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Skeletal Muscle Lipid Metabolism and Markers of Insulin Resistance in Young Male Low Birth Weight Offspring in Combination With a Postnatal Western Diet

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

SKELETAL MUSCLE LIPID METABOLISM AND MARKERS OF INSULIN RESISTANCE IN YOUNG MALE LOW BIRTH WEIGHT OFFSPRING IN COMBINATION WITH A POSTNATAL WESTERN DIET

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by

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Abstract

Low birth weight offspring are at increased risk for developing metabolic syndrome in later life, specifically its precursor, insulin resistance (IR). Reduced mitochondrial lipid metabolism is implicated in IR pathogenesis, promoting accumulation of acylcarnitines, and potentiating alterations in phosphorylation status of insulin signaling intermediates. While high-energy Western diets are classically implicated in IR progression, the *in utero* environment was recently highlighted as a major programming mechanism of later life IR. Using a guinea pig model of placental insufficiency, we investigated how an adverse *in utero* environment impacts later life mitochondrial lipid metabolism and IR progression, as well as its interaction with a postnatal Western diet. Markers of mitochondrial dysfunction and reduced lipid metabolism, including acylcarnitine accumulation, were observed in conjunction with disrupted phosphorylation of insulin signaling intermediates in skeletal muscle of low birth weight offspring. Additionally, a postnatal Western diet unmasked low birth weight-induced mitochondrial dysfunctions, promoting further acylcarnitine accumulation.

Keywords

Intrauterine Growth Restriction, Low Birth Weight, Metabolic Syndrome, Skeletal Muscle, Insulin Resistance, Mitochondria, Lipid Metabolism, Western Diet, Fetal Programming

Co-Authorship Statement

A portion of Chapter 1 (Introduction – Literature Review) was reproduced (adapted) from a Review Article:

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Kristyn Dunlop and Megan Cedrone researched pertinent material from the scientific literature and wrote the review article. James F Staples and Timothy RH Regnault assisted with editing and revisions to the manuscript drafts. All authors approved the final version of the review article.

Chapter 2:

Timothy RH Regnault and Kristyn Dunlop designed the experiments. Joyce Liu, Kristina Wiggers and Alexandra Blake assisted with data input and processing. Kristyn Dunlop, Jacky Chiu and Ousseynou Sarr assisted with Computed Tomography, Glucose Tolerance and Animal Handling. Kristyn Dunlop analyzed and interpreted the data, and wrote the manuscript.

Chapter 3:

Timothy RH Regnault and Kristyn Dunlop designed the experiments. Kristyn Dunlop, Jacky Chiu and Ousseynou Sarr assisted with Animal Handling. Joshua Findlay performed the immunoblots for IRβ, IRS-1, Akt, and JNK. Acylcarnitine and Amino Acid measurements were conducted by The Analytical Facility for Bioactive Molecules at the Hospital for Sick Children. Kristyn Dunlop analyzed and interpreted the data, and wrote the manuscript.

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Dedication

To Mom, Nana, and Papa with love. Thank you from the bottom of my heart; I would not be where I am today without you and your never-ending support!

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List of Abbreviations

AGR – Absolute Growth Rate

AMPK – 5'-Adenosine Monophosphate-activated Protein Kinase

AS160 – Akt Substrate of 160 kDa

ATP – Adenosine Triphosphate

AUGC – Area Under the Glucose Curve

BHT – Butylated Hydroxytoluene

BMI – Body Mass Index

CD – Control Diet

CPT – Carnitine Palmitoyltransferase

CT – Computed Tomography

DAG - Diacylglycerol

DOHaD – Developmental Origins of Health and Disease

EPOCH – Evaluating Processes of Care & the Outcomes of Children in Hospital

$FADH_2$ – Flavin Adenine Dinucleotide

FAT/CD36 – Fatty Acid Translocase/Cluster of Differentiation 36

FATP – Fatty Acid Translocator Protein

FDG – ^{18}F-fluoro-deoxy-glucose

FGR – Fractional Growth Rate

FOXO – Forkhead/Winged Helix Transcription Factor

GLUT – Glucose Transporter

HDL – High Density Lipoprotein

HMOX1 – Heme Oxygenase 1

HOMA-IR – Homeostatic Model Assessment of Insulin Resistance

IKKβ – Inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) Kinase

IMTG – Intramyocellular Triglycerides

IPGTT – Intraperitoneal Glucose Tolerance Test

IR – Insulin Resistance

IRS – Insulin Receptor Substrate

ITT – Insulin Tolerance Test

IUGR – Intrauterine Growth Restriction

JNK – c-Jun N-terminal Kinase

KT – 3-ketoacyl CoA Thiolase

LBW – Low Birth Weight

LC-MS/MS – Liquid Chromatography Tandem Mass Spectrometry

LCAD – Long Chain Acyl-CoA Dehydrogenase

MCAD – Medium Chain Acyl-CoA Dehydrogenase

MetS – Metabolic Syndrome

miRNA - microRNA

NADH – Nicotinamide Adenine Dinucleotide

NBW – Normal Birth Weight

NRF-1 – Nuclear Respiratory Factor-1

PCA – Principle Components Analysis

PDK – Pyruvate Dehydrogenase Kinase

PET – Positron-Emission Tomography

PGC-1α – Peroxisome Proliferator-Activated Recetpor-Gamma Co-activator 1-Alpha

PI3-Kinase – Phosphatidylinositol 3-kinase

PKB/Akt – Protein Kinase B/Akt

PKC-θ – Protein Kinase C-θ

PND – Postnatal Day

PPARα – Peroxisome Proliferator-Activated Receptor Alpha

PTEN – Phosphatase and Tensin Homologue Deleted on Chromosome 10

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

ROS – Reactive Oxygen Species

SEM – Standard Error of the Mean

SGA – Small for Gestational Age

SIRT - Sirtuin

SUV – Standard Uptake Value

TCA – Tricarboxylic Acid

TG - Triglyceride

TLC-FID – Thin Layer Chromatography-Flame Ionization Detection

VLCAD – Very-Long Chain Acyl-CoA Dehydrogenase

WD – Western Diet

WHO – World Health Organization

Chapter 1: Introduction – Literature Review

Excerpts of this chapter have been previously published: Dunlop K, Cedrone M, Staples JF, Regnault TR. Altered Fetal Skeletal Muscle Nutrient Metabolism Following an Adverse *In Utero* Environment and the Modulation of Later Life Insulin Sensitivity. Nutrients. 2015 Feb 12;7(2):1202-16. doi: 10.3390/nu7021202.

1.1 Metabolic Syndrome

Throughout the world, non-communicable diseases now represent one of the greatest strains on the global community. Furthermore, as our population ages, the burden of treating these non-communicable diseases on the health care system continues to increase, placing strain on the economy as well. Non-communicable diseases, including heart disease, stroke and diabetes are among the top six leading causes of death in Canada, according to a 2014 survey conducted by Statistics Canada.[1] Diabetes has been shown to lead to cardiovascular diseases, including heart disease and stroke, highlighting diabetes as a likely contributor to the development of these non-communicable diseases.[2] Of particular interest, other chronic diseases with a high prevalence in Canada include hypertension, dyslipidemia, impaired glucose tolerance and obesity, which together describe hallmarks of the metabolic syndrome (MetS).[3] In Canada, it was estimated that 1 in 5 individuals aged 18-79 displayed observable manifestations of MetS between 2009 and 2011,[4] a number that is only expected to continue to increase as our population ages. Clinically, MetS is defined as a cluster of risk factors including central obesity, hyperglycemia, dyslipidemia and hypertension, which predispose the patient to developing cardiovascular disease, type 2 diabetes, non-alcoholic fatty liver disease, and other age-related metabolic diseases.[3,5,6] The clinical presentation of MetS remains highly contested, with various international organizations providing different diagnostic criteria. The World Health Organization (WHO) guidelines for the diagnosis of MetS include the following parameters: waist circumference greater than 102cm in men and greater than 88cm in women; fasting glucose levels greater than 110 mg/dL; blood pressure of 140/90 mmHg or greater;

triglyceride levels of 150 mg/dL; HDL cholesterol levels less than 35 mg/dL in men and less than 39 mg/dL.[7] However, before these overt features of MetS present, peripheral insulin resistance often manifests, representing a key early feature of disease progression.[8,9]

Of growing concern is the rapidly increasing incidence of not only MetS, but also insulin resistance and type 2 diabetes. As of 2008/2009 approximately 1 in 11 Canadians were living with insulin resistance or diabetes.[2] The emergence of insulin resistance has recently been highlighted as a critical pathophysiological event underlying the development of components of MetS, disrupting the metabolic capacity of the patient and perpetuating the development of further metabolic disease.[8] Additionally, a particularly concerning trend is the growing incidence of insulin resistance and type 2 diabetes in the adolescent population. In the past two decades alone, the incidence of type 2 diabetes in the adolescent population has increased worldwide,[10] highlighting that research needs to be conducted not only to determine effective treatments for management of insulin resistance and MetS, but also for early, more effective prevention strategies targeting youth.

1.2 Low Birth Weight (LBW) and Intrauterine Growth Restriction (IUGR)

As part of our investigation into the pathogenesis of MetS, we will be focusing on the LBW population, as epidemiological studies have highlighted an association between LBW secondary to IUGR and increased risk of MetS development in later life.[11-13] LBW is classically defined as an infant born with a weight less than 2500g or below the 10th

percentile for gestational age.[14] However, these arbitrary cutoffs do not account for the fact that some babies outside these cutoffs may have been exposed to an adverse *in utero* environment and grown inappropriately for their genetic potential.[15-19] While these infants are not usually classified as LBW according to the clinical definitions, they may still have experienced metabolic adaptions to a suboptimal *in utero* environment that increases their risk of later life metabolic disease, highlighting that a continuum of risk exists among a range of birth weights.[20,21]

The prevalence of LBW in Canada has been holding steady at approximately 6% between 2000 and 2007.[22] However, worldwide a modest increase in the percentage of births classified LBW has been observed over the past decade.[22] LBW infants are often considered to be "small for gestational age" (SGA), a clinical definition applied when the infant is born with a weight below the 10th percentile for gestational age.[23] However, current growth charts often used to classify these SGA infants do not take important factors such as race and ethnicity into account, highlighting the need for personalized growth curves depending on sex, ethnicity and other factors that may impact growth potential of the fetus in order to accurately represent the LBW population.[24-26] The LBW population is particularly important, since recent evidence suggests these infants are at greater risk for perinatal mortality and morbidity, as well as long-term metabolic disease.[13,27,28]

A particular cause of LBW is the suitability of the *in utero* environment. An adverse *in utero* environment, often characterized by suboptimal nutrient transfer to the fetus, culminates in IUGR. IUGR is a major obstetrical concern, and is the endpoint of a continuum of conditions that result in the failure of the fetus to attain its inherent growth

potential, leading to a low weight at birth.[29] The etiology of IUGR is multifactorial, with adverse environmental or genetic and epigenetic factors likely playing a role in the abnormal growth and development of the fetus. One of the most important environmental factors regulating fetal growth is nutrient delivery to the fetus which occurs via placental diffusion and transport.[30] In developed countries, a main cause of IUGR is placental insufficiency, ultimately affecting 5-10% of all pregnancies.[23] Placental insufficiency is characterized by a reduced functional capacity of the placenta, and is typically associated with poor placental vascular development, which prevents adequate nutrition and oxygen from reaching the developing fetus, resulting in a hypoxic, nutrient deprived *in utero* environment.[31,32] Placental insufficiency may develop due to maternal vascular disease, pre-eclampsia, placenta praevia or other placental anomalies that prevent proper development and/or vascularization of the placenta.[30] Interestingly, it should be noted that independent of reductions in nutrient supply, hypoxia alone has been shown to have a significant impact on fetal growth, emphasizing hypoxia as a key contributor to impaired fetal growth and potentially IUGR.[32]

With exposure to a hypoxic *in utero* environment, the fetus undergoes key adaptations to ensure survival. A critical component of this adaptation in the acute setting has been considered a redistribution of fetal cardiac output towards essential organs such as the brain, heart and adrenal glands, at the expense of other organ systems, including the lungs, kidney, liver and skeletal muscle.[33-35] With prolonged hypoxia, animal and human studies have highlighted a redistribution of blood flow toward the brain, as well as increased blood flow towards adrenal glands,[36-39] and it is inferred from this redistribution that the brain continues to receive sufficient perfusion and nutrient supply

to maintain relative growth. This brain sparing effect is visible at birth, with the size of the fetal head being larger than that of the abdomen, giving rise to an observable asymmetrical growth restriction. Concurrent with the overall reduction in body weight and brain sparing effect, fetuses exposed to hypoxic *in utero* environments also display a reduced muscle mass compared with normal birth weight offspring,[40,41] and are predisposed to altered insulin sensitivity.[8,12,13,42] Furthermore, rodent models of low birth weight have also demonstrated a decrease in skeletal muscle mass at birth and throughout life,[43] similar to the altered muscle to fat ratio observed in older men who were LBW at birth.[44] With this altered body composition there are also metabolic changes to the offspring, resulting in what has been proposed as a "thrifty phenotype".[12,13] This "thrifty phenotype" encompasses a collection of metabolic adaptations initiated to aid in fetal survival when challenged with nutrient deprivation *in utero*.[45]

1.3 The "Thrifty Phenotype" Hypothesis and The Developmental Origins of Health and Disease

The "thrifty phenotype" hypothesis was first put forth by Forshdahl, Barker and Colleagues in order to explain the risks associated with metabolic adaptations *in utero*. Over time, this hypothesis came to be known as the "Forsdahl-Barker hypothesis", then later as the Developmental Origins of Health and Disease hypothesis, as further evidence strengthening the association between growth *in utero* and later life disease risk was discovered.

The first associations between growth *in utero* and later life disease presentation were observed by conducting geographical studies of lifestyle factors and cardiovascular disease risk in Norway and England. In the 1970s, Forsdahl used official statistical data in Norway to report a greater risk of coronary heart disease in adulthood when adolescents were exposed to poor living conditions in early life.[46,47] In the 1980s, Barker and colleagues highlighted the emergence of paradoxical patterns whereby cardiovascular disease rates were twice as high in poorer socioeconomic areas of Britain, highlighting that adult lifestyle had a limited ability to predict cardiovascular disease risk.[48] Further investigation into these paradoxical patterns revealed a parallel between coronary heart disease prevalence and death rate among newborns, providing the first clue that cardiovascular disease risk may be linked to fetal growth.[49] These geographical studies provided the evidence required for Barker and colleagues to develop the hypothesis that poor nutrition *in utero* and during early infancy had the ability to induce coronary heart disease and an increased risk of stroke in adult life.[48-51] While no biological mechanisms were put forth by Forsdahl or Barker at this time, Forsdahl's observation that poverty during adolescence followed by a period of prosperity allowed for speculation that a form of permanent damage could be caused by early nutritional deficits that is maladaptive when exposed to later-life nutrient excess.[47]

Over time, further epidemiological studies have been conducted, highlighting associations between growth *in utero* and later life insulin resistance and obesity,[12,52] both of which are important risk factors for development of MetS. Examination of the birth records compiled by midwives in Hertfordshire from 1911 onwards allowed for associations to be made within this population who still resided in the area 50-70 years

later. Studies conducted in this population highlighted impaired glucose tolerance in LBW men at an average age of 64, as well as presence of dyslipidemia and other components of MetS.[12] Epidemiological studies conducted in Helsinki, Finland also highlighted a striking association between thinness at birth as a marker of suboptimal growth *in utero* and body weight in adolescence. Children who were light and thin at birth, but who were caught-up in weight by adolescence so they had an average or above average body mass index had the highest incidence of cardiovascular disease, highlighting rapid postnatal growth as another important contributor to later life disease risk.[53,54]

Once this striking epidemiological data was presented to the scientific community, research has continued with animal models in order to better understand the molecular mechanisms associated with these observations. With the expansion of this field of research, the "thrifty phenotype" hypothesis was expanded into the Developmental Origins of Health and Disease Hypothesis in order to encompass the vast importance of early life environment and its implications for later life disease risk (Figure 1.1).[55]

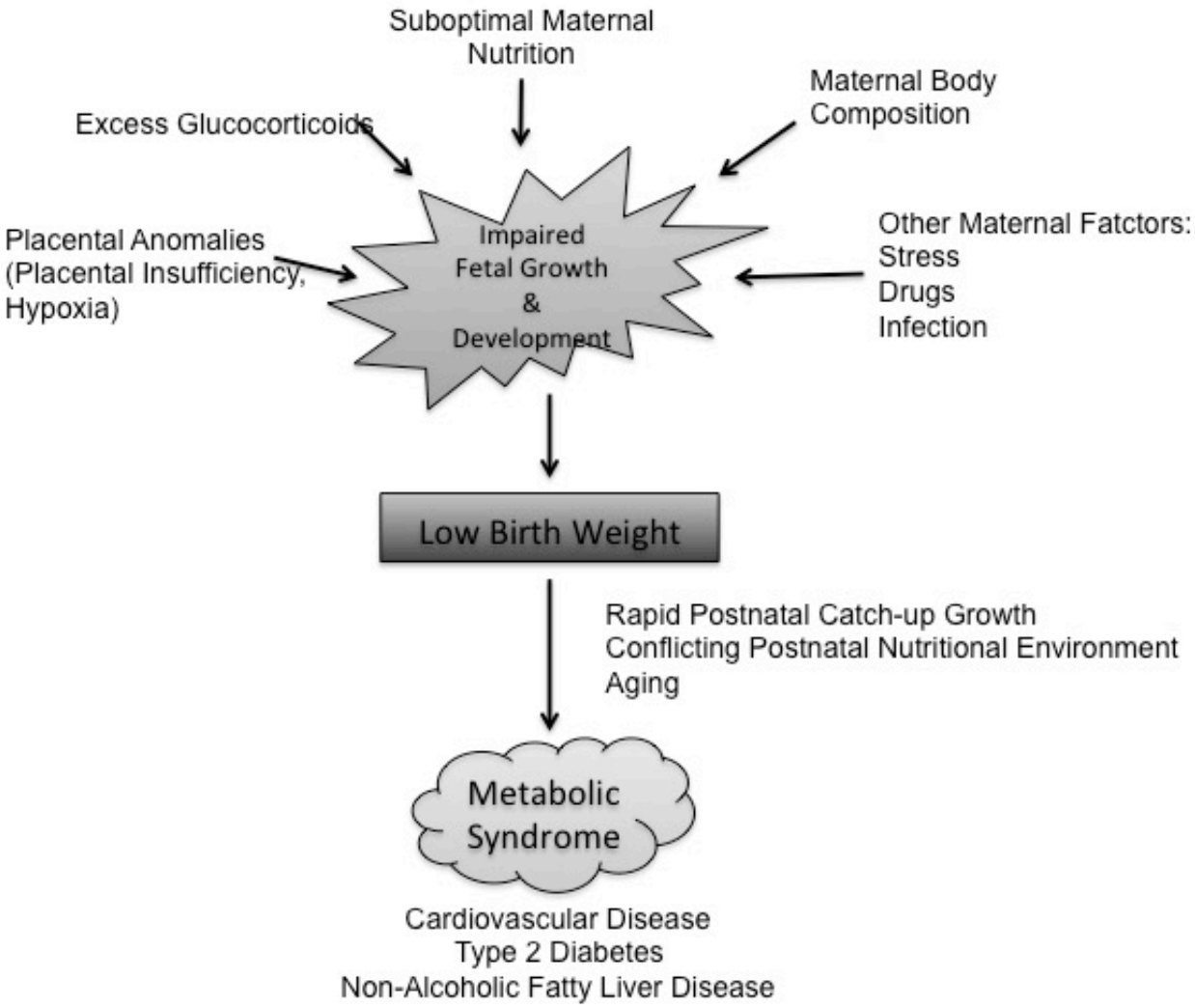

Figure 1.1: Factors That May Contribute to Low Birth Weight and The Developmental Origins of Health and Disease. An *in utero* insult, or collection of insults, generally results in intrauterine growth restriction, impaired fetal growth and development, and a low birth weight outcome. This predisposes the infant to a higher risk of developing metabolic diseases as they age and interact with their postnatal environment, experiencing rapid postnatal catch-up growth that may exacerbate the effects of programmed metabolic disease.

While the "thrifty phenotype" continues to represent the observable phenotype associated with IUGR, theories of an accompanying altered metabolic phenotype are now emerging. Fetal programming and developmental plasticity theories have developed, postulating that altered oxygen and nutrient transfer during critical windows of development, when the fetus is most sensitive to its environment, are associated with permanent alterations in structure and metabolism, and a fixed functional capacity of vital organs in postnatal life.[13] In humans, organogenesis occurs predominantly *in utero*, and is associated with a high degree of organ plasticity in order to promote metabolic development.[56] However, plasticity of the organs is decreased in postnatal life; therefore, adaptations to these metabolic organs initiated *in utero* in order to aid in fetal survival may persist into adulthood, increasing the propensity for these offspring to develop metabolic disease as they interact with their postnatal environment.[13,57]

Markers of altered fetal growth, including low weight at birth and asymmetric growth, are most widely used as indicators of IUGR or a hypoxic *in utero* environment. However, more subtle adaptations at the physiological level may be the drivers underlying the observable later life phenotypes, such as changes in skeletal muscle metabolic function. Once the organs have fully developed *in utero,* the IUGR fetus faced with a postnatal environment characterized by nutrient excess may develop long-term adverse metabolic consequences.[8,12,13] Unfortunately, the mechanisms underlying these alterations in the metabolic capacity of the IUGR fetus and their propensity towards the development of later life insulin resistance and MetS remain poorly defined.

1.4 Skeletal Muscle Insulin Resistance

Skeletal muscle is the primary location for insulin-stimulated glucose uptake, accounting for up to 70% of whole body glucose disposal,[58] and is a key regulator of whole body energy metabolism,[59] with other metabolic organs, including liver, adipose tissue and pancreas also involved in the insulin response and pathogenesis of insulin resistance. The primary metabolic objective in the skeletal muscle is production of adenosine triphosphate (ATP) for contractile purposes; however, skeletal muscle is also responsible for the production and storage of glycogen, an insulin dependent process that provides the cells with glucose for ATP production when circulating levels are low. β-oxidation, a process whereby free fatty acids are broken down to provide muscle with carbon chain substrates, is also important for skeletal muscle ATP production. Since the skeletal muscle is a critical producer of ATP and an important location for glucose and fat metabolism, determining the propensity of skeletal muscle towards developing insulin resistance is a key determinant in the pathophysiological progression towards metabolic syndrome.

Depressed insulin sensitivity, or insulin resistance, is a metabolic state in which peripheral tissues, such as skeletal muscle, are no longer responsive to the anabolic effects of insulin, thereby displaying reduced insulin-stimulated glucose uptake and perpetuating a state of hyperglycemia.[60] Insulin resistance at the level of the skeletal muscle has been associated with a modulation of the serine/threonine phosphorylation status of insulin receptor substrates (IRS).[61-63] A relative increase in the serine phosphorylation of IRS-1, the predominant isoform in skeletal muscle,[64] at Ser^{307} reduces its ability to activate or complex with phosphatidylinositol 3-kinase (PI3-kinase). This

activation failure impairs downstream phosphorylation of protein kinase B (Akt) at Ser^{473} and Thr^{308}, and Akt Substrate of 160 kDa (AS160) at Thr^{642}, ultimately leading to a reduction in glucose uptake into skeletal muscle cells through glucose transporter 4 (GLUT4) transporters (Figure 1.2).[61,63,65]

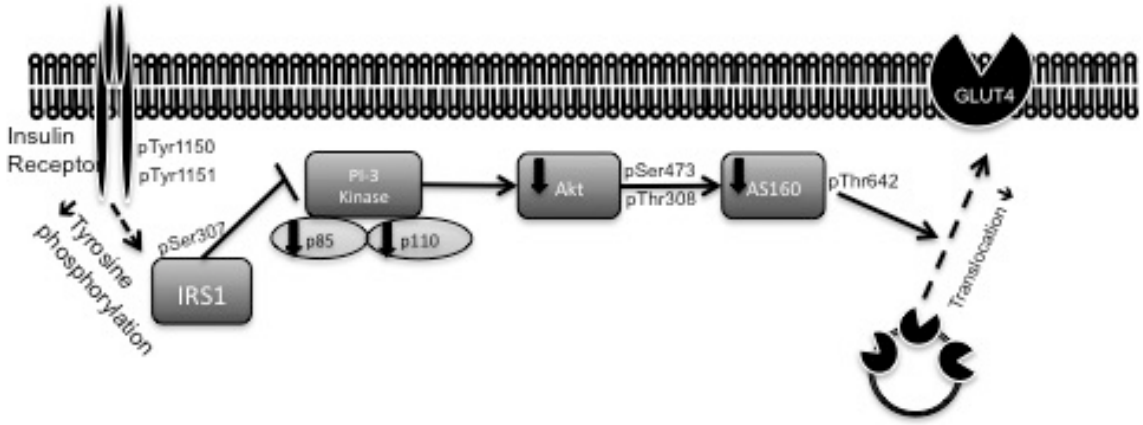

Figure 1.2: Molecular Manifestations of Insulin Resistance. Modulations of the phosphorylation status, including increased inhibitory phosphorylation or decreased activating phosphorylation, impair the ability of GLUT4 to translocate to the plasma membrane, preventing adequate glucose uptake and perpetuating hyperglycemia.

1.4.1 Skeletal Muscle Insulin Resistance in IUGR Offspring

A hypoxic *in utero* environment, commonly associated with IUGR, is known to negatively influence the fetus during critical periods of development.[8,12,13] Interestingly, IUGR offspring in animal models display improved insulin sensitivity in very early postnatal life, as assessed by intravenous glucose tolerance challenge; however, a shift towards impaired glucose clearance and decreased insulin sensitivity is evident as these offspring age.[66-68] The timing of this shift in insulin sensitivity appears to be sex-specific, with insulin action being impaired earlier in males and later in females.[66] These animal studies highlight that sexually dimorphic effects occur with adverse *in utero* environments, and in the case of insulin resistance, studies suggest males appear to be more susceptible to a programmed later life insulin resistance.[66,69]

Interestingly however, this sex-specific programming of insulin resistance may not exist in humans. In a mixed cohort of offspring aged 25 who were growth restricted *in utero*, a significantly lower insulin-stimulated glucose uptake was observed compared to controls, as well as a higher plasma insulin concentration, suggesting the development of insulin resistance irrespective of sex. Of note, these observations were in conjunction with a normal glucose tolerance challenge, thus representing an early phase in the pathogenesis to insulin resistance and MetS.[70] However, by age 64, men who were born small exhibited a strong link with impaired glucose tolerance and type 2 diabetes,[52] while data concerning women born small is not widely available. Therefore, understanding the early steps in the progression towards insulin resistance in the LBW population, and any sex-specific effects, is of critical importance for mitigating the risk of these offspring to the development of later life type 2 diabetes.

Animal models of IUGR, as well as human studies, have been used in order to identify the molecular changes occurring at the level of the insulin-signaling cascade that may be underlying the reduced insulin sensitivity in this population.[65,71] In early postnatal life, expression of the insulin receptor is increased in LBW sheep, as a compensatory mechanism for the low insulin and glucose levels experienced *in utero*.[72] However, by young adulthood skeletal muscle expression levels of the insulin receptor in LBW offspring are no different to normal birth weight controls, highlighting that the later life alterations in insulin sensitivity are likely due to a defect down-stream of the receptor that affects the ability of GLUT4 to translocate to the membrane and take glucose into the cell.[71] Analysis of key intermediates in the insulin signaling cascade has demonstrated sex-specific alterations in animal models of IUGR that may be responsible for altered GLUT4 expression and reduced glucose uptake that is associated with states of insulin resistance.[41,65,71,73]

In rodent models of placental insufficiency, young adolescent male offspring show altered GLUT4 transport in conjunction with an increased phosphorylation of IRS-1,[65] which is known to blunt the physiological response to insulin[63] by reducing the coupling efficiency of the insulin receptor and IRS-1.[74] IRS-1 is also known to complex with PI3-kinase, and in young male LBW offspring, reduced expression of the p85 regulatory subunit and p110β catalytic subunit of PI3K has also been observed in rodent, as well as larger animal (e.g., sheep) models of placental insufficiency.[67,71,72] Downstream of IRS-1, the total Akt levels were not altered by insulin infusion in growth restricted males;[65,71] however, phosphorylation of this intermediate was increased by insulin, suggesting a compensatory mechanism may be at play to maintain this

physiological response to exogenous insulin.[65] Although Akt's involvement in the insulin cascade appears to be unaffected, downstream AS160 shows reduced phosphorylation in response to insulin,[65] suggesting a functional impairment that impacts GLUT4 translocation to the plasma membrane.[75]

Human data has suggested similar molecular alterations are present in young LBW males, including reduced expression of the p85 and p110β subunits of PI3-kinase and reduced skeletal muscle GLUT4 content.[40,71] These alterations occur in conjunction with a blunted phosphorylation of Akt in response to insulin infusion, but with maintenance of glucose tolerance and whole body insulin sensitivity.[40,71] Taken together, changes in the molecular expression of key insulin signaling intermediates in skeletal muscle of LBW offspring may precede development of whole body insulin resistance and glucose intolerance, representing an early defect in metabolism, or pre-diabetic state, that could be indicative of future metabolic disease.[71] However, the mechanisms by which the *in utero* environment may be modulating these changes in insulin sensitivity remain ill defined.

