METABOLIC AND CARDIORENAL ADAPTATIONS TO PREGNANCY IN FEMALES BORN SMALL ON A HIGH FAT DIET AND BENEFITS OF ENDURANCE EXERCISE TRAINING

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This thesis is dedicated to my parents,

Latipah and Mahizir,

for your unconditional love and support

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ABSTRACT

Uteroplacental insufficiency is the major cause of intrauterine growth restriction in Western society and is often associated with adult metabolic, cardiovascular and renal diseases. Recent studies have reported that these phenotypes are exacerbated with "second hits" such as pregnancy and obesity in women born small. Pregnancy is the greatest physiological challenge facing women and, indeed, females born small are at increased risk of gestational diabetes and hypertension. Interestingly, there has been a significant increase in the number of reproductive age women who are either overweight or obese over the past few decades. Being obese or overweight is suggested to exacerbate the pregnancy complications in women born small. Importantly, exercise is reported to prevent or delay the metabolic and cardiovascular dysfunction in individuals born small. Thus, the development of targeted interventions in growth-restricted females may prevent them from developing metabolic and cardiorenal dysfunctions during pregnancy.

Uteroplacental insufficiency, resulting in growth restriction, was induced by bilateral uterine vessel ligation (Restricted) or sham (Control) surgery on embryonic day 18 (E18) in Wistar-Kyoto rats. Female offspring consumed a Chow or a high fat diet (HFD; 43% kcal from fat) from 5 weeks and were mated at 20 weeks with normal male. Systolic blood pressure was measured by tail cuff in all rats prior to pregnancy (week 18). Female rats remained Sedentary or exercised on treadmills for 4 weeks before pregnancy and throughout pregnancy or only during the last two thirds of pregnancy. Rats were individually placed in an indirect open-circuit calorimeter chamber (CLAMS; 24 hours) at E16 to measure their energy expenditure and spontaneous physical activity. Systolic blood pressure was measured by tail cuff and non-fasted glucose tolerance test was performed at E18. At E19, rats were individually placed in a metabolic cage to collect urine and blood was taken by tail vein to calculate estimated glomerular filtration rate (eGFR) via measurement of urinary and plasma creatinine. Maternal plasma, pancreas, and heart were collected at E20. Fetuses and placentae were weighed and sex was confirmed using qPCR (SRY).

In Chapter 3, the effect high fat feeding on the metabolic adaptations during pregnancy in rats born small was explored as was whether exercise before and during pregnancy is more beneficial in preventing these complications than exercise during pregnancy alone. Control and Restricted rats consuming a HFD were significantly heavier with more dorsal fat (+40%) and higher plasma leptin concentrations (+80%) compared to Chow-fed rats, irrespective of exercise interventions ($P<0.05$). Compared to Sedentary, both exercise interventions increased oxygen consumption (VO_2) and respiratory exchange ratio (RER) in Restricted Chow-fed rats only ($P<0.05$). HFD induced glucose intolerance in Control females and exacerbated glucose intolerance in Restricted females that remained

Sedentary throughout the study ($P<0.05$). The development and exacerbation of glucose intolerance in Control and Restricted females were prevented by exercise initiated prior to and continued during pregnancy. Furthermore, exercise before and during pregnancy increased insulin secretion in Chow-fed females and increased β-cell mass in HFD Control and Restricted females ($P<0.05$). No differences in spontaneous physical activity were detected across the groups. Of interest, metabolic dysfunction in Control and Restricted females was not improved by exercise initiated during pregnancy.

In Chapter 4, we determined if high fat feeding exacerbates the known adverse cardiorenal adaptations to pregnancy in rats born small and whether exercise before and during pregnancy is more beneficial in preventing these complications than exercise during pregnancy alone. Sedentary Control females on a HFD altered renal function (increased eGFR; $P<0.05$) and this was not affected by exercise. Compared to Control, Restricted females that remained Sedentary also had an increased eGFR ($P<0.05$), which was not influenced by HFD or restored by both exercise interventions. No changes in pre-pregnancy systolic blood pressure were identified in all experimental groups. When pregnant, Restricted Chow-fed rats and both Control and Restricted females on a HFD had an impaired cardiovascular adaptation, with a greater reduction in systolic blood pressure during late gestation ($P<0.05$), and only exercise initiated before and continued during pregnancy prevented this. Additionally, Control and Restricted rats that exercised prior to and during pregnancy had an increased heart weight (normalized to tibial length) irrespective of diet at E20, indicative of physiological cardiac hypertrophy.

Finally, Chapter 5 of the thesis investigated the effect of high fat feeding and endurance exercise training on the fetal outcomes in rats born small. Maternal growth restriction and HFD did not affect male and female fetal and placental weights in mothers that remained Sedentary throughout the study. Exercise initiated before and continued during pregnancy increased fetal weight in both male and female fetuses of mothers on a Chow diet, but not in mothers on a HFD. No difference in male and female fetal and placental weights were observed when mothers exercised during pregnancy only.

In summary, HFD revealed and exacerbated glucose intolerance in pregnant females born of normal birth weight and born small, respectively. These were prevented by the lifestyle intervention of exercise, potentially due to improved β-cell mass. The improved glucose intolerance in Chow-fed Restricted that initiated exercise prior to pregnancy was likely contributed by increase in glucose-stimulated insulin secretion. This study also suggests that females born small have altered cardiorenal adaptations to pregnancy. Although impaired metabolic and cardiovascular adaptations during pregnancy were prevented by exercise prior to and during pregnancy, renal function was not affected by both exercise interventions. This study highlights that modifiable risk factors, such as diet and exercise, can have beneficial effects in the mother during pregnancy; particularly for females born small. By identifying females who are at high risk, especially in women born small and overweight/obese women, will ensure

preventative intervention strategies to minimise adverse outcomes during pregnancy and in later life. Ultimately, these approaches may reduce the perpetuating cycle of metabolic and cardiorenal disease risk to future generations.

DECLARATION

This is to certify that:

i. The thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface;

ii. Due acknowledgement has been made in the text to all other material used;

iii. The thesis is less than 100, 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Nurul Dayana Mahizir

December 2017

PREFACE

All work carried out in the preparation of this thesis was my own except for those outlined below:

The uteroplacental insufficiency surgeries for Chapters 3-5 were performed by Andrew Jefferies and Kristina Anevska (The University of Melbourne, Parkville, VIC, Australia).

I was given assistance with animal experiments presented in Chapters 3-4 from Andrew Jefferies and Kristina Anevska, who helped with IPGTT and blood pressure procedures which require 2 persons. Kristina Anevska and Yeukai Mangwiro assisted with exercise training presented in Chapters 3-5.

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Fetal sex determination procedure in Chapter 5 was performed by Sogand Hosseini.

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PUBLICATIONS, PRESENTATIONS AND AWARDS

Published Review

Mahizir, D, Briffa, JF, Hryciw, DH, Wadley, GD, Moritz, KM & Wlodek, ME (2015). Maternal obesity in females born small: Pregnancy complications and offspring disease risk. *Mol Nutr Food Res* **60,** 8-17 (see Appendix 2).

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Mahizir, D, Mangwiro, YTM, Cuffe, JSM, Briffa, JF, Hosseini, SS, Anevska, K, Jefferies, AJ, Romano, T, Wadley, GD, Moritz, KM & Wlodek ME. Effects of maternal high fat diet on cardiorenal function and placental angiogenesis and vasculogenesis in females born small and the impact of exercise interventions.

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Metabolic and cardiorenal adaptations to pregnancy in females born small on a high fat diet and the benefits of endurance exercise training. November 22, 2016. Final PhD Seminar.

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Victorian Obesity Consortium, Melbourne, Australia, December 13, 2016.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 4

Chapter 5

Chapter 6

LIST OF TABLES

LIST OF ABBREVIATIONS

AMPK	5'-adenosine monophosphate-activated protein kinase
AngII	angiotensin II
AUC	area under the curve
BMI	body mass index
Ca^{2+}	calcium
$CHCl_3$	chloroform
CLAMS	indirect open-circuit calorimeter
CV	calorific value
DAB	3,3'-diaminobenzidine
dH2O	distilled water
DTT	dithiothreitol
E	embryonic day
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eRPF	effective renal plasma flow
et al.	and associates
EtOH	ethanol
Exercise	exercise before and during pregnancy
ExPregOnly	exercise during pregnancy only
F0	initial generation
F1	first generation
F2	second generation
FOV	field of view
G-6-PDH	glucose-6-phosphate dehydrogenase
GFR	glomerular filtration rate
GLP-1	glucagon-like-peptide-1
GLUT	glucose gransporter
GLUT4	glucose gransporter 4
GSK3	glycogen synthase kinase 3
HCl	hydrochloric acid
HFD	high fat diet
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor 2
IP	intraperitoneal

IPGTT	intraperitoneal glucose tolerance test
IRS-1	insulin receptor substrate-1
K	potassium
K_f	ultrafiltration coefficient
KOH	potassium hydroxide
MAPK	mitogen-activated protein kinase
MeOH	methanol
MgCl2	magnesium chloride
n	number
Na	sodium
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP+	nicotinamide adenine dinucleotide phosphate
NBF	neutral buffered formalin
NIBP	non-invasive blood pressure
NO	nitric oxide
NSB	non-specific binding
PCA	perchloric acid
Pdx-1	pancreatic duodenal homeobox-1
PGI2	prostacyclin
PI-3K	phosphoinositide 3-kinase
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PKCδ	protein kinase C δ
PKD-1	phosphoinositide-dependent kinase-1
PL	placental lactogen
PN	postnatal day
PP-cells	pancreatic polypeptide cells
PRL	prolactin
QC	quality control
RER	respiratory exchange ratio
Sed	sedentary
TBST	tris-buffered saline with Tween 20
TRIS	tris (hydroxymethyl) aminomethane
UPI	uteroplacental insufficiency
VCO_2	carbon dioxide production
Vd	volume density

VLDL	low -density lipoprotein
VO_2	oxygen consumption
vs.	versus
WKY	Wistar Kyoto
β-cell	beta cell

LIST OF SYMBOLS

<	less than
>	greater than
≥	equal or greater than
≤	equal or less than
α	alpha
β	beta
Δ	delta
γ	gamma
↑	increase
↓	decrease
↔	no change
÷	divided by
×	multiplied by
%	percent
~	approximately
±	plus and minus

LIST OF UNITS

°C	degrees celcius
cm	centimetres
cm^3	centimetres cubed
cpm	counts per minute
G	gauge
g	gram
h	hour
kg	kilogram
kΩ	ohms
l	liter
M	molar
mg	milligram
ml	millilitre
mm	millimetres
mmHg	millimeter of mercury
ng	nanogram
nm	nanometre
nmol	nanomole
pg	picogram
rpm	revolutions per minute
U	unit
umol	micromole
$v.v^{-1}$	volume per volume
$w.v^{-1}$	weight per volume
µl	microlitre

CHAPTER 1

Review of Literature

1.1 Introduction

The prevalence of obesity is escalating rapidly and is considered as the epidemic of 21st century. It is clinically defined as having a body mass index (BMI) over 30 and is associated with an increased risk of developing a number of co-morbidities including metabolic and cardiovascular disease and nephropathy (Eckel *et al.*, 2005). One subset of the population who are predisposed to developing obesity are children born small for gestational age, which occurs in 10% of pregnancies worldwide (Martin *et al.*, 2017). Epidemiological studies reported that these growth-restricted children have an increased susceptibility to type 2 diabetes, insulin resistance, hypertension and cardiovascular disease (Barker *et al.*, 1989a; Barker *et al.*, 1989b; Hales *et al.*, 1991; Eriksson *et al.*, 2000). Most importantly, increasing evidence has suggested that women born small are at risk of adverse pregnancy outcomes.

Pregnancy is the greatest physiological challenge facing women that results in alterations in maternal physiology and metabolism to assist in fetal growth and development, which is modulated by a number of key molecules (Herrera, 2000; Carlin & Alfirevic, 2008). In an obese mother, the pregnancy adaptations become adverse which predispose them to a number of complications (Mahizir *et al.*, 2016). Indeed, pregnant women who are overweight or obese have higher risk of gestational hypertension, diabetes and preeclampsia (Sebire *et al.*, 2001; Cunningham & Teale, 2013). Given that growth-restricted females are at higher risk of cardiovascular and metabolic disease during their pregnancy, it is likely that maternal obesity may exacerbate the phenotypes. By investigating the underlying mechanisms of disease development during pregnancy in growth-restricted and/or obese females, it will assist in the development of future strategies for the prevention and therapy of diseases.

The following review will discuss and provide rationale for the subsequent studies that aimed to determine whether maternal obesity exacerbates the adverse pregnancy adaptations in growth restricted females and the impact of lifestyle intervention of exercise on pregnancy outcomes.

1.2 Developmental Origins of Health and Disease

Fetal origins of adult disease hypothesis was first described by David Barker and colleagues in 1989, which proposed that insults during critical stages of fetal development program an increased susceptibility for cardiovascular disease in adulthood (Barker *et al.*, 1989a; Barker *et al.*, 1989b). Following these early studies, a plethora of epidemiological and animal studies have emerged investigating the impact of early life on the development of adult disease (Hales *et al.*, 1991; Lucas *et al.*, 1997; Forsen *et al.*, 2000; Eriksson *et al.*, 2006). Low birth weight in particular is associated with increased risk of glucose tolerance (Hales *et al.*, 1991; Phipps *et al.*, 1993; McCance *et al.*, 1994), type 2 diabetes (Hales *et al.*, 1991; Lithell *et al.*, 1996; Phillips *et al.*, 2005), cardiovascular disease (Barker & Martyn, 1992) and obesity (Yliharsila *et al.*, 2007).

1.2.1 Intrauterine Growth Restriction

Babies born small at birth are defined as having birth weight less than 10th percentile for gestational age or less than 2.5 kg at term (Wollmann, 1998). It accounts for ~10% of births in the Western countries (Hamilton *et al.*, 2015). Although low birth weight is often used as an indicator of intrauterine growth restriction, other measurements including ponderal index (birth weight/length^3) and head circumference are proposed to be better markers. Indeed, some studies have reported associations between ponderal index and head circumference with coronary heart disease in adulthood (Barker *et al.*, 1993a; Barker *et al.*, 1993b; Forsen *et al.*, 1997; Eriksson *et al.*, 1999; Eriksson *et al.*, 2001). Impaired fetal nutrition during first and second trimesters often results in offspring with whole-body (symmetrical) growth restriction and it affects only 20-30% of low birth weight cases (Wollmann, 1998). In contrast, fetal growth restriction that occurs during the third trimester causes asymmetric growth restriction, with reduced weight and ponderal index but no alteration to head size (brain sparing) in the offspring (Wollmann, 1998). It occurs in 70-80% of low birth weight cases and asymmetric growth restricted offspring tend to have worse health outcomes later in life (Wollmann, 1998).

Intrauterine growth restriction can be caused by a number of factors including genetic factors, maternal malnutrition, smoking, alcohol consumption, drug abuse or stress (Albu *et al.*, 2014). While maternal undernutrition is the main cause of low birth weight in the Third world countries, inadequate placental perfusion is considered as one of the major causes of intrauterine growth restriction in the Western world (Haggarty *et al.*, 2002; Henriksen & Clausen, 2002). The placenta plays an important role as the interface between the mother and fetus. It is responsible for oxygen and nutrient transfer from the maternal blood system to the developing fetus (Nayak & Giudice, 2003). In normal placentation, adequate trophoblast invasion of the uterine vasculature is important to increase uteroplacental blood

flow to ensure sufficient oxygen and nutrient supply to the fetus (Nayak & Giudice, 2003). However, inadequate placentation causes intrauterine growth restriction and low birth weight (Vuguin, 2007).

1.2.2 Thrifty Phenotype Hypothesis

In a suboptimal *in utero* environment, fetus adapts to enhance immediate survival by ensuring nutrient supply to the most vital organs such as brain but at the detriment of other organs such as the pancreas and kidney (Hales & Ozanne, 2003). The thrifty phenotype hypothesis proposes that early life metabolic adaptations happen by selecting appropriate trajectory of growth in response to the *in utero* environment, which continue during the postnatal period. However, the metabolic and physiology adaptations of the offspring become detrimental when postnatal nutrition is mismatched and this may adversely impact the offspring health (Hales & Ozanne, 2003). For instance, offspring that are exposed to poor nutrition *in utero* environment may experience postnatal catch-up growth when postnatal nutrition becomes abundant. The programmed metabolic adaptations that occurred *in utero* will be detrimental and result in increased risk of obesity and impaired glucose tolerance later in life (Hales & Ozanne, 2003).

1.2.3 Animal Models of Low Birth Weight

A number of animal models have been used to investigate the underlying mechanisms involved in intrauterine growth restriction and programming of adult diseases. Numerous studies have found a link between low birth weight and metabolic and cardiorenal disease in many animal species including rodent, sheep and guinea pigs (Vuguin, 2007). The most commonly used animal models responsible for human low birth weight are maternal undernutrition (Vuguin, 2007) and uteroplacental insufficiency (Wigglesworth, 1964). Rodents are often used for fetal programming studies due to their shorter gestation time, relatively easy to manipulate and cost-effective. Importantly, as mammals, rodents share similar genes, biochemical pathways, organs and physiology to humans (Vuguin, 2007). However, one of the major drawbacks is that some of the rodent organs in particular brain and kidney are underdeveloped when compared to humans at birth (Vuguin, 2007).

The impact of maternal undernutrition was studied using a global nutrient restriction (equal reductions of all nutrients in the diet) or by reducing specific nutrients in the diet (low protein diet) to induce growth restriction in the offspring (Aerts & Van Assche, 2006; Van Abeelen *et al.*, 2012). Although animal models of maternal undernutrition are more reflective of intrauterine growth restriction in the Third world countries, uteroplacental insufficiency animal models are more translatable to human growth restriction in the developed countries. Wigglesworth was the first to develop the model of uteroplacental insufficiency by bilateral uterine vessel ligation in the rats (Wigglesworth, 1974). This

model caused a reduction in oxygen and nutrient delivery to the fetus and thus altered fetal growth and development (Wigglesworth, 1974). Our laboratory performed bilateral uterine vessel ligation surgery on day 18 of gestation, with term at 22 days (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wlodek *et al.*, 2008; Mazzuca *et al.*, 2010; Siebel *et al.*, 2010; Gallo *et al.*, 2012b; Cheong *et al.*, 2016a) to mimic late gestation uteroplacental insufficiency in humans. Offspring are born 10-15% smaller (Wlodek *et al.*, 2005), which is similar to the definition of low birth weight in humans. Furthermore, as a consequence of bilateral uterine vessel ligation surgery, offspring have organ deficits and are more susceptible to diseases, which reflect the conditions observed in humans (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wlodek *et al.*, 2008; Mazzuca *et al.*, 2010; Siebel *et al.*, 2010; Gallo *et al.*, 2012b; Cheong *et al.*, 2016a).

1.3 Metabolic Dysfunction

Diabetes is a major health problem with an exponential increase in its incidence over the recent years. Of particular interest, about 90% of humans with diabetes mellitus are diagnosed with type 2 diabetes (Olokoba *et al.*, 2012). Patients with type 2 diabetes are normally present with chronic hyperglycemia, resulting from altered insulin secretion and impaired insulin action (Alberti & Zimmet, 1998). Complications arising from diabetes are often severe, which if left untreated can lead to premature death. As low birth weight is associated with high risk for type 2 diabetes, understanding the pathophysiology of this disease is important for future interventions

1.3.1 Normal Glucose Homeostasis

Glucose homeostasis is primarily controlled by the release of the enzyme insulin from β-cells of the pancreas in response to elevated circulating glucose concentrations in the blood (Cavaghan *et al.*, 2000). Insulin, a peptide hormone acts in the muscle to promote glucose uptake (Dugani & Klip, 2005) and at the liver to inhibit hepatic glucose output via decreased gluconeogenesis and glycogenolysis (Radziuk & Pye, 2001), concurrently promoting storage of glucose as glycogen.

After its release, insulin bind to the insulin receptor on the cell surface. This induces a conformational change resulting in the autophosphorylation and initiation of its tyrosine kinase activity (Pessin & Saltiel, 2000). Receptor activation leads to tyrosine phosphorylation of key tyrosine residues on insulin receptor substrate-1 (IRS-1) proteins (Pessin & Saltiel, 2000). This results in the recruitment of a lipid kinase termed phosphoinositide 3-kinase (PI-3K) through its SRC homology 2 domains. PI-3K is a heterodimer containing p85 regulatory subunit and p110 catalytic subunit (White, 1998). Once activated, the catalytic subunit phosphorylates phosphoinositides lipids at the 3’ position of the inositol ring or proteins at the serine residues. PI-3K activates phosphoinositide-dependent kinase-1 (PKD-1) which activates Protein Kinase B (PKB) (Khan & Pessin, 2002). PKB, in turn, deactivates glycogen synthase kinase 3 (GSK3), leading to activation of glycogen synthase thus promoting glucose storage as glycogen. Activation of PKB also results in translocation of GLUT vesicles from their intracellular pools to the plasma membrane, which allows uptake of glucose into the cell. Once glucose enters the cell, the enzyme hexokinase catalyses the first step of glucose metabolism by phosphorylating glucose to glucose-6-phosphate, which can then undergo glycolysis to produce energy or stored as glycogen in muscle and liver for later use (Radziuk & Pye, 2001; Saltiel & Kahn, 2001; Shulman, 2004). Glucose is then oxidised into energy through glycolysis or alternatively undergo glycogenesis to form glycogen for storage (Radziuk & Pye, 2001; Saltiel & Kahn, 2001). Up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle, whereas adipose tissue accounts for only a small fraction (Klip & Paquet, 1990).

Insulin resistance occurs when tissues have reduced the ability to respond to insulin and often precedes the development of type 2 diabetes (Cavaghan *et al.*, 2000; Donath & Halban, 2004). In the early stages of insulin resistance, pancreatic β-cells compensates by hypersecreting insulin to maintain normal glucose homeostasis (Flanagan *et al.*, 2000). After a prolonged period of hypersecretion, β-cell exhaustion could ensue and eventually the pancreas may no longer able to secrete sufficient insulin to maintain glucose homeostasis which eventually leads to hyperglycemia, the clinical onset of type 2 diabetes (Cavaghan *et al.*, 2000; Donath & Halban, 2004). As pancreas is the only organ in the body that produces insulin, the next section of this literature review will focus on the pancreas development and function.

1.3.2 Pancreas Development

The pancreas develops from a multipotent endodermal cell population that gives rise to exocrine and endocrine cells. The endocrine cells are grouped into islets of Langerhans, which contribute to approximately 4% of the total rat pancreas (Fowden & Hill, 2001). Within the pancreas, there are 4 endocrine cell types (α, β, δ and Pancreatic Polypeptide (PP)-cells). The α-cells are responsible for producing glucagon acting on the liver to increase blood glucose levels through the stimulation of hepatic glycogenolysis (Slack, 1995; Fowden & Hill, 2001). The δ-cells and PP-cells each secrete somatostatin and pancreatic polypeptide, respectively (Slack, 1995; Fowden & Hill, 2001). Of interest, β-cells which make up the majority of cells within the islets, are the key regulators of insulin and are important for optimal glucose homeostasis (Slack, 1995)(Figure 1.1).

Pancreatic development in the rodent and human are similar. There are 3 processes that regulate the development of the pancreatic islets: (i) neogenesis from the duct epithelia, (ii) proliferation of cells for endocrine differentiation and (iii) apoptosis of cells to remodel the developing islets. The transcription factor pancreatic duodenal homeobox-1 (Pdx-1) is a key regulator that involves in pancreatic development, initiating and regulating endocrine cell differentiation (Grapin-Botton *et al.*, 2001). In humans, fetal β-cells are classed as true endocrine cells by the end of the first trimester of pregnancy (Hill & Duvillie, 2000; Fowden & Hill, 2001). However, in rodent, this maturation is only achieved during the last third of pregnancy (Piper *et al.*, 2004). An accelerated growth of various islet cell types in the rodent fetuses is achieved by high cell proliferation during this period (Kaung, 1994). Following birth, the restructuring of pancreatic endocrine tissue continues, and this postnatal islet remodelling lasts for four weeks in the rat while continuing into infancy in humans (Fowden & Hill, 2001). This occurs in the rat via a transient wave of islet cell apoptosis shortly following birth (Scaglia *et al.*, 1997; Hill & Duvillie, 2000).

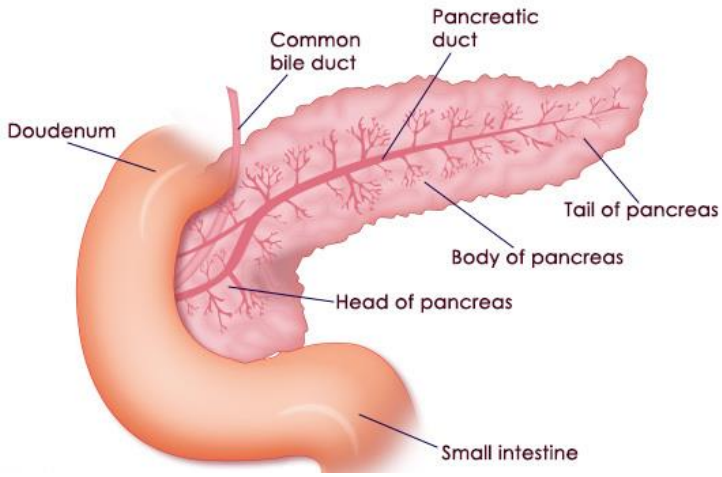


Figure 1.1 Schematic representation of human pancreas

Pancreatic islets house α, β, δ and PP-cells that secrete enzymes and hormones. Importantly, pancreatic β-cells are the key regulators of insulin and are important for optimal glucose homeostasis (Picture from http://www.buzzle.com/images/diagrams/human-body/location-of-pancreas.jpg).

1.4 Low Birth Weight and Metabolic Dysfunction

1.4.1 Human Epidemiological Studies

Hales and colleagues first reported the link between low birth weight and development of type 2 diabetes in later life (Hales *et al.*, 1991). Over 300 men aged 64 years from Hertfordshire, United Kingdom that were born between 1920 and 1930 were investigated (Hales *et al.*, 1991). Those who had low birth weights and weights at one year of age were more likely to develop impaired glucose tolerance and type 2 diabetes in later life (Hales *et al.*, 1991). Similar associations were observed in men and women from Preston, UK, where the prevalence of impaired glucose tolerance or type 2 diabetes fell from 27% in subjects who weighed 2.5 kg or less at birth to 6% in those who weighed 3.4 kg or more at birth (Phipps *et al.*, 1993). Both of these studies proposed that the increased susceptibility of type 2 diabetes in those who were born of low birth weight is due to altered growth of the endocrine pancreas *in utero* environment (Hales *et al.*, 1991; Phipps *et al.*, 1993). However, when glucose tolerance tests were performed in younger cohort of men and women born between 1931 and 1939 from the Hertfordshire Cohort Study, birth weight was again inversely related to the prevalence of type 2 diabetes but no association was reported between weight at one year of age and type 2 diabetes (Phillips *et al.*, 2005). The differences in the finding are possibly due to the small number of subjects in the later study.

The Dutch Hunger Winter from 1944 to 1945 provides a unique opportunity to study effects of maternal nutrition on the offspring metabolic outcomes (Ravelli *et al.*, 1998; Roseboom *et al.*, 2001). The official daily adult rations during the end of World War II in 1944 dropped to below 1000 calories per day during the height of the famine compared to 1800 calories per day before the war happened (Ravelli *et al.*, 1998; Roseboom *et al.*, 2001). However, women continued to conceive and deliver babies under these harsh conditions. The Dutch Famine Cohort Study studied adults who were born around the time of the Dutch famine in a university hospital, in Amsterdam, the Netherlands (Ravelli *et al.*, 1998). Mothers who were exposed to famine during mid and late gestation delivered babies that were lighter, shorter and thinner with smaller heads and placentas than babies who were not exposed to famine (Roseboom *et al.*, 2001). Furthermore, famine during mid and late gestation caused glucose intolerance in adulthood, exhibited by increased in 2 hours plasma glucose concentrations, which may predispose them to type 2 diabetes later in life (Ravelli *et al.*, 1998). These studies provide an insight that maternal undernutrition programs the development of type 2 diabetes in their offspring. In contrast, the findings from famine exposure during the Leningrad siege that happened between 1941 to 1944 did not show any association between birth weight and metabolic disease risk (Stanner & Yudkin, 2001). The siege in Leningrad lasted for 28 months with food ratios dropped below 1000 calories and to 300 calories from November 1941 to February 1942. This study found that individuals who were exposed to siege during gestation did not develop glucose intolerance in adulthood which is different to the Dutch

Hunger Winter Famine study (Stanner & Yudkin, 2001). The inconsistency between these findings is likely due to the different nutritional environments during the postnatal period in both studies. Following the Dutch Hunger Winter, the food supply was restored to normal levels in a short period, where they were exposed to normal nutrition during their postnatal life (Ravelli *et al.*, 1998; Roseboom *et al.*, 2001). Conversely, in Leningrad, the children were exposed to poor nutritional environment in utero and in their early postnatal years (Stanner & Yudkin, 2001). These findings suggest that a mismatch in nutritional environment between the intrauterine and postnatal period may influence the metabolic outcomes of growth-restricted babies in adulthood, which support the thrifty phenotype hypothesis.

Altered postnatal growth can also influence the disease outcomes of growth-restricted babies in adulthood. Growth-restricted babies often experience catch up growth in the first 6 to 12 months of age and to as late as 2 years after birth when the postnatal nutritional environment is improved (Eriksson *et al.*, 1999; Simmons, 2005; Eriksson *et al.*, 2006). They will accelerate their growth trajectory to match the growth of normal weight babies to compensate for their low birth weight. Previous studies have reported that children born small for gestational age, who had a high childhood fat mass, had an increased risk of developing diabetes in later life (Whincup *et al.*, 1997; Bhargava *et al.*, 2004) and present with insulin resistance at 3 years (Mericq *et al.*, 2005). Another study in a cohort of men and women born between 1924 and 1933 from Helsinki demonstrated that the risk of type 2 diabetes reduced with increasing birth weight and rose with increasing BMI between age 3 and 11 years (Barker, 2002). Furthermore, individuals who had type 2 diabetes later in life were associated with accelerated growth in height, weight and BMI between the ages of 7 to 15 years (Forsen *et al.*, 2000). These studies suggest that accelerated catch-up growth during postnatal life is an additional independent risk factor for disease development in growth-restricted individuals. Therefore, a combination of adverse prenatal and postnatal environment can lead to an exacerbation of the programmed metabolic disease in these individuals.

The underlying mechanisms that cause the development of metabolic disease in growth-restricted individuals are still not clear. Some studies demonstrated that impaired glucose tolerance and type 2 diabetes in growth-restricted individuals were caused by impaired insulin sensitivity and insulin secretion (Jaquet *et al.*, 2000; Veening *et al.*, 2003). Muscle insulin resistance, in particular, has been demonstrated in individuals with low birth weight. Indeed, insulin resistance in growth restricted humans were associated with reduced expression of insulin signaling proteins in skeletal muscle and impaired regulation of skeletal muscle and adipose tissue GLUT4 gene expression following insulin stimulation (Jaquet *et al.*, 2001; Ozanne *et al.*, 2005). As glucose is primarily uptake by skeletal muscle, impairments in the expression of insulin signaling proteins may contribute to muscle insulin resistance and thus lead to glucose intolerance or type 2 diabetes. However, other organs that are involved in

glucose metabolism and regulation including pancreas and liver have not been properly investigated in humans.

1.4.2 Experimental Animal Studies

As mentioned previously, low birth weight as a consequence of maternal undernutrition and uteroplacental insufficiency are the most used animal models. A rat study on a global nutrient restriction (50% caloric restriction) in the last trimester of pregnancy resulted in a 16% reduction in birth weight than the control offspring and was accompanied by impaired insulin secretion and reduced β-cell mass (Garofano *et al.*, 1997). Although the rats had a catch-up growth during lactation, β-cell mass deficit and impaired insulin secretion were sustained (Garofano *et al.*, 1997). Similarly, 50% reduction in caloric restriction during pregnancy reduced β-cell mass and insulin secretion in response to glucose stimulation in islet in both male and female offspring at 3 months of age (Theys *et al.*, 2011). However, only male offspring demonstrated pancreatic islet mitochondrial dysfunction (Theys *et al.*, 2011). This study suggests that, as regards pancreatic mitochondrial dysfunction, male offspring appear to be more severely affected by inadequate nutrition *in utero* and therefore may have greater risk of metabolic dysfunction in later life than females. Offspring that were exposed to low protein diet (8%) throughout gestation developed glucose intolerance and had lower insulin secretion in adulthood (Dahri *et al.*, 1991). These adverse metabolic modifications were irreversible even when they were exposed to a normal diet after birth (Dahri *et al.*, 1991). Further studies in male rat offspring of low protein diet dams demonstrated an age-dependent loss in glucose tolerance. Specifically, they had improved glucose tolerance and reduced plasma insulin concentrations in early life (6 weeks to 3 months), which is indicative of enhanced insulin sensitivity (Ozanne *et al.*, 1998). Nevertheless, when they reached 15 months of age glucose intolerance was evident (Hales & Ozanne, 2003) and by 17 months of age they developed frank diabetes and insulin resistance (Petry *et al.*, 2001; Hales & Ozanne, 2003).

In sheep, placental insufficiency is induced by removal of placentation sites (carunclectomy) prior to pregnancy or injection of microspheres during late gestation (Robinson et al., 1979; Cheung et al., 2004). Placental restriction in sheep reduced birth weight (24%), and also caused catch-up growth and increased adiposity at 6 weeks of age (De Blasio *et al.*, 2007a). Fetal sheep and young lambs were also demonstrated to have fasting hypoinsulinemia and impaired glucose-stimulated insulin production at 5 weeks of age and before birth (De Blasio *et al.*, 2007b; Owens *et al.*, 2007). By adulthood, the defects in β-cell function become apparent in growth-restricted male sheep despite evidence of compensatory increased in β-cell mass (Gatford *et al.*, 2008).

In rats, growth-restriction induced through bilateral uterine ligation surgery resulted in offspring born 10–15% smaller than control animals (Simmons *et al.*, 2001; Boloker *et al.*, 2002; Styrud *et al.*, 2005).

These offspring had reductions in β-cell mass at birth (De Prins & Van Assche, 1982; Styrud *et al.*, 2005) with a similar decrease in pancreatic insulin content (Styrud *et al.*, 2005), however, glucose tolerance was normal at 3 months of age (Styrud *et al.*, 2005). Similarly, a study by Simmons et al. reported that although male growth-restricted Sprague Dawley rats had normal β-cell mass, islet size, and pancreatic weight, they exhibited mild insulin resistance and β-cell secretory defects at 1 week of age (Simmons *et al.*, 2001). Adequate compensatory insulin secretion was demonstrated in these rats for several weeks (Simmons *et al.*, 2001). By 15 weeks of age, these rats had reduced β-cell mass and decreased pancreatic insulin content as well as a reduced insulin response to glucose, and at 26 weeks these offspring were diabetic and obese. Additionally, other uteroplacental insufficiency studies recorded fasting hyperglycemia, early onset insulin resistance, obesity, and impaired glucose tolerance in the growth-restricted Sprague Dawley rats (Simmons *et al.*, 2001; Lane *et al.*, 2002; Selak *et al.*, 2003; Vuguin *et al.*, 2004; Simmons, 2007; Nusken *et al.*, 2008). Similarly, findings from our laboratory reported that male Wistar Kyoto (WKY) rats that were exposed to uteroplacental insufficiency develop impaired glucose tolerance and were hyperinsulinemic at 6 months of age, which was associated with a 40–45% reduction in β-cell mass (Siebel *et al.*, 2008; Wadley *et al.*, 2008; Siebel *et al.*, 2010; Laker *et al.*, 2011). Interestingly, growth-restricted female rats exhibited normal glucose tolerance regardless of reductions in basal insulin concentrations and pancreatic β-cell mass (Wadley *et al.*, 2008; Gallo *et al.*, 2012b). Importantly, our model of uteroplacental insufficiency was not confounded by obesity. Therefore, our model is ideal for investigating further mechanisms of developmental programming of metabolic disease.

1.5 Kidney Physiology

The kidneys are the principal excretory organs in human through which metabolic waste products are filtered and eliminated from the blood (Atherton, 2015). They also play a vital role in maintaining electrolyte balance as well as regulating blood pressure through the renin–angiotensin–aldosterone system (Hall, 1991) and erythrocyte production through production of erythropoietin (Dunn *et al.*, 2007). Normal kidney function is dependent on an adequate blood supply (20% of cardiac output), filtration of plasma and the ability to modify the composition of the filtrate through reabsorption and secretion from and into nephron tubules, respectively (Atherton, 2015).

1.5.1 Kidney Development

In utero, kidney development occurs through a series of stages, known as the pronephros, mesonephros and metanephros. Only the metanephros persists as the definitive adult kidney while both the pronephoros and mesenephros serve as transient organs (Rosenblum, 2008). The pronephros, originates from the intermediate mesoderm, forms the pronephric tubules and ducts (precursors of Wolffian ducts) at embryonic day (E) 21-22 in humans and E8 in rodents (Boyle & de Caestecker, 2006; Moritz *et al.*, 2008). As the pronephros regresses around E25 in humans and E9 in rodents, the mesonephros begins to develop (Moritz *et al.*, 2008). The mesonephros forms in a cranial to caudal direction to the pronephric tubules and gives rise to Wolffian duct (Boyle & de Caestecker, 2006). In females, the mesonephric kidney degenerates while the mesonephric tubules in males develop into reproductive organs including rete testis, efferent ducts, epididymis, vas deferens, seminal vesicle and prostate (Rosenblum, 2008). The metanephros development starts when the ureteric bud extends from the caudal end of the Wolffian duct into the adjacent metanephric mesenchyme cells at 5 weeks of gestation in human (Shah *et al.*, 2004) and E10 in rodents (Boyle & de Caestecker, 2006). Through branching morphogenesis, the ureteric bud undergoes repetitive branching and elongation and forms the renal collecting system consists of the cortical and medullary collecting ducts, the renal calyces and the renal pelvis (Figure 1.2) (Moritz *et al.*, 2008; Rosenblum, 2008). The remaining region of the ureteric bud that does not invade the metanephric mesenchyme forms the ureter (Moritz *et al.*, 2008). The tips of the ureteric bud stimulate the metanephric mesenchyme cells to condense and aggregate around each branch of ureteric bud to form renal vesicles (Shah *et al.*, 2004; Boyle & de Caestecker, 2006; Uhlenhaut & Treier, 2008). These renal vesicles differentiate into comma-shaped and S-shaped bodies that lengthen and eventually form the components of the nephron (proximal tubules, distal tubules, loop of Henle and renal corpuscle; Figure 1.2) (Shah *et al.*, 2004; Moritz *et al.*, 2008).

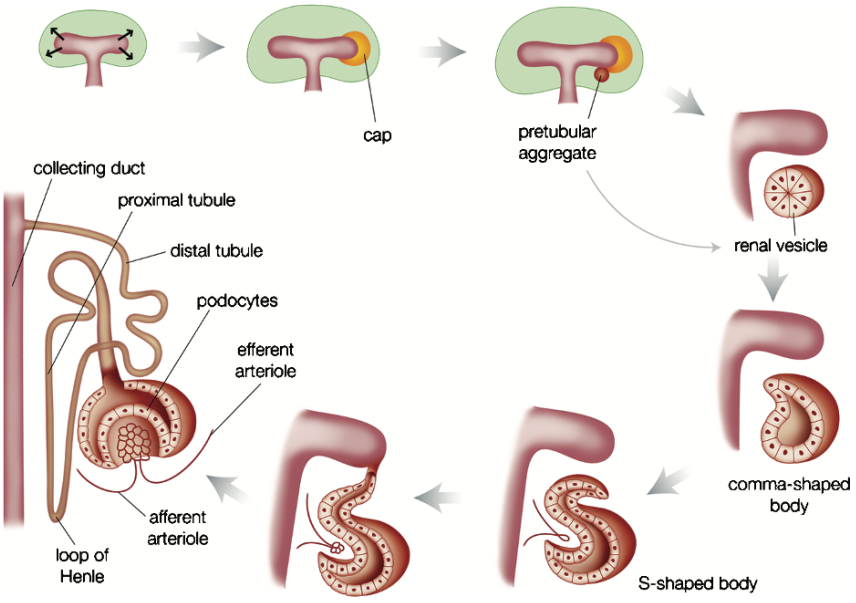


Figure 1.2 Kidney development

Schematic representation of branching morphogenesis and nephrogenesis that gives rise to nephron and collecting duct. Adapted from (Moritz *et al.*, 2008).

The timing of nephrogenesis varies between species, with nephrogenesis begins around 5 weeks of gestations in human and ceases around 36 weeks so that nephrogenesis is complete at birth (Fetterman *et al.*, 1965). In rodents, nephrogenesis begins mid-gestation and completes by postnatal day 14 (Moritz *et al.*, 2003; Moritz *et al.*, 2008). These findings implicate that final nephron number is determined during fetal kidney development *(in utero* period in humans and pre- and postnatal period in rats) and is irreversible.

1.5.2 Structure and Function of the Nephron

The nephron is the functional unit of kidney. Each nephron is composed of renal corpuscle and renal tubule (Figure 1.3). Renal filtration first takes place in glomerulus, a convoluted knot of capillaries located at the beginning of a nephron (Scott & Quaggin, 2015). Blood enters the glomerular capillaries through an afferent arteriole, which branch from renal arteries and leaves by an efferent arteriole into peritubular capillaries. Peritubular capillaries are tiny blood vessels that surround the tubular components of the kidneys and involve in the exchange with the filtrate passing through tubules.

The filtrate that has passed through the glomerular capillaries enters the Bowman's capsule and flow into a connected series of epithelial tubules starting from the proximal convoluted tubule, the loop of Henle, the distal tubules and finally, a collecting duct (Scott & Quaggin, 2015). The distal tubule and

glomerular afferent arteriole form the juxtaglomerular apparatus that play a vital role in regulating blood pressure and the filtration rate of the glomerulus (McMahon, 2016).

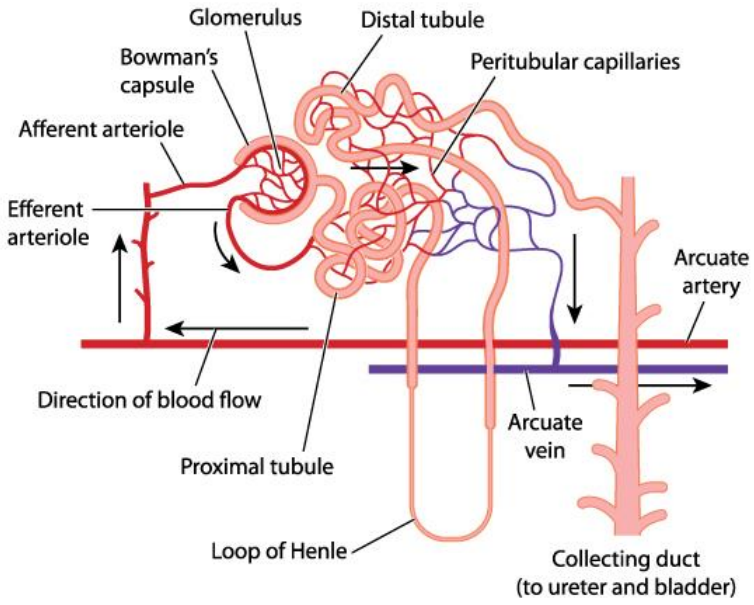


Figure 1.3 Anatomy of a kidney nephron

Renal filtration first takes place in renal corpuscle and the filtrates are passed into a connected series of renal tubules. Adapted from http://www.medicalsciencenavigator.com/tag/kidney-nephron-function/.

1.5.2.1 Glomerular Filtration

In the glomerulus, about 20% of blood is filtered across the glomerular capillaries into Bowman's capsule (Atherton, 2015). The passage of blood cell and other large molecules are limited, but water and neutral or cationic molecules with molecular diameter less than 7 nm are freely filtered. Albumin, the smallest negatively charged plasma protein (molecular diameter about 7 nm) can pass through the filtration barrier but only in small amounts due to the presence of negatively charged glycoproteins on the filtration barriers. The formation of an ultrafiltrate through glomerular filtration is dependent on intra-glomerular hydrostatic pressure that promotes fluid movement out of the glomerular capillary and plasma colloid osmotic pressure and Bowman's capsule hydrostatic pressure that oppose fluid movement from the glomerular capillary (Atherton, 2015). Thus, net ultrafiltration pressure (glomerular filtration rate (GFR)) is the product of outward pressure minus the inward pressure and depend on the ultrafiltration coefficient (K_f) that takes into account the surface area of the glomerular capillary and the permeability per unit of surface area.

GFR = K_f (inra-glomerular hydrostatic pressure) - (Bowman's capsule hydrostatic pressure + plasma colloid osmotic pressure).

There are many factors that can change GFR through alteration in renal plasma flow (Atherton, 2015). While renal plasma flow is considered as an important determinant of GFR, the location of the afferent and efferent arteriole, of which resistance can change independently, allow for changes in GFR that are in parallel or opposite to renal plasma flow. Sympathetic nerve activation and Angiotensin II cause vasoconstriction in the efferent arteriole and lead to reduced renal plasma flow. However, the effect is predominant on the efferent arteriole and thus the change in renal plasma flow is not accompanied by an equivalent change in GFR except at high stimulation. Endothelin, a vasoconstrictor and nitric oxide (NO), a vasodilator secreted from the endothelial cells lining the renal arterioles, respectively, reduce and increase renal plasma flow and GFR. Importantly, kidneys sustain renal plasma flow and GFR despite increments in blood pressure (within 80-200 mmHg) through the renal myogenic response and tubuloglomerular feedback (Atherton, 2015). The myogenic feedback mechanism ensures that the stretching of muscle activates the contraction of the muscle in response to increase in blood pressure to maintain renal plasma flow and GFR. Conversely, the tubuloglomerular feedback depends on the macula densa, a group of specialized tubular epithelial cells in the ascending loop of Henle that can detect changes in the tubular fluid flow and sodium load. These changes initiate a sequence of events that dilate or constrict the afferent arteriole.

GFR is considered as the gold standard measure of kidney function (Levey & Inker, 2016) and is thought to be best reflected by the renal clearance of inulin. Inulin is a non-endogenous polysaccharide of fructose that is freely filtered and is neither reabsorbed nor secreted by the kidneys. The accurate measurement of GFR using inulin is achieved by infusing inulin over some time to achieve a steady state of plasma concentrations (Traynor *et al.*, 2006). However, this method is not practical in clinical setting as it is expensive and time-consuming. As a result, creatinine clearance measured using endogenous plasma creatinine and 24 hours urinary creatinine concentrations are used instead to assess GFR (Levey & Inker, 2016). Creatinine is a breakdown product of creatinine phosphate in muscle that is freely filtered and is not reabsorbed, but around 5-10% is secreted by the tubules (Graves, 2008).

Creatinine clearance = (urine creatinine × urine volume) / plasma creatinine

Despite free filtration of small molecules at the glomerulus, urine content is generally low in essential nutrients such as sodium, glucose and water, but high in waste products including urea, creatinine and phenol. There are a variety of transporters located on the plasma membrane of tubular epithelial cells for reabsorption of important substances and excretion of wastes (Wallace, 1998).

1.5.3 Nephron Endowment and Blood Pressure

The importance of the kidneys on long-term blood pressure control is well established. Animal and human studies demonstrated that nephron number was significantly less in individuals with hypertension than normotensive controls (Skov *et al.*, 1994; Keller *et al.*, 2003). This was associated with individual glomerular hypertrophy, and despite normal total filtration surface area, blood pressure remained elevated. Importantly, Brenner *et al.* suggested that reduced nephron endowment and/or filtration surface area reduce renal sodium excretion leading to increase in plasma volume, intra-glomerular and systemic blood pressure (Brenner *et al.*, 1988). In a setting of fewer nephrons, sodium excretion is initially maintained through renal hyperfiltration. This leads to dilation of glomerular capillaries (hypertrophy) that eventually adhere to the Bowman's capsule (Brenner & Chertow, 1994). This results in hardening of glomerular capillaries, or known as glomerulosclerosis (Brenner & Chertow, 1994) and subsequently leads to further loss of functional nephrons. In a setting of low birth weight humans, individuals are born with congenital defect in nephron number. Since no new nephrons are formed after birth in humans, these individuals may be predisposed to an increased susceptibility to hypertension and kidney disease in adulthood. Indeed, human studies demonstrated that deficits in renal mass program and increased susceptibility to adult hypertension and kidney disease (Brenner *et al.*, 1988; Brenner & Chertow, 1994; Zandi-Nejad *et al.*, 2006).

1.6 Low Birth Weight and Cardiorenal Disease

1.6.1 Human Epidemiological Studies

The association between low birth weight and cardiovascular disease risk in human has been widely investigated. Barker and colleagues were the first to study the link between birth weight and cardiovascular disease by tracing 5,654 men born between 1911 and 1930 from six districts of Hertfordshire, England (Barker *et al.*, 1989b). The study demonstrated that men with the lowest weights at birth and at one year of age had a greater death rate from ischemic heart disease (Barker *et al.*, 1989b). They also proposed that measures that promote prenatal and postnatal growth may reduce the death rates from ischemic heart disease among low birth weight individuals (Barker *et al.*, 1989b). A follow-up study was conducted in 5585 women born between 1911 and 1930 in Hertfordshire, England (Osmond *et al.*, 1993). Similar observations were reported with the highest death rates from cardiovascular disease was reported among low birth weight women (Osmond *et al.*, 1993). These studies suggest that the relationship between cardiovascular disease and birth weight are similar in men and women.

These findings lead to a myriad of studies that investigated the link between birth weight and risk of elevations in blood pressure, a known risk factor for both ischemic heart disease and stroke. A study in both men and women aged 46 to 54 years demonstrated that systolic and diastolic blood pressure rose as placental weight increased and reduced as birth weight increased (Barker *et al.*, 1990). Difference between placental and fetal size in blood pressure outcomes suggest that there are circulatory adaptations in the fetus in the suboptimal *in utero* environment which may lead to altered arterial structure and subsequently cause hypertension in adulthood. Similarly, another study found that systolic blood pressure was higher in low birth weight individuals at all ages (early months of life up to the age of 71 years) and the strength of this relationship increased with age (Law *et al.*, 1993).

Similar associations between birth weight and kidney disease have been observed. The population-based Nord Trøndelag Health Study recruited 7,457 adults aged 20 to 30 years born between 1967 and 1977 in Nord Trøndelag County, Norway to investigate the effect of intrauterine growth restriction on young adult kidney function (Hallan *et al.*, 2008). Young adults who had lower weight at birth were reported to have low-normal kidney function, demonstrated by reduced creatinine clearance in these individuals (Hallan *et al.*, 2008). Furthermore, creatinine clearance increased by 7.2 mL/min and 5.7 mL/min in men and women respectively for every kilogram increase in birth weight (Hallan *et al.*, 2008). Another study reported that men aged 18-75 years who were born with weight less than 2.5 kg had a greater prevalence of chronic kidney disease, defined as low glomerular filtration rate or increased urine albumin/creatinine ratio (Li *et al.*, 2008). However, no association between low birth weight and

chronic kidney disease was reported in women (Li *et al.*, 2008). One of the drawbacks of this study was that birth weights were self-reported and there were no records of gestational ages of the participants (Li *et al.*, 2008).

As discussed previously, altered postnatal growth can affect disease outcomes in individuals with low birth weight. Indeed, men and women from Helsinki, Finland who had low weight at birth and experienced accelerated growth from birth to 7 years of age, had increased risk of hypertension later in life (Eriksson *et al.*, 2000). Furthermore, low birth weight, height and BMI at birth and at one year of age were associated with increased risk of coronary heart disease in adulthood but when these individuals had rapid weight gain after one year of age, the risk of coronary heart disease later in life was amplified (Eriksson *et al.*, 2001). Interestingly, a separate study on adults born between 1934 and 1944 in Helsinki demonstrated that risk of stroke progressively reduced with increased weight between birth and 2 years of age in low birth weight individuals (Osmond *et al.*, 2007). However, catch up growth after 2 years of age was associated with increased risk of cardiovascular events in adulthood (Barker *et al.*, 2005). These studies suggest that catch up growth during early postnatal life may protect low birth weight babies from developing adverse cardiovascular outcomes later in life. Therefore, improvement in fetal growth during early postnatal life could lead to substantial reduction in the incidence of cardiovascular disease.

1.6.2 Experimental Animal Studies

In animal study, maternal protein restriction throughout pregnancy leads to a reduction in the nephron number and GFR in growth-restricted male rats (Woods *et al.*, 2001). This resulted in adult male offspring with a conscious, resting mean arterial blood pressure that is ~10 mmHg higher than controls (Woods *et al.*, 2001). Similar outcomes were reported in female rats, whereby maternal protein-restriction during pregnancy increased systolic blood pressure at 9 weeks of age and was sustained at 21 weeks (Langley & Jackson, 1994). Another recent study demonstrated that the combination of prenatal and postnatal protein restriction reduced the nephron number (31%) as well as estimated renal plasma flow, GFR and mean arterial pressure in male rats at 19 weeks of age (Hoppe *et al.*, 2007). In contrast, global maternal undernutrition during the second half of pregnancy was demonstrated to have no effect in blood pressure in adult female rat offspring (Holemans *et al.*, 1999).

Growth restriction induced by placental embolization in fetal sheep delayed cardiomyocyte maturation and increased vasoconstrictor responsiveness in coronary arteries (Bubb *et al.*, 2007), and reduced nephron number by 24% (Zohdi *et al.*, 2007) at embryonic day 130 (term 147 days). In rats, placental insufficiency induced during late gestation resulted in male offspring with reduced nephron

number (-30%) and increased glomerular size at postnatal day 21(Sanders *et al.*, 2004). These outcomes persisted throughout adulthood and were accompanied by renal hyperfiltration and elevated urinary albumin excretion at 12 weeks and 6 months of age without affecting the blood pressure (Sanders *et al.*, 2004). In our laboratory, we reported that growth-restricted male offspring had reduced nephron number (-27%) and increased systolic blood pressure (9 mmHg) by 22 weeks of age (Wlodek *et al.*, 2008). Similarly, subsequent study from our laboratory reported that growth-restricted females exhibited reduced nephron number from 6 months of age (Moritz *et al.*, 2009a). However, despite similar magnitude of nephron deficit to those seen in male offspring subjected to the same intrauterine perturbation, female offspring blood pressure remained normal, even at 18 months of age (Moritz *et al.*, 2009a). Additionally, plasma creatinine levels were elevated, and compensatory glomerular hypertrophy emerged at 18 months of age in growth-restricted females (Moritz *et al.*, 2009a).

1.7 Maternal Adaptations to Pregnancy

Pregnancy is the greatest physiological challenge facing women that results in alterations in maternal physiology and metabolism to assist in fetal growth and development, which is modulated by a number of key molecules. These changes are necessary to facilitate maternal cardiac output and to maintain uteroplacental perfusion of vital organs and fetal demands (Hill & Pickinpaugh, 2008). However, in some instances, the body does not adapt properly to these physiological changes to pregnancy, thereby resulting in pregnancy-related maternal-fetal diseases (Hill & Pickinpaugh, 2008).

1.7.1 Metabolic Adaptations

Glucose the primary nutrient crossing the placenta, is important for fetal and placental growth (Nolan & Proietto, 1994; Herrera, 2000). During pregnancy, glucose homeostasis in the mother is altered so that there is a progressive increase in insulin resistance and gluconeogenetic activity to sustain glucose transfer to the fetus (Herrera *et al.*, 1969). Lipid metabolism is also altered in pregnancy with a significant increase in plasma cholesterol and triglyceride concentrations due to enhanced lipolytic activity and reduced lipoprotein lipase activity of adipose tissue during late gestation (Knopp *et al.*, 1970; Merzouk *et al.*, 2000). During the first and second trimester, the mother is in an anabolic state whereby an increase in lipogenesis activity and adipose tissue lipoprotein lipase activity causes the mothers fat depots to accumulate (Herrera, 2000). The mother then shifts into a catabolic state during late pregnancy when fetal growth accelerates (Lopez-Luna *et al.*, 1986). The increase in maternal insulin resistance during pregnancy is balanced by an increased in maternal pancreatic β-cells mass (Rieck & Kaestner, 2010). Studies in rodents demonstrated a 3-4 fold increase in β-cell mass during pregnancy as a result of increased proliferation and division of preexisting β-cells (Teta *et al.*, 2007; Abouna *et al.*, 2010; Toselli *et al.*, 2014). Maternal pancreatic β-cell proliferation is directly regulated by prolactin (PRL) and placental lactogen (PL) (Brelje & Sorenson, 1991; Parsons *et al.*, 1992; Brelje *et al.*, 1993) that signal through to the prolactin receptor (Amaral *et al.*, 2004; Huang *et al.*, 2009). The β-cell population contracts back to its pre-pregnancy levels at the end of pregnancy through increased rates of β-cell apoptosis, decreased proliferation and reduced β-cell size (Scaglia *et al.*, 1995).

1.7.2 Cardiorenal Adaptations

Cardiovascular adaptations to pregnancy begin as early as 6 weeks of gestation. Maternal blood volume expands and peaks at 30-50% by 32 weeks in humans and 30% by E20 in rats (Atherton *et al.*, 1982; Granger, 2002; Torgersen & Curran, 2006; Hill & Pickinpaugh, 2008) et al., 1982). However, there is a disproportionate increase in plasma (~40%) to red blood cell (~20%) volume, causing physiological anemia (Peck & Arias, 1979; Hytten, 1985). During pregnancy, there is a change in systemic blood

pressure. The increased progesterone, nitric oxide, relaxin, and prostaglandins lead to the systemic vasodilation at pregnancy onsets (Carbillon *et al.*, 2000; Torgersen & Curran, 2006; Conrad, 2011). Systemic vascular resistance remains low in the first and second trimesters, and later begins increasing in the third trimester (Torgersen & Curran, 2006). These changes contribute to an early fall in systemic blood pressure by ~15 mmHg, returning to pre-pregnant levels by term (Hill & Pickinpaugh, 2008). In human pregnancy, increases in both stroke volume and heart rate contribute to the increase in cardiac output (Yeomans & Gilstrap, 2005; Torgersen & Curran, 2006; Chang & Streitman, 2012). Cardiac output rises up to 50% of pre-pregnancy level by the third trimester and remains stable toward term (Hunter & Robson, 1992). Stroke volume is the primary determinant of cardiac output during pregnancy (Hunter & Robson, 1992). Stroke volume increases sharply from early pregnancy resulting in a 32% rise by 20 weeks (Ouzounian & Elkayam, 2012). Meanwhile, heart rate increases by 11-12% by mid-pregnancy. The progressive increase in stoke volume during early pregnancy is mainly attributed to the increase in preload due to plasma volume expansion.

Despite increased levels of circulating renin during pregnancy, pregnant women are resistant to the pressor effects of Angiotensin II (AngII) (Luppi, 1999). Increased nitric oxide production may antagonise the vasoactive effects of AngII and noradrenalin (Rosenfeld, 2001). Maternal blood flow is enhanced to the uterus and kidneys, each receiving ~20% of cardiac output (Chang & Streitman, 2012). These hemodynamic changes allow for optimal perfusion and oxygenation of vital tissues to meet the demands of the developing fetus. Dilation of the renal vasculature soon after conception results in increased effective renal plasma flow (eRPF) and GFR, peaking at 50-85% and 40-65% above normal, respectively by mid human pregnancy (Lindheimer *et al.*, 2001; Granger, 2002). In pregnant rats, eRPF and GFR increases are smaller at ~20-40% (Granger, 2002). Since eRPF increases are greater than GFR, the filtration fraction is often reduced during pregnancy (Yeomans & Gilstrap, 2005). Renal expression of nitric oxide synthase elevated (Gandley *et al.*, 2001) and its inhibition attenuates the renal hyperfiltration usually seen (Danielson & Conrad, 1995). The peptide hormone relaxin has also been postulated as key mediator of reduced renal vascular resistance and subsequent hyperfiltration (Danielson *et al.*, 2000).Increased GFR leads to increased urine flow and volume, as well as elevated clearance of creatinine, urea, uric acid and glucose (Torgersen & Curran, 2006). Despite the increased flow, increased activity of the renin-angiotensin-system acts on the kidneys to promote sodium and water retention, aiding in the accumulation of ~ 7 L of fluid and providing sufficient amounts of water to the feto-placental unit (Sibai & Frangieh, 1995; Luppi, 1999). Marked increases in GFR may result in proteinuria, particularly in pregnancies complicated by maternal hypertension and/or preeclampsia. Finally, plasma electrolyte and protein levels (albumin) are also decreased during pregnancy, due to increase in renal excretion and changes in charge selectivity of the glomerular membrane that reduce colloid oncotic pressure (Hill & Pickinpaugh, 2008).

1.7.3 Pregnancy and Low Birth Weight

In some instances, the physiological challenge of pregnancy may reveal an underlying predisposition to disease and complications arise with both short- and long-term adverse health effects for the mother (Clifton & Murphy, 2004). In females that were born growth-restricted, pregnancy may exacerbate their risk of cardiovascular and metabolic disease due to an increase in both maternal and fetal demands. Indeed, epidemiological studies associate a low birth weight with a higher risk of developing preeclampsia during later pregnancy (Klebanoff *et al.*, 1999; Zetterstrom *et al.*, 2007). Furthermore, women born with a low birth weight were also more susceptible to gestational diabetes mellitus (GDM) during pregnancy compared to women that were born of normal weight (Seghieri *et al.*, 2002). A study in female rats born small also identified a higher risk of developing GDM in pregnancy as well as an increased risk of their offspring developing an altered metabolic phenotype (Boloker *et al.*, 2002). Likewise, we have previously demonstrated that growth-restricted female rats during late pregnancy develop glomerular hypertrophy and glucose intolerance, despite a normal plasma insulin response (Gallo *et al.*, 2012b).

