of glomeruli in the runts.

4.6.4 Kidney, Salt and Blood Pressure

Fetal/early programming might predispose the low birth weight offspring to salt-induced hypertension. If so, the mechanism is not fully elucidated. The kidney plays a major role in the pathogenesis of hypertension, primarily through the long-term regulation of arterial pressure via pressure-natriuresis. For example, if there are impairments in the renin-angiotensin (RAS)-aldosterone system and/or atrial natriuretic peptide, then salt loading will cause changes in renal perfusion pressure, which can result in an elevation of arterial BP in order to maintain a balance between normal sodium intake and excretion (Guyton, Coleman et al. 1972; Guyton, Coleman et al. 1972). Additionally, reduced nephron number has been proposed to explain alteration in the pressure-natriuresis system. That is, a reduction in nephron number leads to reduced total glomerular filtration surface area, with subsequent sodium retention and increase in blood volume, and consequent development of hypertension (Brenner, Garcia et al. 1988; Brenner and Chertow 1994;

Mackenzie, Lawler et al. 1996). Indeed, there are reports in humans and some animal models that fetal/early developmental stresses can program salt sensitivity later in life (Vehaskari, Aviles et al. 2001; Woods, Weeks et al. 2004; Vehaskari and Woods 2005; de Boer, Ijzerman et al. 2008; Simonetti, Raio et al. 2008), and one or both of the above mechanisms have been implicated. However, in this study, both groups of animals demonstrated salt sensitivity, and the degree of salt sensitivity was similar between the groups. These data suggest that abnormality in the pressure-natriuresis system via RAS-aldosterone system and/or atrial natriuretic peptide may not be key mechanisms regulating fetal/early programming of BP in this swine model. Our results are in agreement with those of other researchers (Langley-Evans and Jackson 1996; Zimanyi, Bertram et al. 2004) who also did not observe fetal/early programming of salt sensitivity in their rat models. In those studies (Langley-Evans and Jackson 1996; Zimanyi, Bertram et al. 2004), as with this study, all animals demonstrated salt-sensitive elevation in BP when challenged with an increase in salt (NaCl) intake, thus implying that the RAS-aldosterone system is resistant to early programming mechanisms. It is also likely that the runts have successfully accommodated for their lower nephron number at this age, and have not yet progressed to glomerular compromise that would lead to altered salt sensitivity.

4.6.5 Conclusions

In conclusion, in this naturally occurring runt Yucatan miniature pig model, we found that small birth weight and catch-up growth led to higher BP early in adulthood. This higher BP was associated with smaller nephron number, which also correlated negatively

with BP. However, despite these profound findings early in adulthood, BP elevations were modest. The animals are still in the early stages of adulthood; therefore, waiting longer might have led to overt hypertension as a result of the reduced nephron number compounded by age-related glomerular damage. However, it is also possible that feeding pigs a Western-style diet that is high in salt throughout life might also induce greater BP differences, and consequently early glomerular damage in the young adult pig; this hypothesis was tested in the follow-up study (**Chapter6**).

CHAPTER 5. LOW BIRTH WEIGHT YUCATAN MINIATURE SWINE THAT EXPERIENCE EARLY CATCH-UP GROWTH HAVE IMPAIRED LIPID METABOLISM AS YOUNG ADULTS

5.1 ABSTRACT

Background and Objectives: Many studies report inverse relationships between birth weight and risk for cardiovascular diseases (CVD). Few studies assessed the risk relationship with lipid parameters. The aim of this study was to evaluate lipid metabolism in low birth weight Yucatan miniature pigs.

Methods: From the same litter, runt piglets (<850 g) (N=6) were paired with the largest same sex littermates (>1100 g) (N=6) and taken from the 6 sows at 3 d of age. During the first 4 weeks, piglets were fed milk replacer *ad libitum*; thereafter pigs were fed standard diet *ad libitum* for 5 h/d. Body weights, body measurements and feed intakes were recorded throughout the study. Monthly fasted blood samples were collected throughout the study, and lipid assays performed. At 9 mo of age, pigs were surgically fitted with venous catheters and a blood pressure telemeter. Following at least 1 wk recovery from surgery, an oral fat tolerance test was performed to assess postprandial lipid metabolism. At the end of the study, after an overnight fast, tissues were collected, backfat thickness was measured, and the aorta was stained for plaque using Sudan IV.

Results: The runts demonstrated catch-up growth in body weight and abdominal circumference prior to sexual maturity (7 mo-old). Furthermore, in comparison to their

large littermates, the runts had significantly ($P<0.05$) more subcutaneous fat at 10 mo old. There were significant ($P<0.05$) inverse correlations between birth weight and plasma triglycerides (TG) levels, where, from 5 mo old until 10 mo old, there were consistently higher levels of fasting plasma TG in the runts relative to the large littermates. Furthermore, an oral fat challenge indicated that postprandial lipid metabolism was significantly impaired in the runts relative to large littermates. Additionally, hepatic TG and total cholesterol levels were more elevated in the runts. However, there was no difference in the level of plasma cholesterol between the groups and there was no evidence of fatty streaks in the aortas of the runts or large littermates.

Conclusion: Fetal and early postnatal adaptations are associated with altered lipid metabolism in the runt Yucatan miniature swine during adolescence and adulthood. Furthermore, early catch-up growth is associated with increased obesity in the runt pig, which in turn associated with elevated plasma lipid content.

5.2 INTRODUCTION

Coronary heart disease (CHD), the second most common contributor to cardiovascular diseases (CVD) after hypertension, represents the leading cause of death in industrialized societies (AHA 2008; WHO 2008), accounting for over 20% of deaths in Canada (StatsCanada 2007; AHA 2008; AHA 2008). A large body of evidence from work in both animal models and human trials has established that plasma lipids play a causal role in CHD and CVD via their role in atherosclerosis (Cullen and Assmann 1999; Wilson 2004). Furthermore, recent epidemiological and animal studies have demonstrated inverse relationships between birth weight and CVD (Barker and Osmond 1986; Armitage, Khan et al. 2004; McMillen and Robinson 2005; Yajnik and Deshmukh 2008); with the postulation that fetal undernutrition is an independent risk factor for the susceptibility to the development of CHD and CVD (Barker 1995; Barker 1999). Specifically, some studies have found that low birth weight was associated with dyslipidaemia, such as hypertriglyceridaemia, in adulthood resulting from impaired lipid metabolism (Barker, Gluckman et al. 1993; Barker 1999; Huxley, Owen et al. 2004; Poston 2006). This fetal origin of adult disease (FOAD) hypothesis proposed that adverse *in utero* conditions could result in altered "programming" of metabolic and physiological functions, which can have lifelong effects and increase the risk of chronic disease onsets.

Mechanistically, the FOAD hypothesis suggests that altered "programming" occurs due to the concept of 'brain-sparing', that is, in response to the poor intrauterine nutritional environment, the fetus adapts by preferentially partitioning the supply of nutrients to

organs such as the brain at the expense of visceral organs, including the liver and muscle (Barker 1993). Indeed, animal experiments have demonstrated that fetal undernutrition can permanently change lipid metabolism through alterations of the microstructure of the liver (Ozanne 2001). Additionally, recent research indicates that the altered "programming" may extend beyond the *in utero* environment. That is, in humans, whereas most organs such as the kidney are said to have completed their critical period of development prior to birth, there are a few organs, such as the liver, that are believed to continue to be plastic after birth (Barker 2003). Furthermore, there is an accumulating body of data that indicates that postnatal growth rate is just as important as fetal growth rate in the process of chronic disease outcomes. Specifically, it has been argued that the change in body size (i.e., body fat deposition) between birth and adulthood is most important for the manifestation of CVD outcomes (Lucas, Fewtrell et al. 1999; Barker, Eriksson et al. 2002; Yajnik 2002; Wells, Chomtho et al. 2007).

The aim of this study was to evaluate the effect of birth weight, as a proxy for IUGR, along with postnatal growth rate on lipid metabolism as a biomarker of CHD in the Yucatan miniature pig.

5.3 MATERIALS AND METHODS

All animal procedures were approved by Memorial University Animal Care Committee, and carried out in accordance with the Canadian Council on Animal Care Guidelines.

5.3.1 Animals and study protocol:

Six pairs of runt and large littermates (3 female and 3 male pairs) of Yucatan miniature pigs from six sows from the Memorial University of Newfoundland swineherd were studied (average litter size was 7 ± 1). One day after a sow gave birth, the entire litter was weighed. The mean birth weight of all piglets born in the 6 litters used in this study was 0.92 ± 0.08 kg (average body weight per litter ranged from 0.83 to 1.03 kg). Within each litter, a runt was defined as a piglet weighing less than 850 g. A same-sex larger littermate weighing at least 300 g more than the runt was chosen as a littermate control. The runt and the larger same-sex littermate were taken from the sow at 3 d old. The pair was housed together, but individually fed rehydrated sow milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) eight to ten times daily *ad libitum*, with all intakes recorded. The piglets were housed in pens containing straw bedding and infrared heat lamps. Pigs were weaned at 4 wk old and siblings remained group-housed throughout the trial; however, each pig was fed *ad libitum* separately for 5 h (1200-1700 h) daily and intakes were recorded. The pigs were fed standard pellet pig grower diet (Eastern Farmers Co-op, St. John's, NL, Canada) containing typical levels of protein (21%) and NaCl (0.5%). All animals had 24-h *ad libitum* water access and were maintained on a 12-h day-night cycle (lights on 0700 – 1900 h).

5.3.2 Swine body measurements and monthly blood samples:

Serial growth measurements were taken throughout the study. During the milk-feeding phase (birth to 1 mo-old), body weight, snout to rump length, crown to rump and abdominal circumference were measured at least twice weekly; thereafter until the end of

the study, these measurements were made bimonthly. Using jugular venipuncture, monthly blood samples were collected for all animals from 1 to 9 mo-old. Overnight fasted blood samples were collected in EDTA tubes (BD; Franklin Lakes, NJ, USA) and immediately centrifuged at 4 000 x g for 15 min at 4°C for separation and collection of plasma. Lipid assays, as described below, were performed on fresh samples stored at 4°C and the remainder of the plasma was stored at -80°C.

5.3.3 Oral fat tolerance test:

At 9 mo old, the animals underwent surgery for implantation of blood sampling catheters, which were used for the oral fat tolerance test (as well as intravenous glucose tolerance and insulin sensitivity tests – see **Chapter 2**), and for implantation of a radio-telemeter for BP measurement (see **Chapter 3** and **Chapter 4**). Animals were allowed to recover for 4 to 5 days after surgery before any *in vivo* testing began, with the fat tolerance test performed at least 2 days after the diabetes tests, i.e., 8 to 10 days post-surgery. Prior to the fat tolerance test, the animals were fasted overnight for 12 to 14 h. On the day of the test, a 10 mL baseline blood sample (EDTA tube) was collected; immediately thereafter the pigs were given the high fat meal bolus (1.5 g fat/kg body weight), which consisted primarily of margarine (25% of total fat in the meal; Central Dairies, St. John's, NL, Canada) and lard (75% of the total fat in the meal; Loblaw Inc., Toronto, ON, Canada), combined with some ground pig grower diet (about 5% of the total meal bolus) and sucrose (about 0.4% of the total meal; Lantic, Montreal, QC, Canada) added for palatability. The pigs were allowed 2 hours to consume all the meal, and on average the

meal was completely consumed within 1 hour. One hour after the meal was initially offered a blood sample was collected, which was considered as the 1 h time point. Thus, in addition to the baseline sample, blood samples (10 mL) were collected at time 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after the meal was initially given. After each blood sample the catheter was flushed with 3-5 mL non-heparinized saline, and at the end of the experiment the catheter was flushed with 5 mL of 0.2% heparinized saline. Water was available during the test. Each blood sample was immediately separated by centrifugation at 4 000 x g for 15 min at 4°C with the plasma fractions collected and stored at 4°C until the next day when most of the plasma samples were further centrifuged at 15 500 x g for 20 min at 12°C, for separation into chylomicron (CM) and chylomicron-free (CMF) fractions (McAteer, Grimsditch et al. 2003). Plasma, CM and CMF fractions were analyzed for triglyceride (TG) concentrations using an enzymatic assay kit (Diagnostics Chemicals, Saint John, NB, Canada). The TG responses during the fat tolerance test were quantified as the total area under the curve (AUC) – calculated from baseline to final TG measurements for each fraction, and the peak TG, and time to peak TG for each fraction.

5.3.4 Necropsy:

At the end of the study (i.e., 1 month post surgery) the pigs were anaesthetized with 105-mg/kg sodium pentobarbital (Euthanyl, Biomeda-MTC Cambridge ON, Canada) and ventilated and maintained with 0.5 – 1 % halothane gas mixed with oxygen. Internal organs were removed from the animals and samples stored in 10% neutral buffered formalin and/or liquid nitrogen for later analyses. The animals died of exsanguination

after removal of the liver. Blood samples were collected in EDTA tubes and assayed for lipids as described below.

5.3.5 Plasma lipid analyses

Most of the plasma lipid assays were performed on fresh unfrozen (4°C) plasma samples with additional plasma samples stored at –20°C for other analyses (e.g., see **Chapter 4**) and for repeated lipid assays as necessary. Monthly plasma samples were analyzed for concentrations of total cholesterol, high-density lipoprotein (HDL)-cholesterol, and TG were determined using enzymatic reagent kits (Diagnostics Chemicals, Saint John, NB, Canada, and Stanbio Laboratories, Boerne, TX, USA). HDL was precipitated using HDL reagent # 200-26A (Diagnostics Chemicals, Saint John, NB). The concentration for plasma low-density lipoprotein (LDL)-cholesterol was calculated from the concentrations of total cholesterol, HDL-cholesterol and TG (Friedewald, Levy et al. 1972). Additionally, aliquots of blood samples collected at the end of the study (i.e., necropsy) were separated into lipoprotein fractions; plasma was centrifuged at 15 500 x g for 20 min at 12°C to separate any chylomicrons (McAteer, Grimsditch et al. 2003), followed by separation of the infranatant into lipoproteins fractions, i.e., very-low-density lipoprotein (VLDL), LDL and HDL, by sequential density ultracentrifugation (Salter, Mangiapane et al. 1998). Each fraction was analysed for TG and total cholesterol.

5.3.6 Liver lipid analyses:

Lipids were extracted from liver samples using a mixture of methanol and chloroform (2:1, v/v; (Folch 1956)). For the determination of the concentration of lipid in the

samples, aliquots of the lipid extracts were dried and the lipids were re-dissolved using isopropanol, and then analyzed for concentrations of TG and total cholesterol by enzymatic assays as described above for plasma samples.

5.3.7 Aorta plaque assay:

Atherosclerosis begins as deposits of cholesterol and its esters, referred to as fatty streaks, in the intima of large muscular arteries (McGill, McMahan et al. 2000). These fatty streaks have been documented to appear as early as adolescence (McGill, McMahan et al. 2000). Thus to assess fatty streak formation in the pigs in this study, we used a classical process of gross anatomic staining with Sudan IV, based on the method of Holman et al. (Holman, Mc et al. 1958). Briefly, at necropsy, the entire intact aorta (i.e., from the ascending aorta to the abdominal aorta) was removed, rinsed with saline, trimmed of arteries, opened longitudinally along the ventral surface, pinned out flat, and fixed with neutral-buffered formalin (10% v/v). After fixation, to determine if there were atherosclerotic lesions, the intact aorta was gross stained with a Herxheimer's solution, which is a mix of 5 mg/mL Sudan IV Red; 70% (v/v) ethyl alcohol and acetone (1:1, v/v), and then rinsed with 70% (v/v) ethyl alcohol.

5.3.8 Statistical analyses:

Within dietary treatment, the effects of birth weight on plasma and hepatic lipid concentrations were compared using two-way ANOVA (sex and group); if no sex effect was found we reduced the model to a paired-t-test using littermates as pairs. All values are expressed as mean ± SD. A value of $P < 0.05$ was considered significant.

5.4 RESULTS

5.4.1 IUGR swine experienced catch-up growth prior to sexual maturity:

In this study, birth weights of runts averaged about 30% lower than the large littermates, and the weight differences remained significantly lower for the runts until the end of sexual maturity at 7 mo old (Table 5.1). Indeed, as highlighted in Table 5.1, in terms of fractional growth rates, the runts experienced significant catch-up growth before sexual maturity. Furthermore, as documented in Table 5.1, the runts experienced greater relative growth in abdominal circumference as early as 1 mo old. In other terms, from birth until 4 mos old, the runt had significantly greater abdominal circumference per bodyweight. (Table 5.1), and abdominal circumference measurements through the study were correlated with percent visceral fat (measured at the end of study), i.e., $R = 0.76$, $P = 0.004$. The data in Table 4.1 **(Chapter 4)**, showed that after the neonatal period (i.e., >1 mo old) and prior to the end of sexual maturity (i.e., 7 mo old), the average daily feed intake (ADFI) was significantly greater for the runts. However, throughout the study, runts and large littermates demonstrated similar feed efficiency. Thus, these data suggest that catch-up growth in runts was due to greater feed intake. By the end of the study at 10 mo old, there were no significant differences in bodyweights or lengths between the groups, and the runts had more subcutaneous fat compared to the large littermates (0.90 ± 0.19 vs. 0.82 ± 0.17 mm backfat/kg body weight, $P = 0.04$). Although we were able to measure backfat thickness in all the pigs in this study, this was not the case for carcass fat. Due to the very thick, naturally formed hide or 'armour' on the male pigs, it was impossible to grind those carcasses in our grinding equipment; consequently we only

measured carcass fat in the female pigs in this study. Nevertheless, the backfat measurements were verified by chemical analysis of carcass fat in females, showing that backfat measurements were positively correlated to percent carcass fat (R = 0.40, P = 0.05, N = 6). A similar significant correlation was also observed in the Western-style diet study (see **Chapter** 7), where we included carcass fat measurements for both males and females in the correlation (N = 11). (Interestingly, none of the male pigs fed the HSFS diet developed the typical thick outer skin layer as seen in the male pigs fed the regular diet). Furthermore, in this study, we found that backfat and carcass fat content were correlated with some plasma lipid parameters such as total cholesterol (Table 5.2).

5.4.2 IUGR swine experienced alteration in fasted and postprandial plasma lipid parameters:

5.4.2.1 Monthly fasted plasma lipid in Yucatan miniature pigs:

From about 3 mos old until the end of the study at 10 mos old, plasma fasting TG levels were elevated in runts compared to large littermates (Figure 5.1 **A**). At 6 mos old there was a significant inverse correlation between birth weight and plasma TG levels (Figure 5.2). Conversely, throughout the study, there were no differences in the levels of plasma total cholesterol between groups (Figure 5.1**C** and **D**) nor were there any significant correlations between birth weight and total cholesterol concentrations at any time point (data not shown), including at necropsy. At the end of the study, at 10 mos old, further evaluation of the plasma total cholesterol profile in these animals also indicated no difference between runts and large littermates (Figure 5.3).

5.4.2.2 Oral fat tolerance test – postprandial lipid in Yucatan miniature pigs:

Because pigs, like humans, spend a large percentage of their waking hours in the postprandial state, elevated fasting plasma TG may be indicative of delayed TG clearance. Because of the consistently higher fasting plasma TG in the runts relative to large littermates (Figure 5.1), we also performed an oral fat tolerance test. Results of the fat tolerance test revealed that postprandial TG metabolism was altered in the runts compared to the large littermates (Figure 5.4). For the chylomicron plasma fraction, runts had a greater AUC (Figure 5.4**A** and **B**) and greater peak TG concentration (Figure 5.4**C**), but similar time to TG peak, compared to the large littermates (Figure 5.4**D**). As expected, the CMF fractions showed no difference in TG levels between the groups (data not shown).

5.4.3 IUGR influence on hepatic lipid content:

At 10 mos old, the liver TG and total cholesterol contents were significantly higher in the runts compared to the large littermates (Figure 5.5).