1.5 Origins of Skeletal Muscle Insulin Resistance

Traditional factors identified in the development of insulin resistance have been poor diet and sedentary lifestyle. The increasing prevalence of a "Western", or energy-dense, high-fat/high-sugar diet, has been implicated as a key factor contributing to the pathogenesis of insulin resistance, promoting accumulation of fat within the skeletal muscle associated with a state of lipid overload and impacting mitochondrial function,[76-

[78] leading to a diminished capacity for mitochondrial fatty acid oxidation associated with mitochondrial dysfunction.[79]

1.5.1 Lipid Overload

Consumption of this energy-dense diet generates a surplus of free fatty acids, ultimately leading to ectopic lipid accumulation in non-adipose tissues such as skeletal muscle.[80] Once in the skeletal muscle, excess fatty acids are activated to form their acyl-CoA derivatives, which can be esterified into diacylglycerol (DAG), metabolized into ceramide, or conjugated to acylcarnitine for entry into the mitochondria to undergo β-oxidation.[81] Excess lipids may also be stored in lipid droplets as triacylglycerols, generating a pool of substrates termed intramyocellular triglycerides (IMTG).[77] IMTG content is known to increase with percentage body fat, and to be elevated in obese or type 2 diabetic individuals.[82] IMTGs are broken down by lipases to undergo oxidation; however, disturbances in the rate of breakdown or oxidation may lead to accumulation of toxic lipid intermediates and subsequent insulin resistance.[77,83] The type of triglyceride (saturated *vs.* unsaturated) that make up the skeletal muscle lipid pool is also important, since unsaturated triglycerides may be destined to accumulate as IMTG, whereas saturated triglycerides may be broken down into DAGs.[84] Additionally, a higher proportion of saturated fatty acids within this lipid pool has recently been associated with insulin resistance.[84]

In rodent lipid infusion studies, insulin resistance develops secondary to increased concentrations of long-chain acyl-CoAs in the skeletal muscle.[61] This increased lipid availability has been associated with reduced levels of skeletal muscle β-oxidation,[85,86]

leading to accumulation of toxic lipid metabolites including DAG[61] and ceramide.[87] While specific changes in long-chain acyl-CoA levels have not been reported in IUGR or LBW offspring, reductions in enzymes involved in β-oxidation have been reported,[88] suggesting that accumulation of long-chain acyl-CoAs secondary to a reduction in oxidative capacity may be involved in the pathogenesis of insulin resistance.

Accumulation of these lipid metabolites in the skeletal muscle has been associated with increases in stress-induced kinases, such as protein kinase C (PKC)-θ or ε, isoforms known to act upstream of c-Jun N-terminal kinase (JNK) and IκB (inhibitor of NFκB) kinase (IKKβ). Notably, JNK and IKKβ are two central serine/threonine kinases mediating phosphorylation of IRS-1 at Ser^{307}. Increased inhibitory serine phosphorylation of IRS-1 induces a subsequent reduction in Ser^{473} phosphorylation of Akt, and reduced insulin-stimulated glucose uptake,[61,81,87,89] similar to the molecular alterations that have been observed in the insulin signaling pathway in skeletal muscle of the LBW population.[65,71] PKC-θ activation itself (translocation from the cytosol to the plasma membrane) has also been reported to occur during a state of lipid overload in skeletal muscle, and may represent an alternative pathway mediating alterations in the serine/threonine phosphorylation status of key insulin signaling intermediates.[61,83] Direct evidence that these changes may be happening in LBW, IUGR offspring as they age is still lacking, but if occurring, presents a potential pathway where *in utero* induced changes in skeletal muscle metabolic capacity may play a contributing role to later life insulin resistance when challenged with a postnatal high fat diet.

1.5.2 Mitochondrial Dysfunction

Recently, reports implicating mitochondrial lipid metabolism in the pathogenesis of insulin resistance have emerged in addition to the traditional lipid overload theories described above. Skeletal muscle relies heavily on fatty acid β-oxidation to generate energy, using this method to provide up to 90% of its total energy demand.[79] Therefore, any alterations in fatty acid oxidation may impair skeletal muscle oxidative capacity. Certainly in rodent IUGR studies, muscle oxidative capacity is adversely affected in growth-restricted animals, preventing ATP generation,[43,90] and as such could set the stage for impaired mitochondrial function when challenged with a postnatal Western diet.

Mitochondrial metabolism represents a complex series of enzymatic events initiated to oxidize lipids to carbon dioxide, with the ultimate goal of producing energy in the form of ATP. A definition of mitochondrial dysfunction has recently been proposed that implicates decreased mitochondrial oxidation of substrates resulting from a global reduction in oxidative phosphorylation.[91] This decreased oxidation characteristic of mitochondrial dysfunction may be induced due to decreased protein content or activity of oxidative proteins and/or reduced mitochondrial biogenesis or content.[91] Mitochondrial dysfunction has also been associated with accumulation of toxic lipid intermediates as described above, as well as accumulation of acylcarnitine intermediates, in association with alterations in insulin signaling.[92] However, the exact mechanisms linking mitochondrial dysfunction and insulin signaling remain complex and poorly understood.

In order to fully comprehend the scope of mitochondrial dysfunction and its role in the pathogenesis of insulin resistance, knowledge of normal mitochondrial lipid metabolism is essential. The process begins with long-chain fatty acid generation from

lipolysis in adipose tissue, or breakdown of triglycerides in very low-density lipoproteins or chylomicrons.[93] Once these long-chain fatty acids are formed they are taken up into skeletal muscle either through passive diffusion, or the coordinated actions of fatty acid translocase/cluster of differentiation 36 (FAT/CD36) and the family of fatty acid transport proteins (namely FATP1 and 4).[93] While the majority of fatty acid chains will be oxidized in the mitochondria, a subset of very-long chain fatty acids may be preferentially diverted towards the peroxisomes to undergo β-oxidation in this location, generating the reactive oxygen species hydrogen peroxide as a signaling intermediate.[94]

To initiate the oxidative process in the mitochondria, fatty acyl-CoAs must enter the mitochondrial matrix through the carnitine palmitoyltransferase (CPT) system.[95] This system consists of subcellular CPT1, which exchanges the Co-A moiety of the fatty acid for carnitine, forming an acylcarnitine. Once the acylcarnitine has crossed into the mitochondria through acylcarnitine translocase, it is converted once again to its acyl-CoA form by CPT2, in order to continue through the oxidative process.[95] It is important to note that CPT1 is allosterically regulated by malonyl-CoA, an intermediate derived from glucose metabolism and the first step of lipogenesis, thus providing an important control mechanism for regulating oxidative rate, making CPT1 the control point, or rate-limiting enzyme of mitochondrial oxidation.[93]

Now that the fatty acyl-CoAs have been transported into the mitochondria, they undergo a series of enzymatic reactions collectively termed β-oxidation. This process begins when acyl-CoA dehydrogenase enzymes selectively oxidize the acyl group of the fatty acid depending on the length of the chain. For example, very-long chain acyl-CoA dehydrogenase (VLCAD) acts on fatty acid chains greater than 22 carbons long, whereas

medium chain acyl-CoA dehydrogenase acts on fatty acids with a chain length between 6 and 12 carbons.[96] Following dehydrogenation, the carbon chain then undergoes hydration and another oxidation step, followed by thiolysis catalyzed by 3-ketoacyl CoA thiolase (KT), ultimately releasing acetyl-CoA.[96] This 4-step cycle continues, generating 2 carbon acetyl-CoA molecules at each iteration, until the entire long-chain has been reduced to an acetyl-CoA intermediate. These acetyl-CoA intermediates generated from β-oxidation are then fed into the tricarboxylic acid (TCA) cycle, a series of enzymatically driven reactions that generate carbon dioxide and the electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$).[97] NADH and $FADH_2$ then enter the electron transport chain, where the energy from the electrons carried on these intermediates is harnessed to pump protons across the mitochondrial membrane to generate the electrochemical gradient required for ATP synthesis through the process of oxidative phosphorylation (Figure 1.3).[95,97]

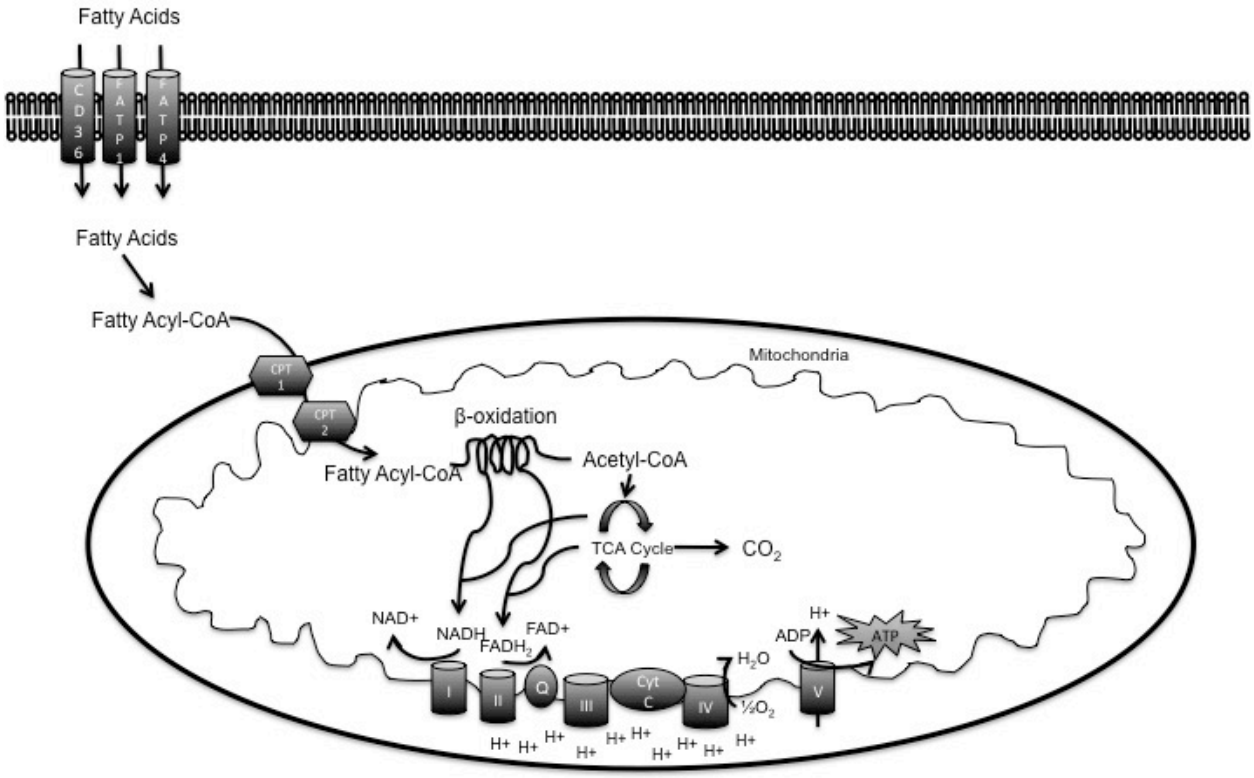

Figure 1.3: Mitochondrial Lipid Oxidation Pathway. Fatty acids enter the cell via fatty acid transporters, and are then transported into the mitochondria through the co-ordinated actions of CPT1 and CPT2. Once in the mitochondria, the fatty acyl-CoA undergoes β-oxidation, successively reducing the fatty acid chain by 2 carbons and generating acetyl-CoA intermediates for use in the TCA Cycle. The TCA cycle then generates CO_2 and the reducing co-factors NADH and $FADH_2$, which are fed through the complexes of the electron transport chain, with the ultimate goal of generating energy in the form of ATP.

Defects along this oxidative pathway have the potential to allow for accumulation of acylcarnitine metabolites, which have recently emerged as strong indicators of mitochondrial dysfunction and increased nutrient load.[62,98] Acylcarnitine accumulation in tissue is indicative of impairment in the utilization of the substrates generated through β-oxidation for downstream energy production, and the presence of fatty acids that are incompletely oxidized.[99] The recent interest in the field of metabolomics, which uses mass spectrometry techniques to determine how metabolic intermediates are altered in disease states, has allowed for profiling of these acylcarnitine intermediates. The surge in metabolomics research has highlighted a method well suited to further investigation of the relationship between mitochondrial dysfunction, incomplete β-oxidation and the pathogenesis of insulin resistance. For example, high-fat feeding in rodents has been shown to induce a lipid profile high in even-chain acylcarnitines. Accumulation of these even-chain acylcarnitine species ranging in length from 6 carbon chains (C6) to 22 carbon chains (C22) indicates that a large proportion of the fatty acids entering the mitochondria are only partially degraded for use as metabolic substrates.[62,100] Of interest, recent evidence has suggested that one even-chain acylcarnitine in particular, C14 carnitine, which can accumulate in situations of incomplete β-oxidation, has the ability to activate pro-inflammatory pathways, as well as an induction of JNK which may modulate the serine/threonine phosphorylation status of insulin signaling intermediates, perpetuating a state of insulin resistance.[89,101] Mitochondrial dysfunction has also been observed in rodent models of diabetes, including the Zucker Diabetic Fatty rat, further supporting the idea that incomplete fatty acid oxidation and accumulation of acylcarnitine intermediates may be implicated in the pathogenesis of insulin resistance.[62,102] Currently,

the impact of high fat feeding in LBW, IUGR offspring upon acylcarnitine production and mitochondrial dysfunction has not been reported. However, short-term high fat feeding trials in human LBW offspring show that LBW is associated with a reduced degree of metabolic plasticity,[103] suggesting that *in utero*-induced changes to mitochondrial oxidation may be unique in LBW offspring. Given the impact of diet alone upon acylcarnitine accumulation, a LBW situation in conjunction with poor postnatal diet may potentially exacerbate acylcarnitine accumulation and mitochondrial dysfunction.

1.5.3 Regulation of Mitochondrial Function and Biogenesis

The regulation of mitochondrial biogenesis and respiration is an important element determining oxidative capacity and the subsequent propensity for development of insulin resistance. The genes and enzymes involved in the oxidative process must be tightly regulated in order to adequately oxidize incoming fatty acids, generating co-factors and TCA substrates to generate ATP for cellular demand. This is accomplished by a tight co-ordination and interactions between a number of transcription factors regulating the enzymes involved in mitochondrial β-oxidation, including peroxisome proliferator-activated receptors, their co-activators, sirtuins, and the forkhead/winged helix transcription factors that each mediate an important aspect of mitochondrial biogenesis and oxidative function.

One such regulator of lipid oxidation is peroxisome proliferator-activated receptor alpha (PPARα), which is mainly expressed in tissues with a high oxidative capacity such

as the liver, heart, and skeletal muscle.[79] PPARα is activated upon binding of neutral lipids, promoting fatty acid uptake, and is involved in the induction of oxidative target genes with a PPARα response element, including CD36, CPT1b, VLCAD, MCAD and KT.[100,102,104,105] As such, increased activity of PPARα promotes mitochondrial β-oxidation. However, in order for full activation of PPARα and induction of its gene targets, co-ordination with peroxisome proliferator-activated receptor-gamma co-activator 1-alpha (PGC-1α) is required as a critical component of the PPARα-activated transcriptional machinery.[100] High levels of PGC-1α activity not only activate genes related to β-oxidation through the actions of PPARα, but also activate genes involved in the TCA cycle, including α-ketoglutarate dehydrogenase and succinate dehydrogenase.[105,106] Additionally, PGC-1α in complex with nuclear respiratory factor-1 (NRF-1) has the ability to induce transcription of components of oxidative phosphorylation, including subunits of complexes I through V.[106] Furthermore, this highlights PGC-1α as a key regulator of optimal mitochondrial function that promotes the complete oxidation of fatty acids.[105] Loss of PGC-1α has been implicated in the pathogenesis of insulin resistance in the context of high-fat diets, such that consumption of a high-fat diet decreases muscle PGC-1α expression, leading to a reduction in the coupling efficiency between β-oxidation and the TCA cycle, and coinciding with a shift from complete to incomplete β-oxidation, accumulation of acylcarnitines and altered insulin sensitivity.[100,105] Recent studies have also reported reduced levels of PGC-1α mRNA[107,108] and protein in the soleus[108] and gastrocnemius[109] muscles of young adolescent LBW offspring, as well as alterations in the methylation status of the PGC-1α promoter of both rodent[109] and human[110] models of IUGR. However, expression levels of

mitochondrial regulators of β-oxidation following consumption of a high-fat diet, combined with a LBW situation remain to be fully elucidated.

Another important family of transcription factors involved in the regulation of mitochondrial biogenesis as it relates to oxidative capacity is the sirtuins (SIRTs). SIRTs are responsible for cellular energy sensing, as they are modulated by the availability of metabolic cofactors, including NAD+ and NADH, therefore acting as a regulatory mechanism for the mitochondrial oxidative process in response to nutrient availability.[111] Seven SIRT isoforms have been identified in mammals; however, in terms of the regulation of overall mitochondrial function, the most notable isoforms are SIRT1 and SIRT3.[112] SIRT1 is predominantly localized to the nucleus, as well as the cytosol and mitochondria,[113] where it deacetylates target proteins involved in lipid metabolism and energy balance.[111] SIRT1 is activated during situations in which cellular energy is low (high NAD+/NADH ratio) in order to induce fatty acid oxidation by deacetylating PGC-1α and PPARα, allowing a global induction of oxidative genes with the ultimate goal of increasing ATP production.[105,111,113] On the other hand, SIRT3 is localized exclusively to the mitochondria and may have the potential to deacetylate proteins involved in the electron transport chain, highlighting SIRT3 as an important regulator of ATP synthesis and mitochondrial energy metabolism.[114,115] Recent reports suggest SIRT3 may also regulate metabolism at the oxidative level by initiating deacetylation of long-chain acyl-CoA dehydrogenase (LCAD) in order to promote β-oxidation.[116] Therefore, dysregulation of mitochondrial biogenesis or oxidative function due to decreased availability or activity of the above-mentioned factors, including PPARα, PGC-1α,

SIRT1 and/or SIRT3, has the potential to promote mitochondrial dysfunction and reduced lipid oxidation (Figure 1.4).

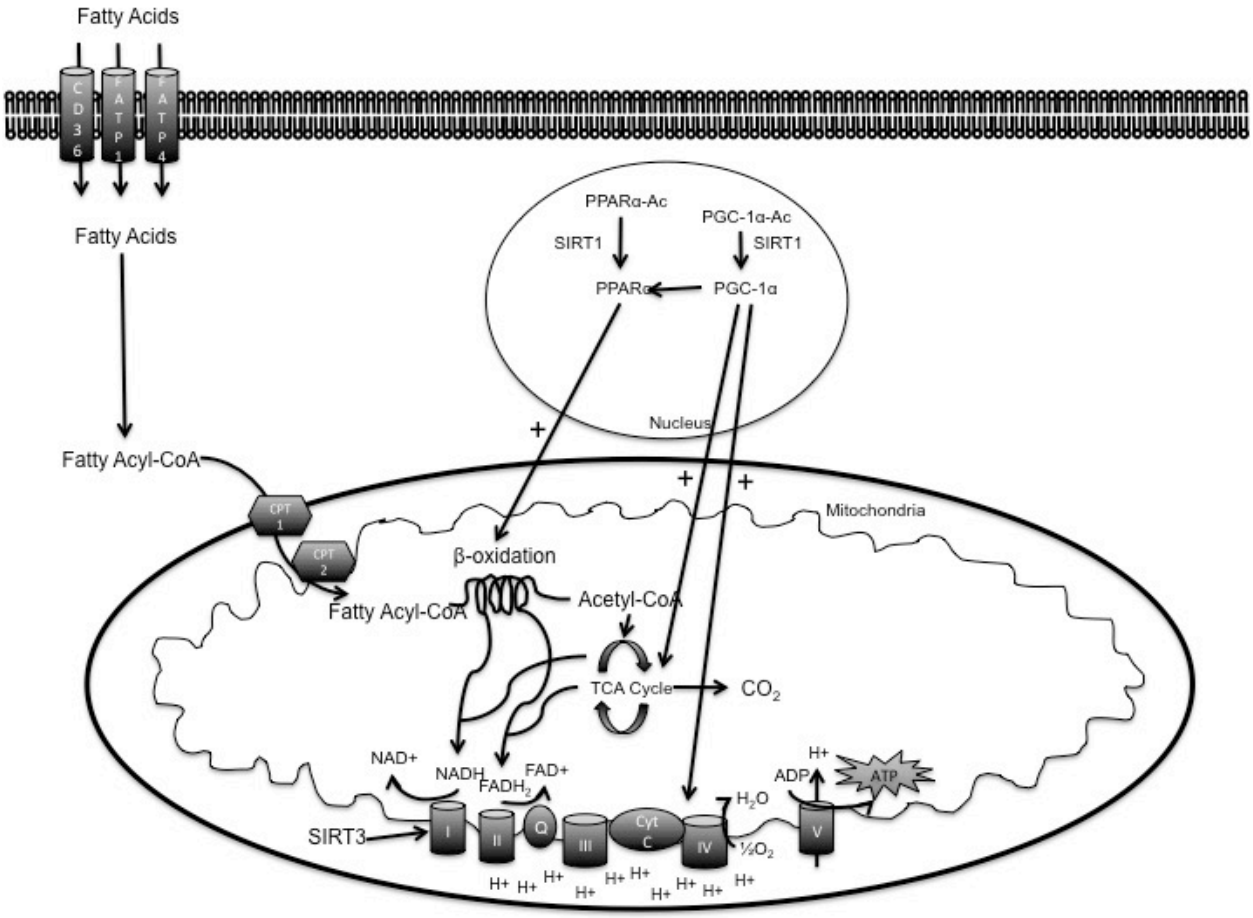

Figure 1.4: Regulation of Mitochondrial Lipid Oxidation. In order for lipids to be completely oxidized, tight co-ordination between a number of transcription factors is required. In resting states, PGC-1α and PPARα are acetylated, preventing their co-ordination. To induce the gene targets involved in β-oxidation and increase the oxidation rate, deacetylation by SIRT1 is required to allow PGC-1α and PPARα to complex together. PGC-1α on its own, in its deacetylated state, is also able to induce genes involved in the TCA cycle and electron transport chain, promoting complete lipid oxidation. SIRT3, which resides exclusively in the mitochondria, is responsible for deacetylating protein targets in the electron transport chain, in order to maintain its activity and promote ATP generation.

Unfortunately, the link between mitochondrial dysfunction and insulin resistance remains unclear. Is mitochondrial dysfunction the insult that induces insulin resistance, or does a primary insult of insulin resistance promote mitochondrial dysfunction? While this relationship remains complex and elusive, important insight into how insulin signaling and mitochondrial metabolic pathways intersect has been brought forth in recent years. One important transcription factor in this relationship between insulin signaling and mitochondrial metabolism has been identified, namely the forkhead/winged helix transcription factor FOXO1.[92] FOXO1 is important in regulating cell proliferation, as well as mediating the expression of hundreds of genes involved in gluconeogenesis, lipid metabolism and oxidative stress.[117] Recently, FOXO1 has been identified as a substrate of Akt, containing three phosphorylation sites for Akt-mediated phosphorylation (Thr^{24}, Ser^{256}, Ser^{319}).[118] Phosphorylation of these residues, secondary to Akt activation through the insulin signaling cascade, promotes FOXO1 nuclear exclusion and translocation to the cytosol, inhibiting FOXO1-mediated gene expression and regulating rates of gluconeogenesis and lipid metabolism in response to nutrient availability.[119] In situations of insulin resistance, impairment of IRS and reduced activation of Akt allows for hyperactivation of FOXO1, which has the ability to disrupt the electron transport chain by inducing expression of heme oxygenase 1 (HMOX1) which depletes heme availability, an essential cofactor facilitating electron transport.[92] By disrupting mitochondrial oxidative phosphorylation, the NAD+/NADH ratio decreases, blunting the activation of SIRT1 and PGC-1α-mediated mitochondrial biogenesis and impairing fatty acid oxidation, promoting a state of mitochondrial dysfunction.[92]

However, whether insulin resistance is the result of accumulation of intermediates associated with mitochondrial dysfunction remains unclear.

1.6 Rationale, Hypothesis, Objectives

1.6.1 Rationale and Hypothesis

Recent evidence has suggested that infants of an adverse *in utero* environment represent a unique population who appear to be at greater risk for development of insulin resistance and other components of MetS.[12,13,49-52] Adaptive, programmed changes in metabolic development and function initiated to aid in fetal survival are thought to lead to structural and metabolic deficits in later life.[8] However, the molecular alterations associated with these programmed changes and their role in promoting later life metabolic disease remain unclear. While defects in the skeletal muscle metabolism of LBW offspring have been highlighted in both human and animal studies, including alterations in insulin sensitivity and oxidative phosphorylation,[40,43,71,120] the *in utero* contributors to the origins of insulin resistance and the relative contribution of *in utero*-induced alterations to skeletal muscle fatty acid metabolism and mitochondrial function on the progression of insulin resistance remain ill defined.

Further, and more concerning, are recent studies suggesting that these LBW offspring are unable to lose weight as efficiently as normal birth weight offspring when maintained on a 40%-calorie restricted diet,[43] and that PGC-1α deficiencies associated with IUGR appear resistant to exercise intervention.[121] These studies highlight that these

offspring, while being at a higher risk of developing aspects of metabolic disease, may be resistant to current intervention practices. Additionally, when these infants with programmed alterations in skeletal muscle development are faced with a postnatal environment of nutrient excess, they appear to be at greater risk for aberrant skeletal muscle function.[8,13] Therefore, understanding the unique relationships and subtle metabolic differences between low and normal birth weight infants and how they interact with their postnatal nutritional environment is an important step in determining methods to improve skeletal muscle insulin sensitivity in order to mitigate the risk of these LBW offspring developing MetS in later life. As such, ***we postulate that IUGR is associated with in utero-induced alterations in skeletal muscle lipid oxidation, which impair insulin sensitivity, independent of postnatal diet. Early exposure to a poor postnatal diet may have the potential to exacerbate the already dysfunctional system, further increasing the risk of developing insulin resistance and progression to MetS in young adulthood.***

1.6.2 Objectives

The first objective of the study is to assess growth and metabolic parameters of guinea pig offspring of an adverse *in utero* environment independently, and in conjunction with a postnatal high-fat/high-sugar diet, in order to investigate the relative contributions of the components of the *in utero* environment and postnatal diet on glucose tolerance and body composition as they relate to early markers of the pathogenesis of MetS.

The second objective of the study is to investigate the impact of an adverse *in utero* environment upon skeletal muscle lipid metabolism independently, and in conjunction with a postnatal high-fat/high-sugar diet, focusing on mitochondrial lipid oxidation, acylcarnitine accumulation and mitochondrial dysfunction, as a contributor to the pathogenesis of insulin resistance.

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Chapter 2:

Growth and Body Composition of Guinea Pig Offspring are Altered in Response to Low Birth Weight and Postnatal Exposure to a "Western" Diet

Data presented in this chapter is part of a larger work profiling our entire cohort, which is currently in preparation for submission.

2.1 Introduction

Metabolic syndrome (MetS) is characterized by a cluster of risk factors, including hyperglycemia, central obesity, hypertriglyceridemia, dyslipidemia and high blood pressure,[1,2] which ultimately increases the patient's risk of developing cardiovascular disease, type 2 diabetes, and other age-related metabolic diseases.[3] An early marker of disease progression is insulin resistance, as it is typically observable before overt manifestations become apparent.[4] Insulin resistance is a metabolic state in which peripheral tissues are no longer sensitive to the anabolic effects of insulin, perpetuating a state of hyperglycemia, further validating insulin resistance as a critical precursor to the pathophysiological development of MetS.[4]

The prevalence of MetS and its associated morbidities are steadily increasing in developed countries, with recent surveys estimating that 1 in 5 Canadians aged 18-79 display observable manifestations of MetS.[5] Classically, the origins of MetS and insulin resistance have largely been attributed to lifestyle factors, including consumption of energy-dense diets and sedentary behavior.[6] The increasing prevalence of the symptoms associated with MetS mirrors the increase in obesity rates, which is largely attributable to increased consumption of energy-dense, high-fat/high-sugar, "Western" diets and their propensity to induce a state of insulin resistance.[6] These Western diets, with their high percentage of saturated fatty acids and low percentage of poly-unsaturated fatty acids,[7] alter lipid metabolism, induce an inflammatory state, and promote altered insulin sensitivity, increasing the patient's risk for developing cardio-metabolic disease.[6,8] The Western diet is also characterized by a high percentage of added sugars,[9] including fructose, which alters carbohydrate and glucose metabolism and impact insulin

sensitivity, likely further contributing to the development of a state of insulin resistance.[10,11] These increases in fats and sugars independently, and in combination, alter growth trajectory, favouring fat deposition and accelerating development of obesity and metabolic disease, promoting MetS progression.

Recently, human epidemiological studies have highlighted that an adverse *in utero* environment that restricts fetal growth, culminating in intrauterine growth restriction (IUGR) and low birth weight (LBW), increases the propensity for the offspring to develop age-related metabolic and cardiovascular diseases in later life.[12] Indeed, the recent EPOCH study highlighted higher fasting insulin levels and homeostatic model assessment of insulin resistance (HOMA-IR) values indicative of impaired insulin sensitivity as early as 10 years of age in IUGR-exposed children, independent of both childhood body mass index (BMI) and BMI trajectory following a period of "catch-up growth" during the first year of life.[13] Similarly, reports of a 7-fold increased risk for development of insulin resistance by the age of young adulthood have also been observed.[14] Furthermore, this pattern holds throughout the life-span, with low birth weight males at age 64 exhibiting a strong association with impaired glucose tolerance, a marker of whole-body insulin resistance and type 2 diabetes.[15] Additionally, animal models of low birth weight generated through a variety of means, including caloric restriction, maternal protein restriction and uterine artery ligation, have confirmed the impact of an adverse *in utero* environment on an increased incidence of cardiovascular disease, visceral obesity and hypercholesterolemia in these offspring.[4,16-20] Overall, these human and animal studies highlight that metabolic adaptations to an adverse *in utero*

environment are associated with a predisposition for development of aspects of MetS in later life.