1.8 Obesity and Pregnancy

In Australia, approximately 26% of pregnant women were identified to be overweight and 20% were obese (AIHW, 2015). Obesity during pregnancy is associated with a number of obstetric complications including GDM, hypertension and pre-eclampsia (Doherty *et al.*, 2006; McIntyre *et al.*, 2012; Cunningham & Teale, 2013; Dodd *et al.*, 2014). Of particular concern is maternal obesity does not only affect the mother but the offspring have higher risk to be born large for gestational of age and expose to metabolic and cardiovascular diseases later in life (Catalano & Ehrenberg, 2006).

In an obese mother, the pregnancy adaptations differ from what occurs in healthy pregnant women. For example, glucose metabolism is significantly altered with an increase in peripheral and hepatic insulin resistance during the first trimester of pregnancy compared to normal weight pregnant women (Catalano, 2010). Therefore, is not surprising that overweight or obese women, who are prone to beta-cell dysfunction and compromised glucose tolerance pre-pregnancy, are at increased risk of GDM. In Australia, it has been reported that 8-10% of pregnant women has GDM (Moses *et al.*, 2011). Importantly, the incidence of GDM is higher in overweight or obese pregnant women (Sermer *et al.*, 1995; Jensen *et al.*, 2001) with a 2-10 fold increase (Bianco *et al.*, 1998; Kumari, 2001; Sebire *et al.*, 2001; Ramachenderan *et al.*, 2008; Cunningham & Teale, 2013). Indeed, a study reported that women who were overweight or obese during their pregnancy developed glucose intolerance and had higher risk of GDM (Yogev *et al.*, 2004). Another study reported that an increase in BMI in pregnant women was linked to an increased risk of GDM in their next pregnancy and pregnant women with lower BMI had a reduced risk for GDM in their next pregnancy (Ehrlich *et al.*, 2011). Furthermore, GDM has also been associated with long-term health complications in the mother (Ramachenderan *et al.*, 2008; Clausen *et al.*, 2009; Yogev & Visser, 2009). Clearly, there is a linear association between BMI and the incidence of GDM. The mechanisms underlying this adverse pregnancy outcome are poorly understood. However, abdominal fat accumulation in obese women during pregnancy is likely to be associated with an increase in inflammatory cytokine production, leading to insulin resistance (Ramsay *et al.*, 2002).

Also, obese and overweight pregnant women are at higher risk of developing preeclampsia and pregnancy-induced hypertension. Women with a BMI greater than 30 before pregnancy had 2 to 3 fold higher risk of being diagnosed with hypertension or preeclampsia during their pregnancy (Sibai & Frangieh, 1995; Sibai *et al.*, 1997; Sattar *et al.*, 2001; Mostello *et al.*, 2010). An epidemiological study by Bianco *et al*. showed a 4-fold increased risk for pre-eclampsia in 612 morbidly obese pregnant women (Bianco *et al.*, 1998). Another study of 159 morbidly obese pregnant women and 300 normal weight women reported that 28.8% of morbidly obese women developed hypertension during gestation compared to 2.9% in the normal weight women (Kumari, 2001). Finally, a meta-analysis reported that with each 5-7kg/m^2 increase in pre-pregnancy BMI, the incidence of pre-eclampsia during pregnancy

is doubled (O'Brien *et al.*, 2003). Given that growth-restricted females are at higher risk of cardiovascular and metabolic disease during their pregnancy, it is likely that maternal obesity may exacerbate the phenotypes. However, there are limited studies investigating the effects of maternal obesity in growth-restricted mothers and the subsequent effects in their offspring.

1.8.1 The Effects of Maternal Obesity on Offspring Health

It is well established that maternal obesity can lead to increased fetal growth, which can cause the offspring being born macrosomic (Ehrenberg *et al.*, 2004). However, recent findings suggest that offspring born to obese mother can also be small for gestational of age or born with a normal birth weight (McIntyre *et al.*, 2012; Anderson *et al.*, 2013). Of particular note, being small or large for gestational age due to maternal obesity predispose the offspring to obesity in adulthood (Drake & Reynolds, 2010). Several animal studies have investigated the relationship between maternal obesity and the development of obesity in the offspring (Bayol *et al.*, 2007; Samuelsson *et al.*, 2008; Rajia *et al.*, 2010). In rats, exposure to maternal obesity during pregnancy and lactation increased the risk of obesity later in life (Bayol *et al.*, 2007; Rajia *et al.*, 2010). The risk of obesity was further exacerbated when the offspring were exposed to high fat diets post-weaning (Bayol *et al.*, 2007; Chen *et al.*, 2008). These clearly indicate that maternal obesity increased the obesity risk in their offspring. There are a number of mechanisms that may explain the programming effects of maternal obesity on offspring obesity risk including programming of appetite dysregulation (Bayol *et al.*, 2007; Samuelsson *et al.*, 2008; Ross & Desai, 2014) and altered adipogenesis (Muhlhausler *et al.*, 2007; Bayol *et al.*, 2008).

In addition to increasing the risk of offspring obesity, maternal obesity and overnutrition also program metabolic dysfunction in their offspring. Limited human studies have examined the link between maternal obesity, offspring insulin resistance and other adverse metabolic outcomes. For instance, the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study reported that an increase in maternal BMI is linked with fetal hyperinsulinemia, which is independent of maternal glycemia (Group HSCR, 2010). Importantly, there is evidence that demonstrates that babies of obese mothers develop insulin resistance in utero, indicating that maternal obesity is an important predictor of metabolic disease in their offspring (Catalano *et al.*, 2009). The effects maternal obesity has on insulin sensitivity then persists into later life with children of overweight women having an increased risk of developing insulin resistance by 11 years of age (Boney *et al.*, 2005)and in early adulthood (early 20s) (Mingrone *et al.*, 2008). Therefore, these findings support the association between maternal obesity and altered glucose-insulin homeostasis in the offspring.

Indeed, in animal studies, maternal obesity or overnutrition during both pregnancy and lactation is linked to increased insulin and glucose in the offspring and these features were exacerbated when the

offspring were also exposed to a high fat diet post-weaning (Bayol *et al.*, 2008; Samuelsson *et al.*, 2008; Shankar *et al.*, 2008; Nivoit *et al.*, 2009; Tamashiro *et al.*, 2009). Studies suggest that alteration in glucose-insulin homeostasis in offspring of obesity mothers is likely due to β-cell failure (Han *et al.*, 2005; Srinivasan *et al.*, 2006; Cerf *et al.*, 2009; Cerf *et al.*, 2012). Evidence in rat studies demonstrated that exposure to a high fat diet (40% calories from fat) during pregnancy and lactation caused hyperglycemia and insulin resistance with compromised β -cell development and function in the offspring (Cerf *et al.*, 2009; Cerf *et al.*, 2012). Additionally, offspring of obese mice develop insulin resistance at 3 months of age but by 6 months of age male offspring developed frank diabetes with reduced plasma insulin and pancreatic insulin content, indicative of β-cell exhaustion (Samuelsson *et al.*, 2008). Other animal studies recorded similar observations suggesting that there is an age-related decline in β -cell function in offspring of obese mothers that leads to altered glucose and insulin homeostasis (Han *et al.*, 2005; Srinivasan *et al.*, 2006). In sheep, maternal obesity is linked to an increased fetal pancreatic weight and an increased number of insulin-positive cells per unit area, which is indicative of accelerated β-cell maturation (Ford *et al.*, 2009)A further study showed that offspring of high fat dam had reduced β-cell numbers and this was associated with an increased in β-cell apoptotic rate (Zhang *et al.*, 2011).Therefore, these changes may predispose the offspring to premature postnatal β-cell function loss and this will subsequently lead to elevated risk of metabolic disease in adulthood.

Taken together, these findings suggest that maternal obesity or overnutrition during pregnancy have an adverse effect on offspring metabolic outcomes (Schaefer-Graf *et al.*, 2008). Thus, further studies are required to understand the underlying mechanisms that predispose the offspring of obese mothers to metabolic diseases and whether these adverse phenotypes can be modified by lifestyle interventions.

1.9 Effects of Exercise

Exercise has been a long-recognized lifestyle intervention associated with the prevention of adult diseases in human. A myriad of epidemiological studies reported that regular exercise training in non-pregnant individuals improves their insulin sensitivity (Bruce *et al.*, 2004; Hawley, 2004; Hawley & Lessard, 2008), glucose tolerance (Tuomilehto *et al.*, 2001; Bruce *et al.*, 2006), cardiac function as well as lower blood pressure in individuals with hypertension (Batty *et al.*, 2002; Tanasescu *et al.*, 2003). It has been suggested that exercise promotes numerous molecular and cellular changes in several tissues in particular skeletal muscle and heart and these adaptations are likely to underpin many of the beneficial actions of exercise. Although the benefits of exercise intervention are well characterized in non-pregnant individuals, the effects of exercise intervention on pregnancy especially in those at higher risk of pregnancy complications are not conclusive.

1.9.1 Effects of Exercise in Pregnancy

The American College of Obstetrics and Gynecologists recommends that pregnant women should engage in moderate-intensity exercise at least 20 to 30 minutes on most or all days of the week (ACOG Committee Obstetric Practice, 2015). This is largely due to an increasing number of studies that reported positive outcomes of exercise training on both maternal and fetal health. Epidemiological studies have shown that exercise is associated with reduced risk of GDM (Dempsey, 2004; Oken *et al.*, 2006; Zhang *et al.*, 2006; Tobias *et al.*, 2011; Barakat *et al.*, 2012), excessive gestational weight gain (Stuebe *et al.*, 2009; Barakat *et al.*, 2012) and pregnancy-induced hypertension and preeclampsia.

In a prospective cohort study, over 1000 healthy pregnant women were recruited to investigate the link between GDM risk and maternal physical activity performed one year before pregnancy and during early pregnancy (Dempsey, 2004). The study found that women who participated in any physical activity one year before pregnancy had a 56% reduced risk of GDM compared to inactive women (Dempsey, 2004). Physical activity performed during early pregnancy was also associated with a 36% reduced risk of GDM and the risk was further reduced (69% reduction) when physical activity was performed both before and during early pregnancy (Dempsey, 2004). Similar link was demonstrated in other studies, where risk of GDM and glucose intolerance was reduced in women who involved in any physical activity in the year before pregnancy (Oken *et al.*, 2006; Zhang *et al.*, 2006; Redden *et al.*, 2011) as well as in women who exercised both before and during pregnancy (Oken *et al.*, 2006). Furthermore, a supervised physical activity program from early gestation to the end of the third trimester in healthy pregnant women was also reported to improve glucose tolerance and prevent excessive maternal weight gain and GDM (Barakat *et al.*, 2012). However, other recent studies that investigated the effects of 12 weeks of exercise intervention in the second trimester of pregnancy failed to associate

the beneficial effects of exercise with a reduced risk of GDM (Stafne *et al.*, 2012; Nobles *et al.*, 2015). From these studies, it can be postulated that a beneficial effects of exercise on GDM prevention may be limited to women who engaged in exercise prior to and/ or during early pregnancy than those who started exercise during the second and third trimesters. This is partly because chronic changes in the regulation of skeletal muscle glucose uptake are already adapted in women who started exercise before or early pregnancy and thus, they may be better at handling the metabolic changes that largely happened during late gestation.

In studies that are examining the effects of exercise and the risk of gestational hypertension and preeclampsia, the evidences however were not as conclusive. Some studies found no clear link between preeclampsia risk and physical activity performed before pregnancy (Hegaard *et al.*, 2010; Tyldum *et al.*, 2010) and early pregnancy (Osterdal *et al.*, 2009; Vollebregt *et al.*, 2010). Findings from a cohort study on Danish women even suggest that leisure time physical activity exceeding 270 minutes per week during early pregnancy may increase risk of severe preeclampsia (Osterdal *et al.*, 2009). Nevertheless, there are studies that demonstrated the beneficial effects of exercise performed before and/or early pregnancy on the risk of preeclampsia (Sorensen *et al.*, 2003; Saftlas *et al.*, 2004; Rudra *et al.*, 2008; Spracklen *et al.*, 2016). Similar patterns were observed on the effects of exercise and the risk of gestational hypertension alone with some studies found positive effects (Martin & Brunner Huber, 2010; Fortner *et al.*, 2011; Barakat *et al.*, 2016) and some studies reported no difference (Saftlas *et al.*, 2004; Vollebregt *et al.*, 2010; Chasan-Taber *et al.*, 2015). The inconsistencies in the findings from all these studies may be due the differences in activity assessment with some studies included all types of leisure time physical activity such as sports, transportation and walking as a measure of total physical activity (Rudra *et al.*, 2008; Hegaard *et al.*, 2010) and some studies were more selective (Saftlas *et al.*, 2004; Osterdal *et al.*, 2009; Barakat *et al.*, 2016).

There has been a lot of concern regarding the effects of maternal exercise on the fetus growth and development as exercise may divert maternal blood flow and nutrients from uteroplacental circulation to working muscles (Currie *et al.*, 2014). In a study that investigated the effects of 12 weeks of exercise intervention during pregnancy, no incidence of adverse birth outcomes including small and large for gestational age, low birth weight, and preterm birth were reported (Currie *et al.*, 2014; Nobles *et al.*, 2015). Some studies even demonstrated that maternal exercise reduced risk of delivering macrosomic infants (Currie *et al.*, 2014; Barakat *et al.*, 2016). However, the mechanisms underlying the effects of exercise on fetal growth and development are still not clear and need to be explored.

It is important to note that most lifestyle intervention studies were conducted in normal weight pregnant women, and the findings cannot be extrapolated directly to obese pregnant women. Recently however, there have been a number of intervention studies (Artal *et al.*, 2007; Claesson *et al.*, 2008; Ong *et al.*,

2009; Mottola *et al.*, 2010; Oostdam *et al.*, 2012; Dodd *et al.*, 2014), systematic reviews (Dodd *et al.*, 2008; Dodd *et al.*, 2010; Sui *et al.*, 2012) and a meta-analysis (Oteng-Ntim *et al.*, 2012) that have investigated the effects of exercise and other lifestyle modifications on pregnancy outcomes in overweight and obese pregnant women. There is some evidence that suggests that exercise can be helpful in improving fitness (Saftlas *et al.*, 2004; Ong *et al.*, 2009) and glucose tolerance (Ong *et al.*, 2009; Oteng-Ntim *et al.*, 2012; van Poppel *et al.*, 2013) and limiting weight gain (Artal *et al.*, 2007; Claesson *et al.*, 2008; Mottola *et al.*, 2010; Oteng-Ntim *et al.*, 2012; Sui *et al.*, 2012) in obese pregnant women. However, not all of these studies have found a positive effect from antenatal exercise (Oostdam *et al.*, 2012; Dodd *et al.*, 2014). In fact, a systematic review found that, apart from limiting gestational weight gain, the benefits of antenatal exercise in overweight and obese women on maternal and perinatal outcomes remain unproved (Sui *et al.*, 2012). Nonetheless, it is important to stress that no negative effects have so far been reported (Sui *et al.*, 2012).

In animal studies, maternal exercise was reported to reduce the metabolic risk caused by maternal obesity in both the mother and offspring (Raipuria *et al.*, 2015; Vega *et al.*, 2015). A recent study on rats reported that voluntary wheel running before and during pregnancy prevented the increase in insulin, glucose, insulin resistance (HOMA-IR) and triglyceride during lactation in obese dams (Vega *et al.*, 2015). Additionally, voluntary exercise before and during pregnancy reduced glucose and insulin concentrations in male offspring (postnatal day 19) of obese rat mother (Raipuria *et al.*, 2015) as well as prevented glucose intolerance induced by maternal obesity in female offspring (24 weeks) of C57BL/6 mice (Laker *et al.*, 2014). Of particular concern, there are very limited animal studies that investigating the effects of exercise intervention in maternal obesity and most of the studies utilised a poorly controlled voluntary wheel running as the exercise intervention. Therefore, a well-controlled intervention animal study using a motorised treadmill exercise is required as precise exercise intensity and duration can be controlled.

1.9.2 Effects of Exercise in Low Birth Weight

Since physical activity is associated with reduced risk of GDM, pregnancy induced hypertension and preeclampsia, any possible interventions approach that may prevent the altered pregnancy adaptations in women with low birth weight are of important interest. Eriksson and colleagues have reported that regular and moderate exercise protected elderly people who were born small from glucose intolerance and thus prevents the development of impaired glucose tolerance (Eriksson *et al.*, 2004). This suggests that implementing exercise training in offspring born small may prevent them from developing severe diseases later in life. Likewise, an observational study reported that physical activity decreased the risk of insulin resistance in adolescents with low birth weight (Ortega *et al.*, 2011).

In rats, exercise from 3 to 8 weeks of age in female offspring exposed to maternal nutrient restriction during gestation demonstrated subtle metabolic improvements in response to intravenous glucose tolerance and insulin tolerance tests, as well as improved GLUT4 translocation to the plasma membrane (Garg *et al.*, 2009). Others have shown that low levels of voluntary wheel running from 8-35 weeks of age in male rats exposed to maternal undernutrition were protected from prenatally induced obesity (Miles *et al.*, 2009) and restored expression of skeletal muscle GLUT4 and protein kinase C δ (PKCδ) protein expression to control levels (Huber *et al.*, 2009). Additionally, studies from our lab reported that growth restricted male rats that underwent four weeks of exercise training by treadmill from 5 to 9 weeks of age had increased β-cell mass in adulthood (6 months), restoring 60-68% deficits (Laker *et al.*, 2011). The findings of these studies suggest that exercise training has beneficial effects on low birth weight individuals however; it is unknown whether exercise can improve maternal outcomes in low birth weight pregnant females.

1.10 Overview

There has been a significant increase in the number of overweight or obese pregnant women in the past two decades. Women who are overweight or obese often experience pregnancies complicated with GDM, preeclampsia and pregnancy induced hypertension. Of particular concern, maternal obesity does not only affect the mother but fetal growth and development can also be compromised. Although it is well established that being born small for gestational age is one of the risk factors for developing obesity, there are currently no studies that have examined the effects of maternal obesity in growth-restricted mothers. Importantly, evidence from epidemiological studies demonstrated that women born small for gestational age have adverse pregnancy adaptations, which predispose them to GDM and hypertension and preeclampsia. Thus, the combination of maternal obesity and low birth weight may exacerbate the pregnancy outcomes (Figure 1.4). Recently, there has been much interest in the development of lifestyle interventions targeting pregnant women. Exercise, in particular, is associated with the prevention of metabolic and cardiovascular disease in pregnant and non-pregnant women. Furthermore, the benefits of exercise to pregnancy outcomes are much greater when performed before and during pregnancy. Thus, the development of targeted interventions in growth-restricted mothers may prevent them from developing cardiorenal and metabolic dysfunction during pregnancy (Figure 1.4).

A recent study from our laboratory investigated the metabolic and cardiovascular adaptations during pregnancy in growth restricted females by measuring renal function and tail-cuff blood pressure as well as performing glucose tolerance test during late gestation (Gallo *et al.*, 2012b). It was discovered that growth restricted females that were exposed to these physiological measurements during late gestation had 5-6% smaller offspring (Gallo *et al.*, 2012b; Cheong *et al.*, 2016a; Cheong *et al.*, 2016b). Therefore, it is important to characterise the effects of physiological measurements that were conducted during late gestation on the fetal growth and whether exercise intervention can prevent this outcome.

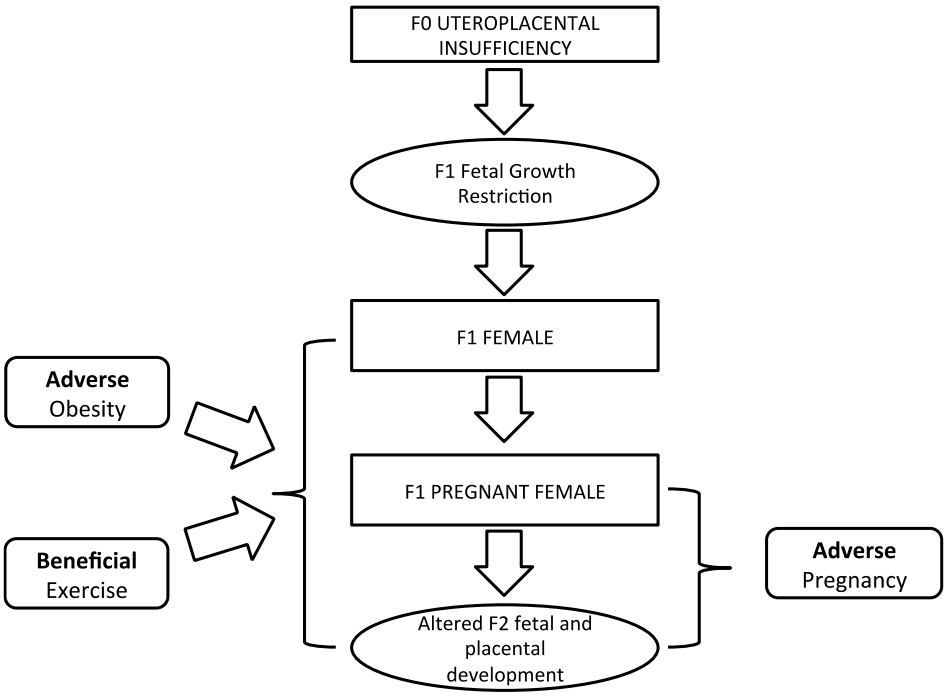


Figure 1.4 Lifestyle challenges that may exacerbate growth restricted females metabolic and cardiovascular dysfunctions.

Metabolic and cardiovascular dysfunctions in growth restricted F1 female can be exacerbated with second hits such as pregnancy and obesity and endurance exercise may prevent these complications.

1.10.1 Aims and Hypotheses

Chapter 3 Effects of Exercise and High Fat Diet on Metabolic Adaptations to Pregnancy in Females Born Small

The overall aim of this study was to determine if a high fat diet exacerbates the known adverse metabolic adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these complications.

Specific aims of Chapter 3 were:

- ***i.*** To determine if a high fat diet exacerbates impaired glucose tolerance and insulin secretion in late gestation in growth restricted females;
- ***ii.*** To determine whether exercise before and during pregnancy is more beneficial in preventing impaired glucose tolerance and insulin secretion in late gestation in growth restricted females than exercise during pregnancy alone;
- ***iii.*** To determine whether the exacerbation and prevention of impaired glucose tolerance and insulin secretion in late gestation in growth restricted females is associated with alterations in pancreatic β-cell and islet morphology;
- ***iv.*** To determine whether the exacerbation and prevention of impaired glucose tolerance and insulin secretion in late gestation in growth restricted females is associated with alterations in plasma cytokines and skeletal muscle and liver triglycerides;
- ***v.*** To determine whether high fat diet and exercise alter energy expenditure and spontaneous physical activity in late gestation in growth restricted females.

It is hypothesised that high fat diet will exacerbate the known adverse metabolic adaptations to pregnancy in growth restricted females in late gestation and endurance exercise training will prevent the adverse pregnancy outcomes.

Chapter 4 Effects of Exercise and High Fat Diet on Cardiorenal Adaptations to Pregnancy in Females Born Small

The overall aim of this study was to determine if a high fat diet exacerbates the known adverse cardiorenal adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these altered adaptations.

Specific aims of Chapter 4 were:

- ***i.*** To determine if a high fat diet exacerbates renal dysfunction and unmasks high blood pressure in late gestation in growth restricted females;
- ***ii.*** To determine whether exercise before and during pregnancy is more beneficial in preventing renal dysfunction and prevents the emergence of high fat diet induced high blood pressure in late gestation in growth restricted females than exercise during pregnancy alone.

It is hypothesized that growth restricted females on a high fat diet will develop high blood pressure and renal dysfunction in late gestation, and exercise before and during pregnancy will be more beneficial in preventing the adverse pregnancy outcomes than exercise during pregnancy alone.

Chapter 5 Effects of High Fat Diet and Exercise on Fetal Outcomes

The overall aim of this study was to investigate the effect high fat diet, endurance exercise training and physiological measurements performed during late gestation on the fetal outcomes in growth restricted mothers.

Specific aims of Chapter 5 were:

- ***i.*** To determine if high fat diet alters fetal and placental weights and placental efficiency in growth restricted mothers in late gestation;
- ***ii.*** To determine if physiological measurement performed during late gestation of pregnancy reduces fetal and placental weights and placental efficiency in growth restricted mothers in late gestation;
- ***iii.*** To determine whether endurance exercise training alters fetal outcomes in growth restricted mothers in late gestation;
- ***iv.*** To determine the sex specific difference in fetal and placental outcomes.

It is hypothesised that high fat diet and physiological measurements performed during late gestation in growth restricted mothers will adversely affect fetal and placental growth in late gestation and endurance exercise training will prevent the adverse fetal outcomes.

CHAPTER 2

General Methods

2.1 Study Overview

All experiments were approved by The University of Melbourne Animal Ethics Committee (AEC #1212639) and conducted in accordance with the National Health and Medical Research Council of Australia, *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC, 2004) and *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes* (NHMRC, 2008). Wistar Kyoto (WKY) rats (obtained from Biological Research Facility, The University of Melbourne) were housed in plastic cages with stainless steel lids in an environmentally controlled room (temperature 22 degree Celsius (°C)) with a 12 hours light/dark cycle and 45-55% relative humidity at the Biological Research Facility, The University of Melbourne. All rats had access to standard chow consisting of 19% protein, 76.4% carbohydrate and 4.6% fat (Specialty feeds, Glenforrest, WA, Australia) and tap water *ad libitum*.

2.1.1 Experimental Timeline

A brief experimental timeline is presented in Figure 2.1. F0 female rats were mated at 18-24 weeks and exposure to sham or uteroplacental insufficiency at day 18 of gestation resulted in F1 Control and Restricted offspring. At 5 weeks, female F1 rats were weaned and given access to either a chow or high fat diet (HFD). F1 females were randomly allocated to sedentary, exercise before and during pregnancy (Exercise) or exercise during pregnancy only (ExPregOnly) groups at 16 weeks. Systolic blood pressure by tail cuff was performed at 19 weeks and rats were mated with F0 (normal) males at 20-24 weeks. Physiological measurements consisted of 24 hours in a metabolic cage, 24 hours in an indirect open-circuit calorimeter (CLAMS), tail-cuff blood pressure and non-fasted intraperitoneal glucose tolerance test were performed during late gestation. A second cohort of pregnant F1 females was generated where none of these physiological measurements were performed to investigate the possible effect of these measurements on F2 fetal body and placental weights. On day 20 of gestation, *post mortem* was performed on the pregnant rats and in F2 male and female fetuses. All experimental groups (1/litter/group; n = 8-10/group) are summarised in Table 2.1.

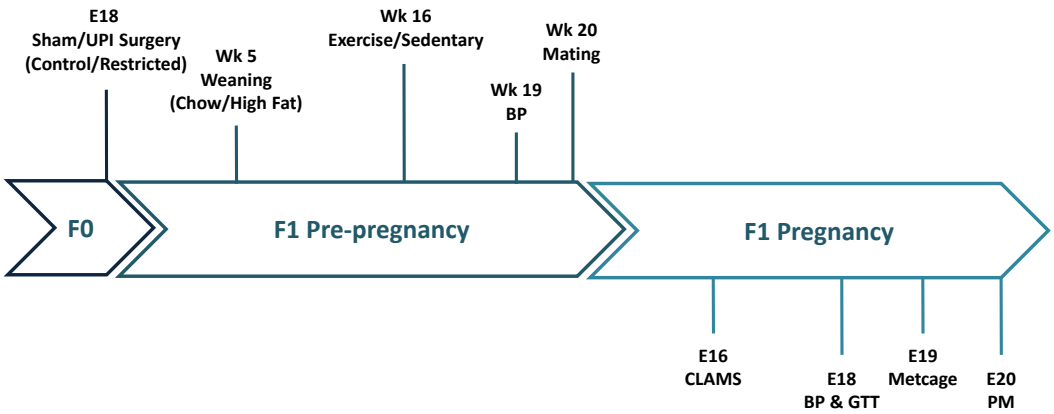


Figure 2.1 Experimental timeline

F0 females were mated and uteroplacental insufficiency (UPI) surgery was performed on gestational day 18 (E18). F1 female offspring were randomly allocated to a chow or high fat diet at week 5 and Sedentary or Exercise group at week 16. Systolic blood pressure (BP) was measured at week 19 and rats were mated at week 20. Physiological measurements (CLAMS on E16, non-fasted intraperitoneal glucose tolerance test (GTT) and tail cuff blood pressure (BP) on E18 and 24 hours metabolic cage measurement (Metcage) on day 19) were performed during late gestation. A *post mortem* was conducted on E20 on the pregnant rats and in F2 fetuses.

Table 2.1 Experimental groups

C- Control, **R**- Restricted, **Ch**- Chow, **HF**- High fat, **Sed**- Sedentary, **PrePregEx**- Exercise before and during pregnancy, **PregEx**- Exercise during pregnancy only, **N**- No physiology measures, **M**- Physiology measures (1 per litter per group; *n*= 8-10 per group).

Group	No Physiology Measures		Physiology Measures	
	Control	Restricted	Control	Restricted
Chow + Sedentary	C-Ch-Sed-N	R-Ch-Sed-N	C-Ch-Sed-M	R-Ch-Sed-M
High Fat + Sedentary	C-HF-Sed-N	R-HF-Sed-N	C-HF-Sed-M	R-HF-Sed-M
Chow + Exercise	C-Ch-Exercise-N	R-Ch-Exercise-N	C-Ch-Exercise-M	R-Ch-Exercise-M
High Fat + Exercise	C-HF-Exercise-N	R-HF-Exercise-N	C-HF-Exercise-M	R-HF-Exercise-M
Chow + Pregnancy Exercised Only	C-HF-PregExOnly-N	R-HF-PregExOnly-N	C-HF-PregExOnly-M	R-HF-PregExOnly-M
High Fat + Pregnancy Exercised Only	C-HF-PregExOnly-N	R-HF-PregExOnly-N	C-HF-PregExOnly-M	R-HF-PregExOnly-M

2.2 Generation of F1 Offspring

2.2.1 Mating

Virgin female rats were examined for appropriate estrous cycle for mating using a rat vaginal impedance checker (model MK-10B; Mukomachi Kikai, Osaka, Japan) (Bartos, 1977). Electrical impedance reading of >7kΩ at 1430 hour suggested that the female was in proestrus and would likely to enter estrus overnight. One to two proestrus females were then housed with a WKY male overnight for mating. The presence of sperm in vaginal smears the following morning was considered as day 1 of pregnancy (E1) (Wlodek *et al.*, 2005; O'Dowd *et al.*, 2008).

2.2.2 Uteroplacental Insufficiency Surgery

On day 18 of pregnancy (E18), F0 female rats were weighed and the abdomen was examined to confirm pregnancy. F0 pregnant rats were then randomly allocated into Control (sham surgery) or Restricted (uteroplacental insufficiency surgery) group. Uteroplacental insufficiency in Restricted group was induced by bilateral uterine vessel ligation (artery and vein), which reduced oxygen, nutrient and blood supply to the developing fetuses (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Gallo *et al.*, 2012a; Cheong *et al.*, 2016a). Prior to surgery, the pregnant rats were anaesthetised with 4% isofurane (Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia) and 650 $ml.min^{-1}$ oxygen flow (reduced to 3.2% isoflurane and 250 $ml.min^{-1}$ oxygen flow when suturing) and the absence of corneal and pedal reflexes indicated that the rats were completely unconscious. A 2-3 cm vertical midline abdominal incision was made through the skin and the underlying muscle layer to expose the cervical end of the uterus. The uterine horns containing the fetuses were carefully taken out from the abdominal cavity and placed onto gauze soaked in sterile saline (0.9% sodium chloride, Baxter Healthcare International, Deerfield, IL, USA). Both sides (left and right) of uterine arteries and veins were ligated near the cervical end using 4-0 sterile silk suture (Ethicon Inc, Piscataway, NJ, USA; Figure 2.2). The surface of the uterus was regularly flushed with sterile saline to keep it moist throughout the surgery and placed back into the abdominal cavity after the vessel ligation. The muscle layer was sutured with 4-0 chromic catgut and the skin layer was sutured using double single stitching with sterile 4-0 silk (Johnson & Johnson Medical, North Ryde, NSW, Australia). Pregnant rats that were in Control group were exposed to identical conditions except the uterine vessels were not ligated to control for the effects of surgical and anaesthetic procedures that may affect fetal growth and development. Rats were then individually housed and left to deliver naturally at term on E22.

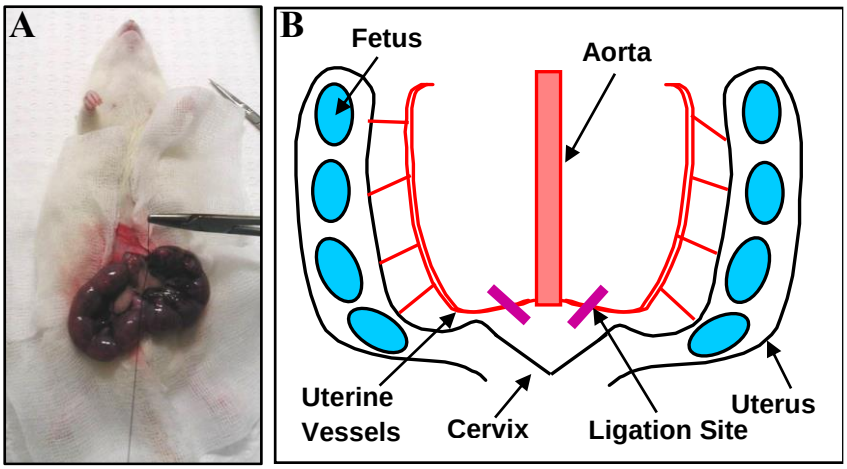


Figure 2.2 Uteroplacental insufficiency surgery

Uteroplacental insufficiency was induced by bilateral uterine vessel (artery and vein) ligation on E18 of pregnancy. (A) Photograph during surgery (B) Schematic diagram. Photographs reproduced with permission from Wlodek.

2.2.3 F1 Postnatal Body Weights and Growth Profile Measurements

After birth on postnatal day 1 (PN1), the number of pups and sex of individual offspring were determined and body weights were recorded. As the individual pup sex on PN1 was not identifiable, birth weight was taken as the litter average and separated by sex. On PN7, each pup underwent toe clipping using a pair of fine scissors for identification purposes. Body weights were taken and body dimensions (crown-to-rump length, head length, head width and hind limb length) were measured using digital vernier calipers accurate to 0.01 mm (Figure 2.3) at postnatal day 7, 14, 35 and 15, 19 and 20 (mating) weeks. All female offspring were weaned at PN35 and were housed in a group of four. Only one female from each litter was allocated to one of the 24 experimental groups. During pregnancy, body weights were recorded at gestational day 20 and gestational weight gain (E0-E20) was calculated.

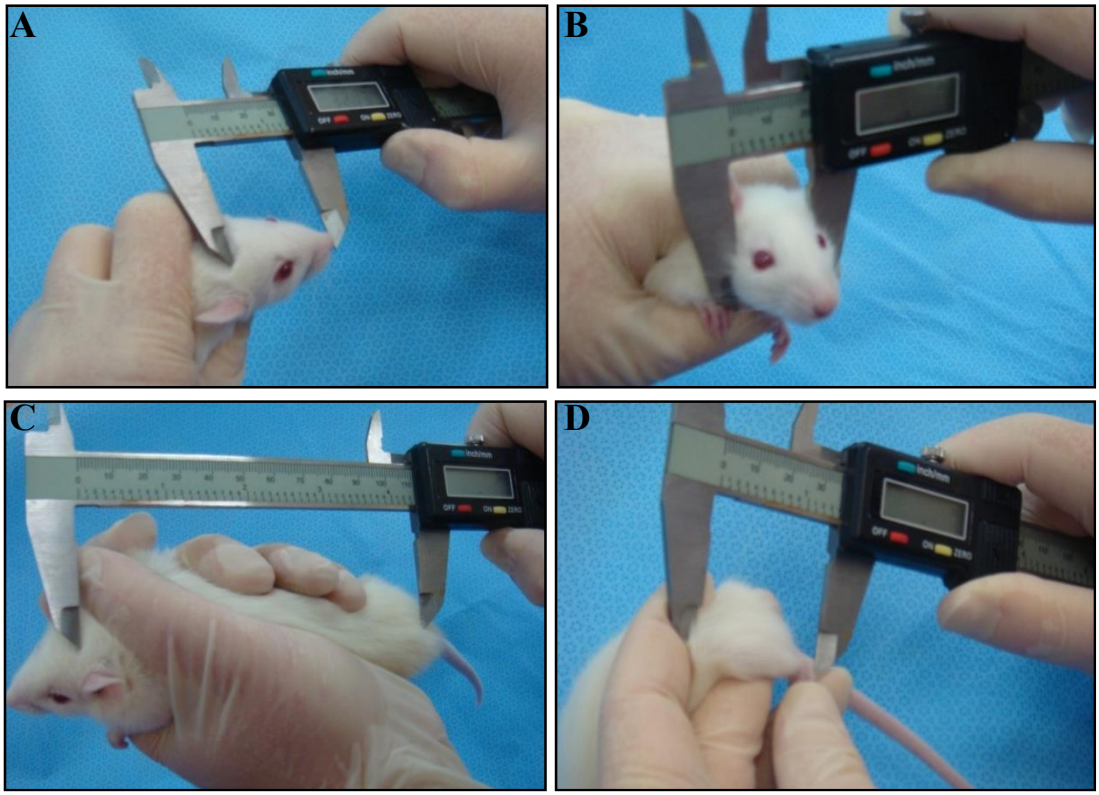


Figure 2.3 Body dimension measurements

Measurements of (A) head length, (B) head width, (C) crown-to-rump length and (D) hind limb length on postnatal day 35. Photographs reproduced with permission from Wlodek.

2.3 Diet Protocol for F1 Offspring

At weaning, F1 female offspring were randomly allocated to receive *ad libitum* access to a standard chow diet (AIN93G (Appendix 1), Specialty Feeds, Glen Forrest, WA, Australia) or commercial high fat diet. The HFD group was offered two types of commercially available high fat pellet diets; SF03–020 (Appendix 1) and SF01-028 (Appendix 1) (Specialty Feeds, Glen Forrest, WA, Australia) and standard chow (Raipuria *et al.*, 2015). Both high fat diets are based on the AIN93G diet and have similar macronutrient and micronutrient contents (Table 2.2).

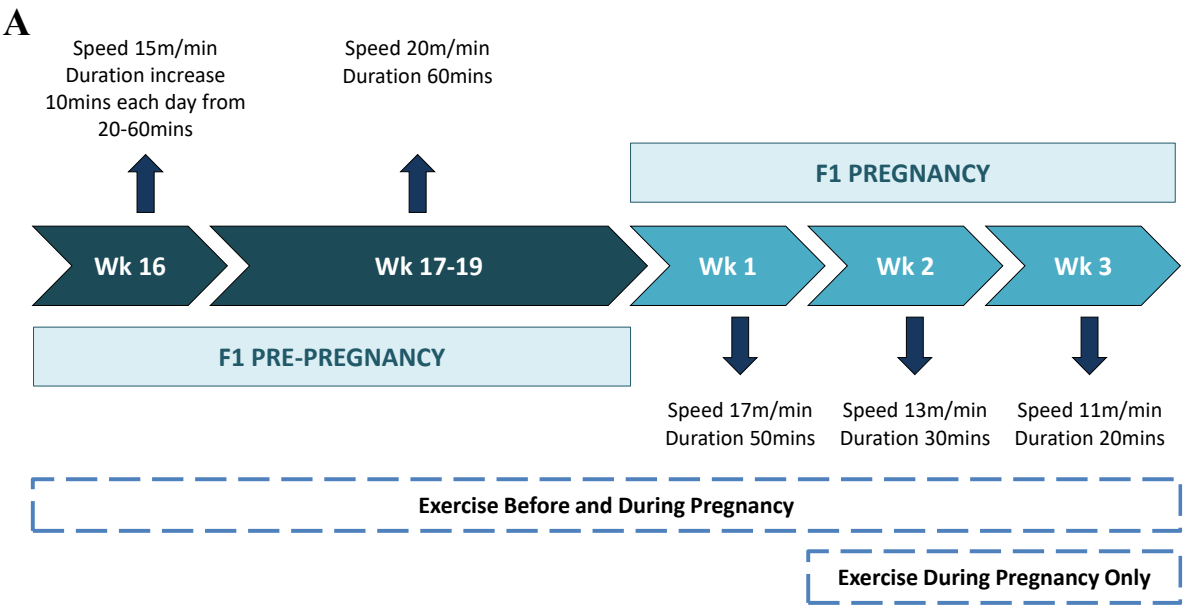
Table 2.2 Calculated nutritional parameters of the diets

F1 female offspring were exposed to either standard chow or high fat diet (43% kcals from fat) on postnatal day 35.

Calculated Nutritional Parameters	Standard AIN93G	High Fat SF03-020	High Fat SF01-028
Protein	19.4%	19.4%	19.0%
Total Fat	7.0%	23.0%	22.60%
Total Carbohydrate	56.9%	56.9%	56.9%
Crude Fibre	4.7%	4.7%	4.7%
AD Fibre	4.7%	4.7%	4.7%
Digestible Energy	16.1 MJ/Kg	20 MJ/Kg	19 MJ/Kg
% Total calculated digestible energy from lipids	16.0%	43.0%	43.0%
% Total calculated digestible energy from protein	21.0%	17.0%	17.0%

2.4 Exercise Protocol

At 16 weeks of age, F1 female offspring were randomly allocated to Sedentary, Exercise before and during pregnancy or Exercise during pregnancy only groups. Rats were exercised on a motorised treadmill (Columbus Instruments, Columbus, OH, USA) five days a week followed by two days of rest. Compressed air was blown on the base of the rats' tails to encourage them to run during the exercise training. On the first day or training, rats were trained for 20 minutes at a speed of 15 m/min and 0 degree incline for acclimatisation. An additional of 10 minutes was applied to the duration time on each subsequent day until day 5 of week 1. On day 1 of week 2 and thereafter, rats ran for 60 minutes a day at a speed of 20 m/min with 0 degree incline (Laker *et al.*, 2011; Laker *et al.*, 2012a; Laker *et al.*, 2012b; Asif *et al.*, 2017). During pregnancy, rats exercised in progressively shorter duration and speed (17 m/min for 50 minutes in week 1, 13 m/min for 30 minutes in week 2, 11 m/min for 20 minutes in week 3) (Amorim *et al.*, 2009). Rats in Exercise group started their exercise training four weeks before conception and continued during pregnancy while rats in ExPregOnly group initiated exercise training from week 2 of pregnancy and continued until *post mortem* on E20 (Figure 2.4).



B

Figure 2.4 Exercise protocol timeline and treadmill running

(A) Exercise rats initiated exercise training four weeks before pregnancy and continued during pregnancy while ExPregOnly rats started exercise training on the second week of gestation. (B) Photograph of rats performing exercise training on a motorised treadmill. Photographs reproduced with permission from Wlodek.

2.5 Metabolic Cage Measurements

Pregnant F1 females were acclimatised to metabolic cages by placing them in for 8 hours on E8 to minimise stress and associated behavioural changes (Moritz *et al.*, 2009b; Gallo *et al.*, 2012a; Gallo *et al.*, 2012b; Cheong *et al.*, 2016a). Final measurements were taken on E11-12 and E19-20. Rats were weighed and placed individually in metabolic cages and were allowed *ad libitium* access to known amount of food and water throughout the experiment (Figure 2.5). Urine and feces were collected in removable clean containers placed at the bottom of the metabolic cage. After 24 hours, the remaining food and water, as well as urine volume were recorded. Urine and feces were collected and stored in eppendorf tubes at -20°C for later biochemical analyses with plasma samples that were obtained after the rats were removed from metabolic cages on the same day.

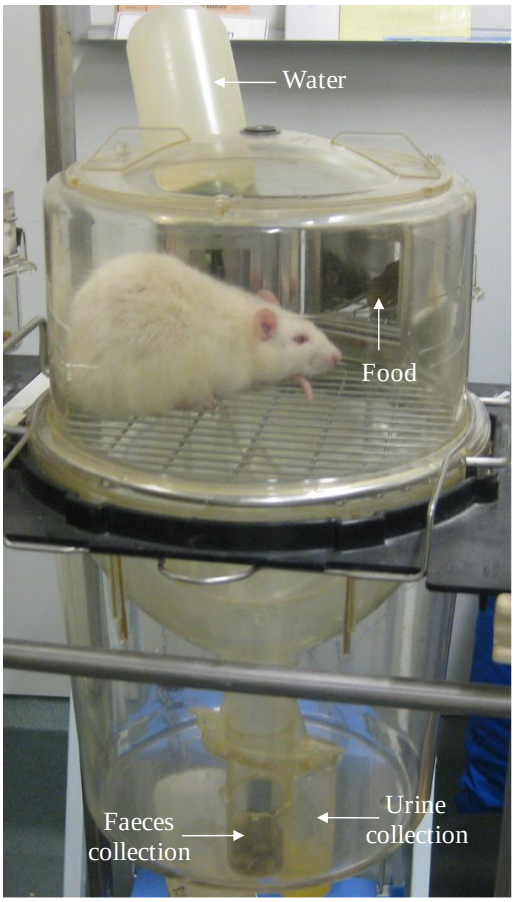


Figure 2.5 Metabolic cage set-up

Rats were placed individually in metabolic cage for measurement of food and water intake, and urine and feces collection. Photographs reproduced with permission from Wlodek.

2.6 Basal Activity and Oxygen Consumption

Energy expenditure and spontaneous physical activity were measured using an indirect open-circuit calorimeter (Comprehensive Laboratory Animal Monitoring System (CLAMS); Columbus Instruments, Columbus, OH, USA) on gestational day 16. The calorimeter was calibrated with gases of known concentration and rats were weighed and placed individually in sealed chambers. The calorimeter airflow rate was then adjusted according to animal weight to ensure that the changes in the composition of the expired gases were more than 0.05%. Rats were conscious and unrestrained during the measuring period (30 hours) and were allowed *ad libitium* access to food and water. The expired air was analysed for 60 seconds every 10 minutes using an electrochemical oxygen analyser and carbon dioxide sensor. Controlling software (Oxymax for Windows, Columbus Instruments, Columbus, OH, USA) provided specific calorimetric measurement and recording of oxygen consumption (VO_2), carbon dioxide production (VCO_2) and respiratory exchange ratio (RER) minute by minute. RER is the ratio between VCO_2 and VO_2 and indirectly determine the relative contribution of carbohydrate and lipids to overall energy expenditure (Simonson & DeFronzo, 1990). A high RER indicates that carbohydrates are being predominantly used, whereas a low RER suggests lipid oxidation (Simonson & DeFronzo, 1990).

Rate of heat production was derived by assessment of the exchange of oxygen and carbon dioxide during the metabolic process. The relationship between VO_2 and VCO_2 provided the calorific values, which revealed the energy content of the food, utilised by the rats. This calorific value (CV) is then applied to the volume of gases exchanged to compute heat. CV is derived from RER which is then used with the observed oxygen consumption (VO_2) to calculate heat.

$$\textbf{CV} = 3.815 + 1.232 \text{ X RER}$$

$$\textbf{Heat} = \text{CV x } VO_2$$

Spontaneous physical activity was also monitored concurrently during the 30-hour period in CLAMS by dual-axis detection (*x*-axis and *z*-axis) using infrared photocell technology. Each interruption of infrared beams in *x*-axis accrued a count of activity and *z*-axis counted rearing or jumping (Figure 2.6). Data was collected at the end of experiment and analysed by taking the average of the key parameters and split according to 12 hours light and dark phases. The first 6-hour was considered as familiarisation period and therefore not included as part of the analysis.

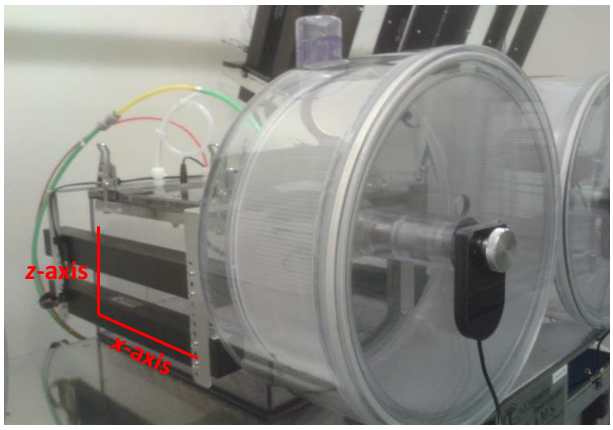


Figure 2.6 CLAMS set up

Rats were placed in CLAMS for 30 hours for measurement of VO_2, VCO_2, RER and heat production. The CLAMS chamber is equipped with *x*-axis and *x*-axis infrared beams wheel to determine spontaneous physical activity.

2.7 Systolic Blood Pressure by Tail Cuff Plethysmography

Non-invasive tail cuff plethysmography was used to measure systolic blood pressure in rats at 19 weeks of age and gestational day 18 (Moritz *et al.*, 2009b; Gallo *et al.*, 2012a; Gallo *et al.*, 2012b; Cheong *et al.*, 2016a). Rats were placed individually in a small cage (16x33x13 cm^3) with a stainless steel lid and warmed under a 38-40°C heat lamp (constructed within the Department of Physiology, The University of Melbourne) for 10 minutes (Figure 2.7 B). This improved blood circulation through the tail veins for adequate recording of the signal to noise ratio. Rats were then taken out from the cage and wrapped in a towel, exposing only the tail so that the pressure cuff can be positioned with the pulse transducer (non-invasive blood pressure (NIBP) Controller, MLT125R, ADInstruments Pty. Ltd., Castle Hill, NSW, Australia) aligned directly over the caudal artery (Figure 2.7 E). The tail pressure cuff and pulse transducer were adjusted until a clear measure of tail pulse pressure was detected using the software program (LabChart6, ADInstruments Pty. Ltd.). The cuff was inflated using the NIBP controller to a maximum pressure of 220 mmHg to occlude blood flow. Pressure was then slowly released by deflating the NIBP until 40 mmHg was reached. The systolic blood pressure was recorded as the cuff pressure at which the pulse in the caudal artery was first restored (Figure 2.7). This process was repeated 10 consecutive times and the final systolic blood pressure reading was taken from the average of the last five measurements. The cuff pressure and caudal artery pulse signal outputs were recorded by a digital recording system (Powerlab4, ADInstruments) that was connected to a computer and software program (LabChart6, ADInstruments).

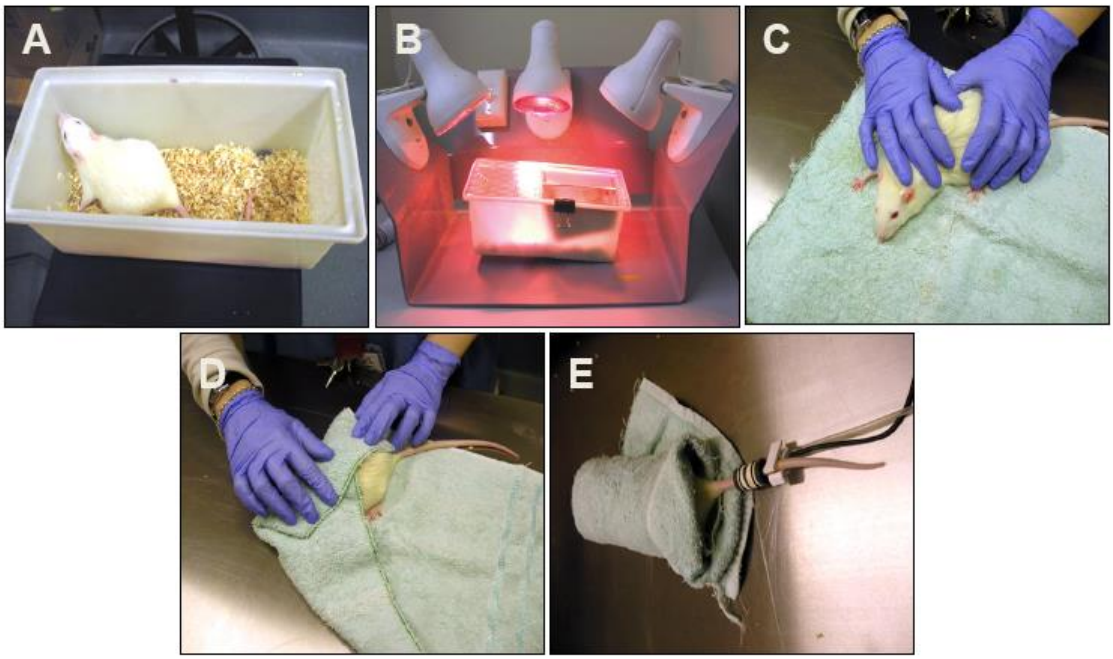


Figure 2.7 Tail-cuff systolic blood pressure procedure

Rats were individually placed in a small cage (A) and warmed under a heat lamp for 10 minutes (B). Rats were taken out from the cage and restrained in a towel (C and D). The tail was inserted through the pressure cuff with the pulse transducer aligned directly over the caudal artery (E). Photographs reproduced with permission from Wlodek.

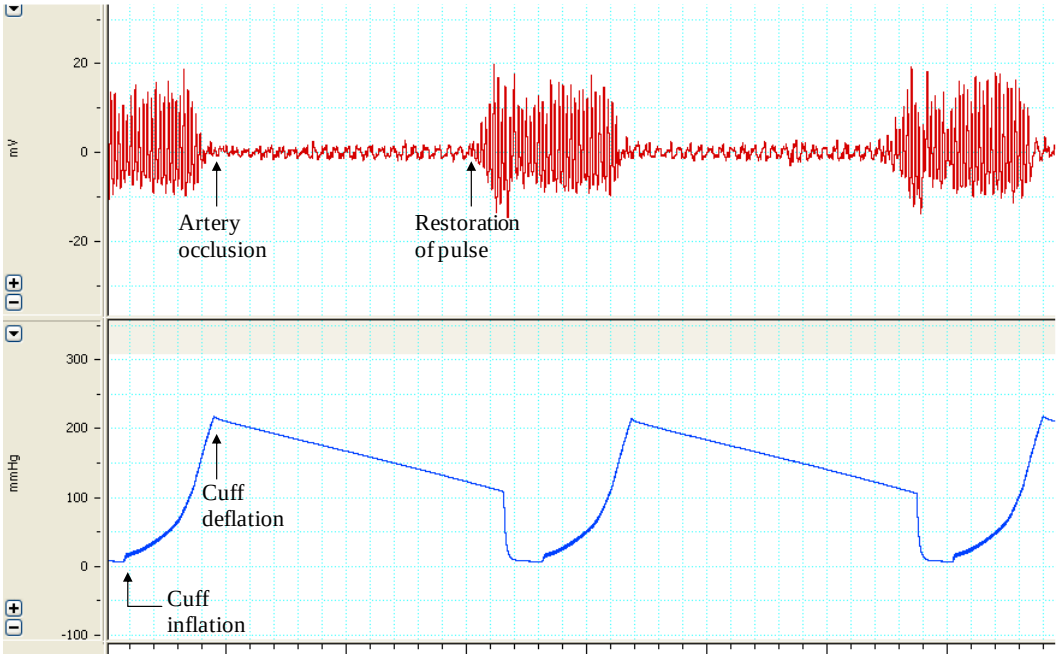


Figure 2.8 Tail-cuff systolic blood pressure trace recording

Tail-cuff was inflated to a maximum of 220 mmHg (bottom panel) to occlude blood flow and subsequent pulse pressure (top panel). Tail cuff was then slowly deflated to 40 mmHg. Systolic blood pressure was taken as the cuff pressure corresponding to the restoration of the first caudal artery pulse.

2.8 Intraperitoneal Glucose Tolerance Test

A non-fasted intraperitoneal glucose tolerance test (IPGTT) was performed in pregnant rats on gestational day 18 (starting time between 0900-1000 hour) to prevent any fetal compromise associated with fasting (Siebel *et al.*, 2008; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Gallo *et al.*, 2012b; Tran *et al.*, 2012; Tran *et al.*, 2013; Cheong *et al.*, 2016a; Cheong *et al.*, 2016b). All rats were weighed and placed individually in a small cage (16x33x13 cm^3) with a stainless steel lid. Rats were placed under a heat lamp (38-40°C) for 5 minutes before each blood sample collection to improve blood circulation in the tail. Blood samples (300 µl) were taken via tail vein 5 minutes prior to the glucose administration and blood glucose was measured using a glucometer (Accu-chek Performa, Roche, Mannheim, Germany). A bolus of glucose injection (1 $g.kg^{-1}$ body weight, 50% $w.v^{-1}$, Pharmlab, Lane Cove, NSW, Australia) was administered through an intraperitoneal route and tail vein blood samples were collected (300 µl) and blood glucose was measured at 10, 20, 30, 45, 60, 90 and 120 minutes. Upon completion of the IPGTT experiment, rats were placed back into their cages, provided with food and water *ad libitum* and monitored for any complications. Blood samples were centrifuged at 3000 rpm at 4°C for 15 minutes and plasma was transferred into 0.6 ml labelled eppendorf tubes and stored at -20°C for further analysis.

2.9 Post Mortem and Tissue Collection

Post mortem analysis was performed on all rats in the morning of gestational day 20. Rats were anaesthetised with an overdose intraperitoneal injection of 1:1 mixed solution of Illium Xylazil-20 (30 $mg.kg^{-1}$; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and Ketamine (100 $mg.kg^{-1}$; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia). Rats were considered as fully anaesthetised by the absence of corneal and pedal reflexes. A vertical midline abdominal incision was made towards the lower abdomen to expose the uterine horns containing the fetuses. Amniotic fluid was collected from each fetal sac and stored separately in individual tubes. Then fetal blood was collected via cardiac puncture and pooled by sex. Each fetus and placenta were excised from the uterus and individually weighed. Placentae were then separated into basalis and labyrinth regions and weighed. Fetal organs including brain, heart, pancreas, liver, left and right kidneys were dissected, weighed and either snap frozen in liquid nitrogen and stored at -80°C or immersion fixed in 10% Neutral Buffer Formalin (NBF, Sigma-Aldrich Co., St Louis, MO, USA).

Following fetal *post mortem*, the abdominal and chest cavity of the mother was cut open and blood was collected via cardiac puncture using heparinised 3 ml syringes with a 23G needle (Terumo Philippines Corporation, Laguna, Manila, Philippines) and transferred into heparinised 1.7 ml eppendorf tubes. Blood samples were used to measure glycated haemoglobin (HbA1c) and the rest were centrifuged at 3000 rpm at 4°C for 15 minutes. Plasma was then collected and transferred into 0.6 ml labelled eppendorf tubes and stored at -20°C. Major maternal organs including brain, heart, adrenals, pancreas, liver, left and right kidneys, dorsal fat, reproductive organs and individual hind limb muscles (soleus, plantaris, gastrocnemius, extensor digitorum longus (EDL), and tibialis cranialis) were rapidly dissected, weighed and either snap frozen in liquid nitrogen and stored at -80°C or immersion fixed in 10% NBF. A piece (~1 cm^3) of the hepatic end of the pancreas was cut and fixed in 10% NBF for later histological analysis (Section 2.13).

2.10 Insulin Radioimmunoassay

Plasma insulin concentrations from IPGTTs were measured in duplicate using a rat insulin radioimmunoassay kit with an assay sensitivity range of 0.1-10 $ng.ml^{-1}$ (Millipore, Abacus Australian Laboratory Services, Brisbane, QLD, Australia). The intra-assay and inter-assay variation were 1.4-4.6% and 8.5-9.4%, respectively. The kit was performed as per manufacturer's instructions but modified by reducing all volumes of reagents and samples by half (Siebel *et al.*, 2008; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Tran *et al.*, 2012; Tran *et al.*, 2013; Cheong *et al.*, 2016a). This assay is based on the competitive binding between the radio-labelled and unlabelled insulin antigen (within plasma samples) for the binding sites on the insulin antibodies. A known concentration of labelled insulin tracer antigen (radio-labelled with a gamma radioactive isotope of iodine attached to the tyrosine (^{125}I-insulin) is incubated with a known amount of antibody for that antigen. When unlabelled insulin antigen (plasma sample) is added, competition between the labelled antigen tracer and unlabelled antigen for the limited binding sites on the antibody occur. The amount of labelled antigen tracer bound to the antibody decreases as the concentration of unlabelled antigen increases. The radioactivity is then measured using a gamma counter after separating the unbound fractions from the bound antigens. The known rat insulin standards (0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 $ng.ml^{-1}$) provided in the kit were used to create a standard curve to calculate the insulin concentrations in the plasma samples.