At the end of the study at 10 mos old, using the Sudan IV staining technique, we observed no visible evidence of fatty streak formation in the aorta of any of the pigs (data not show).

Table 5.1: Growth rates in the runt and large Yucatan miniature swine.

	Runt	Large	P-value	Runt/ Large
		Body Weight (kg)		
Birth	0.73 ± 0.11	1.11 ± 0.13	<0.0001	0.66
1 mo old (weaning)	4.60 ± 0.53	6.04 ± 1.03	0.007	0.77
4 mo old	29.54 ± 2.69	34.19 ± 2.50	0.013	0.87
7 mo old	55.70 ± 5.37	60.93 ± 3.58	0.113	0.92
9 mo old[1]	66.83 ± 6.83	71.49 ± 4.14	0.243	0.94
		Fractional Growth Rate ($g_* kg$ body $wt^{-1}_* d^{-1}$)		
Birth to 1 mo old	146.3 ± 27.9	116.5 ± 8.6	0.025	1.25
1 to 4 mo old	56.9 ± 6.9	46.2 ± 4.7	0.015	1.24
4 to 7 mo old	10.4 ± 2.2	8.5 ± 3.2	0.260	1.22
7 to 9 mo old[1]	3.7 ± 0.8	4.5 ± 1.2	0.267	0.82
		Crown-Rump Length (cm)		
Birth	24.2 ± 1.7	27.4 ± 1.2	0.0001	0.88
1 mo old (weaning)	44.8 ± 2.5	48.1 ± 1.7	0.020	0.93
4 mo old	84.7 ± 3.6	91.0 ± 3.3	0.020	0.93
7 mo old	104.8 ± 3.0	110.2 ± 4.7	0.051	0.95
9 mo old[1]	107.3 ± 3.9	111.5 ± 3.3	0.097	0.96
		Fractional Growth Rate ($cm_* m$ body $length^{-1}_* d^{-1}$)		
Birth to 1 mo old	28.1 ± 4.1	25.0 ± 3.2	0.004	1.12
1 to 4 mo old	9.9 ± 1.3	9.8 ± 1.0	0.843	1.01
4 to 7 mo old	2.7 ± 0.6	2.3 ± 0.3	0.259	1.17
7 to 9 mo old[1]	2.3 ± 3.2	1.8 ± 3.2	0.068	1.27

Abdominal Circumference (cm)				
Birth	23.4 ± 1.6	28.0 ± 1.4	0.0002	0.84
1 mo old (weaning)	42.1 ± 2.7	46.6 ± 1.9	0.013	0.90
4 mo old	77.7 ± 4.5	81.9 ± 4.6	0.005	0.95
7 mo old	96.3 ± 6.0	100.2 ± 6.4	0.198	0.96
9 mo old[1]	99.2 ± 6.4	102.8 ± 4.8	0.159	0.97
Abdominal Circumference per Body Weight ($cm * kg^{-1}$)				
Birth	32.7 ± 5.3	25.5 ± 3.6	0.004	1.28
1 mo old (weaning)	8.9 ± 0.9	7.5 ± 0.8	0.008	1.20
4 mo old	2.7 ± 0.2	2.4 ± 0.2	0.048	1.10
7 mo old	1.7 ± 0.1	1.6 ± 0.2	0.159	1.06
9 mo old[1]	1.7 ± 0.2	1.6 ± 0.2	0.391	1.04
Fractional Growth Rate ($cm * m * abdominal\ circumference^{-1} * d^{-1}$)				
Birth to 1 mo old	27.1 ± 5.4	21.7 ± 2.7	0.036	1.24
1 to 4 mo old	9.1 ± 1.0	8.4 ± 1.1	0.258	1.08
4 to 7 mo old	2.8 ± 0.5	2.6 ± 0.9	0.561	1.08
7 to 9 mo old[1]	1.2 ± 2.2	1.8 ± 2.7	0.681	0.67

[1]Note – body measurements were not conducted post-surgery, i.e., these measurements were only available from birth to 9 mos old. Each value represents the mean ± SD in n = 6 pigs. Significant differences ($P < 0.05$) between dietary treatments were assessed by paired t-test.

Table 5.2: Relationship between monthly plasma lipid levels and gross body fat at necropsy in Yucatan miniature swine.

Age at plasma lipid measurements	Correlation coefficient(R)	P-value
Backfat at 10 mo to Plasma Cholesterol		
3 mo old	0.545	0.067
5 mo old	0.659	0.020
7 mo old	0.500	0.100
10 mo old	0.360	0.306
Visceral fat at 10 mo to Plasma Cholesterol		
3 mo old	0.661	0.019
5 mo old	0.790	0.002
7 mo old	0.746	0.005
10 mo old	0.624	0.054
Backfat at 10 mo to Plasma Triglyceride		
3 mo old	0.525	0.080
5 mo old	0.162	0.634
7 mo old	0.563	0.057
10 mo old	-0.182	0.614
Visceral fat at 10 mo to Plasma Triglyceride		
3 mo old	0.151	0.639
5 mo old	0.116	0.734
7 mo old	0.211	0.511
10 mo old	-0.304	0.394

N = 12.

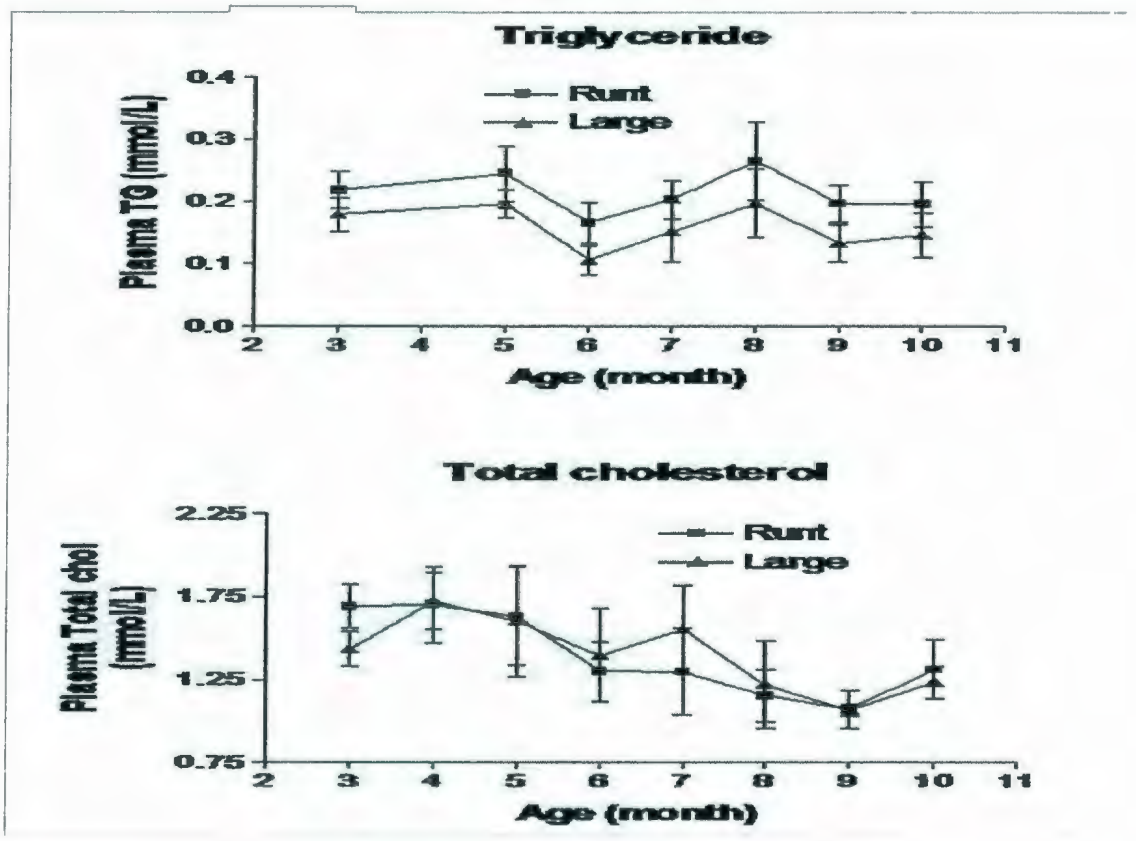

Figure 5.1: Fasting plasma lipid levels over time in Yucatan miniature pigs.

Monthly fasting plasma triglyceride (TG) and total cholesterol concentrations in the groups, from 3 mos old to 10 mos old. Each symbol represents the mean ± SD in n = 6 pigs.

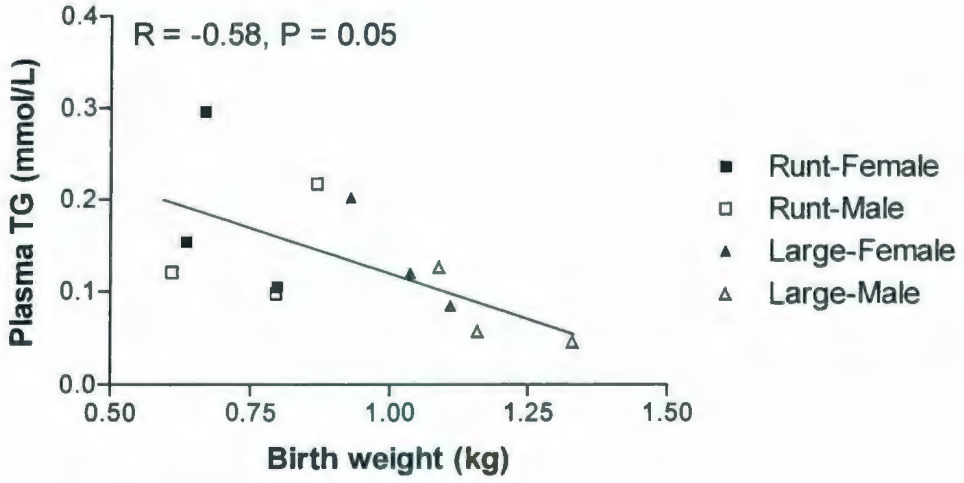

Figure 5.2: The relationship between birth weight and fasting plasma triglyceride in Yucatan miniature pigs at 6 mos old.

Each symbol represents a pig.

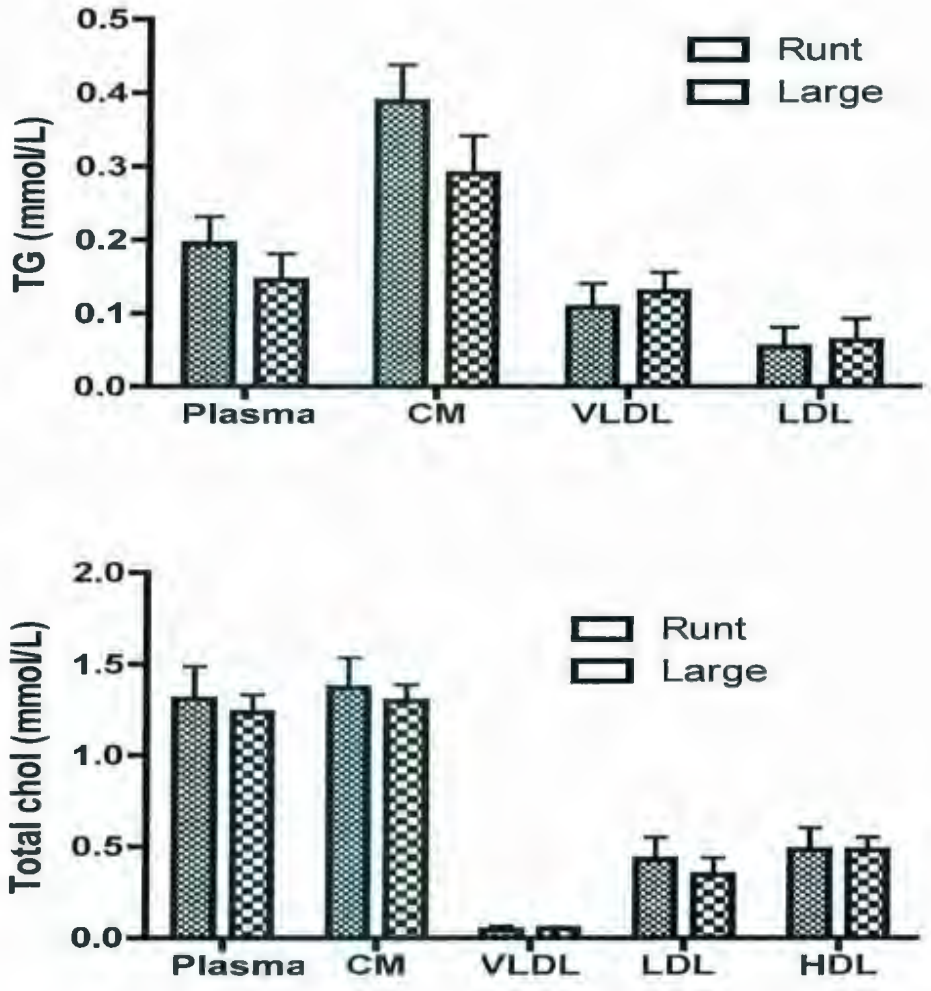

Figure 5.3: Plasma lipid profile in 10 mo old Yucatan miniature pigs.

Plasma sample were separated into lipoprotein fractions: CM = chylomicron; VLDL= very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein. Plasma and lipoprotein fractions were analyzed for triglyceride (TG) [top graph] and total cholesterol (chol) [bottom graph]. Note: no TG was detected in the HDL fractions. Each symbol represents the mean ± SD in n = 6 pigs. No significant differences were observed between groups.

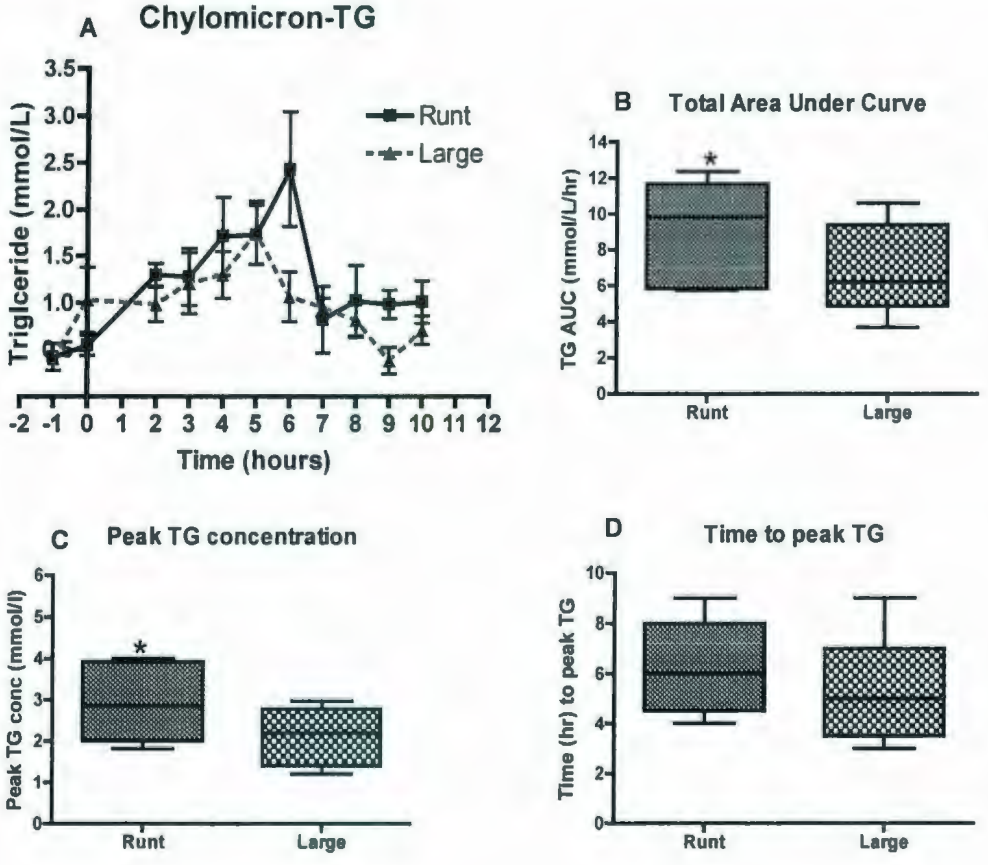

Figure 5.4: The effects on postprandial lipid metabolism in Yucatan miniature swine: the results of oral fat tolerance test in 9.5-mo old pigs.

For Figure A, each symbol represents the mean ± SD in n = 6 pigs. For Figure B-D, the middle line represents the median value for the group (n = 6), the top and bottom of the boxes represent the 25th and 75th percentiles, and the 'whiskers' represent the 10th and 90th percentiles. * = $P < 0.05$. TG = triglyceride; AUC = area under the curve.

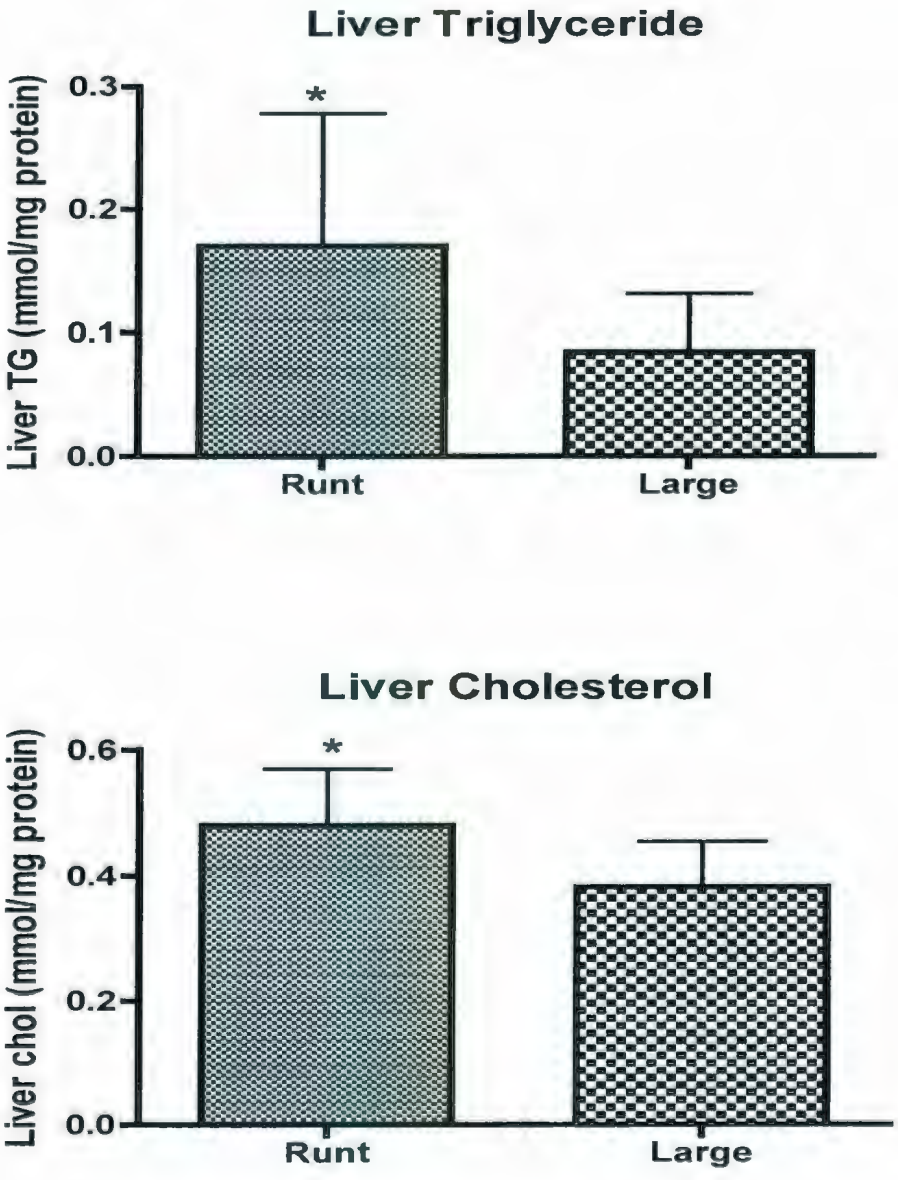

Figure 5.5: Hepatic triglyceride and total cholesterol concentrations in 10-months-old Yucatan miniature pigs.