The Developmental Origins of Health and Disease (DOHaD) hypothesis has developed immensely over the last thirty years to postulate how adaptations to limited nutrient and oxygen supply, as a consequence of reduced placental blood flow to the fetus, results in an adverse *in utero* environment which would have the ability to permanently program structural and functional alterations to major metabolic organs.[4] These adaptive responses during critical periods of *in utero* development are thought to program a "thrifty phenotype" which aids in immediate survival of the fetus. One overt manifestation of this adaptive growth *in utero* is a leaner phenotype at birth as a consequence of reduced muscle mass,[21,22] which has been associated with a programmed predisposition to altered insulin sensitivity in later life.[4,12,14,20] Since organogenesis occurs predominantly *in utero* and is followed by a period of rapid fetal growth and loss of organ plasticity shortly after birth,[23,24] environmental insults during this sensitive period of development have the potential to result in a fixed functional capacity of vital metabolic organs.[24] As such, these adaptive responses in response to an adverse *in utero* environment have the potential to induce a propensity for life-long metabolic consequences as these offspring interact with their postnatal environment.[12,20]

Additionally, IUGR and LBW are associated with a period of rapid postnatal catch-up growth which further modulates later-life disease risk.[12] The cessation of growth experienced *in utero* is suggested to slow the genetic growth program of the fetus, delaying the loss of proliferative capacity typically experienced following birth, allowing for rapid catch-up growth to occur in early life.[25] However, this period of rapid growth in

early life has been associated with increased childhood adiposity,[26] as well as an increased strain on vital metabolic organs that contribute to the development of MetS.[27] In comparison to the nutrient deprivation experienced *in utero,* introduction to a postnatal environment characterized by relative nutrient excess also has the potential to induce postnatal catch-up growth and alter the metabolic capacity of vital organs, increasing the offspring's risk for developing metabolic disease with age.[24]

Recently, evidence has suggested that *in utero* influences and secondary insults in adult life may have a synergistic effect, further exacerbating an already dysfunctional system and promoting earlier onset of MetS.[12] While some studies have suggested the prevalence of childhood obesity has begun to plateau in the United States,[28] it still remains a concern worldwide. Indeed, using the WHO guidelines for classifying obesity in children, it was estimated that approximately 33% of children in Canada aged 5 to 11 years were overweight or obese between 2009 and 2011.[29] This increased incidence of childhood obesity can largely be attributed to early introduction of energy-dense diets and sedentary behavior;[28] however genetics and the contribution of the *in utero* environment cannot be negated. Ultimately, altered childhood growth and body composition, namely obesity, have the potential to influence later-life disease risk, putting these children at increased risk of developing symptoms associated with MetS at an earlier age.[30] While the increasing prevalence of childhood obesity and poor food choices in our society continues to expand, the postnatal effects of consumption of an energy-dense Western diet still remain largely unexplored in LBW offspring. As such, we sought to assess body composition, growth parameters and glucose tolerance as a marker of the MetS phenotype in guinea pig offspring of an adverse *in utero* environment. Additionally, we

investigated body composition, growth and glucose tolerance in offspring of an adverse *in utero* environment in conjunction with a postnatal high-fat/high-sugar diet in order to investigate the relative contributions of the components of the diet on progression of the MetS phenotype.

2.2 Materials and Methods

2.2.1 Ethics Statement

Animal care, maintenance and surgical procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were approved by The University of Western Ontario Animal Use Subcommittee (Appendix II).

2.2.2 Animal Handling

Time-mated pregnant Dunkin-Hartley guinea pigs (Charles River Laboratories, Wilmington, MA, USA) were housed in a constant temperature (20°C) and humidity (30%) controlled environment with 12h light/dark cycle. All animals had *ad libitum* access to standard guinea pig chow (LabDiet diet 5025) and tap water.

All pregnant guinea pigs underwent uterine artery ablation, as previously described,[31] to induce uteroplacental insufficiency and IUGR. At mid-gestation (~32 days, term ~67 days), sows were anesthetized in an anesthetic chamber (4-5% isoflurane with 2L/min O_2, followed by 2.5-3% isoflurane with 1L/min O_2 for maintenance). Immediately following induction of anesthesia, a subcutaneous injection of Rubinol (Glycopyrrolate, 0.01mg/kg, Sandoz Can Inc., Montreal, QC) was administered. A midline incision was made below the umbilicus, exposing the bicornate uterus. Arterial vessels feeding each horn of the uterus were identified, and every second branch was cauterized using an Aaron 2250 electrosurgical generator (Bovie Medical, Clearwater,

FL), then the abdominal cavity closed. Immediately following surgery, a subcutaneous injection of Temgesic (Buprenorphine, 0.025mg/kg, Schering-Plough Co., Kenilworth, NJ) was administered, and regular post-operative monitoring undertaken. Sows delivered spontaneously at term (approximately 67-69 days),[31] at which time pup weight, length, abdominal circumference and biparietal distance were measured. At the end of the pupping period, male guinea pigs were allocated to the low birth weight (LBW) group if birth weight was below the 25th percentile, and the normal birth weight (NBW) group if birth weight was between the 25th and 75th percentile (Figure 2.1). Based on these criteria, LBW pups were below 80g, and NBW pups were above 90g, in accordance with previously reported data.[19,32]

Pups remained with their dams during the 15-day lactation period. Five days prior to weaning, all pups were introduced to small amounts of a control diet (Harlan Laboratories TD.110240: Table 2.1) and weaned on postnatal day (PND)15 into individual cages. At this time, guinea pig offspring were randomized to either a control (CD, Harlan Laboratories TD.110240: Table 2.1) or a Western (WD, Harlan Laboratories TD.110239: Table 2.1) diet. To avoid litter effects, only one male LBW and one male NBW animal from a single litter was assigned to each diet. From the time of weaning, feed intake was measured daily, and body weights were measured daily until PND50, then twice weekly until PND145, corresponding to young adulthood.[16,33]

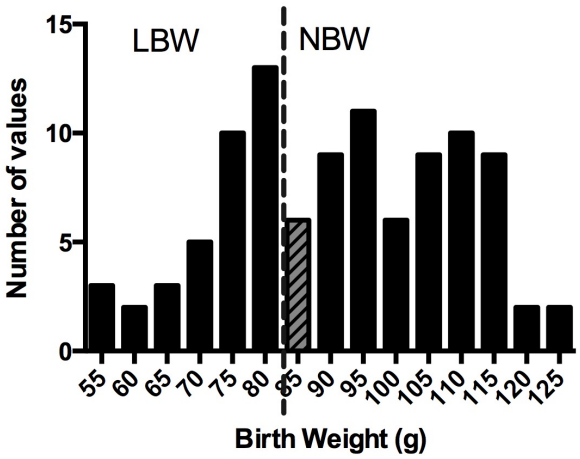

Figure 2.1: Birth Weight Distribution. Pregnant sows underwent uterine artery ablation at mid-gestation in order to induce placental insufficiency and intrauterine growth restriction. Briefly, in anesthetized sows, a midline incision was made below the umbilicus, exposing the bicornate uterus. Arterial vessels feeding each horn of the uterus were identified, and every second branch was cauterized, reducing placental blood flow, oxygen and nutrient transfer to the fetus. Following spontaneous pupping, pups born below the 25th percentile (dashed line) were classified as low birth weight (LBW), and those above the 25th percentile were classified as normal birth weight (NBW). Animals between 80g and 90g (grey, hatched bar) were excluded from analyses.

Component	Control Diet (CD)	Western Diet (WD)
Total protein (isolated soy protein, %kcal)	22	21
Total Fat (%kcal)	18	46
Saturated Fatty Acids	15	70
Poly-unsaturated Fatty Acids	61	4
Mono-unsaturated Fatty Acids	24	26
Fatty Acid Composition (%weight)		
C12:0	1.26	17.90
Myristic Acid (C14:0)	1.28	9.33
Palmitic Acid (C16:0)	14.74	21.64
Stearic Acid (C18:0)	5.93	16.96
Oleic Acid (C18:1*cis*9)	25.60	26.72
Linoleic Acid (C18:2*n-6*)	46.04	7.45
Alpha Linoleic Acid (C18:3*n-3*)	5.15	-
Total Carbohydrate (%kcal)	60	33
Sucrose	11	22
Fructose	-	7.6
Cholesterol (%kcal)	-	0.25
Density (kcal/g)	**3.4**	**4.2**

Table 2.1: Composition of the Postnatal Diets.

2.2.3 Analysis of Growth and Feed Intake

Absolute growth rate (AGR: g/day), percent increase in body weight, and fractional growth rate (FGR: AGR/birth weight)[16] were calculated for the 15-day lactation period for n=20 NBW and n=18 LBW offspring.

The post-weaning period was divided into three intervals: weaning-PND50 representing the period of maximal growth, PND51-110 representing the period of adolescent growth, and PND111-145 representing the plateau period in young adulthood. For each interval, the AGR, average food intake (g/day/kg body weight), calorie consumption (calories/day) and efficiency (g gained/day/calories consumed/day) were calculated for n=11 NBW/CD, n=11 LBW/CD, n=9 NBW/WD and n=7 LBW/WD offspring.

2.2.4 Computed Tomography (CT) Measurements

At approximately PND110, *in vivo* computed tomography scans were performed, as previously described,[19] in order to quantify total adipose tissue, bone and muscle volume of the whole body (from proximal tibia to base of skull).[34] Briefly, anesthesia was induced in an anesthetic chamber using 4-5% isoflurane with 2L/min O_2, and maintained with 1.5-3% isoflurane. Images were taken using a Discovery CT750 HD scanner (GE Healthcare, Mississauga, ON) at 1.25mm slices, with voltage and current set to 80kV and 100mA, respectively. Using a MATLAB developed program (The Mathworks Inc., Natick, MA), bone, muscle and adipose tissue were differentiated by

thresholding the CT image slides of the guinea pig.[35] Using pixel size and thickness of each CT image, volumes (cm^3) of bone, muscle and adipose tissue type were calculated for each tissue type and expressed as a percentage of total body volume for n=4-5 animals per group.

2.2.5 Glucose Tolerance Testing

At PND60 and PND120, intraperitoneal glucose tolerance tests (IPGTTs) were performed on n=12 NBW/CD, n=12 LBW/CD, n=9 NBW/WD and n=7 LBW/WD offspring to assess whole body glucose tolerance. Following an overnight (16h) fast, a bolus injection of 0.5% dextrose (1g/kg body weight)[16,36] was administered intraperitoneally. Blood was sampled at the peripheral ear vein at times 0, 10, 20, 50, 80, 110, 140, 170, and 200 minutes following injection. Blood glucose levels were measured using a Bayer Contour hand-held glucometer (Bayer Diabetes, Mississauga, ON). Area under the glucose curve was calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA) for each animal as a measure of glucose clearance.[37]

2.2.6 Statistical Analyses

All data presented are mean ± standard error (SEM). Statistical significance during the lactation period was determined using a two-tailed unpaired *t*-test. Two-way ANOVA followed by post-hoc Tukey's test was performed to assess the effects of birth weight and postnatal diet. Two-way ANOVA Repeated Measures, followed by post-hoc

Tukey's test, were performed to assess the effects of birth weight and postnatal diet over the course of the experimental period. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant.

2.3 Results

2.3.1 Uterine Artery Ablation Results in Growth Restriction of LBW Offspring

At the time of birth, LBW offspring displayed indications of growth restriction. Birth weight/birth length, a measure of leanness,[16] was significantly ($p<0.001$) reduced in LBW offspring, compared to NBW offspring (Table 2.2). Biparietal diameter was significantly ($p<0.05$) decreased in LBW offspring, compared to their NBW counterparts (Table 2.2). Crown-rump distance was significantly ($p<0.01$) reduced in LBW offspring (Table 2.2). Abdominal circumference was reduced ($p=0.0544$) in LBW offspring (Table 2.2).

	Bi-parietal Distance (cm)	Crown-Rump Length (cm)	Abdominal Circumference (cm)	Ponderal Index (g/cm^3)	Birth Weight/ Birth Length (g/cm)	Absolute Growth Rate (g/day)	%Increase in Body Weight	Fractional Growth Rate
NBW	2.16 ± 0.035	15.49 ± 0.32	11.56 ± 0.17	0.029 ± 0.002	6.7 ± 0.21	9.50 ± 0.26	115.7 ± 3.79	0.092 ± 0.002
LBW	2.04 ± 0.039	13.97 ± 0.38	10.98 ± 0.24	0.029 ± 0.002	5.46 ± 0.22	8.29 ± 0.24	149.6 ± 6.14	0.113 ± 0.004
	*	**	p=0.0544	ns	***	**	****	****

Table 2.2: Birth Characteristics and Lactation Period Growth. Following spontaneous birth, pup weights, length, biparietal distance, and abdominal circumference were measured as indicators of growth restriction. Pup weight was measured daily throughout the 15-day lactation period. Data presented are mean ± standard error (SEM) for n=20 normal birth weight (NBW) and n=18 low birth weight (LBW) offspring. Statistical significance was determined by two-tailed unpaired *Student's* t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns = not significant.

2.3.2 Growth Performance During the Lactation Period

During the lactation period, LBW offspring exhibited an altered growth trajectory, including a 13% reduction ($p<0.01$) in absolute growth rate (AGR; Table 2.2). However, LBW offspring showed a 149.6% increase in their body weight over the course of the lactation period, a 29% ($p<0.0001$) greater increase than the NBW offspring (Table 2). The fractional growth rate was also 23% greater ($p<0.0001$) in LBW offspring, compared to NBW (Table 2.2).

2.3.3 Growth Performance and Energy Intake Post-weaning

During the post-weaning period, NBW/CD offspring had an AGR of 7.38 ± 0.323 g from weaning until PND50, an AGR of 4.62 ± 0.23 g from PND51-110, and an AGR of 2.87 ± 0.47 g from PND111-145 (Figure 2.2A), ultimately reaching a body weight of 825.2 ± 28.3 g at the time of putdown. During the period from weaning until PND50, NBW/WD offspring displayed a 27% reduction ($p<0.01$) in AGR, compared to NBW/CD offspring, whereas LBW/WD offspring displayed a 34% reduction in AGR compared to NBW/CD offspring ($p<0.001$; Figure 2.2A). No significant differences in mean AGR were observed for the period from PND51-110. From PND111 to the time of putdown, LBW/CD offspring displayed a 52% reduction ($p<0.05$) in AGR, compared to NBW/CD offspring (Figure 2.2A). An overall reduction ($p<0.01$) in total mean AGR was also observed in our WD-fed groups, independent of time. Additionally, there was a significant ($p<0.01$) interaction between time and our experimental groups (Figure 2.2A). WD-fed offspring remained significantly ($p<0.05$) lighter than CD-fed offspring at the

time of putdown, with body weights of NBW/WD: 732.3 ± 26.8 g; LBW/WD: 708.8 ± 19.8 g vs NBW/CD: 825.2 ± 28.3 g; LBW/CD: 747.9 ± 38.1 g. Compared to the NBW/CD group, male LBW/WD animals had significantly ($p<0.05$) lower body weights at putdown.

No significant differences in average food intake were observed throughout the experimental period (Figure 2.2B). However, calorie consumption was significantly ($p<0.0001$) higher in WD-fed offspring, independent of time (Figure 2.2C). From weaning-PND50, calorie consumption was 23% higher in NBW/WD ($p<0.05$) and 36% higher in LBW/WD ($p<0.001$) offspring compared to NBW/CD. From PND51-110, calorie consumption was 26% higher in NBW/WD ($p<0.05$) compared to NBW/CD, and from PND111-PND145, calorie consumption was 34% higher in NBW/WD ($p<0.05$) and 55% higher in LBW/WD ($p<0.001$) when compared to NBW/CD (Figure 2.2C).

The feeding efficiency (grams gained/day/calories consumed/day) was significantly ($p<0.0001$) decreased in WD-fed offspring, in addition to a significant ($p<0.05$) interaction with time (Figure 2.2D). From weaning-PND50, feeding efficiency was reduced by 39% in NBW/WD ($p<0.0001$) and by 52% in LBW/WD ($p<0.0001$) offspring compared to NBW/CD. From PND51-110, NBW/WD males showed a 35% reduction in ($p<0.01$) feed efficiency compared to NBW/CD, whereas LBW/CD offspring showed a 50% reduction in ($p<0.01$) feed efficiency from 111-PD, when compared to NBW/CD (Figure 2.2D).

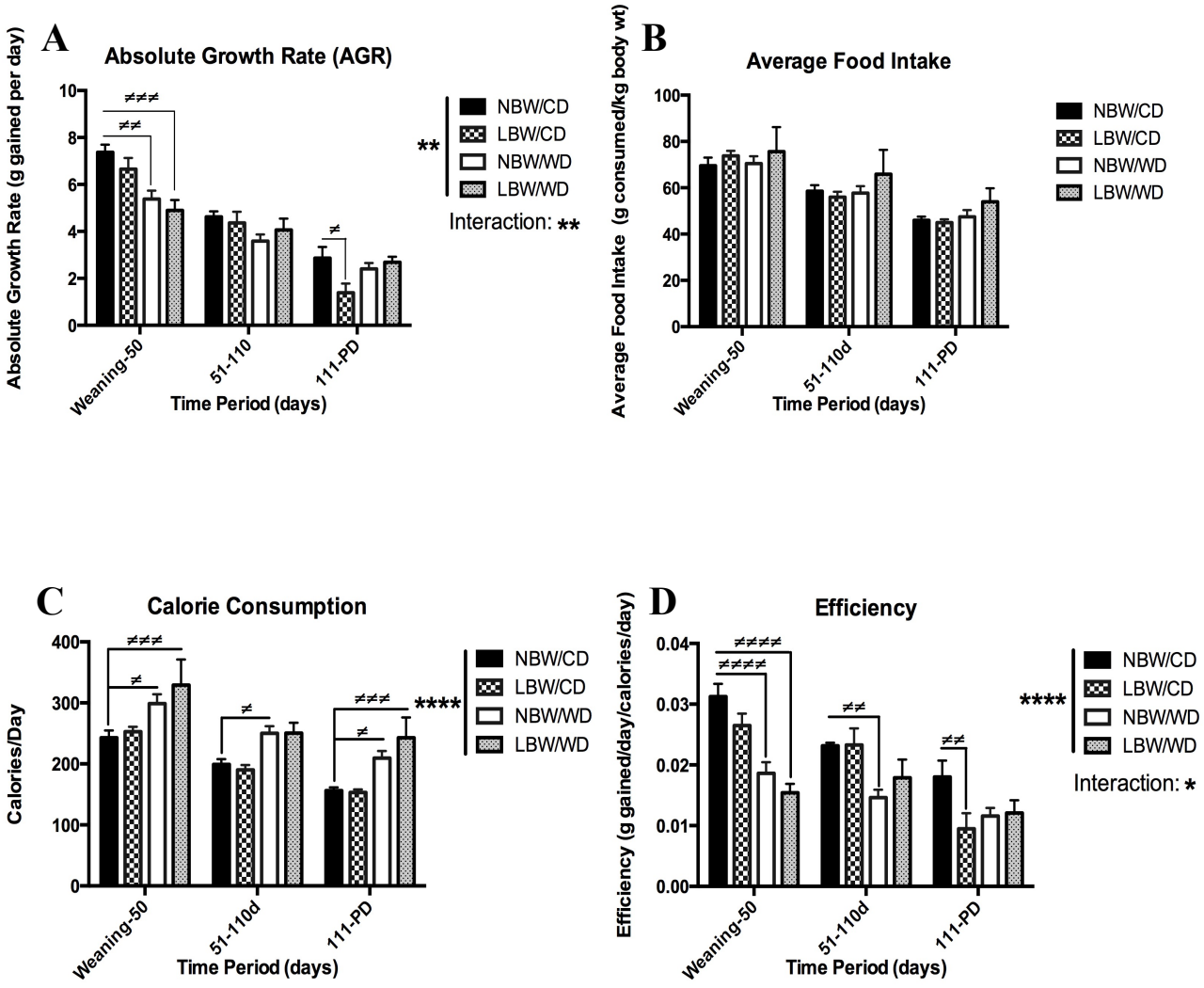

Figure 2.2: Post-weaning Growth and Feeding Behaviour. A) Absolute growth rate (AGR) = grams gained per day. B) Average food intake = grams of food consumed/kilogram body weight. C) Calorie consumption = calories consumed per day. D) Feeding efficiency = grams gained per day/calories consumed per day. Data presented are the mean ± standard error (SEM) for n=11 NBW/CD, n=11 LBW/CD, n=9 NBW/WD and n=7 LBW/WD offspring. Statistical significance was determined by Two-Way ANOVA repeated measures, followed by post-hoc Tukey's Test. Interaction: *$p<0.05$, **$p<0.01$ significant interaction between experimental group and time; **$p<0.01$, ****$p<0.0001$ significant effect of experimental group, independent of time; ≠$p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$ by post-hoc Tukey's Test.

2.3.4 Body Composition and Glucose Tolerance

Whole-body adiposity, as measured by computed tomography at PND110, was significantly ($p<0.01$) reduced in WD-fed offspring, independent of birth weight (Figure 2.3). At tissue collection, mesenteric fat pad depot weights were significantly ($p<0.001$) reduced in WD-fed offspring at the time of putdown (Table 2.3). Post-hoc analysis demonstrated a significant ($p<0.01$) reduction of mesenteric fat pad mass in NBW/WD and LBW/WD, when compared to NBW/CD offspring (Table 2.3).

No significant differences were observed in whole body glucose tolerance following a bolus administration of glucose at PND60 (Figure 2.4A) or PND120 (Figure 2.4C). The clearance of glucose, as measured by the area under the curve, was also not significantly altered by birth weight or postnatal diet at PND60 (Figure 2.4B) or PND120 (Figure 2.4D).

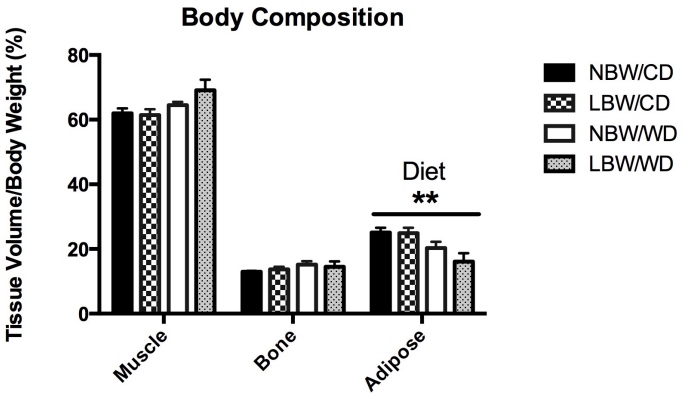

Figure 2.3: Body Composition as Assessed by Computed Tomography Analysis. Volumes occupied by muscle, bone and adipose tissue in the area between the proximal tibia to base of skull was calculated using a MATLAB-derived application, normalized to total body volume and expressed as a percentage. Data presented are the mean ± standard error (SEM) for n=4-5 animals per group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. **p<0.01 significant effect of diet, independent of birth weight.

	Inguinal Fat Mass	Brown Fat Mass	Epididymal Fat Mass	Mesenteric Fat Mass	Perirenal Fat Mass	Retroperit-oneal Fat Mass	Leg Fat Mass
Males							
NBW/CD	0.011 ± 0.0007	0.010 ± 0.0010	0.012 ± 0.0009	0.015 ± 0.0010	0.007 ± 0.0009	0.009 ± 0.0004	0.002 ± 0.00009
LBW/CD	0.012 ± 0.0009	0.007 ± 0.0007	0.011 ± 0.0008	0.013 ± 0.0010	0.006 ± 0.0008	0.008 ± 0.0010	0.002 ± 0.00008
NBW/WD	0.011 ± 0.0006	0.009 ± 0.0010	0.011 ± 0.0008	0.010 ± 0.0010 ##	0.005 ± 0.0004	0.008 ± 0.0005	0.002 ± 0.00017
LBW/WD	0.011 ± 0.0010	0.009 ± 0.0016	0.009 ± 0.0009	0.009 ± 0.0010 ##	0.006 ± 0.015	0.008 ± 0.0010	0.002 ± 0.00019
	ns	ns	ns	Diet: ***	ns	ns	ns

Table 2.3: Adipose Tissue Depot Weights. Data presented are mean ± standard error (SEM) for n=11 NBW/CD, n=11 LBW/CD, n=9 NBW/WD, n=7 LBW/WD offspring. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. Diet: ***p<0.001, significant effect of diet, independent of birth weight. ##p<0.01, compared to NBW/CD by post-hoc Tukey's Test. ns = not significant.

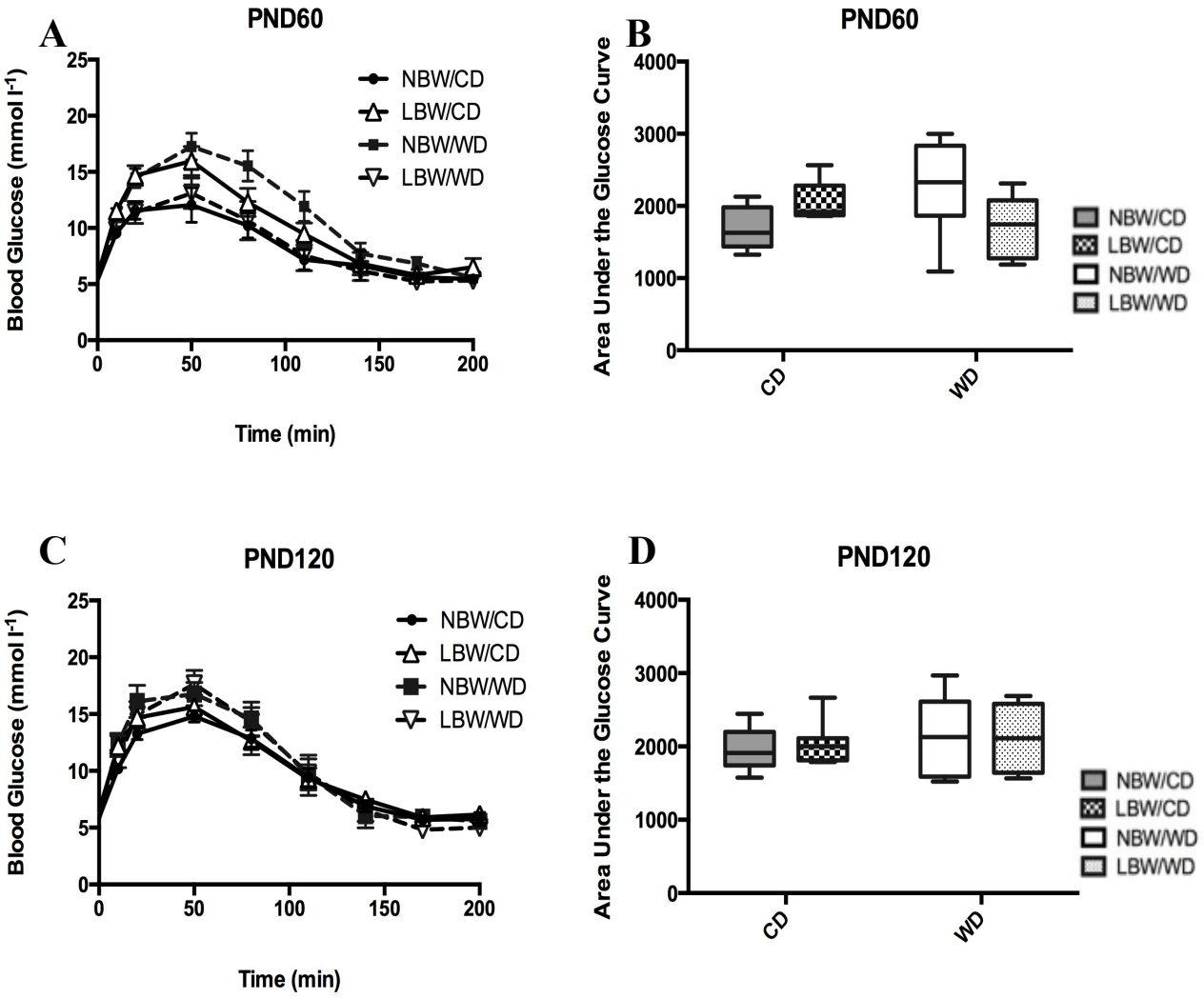

Figure 2.4: Glucose Tolerance. A) Blood glucose values (mmol l^{-1}) following an intraperitoneal injection of 1g/kg of 50% dextrose solution at PND60. B) The area under the glucose curve (AUGC) as a measurement of glucose clearance at PND 60. C) Blood glucose values (mmol l^{-1}) at PND120. D) The area under the glucose curve (AUGC) at PND 120. Data presented are the mean ± standard error (SEM) or min. to max. box and whisker plots for n=12 NBW/CD, n=12 LBW/CD, n=9 NBW/WD, n=7 LBW/WD. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test.

2.4 Discussion

Early-life growth and development, especially the contribution of the *in utero* environment as a key contributor to later life metabolic outcome, has continued to gain recognition and support in the global health and scientific community.[38] Human and animal studies have highlighted that insults affecting growth trajectory *in utero* and early postnatal life have the potential to alter the programmed metabolic development of the offspring, increasing their risk of developing serious metabolic consequences as they interact with their postnatal environment. Therefore, the aim of the current study was to assess growth and metabolic parameters of guinea pig offspring subjected to an adverse *in utero* environment, and to assess the impact of the easily accessible Western diet in the postnatal period on altered growth and metabolic parameters associated with a developing MetS phenotype.