Borosilicate glass tubes (12×75 mm tubes, Livingstone International Pty. Ltd., Rosebury, NSW, Australia) were labelled as 1-n. 100 µl of assay buffer was added to the non-specific binding (NSB) tubes (3-4) and 50 µl was added to Reference (B_0) tubes (5-6). Insulin standards and Quality Controls (QCs) were added to tubes 7-24 as per Figure 2.8. 25 µl of unknown plasma samples were diluted 1:2 with 25 µl of assay buffer and added to tubes 25-n in duplicates. 50 µl of hydrated ^{125}I-Insulin antigen tracer was added to all tubes (1-n) and followed by 50 µl of rat insulin antibody to all tubes except total count (1-2) and NSB (3-4) tubes. All tubes were vortex and covered with foil and incubated overnight at 4°C for 20-24 hours. On the next day, 500 µl of Precipitating Reagent was added to all tubes except total count tubes (1-2). All tubes were vortex until well mixed and incubated for 20 minutes at 4°C. All tubes except total count tubes (1-2) were centrifuged at 4°C for 20 minutes at 3,900 rpm. The supernatant from all tubes except total count tubes (1-2) were decanted and the remaining pellets were counted in a gamma counter for 1 minute (Department of Zoology, The University of Melbourne, Parkville, VIC, Australia).

Day 1						Day 2		
Set Up	**Step 1**	**Step 2-3**	**Step 4**	**Step 5**	**Step 6**	**Step 7**	**Step 8**	**Step 9-11**
Tube #	Assay Buffer	Standards/ QCs/ Unknown Samples	Add I^{125} Insulin Tracer	Add Rat Insulin Antibody	Vortex, Cover with foil and Incubate for 20-24 hours at 4°C	Add Precipitating Agent	Vortex and Incubate for 20 minutes 4°C	Centrifuge at 4°C for 20 minutes, decant and count
1,2			50 µl					
3,4	100 µl		50 µl			500 µl		
5,6	50 µl		50 µl	50 µl		500 µl		
7,8		50 µl of 0.1 ng/ml	50 µl	50 µl		500 µl		
9,10		50 µl of 0.2 ng/ml	50 µl	50 µl		500 µl		
11,12		50 µl of 0.5 ng/ml	50 µl	50 µl		500 µl		
13,14		50 µl of 1.0 ng/ml	50 µl	50 µl		500 µl		
15,16		50 µl of 2.0 ng/ml	50 µl	50 µl		500 µl		
17,18		50 µl of 5.0 ng/ml	50 µl	50 µl		500 µl		
19,20		50 µl of 10.0 ng/ml	50 µl	50 µl		500 µl		
21,22		50 µl of QC1	50 µl	50 µl		500 µl		
23,24		50 µl of QC2	50 µl	50 µl		500 µl		
25,26	25 µl	25 µl of unknown	50 µl	50 µl		500 µl		
27-n	25 µl	25 µl of unknown	50 µl	50 µl		500 µl		

Figure 2.9 Insulin radioimmunoassay procedure

Assay buffer was added to appropriate tubes containing standards, QCs and unknown plasma samples prior to the addition of ^{125}I-insulin tracer and antibody. Tubes were incubated overnight at 4°C for 20-24 hours and precipitating reagent were added the following day. Tubes centrifuges and supernatant was decanted. The remaining pellet was counted using a gamma counter.

2.10.1 Determination of Plasma Insulin Concentrations

Insulin concentrations for the unknown plasma samples were calculated using a weighted log/logit graph. Averages of the raw values in counts per minute (cpm) of all duplicates were calculated. The average NSB counts were subtracted from each average counts (except for total counts) and were used in the final calculations. The percentage of tracer bound was calculated by dividing total binding counts by total counts and multiplied by 100 ((B_0/total counts) x 100)). The percentage of total binding (%B/B_0) for each insulin standards and unknown samples were then calculated. A standard log-curve was created by plotting percentage bound (%B/B_0) for each insulin standard on the *y*-axis against the known concentrations of the insulin standards on the *x*-axis. The insulin concentration of the unknown plasma samples and QCs were calculated from the standard curve and final values were expressed as $ng.ml^{-1}$.

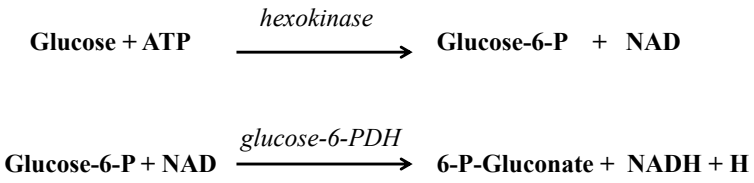
2.11 Plasma Glucose Analysis

2.11.1 Pretreatment of Glucose Samples

Plasma samples obtained from IPGTT (25 µl) were deproteinised with 25 µl of 3M perchloric acid (PCA, Sigma-Aldrich Co., Castle Hill, NSW, Australia), vortex until mixed and centrifuged at 13,000 rpm for 3 minutes at 4°C. 30 µl of supernatant was retrieved and neutralised with 7.5 µl of 6M potassium hydroxide (KOH, Sigma-Aldrich Co., Castle Hill, NSW, Australia), vortex until mixed and centrifuged at 13,000 rpm for 3 minutes at 4°C. The remaining supernatant (~25-30 µl) was transferred into a 0.6 ml labelled eppendorf tube and stored at -20°C until further analysis.

2.11.2 Glucose Assay

Plasma glucose concentrations were measured in duplicate using a scaled down version of the enzymatic fluorometric analysis (Passonneau & Lauderdale, 1974; Siebel *et al.*, 2008; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Tran *et al.*, 2012; Tran *et al.*, 2013). This involves a two-step reaction where glucose is converted to 6-P-Gluconate yielding a NADH molecule, which can be measured flurometrically at 355 nm absorption/460 nm emission. The amount of NADH formed is directly proportional to the amount of glucose present and can be measured by the change in fluorescence.



To determine plasma glucose concentrations, previously treated plasma samples were diluted 1:8 with dH_2O (5 µl of treated sample + 35 µl of dH_2O). 5 µl of treated plasma, quality controls (QCs), blank, glucose standards (250, 500 and 1000 µM; Sigma-Aldrich Co., Castle Hill, NSW, Australia) and NADH standards (100, 200, 400, 800 and 1600 µM, Sigma-Aldrich Co., Castle Hill, NSW, Australia) were pipetted into a black 96 well microplate (PerkinElmer, Glen Waverly, VIC, Australia) in duplicate. 300 µl of freshly made cocktail reagent containing 100 mM Tris buffer (Sigma-Aldrich Co., Castle Hill, NSW, Australia), 50 mM hydrochloric acid (HCl; Ajax Finechem, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia), 1 mM magnesium chloride ($MgCl_2$; Sigma-Aldrich Co., Castle Hill, NSW, Australia), 0.5 mM dithio-thrietol (DTT, Sigma-Aldrich Co., Castle Hill, NSW, Australia), 0.3 mM adenosine triphosphate (ATP; Sigma-Aldrich Co., Castle Hill, NSW, Australia), 0.05 mM nicotinamide adenine dinucleotide phosphate (NADP+; Sigma-Aldrich Co., Castle Hill, NSW,

Australia), 0.1 $U.ml^{-1}$ glucose-6-phosphate dehydrogenase (G6P-DH, Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia), 1 $U.ml^{-1}$ hexokinase (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) and Milli-Q water was added to each well using a multichannel pipette. The plate was then covered with foil and incubated in the dark at room temperature for 30 minutes. Fluorescence (355nm absorption/460nm emission) was measured using the Fluoroscan Ascent plate reader and Ascent software (Thermo Fisher Scientific, Waltham, MA, USA).

2.11.3 Determination of Plasma Glucose Concentrations

NADH standards (100, 200, 400, 800 and 1600 μM) were measured at wavelength 340 nm on a nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) and the Beer-Lambert Law (measured absorbance at 340 nm / 6.22 x 1000) was used to calculate the actual concentration of the standards. Plotting the actual NADH concentrations on the *x*-axis against the NADH fluorescence of the standard on the *y*-axis generated a linear standard curve. The most accurate glucose standard (±5%) was then selected by comparing the glucose standard fluorescence to the NADH standard curve and used to calculate the glucose concentration of the unknown plasma samples from the fluorescence (F) values measured using the Fluoroscan Ascent plate reader. The final values was multiplied by the dilution factor and plasma glucose concentrations were expressed in $mmol.l^{-1}$.

$$\textbf{Glucose Conc.} = \frac{\text{F of unknown sample}}{\text{F of glucose standard}} \times \text{Conc. of glucose std.} \times \text{dilution factor}$$

2.12 Glucose and Insulin Data Assessment

Glucose and insulin area under the curve (AUC) were calculated as the total AUC from basal to 120 minutes for IPGTT using GraphPad Prism 7 Software (GraphPad Software Inc. La Jolla, CA, USA) (Figure 2.10) (Siebel *et al.*, 2008; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Tran *et al.*, 2012; Tran *et al.*, 2013; Cheong *et al.*, 2016a). The ratio of insulin AUC to glucose AUC, which is an indicative of insulin secretory response to glucose, was calculated by dividing the total insulin AUC by the total glucose AUC.

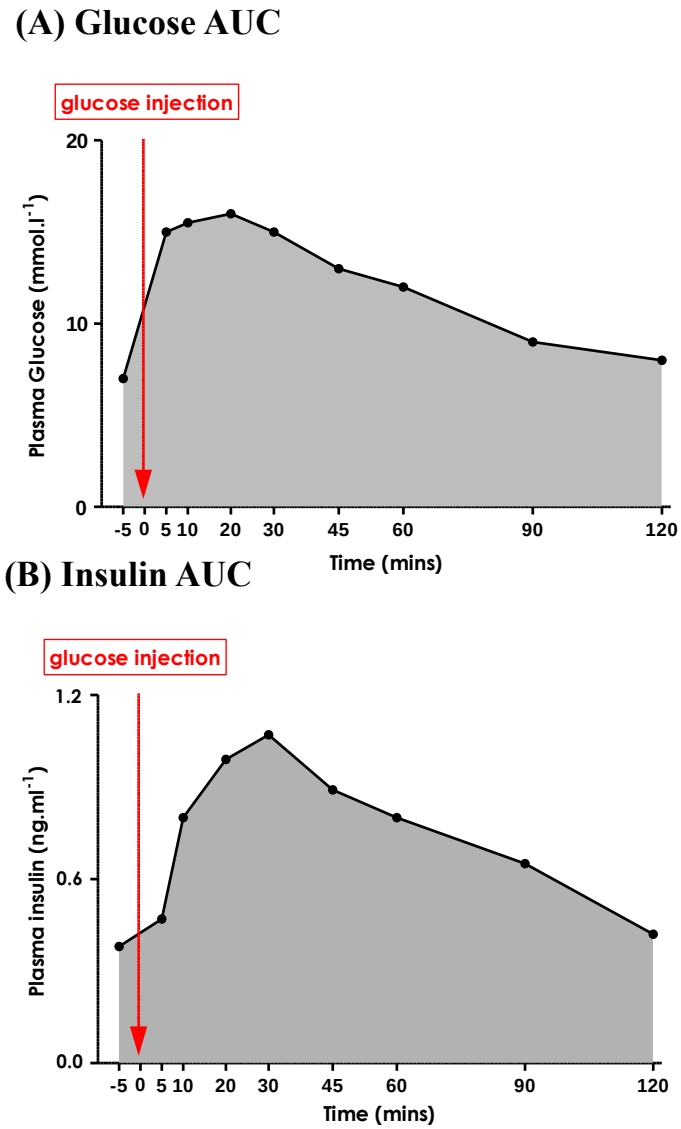


Figure 2.10 Glucose and insulin area under the curve

(A) Glucose and (B) insulin AUC were calculated as the total AUC from basal to 120 minutes for IPGTT.

2.13 Pancreatic Histological Analyses

The fixed pancreatic tissue from *post mortem* (Section 2.9) was processed overnight (Department of Anatomy and Cell Biology Histology Facility, The University of Melbourne, VIC, Australia) and embedded into tissue embedding medium of paraffin wax (Leica Microsystems, North Ryde, NSW, Australia) in medium sized blocks. Following embedding, the pancreatic tissue was exhaustively sectioned at 5μm thickness on a Leica ultracut microtome (Leica Microsystems, North Ryde, NSW, Australia) to avoid any potential bias due to regional variation in islet distribution and cell composition. Two consecutive sections (every 30^{th} and 31^{st} sections) were collected throughout the whole pancreas piece and mounted onto positively charged glass slides (SuperFrost Plus ® Menzel GmbH & Co. KG, Lower Saxony, Germany). About 20 to 25 slides were generated and selected sections were immunostained to characterise pancreatic *β*-cell and islet mass. All tissue blocks and slides were code-blinded to prevent potential bias.

2.13.1 Pancreatic Immunohistochemistry

Three pancreas sections of equal distance apart (every 150^{th} section or 150 μm apart) were stained for insulin to identify and localise *β*-cells (Siebel *et al.*, 2010; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Tran *et al.*, 2012; Tran *et al.*, 2013; Cheong *et al.*, 2016a). The slides were dewaxed in histolene (Grale Scientific, Ringwood, VIC, Australia) for 2 x 5 minutes and rinsed in 100% ethanol (EtOH) (Merck Pty Ltd, Kilsyth, VIC, Australia) for 1 minute. The slides were then rehydrated with 90% EtOH for 2 minutes followed by 3 minutes in 70% EtOH. A circle was drawn around the pancreas section on each slide using a PAP pen (Dako Australia, Botany, NSW, Australia) and the slides were rehydrated in Tris-buffered saline with Tween 20 (TBST; 1:10 dilution; 0.05 $mol.l^{-1}$ Tris-HCl, 0.3$mol.l^{-1}$ NaCl, 0.1% Tween 20, 0.01% preservative pH 7.6; DakoCytomation, Cambridgeshire, UK) for 15min. Slides were incubated with 1:10 dilution of 3% w/w hydrogen peroxidase (200 μl per section/slide; Sigma-Aldrich Co., Castle Hill, NSW, Australia) for 10 minutes to inhibit endogenous peroxidase and then washed in TBST for 5 minutes. To block non-specific binding, pancreas sections were individually incubated for 20 minutes with Protein Block Serum-Free (100 μl per section/slide; DakoCytomation, Cambridgeshire, UK). One pancreas section from each slide was then incubated at room temperature for 1 hour with 100 μl of polyclonal guinea-pig anti-insulin primary antibody diluted 1:200 in antibody diluent (Dako North America Inc., CA, USA). Another pancreas section served as a negative control by incubating with 100 μl of rabbit immunoglobulin fraction diluted 1:200 in antibody diluent (Dako Denmark, Glostrup, Denmark). The slides were washed with TBST for 5 minutes, followed by incubation with 100 μl of peroxidase-conjugated anti-guinea-pig secondary antibody diluted 1:40 in antibody diluents (Dako Denmark, Glostrup, Denmark) tagged with immunoperoxidase for 30 minutes. Slides were then washed with TBST for 5 minutes and incubated for 4 minutes with 200 μl 3,3'-diaminobenzidine (DAB; Sigma-

Aldrich Co., Castle Hill, NSW, Australia) solution which stains *β*-cells brown. All slides were washed with TBST and distilled water for 5 minutes each and counterstained with Mayer's haematoxylin (Amber Scientific supplied by Grale Scientific, Ringwood, VIC, Australia) for 2 minutes and rinsed clear under running tap water. Followed by 3 dips in Scott's tap water (Amber Scientific supplied by Grale Scientific, Ringwood, VIC, Australia). Pancreas sections were dehydrated in gradual increases of ethanol washes (70%, 90% and 100% EtOH) and incubated twice in histolene for 1 minute each and cover slipped with (22mm x 40mm, Mikro-Glass Australia, Grale Scientific, Ringwood, VIC, Australia) with DPX mountant (VWR International Ltd, Poole, England). All slides were left to dry overnight in fume-hood.

2.13.2 β-cell Counting

A Light Zeiss microscope, camera and software (AxioCam MRc5, Carl Zeiss Pty Ltd, North Ryde, NSW, Australia) were used to visualise the stained pancreas sections at x20 magnification (Figure 2.11). 40 fields of views (field of view (FOV) is 700 grid) were selected and the number of grid points on the pancreas tissue, islet and insulin positive *β*-cells were recorded. The FOV was moved in a systemic order (vertical down, then horizontal steps) to prevent overlapping with the previous FOV.

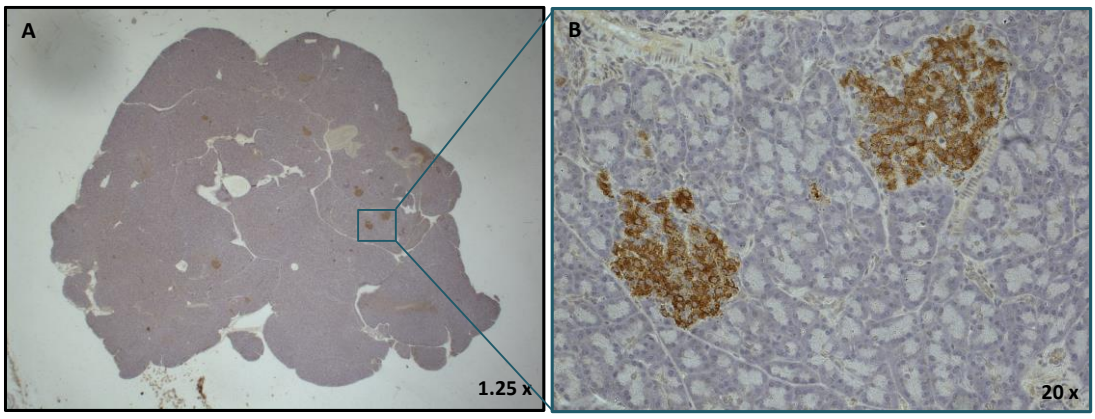


Figure 2.11 Histological image of rat pancreas
Image of whole rat pancreas at x1.25 magnification (A) and individual islet with *β*-cells visible by DAB staining at x20 magnification (B). Photographs with permission from Wlodek.

Relative islet and β-cell volume density were quantified by point-counting (700 points/field, Vd equals the number of intercepts on insulin positive cells as a proportion of intercepts on a pancreas). As 1 cm^3 tissue weighs 1g, Vd and pancreatic weight were multiplied to determine absolute islet and β-cell mass (Bonner-Weir, 2001). Relative islet and β-cell mass were determined by dividing absolute β-cell and

islet mass with body weight (Bonner-Weir, 2001; Siebel *et al.*, 2010; Laker *et al.*, 2011; Gallo *et al.*, 2012a; Tran *et al.*, 2012; Tran *et al.*, 2013; Cheong *et al.*, 2016a). For each rat, the average of relative islet and β-cell mass from 3 pancreas sections was used as the final result.

β-cell mass = V_d b-cell per pancreas ´ pancreas weight (mg)

Islet mass = V_d islet per pancreas ´ pancreas weight (mg)

% β-cell per pancreas = V_d b-cell per pancreas ´ 100

% Islet per pancreas = V_d islet per pancreas ´ 100

% β-cell per islet = V_d b-cell per islet / V_d islet per pancreas ´ 100

2.14 Plasma Cytokines Analyses

Plasma leptin and MCP-1 concentrations were measured in duplicate using a 96 well plate enzyme-linked immunosorbent assay (Rat ELISA) as per manufacturer's instructions with an assay sensitivity of 0.022ng.ml^{-1} (Figure 2.11A; Signosis Inc,. Santa Carla, CA, USA). The intra-assay and inter-assay variation were 1.4-4.6% and 8.5-9.4%, respectively (Tran *et al.*, 2013; Tran *et al.*, 2015). Plasma adiponectin concentrations were also measured in duplicate using a 96 well ELISA plate as per manufacturer's instructions (R&D Systems, Minneapolis, MN, USA ; Figure 2.11 B) and the limit of sensitivity of this assay was 0.4ng.ml^{-1} (20µl sample size). The reported intra-assay variation was 0.4 - 1.6% while inter-assay variation was 6.5 – 7.8% (Tran *et al.*, 2013; Tran *et al.*, 2015).

Specific capture antibody is coated on a polystyrene microtiter plate. When samples with an unknown amount of antigen are added to the plate, capture antibody immobilise the protein antigen upon binding during incubation. A specific biotinylated detection antibody is then added and formed a complex with the immobilised antigen to enable detection by a secondary antibody that is linked to an enzyme through bioconjugation. The plate is washed between each step with a mild detergent wash solution to remove any proteins or antibodies that are not specifically bound. After the final wash, an enzymatic substrate solution is added to produce a detectable signal (blue colour). The intensity of the colour is directly proportional to the concentration of antigen in the samples. The enzymes activity was measured on the xMark Microplate Absorbance Spectrophotometer using Microplate Manager 6 software at 450 nm and a correction wavelength of 570 nm to remove background. The concentration of leptin and adiponectin in the plasma were determined by comparing the absorbance to a standard curve.

A

	Step 1	Step 2-3	Step 4	Step 5-6	Step 7	Step 8-9	Step 10	Step 11	Step 12	Step 13
Well #	**Standards/ Controls/ Samples**	Seal, Agitate, Incubate 1 hour at room temperature Wash 3x with 200 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Detection Antibody**	Seal, Agitate, Incubate 1 hour at room temperature Wash 3x with 200 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Enzyme Solution**	Seal, Agitate, Incubate 45 minutes at room temperature Wash 3x with 200 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Substrate**	Seal, Agitate, Incubate 10-30 minutes at room temperature	**Stop Solution**	Read Absorbance at 450 nm and 590 nm
A1, A2	Blank		100 µl		100 µl		100 µl		50 µl	
B1,B2	50 µl of 63 pg/ml Leptin/ MCP-1 Standard		↓		↓		↓		↓	
C1, C2	50 µl of 125 pg/ml Leptin/ MCP-1 Standard									
D1, D2	50 µl of 250 pg/ml Leptin/ MCP-1 Standard									
E1, E2	50 µl of 500 pg/ml Leptin/ MCP-1 Standard									
F1, F2	50 µl of 1000 pg/ml Leptin/ MCP-1 Standard									
G1, G2	50 µl of Sample									
H1, H2	50 µl of Sample									
	↓									

B

	Step 1	Step 2-3	Step 4	Step 5-6	Step 7	Step 8-9	Step 10	Step 11	Step 12	Step 13
Well #	**Standards/ Controls/ Samples**	Seal, Agitate, Incubate 2 hours at room temperature Wash 3x with 300 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Detection Antibody**	Seal, Agitate, Incubate 2 hours at room temperature Wash 3x with 300 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Enzyme Solution**	Seal, Agitate, Incubate 20 minutes at room temperature Wash 3x with 200 µl Wash Buffer Remove remaining liquid by inverting the plate against paper towels	**Substrate**	Seal, Agitate, Incubate 20 minutes at room temperature	**Stop Solution**	Read Absorbance at 450 nm and 590 nm
A1, A2	Blank		100 µl		100 µl		100 µl		50 µl	
B1,B2	100 µl of 156 pg/ml Adiponectin Standard		↓		↓		↓		↓	
C1, C2	100 µl of 313 pg/ml Adiponectin Standard									
D1, D2	100 µl of 625 pg/ml Adiponectin Standard									
E1, E2	100 µl of 1250 pg/ml Adiponectin Standard									
F1, F2	100 µl of 2500 pg/ml Adiponectin Standard									
G1, G2	100 µl of 5000 pg/ml Adiponectin Standard									
H1, H2	100 µl of 10000 pg/ml Adiponectin Standard									
A3, A4	100 µl of Sample									
	↓									

Figure 2.12 Assay standard procedure for ELISA

Plasma leptin and MCP-1 (A) and adiponectin (B) concentrations were measured using ELISA as per manufacturer's instructions. Standards, QCs and unknown plasma samples are added to appropriate wells and incubated before addition of detection antibody and enzyme solution. The plate is washed with a mild detergent wash buffer between each step. After the final wash, enzyme substrate is added to produce a detectable signal. The enzymes activity was measured on the spectrophotometer at 450 nm.

2.15 Skeletal Muscle and Liver Triacylglycerol Analysis

Frozen gastrocnemius muscle and liver were crushed in a mortar filled with liquid nitrogen using a pestle. About 15-20 mg of crushed muscle and liver were weighed and transferred into a 16 x 100 mm borosilicate glass tube containing 2.5 ml $CHCl_3$:MeOH (2:1 v/v, Sigma, St Louis, MO, USA) (Folch *et al.*, 1957; Laker *et al.*, 2012a; Tran *et al.*, 2012). The tissues were homogenised using a Polytron homogeniser (Capital Scientific, Austin, TX, USA) and 0.5 ml of $CHCl_3$ and 1.5 ml distilled water were then added to each tube. The tubes were placed on a shaker for 10 minutes and centrifuged at 2000 rpm for another 10 minutes. 1.5 ml of the lower chloroform phase that contained dissolved lipids was removed and transferred to a clean glass tube. The chloroform was evaporated under nitrogen at 40°C to complete dryness and the lipid was dissolved in 250 µl of 100% EtOH and vortex vigorously until well mixed. Triacylglycerol concentrations were determined using calorimetric analysis (TAG GPO-PAP, Roche Diagnostics Australia, Castle Hill, NSW, Australia) as per manufacturer's instructions. 10 µl of glycerol standards (0, 2, 5, 10, 20, 50, 100 & 200 nmol) and samples were added to a 96 well plate in duplicate followed by 300 µl of TAG GPO-PAP reagent. The plate was then incubated at 37°C for 20 minutes and read on a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 490 nm. The concentrations of triacylglycerol of the samples were determined by comparing the absorbance to a standard curve.

2.16 Urine and Plasma Biochemical Analyses

Urinary and plasma concentrations of sodium, potassium, creatinine, albumin and total protein content were measured using a COBAS Integra 400 (Roche Diagnostics, Castle Hill, NSW, Australia). 120 µl of urine and 100 µl of plasma were transferred into micro test tubes (Kartell, Noviglio, MI, Italy). Samples were then placed in mounting trays into the analyser and left to run for 45 minutes. A comprehensive spreadsheet was generated once the analysis was completed. Urinary excretions were then calculated as described in Table 2.3.

Table 2.3 Calculation of urinary excretions

Urine flow rate, osmolality and excretions including Na^+, K^+, creatinine, albumin and total protein were measured and calculated over 24h. Creatinine clearance were calculated in animals with a urine and plasma sample obtained within 24h.

Urinary excretion	**Calculation**
Urine flow rate *(l.24 h^{-1})*	urine volume *(l)* ÷ time in met cage *(h)* × 24 hours
Na^+, K^+, creatinine *(mmol.l^{-1}(24 h)$^{-1}$)*	concentration *(mmol.l^{-1})* × urine flow rate *(l.24 h^{-1})*
Albumin, Total protein *(mg.l^{-1}(24 h)$^{-1}$)*	concentration *(mg.l^{-1})* × urine flow rate *(l.24 h^{-1})*
Creatinine clearance *(ml.min^{-1})*	urine creatinine *(µmol.l^{-1})* × urine flow rate *(ml.min^{-1})* ÷ plasma creatinine *(µmol.l^{-1})*

2.17 Statistical Analyses

To reflect the aims and hypotheses, the effect of uteroplacental insufficiency (Control and Restricted), diet (Chow and High Fat) and exercise (Sedentary, Exercise and ExPreg) were examined on data from physiological measurements (CLAMS, 24 hours metabolic cage, tail cuff blood pressure and non-fasted glucose tolerance test). Similarly, the effect of uteroplacental insufficiency (Control and Restricted), diet (Chow and High Fat), exercise (Sedentary, Exercise and ExPreg) and physiology measures (No physiology measures and Physiology measures) were examined on maternal body and organ weights, plasma cytokines concentrations, pancreatic and skeletal muscle analyses as well as fetal body and organ weights. A two-way ANOVA was first conducted to identify differences between Treatment and Diet within each Exercise. To determine differences between Exercise, the data was split by Diet and a two-way ANOVA conducted to report Exercise effects within Treatments in each Diet. If a main Exercise effect was present, a one-way ANOVA with a Duncan's post-hoc test was used to identify Exercise differences. If an interaction was observed, the data was further split to identify Treatment effects within each Exercise using a Student's unpaired t-test and a one-way ANOVA determined Exercise effects in Control and Restricted groups. To identify any Physiology measures differences, the data was split by Diet and a two-way ANOVA was conducted to report Physiology measures effects within Treatment within each Diet. All data are presented as mean ± SEM and $P<0.05$ was considered statistically significant. All statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

A power calculation was performed before the study was initiated and sample size of 8 (N= 8) was sufficient to get statistical significant difference with 80% power in this study. All the data presented in this study were also tested for normality and they fit within a standard normal distribution. This statistical approach was approved by Doctor Sue Finch, a statistical consultant from School of Mathematics and Statistics, The University of Melbourne.

CHAPTER 3

Effects of Exercise and High Fat Diet on Metabolic Adaptations to Pregnancy in Females Born Small

3.1 Introduction

The prevalence of obesity is escalating rapidly and is considered as the epidemic of 21st century. Since 1980, obesity rates worldwide have more than doubled with 2.1 billion people, or nearly 30% of the global population, were classified as being obese or overweight in 2013 (Ng *et al.*, 2014). It is clinically defined as having a body mass index (BMI) over 30 and is associated with an increased risk of developing a number of co-morbidities including metabolic and cardiovascular disease and nephropathy (Eckel *et al.*, 2005). One subset of the population who are predisposed to developing obesity are children born small for gestational age, which occurs in 10% of pregnancies worldwide (Martin *et al.*, 2017). Epidemiological studies reported that these growth-restricted children have an increased susceptibility to type 2 diabetes, insulin resistance, hypertension and cardiovascular disease (Barker *et al.*, 1989a; Barker *et al.*, 1989b; Hales *et al.*, 1991; Eriksson *et al.*, 2000).

Uteroplacental insufficiency is the most common cause of intrauterine growth restriction in developed countries and it is characterized by inadequate blood flow to the placenta which disrupts oxygen and nutrient supply to the fetus (Henriksen & Clausen, 2002). We have previously demonstrated that there are clear sex-specific differences as a consequence of uteroplacental insufficiency where growth restricted males have more severe outcomes than females. At 6 months of age, growth restricted males have glucose intolerance and pancreatic β-cell mass, whereas females appear to be protected (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008). Interestingly, when these growth-restricted females get pregnant, they developed glucose intolerance during late gestation suggesting that they have adverse adaptations to pregnancy (Gallo *et al.*, 2012b).

Pregnancy is the greatest physiological challenge facing women that results in alterations in maternal physiology and metabolism to assist in fetal growth and development, which is modulated by a number of key molecules (Herrera, 2000; Carlin & Alfirevic, 2008). Glucose homeostasis in the mother is altered so that there is a progressive increase in insulin resistance and gluconeogenetic activity to sustain glucose transfer to the fetus (Herrera, 2000). In an obese mother, the pregnancy adaptations become

adverse which predispose them to a number of complications (Mahizir *et al.*, 2016). Indeed, pregnant women who are overweight or obese have higher risk of GDM (Cunningham, 1991; Sebire *et al.*, 2001). Given that growth restricted females are at higher risk of metabolic disease during their pregnancy, it is likely that maternal obesity may exacerbate the phenotypes. However, there are limited studies investigating the effects of maternal obesity in growth-restricted mothers and the subsequent effects in their offspring.

There has been much interest in the development of lifestyle interventions targeting overweight and obese pregnant women. Of particular interest, epidemiological studies demonstrated that exercise training during pregnancy in overweight and obese women prevented them from developing GDM (Artal *et al.*, 2007; Garnaes *et al.*, 2016). However, there are studies that failed to associate the beneficial effects of exercise with a reduced risk of adverse pregnancy outcomes in overweight and obese women (Dodd *et al.*, 2014; Seneviratne *et al.*, 2016). Lack of consistent evidence regarding the benefits of exercise in pregnant obese or overweight women suggests that interventions during pregnancy alone may not be enough to ameliorate the adverse effects obesity has on the mother. Therefore, an exercise intervention before and during pregnancy may be more beneficial. Indeed, in non-obese pregnant women who were involved in exercise training one year before pregnancy had a reduced risk of developing GDM (Dempsey, 2004; Oken *et al.*, 2006; Zhang *et al.*, 2006), and the effects were greater in women who exercised before and during pregnancy (Dempsey, 2004). Similar observations were reported in animal studies where moderate exercise training before and during pregnancy reduced the metabolic and cardiovascular risk caused by maternal obesity in both the mother and offspring (Raipuria *et al.*, 2015; Vega *et al.*, 2015). Together, these findings propose that these lifestyle interventions are more beneficial if they are performed before the reproductive years. Thus, the development of targeted interventions in growth-restricted mothers may prevent them from developing metabolic dysfunction during pregnancy.

3.2 Aims and Hypotheses

The overall aim of this study was to determine if a high fat diet exacerbates the known adverse metabolic adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these complications.

Specific aims of Chapter 3 were:

- ***i.*** To determine if a high fat diet exacerbates impaired glucose tolerance and insulin secretion in late gestation in growth restricted females;
- ***ii.*** To determine whether exercise before and during pregnancy is more beneficial in preventing impaired glucose tolerance and insulin secretion in late gestation in growth restricted females than exercise during pregnancy alone;
- ***iii.*** To determine whether the exacerbation and prevention of impaired glucose tolerance and insulin secretion in late gestation in growth restricted females is associated with alterations in pancreatic β-cell and islet morphology;
- ***iv.*** To determine whether the exacerbation and prevention of impaired glucose tolerance and insulin secretion in late gestation in growth restricted females is associated with alterations in plasma cytokines and skeletal muscle and liver triglycerides;
- ***v.*** To determine whether high fat diet and exercise alter energy expenditure and spontaneous physical activity in late gestation in growth restricted females.

It is hypothesised that high fat diet will exacerbate the known adverse metabolic adaptations to pregnancy in growth restricted females in late gestation and endurance exercise training will prevent the adverse pregnancy outcomes.

3.3 Materials and Methods

Experimental design was described in Chapter 2.1. Only the specific endpoints of this chapter are briefly described in this section.

3.3.1 Intraperitoneal Glucose Tolerance Test and Plasma Analyses

A non-fasted IPGTT was performed in pregnant rats on E18 to prevent any fetal compromise associated with fasting. Blood samples were taken prior to and at 10, 20, 30, 45, 60, 90 and 120 minutes after an intraperitoneal bolus injection of glucose. Plasma was stored at -20°C until further analysis.

Plasma insulin and glucose concentrations were measured in duplicate using a rat insulin radioimmunoassay kit (Millipore, Abacus Australian Laboratory Services, Brisbane, QLD, Australia) and scaled down version of the enzymatic fluorometric analysis respectively. Plasma leptin (Signosis Inc) and adiponectin (R&D Systems) concentrations were measured using ELISA as per the manufacturer's instructions. Glucose and insulin area under the curve (AUC) were calculated as the total AUC from basal to 120 minutes using the trapezoidal model.

3.3.2 Energy Expenditure and Basal Activity

Energy expenditure, respiratory exchange ratio and spontaneous physical activity were measured using an indirect open-circuit calorimeter (CLAMS) on E16. Rats were weighed and individually placed in sealed chambers for 30 hours with free access to food and water. Oxygen consumption (VO_2), respiratory exchange ratio (RER), heat production, and spontaneous physical activity (dual axis detection; *x* and *y*) were recorded during light and dark cycles. Data were analysed by taking the average of the key parameters and split according to 12 hours light and dark phases.

3.3.3 Post Mortem Tissue Collection

At E20, rats were anaesthetised with intraperitoneal injection of ketamine (100 $mg.kg^{-1}$) and Illium Xylazil-20 (30 $mg.kg^{-1}$) and a cardiac puncture was performed. Heart, kidneys, liver, pancreas, adrenals, fats and skeletal muscle were excised and weighed. A piece of the hepatic end of the pancreas was cut and fixed in 10% neutral buffered formalin for histological analyses.

3.3.4 Pancreatic Islet, β-cell Morphology and Immunohistochemistry

Fixed pancreatic tissue was processed, embedded in paraffin wax and exhaustively sectioned at 5 μm thickness. Three pancreas sections of equal distance apart were immunostained using a guinea pig

polyclonal anti-insulin antibody. Random systemic point counting of 40 fields of view was used to determine relative islet and β-cell volume density (V_d) using a 700 point grid (700 points/field, V_d equals the number of intercepts on an islet of insulin positive cells as a proportion of intercepts on a pancreas). As 1 cm^3 tissue weighs 1 g, V_d and pancreatic weight were multiplied to determine absolute islet and β-cell mass.

3.3.5 Skeletal Muscle and Liver Triacylglycerol Analysis

Triacylglycerol was extracted from gastrocnemius muscle and liver in CHCl3-MeOH (2:1 vol/vol), and distilled water was added to separate the phases. The organic extracts were dried down, reconstituted in ethanol, and assayed for triacylglyerol (total glycerol) by measuring the glycerol liberated after enzymatic hydrolysis of triacylglycerol (GPO-PAP, Roche Diagnostics, Castle Hill, NSW, Australia).

3.3.6 Statistical Analyses

All data were analysed using a three-way ANOVA to determine main effects of uteroplacental insufficiency (Control and Restricted), diet (Chow and High fat) and exercise (Sedentary, Exercise and ExPreg). If significant interactions were detected, Student's unpaired *t*-test or a one-way ANOVA was performed with Student-Newman-Kewls post hoc test where appropriate. All data are presented as mean ± SEM with n representing the number of animals per litter from each group. $P<0.05$ was considered statistically significant.

3.4 Results

3.4.1 F1 Litter Size and Postnatal Body Weights

Postnatal body weights from PN1 to PN35 were combined into Control and Restricted groups and siblings averages were used. Body weights were also combined at 15 weeks of age into treatment and diet groups and at 19 weeks and mating into treatment, diet and exercise (Sedentary vs ExPrePreg) groups. Uteroplacental insufficiency in F0 female reduced total (male and female) litter size at PN1 (P<0.05; Figure 3.1 A) and body weight (-17%, P<0.05; Figure 3.1 B). Restricted females remained lighter at PN7, 14 and 35 (P<0.05; Figure 3.1 B), at 15 weeks irrespective of diet (P<0.05, Figure 3.2 A), and at 19 and 20 (mating) weeks irrespective of diet and exercise (P<0.05, Figures 3.2 B & C). HFD increased body weight at 15 weeks in both Control (+3%) and Restricted (+8%) females compared with Chow counterparts (P<0.05, Figure 3.2 A). The increased in body weight were sustained at 19 and 20 weeks in both Control and Restricted on a HFD compared to Chow irrespective of exercise intervention (P<0.05, Figures 3.2 B & C). Female rats that exercised before and during pregnancy were heavier at 19 weeks regardless of treatment and diet (P<0.05, Figure 3.2 A) and at mating in Control and Restricted females on a HFD only (P<0.05, Figure 3.2 B) compared with Sedentary rats.

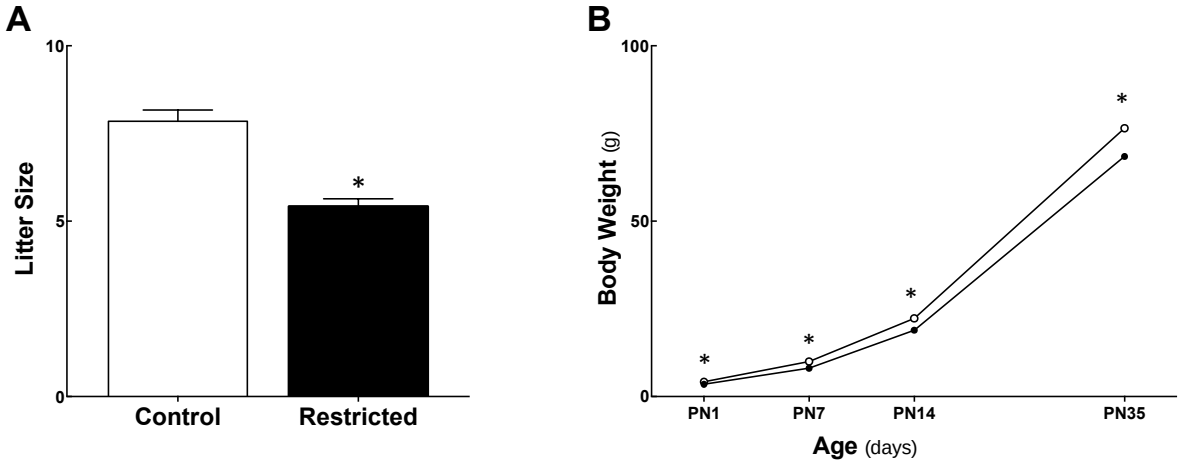


Figure 3.1 Total litter size at PN1 and female body weights at PN1, 7, 14 and 35

Control (white bar and circles) and Restricted (black bar and circles) (A) total litter size and (B) female body weight at PN1, 7, 14 and 35. All values are expressed as mean ± SEM; 47-59 per group from separate litters. *P<0.05 vs Control.

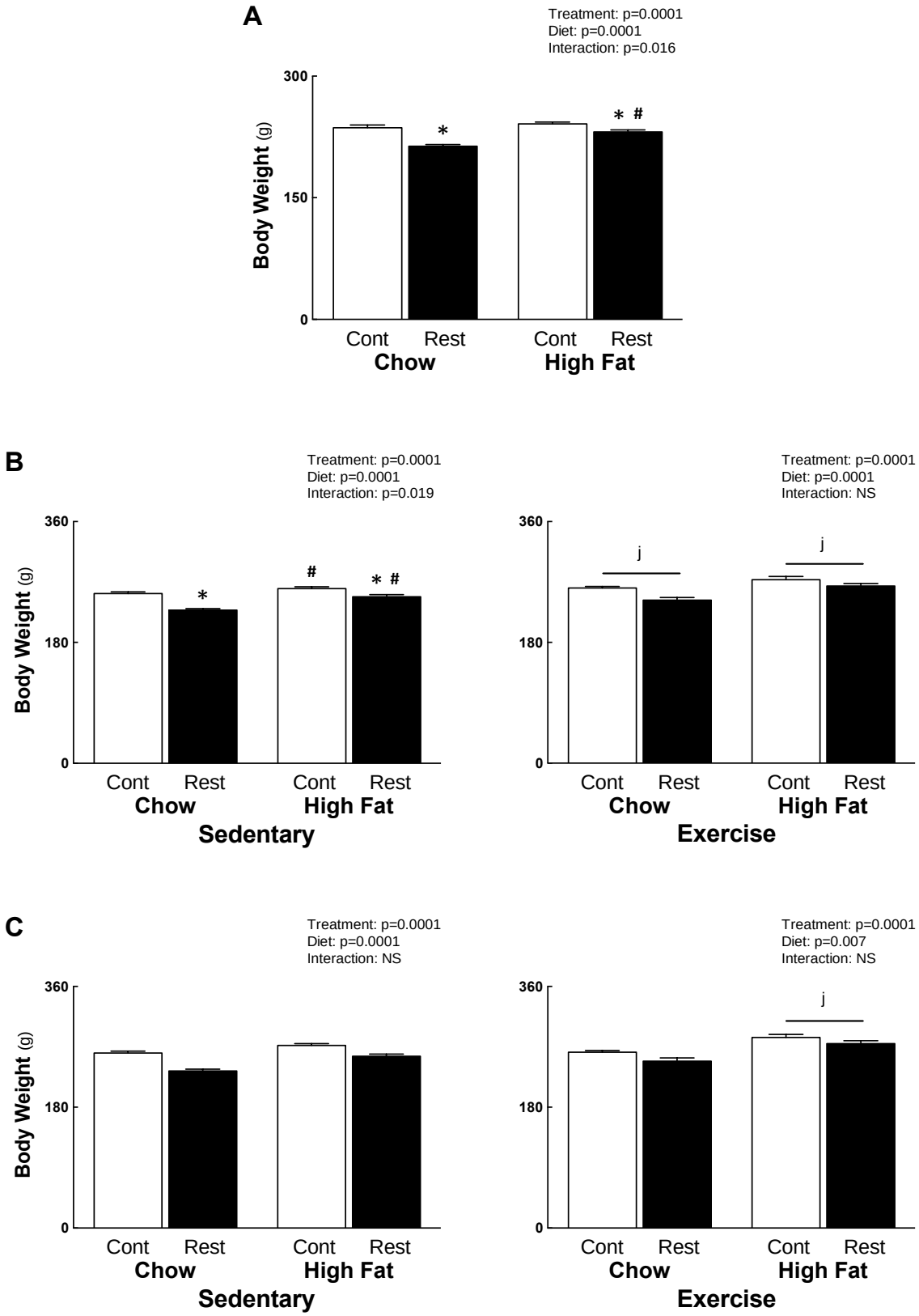


Figure 3.2 Body weights at week 15, 19 and 20 (mating)

Control (white bars) and Restricted (black bars) females body weights at (A) 15; (B) 19; and (C) 20 weeks of age. All values are expressed as mean ± SEM; 15-39 per group from separate litters. **P*<0.05 vs Control; #*P*<0.05 vs Chow; φ*P*<0.05 vs Sedentary.

3.4.2 Gestational Body Weights and Weight Gain

3.4.2.1 Group Effect

In no physiology measures groups, Restricted females were lighter than Control counterparts irrespective of diet and exercise interventions. Restricted females on a Chow and HFD that remained Sedentary throughout the study had lower gestational weight gain compared to Control counterparts (P<0.05, Figure 3.4 A).

In physiology measures groups, Restricted females were lighter than Control counterparts irrespective of diet and exercise interventions. Chow-fed Restricted females that were Sedentary had lower gestational weight gain (P<0.05, Figure 3.4 B).

3.4.2.2 Diet Effect

In no physiology measures groups, HFD increased both Control and Restricted body weights at E20 (+8-12%, P<0.05, Figure 3.3 A). The increase was sustained with both Exercise and ExPregOnly (P<0.05, Figure 3.3 A). In both exercise interventions, high fat feeding increased gestational weight gain in Control and Restricted females (P<0.05, Figure 3.4 A).

In physiology measures groups, HFD increased both Control and Restricted body weights at E20 (+8-12%, P<0.05, Figure 3.3 B). The increase was sustained with both Exercise and ExPregOnly (P<0.05, Figure 3.3 B). Sedentary- Control and Restricted females on a HFD had an increase in gestational weight gain compared to Chow counterparts (P<0.05, Figure 3.4 B). The increase was sustained with both Exercise and ExPregOnly (P<0.05, Figure 3.4 B).

3.4.2.3 Exercise Effect

In no physiology measures groups, Exercise increased body weights of Restricted females on a Chow diet compared with Sedentary (P<0.05, Figure 3.3 A). ExPregOnly reduced body weights of Chow-fed Restricted females compared with Sedentary counterparts (P<0.05, Figure 3.3 A). Exercise increased body weight of Control and Restricted females on a HFD compared with Sedentary (P<0.05, Figure 3.3 A). ExPregOnly reduced gestational weight gain in Chow-fed Control and Restricted females compared with Sedentary (P<0.05, Figure 3.4 A).

In physiology measures groups, Exercise increased body weight of Control and Restricted females on a HFD compared with Sedentary (P<0.05, Figure 3.3 A).

3.4.2.4 Physiology Measures Effect

Physiological measurements performed during pregnancy had no effect on body weights in all groups (Figure 3.4). Physiology measures reduced gestational weight gain in Sedentary-Control and Restricted rats on a Chow diet ($P<0.05$, Figure 3.4). Physiology measures increased gestational weight gain in Chow-fed Control and Restricted females that ExPregOnly ($P<0.05$, Figure 3.4).

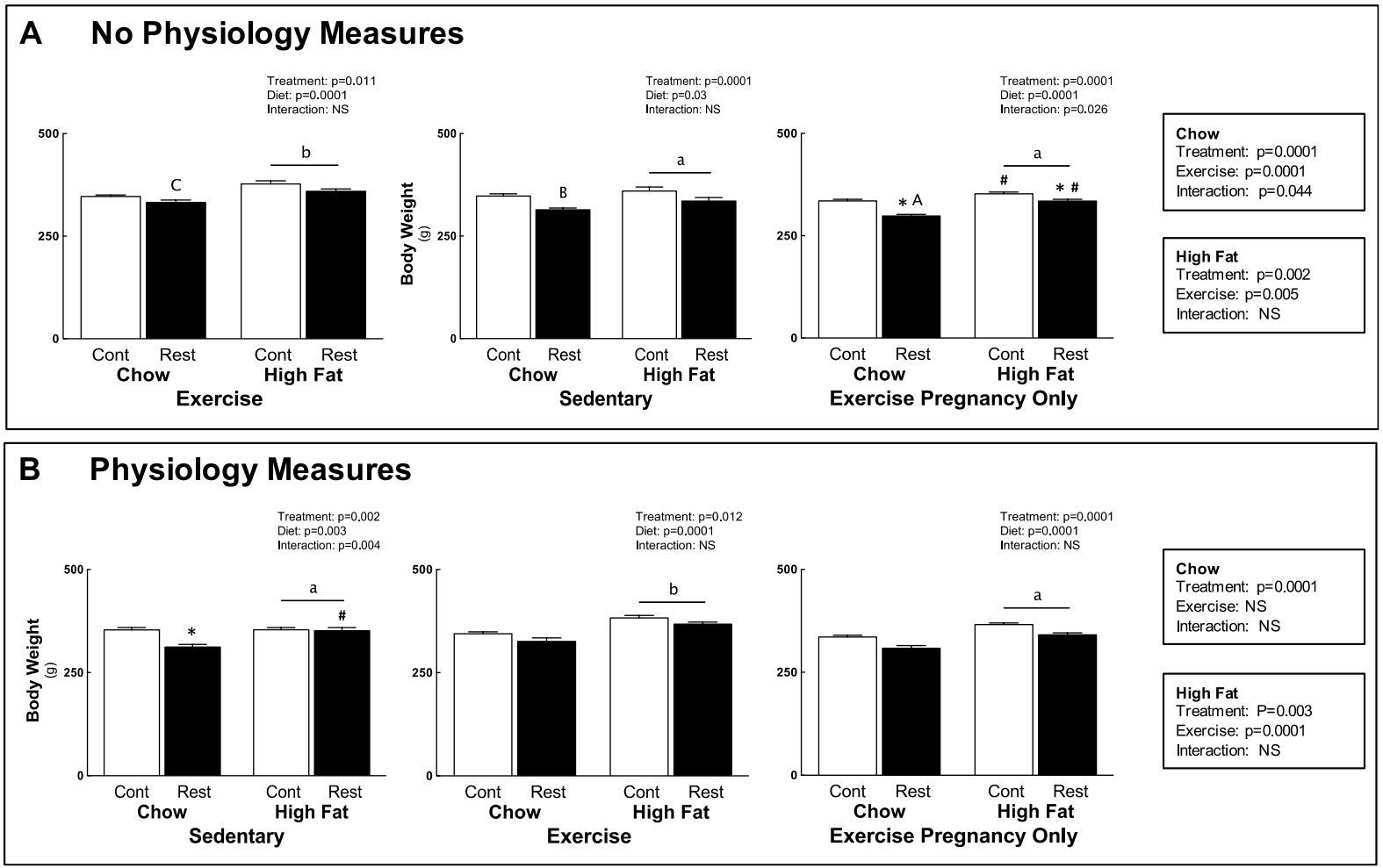


Figure 3.3 Body weights at *post mortem* (E20)

Control (white bars) and Restricted (black bars) body weights at *post mortem* (E20). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; 'a' is different to 'b' and 'c' ($P<0.05$).

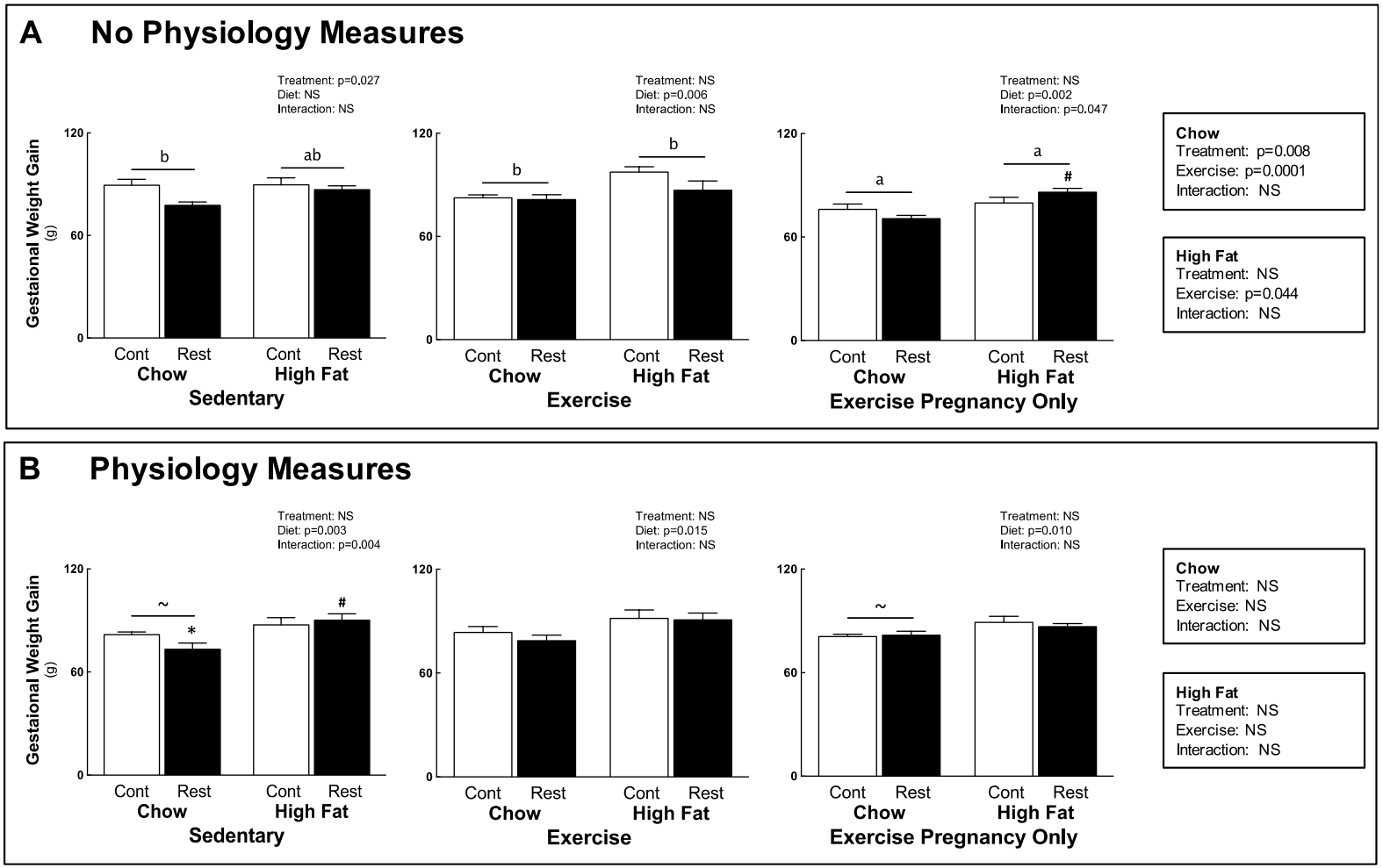


Figure 3.4 Gestational weight gain at *post mortem* (E20)

Control (white bars) and Restricted (black bars) gestational weight gain at *post mortem* (E20). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; ~ $P<0.05$ vs No physiology measures; 'a' is different to 'b' but not different to 'ab' ($P<0.05$).

3.4.3 Organ Weights

No effects of treatment, diet, exercise and physiology measures were demonstrated in liver, pancreas and hind limb leg muscle weights corrected to tibia length in all groups (Table 3.1).

3.4.3.1 Group Effect

In no physiology measures groups, Chow-fed Restricted females that were Sedentary had a reduction in relative dorsal and retro fat weights compared with Control counterparts ($P<0.05$, Table 3.1).
In physiology measured groups, Sedentary-Restricted females on a HFD had a reduction in relative retro fat weight compared with Control counterparts ($P<0.05$, Table 3.1).

3.4.2.2 Diet Effect

HFD increased relative dorsal fat, retro fat and total fat weights in both Control and Restricted irrespective of exercise and physiology measures (+30-50%, $P<0.05$, Table 3.1).

3.4.2.3 Exercise Effect

In no physiology measures groups, both Exercise and ExPregOnly reduced relative dorsal, retro and total fat weights in Chow-fed Control females ($P<0.05$, Table 3.1 and Figure 3.5 A).

In physiology measures groups, relative dorsal and total fat weights were reduced in Control females on a Chow diet that Exercise and ExPregOnly compared to Sedentary counterparts ($P<0.05$, Table 3.1 and Figure 3.5 B). Exercise increased relative dorsal fat weight in Control females on a HFD ($P<0.05$, Table 3.1). ExPregOnly reduced relative retro and total fat weights in Restricted females on a HFD compared with Sedentary counterparts ($P<0.05$, Table 3.1).

3.4.2.4 Physiology Measures Effect

Physiological measurements performed during pregnancy increased relative adrenal weight in all of the groups ($P<0.05$, Table 3.1).

Table 3.1 Organ weights at E20

Adrenal, liver, pancreas, hind limb leg muscles, dorsal and retro fat weights corrected to tibia length at E20. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures; 'a' is different to 'b' but not different to 'ab' ($P<0.05$).

		Chow		High Fat		Two-way Anova		
		Control	Restricted	Control	Restricted	Treatment	Diet	Interaction
Adrenal Weight/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	1.38 ± 0.07	1.50 ± 0.08	1.63 ± 0.03	1.46 ± 0.10	NS	NS	NS
	Exercise	1.79 ± 0.09	1.91 ± 0.05	1.88 ± 0.05	1.98 ± 0.07	NS	NS	NS
	ExPreg	1.78 ± 0.05	1.67 ± 0.07	1.68 ± 0.06	1.85 ± 0.06	NS	NS	NS
Physiology Measures	Sedentary	1.72 ± 0.05~	1.68 ± 0.03~	1.76 ± 0.06~	1.75 ± 0.09~	NS	NS	NS
	Exercise	2.03 ± 0.06~	1.94 ± 0.07~	2.03 ± 0.04~	2.04 ± 0.04~	NS	NS	NS
	ExPreg	1.93 ± 0.09	1.73 ± 0.10	1.92 ± 0.05~	2.03 ± 0.09~	NS	NS	NS
Liver Weight/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	317.93 ± 7.97	313.54 ± 6.82	330.77 ± 6.61	324.05 ± 7.54	NS	NS	NS
	Exercise	349.79 ± 7.05	333.99 ± 7.62	356.84 ± 6.54	346.13 ± 5.43	NS	NS	NS
	ExPreg	335.13 ± 8.25	304.14 ± 4.68	324.91 ± 5.93	331.77 ± 4.89	NS	NS	NS
Physiology Measures	Sedentary	335.13 ± 5.42	315.90 ± 6.32	341.88 ± 7.38	334.17 ± 10.61	NS	NS	NS
	Exercise	357.83 ± 6.63	350.61 ± 7.12	363.04 ± 10.18	363.47 ± 6.72	NS	NS	NS
	ExPreg	349.95 ± 5.65	335.57 ± 7.51	363.58 ± 8.61	339.69 ± 6.94	NS	NS	NS
Pancreas/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	24.03 ± 1.82	25.20 ± 1.05	27.47 ± 1.58	26.96 ± 1.08	NS	NS	NS
	Exercise	28.70 ± 0.73	27.76 ± 0.65	29.41 ± 1.68	29.56 ± 1.51	NS	NS	NS
	ExPreg	25.44 ± 1.54	23.09 ± 1.91	27.86 ± 1.05	26.33 ± 1.78	NS	NS	NS
Physiology Measures	Sedentary	24.42 ± 1.55	26.07 ± 0.66	27.83 ± 1.24	28.85 ± 0.81	NS	NS	NS
	Exercise	27.98 ± 0.42	22.58 ± 1.79	28.84 ± 1.55	28.66 ± 0.80	NS	NS	NS
	ExPreg	24.23 ± 1.45	22.22 ± 1.96	24.56 ± 1.46	25.11 ± 1.73	NS	NS	NS

Table 3.1 Organ weights at E20

Adrenal, liver, pancreas, hind limb leg muscles, dorsal and retro fat weights corrected to tibia length at E20. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures; 'a' is different to 'b' but not different to 'ab' ($P<0.05$).