Each symbol represents the mean ± SD in n = 6 pigs.* = $P < 0.05$.

5.5 DISCUSSION

The major findings of the present work reveal that during early development, fetal/early adaptation in the IUGR Yucatan miniature swine has a deleterious impact on lipid metabolism during adolescence and early adulthood. Small birth weight with early catch-up growth also leads to obesity, which may play a role in the development of dyslipidaemia, possibly via altered hepatic lipid metabolism.

5.5.1 Growth pattern and body composition as a marker of obesity

Research has suggested that the pattern of growth (i.e., restricted fetal growth followed by early postnatal catch-up growth) may be an important contributor to the underlying susceptibility to cardiovascular diseases later in life. In this study *ad libitum* feed intake allowed us to demonstrate that the runts had greater food intake compared to their large littermate siblings, and consequently experienced early catch-up growth (i.e., prior to sexual maturity). In this study, we found that early catch-up growth was associated with increased obesity in the runt Yucatan miniature pig, as measured by backfat thickness. In pigs, backfat thickness measurement is routinely used as an indicator of body fatness because it is highly, positively correlated with whole body or carcass fatness (Doornenbal 1967; Fortin 1985). Indeed, in this study backfat thickness measurement was significantly correlated with chemically determined carcass fatness ($R = 0.40$, $P = 0.05$). Furthermore, epidemiological (Eriksson, Forsen et al. 1999; Nobili, Alisi et al. 2008) and animal (Anguita, Sigulem et al. 1993; Breier, Vickers et al. 2001; Parsons, Power et al. 2001; Ozanne and Hales 2004) studies have shown that rapid catch-up growth following intra-uterine growth restriction leads to increased obesity, which was associated with an

elevation in plasma free fatty acid concentrations as well as other lipids (Remacle, Bieswal et al. 2004; Boden 2008). In humans, serum lipid concentrations are strongly correlated with total body fat content (Choi, Pai et al. 2002) and with anthropometric parameters such as waist circumference (Anderson, Sobocinski et al. 1988). In this study, we similarly found that body fat composition measurements (i.e., backfat and viscera fat) were correlated with some plasma lipid parameters (Table 5.2). Interestingly, although we observed consistently higher TG concentrations in the runts relative to the large littermates, there was no significant relationship between TG levels and body fat content, while the reverse relationship was observed with total cholesterol. This unusual observation should be explored in more details in follow-up studies, after all, evidence suggest that increased body weight (i.e., increased adiposity) is associated with increased TG and HDL cholesterol (Cullen 2000; Choi, Pai et al. 2002).

5.5.2 Plasma lipid metabolism:

Although pigs have lower plasma triglyceride concentrations in comparison to humans (Larsen, Rolin et al. 2002; Olsen, Bladbjerg et al. 2002), in comparison to rats and mice, pigs still represent one of the most relevant species for modeling lipoprotein metabolism in humans (Larsen, Rolin et al. 2002; Sebert, Lecannu et al. 2005). We observed that from 5 mos old until 10 mos old, the runts had consistently higher plasma TG levels relative to the large littermates. Conversely, there was no difference in total cholesterol levels between the groups in whole plasma or any of its fractions (Figure 5.3). However, hepatic lipid analyses revealed significantly higher levels of both TG and total cholesterol in the runts. In the human literature, although researchers have previously reported associations

between plasma lipid concentrations and birth weight, the data are notably inconsistent (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004), with most of the research indicating no association between plasma cholesterol levels and birth weight, similar to what was observed in this study. We did, however, observe significant associations between birth weight and plasma TG concentration. Indeed, two large systematic reviews from human research concluded that birth weight was only consistently associated with TG levels (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004). Because of the inconsistency in the association between lipid profile and birth weight, birth size (i.e., body weight or length) may not be an appropriate marker for assessing the effects of intrauterine conditions on lipid metabolism (Barker 2003). People who were conceived during the Dutch famine had a more atherogenic profile (higher ratio of LDL and HDL) than those who were not exposed to the famine *in utero*, but the atherogenic profile was found to be independent of birth size (Roseboom, van der Meulen et al. 2000). Such observations suggest that developmental programming of lipid metabolism may be more associated with microstructural changes (Ozanne 2001)rather than overt changes in developing organs during fetal/early development.

Body measurements, however, such as abdominal circumference, are said to be a reflection of liver size and/or abdominal fat, and are therefore better markers of CVD risk outcomes (Barker 2003; Lauren, Jarvelin et al. 2003). In this study, at 10 months old, we found no difference in liver size between runts and large littermates (data not shown); suggesting that the increase abdominal circumference in the runts is more indicative of abdominal fat. For instance, epidemiological studies have found that total and LDL

cholesterol levels were not related to birth weight, but were instead strongly inversely related to abdominal circumference at birth (Lauren, Jarvelin et al. 2003). However, in this study we observed no significant association between abdominal circumference at birth and monthly plasma total cholesterol or TG levels (data not shown). We found however that the runts experienced early abdominal circumference 'growth' rate (Table 5.1), and at the end of the study we found that abdominal circumference measurements throughout the study were highly correlated with measured percent visceral fat content ($R = 0.76$, $P = 0.004$), which in turn was significantly correlated to monthly total cholesterol and TG levels (Table 5.2). In terms of CVD risk factors, intra-abdominal fat is a more important risk factor than overall weight (Rimm, Stampfer et al. 1995; Montague and O'Rahilly 2000). Intra-abdominal fat increases the clusters of risk factors associated with the metabolic syndrome (i.e.., glucose intolerance, low HDL-cholesterol concentration, elevated TG, hypertension and obesity) (Reaven 1988; Despres 1993). Although none of the pigs in this project presented with glucose intolerance (data not shown), in this study, we found elevated TG levels and increased obesity in the runts. Furthermore, relative to large littermates, runts had higher blood pressure levels as young adults (**Chapter 4**). Overall, the data suggest that the early increase in abdominal circumference in the runts is partly due to increased deposition of intra-abdominal fat, which could contribute to elevated plasma triglyceride levels observed in the runts.

5.5.3 Postprandial lipid metabolism:

Alterations in plasma lipid and lipoprotein concentrations are associated with an increased risk of CHD, partly through a delay in postprandial TG clearance, leading to the

formation of aortic plaque and subsequent atherosclerosis. Most of the assessment of plasma lipids levels is based on blood sampling conducted in the fasting state (Paglialunga and Cianflone 2007). Although fasting TG levels can often be indicative of delayed postprandial TG clearance, this may not always be the case. Thus, in this study, we performed an oral fat tolerance test to provide a more definitive assessment of postprandial TG clearance.

Postprandial lipid metabolism involves several processes, including lipid digestion, absorption, lipolysis and hepatic remnant clearance (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007). In brief, digestion of most dietary fat requires pancreatic lipase for hydrolysis of the fat into free fatty acids and monacylyglycerols; thus if there is any impairment in the secretion of pancreatic lipase and/or reduction in its activity, this could decrease fat digestion. During absorption, most dietary fatty acids enter the circulation as chylomicrons via the lymph. Once in circulation, lipoprotein lipase acts on the chylomicron to release fatty acids, which are taken up by adipose tissue (storage) or muscle (oxidized), and eventually the liver takes up the chylomicron remnant. Consequently, the main lipid abnormality found in postprandial blood is hypertriglyceridaemia, and the magnitude of the postprandial triglyceridaemic response is said to play a role in the aetiology and progression of CHD (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007).

In this study, the results of the fat tolerance test indicated that the runts had a lower ability to metabolize dietary lipids, probably due to impairment in mechanisms at certain stages of lipid metabolism. First, the similar time to reach peak chylomicron-TG concentrations

for both groups suggested that the runts did not have problems with digestive processes such as pancreatic lipase secretion and/or activity, or packaging of chylomicrons. The packaging of long chain dietary fatty acid into chylomicrons is tightly regulated by microsomal triglyceride transfer protein (MTP) (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007). However, the time to reach peak chylomicron-TG concentration was not significantly different between groups, this suggest that impairment in lipid metabolism in the runts is likely at later stages of lipid metabolism.

Second, the larger area under the curve, and more specifically, the higher peak chylomicron-TG concentrations suggested that the runts had a lower ability to clear circulating chylomicron-TG. In the postprandial state, the clearance of circulating chylomicrons is largely dependent on adipocyte and muscle lipoprotein lipase (LPL) activities (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007); thus, the reduced ability of the runts to clear circulating chylomicron-TG suggest these animals have lower LPL activity relative to their large littermates. This impaired LPL activity could also be coupled with impaired hepatic remnant clearance, possibly due to reduced expression of hepatic ApoE receptors. However, in this study, the results of the fat tolerance test can only suggest these aspects of lipid metabolism are impaired. We conclude that runt pigs have problems clearing postprandial TG, but without further detailed investigation, we are not able to specifically determine which of these mechanisms were impaired in the runts.

The magnitude of postprandial TG concentration and the slow return to fasting state are independent predictor of CHD (Cullen 2000). Thus, with the chronically high fasting and

postprandial (i.e., during the oral fat tolerance test) TG levels in the runts, we investigated whether this would lead to the initiation of arterial fatty streaks, precursors of plaques. However, at the end of the study at 10 mo old, using the Sudan IV staining technique, we observed no visible evidence of fatty streak formation in the aorta of any of these animals, probably because the pigs are still fairly young (i.e., early adulthood), or other possibilities.

5.5.4 Hepatic lipid metabolism:

"Measures related to fetal liver development may be needed to understand the developmental origins of elevated serum cholesterol concentrations" (Barker 2003). Hepatic lipid metabolism has an important influence on overall plasma lipid levels (Nguyen, Leray et al. 2008). Some researchers have suggested that delayed clearance of TG after a high fat meal might be related to the presence of a large pool of endogenous TG in the liver and plasma levels (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007). Indeed, studies have shown a close association between the degree of alimentary lipidemia and fasting plasma TG (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007). In this study, we observed that the runts had prolonged increases in postprandial plasma TG concentrations, as indicated by the oral fat test as well as the monthly fasting plasma TG levels. From 3 mos old until the end of the study at 10 mo old, we found that fasting concentrations of plasma TG were consistently higher for runts compared to large littermates. Moreover, runts also had significantly higher hepatic concentrations of total cholesterol and TG, which may be related to the chronically higher plasma TG levels in runts. Potentially, there could be impairments in lipid metabolism in the liver (e.g., ApoE,

which is synthesised in the liver, is required for the normal catabolism of triglyceride-rich lipoproteins), which would be reflected in higher lipid content and elevated fasting and postprandial TG levels in the runt. Indeed, alterations in lipid metabolism may be due to changes in the structure of the liver (Desai, Byrne et al. 1997; Roberts, Nava et al. 1999; Ozanne 2001; McMillen and Robinson 2005). Therefore, the next step is to analyze the overall liver structure and functions of the pigs in this study to determine if there are difference between runts and large littermates, which could in part explain the higher lipid levels in the runts.

In summary, the results of this study indicate that intrauterine growth restriction followed by early postnatal growth rate leads to increased obesity and altered lipid metabolism in Yucatan miniature swine during adolescence and adulthood. Such altered metabolism seems to involve the latter stages of lipid processing which likely involves the liver as well as the muscle/adipose. The most significant result is the altered triglyceride clearance, as this was in the chylomicron fraction it likely involves LPL which is in muscle/adipose tissue and not a liver enzyme.

Developmental Origins of Cardiovascular Diseases: Effects of a Post-weaning Western-style diet

CHAPTER 6 *Early Programming of Adult Blood Pressure in the Low Birth Weight Yucatan Miniature Pig is Exacerbated by a Post-weaning Western-style Diet*

CHAPTER 7 *Impact of Birth weight and a Post-weaning Chronic High Calorie diet on Lipid Metabolism in Young Adult Yucatan Miniature Swine*

CHAPTER 6. EARLY PROGRAMMING OF ADULT BLOOD PRESSURE IN THE LOW BIRTH WEIGHT YUCATAN MINIATURE PIG IS EXACERBATED BY A POST-WEANING WESTERN-STYLE DIET

6.1 ABSTRACT

***Background and Objectives*:** We have previously shown that blood pressure (BP) is elevated in low birth weight Yucatan miniature pigs, and is negatively associated with nephron endowment. The aim of this study was to evaluate the impact of a post-weaning Western-style diet on fetal/early programming of BP, including the effectiveness of an acute low salt (0.5% NaCl) challenge.

Methods: Runt piglets (<850 g) (N=6) were paired with the largest same sex littermate (>1100 g) (N=6) and taken from the sow at 3 d of age. During the first 4 wk, piglets were fed milk replacer *ad libitum*; thereafter pigs were fed a Western-style diet high in fat, sugar and salt *ad libitum* for 5 h/d. Body weights and feed intakes were recorded throughout the study. Additionally, monthly fasted blood samples were taken from all animals and plasma creatinine and blood urea nitrogen measured. At 11 mo of age, BP telemeters were implanted via the femoral artery to measure continuous BP, heart rate (HR) and locomotor activity in unrestrained pigs fed: 1) high-salt-fat-sugar diet (4.5% NaCl), and 2) after a 7-d standard salt challenge (0.5% NaCl). At the end of the study at 12 mos old, kidneys were removed and nephron numbers and average glomerular size were determined using unbiased stereological techniques.

Results: Prior to sexual maturity (4-7 mo-old), the runts had greater ($P<0.05$) relative feed intake, and experienced significant catch-up growth. Diastolic BP was higher in the runts compared to their larger siblings (107.5 ± 11.3 vs. 96.5 ± 5.6, $P < 0.05$). However, chronic ingestion of a Western-style diet caused systolic BP to be similarly elevated in both runts and large littermates. The runts were also shown to have less nephron numbers when compared to the large littermates ($250,636 \pm 68,852$ vs. $338,662 \pm 44,861$), but glomerular size was not different between the groups. The acute standard salt challenge reduced BP in both groups but there was no difference in the degree of change between runts and large littermates.

***Conclusion*:** The previously observed higher diastolic BP in the naturally occurring low birth weight Yucatan miniature swine was exacerbated by a post-weaning Western-style diet that is high in salt and saturated fat; systolic BP was also elevated as a result of the Western-style diet but no differences between runt and large littermates were observed.

6.2 INTRODUCTION

A poor perinatal environment leads to reduced nephron endowment in the offspring that has a detrimental impact on blood pressure (BP) later in life (see reviews: (Hughson, Farris et al. 2003; Adair and Dahly 2005; McMillen and Robinson 2005; Bagby 2007; Schreuder and Nauta 2007). Additive effects of perinatal environment and postnatal diet may affect the induction of adult chronic diseases such as hypertension. These findings suggest that postnatal dietary factors are important accelerators in the aetiology of these diseases (Petry, Ozanne et al. 1997; Vickers, Breier et al. 2000; Carlstrom, Sallstrom et al. 2007). Indeed, the underlying cause of high BP is multifactorial, but includes factors such as excessive consumption of dietary sodium, which is typical in individuals consuming a Western-style diet. The typical Western diet is high in salt (NaCl), saturated fat and simple carbohydrates, and accounts for over 30% of the world's cardiovascular disease events (AHA 2008; AHA 2008; WHO 2008). Recently, some studies have suggested that dietary salt intake is important for the development of hypertension in those with congenitally reduced nephron endowment (Manning and Vehaskari 2005; Magalhaes, da Silveira et al. 2006; Carlstrom, Sallstrom et al. 2007; de Boer, Ijzerman et al. 2008; Simonetti, Raio et al. 2008). Conversely, there are a few studies that failed to note any association between fetal/early programming and salt sensitivity (Langley-Evans and Jackson 1996; Zimanyi, Bertram et al. 2004); **Chapter 4**). There are potentially many reasons for the discrepancy regarding salt sensitivity and fetal/early programming among these studies. For instance, in our previous study (**Chapter 4**), it is possible that due to their age (young adult), the runt pigs could adequately compensate for the reduced

nephron endowment without any noticeable glomerular compromise that would lead to altered salt sensitivity, in which case, perhaps long-term exposure to a nutritionally poor diet, which is high in salt would have a greater impact on BP and nephron (glomerular) morphology.

In the previous study (**Chapter** 4), we reported significant, but very modest increases in BP values in the runts in comparison to their large littermates, and possibly because of their relatively young age (9.5 mos old), these BP values were still within the normal range. Nevertheless, in that study we found that BP in the low birth weight Yucatan miniature pig was associated with nephron endowment (**Chapter 4**). Impaired nephrogenesis as a result of fetal growth restriction is believed to result in a deficit in the kidney's ability to excrete dietary sodium, thus contributing to the development of hypertension, cardiovascular diseases and renal disease (Brenner and Chertow 1994; Mackenzie, Lawler et al. 1996; Bagby 2007; Schreuder and Nauta 2007). However, in our study (**Chapter 4**), a 7-day acute high salt (4.5 % NaCl) challenge revealed no difference in the degree of salt sensitivity between low and large birth weight pigs. Alternatively, others have suggested that chronic salt loading may be necessary to illustrate the effects of salt on nephron endowment and BP (Carlstrom, Sallstrom et al. 2007). Indeed, research has shown that lifestyle factors, including postnatal nutrition, may influence the delay or hasten the onset of chronic disease outcomes in low birth weight offspring (Vickers, Breier et al. 2000; Armitage, Taylor et al. 2005; Khan, Dekou et al. 2005; Mitra, Alvers et al. 2009). Given the noticeably reduced nephron endowment in the runt Yucatan miniature pigs and its association with BP values in these animals, it is possible that long-

term salt loading may exacerbate BP outcomes. Therefore, in this follow-up study we wanted to examine whether early lifelong exposure to a Western-style diet that was high in salt, as well as saturated fat and sugar, would exacerbate BP outcomes in the runt.

6.3 MATERIALS AND METHODS

All animal procedures described below were approved by Memorial University of Newfoundland's Institutional Animal Care Committee, and carried out in accordance with guidelines set by the Canadian Council on Animal Care.

6.3.1 Animals and experimental protocol:

Twelve (6 females and 6 males) Yucatan miniature pigs from Memorial University swineherd were studied. Specifically, six litters (average litter size was 8 ± 1) from six sows allowed to farrow normally at term (114 ± 3 d) were used. One day after a sow gave birth, the entire litter was weighed. The mean birth weight of all piglets born in the 6 litters used in this study was 0.98 ± 0.12 kg (average body weight per litter range from 0.78 to 1.10 kg). Within each litter, a runt piglet was defined as a piglet weighing less than 850 g. A same sex larger littermate weighing at least 300 g more than the runt was chosen as a littermate control. The runt and larger same sex littermate were taken from the sow at 3 d old. The pair was housed together, but individually provided with rehydrated sow milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) eight to ten times daily *ad libitum*, with intakes recorded. The piglets were housed in pens containing straw bedding and infrared heat lamps. Pigs were weaned at 4 wk old and

remained group-housed (runt and littermate siblings); however, throughout the study, each pig was fed separately for 5 h (1200-1700 h) *ad libitum* daily with intakes recorded. From weaning, at 1 mo old, to the end of the study, the animals were placed on a high-salt-fat-sugar (HSFS) diet. This HSFS diet (Table 6.1) was made by the addition of table salt (40 g/kg; Windsor Co., Windsor, ON, Canada), hydrogenated margarine (50 g/kg; Central Dairies, St. John's, NL, Canada), lard (150 g/kg; Loblaw Inc., Toronto, ON, Canada) and granulated sugar (100 g/kg; Lantic, Montreal, QC, Canada) to standard pig diet (Eastern Farmers Co-op, St. John's, NL, Canada), thus, providing 50% of caloric intake from fat, 40% from carbohydrate and 10% from protein. All animals had 24-h *ad libitum* water access and were maintained on a 12-h day-night cycle (lights on 0700 – 1900 h).