We chose to utilize a guinea pig model to investigate developmental programming and the importance of the *in utero* environment on metabolic outcomes of the offspring since, similar to humans and sheep, the guinea pig is a precocious species that develops predominantly prenatally and is born relatively mature.[39] Unlike other rodent models, the guinea pig has a relatively long gestation (approximately 67-69 days),[31] allowing for fetal adaptations to an adverse *in utero* environment. Invasion and endocrine control of the placenta is also similar between human and guinea pig, further validating the use of this model to investigate the fetal adaptations to chronic perturbations in placental blood flow.[40,41] The uterine artery ablation technique employed has been shown to decrease delivery of oxygen and nutrients to the fetus via the placenta,[31] recapitulating the most common type of placental insufficiency characterized

by placental hypoxia and abnormal uterine artery Doppler waveforms typically observed in obstetrical clinics.[42] Also of note, guinea pig lipid metabolism, fat deposition, as well as metabolic responses to a high-fat diet, are similar to humans,[43-45] further validating our guinea pig model for investigating the relative impact of diet on aspects of postnatal metabolism.

In our cohort, the uterine artery ablation surgery induced IUGR, and LBW offspring below the 25th percentile displayed markers of growth restriction. Specifically, LBW offspring exhibited a leaner phenotype as evidenced by the reduced birth weight/birth length ratio. Similar reports of a lean phenotype have been observed in other animal models of IUGR.[16] Additional markers included a reduced biparietal diameter, abdominal circumference, and crown-rump length, in accordance with other guinea pig models of IUGR.[16,18,31] Therefore, IUGR induces an altered body composition as early as the time of birth when LBW offspring are compared to their NBW littermates.

During early life, the LBW offspring displayed an altered growth trajectory when compared to NBW offspring. While the absolute growth rate (g/day) was lower in LBW offspring, their percent increase in body weight and fractional growth rate throughout the lactation period were greater than the NBW offspring. This accelerated growth trajectory is characteristic of the period of rapid postnatal catch-up growth often observed in human offspring of adverse *in utero* environments,[12] and has also been observed in animal models of IUGR.[16,46] Fractional growth rate is commonly used as a marker of the interaction between an offspring's genetic growth potential and the nutritional environment experienced. An increased fractional growth rate in early postnatal life has been shown to be predictive of increased visceral obesity and impaired glucose tolerance

in later life, early markers of MetS pathogenesis.[47,48] Therefore, this accelerated growth trajectory observed in our LBW offspring during the lactation period may be indicative of rapid postnatal catch-up growth that predisposes these offspring to development of age-related metabolic diseases.

Of particular concern to the global health community is the increased incidence of worldwide childhood obesity along with the emergence of risk factors associated with MetS in children and young adults.[49] Independent of weight at birth, a high intake of saturated fatty acids and simple sugars in addition to a lack of poly-unsaturated fatty acids have been implicated in MetS pathogenesis.[7,50] Diets high in saturated fats have the propensity to promote ectopic lipid accumulation, disrupting normal metabolic function and perpetuating a state of insulin resistance as an early indicator of MetS progression.[51] Additionally, a high intake of simple sugars, namely glucose and fructose, is known to disrupt whole body glucose homeostasis, altering insulin action in addition to promoting ectopic lipid accumulation, two situations that have to the potential to promote insulin resistance and progression of MetS.[52] Furthermore, decreased consumption of poly-unsaturated fatty acids also contributes to insulin resistance and a MetS phenotype by altering the composition of skeletal muscle phospholipids and influencing insulin action.[52] With the goal of recapitulating the adverse metabolic consequences observed in children and young adults, the composition of our WD replicates a "classic" WD, as defined by a relatively large contribution of saturated fats, and added simple sugars, namely fructose.

Following weaning, LBW offspring on a CD (LBW/CD) continued to display an altered growth trajectory, such that at the time of putdown, there was no significant

difference in weight between NBW/CD and LBW/CD offspring. This highlights that the LBW/CD experienced a period of rapid postnatal growth that may be associated with an increased development of risk factors associated with MetS.[53] It is interesting to note that these LBW/CD offspring did display a reduced absolute growth from PND111 to the time of putdown, in addition to a reduced weight gain during this period despite consuming a similar number of calories per day when compared to NBW/CD offspring. This reduced weight gain during this period highlights the possible emergence of metabolic deficiencies impairing efficient metabolism of caloric intake. While this reduction in growth during the plateau phase did occur, these offspring still reached similar weights as their NBW counterparts, further supporting the notion that these offspring experienced an early phase of rapid growth, a phenomena characteristic of LBW postnatal growth following development in an adverse *in utero* environment.

In our cohort, WD-fed offspring remained significantly lighter than CD-fed offspring throughout the duration of the post-weaning period, even at the time of putdown. During the period of maximal growth, from weaning until PND50, WD-fed offspring, irrespective of birth weight, displayed a reduced AGR, despite increased calorie consumption. This discrepancy, in addition to the reduced feed efficiency, highlights an inefficiency affecting the ability of these animals to gain weight despite consuming a more calorie-dense diet. Additionally, the LBW/WD offspring remained significantly lighter throughout the duration of the experimental period, highlighting that the consumption of a postnatal WD stunted the accelerated growth associated with adverse growth *in utero.* Interestingly, this reduced body weight in the WD-fed offspring occurred in conjunction with altered body composition and adiposity. At the whole body

level, WD-fed offspring displayed reduced adiposity when assessed by computed tomography. Additionally, when measured at the time of putdown, mesenteric fat pad mass, a measure of visceral adiposity,[54] was also reduced in the WD-fed offspring. This reduction in adipose tissue mass, and overall reduction in adiposity, in addition to the reduced body weight observed in these offspring, highlight that components of the postnatal WD alters the body composition and metabolic profile of these offspring.

The diet of modern society is characterized by excess fats and sugars, particularly fructose and saturated fats, which have been associated with not only a rise in obesity rates, but also higher incidence of insulin resistance and type 2 diabetes.[7,9] While fructose is typically associated with an increase in adiposity and insulin resistance,[54] rodent studies that introduce fructose from an early age have observed similar reductions in body weight and adiposity.[55-57] It has been suggested that early introduction of fructose in the diet is associated with development of fructose intolerance and subsequently an impairment of weight gain.[58,59] Early in life, intestinal levels of GLUT5, the main fructose transporter, are reported to be low, impairing fructose transport and contributing to the development of fructose intolerance should administration of fructose be in large quantities during this period of development.[60]

While recent evidence suggests that the excess fat consumed in energy-dense diets may be repartitioned to other tissues, namely liver, negatively impacting metabolic health,[61] excess consumption of fructose on its own has also been associated with alterations in liver metabolism. In non-human primates fed a high-fructose diet for 7 years, poor hepatic health as assessed by serum biochemistry was associated with histological liver damage, without significant increases in body weight.[62] The

consumption of a fructose-rich diet for greater than the equivalent of one year also resulted in development of lipidosis in the liver, promoting development of hepatic steatosis and metabolic disease.[62] Human subjects consuming fructose have also been shown to display significantly higher rates of hepatic de novo lipogenesis in comparison to those consuming glucose alone,[63] highlighting the liver as an important metabolic organ impacted by fructose consumption that may perpetuate the MetS phenotype. Consumption of fructose and saturated fatty acids in combination has also been reported to alter body composition and endocrine function of adult offspring. Studies where rodents were fed 30% fructose with or without addition of saturated fats displayed reduced fat accumulation and impaired insulin sensitivity in male offspring weaned onto the fructose plus saturated fat diet for 12 weeks.[64] This study highlights that an early exposure to a combination of fructose and saturated fat modulates adiposity and endocrine function, promoting a MetS phenotype.[64]

Another important hallmark in the pathogenesis of MetS is dysregulation of glucose homeostasis, namely the development of hyperglycemia and glucose intolerance.[6] Typically, studies assessing glucose homeostasis as a component of the MetS phenotype focus on the adult period,[65,66] with few studies investigating the impact of being weaned onto a poor diet on the development of glucose intolerance. Studies have shown that children growing up in families consuming poor diets are exposed earlier in life to these energy-dense diets, and continue this consumption pattern throughout postnatal life.[67,68] Therefore, an important aspect of our study was to investigate the impact of being weaned onto a poor diet on glucose tolerance as a component of the MetS phenotype. In our cohort, whole-body glucose tolerance as

assessed by IPGTT was not affected by birth weight or postnatal diet. However, at this age similar results have been observed in other models of young IUGR offspring, namely those in sheep and rodents.[46,69] The young age of our offspring, approximately equivalent to 18-20 years in the adult,[33] may explain the maintenance of glucose homeostasis we have observed. In a study examining 20-year-old men who were born low birth weight, glucose tolerance was similarly maintained, however reduced insulin sensitivity was also observed.[70] Maintenance of glucose tolerance has also been observed in young offspring of type 2 diabetic mothers; however progression to a state of insulin resistance does occur with age.[71] Therefore, the young age of our offspring highlights that the progression towards a MetS phenotype may be in its early stages. It is important to note that reductions in peripheral insulin-stimulated glucose uptake, namely in liver, heart, adipose tissue and skeletal muscle, are typically observed before overt whole-body changes in glucose tolerance.[72] Young LBW men with normal whole body glucose disposal have also been observed to display reduced forearm glucose uptake, and molecular changes in insulin signaling, highlighting an early step in disease pathogenesis that may continue to fail with age. [21,73,74] Overall, while we do not observe overt features of whole-body glucose intolerance at this stage, there may be underlying alterations at the physiological level that predispose these offspring to development of later life MetS as they continue to interact with their postnatal environment.

While our animals remain small and non-obese at this young age, they do show evidence of metabolic disruptions. Previous studies from our laboratory have demonstrated increased aortic wall stiffening in LBW offspring,[75] which was further exacerbated by postnatal consumption of a WD.[32] These LBW-induced alterations are an

early sign of vascular dysfunction that may be associated with risk factors for the development of later life cardiovascular disease. Additionally, we have observed altered adipose tissue lipid metabolism in conjunction with increased visceral adiposity in the LBW offspring,[19] predisposing these offspring to later life dyslipidemia associated with MetS. Therefore, the presence of these previously characterized metabolic disruptions, in addition to the altered growth and metabolic parameters assessed here, support the theory that the *in utero* environment and growth trajectory are important players in determining postnatal disease risk.

In summary, our data suggest that body composition, growth, and feed efficiency are altered following development in an adverse *in utero* environment. Additionally, interaction with a postnatal nutritional environment characterized by nutrient excess impairs growth and further alters the body composition of the offspring. This altered growth and body composition is associated with an increased predisposition for these offspring to develop age-related metabolic diseases, supporting the concept of developmental programming of postnatal metabolism.

2.5 References

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Chapter 3:

Indicators of Skeletal Muscle Mitochondrial Dysfunction and Impaired Insulin Signaling Occurs in Low Birth Weight Guinea Pigs, and Following Postnatal Exposure to a "Western" Diet

Data presented in this chapter is in preparation for submission.

3.1 Introduction

Epidemiological studies have highlighted that an adverse *in utero* environment that restricts fetal growth, culminating in intrauterine growth restriction (IUGR) and low birth weight (LBW), increases the propensity for the offspring to develop age-related metabolic and cardiovascular diseases in later life.[1-4] Before overt manifestations of metabolic syndrome (MetS) are apparent, insulin resistance is typically observed, highlighting that the development of insulin resistance is a critical precursor to the pathophysiological development of MetS and establishing its use as an early marker of metabolic disease development and progression.[5] Insulin resistance is classically characterized as a metabolic state in which peripheral tissues, particularly skeletal muscle, are no longer responsive to insulin, preventing insulin-stimulated glucose uptake and perpetuating a state of hyperglycemia.[6] Insulin sensitivity is primarily assessed in skeletal muscle, since this is the location for the majority of insulin-stimulated glucose uptake, accounting for up to 70% of whole body glucose clearance.[7]

Typically, insulin resistance development is associated with poor diet choices and sedentary lifestyles;[8,9] however, the *in utero* environment is now recognized as a key player in insulin resistance pathogenesis.[3,5,10] Indeed, LBW offspring have been shown to display a 7-fold increased risk of being insulin resistant by young adulthood.[11] More recently, the EPOCH study highlighted higher fasting insulin levels and homeostatic model assessment of insulin resistance (HOMA-IR) values indicative of impaired insulin sensitivity as early as 10 years of age in IUGR-exposed children.[12] These studies highlight that metabolic adaptations initiated *in utero* can have life-long metabolic consequences.

The origins of skeletal muscle insulin resistance in the adult population are complex and multifactorial, however energy-dense, high-fat/high-sugar "Western" diets have been implicated as contributing factors in the pathophysiological progression to insulin resistance.[9] Consumption of this diet generates a surplus of circulating fatty acids, ultimately leading to ectopic lipid accumulation in non-adipose tissues, such as skeletal muscle, and impaired mitochondrial function.[13] Skeletal muscle lipid accumulation in animal models has been associated with impairments in insulin signaling, with long-chain acyl-CoAs, triglycerides (TG) and diacylglycerol (DAG) highlighted as key mediators of this phenomenon. The saturation of the lipids making up the TG pool is also a critical factor contributing to skeletal muscle insulin resistance. A higher proportion of saturated fatty acids in the TG pool reduces lipid oxidation, allowing accumulation of DAG and ceramide.[14] Accumulation of these lipotoxic intermediates has been associated with classical markers of insulin resistance.[15] These markers include reductions in the phosphorylation of the insulin receptor and decreased activation of phosphoinositide-3 (PI-3) kinase, which is responsible for phosphorylating and activating Protein Kinase B (Akt). Activation of Akt requires phosphorylation at both Thr^{308} and Ser^{473}; therefore a reduction in phosphorylation at either site may be indicative of impaired signaling.[16] Active Akt is responsible for phosphorylating and activating the Akt substrate of 160 kDa (AS160) at Thr^{642}, allowing insertion of glucose transporters (GLUT4) into the plasma membrane.[15,17] Decreases in the phosphorylation and activation of the above intermediates occur in conjunction with an increase in the inhibitory phosphorylation of Insulin Receptor Substrate-1 (IRS-1) at Ser^{307}, ultimately resulting in a decrease in insulin-stimulated glucose uptake through GLUT4 transporters.[15] Insulin resistance can

occur at any level of the insulin signaling cascade, from the initial binding of insulin to its receptor to alterations in the phosphorylation cascade responsible for initiating glucose uptake.[18] When these molecular alterations associated with insulin resistance occur in conjunction with hyperinsulinemia and/or maintenance of whole body glucose tolerance, it is associated with a pre-diabetic state characteristic of an early stage of disease pathogenesis.[19,20]

An alternate theory for development of impaired insulin sensitivity implicates alterations in skeletal muscle mitochondrial metabolism. It has been recently demonstrated that adults with high intakes of high-fat food and reduced physical activity display impaired mitochondrial function.[21] Mitochondrial dysfunction is defined as a decrease in mitochondrial substrate oxidation resulting from a general decrease in oxidative phosphorylation.[22] In order to initiate the oxidation process, long-chain acyl-CoAs in the skeletal muscle must enter the mitochondria as acylcarnitines. Acylcarnitines are formed when long-chain acyl-CoAs have their CoA exchanged for carnitine, catalyzed by subcellular carnitine palmitoyltransferase 1 (CPT1), which is rate limiting step of oxidation, and classically used as a surrogate marker of oxidative capacity.[13,21,23,24] Once the acylcarnitines have crossed into the mitochondria via carnitine acetyltransferase, they are converted back to acyl-CoAs by carnitine palmitoyltransferase 2 (CPT2) so the long-chain acyl-CoAs can undergo β-oxidation.[24] The process of β-oxidation successively reduces the length of the acyl-CoA carbon chain by 2 carbons through the actions of a series of enzymatic reactions catalyzed by very long-chain acyl-CoA dehydrogenase (VLCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and 3-ketoacyl-CoA thiolase (KT) among others.[21,25] Acetyl-CoA, the products

of β-oxidation, are then fed through the TCA cycle, generating carbon dioxide and electron carriers such as NADH and $FADH_2$. These electron carriers then enter the electron transport chain in order to generate ATP as an energy source through oxidative phosphorylation.[26] Accumulation of acylcarnitines in tissue is reported to be indicative of impairment in the utilization of the substrates generated through β-oxidation, and the presence of fatty acids that are incompletely oxidized.[23] It has also been suggested that these acylcarnitines, when present in excessive amounts, have the ability to promote alterations in the phosphorylation status of key insulin signaling intermediates through the actions of stress-induced kinases,[27] highlighting acylcarnitine accumulation secondary to mitochondrial dysfunction as key mediators of the pathogenesis of insulin resistance.

Recently, evidence has suggested that *in utero* influences and secondary insults in adult life may have a synergistic effect, further exacerbating an already dysfunctional system and promoting earlier onset of MetS.[10] Of late, an increased incidence of childhood obesity has largely been attributed to early introduction of energy-dense diets and sedentary behavior.[28] However, genetics and the contribution of the *in utero* environment to epigenetic alterations to metabolic function cannot be negated when assessing whether these children are at increased risk of developing symptoms associated with MetS at an earlier age.[29] Therefore, the present study is aimed to identify alterations in skeletal muscle mitochondrial lipid metabolism following IUGR independently, and in conjunction with early postnatal exposure to a Western diet.

3.2 Materials and Methods

3.2.1 Ethics Statement

Animal care, maintenance and surgical procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were approved by The University of Western Ontario Animal Use Subcommittee (Appendix II).

3.2.2 Animal Handling

Time-mated pregnant Dunkin-Hartley guinea pigs (Charles River Laboratories, Wilmington, MA, USA) where housed in a constant temperature (20°C) and humidity (30%) controlled environment with 12h light/dark cycle. All animals had *ad libitum* access to standard guinea pig chow (LabDiet diet 5025) and tap water.

All pregnant guinea pigs underwent uterine artery ablation, as previously described,[30] to induce uteroplacental insufficiency and intrauterine growth restriction (IUGR). At mid-gestation (~32 days, term ~67 days), sows were anesthetized in an anesthetic chamber (4-5% isoflurane with 2L/min O_2, followed by 2.5-3% isoflurane with 1L/min O_2 for maintenance). Immediately following induction of anesthesia, a subcutaneous injection of Rubinol (Glycopyrrolate, 0.01mg/kg, Sandoz Can Inc., Montreal, QC) was administered. A midline incision was made below the umbilicus, exposing the bicornate uterus. Arterial vessels feeding each horn of the uterus were identified, and every second branch was cauterized using an Aaron 2250 electrosurgical

generator (Bovie Medical, Clearwater, FL). Immediately after surgery, a subcutaneous injection of Temgesic (Buprenorphine, 0.025mg/kg, Schering-Plough Co., Kenilworth, NJ) was administered, and monitoring continued following surgery. Sows delivered spontaneously at term, at which time pup weight, length, abdominal circumference and biparietal distance were measured. Following the pupping period, all birth weights were collated and if birth weight was below the 25th percentile these guinea pigs were allocated to the low birth weight (LBW) group, and if birth weight was between the 25th and 75th percentile these pups were allocated to the normal birth weight (NBW) group (Figure 2.1). Based on these criteria, LBW pups were below 80g, and NBW pups were above 90g, in accordance with previously reported data.[31,32] Low body weight is often recognized as a crude measure of an adverse *in utero* growth environment. An additional measure of adverse growth *in utero*, and potentially more reflective of the growth conditions experienced *in utero* is birth weight/birth length,[33] which was also measured (Chapter 2).

Pups remained with their dams during the 15-day lactation period. Five days prior to weaning, all pups were introduced to a synthetic control diet (Harlan Laboratories TD.110240: Table 2.1) and weaned on PND15 into individual cages. At this time, guinea pig offspring were randomized to either a control (CD, Harlan Laboratories TD.110240: Table 2.1) or a Western (WD, Harlan Laboratories TD.110239: Table 2.1). To avoid litter effects, only one LBW and one NBW animal from a single litter was assigned to each diet. Only male pups were examined for this study in order to prevent confounding factors related to estrous cycle and hormone profile of female offspring.

3.2.3 Assessment of Whole-Body Glucose Tolerance

At PND60 and PND120, intraperitoneal glucose tolerance tests (IPGTTs) were performed to assess whole body glucose tolerance as previously described in section 2.2.5.

3.2.4 Assessment of Skeletal Muscle Glucose Uptake

At PND50 and PND110, pups underwent positron-emission tomography (PET) scans in order to assess *in vivo* glucose uptake at the level of the skeletal muscle. Prior to scanning, body weights and blood glucose measurements were taken. Throughout the duration of the scans, physiological parameters including heart rate, respiration rate and oxygen saturation were monitored to ensure well-being of the animal. Pups were anesthetized in an anesthetic chamber (4-5% isoflurane with 2L/min O_2) then transferred to a tight fitting nose cone (2.5-3% isoflurane with 1L/min O_2 for maintenance). While anesthetized, pups were injected with ~25kBq/kg (0.2-0.3mL) of ^{18}F-fluoro-deoxy-glucose (FDG) via the pedal vein. Following injection, pups were allowed to recover for 40 minutes, before being anesthetized again (4-5% isoflurane with 2L/min O_2) and placed on a micro-PET scanner (GE eXplore Vista, GE Healthcare) fitted with a nose cone (2.5-3% isoflurane with 1L/min O_2 for maintenance). Emission scans for the leg regions were conduced for 20 minutes, then reconstructed in 3D mode. Resulting images were analyzed with a custom MATLAB-designed program (MATLAB; MathWorks Inc., MA, USA), where regions of interest were drawn around the muscles of the hind-limb.

Measurements of Standard Uptake Value (SUV) were obtained by applying FDG dosage and body weight of the pup to the following equation:[34,35]

$$SUV = \frac{Activity\ in\ Tissue}{(Intrinsic\ Activity/Body\ Weight)}$$

3.2.5 Tissue Collection

At PND145, pups were sacrificed by CO_2 inhalation[36] following an overnight fast. Gastrocnemius muscle, a representative muscle with mixed composition of oxidative and glycolytic fibers,[37] was removed and trimmed of excess fat and connective tissue, then flash frozen in liquid nitrogen for subsequent analysis.

3.2.6 Protein Extraction and Immunoblotting

Gastrocnemius samples (~50mg each) were homogenized in 0.5mL ice-cold lysis buffer (pH 7.4, 50mM Tris-HCl, 1% NP-40, 0.5% Sodium-deoxycholate, 150mM NaCl, 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Cocktail set 1, Calbiochem, Bilerica, MA) and phosphatase inhibitors (1 M NaF, 0.2 M Na_2VO_4, 0.2 M NaPP and 1 M Na_2Gly). Homogenates were incubated on ice for 15 minutes, then subjected to sonication with 3-5 bursts at 30% output (MISONIX: Ultrasonic liquid processor), followed by centrifugation at 15,000g for 30 min at 4°C. The supernatant was collected and Pierce BCA Protein Assay (Thermo Scientific, Waltham MA) was employed for protein quantification.

Protein samples were separated using 7.5%, 10% or 12% Bis-Tris gels, and transferred onto Immobilon transfer membranes (EMD Millipore, Billerica, MA) via wet transfer. Consistent protein loading and sufficient transfer of proteins was confirmed using 1× Amido Black stain and images were captured using a Versadoc System (Bio-Rad, Mississauga, Ontario). Membranes were blocked overnight at 4°C with Tris-buffered saline (TBS)/0.1% Tween 20 (Thermo Fisher Scientific, Waltham, MA) containing 5% skim milk, 5% bovine serum albumin (BSA; AMRESCO, Solon, Ohio) or 10% BSA as indicated in Table 3.1. Following blocking, primary antibodies were applied for 1h at room temperature at dilutions specified in Table 3.1. The blots were then washed in TBS/0.1% Tween 20 and incubated at 1h room temperature with the appropriate secondary conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS/0.1% Tween 20 containing 5% skim milk or BSA. The blots were then washed in TBS/0.1% Tween 20 and protein bands were revealed by Luminata Forte Western Blot HRP Substrate (EMD Millipore, Billerca, MA) and captured using a Versadoc System (Bio-Rad, Mississauga, Ontario). All images were visualized using Quantity One software (Bio-Rad, Mississauga, Ontario), and densitometry values (arbitrary units) were determined using Image Lab 4.0 software (Bio-Rad, Mississauga, Ontario). The abundance of target proteins was expressed relative to Amido Black densitometry values for proteins of the same molecular weight.[38]

3.2.7 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from gastrocnemius tissue by homogenizing ~20mg in 1mL Trizol (Invitrogen, Carlsbad, CA) and following the manufacturers protocol. Total RNA was subsequently treated with deoxyribonuclease for 30 minutes to eliminate genomic DNA. Quantification of RNA was performed with the ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Rochester, NY). Each sample was also assessed for RNA integrity using 1.2% agarose electrophoresis with RedSafe™ (iNtRON Biotechnology, Sangdaewon-Dong, Joongwon-Ku, Sungnam, Kyungki-Do, 462-120, KOREA). Complementary DNA was synthesized from 2μg of purified RNA, reverse-transcribed using M-MLV Reverse Transcriptase (Life Technologies, Burlington, ON) and a C1000 Thermal Cycler (Bio-Rad, Mississauga, ON). All primers were designed from guinea pig (*cavia porcellus*) sequences using NCBI/Primer-BLAST tool (Table 3.2). Standard curves for each primer pair (three pairs for each gene) were generated from serial dilutions of cDNA for determination of primer efficiencies. PCR efficiencies for each primer set were 90-105% (Table 3.2). Melting curve analysis and presence of a single amplicon at the expected size in a 1.8% agarose gel were used to confirm amplification of a single product. cDNA products were used as templates for qRT-PCR assessment of gene expression using the SYBR green system (SensiFast™ Sybr No-Rox Mix; Bioline, London, United Kingdom) on a Bio-Rad CFX384 Real-Time system instrument (Bio-Rad, Mississauga, ON). Each sample was run in triplicate. Forty cycles of amplification were performed, with each cycle consisting of: denaturation at 95°C for 15s, annealing at the pre-determined temperature for each primer set denoted in Table 3.2 for 20s, and extension at 72°C for 20s. Control samples containing no cDNA were used

to confirm absence of DNA contamination. The transcript level of target genes was normalized to β-actin; there were no differences in the expression of β-actin between experimental groups. The fold expression of each individual target gene was determined by the $2^{-\Delta Ct}$ method.[39]

3.2.8 Thin Liquid Chromatography and Gas Chromatography

Total lipids were extracted from gastrocnemius muscle (~160mg), using a protocol adapted from Klaiman et al.[40] Briefly, tissue samples were homogenized in 850µl 1:1 chloroform:methanol (v/v) containing 0.1% butylated hydroxytoluene (BHT).[41] Following homogenization, 8.5mL of 2:1 chloroform:methanol (v/v)+0.1% BHT was added to each sample. 1.7mL of 0.25% KCl was added to separate the aqueous solutes, then incubated at 70°C for 10 minutes. Once the samples were cooled, they were centrifuged at 2000rpm for 5 minutes, and the aqueous layer was removed. The remaining solution, containing lipids, was dried under a gentle stream of N_2, and resuspended in 1mL chloroform. 20µL of total lipids were removed for subsequent analyses and diluted to a concentration of 2µg/µL. The remaining sample was dried again under a gentle stream of N_2.

Separation and analysis of lipid classes was accomplished by thin layer chromatography-flame ionization detection (TLC-FID) using the Iatroscan MK-6 TLC/FID Analyzer System (Shell-USA, Federicksburg, VA). The system is composed of 10 quartz rods that are 15cm in length and 0.9mm in diameter, coated with a 75µm layer of porous silica-gel particulates that are 5µm in diameter[42] (Shell-USA, Federicksburg,

VA). Prior to application of samples, chromarods were repeatedly blank scanned in order to burn off any impurities and activate the rods. 1µl of the 2µg/µL lipid sample or the reference standard was spotted onto the rods. Rods were developed in a TLC chamber with benzene:chloroform:formic acid (70:30:0.5 v/v/v) to separate the neutral lipids until the solvent front reached the 100 mark on the rod holder. Rods were subsequently removed and dried at 60°C in a rod dryer (TK8, Iatron Inc, Tokyo, Japan) for 5 minutes. Rods were analyzed using the following settings: 2L/min atmospheric air, 160mL/min hydrogen, scanning speed of 3s/cm. Area under the curve for the peaks of interest were integrated using PeakSimple 3.29 Software (SRI Instruments, CA) and expressed as a percentage of the total area under the curve for all peaks analyzed.

In preparation for gas chromatography, the total lipids were fractioned using Supelco Supelclear LC-NH_2 sPE columns (Supelco, Bellefonte, PA). Total lipid samples were resuspended in chloroform to a concentration of 10mg/mL. Once the columns were conditioned, 300µL of suspended sample was added and allowed to flow through the column, then centrifuged at 1000rpm for 1min. Neutral lipids were eluted with 1.8mL of 2:1 chloroform:isopropanol (v/v), followed by centrifugation at 1000rpm for 1min. Non-esterified fatty acids were eluted with 1.6mL 98:2 isopropyl ether:acetic acid (v/v), followed by centrifugation at 1000rpm. Phospholipids were eluted with 3mL of methanol, followed by centrifugation at 1000rpm for 1min. Each sample was subsequently dried under a gentle flow of N_2. 300µg of each sample was then methylated by adding 200µL of Meth-HCl to the dried sample and incubated at 90°C for 45min. 800µL of water, followed by 500µL of hexane was added to each sample, with the hexane layer extracted and put in a fresh tube. Following hexane extraction, samples

were dried under a gentle flow of N_2, and resuspended in dichloromethane for injection into a 6890N gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) with a J&W Scientific High Resolution Gas Chromatography Column (DB-23, Agilent Technologies) and a flame ionization detector. Fatty acids were identified by comparison of the relative retention time with known standards (Supelco 37 component FAME mix, and Supelco PUFA No.3, from Menhaden Oil).