		Chow		High Fat		Two-way Anova		
		Control	Restricted	Control	Restricted	Treatment	Diet	Interaction
Hind Limb Leg Muscle Weight/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	54.09 ± 1.30	51.07 ± 1.19	56.70 ± 1.18	52.07 ± 0.69	NS	NS	NS
	Exercise	55.94 ± 1.37	54.16 ± 1.21	55.58 ± 0.66	54.96 ± 1.88	NS	NS	NS
	ExPreg	51.57 ± 1.33	50.86 ± 1.47	53.04 ± 1.23	53.37 ± 0.96	NS	NS	NS
Physiology Measures	Sedentary	56.29 ± 1.12	50.18 ± 1.40	53.66 ± 1.48	51.71 ± 1.32	NS	NS	NS
	Exercise	57.39 ± 0.99	53.11 ± 1.85	58.10 ± 0.50	56.34 ± 1.05	NS	NS	NS
	ExPreg	50.74 ± 1.53	48.61 ± 1.67	54.17 ± 1.08	50.49 ± 1.50	NS	NS	NS
Dorsal Fat/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	203.97 ± 8.55^{b}	190.44 ± 6.80	268.05 ± 11.21	274.97 ± 19.20	NS	p=0.0001	NS
	Exercise	164.49 ± 4.40^{a}	187.23 ± 8.45	271.22 ± 19.14	235.09 ± 14.43	NS	p=0.0001	NS
	ExPreg	181.02 ± 7.63^{a}	171.30 ± 7.31	267.60 ± 11.14	255.40 ± 10.42	NS	p=0.0001	NS
Physiology Measures	Sedentary	219.08 ± 12.02^{b}	171.54 ± 10.75	248.22 ± 9.22^{a}	275.98 ± 12.38^{ab}	NS	p=0.0001	NS
	Exercise	169.17 ± 6.57^{a}	176.98 ± 11.00	289.82 ± 11.10^{b}	282.76 ± 13.60^{b}	NS	p=0.0001	NS
	ExPreg	185.18 ± 7.05^{a}	158.52 ± 8.26*	276.77 ± 11.62^{ab}	248.34 ± 6.54^{a}	NS	p=0.0001	NS
Retro Fat/ Tibia Length (mg.mm^{-1})								
No Physiology Measures	Sedentary	332.75 ± 13.30^{b}	284.67 ± 12.32	423.54 ± 22.87	380.98 ± 26.90	p=0.035	p=0.0001	NS
	Exercise	268.17 ± 7.84^{a}	285.44 ± 12.45	396.32 ± 22.13	367.57 ± 22.14	NS	p=0.0001	NS
	ExPreg	296.49 ± 8.27^{a}	265.83 ± 7.15*	438.83 ± 13.81	396.16 ± 15.54	p=0.002	p=0.0001	NS
Physiology Measures	Sedentary	346.45 ± 13.68	279.35 ± 11.76	407.90 ± 20.28	442.52 ± 19.55^{a}	NS	p=0.0001	NS
	Exercise	280.80 ± 11.02	278.55 ± 19.49	438.69 ± 24.59	422.48 ± 15.78^{ab}	NS	p=0.0001	NS
	ExPreg	311.60 ± 13.37	268.60 ± 10.17	482.57 ± 15.46	399.39 ± 10.73^{b}	p=0.001	p=0.0001	NS

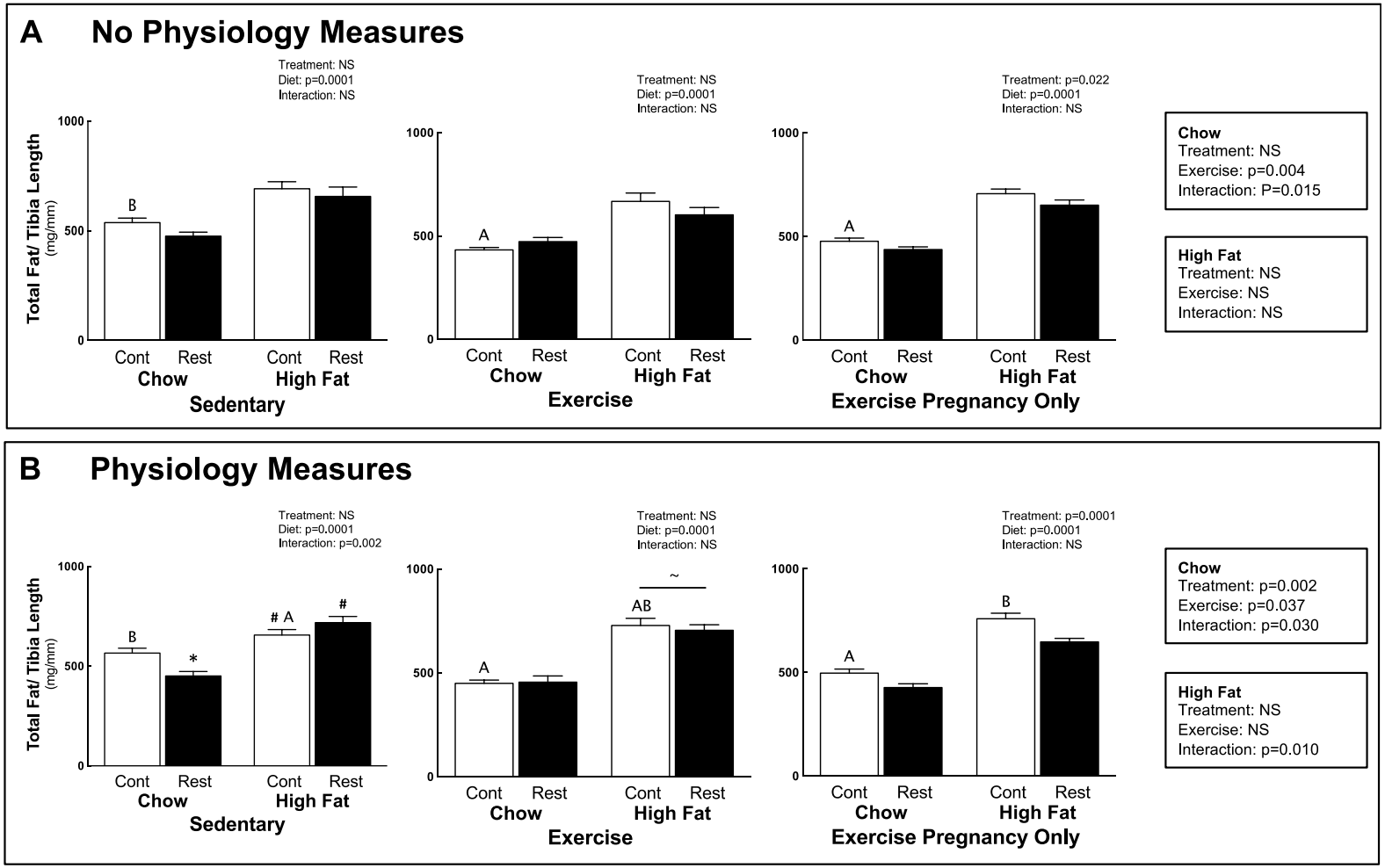


Figure 3.5 Total fat weights at E20

Control (white bars) and Restricted (black bars) total fat weights at E20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; ~ $P<0.05$ vs No physiology measures; 'A is different to 'B' but not different to 'AB' ($P<0.05$).

3.4.4 Food and Energy Intake

HFD increased food intake in all of the groups except in Restricted females that exercised during pregnancy only (+16-20%, $P<0.05$, Figure 3.6 A). Energy intake was also increased by HFD in both Control and Restricted females regardless of exercise interventions (+10-27%, $P<0.05$, Figure 3.6 B).

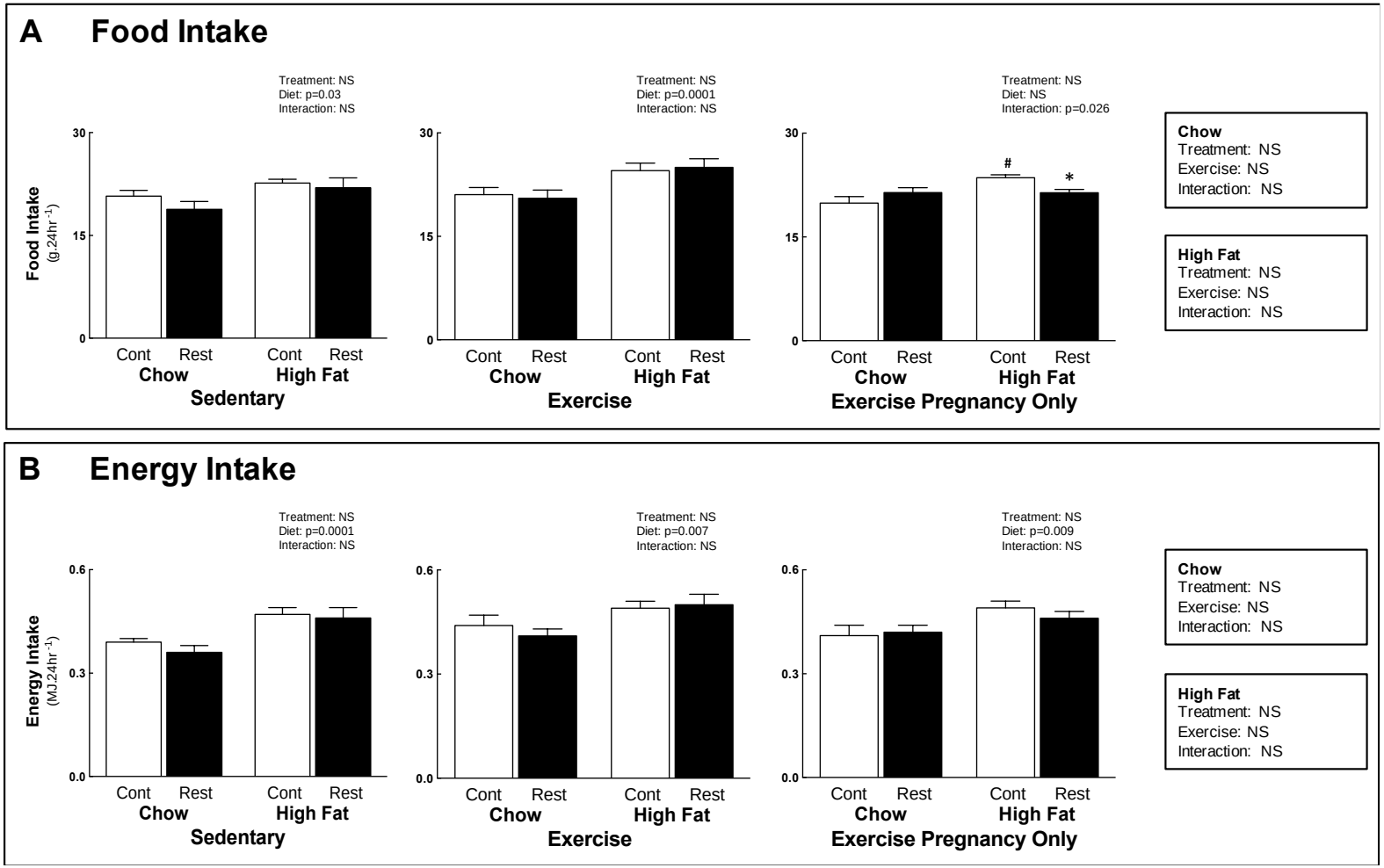


Figure 3.6 Food and Energy Intake

Control (white bars) and Restricted (black bars) (A) food and (B) energy intake for 24 hours. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow.

3.4.5 Metabolic Profile

3.4.5.1 Group Effect

Basal glucose and insulin and HbA1c were not different between Control and Restricted females irrespective of diet and exercise (Figures 3.7 and 3.9). During IPGTT, glucose area under the curve (AUC) that measures glucose tolerance, was increased in Sedentary Restricted females on a Chow (+18%) and High fat (+14%) diet compared to Control counterparts (P<0.05, Figure 3.8 A). Glucose stimulated insulin secretion as determined by insulin AUC was not affected by uteroplacental insufficiency regardless of diet and exercise interventions (Figure 3.8 B).

3.4.5.2 Diet Effect

Basal glucose and insulin and HbA1c were not affected by HFD in all of the experimental groups (Figure 3.7 and Figure 3.9). HFD increased glucose AUC in both Control (+14%) and Restricted (+31%) females that remained Sedentary throughout the experiment (P<0.05, Figure 3.8 A). The effect was sustained when Control and Restricted females exercised during pregnancy only but not in Control and Restricted that exercised prior to and during pregnancy (P<0.05, Figure 3.8 A). Glucose stimulated insulin secretion in Control and Restricted were not affected by diet irrespective of exercise interventions (Figure 3.8 B).

3.4.5.3 Exercise Effect

Exercise interventions did not affect basal glucose and HbA1c in all of the groups (Figure 3.7 A and Figure 3.9). Basal insulin in Control and Restricted females on a HFD were not affected by both exercise interventions (Figure 3.7 B). Exercise but not ExPregOnly increased basal insulin in Control (+32%) and Restricted (+67%) on a Chow diet compared with Sedentary counterparts (P<0.05, Figure 3.7 B). Exercise, but not ExPregOnly, reduced glucose AUC in Restricted females on a Chow compared with Sedentary counterparts (P<0.05, Figure 3.8 A). Exercise, but not ExPreg, reduced glucose AUC in both Control and Restricted on a HFD compared with Sedentary counterparts (P<0.05, Figure 3.8 A). Exercise increased glucose stimulated insulin secretion in Control (+18%) and Restricted (+47%) females on a Chow diet compared with Sedentary (P<0.05, Figure 3.8 B). Glucose stimulated insulin secretion was not affected by ExPregOnly in both Control and Restricted on a Chow or HFD (Figure 3.8 B).

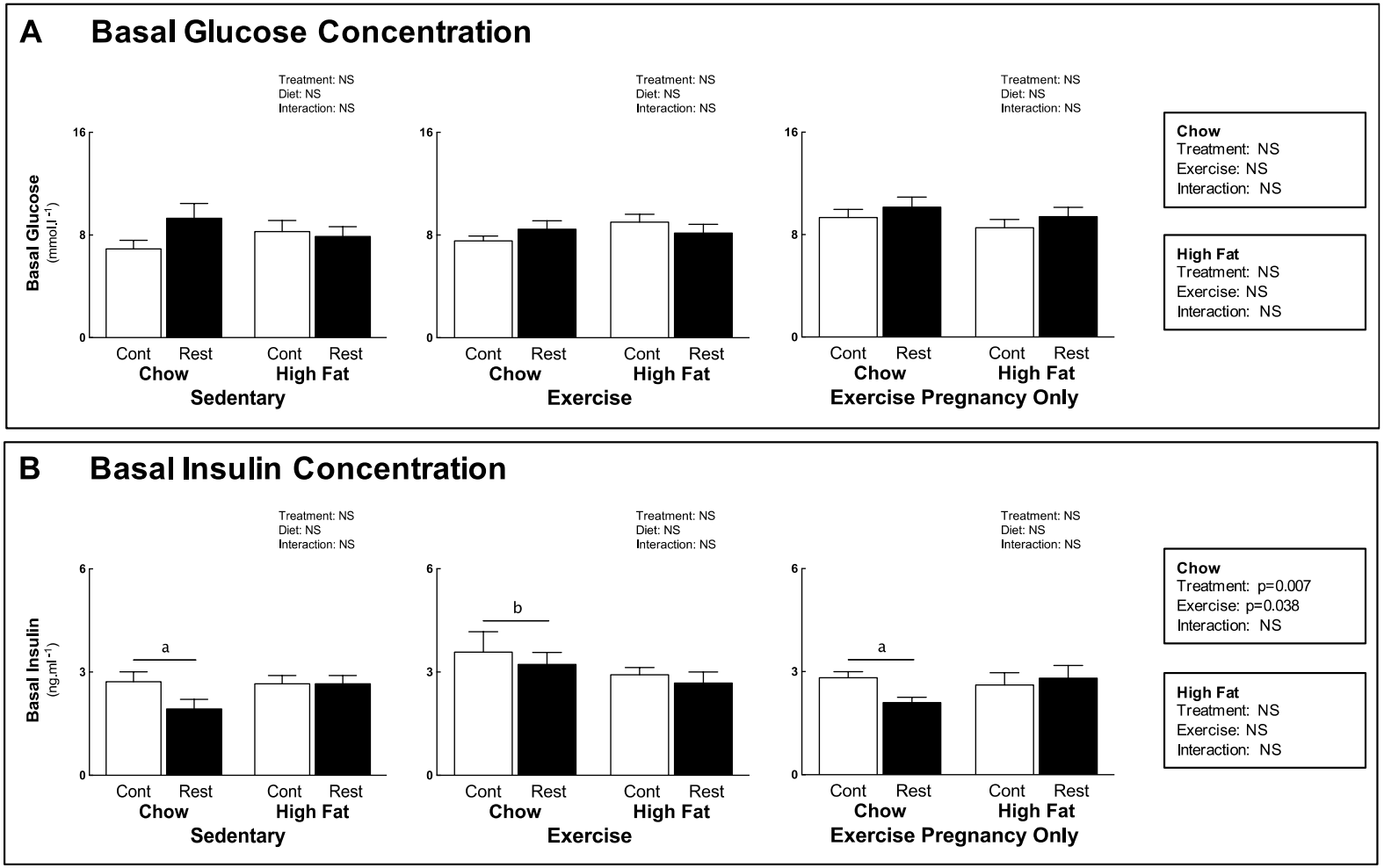


Figure 3.7 Basal glucose and insulin prior to IPGTT

Control (white bars) and Restricted (black bars) basal (A) glucose and (B) insulin prior to IPGTT. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' (P<0.05).

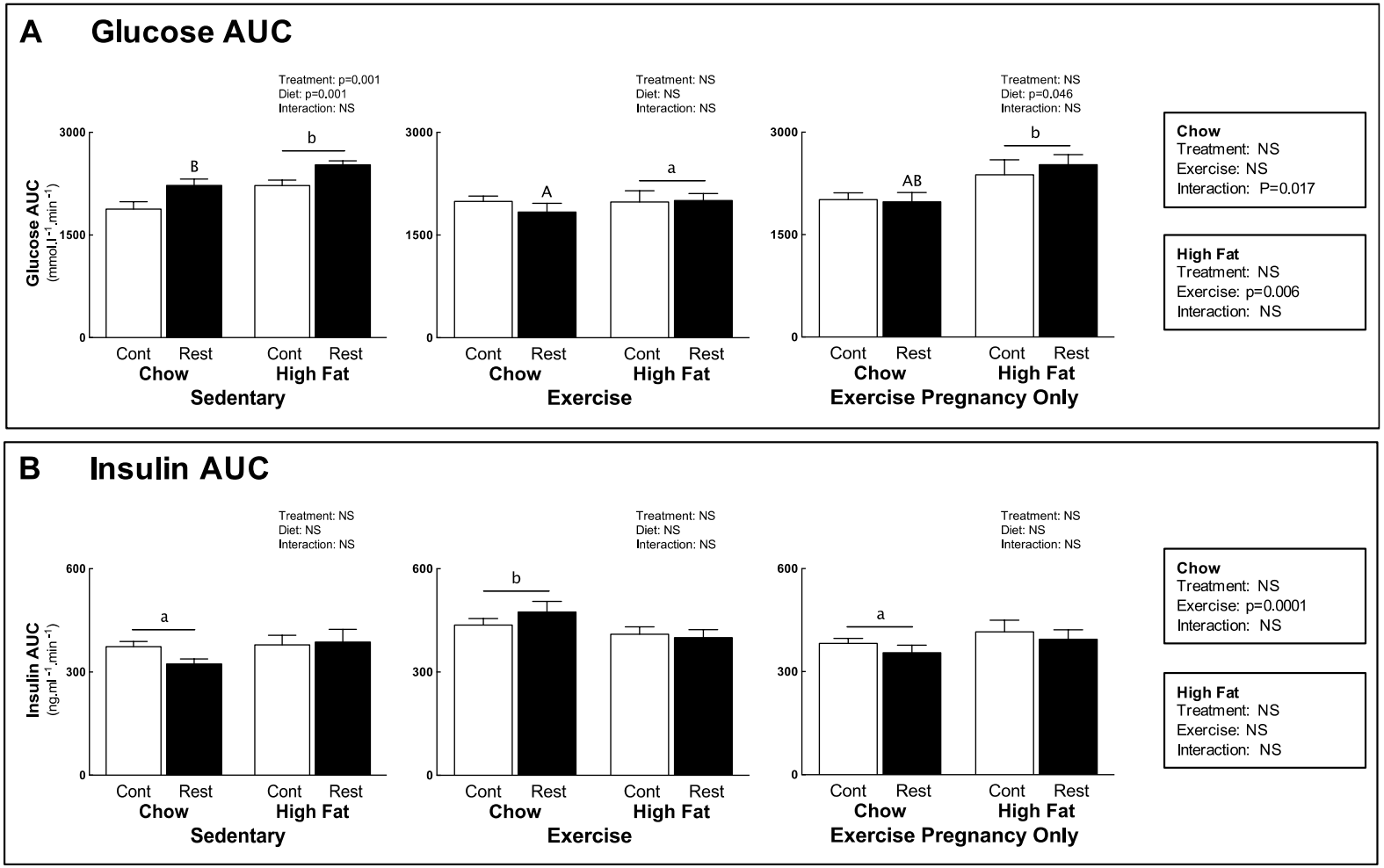


Figure 3.8 Glucose and insulin AUC during IPGTT

Control (white bars) and Restricted (black bars) (A) glucose and (B) insulin area under the curve during IPGTT. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a/A' is different to 'b/B' but not different to 'ab/AB' (P<0.05).

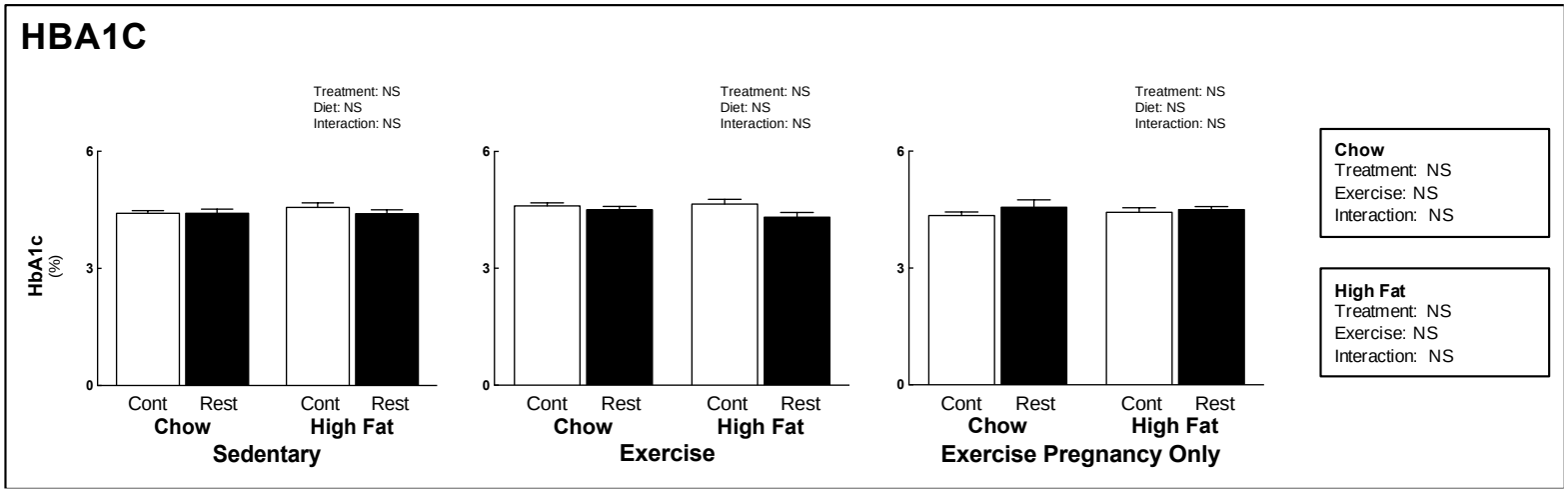


Figure 3.9 HbA1c at E20

Control (white bars) and Restricted (black bars) HbA1c at E20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

3.4.6 Pancreatic Morphometry

3.4.6.1 Treatment Effect

β-cell and islet mass were not different between Control and Restricted regardless of diet, exercise and physiology measures (Figures 3.10 and 3.11).

3.4.6.2 Diet Effect

β-cell and islet mass were not affected by HFD in any of the experimental (Figures 3.10 and 3.11).

3.4.6.3 Exercise Effect

Exercise, but not ExPregOnly, increased pancreatic β-cell and islet mass in both Control and Restricted females on a HFD compared with Sedentary (+34%-44%, $P<0.05$, Figures 3.10 and 3.11).

3.4.6.4 Physiology Measures Effect

β-cell and islet mass were not affected by physiology measures in all of the experimental groups (Figures 3.10 and 3.11).

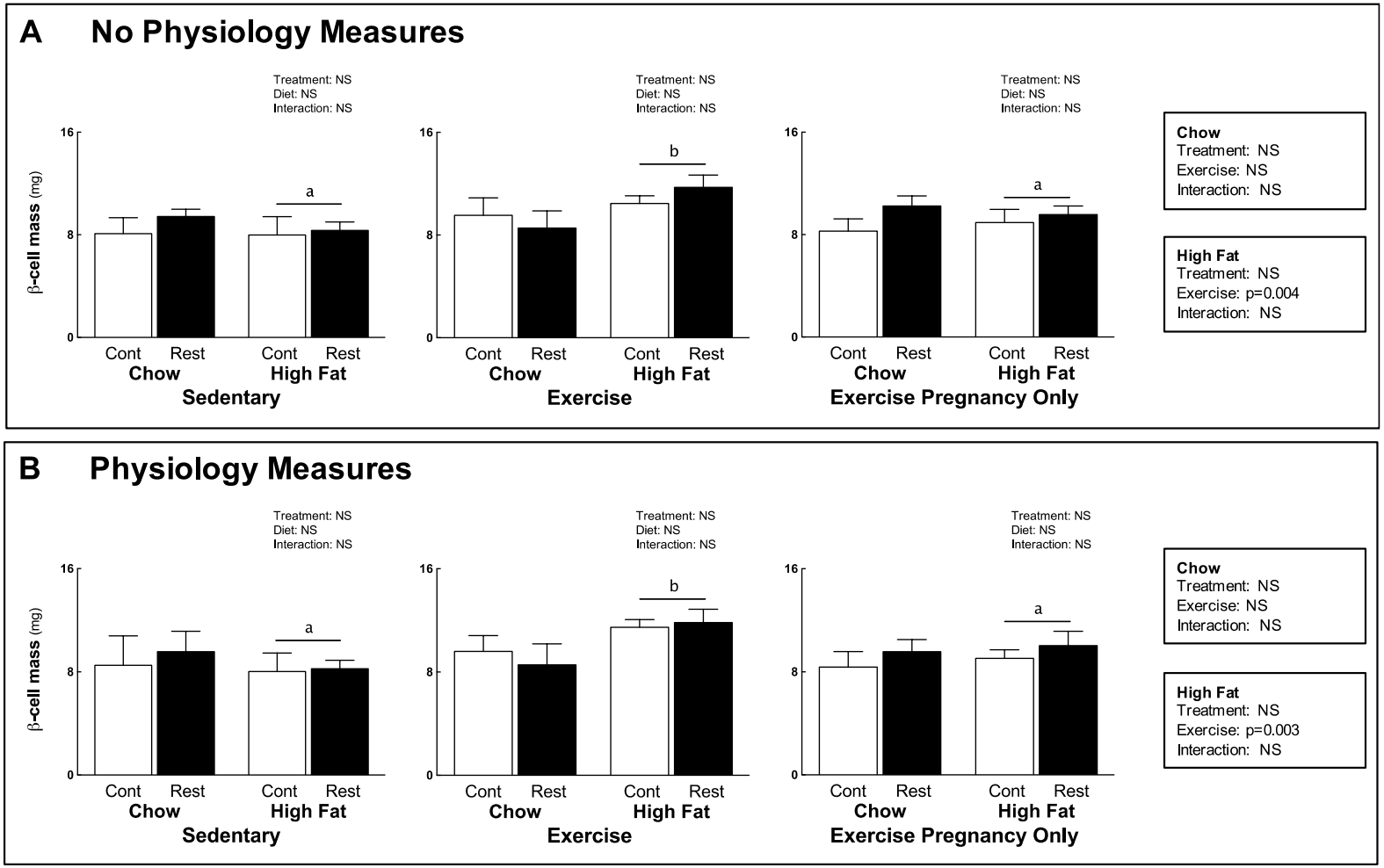


Figure 3.10 β-cell mass at E20

Control (white bars) and Restricted (black bars) β-cell mass at E20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 6-7 per group from separate litters. 'a' is different to 'b' (P<0.05).

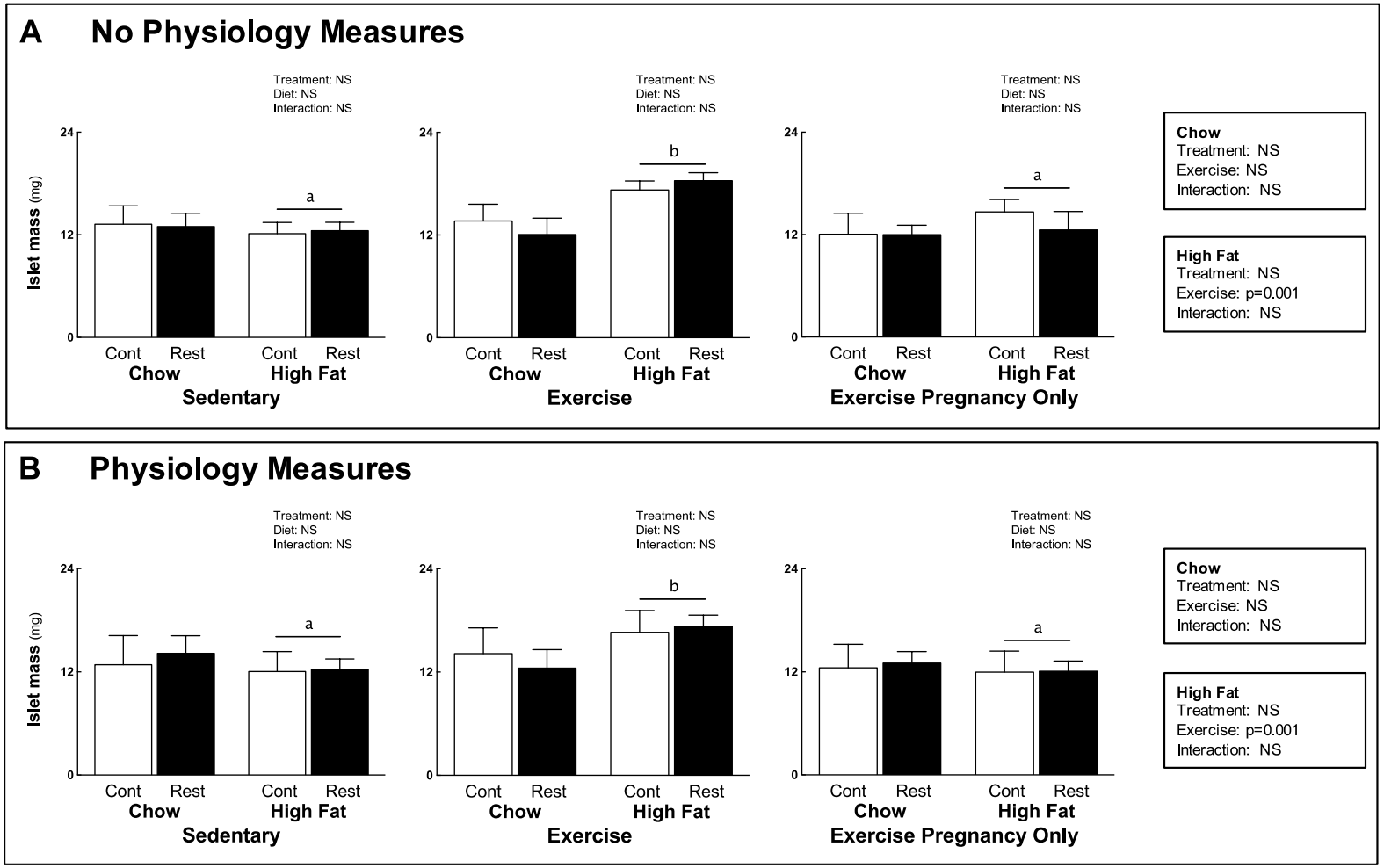


Figure 3.11 Islet mass at E20

Control (white bars) and Restricted (black bars) islet mass at E20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 6-7 per group from separate litters. 'a' is different to 'b' (P<0.05).

3.4.7 Basal Acitivity and Energy Expenditure

3.4.7.1 Treatment Effect

Restricted females that exercised prior to and during pregnancy had an increased in oxygen consumption during dark cycle irrespective of diet (+13%, $P<0.05$, Figure 3.12 A). During light cycle, oxygen consumption was increased in Restricted females that were Sedentary regardless of diet (+4-8%, $P<0.05$, Figure 3.12 B). The effect was sustained in Exercise groups ($P<0.05$, Figure 3.12 B), but not in ExPregOnly groups. Carbon dioxide production during dark cycle was increased in Restricted females that Exercise and ExPregOnly irrespective of diet ($P<0.05$, Figure 3.13 A). Restricted females had an increased in carbon dioxide production during light cycle when Exercise regardless of diet ($P<0.05$, Figure 3.13 B).

Respiratory exchange ratio (RER) during dark and light cycle was not different between Control and Restricted irrespective of diet and exercise (Figure 3.14). Heat production during dark cycle was reduced in Restricted females on a Chow diet (-9%, $P<0.05$, Figure 3.15 A) and increased in High fat-fed Restricted females (+8%, $P<0.05$, Figure 3.15 A) that were Sedentary. Exercise-Restricted females had an increased in dark cycle heat production in females irrespective of diet ($P<0.05$, Figure 3.15 A). Heat production during light cycle was not affected by treatment regardless of diet and exercise interventions (Figure 3.15 B).

Total x-activity and z-activity as well as ambulatory x-activity during dark and light cycle were not different between Control and Restricted irrespective of diet and exercise (Figures 3.16, 3.17 and 3.18).

3.4.7.2 Diet Effect

Oxygen consumption during dark cycle was reduced in both Control (-4%) and Restricted (-14%) females on a HFD that Exercise ($P<0.05$, Figure 3.12 A). HFD reduced oxygen consumption in both Control and Restricted females that Exercise and ExPregOnly ($P<0.05$, Figure 3.12 B). In Exercise and ExPregOnly groups, HFD reduced carbon dioxide production in Control and Restricted females during dark and light cycle ($P<0.05$, Figures 3.13 A and B).

HFD reduced RER during dark and light cycle in Control and Restricted females that were Sedentary (-5-9%, $P<0.05$, Figure 3.14). The effect was sustained in Exercise and ExPregOnly groups ($P<0.05$, Figure 3.14). During dark cycle, HFD increased heat production in Sedentary-Restricted ($P<0.05$, Figure 3.15 A). HFD increased heat production during light cycle in Control and Restricted females that were Sedentary ($P<0.05$, Figure 3.15 B).

Total x-activity and z-activity as well as ambulatory x-activity during dark and light cycle were not affected by diet in all groups (Figures 3.16, 3.17 and 3.18).

3.4.7.3 Exercise Effect

During dark cycle, oxygen consumption was not affected by exercise interventions regardless of treatment and diet (Figure 3.12 A). Both Exercise and ExPregOnly increased oxygen consumption during light cycle in Control and Restricted females on a Chow diet compared with Sedentary counterparts ($P<0.05$, Figure 3.12 B). Carbon dioxide production during dark cycle was increased in Chow-fed Restricted females that Exercise and ExPregOnly compared with Sedentary ($P<0.05$, Figure 3.13 A). Compared to Sedentary, Exercise and ExPregOnly increased carbon dioxide production during light cycle in both Control and Restricted on a Chow diet ($P<0.05$, Figure 3.13 B).

Exercise increased dark and light cycle RER in Chow-fed Restricted females compared with Sedentary ($P<0.05$, Figure 3.14). RER was also increased in Restricted females on a Chow diet that ExPregOnly during dark but not light cycle ($P<0.05$, Figure 3.14). Exercise increased heat production during dark cycle in Restricted females on a Chow diet (+20%, $P<0.05$, Figure 3.15 A). During light cycle, Exercise and ExPregOnly increased heat production in Control and Restricted regardless of diet compared with Sedentary counterparts ($P<0.05$, Figure 3.15 B).

Exercise, but not ExPregOnly, increased total x-activity during dark cycle in Control and Restricted on a HFD compared with Sedentary ($P<0.05$, Figure 3.16 A). Total z-activity and ambulatory x-activity were not affected by both exercise interventions in all groups (Figures 3.17 and 3.18).

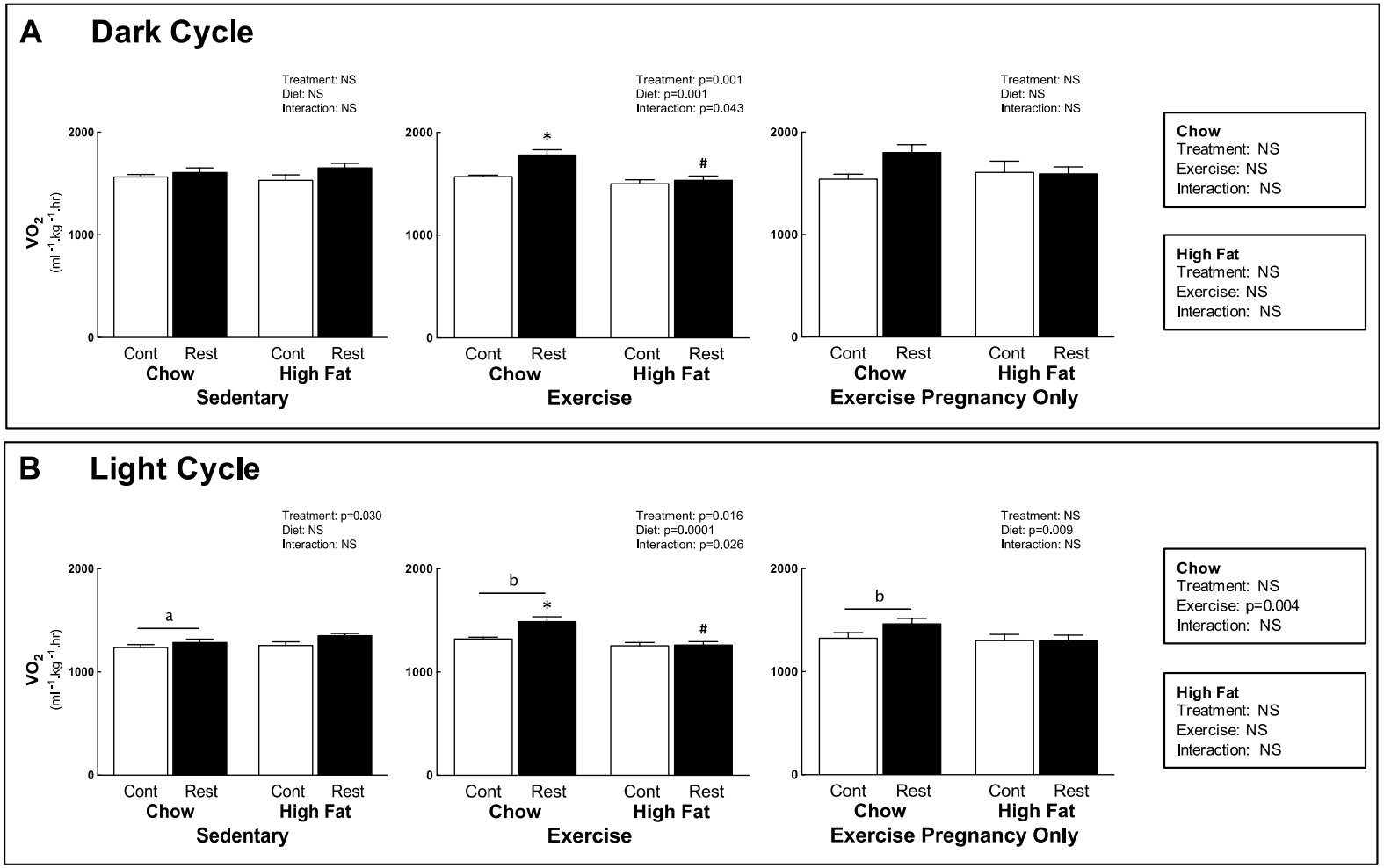


Figure 3.12 Oxygen consumption during dark and light cycle

Control (white bars) and Restricted (black bars) oxygen consumption during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; 'a' is different to 'b' ($P<0.05$).

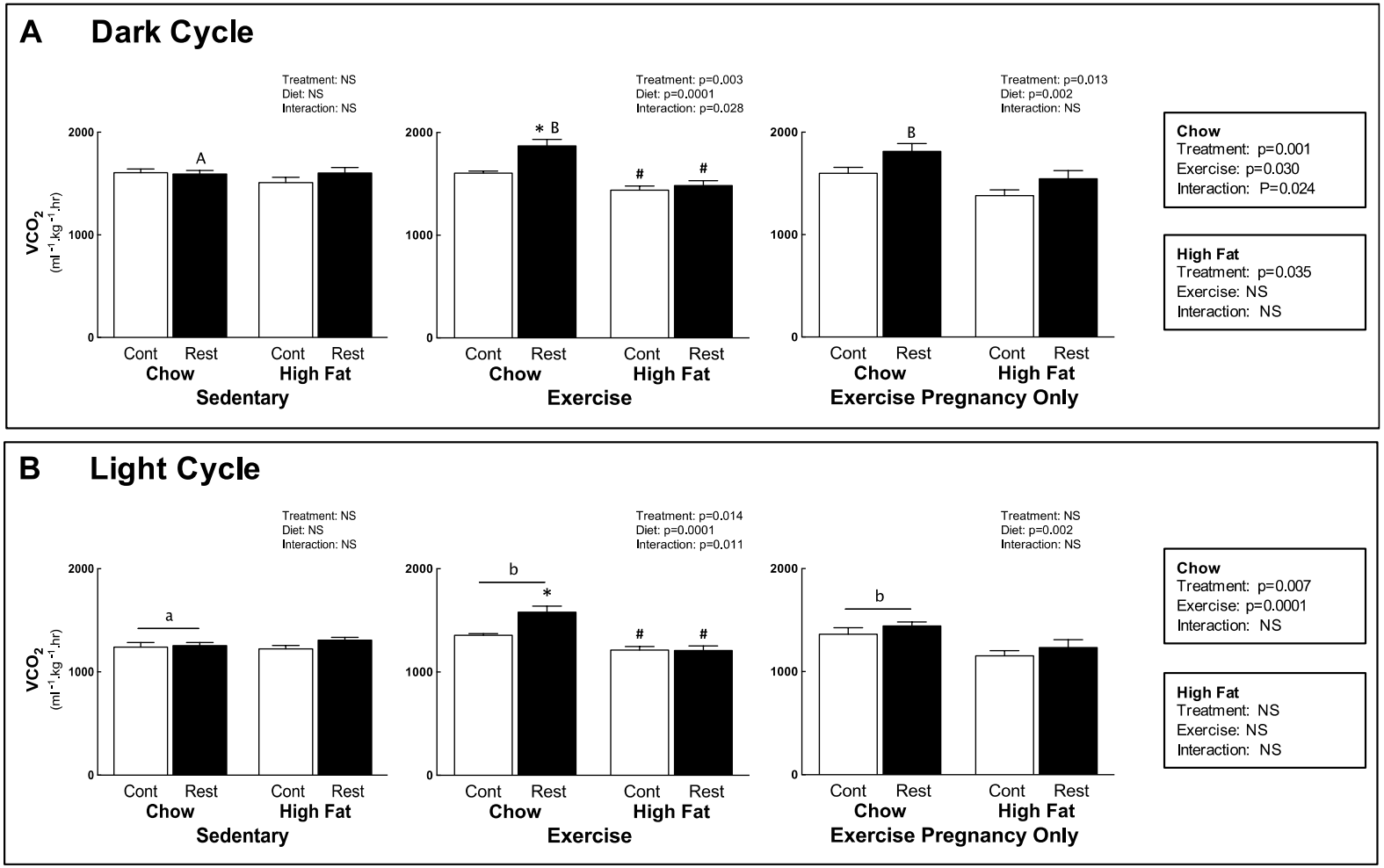


Figure 3.13 Carbon dioxide production during dark and light cycle

Control (white bars) and Restricted (black bars) carbon dioxide production during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; 'a/A' is different to 'b/B' ($P<0.05$).

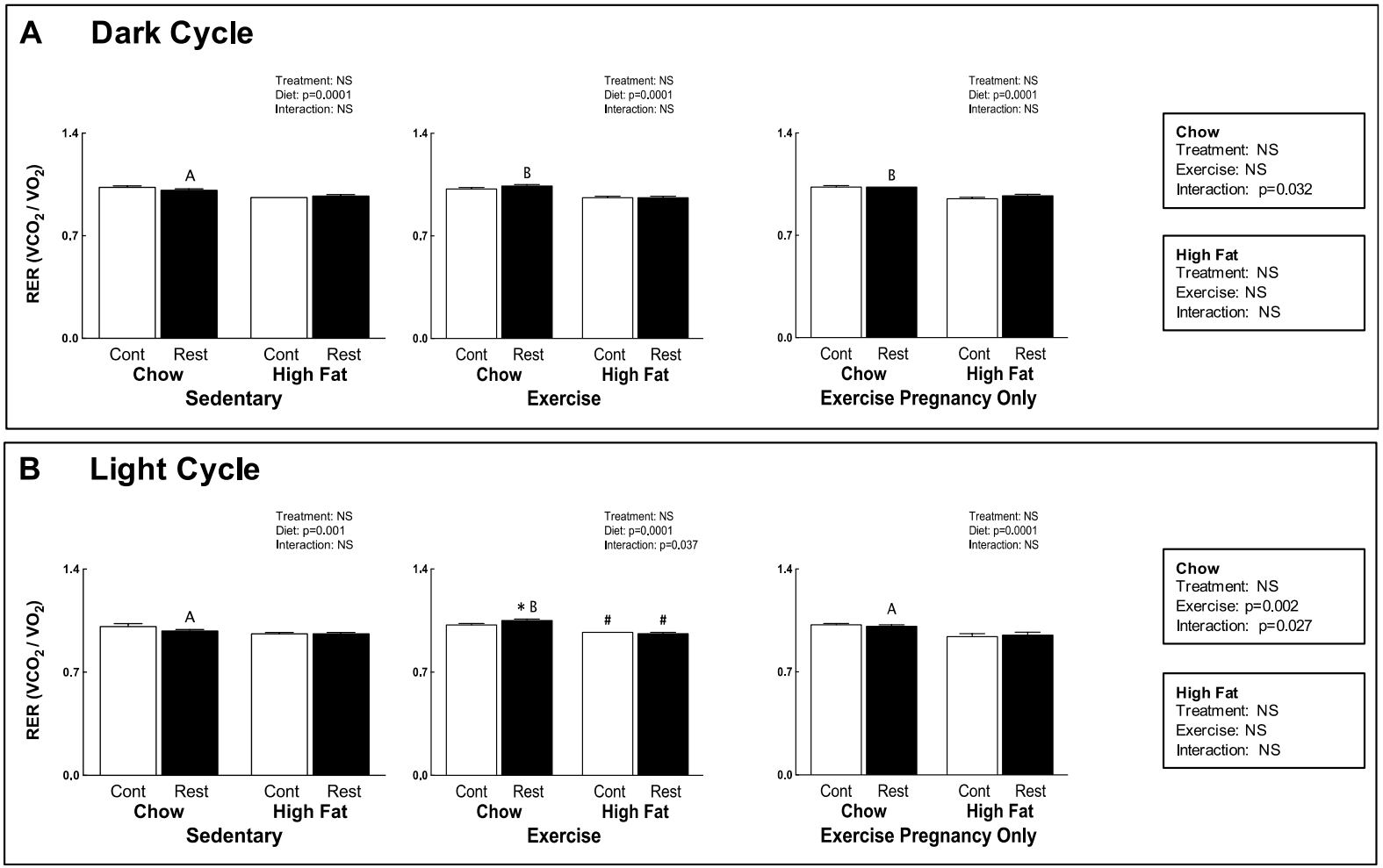


Figure 3.14 Respiratory exchange ratio during dark and light cycle

Control (white bars) and Restricted (black bars) respiratory exchange ratio during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; 'a/A' is different to 'b/B' ($P<0.05$).

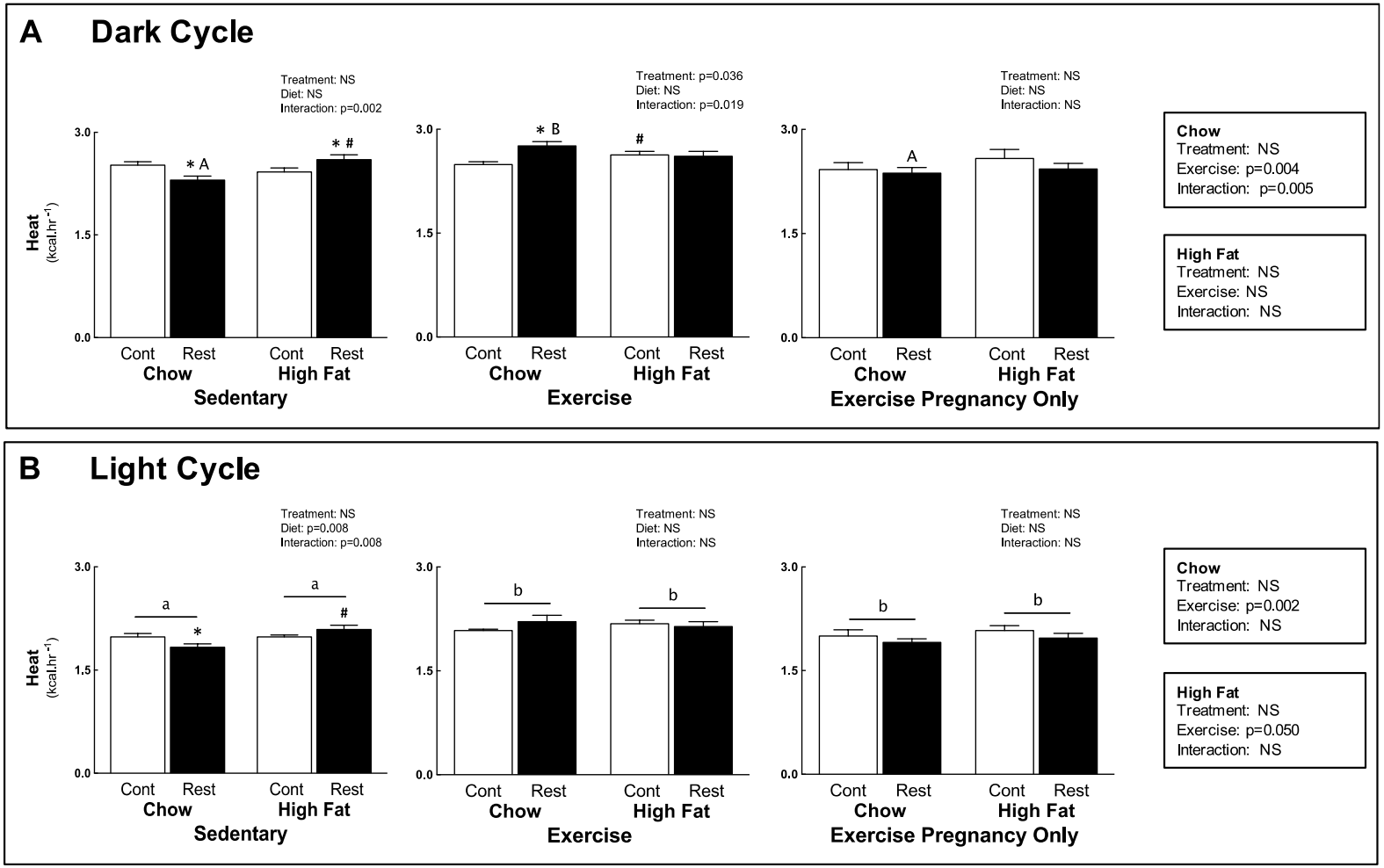


Figure 3.15 Heat production during dark and light cycle

Control (white bars) and Restricted (black bars) heat production during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; 'a/A' is different to 'b/B' ($P<0.05$).

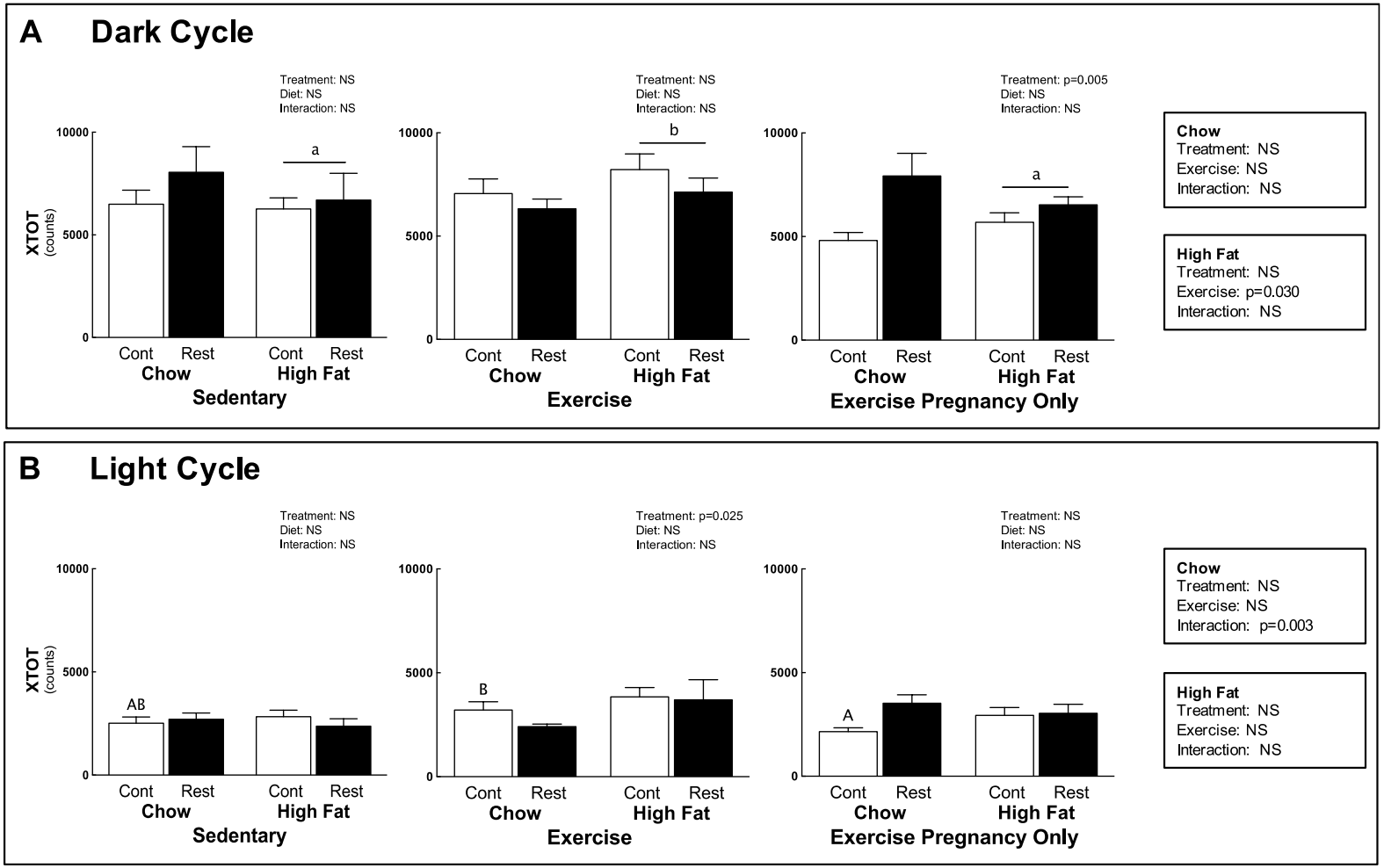


Figure 3.16 Total *x*-activity during dark and light cycle

Control (white bars) and Restricted (black bars) total *x*-activity during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a/A' is different to 'b/B' but not different to 'AB' (P<0.05).

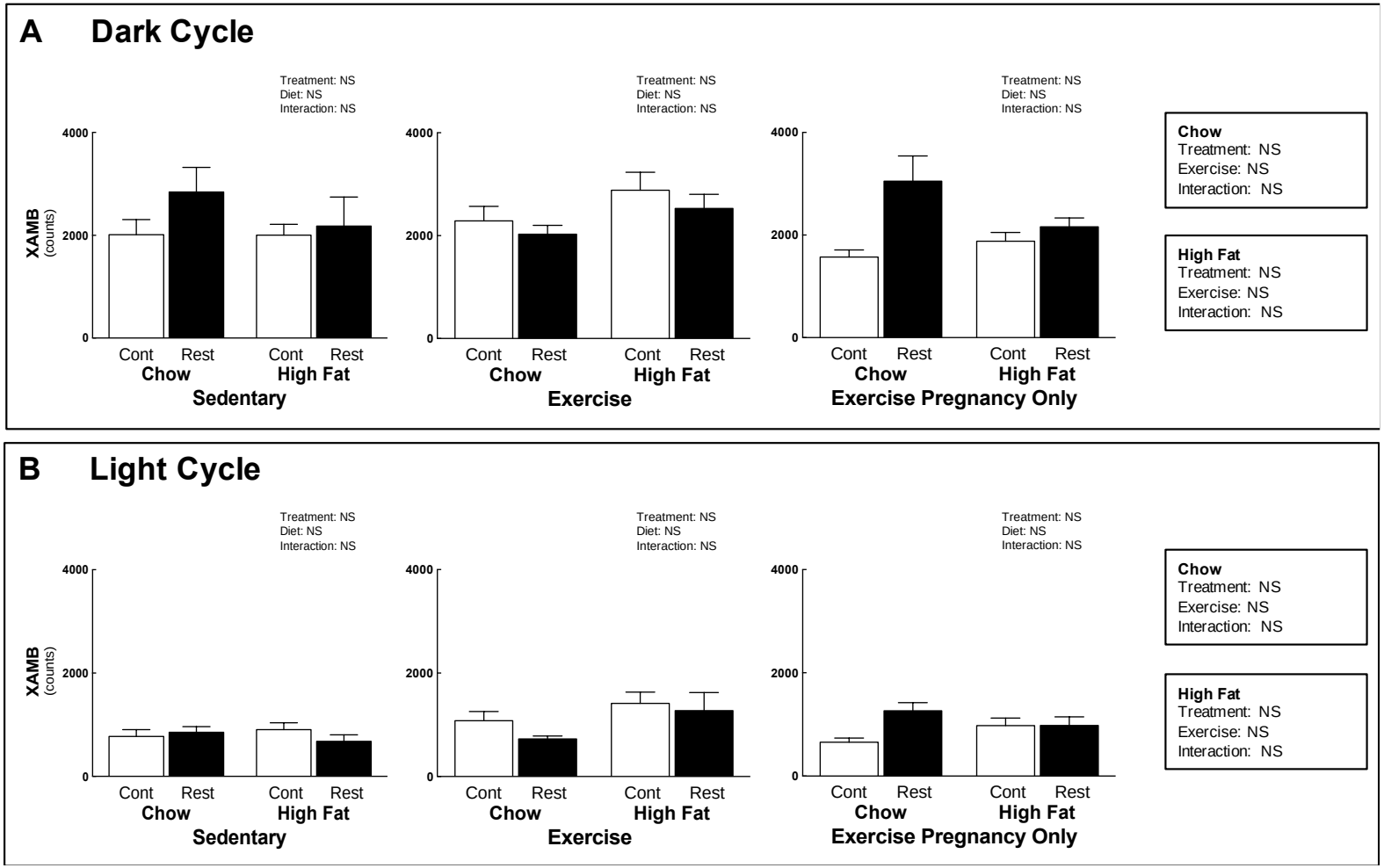


Figure 3.17 Ambulatory *x*-activity during dark and light cycle

Control (white bars) and Restricted (black bars) ambulatory *x*-activity during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

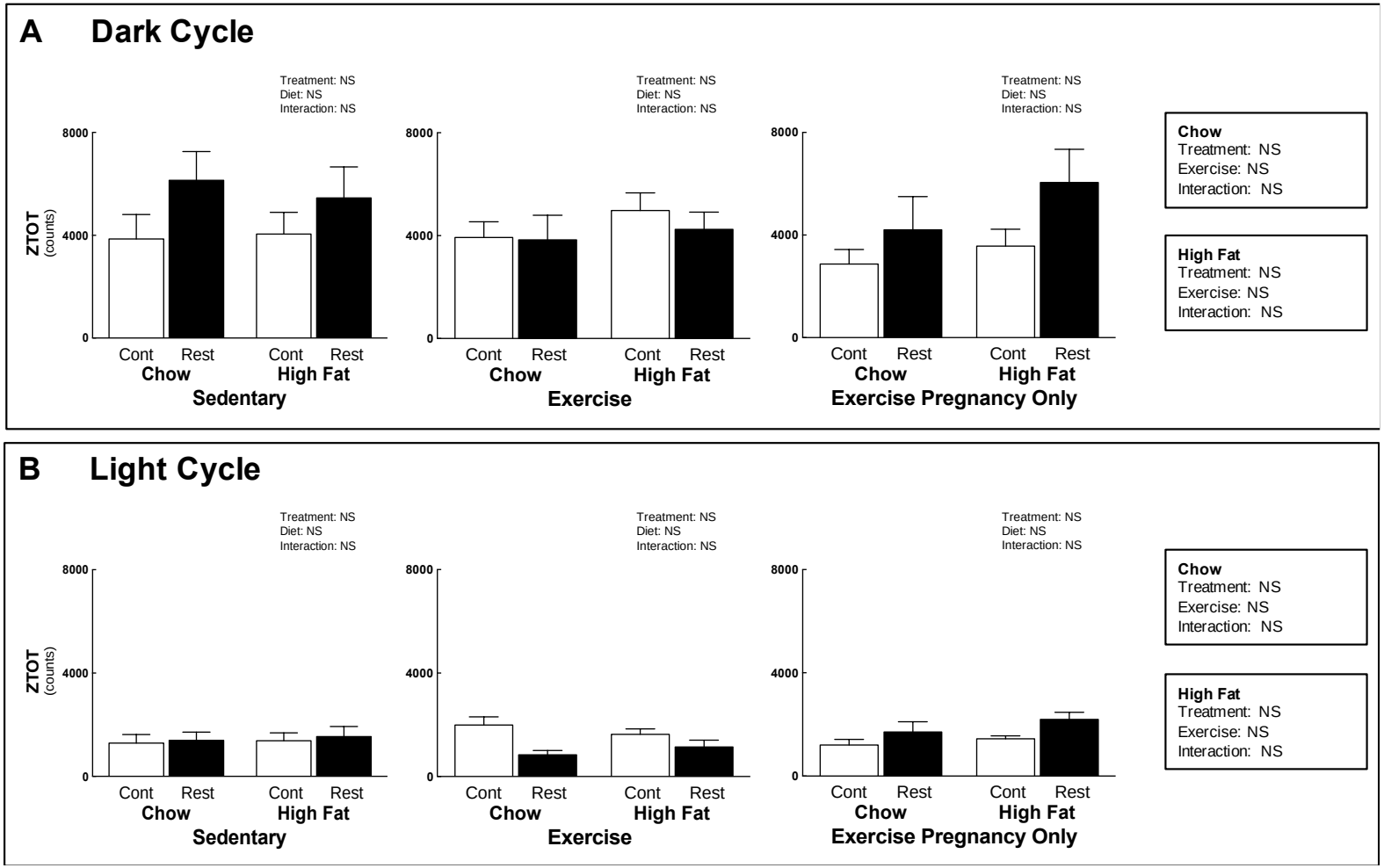


Figure 3.18 Total *z*-activity during dark and light cycle

Control (white bars) and Restricted (black bars) ambulatory *z*-activity during (A) dark and (B) light cycle. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

3.4.8 Cytokines and Triglycerides

3.4.8.1 Treatment Effect

Leptin, adiponectin and MCP-1 plasma concentrations were not different between Control and Restricted irrespective of diet and exercise (Table 3.2). Restricted increased muscle triglyceride concentration in females that were Sedentary irrespective of diet, exercise and physiology measures (P<0.05, Figure 3.19).