6.3.2 Swine body measurements:

Serial growth measurements were taken throughout the study. During the milk-feeding phase (birth to 1 mo old), body weight, crown to rump length, and abdominal circumference were measured at least twice weekly; thereafter, until the end of the study, these measurements were made twice monthly.

6.3.3 Biochemical analyses:

Using the jugular venipuncture technique, monthly blood samples were collected for all animals from 1 to 11 mo of age. Blood samples were collected in EDTA tubes (BD; Franklin Lakes, NJ, USA) and immediately centrifuged at 4°C for separation and collection of plasma, which was stored at –20°C until later analysis for creatinine and

plasma urea nitrogen (PUN) using enzymatic assays (Bioassay Systems, Hayward, CA, USA).

6.3.4 Radiotelemetric measurement of haemodynamics:

At 11 mo old, with an average weight of 62 ± 13 kg, the animals underwent surgery for implantation of arterial BP telemeter (TA11PA-D70; Data Sciences International, St. Paul, MN, USA) and blood sampling catheters, as previously described (**Chapter 2**). After surgery, each animal was housed individually. The telemetry system (Data Sciences International; St. Paul, MN, USA) was set up to monitor haemodynamics and spontaneous locomotor activity in the pigs, as previously described (**Chapter 2 and Chapter 3**). Individual haemodynamics and activity data were exported from the data acquisition software and transferred to Microsoft Excel 2000® spreadsheet program for calculations (**Chapter 3**). All measured parameters (BP, heart rate and activity) were sampled every 30 s and data analysed over 24-h and 12-h periods (Van Vliet, Chafe et al. 2003).

6.3.5 Experimental Protocols

6.3.5.1 Part 1: Baseline 24-h haemodynamics:

Pigs were allowed at least 8 d recovery from surgery followed by at least 48 h of continuous BP recordings at 30 s intervals, while on the HSFS (4.5% NaCl) diet. Pigs were maintained on a 12-h light-dark cycle (lights on 0700 to 1900 h). The recording room had a radio turned on at a low volume to reduce the impact of environmental noise. Systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure

(MAP), pulse pressure (PP), heart rate (HR) and locomotor activity were recorded for each animal.

6.3.5.2 Part 2: Salt (NaCl) challenge study:

After the 48-h baseline BP recordings the pigs were given a standard salt (0.5% NaCl) version of HSFS for 7 d, with continuous BP recordings for the last 48 h on this diet. At the end of the salt challenge the pigs' diets reverted to the regular HSFS (4.5% NaCl) diet until necropsy.

6.3.6 Necropsy:

At the end of the recording period, all pigs were anaesthetized using an intravenous infusion of sodium pentobarbital and killed by exsanguination with organ removal, as previously described (**Chapter 4**). Their hearts were removed and weighed. Relative cardiac mass was assessed by calculation of the ratio of the cardiac weight to the whole body weight. Both kidneys were removed and weighed; the right kidney was cut in half along its longitudinal axis, immediately frozen in liquid nitrogen and stored at –80°C, while the left kidney was removed and immediately perfuse-fixed with 10% buffered formalin, then immersion-fixed in more formalin until histology was performed for nephron measurements, as previously described (**Chapter 4**).

6.3.7 Statistical analyses:

All values are expressed as mean ± SD. Statistical analyses indicated significant sex effects for most parameters studied. However, because there were only n=3 per sex for each treatment, all results below were reported only as overall (combined male and

female) group means, with sex effect accounted for in the ANOVA-GLM (Minitab v.15.1, Minitab Inc., State College, PA, USA), where the GLM analysis model included treatment, sex and the interaction between the two; reported P-values represent treatment means adjusted for sex effect. Haemodynamic values can be affected by various parameters, thus multiple regression analyses were conducted using birth weight, current weight, kidney weight (and nephron/kidney), ventricle weights, subcutaneous fat measurement, fractional growth rate and activity level as covariates in the GLM analysis (model included treatment, sex and interaction between the two). For within treatment comparisons, statistical comparisons were made by paired t-tests using Prism 4 (GraphPad Software Inc., CA, USA). $P < 0.05$ was considered significant.

6.4 RESULTS

6.4.1 Effects of birth weight on growth and feed intake:

Prior to sexual maturity (between 4 to 7 mo old), the runts remained significantly smaller than the large littermates. However, by 7 mos old, there were no differences ($P > 0.05$) in bodyweights between the groups, indicating that the runts had experienced catch-up growth (Table 6.2). Indeed, as shown in Table 6.2, from 1 to 4 mo old ($P = 0.127$) and from 4 to 7 mo old ($P = 0.068$), the fractional growth rates of the runts tended to be higher than the large littermates. However, this tendency towards higher fractional growth rates for the runts seemed to be the result of greater relative feed intake as opposed to improved feed efficiency (Table 6.2). At the end of the study, at 12 mo old, backfat measurements

showed that the runts had more subcutaneous fat compared to the large littermates (0.95 ± 0.13 vs. 0.84 ± 0.12 mm backfat/kg bodyweight, P = 0.04). Thus, catch-up growth in the runts seemed to include increased adiposity.

6.4.2 Telemeter haemodynamic recordings:

As shown in Table 6.3, even on a post-weaning high-salt-high-calorie diet, at 12 mo of age, the runts had greater (P<0.05) elevation in diastolic BP compared to their larger littermates, with a tendency towards an inverse correlation between birth weight and diastolic BP (P = 0.08, Figure 6.1). However, no differences were observed for systolic BP (Table 6.3) and no association was evident between birth weight and systolic BP (Figure 6.1). As in the previous study (**Chapter 4**), we observed a significant relationship between nephron number and birth weight (Figure 6.1, P <0.05), where the runts had fewer nephrons compared to the large littermates (P < 0.05, Table 6.4). However, the association between nephron number and BP was weak for diastolic BP (i.e., R = -0.55, P =0.06) and absent for systolic BP (i.e., R = -0.12, P = 0.72).

6.4.3 Salt challenge:

The low salt challenge in this study showed that most of the effects of the HSFS diet on haemodynamics were due to the salt content of the HSFS diet. Indeed, 7 d on a standard salt (0.5% NaCl) version of the HSFS diet led to significant decreases in BP values in both runts and large littermates (Figure 6.2, **Column A**). Interestingly, in this study, on both the standard salt (i.e., acute salt challenge) and high salt (i.e., chronic salt challenge) versions of the HSFS diet, the diastolic BP remained more elevated in the runts compared

to the large littermates (Figure 6.2, $P < 0.05$). However, the change in BP as a result of the salt challenge (Figure 6.2, **Column B**) showed that the degree of salt sensitivity was not significantly different between groups, suggesting that acute salt sensitivity is not associated with birth weight in these pigs. Indeed, as in the previous study (**Chapter 4**), there were no significant correlations between the degree of salt sensitivity (i.e., change in BP after the acute salt challenge) and either birth weight (i.e. DAP: $R = -0.36$, $P = 0.25$), or nephron number (i.e. DAP: $R = -0.13$, $P = 0.68$).

Table 6.1: Composition of the high-salt-fat–sugar (HSFS) diet fed to the Yucatan miniature pigs post-weaning.

	HSFS diet (High salt)	HSFS diet (Standard salt)
Energy distribution (% total energy)		
Carbohydrates	40	40
Complex	30	30
Sugar	10	10
Fat	50	50
Protein	10	10
Ingredients (g/ kg DM)		
Wheat shorts	264.3	264.3
Canola	32.3	32.3
Meat meal	12.5	12.5
Limestone	8.58	8.58
Corn gluten feed	26.4	26.4
Ground barley	196.0	196.0
Oats	115.5	115.5
Vitamin mix	0.5	0.5
Mineral mix	0.7	0.7
Lard[€]	150.0	150.0
Margarine[£]	50.0	50.0
Sugar[¥]	100.0	100.0
Salt*	40.0	4.7

HSFS diet = High-salt-fat-sugar diet. HSFS diet was made by the addition of salt* (40 g/kg; Windsor, Clarkson, ON), hydrogenated margarine[£] (50 g/kg; Central Dairies, St. John's, NL),

lard[€] (150 g/kg; Loblaw Inc., Toronto, ON) and granulated sugar[¥] (100 g/kg; Lantic, Montreal, QC) to ground pig grower diet (Eastern Farmers Co-op, St. John's, NL). DM = dry matter.

Table 6.2: Growth and feeding characteristics of the animals studied.

	Runt	Large	P-value	Runt/ Large
Body Weight (kg)				
Birth	0.77 ± 0.09	1.14 ± 0.09	<0.0001	0.68
1 mo old (weaning)	3.87 ± 0.60	5.93 ± 0.50	<0.0001	0.66
4 mo old	15.69 ± 3.28	20.40 ± 1.82	0.021	0.77
7 mo old	35.54 ± 4.58	40.14 ± 3.92	0.152	0.89
12 mo old	61.74 ± 9.95	68.18 ± 15.96	0.439	0.93
Fractional Growth Rate (g• kg body wt^{-1}• day^{-1})				
Birth to 1 mo old	108.4 ± 23.7	115.9 ± 19.3	0.596	0.96
1 to 4 mo old	33.7 ± 7.5	27.3 ± 4.8	0.127	1.24
4 to 7 mo old	15.3 ± 3.1	11.5 ± 2.7	0.068	1.37
7 to 12 mo old	5.4 ± 1.0	4.7 ± 1.6	0.445	1.33
Average Daily Feed Intake (kg body wt $^{-1}$)				
Birth to 1 mo old (mL)	348.6 ± 55.3	372.3 ± 43.8	0.477	0.94
1 to 4 mo old (g)	45.4 ± 3.4	40.1 ± 1.9	0.014	1.14
4 to 7 mo old (g)	30.5 ± 4.1	26.5 ± 3.3	0.075	1.16
7 to 12 mo old (g)	22.4 ± 1.4	20.6 ± 4.2	0.286	1.10
Feed Efficiency (body wt gain (g)• feed (kg) $^{-1}$)				
Birth to 1 mo old	137.5 ± 13.1	127.5 ± 9.4	0.133	1.08
1 to 4 mo old	340.5 ± 35.8	329.6 ± 55.6	0.696	1.06
4 to 7 mo old	299.1 ± 21.8	280.1 ± 31.1	0.166	1.08
7 to 12 mo old	199.8 ± 34.4	190.0 ± 49.9	0.445	1.15

Each value represents the mean ± SD in n = 6 pigs. Significant differences ($P < 0.05$) between dietary treatments were assessed by GLM.

Table 6.3: Summary of basic haemodynamics for the Yucatan miniature pigs on post-weaning high-salt-fat-sugar (4.5% NaCl) diet.

Period	Variable	Runt	Large	P value
24 h	SAP (mm Hg)	151.3 ± 9.9	152.1 ± 6.3	ns (0.879)
	MAP (mm Hg)	129.1 ± 8.8	122.8 ± 4.9	ns (0.120)
	DAP (mm Hg)	107.7 ± 12.1	97.2 ± 4.5	ns (0.060)
	PP (mm Hg)	43.6 ± 14.9	55.0 ± 3.7	ns (0.134)
	HR (beats min^{-1})	94.7 ± 6.2	91.2 ± 5.1	ns (0.185)
Light period	SAP (mm Hg)	150.4 ± 9.6	151.4 ± 7.6	ns (0.867)
	MAP (mm Hg)	128.5 ± 7.6	122.2 ± 5.9	ns (0.092)
	DAP (mm Hg)	107.5 ± 11.3	96.5 ± 5.6	0.042
	PP (mm Hg)	42.9 ± 15.4	54.9 ± 3.1	ns (0.124)
	HR (beats min^{-1})	101.3 ± 8.8	96.1 ± 8.0	ns (0.195)

Dark period	SAP (mm Hg)	152.2 ± 10.5	152.9 ± 8.1	ns (0.907)
	MAP (mm Hg)	129.6 ± 10.4	123.4 ± 5.9	ns (0.215)
	DAP (mm Hg)	107.9 ± 13.7	97.8 ± 4.6	ns (0.110)
	PP (mm Hg)	44.3 ± 14.5	55.1 ± 5.5	ns (0.158)
	HR (beats min^{-1})	88.1 ± 4.9	86.4 ± 3.3	ns (0.471)

SAP: systolic arterial pressure; MAP: mean arterial pressure; DAP: diastolic pressure; HR: heart rate; PP: pulse pressure. Each value represents the mean ± SD in n = 6 pigs. Significant differences between dietary treatments were assessed by GLM with $P < 0.05$ as significant; ns = not significant.

Table 6.4: Renal and cardiac parameters in runt and large littermate Yucatan miniature swine at the end of the study at 12 months old.

	Runt	Large	P-value
Renal Parameters			
Total Kidney wt (kg)	0.153 ± 0.018	0.173 ± 0.028	ns (0.187)
Total kidney/body wt ratio (%)	0.252 ± 0.034	0.258 ± 0.038	ns (0.705)
Nephron number per kidney	250,636 ± 68,852	338,662 ± 44,861	0.016
Average glomerular surface area (μm^2)	23,235 ± 3241	22,249 ± 2,190	ns (0.533)
Average glomerular volume ($\mu m^3 \times 10^6$)	2.91 ± 0.65	2.73 ± 0.40	ns (0.560)
Total glomerular volume ($\mu m^3 \times 10^6$)	66.75 ± 20.30	73.83 ± 13.72	ns (0.530)
Plasma urinary nitrogen (PUN) (mmol/L)	2.0 ± 0.4	1.7 ± 0.3	ns (0.228)
Plasma creatinine (μmol/L)	96.9 ± 12.4	110.0 ± 14.1	ns (0.144)
Cardiac Parameters			
Heart wt (kg)	0.165 ± 0.022	0.178 ± 0.030	ns (0.449)
Heart/body wt (%)	0.270 ± 0.028	0.265 ± 0.017	ns (0.646)
Left ventricle (kg)	0.117 ± 0.019	0.123 ± 0.022	ns (0.655)
Right ventricle (kg)	0.036 ± 0.014	0.042 ± 0.006	ns (0.406)
Right/left ventricle	0.320 ± 0.114	0.344 ± 0.017	ns (0.618)

Each value represents the mean ± S.D. in n= 6 pigs. Significant difference between dietary treatments were assessed by GLM with $P < 0.05$ as significant; ns = not significant.

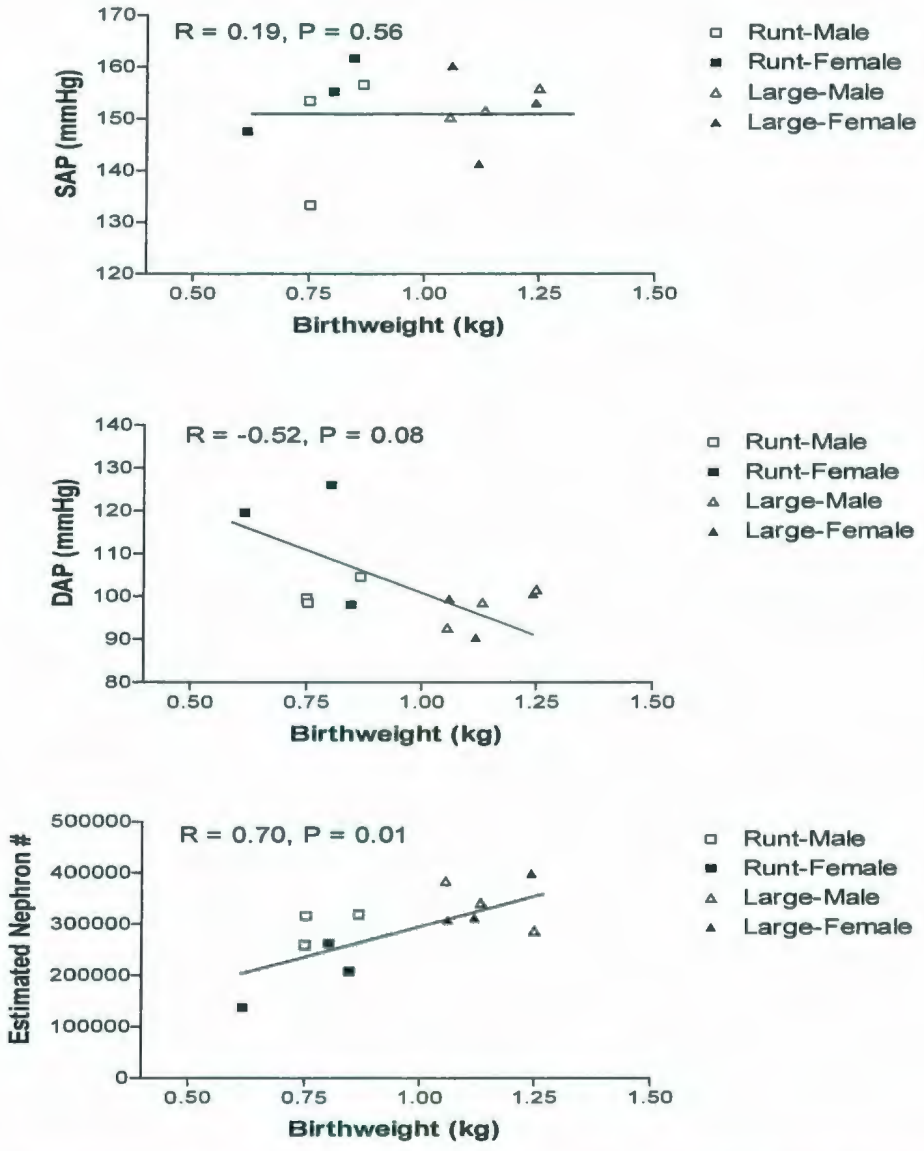

Figure 6.1: Relationship between birth weight and blood pressure and nephron numbers.

Blood pressure parameters were measured at about 12 mos old in all pigs, and then correlated to birth weights. The kidneys were removed from the animals about 3 weeks later and the nephrons counted and then correlated to birth weight. SAP = Systolic arterial

pressure (mmHg); DAP = Diastolic arterial pressure (mmHg). Each symbol represents a pig.