3.2.9 Extraction and LC-MS/MS Method for the Analysis of Amino Acids and Acylcarnitines

Acylcarnitine and amino acid analysis was performed by the Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto, Canada.

Ground tissue was weighed out and a solution of 1:1 water:methanol was added to yield 25mg/mL. Samples were then homogenized and kept on ice. An equivalent of 5mg of homogenized tissue was added to an Eppendorf tube with an acylcarnitine internal standard (IS) mixture (NSK-B, Cambridge Isotopes) and an amino acid IS mixture (Arginine-$^{13}C_6$, ADMA-d_7, citrilline-d_7, glutamic acid-d_2, ornithine-d_7, and leucine-d_{10}, Cambridge Isotopes and CDN Isotopes). Samples and standard mixtures were then acidified with 60µL of 0.1% formic acid and then protein was precipitated using 1mL of 0.3% formic acid in acetonitrile. Tubes were then vortexed and centrifuged at 10,000g for 10 minutes at 4°C. Supernatants were transferred to conical glass tubes and the remaining pellet in the Eppendorf was re-suspended in an additional 1mL 0.3% formic acid in acetonitrile and vortexed and centrifuged again. The combined supernatants were

dried under a gentle flow nitrogen gas. Samples and standards were derivatized with 100µL butanolic-HCL 3N for 20 minutes at 65°C. Solvent was then removed under a gentle flow nitrogen gas and samples were reconstituted in 200µL MPB (see below) and transferred to auto sampler vials. 10µL of the reconstituted sample was diluted 50x in the same solution for amino acid analysis.

Extracted samples were injected onto a Kinetex HILIC 50 x 4.6 mm 2.6 µm column (Phenomenex) connected to an Agilent 1290 HPLC system attached to a Q-Trap 5500 mass spectrometer (AB Sciex, Framingham, MA). Injected samples were eluted with a gradient of mobile phases MPA: 90/10 5 mM ammonium formate pH 3.2/acetonitrile and MPB: 10/90 5mM ammonium formate pH 3.2/acetonitrile. For Acylcarnitines, the gradient started at 4% MPA for 1 minute, increasing to 45% for 0.65 min, holding for 0.05 min and then returning to 4% MPA for the remaining 3 minutes. For amino acids, the 50 x diluted samples were eluted with a gradient of MPA starting at 4% for 2.5 minutes and increasing to 75% MPA until 6 minutes, holding at 75% MPA for 1 minute, and then returning to 4% over the final 2.5 minutes. Mass transitions were monitored for each sample and compared against known standard mass transitions for species analyzed.[43] Data was collected and analyzed using Analyst v1.6 (AB Sciex, Framingham, MA). Qualitative area ratios were determined for each species examined.[43]

3.2.10 Statistical Analyses

Data presented are mean ± standard error (SEM) or min. to max. box and whisker plots. Statistical significance was determined by two-way ANOVA followed by post-hoc

Tukey's test to assess the effects of birth weight and postnatal diet, using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Tests of correlation were performed with the Spearman rank-order test. Values of $p<0.05$ were considered statistically significant.

A principle component (PC) analysis (PCA) of the acylcarnitine and amino acid measurements was performed (SPSS Statistics, version 21.0; IBM SPSS, Armonk, NY) to reduce the dimensionality of the data set while retaining as much of the variance as possible. The metabolic PCs, defined as linear, orthogonal (uncorrelated) combinations of the original metabolites, were ordered according to their decreasing ability to explain variance in the original data set. Only principle components with eigenvalues greater than 1 were retained. The cumulative variance explained by the 9 PCs included in the resultant analysis was 87.6%. The data set was then rotated using an orthogonal varimax procedure. To facilitate biological interpretation of the PCs, the associated weight loading factors for each metabolite were examined and metabolites with a loading score greater than 0.5 are reported for each factor. Tests of main effects of birth weight, diet, and birth weight by diet interactions were performed using two-way ANOVA. Associations between factor scores and immunoblot measurements are reported as Spearman rank correlation coefficients.

Protein	Species	Dilution	Blocking Solution	Company	Catalogue No.
IRβ	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	3025
pIRβ (Tyr 1150/1151)	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	3024
IRS1	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	2382
pIRS1 (Ser 307)	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	2381
p85	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	4257
p110	Rabbit Monoclonal	1:500	5% BSA	Cell Signaling Technology	3011
Pan AKT	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	4691
pAKT (Ser 473)	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	4060
pAKT (Thr 308)	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	2965
AS160	Rabbit Monoclonal	1:1000	5% BSA	Cell Signaling Technology	2670
pAS160 (Thr 642)	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	4288
GLUT4	Mouse Monoclonal	1:1000	5% Skim Milk	ABCAM	ab166704
GLUT2	Rabbit Monoclonal	1:1000	5% Skim Milk	Santa Cruz Biotechnology	sc-9117
GLUT5	Mouse Monoclonal	1:4000	5% Skim Milk	Pierce Biotechnologies	MA1-036
JNK	Mouse Monoclonal	1:1000	5% BSA	Santa Cruz Biotechnology	sc-7345
pJNK (Thr 183/Tyr 185)	Mouse Monoclonal	1:1000	10% BSA	Santa Cruz Biotechnology	sc-6254
IKKβ	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	2678
pIKKβ (Ser 176/180)	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	2694
PKC-θ	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	2059
CD36	Rabbit Polyclonal	1:500	5% Skim Milk	Santa Cruz Biotechnology	sc-9154
FATP1 (ACSVL5)	Rabbit Polyclonal	1:1000	5% Skim Milk	Santa Cruz Biotechnology	sc-25541
FATP4	Rabbit	1:1000	5% Skim	Santa Cruz	sc-25670

(ACSVL4)	Polyclonal		Milk	Biotechnology	
CPT1	Mouse Monoclonal	1:500	5% Skim Milk	Santa Cruz Biotechnology	sc-393070
PPARα	Mouse Monoclonal	1:500	5% Skim Milk	ABCAM	ab2779
PGC1α	Rabbit Polyclonal	1:500	5% Skim Milk	Santa Cruz Biotechnology	sc-13067
SIRT1	Mouse Monoclonal	1:2000	5% Skim Milk	Santa Cruz Biotechnology	sc-74465
SIRT3	Rabbit Polyclonal	1:1000	5% Skim Milk	ABCAM	ab86671
FOXO1	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	9454
pFOXO1 (Ser256)	Rabbit Polyclonal	1:1000	5% BSA	Cell Signaling Technology	9461
Anti-Rabbit Secondary	Donkey	1:10,000	-	Santa Cruz Biotechnology	sc-2077
Anti-Mouse Secondary	Donkey	1:10,000	-	Santa Cruz Biotechnology	sc-2314
Anti-Goat Secondary	Donkey	1:20,000	-	Santa Cruz Biotechnology	sc-2020

Table 3.1: Specifications and Catalog Numbers of Antibodies Used for Immunoblotting.

Gene	Accession No.	Anneal Temp (°C)	Forward Sequence (5'è3')	Reverse Sequence (3'è5')	Efficiency
VLCAD	XM_003466183.2	59	CAAACTGGCAGTGACGGCT	TTGGTGGGGGTCAGACTGTA	91.2%
MCAD	XM_003479087.2	59	CGAGTTGACCGAACAGCAGA	CAACAGGCATTTGCCCCAAG	94.4%
KT	XM_003464099.2	59	TAAGGTCCTACGCAGTGGTTG	CTCCATAAGCCCTCTTCCCAC	90.8%
PDK4	XM_003475111.2	59	GCAGTGGTCCAAGATGCCTT	TGGTGTTCAACTGTTGCCCT	94.2%
CPT1b	XM_003461559.1	59	AGCTCCCCATTCCTAGCAGA	CGCTGAGCATTCGTCTCTGA	96.7%
PGC1α	XM_003467408.2	56	CAAGACCAGTGAATTGAGGG	CATCCTTTGGGGTCTTTGAG	92.5%
PPARα	NM_001173004.1	56	AGATCCAGAAAAAGAACCGC	TTTTGCTTTCTCAGACCTCG	91.8%
SIRT1	XM_005005505.1	58	TTGCAACTGCATCTTGCCTG	TCATGGGGTATGGAACTTGGAA	103.8%
SIRT3	XM_004999541.1	58	CATGGCGGATCTGCTACTCA	AGGCTGCATGTTGTGGTTTG	93.8%
β-actin	NM_001172909.1	59	AAGAGATGTGGCCTCAAAGC	CAGGAACAGGCCGTAGAGTG	100.6%

Table 3.2: Primers Used for Analysis of Gene Expression by qRT-PCR.

3.3 Results

3.3.1 Glucose Homeostasis is Maintained at 4 Months of Age

Intraperitoneal glucose tolerance challenges were performed to assess whole body glucose tolerance. No significant differences were observed in whole body glucose handling following a glucose load at either PND 60 (Figure 3.1A) or PND120 (Figure 3.1C). The whole body clearance of glucose, as measured by the area under the curve, was also not significantly altered by birth weight or postnatal diet at either PND60 (Figure 3.1B) or PND120 (Figure 3.1D).

Glucose uptake at the level of the skeletal muscle was assessed using ^{18}F-FDG PET and measuring the standard uptake value of radioactivity observed during the scans. No significant differences in skeletal muscle glucose uptake were observed due to birth weight or postnatal diet at either PND50 (Figure 3.2A) or PND110 (Figure 3.2B).

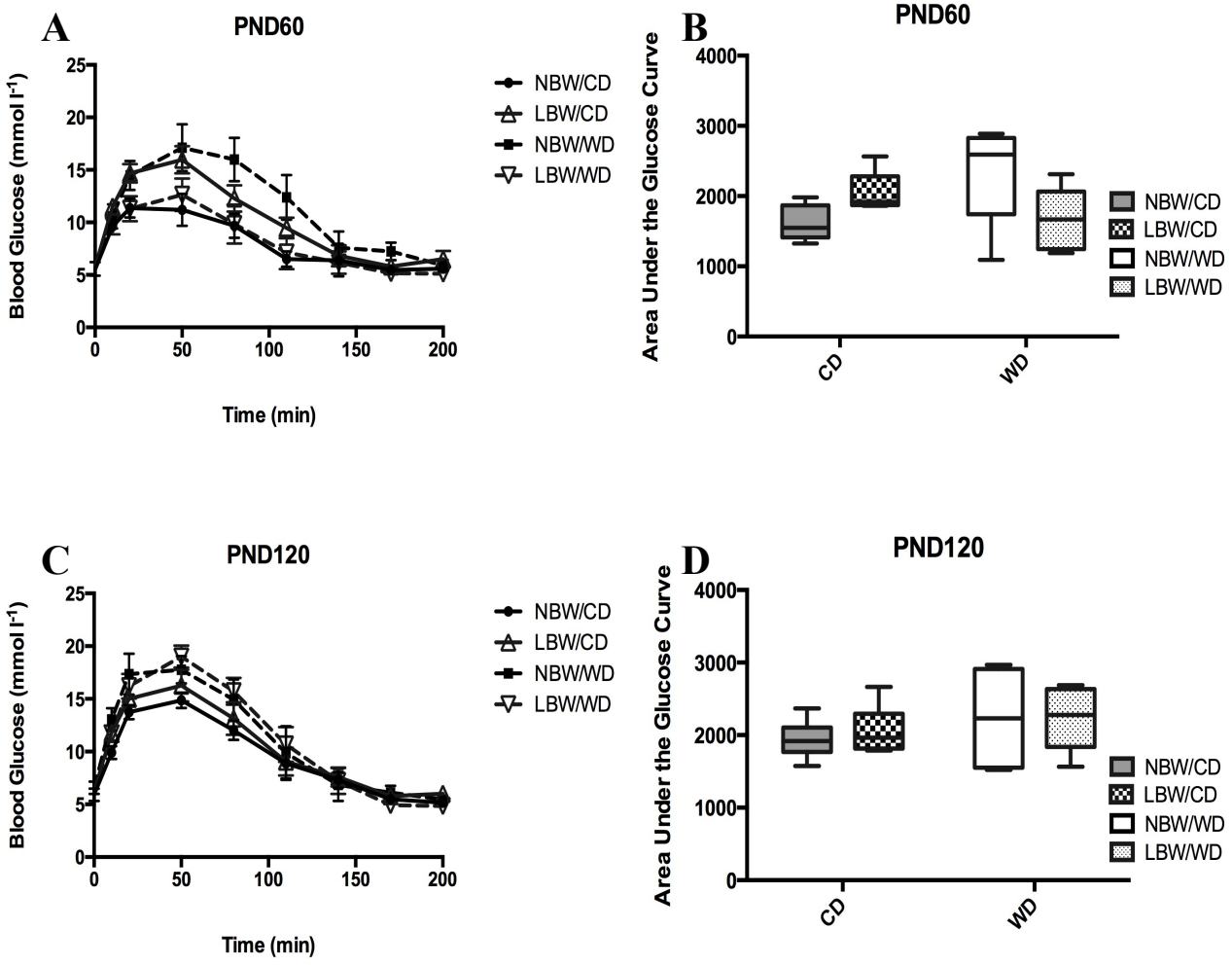


Figure 3.1: Whole-Body Glucose Tolerance. A) Blood glucose values (mmol l^{-1}) following an intraperitoneal injection of 1g/kg of 50% dextrose solution at PND60. B) The area under the glucose curve (AUGC) as a measurement of glucose clearance at PND 60. C) Blood glucose values (mmol l^{-1}) at PND120. D) The area under the glucose curve (AUGC) at PND 120. Data presented are the mean ± standard error (SEM) or min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test.

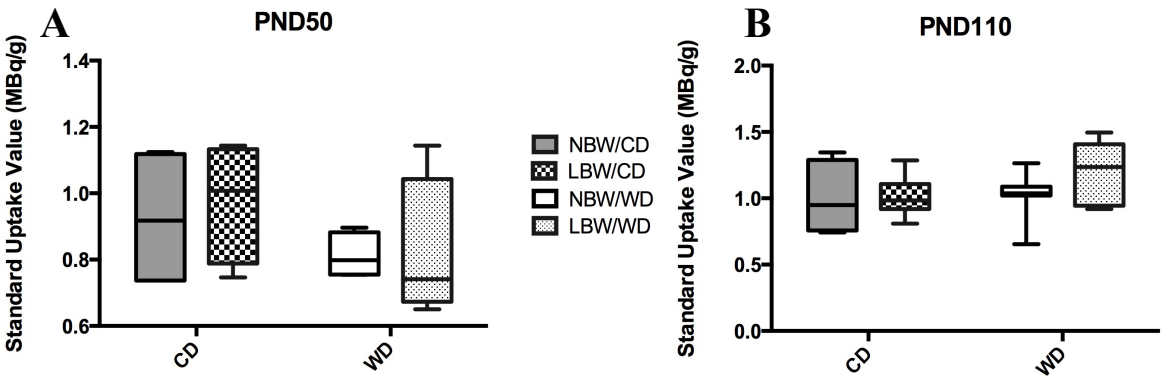


Figure 3.2: Skeletal Muscle Glucose Uptake at A) Postnatal Day 50 and B) Postnatal Day 110. Standard Uptake Value (SUV) = image derived radioactivity / (injected activity/body weight). Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test.

3.3.2 Skeletal Muscle Lipid Profile

Using thin layer chromatography-flame ionization detection (TLC-FID), separation and analysis and lipid classes in gastrocnemius muscle was accomplished. There were no significant differences in the accumulation of triglycerides (TG), cholesterol (Chol), or diacylglycerol (DAG) due to birth weight or postnatal diet (Figure 3.3).

The intermuscular fatty acid pool was profiled using gas chromatography. In the neutral lipid fraction, there was a significant ($p<0.001$) increase in C14:0 in WD-fed animals, a significant ($p<0.001$) decrease in C18:2n6c in WD-fed animals, and a significant ($p<0.01$) decrease in C18:3n3 in WD-fed animals (Figure 3.4A). In the phospholipid fraction there was a significant ($p<0.001$) decrease in C16:0 and a significant ($p<0.01$) increase in C18:1n7 in WD-fed animals (Figure 3.4B). No alterations in the fatty acid composition were influenced by birth weight alone.

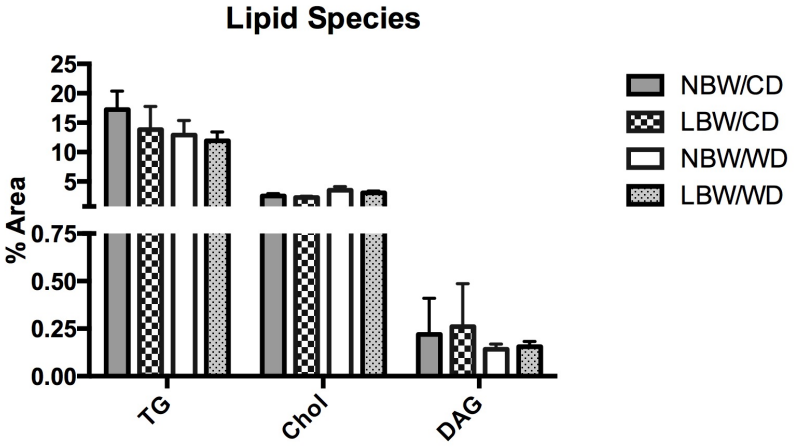


Figure 3.3: Relative Abundance of Lipid Species in Gastrocnemius Muscle. Measured by thin liquid chromatography-flame ionization detection. Data presented are mean ± standard error (SEM) for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test.

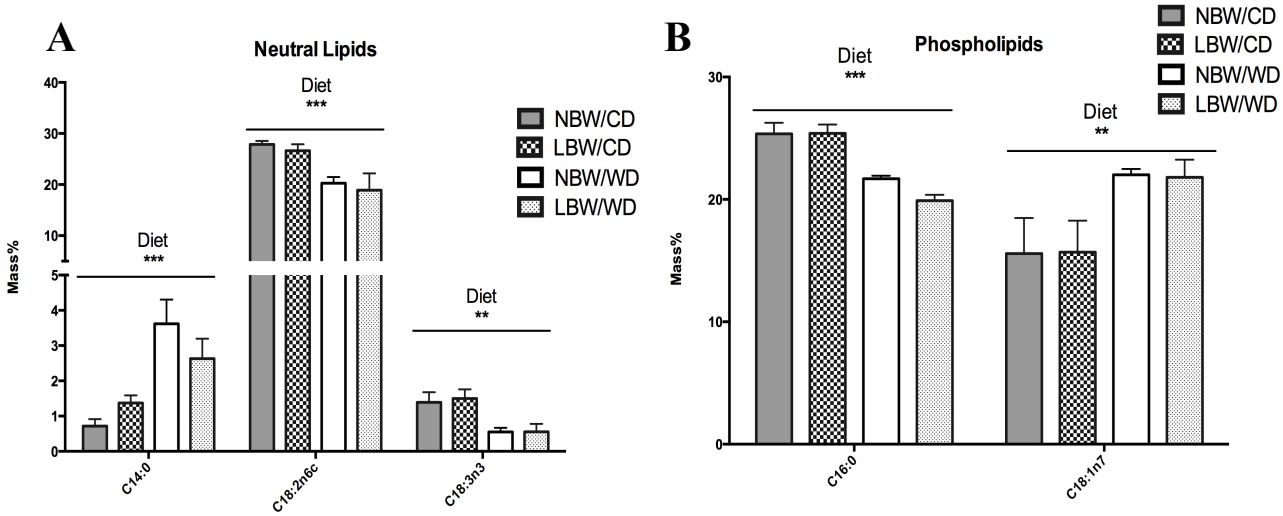


Figure 3.4: Fatty Acid Profile of Lipids in Gastrocnemius Muscle. A) Neutral lipid fraction and B) Phospholipid fraction, measured by gas chromatography. Data presented are mean ± standard error (SEM) for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Two-Way ANOVA followed by post-hoc Tukey's Test.

3.3.3 Skeletal Muscle Insulin Signaling is Disrupted in WD-fed and LBW offspring

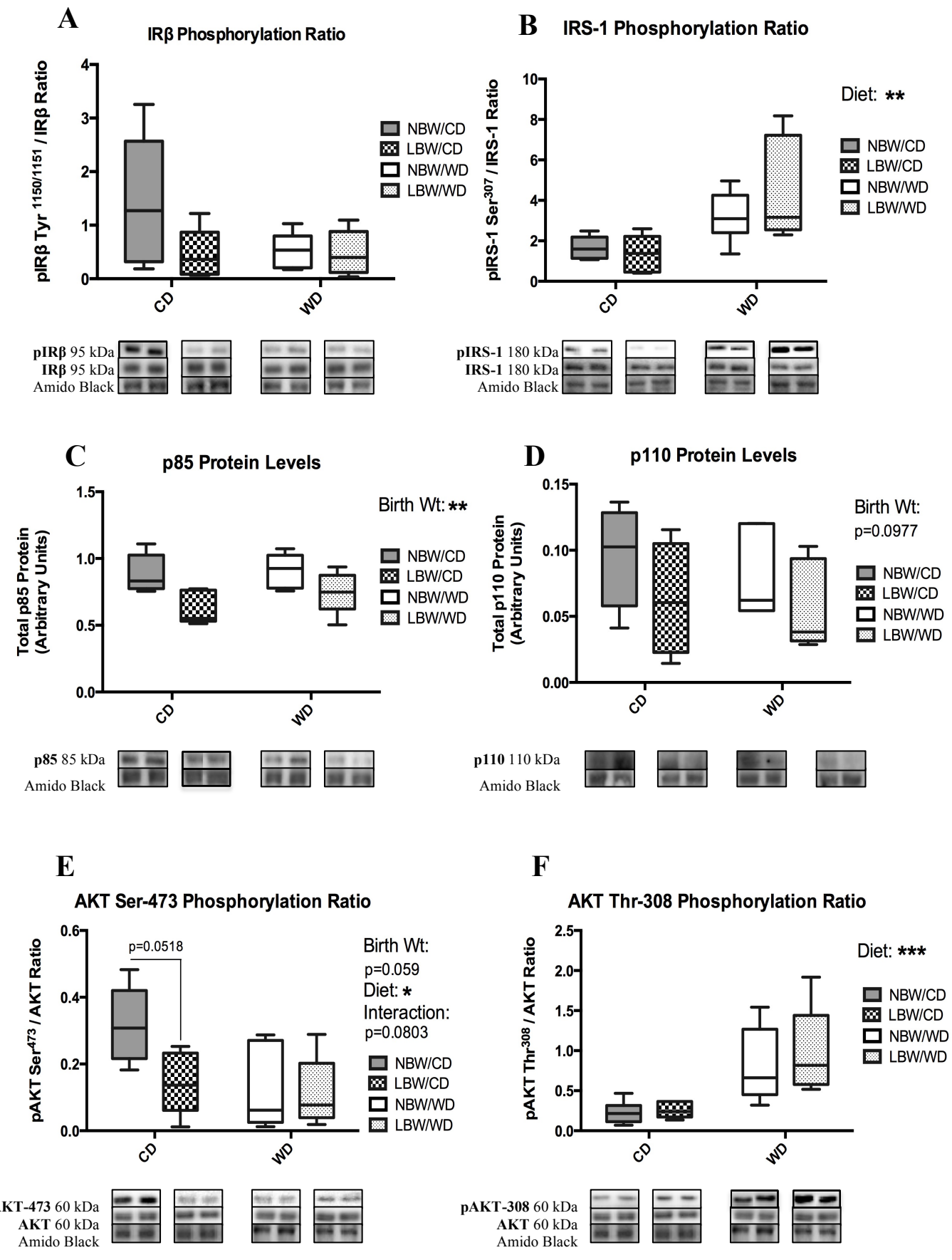
The phosphorylation of the β-subunit of the insulin receptor (IRβ) is required for its activation and propagation of downstream signaling events.[44] Skeletal muscle of WD-fed, and LBW offspring displayed a 46% ($p=0.1729$) and 67% ($p=0.1460$) reduction of phosphorylation of IRβ, respectively (Figure 3.5B).

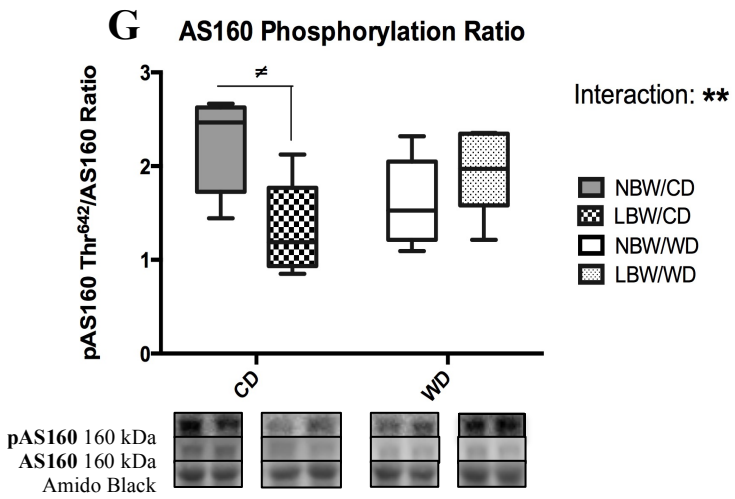
In contrast to tyrosine phosphorylation, which activates IRS-1, serine phosphorylation is inhibitory, preventing activation of PI3-kinase and downstream signaling events.[44] In WD-fed offspring, there was a significant ($p<0.01$) increase in inhibitory serine phosphorylation of IRS-1 (Figure 3.5C).

Full activation of PI3-kinase requires coordination between the p85 regulatory subunit and the p110 catalytic subunit,[45] in order to phosphorylate downstream components of the insulin signaling pathway. Total protein levels of the p85 regulatory subunit were significantly ($p<0.01$) reduced in LBW-offspring, irrespective of postnatal diet (Figure 3.5D). Total protein levels of the p110 catalytic subunit were reduced by 37% ($p=0.0977$) in LBW-offspring, irrespective of postnatal diet (Figure 3.5E).

Protein Kinase B/Akt is phosphorylated by PI3-kinase at two resides, Ser^{473} and Thr^{308}, that are both required for full activation of this protein.[46] At the Ser^{473} residue, phosphorylation was significantly ($p<0.05$) reduced in WD-fed offspring, and reduced ($p=0.059$) in LBW-offspring, irrespective of postnatal diet (Figure 3.5F). Additionally, a potential interaction between birth weight and postnatal diet ($p=0.0803$) was observed. At the Thr^{308} residue, there was a significant ($p<0.001$) increase in phosphorylation in WD-fed offspring (Figure 3.5G)

One of the final steps required for GLUT4 translocation is activation of Akt Substrate of 160 kDa (AS160), which is accomplished by Akt-mediated phosphorylation at Thr^{642}.[47] Phosphorylation of AS160 at Thr^{642} was significantly ($p<0.05$) reduced in LBW/CD offspring when compared to NBW/CD offspring (Figure 3.5H). Additionally, a significant ($p<0.01$) interaction between birth weight and postnatal diet was observed.


A
IRβ Phosphorylation Ratio
pIRβ Tyr 1150/1151 / IRβ Ratio
NBW/CD
LBW/CD
NBW/WD
LBW/WD
CD
WD
pIRβ 95 kDa
IRβ 95 kDa
Amido Black
B
IRS-1 Phosphorylation Ratio
pIRS-1 Ser307 / IRS-1 Ratio
Diet: **
pIRS-1 180 kDa
IRS-1 180 kDa
C
p85 Protein Levels
Total p85 Protein (Arbitrary Units)
Birth Wt: **
p85 85 kDa
D
p110 Protein Levels
Total p110 Protein (Arbitrary Units)
Birth Wt: p=0.0977
p110 110 kDa
E
AKT Ser-473 Phosphorylation Ratio
pAKT Ser473 / AKT Ratio
p=0.0518
Birth Wt: p=0.059
Diet: *
Interaction: p=0.0803
pAKT-473 60 kDa
AKT 60 kDa
F
AKT Thr-308 Phosphorylation Ratio
pAKT Thr308 / AKT Ratio
Diet: ***
pAKT-308 60 kDa
AKT 60 kDa



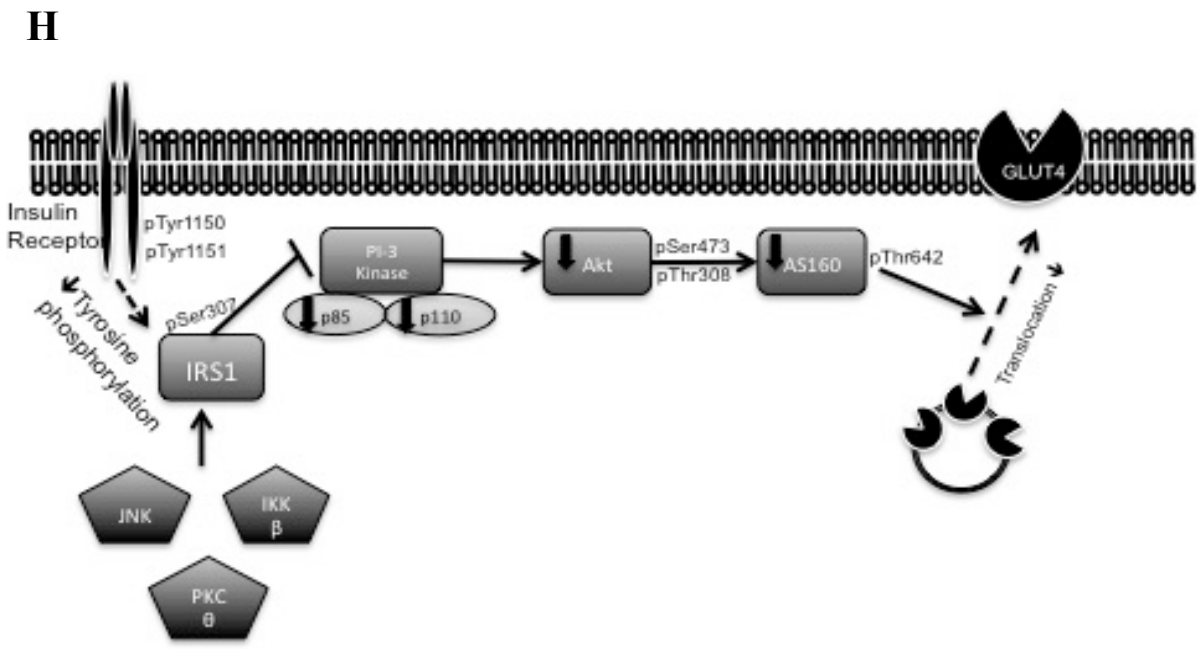


Figure 3.5: Expression and Phosphorylation of Key Proteins Involved in Insulin Signaling. A) Insulin Receptor phosphorylation, B) Insulin Receptor Substrate-1 (IRS-1) phosphorylation, C) p85 subunit of PI3-Kinase, D) p110 subunit of PI3-Kinase, E) Protein Kinase B/Akt phosphorylation at Ser-473, F) Protein Kinase B/Akt phosphorylation at Thr-308, G) Akt Substrate of 160 kDa (AS160) phosphorylation, H) Working schematic for molecular manifestations of insulin resistance. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ significant main effect of birth weight, diet or interaction; ≠$p<0.05$ by post-hoc Tukey's Test.