3.4.8.2 Diet Effect

Adiponectin and MCP-1 plasma concentrations as well as muscle triglyceride concentration were not affected by diet in all groups (Table 3.2 and Figure 3.19). Leptin plasma concentration was increased in Control and Restricted on a HFD (+82-84%, P<0.05, Table 3.2). The effect was sustained with both exercise interventions and physiology measures (P<0.05, Table 3.2).

3.4.8.3 Exercise Effect

Leptin, adiponectin and MCP-1 plasma concentrations were not affected by exercise interventions in all experimental groups (Table 3.2). In no physiology measures groups, ExPregOnly increased muscle triglyceride concentration in Control and Restricted on a Chow diet compared with Exercise (P<0.05, Figure 3.19 A). Control and Restricted females on a HFD that exercised before and during pregnancy had reduced muscle triglyceride concentration compared with Sedentary counterparts (-12-43%, P<0.05, Figure 3.19 A). Muscle triglyceride concentration in High fat-fed Control and Restricted was not affected by ExPregOnly compared with Sedentary (Figure 3.19 A). In physiology measures groups, Exercise but not ExPregOnly reduced muscle triglyceride concentration in Restricted females on a Chow diet (-42%, P<0.05, Figure 3.19 B).

3.4.8.4 Physiology Measures Effect

Leptin, adiponectin and MCP-1 plasma concentrations as well as muscle triglyceride concentration were not affected by physiology measures in all groups (Table 3.2 and Figure 3.19).

Table 3.2 Plasma cytokines concentrations at E20

Plasma leptin, adiponectin and MCP-1 concentrations at E20. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

		Chow		High Fat		Two-way Anova		
		Control	Restricted	Control	Restricted	Treatment	Diet	Interaction
Leptin (pg.ml^{-1})								
No Physiology Measures	Sedentary	4529 ± 267	8315 ± 465	4150 ± 256	7550 ± 422	NS	p=0.0001	NS
	Exercise	4235 ± 401	8027 ± 333	4383 ± 270	7721 ± 275	NS	p=0.0001	NS
	ExPreg	4715 ± 307	8078 ± 408	4120 ± 368	7719 ± 434	NS	p=0.0001	NS
Physiology Measures	Sedentary	4519 ± 178	8254 ± 437	4373 ± 270	8552 ± 223	NS	p=0.0001	NS
	Exercise	4561 ± 185	8171 ± 254	4232 ± 216	8198 ± 383	NS	p=0.0001	NS
	ExPreg	4679 ± 424	9406 ± 312	5035 ± 453	8384 ± 480	NS	p=0.0001	NS
Adiponectin (pg.ml^{-1})								
No Physiology Measures	Sedentary	5464 ± 157	5638 ± 172	5833 ± 122	5608 ± 221	NS	NS	NS
	Exercise	5545 ± 208	5386 ± 206	5980 ± 221	5339 ± 365	NS	NS	NS
	ExPreg	5623 ± 173	5627 ± 165	6015 ± 124	5657 ± 167	NS	NS	NS
Physiology Measures	Sedentary	4773 ± 146	5108 ± 197	4944 ± 202	5398 ± 189	NS	NS	NS
	Exercise	4499 ± 294	4677 ± 299	5142 ± 237	5183 ± 137	NS	NS	NS
	ExPreg	5207 ± 167	5223 ± 265	5673 ± 304	5358 ± 291	NS	NS	NS
MCP-1 (pg.ml^{-1})								
No Physiology Measures	Sedentary	253 ± 20	285 ± 27	241 ± 23	244 ± 10	NS	NS	NS
	Exercise	266 ± 18	234 ± 26	267 ± 23	244 ± 15	NS	NS	NS
	ExPreg	277 ± 37	261 ± 26	264 ± 20	251 ± 22	NS	NS	NS
Physiology Measures	Sedentary	242 ± 10	233 ± 24	242 ± 27	254 ± 24	NS	NS	NS
	Exercise	243 ± 37	244 ± 26	264 ± 16	232 ± 14	NS	NS	NS
	ExPreg	266 ± 30	264 ± 18	250 ± 15	260 ± 22	NS	NS	NS

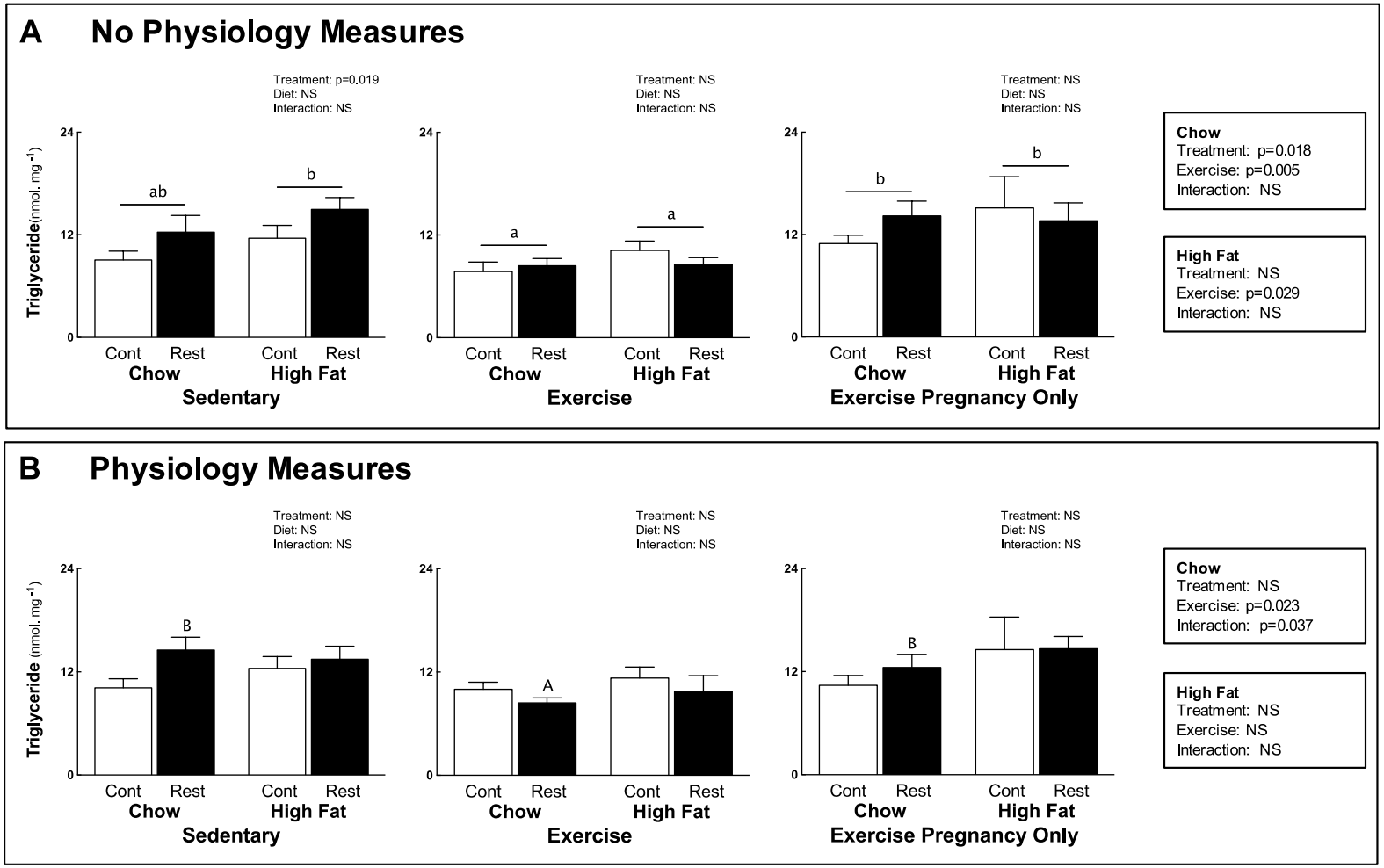


Figure 3.19 Muscle triglyceride concentrations

Control (white bars) and Restricted (black bars) muscle triglyceride concentrations. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a/A' is different to 'b/B' but no different to 'c' (P<0.05).

3.5 Discussion

Although it is well characterised that low birth weight females have an increased risk of obesity during adulthood, the combined effects of intrauterine growth restriction and obesity on the pregnancy adaptations is not studied. This present chapter investigated the effects of HFD on the metabolic and pancreatic adaptations to pregnancy and the beneficial effects of lifestyle intervention of exercise. We demonstrated that HFD exacerbated the pre-existing glucose intolerance in growth-restricted females during late gestation despite normal insulin secretion in response to glucose load. This alteration in glucose control in females born small occurred in the presence of normal β-cell mass. Epidemiological studies extrapolate that alteration in glucose control during gestation increases the likelihood of women to develop type 2 diabetes in later life (Kim *et al.*, 2002; Ben-Haroush *et al.*, 2004; Lauenborg *et al.*, 2004; Feig *et al.*, 2008; Bellamy *et al.*, 2009). Importantly, a novel finding that emerges from this study is that the alteration in glucose control was prevented by lifestyle intervention of exercise initiated before pregnancy potentially due to improved β-cell mass.

3.5.1 Maternal Growth Profile and Organ Weights

Restricted females were born lighter than Control and remained smaller at PN 7, 14, 35, Week 15, 19, 20 and *post mortem*. These findings are in contrast to our recent published studies showing that growth restricted females were lighter than Control at PN1, 6, 14 and 35 but caught up to Controls body weights at 4 months (Gallo *et al.*, 2012b). Differences in growth profile between cohorts of the same animal models may be due to the variations in the genetic pool of the same species across the time and thus, comparison between studies is challenging. Another possibility is potentially due to the higher degree of animal handling performed in the current study compared to an earlier study (Gallo *et al.*, 2012b). Specifically, in this study, all female rats were handled daily throughout pregnancy for exercise training. Importantly, both Control and growth-restricted females on a HFD also exhibited reduced systolic blood pressure during late gestation (Figure 6.1). Importantly, growth-restricted females body weights did not exceed Control females at all ages suggest that our data were not compromised by changes in maternal weight or obesity.

Consumption of HFD increased body weights in both Control and Restricted at all ages. This is parallel with increased in gestational weight gain, relative total fat weights and plasma leptin concentrations at E20. However, plasma adiponectin concentrations as well as liver and muscle triglycerides at E20 were not altered by HFD perhaps due to the percentage of total fat in high fat pellet diets used in this study was not high enough to elevate the adiponectin and triglyceride concentrations. Although evidence from epidemiological and animal studies demonstrates that intrauterine growth restriction is associated with higher risk of obesity (Ravelli *et al.*, 1999; Ong *et al.*, 2000; Parsons *et al.*, 2001; Howie *et al.*, 2012),

we found no difference in body and relative fat weights between Control and Restricted. The potential mechanisms for the development of obesity in growth-restricted individuals include enhanced appetite mechanisms and/or reduced energy expenditure, which lead to rapid catch up growth postnatally (Desai *et al.*, 2005; Desai & Ross, 2011; Fukami *et al.*, 2012). The absence of postnatal catch up growth in growth-restricted females in the present study possibly due to unaltered food intake and increased energy expenditure in growth-restricted females may explain the similar body weight observed in both Control and Restricted females in response to a HFD.

In the current study, lifestyle intervention of exercise did not promote weight loss in Control and Restricted females on a Chow and HFD diet. While regular exercise is considered a central component of weight loss, growing evidence corroborate that exercise has negligible impact on body weight in females (Donnelly *et al.*, 2003; Nassis *et al.*, 2005; Church *et al.*, 2009). It has been suggested that the most effective way of losing weight is through a caloric restricted diet intervention or a combination of exercise and diet intervention. Indeed, a recent randomised controlled trial reported that obese women who involved in aerobic exercise program had 2.4% reduction in body weights while combination of dietary and exercise interventions lead to 10.8% weight loss in obese women (Foster-Schubert *et al.*, 2012). Although exercise did not promote weight loss, relative total fat weights at E20 were reduced in Control and Restricted females when exercise was initiated before and continued during pregnancy. Similar outcomes were reported in other studies in which exercise training has been demonstrated to improve body composition and lipid profiles in the absence of weight loss (Guo *et al.*, 2011; Kim *et al.*, 2014; Mendelson *et al.*, 2015).

3.5.2 Energy Expenditure and Spontaneous Physical Activity

Obesity is associated with high metabolic rate, both in resting conditions and over 24 hours (Ravussin *et al.*, 1986; Weyer *et al.*, 1999). Indeed, in the present study, Control and Restricted on a HFD had an increased in metabolic rate compared with females on a Chow diet. The increased in metabolic rate in these rats can be contributed by an increased in body weight as heavier individuals tend to have larger fat-free mass (Speakman & Westerterp, 2010). The link between fat-free mass and metabolic rate is well established and is considered as the best predictor of resting energy expenditure (Cunningham, 1991; Nelson *et al.*, 1992). Furthermore, we also demonstrated that HFD reduced respiratory exchange ratio in both Control and Restricted, which indicates a greater proportional contribution of fatty acid metabolism for energy expenditure in these rats. Variability in substrate oxidation may be another mechanism underlying a predisposition to weight gain. Longitudinal studies report that individuals who rely less on lipid as an energy substrate have a greater tendency to gain body weight and body fat relative to those who oxidize lipid more readily (Westerterp, 2009; Astrup, 2011). Thus, the minor

increase in body weight and non-significant increase in triglyceride levels in females on a HFD in this current study perhaps can be explained by an increased in fat oxidation in these rats.

Exercise training may have a major impact on the total energy expenditure and energy balance. Muscle contractions associated with exercise training requires energy to synthesise and hydrolyse adenosine triphosphate (ATP) (Li *et al.*, 2009). As a result, exercise will increase energy expenditure above the basal energy expenditure as observed in this study, in which, both exercise interventions increased metabolic rate in Control and Restricted females irrespective of diet. Exercise also leads to an increased in carbohydrate or fat oxidation by the working muscle. The relative contribution of these substrates as the fuel sources largely depends on the exercise intensity and duration (Romijn *et al.*, 1993; van Loon *et al.*, 2001) and individuals training status (van Loon *et al.*, 1999). In trained individuals, low to moderate intensity exercise increases fat oxidation. The contribution of carbohydrate oxidation to total energy expenditure becomes greater, with muscle glycogen becoming the most important carbohydrate source when the exercise intensity increases (Romijn *et al.*, 1993). However, we found no difference in the substrate oxidation between sedentary rats and rats that underwent endurance exercise training before and during pregnancy as well as during pregnancy only. Non-significant in RER observed in this study maybe due to lack of stability in oxygen and carbon dioxide pool sizes in the body during the course of 24 hours in an open-circuit indirect calorimetry which affect the interpretation of short-term changes in RER (Arch *et al.*, 2006). Importantly, we also demonstrated that spontaneous physical activity was not difference between sedentary and exercise groups suggest that our data were not compromised by the presence of physical activity in sedentary rats.

3.5.2 Effects of High Fat Diet on Metabolic Adaptations to Pregnancy

HbA1c, a measure of glycosylated hemoglobin was not different across the groups during late gestation. In human, HbA1c is considered as the most reliable measure of diabetes outside pregnancy as it reflects the average glucose levels over the previous 2-3 months. However, the usage of HbA1c as a diagnostic tool for diabetes during pregnancy is controversial. This is due to the nature of pregnancy that causes anemia and increases turnover of erythrocytes (Rafat *et al.*, 2012). Since HbA1c levels depend upon the erythrocytes survival time, changes that occur during pregnancy can hamper the accuracy of HbA1c measurements (Jiao *et al.*, 1998). Therefore, it has been suggested that glucose tolerance test is a more accurate tool to detect diabetes during gestation.

In response to IPGTT, growth-restricted females developed glucose intolerance during late gestation in the presence of normal β-cell mass and insulin secretion. This finding was similar to our previous study in which physiological changes in pregnancy affect growth-restricted females metabolic function as they demonstrated first onset of glucose intolerance during late gestation (Gallo *et al.*, 2012b). A novel

finding that emerges from this study was that HFD exacerbated glucose intolerance in growth-restricted females as well as revealed glucose intolerance in females born of normal birth weight during late gestation. It is well established that obesity is often associated with an increased risk of metabolic dysfunction during pregnancy and in later life (Baeten *et al.*, 2001; Sebire *et al.*, 2001). The risk of developing GDM is increased 1.3–3.8 times in obese women compared to women with normal BMI (Kim *et al.*, 2013). In the non-pregnant state, obesity is associated with a chronic, low-grade inflammatory state, termed 'metainflammation', or metabolically induced inflammation (Gregor & Hotamisligil, 2011). Leptin, in particular, is one of the most abundant adipocytokines produced by adipocytes and plays a major role in the chronic pro-inflammatory pathway leading to diabetes. Indeed, epidemiological and animal studies demonstrate that obese humans and rodents develop leptin resistance that may directly contribute to the reduction of lipid oxidation in insulin-sensitive organs, leading to accumulation of lipids and insulin resistance (van den Hoek *et al.*, 2008; Zhang *et al.*, 2010). Thus, increased in plasma leptin concentrations in the present study may have contributed to the development and exacerbation of glucose intolerance in Control and Restricted females on a HFD during late gestation.

Another possible mechanism that is often associated with GDM is inadequate β-cell compensation for insulin resistance that occurs during pregnancy. In a normal pregnancy, adaptations in glucose metabolism and insulin sensitivity ensure a continuous supply of nutrients to developing fetus. Despite increased gluconeogenic activity, glucose is the most abundant substrate able to cross the placenta and is thus largely responsible for the maternal hypoglycemia. In counterbalance to this hypoglycemic state, the placenta secretes hormones that increase maternal insulin resistance and hepatic glucose production, thus raising glucose levels in the maternal circulation (Herrera, 2000). Maternal pancreatic β-cell mass adapts to this changing insulin demands in the body through β-cell proliferation, hypertrophy and neogenesis (Rieck & Kaestner, 2010). In our previous study, we demonstrated that growth-restricted females had β-cell deficits with no apparent metabolic dysfunction at 4 months of age (Gallo *et al.*, 2012b). Adverse pregnancy adaptations in growth-restricted females lead to glucose intolerance during late gestation despite normal β-cell mass (Gallo *et al.*, 2012b). It has been proposed that growth-restricted females may adapt to adverse pregnancy condition by increasing β-cell proliferation but the compensation was not sufficient to protect them from adverse metabolic control during pregnancy. Similarly, in the present study, HFD revealed and exacerbated glucose intolerance in Control and Restricted females respectively in the presence of normal β-cell mass during late gestation. It is possible that similar mechanisms may have happened in these rats, thus future study should investigate the effects of HFD on β-cell mass and function in Control and Restricted females before pregnancy. Furthermore, in rodents, β-cell mass increases by more than 2-fold during mid-pregnancy and declines from late gestation to one week post-partum so that β-cell mass returns to pre-pregnant levels by term (Rieck & Kaestner, 2010; Xue *et al.*, 2010). The lack of difference observed between Control and

Restricted as well as Chow and HFD females β-cell mass, may also reflect a delayed restoration to pre-pregnant values in Restricted females or Control and Restricted females on a HFD. Hence, assessment of pancreatic islet and β-cell morphology after pregnancy, at a later stage in life, would also be a focus of future work. This would enable us to determine whether any alterations that may have occurred during pregnancy would be evident in the long term. Furthermore, pancreatic analysis such as quantifying β-cell mass or proliferation rate during mid pregnancy, at the peak of pancreatic morphological changes, may identify a period more susceptible to the challenge of pregnancy and may explain the loss of glucose tolerance in females born small and exposed to HFD.

3.5.3 Effects of Exercise on Metabolic Adaptations to Pregnancy

Exercise training initiated 4 weeks before and continued during pregnancy prevented the development of glucose intolerance in growth-restricted females by increasing glucose-stimulated insulin secretion. During pregnancy, maternal insulin demands increase due to the insulin resistance state. Maternal islets adapt to this increased demand through enhanced insulin secretion per β-cell (Retnakaran *et al.*, 2008). Exercise performed before and during pregnancy in this study may further enhance the insulin secretion in growth-restricted females and thus protect them from developing adverse metabolic function during pregnancy.

Another important finding from this study was exercise training initiated before and continued during pregnancy prevented the development and exacerbation of glucose intolerance in Control and Restricted females on a HFD respectively. The prevention of glucose intolerance during late gestation in these rats was mainly contributed by increased in β-cell mass. β-cell mass is tightly regulated through a balance of β-cell birth through β-cell proliferation and islet neogenesis from precursor cells and β-cell death through apoptosis (Taylor, 1999). Evidence from epidemiological and animal studies demonstrated that exercise has beneficial effects on both of these mechanisms (Narendran *et al.*, 2015). Physical activity increases circulating levels of growth hormone, insulin-like growth factor 1, glucagon-like peptide 1, interleukin-6 and interukin-1 receptor agonist, all of which are thought to have a direct and indirect effects on β-cell proliferation (Ronsen *et al.*, 2002; Choi *et al.*, 2006; Park *et al.*, 2007; Narendran *et al.*, 2015). Furthermore, exercise reduces β-cell death through reduction in plasma glucose and serum lipids (Solomon *et al.*, 2009; Karstoft *et al.*, 2013) as chronic exposure of pancreas islets to hyperglycemia induces inflammation, which impairs glucose-stimulated insulin secretion and augments β-cell apoptosis (Donath *et al.*, 1999). Thus, exercise performed before and during pregnancy in this study may augment pancreatic β-cell mass in Control and Restricted on a HFD through an increased in β-cell proliferation and decreased in β-cell apoptosis.

Of particular importance, we also demonstrated that exercise initiated during second week of pregnancy had no effects on the adverse metabolic phenotypes in both Control and Restricted females. Similar outcomes were reported in human studies in which beneficial effects of exercise on GDM prevention are limited to women who engaged in exercise prior to and/or during early pregnancy than those who started exercise during the second and third trimesters (Dempsey, 2004; Oken *et al.*, 2006; Zhang *et al.*, 2006; Redden *et al.*, 2011; Stafne *et al.*, 2012; Nobles *et al.*, 2015). These findings suggest that the beneficial effects of exercise on the metabolic control is more apparent when performed prior to pregnancy. In our previous study we demonstrated that four weeks of exercise training early in life restores deficits in pancreatic β-cell mass associated with growth restriction in adult male rats (Laker *et al.*, 2011). Therefore, for future studies, it will be important to determine when exercise training programs the increase in pancreatic β-cell mass by investigating the effects of exercise performs prior to pregnancy only.

3.5.4 Conclusions

Uteroplacental insufficiency did not alter pancreatic β-cell mass during late gestation. Nevertheless, despite having normal pancreatic β-cell mass, growth restricted females demonstrated an increased in plasma glucose in response to IPGTT indicates loss of glucose tolerance compared with Control. When challenged with HFD, glucose intolerance in growth-restricted was exacerbated during late gestation. We also found that HFD revealed glucose intolerance in females born of normal birth weight. The exacerbation and development of glucose intolerance in Restricted and Control females respectively, were presence despite having normal pancreatic β-cell mass and insulin secretion during late gestation. Importantly, exercise initiated prior to and continued during pregnancy prevented the development of glucose intolerance in growth-restricted females on a Chow diet by increasing glucose-stimulated insulin secretion (Figure 3.20). Additionally, the development and exacerbation of glucose intolerance in Control and Restricted females respectively were also prevented when these females exercised before and during pregnancy through increased in pancreatic β-cell mass. However, metabolic dysfunction in Control and Restricted females during late gestation was not improved by exercise initiated during pregnancy (Figure 3.20). These findings suggest that the exercise may has important role in improving pancreatic adaptations during pregnancy which prevent metabolic dysfunction in these females. Thus, it will be important to further investigate the mechanisms underlying the effects of exercise on pancreatic β-cell mass.

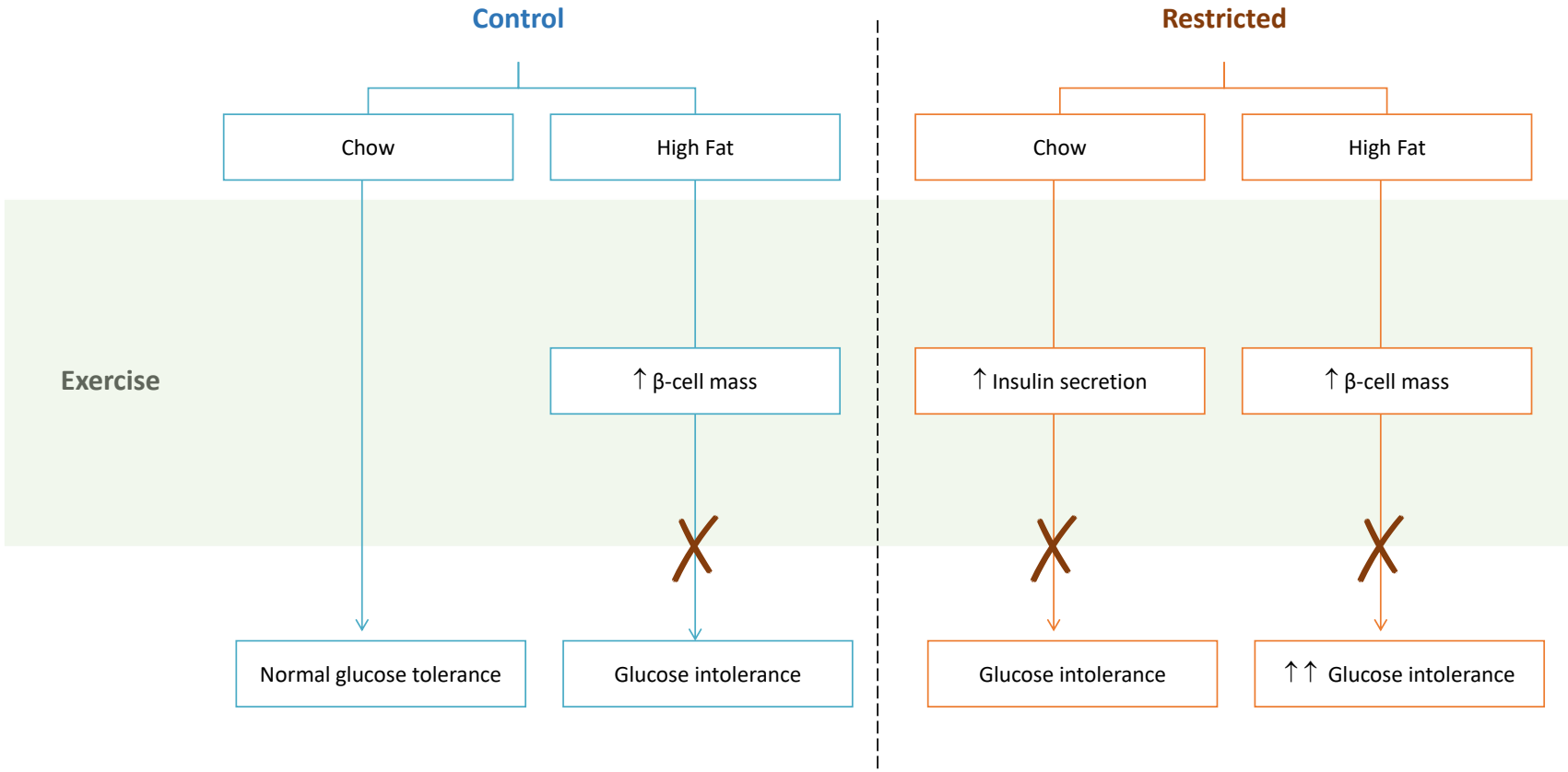


Figure 3.20 Effects of high fat diet and exercise on metabolic adaptations during later pregnancy

Uteroplacental insufficiency leads to loss of glucose tolerance during late gestation. HFD revealed and exacerbated glucose intolerance in Control and Restricted females during late gestation. Exercise before and during pregnancy prevented glucose intolerance in growth-restricted females on a Chow diet by increasing insulin secretion. Exercise before and during pregnancy also prevented the development and exacerbation of glucose intolerance in Control and Restricted by increasing pancreatic β-cell mass.

Although we have clearly shown that exercise before and during pregnancy improved pancreatic adaptations, what remains to be elucidated is when and how these adaptations occurred. It will be important in future studies to identify the mechanisms responsible for the apparent beneficial adaptations in the pancreas (*i.e* restored pancreatic β-cell mass) by investigating pancreatic morphology and function at different time points during and before gestation. Future studies should also consider generating an additional cohort for islet isolation experiments to determine whether *Pdx-1* expression, which is important for maintenance of β-cell mass and function, was improved by exercise interventions. Thus, understanding the mechanisms when pancreatic changes occur may allow targeted therapeutic strategies for pregnancies that are complicated by obesity and intrauterine growth restriction. One of the weaknesses of this study was, we were not able to conduct a fasted IPGTT, thus insulin resistance in these females was not characterized. This is because we were interested in fetal outcomes from this study and fasting will compromise the fetal development. Another consideration for future studies is to generate another cohort of pregnant rats to determine whether insulin resistance accompanies the development and exacerbation of glucose intolerance in Control and Restricted females.

Overall, HFD revealed and exacerbated glucose intolerance in pregnant females born of normal birth weight and born small respectively. These were prevented by the lifestyle intervention of exercise, potentially due to improved β-cell mass. This study highlights that modifiable risk factors such as diet and exercise can have beneficial effects in the mother during pregnancy particularly for females born small.

CHAPTER 4

Effects of Exercise and High Fat Diet on Cardiorenal Adaptations to Pregnancy in Females Born Small

4.1 Introduction

The developmental origins of health and disease hypothesis proposes that perturbations during critical prenatal and early postnatal periods program the developing fetus for adverse cardiovascular and metabolic outcomes in adulthood (Barker, 1995; McMillen & Robinson, 2005). Adverse *in utero* environment restricts fetal growth alters the development of key organs such as nephron (Wlodek *et al.*, 2007; Wlodek *et al.*, 2008). Uteroplacental insufficiency is the most common cause of intrauterine growth restriction in the Western world, affecting ~ 8 % of pregnancies. (Henriksen & Clausen, 2002) It is characterised by poor placental vascularisation leading to compromised delivery of nutrients and oxygen, and becomes most apparent during the third trimester when fetal demands are at their greatest (Wigglesworth, 1964).

Our laboratory has utilised a rat model that mimics this condition, whereby the uterine vessels are bilaterally ligated during late gestation resulting in offspring that are born lighter than those exposed to a sham surgery (Wlodek *et al.*, 2007; Wlodek *et al.*, 2008). Our model of uteroplacental insufficiency is associated with a sexually dimorphic phenotype, whereby male offspring generally present with more severe disease outcomes. The sexes were similarly growth restricted, as was the magnitude of nephron deficit, but only males had increased in blood pressure (Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wadley *et al.*, 2008; Wlodek *et al.*, 2008; Moritz *et al.*, 2009b). Importantly, however, Restricted female offspring exhibited uterine artery endothelial dysfunction and increased wall stiffness (Mazzuca *et al.*, 2010), which may, in turn, compromise their own pregnancy adaptations.

Pregnancy is an intricate physiological state with profound cardiovascular and renal adaptations essential to support growth and development of the fetus. By late pregnancy, maternal blood volume expands by up to 50% in humans and 30% in rats, with similar increases in cardiac output (Torgersen & Curran, 2006; Hill & Pickinpaugh, 2008). The hypervolemic state is secondary to reductions in peripheral vascular tone and together these factors allow for increased uteroplacental blood flow while maintaining maternal blood pressure (Torgersen & Curran, 2006). In order to support the cardiovascular changes and maintain fluid homeostasis, renal processes must adapt accordingly (Torgersen & Curran,

2006). Dilation of the renal vasculature permits greater blood flow to the maternal kidneys, with GFR reaching peak levels at mid gestation. In turn, increased solute filtration reduces plasma osmolality and viscosity, considered to aid uteroplacental perfusion. During the last week of normal rat pregnancy, active electrolyte reabsorption by renal tubules increases water retention to assist with plasma volume expansion (Atherton *et al.*, 1982; Yeomans & Gilstrap, 2005; Torgersen & Curran, 2006). Of interest to the current study, small birth weight women, compared with those born of normal weight, were more likely to develop hypertension during late pregnancy (Klebanoff *et al.*, 1999), likely attributed to alterations in maternal vascular remodeling and/or deficits in nephron endowment. However, our previous study demonstrated no difference in blood pressure during late gestation in growth-restricted female rats despite having low nephron number (Gallo *et al.*, 2012b). It has been suggested that a second-hit such as high fat diet may unmask or exacerbate disease phenotypes associated with poor conditions in utero in growth-restricted females. Indeed, in an obese mother these pregnancy adaptations become adverse, which predispose them to a number of complications (Mahizir *et al.*, 2016). Given that growth restricted females are at higher risk of cardiorenal disease during their pregnancy, it is likely that maternal obesity may exacerbate the phenotype.

There has been much interest in the development of lifestyle interventions targeting overweight and obese pregnant women. However, there are lack of consistent evidence regarding the benefits of exercise during pregnancy in obese or overweight women suggests that interventions during pregnancy alone may not be enough to ameliorate the adverse effect obesity has on the mother (Artal *et al.*, 2007; Dodd *et al.*, 2010; Garnaes *et al.*, 2016). Indeed, human and animal studies demonstrated that the risks of having adverse pregnancy adaptations in overweight or obese females were reduced significantly if exercise training was performed before and during pregnancy (Dempsey, 2004; Vega *et al.*, 2015). Thus, the development of targeted interventions in growth-restricted females may prevent them from developing cardiovascular and renal dysfunction during pregnancy.

4.2 Aims and Hypotheses

The overall aim of this study was to determine if a high fat diet exacerbates the known adverse cardiorenal adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these altered adaptations.

Specific aims of Chapter 4 were:

- ***i.*** To determine if a high fat diet exacerbates renal dysfunction and unmasks high blood pressure in late gestation in growth restricted females;
- ***ii.*** To determine whether exercise before and during pregnancy is more beneficial in preventing renal dysfunction and prevents the emergence of high fat diet induced high blood pressure in late gestation in growth restricted females than exercise during pregnancy alone.

It is hypothesized that growth restricted females on a high fat diet will develop high blood pressure and renal dysfunction in late gestation, and exercise before and during pregnancy will be more beneficial in preventing the adverse pregnancy outcomes than exercise during pregnancy alone.

4.3 Materials and Methods

Experimental design was described in Chapter 2.1. Only the specific endpoints of this chapter are briefly described in this section.

4.3.1 Systolic Blood Pressure and Renal Excretion

Systolic blood pressure was measured by tail-cuff plethysmography at E18 in pregnant rats that were acclimatised to the restraint procedure. Rats were placed in metabolic cages at E12 and E19 for determination of 24 hours food and water intake, and renal excretions, after training. Measurements of urinary sodium, creatinine, total protein, potassium, and albumin were measured using a COBAS Integra 400, according to the manufacturer's instructions. Plasma samples were collected at post mortem from pregnant rats to calculate creatinine clearance.

4.3.2 Post Mortem Tissue Collection

At E20, rats were anaesthetised with intraperitoneal injection of ketamine (100 $mg.kg^{-1}$) and Illium Xylazil-20 (30 $mg.kg^{-1}$) and a cardiac puncture was performed to collect blood. Heart and kidneys were excised and weighed.

4.3.3 Statictical Analyses

All data were analysed using a three-way ANOVA to determine main effects of uteroplacental insufficiency (Control and Restricted), diet (Chow and High fat) and exercise (Sedentary, Exercise and ExPreg). If significant interactions were detected, Student's unpaired *t*-test or a one-way ANOVA was performed with Student-Newman-Kewls post hoc test where appropriate. All data are presented as mean ± SEM with n representing the number of animals per litter from each group. $P<0.05$ was considered statistically significant.

4.4 Results

4.4.1 Heart and Kidney Weights

4.4.1.1 Group Effect

In no physiology measures groups, relative heart (-3-8%, $P<0.05$, Figure 4.1 A) and left ventricular weights (-4-8%, $P<0.05$, Figure 4.2 A) were lower in Restricted Sedentary females irrespective of diet. In physiology measures groups, relative heart ($P<0.05$, Figure 4.1 B), left ventricular ($P<0.05$, Figure 4.2 B) and kidney weights ($P<0.05$, Figure 4.3 B) were lower in Restricted Sedentary females irrespective of diet.

4.4.1.2 Diet Effect

High fat diet had no effect on relative heart and left ventricular weights (Figures 4.1 and 4.2). In no physiology measures groups, relative kidney weight was lower in Control and Restricted females on a HFD irrespective of exercise interventions ($P<0.05$, Figure 4.3).

4.4.1.3 Exercise Effect

In no physiology measures groups, Control and Restricted females that exercised before and during pregnancy had an increase in relative heart (+4-8%), left ventricular (+7-12%) and kidney (+8-9%) weights irrespective of diet ($P<0.05$, Figures 4.1 A, 4.2 A and 4.3 A). In physiology measures groups, exercise before and during pregnancy increased relative heart (+5-10%) weight in Control and Restricted females irrespective of diet ($P<0.05$, Figure 4.1 B). Relative kidney weight was increased in High fat fed-Control and Restricted females that exercised before and during pregnancy (+6%, $P<0.05$, Figure 4.3 B). Control and Restricted females relative heart, left ventricular and kidney weights were not affected by exercise initiated during pregnancy regardless of diet and physiology measures (Figures 4.1, 4.2 and 4.3).

4.4.1.4 Physiology Measures Effect

Control and Restricted females relative heart, left ventricular and kidney weights were not affected by physiological measurements conducted during pregnancy (Figures 4.1, 4.2 and 4.3).

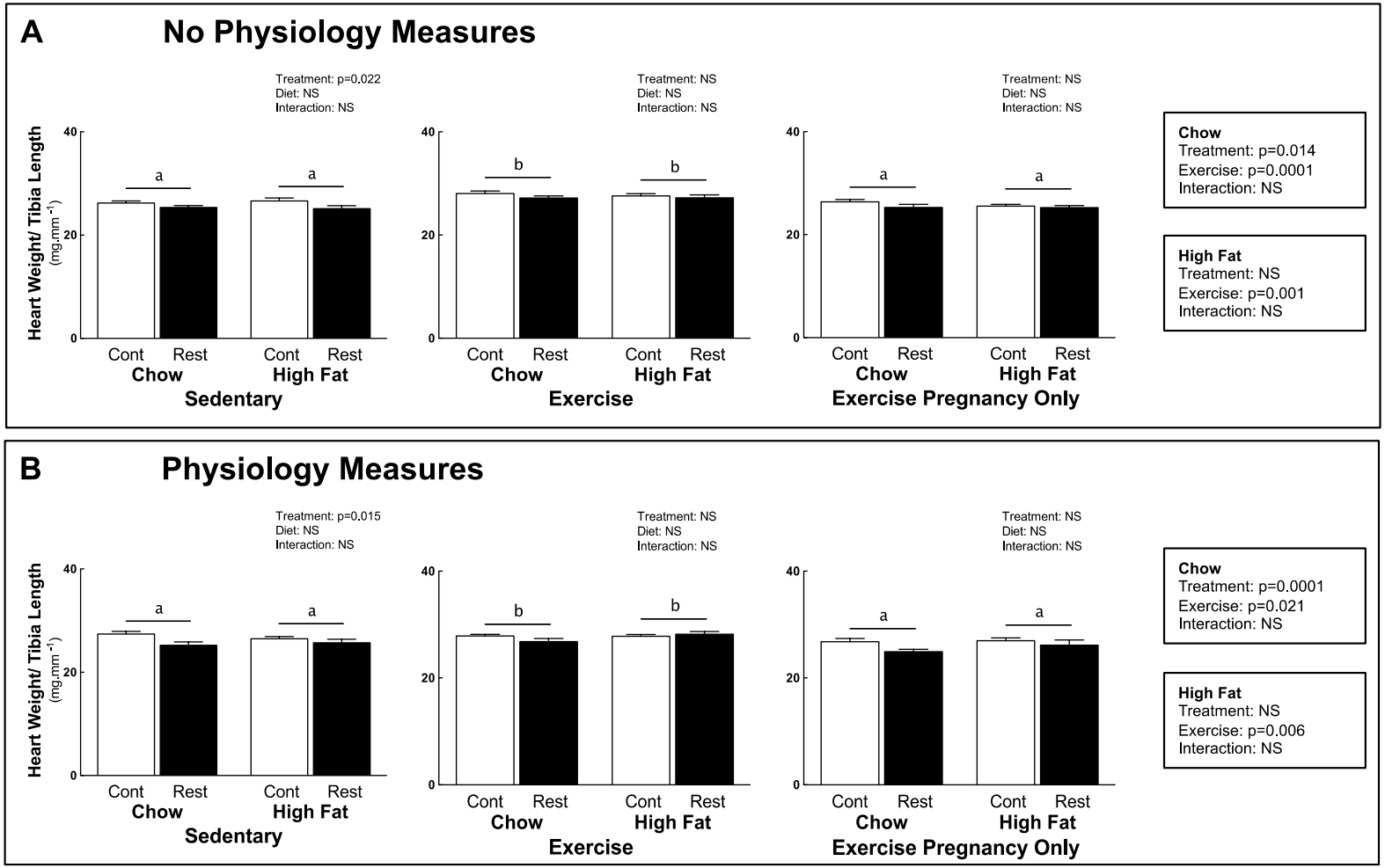


Figure 4.1 Heart weight at *post mortem* (E20)

Control (white bars) and Restricted (black bars) heart weight at *post mortem* (E20). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' ($P<0.05$).

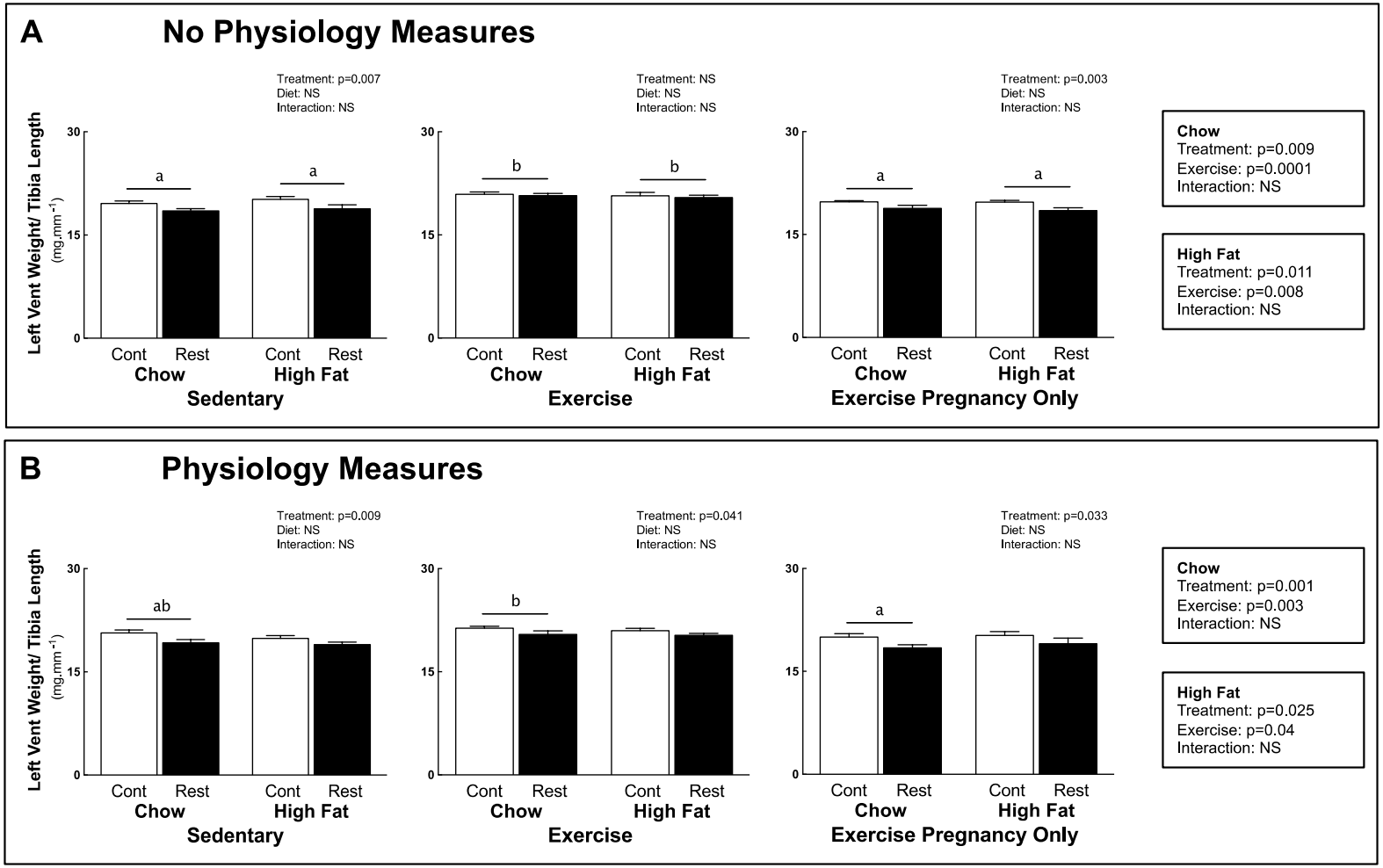


Figure 4.2 Left ventricular weight at *post mortem* (E20)

Control (white bars) and Restricted (black bars) left ventricular weight at *post mortem* (E20). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' but not different to 'ab' (P<0.05).

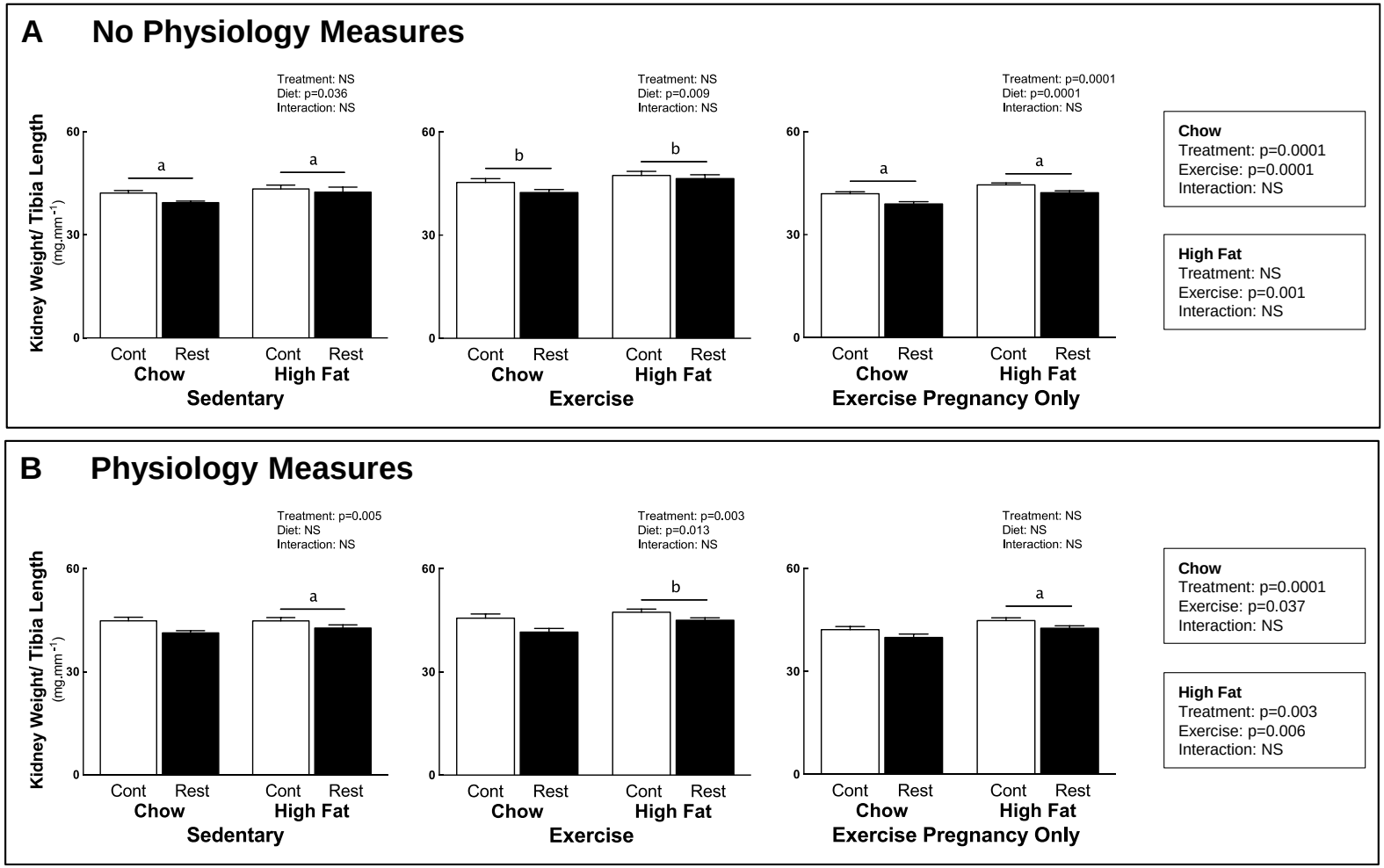


Figure 4.3 Kidney weight at *post mortem* (E20)

Control (white bars) and Restricted (black bars) kidney weight at *post mortem* (E20). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. (A) No physiological measures and (B) physiological measures cohorts. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' (P<0.05).

4.3.2 Systolic Blood Pressure

Systolic blood pressure at 19 weeks of age prior to pregnancy was not affected by intrauterine growth restriction, high fat diet nor exercise interventions (Figure 4.4 A). At E19, systolic blood pressure was reduced in Restricted Sedentary on a Chow diet compared with Control females (-10 mmHg, $P<0.05$, Figure 4.4 B). Exercise before and during pregnancy but not exercise initiated during pregnancy increased systolic blood pressure in Chow-fed Restricted females at E19 compared with Sedentary females ($P<0.05$, Figure 4.4 B).

Compared with pre-pregnancy, there was a greater reduction in systolic blood pressure during late gestation in Chow-fed Restricted females and both Control and Restricted females on a HFD (-17-22 mmHg, $P<0.05$, Figure 4.5). Exercise before and during pregnancy, but not exercise initiated during pregnancy, prevented the reduction of systolic blood pressure during late gestation in Chow-fed Restricted females and both Control and Restricted females on a HFD (Figure 4.5)

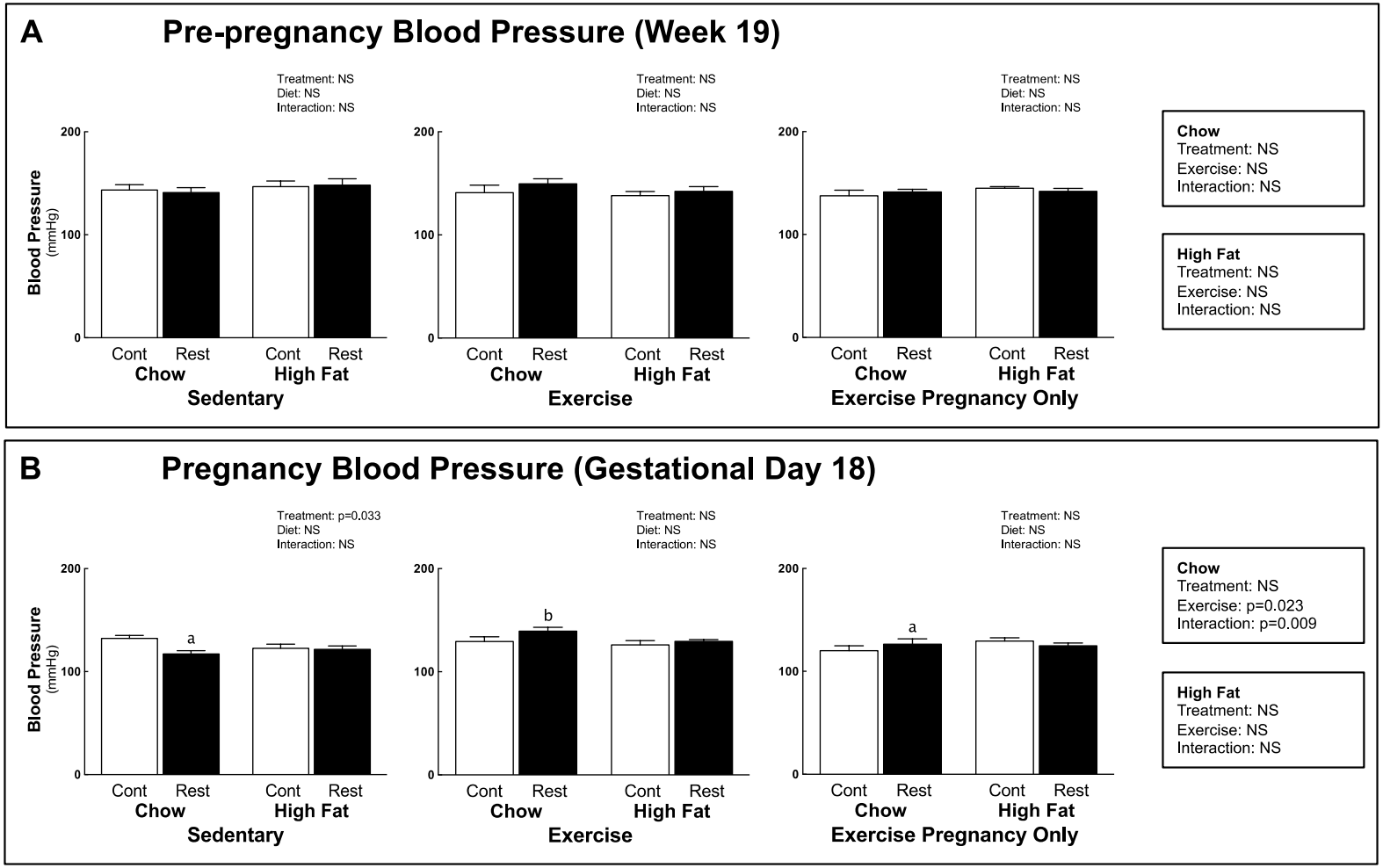


Figure 4.4 Systolic blood pressure

Control (white bars) and Restricted (black bars) systolic blood pressure at (A) 19 weeks (before pregnancy) and (B) E19 (during pregnancy). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' (P<0.05).

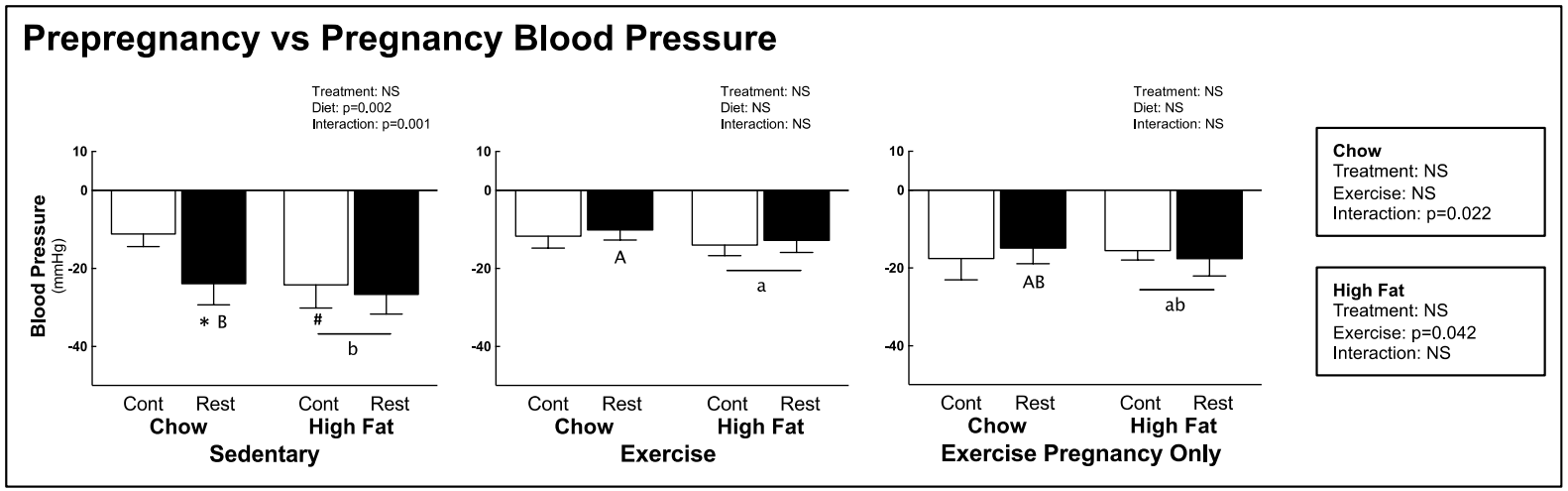


Figure 4.5 Difference in systolic blood pressure between 19 weeks (before pregnancy) and E19 (during pregnancy)

Control (white bars) and Restricted (black bars) difference in systolic blood pressure between 19 weeks (before pregnancy) and E19 (during pregnancy). Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. **P*<0.05 vs Control; #*P*<0.05 vs Chow; 'a/A' is different to 'b/B' but not different to 'ab/AB' (*P*<0.05).

4.3.3 Water Intake and Renal Excretion

At E12, water intake, urine flow rate, urinary sodium, potassium and albumin excretions and creatinine clearance were not affected by intrauterine growth restriction and high fat diet (Figure 4.6 A - 4.12 A). Urinary sodium and potassium excretions at E12 were increased in females that exercise before and during pregnancy (Figures 4.11 A and 4.12 A). Urinary total protein excretion was reduced in growth-restricted mothers on a Chow diet irrespective of exercise interventions (Figure 4.9 A).

At E20, consumption of HFD lead to a reduction in water intake (Figure 4.6 B) and urine flow rate (Figure 4.7 B) in Control and Restricted females that were Sedentary (-14-23%, $P<0.05$). Urinary albumin excretion was also reduced in Sedentary Control and Restricted females on a HFD compared with females on a Chow diet (-36-42%, $P<0.05$, Figure 4.8 B). Urinary total protein excretion at E20 was reduced in growth-restricted mothers on a Chow diet irrespective of exercise interventions (Figure 4.9 B). Both exercise interventions had no effects on urinary total protein excretion at E20 in all groups (Figure 4.9 B).

Creatinine clearance at E20 was increased in Restricted females on a Chow diet compared with Control females (+56%, $P<0.05$, Figure 4.10 B). Exercise initiated during pregnancy, but not exercise before and during pregnancy, increased creatinine clearance in Chow-fed Restricted females at E20 ($P<0.05$, Figure 4.10 B). HFD increased creatinine clearance in Control but not Restricted females that were Sedentary compared with females on a Chow diet (+44%, $P<0.05$, Figure 4.10 B).

Urinary sodium excretion at E20 was not affected by intrauterine growth restriction and high fat diet (Figure 4.11 B). Both Control and Restricted females that exercised before and during pregnancy, but not exercise before and during pregnancy, had an increased in urinary sodium excretion at E20 ($P<0.05$, Figure 4.11 B). Urinary potassium excretion at E20 was increased in Restricted females on a Chow diet compared with Control females (+35%, $P<0.05$, Figure 4.12 B). HFD increased urinary potassium excretion in Control but not Restricted females that were Sedentary compared with females on a Chow diet (+44%, $P<0.05$, Figure 4.12 B). Exercise initiated before and continued during pregnancy, but not exercise before and during pregnancy, increased urinary potassium excretion in both Control and Restricted females irrespective of diet compared with Sedentary females ($P<0.05$, Figure 4.12 B).