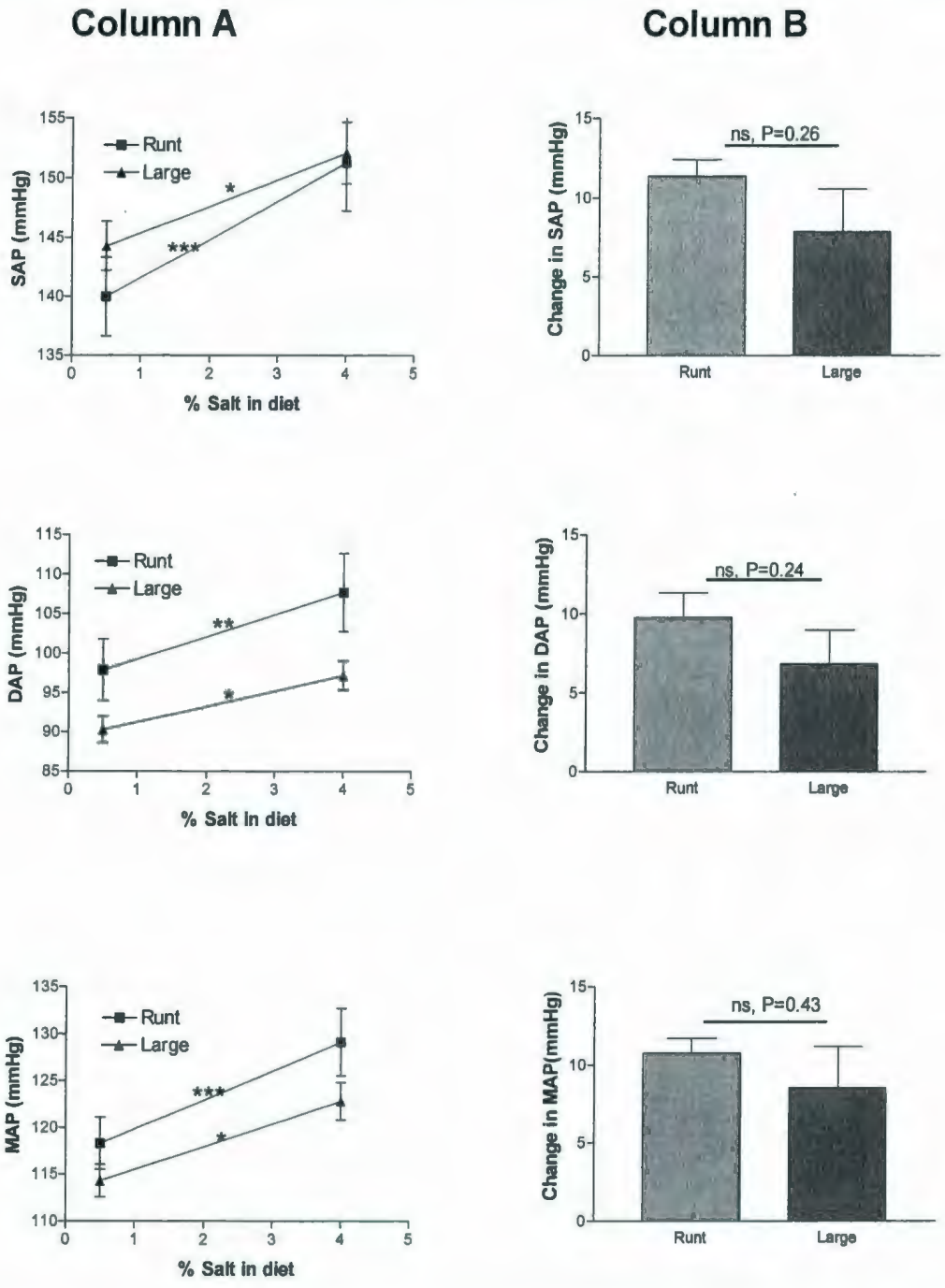

Figure 6.2: Effects of HSFS low salt (0.5% NaCl) diet on blood pressure parameters in runts and large littermates.

Column A: On each graph, the mean BP for the animals on the high salt (4.5% NaCl) version of the HSFS diet, and after 7 d on a low salt (0.5% NaCl) version of the HSFS diet are presented. DAP = Diastolic arterial pressure (mmHg); MAP = Mean arterial pressure (mmHg); SAP = Systolic arterial pressure (mmHg). Each symbol represents the mean ± S.D. in n = 6 pigs. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ indicates a differences with change in salt intake within groups, ns = not significant ($P > 0.05$). **Column B**: Each graph shows the comparison of the difference between diets as the diet changed from 0.5% to 4.5% NaCl for the runts and the large littermates.

6.5 DISCUSSION

Diet plays a pivotal role in the aetiology of CVD and hypertension, which include hypertension. As such, diet can be used to modify the onset and/or degree of hypertension and examine its potential target organ damage (Franco and Oparil 2006). In a previous study (**Chapter 4**), we found that low birth weight piglets had reduced nephron endowment, which correlated to increased BP levels; but, there were only modest increases in BP in the runt pigs and no association between acute salt sensitivity and nephron endowment. Other researchers, however, have reported associations between birth weight and salt sensitive BP (Manning and Vehaskari 2005; Magalhaes, da Silveira et al. 2006; Carlstrom, Sallstrom et al. 2007; de Boer, Ijzerman et al. 2008; Simonetti, Raio et al. 2008). Thus, a question that we wanted to address was whether early exposure to chronic salt loading would lead to greater impact on BP in the low birth weight pig. Therefore, in this study, as part of assessing the effects of birth weight on BP, a post-weaning Western-style diet that was high in salt (4.5% NaCl), as well as fat and sugar, was used to accelerate the onset of hypertension in Yucatan miniature pigs. The major findings of the present work are: 1) runts experienced catch-up growth prior to sexual maturity; and, 2) nephron numbers were significantly reduced in the runts; 3) diastolic BP remained more elevated in the runts when fed a post-weaning high-salt-fat-sugar diet; and 4) runts did not show worsened salt sensitivity in response to an acute lower salt (0.5% NaCl) intake.

6.5.1 Catch-up growth, obesity and blood pressure

Based on bodyweights and fractional growth rates, runt Yucatan miniature pigs experienced catch-up growth prior to sexual maturity (4 to 7mo-old) when fed a Western-style diet. Similar to our previous study using grower diet, this catch-up growth was due to greater feed intake in the runts rather than enhanced feed efficiency (Table 6.2). Furthermore, measurements of body composition collected at the end of the study indicated that the runts were more obese compared to their littermate siblings, suggesting that early catch-up growth in the runt associated with increased obesity. Other researchers have also found increased obesity resulting from early accelerated growth, leading to reduced lifespan in the animals, suggesting that early catch-up growth is a risk factor for the onset of cardiovascular diseases (Ozanne and Hales 2004; Mitra, Alvers et al. 2009). Moreover, some researchers have suggested that intrauterine growth restriction and later obesity are independent risk factors for the development of hypertension (Petry, Ozanne et al. 1997). There have been various postulated mechanisms regarding the contribution of obesity to elevation in BP and target kidney damage. In this study we peripherally address a couple of these hypotheses, namely, hormonal regulation of obesity and hypertension as well as obesity compression of the kidney.

The mechanisms underlying the development of hypertension via obesity are complex, but hyperleptinaemia, hyperinsulinaemia and adipose tissue renin-angiotensin aldosterone system (RAS) are believed to be major contributors (Vickers, Breier et al. 2000; Davy and Hall 2004). In this study, the runts had more ($P < 0.05$) subcutaneous fat compared to the large littermates, and this resulted in significant correlation between

subcutaneous fat and diastolic BP ($R = 0.68$, $P = 0.02$), as well as mean arterial BP ($R = 0.70$, $P = 0.01$). However, fasting plasma insulin levels and intravenous insulin sensitivity tests in these pigs revealed no difference between the groups (data not shown). Unfortunately, we did not measure leptin levels in these pigs; however, others (Poore and Fowden 2004) have found a reduction in plasma leptin concentration in naturally occurring low birth weight domestic pigs (in females as early as 3 mo old and males at 12 mo old) which coincided with greater fat depth in the low compared to the high birth weight pigs. Furthermore, leptin secretion is regulated by hormones such as insulin (Poore, Forhead et al. 2002; Poore and Fowden 2004); thus, in the present study, the greater fat depth combined with the lack of elevation of insulin levels in the runts would suggest that leptin secretion may also not be enhanced in these pigs, although direct measurements of plasma leptin could confirm this assumption. Therefore, hyperinsulinaemia, and possibly hyperleptinaemia are not likely contributors to the elevated BP noted for the runts in this study; of course, further tests are required to draw conclusive results.

Increased metabolic demand of obesity may contribute to glomerular hyperfiltration/hypertrophy and lead to kidney damage such as glomerulosclerosis; this risk becomes substantially greater when compounded with reduced nephron number (Bagby 2007; Griffin, Kramer et al. 2008). Specifically, it has been reported that excessive adipose tissue activates RAS by compressing the medulla, leading to chronic renal vasodilation and an increased glomerular filtration rate (GFR), thereby damaging nephron function (Davy and Hall 2004; Griffin, Kramer et al. 2008). In this study, the similarity in creatinine concentrations between the groups suggested that there was more

likely increased GFR (i.e., nephron hyperfiltration) in the runts, compensating for the lower number of glomeruli (i.e., nephrons). Furthermore, there was no significant difference in glomerular size between the groups, suggesting a lack of enlargement of glomeruli in runts congenitally endowed with less nephrons. Thus, these data suggest that the degree of obesity in the runt pigs did not seem to have resulted in notable compression of the kidneys (i.e. kidney damage such as glomerulosclerosis).

6.5.2 Nephrogenesis and the low birth weight Yucatan miniature pig

In this study, as with a previous study (**Chapter 4**), we observed that there was fetal/early programming of nephrogenesis; because, in both studies, the number of nephrons were significantly lower in the runts compared to the large littermates (Table 6.4), and similar to the previous study, there was a significant association between nephron number and birth weight (Figure 6.1). These data are consistent with the concept that nephrogenesis is completed early in development (Bauer, Walter et al. 2002; Adair and Dahly 2005; Bagby 2007; Schreuder and Nauta 2007). However, in comparison to our previous study (**Chapter 4**), in this study we observed that a post-weaning HSFS diet intensified the overall effects on BP in all of the pigs, but weakened or masked the association between nephron number and BP. Nevertheless, there was still an observable trend ($P = 0.06$) in the correlation between DAP and nephron number. Furthermore, based on the literature (Brenner, Garcia et al. 1988; Guyton 1996), chronic salt loading should result in kidney damage with further reduction in nephrons. However, as mentioned above, we saw no difference in glomerular size between the groups, suggesting that

although impaired nephrogenesis resulted in reduced nephron number in the runts, with some evidence of hyperfiltration, a chronic post-weaning Western-style diet did not seem to lead to progressively greater kidney damage in runt Yucatan miniature pigs in comparison to the large littermates. However, comparison of the glomerular volume in runts in the previous study (on standard grower diet; **Chapter 4**) to runts in the current study (i.e., Western-style diet) indicated ~15% larger glomerular volume for the animals on the Western-style diet (e.g. 2.52 ± 0.53 vs. $2.91 \pm 0.65 \times 10^6$ μm^3, for runts on the standard grower diet and HSFS diet, respectively); large littermates fed the grower diet and HSFS diets also had ~12% greater glomerular volumes (2.43 ± 0.82 vs. $2.73 \pm 0.40 \times 10^6$ μm^3, for large littermates on the standard grower diet and HSFS diet, respectively). Therefore, overall, these data suggest that the Western-style diet could potentially lead to more kidney damage in all the pigs. Understandably, to draw more definitive conclusions about the effects of the Western-style diet on the kidneys in these animals would require more detailed analyses of the kidneys, such as with the expertise of a pathologist who could evaluate any potential kidney damage.

6.5.3 Post-weaning Western-style (HSFS) diet and blood pressure parameters

A post-weaning HSFS diet increased the systolic BP in the large littermates more than it did in the runts, thereby masking the previously observed (**Chapter 4**) effects of early programming on this BP parameter. Such data suggest that post-weaning nutrition has a greater impact on systolic BP than fetal/early programming. In contrast, a post-weaning HSFS diet similarly increased diastolic BP in runts and large littermates, such that the

effect of fetal/early programming on diastolic BP was still evident. Furthermore, DAP remained significantly more elevated in the runts both on the low and high salt versions of the HSFS diet, and there tended to be a correlation between DAP and nephron number, unlike SAP; thus, these data further suggest that fetal/early programming had a more dominant role than post-weaning diet on diastolic BP. Other researchers have also reported differential effects of prenatal versus postnatal diet environment on systolic and diastolic BP (Khan, Dekou et al. 2005). Khan et al. (2005) found that both systolic and diastolic BP were elevated in rat offspring that were exposed to a fat-rich diet only during gestation. Conversely, regardless of birth weight, only the systolic BP was elevated in rats that were given a fat-rich diet during the postnatal period only. Thus, those data, as with our data, would suggest that diastolic BP is modulated by the intrauterine environment (i.e., fetal undernutrition resulting in low birth weight), unlike systolic BP, which seems to be more associated with postnatal diet. This may not be surprising considering that there are different mechanisms regulating systolic and diastolic blood pressure (Guyton 1996; Beevers, Lip et al. 2001; Tin, Beevers et al. 2002).

The BP recorded while the heart muscle is contracting is the systolic pressure, whereas the BP recorded in between heart muscle contractions is the diastolic pressure. In humans, hypertension in most individuals below the age of 65 tends to be due to elevation in diastolic pressure. Although these people may also most often have elevation in systolic pressure, it is believed that it is the chronic elevation in diastolic pressure which is more detrimental. That is, elevation in diastolic pressure is what eventually causes damage in organs such as the heart and kidneys (Banegas, de la Cruz et al. 2002; Tin, Beevers et al.

2002). Systolic hypertension is believed to be mostly due to hardening and loss of elasticity of the major arteries, which was thought to be unavoidable with age (Tin, Beevers et al. 2002). Considering the high fat (saturated and trans fat) content of the HSFS diet, it is feasible that chronic exposure to the fat component, in addition to the high salt component, could contribute to increased systolic BP in all of the animals, due to diet-related impairment in the vascular system (Martyn, Barker et al. 1995; Hoet, Ozanne et al. 2000; Martyn and Greenwald 2001). In spite of the lack of any plaque formation in any of our pigs, further studies are required to ascertain any association between diet and the vascular function in these pigs.

6.5.4 Acute versus chronic salt sensitivity of blood pressure

The diet for this study was designed to evaluate the general effects of a poor post-weaning diet on the fetal origins of the later development of cardiovascular disease. The general composition mimics the average Western-style diet, which tends to be high not only in saturated fats, but also in sugar and salt. Interestingly, the acute standard salt (0.5% NaCl) challenge at the end of this study showed that most of the effect of the HSFS diet on BP was due to the salt content of the HSFS diet. Comparison of the BP values on the 7-day low salt challenge to the previous study with pigs on chronic standard diet with the same salt content (**Chapter 4**) showed that more than 95% of the BP values observed in the pigs in this study were due to the salt content of this Western-style diet. That is, BP values on the standard salt (0.5% NaCl) version of this diet during the salt challenge (e.g., MAP: 118.3 ± 6.9 and 114.3 ± 4.3 mmHg for runts and large littermates, respectively) were similar to the BP values we observed in the previous study using standard (0.5%

NaCl) grower diet (e.g., MAP: 115.7 ± 7.2 and 110.6 ± 8.0 mmHg for runt and large littermates, respectively) (**Chapter 4**). Indeed, this conclusion is supported by an elegant study done in rats (Song, Hu et al. 2004), where the researchers separately examined the effects of dietary fat, NaCl, sugar, and combination of these components on systemic BP, and found that NaCl had the single most significant effect on BP, followed by the fat content of the diet.

In this study, as in our previous study (**Chapter 4**), an acute salt challenge did not show a greater (or lesser) degree of salt sensitivity in the runts. However, as mentioned above, chronic salt loading resulted in persistently higher diastolic BP in the runts. There are different forms of salt sensitivity of BP, as evident by the wide range of time periods over which salt-induced changes in BP have been noted to occur (Weinberger 1996; Obarzanek, Proschan et al. 2003; Farquhar, Paul et al. 2005; Van Vliet, Chafe et al. 2006; Van Vliet and Montani 2008). Changes in dietary salt intake can lead to rapid changes in BP within days to several weeks, known as acute salt sensitivity. At the other end, ingestion of high salt may require a much longer time period (i.e., months to years) before more permanent changes in BP are noted, also known as chronic salt sensitivity. Both forms of salt sensitivity, and the possible interaction between them, are suggested to contribute to systemic hypertension (Van Vliet, Chafe et al. 2006; Van Vliet and Montani 2008; Montani and Van Vliet 2009). Acute and chronic salt sensitivity of blood pressure may be controlled by different mechanisms (Van Vliet, Chafe et al. 2006; Van Vliet and Montani 2008; Montani and Van Vliet 2009). Certainly, there are many mechanisms that appear to contribute to salt sensitivity, including blunted activity of the renin-angiotensin

aldosterone system, deficiency in expression of arterial natriuretic peptide and blunted arterial baroreflex sensitivity (Strazzullo, Barbato et al. 2001; Franco and Oparil 2006). However, ultimately, an acute or chronic increase in BP is said to be due to alterations in cardiac output or peripheral vascular resistance or both (Farquhar, Paul et al. 2005). In most instances, the underlying mechanism of salt-induced increases in BP is said to be related to an expansion of extracellular volume due to an increase in salt ingestion (known as volume-loading hypertension) (Guyton, Coleman et al. 1972; Guyton, Coleman et al. 1972; Guyton 1996). In short-term salt loading experiments that range from days to weeks, the initial rise in BP is said to be attributed to a volume-induced increase in cardiac output, followed by a secondary increase in peripheral vascular resistance (Guyton, Coleman et al. 1972; Guyton, Coleman et al. 1972; Guyton 1996). Although it is possible that these mechanisms are operative in the present study, we did not assess extracellular or plasma volume. However, because the chronic salt ingestion from the HSFS diet led to acutely reversible BP levels after only 7 days on the low salt version of the diet, this might suggest that these pigs did not yet develop the irreversible form of chronic salt sensitivity; although more studies designed to specifically examine this are needed.

In this study, one of our proposed hypotheses was that early lifelong exposure to a high salt diet would lead to early onset of hypertension with target organ damage to the kidneys. However, as noted earlier in the discussion, chronic salt ingestion, as part of the Western-style diet, did not result in notable kidney damage in any of the pigs in this study, when compared to the previous study (**Chapter 4**).

6.5.5 Summary

In summary, feeding a post-weaning Western-style diet, which is high in salt and saturated and trans fats, suggested that diastolic BP is prenatally programmed in Yucatan miniature swine whereas systolic BP is more sensitive to postnatal diet. Systemic hypertension is said to be determined more by diastolic BP rather than systolic BP (Tin, Beevers et al. 2002), and the evidence from this study supports that theory for the Yucatan miniature pig; that is, there was a consistent association of diastolic BP to various parameters (e.g., nephron number and salt challenge), unlike systolic BP. Therefore, overall, the data from this study suggest that in comparison to a post-weaning Western-style diet, fetal/early programming still had the greater impact on long-term BP regulation.

CHAPTER 7. THE IMPACT OF BIRTH WEIGHT AND A POST-WEANING CHRONIC HIGH CALORIE DIET ON LIPID METABOLISM IN YOUNG ADULT YUCATAN MINIATURE SWINE

7.1 ABSTRACT

Background and Objectives: Part of the increased prevalence of cardiovascular diseases in industrialized societies is attributed to nutritionally poor diets that are high in fat, refined sugar and salt. Furthermore, the 'fetal origins hypothesis' suggests that the fetal nutritional environment contributes to the risk of developing chronic metabolic diseases in adulthood. We have previously observed altered lipid metabolism in low birth weight Yucatan miniature pigs due to fetal/early 'programming' events. In this follow-up study, we investigated the impact of a post-weaning chronic high-fat-sugar-salt diet on this early programming of dyslipidaemia in this pig model.

Methods: Runt piglets (<850 g) (n=6) were paired with the largest same sex littermates (>1100 g) (n=6) and taken from the sow at 3 d old. During the first 4 weeks, piglets were fed milk replacer *ad libitum*; thereafter pigs were fed a high-fat-sugar-salt diet *ad libitum* for 5 h/d. From 1-mo old to the end of the study at 12 mo old, monthly blood samples were collected and lipid assays performed. At 11 mos old, pigs were surgically fitted with venous catheters and a blood pressure telemeter. Following recovery

from surgery, an oral fat tolerance test was performed to assess lipid metabolism. At the end of the study, tissues were collected after an overnight fast.

Results: The runts demonstrated catch-up growth in body weight and length prior to sexual maturity at 7 mos old. From 3 to 6 mo old, both groups demonstrated a significant ($P < 0.05$) decline in plasma triglyceride (TG) levels over time. Throughout the study, plasma TG levels remained more elevated in the runts in comparison to the large littermates; however, there were no differences in plasma cholesterol levels between groups. The plasma TG area-under-the-curve from the oral fat tolerance test was significantly greater ($P < 0.05$) for the runts, suggesting that postprandial lipid metabolism was impaired in the runt relative to the large littermates even on this high-salt-fat-sugar diet.