3.3.4 Glucose Transporter Profiles are Altered in WD-fed and LBW Offspring

While there were no significant differences observed in total skeletal muscle GLUT4 expression (Figure 3.6A), total GLUT2 expression was significantly ($p<0.001$) reduced in WD-fed offspring. However, birth weight significantly ($p<0.01$) increased expression of GLUT2 (Figure 3.6B). When compared to NBW/CD offspring, LBW/CD offspring showed a significant ($p<0.05$) increase in GLUT 2 expression. Alternatively, total GLUT5 expression was significantly increased in WD-fed offspring ($p<0.01$), as well as LBW offspring ($p<0.05$; Figure 3.6C). Similar to GLUT2 expression levels, GLUT5 was significantly ($p<0.05$) increased in LBW/CD offspring when compared to NBW/CD offspring.

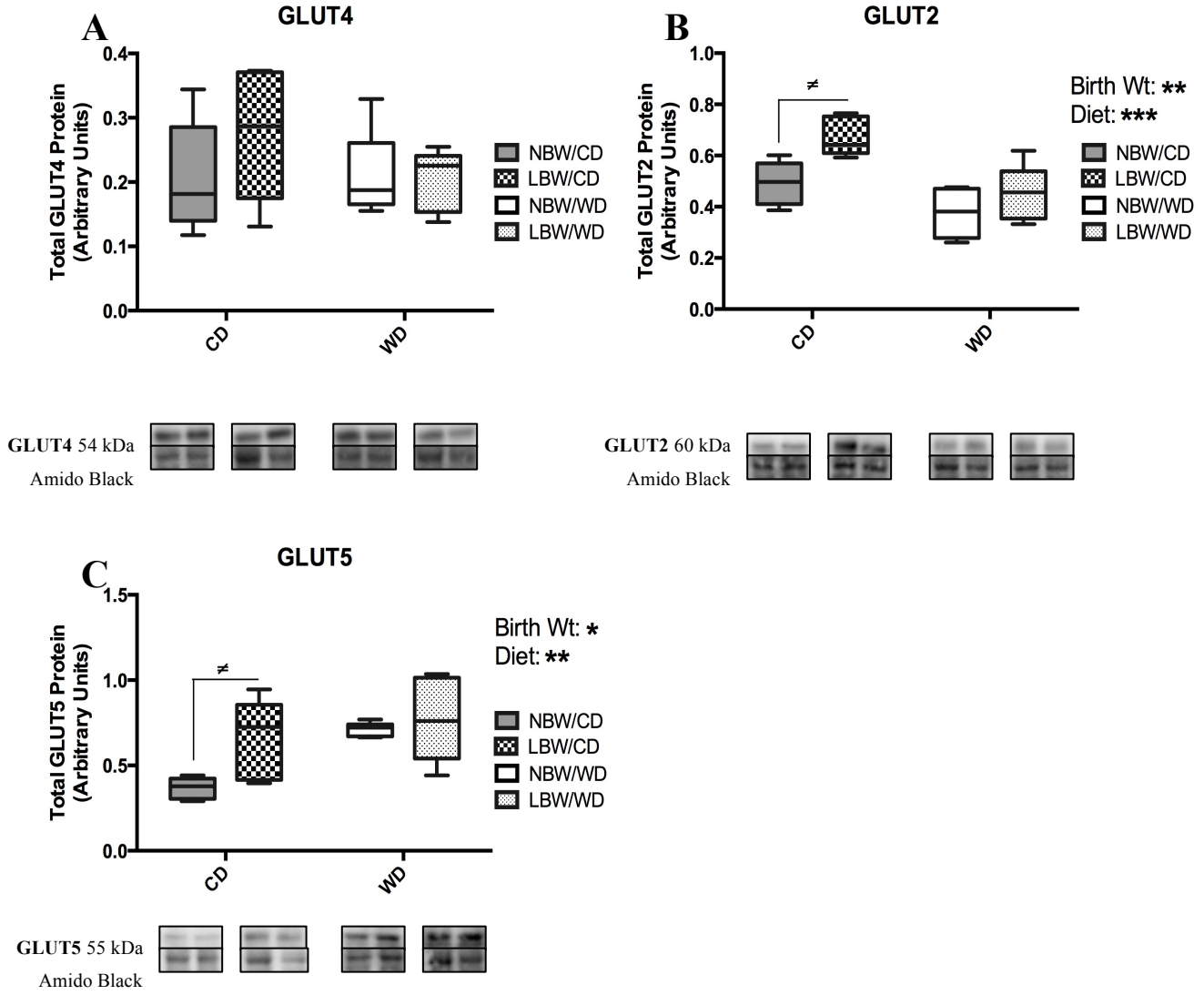


Figure 3.6: Expression of Glucose Transporters. A) GLUT4, B) GLUT2, C) GLUT5. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ significant main effect of birth weight, diet or interaction; ≠$p<0.05$ by post-hoc Tukey's Test.

3.3.5 Disruption of FOXO1 Signaling Occurs in WD-fed Offspring

In skeletal muscle of WD-fed offspring, there were significantly ($p<0.001$) reduced total protein levels of the transcription factor FOXO1 (Figure 3.7A). Additionally, there was significantly ($p<0.05$) increased phosphorylation of FOXO1 at Ser^{256}, promoting nuclear export and preventing activation of its target genes[48] (Figure 3.7B), a pattern not observed in LBW offspring.

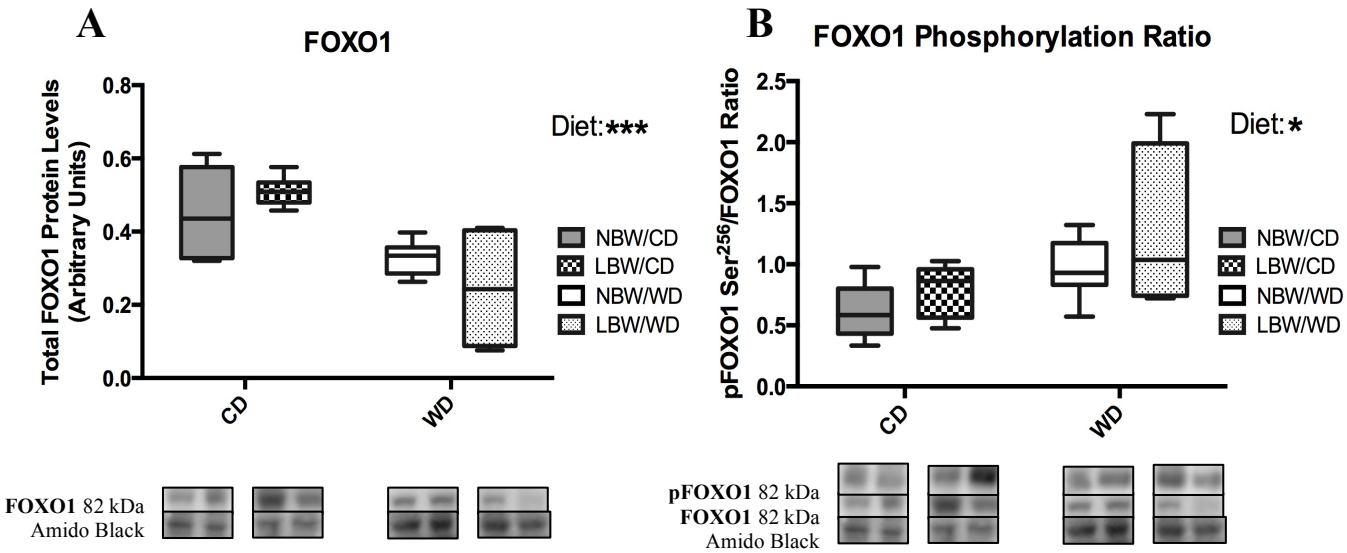


Figure 3.7: Expression of A) Total Levels and B) Phosphorylation of FOXO1 Protein. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, ***$p<0.001$, significant main effect of diet.

3.3.6 Stress-Induced Kinases are Activated in WD-fed and LBW Offspring

Stress-induced kinases, including JNK, IKKβ and PKC-θ, are known to be activated in situations of oxidative stress or lipid accumulation.[24,49] A differential pattern of stress-induced kinase activation was present in WD-fed vs LBW offspring. Activation of JNK through phosphorylation at Thr^{183}/Tyr^{185} was significantly ($p<0.0001$) increased only in WD-fed offspring (Figure 3.8A), whereas phosphorylation levels were minimal in LBW/CD offspring. Activation of JNK has been documented to promote inhibitory serine phosphorylation of IRS-1.[50] In our study, an increased JNK phosphorylation ratio correlated with an increased IRS-1 serine phosphorylation ratio ($r=0.554$, $p=0.006$, Figure 3.14C).

Furthermore, the activation of IKKβ through phosphorylation at $Ser^{176/180}$ was significantly ($p<0.0001$) increased in WD-fed and LBW offspring (Figure 3.8B). A significant ($p<0.0001$) interactive effect between birth weight and postnatal diet was also observed.

Lastly, total protein levels of PKC-θ were significantly ($p<0.01$) higher in LBW-offspring, irrespective of postnatal diet. When compared to NBW/CD offspring, LBW/CD offspring showed a robust ($p<0.05$) increase in total PKC-θ protein levels.

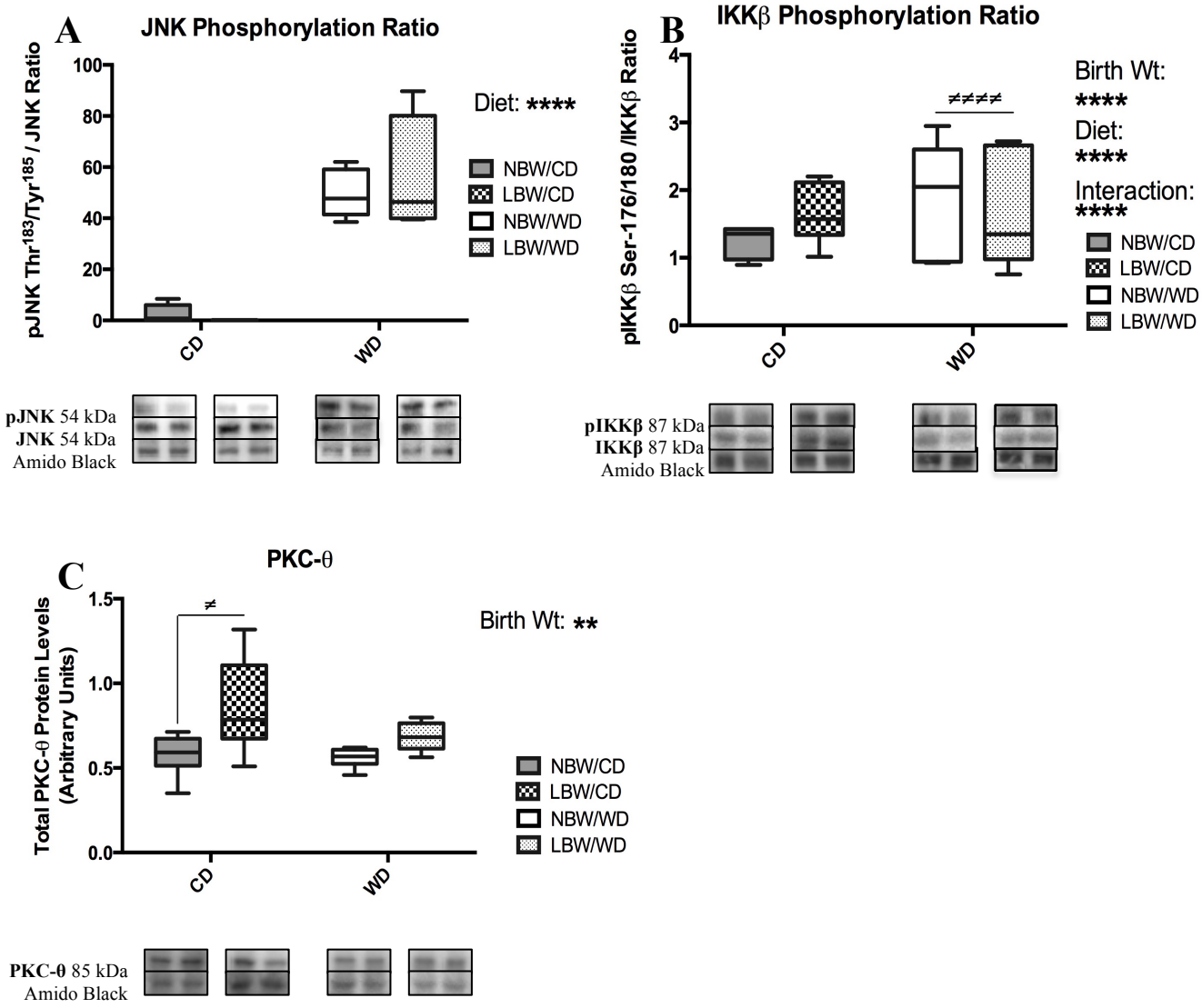


Figure 3.8: Expression and Phosphorylation of Key Stress-Induced Kinases. A) JNK phosphorylation, B) IKKβ phosphorylation, C) Total PKC-θ levels. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significant main effect of birth weight, diet or interaction; ≠p<0.05, ≠≠≠≠p<0.0001 by post-hoc Tukey's Test.

3.3.7 Fatty Acid Transporters are Differentially Altered in WD-fed and LBW Offspring

CD36, one transporter by which fatty acids can enter skeletal muscle, exhibited significantly ($p<0.0001$) reduced total protein levels in WD-fed offspring (Figure 3.9A). However, birth weight significantly ($p<0.05$) increased protein levels of CD36, such that LBW/CD offspring showed significantly ($p<0.05$) increased expression when compared to NBW/CD offspring (Figure 3.9A).

In contrast, FATP1 was significantly ($p<0.01$) increased in WD-fed offspring (Figure 3.9B). Additionally, a significant ($p<0.01$) interactive effect between birth weight and postnatal diet was observed, such that LBW/WD offspring displayed significantly ($p<0.001$) increased FATP1 expression when compared to LBW/CD offspring (Figure 3.9B). Expression levels of FATP4 were not altered by birth weight or postnatal diet (Figure 3.9C).

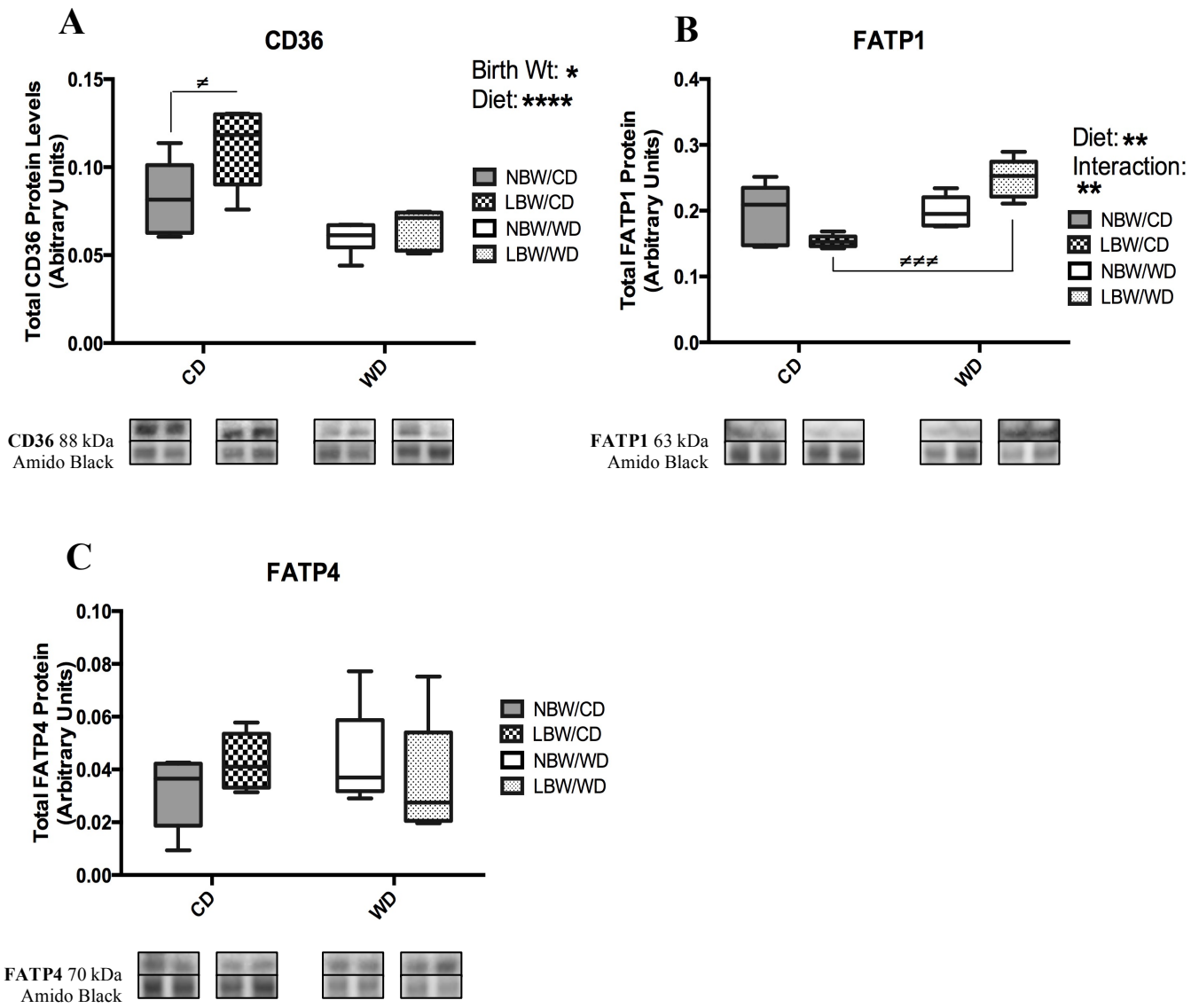


Figure 3.9: Fatty Acid Transporters. A) CD36, B) FATP1, C) FATP4. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, **$p<0.01$, ****$p<0.0001$ significant main effect of birth weight, diet or interaction; ≠$p<0.05$, ###$p<0.001$ by post-hoc Tukey's Test.

3.3.8 Mitochondrial β-oxidation is Disrupted in LBW Offspring

The rate limiting step of mitochondrial β-oxidation, CPT1-catalyzed transport of acylcarnitines into the mitochondria, is often used as a marker of mitochondrial oxidative capacity.[51] We observed a significant ($p<0.05$) decrease in CPT1b mRNA transcript abundance in LBW offspring, irrespective of postnatal diet (Figure 3.10A). A significant ($p<0.05$) decrease was also observed at the protein level in LBW offspring (Figure 3.10B).

Up-regulation of PDK4 inactivates the pyruvate dehydrogenase complex, initiating a switch from glucose catabolism to lipid oxidation.[52] However, PDK4 mRNA expression levels were not altered by birth weight or postnatal diet (Figure 3.10C). To initiate lipid oxidation and the cycle of β-oxidation, acyl-CoA must be dehydrogenated, which is accomplished by enzymes specific to the length of the chain being oxidized.[25] Both VLCAD, which dehydrogenates the very-long acyl-CoA chains, and MCAD, which dehydrogenates the medium acyl-CoA chains, mRNA levels were not altered by birth weight or postnatal diet (Figures 3.10D and 3.10E, respectively). The final enzyme in the β-oxidation cycle is KT, which catalyzes the removal of the 2-carbon acetyl-CoA that is fed into downstream TCA cycle.[25] KT mRNA levels were significantly ($p<0.05$) decreased in skeletal muscle of LBW offspring, irrespective of postnatal diet (Figure 3.10F).

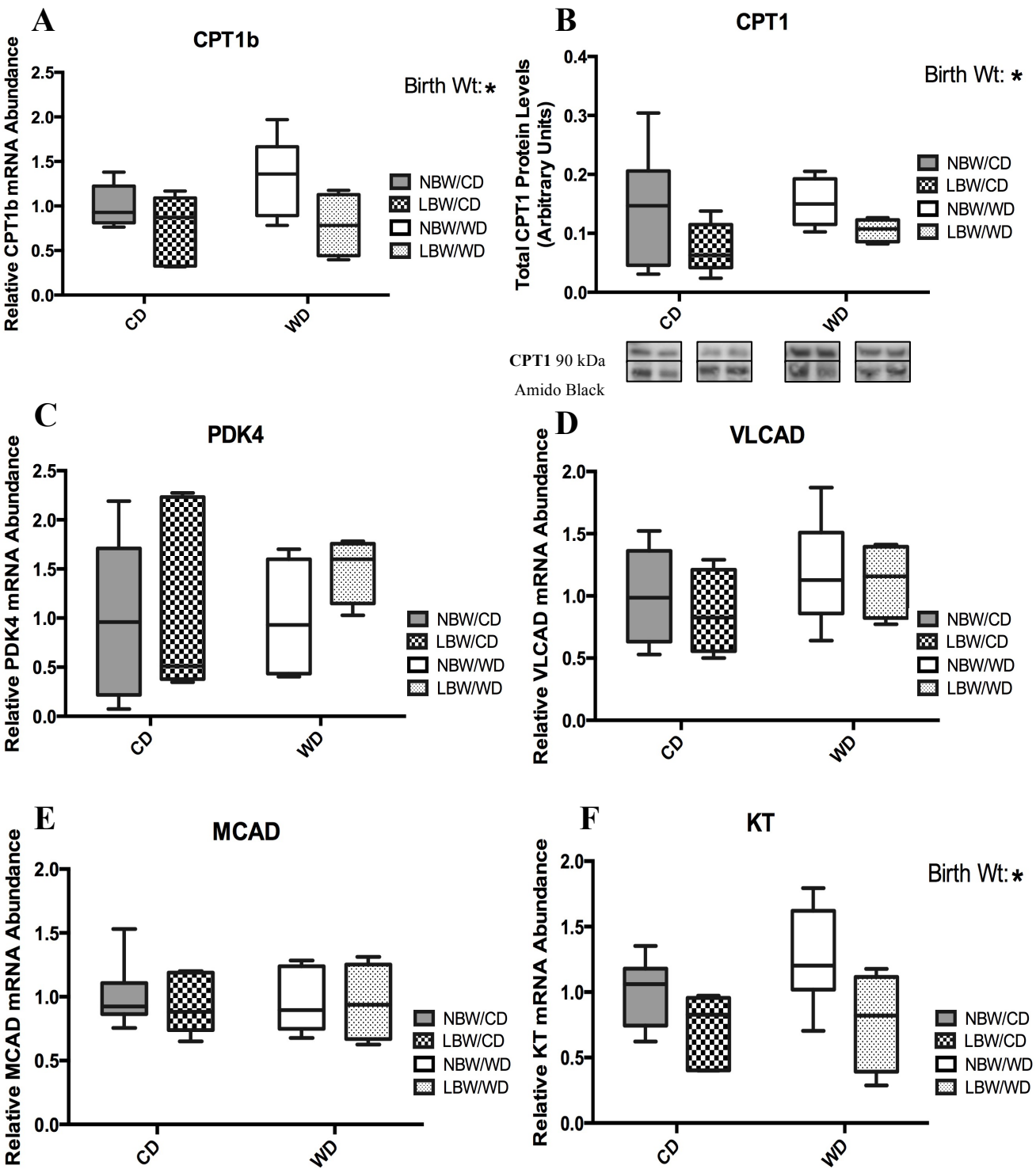


Figure 3.10: Expression of Key Enzymes Involved in Mitochondrial β-Oxidation. A) CPT1b mRNA expression, B) CPT1 protein expression, C) PDK4 mRNA expression, D) VLCAD mRNA expression, E) MCAD mRNA expression, F) KT mRNA expression. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD,

n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, significant main effect of birth weight.

3.3.9 Transcription Factors Regulating Mitochondrial β-oxidation are Reduced in WD-fed and LBW Offspring

PGC-1α and PPARα are well-known transcription factors that regulate aspects of mitochondrial lipid oxidation.[53] While skeletal muscle PGC-1α mRNA expression was similar among groups (Figure 3.11A), protein levels were significantly ($p<0.001$) reduced in WD-fed offspring, and reduced by 15% ($p=0.0797$) in LBW offspring (Figure 3.11B).

A similar pattern was observed for PPARα, where mRNA expression was not different among groups (Figure 3.11C). However, protein levels were similarly significantly ($p<0.001$) reduced in WD-fed offspring, and reduced by 26% ($p=0.0845$) in LBW offspring (Figure 3.11D).

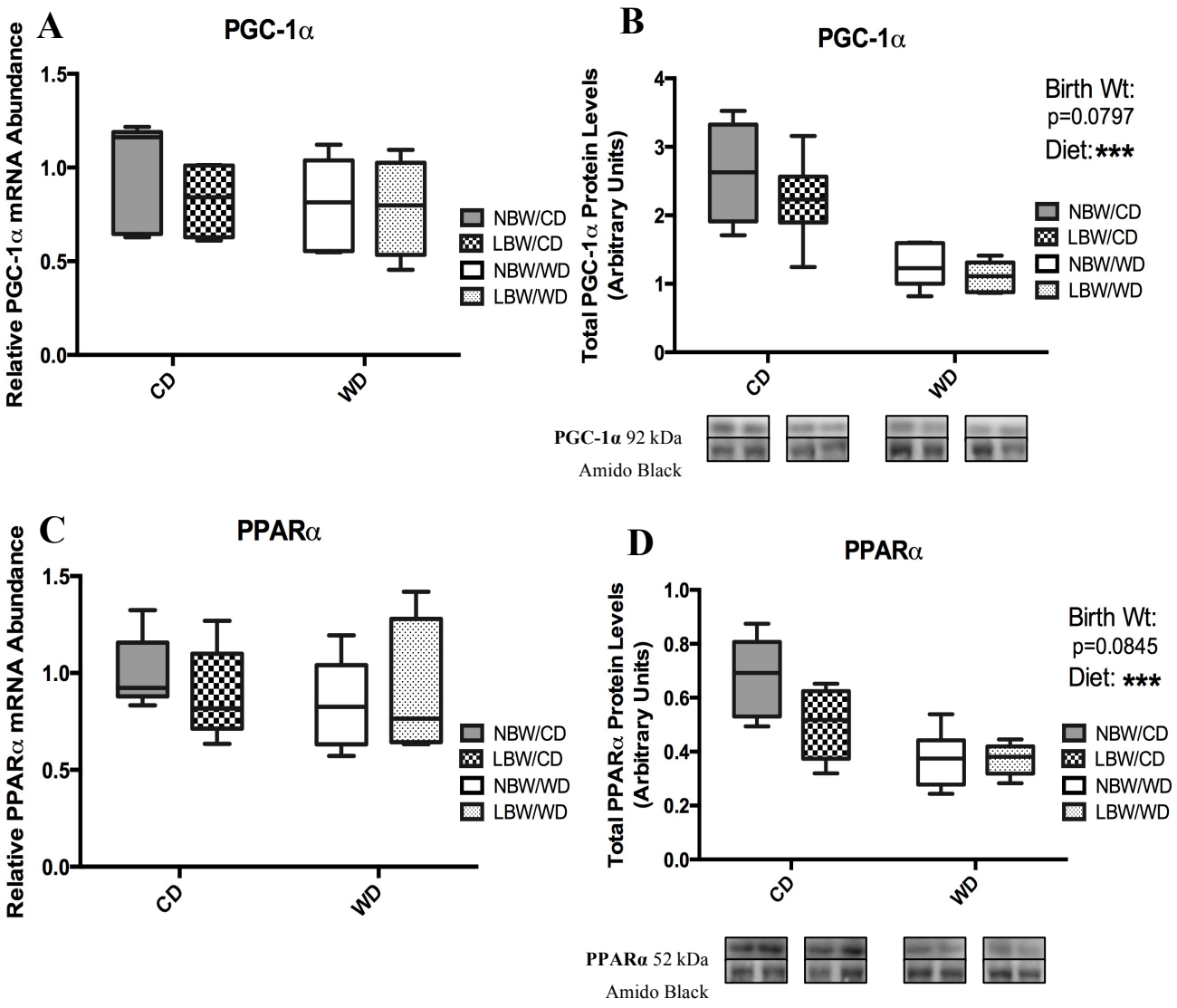


Figure 3.11: Expression of Transcription Factors Regulating Mitochondrial β-Oxidation. A) PGC-1α mRNA expression, B) PGC-1α protein expression, C) PPARα mRNA expression, D) PPARα protein expression. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. ***$p<0.001$, significant main effect of diet.