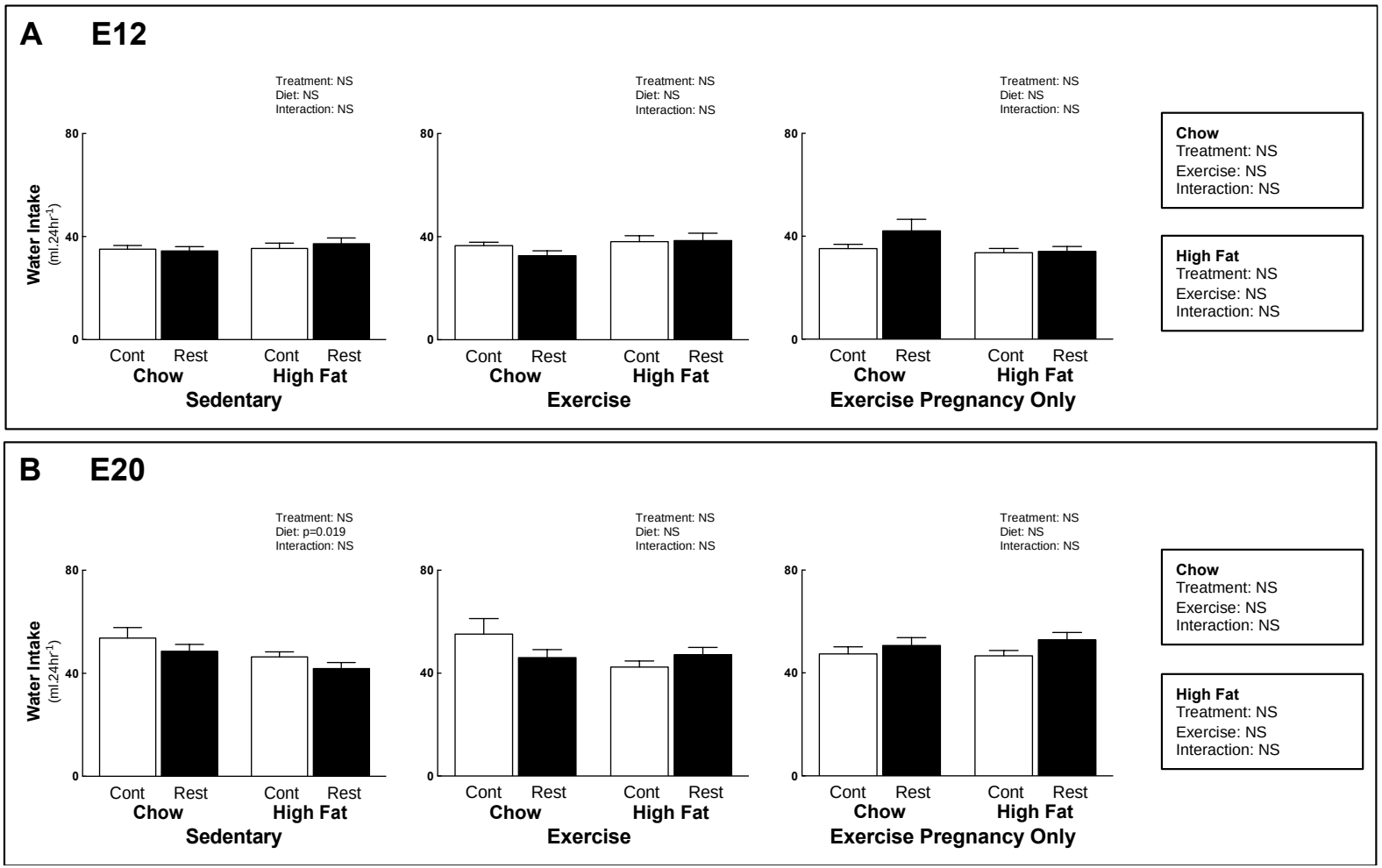


Figure 4.6 Water intake at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours water intake at (A) E12-13 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

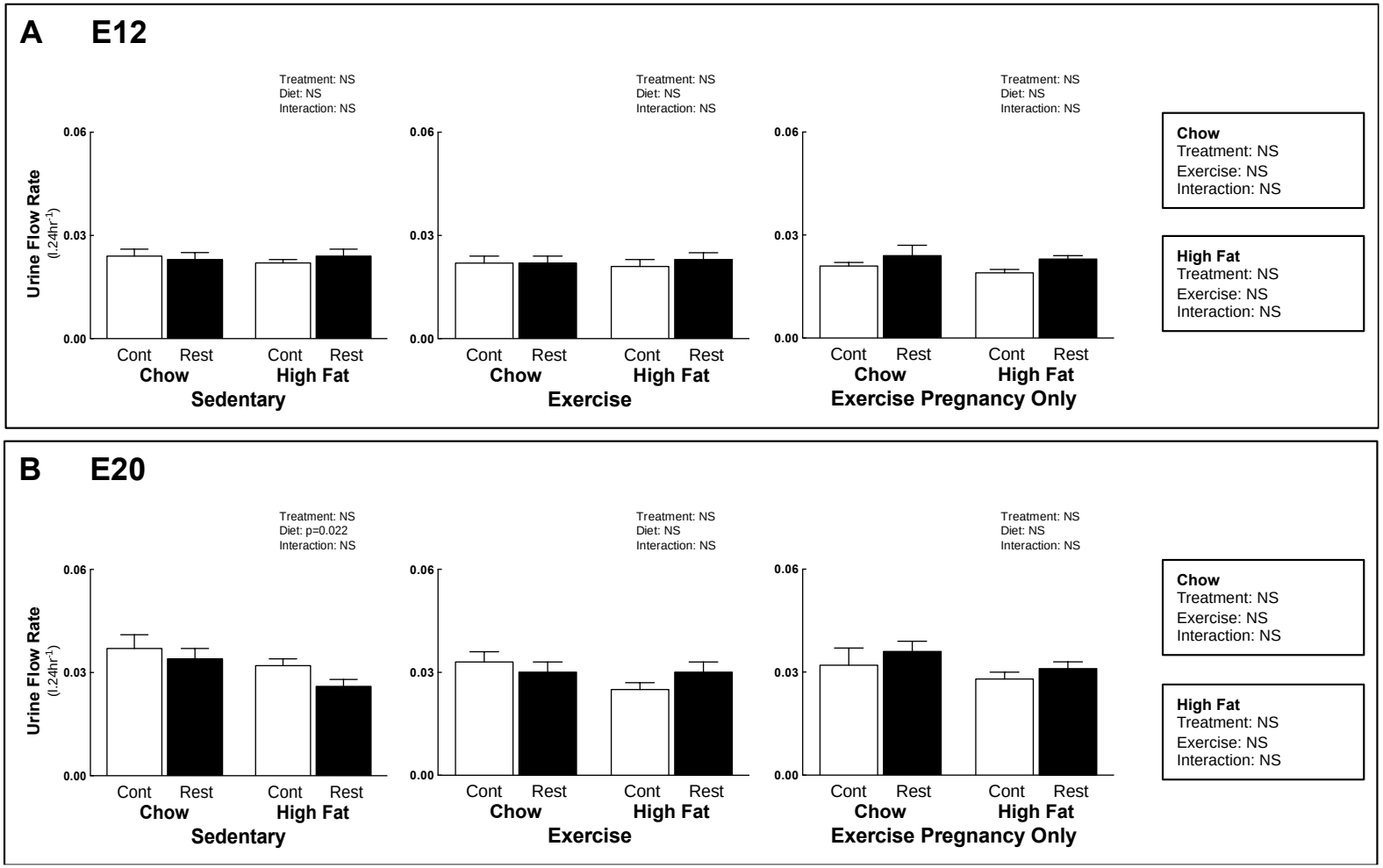


Figure 4.7 Urine Flow Rate at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours urine flow rate at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

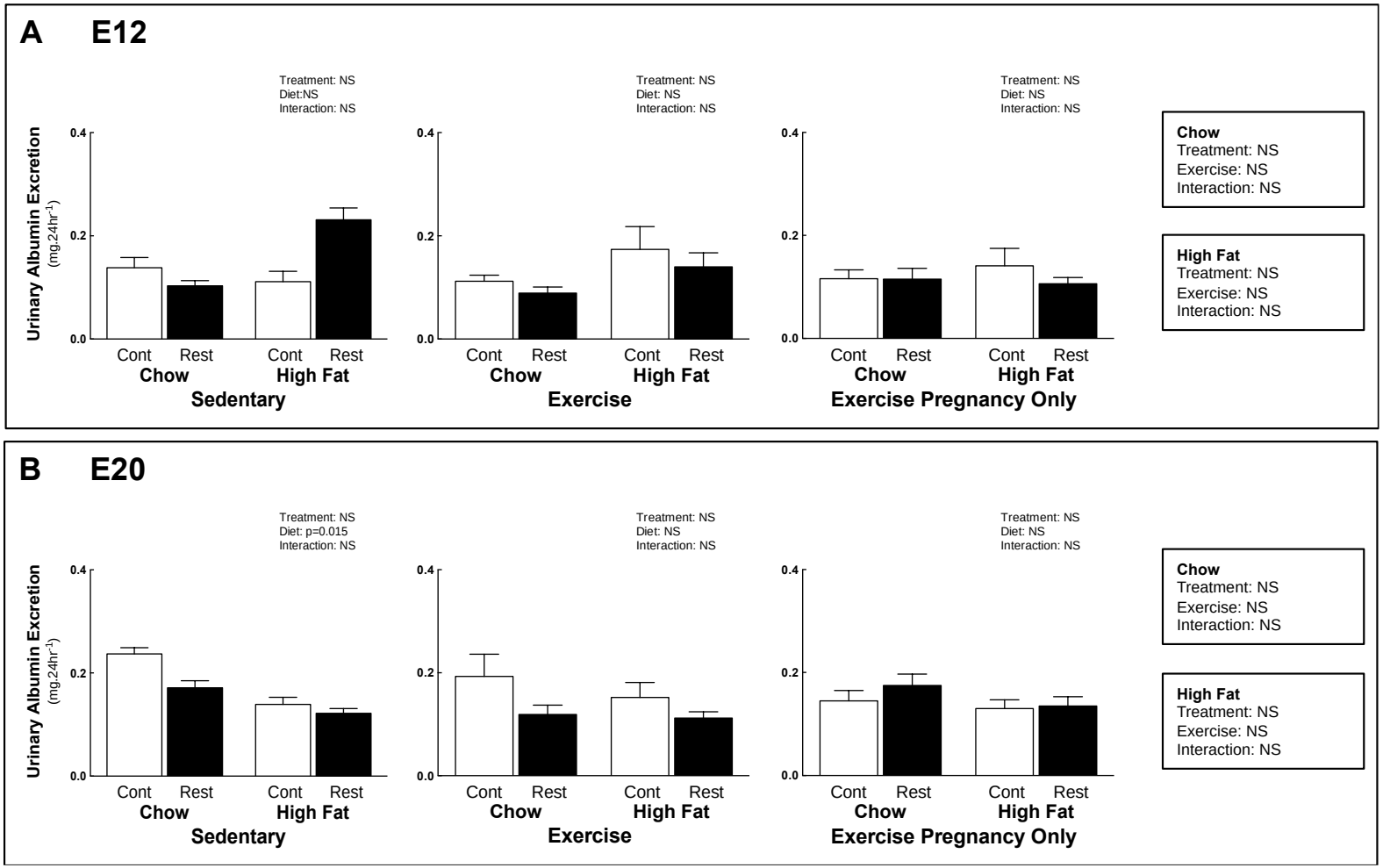


Figure 4.8 Urinary albumin excretion at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours urinary albumin excretion at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

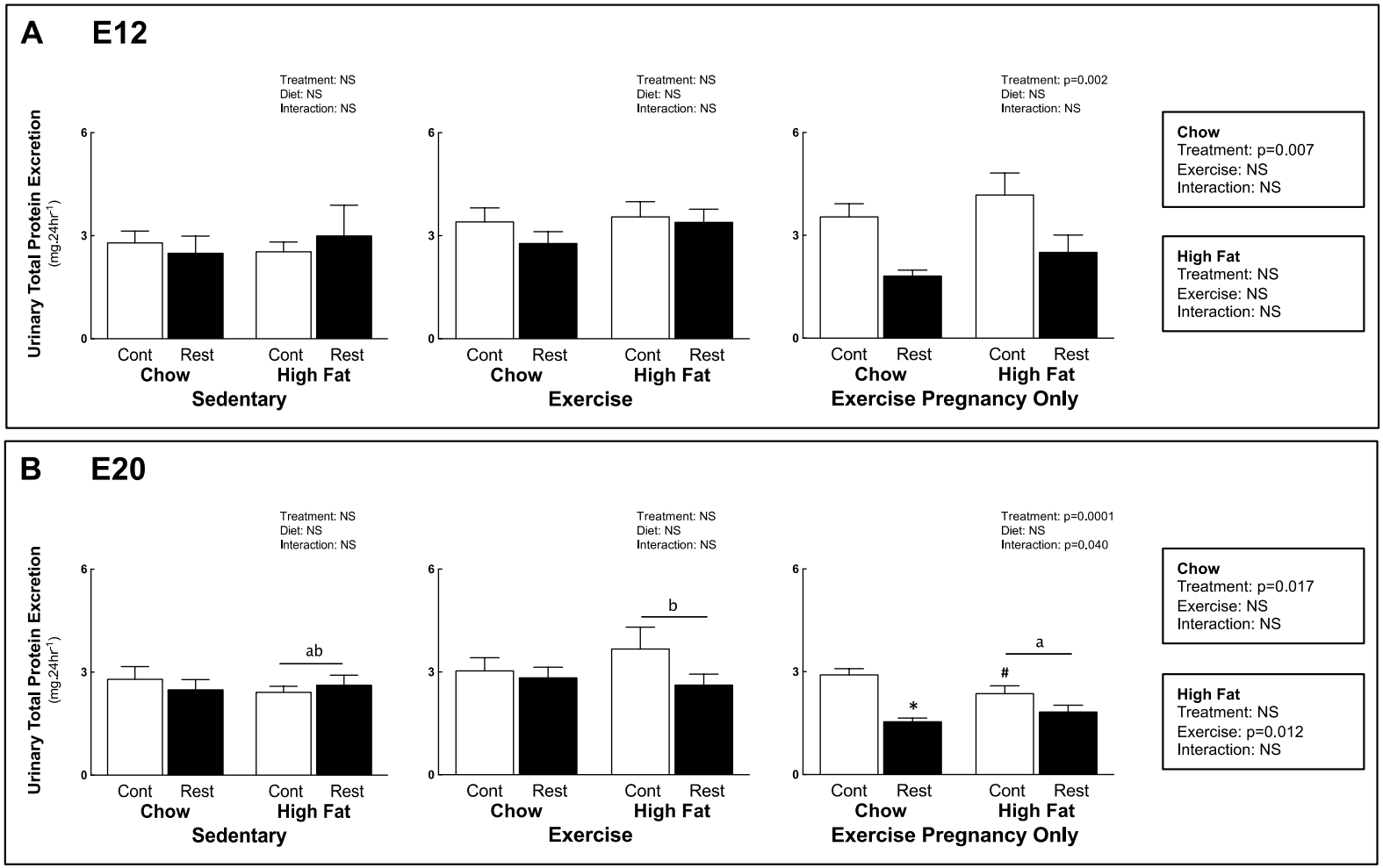


Figure 4.9 Urinary total protein excretion at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours urinary total protein excretion at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. **P*<0.05 vs Control; #*P*<0.05 vs Chow; 'a' is different to 'b' but not different to 'ab' (*P*<0.05).

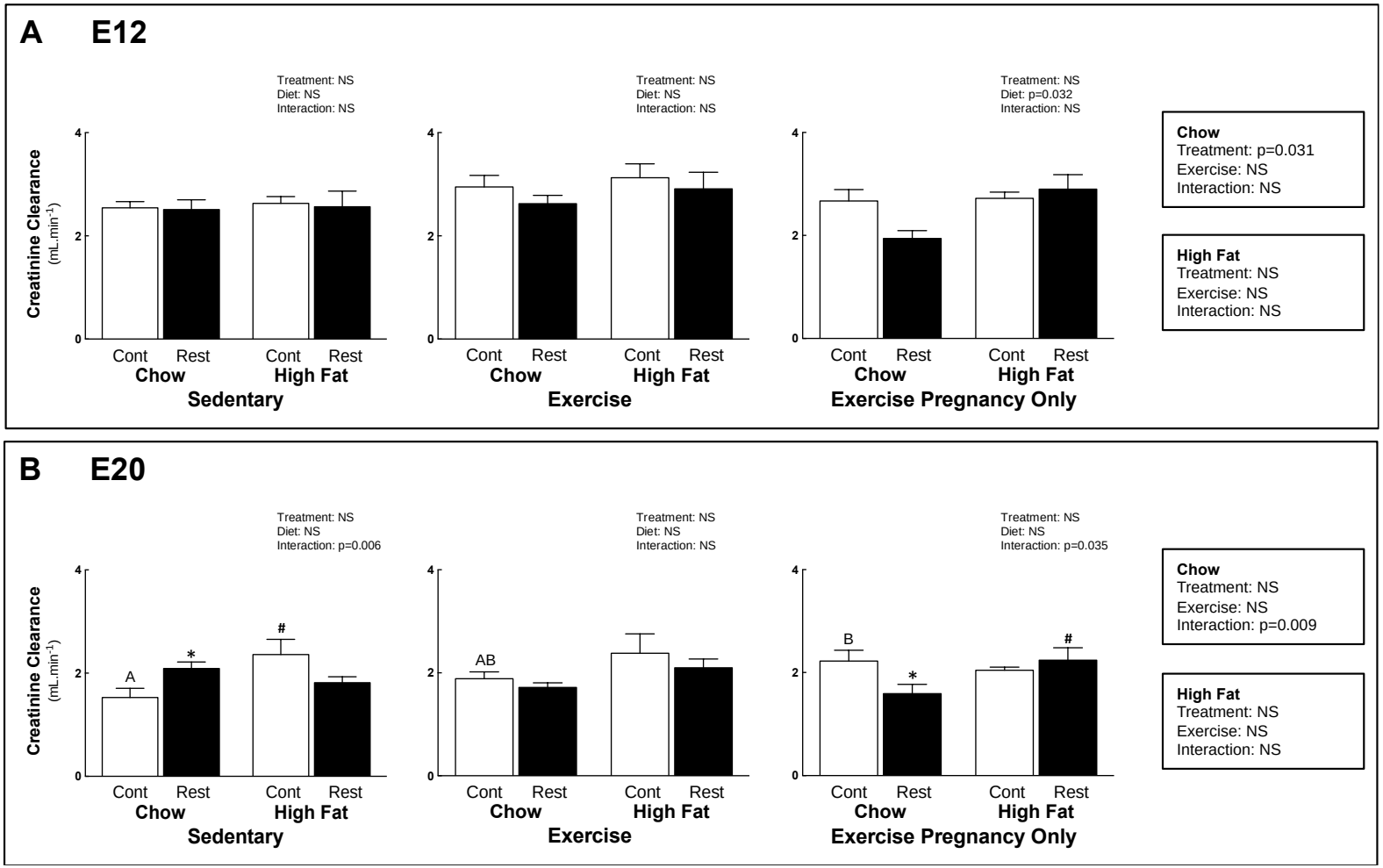


Figure 4.10 Creatinine clearance at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours creatinine clerance at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. **P*<0.05 vs Control; #*P*<0.05 vs Chow; 'A' is different to 'B' but not different to 'AB' (*P*<0.05).

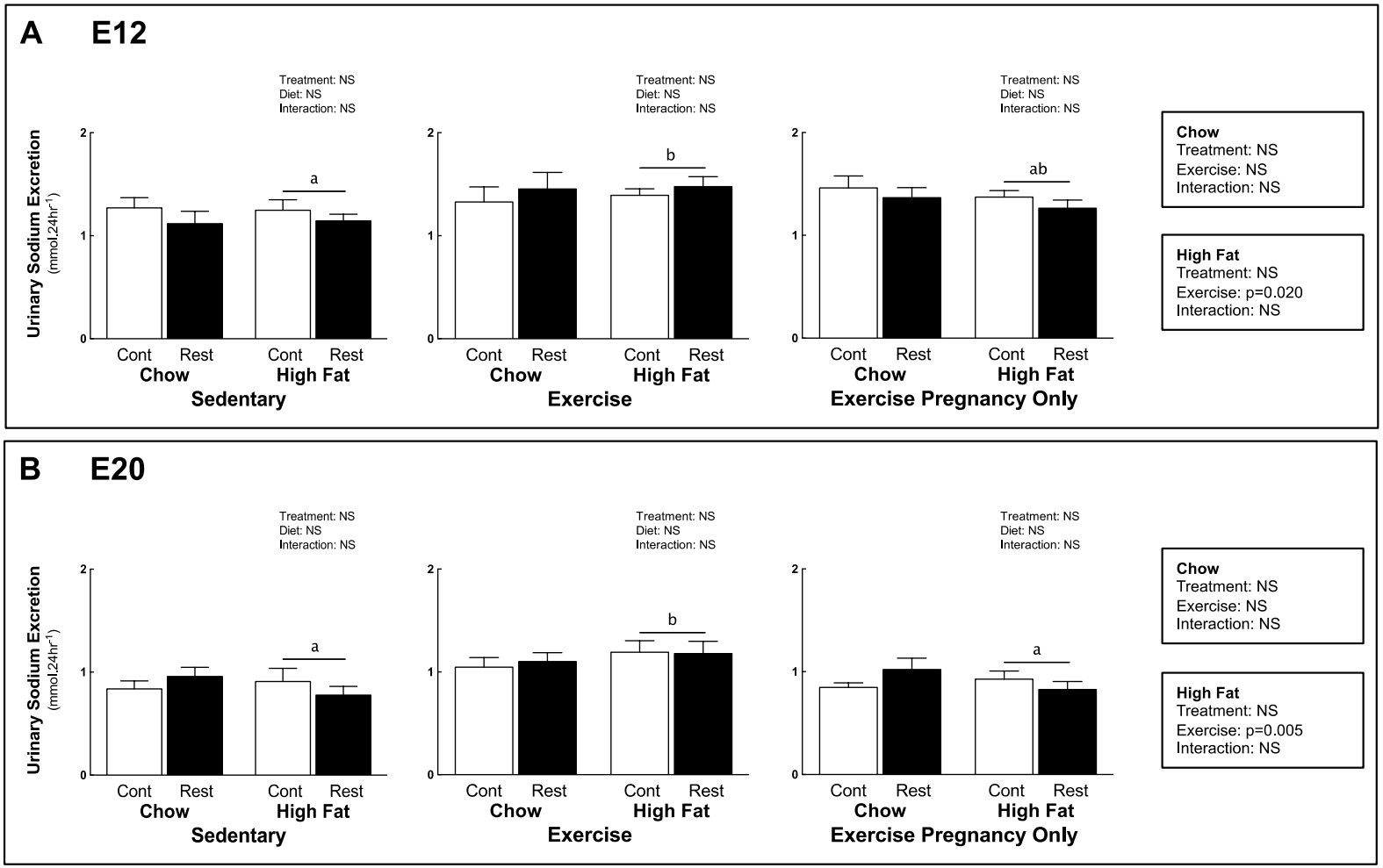


Figure 4.11 Urinary sodium excretion at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours urinary sodium excretion at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. 'a' is different to 'b' but not different to 'ab' (P<0.05).

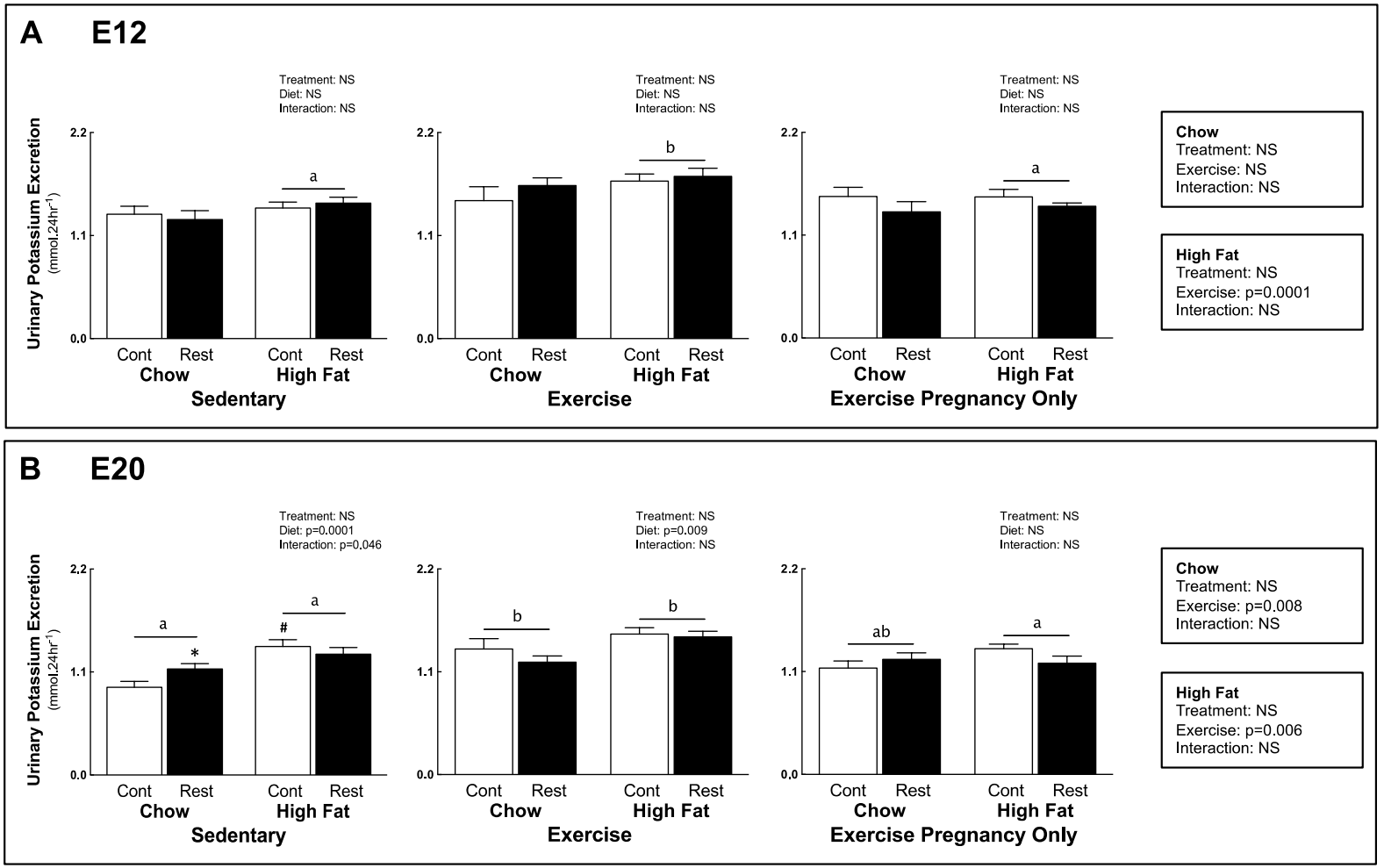


Figure 4.12 Urinary potassium excretion at E11-12 and E19-20

Control (white bars) and Restricted (black bars) 24 hours urinary potassium excretion at (A) E11-12 (B) E19-20. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. *P<0.05 vs Control; #P<0.05 vs Chow; 'a' is different to 'b' but not different to 'ab' (P<0.05).

4.4 Discussion

This study determined the impact of intrauterine growth restriction and high fat diet on the cardiorenal adaptations of pregnancy. Growth-restricted females, induced by uteroplacental insufficiency, and High fat-fed Control females presented with altered renal function during late gestation. The present study also demonstrated that growth-restricted Chow-fed rats, and both Control and growth-restricted females on a HFD had adverse blood pressure adaptations to pregnancy. Importantly, both blood pressure and renal function were affected by exercise prior to and during pregnancy, but not exercise initiated during pregnancy.

4.4.1 Exercise Induced Physiological Cardiac Hypertrophy

Exercise initiated prior to and continued during increased heart and left ventricular weights in both Control and Restricted females irrespective of diet. The magnitude of this change (~10%) suggests that the increased heart and left ventricular weights in these rats are likely to be structural and functional changes that are physiologically relevant (Hickson *et al.*, 1979; Hickson *et al.*, 1983). Similar outcomes were also demonstrated in our previous study in which four weeks of exercise early (5 to 9 weeks) and later in life (20 to 24 weeks) increased absolute and relative heart mass at 24 weeks of age in both Control and Restricted male rats in the presence of normal blood pressure (Wadley *et al.*, 2016).

Physiological hypertrophy differs in both structural and molecular characteristics to pathological hypertrophy, which is associated with pressure or volume overload (Norton *et al.*, 1997; Aeschbacher *et al.*, 2001; Badenhorst *et al.*, 2003). Physiological hypertrophy, for example, appears to be regulated through an IGF-1−PI3-K−Akt signalling cascade and is characterised by an increase in left ventricular volume (McMullen *et al.*, 2003; McMullen *et al.*, 2004), while pathological hypertrophy, is activated through G-protein-coupled receptors by a variety of cardiac paracrine or autocrine factors and is characterised by an increase in left ventricular wall thickness (Akhter *et al.*, 1998; McMullen & Jennings, 2007). Physiological cardiac hypertrophy may be beneficial for the prevention of cardiovascular complications that may develop at later age (Regina *et al.*, 2001; Vehaskari *et al.*, 2001), particularly in offspring exposed to altered prenatal and postnatal growth that have lower cardiomyocyte number in early life (Black *et al.*, 2012) and develop a degree of pathological left ventricular hypertrophy in adulthood (Wlodek *et al.*, 2008). Recently, a study demonstrated that juvenile exercise induced sustained cardiac hypertrophy and increase in cardiomyocyte number in adulthood in normal male rats (Asif *et al.*, 2017). It is therefore possible that the “reprogramming” effect of exercise on heart size may help prevent the potential onset of hypertension and cardiovascular

disease. Indeed, in one large epidemiological study, physical activity was inversely related to later development of hypertension (Fagard, 2005) and has important implications for humans born small.

4.4.2 Blood Pressure Adaptations to Pregnancy

Systolic blood pressure was not different between Control and Restricted females prior to pregnancy (19 weeks of age), but was reduced significantly in growth-restricted females during late gestation. These findings were different to our previous study in which systolic blood pressure at late gestation was not different between pregnant and non-pregnant growth-restricted females (Gallo *et al.*, 2012a). Differences in the growth pattern of growth-restricted females between the present and previous study may explain the different outcomes in their systolic blood pressure before and during pregnancy. Conversely, an epidemiological study reported increased risk of hypertension during late pregnancy in low birth weight women compared with those born of normal weight, likely attributed to alterations in maternal vascular remodeling and/or deficits in nephron endowment (Klebanoff *et al.*, 1999). Another finding to emerge from this study was that a high fat diet did not alter systolic blood pressure in both Control and Restricted females prior to pregnancy (19 weeks of age), which was in contrast to evidence from human studies that reported increased risk of hypertension in obese individuals (Brown *et al.*, 2000; Droyvold *et al.*, 2005; Shihab *et al.*, 2012). This is possibly due to the lack of increased body weights in both Control and Restricted females on a HFD after 14 weeks. Indeed, a recent study reported that 15 weeks of HFD was not sufficient to significantly result in an elevation of systolic blood pressure in rats (Marques *et al.*). Thus, systolic blood pressure is likely to alter if the duration of rats being on a HFD is longer.

Similar to growth-restricted females on a Chow diet, systolic blood pressure was also reduced during late gestation when compared to pre-pregnancy in both Control and Restricted on a HFD (Figure 4.13). In normal human pregnancies, blood pressure often decreases during early pregnancy, reaching its lowest point between 24 and 32 weeks gestation, followed by a progressive increase until term (Volman *et al.*, 2007; Grindheim *et al.*, 2012; Mahendru *et al.*, 2014). This mid-trimester drop in blood pressure occurs partly due to increase in relaxin, progesterone and prostaglandins that relax the walls of maternal blood vessels, thus decreasing systemic vascular resistance (Anderson *et al.*, 1976; Kristiansson & Wang, 2001). This change, coupled with a major portion of maternal blood flow and cardiac output directed toward the uteroplacental circulation provides a basis for the decrease in maternal systemic blood pressure (Robson *et al.*, 1989; Hunter & Robson, 1992; Mabie *et al.*, 1994). Systemic vascular resistance is lowest in the first and second trimesters, then gradually increases by term; therefore, both

systolic and diastolic blood pressure tend to increase during the third trimester term (Volman *et al.*, 2007; Grindheim *et al.*, 2012; Mahendru *et al.*, 2014). Thus, it can be extrapolated that Restricted females on a Chow diet and both Control and Restricted on a HFD may have delayed cardiovascular adaptations to pregnancy, possibly due to alterations in pregnancy hormones and/or systemic vascular resistance which caused their blood pressure to remain lower during late gestation. Indeed, a human study reported that high serum concentrations of progesterone and relaxin during early trimester were related to lower mean systolic blood pressures in the second and third trimester (Kristiansson & Wang, 2001). Therefore, measurements of blood pressure and pregnancy hormones such as progesterone and relaxin during early pregnancy and post pregnancy, with more dynamic time-points, may reveal differences in cardiovascular function in these pregnant females. Other possible mechanisms that may contribute to the reduction of blood pressure during late gestation is the renin-angiotensin-aldosterone system (RAAS) (Figure 4.13), which plays a major role in regulating blood pressure during pregnancy (Lumbers & Pringle, 2014). Reduction in the complex integration of the secretions and actions of the circulating maternal RAAS in pregnancy may also contribute to the greater reduction in systolic blood pressure during late gestation in the present study (Lumbers & Pringle, 2014).

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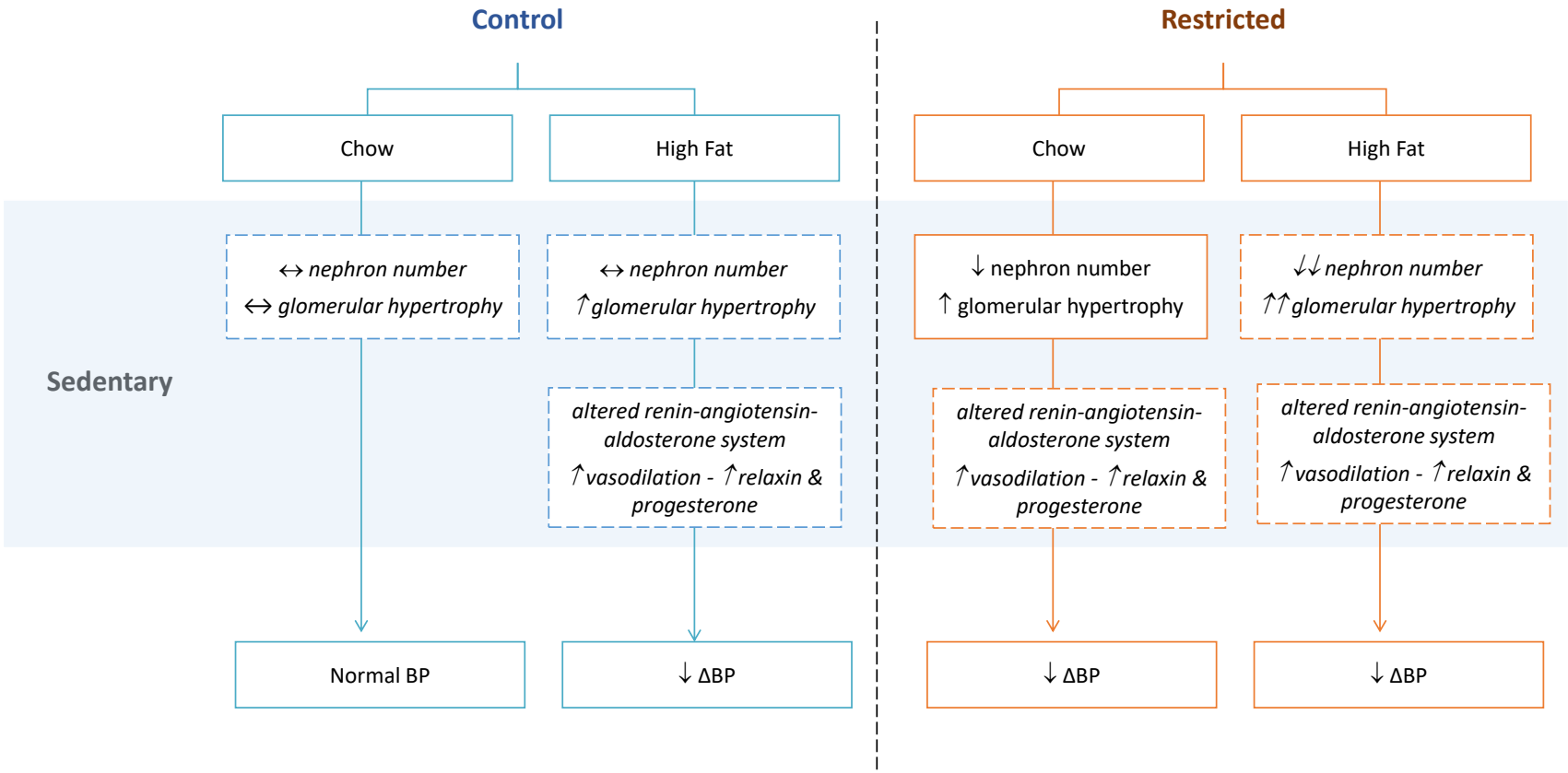

Figure 4.13 Effect of IUGR and high fat diet on blood pressure adaptations during late gestation

Growth-restricted Chow-fed rats, and both Control and growth-restricted females on a HFD had an adverse cardiovascular adaptations to pregnancy with a greater reduction in systolic blood pressure during late gestation. Solid-lined boxes are findings demonstrated in this current and past study from our laboratory, whereas dashed-line boxes with italicized font represent hypothesized mechanisms that may contribute to the outcomes reported in this study. Delta represent the difference in systolic blood pressure between pre-pregnancy and during late pregnancy and arrows in boxes reflect the directionality of change.

Importantly, this study also demonstrated that exercise training before and during pregnancy prevented the reduction in systolic blood pressure during late gestation when compared with pre-pregnancy in Chow-fed Restricted females and both Control and Restricted females on a HFD (Figure 4.14). However, exercise initiated during second week of pregnancy had no effect on systolic blood pressure during late gestation. Exercise-induced physiological left ventricular hypertrophy, as demonstrated in females that initiated exercise before and continued during pregnancy but not in females that exercised during pregnancy only, may contribute to the prevention of adverse blood pressure adaptations in these rats. Although it is well established that exercise-induced cardiac hypertrophy has favorable effects on heart in animal studies (Scheuer *et al.*, 1982; Konhilas *et al.*, 2006; Ooi *et al.*, 2014; Powers *et al.*, 2014), no human studies to date have investigated the association between exercise-induced cardiac hypertrophy and blood pressure.

It is important to note that systolic blood pressure was measured using tail cuff plethysmography in order to obtain repeated measures in the same animals at different time points from this large cohort of rats prior to and during pregnancy. The tail cuff approach allows for non-invasive, inexpensive and high throughput measures of systolic blood pressure, however has a tendency to overestimate and distort responses as well as exert stress upon the animal (Van Vliet *et al.*, 2000). Future studies will require more rigorous methods, such as tail artery catheter or ideally telemetry studies, to more accurately characterise the effect of uteroplacental insufficiency, high fat diet and exercise training on blood pressure, but this was not practical for this long-term study. Furthermore, although previous study reported the association between kidney nephron deficits and high blood pressure in growth-restricted offspring (Wlodek *et al.*, 2007; Wlodek *et al.*, 2008), kidney nephron number in this current study was not characterized. Therefore, future studies are required to characterise the structural changes in nephron number or glomerular volume that may have occurred within the kidney following high fat diet and exercise training to determine whether these changes have functional and/or physiological consequences on the cardiovascular adaptations to pregnancy.

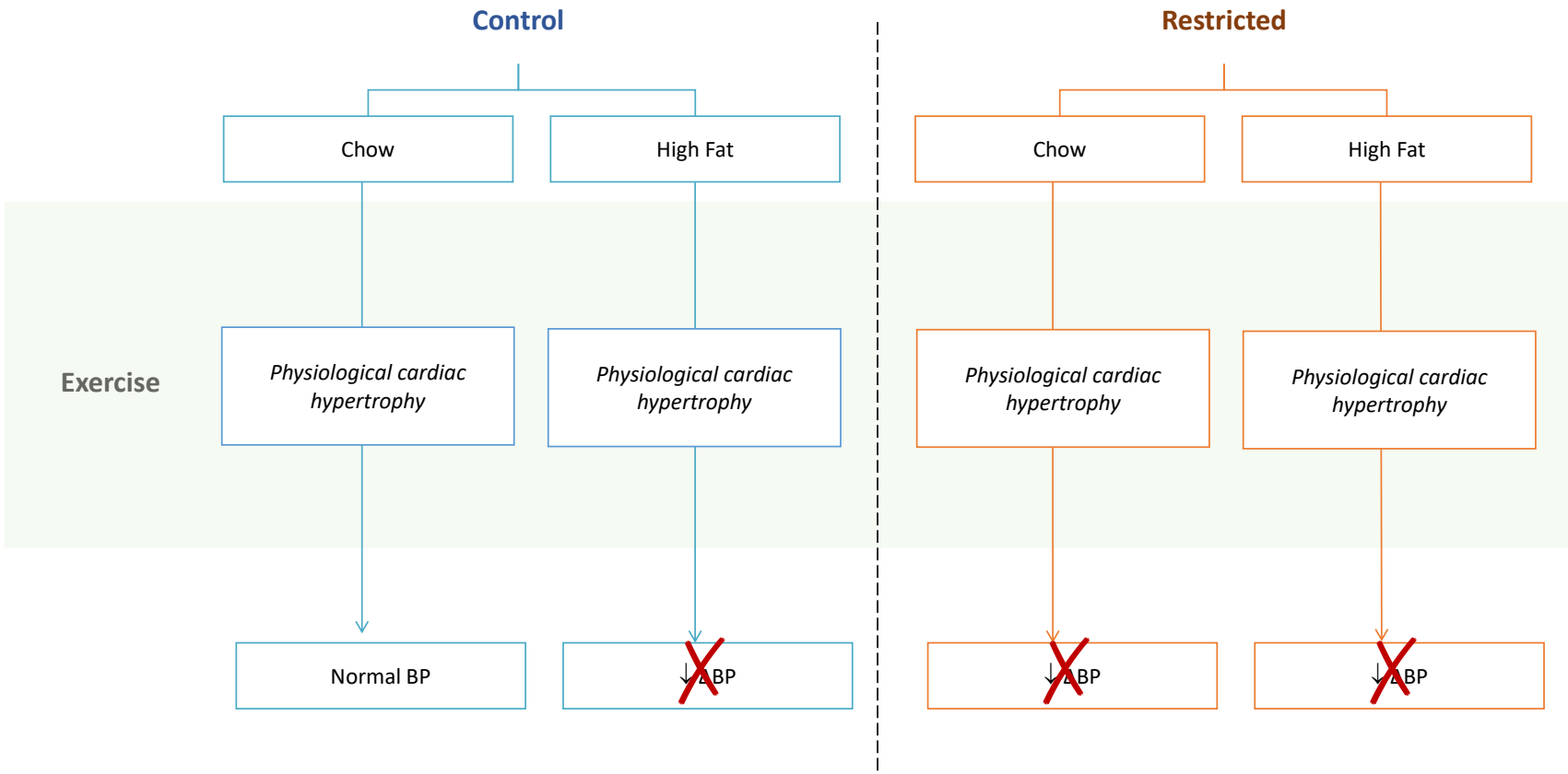

Figure 4.14 Effect of exercise before and during pregnancy on blood pressure adaptations during late gestation

Exercise before and during pregnancy prevented adverse cardiovascular adaptations to pregnancy in growth-restricted Chow-fed rats, and both Control and growth-restricted females on a HFD. Delta represent the difference in systolic blood pressure between pre pregnancy and during late pregnancy and arrows in boxes reflect the directionality of change.

4.4.3 Renal Function

During pregnancy, the kidney undergoes significant anatomical and physiologic changes with a marked vasodilation and increase in glomerular filtration rate as early as first trimester in humans (Cheung & Lafayette, 2013). Tubular function and handling of water and electrolytes are also altered, leading to mild increases in proteinuria, lower serum osmolality, and reductions in serum sodium levels (Cheung & Lafayette, 2013). In the current study, renal function, including creatinine clearance that is indicative of estimated glomerular filtration rate (eGFR), water intake, urine flow rate, urinary sodium, potassium, albumin and total protein were not different across the groups at E12. Of particular importance, the changes in these renal parameters from E12 to E20 mimic changes in renal adaptations in human pregnancy (Cheung & Lafayette, 2013).

Despite no changes in renal function at E12, eGFR was significantly increased in growth-restricted females compared with Control females during late gestation (E20) (Figure 4.15). Previous studies have established that while both growth-restricted male and female are equally affected by nephron deficits, only males develop renal dysfunction whilst females are relatively protected (Langley-Evans *et al.*, 1999; Wlodek *et al.*, 2008; Moritz *et al.*, 2009a; Gallo *et al.*, 2012a; Zohdi *et al.*, 2012). However, substantial changes in the kidney hemodynamics during pregnancy may have unmasked the glomerular hyperfiltration in the kidneys of growth-restricted females in the current study. The increased in eGFR was possibly contributed to by the increased glomerular size, as a compensatory mechanism due to low nephron number in these rats. Indeed, our laboratory reported that early onset glomerular hypertrophy was evident in growth-restricted females at 4 months of age during pregnancy, who have nephron deficits (Gallo *et al.*, 2012a). Similarly, eGFR was also increased in Control females on a HFD compared with Control females on a Chow diet during late gestation (Figure 4.14). Compared to growth-restricted females, Control females on a HFD were born with normal nephron number. However, studies have demonstrated that sufficient increases in body mass can lead to obesity-related nephropathy despite a normal number of nephrons, which lead to glomerular hypertrophy (Kambham *et al.*, 2001; Ahmed & Khalil, 2007; D'Agati & Markowitz, 2008). Even in the absence of apparent renal disease or injury, glomerular size is often larger in obese humans and rats (Nyengaard & Bendtsen, 1992; Hoy *et al.*, 2010; Puelles *et al.*, 2012; Tsuboi *et al.*, 2013). The increased glomerular size in obese individuals is paralleled by a stepwise increase of glomerular filtration rate (Ribstein *et al.*, 1995; Chagnac *et al.*, 2000; Cheung & Lafayette, 2013). These findings suggest that the increased eGFR in Control females on a HFD in the current study was possibly due to increased glomerular size. Although eGFR was increased in growth-restricted females on a Chow diet and Control females on a HFD, no

difference in urinary sodium excretion was demonstrated (Figure 4.15). During pregnancy, a remarkable capacity to regulate sodium and potassium persists, which may explain the lack of difference in urinary sodium excretion in these rats (Mitch & Wilcox, 1982). However, despite well-controlled electrolyte balance during pregnancy, urinary potassium excretion remains increased during late gestation in these rats. Greater potassium loss, through renal excretion, is often associated with impaired insulin secretion and decreased peripheral glucose utilization resulting in glucose intolerance and hyperglycemia (Wilcox, 1999). As discussed in Chapter 3, both growth-restricted females on a Chow diet and Control females on a HFD developed glucose intolerance during late gestation. Therefore, the increased urinary potassium secretion in these rats can be due to the altered metabolic function that occurred during late pregnancy.

As both intrauterine growth restriction and high fat diet independently increased eGFR, it was expected that the combined effect of IUGR and HFD would exacerbate glomerular hyperfiltration. Interestingly, this study reported no difference in eGFR in growth-restricted females on a HFD during late gestation (Figure 4.15). It is likely that the lack of difference in eGFR in these rats was merely protective adaptations to adverse renal changes during pregnancy, which is contributed to by a further increase in the glomerular size in order to meet pregnancy demands. However, due to time constraints, glomerular size was not characterized in this study and, thus, will be important to investigate in the future. Similar to eGFR, urinary sodium and potassium excretion were also not altered in growth-restricted females on a HFD, suggesting that they have normal renal function during late gestation.

Of particular importance, this current study demonstrated that a high fat diet reduced water intake and, subsequently, urine flow rate in both Control and Restricted females at E20. In normal human pregnancies, the threshold for stimulating the osmoreceptors for antidiuretic hormone and thirst are significantly reduced during early pregnancy (Davison *et al.*, 1988). However, no study to date has examined the impact of a high fat diet on thirst regulation during pregnancy as well as in non-pregnant individuals. Similarly, a high fat diet also had an effect on urinary albumin excretion during late gestation in which both Control and growth-restricted females excreted less albumin compared to females on a Chow diet. Pregnancy itself is a state of decreased serum albumin and serum albumin concentrations fall as pregnancy progresses (Maher *et al.*, 1993). This decrease is generally thought to be related to the dilutional effect of the increased plasma volume or due to the increased serum estrogen and progesterone concentrations that occur during pregnancy (Cheung & Lafayette, 2013). On the other hand, serum albumin concentrations were negatively correlated with BMI in non-pregnant humans (Babaei *et al.*, 2015). Low-grade chronic inflammation in obese individuals is reported to suppress albumin synthesis and, thus, reduces the concentration of serum albumin (Don &

Kaysen, 2004; Kaysen *et al.*, 2004). The combined effect of pregnancy and a high fat diet in the current study may have contributed to the reduction in urinary albumin excretion in both Control and growth-restricted females during late gestation. Nevertheless, future studies need to characterize renal function post pregnancy to determine whether these changes are a protective response during pregnancy that does not progress into the "vicious cycle" of future renal damage and systemic hypertension.

In the current study, both exercise before and during pregnancy as well as exercise during pregnancy only had no effect on the renal function. This finding is in contrast to human studies, as most of the studies reported beneficial effects of exercise in preventing adverse renal adaptations during pregnancy (Sorensen *et al.*, 2003; Saftlas *et al.*, 2004; Rudra *et al.*, 2008). It is possible that the exercise intensity and timing used in the current study was not sufficient enough to have beneficial effects on renal function in pregnant rats.

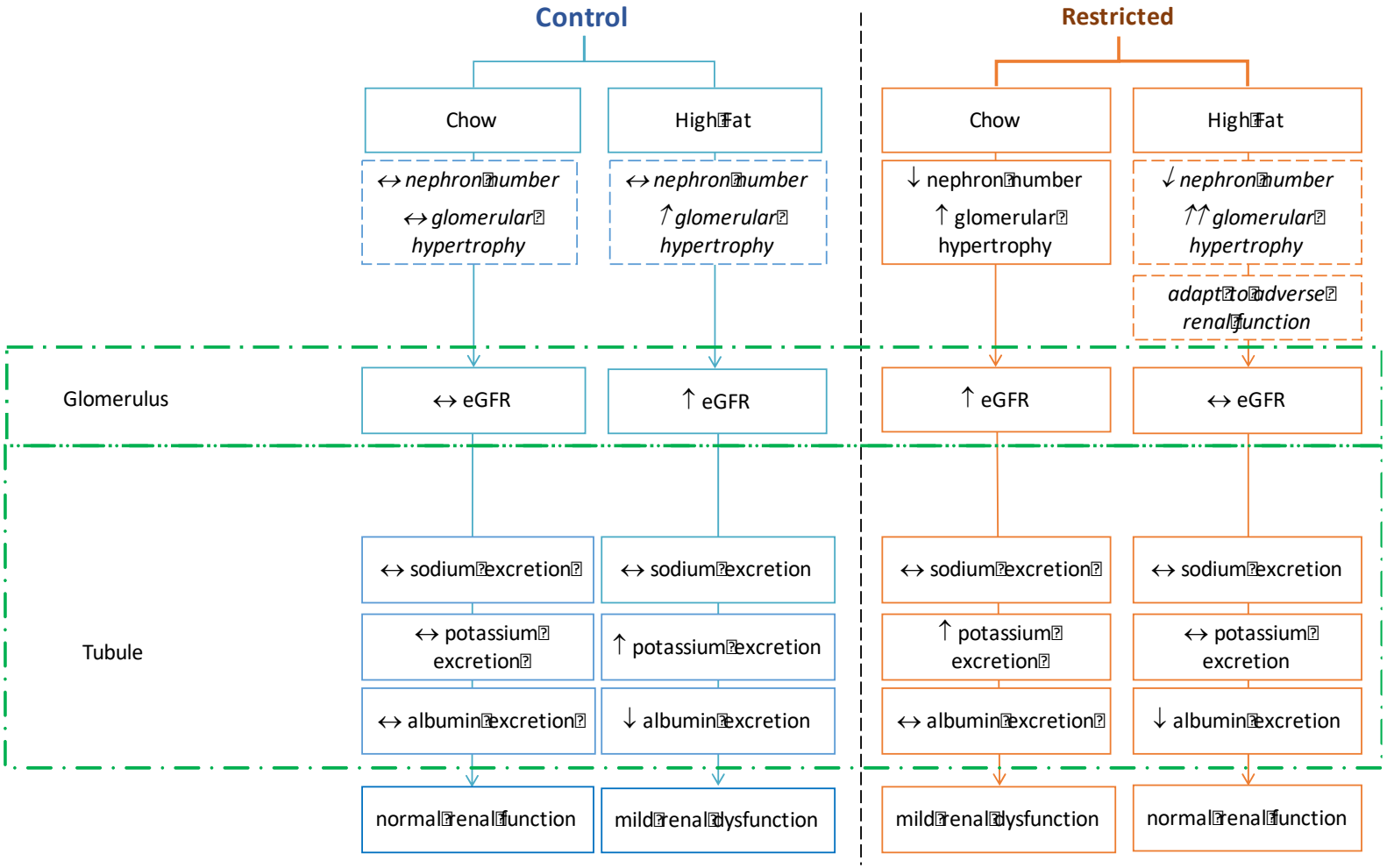

Figure 4.15 Effect of IUGR and high fat diet on renal adaptations during late gestation

IUGR and HFD independently altered renal function as evident in the increased of eGFR as assessed by creatinine clearance and urinary potassium excretion during late gestation. The combined effect of IUGR and HFD had no difference in eGFR and other renal parameters. Solid-lined boxes are findings demonstrated in this current and past study from our laboratory, whereas dashed-line boxes with italicized font represent hypothesized mechanisms that may contribute to the outcomes reported in this study. Arrows in boxes reflect the directionality of change.

4.4.4 Conclusions

In summary, findings from the present study have demonstrated that intrauterine growth-restriction and obesity limit the renal and cardiovascular adaptations of pregnancy. Renal function in growth-restricted females and females on a HFD was altered, as evident by the increased eGFR and urinary potassium excretion during late gestation. Interestingly, the combined effect of IUGR and HFD was reported to have no difference in eGFR possibly as protective adaptations to the adverse renal changes during pregnancy. High fat diet alone reduced water intake, urine flow rate and urinary albumin excretion in both Control and growth-restricted females during late gestation. However, both exercise before and during pregnancy as well as exercise initiated during second week of pregnancy had no effect on renal function in this study. Of particular importance, growth-restricted Chow-fed rats as well as Control and growth-restricted females on a HFD had adverse cardiovascular adaptations to pregnancy, with a greater reduction in systolic blood pressure during late gestation. Importantly, exercise before and during pregnancy prevented these adverse cardiovascular adaptations possibly by inducing physiological cardiac hypertrophy.

Overall, pregnant females born small and on a HFD are at a greater risk of cardiorenal alterations during pregnancy. Both blood pressure and renal function was affected by exercise prior to and during pregnancy, but no exercise during pregnancy only. Thus, this study highlights that modifiable risk factors, such as diet and exercise, can have beneficial effect on the mother during pregnancy, which may have long term health implications.

CHAPTER 5

Effects of High Fat Diet and Exercise on Fetal Outcomes

5.1 Introduction

Intrauterine growth restriction affects 10% of pregnancies worldwide and is associated with the development of adult-onset diseases in offspring (Wollmann, 1998). Females are generally less susceptible to programmed disease development compared to growth-restricted males although similar organ deficits are reported in both sexes (Gallo *et al.*, 2012a). However, physiological challenges of pregnancy unmask cardiovascular (Klebanoff *et al.*, 1999; Zetterstrom *et al.*, 2007) and metabolic (Seghieri *et al.*, 2002) disease state in growth-restricted females. Consistent with this, we have previously demonstrated that growth-restricted female rats develop glucose intolerance for the first time during late gestation, despite a normal plasma insulin response (Gallo *et al.*, 2012b). Importantly, we also demonstrated that physiological measurements conducted on growth-restricted females throughout pregnancy is a form of stress, as evidenced by increased glucocorticoid concentrations in the mother, which reduced F2 fetal weight at E20 (Gallo *et al.*, 2012b). This suggests maternal 'second hits' such as stress and obesity may unmask or exacerbate pregnancy complications and subsequently affect the growth and development of her offspring.

Although it is well established that being born small for gestational age is one of the risk factors for developing obesity, there are currently no studies that examine the effect of maternal obesity in growth-restricted mothers. Maternal obesity has been associated with a number of obstetric complications including GDM, hypertension and pre-eclampsia (Doherty *et al.*, 2006; McIntyre *et al.*, 2012; Cunningham & Teale, 2013). Of particular concern, maternal obesity does not only affect the mother but it is also associated with increased fetal growth, which can lead to offspring being born macrosomic (Ehrenberg *et al.*, 2004). Nevertheless, recent findings suggest that offspring born to an obese mother can also be small for gestational age or born with a normal birth weight (McIntyre *et al.*, 2012; Anderson *et al.*, 2013). Of particular note, being small or large for gestational age due to maternal obesity predispose the offspring to obesity and metabolic diseases in adulthood (Catalano & Ehrenberg, 2006).

It is well established that exercise during pregnancy promotes optimization of gestational weight gain (Stuebe *et al.*, 2009) and prevention of pregnancy-related disorders (Barakat *et al.*, 2013; Aune *et al.*, 2014). However, the influence of maternal exercise on fetal outcomes is poorly defined with conflicting

reports. Early human studies suggested chronic redirection of calories and blood flow to working muscles during maternal exercise may attenuate fetal growth elevating the risk of premature or small for gestational age newborn, especially at high exercise intensities (Clapp & Dickstein, 1984; Clapp & Capeless, 1990; Bell *et al.*, 1995). In contrast to previous findings, more recent studies suggest that prenatal exercise may reduce the prevalence of large for gestational age without increasing the risk of having a small for gestational age baby (Hopkins & Cutfield, 2011; Ferraro *et al.*, 2012). Thus, the development of targeted interventions in growth-restricted and obese mothers may have beneficial effects on fetal growth and development.

5.2 Aims and Hypotheses

The overall aim of this study was to investigate the effect high fat diet, endurance exercise training and physiological measurements performed during late gestation on the fetal outcomes in growth restricted mothers.

Specific aims of Chapter 5 were:

- ***i.*** To determine if high fat diet alters fetal and placental weights and placental efficiency in growth restricted mothers in late gestation;
- ***ii.*** To determine if physiological measurement performed during late gestation of pregnancy reduces fetal and placental weights and placental efficiency in growth restricted mothers in late gestation;
- ***iii.*** To determine whether endurance exercise training alters fetal outcomes in growth restricted mothers in late gestation;
- ***iv.*** To determine the sex specific difference in fetal and placental outcomes.

It is hypothesised that high fat diet and physiological measurements performed during late gestation in growth restricted mothers will adversely affect fetal and placental growth in late gestation and endurance exercise training will prevent the adverse fetal outcomes.

5.3 Material and Methods

Experimental design was described in Chapter 2.1. Only the specific endpoints of this chapter are briefly described in this section.

5.3.1 Post Mortem Tissue Collection

At E20, rats were anaesthetised with intraperitoneal injection of ketamine (100 $mg.kg^{-1}$) and Illium Xylazil-20 (30 $mg.kg^{-1}$) and a cardiac puncture was performed. Amniotic fluid was collected from each fetal sac and stored separately in individual tubes. Then fetal blood collection was collected via cardiac puncture and pooled within a litter. Each fetus and placenta were excised from the uterus and individually weighed. Fetal organs including brain, heart, pancreas, liver, left and right kidneys were dissected, weighed and either snap frozen in liquid nitrogen and stored at -80°C or immersion fixed in 10% neutral buffer formalin.

5.3.2 Statistical Analyses

All data were analysed using a five-way ANOVA to determine main effects of uteroplacental insufficiency (Control and Restricted), diet (Chow and High fat) and exercise (Sedentary, Exercise and ExPregOnly), physiology measures (No physiology measures and Physiology measures) and sex (Male and Female). 2-way ANOVAs were conducted to examine the interactions between Treatment and Diet, Treatment and Exercise, Diet and Exercise, Treatment and Physiology Measures, Diet and Physiology Measures, and Exercise and Physiology Measures. If significant interactions were detected, Student's unpaired *t*-test or a one-way ANOVA was performed with Student-Newman-Kewls post hoc test where appropriate. All data are presented as mean ± SEM with n representing the number of animals per litter from each group. $P<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Fetal Body, Organ and Placental Weights from Dams with No Physiology Measures

F2 total litter size was reduced in growth-restricted mothers on a Chow diet in Sedentary, Exercise and ExPregOnly groups compared to Control counterparts (Figure 5.1 A). High fat diet and both exercise interventions had no effect on F2 total litter size at E20 (Figure 5.1 A).

Maternal birth weight (Restricted) and high fat feeding had no effect on male and female fetal weights at E20 (Figures 5.2 A and 5.3 A). Control and Restricted mothers on a Chow diet who exercised prior to and continued during pregnancy had heavier male and female fetuses (+6-8%, $P<0.05$, Figures 5.2 A and 5.3 A). Exercise initiated during pregnancy had no effect on male and female fetal weights (Figures 5.2 A and 5.3 A). Placental weight of male fetuses was not affected by maternal birth weight, HFD and either Exercise and ExPregOnly (Figure 5.4 A). HFD increased placental weight of female fetuses in Control and Restricted mothers that exercised during pregnancy only ($P<0.05$, Figure 5.5 A). Control and Restricted mothers on a Chow diet that initiated exercise during pregnancy had female fetuses with lighter placenta ($P<0.05$, Figure 5.5 A). Both exercise interventions reduced placental weight of female fetuses of Control and Restricted mothers on a HFD ($P<0.05$, Figure 5.5 A). Fetal to placental weight ratio of male and female fetuses was not affected by maternal birth weight and high fat feeding (Figures 5.6 A and 5.7 A). Both Exercise and ExPregOnly increased male fetal to placental weight ratio of Control mothers on a Chow diet (Figure 5.6 A). Both exercise interventions increased female fetal to placental weight ratio of Control and Restricted mothers on a HFD (+5-6%, $P<0.05$, Figure 5.7 A).