***Conclusion*:** The effects of fetal/early programming persist despite a post-weaning chronic diet high in trans and saturated fats demonstrating that intrauterine growth restriction, along with early catch-up growth are implicated in the impaired lipid metabolism in this Yucatan miniature swine model.

7.2 INTRODUCTION

The typical Western diet is rich in saturated and trans fatty acids, refined sugars and salt, and has been implicated as the underlying contributor to many chronic metabolic diseases (Cordain, Eaton et al. 2005)For instance, poor lifestyle choices such as high dietary fat intake, especially saturated fatty acids, are known to be at least partly responsible for the burden of obesity and cardiovascular diseases (CVD) in industrialized societies (Hill, Melanson et al. 2000; Hu, Manson et al. 2001; Jakobsen, O'Reilly et al. 2009)Recent efforts aimed at trying to understand the cause for the continued rise in obesity and other metabolic diseases have shifted to a focus on the role of developmental contributions (Barker and Osmond 1986; Barker, Osmond et al. 1989; McMillen and Robinson 2005), including the interactions between fetal/early development and postnatal diets (Vickers, Breier et al. 2000; Khan, Dekou et al. 2005; Jimenez-Chillaron, Hernandez-Valencia et al. 2006; Mitra, Alvers et al. 2009). Unfortunately, this focus is almost exclusively on the effects of maternal nutrition status (under- or over-nutrition) on the fetal or neonatal nutritional environment (Frazier, Mason et al. 2008). Although the perinatal environment is critical, the postnatal and childhood nutritional environments are proving to be just as important for the long-term determinants of risk factors related to the onset of chronic adult diseases.

The predictive adaptive response hypothesis proposes that peri-and post-natal nutrition status developmentally programs the organism's adult metabolic state (Gluckman, Hanson et al. 2005).This window of developmental programming, however, is not clearly

defined. For instance, although the childhood stage is a period of rapid growth and development, there is little research on whether a developmental window remains open during childhood and adolescence allowing nutritional experiences during this time to substantively shape the subsequent adult metabolic state (Frazier, Mason et al. 2008).. After all, children's diets in contemporary Western society tend to be replete with higher fat and sugar foods, as well as poor in nutrient density, and such diets contribute to obesity (Moreno and Rodriguez 2007; Frazier, Mason et al. 2008). Yet, despite this evidence, most studies that examine the interactions between fetal/early development and postnatal diets tend to be more focused on infant nutrition rather than the effects of dietary experiences after weaning.

In a previous study (**Chapter 5**) we found that intrauterine growth restricted Yucatan miniature pigs (i.e., runts) that underwent early catch-up growth developed impaired lipid metabolism. Thus, the aim of this study was to investigate the impact of both birth weight (i.e., fetal/early programming) and a post-weaning chronic high calorie diet on lipid metabolism and evidence for CVD. That is, will a post-weaning diet that is high in saturated fat and refined sugar exacerbate the effects of fetal/early programming?

7.3 MATERIALS AND METHODS

All animal procedures described below were approved by Memorial University Animal Care Committee, and carried out in accordance with the Canadian Council on Animal Care Guidelines.

7.3.1 Animals and experimental protocol:

The same animals used in **Chapter 6** we also used for the experiments in this chapter. That is, 6 pairs (3 females and 3 males) of Yucatan miniature pigs from 6 sows from the Memorial University of Newfoundland swineherd were studied. At 3 d old, the runt and the larger same sex littermate were taken from the sow, housed together, but individually fed rehydrated sow milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) eight to ten times daily *ad libitum*, with intakes recorded. Pigs were weaned at 4 wk old and remained group-housed in terms of runt, large littermate and sow-fed siblings; however, throughout the study, each pig was fed separately for 5 h (1200-1700 h) *ad libitum* daily and intakes recorded. At weaning the animals were placed on a high-salt-fat-sugar (HSFS) diet (see **Chapter** 6 for diet details). All animals had 24-h *ad libitum* water access and maintained on a 12-h day-night cycle (lights on 0700 – 1900 h).

7.3.2 Swine body measurements and monthly blood samples:

Body weight, body length and abdominal circumference were routinely measured throughout the study. Monthly overnight fasted blood samples were collected in EDTA tubes (BD; Franklin Lakes, NJ, US) and immediately centrifuged at 4000 x g for 15 min at 4°C for separation and collection of plasma. Plasma samples were analyzed for lipids as described below.

7.3.3 Oral fat tolerance test:

At 11 mos old, the pigs underwent surgery for implantation of blood sampling catheters and blood pressure radio-telemeter (see **Chapter 2** and **Chapter 3** for details). One week after surgery, an oral fat tolerance test was performed in these pigs - see **Chapter 5** for a

detailed description of the test and the high fat meal bolus. Whole plasma, plasma-chylomicron and chylomicron-free fractions were analyzed for TG concentrations using an enzymatic assay kit (Diagnostics Chemicals, Saint John, NB, Canada). The TG responses were quantified as the total area under the curve (AUC) – calculated from baseline to final TG measurements for each fraction and the peak TG levels and time to reach peak TG levels for each fraction.

7.3.4 Necropsy:

One month post surgery (i.e., 12 mos old) the pigs were anaesthetized with 105 mg/kg sodium pentobarbital (Euthanyl, Biomeda-MTC Cambridge ON, Canada) and ventilated and maintained with 0.5 – 1% halothane gas mixed with oxygen. Internal organs were removed from the animals and samples stored in 10% neutral buffered formalin and/or liquid nitrogen for later analyses. The animals died of exsanguination upon removal of the liver. Blood samples were collected in EDTA tubes and assayed for lipids as described below.

7.3.5 Lipid analyses:

Monthly plasma samples were analysed for total cholesterol, high-density lipoprotein (HDL)-cholesterol, and TG using enzymatic reagent kits (Diagnostics Chemicals, Saint John, NB, Canada, and Stanbio Laboratories, Boerne, TX, USA). HDL was precipitated using HDL reagent # 200-26A (Diagnostics Chemicals, Saint John, NB). The concentration for plasma low-density lipoprotein (LDL)-cholesterol was calculated from the concentrations of total cholesterol, HDL-cholesterol and TG (Friedewald, Levy et al.

1972). Additionally, aliquots of blood samples collected at the end of the study were separated into chylomicron (McAteer, Grimsditch et al. 2003), very low density lipoprotein (VLDL), LDL and HDL fractions (Salter, Mangiapane et al. 1998), and each fraction was analyzed for TG and total cholesterol.

Lipids were extracted from homogenized liver, carcass and viscera samples using a mixture of methanol and chloroform (2:1, v/v; (Folch 1956)). Liver lipid was analyzed for levels of TG and total cholesterol.

7.3.6 Gross staining for evidence of aortic plaque:

Similarly to **Chapter 5**, in this study at 12 mo old, the entire intact aorta was removed and stained with Sudan IV using the method of Holman et al. (Holman, Mc et al. 1958).

7.3.7 Statistical analyses:

Within dietary treatment, the effects of birth weight on plasma and hepatic lipid concentrations were compared using two-way ANOVA (sex and group); if no sex effect was found we reduced the model to a paired-t-test using littermates as pairs. All values are expressed as mean ± SD. A value of $P < 0.05$ was considered significant.

7.4 RESULTS

7.4.1 Early programming and the effects of a post-weaned Western-style diet on growth in the Yucatan miniature runt.

As shown in Table 7.1, during the neonatal period (i.e., birth to 1 mo old) body weight and length remained significantly lower for the runts relative to their large littermate siblings, and these differences remained significant until sexual maturity, which occurs between 4 to 7 mos old. By the end of the study at 12 mo old, the runts had more subcutaneous fat (0.95 ± 0.13 vs. 0.84 ± 0.12 mm backfat/kg body weight, $P = 0.04$), with a positive correlation between backfat thickness measurements and carcass fat content ($R = 0.64$, $P = 0.04$, $N = 11$ (missing 1 sample)). The runts also experienced early abdominal 'catch-up growth' (Table 7.1), with significant correlations between abdominal circumference and visceral fat ($R = 0.59$, $P = 0.04$) at the end of the study at 12 mo old. Furthermore, abdominal circumference was also correlated to backfat thickness ($R = 0.87$, $P = 0.0002$) and to carcass fat content ($R = 0.67$, $P = 0.02$) validating abdominal circumference as a measure of obesity in pigs; however, there was no difference in visceral fat content between runts and large littermates (both at 32%) at 12 mo old. Interestingly, there were significant correlations between visceral fat content and monthly total cholesterol levels, which started at 4 mo old ($R = 0.64$, $P = 0.03$) and continued until 12 mo old ($R = 0.80$, $P = 0.002$), and some significant correlations were observed between measures of abdominal circumference and plasma lipid levels (e.g., at 6 mo old, abdominal circumference was positively correlated to total cholesterol levels ($R = 0.75$, $P = 0.03$) and to TG ($R = 0.74$, $P = 0.03$)). However, throughout the study, there were no differences ($P > 0.05$) in total cholesterol levels between groups (Figure 5.1). In contrast, visceral and carcass fat contents were not correlated to monthly TG levels, and neither carcass fat nor backfat measurements were related to monthly total cholesterol levels (data not shown).

7.4.2 Fasted and postprandial plasma lipids:

In comparison to the results seen in **Chapter 5** with a control diet, the post-weaning consumption of a high-salt-fat-sugar diet resulted in about 70% elevation in fasting plasma TG levels in all the pigs. Interestingly, as documented in Figure 5.1, from 3 to 6 mo old, there was a significant ($P = 0.001$) steady decline in plasma TG levels overtime in both groups, which plateau at 6 mo old (i.e., $P = 0.31$ for TG level over time from 6 to 12 mo old). However, in comparison to the large littermates, TG levels remained more elevated in the runts throughout the study (Figure 5.1); this included a trends for an inverse relationships between birth weight and plasma TG concentrations (e.g., at 5 mo old, $R = 0.52$, $P = 0.08$). However, throughout the study, there were no significant differences in total cholesterol levels between groups. Oral fat tolerance test results (Figure 7.2), suggested that there might have been impairment in postprandial lipid metabolism in the runts relative to the large littermates; however, there was no significant difference between runts and large littermates (6.11 ± 2.58 vs. 4.30 ± 0.58 mmol/L/h, $P = 0.16$). Furthermore, there were no differences in peak TG concentrations (Figure 7.2**C**) or in time to reach TG peak concentrations (Figure 7.2**D**). There were also no differences in CMF-TG (data not shown) data from the fat tolerance test. In addition, lipoprotein cholesterol levels at 12 mo old (Table 7.2) did not differ between groups.

7.4.3 IUGR and hepatic lipid content:

At 12 mo old, there was no difference in liver size between the runts and large littermates whether expressed in absolute size (1.0 ± 0.1 vs. 1.2 ± 0.3 kg, P = 0.30), or when expressed relative to body weight (17.1± 3.4 vs.17.4 ± 5.3 g liver/kg body weight, P = 0.85), and there were no differences in hepatic TG or total cholesterol levels between runts and large littermates (Table 7.2).

Table 7.1: Growth parameters in the Yucatan miniature swine fed a high-fat-sugar-salt diet.

	Runt	Large	P-value	Runt/ Large
Body Weight (kg)				
Birth	0.77 ± 0.09	1.14 ± 0.09	<0.0001	0.68
1 mo old (weaning)	3.87 ± 0.60	5.93 ± 0.50	<0.0001	0.66
4 mo old	15.69 ± 3.28	20.40 ± 1.82	0.021	0.77
7 mo old	35.54 ± 4.58	40.14 ± 3.92	0.152	0.89
11 mo old	59.59 ± 8.07	64.37 ± 10.87	0.357	0.97
Fractional Growth Rate (g_{*} kg body $wt^{-1}{}_{*} d^{-1}$)				
Birth to 1 mo old	108.4 ± 23.7	115.9 ± 19.3	0.596	0.96
1 to 4 mo old	33.7 ± 7.5	27.3 ± 4.8	0.127	1.24
4 to 7 mo old	15.3 ± 3.1	11.4 ± 2.7	0.068	1.37
7 to 11 mo old	5.4 ± 1.2	4.7 ± 1.6	0.319	1.36
Crown-Rump Length (cm)				
Birth	24.0 ± 1.3	27.7 ± 2.0	0.007	0.87
1 mo old (weaning)	41.9 ± 2.5	49.0 ± 2.6	0.011	0.86
4 mo old	71.5 ± 6.8	78.3 ± 3.8	0.065	0.89
7 mo old	94.8 ± 4.2	97.7 ± 5.7	0.192	0.98
11 mo old	111.0 ± 8.4	114.8 ± 5.1	0.183	0.97
Fractional Growth Rate (cm_{*} m body $length^{-1}{}_{*} d^{-1}$)				
Birth to 1 mo old	24.8 ± 3.4	25.6 ± 3.7	0.764	0.97
1 to 4 mo old	7.3 ± 2.3	6.5 ± 0.3	0.704	1.13
4 to 7 mo old	3.8 ± 1.2	2.8 ± 0.7	0.132	1.37
7 to 11 mo old	1.4 ± 0.2	1.6 ± 0.2	0.421	0.86

Abdominal Circumference (cm)				
Birth	24.5 ± 0.9	28.2 ± 1.6	0.008	0.87
1 mo old (weaning)	38.6 ± 2.2	43.5 ± 2.1	0.016	0.89
4 mo old	65.3 ± 6.8	69.8 ± 3.0	0.166	0.93
7 mo old	80.6 ± 5.4	85.6 ± 3.7	0.091	0.94
11 mo old	90.8 ± 5.4	93.2 ± 5.3	0.738	0.98
Abdominal Circumference per Body Weight ($cm * kg^{-1}$)				
Birth	32.1 ± 4.2	24.7 ± 1.7	0.002	1.30
1 mo old (weaning)	10.0 ± 1.0	7.4 ± 0.5	0.004	1.37
4 mo old	4.2 ± 0.6	3.4 ± 0.3	0.038	1.22
7 mo old	2.3 ± 0.2	2.1 ± 0.1	0.178	1.06
11 mo old	1.9 ± 0.3	1.6 ± 0.1	0.083	1.16
Fractional Growth Rate ($cm * m * abdominal\ circumference^{-1} * d^{-1}$)				
Birth to 1 mo old	19.8 ± 2.1	18.5 ± 3.2	0.428	1.07
1 to 4 mo old	7.3 ± 1.8	6.6 ± 0.7	0.498	1.10
4 to 7 mo old	2.9 ± 0.5	2.6 ± 0.5	0.408	1.12
7 to 11 mo old	1.1 ± 0.3	0.9 ± 1.1	0.751	1.30

[1]Note – swine body measurements were not collected post surgery; therefore these measurements were only available from birth to 11 mos old. Each value represents the mean ± SD in n = 6 pigs. Significant differences ($P < 0.05$) between dietary treatments were assessed by paired t-test.

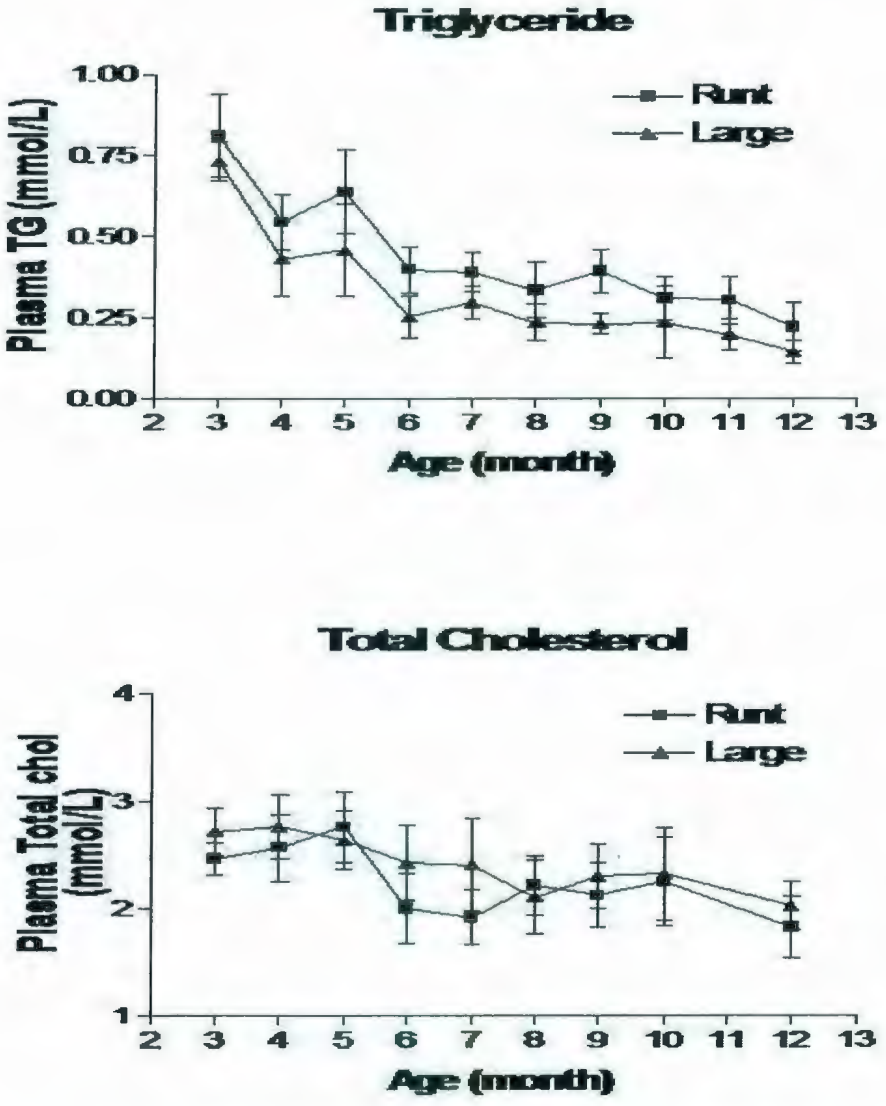

Figure 7.1: Fasting plasma lipids over time in Yucatan miniature pigs fed a high-fat-sugar-salt diet.

Monthly fasting plasma triglyceride (TG) and total cholesterol concentrations in the runts and large littermates, from 3 to 12 mos old. Each symbol represents the mean ± SD in n = 6 pigs.

Table 7.2. Summary of lipid profile at 12 mos old of runt and large littermate Yucatan miniature pigs fed a high-fat-sugar-salt diet.

Lipid Component	Runt	Large
Plasma total cholesterol	1.83 ± 0.69	2.03 ± 0.55
Chylomicron cholesterol	1.89 ± 0.70	2.12 ± 0.67
Plasma VLDL cholesterol	0.13 ± 0.13	0.11 ± 0.19
Plasma LDL cholesterol	0.47 ± 0.18	0.51 ± 0.16
Plasma HDL cholesterol	0.36 ± 0.17	0.39 ± 0.23
Plasma triglyceride	0.17 ± 0.15	0.20 ± 0.16
Chylomicron triglyceride	0.41 ± 0.35	0.50 ± 0.34
Plasma VLDL triglyceride	0.07 ± 0.04	0.14 ± 0.04
Plasma LDL triglyceride	0.02 ± 0.01	0.08 ± 0.09
Liver triglyceride	0.17 ± 0.07	0.30 ± 0.23
Liver total cholesterol	0.44 ± 0.20	0.46 ± 0.25

VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein. Each value represents the mean ± SD in n = 6 pigs.