3.3.10 Sirtuins Regulating Mitochondrial Biogenesis are Reduced in WD-fed Offspring

Skeletal muscle sirtuins are a family of transcription factors regulating mitochondrial biogenesis and function.[54] SIRT1, which is predominantly found in the cytosol, is a key player involved in regulation of lipid oxidation.[55] While mRNA expression levels of SIRT1 were not different between experimental groups (Figure 3.12A), SIRT1 protein levels were significantly ($p<0.001$) reduced in WD-fed offspring (Figure 3.12B). Additionally, LBW/CD offspring displayed a significant ($p<0.05$) increase in SIRT1 protein levels when compared to NBW/CD offspring, and a significant ($p<0.05$) interactive effect between birth weight and postnatal diet was also observed (Figure 3.12B).

SIRT3, which is exclusively located in the mitochondria, is a key player in regulating mitochondrial oxidation at the level of β-oxidation and the electron transport chain.[56] Similar to SIRT1, SIRT3 mRNA expression was not affected by birth weight or postnatal diet (Figure 3.12C). However, protein levels of SIRT3 were significantly ($p<0.05$) reduced in WD-fed offspring (Figure 3.12D).

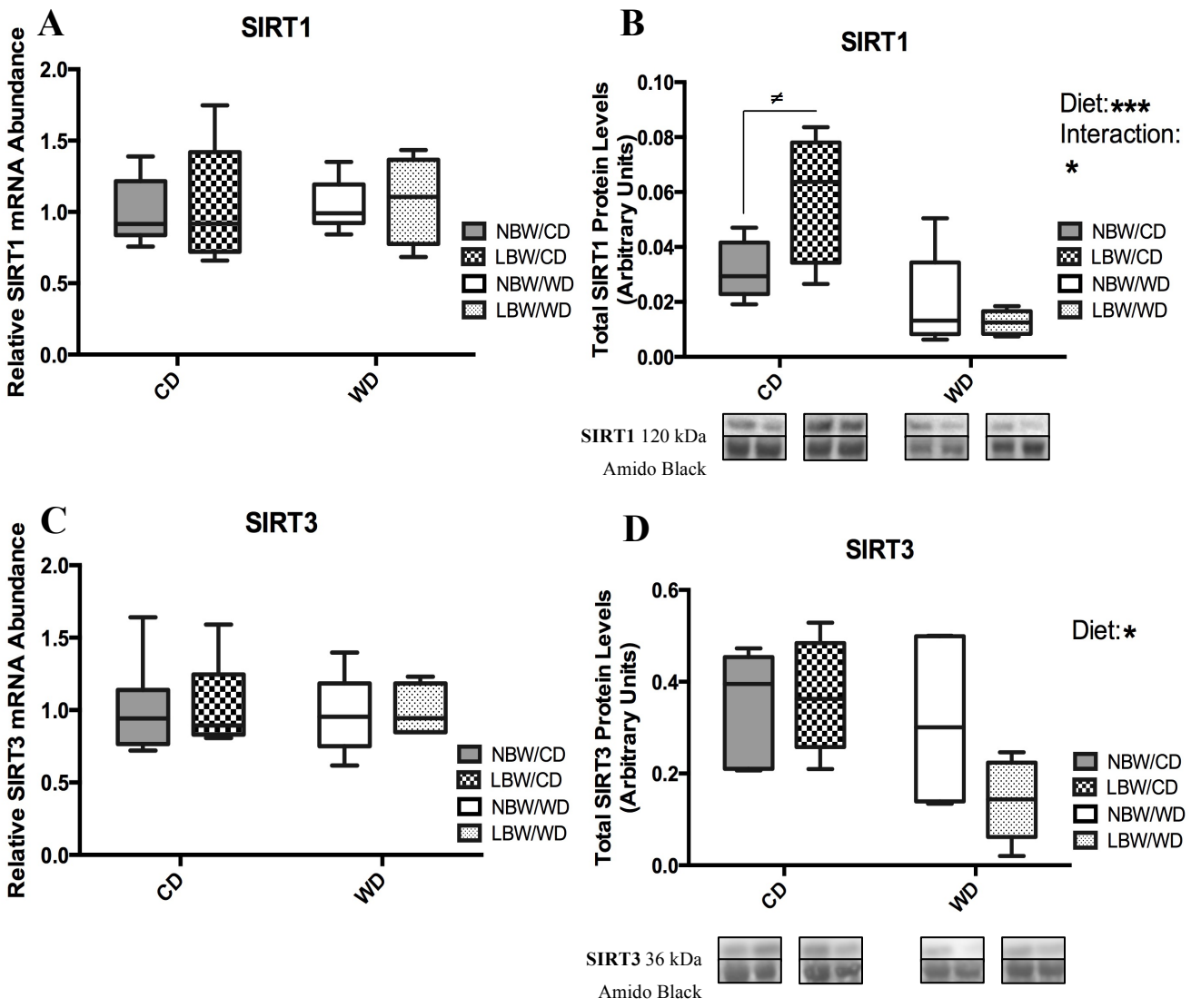


Figure 3.12: Expression of Skeletal Muscle Sirtuins. A) SIRT1 mRNA expression, B) SIRT1 protein expression, C) SIRT3 mRNA expression, D) SIRT3 protein expression. Data presented are min. to max. box and whisker plots for n=6 NBW/CD, n=6 LBW/CD, n=6 NBW/WD, n=5 LBW/WD. Representative blots of the respective proteins are below each group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *$p<0.05$, ***$p<0.001$, significant main effect of diet or interaction. ≠$p<0.05$, by post-hoc Tukey's Test.

3.3.11 Markers of Mitochondrial Dysfunction are Present in WD-fed and LBW Offspring

Recently, acylcarnitine metabolites have emerged as strong markers of mitochondrial stress and dysfunction.[21,23,57,58] Accumulation of even-chain acylcarnitines represents partially oxidized intermediates of β-oxidation, whereas accumulation of odd-chain acylcarnitines represents catabolism of amino acids.[21]

In gastrocnemius muscle, medium-chain acylcarnitines (C6-C12) were significantly ($p<0.05$) increased following consumption of a postnatal WD (Figure 3.13A). Additionally, a significant ($p<0.05$) effect of birth weight upon accumulation of C8 and C10 was also observed (Figure 3.13A). While not reaching statistical significance, a potential interactive effect between birth weight and postnatal diet was observed in C8-C12 species, with LBW/WD offspring tending to exhibit increased accumulation of C8 and C12 ($p=0.0622$ and $p=0.0554$, respectively) when compared to NBW/WD offspring (Figure 3.13A). Levels of C2 acylcarnitine, the end product of complete β-oxidation,[25] were significantly ($p<0.05$) reduced in WD-fed offspring.

Similarly, long-chain acylcarnitines (C14-C18:1) were significantly ($p<0.0001$) increased following consumption of a postnatal WD (Figure 3.13B). Significant ($p<0.05$) effects of birth weight were observed in C14:1, C16, C18 species, whereas birth weight alone tended to induce accumulation of C18:1 and C18:3 species ($p=0.0763$ and $p=0.0906$, respectively; Figure 3.13B). A significant ($p<0.05$) interactive effect between birth weight and postnatal diet was observed for C14:1 acylcarnitine. In LBW/WD offspring, significant ($p<0.05$) accumulation of C14:1 and C16 was noted, while C18

tended ($p=0.0592$) to accumulate, when compared to NBW/WD offspring (Figure 3.13B). A significant ($p<0.01$) reduction of C20:4 acylcarnitine levels was also observed in WD-fed offspring (Figure 3.13B)

Inhibition of NADH-linked β-hydroxy fatty acyl-CoA oxidation is known to promote accumulation of hydroxyacylcarnitines.[59] We observed significant ($p<0.05$) accumulation of the 3-β-hydroxylated forms of C12, C14, C16, C18 and C18:1 in response to WD-feeding (Figure 3.13C). Birth weight also induced a significant ($p<0.05$) accumulation of C16-OH (Figure 3.13C). A potential interactive effect between birth weight and postnatal diet ($p=0.0615$) was observed in C12-OH species (Figure 3.13C).

A cluster of amino acids has recently been identified that strongly predict risk of developing diabetes and impaired insulin action, including the branched-chain amino acids (leucine, isoleucine, valine), as well as tyrosine and phenylalanine.[58] In our model, we observed increased ($p=0.0572$) levels of leucine in LBW offspring, irrespective of postnatal diet (Figure 3.13D). We also observed reduced ($p=0.0734$) levels of isoleucine in gastrocnemius muscle of WD-fed offspring (Figure 3.13D). Lastly, we observed a significant ($p<0.05$) accumulation of phenylalanine in WD-fed offspring, and a trend ($p=0.0878$) towards accumulation in LBW offspring (Figure 3.13D).

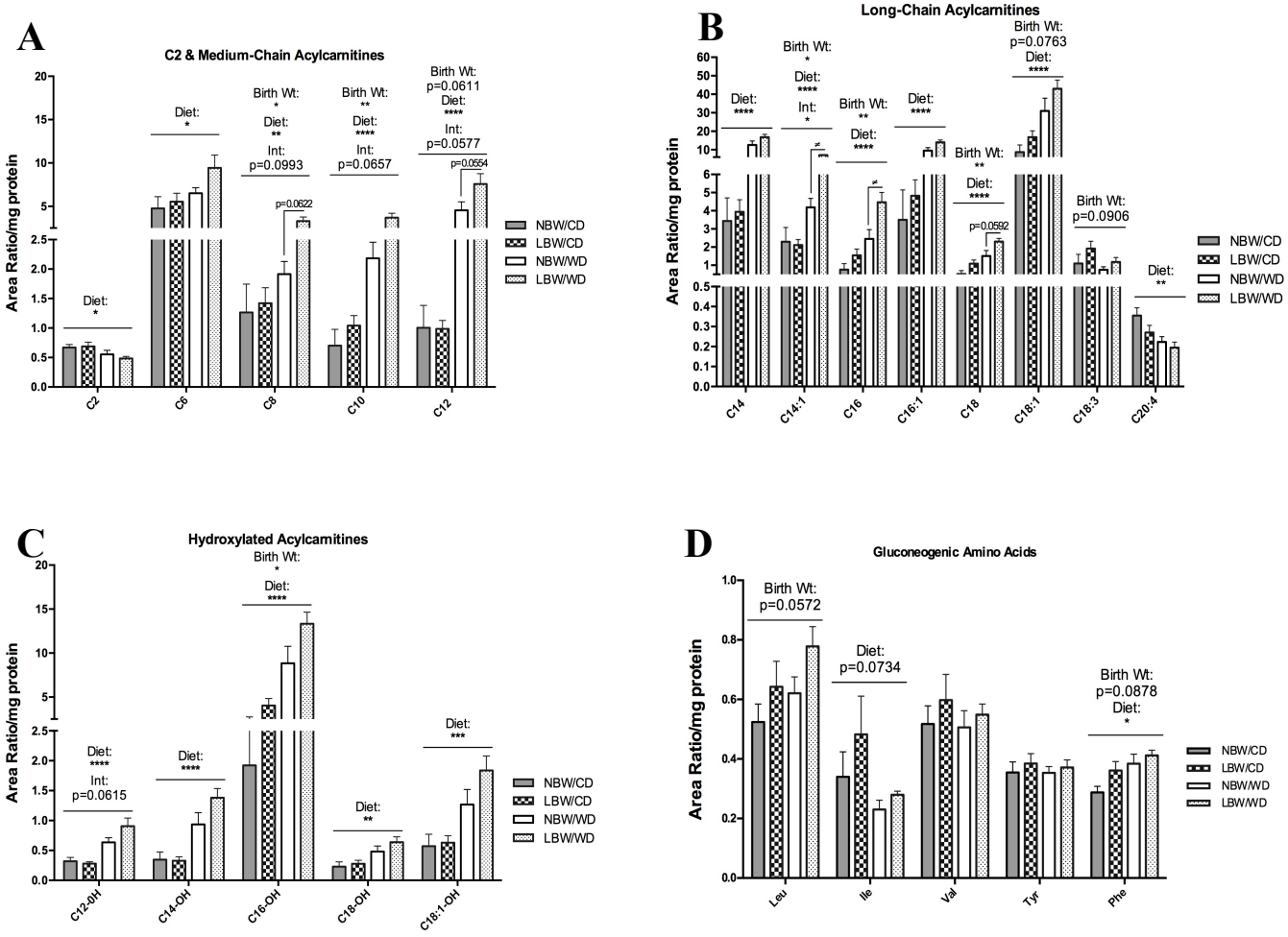


Figure 3.13: Acylcarnitine and Amino Acid Levels in Gastrocnemius Muscle. A) C2 & medium-chain acylcarnitines, B) long-chain acylcarnitines, C) hydroxylated acylcarntines, D) gluconeogenic amino acids as measured by LC-MS/MS. Data presented are mean ± standard error (SEM) for n=5-8 animals per group. Statistical significance was determined by Two-Way ANOVA, followed by post-hoc Tukey's Test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, significant main effect of birth weight, diet, or interaction. ≠p<0.05, by post-hoc Tukey's Test. Common names for the acylcarnitine short hands can be found in Appendix I.

3.3.12 Principle Components Analysis (PCA) of Acylcarntine and Amino Acid Levels

We employed PCA as an unbiased data reduction strategy to determine a model of how acylcarnitine and amino acid metabolism relates to the observed alterations in insulin signaling described above. Nine factors were identified, explaining 87.6% of the total variance in the model. Features of the top 4 PCs are described in Table 3.3.

The first PC (PC1; medium- and long-chain acylcarnitines) was composed of even-chain acylcarnitines ranging from C8 through C18, in addition to the unsaturated acylcarnitines C14:1, C16:1 and C18:1. Additionally, C2 (acetylcarnitine) was also a part of this component, however the negative loading factor suggests that levels of C2 would decrease as levels of the other components of this cluster increase, similar to the accumulation profile we observed in Figure 3.13. A significant ($p<0.0001$) main effect of diet was observed for the components of this factor, highlighting postnatal diet as a major contributor to the acylcarnitine species associated with this component. PC2 (hydroxylated acylcarnitines) was composed predominantly of the hydroxylated acylcarnitines derived from C4, C12-C18 and C18:1, with contributions from C4 as well as C6. A potential contribution ($p=0.076$) of postnatal diet was observed for this factor. PC3 (poly-unsaturated long-chain acylcarnitines) was composed of C18:2, C18:3, C20:4 and C22:5 acylcarnitine species, with a significant ($p=0.022$) contribution of postnatal diet to this cluster. PC4 (gluconeogenic amino acids) was composed of the amino acids leucine, isoleucine, valine, tyrosine and phenylalanine. Birth weight was a potential ($p=0.075$) contributor to the species observed in this factor.

Since accumulation of medium- and long-chain acylcarnitines is a well-documented marker of mitochondrial dysfunction,[21,22] we decided to focus further analysis on PC1. PC1 scores were moderately correlated with increased JNK phosphorylation as a measure of its activation ($r=0.658$, $p=0.001$; Figure 3.14A). Additionally, PC1 scores were modestly correlated with increased inhibitory serine IRS-1 phosphorylation ($r=0.466$, $p=0.025$; Figure 3.14B).

		P-values for Main Effect		
Factor	**Metabolite Description**	**Birth Weight**	**Diet**	**Birth Weight and Diet**
PC1, medium- & long-chain acylcarnitines; eigenvalue 12.6 (43.40%)	C2 (-0.715) C8 (0.556) C10 (0.797) C12 (0.886) C14 (0.801) C16 (0.929) C18 (0.836) C14:1 (0.766) C16:1 (0.877) C18:1 (0.921)	p=0.273	p<0.0001	p=0.469
PC2, hydroxylated acylcarnitines; eigenvalue 3.9 (13.71%)	C4 (0.865) C6 (0.784) C4-OH (0.721) C12-OH (0.723) C14-OH (0.783) C16-OH (0.741) C18-OH (0.814) C18:1-OH (0.764)	p=0.415	p=0.076	p=0.569
PC3, poly-unsaturated long-chain acylcarnitines; eigenvalue 3.3 (11.36%)	C18:2 (0.807) C18:3 (0.813) C20:4 (0.716) C22:5 (0.925)	p=0.775	p=0.022	p=0.996
PC4, gluconeogenic amino acids; eigenvalue 2.2 (7.53%)	Leu (0.723) Ile (0.603) Val (0.801) Tyr (0.554) Phe (0.673)	p=0.075	p=0.587	p=0.459

Table 3.3: Results of the Exploratory PCA on Acylcarnitine Levels. Eigenvalues and percent contribution to total sample variance is presented for the first 4 principle components of the model. Individual features of each component are displayed, along with rotated loading coefficients when greater than 0.5. Values in parentheses next to each metabolite are the loading coefficient from the PCA model, which is a measure of the importance of each metabolite to the factor. Parenthetical values next to the eigenvalue represent the percentage of the total sample variation for each factor. Common names for the acylcarnitine short hands can be found in Appendix I.

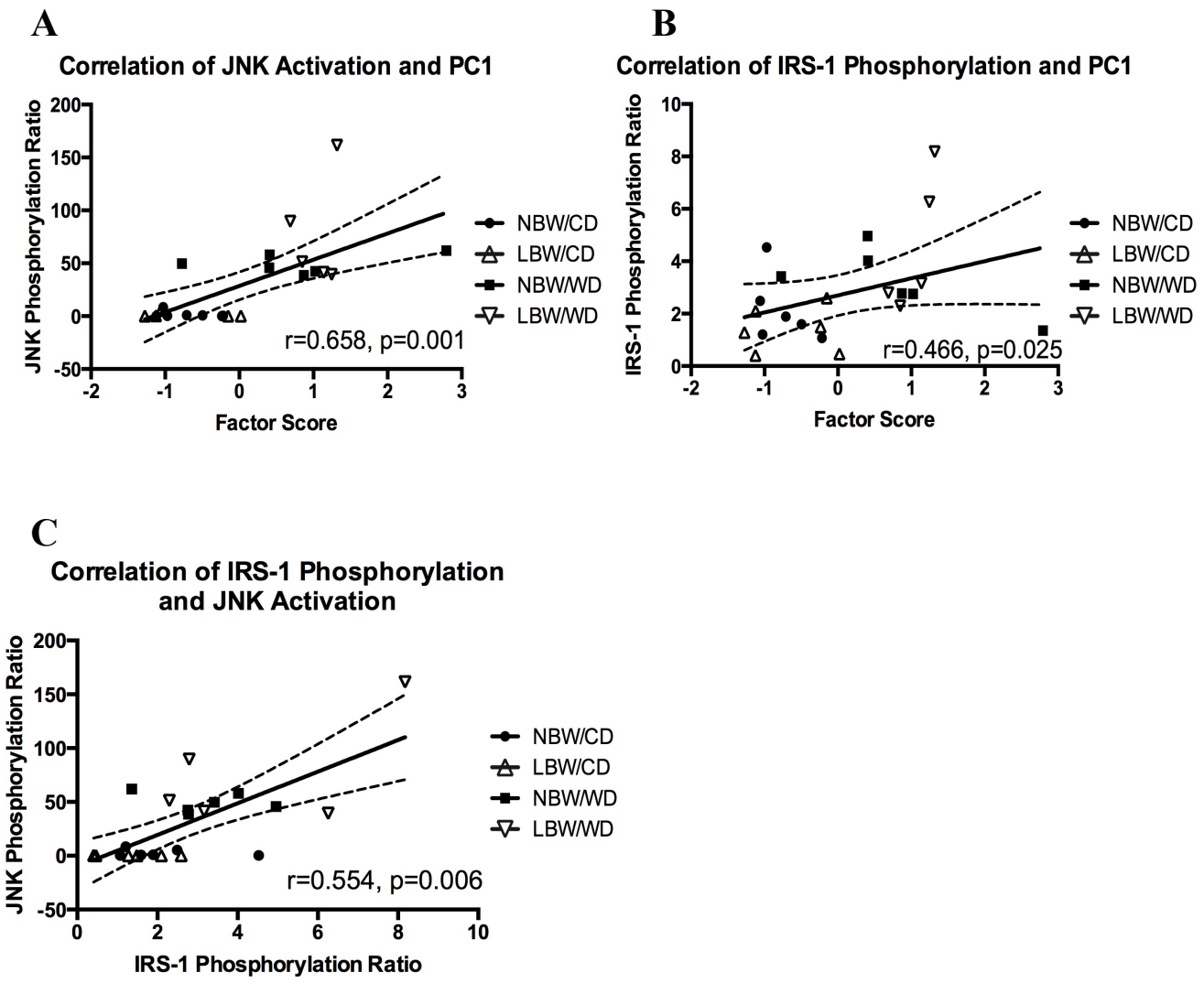


Figure 3.14: Correlation Analyses. A) Correlation of JNK phosphorylation and Factor 1 score, B) Correlation of IRS-1 phosphorylation and Factor 1 score, C) Correlation of IRS-1 phosphorylation and JNK phosphorylation. Regression lines with 95% confidence intervals of the mean are shown.

3.4 Discussion

With the recent surge of research investigating the developmental origins of health and disease, the contribution of the *in utero* environment and its ability to modulate growth is a well-established determinant of later life disease risk. However, the mechanisms by which adverse growth *in utero* may induce later life insulin resistance and the progression to MetS remain elusive. In this study, we sought to determine the relative contribution of the *in utero* environment upon disrupted skeletal muscle lipid metabolism as a component of the pathophysiological progression of insulin resistance. Additionally, with the increasing availability of high-fat/high-sugar diets and their early introduction during the postnatal period, we sought to determine whether early consumption of these diets would have a synergistic effect following a LBW outcome, promoting early markers of insulin resistance.

3.4.1 Skeletal Muscle Insulin Signaling and Glucose Homeostasis in LBW offspring

While we did not see alterations in whole-body glucose tolerance or skeletal muscle glucose uptake in our cohort, we did observe molecular disruptions to the skeletal muscle insulin signaling cascade, a documented component of pre-diabetes.[9,20] LBW was associated with a reduction in the phosphorylation of the insulin receptor, as well as decrease in total protein content of the p85 and p110 subunits of PI3-kinase, highlighting impaired activation, in addition to reductions in phosphorylation of Akt at Ser^{473}, and AS160 at Thr^{642}. Similar to our observations, reduced levels of p85 and p110 have been observed in both animal models of IUGR, as well as young adult men born LBW.[60-62]

This impairment of PI3-kinase actions in these other models of IUGR occurred in conjunction with impaired phosphorylation of Akt and AS160 in response to insulin stimulation.[63] In young adulthood, the molecular alterations observed in human offspring also occurred with maintenance of whole-body glucose tolerance, similar to what we have observed in our cohort.[60,61] It has been postulated that changes in expression and phosphorylation of the insulin signaling intermediates precedes the development of overt insulin resistance and glucose intolerance,[61] providing a molecular signature associated with early stages of disease progression.[64] Therefore, the persistent changes in the insulin signaling pathway, in conjunction with maintenance of glucose homeostasis highlight that at this young age, our guinea pigs are in an early-stage of insulin resistance pathogenesis resembling a pre-diabetic state;[20] however, we speculate that with time insulin sensitivity will decrease and disrupt glucose homeostasis, similar to human reports[4,61] and animal models of older LBW offspring.[65]

While whole-body glucose tolerance is maintained in young adolescent men born LBW, reduced glucose uptake in the muscles of the forearm have been observed.[60] This reduced glucose uptake is typically associated with reductions in skeletal muscle GLUT4 content,[60,61,63] reducing insulin-stimulated glucose uptake into the muscle. Interestingly, we did not observe any significant differences in total GLUT4 protein content in our cohort, nor did we observe deficiencies in skeletal muscle glucose uptake as measured by ^{18}F-FDG PET scans. However, we observed significant increases of GLUT2 and GLUT5 total protein content in LBW offspring. Additionally, we observed significant decreases in GLUT2 content in WD-fed animals, in conjunction with significant increases in GLUT5 content, which may be able to provide an avenue for compensation in order to

maintain skeletal muscle glucose uptake following consumption of a postnatal WD. In the skeletal muscle the more significantly expressed GLUTs include GLUT4, GLUT5 and GLUT12;[66] however, GLUT2 is a low-affinity glucose transporter, known to facilitate minimal glucose uptake into skeletal muscle in order to maintain glucose homeostasis.[67] Additionally, GLUT5, while typically considered to be the main fructose transporter in skeletal muscle,[68] also has the ability to facilitate glucose uptake.[66] It is even responsive to the actions of insulin, activating the GLUT5 promoter region to induce its expression in response to the hormonal milieu.[66] Therefore, the observed increases in total GLUT5 protein content of LBW and WD-exposed offspring highlight a potential compensatory mechanism at play in our model, similar to that observed in the muscle of type 2 diabetic patients, aimed at maintaining glucose homeostasis at this age.[69]

3.4.2 Mechanisms Underlying Molecular Alterations in Insulin Signaling

We next sought to assess the mechanisms by which the alterations in skeletal muscle insulin signaling may occur. Literature investigating the mechanisms underlying insulin resistance pathogenesis secondary to high-fat feeding highlighted three candidate stress-induced kinases that may be activated in situations of oxidative stress or lipid overload.[24] Following chronic exposure to high-fat diets, activation of JNK through phosphorylation at $Thr^{183/}Tyr^{185}$ has been associated with increased inhibitory serine phosphorylation of IRS-1 at Ser^{307}.[50] Similarly, in our offspring exposed to postnatal WD diets, we saw a robust activation of JNK, which was modestly correlated with the

observed inhibitory serine phosphorylation of IRS-1. However, phosphorylation of JNK was not detectable in our LBW/CD offspring, prompting further investigation into other stress-kinases that may be activated in LBW situations. In addition to significant activation of IKKβ in our WD-fed offspring, we also observed significant activation in our LBW/CD offspring, in addition to a LBW-specific increase in total levels of PKC-θ. Each of these stress-kinases have been well documented to have the ability to promote inhibitory serine phosphorylation of IRS-1 in situations of oxidative stress,[49] disrupting downstream insulin signaling events. Overall, we observed differential activation of stress-induced kinases in LBW and WD-fed offspring, highlighting that activation of stress-induced kinases may be environment specific depending on the mechanism by which the stress occurs. Additionally, in LBW/WD offspring, where activation of stress-kinases due to a LBW outcome and postnatal consumption of a WD may occur, an interactive effect is possible, promoting further disruption to skeletal muscle insulin signaling.

Activation of stress-kinases is also known to occur secondary to lipid overload and accumulation of toxic lipid intermediates, including triglycerides, DAG and ceramides.[15,49] Therefore, we sought to determine the relative contribution of lipotoxic intermediates to the alterations in insulin signaling and the activation of stress-kinases observed above. We did not observe any significant accumulation of triglycerides or DAG in the skeletal muscle of WD-fed offspring, contrary to other high-fat diet-induced insulin resistance models.[14,15] Interestingly, altered insulin sensitivity in the absence of DAG accumulation has been observed in pre-diabetic men,[70] highlighting alternative

mechanisms may be at play early in disease progression, namely accumulation of other lipotoxic intermediates such as ceramides or acylcarnitines.[49]

We also profiled the composition of the fatty acids present in the skeletal muscle by gas chromatography. A higher percentage of saturated fatty acids in the neutral lipid fraction has been associated with reduced insulin sensitivity,[14] similar to the accumulation of C14:0 we observed in our WD-fed offspring. We also observed reductions in the omega-6 and omega-3 poly-unsaturated fatty acids in the neutral lipid fraction, which have also been implicated in reduced insulin sensitivity.[14,71] Unsaturated fatty acids have the ability to protect against lipotoxicity by promoting uptake into an inert triglyceride pool that is not esterified into intermediates, namely ceramide.[14] Additionally, a higher proportion of saturated fatty acids in this lipid pool have been associated with higher ceramide content due to de novo ceramide synthesis.[72] These alterations in fatty acid composition favouring ceramide accumulation have the potential to reduce insulin sensitivity by reducing Akt activation.[49] Overall, the alterations we observed in the skeletal muscle of WD-fed offspring mirrored the composition of the dietary fats present in the WD (Chapter 2, Table 2.1). Additionally, we did not observe any alterations in the fatty acid profile due to birth weight alone, prompting further investigation into mitochondrial metabolism as an alternative theory to explain the observed defects in insulin signaling.

3.4.3 Mitochondrial Oxidation in Response to LBW and Postnatal WD

The first step in the mitochondrial oxidation of lipids is entry of fatty acids into the skeletal muscle through the coordinated actions of the fatty acid transporters FAT/CD36, FATP1 and FATP4.[73] In our model, total protein levels of CD36 were significantly reduced in offspring consuming a postnatal WD; however FATP1 was significantly increased in these same offspring. Conversely, in LBW/CD offspring, CD36 levels were significantly increased, but FATP1 levels were significantly decreased. Not only does this data suggest differential expression of fatty acid transport proteins in response to birth weight and postnatal diet, but also highlights a mechanism by which fatty acid transport into the skeletal muscle can continue without impediment. Additionally, recent evidence suggests both CD36 and FATP1 are localized to the mitochondria in addition to the plasma membrane,[74,75] providing a concentrated supply of long-chain fatty acids for entry into mitochondria and subsequent oxidation. Therefore, the differential expression of FATP1 and CD36 also support the notion that entry of fatty acids into the cell for oxidation is not compromised at this stage of disease progression, preventing accumulation of fatty acids as triglycerides or toxic lipid intermediates.