Relative brain, heart, kidney and liver weights of male and female fetuses were not affected by maternal birth weight, high fat feeding and exercise interventions (Table 5.1).

5.3.2 Fetal Body and Placental Weights from Dams with Physiology Measures

F2 total litter size was not affected by intrauterine growth restriction and high fat diet in Sedentary mothers (Figure 5.1 B). Both exercise interventions had no effect on F2 total litter size at E20 (Figure 5.1 B).

Maternal birth weight and high fat feeding had no effect on male and female fetal weight at E20 (Figures 5.2 B and 5.3 B). Control and Restricted mothers on a HFD who exercised prior to and continued during pregnancy had heavier male and female fetuses (+3-7%, $P<0.05$, Figures 5.2 B and 5.3 B). Exercise also increased male fetal weight of Restricted mothers on a Chow diet (+8%, $P<0.05$, Figure 5.2 B).

Placental weight of male and female fetuses was not affected by maternal birth weight, HFD and both exercise interventions (Figures 5.4 B and 5.5 B). Fetal to placental weight ratio of male and female fetuses was not affected by maternal birth weight, high fat feeding and both exercise interventions (Figures 5.6 B and 5.7 B).

Relative brain, heart, kidney and liver weights of male and female fetuses were not affected by maternal birth weight, high fat feeding and exercise interventions (Table 5.1).

5.3.3 Sex Effect

F2 male fetuses were heavier than female fetuses despite no difference in placenta weight at E20 (3-5%, $P<0.05$, Figures 5.2 and 5.3). F2 male fetal to placenta weight ratio was higher than female at E20 ($P<0.05$, Figures 5.6 and 5.7).

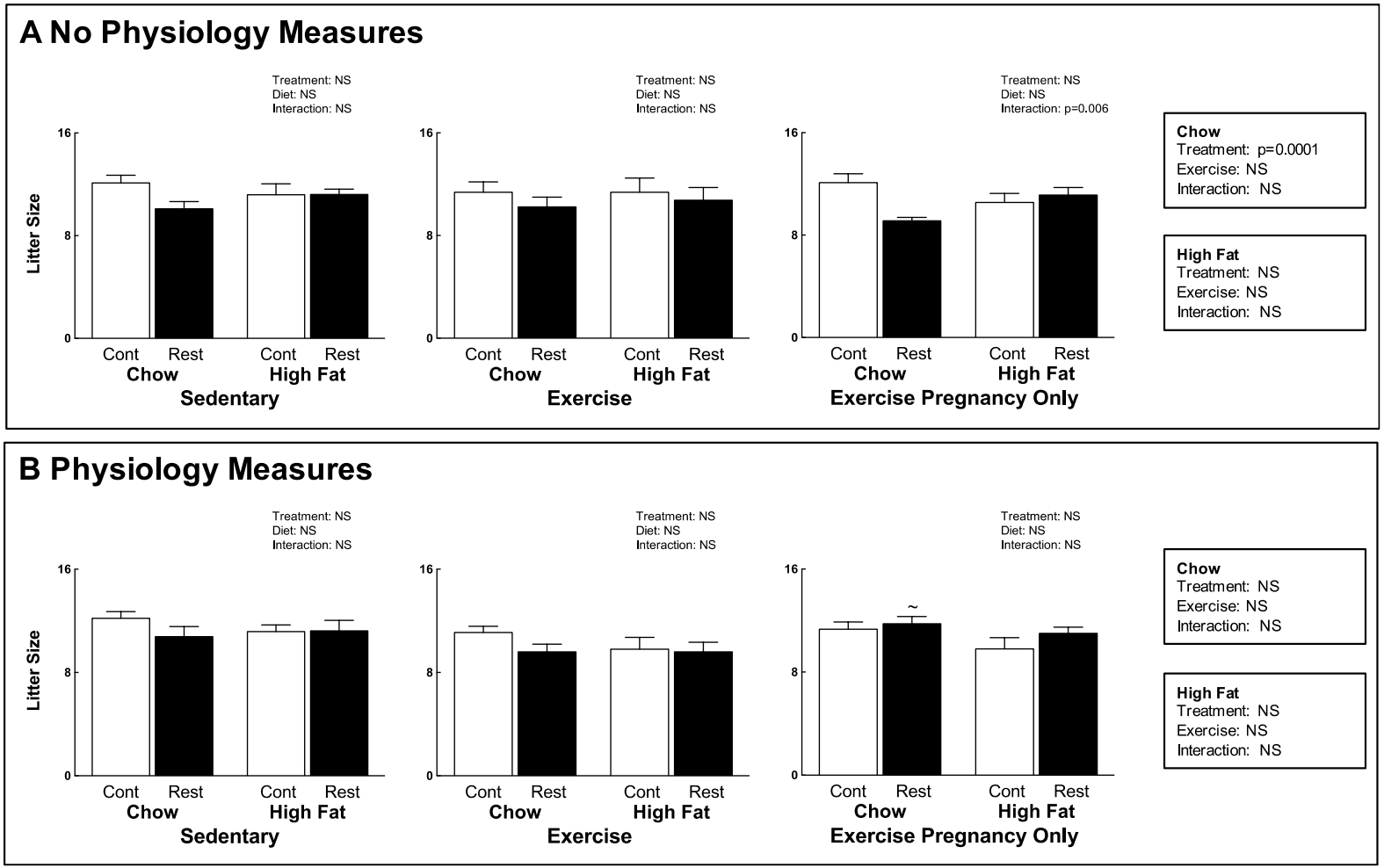

Figure 5.1 F2 litter size

Control (white bars) and Restricted (black bars) F2 litter size at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-way ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures.

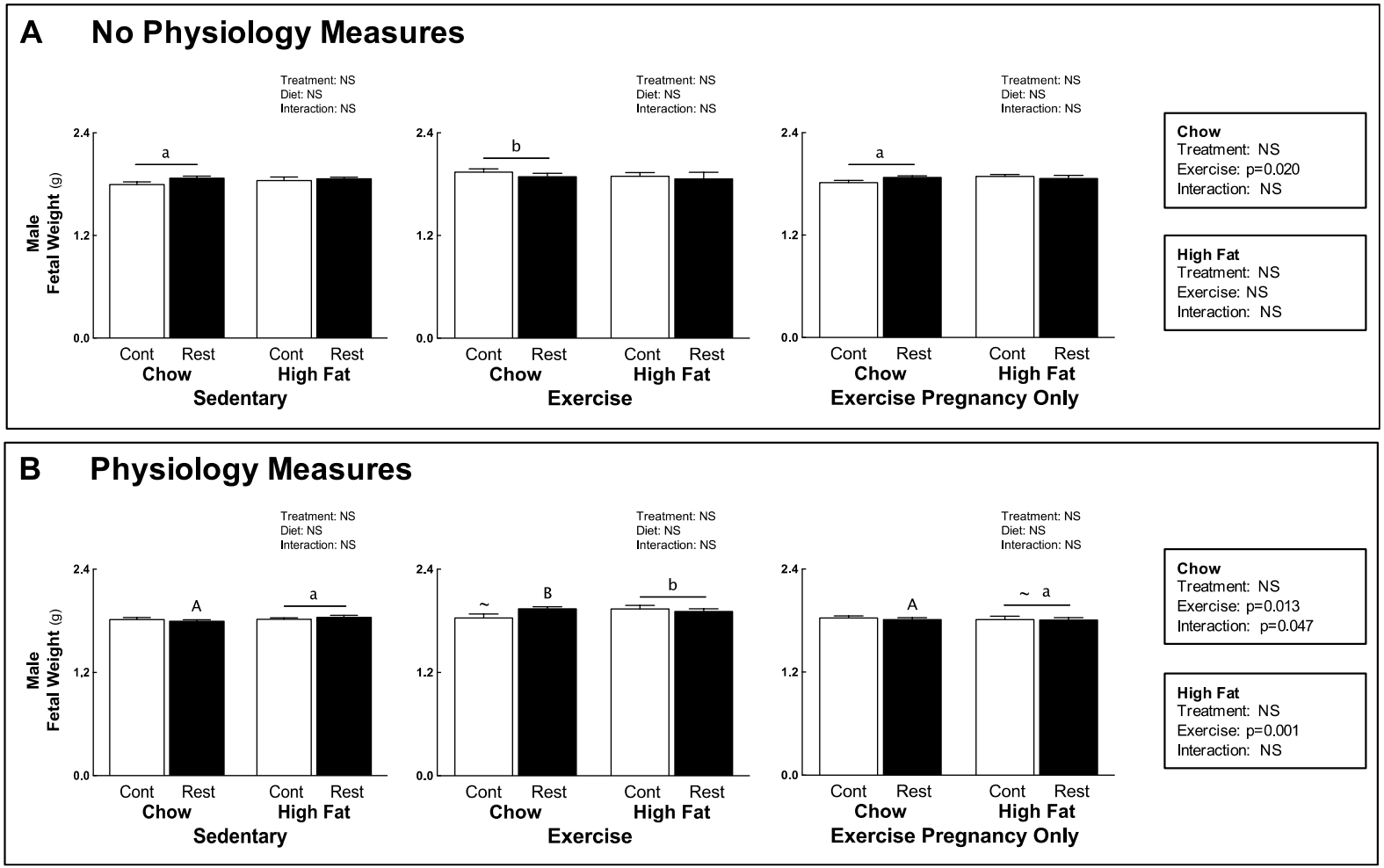

Figure 5.2 F2 male fetal weights

Control (white bars) and Restricted (black bars) F2 male fetal weight at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures; 'a/A' is different to 'b/B' ($P<0.05$).

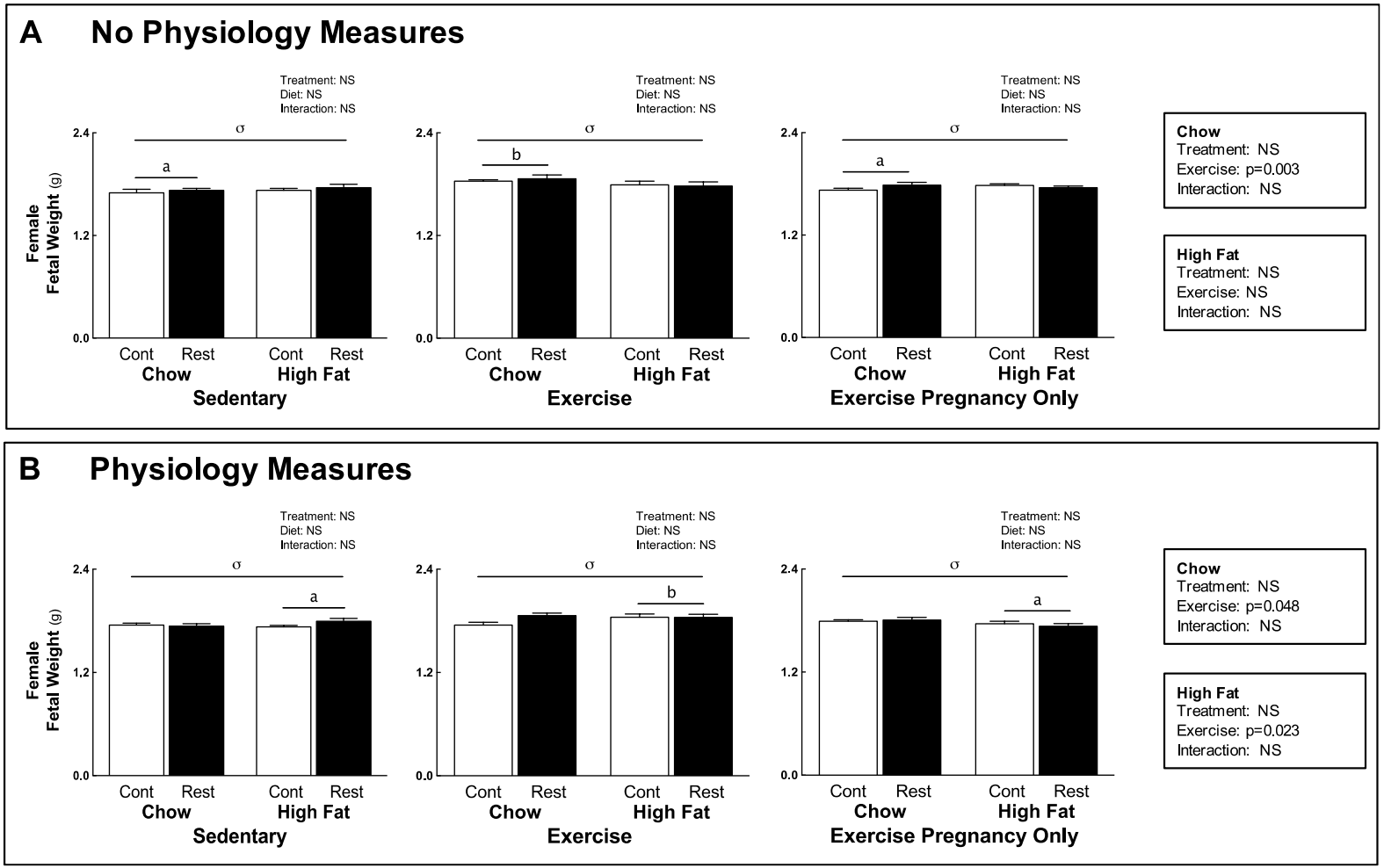

Figure 5.3 F2 female fetal weight

Control (white bars) and Restricted (black bars) F2 female fetal weight at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. σ $P<0.05$ vs Male; 'a' is different to 'b' ($P<0.05$).

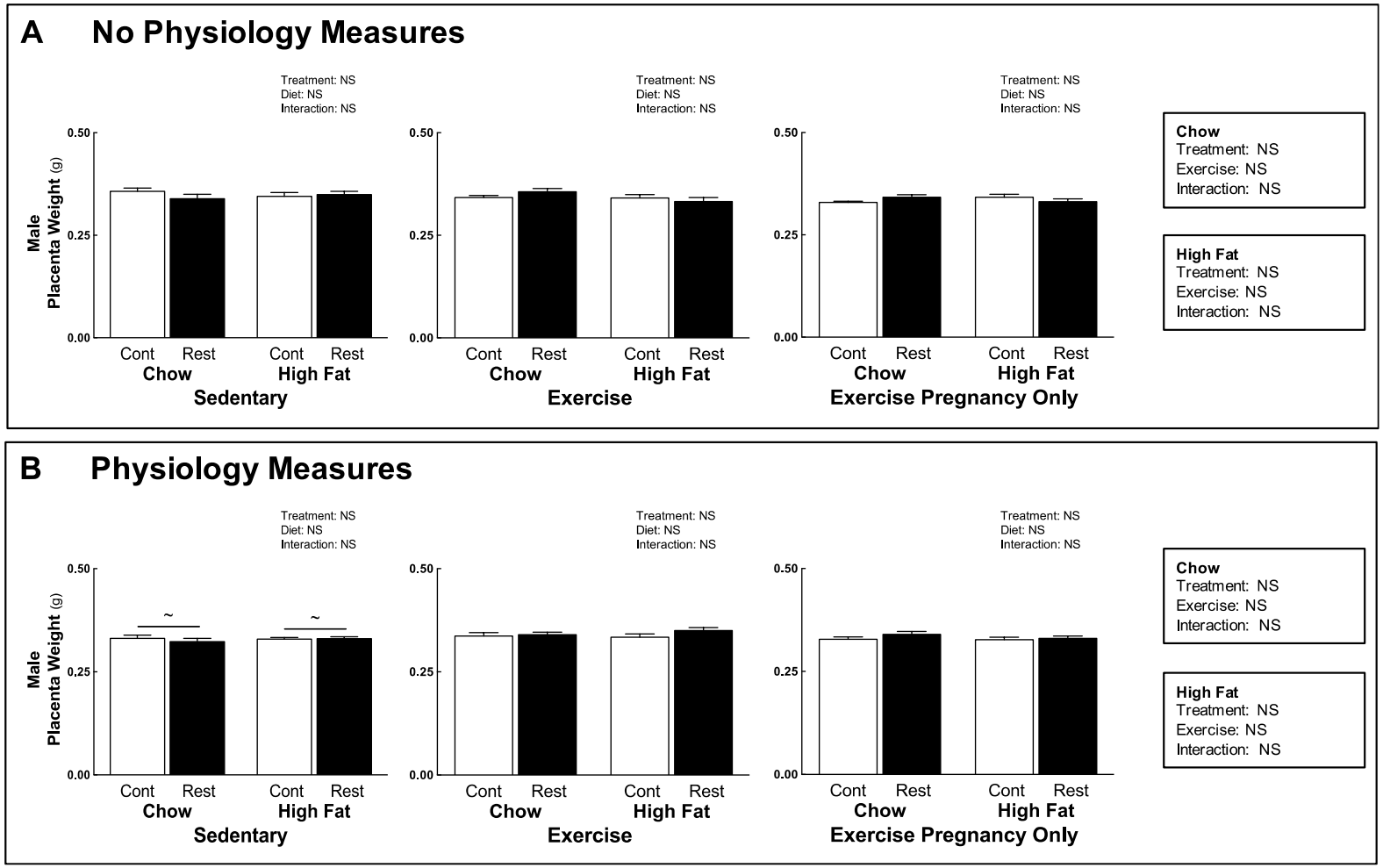

Figure 5.4 F2 male placenta weight

Control (white bars) and Restricted (black bars) F2 male placenta weight at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ P<0.05 vs No physiology measures.

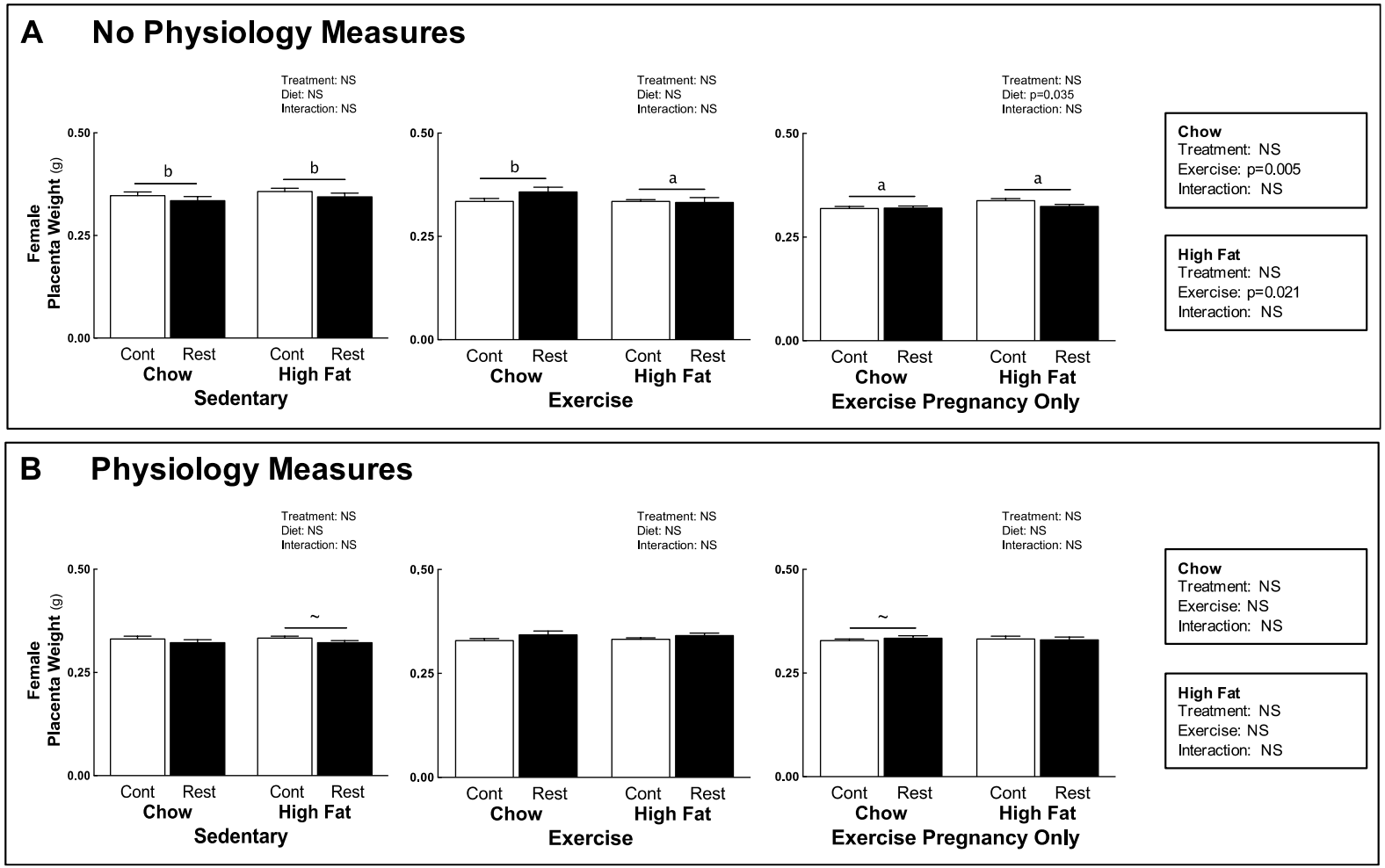

Figure 5.5 F2 female placenta weight

Control (white bars) and Restricted (black bars) F2 female placenta weight at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures; 'a' is different to 'b' ($P<0.05$).

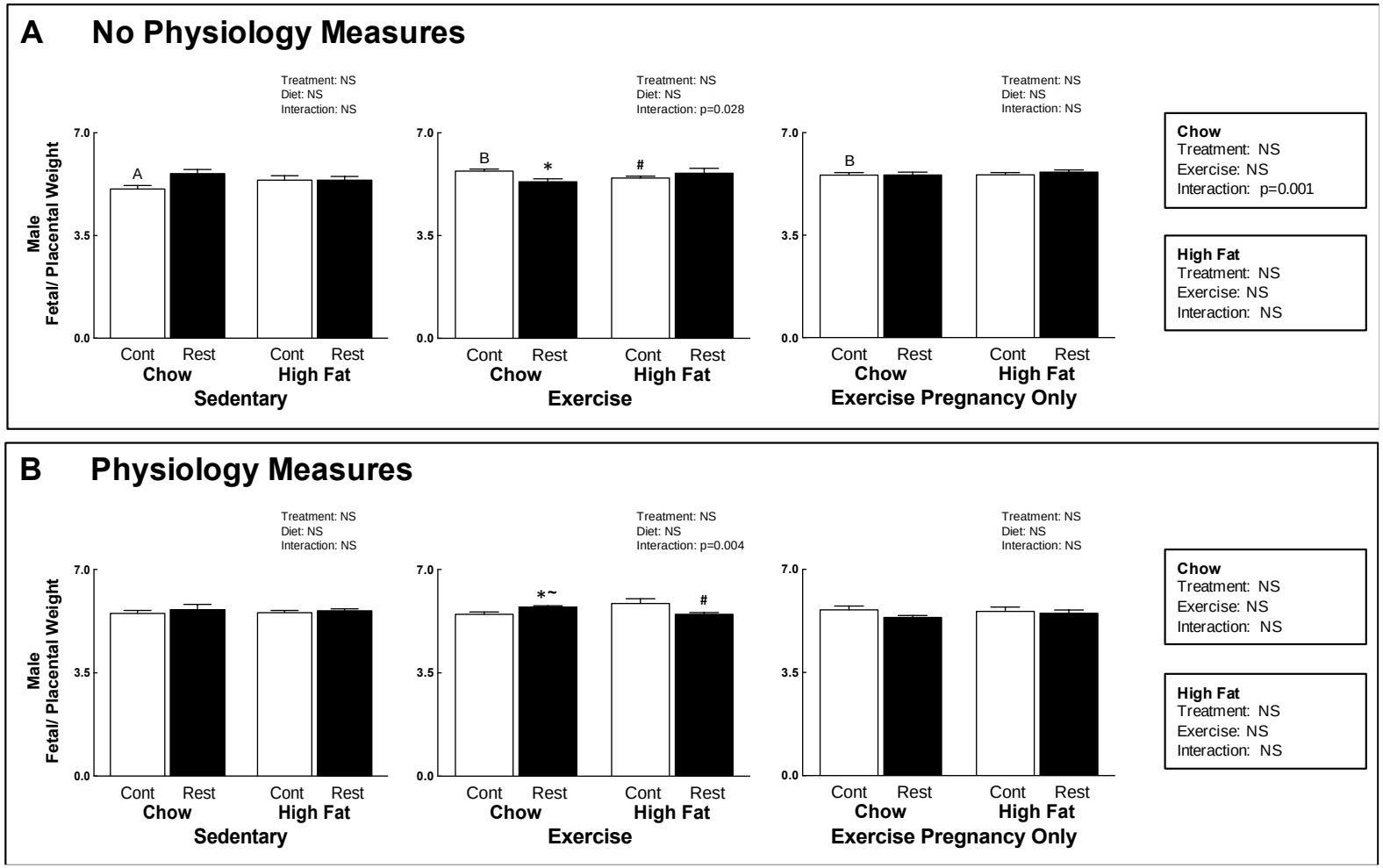

Figure 5.6 F2 male fetal to placenta weight ratio

Control (white bars) and Restricted (black bars) F2 male fetal to placental weight ratio at E20. (A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. * $P<0.05$ vs Control; # $P<0.05$ vs Chow; ~ $P<0.05$ vs No physiology measures; 'A' is different to 'B' ($P<0.05$).

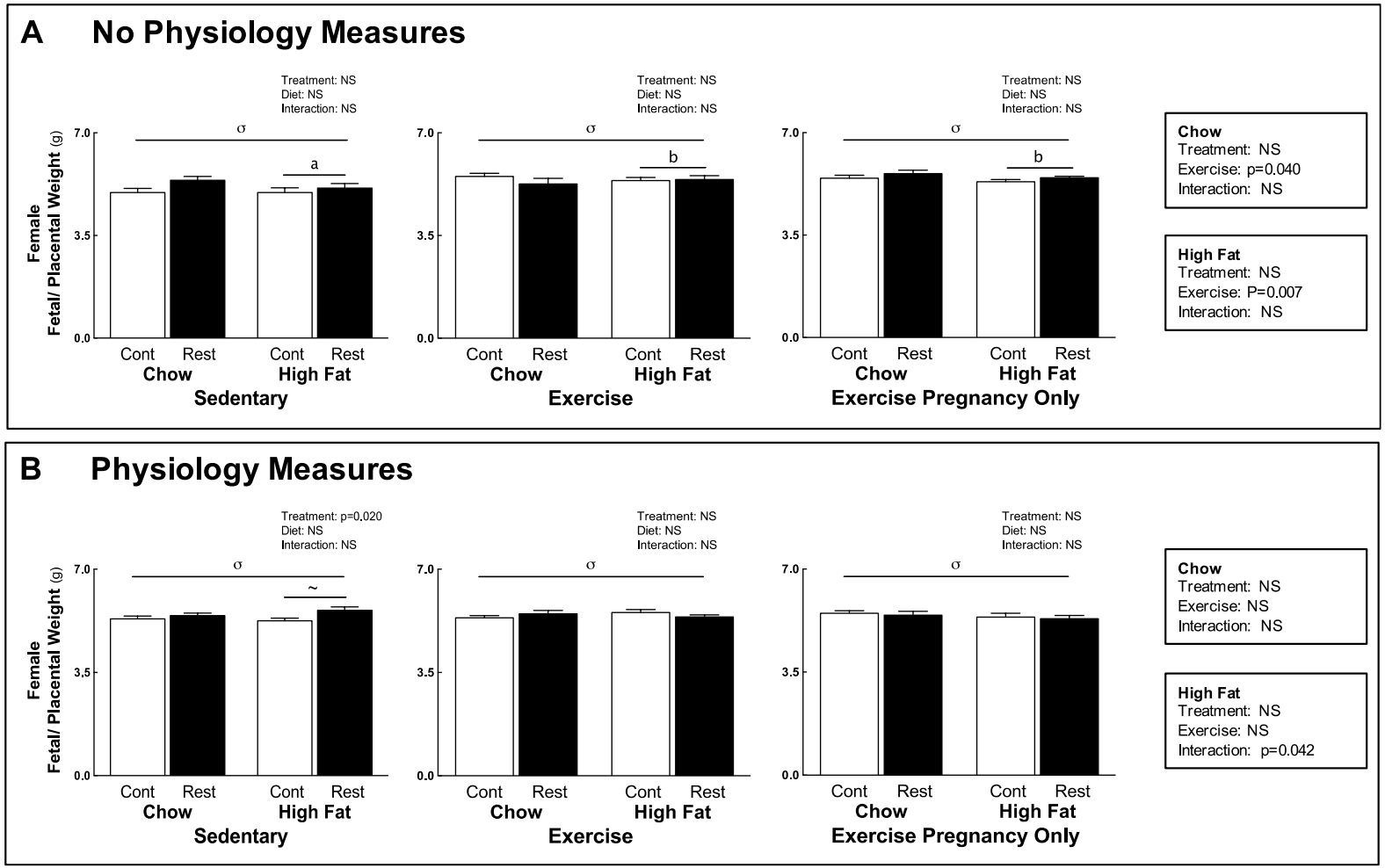

Figure 5.7 F2 female fetal to placenta weight ratio

Control (white bars) and Restricted (black bars) F2 female fetal to placental weight ratio at E20(A) No physiology measures and (B) Physiology measures cohorts. Results from the two-way ANOVA of treatment and diet separated by exercise is located on the top right of each graph panel and two-ways ANOVA of treatment and exercise separated by diet is located on the right side of the graphs. All values are expressed as mean ± SEM; 8-12 per group from separate litters. ~ $P<0.05$ vs No physiology measures; σ $P<0.05$ vs Male; 'a' is different to 'b' ($P<0.05$).

Table 5.1 Relative male and female fetal organ weights at post mortem

Relative fetal brain, heart, liver and kidney weights at *post mortem*. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

			Chow		**High Fat**		**Two-way Anova**		
			Control	Restricted	Control	Restricted	Treatment	Diet	Interaction
Relative Brain Weight (% body weight)									
No Physiology Measures	Male	Sedentary	*6.683 ± 0.155*	*6.645 ± 0.105*	*6.545 ± 0.152*	*6.607 ± 0.105*	NS	NS	NS
		Exercise	*6.613 ± 0.096*	*6.601 ± 0.085*	*6.556 ± 0.147*	*6.546 ± 0.247*	NS	NS	NS
		ExPreg	*6.746 ± 0.049*	*6.511 ± 0.117*	*6.547 ± 0.106*	*6.471 ± 0.140*	NS	NS	NS
	Female	Sedentary	*6.845 ± 0.089*	*6.713 ± 0.122*	*6.688 ± 0.084*	*6.805 ± 0.174*	NS	NS	NS
		Exercise	*6.617 ± 0.094*	*6.641 ± 0.084*	*6.822 ± 0.182*	*6.821 ± 0.181*	NS	NS	NS
		ExPreg	*6.980 ± 0.076*	*6.600 ± 0.123*	*6.752 ± 0.082*	*6.766 ± 0.124*	NS	NS	NS
Physiology Measures	Male	Sedentary	*6.734 ± 0.124*	*6.802 ± 0.108*	*6.582 ± 0.085*	*6.434 ± 0.148*	NS	NS	NS
		Exercise	*6.572 ± 0.145*	*6.645 ± 0.086*	*6.5557 ± 0.088*	*6.520 ± 0.152*	NS	NS	NS
		ExPreg	*6.666 ± 0.097*	*6.514 ± 0.160*	*6.856 ± 0.114*	*6.721 ± 0.125*	NS	NS	NS
	Female	Sedentary	*6.832 ± 0.057*	*7.125 ± 0.108*	*6.829 ± 0.094*	*6.745 ± 0.073*	NS	NS	NS
		Exercise	*6.965 ± 0.220*	*6.735 ± 0.131*	*6.618 ± 0.136*	*6.527 ± 0.159*	NS	NS	NS
		ExPreg	*6.679 ± 0.133*	*6.664 ± 0.262*	*6.791 ± 0.133*	*6.883 ± 0.124*	NS	NS	NS
Relative Heart Weight (% body weight)									
No Physiology Measures	Male	Sedentary	*0.466 ± 0.015*	*0.427 ± 0.018*	*0.423 ± 0.018*	*0.423 ± 0.015*	NS	NS	NS
		Exercise	*0.431 ± 0.010*	*0.409 ± 0.019*	*0.423 ± 0.024*	*0.408 ± 0.017*	NS	NS	NS
		ExPreg	*0.483 ± 0.044*	*0.440 ± 0.013*	*0.448 ± 0.009*	*0.448 ± 0.018*	NS	NS	NS
	Female	Sedentary	*0.478 ± 0.014*	*0.441 ± 0.016*	*0.460 ±0.021*	*0.437 ± 0.012*	NS	NS	NS
		Exercise	*0.439 ± 0.014*	*0.442 ± 0.014*	*0.451 ± 0.022*	*0.432 ± 0.016*	NS	NS	NS
		ExPreg	*0.438 ± 0.014*	*0.469 ± 0.019*	*0.450 ± 0.016*	*0.460 ± 0.016*	NS	NS	NS
Physiology Measures	Male	Sedentary	*0.420 ± 0.011*	*0.425 ± 0.014*	*0.432 ±0.016*	*0.402 ± 0.017*	NS	NS	NS
		Exercise	*0.407 ± 0.017*	*0.429 ± 0.014*	*0.407 ± 0.015*	*0.446 ± 0.011*	NS	NS	NS
		ExPreg	*0.433 ± 0.011*	*0.438 ± 0.010*	*0.425 ± 0.023*	*0.428 ± 0.016*	NS	NS	NS
	Female	Sedentary	*0.450 ± 0.009*	*0.416 ± 0.012*	*0509 ± 0.071*	*0.424 ± 0.010*	NS	NS	NS
		Exercise	*0.433 ± 0.013*	*0.429 ± 0.008*	*0.418 ± 0.014*	*0.412 ± 0.018*	NS	NS	NS
		ExPreg	*0.443 ± 0.017*	*0.443 ± 0.011*	*0.410 ± 0.019*	*0.441 ± 0.009*	NS	NS	NS

Table 5.1 Relative male and female fetal organ weights at *post mortem*

Relative fetal brain, heart, liver and kidney weights at *post mortem*. All values are expressed as mean ± SEM; 8-12 per group from separate litters.

			Chow		High Fat		Two-way Anova		
			Control	Restricted	Control	Restricted	Treatment	Diet	Interaction
Relative Liver Weight (% body weight)									
No Physiology Measures	Male	Sedentary	*6.770 ± 0.178*	*6.923 ± 0.159*	*7.057 ± 0.094*	*7.063 ± 0.056*	NS	NS	NS
		Exercise	*7.004 ± 0.101*	*7.094 ± 0.120*	*7.084 ± 0.101*	*6.969 ± 0.187*	NS	NS	NS
		ExPreg	*6.627 ± 0.373*	*7.491 ± 0.297*	*7.129 ± 0.137*	*7.140 ± 0.058*	NS	NS	NS
	Female	Sedentary	*7.028 ± 0.152*	*6.965 ± 0.131*	*7.231 ± 0.114*	*7.205 ± 0.138*	NS	NS	NS
		Exercise	*7.184 ± 0.093*	*7.255 ± 0.073*	*7.195 ± 0.117*	*6.731 ± 0.338*	NS	NS	NS
		ExPreg	*6.890 ± 0.326*	*7.310 ± 0.101*	*7.127 ± 0.124*	*7.134 ± 0.214*	NS	NS	NS
Physiology Measures	Male	Sedentary	*6.683 ± 0.103*	*6.764 ± 0.149*	*6.906 ± 0.094*	*7.059 ± 0.112*	NS	NS	NS
		Exercise	*6.621 ± 0.208*	*6.693 ± 0.115*	*7.003 ± 0.116*	*7.056 ± 0.143*	NS	NS	NS
		ExPreg	*6.720 ± 0.112*	*6.789 ± 0.107*	*7.088 ± 0.089*	*6.796 ± 0.079*	NS	NS	NS
	Female	Sedentary	*6.745 ± 0.133*	*6.759 ± 0.151*	*7.196 ± 0.076*	*7.135 ± 0.118*	NS	NS	NS
		Exercise	*6.777 ± 0.158*	*7.103 ± 0.155*	*7.191 ± 0.088*	*7.160 ± 0.104*	NS	NS	NS
		ExPreg	*6.988 ± 0.087*	*6.972 ± 0.063*	*7.400 ± 0.131*	*7.093 ± 0.097*	NS	NS	NS
Relative Kidney Weight (% body weight)									
No Physiology Measures	Male	Sedentary	*0.674 ± 0.018*	*0.691 ± 0.020*	*0.707 ± 0.025*	*0.687 ± 0.017*	NS	NS	NS
		Exercise	*0.724 ± 0.008*	*0.704 ± 0.024*	*0.699 ± 0.025*	*0.707 ± 0.025*	NS	NS	NS
		ExPreg	*0.695 ± 0.016*	*0.707 ± 0.018*	*0.712 ± 0.013*	*0.694 ± 0.016*	NS	NS	NS
	Female	Sedentary	*0.643 ± 0.027*	*0.702 ± 0.012*	*0.712 ± 0.020*	*0.737 ± 0.019*	NS	NS	NS
		Exercise	*0.716 ± 0.007*	*0.694 ± 0.018*	*0.783 ± 0.035*	*0.707 ± 0.037*	NS	NS	NS
		ExPreg	*0.720 ± 0.014*	*0.745 ± 0.026*	*0.740 ± 0.017*	*0.720 ± 0.022*	NS	NS	NS
Physiology Measures	Male	Sedentary	*0.716 ± 0.017*	*0.707 ± 0.012*	*0.682 ± 0.017*	*0.710 ± 0.028*	NS	NS	NS
		Exercise	*0.665 ± 0.019*	*0.721 ± 0.014*	*0.739 ± 0.019*	*0.697 ± 0.019*	NS	NS	NS
		ExPreg	*0.648 ± 0.03.6*	*0.972 ± 0.016*	*0.685 ± 0.025*	*0.679 ± 0.014*	NS	NS	NS
	Female	Sedentary	*0.695 ± 0.032*	*0.701 ± 0.017*	*0.723 ± 0.020*	*0.781 ± 0.026*	NS	NS	NS
		Exercise	*0.731 ± 0.023*	*0.760 ± 0.019*	*0.749 ± 0.016*	*0.751 ± 0.026*	NS	NS	NS
		ExPreg	*0.714 ± 0.030*	*0.686 ± 0.014*	*0.696 ± 0.018*	*0.701 ± 0.024*	NS	NS	NS

5.5 Discussion

5.5.1 Effects of Growth Restriction and High Fat Diet on Fetal Outcomes

In the current study, F2 fetal body and placental weights were characterized for insight into the transgenerational consequences of maternal growth-restriction and high-fat feeding. This study demonstrated no changes in fetal and placental weights in both male and female fetuses of growth-restricted mothers, which is similar to our previous study (Gallo *et al.*, 2012a). Other studies investigating transmitted characteristics to F2 offspring from F1 growth restricted mothers, due to maternal protein restriction models, similarly report that F2 birth weight is largely unaffected (Zambrano *et al.*, 2005; Torrens *et al.*, 2008; Harrison & Langley-Evans, 2009). Fetal and placental weights in both male and female fetuses of Control and Restricted mothers remained similar, despite the mothers being exposed to physiological measurements throughout pregnancy. This was in contrast in our previous study that reported a 5-6% reduction in male and female fetal weight of mothers who were born small and exposed to physiological measurements during late pregnancy (Gallo *et al.*, 2012a), which is likely contributed by maternal stress and elevated glucocorticoids. Indeed, findings from animal studies and humans have reported reduced weight at birth (Kutzler *et al.*, 2004; Davis *et al.*, 2009), or no change (Moritz *et al.*, 2009b), with prenatal exposure to excess glucocorticoids. No difference in fetal and placenta weights in this study was possibly due to daily handling of the mothers throughout pregnancy, which may reduce the stress levels, whereas in the previous study the mothers were only handled on limited days of gestation (Gallo *et al.*, 2012a). The rats in the current study were either on a sedentary treadmill or exercise for 5 days per week during pregnancy and thus, became accustomed to the experimental procedures performed during pregnancy.

Although it is well established that maternal obesity is associated with increased fetal growth (Jensen *et al.*, 2003; Ehrenberg *et al.*, 2004; Abenhaim *et al.*, 2007; Nohr *et al.*, 2008), the current study reported no difference in fetal and placental weights in fetuses of mothers on a HFD. This is possibly due to the modest increased in body weight demonstrated in mothers on a HFD. Therefore, greater changes in the fetal growth may have been observed if the mothers on a HFD had gained more weight. Importantly, recent human studies suggest that offspring born to obese mothers can also be small for gestational age or born with a normal birth weight (McIntyre *et al.*, 2012; Anderson *et al.*, 2013; Radulescu *et al.*, 2013) with supporting evidence from an animal study (Luzzo *et al.*, 2012), underscoring the complexity of the maternal obesity paradigm. Of particular note, offspring from obese mothers are predisposed to obesity and adverse metabolic outcomes later in life, which is independent of their birth weight (Bayol *et al.*, 2007; Tamashiro *et al.*, 2009; Drake & Reynolds, 2010; Group HSCR, 2010). This is partly contributed to by increased metabolic inflammation in obese mothers (Madan *et al.*, 2009; Basu *et al.*, 2011) that extend to the placenta (Challier *et al.*, 2008), and thus exposes the fetus to an inflammatory

environment during development. Therefore, although there were no changes in fetal and placenta weights in fetuses of mothers on a HFD, exposure to an adverse environment *in utero* may program long-term consequences for the offspring, predisposing them to the development of metabolic and cardiovascular diseases in adulthood.

5.5.2 Effects of Exercise Interventions on Fetal Outcomes

Human studies on the effect of physical exercise on fetal growth and development have reported varying results likely due to differences in the type, intensity, duration and frequency of maternal exercise. For example, some studies report poor (Clapp & Capeless, 1990; Hopkins *et al.*, 2010; Juhl *et al.*, 2010), improved (Hatch *et al.*, 1993) and normal (Kardel & Kase, 1998; Owe *et al.*, 2009) infant growth.

No physiology measures

In the present study, the lifestyle intervention of exercise was demonstrated to affect fetal weight, which was dependent upon the timing of exercise initiation. Exercise initiated before and continued during pregnancy increased male and female fetal weight of both Control and Restricted mothers on a Chow diet. However, no effect in fetal weight was reported in mothers who initiated exercised during the second week of pregnancy. Differences in these findings suggest that beginning a moderate intensity exercise regimen before or in early pregnancy, during the hyperplastic phase of placental growth, may be an important mechanism for improving placental functional capacity, which in turn increases nutrient delivery to and the overall growth rate of the fetus later in gestation (Rabkin *et al.*, 1990; Hatch *et al.*, 1993; Clapp *et al.*, 2000a). However, the mechanisms by which regular exercise improves placental growth is unknown, but it is probable that it is linked to the intermittent reductions in uterine blood flow that occur during sustained exercise, as well as to the markedly expanded blood volume found in regularly exercising pregnant women (Clapp & Rizk, 1992; Clapp *et al.*, 2000b). Interestingly, these effects of exercise on fetal weight were not demonstrated in fetuses of Control and Restricted mothers on a HFD. It is possible that mothers on a HFD already had an adequate nutrient transfer to the fetus even in a condition of increased metabolic demand due to exercise training (Barakat *et al.*, 2015).

Both exercise interventions reduced placental weight of female fetuses of Control and Restricted mothers on a HFD. This resulted in increased fetal-to-placental weight ratio, which is an indication of improved placental efficiency. These findings suggest that female placentae may have a greater ability to adapt to adverse *in utero* environment than male placentae. A human study has reported that males grow faster *in utero* and are heavier at birth than females, with equivalent placental size (Misra *et al.*, 2009). Similarly, in this present study, male fetuses were heavier than females with no differences in placental weight. However, a consequence of growing more quickly and being larger *in utero* is that males are left with less reserve placental capacity to draw upon if sub-optimal conditions arise which

places them at increased risk of under nutrition (Eriksson *et al.*, 2010). These adverse conditions can restrict growth and lower birthweight, both of which have been linked to increased risk of adult-onset disorders in males such as metabolic and cardiovascular diseases (Barker, 2002; Cheong *et al.*, 2016c).

Physiology Measures

In physiology measures cohort, exercise initiated before and continued during pregnancy increased male and female fetal weight of both Control and Restricted mothers on a HFD despite no changes in placental weight. It is possible that the physiological measurements conducted during pregnancy induced some degree of stress on the mothers, as reported in our previous study (Gallo *et al.*, 2012a). Physiological measurements conducted on the mothers during pregnancy may have programmed different adaptation responses which cause changes in nutrient handling, whereby nutrient delivery to the fetuses of mothers on a HFD increased. However, further studies have to be conducted to further elucidate the placental mechanisms that contributed to the different fetal outcomes demonstrated in mothers who were exposed to physiological measurements during pregnancy.

To explain the fetal and placental weights and placental efficiency changes, our laboratory has explored potential mechanisms in the transport region of the placenta, the labyrinth, in sex-specific fashion (Yeukai & Wlodek, unpublished). Briefly, the insulin-like growth factor (IGF) system has been identified to play a role in the increased in fetal weight of the mothers that exercised before and during pregnancy. It is suggested that changes in IGF family gene and protein expression together with fetal plasma IGF concentration provide explanation in part for the increased in fetal weight in response to exercise. Furthermore, the placental glucose and amino acid transporters have been explored as other potential mechanisms that link to maternal metabolic phenotype reported in Chapter 3 to the fetal growth findings in the current study. The important finding has been that the vascular spaces of the placenta are altered which may have affected placental angiogenesis and vasculogenesis pathways.

5.5.3 Conclusions

In summary, findings from the present study demonstrate that intrauterine growth-restriction and high fat feeding have no effects on fetal and placental weights in both male and female fetuses. Importantly, exercise initiated before and during pregnancy increased fetal weight in both male and female fetuses of mothers on a Chow diet, but not in mothers on a HFD. Exercise interventions had no effect on male placental weight but reduced placental weight of female fetuses of mothers on a HFD. These data highlight that exercise interventions had a differential effect on male and female fetuses and were dependent upon the diet and the timing of the initiation of the exercise.

CHAPTER 6

General Discussion

6.1 Overview

The developmental origins of health and disease hypothesis proposed that insults during critical stages of fetal development program an increased susceptibility for metabolic and cardiorenal diseases in adulthood (Barker *et al.*, 1989a; Hales *et al.*, 1991; Eriksson *et al.*, 2000). Uteroplacental insufficiency is the leading cause of growth restriction in Western societies, characterised by reduced uteroplacental perfusion of nutrient and oxygen delivery to the developing fetus (Haggarty *et al.*, 2002; Henriksen & Clausen, 2002). A rat model of uteroplacental insufficiency was utilised in this thesis whereby pregnant dams underwent bilateral uterine vessel ligation surgery during day 18 of a 22-day pregnancy. This resulted in reduced nutrient and oxygen perfusion to the fetus, leading to a 10-15% reduction in birth weight (Wlodek *et al.*, 2005; Wlodek *et al.*, 2007; Siebel *et al.*, 2008). We have previously demonstrated that there are apparent sex-specific differences as a consequence of uteroplacental insufficiency, whereby growth restricted males have more severe metabolic and cardiovascular outcomes than females. Growth-restricted male rats developed glucose intolerance, hypertension, glomerular hypertrophy and pancreatic β-cell mass and nephron deficits (Wlodek *et al.*, 2007; Siebel *et al.*, 2008; Wadley *et al.*, 2008; Wlodek *et al.*, 2008; Siebel *et al.*, 2010; Laker *et al.*, 2011). In contrast, growth-restricted females were protected against the development of adverse functional outcomes, despite comparable pancreatic β-cell mass and nephron deficits (Moritz *et al.*, 2009a; Gallo *et al.*, 2012b). It has been suggested that second-hits such as pregnancy, a high fat/salt diet and ageing may unmask or exacerbate disease phenotypes in females that were born small. Indeed, a recent study from our laboratory demonstrated that pregnancy unmasks glucose intolerance and glomerular hypertrophy in growth-restricted females during late gestation (Gallo *et al.*, 2012b).

Of particular importance, human studies have reported that individuals born small for gestational age are at higher risk of obesity (Martin *et al.*, 2017). In obese females, the pregnancy adaptations become adverse and are associated with an increased risk of short- and long-term metabolic and cardiovascular dysfunctions in both mother and offspring (Cunningham, 1991; Sebire *et al.*, 2001; Mahizir *et al.*, 2016). Given that growth restricted females are at higher risk of cardiovascular and metabolic diseases during their pregnancy, it is

likely that maternal obesity may exacerbate the phenotypes. Therefore, the development of targeted lifestyle intervention in growth-restricted and/or obese mothers is important in preventing them from developing any adverse metabolic and cardiorenal adaptations to pregnancy. Certainly, in epidemiological and animal studies, exercise was reported to reduce the risk of metabolic and cardiovascular dysfunctions in normal weight females during pregnancy (Dempsey, 2004; Zhang *et al.*, 2006; Falcao *et al.*, 2010). However, evidence regarding the benefits of exercise in pregnant overweight or obese females is not consistent with some studies reporting a reduced risk of GDM and hypertension (Artal *et al.*, 2007; Garnaes *et al.*, 2016) and other studies demonstrated that there are no beneficial effects of exercise training possibly due to lack of compliance with the interventions (Dodd *et al.*, 2014; Seneviratne *et al.*, 2016).

Therefore, this thesis aims to investigate if a HFD exacerbates the known adverse metabolic and cardiorenal adaptations to pregnancy in rats born small and whether exercise before and during pregnancy is more beneficial in preventing these complications than exercise during pregnancy alone.

Specific aims and major findings for each Chapter are summarised as follows:

Chapter 3 Effects of Exercise and High Fat Diet on Metabolic Adaptations to Pregnancy in Females Born Small

The overall aim of this study was to determine if a HFD exacerbates the known adverse metabolic adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these complications. The major findings of this study were:

- Growth-restricted females were born 10-15% lighter compared with normal birth weight Controls and remained lighter at PN6, 14 and 35, at mating and *post mortem.*
- Growth-restricted females developed loss of glucose control when pregnant despite normal first and second phase insulin elevation in response to an IPGTT.
- HFD increased body and total fat weights and plasma leptin concentration in both Control and Restricted females at E20.
- HFD resulted in glucose intolerance in Control females and exacerbated glucose intolerance in Restricted females, during late gestation.
- Exercise before and during pregnancy prevented glucose intolerance in Restricted females by increasing insulin secretion.
- Exercise before and during pregnancy prevented the emergence and exacerbation of glucose intolerance by increasing pancreatic β-cell mass in both Control and Restricted females on HFD.
- Exercise during pregnancy only had no effect on metabolic function during late gestation in all groups.

This study demonstrated that HFD resulted in glucose intolerance in females born of normal birth weight. Importantly, females born small are at a greater risk of developing glucose intolerance when exposed to a HFD. The altered metabolic adaptation measured in both Control and Restricted females on a HFD, was prevented by the lifestyle intervention of exercise associated with improved β-cell mass but this was only effective when exercise occurred prior to and during pregnancy. Exercise initiated during pregnancy could not prevent metabolic disease in late gestation.

Chapter 4 Effects of Exercise and High Fat Diet on Cardiorenal Adaptations to Pregnancy in Females Born Small

The overall aim of this study was to determine if a HFD exacerbates the known adverse cardiorenal adaptations to pregnancy in rats born small and whether endurance exercise training can prevent these complications. The significant findings of this study were:

- Growth-restricted females had an increased in eGFR and urinary potassium excretion during late gestation.
- HFD caused an increase in eGFR and urinary potassium excretion in Control females during late gestation.
- HFD did not alter renal function in growth-restricted females during late gestation.
- Both exercise before and during pregnancy as well as exercise initiated during the second week of pregnancy had no effect on renal function during late gestation in all groups.
- Growth-restricted Chow-fed rats and both Control and growth-restricted females on a HFD had a greater reduction in systolic blood pressure during late gestation.
- Exercise before and during pregnancy prevented the reduction in systolic blood pressure during late gestation in growth-restricted Chow-fed rats, as well as both Control and growth-restricted females on a HFD.
- Exercise before and during pregnancy induced physiological cardiac hypertrophy in both Control and Restricted, irrespective of diet.
- Exercise during pregnancy only had no effects on systolic blood pressure and heart weight during late gestation in all groups.

In summary, pregnant females born small and on a HFD are at a greater risk of cardiorenal alterations during pregnancy. Although cardiovascular dysfunction was prevented by exercise prior to and during pregnancy, renal dysfunction was not affected by exercise interventions. This study suggests that exercise prior to and during pregnancy is more beneficial in preventing altered blood pressure adaptations than exercise during pregnancy only.

Chapter 5 Effects of Exercise and High Fat Diet on Fetal Outcomes

The overall aim of this study was to investigate the effects on the fetal outcomes in growth restricted mothers exposed to a high fat diet, endurance exercise training and physiological measurements that were performed during late gestation. The major findings of this study were:

- Male and female fetal and placental weights and placental efficiency were not affected by maternal birth weight at E20.
- Male and female fetal and placental weights and placental efficiency were not affected by maternal high fat feeding at E20.
- Exercise initiated before and continued during pregnancy increased fetal weight in both male and female fetuses of Control and Restricted mothers on a Chow diet, but not in fetuses of Control and Restricted mothers on a HFD.
- Exercise interventions had no effect on male placental weight but reduced placental weight of female fetuses of Control and Restricted mothers on a HFD.
- Male fetuses were heavier than female fetuses at E20.

Findings from this Chapter demonstrate that both intrauterine growth-restriction and HFD have no effects on fetal and placental weights in both male and female fetuses. These data highlight that exercise interventions had a differential effect on growth in male and female fetuses that were dependent upon the initiation of the exercise and diet. There was also a sex-specific difference in this current study with male fetuses reported to be heavier than female fetuses.

6.2 Intrauterine Growth Restriction and High Fat Diet on Pregnancy Adaptations

Pregnancy is a stressor in women which results in alterations in maternal physiology and metabolism to support fetal growth and development (Hill & Pickinpaugh, 2008). These changes are necessary to facilitate maternal cardiac output and to maintain uteroplacental perfusion of vital organs and fetal demands (Hill & Pickinpaugh, 2008). In some instances, the body does not adapt properly to these physiological changes to pregnancy, thereby resulting in pregnancy related maternal-fetal diseases particularly in growth-restricted and obese women (Klebanoff *et al.*, 1999; Seghieri *et al.*, 2002; Zetterstrom *et al.*, 2007; Mouzon & Lassance, 2015). However, most research into the detrimental impact of maternal growth restriction and obesity has primarily focused on the programming of cardiovascular and metabolic outcomes in the offspring. Studies have overlooked the vital role of adequate adaptations in the metabolic and cardiorenal systems during pregnancy particularly in growth restricted mothers that are overweight or obese. Studies in Chapter 3 and 4 of this thesis have addressed this gap in the field and demonstrated that growth restriction and obesity indeed impair the metabolic and cardiorenal adaptations that occur in pregnancies.

Chapter 3 demonstrated that growth-restricted females developed glucose intolerance during late gestation associated with the absence of β-cell deficits, altered intramuscular triglycerides and muscle mitochondrial biogenesis (Figure 6.1). In our previous study, non-pregnant growth-restricted females were reported to have β-cell deficits and subsequently reduced basal insulin secretion at 4 months of age (Gallo *et al.*, 2012b). Pregnancy changes upregulate pancreatic β-cells in growth-restricted females to match increases in insulin demand during late pregnancy (Gallo *et al.*, 2012b). However, similar to the present study, this did not protect growth-restricted females from adverse metabolic adaptations during pregnancy (Gallo *et al.*, 2012b). A novel finding that emerges from this study is that HFD exacerbated glucose intolerance in growth-restricted females and revealed glucose intolerance in females born of normal birth weight during late gestation. Given these findings, overweight or obese women with low birth weight will have an exacerbated risk of developing GDM during pregnancy compared to women who were obese but born of normal birth weight.

In humans, it is well established that there is a significantly increased risk of gestational hypertension and preeclampsia among women with GDM (Suhonen & Teramo, 1993; Ros *et al.*, 1998; Jensen *et al.*, 2000; Vambergue *et al.*, 2002; Bryson *et al.*, 2003; Schneider *et al.*, 2012). This is partly due to a higher degree of insulin resistance and glucose tolerance

demonstrated in women with GDM (Coustan, 2013). Indeed, recent studies support an association between preeclampsia and gestational hypertension with increased insulin resistance and insulin secretion in human pregnancy (Fuh *et al.*, 1995; Lorentzen *et al.*, 1998; Innes & Wimsatt, 1999). Hyperinsulinemia is associated with a reduction in urinary sodium excretion, increased plasma epinephrine, overproduction of very-low-density lipoprotein (VLDL) in the liver and by endothelial cells, decreased prostacyclin (PGI2) production and platelet activation (Berkowitz, 1998; Innes & Wimsatt, 1999). All these processes are involved in an increase in blood pressure (Berkowitz, 1998; Innes & Wimsatt, 1999). Other authors have also demonstrated that altered glucose control as a result of GDM causes arteriosclerosis and glomerular filtration dysfunction, which can result in a predisposition for preeclampsia and gestational hypertension (Heitritter *et al.*, 2005; Powe *et al.*, 2011; Guimaraes *et al.*, 2014). However, in the present study, systolic blood pressure and urinary protein excretion were not increased and eGFR was reduced in females that developed glucose intolerance during late gestation. Instead, we reported a greater reduction in systolic blood pressure, an increase in eGFR and no difference in urinary protein excretion in female rats that had glucose intolerance during late gestation (Figure 6.1). The current study suggests that altered cardiovascular and renal adaptations to pregnancy were contributed by different mechanisms that caused the emergence and exacerbation of glucose intolerance in growth-restricted females and females on a HFD during late gestation.

Of particular note, systolic blood pressure and renal function of growth-restricted females were not different despite reduced nephron number and early-onset glomerular hypertrophy during pregnancy (Gallo *et al.*, 2012b). In contrast, this present study demonstrated that growth-restricted females had reduced systolic blood pressure and increased in eGFR during late gestation. Although nephron number was not assessed in the current study, it was presumed to be decreased given the findings in our previously published data (Moritz *et al.*, 2009a; Gallo *et al.*, 2012b). The disparities in blood pressure and renal function between cohorts of the same model are potentially due to the higher degree of animal handling performed in the current study compared to an earlier study (Gallo *et al.*, 2012b). Specifically, in this study, all female rats were handled daily throughout pregnancy for exercise training. Importantly, both Control and growth-restricted females on a HFD also exhibited reduced systolic blood pressure during late gestation (Figure 6.1). It is extrapolated that this reduction in blood pressure is possibly due to alterations in pregnancy hormones and/or systemic vascular resistance which may have delayed the blood pressure adaptation to pregnancy. Indeed, a human study reported that high serum concentrations of progesterone and relaxin during early trimester were related to lower mean systolic blood pressures in the second and third trimester (Kristiansson & Wang, 2001). Therefore, measurements of systolic blood pressure and pregnancy hormones during early

pregnancy and post-pregnancy, with more dynamic time-points, may reveal differences in cardiovascular function in these pregnant females.

Chapter 4 also reported that eGFR was increased in growth-restricted females and females on a HFD during late gestation. Substantial changes in the kidney hemodynamics during pregnancy may have unmasked the glomerular hyperfiltration in the kidneys of these rats which we reported in Chow-fed Restricted pregnant rats (Gallo *et al.*, 2012b). The increased in eGFR was possibly contributed to by the increased glomerular size, as a compensatory mechanism due to low nephron number in these rats. As both intrauterine growth restriction and HFD independently increased eGFR, it was expected that the combined effect of IUGR and HFD would exacerbate glomerular hyperfiltration. Interestingly, this study reported no difference in eGFR in growth-restricted females on a HFD during late gestation. It is likely that the lack of difference in eGFR in these rats was merely a protective adaptation to adverse renal changes during pregnancy, which is contributed to by a further increase in the glomerular size and volume to meet pregnancy demands. However, due to time constraints, glomerular size was not characterized in this study and, thus, will be important to investigate in the future.