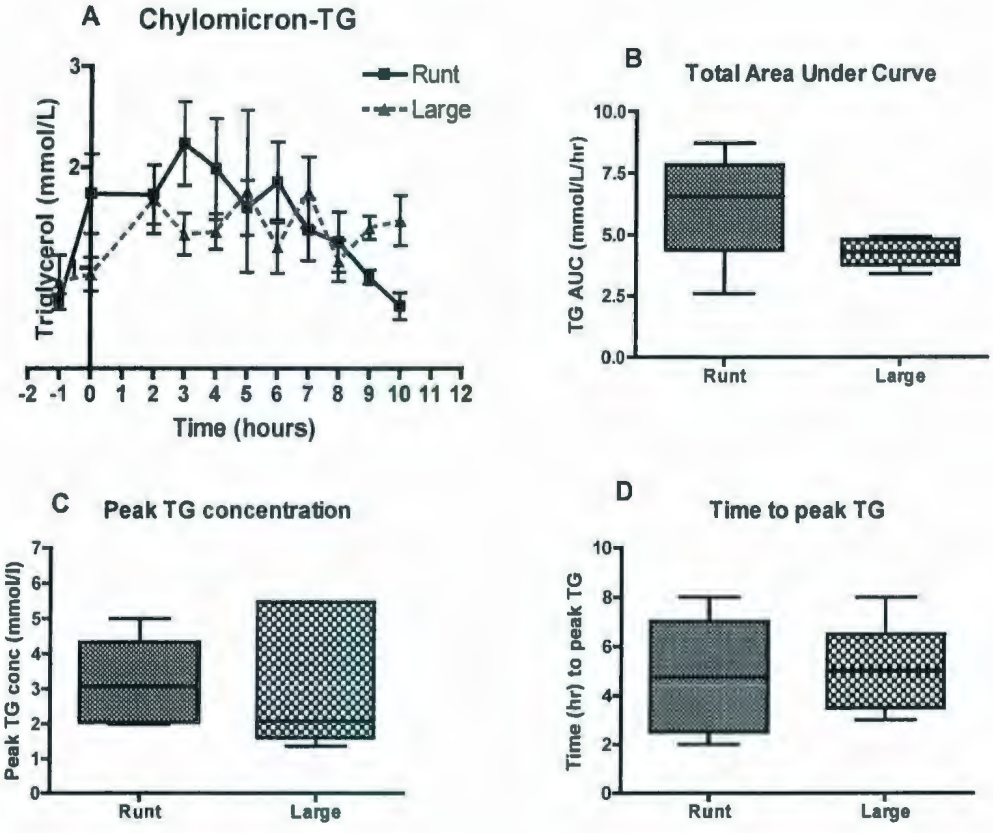

Figure 7.2. Plasma chylomicron-triglyceride (TG) concentrations in response to a bolus fat tolerance test in runt and large littermate Yucatan miniature pigs fed a high-fat-sugar-salt diet.

For Figure A, each symbol represents the mean ± SD in n = 6 pigs. For Figure B-D, the middle line represents the median value for the group (N = 6), the top and bottom of the boxes represent the 25th and 75th percentiles, and the 'whiskers' represent the 10th and 90th percentiles. TG = triglyceride; AUC = area under the curve.

7.5 DISCUSSION

The main objective of this study was to evaluate the effects of a Western-style diet on lipid metabolism in low versus high birth weight Yucatan miniature pigs. In other words, does a high salt-fat-sugar (HSFS) diet exacerbate the previously observed dyslipidaemia in small birth weight pigs? In this study, because the HSFS diet led to dyslipidaemia in all pigs, we found no strong differences in lipidaemic parameters between runts and large littermates.

The original description of the fetal origins hypothesis proposed that poor nutrition in early life increases the effects of the Western diet in later life (Barker and Osmond 1986). The results of the present study indicate that a post-weaning high-fat-sugar-salt diet elevated overall lipid concentrations in all the pigs, with some evidence that early programming also had an impact on later lipid metabolism. Interestingly, in this study, we saw that in both groups, plasma TG levels steadily declined during the period from 3 to 6 mo-old. Studies in children and adolescents have shown that serum lipid and lipoprotein concentrations tend to be elevated during early childhood, but do not reflect values in the later years of childhood or adult values (Kwiterovich, Barton et al. 1997; Friedman, Morrison et al. 2006; Daniels and Greer 2008). That is, childhood plasma lipid values decrease during pubertal development and then may increase thereafter during adulthood, implying that lipid concentrations are age-dependent. This observation is said to have important implications for the timing of lipid screening as a predictor of chronic disease risks (Friedman, Morrison et al. 2006; Daniels and Greer 2008). The decrease in lipid concentrations during adolescence is likely due to effects of sex hormones (Kwiterovich,

Barton et al. 1997).The early elevated TG concentration was only observed with pigs fed the high-fat-sugar-salt diet, unlike the pigs fed the regular diet (**Chapter 5**), indicating that this persistent elevated TG concentration in early childhood is a reflection of the diet, and therefore may not be related to the fetal programming hypothesis.

In this study, at 3 d old, the runts had significantly smaller abdominal circumference compared to their large littermates. But, by the end of sexual maturity at 7 mos old, the runts had faster growth rates, with resulting catch-up growth in body size, including abdominal circumference. While we found no significant correlations between abdominal circumference at birth and plasma lipid concentrations at any time point throughout the study, there were significant correlations between visceral fat measurements and plasma total cholesterol levels, which started at the beginning of sexual maturity (i.e., 4 mo old); additionally, abdominal circumference was significantly correlated to plasma total cholesterol levels and TG levels at 6 mo old. Studies have indicated that body proportions such as abdominal circumference at birth, rather than birth weight are a better indicator of the relationship between prenatal growth and adult lipid metabolism (Barker, Martyn et al. 1993; Barker 2003). For instance, an epidemiologic study in the United Kingdom found that small abdominal circumference at birth predicted higher total cholesterol and LDL-cholesterol levels during adulthood, whereas the association with birth weight was weak (Barker, Martyn et al. 1993). Abdominal circumference at birth is said to reflect liver size, and the liver has a major role in the regulation of cholesterol and other lipid metabolism (Roberts, Nava et al. 1999). An inference is that impaired liver growth *in utero* resets cholesterol homeostasis towards a more atherogenic profile (Barker 2003). In

this study, at 12 mos old, we found no difference in liver size between runts and large littermates or in hepatic lipid content. This suggest that the increased abdominal circumference measurements that were noted in this study is most likely related to abdominal adiposity (hence, visceral adiposity); this was confirmed by the strong correlation between abdominal circumference and visceral fat content at necropsy, suggesting that abdominal circumference was more indicative of visceral fat content rather than liver size in this Yucatan miniature pig model. Indeed, studies have shown that an increase in visceral adiposity, rather than other body fat storage sites, has the strongest correlations with more deleterious effects on cardiovascular and other metabolic health (Ross, Aru et al. 2002).

Even though the addition of fat and sugar in the HSFS diet diluted the other nutrients (i.e., the protein content was reduced to 10%), the similarity in the effects of the HSFS diet to that of the regular diet in **Chapter 5** on overall growth patterns suggested that this calorie-rich and nutrient-dilute diet still facilitated catch-up growth in runts. Comparison of the growth rate of the pigs in this study to those in **Chapter 5** showed that these pigs grew slower because of the lower dietary protein content; generally when growing pigs are fed a high-energy feed on an *ad libitum* basis, they will eat to meet their energy requirement and, therefore, energy content of the diet is the main factor controlling voluntary feed intake (NRC 1987). However, the over growth patterns remained similar between the studies, and in both studies, runts experienced catch-up growth prior to sexual maturity at 7 mo old. In other words, despite the low protein content of the post-weaned HSFS diet (10% vs. 18% in a typical diet for domestic swine), the runts still had

significant catch-up growth in body weight and length by the end of sexual maturity at 7 mo old (Table 7.1). In terms of micronutrients, it is worth noting that the vitamin and mineral contents in the HSFS were within NRC (NRC 1998) recommendations for swine and, no clinical signs of micronutrient deficiency (NRC 1979; NRC 1998) were observed during the study.

Overall, the results of this study suggested that it is not birth weight or size *per se* which had the strongest influence on adulthood lipid profile; instead, body composition, specifically visceral adiposity, is more predictive of adolescent and adult lipid levels. Because almost all of the lipid measurements were higher in pigs fed the HSFS diet, compared to the standard diet-fed pigs previously, these data suggest that diet has the greatest effect on dyslipidaemia in Yucatan miniature pigs and that being born larger confers no protection against the effects of a poor diet.

CHAPTER 8. GENERAL DISCUSSION

The overall project objective was to determine whether the naturally occurring, intrauterine growth restricted Yucatan miniature swine (i.e., runts) represents a suitable model to study developmental origins of adult health and diseases (DOHaD) by assessing biological indicators of cardiovascular diseases (CVD). The aim of this chapter is to summarize the main results of this thesis and provide some proposals for future studies and experiments in this Yucatan miniature swine model.

Figure 8.1 provides a simplified overview of the main outcomes related to DOHaD that were observed in the runt Yucatan miniature pig model in this thesis. Overall, the main results of this thesis fall under the umbrella of developmental origins of CVD, but can be subcategorized into several specific areas: 1) the metabolic consequences of catch-up growth following intrauterine growth restriction (IUGR), 2) developmental origins of high blood pressure, which includes underlying mechanisms such as altered tissue differentiation and organ development, 3) developmental origins of coronary heart disease, involving altered metabolic regulation, leading to outcomes such as a dyslipidaemic plasma profile, and 4) the imposition of a diet high in saturated and trans fats, sodium and simple carbohydrates (typical of a Western-style diet) on early programming events, with resulting long-term adverse metabolic consequences. The results of each area will be discussed below.

8.1 IUGR, catch-up growth and chronic diseases

Although there is very limited information on this subject, it has been widely assumed that catch-up growth is desirable for low birth weight children (Victora, Barros et al. 2001). But, catch-up growth, following low birth weight, is receiving increased attention as a key player in the development of chronic metabolic diseases (Eriksson, Forsen et al. 1999; Fagerberg, Bondjers et al. 2004; Nobili, Alisi et al. 2008). One of the earliest epidemiological studies documenting the adverse effects of catch-up growth in humans was conducted by Barker and colleagues (Eriksson, Forsen et al. 1999), wherein they found that the highest death rates from coronary heart disease occurred in males who had low birth weight and were thin at birth but by 7 years old, their weight had caught up to an average or above average body mass. Similarly, studies such as the British birth cohort study have shown that men who were light at birth have the greater risk of developing obesity if they grew rapidly during childhood (Parsons, Power et al. 2001). Accordingly, the results of this project are in agreement with these epidemiological studies, as well as other animal studies (Parsons, Power et al. 2001), showing that being born small combined with accelerated increase in body weight is associated with even greater susceptibility to chronic metabolic diseases later in life.

In this thesis, catch-up growth was observed in runts fed both the standard grower diet (**Chapter 4** and **5**) and the HSFS diet (**Chapter 6** and 7), and was shown to be the result of greater feed intake rather than enhanced feed efficiency. Moreover, catch-up growth occurred in the runts prior to sexual maturity at 7 mo old, independent of the post-weaning diet, implying that early catch-up growth in this runt model is likely due to

fetal/early programming of food intake regulation. Furthermore, also independent of the diet formulation, catch-up growth was associated with increased adiposity when measured in adulthood. Indeed, other animal studies have shown that low birth weight offspring that experienced early catch-up growth have a preferential deposition of body fat instead of protein mass, with resulting increase in the risk for development of metabolic diseases later in life (Vickers, Breier et al. 2000; Kind, Clifton et al. 2003; Druet and Ong 2008), and elevated plasma leptin levels have been shown to precede an increase in adiposity in IUGR offspring (Kay's and Hindmarsh 2006; Vickers, Krechowec et al. 2007; Dulloo 2008).

The mechanistic basis of catch-up growth after IUGR is poorly understood, but altered programming of activity of neuroendocrine and endocrine axes, which influence growth, appetite and satiety, and metabolic efficiency, are implicated in this phenomenon (Yajnik, Fall et al. 1995; Cameron and Demerath 2002; Davy and Hall 2004; Kay's and Hindmarsh 2006). In this project, the results from both studies (i.e., standard diet and Western-style diet) demonstrated increased food intake in the runts relative to the large littermates, suggesting early programming of food intake regulation in the low birth weight offspring. Similarly, other researchers have reported hyperphagia following spontaneous or experimental fetal restriction animal models (e.g., (Greenwood, Hunt et al. 1998; Vickers, Breier et al. 2000; Kind, Clifton et al. 2003; Mitra, Alvers et al. 2009)). This has led to the suggestion that adverse prenatal environments permanently alter the set point and activity of neuroendocrine mechanisms regulating satiety and appetite (Ong, Preece et al. 2002; Kay's and Hindmarsh 2006; Vickers, Krechowec et al. 2007). The alteration in

neuroendocrine regulation of appetite may involve alterations in leptin and neuropeptide Y (NPY) concentrations. Leptin is secreted by adipocytes in direct proportion to the amount of adipose tissue in the body; whereas NPY hormone, a potent stimulator of feeding behaviour, is secreted by the hypothalamus. Leptin acts at the hypothalamus to regulate appetite and energy homeostasis (Ahima and Flier 2000; Haynes 2005; Friedman 2009). Under normal physiological conditions, increased leptin signalling in the hypothalamus is associated with reduced NPY and agouti-related (AgRP) protein production, but increased cocaine-and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) production; these leptin-induced changes lead to decreased food intake and increased energy expenditure (Schwartz, Seeley et al. 1997; Hahn, Breininger et al. 1998; Haynes 2005). However, most low birth weight offspring that experienced catch-up growth tend to have elevated leptin with increased appetite, which has been attributed to leptin resistance (Jaquet, Leger et al. 1999; Dulloo 2008). Furthermore, it has been suggested that obesity in IUGR offspring could be due to programming events that results in leptin resistance caused by decreased level of leptin receptor expression and/or alterations in leptin signalling in the hypothalamus (Vickers 2007; Dulloo 2008). Analyses of these hormones in the Yucatan miniature pig model may provide an overall better indication of the underlying mechanisms controlling feeding behaviour and catch-up growth in the runts.

Besides leptin, other hormones have also been shown to be associated with adverse metabolic effects of catch-up growth. Adverse effects of catch-up growth may also reflect persistent changes in secretion of hormones that were established *in utero* in response to

undernutrition, including anabolic hormones such as insulin, insulin-like growth factor 1 (IGF-1), and growth hormone (Barker, Martyn et al. 1993; Eriksson, Forsen et al. 1999; De Blasio, Gatford et al. 2007). For instance, increased adiposity following IUGR may be driven in part by increased IGF and insulin action, but the extent of the influence of IGF and insulin on early catch-up growth, and whether IGF production is altered is still unknown (Kay's and Hindmarsh 2006; De Blasio, Gatford et al. 2007). In sheep, increased growth and adiposity are associated with increased sensitivity to insulin and IGF-1. Were an animal to be more sensitive to these hormones, then more nutrients would be taken up into tissues to promote growth, but excess nutrients would be stored as fat in adipocytes (De Blasio, Gatford et al. 2007). In this project, measurement of plasma insulin levels through the first 12 months of life revealed no differences between runts and large littermates (McKnight 2008), suggesting that insulin levels may not be related to catch-up growth or adverse effects of catch-up growth in runt Yucatan miniature swine. Furthermore, other researchers (Ritacco, Radecki et al. 1997) have shown that catch-up growth in runt domestic pigs was not mediated by IGF-1; presumably, this is also true for runt Yucatan miniature pigs, but of course this needs to be validated in this strain of pig. Indeed, the results in this thesis suggest that catch-up growth occurs because of increased feed intake rather than increased efficiency, so the leptin hypothesis, instead of the anabolic hormone hypothesis, may be more relevant to this model. However, all these hormones should be assessed in this Yucatan pig model in order to provide support for either theory.

Low birth weight offspring that experience early catch-up growth have a preferential deposition of body fat instead of protein mass with resulting increase in the risk for development of metabolic diseases later in life (Robinson, Wheeler et al. 1991; Eriksson, Forsen et al. 1999; Vickers, Breier et al. 2000; Kind, Clifton et al. 2003; Druet and Ong 2008). For instance, epidemiological studies show that babies that are thin at birth have less muscle mass and when they later develop a higher body mass in childhood, they have a disproportionately higher fat mass (Robinson, Wheeler et al. 1991; Eriksson, Forsen et al. 1999). An increase in myostatin concentration in low birth weight animals may be one explanation for the reduced muscle mass seen at birth. Myostatin is a putative bodyweight/skeletal muscle mass signaller, which acts as a negative regulator of skeletal muscle mass (Lee and McPherron 2001). In this project we collected muscle tissues from all the animals at the end of the study, but unfortunately, time constraints prevented the analysis of these tissues as part of the thesis; but follow-up studies should include analysis of myostatin concentration as one component of understanding the regulation of catch-up growth in this swine model.

In summary, the results of this project are in agreement with results of other human and animal studies, showing that IUGR is associated with hyperphagia resulting in accelerated growth rate and with adverse metabolic outcomes such as increased adiposity, which in turn is a risk factor for development of, and/or contributor to, other chronic metabolic diseases such as CVD. Therefore, understanding the mechanisms that promote increased postnatal growth and adiposity in intrauterine growth restricted infants and experimental animal models require further investigation. The Yucatan miniature pig has proven to be a

good candidate animal model for such investigations. The preceding section provides some ideas for future direction that will help elucidate the underlying mechanism of catch-up growth in the low birth weight offspring, particularly the runt Yucatan pig.

8.1.1 Future directions: studies about IUGR, catch-up growth and chronic diseases in runt Yucatan miniature pigs

One of the main objectives of a future study should be to determine whether altered programming of activity of neuroendocrine and endocrine axes is involved with appetite regulation, catch-up growth and obesity in the runt Yucatan miniature pig. Are differences in leptin levels between runts and large littermates apparent? If so, what are the underlying mechanisms by which leptin regulates catch-up growth with subsequent obesity? The suggested approach involves measurement of plasma, adipocyte and hypothalamic leptin levels, as well as other neuroendocrine hormones such as NPY. These parameters should be measured in situations where catch-up growth is known to occur (such as *ad libitum* feeding, as documented in this thesis) and compared against conditions where catch-up growth is inhibited (such as with feed restriction). Using other animal models, researchers have examined the consequences of *ad libitum* versus restricted feed intake on growth of low and high birth weight male lambs during the neonatal period (Greenwood, Hunt et al. 1998). The researchers found that low birth weight lambs grew at absolute rates equivalent to that of high birth weight lambs if fed milk *ad libitum* in the first few months of life. In our present project, the pigs were fed *ad libitum* on both the regular diet feeding and the HSFS diets. Both studies could have been

enhanced by adding a calorically restricted group; this would allow us to assess the impact of a Western-style diet on catch-up growth and fetal programming.

In this proposed future study, if leptin and other endocrine and neuroendocrine hormones were found to be different between catch-up and non-catch-up growth situations, then the investigation should be explored further at the molecular level, i.e., examination of leptin receptor expression in adipocytes and the hypothalamus to test for leptin resistance in low birth weight offspring. As mentioned above, this proposed study (or series of studies) should also measure other endocrine hormones such as IGF-1, primarily to validate the findings from other studies (mentioned above) in this Yucatan miniature pig model.

As presented in the studies in this thesis, the runts and large littermate siblings were taken from the sow soon after birth, and throughout the studies for 5 hours daily, the animals had *ad libitum* food intake. Consequently, we were able to demonstrate increased food intake in the runts with resulting catch-up growth and increased obesity. Furthermore, in these studies, fractional growth rate measurements indicated that catch-up growth started during the neonatal period and was completed before the pigs reached sexual maturity at 7 mos old. Therefore, future studies on catch-up growth in the Yucatan miniature swine should focus on the neonatal phase, as it appears that the altered programming effects of neuroendocrine and endocrine activity axes occur this early in life and sets up later obesity development.

8.2 Developmental origins of cardiovascular disease

8.2.1 Developmental origins of high blood pressure

In this project, BP values in the pigs tended to be inversely correlated with birth weight (**Chapter 4**), similar to findings reported in epidemiological and other animal studies. Moreover the higher BP values in the runts were significantly related to reduce nephron endowment (**Chapter 4** and **6**). Although the underlying mechanisms are not fully known, it is now established that alterations in kidney development and function play pivotal roles in early programming of BP. For instance, low nephron endowment has been found to be common to a large number of developmental programming models and species ((Hughson, Farris et al. 2003; Vehaskari and Woods 2005; Schreuder and Nauta 2007; Moritz, Wintour et al. 2008); **Chapter 4** and **6**). However, recent research suggests that reduced nephron number alone is not sufficient for manifestation of disease (Bagby 2007; Griffin, Kramer et al. 2008; Moritz, Wintour et al. 2008); other changes within the kidney (e.g. compensatory alterations such as hyperfiltration) or in the postnatal environment (such as high-salt or high-fat diet after birth) appear to be necessary for development of hypertension and cardiovascular disease (Moritz, Wintour et al. 2008). Indeed, the results in this thesis suggest that in order to maintain normal plasma creatinine levels, the lower number of nephrons in the runts must lead to nephron hyperfiltration (**Chapter 4** and **6**). In addition, increased adiposity, resulting in part from early catch-up growth, was shown to be strongly associated with increased blood pressure values in the runts (**Chapter 4** and DAP in **Chapter 6**). In **Chapter 6** we demonstrated that a post-weaning HSFS diet resulted in significant elevation in blood pressure; DAP was

controlled by fetal/early programming events, whereas SAP was influenced more by postnatal diet. These results help to illustrate the complexity of blood pressure regulation. Overall, the results of this thesis are in agreement with other studies in the literature, highlighting the pivotal role of the kidney, especially nephron endowment, in long-term blood pressure regulation.

In terms of developmental programming of blood pressure via impaired nephrogenesis, the query has now shifted to a focus on understanding the underlying mechanisms that lead to impaired nephrogenesis with resulting low nephron endowment. There have been a few postulated mechanisms to explain the reduced nephron endowment in developmental programming models and animal species , such as delayed branching of the ureteric bud into the surrounding metanephric mesenchyme, decreased rates of branching morphogenesis, increased apoptosis, and early cessation of nephrogenesis (Moritz, Wintour et al. 2008; Moritz, Singh et al. 2009). Decrease nephron number may be due to changes in the expression of genes that are critical for normal branching morphogenesis in the kidney (Moritz 2006). Mammalian kidney development involves interactions between uretic bud and the surrounding metanephric mesenchyme. Branching of the uretic tree may be a critical process that determines the final number of nephrons, as new nephrons only form adjacent to uretic tips (Singh, Moritz et al. 2007). Because of the reduction in nephron endowment in this model and the association to runting, future studies in the Yucatan miniature pig should explore the underlying mechanisms of impaired nephrogenesis.

Although our results are in agreement with others in the literature, showing that developmental programming of reduced nephron number may program hypertension, it was recently pointed out by Moritz et al (Moritz, Wintour et al. 2008) that caution should be taken when making such associations. They suggested that "a reduced nephron endowment and hypertension may be simply coincident, and it is possible that compensatory changes in tubular function and/or renal hormonal systems must occur concomitantly for hypertension to develop" (Moritz, Wintour et al. 2008). The point is that some studies have shown that hypertension can be developmentally programmed independent of reduced nephron number (Moritz, Wintour et al. 2008). In such cases, the suggested potential mechanisms include the renin-angiotensin system, renal sympathetic innervations, and the tubular transport mechanism in the kidney (Denton 2006; Moritz, Wintour et al. 2008). We addressed the renin-angiotensin system by examining acute salt-sensitivity, where we found that there was no difference in the degree of salt sensitive blood pressure between runts and large littermates (**Chapter 4** and **6**). Those data suggested that any impairment in the renin-angiotensin system may not be related to developmental programming of blood pressure in the Yucatan miniature pig model. However, the other mechanisms were not explored in this thesis, and future studies in this Yucatan miniature pig model should also investigate the potential contribution of these mechanisms to developmental programming of blood pressure.

8.2.2 Future directions: studies on developmental origins of blood pressure in runt Yucatan miniature pigs

Both in our animal model and in general, there are still many significant unanswered questions related to developmental programming of blood pressure. More specifically there are still many unknowns regarding the association between congenital nephron endowment and blood pressure regulation, such as: 1) what are the underlying mechanisms that regulate reduced glomeruli (and therefore nephrons) at critical periods of development? 2) Are the remaining nephrons truly hyperfunctioning (to fully answer this question will require more definitive analytical techniques than presented in this thesis)? 3) Are the types of nephron affected important for development of hypertension (i.e., are there differences in cortical (short-loop) or juxtamedullary (long-loop) nephron)? 4) What are the structural and functional effects of programming on the renal medulla?

To address all the above mentioned issues would require a series of studies. In terms of prioritizing these queries, based on the results of this thesis, the first study should be about refining the results observed in this project. In this thesis, using unbiased stereological techniques, reduced nephron number was clearly demonstrated in the runts fed both the standard diet and HSFS diet, suggesting that impaired nephrogenesis is an important component of developmental programming of blood pressure in the Yucatan miniature pig model. Measurement of monthly plasma creatinine levels indicated no difference between runts and large littermates; therefore, we concluded that that there is likely hyperfiltration of nephrons in the runts because the runts had fewer nephrons but were still able to maintain the same plasma creatinine levels as the large littermates. One

of the main objectives of a future study should be to determine whether there is truly hyperfiltration of nephron in the reduced nephron-endowed runts. To answer this question involves assessment of glomerular filtration rate (GFR) which is a measure of the fluid filtration rate of the kidney. Creatinine, which is the by-product of creatine phosphate (in muscle), is already at a steady-state concentration in the blood, and is freely filtered by the kidney (although there is a small amount secreted by the kidney, so there is a small margin of error); therefore, in clinical setting creatinine clearance is routinely used as an approximation of GFR (Schwartz and Furth 2007). However, the gold standard method for assessing GFR in animal models uses inulin (a fructose polysaccharide), an exogenous chemical that is freely filtered but not reabsorbed or secreted by the kidneys. The amount of inulin filtered per unit time by the glomeruli equals the volume of plasma filtered/time (GFR) times the plasma concentration (P_{in}). The amount of fluid exiting from the ureter per unit time equals the urine flow rate (V) times the urine concentration (U_{in}), i.e., GFR = $[U_{in}x\ V]/P_{in}$. Overall, plasma and urine concentrations of inulin and the urine flow rate must be measured in order to calculate GFR. Therefore, proper assessment of GFR also requires complete collection of urine.

The issue regarding different types of nephrons could also be addressed in the same study that assesses GFR. Results of studies in other animal models show that the long-loop nephrons are more susceptible to hypertensive damage (Olson, Wilson et al. 1986). The histological procedures we employed in this study (unbiased stereological dissector method) assessed glomeruli as an indicator of nephron anatomy, which are more indicative of cortical nephrons; therefore, future histological analyses should assess not

only total nephrons, but also the types of nephrons. The assessment of nephron types would require the use of histological cutting and assessment techniques that require advanced skills compared to the unbiased stereology method used in this thesis. As such, future research in this area would benefit from collaboration with a researcher(s) who is an expert in kidney pathology and assessment.

Salt-sensitive blood pressure in the low birth weight offspring is a growing area of research interest in developmental programming of blood pressure. Studies have suggested that part of developmental programming of blood pressure may involve structural and functional programming effects on the renal medulla (regulates the final excretion of NaCl and water). That is, the renal medulla plays a pivotal role in long-term control of systemic blood pressure and hypertension through control of the pressure natriuresis/diuresis system (Cowley 2008); therefore, developmental programming of salt-sensitive blood pressure will likely be the result of impairment in renal medulla. In this thesis we found that acute salt challenges did not result in a greater degree of salt sensitive blood pressure in the runts; however, chronic salt loading resulted in persistently higher DAP values in the runts. It would be interesting to determine if there are differences in renal medulla function and structure in runts on acute versus chronic salt loading. Assessment of renal medulla function in part requires measurement of renal GFR and renal medullary blood flow. Furthermore, assessing structural changes in renal medulla requires histological expertise as mentioned above for nephron types.

But ultimately, future research about developmental programming of blood pressure in this Yucatan miniature pig model should also focus on understanding the underlying

mechanisms that result in low nephron endowment. Studying these mechanisms involves a molecular approach. For instance, metanephric organ culture techniques have been used to study the mechanisms by which glucocorticoids may affect branching morphogenesis during nephrogenesis (Singh, Moritz et al. 2007).

8.2.3 Developmental origins of coronary heart disease

A dyslipidaemic plasma lipid profile is an established hallmark of coronary heart disease (CHD), and some epidemiological and animal studies have shown that low birth weight was associated with dyslipidaemic profile (Barker and Osmond 1986; Barker, Martyn et al. 1993; Barker 1995; Huxley, Owen et al. 2004; McMillen and Robinson 2005). Similarly, in this thesis (**Chapter 5**) we found that low birth weight was associated with higher plasma TG levels, in both the fasted and postprandial states. Indeed, the postprandial lipid assessment test (i.e., oral fat tolerance test) suggested that there might be impaired lipid metabolism in the runts. For instance, we noted a higher peak chlylomicron-TG concentration in the runts, suggesting lower TG clearance. Other studies have shown that lipoprotein lipase (LPL) and apoE are involved in modulating the risk of dyslipidaemia and CHD (Watts, Mamo et al. 1998; McGladdery and Frohlich 2001; Paglialunga and Cianflone 2007). LPL, required for hydrolysis of TG from TG-rich lipoprotein, is important for maintaining optimal plasma lipid levels. ApoE plays a major role in regulating the metabolism of chylomicrons, VLDL, and HDL, partly through regulating uptake of chylomicron remnants, clearance of VLDL remnants and removal of excess cholesterol from peripheral tissues (Watts, Mamo et al. 1998; McGladdery and Frohlich 2001; Paglialunga and Cianflone 2007).

As discussed in **chapter 5,** in the postprandial state, the clearance of circulating chylomicrons is largely dependent on adipocyte and muscle LPL activities (Watts, Mamo et al. 1998; Paglialunga and Cianflone 2007); thus, the reduced ability of the runts to clear circulating chylomicron-TG suggests these animals have lower LPL activity relative to their large littermates. This impaired LPL activity could also be coupled with impaired hepatic remnant clearance, possibly due to reduced expression of hepatic ApoE receptors. Unfortunately, we were not able to test these hypotheses in this thesis. Therefore, follow-up experiments in this model should include examination of the mechanisms that contribute to impaired lipid metabolism in the runts, possibly by investigating the expression of LPL and ApoE in runt and large littermate (McGladdery and Frohlich 2001; Ruiz, Labayen et al. 2008). In the studies in this thesis, plasma samples were routinely collected throughout; therefore, we have the samples to perform these analyses. The results of such tests would help to strengthen the interpretation of the results from the oral fat tolerance test.

Furthermore, we should also investigate whether altered lipid metabolism in the runts leads to vascular dysfunction in these animals. Endothelial dysfunction is an early indicator of CVD development. Endothelial function is particularly sensitive to high saturated fat intake and subsequent elevation of plasma lipids. Deficiency of the vascular system (both function and structural) has been implicated in fetal/early programming. For example, deficiency in the elastin in the walls of the aorta and large arteries could lead to arterial stiffness with resulting elevation in blood pressure. In fact, vascular function

analyses were performed on the pigs in this project; however, the results were not included in this thesis.

8.3 Impact of post-weaning Western-style diet on early programming events

The predictive adaptive response hypothesis proposes that peri-and post-natal nutrition status developmentally programs the organism's adult metabolic state (Gluckman, Hanson et al. 2005). In this thesis, we attempted to assess the impact of a post-weaning HSFS diet on fetal/early programming of chronic diseases; the results indicated mixed effects. We found that while DAP was controlled primarily by fetal/early programming events, SAP was determined by postnatal diet (**Chapter 6**). Conversely, all aspects of lipid metabolism that we assessed were dominated by postnatal diet; that is, a post-weaning HSFS diet masked any fetal/early programming effects so no significant difference in lipid parameters (plasma lipids and lipoproteins, TG clearance or hepatic lipids) were noted between runts and large littermates (**Chapter 7**). The lipid results were partly unexpected, as other researchers have reported additive effects of fetal/early programming and postnatal high calorie diets (Vickers, Breier et al. 2000; Khan, Dekou et al. 2005; Jimenez-Chillaron, Hernandez-Valencia et al. 2006; Mitra, Alvers et al. 2009).

Part of the limitation of the HSFS diet was the dilution of required nutrients with added calories (which is typical in a Western-style diet). As discussed in **Chapter 7**, the protein content of the diet was lower than normal (10% vs. the normal 18%) by design; nevertheless, we still observed hyperphagia and resulting catch-up growth and obesity in

the runts (albeit at a slower rate), indicating that fetal/early programming was still evident despite the diet. Moreover, other essential nutrients that are likely involved in early programming, such as folate and vitamin B_{12} (Szeto, Das et al. 2009), were also diluted by calories. Folate, vitamin B12, vitamins A and D are known to have an impact on gene expression, where the current theory postulates that epigenetic effect of these nutrients during early development is the leading underlying mechanism for DOHaD (Waterland 2006; Burdge, Hanson et al. 2007; Szeto, Das et al. 2009). In order to investigate the mechanisms, a future study would have to isolate the effects of individual nutrient candidates and control for other components of the diet.

The HSFS diet study was conducted concurrent with the standard diet study. While we were designing this project the available literature at the time suggested that a Western-style diet was critical for the observation of early manifestation of fetal/early programming effects (Barker and Osmond 1986; Vickers, Breier et al. 2000). Indeed, the original description of the fetal origins hypothesis proposed that poor nutrition in early life increases the effects of the Western diet in later life (Barker and Osmond 1986). Therefore, the HSFS diet was our "back-up plan" in case we saw no observable evidence of fetal/early programming in runt Yucatan miniature pigs fed the standard grower diet. However, as discussed above and in **Chapter 4** and **5**, in the standard diet study we found clear evidence of fetal/early programming in runts , even when fed a healthy lifelong diet.

8.3.1 Future directions: studies about Western-style diet and DOHaD in runt Yucatan miniature pigs

This project was a hypothesis-generating study. There is still much to be learned regarding the interaction between pre- and post-natal diets and their effects on long-term adult outcomes. Of particular interest are the underlying mechanisms of fetal/early programming and the evaluation of the role of postnatal diet in ameliorating programming effects. The typical Western-style diet is rich in saturated fatty acids (SFA), refined sugars and salt, and is known to be associated with conditions such as dyslipidaemia, obesity and hypertension. Along with lipid abnormalities, endothelial dysfunction is considered an early predictor of cardiovascular diseases. SFA-rich diet has been shown to result in endothelial dysfunction (Brown and Hu 2001), in part due to production of free radicals which decrease endothelium derived vasodilator molecules such as nitric oxide (Erdei, Toth et al. 2006).

Furthermore, the majority of the studies that assess the interaction between peri-and post-natal programming effects still tend to focus on the early postnatal period (i.e., neonatal). However, the window of developmental programming is not clearly defined; for instance, the childhood stage is also a period of rapid growth and development but there is still an unanswered question of whether a developmental window remains open during childhood and adolescence allowing nutritional experiences during this time to substantively shape subsequent adult metabolic states (Frazier, Mason et al. 2008).

8.4 Overall conclusion

The results of this thesis indicate that the naturally occurring IUGR Yucatan miniature swine (i.e., runt) represents a suitable model to study DOHaD. The runt showed qualities similar to the human IUGR infant, i.e., small size at birth likely due to poor *in utero* nutrient supply, an increased rate of postnatal growth (catch-up growth), and organ and metabolic changes which led to the development of adult metabolic diseases such as obesity and CVD. Specifically, the data suggest that catch-up growth and subsequent obesity may be the result of developmental programming of food intake regulation changes in the runts. Furthermore, we found that developmental programming of nephrogenesis seems to be associated with programming hypertension and the impaired lipid metabolism in the runts may also be developmentally programmed. Finally, we also saw that postnatal dietary intake plays an important role as a determinant of chronic disease outcomes; a postnatal Western-style diet that was high in salt, saturated fat and refined sugar had the potential to exacerbate (e.g., diastolic blood pressure) or mask (e.g., systolic blood pressure and lipid profile) fetal/early programming events. Overall, this novel miniature pig is an animal model that can be used to explore mechanisms that contribute to developmental origins of human adult health and diseases.

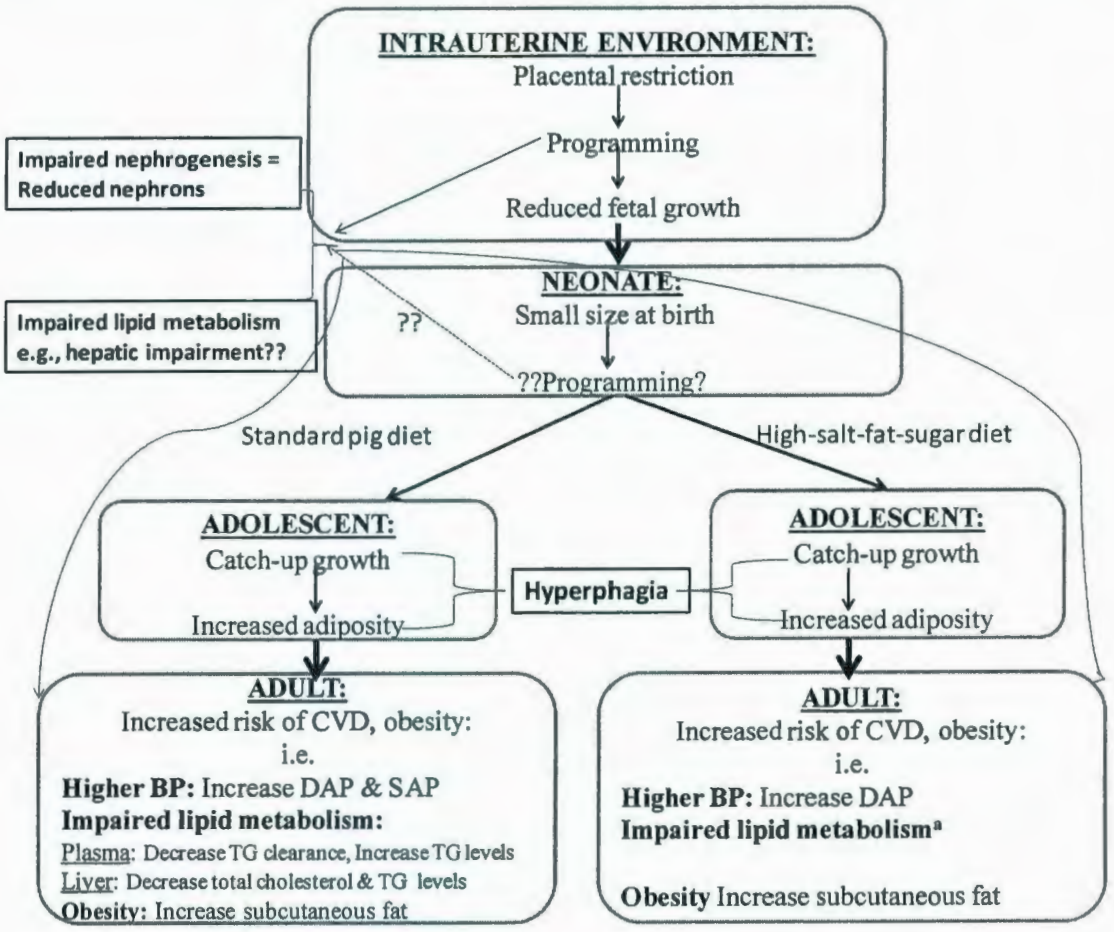

Figure 8.1: Flow diagram highlighting the main outcomes in runt Yucatan miniature pigs fed standard grower diet or the high-salt-fat-sugar diet (Western-style diet).

Question signs (??) indicate potentially programmed events, but we were unable to fully ascertain based on current data. [a]HSFS diet masked fetal/early programming effects, resulting in impaired lipid metabolism in all the pigs (i.e. in both runts and large littermates) (see Chapter 7).

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