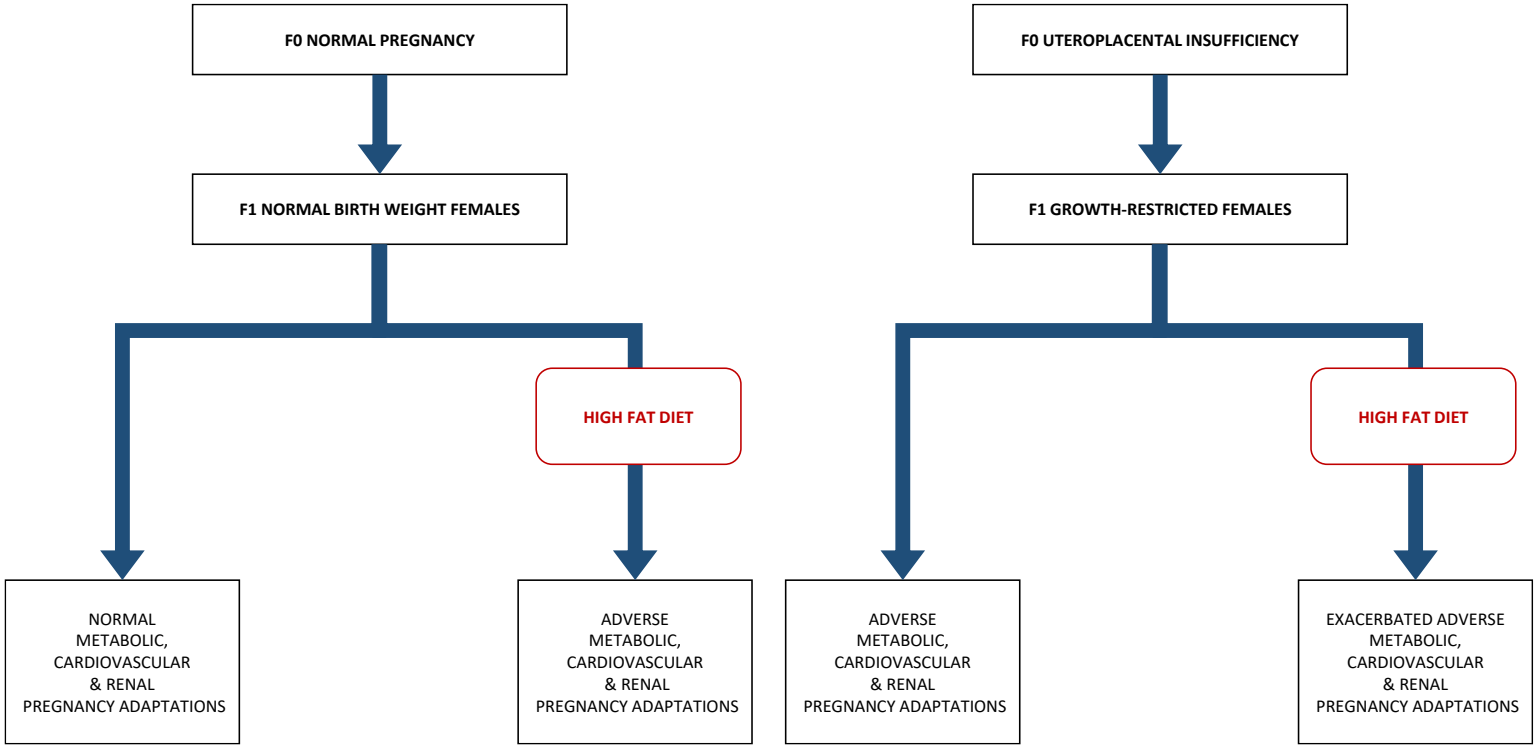

Figure 6.1 The effects of intrauterine growth restriction and high fat diet on metabolic and cardiorenal adaptations to pregnancy

Growth-restricted females and Control females on a HFD demonstrated altered metabolic and cardiorenal adaptations to pregnancy during late gestation. Importantly, HFD was reported to exacerbate adverse metabolic and cardiorenal adaptations to pregnancy.

6.3 Animal Model of Gestational Diabetes Mellitus

We previously demonstrated that growth-restricted virgin females were protected from developing glucose intolerance (at 4 months) (Gallo *et al.*, 2012b). Adaptations in pregnancy lead to impaired glucose intolerance during late gestation in the age-matched growth-restricted pregnant females (Gallo *et al.*, 2012b). Similarly, in current study, we reported a similar finding with growth-restricted females developing glucose intolerance during late gestation in the absence of obesity. This phenotype of the emergence of glucose intolerance for the first time during pregnancy is consistent with the human condition of GDM. Our rat model of GDM is therefore ideal to study the impact of treatments to prevent GDM and its consequences to the mother and offspring. Importantly, our model is not confounded by the non-physiological approaches used to induce GDM in other models.

Gestational diabetes mellitus is defined as diabetes diagnosed during pregnancy that is not overt diabetes (American Diabetes Association, 2014). In addition to the acute health concerns of hyperglycemia, women diagnosed with GDM during pregnancy have an increased incidence of complications during pregnancy as well as an increased risk of developing type 2 diabetes (T2D) later in life (Carr *et al.*, 2008; Retnakaran *et al.*, 2009). Furthermore, children born to mothers diagnosed with GDM have increased incidence of perinatal complications, including hypoglycaemia, respiratory distress syndrome, and macrosomia, as well as an increased risk of being obese or developing T2D as adults (Crowther *et al.*, 2005; Dabelea, 2007; Damm, 2009; Landon *et al.*, 2009).

Although there are many animal models of type 2 diabetes, not many of them can be utilised to study GDM. A variety of risk factors, including weight, ethnicity, genetics, and family history contribute to the likelihood of developing GDM, making the generation of animal models that fully recapitulate the disease difficult. Animal models established using Streptozotocin (STZ) protocol, through continuous infusion of glucose and diet-induced obesity in pregnancy are the widely used animal models to investigate GDM (Lopez-Soldado & Herrera, 2003; Holemans *et al.*, 2004). However, it has been reported that STZ causes a permanent ablation of pancreatic β-cell and insulin deficiency (Guz *et al.*, 2001). Thus STZ administration does not interrogate the β-cell compensatory mechanisms and physiology that underlie GDM. Additionally, STZ-induced models of diabetes during pregnancy often result in severe elevation of blood glucose, whereas GDM is characterized by more mild glucose intolerance (Lopez-Soldado & Herrera, 2003).

Obesity and HFD are known risk factors for GDM among pregnant women (Jensen *et al.*, 2000; Sebire *et al.*, 2001; Cunningham & Teale, 2013). As the ability of HFD to disrupt β-cell function and confer insulin resistance and diabetes is also a well-established phenomenon in animal models, several models of GDM have been created using high-fat feeding in pregnant animals (Holemans *et al.*, 2004; Ford *et al.*, 2009; Liang *et al.*, 2010; Musial *et al.*, 2017). However, in this model the first onset of glucose intolerance was not consistent across the studies and some studies even reported the first onset of glucose intolerance before pregnancy and thus, does not accurately represent most cases of GDM in human (Holemans *et al.*, 2004; Ford *et al.*, 2009; Liang *et al.*, 2010; Musial *et al.*, 2017). Furthermore, all HFD-based models of GDM create a condition similar to type 2 diabetes where peripheral insulin resistance and increased adiposity are strong drivers of disease (Holemans *et al.*, 2004; Ford *et al.*, 2009; Liang *et al.*, 2010; Musial *et al.*, 2017). While some patients may present with GDM due to pre-existing obesity, many manifest with the disease despite being lean prior to pregnancy. Another GDM animal model utilises continuous glucose infusions directly into pregnant dams during the last week of pregnancy resulting in hyperglycemia and hyperinsulinemia (Gauguier *et al.*, 1990). However, the timing of glucose infusions during late gestation makes it impossible to investigate the underlying mechanism of GDM in the mother but is beneficial to examine the effect of maternal hyperglycemia on the offspring (Gauguier *et al.*, 1990). Therefore, continued development of animal models of GDM is essential for understanding the consequences of this disease as well as providing insights into potential treatments and preventative measures.

6.4 Intrauterine Growth Restriction and High Fat Diet on Long-term Maternal Health and F2 Offspring Outcomes

GDM is one of the most frequent complications of pregnancy that may lead to considerable risk for both the fetus and the mother (Soheilykhah *et al.*, 2011). If untreated, it may progress to type 2 diabetes later in life (Carr *et al.*, 2008; Retnakaran *et al.*, 2008, 2009). A systemic review reported that the cumulative incidence of type 2 diabetes ranged from 2.6% to over 70% in studies that examined women with GDM 6 weeks to 28 years postpartum (Kim *et al.*, 2002). The increased risk of future diabetes risk is directly proportional to the degree of gestational dysglycemia, such that women who develop severe glucose intolerance during gestation incur the greatest risk (Carr *et al.*, 2008; Retnakaran *et al.*, 2008, 2009). Importantly, the risk for type 2 diabetes is further exacerbated by obesity and excessive weight gain during pregnancy (Durnwald, 2015). Our previous study has demonstrated that despite loss of glucose tolerance at 4 months of age during pregnancy, growth-restricted females exhibited normal glucose function later in life at 13 months of age (Tran *et al.*, 2012). Besides type 2 diabetes, women with a history of GDM are also at risk of developing several other traditional cardiovascular risk factors, including hypertension, dyslipidemia, obesity and metabolic syndrome (Bentley-Lewis, 2009; Sullivan *et al.*, 2012; Vrachnis *et al.*, 2012; Xu *et al.*, 2014). The additional insult of a HFD that exacerbated glucose intolerance in growth-restricted females during late gestation in the current study may increase the chance of developing adverse metabolic control later in life. Therefore, future studies should investigate growth-restricted females on a HFD post pregnancy to determine if the HFD unmasks the metabolic and cardiovascular diseases later on in their lives.

While affected women with GDM have an increased risk of developing type 2 diabetes and cardiovascular disease in the years to come (Carr *et al.*, 2008; Retnakaran *et al.*, 2008, 2009), the most pressing concern at the time of diagnosis is that GDM is associated with an increased risk of adverse obstetrical outcomes, including macrosomia, shoulder dystocia, birth injury, prematurity, perinatal mortality, and the need for caesarian section (King, 1998; Buchanan & Xiang, 2005). The common feature underlying these risks is fetal overgrowth, which may be partly driven by maternal hyperglycemia. Specifically, maternal hyperglycemia leads to fetal hyperglycemia, which stimulates fetal insulin secretion. While the metabolic effects of this insulin secretory response will lower blood glucose levels in the fetus, the concomitant anabolic effects of insulin can cause excessive fetal growth. First proposed by Pedersen in 1951, this model of fuel-mediated macrosomia has since been supported through the work of numerous investigators and is well-accepted as the basis for macrosomic risk in diabetes in pregnancy (Macfarlane & Tsakalakos, 1988; King, 1998; Buchanan & Xiang, 2005). An

additional possibility is that changes in placental nutrient transfer capacity and metabolism contribute to fetal overgrowth in diabetic pregnancies. Increased placental weights and fetal to placental ratios have been reported in pregnancies complicated by GDM (Retnakaran *et al.*, 2009), even in the presence of optimal maternal glycemic control throughout the third trimester (King, 1998). The increased placental mass could then augment placental nutrient exchange by increasing the surface area available for substrate transfer.

However, Chapter 5 demonstrated no changes in fetal and placental weights of the Chow-fed growth-restricted mothers and Control and Restricted mothers on a HFD that developed glucose intolerance during late gestation. It is important to note that fetal weight in the current study was measured at E20; thus, the effects of growth restriction and HFD on birth weight are unknown and need to be explored in future studies. Although no difference in fetal and placental weights was reported, it is possible that these offspring will still have high risk of developing adult diseases later in life as reported in human studies (Pettitt *et al.*, 1991; Dabelea, 2007; Damm, 2009). Indeed, studies in rats have recently shown altered glucose and insulin metabolism in F2 offspring that were born of normal weight, from mothers exposed to dietary protein restriction throughout pregnancy (Zambrano *et al.*, 2005; Torrens *et al.*, 2008). In fact, studies from our lab also reported that F2 offspring whose mothers were born small at birth had elevated blood pressure, reduced first-phase insulin response and altered pancreatic β-cell mass despite no difference in their birth weight (Tran *et al.*, 2013). This suggests that fetal weight does not always correlate with transgenerational disease outcomes.

6.5 Effects of Exercise Timing on Pregnancy Adaptations

The American College of Obstetrics and Gynecologists recommends that pregnant women should engage in moderate intensity exercise at least 20 to 30 minutes on most or all days of the week (ACOG Committee Obstetric Practice, 2015). This is largely due to an increasing number of studies that reported positive outcomes of exercise training on both maternal and fetal health. Epidemiological studies have shown that exercise is associated with reduced risk of GDM (Dempsey, 2004; Oken *et al.*, 2006; Zhang *et al.*, 2006; Tobias *et al.*, 2011; Barakat *et al.*, 2012), excessive gestational weight gain (Stuebe *et al.*, 2009; Barakat *et al.*, 2012) and pregnancy-induced hypertension and preeclampsia. In the present study, the endurance exercise training protocol that was conducted most closely represents a woman who does moderate intensity aerobic exercise 4-5 days/week, consistent with American College of Obstetricians and Gynecologists recommendations (Artal & O'Toole, 2003).

In Chapter 3, exercise training initiated 4 weeks before and continued during pregnancy prevented the development of glucose intolerance in growth-restricted females by increasing glucose-stimulated insulin secretion (Figure 6.2). During pregnancy, maternal insulin demands increase due to the insulin resistance state. Maternal islets adapt to this increased demand through enhanced insulin secretion per β-cell (Retnakaran *et al.*, 2008). Exercise performed before and during pregnancy in this study may further enhance the insulin secretion in growth-restricted females and thus protect them from developing adverse metabolic function during pregnancy. Another significant finding from this study was exercise training initiated before and continued during pregnancy prevented the development and exacerbation of glucose intolerance in Control and Restricted females on a HFD, respectively. The prevention of glucose intolerance during late gestation in these rats was mainly contributed by increase in β-cell mass. β-cell mass is tightly regulated through a balance of β-cell birth through β-cell proliferation and islet neogenesis from precursor cells and β-cell death through apoptosis (Taylor, 1999). Evidence from epidemiological and animal studies demonstrated that exercise has beneficial effect on both of these mechanisms (Narendran *et al.*, 2015). Physical activity increases circulating levels of growth hormone, insulin-like growth factor 1, placental lactogen, glucagon-like peptide 1, interleukin-6 and interleukin-1 receptor agonist, all of which are thought to have a direct and indirect effect on β-cell proliferation (Ronsen *et al.*, 2002; Choi *et al.*, 2006; Park *et al.*, 2007; Narendran *et al.*, 2015). Furthermore, exercise reduces β-cell death through reduction in plasma glucose and serum lipids (Solomon *et al.*, 2009; Karstoft *et al.*, 2013) as chronic exposure of pancreas islets to hyperglycemia induces inflammation, which impairs glucose-stimulated insulin secretion and augments β-cell apoptosis (Donath *et al.*, 1999). Thus, exercise performed before and during pregnancy in this study may augment

pancreatic β-cell mass in Control and Restricted on a HFD through an increased in β-cell proliferation and decreased in β-cell apoptosis.

Chapter 4 demonstrated that exercise initiated prior to and continued during pregnancy resulted in increased heart weight by ~10% in all groups compared with their sedentary counterparts. The magnitude of this change suggests that the increased in heart weights in these rats was likely exercise-induced physiological cardiac hypertrophy, which would likely be accompanied by functional benefits (Hickson *et al.*, 1979; Hickson *et al.*, 1983). Similar outcomes were also demonstrated in our previous study, in which four weeks of exercise early and later in life increased absolute and relative heart mass at 24 weeks of age in both Control and Restricted male rats in the presence of normal blood pressure (Wadley *et al.*, 2016). Importantly, this study also reported that exercise training before and during pregnancy prevented the greater reduction in systolic blood pressure during late gestation when compared with pre-pregnancy in Chow-fed Restricted females and both Control and Restricted females on a HFD (Figure 6.2). However, the mechanisms that contributed to the prevention of adverse blood pressure to pregnancy in these rats are unknown but it is potentially contributed by exercise-induced cardiac hypertrophy demonstrated in these rats. Although exercise performed before and during pregnancy exhibited beneficial outcomes in metabolic and cardiovascular function of growth-restricted females and females on a HFD, no exercise effects were reported in their renal function. This finding is in contrast to human studies as most of the studies reported beneficial effects of exercise in preventing adverse renal adaptations during pregnancy (Sorensen *et al.*, 2003; Saftlas *et al.*, 2004; Rudra *et al.*, 2008). Crucially, we also demonstrate that exercise initiated during the second week of pregnancy had no effect on the adverse metabolic and cardiorenal phenotypes in both Control and Restricted females (Figure 6.2). Similar outcomes were reported in human studies in which the beneficial effects of exercise on GDM and hypertension prevention are limited to women who engaged in exercise prior to and/or during early pregnancy than those who started exercise during the second and third trimesters (Dempsey, 2004; Oken *et al.*, 2006; Zhang *et al.*, 2006; Redden *et al.*, 2011; Stafne *et al.*, 2012; Nobles *et al.*, 2015). These findings suggest that the beneficial effects of exercise on the metabolic and cardiorenal functions are more apparent when initiated prior to pregnancy. It is likely that pregnancy adaptations have already occurred during early gestation; thus, exercise initiated during pregnancy has no effects on the pregnancy adaptations. Initiation of exercise earlier in pregnancy or of a higher intensity, duration and frequency may have different results. Therefore, for future studies, it will be important to determine the timing of exercise interventions that are beneficial in programming the increase in pancreatic β-cell mass and exercise-induced cardiac hypertrophy.

The implications of exercise training on fetal development are controversial, particularly when the intensity is considered (Brown *et al.*, 2017). Evidence suggests that there is no correlation between exercise and reduced fetal weights if the exercise intensity is light or moderate (Spinillo *et al.*, 1996; Hjollund *et al.*, 2000; Brown, 2002). However, there are studies that have confirmed an association between intense exercise and reduced fetal weights (Spinillo *et al.*, 1996; Artal & Sherman, 1999), while other studies have disagreed with such findings (Sternfeld *et al.*, 1995; Kardel & Kase, 1998). Conversely, others have reported that exercise is related to increased birth weight (Hall & Kaufmann, 1987; Hatch *et al.*, 1998). These conflicting results are possibly related to the intensity of the maternal exercise, familiarity of the animal to the type of exercise, and differences in the type of species used. In the present study, Chapter 5 demonstrated that exercise initiated before and continued during pregnancy increased fetal weight of Control and Restricted mothers on a Chow diet. Mechanisms that contributed to the increased in fetal weight are potentially mediated by the placenta as studies from our lab reported changes in placental insulin-like growth factor system and placental glycogen storage in mothers that exercised before and during pregnancy (Mangwiro & Wlodek, unpublished). However, whether the increase in fetal weight translates to higher birth weight and lead to beneficial or detrimental offspring outcomes remains to be established. Similar to metabolic and cardiorenal outcomes, no difference in fetal and placental weights was detected in the mothers that initiated exercise during the second week of pregnancy.

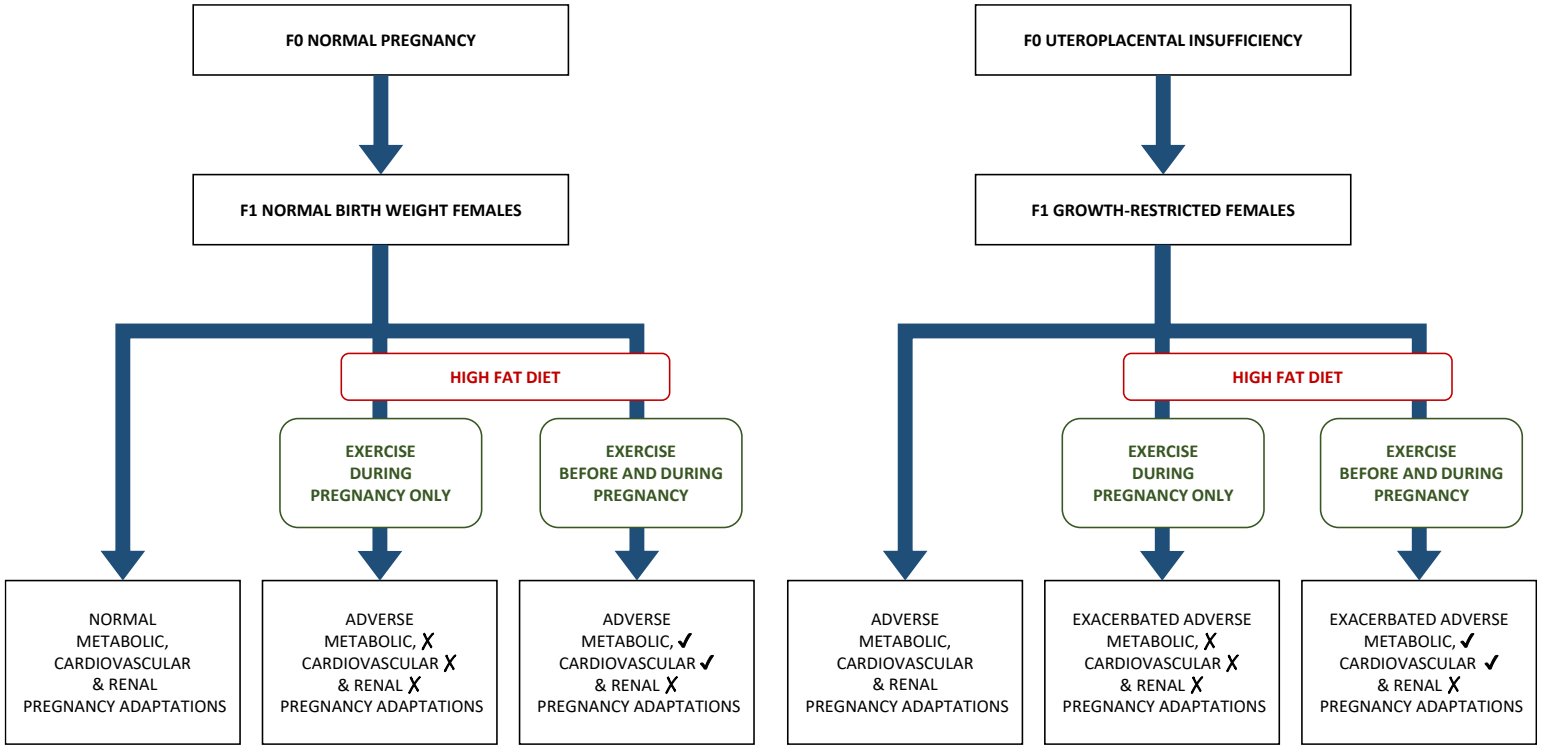

Figure 6.2 The effects of exercise on pregnancy adaptations in growth-restricted females and females on a HFD

Exercise initiated before and continued during pregnancy prevented the emergence and exacerbation of glucose intolerance in chow-fed growth-restricted females and Control and Restricted females on a HFD. Crucially, exercise before and during pregnancy also prevented the altered systolic blood pressure adaptations to pregnancy in chow-fed growth-restricted females and Control and Restricted females on a HFD. However, exercise had no effect on renal function during late gestation in both Control and Restricted irrespective of diet. Exercise initiated during pregnancy had no effect on metabolic and cardiorenal adaptations to pregnancy in all groups. ✓ indicates adverse pregnancy adaptations were prevented with exercise and ✗ indicates adverse pregnancy adaptations were not prevented with exercise.

6.6 Future Directions and Limitations

One of the limitations of this study was HFD that was given to the rats was only able to slightly increase their body weight. Although, the body weight between Chow-fed and High fat-fed rats was significantly difference, the percentage increase was only 12% and therefore, they were not considered as obese. This was perhaps due to the percentage of total fat in high fat pellet diets (43% kcals from fat) used in this study was not high enough to largely increase their body weight at *post mortem* and thus maybe longer durations of high fat feeding was needed to induce obesity. Future studies should use higher fat content to see a large increased in body weight in high fat-fed rats. Indeed, there were studies that utilized high fat diets with 60% kcals from fat to induce obesity in rodents and reported that the animals gain more weight more quickly (Ghibaudi *et al.*, 2002; Johnston *et al.*, 2007). Of particular note, the main finding of this study was that exercise initiated before and continued during pregnancy was more beneficial in preventing altered metabolic and cardiorenal adaptations during pregnancy than exercise initiated during pregnancy. However, the drawback of this study was that the effect of exercise training before pregnancy only was not investigated. Future studies could examine the effect of exercise interventions before pregnancy only on the metabolic and cardiorenal pregnancy adaptations as epidemiological studies reported benefical effects of prepregnancy exercise on GDM (Dempsey, 2004; Tobias *et al.*, 2011) and preeclampsia (Rudra *et al.*, 2008).

Importantly, the mechanisms underlying the programming effect of exercise on the heart and pancreas have yet to be identified. Perhaps future studies should also examine cardiac and β-cell proliferation and apoptotic measures at different time points of pregnancy and with exercise, which may help to identify critical stages during pregnancy that are particularly susceptible to intervention. Due to funding, time constraints and technical limitations for the pancreas analysis in our laboratory, pancreatic islets were not isolated for gene and protein analysis and should be considered in future studies to identify the molecular mechanisms underlying the morphological changes in β-cell mass with exercise initiated before and continued during pregnancy. Additionally, hormones that circulate in high concentrations in pregnancy such as progesterone, cortisol, prolactin, placental lactogen and estrogen have all been reported to influence β-cell function and/or the peripheral tissue sensitivity to insulin. Of particular interest, prolactin and placental lactogens are the two main stimuli that directly involve in β-cell proliferation during pregnancy (Parsons *et al.*, 1992). As exercise before and during pregnancy in the current study increased pancreatic β-cell mass during late gestation, future studies should examine circulating prolactin and placental lactogens as well as β-cell prolactin receptor to determine if these hormones play a role.

As one of the aims of this study was to investigate the effects of growth-restriction, HFD and exercise interventions on the fetal outcomes, many important measurements such as insulin resistance by insulin

challenge and echocardiography that required fasting and anaesthetisation were not conducted on the mothers. Future studies should address these gaps to further characterize their altered metabolic and cardiorenal adaptations during pregnancy. Future studies could also measure whole-body insulin sensitivity by the gold standard euglycaemic clamp technique to provide us with a more accurate measure of insulin resistance (DeFronzo *et al.*, 1979). This involves a steady-state concentration of exogenous insulin infusion with a simultaneous glucose infusion. Glucose would be infused at a rate sufficient to prevent an insulin-induced fall in glucose concentration. The amount of glucose required to maintain basal plasma glucose levels will provide an indication of the level of insulin resistance, with lower glucose infusion rates indicating insulin resistance and vice versa.

One aspect missing in the characterization of cardiovascular adaptation in this study was measurement of cardiac structure and function during pregnancy. In this study, we elected to freeze the hearts for future molecular studies; thus, characterisation of cardiomyocyte number and heart structure are not possible. Future studies should also examine whether stroke volume and cardiac output were also impacted by growth-restriction, obesity and exercise interventions. Assessing cardio-renal function early in pregnancy, during a more dynamic period of reduced vascular resistance, may also identify cardiovascular adaptations during pregnancy. It is important to note that systolic blood pressure was measured using tail-cuff plethysmography to obtain repeated measures in the same animals at different time points from this large cohort of rats. The tail cuff approach allows for non-invasive, inexpensive and high throughput measures of systolic blood pressure. However, it tends to overestimate and distort responses as well as exert stress upon the animal (Van Vliet *et al.*, 2000). Future studies will require more rigorous methods such as tail artery catheter or telemetry studies to more accurately characterise the effect of uteroplacental insufficiency, HFD and exercise training on blood pressure, as this was not practical for this long-term study. Recently, study from our laboratory demonstrated that growth-restricted females exhibited uterine artery-specific endothelial vasodilator dysfunction and increased wall stiffness (Mazzuca *et al.*, 2010) that may, in turn, compromise their cardiovascular adaptations to pregnancy. Therefore, future studies should characterise mesenteric and uterine artery function to determine whether the reduction of blood pressure during late gestation in the current study was related to vascular dysfunction.

The impact of HFD and exercise interventions on glomerular number and size in this study was unknown and thus require further investigations. Although it was presumed that growth-restricted females had nephron deficit and glomerular hypertrophy given the findings in our previously published data (Moritz *et al.*, 2009a; Gallo *et al.*, 2012b), it is important to confirm the findings as both cohorts had different growth trajectories. Importantly, glomerular filtration rate was estimated by calculating the creatinine clearance from plasma creatinine and a 24 hours urine collection. However, studies have reported that the use of creatinine clearance to estimate GFR may not reflect the actual degree of kidney

function of a particular subject (Schwartz & Furth, 2007). Therefore, future studies should measure GFR by the gold standard inulin clearance technique.

6.7 Concluding Remarks

The major findings of this thesis are that growth-restricted females demonstrated loss of glucose tolerance during late gestation compared with Control indicative of a GDM phenotype. When challenged with HFD, glucose intolerance in growth-restricted was exacerbated during late gestation. This study also demonstrated that a HFD revealed glucose intolerance in females born of normal birth weight. The exacerbation and development of glucose intolerance in Restricted and Control females, respectively, were present despite having normal pancreatic β-cell mass and insulin secretion during late gestation. Importantly, exercise initiated prior to and continued during pregnancy prevented the development of glucose intolerance in growth-restricted females on a Chow diet by increasing glucose-stimulated insulin secretion. Additionally, the development and exacerbation of glucose intolerance in Control and Restricted females, respectively, were also prevented when these females exercised before and during pregnancy through increases in pancreatic β-cell mass. However, metabolic dysfunction in Control and Restricted females during late gestation was not improved by exercise initiated during pregnancy. Similarly, growth-restriction and HFD limit the renal and cardiovascular adaptations normally observed in pregnancy. Intrauterine growth restriction and HFD independently altered renal functions during late gestation. Interestingly, the combined effect of IUGR and HFD was reported to have no difference in GFR possibly as a protective adaptation to adverse renal changes during pregnancy. However, both exercise before and during pregnancy as well as exercise initiated during the second week of pregnancy had no effect on renal functions in this study. Of particular importance, growth-restricted Chow-fed rats, and both Control and growth-restricted females on a HFD had an adverse cardiovascular adaptation to pregnancy with a greater reduction in systolic blood pressure during late gestation. Importantly, exercise before and during pregnancy prevented this adverse cardiovascular adaptation possibly by inducing physiological cardiac hypertrophy. Although growth-restriction and HFD affect maternal metabolic and cardiorenal pregnancy adaptations, no effects on fetal and placental weights were reported. There was a sex-specific difference in the current study with male fetuses reported to be heavier than female fetuses, despite no difference in placental weight. Importantly, exercise initiated before and during pregnancy increased fetal weight in both male and female fetuses of mothers on a Chow diet, but not in mothers on a HFD.

Overall this thesis provides valuable insight into the clinical setting, as GDM in pregnant women is associated with an increased risk of maternal and neonatal morbidity and remains a significant obstetric challenge. Importantly, a significant increase over the past few decades in the number of reproductive age women who are either overweight or obese increases the need for targeted interventions. The findings in this thesis demonstrated for the first time that exercise prior to and during pregnancy prevents the development of glucose intolerance and altered blood pressure adaptation to pregnancy in growth-restricted and obese females during late pregnancy. The results of this thesis suggest that

exercise training would be beneficial for maternal pregnancy adaptations in a high-risk population particularly in growth-restricted and/or obese females. However, the exercise interventions need to be initiated before pregnancy to observed major benefits. Thus, by implementing effective interventions in those at high risk will prevent adverse outcomes during pregnancy and in later life. This will not only reduce the costs involved with long-term health care for both women and their babies but importantly, reduce this perpetuating cycle of metabolic disease risk to future generations and decrease the rate of mortality associated with type 2 diabetes.

CHAPTER 7

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Appendix 1

Composition of Chow and High Fat Diets

Specialty Feeds

3150 Great Eastern Hwy
Glen Forrest
Western Australia 6071
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F: +61 8 9298 8700
Email: info@specialtyfeeds.com

Diet
AIN93G — **Standard AIN93G Rodent Diet**

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G. This formulation satisfies the nutritional requirements for growth of rats and mice. Some modifications have been made to the original formulation to suit locally available raw materials.

- We have evidence that vitamin losses and other changes to the diet can occur when irradiated at 25KGy. The diet SF09-091 is formulated with extra vitamins to allow for losses during the irradiation process. Please contact us for more information if the diet is to be irradiated.

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Calculated Nutritional Parameters	
Protein	19.4%
Total Fat	7.0%
Total Carbohydrate	56.9%
Crude Fibre	4.7%
AD Fibre	4.7%
Digestible Energy	16.1 MJ / Kg
% Total calculated digestible energy from lipids	16.0%
% Total calculated digestible energy from protein	21.0%
Ash	4.5%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients	
Casein (Acid)	200 g/Kg
Sucrose	100 g/Kg
Canola Oil	70 g/Kg
Cellulose	50 g/Kg
Wheat Starch	404 g/Kg
Dextrinised Starch	132 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Amino Acids	
Valine	1.26%
Leucine	1.80%
Isoleucine	0.87%
Threonine	0.79%
Methionine	0.84%
Cystine	0.05%
Lysine	1.49%
Phenylanine	0.99%
Tyrosine	1.04%
Tryptophan	0.27%
Histidine	0.60%

Calculated Total Minerals	
Calcium	0.47%
Phosphorous	0.35%
Magnesium	0.08%
Sodium	0.15%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	68 mg/Kg
Copper	7.0 mg/Kg
Iodine	0.2 mg/Kg
Manganese	19 mg/Kg
Cobalt	No data
Zinc	46 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	3.3 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	78 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	103 ug/Kg
Choline	1 470 mg/Kg

Calculated Fatty Acid Composition	
Myristic Acid 14:0	trace
Palmitic Acid 16:0	0.30%
Stearic Acid 18:0	0.14%
Palmitoleic Acid 16:1	0.02%
Oleic Acid 18:1	3.89%
Gadoleic Acid 20:1	0.07%
Linoleic Acid 18:2 n6	1.51%
a Linolenic Acid 18:3 n3	0.98%
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	No data
DHA 22:6 n3	No data
Total n3	0.98%
Total n6	1.51%
Total Mono Unsaturated Fats	3.98%
Total Polyunsaturated Fats	2.50%
Total Saturated Fats	0.50%

Specialty Feeds

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Diet SF03-020

23% Fat, High Simple Carbohydrate 0.19% Cholesterol Semi-Pure Rodent Diet

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G.

- The fat content has been increased to 23%, sucrose content has been increased to improve pellet strength and starch content has been reduced.
- Cholesterol has been added at 0.19%.
- We have evidence that vitamin losses and other changes to the diet can occur during the irradiation process at 25KGy. Please contact us for more information if the diet is to be irradiated.

Calculated Nutritional Parameters

Protein	19.40%
Total Fat	23.00%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	20 MJ / Kg
% Total calculated digestible energy from lipids	43.00%
% Total calculated digestible energy from protein	17.00%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 1.5 Kg, vacuum packed in oxygen- impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave. Note, Irradiation can soften pellets.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients

Casein (Acid)	200 g/Kg
Sucrose	424g/Kg
Canola Oil	50 g/Kg
Cocoa Butter	50 g/Kg
Hydrogenated Vegetable Oil (Copha)	131 g/Kg
Cellulose	50 g/Kg
Pregelled Wheat Starch	50 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg
Cholesterol USP	1.9 g/Kg

Calculated Amino Acids	
Valine	1.26%
Leucine	1.80%
Isoleucine	0.90%
Threonine	0.80%
Methionine	0.80%
Cystine	0.06%
Lysine	1.50%
Phenylanine	1.00%
Tyrosine	1.00%
Tryptophan	0.30%
Histidine	0.60%

Calculated Total Minerals	
Calcium	0.47%
Phosphorous	0.32%
Magnesium	0.09%
Sodium	0.12%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.22%
Iron	73 mg/Kg
Copper	7.1 mg/Kg
Iodine	0.2 mg/Kg
Manganese	19 mg/Kg
Cobalt	No data
Zinc	52 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	2.1 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	80 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7.2 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	103 ug/Kg
Choline	1 470 mg/Kg

Calculated Fatty Acid Composition	
Saturated fats C12 or Less	6.77%
Myristic Acid 14:0	1.80%
Palmitic Acid 16:0	3.11%
Stearic Acid 18:0	3.05%
Oleic Acid 18:1	5.70%
Gadoleic Acid 20:1	0.07%
Linoleic Acid 18:2 n6	1.50%
a Linolenic Acid 18:3 n3	0.74%
EPA 20:5 n3	No data
DHA 22:6 n3	No data
Total n3	0.74%
Total n6	1.50%
Total Saturated Fats	14.93%
Total Monosaturated Fats	5.89%
Total Polyunsaturated Fat	2.24%
Cholesterol	0.19%

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Specialty Feeds

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Diet
SF01-028

23% Fat Semi-Pure Rodent Diet
43% of Energy From Fat

A high fat semi-pure modification of AIN93G.

- Fat content has been increased from around 7% in AIN93G to 23%.
- Calculated energy has increased by around 24% over the base diet. 40% of the total calculated energy is from lipids.
- The triglyceride profile has an increased proportion of saturated and mono-unsaturated fatty acids over the standard diet.
- Other nutritional parameters have remained unchanged.
- The high fat content has resulted in a significant reduction in pellet hardness. The pellets must be handled with great care to avoid breakage.

Calculated Nutritional Parameters

Protein	19.00%
Total Fat	22.60%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	19.9 MJ / Kg
% Total calculated digestible energy from lipids	43.00%
% Total calculated digestible energy from protein	17.00%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 1.5 Kg, trays vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients

Casein (Acid)	200 g/Kg
Sucrose	388 g/Kg
Canola Oil	48 g/Kg
Cocoa Butter	180 g/Kg
Cellulose	50 g/Kg
Wheat Starch	90 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Amino Acids	
Valine	1.10%
Leucine	1.70%
Isoleucine	1.00%
Threonine	0.70%
Methionine	0.70%
Cystine	0.05%
Lysine	1.50%
Phenylanine	0.90%
Tyrosine	1.00%
Histidine	0.60%
Tryptophan	0.10%

Calculated Total Minerals	
Calcium	0.45%
Phosphorous	0.30%
Magnesium	0.09%
Sodium	0.11%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	70 mg/Kg
Copper	6.8 mg/Kg
Iodine	0.2 mg/Kg
Manganese	18 mg/Kg
Cobalt	No data
Zinc	50 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	3.4 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	75 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	100 ug/Kg
Choline	1 700 mg/Kg

Calculated Fatty Acid Composition	
Staurated Fats C12:0 and less	0.09%
Myristic Acid 14:0	0.04%
Palmitic Acid 16:0	4.79%
Stearic Acid 18:0	6.55%
Arachidic Acid 20:0	0.21%
Palmitoleic Acid 16:1	0.04%
Oleic Acid 18:1	8.73%
Gadoleic Acid 20:1	0.08%
Linoleic Acid 18:2 n6	1.50%
a Linolenic Acid 18:3 n3	0.55%
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	Trace
DHA 22:6 n3	No data
Total n3	0.58%
Total n6	1.50%
Total Mono Unsaturated Fats	8.85%
Total Polyunsaturated Fats	2.09%
Total Saturated Fats	11.80%

Calculated data uses information from typical raw material composition. **Diet post treatment by irradiation or auto clave could change these parameters**. It could be expected that individual batches of diet will vary from this figure. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Appendix 2

Publication

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REVIEW

Maternal obesity in females born small: Pregnancy complications and offspring disease risk

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Obesity is a major public health crisis, with 1.6 billion adults worldwide being classified as overweight or obese in 2014. Therefore, it is not surprising that the number of women who are overweight or obese at the time of conception is increasing. Obesity during pregnancy is associated with the development of gestational diabetes and preeclampsia. The developmental origins of health and disease hypothesis proposes that perturbations during critical stages of development can result in adverse fetal changes that leads to an increased risk of developing diseases in adulthood. Of particular concern, children born to obese mothers are at a greater risk of developing cardiometabolic disease. One subset of the population who are predisposed to developing obesity are children born small for gestational age, which occurs in 10% of pregnancies worldwide. Epidemiological studies report that these growth-restricted children have an increased susceptibility to type 2 diabetes, obesity, and hypertension. Importantly during pregnancy, growth-restricted females have a higher risk of developing cardiometabolic disease, indicating that they may have an exacerbated phenotype if they are also overweight or obese. Thus, the development of early pregnancy interventions targeted to obese mothers may prevent their children from developing cardiometabolic disease in adulthood.

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1 Introduction

Obesity is clinically defined as having a BMI over 30 (World Health Organization (WHO) 2015; http://www.who.int/mediacentre/factsheets/fs311/en/), and is associated with an increased risk of developing a number of comorbidities, including cardiovascular and metabolic diseases and nephropathy [1]. A recent report by McKinsey Global Institute stated that obesity is considered one of the top three global social burdens generated by human beings, along with smoking and armed violence, and it is estimated that $2.0 trillion USD is spent worldwide annually as a result of obesity (McKinsey Global Institute 2014; http://www.mckinsey.com/insights/economic_studies/how_the_world_could_better_fight_obesity).

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Abbreviations: **NPY**, neuropeptide Y; **POMC**, proopiomelanocortin

Since 1980, obesity rates worldwide have more than doubled with 2.1 billion people, or nearly 30% of the global population, classified as being obese or overweight in 2013 [2]. Of major concern, there were 42 million children under 5 years of age who are overweight or obese in 2013 according to the WHO (2015).

The dramatic increase in the prevalence of obesity in recent years is suggested to be caused by a poor early life environment, which can have long-term effects on the susceptibility of the developing offspring to develop a wide range of adverse conditions in adulthood. Indeed, there has been a plethora of epidemiological and experimental evidence that strongly suggest that alterations in the in utero environment, due to maternal nutrition, including maternal obesity and maternal undernutrition, programs the developing offspring to develop cardiovascular and metabolic disease later in life [3, 4]. The disturbances during critical stages of development can result in adverse changes in fetal physiology, which predispose the fetus to a number of diseases in adulthood. In fact, other studies relate insults during critical periods of

development to adverse conditions later in life, such as type 2 diabetes [4, 5], hypertension [6, 7], and obesity [8, 9]. The fetus often responds to the poor conditions in an adverse intrauterine environment by undergoing physiological and metabolic adaptations in order to protect the most vital organs, such as brain, at the detriment of other organs [10]. This "thrifty phenotypes hypothesis" suggests that when the postnatal nutritional environment is similar, the individuals would then be able to endure the poor condition, but the adaptations become detrimental when the postnatal nutrition is different than the in utero environment [10, 11].

2 Obesity in pregnancy

Pregnancy is the greatest physiological challenge facing women that results in alterations in maternal physiology and metabolism to assist in fetal growth and development, which is modulated by a number of key molecules [12]. For example, glucose, the primary nutrient crossing the placenta, is important for fetal and placental growth [12, 13]. During pregnancy, glucose homeostasis in the mother is altered so that there is a progressive increase in insulin resistance and gluconeogenetic activity to sustain glucose transfer to the fetus [14]. Lipid metabolism is also altered in pregnancy with a significant increase in plasma cholesterol and triglyceride concentrations due to enhanced lipolytic activity and reduced lipoprotein lipase activity of adipose tissue during late gestation [15, 16]. During the first and second trimester, the mother is in an anabolic state whereby an increase in lipogenesis activity and adipose tissue lipoprotein lipase activity causes the mothers fat depots to accumulate [12]. The mother then shifts into a catabolic state during late pregnancy when fetal growth accelerates [17].

In an obese mother, the pregnancy adaptations differ from what occurs in healthy pregnant women. For example, glucose metabolism is significantly altered with an increase in peripheral and hepatic insulin resistance during the first trimester of pregnancy compared to normal weight pregnant women [18]. Therefore, is not surprising that the incidence of gestational diabetes is higher in overweight or obese pregnant women with a two- to tenfold increase [19–23]. In fact, obese women who were not diagnosed with gestational diabetes or impaired glucose tolerance have higher glucose profiles than normal weight women both during early and late pregnancy, despite consuming a controlled diet [24]. Similarly, women who had a higher gestational weight gain throughout the first 24 wks of pregnancy have a greater risk of developing gestational diabetes [25] and the risk was more pronounced when the women were obese [26]. Clearly, there is a linear association between BMI and the incidence of gestational diabetes. The mechanisms underlying this adverse pregnancy outcome are poorly understood. However, abdominal fat accumulation in obese women during pregnancy is likely to be associated with an increase in inflammatory cytokine production, leading to insulin resistance [27].

Obesity is a chronic low-grade inflammatory condition that is characterized by increased adipocyte mass, an increase in fasting whole body free fatty acids, and glycerol released from adipocytes [28]. White adipose tissue produces several proinflammatory cytokines, such as TNF-α and IL-6, which are increased in obesity [29]. Importantly, the placenta also produces these inflammatory cytokines, with the exception of adiponectin [30, 31]. Similar to the upregulation of inflammatory cytokine expression in obese adipose tissue, there is a two- to threefold increase in proinflammatory cytokine expression (IL-1, TNF-α, and IL-6) in the obese placenta, which is likely due to increased macrophage infiltration compared to a healthy pregnant women [32]. Likewise, the plasma concentrations of C-reactive protein, IL-6, and leptin were also increased in pregnant obese women compared to normal weight women [27, 32]. Obese women who were diagnosed with gestational diabetes were reported to have low serum adiponectin, a marker for increased insulin sensitivity, at 24–28 wk of pregnancy as compared to women with obesity alone [33]. Thus, these studies demonstrate that maternal obesity is associated with increased inflammatory mediator expression in the maternal plasma and placenta, which may contribute to an inflammatory in utero environment for the developing fetus.

3 The effects of maternal obesity on offspring health

It is well established that maternal obesity is associated with increased fetal growth, which can lead to offspring being born macrosomic [34]. However, recent findings suggest that offspring born to obese mother can also be small for gestational age or born with a normal birth weight [35, 36]. Of particular note, being small or large for gestational age due to maternal obesity predispose the offspring to obesity in adulthood [9]. Several animal studies have investigated the relationship between maternal obesity and the development of obesity in the offspring [37–39]. In rats, exposure to maternal obesity during pregnancy and lactation increased the risk of obesity later in life [37, 38]. The risk of obesity was further exacerbated when the offspring consumed a high fat diet postweaning [37, 40]. These clearly indicate that maternal obesity increased the obesity risk in their offspring. There are a number of mechanisms that may explain the programing effects of maternal obesity on offspring obesity risk including programing of appetite dysregulation and altered adipogenesis.

A study reported that the offspring of mice that consumed a high fat diet throughout pregnancy and lactation were hyperphagic from 4 to 6 wk of age before they developed abdominal obesity at 3 months [39]. Similarly, offspring of rats exposed to a junk food diet during both pregnancy and lactation displayed an increased preference for fatty, sugary, and salty foods when compared to offspring exposed to a control diet [37]. Programed changes in the offspring

of obese mothers may be a consequence of changes in hypothalamic functioning, which has an important role in the regulation of appetite. Appetite is primarily regulated by the hypothalamic arcuate nucleus and is composed of two neuron populations that either express the appetite stimulator neuropeptide Y (NPY) or the appetite inhibitor proopiomelanocortin (POMC) [41]. These neurons project into the paraventricular nucleus where they exert their effect on appetite regulation [41]. Importantly, the development of appetite regulation occurs during late gestation and perturbations during these critical periods may lead to a dysregulation in the expression of hypothalamic neuropeptides, increasing the risk of obesity in adulthood [41]. In rats, offspring of dams exposed to a cafeteria diet during pregnancy and lactation have increased hypothalamic NPY signaling [42]. Additionally, offspring born from genetically obese Zucker rats had reduced expression of POMC and lower α-melanocyte stimulating hormone, a cleaved product of POMC [43]. It is suggested that the changes in these neuronal pathways may be due to increased circulating leptin and insulin in the obese state, which are known to play a major role in regulating appetite by stimulating POMC and inhibiting NPY hypothalamic neurons [44, 45]. Therefore, it is postulated that exposure to maternal obesity causes alterations in hypothalamic regulation of appetite in the offspring leading to the development of hyperphagia.

Maternal obesity is also associated with dysfunction in offspring adipose tissue development. Offspring of rat dams fed a "junk food" diet during gestation and lactation demonstrated adipocyte hypertrophy, independent of hyperplasia, with increased expression of adipogenic factor peroxisome proliferator-activated receptor gamma, insulin-like growth factor 1, insulin receptor substrate 1, and vascular endothelial growth factor A mRNA expression at 10 wk of age, indicative of altered adipocyte proliferation [46]. Similarly, maternal overnutrition during late gestation in sheep is also associated with increased expression of genes, which regulate adipogenesis and lipogenesis in fetal perirenal adipose tissue, including lipoprotein lipase, adiponectin, leptin, and peroxisome proliferator-activated receptor gamma [47]. These findings suggest that alterations in adipose gene expression may be one of the underlying mechanisms that increase adiposity in offspring that are exposed to maternal overnutrition.

In addition to increasing the risk of offspring obesity, maternal obesity and overnutrition also program metabolic dysfunction in their offspring. Limited human studies have examined the link between maternal obesity, offspring insulin resistance, and other adverse metabolic outcomes. For instance, the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study reported that an increase in maternal BMI is linked with fetal hyperinsulinemia, which is independent of maternal glycaemia [48]. Importantly, there is evidence that demonstrates that babies of obese mothers develop insulin resistance in utero, indicating that maternal obesity is an important predictor of metabolic disease in their offspring [49]. The effect maternal obesity has on insulin sensitivity then persists into later life with children of overweight women having an increased risk of developing insulin resistance by 11 years of age [4], and in early adulthood (early 20s) [50]. Therefore, these findings support the association of maternal obesity and altered glucose–insulin homeostasis in the offspring.

Indeed, in animal studies, maternal obesity or overnutrition during both pregnancy and lactation is linked to increased insulin and glucose concentrations in the offspring and these features were exacerbated when the offspring consumed a high-fat diet postweaning [39, 46, 51–53]. Studies suggest that alteration in glucose–insulin homeostasis in offspring of obese mothers is likely due to β-cell failure [54–57]. Evidence in rat studies demonstrated consumption a high-fat diet (40% calories from fat) during pregnancy and lactation caused hyperglycemia and insulin resistance with compromised β-cell development and function in the offspring [54, 55]. Additionally, offspring of obese mice develop insulin resistance at 3 months of age, but by 6 months of age male offspring developed frank diabetes with reduced plasma insulin and pancreatic insulin content, indicative of β-cell exhaustion [39]. Other animal studies recorded similar observations, suggesting that there is an age-related decline in β-cell function in offspring of obese mothers that leads to altered glucose and insulin homeostasis [56, 57]. In sheep, maternal obesity is linked to an increased fetal pancreatic weight and an increased number of insulin-positive cells per unit area, which is indicative of accelerated β-cell maturation [58]. A further study showed that offspring of high-fat dams had reduced β-cell numbers and this was associated with an increased in β-cell apoptotic rate [59]. Therefore, these changes may predispose the offspring to a premature loss of β-cell function which would subsequently lead to elevated risk of metabolic disease in adulthood.

Taken together, these findings suggest that maternal obesity or overnutrition during pregnancy have an adverse effect on offspring metabolic outcomes [60]. Thus, further studies are required to understand the underlying mechanisms that predispose the offspring of obese mother to metabolic diseases and whether these adverse phenotypes can be modified by lifestyle interventions.

4 Intrauterine growth restriction

Obesity is a multifactorial disease, with a number of risk factors associated with its development. Exposure to a perturbations in utero, such as maternal malnutrition and placental insufficiency, program an increased risk of developing obesity in later life [61]. Intrauterine growth restriction affects 10% of pregnancies worldwide and is characterized by a term birth weight of less than 2.5 kg [62]. Growth restriction can be caused by genetic factors and maternal stressors, such as maternal smoking, maternal malnutrition, and placental dysfunction [63]. In Western societies uteroplacental insufficiency is the major cause of growth restriction [64–66],

whereas maternal malnutrition is the main cause of babies born small in developing countries [67,68]. Previous epidemiological studies have demonstrated an association between being born small and an increased risk of developing type 2 diabetes, obesity, and hypertension [5, 6, 69]. From these findings the importance of maternal nutrition and its effect on birth weight and subsequent adult diseases was addressed in human studies of famine exposure, particularly the Dutch Hunger Winter of 1944–1945 [70, 71]. The Dutch Hunger Winter study found that growth restriction due to famine exposure in utero is linked to glucose intolerance and abdominal obesity in adults [70, 72]. In contrast, the findings from famine exposure during the Leningrad siege (1941–1944) did not show any association between birth weight and metabolic disease risk [73]. The inconsistency between these findings is likely due to the different nutritional environments during the postnatal period in both studies. Following the Dutch Hunger Winter, the food supply was restored to normal levels in a short period of time, where they were exposed to normal nutrition during their postnatal life [70, 73]. Conversely in Leningrad, the children were exposed to poor nutritional environment in utero and in their early postnatal years [73]. These findings suggest that a mismatch in nutritional environment between the intrauterine and postnatal period may influence the outcomes of growth-restricted babies in adulthood.

Altered postnatal growth can also influence the disease outcomes of growth-restricted babies in adulthood. Growth-restricted babies often experience catch up growth in the first 6 to 12 months of age and to as late as 2 years after birth when the postnatal nutritional environment is improved [74]. They will accelerate their growth trajectory to match the growth of normal weight babies to compensate for their low birth weight. Previous studies have reported that children born small for gestational age, who have a high childhood fat mass, have an increased risk of developing diabetes in later life [75, 76] and present with insulin resistance at 3 years [77]. Another study in a cohort from Helsinki demonstrated that growth-restricted individuals have an exacerbated risk of type 2 diabetes when catch up growth in early postnatal life (6 months of age) is combined with accelerated weight gain during adolescence [78]. These studies suggest that accelerated catch up growth during postnatal life is an additional independent risk factor to disease development in growth-restricted individuals. Therefore, a combination of adverse pre- and postnatal environment can lead to an exacerbation of the programed diseases in these individuals.

Animal models have been extensively used to identify the underlying mechanisms that associate intrauterine growth restriction and the risk of metabolic dysfunction in adulthood. Indeed, many studies using a wide range of animal species, including sheep, rodents and guinea pig, have demonstrated the link between intrauterine growth restriction and metabolic disease [79]. The majority of animal models investigating intrauterine growth restriction have utilized dietary interventions to induce maternal undernutrition [80, 81] and surgical interventions to induce uteroplacental insufficiency [82]. In Wistar rat, exposure to 50% caloric restriction in the last trimester of pregnancy resulted in a 16% reduction in birth weight compared to control offspring [83, 84]. However, the effect caloric restriction has on adiposity is contradictory with this study identifying no change [83, 84], whereas another study identified increased adiposity, which is consistent with the hyperleptinemia observed in these animals [85]. Despite these differences in adiposity, caloric restriction results in catch up growth [86] and alters β-cell morphology and function [83, 84]. Specifically, caloric restriction and low-protein diets (8% protein) reduce β-cell mass [83, 87–89] and insulin content [83, 87]. Interestingly, when these offspring were exposed to a normal diet postnatally, the islet morphology improved, indicating that the in utero environment influences fetal islet development [88, 89]. However, if protein restriction was extended during weaning, these modifications were irreversible [88,89]. Studies in male rat offspring of low-protein diet dams demonstrated an age-dependent loss in glucose tolerance. Specifically, they had improved glucose tolerance and reduced plasma insulin concentrations in early life (6 wk to 3 months), which is indicative of enhanced insulin sensitivity [90]. Nevertheless, when they reached 15 months of age, glucose intolerance was evident [91] and by 17 months of age they developed frank diabetes and insulin resistance [92].

As mentioned previously in developed countries, placental insufficiency is the major cause of intrauterine growth restriction and low birth weight [64–66]. Wigglesworth was the first to describe the model of uteroplacental insufficiency in rats by ligating the uterine vessels during late gestation, which reduced uteroplacental nutrient and oxygen perfusion and thus compromises fetal growth and development [82]. This rat model is equivalent to the degree of birth weight reduction observed in humans in developed countries (10–15% reduction in birth weight), where developmental insults are most apparent during late gestation [93]. Uteroplacental insufficiency surgery in Sprague Dawley rats between embryonic days 16 and 19 (E16-19; term = 22 days) resulted in low birth weight offspring (10–15%) [64, 94, 95]. These offspring had reductions in β-cell mass at birth [95, 96], with a similar decrease in pancreatic insulin content [95], however, glucose tolerance was normal at 3 months of age [95]. In contrast, a study by Simmons et al. reported that male growth-restricted Sprague Dawley rats had normal β-cell mass, islet size, and pancreatic weight at 1 and 7 wk of age [64]. However, at 15 wk of age, these rats had reduced β-cell mass and decreased pancreatic insulin content as well as a reduced insulin response to glucose, and at 26 wk these offspring were diabetic and obese [64]. Additionally, other uteroplacental insufficiency studies recorded fasting hyperglycemia, early onset insulin resistance, obesity, and impaired glucose tolerance in the growth-restricted Sprague Dawley rats [64, 65, 97–100]. Findings from our laboratory reported that male Wistar Kyoto (WKY) rats that were exposed to uteroplacental insufficiency develop impaired glucose tolerance and were

hyperinsulinemic at 6 month of age, which was associated with a 40–45% reduction in β-cell mass [101–104]. Interestingly, growth-restricted female rats exhibited normal glucose tolerance regardless of reductions in basal insulin concentrations and pancreatic β-cell mass [101, 105]. Findings from these studies clearly suggest that there are sex-specific differences where growth-restricted males are more severely affected than females. Thus, "second hits," such as obesity or pregnancy, may exacerbate the adverse metabolic phenotype in growth-restricted females.

4.1 Growth restriction and obesity

Epidemiological studies and animal models link a low birth weight to an increased risk of adult obesity and metabolic syndrome [106, 107]. Early epidemiologic studies demonstrated that growth-restricted babies that experienced accelerated catch up growth have a higher risk of obesity and metabolic syndrome compared to infants that are born small and remain small throughout their life [108, 109]. Importantly, growth-restricted infants with catch up growth during their early postnatal life had reduced lean body mass and elevated abdominal fat [110, 111]. This finding is similar to what is reported in infants with a normal birth weight that exhibit rapid weight gain in the first 2 years of life [112]. Of particular concern, the Dutch Hunger Winter study reported that only women who were exposed to famine in early gestation had increased in body weight, BMI, and waist circumference in adulthood [72]. Likewise, girls aged between 14 and 16 years who were born small had increased central adiposity compared to growth-restricted males [113]. These findings indicate that growth-restricted females are at higher risk of abdominal obesity which is associated with an increased risk of insulin resistance and glucose intolerance.

4.2 Growth restriction and pregnancy

In females that were born growth-restricted, pregnancy may exacerbate their risk of cardiovascular and metabolic disease due to an increase in both maternal and fetal demands. Indeed, epidemiological studies associate a low birth weight with a higher risk of developing preeclampsia during later pregnancy [114, 115]. Furthermore, women born with a low birth weight were also more susceptible to gestational diabetes during pregnancy compared to women that were born of normal weight [116]. A study in female rats born small also identified a higher risk of developing gestational diabetes in pregnancy as well as an increased risk of their offspring developing an altered metabolic phenotype [94]. Likewise, we have previously demonstrated that growth-restricted female rats during late pregnancy develop glucose intolerance, despite a normal plasma insulin response [105]. Given that maternal obesity has adverse effects on glucose homeostasis during pregnancy, it is likely that the metabolic dysfunction in pregnant females who were born growth-restricted will be exacerbated if they consume a high-fat diet. Currently this association has not been investigated, thus future studies should examine this interaction.

So far, most of the developmental programing studies are largely descriptive and there are very limited molecular investigations have been performed in this area. Therefore, additional molecular studies are required to identify the underlying mechanisms that may explain the link of obesity in growth-restricted mothers to their pregnancy outcomes as well as their offspring development.

5 Potential intervention

There has been much interest in the development of lifestyle interventions targeting overweight and obese pregnant women. Of particular interest, epidemiological studies demonstrated that exercise in overweight and obese women prevented them from developing gestational diabetes as well as delivering macrosomic babies [117]. Moderate exercise during pregnancy improved glucose tolerance [118] and reduced fasting insulin [119] in obese pregnant women; thus, lowering their risk for gestational diabetes. Likewise, moderate to vigorous exercise in early pregnancy also improved insulin response and sensitivity, as well as reducing plasma triglyceride concentration in overweight and obese pregnant women [120]. However, there are studies that failed to associate the beneficial effects of exercise with a reduced risk of adverse pregnancy outcomes in overweight and obese women [121, 122]. Lack of consistent evidence regarding the benefits of exercise in pregnant obese or overweight women suggests that interventions during pregnancy alone may not be enough to ameliorate the adverse effect obesity has on the mother and her children. Therefore, an exercise intervention before and during pregnancy may be more beneficial. Indeed, in nonobese pregnant women who were involved in exercise training 1 year before pregnancy had a reduced risk of developing gestational diabetes [123–125], and the effect was greater in women who exercised before and during pregnancy [125]. These findings propose that these lifestyle interventions are more beneficial if they are performed before the reproductive years.

Similarly, in animal studies, maternal exercise reduced the metabolic risk caused by maternal obesity in both the mother and offspring [126, 127]. A recent study on rats reported that voluntary wheel running before and during pregnancy prevented the increase in plasma insulin and glucose, concentrations insulin resistance (HOMA-IR), and plasma triglyceride content during lactation in obese dams [126]. Additionally, voluntary exercise before and during pregnancy reduced glucose and insulin concentrations in male offspring (postnatal day 19) of obese rat mothers [128] and prevented glucose intolerance induced by maternal obesity in female offspring (24 wk) of C57BL/6 mice [128]. Of particular concern, most of the rodent studies investigating the effect of

exercise intervention in maternal obesity utilized a poorly controlled voluntary wheel running as the exercise intervention. Therefore, a well-controlled interventional animal study using a motorized treadmill exercise is required as precise exercise intensity and duration can be controlled.

6 Conclusions

There has been a significant increase in the number of overweight or obese pregnant women in the past two decades. Of particular concern, maternal obesity does not only affect the mother, but the offspring are programed to develop obesity and metabolic disease later in life. Studies suggest that the mechanisms contributing to this is due to appetite dysregulation and enhanced adipogenesis in the offspring. Being born small for gestational age is one of the risk factor for developing obesity. As being born small is associated with increased risk of cardiovascular and metabolic disease during pregnancy, it is suggested that obesity during pregnancy may exacerbate the risk of these diseases. However, there are limited studies investigating the effect of maternal obesity in growth-restricted mothers and the subsequent effects in their offspring. Therefore, it is critical to identify the underlying mechanisms that link obesity in growth-restricted mothers to the development of metabolic diseases in their offspring. This will be fundamental to future strategies for the prevention and therapy of obesity.

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7 References

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