However, we did observe accumulation of acylcarnitine intermediates in skeletal muscle of WD-fed offspring, as well as LBW offspring. These intermediates are known to accumulate in situations of mitochondrial dysfunction and incomplete fatty acid oxidation, where they are indicative of impairment of substrate flux through the TCA cycle and oxidative phosphorylation.[21,57,58] Therefore, we investigated the enzymatic reactions involved in β-oxidation and the regulation of the oxidative process in order to shed light on possible mechanisms underlying the observed accumulation of

acylcarnitines. At both the mRNA and protein level, CPT1, the rate-limiting step of the oxidation process,[25] was reduced in LBW offspring, irrespective of postnatal diet. Additionally, mRNA levels of KT, the enzyme responsible for catalyzing the removal of acetyl-CoA from the fatty acid chain,[25] was reduced in LBW offspring. These defects in the oxidative process would slow down the rate of β-oxidation and impair substrate utilization through the remaining steps of the oxidative process, preventing complete oxidation of the fatty acid chains. However, the reduced levels of these oxidative enzymes were LBW-specific, highlighting that the accumulation of acylcarnitines in the WD-fed offspring is likely due to alternate defects in other components along the oxidative pathway.

3.4.4 Regulation of Mitochondrial Biogenesis and Oxidative Function

In order for fatty acids to be completely oxidized, the tight coordination of a number of transcription factors regulating mitochondrial biogenesis and oxidative function is required. PPARα is one such transcription factor that is highly expressed in oxidative tissues, including skeletal muscle, which is known to induce activation of genes involved in β-oxidation.[24,76] However, in order for full activation of PPARα target genes, coordination with PGC-1α is required.[53,77] PGC-1α also has the ability to independently activate genes involved in the TCA cycle and oxidative phosphorylation, promoting complete oxidation of lipid substrates.[55] Consumption of high-fat diets is known to reduce protein levels of PGC-1α[21,57] reducing lipid oxidation and promoting accumulation of acylcarnitine intermediates indicative of mitochondrial dysfunction. We

observed similar reductions in total protein levels in our WD-exposed offspring. Recent studies have also reported reduced PGC-1α protein levels in gastrocnemius muscle of adolescent low birth weight offspring.[78] While the reductions we observed in our model did not reach statistical significance in the LBW/CD offspring, we did see a 15% reduction in total protein levels, which we postulate would still have the ability to impair complete lipid oxidation in these offspring. Reduced PGC-1α has also been observed in C_2C_{12} muscle cells exposed to hypoxia in culture, a key characteristic of adverse *in utero* environments.[79] The ability of hypoxia alone to modulate the regulation of mitochondrial lipid metabolism highlights a potential role for hypoxia and potentially its association with oxidative stress in an adverse *in utero* environment[80,81] in promoting later life persistent mitochondrial dysfunction.

The sirtuins (SIRTs) are another important family of transcription factors involved in regulating mitochondrial biogenesis and oxidative function. SIRT1, which is predominantly localized to the nucleus or cytosol, promotes deacetylation of target proteins involved in lipid metabolism and energy balance, including PGC-1α and PPARα.[54,82] Reduced levels of SIRT1 protein have been observed in skeletal muscle of type 2 diabetic patients,[83] as well as rats consuming high fat diets.[84] This reduction in SIRT1 has also been associated with hyperacetylation of PGC-1α and PPARα, preventing their ability to complex with one another to induce lipid oxidation.[55] We have observed similar reductions in SIRT1 protein levels in our WD-exposed offspring, highlighting impaired regulation of lipid oxidation through the SIRT1-PGC-1α pathway. Additionally, SIRT3 which is exclusively located in the mitochondria, catalyzes the deacetylation of proteins involved in β-oxidation as well as the electron transport

chain.[56,85,86] In rodents exposed to high fat diets, muscle levels of SIRT3 protein are significantly reduced,[87] similar to the reduced SIRT3 total protein level we observed in gastrocnemius of WD-fed offspring. Since SIRT3 knockouts have been associated with hyperacetylation of SIRT3 target proteins,[88] preventing their activation and promoting mitochondrial dysfunction in addition to insulin resistance,[89] we speculate that the reduced SIRT3 levels observed in our model would be associated with reduced activation of SIRT3 target proteins, preventing complete lipid oxidation.

While FOXO1 protein levels were decreased following postnatal consumption of a WD, FOXO1 phosphorylation at Ser^{256} mediated by Akt [90] was significantly increased. As such, while there are reduced levels of FOXO1, the increased phosphorylation could suggest an increased level of exclusion of the available protein from the nucleus, thus negatively impacting transcription of gluconeogenic and lipid metabolism gene targets.[91] Additionally, the increased phosphorylation, possibly mediated by Akt, suggests that Akt retains its ability to phosphorylate these sites on FOXO1 despite an observed reduction in Ser^{473} phosphorylation level, suggesting a potential compensatory activation of alternative phosphorylation sites on Akt aimed at maintaining its kinase activity.

In terms of the regulation of mitochondrial biogenesis and function, the main effects on the protein expression of these transcription factors were largely attributable to consumption of a postnatal WD, with a trend towards decreasing levels of PPARα and PGC-1α in response to a LBW outcome. However, the alterations observed in the enzymes involved in β-oxidation were exclusively observed in LBW offspring, irrespective of postnatal diet. As such, it is possible that the defects inducing acylcarnitine accumulation in LBW offspring versus WD-exposed offspring occur at

different levels of the oxidative process, namely β-oxidation in LBW offspring, and downstream TCA cycle or oxidative phosphorylation in the WD-exposed offspring. These differential mechanisms, when superimposed on one another in the LBW/WD offspring would have the potential to impair the oxidative process to a greater extent, promoting increased acylcarnitine accumulation, similar to the interactive effect we observed in numerous acylcarnitine species. As such, postnatal consumption of a WD following a LBW outcome may exacerbate mitochondrial dysfunction, promoting increased accumulation of acylcarnitine intermediates.

3.4.5 A Model Linking Mitochondrial Dysfunction and Defective Insulin Signaling

Acylcarnitine accumulation secondary to mitochondrial dysfunction has been shown to activate pro-inflammatory signaling pathways and induce a state of oxidative stress,[27] promoting insulin resistance. Therefore, we sought to use PCA in order to identify a cluster of metabolites that may be implicated in skeletal muscle insulin resistance pathogenesis. The first cluster, composed of medium- and long-chain acylcarnitine species, accounted for more than 40% of the variation of the model, highlighting this cluster of metabolites as important players in the pathogenesis of mitochondrial dysfunction. Medium- and long-chain acylcarnitines are known to accumulate secondary to incomplete lipid oxidation, therefore representing a key manifestation of mitochondrial dysfunction.[21,57,58] The exact mechanisms associated with acylcarnitine accumulation remain poorly defined, however the observed reductions in CPT1, KT, PPARα, PGC-1α, SIRT1 or SIRT3 in our model are likely candidates

modulating mitochondrial lipid metabolism, mitochondrial dysfunction and acylcarnitine accumulation. Additionally, a significant main effect of diet was observed for PC1, highlighting postnatal diet as a key contributor to the accumulation of these metabolites. The metabolites of PC1 were also significantly correlated with activation of JNK, as well as with inhibitory serine phosphorylation of IRS-1. Recent evidence suggests accumulation of C-14 carnitine in particular is able to induce phosphorylation and activation of JNK,[27] highlighting a potential mechanism by which mitochondrial dysfunction and altered insulin signaling may be linked. In our model generated by PCA, accumulation of medium- and long-chain acylcarnitines is also associated with increased activation of JNK, promoting inhibitory serine phosphorylation of IRS-1 and disrupted insulin signaling. Interestingly, acylcarnitine accumulation has been observed in individuals with pre-diabetes who do not display overt reductions in insulin sensitivity,[92] highlighting that our offspring may also be at a pre-diabetic stage since we are seeing molecular disruptions in mitochondrial function and insulin signaling without whole body alterations in insulin sensitivity.

With the knowledge that hypoxia associated with IUGR induces a state of oxidative stress in the fetus,[93-95] we speculate that it is this exposure to oxidative stress that initiates the persistent molecular alterations in insulin signaling and mitochondrial metabolism. Limited nutrient supply *in utero* has been associated with an alteration in the redox state of metabolic tissues of the fetus, altering their metabolic function.[96] Additionally, low oxygen availability has been associated with decreased function of the electron transport chain in fetal mitochondria, generating reactive oxygen species (ROS) that leads to persistent oxidative damage to the mitochondria.[97-99] Mitochondrial

dysfunction itself has also been associated with ROS production,[96] perpetuating a state of oxidative stress that has the potential to activate stress-induced kinases or induce post-translational modifications to important mitochondrial transcription factors.

In summary, IUGR culminating in LBW predisposes the offspring to disrupted insulin signaling and mitochondrial lipid metabolism in skeletal muscle. Additionally, early exposure to a postnatal WD independently induces alterations in mitochondrial lipid metabolism and insulin signaling; however, consumption of this diet following a LBW outcome likely unmasks a LBW-induced metabolic dysfunction, increasing the offspring's risk of developing insulin resistance. Lastly, alterations in mitochondrial function, as observed by acylcarnitine accumulation, occur early in disease progression, without overt disruption of whole-body glucose tolerance, highlighting a pre-diabetic situation in these offspring. Characterization of other modulators, such as the epigenetic drivers, of disrupted mitochondrial metabolism that may underlie the persistent molecular changes observed when exposed to an adverse *in utero* environment will be the subject of future studies.

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Chapter 4: Discussion

4.1 Summary

The importance of the *in utero* environment in modulating growth trajectory and influencing the propensity for development of age-related metabolic diseases is rapidly gaining recognition among healthcare professionals and government agencies. Evidence strongly suggests that infants displaying IUGR, culminating in a LBW, are at greater risk for developing type 2 diabetes, cardiovascular disease and obesity.[1-8] The Developmental Origins of Health and Disease (DOHaD) hypothesis first put forth by Forsdahl, Barker, and expanded upon by others, postulated that development of these chronic, non-communicable, metabolic diseases are the result of a programming mechanism initiated during critical periods of *in utero* development in order to promote fetal survival in poor intrauterine conditions.[5,9-12] Recent evidence has also suggested that secondary insults in postnatal life, including the increasingly accessible "Western" diet, have the potential to exacerbate an already dysfunctional system, further increasing the risk of developing age-related metabolic diseases.[1,13,14] Given the increasing prevalence of non-communicable metabolic diseases, understanding the mechanisms underlying fetal programming of adult disease along with the contribution of postnatal diet on the development of metabolic disorders is of critical importance. While some mechanisms have been postulated, including epigenetic modifications, oxidative stress, and nuclear receptor signaling,[15-17] further delineation of the persistent molecular alterations initiated *in utero* remain to be elucidated.

We chose to focus on the developmental programming of skeletal muscle insulin resistance as it relates to development of later life progression to type 2 diabetes and MetS, since skeletal muscle is the primary location for insulin-stimulated glucose

uptake[18] and a major regulator of whole body energy homeostasis.[19] Overall, the present studies provide strong evidence for the role of the *in utero* environment in modulating skeletal muscle lipid metabolism and insulin signaling as a precursor to the development of insulin resistance. Additionally, the work presented in this thesis, as well as others,[13,14,20,21] provide further support that a postnatal diet characterized by relative nutrient excess has the potential to exacerbate an already dysfunctional system and alter growth trajectory (Chapter 2) and metabolism (Chapter 3) of the offspring.

In the first study (Chapter 2), we sought to characterize the growth and metabolic parameters of offspring born following an adverse *in utero* environment, characterized by reduced oxygen and nutrient supply, as they grew into young adulthood. Furthermore, we also sought to investigate the relative contribution of a consistent, early exposure to a postnatal Western diet on the potential for an increased risk of developing features of age-related metabolic diseases. The study presented evidence that the immediate post-birth growth trajectory of LBW offspring is altered following IUGR, displaying rapid postnatal catch-up growth from weaning through to PND50. This pattern of growth is classically replicated in animal and human IUGR studies, and is traditionally associated with augmenting the offspring's risk for developing age-related metabolic diseases.[4,15,22,23] Few, if any, studies are available that examine this growth pattern when LBW offspring are exposed to a true WD early in life consisting of high fat and high processed sugars. Early exposure to a postnatal Western diet appeared to have a differential effect on growth trajectory, stunting the accelerated catch-up growth post-weaning that is traditionally associated with LBW. Additionally, these WD-fed offspring exhibited a non-obese phenotype and in fact displayed a significantly reduced overall

adiposity. While our offspring displayed maintenance of whole-body glucose tolerance and a non-obese phenotype at this young age, previously published studies in our lab,[24-26] as well as investigation of skeletal muscle lipid metabolism (Chapter 3) highlight that metabolic disturbances are indeed present in these offspring and may be associated with a pre-diabetic phenotype.

The second study (Chapter 3) postulated that adverse *in utero* conditions would impair skeletal muscle mitochondrial function and lipid metabolism, promoting a state of mitochondrial dysfunction, which negatively alters insulin signaling. While whole-body glucose tolerance, as well as skeletal muscle glucose uptake, was maintained, we observed skeletal muscle-specific molecular alterations in insulin signaling and lipid metabolism. Additionally, we observed hallmarks of mitochondrial dysfunction, including an increased level of acylcarnitine intermediates, in WD-fed, as well as in LBW/CD offspring. Furthermore, early consumption of a postnatal WD by LBW offspring from weaning appeared to unmask LBW-induced metabolic disruptions by early adulthood by inducing further accumulation of acylcarnitines. Molecular alterations in skeletal muscle lipid metabolism and insulin signaling are frequently observed early in the pathogenesis of insulin resistance.[8,27] Changes in these two molecular pathways, in conjunction with maintenance of glucose uptake and glucose tolerance, highlights a pre-diabetic phenotype, an early step in disease progression, as previously reported in humans.[7,8,27,28]

Taken together, these two studies provide strong evidence for the role of the *in utero* environment in modulating growth and future disease risk. In both studies, early introduction of a postnatal WD altered growth trajectory and metabolism, potentially

unmasking and exacerbating LBW-induced metabolic dysfunctions. While our offspring outwardly appear healthy due to their non-obese, glucose tolerant phenotype, our studies highlight disruptions in skeletal muscle lipid metabolism and insulin signaling associated with a developing metabolically unhealthy phenotype (Table 4.1)

4.2 Speculations

An adverse *in utero* environment typically observed in situations of IUGR has been associated with hypoxia, reducing the amount of oxygen reaching the developing fetus.[29,30] Fetal hypoxia has been observed to correlate with the incidence of later life cardiovascular disease in high altitude Bolivian populations,[31] and has been replicated in rodent studies as well.[32,33] Skeletal muscle levels of key intermediates of insulin signaling, including Akt and GLUT4, are significantly reduced in adults exposed to prenatal hypoxia,[34,35] further highlighting hypoxia as a key modulator of later life disease risk, possibly by promoting a state of oxidative stress.[36] IUGR has been associated with increased indices of oxidative stress in the fetus,[37-39] while oxidative stress has been postulated to underlie the programming of later life hypertension. Therefore, oxidative stress *in utero* has recently been highlighted as a potential mechanism promoting later life metabolic disease. One mechanism by which this may occur is an altered redox state in fetal tissues in response to limited oxygen and nutrient availability. These low oxygen levels have the potential to decrease activity of the electron transport chain, reducing oxidative phosphorylation, and generating ROS that promote persistent oxidative damage

and mitochondrial dysfunction.[27,40,41] Additionally, mitochondrial dysfunction itself leads to production of ROS, further perpetuating a state of oxidative stress.[17]

Of note, progression of insulin resistance following consumption of a WD is also strongly associated with a state of oxidative stress.[42-44] Mitochondrial dysfunction is also known to occur secondary to high-fat feeding,[43,45,46] increasing mitochondrial stress and decreasing mitochondrial oxidative phosphorylation.[47] This impairment in mitochondrial function promotes ROS generation, as well as acylcarnitine accumulation, both of which have the potential to activate stress-induced kinases that mediate alterations in insulin signaling.[48-51] Of note, the molecular disruptions occurring in skeletal muscle of LBW/CD offspring and NBW/WD offspring, and their associated phenotype, are similar among these two groups. This suggests that a similar trigger may be responsible for inducing the metabolic dysfunctions in both these groups, namely oxidative stress.

	Birth Weight	Diet	Birth Weight/Diet Interaction
Growth & Feeding Behaviour			
AGR	↓	↓	
Food Intake			
Calorie Consumption		↑	
Feed Efficiency	↓	↓	
Body Composition			
Whole-body Adiposity		↓	
Mesenteric Fat Pad Mass		↓	
Glucose Homeostasis			
Whole-body Glucose Tolerance			
Skeletal Muscle Glucose Uptake			
GLUT4			
GLUT2	↑	↓	
GLUT5	↑	↑	
Fatty Acid Uptake			
CD36	↑	↓	
FATP1	↓	↑	↑↑
FATP4			
Mitochondrial Oxidation			
CPT1 (mRNA & protein)	↓		
VLCAD			
MCAD			
PDK4			
KT	↓		
PGC-1α (protein)	↓ p=0.0797	↓	
PPARα (protein)	↓p=0.0845	↓	
Mitochondrial Biogenesis			
SIRT1	↑	↓	
SIRT3		↓	
FOXO1		↓	
pFOXO1		↑	
Mitochondrial Dysfunction			
Acylcarnitines	↑	↑	↑↑
Stress-Induced Kinases			
pJNK		↑	
pIKKβ	↑	↑	↑
PKC-θ	↑		
Insulin Signaling			
pIRβ			
pIRS-1		↑	
p85	↓		
p110	↓p=0.0977		
pAkt (Ser^{473})	↓p=0.0590	↓	↓↓p=0.0803
pAkt (Thr^{308})		↑	
pAS160	↓		↑
Skeletal Muscle Lipid Profile			
Triglycerides			
Diacylglycerol			
Cholesterol			
Saturated Fatty Acids		↑	
Omega 6/Omega 3 Poly-unsaturated Fatty Acids		↓	

Table 4.1: Summary Table of Birth Weight and Diet Effects and Their Interaction on Postnatal Growth, Skeletal Muscle Lipid Metabolism and Insulin Signaling.

4.3 Potential Limitations

Unfortunately, no study is perfect and there are limitations that must be addressed. Firstly, in both studies presented, we chose to examine only male offspring in order to reduce confounding variables associated with estrus cycle and differences in hormonal profiles between females and males. Previous studies have highlighted an increased susceptibility of males to the programming effects of an adverse *in utero* environment,[52,53] further prompting us to limit the current studies to male offspring at this time. However, studies that have examined both males and females have highlighted differential responses between the sexes to the influence of the *in utero* environment on later life insulin sensitivity.[54,55] For example, IUGR offspring in animal models are known to display improved insulin sensitivity in very early postnatal life; however, a shift towards impaired glucose clearance and decreased insulin sensitivity was observed as these offspring aged.[52,56,57] Interestingly, the timing of this shift in insulin sensitivity appears to be sex-specific, with insulin action being impaired earlier in males in comparison to females.[52,55] Therefore, investigation of our female cohort would have provided insight into the mechanisms associated with fetal programming of insulin resistance and how each sex responds to the initial *in utero* insult.

Secondly, our assessment of insulin resistance may have been improved overall. While we were able to extensively profile the expression and phosphorylation of the insulin signaling cascade in the skeletal muscle, we were unable to assess insulin

sensitivity at the whole-body level or skeletal muscle level directly. Unfortunately, due to the low sequence homology between human and guinea pig insulin,[58] assessing insulin levels *in vivo* in the guinea pig is challenging. We have currently been unable to generate confident results for plasma levels of insulin with commercially available ELISAs, despite numerous attempts, therefore limiting our ability to report *in vivo* insulin levels and to generate indices of whole-body insulin resistance. We have attempted to perform whole-body insulin tolerance tests (ITT) using an intraperitoneal injection of 1IU/kg of human insulin, as determined by a dose-response study. To assess insulin sensitivity we measured the percent drop in blood glucose values over the course of the 22-minute ITT, as a reflection of the ability of insulin to reduce blood glucose levels.[59] However, due to a small sample size and inconsistent results, we do not feel confident reporting on the whole-body insulin sensitivity of our cohort at this time. Going forward, further refining our insulin tolerance test protocol and continuing to optimize the measurement of plasma insulin levels in the guinea pig would be important in order to confidently speak to the overall insulin sensitivity of our cohort.

Additionally, all *in vivo* measurements were performed in the basal, fasted state, as were the final tissue collections. While we were able to observe significant alterations in skeletal muscle lipid metabolism and insulin signaling in the basal state in agreement with other studies,[8,60] observations in the insulin-stimulated state would have strengthened our conclusions. It would have been beneficial to observe if the skeletal muscle was able to mount a response to exogenous insulin since insulin resistance is typically characterized by a reduction in glucose uptake in response to insulin stimulation.[61] To address this, performing the ^{18}F-FDG PET scans following insulin

administration would allow us to determine if skeletal muscle was able to take up glucose in response to insulin. As reported by Reichkendler et al, exercise training was observed to increase insulin-stimulated glucose uptake in skeletal muscle, highlighting FDG-PET scans as a useful technique to assess *in vivo* skeletal glucose uptake in response to insulin administration.[62] Also, performing our tissue collections immediately following insulin injection would have allowed us to compare the phosphorylation state of key insulin signaling intermediates in the basal and insulin-stimulated state in order to investigate their activation in response to insulin, and whether insulin-stimulated phosphorylation of key signaling intermediates was blunted.

The fasted-to-fed transition is also important in highlighting metabolic disruptions.[63,64] Studies have shown that during the metabolic transition from a fasted to fed state, diabetic individuals fail to shift from fatty acid to glucose oxidation, depleting TCA cycle intermediates and highlighting a metabolic inflexibility.[65] Therefore, examining some animals in the fed state would allow for a metabolic comparison that may highlight the development of metabolic inflexibility associated with MetS disease progression. Additionally, mitochondria in skeletal muscle are thought to be particularly vulnerable to the nutritional milieu, with the mitochondria of obese animals displaying inabilities to adjust fatty acid influx in response to nutritional status, which is particularly evident in the fed state.[63] It has also been suggested that metabolic inflexibility in response to nutrient status occurs early in disease progression,[63,64] highlighting early detectable markers that may be used to monitor disease progression. Although the fasted state allowed us to assess basal metabolic parameters, it would be beneficial to observe

how these metabolic parameters are altered in the non-fasted state and whether the fasted-to-fed transition highlighted any further impairment in skeletal muscle lipid metabolism.

Finally, considerations must be made when analyzing our protein expression data of the transcription factors involved in regulating mitochondrial β-oxidation, as reductions in protein levels do not always imply reductions in activity. Activity of these transcription factors is tightly regulated by post-translational modifications that facilitate or inhibit the ability of these proteins to activate their target genes. For example, the acetylation status of both PPARα and PGC-1α are known to impact their ability to complex with one another to induce their gene targets. SIRT1 is an important component of this regulation, responsible for deacetylating PGC-1α, allowing it to complex with PPARα to promote lipid oxidation.[66] However, when SIRT1 is not active, PGC-1α remains hyperacetylated, preventing upregulation of lipid oxidation.[67] Therefore, investigation into the activity and post-translational modification of these transcription factors involved in mitochondrial biogenesis and oxidative function may provide insight into the mechanisms by which mitochondrial lipid metabolism is regulated and how an adverse *in utero* environment may induce persistent alterations to these transcription factors.

4.4 Future Directions

As mentioned above, the current studies were limited to male offspring; therefore, further investigation of the programming events underlying insulin resistance in female offspring would be of great value. Preliminary evidence suggests that females are,

similar to the males, glucose tolerant at this young age; however, molecular investigation of skeletal muscle insulin signaling and mitochondrial lipid metabolism remains to be completed. Investigation of the same markers in the females would allow for determination of sexually dimorphic effects of the *in utero* environment on the development of insulin resistance.

Furthermore, it would be worthwhile to conduct another longitudinal study to examine later time points in order to determine if the glucose intolerant phenotype would emerge in the cohort as they aged. Our current time point for sacrifice, approximately PND145, is still within the young adult period for the guinea pig.[68,69] Many non-communicable, metabolic diseases do not present until the effects of aging and insults in later life are compounded with insults that occurred in early life.[1,9] Therefore, due to the young age of our animals, it is possible that overt symptoms of MetS have not yet had time to manifest. Guinea pigs subjected to a high fat diet for 7 months are known to display mild glucose intolerance,[70] highlighting that with continued exposure to a postnatal WD, overt manifestations of MetS are likely to occur. Given the proper funding and resources, a longitudinal study that follows the cohort through to 7-8 months of age, or until observable manifestations of MetS are present, would be of great interest.

Additionally, it would be of interest to determine which component, excess fat or excess sugar, of the postnatal WD had the largest contribution to the diet effects we observed. This could be accomplished by designing diets isocaloric to our WD, with one diet containing excess fats alone and one diet containing excess sugars alone. By introducing these diets to a subset of our cohort from weaning, performing the *in vivo* measurements and molecular analysis, in comparison to a control diet and a Western diet,

we would be able to determine whether it is the excess fat or the excess sugar present in the WD that is generating the effects we observed in these studies. We speculate that the excess fats present in a high fat diet would promote defects in skeletal muscle lipid metabolism,[63,71] whereas excess sugars would have the potential to alter body composition and glucose tolerance.[72-74]

As previously mentioned, the reduced protein expression of the transcription factors regulating mitochondrial biogenesis and oxidative function needs to be explored further. Investigation of post-translational modifications, including phosphorylation and acetylation, that are known to modulate the transcriptional activity of these transcription factors is required in order to associate these changes in protein expression with alterations in function. PGC-1α phosphorylation can be mediated by 5' adenosine monophosphate-activated protein kinase (AMPK) at Thr^{177} and Ser^{538},[75] and is known to promote PGC-1α stability, allowing activation of gene targets;[66] whereas Akt-mediated phosphorylation of PGC-1α at Ser^{570} inhibits transcriptional activity.[76] Therefore, investigation of PGC-1α phosphorylation may provide insight into the activation of this transcription factor. Acetylation of lysine residues of the transcription factors regulating mitochondrial metabolism has also recently been highlighted as an important control mechanism regulating the ability of these transcription factors to form the complexes required to initiate transcription of their gene targets.[77] The acetylation status of PGC-1α and PPARα are tightly regulated by SIRT1, a deacetylase activated in response to nutrient availability.[66] Upon activation, SIRT1 catalyzes the reversible deacetylation of PGC-1α and PPARα, allowing them to complex together in order to induce transcription of their gene targets.[78,79] Therefore, assessing the acetylation status of PGC-1α and

PPARα may provide insight into the activity of SIRT1 as well as the regulation of oxidative gene targets. Additionally, chromatin immunoprecipitation could be employed to investigate the binding of these transcription factors to their target promoters, providing further information regarding how these transcription factors may be inducing the observed reductions in mitochondrial β-oxidation.

While the phosphorylation of FOXO1 was maintained, in conjunction with a reduced Akt phosphorylation level, investigation regarding mechanisms by which this phenomenon may occur would be of value given Akt does play a major role in regulating FOXO1phosphorylation status. In situations of insulin resistance, phosphorylation of FOXO1 is often reduced, resulting in hyperactivation of FOXO1 and disruption of glucose homeostasis.[45] Since we observed enhanced phosphorylation of FOXO1 at Ser^{256} following postnatal WD-consumption, investigation of alternative sites of phosphorylation, namely Thr^{24} and Ser^{319}, which is mediated by other kinases such as serine/threonine kinase SGK [80] would allow for a better understanding of FOXO1 transcriptional regulation of glucose and lipid homeostasis in states of altered insulin sensitivity.

Additionally, it would be of value to assess the regulation of PI3-kinase and its ability to activate Akt. The PI3-kinase signaling pathway is incredibly important in regulating not only glucose homeostasis, but also protein synthesis, proliferation, differentiation and cell survival depending on which downstream targets of Akt are activated.[81] One critical phosphatase that catalyzes the dephosphorylation and deactivation of PI3-kinase is phosphatase and tensin homologue deleted on chromosome 10 (PTEN).[81] An increase in the gene expression and protein levels of PTEN have been

observed in rodent models of type 2 diabetes,[82] suggesting PTEN as an important player in insulin resistance pathogenesis. Dephosphorylation of PTEN at Ser^{380}, Thr^{382}, and Thr^{383} promotes PTEN activity,[83] and PTEN activity has been observed to increase in response to excess free fatty acid availability.[84] Therefore, assessing the phosphorylation and activation of PTEN may provide insight into the regulation of PI3-kinase in response to insulin stimulation, and its ability to facilitate glucose uptake through the Akt/AS160 branch of this signaling pathway.

Lastly, other post-translational and epigenetic mechanisms need to be further explored in order to explain the long-lasting effect of adverse *in utero* growth on later life skeletal muscle function and metabolism. Epigenetic alterations initiated *in utero* are hypothesized to occur in response to adverse environments, which may ultimately promote survival in the short term but may have long-term detrimental effects on metabolic pathways.[15,85,86] Research aimed at determining a molecular mechanism linking environmental cues to altered persistent gene expression and later life disease progression has highlighted DNA methylation as a likely candidate.[66] Hypermethylation of the PGC-1α promoter region has been associated with reduced gene expression in LBW offspring,[87,88] as well as diabetic subjects,[89] preventing PGC-1α expression and impairing mitochondrial oxidative function. Additionally, hypermethylation has been observed in response to hypoxia,[90] highlighting another mechanism by which adverse *in utero* environments may induce permanent epigenetic alterations to metabolic function. Small non-coding RNAs known as microRNAs (miRNA) have also been gaining recognition for their role in disease pathogenesis.[85] Given mRNA was not changed for the transcription factors we investigated but protein levels were decreased, miRNAs are

potential candidates mediating this phenomenon as they initiate post-transcriptional regulation of protein expression.[91] miRNA-34a has recently been observed to be associated with a decrease in SIRT1 translation *in vitro*,[79] whereas miRNA-15b and miRNA-16 have been associated with alterations in insulin signaling proteins in response to diabetes and LBW.[92] Oxidative stress has also been observed to be an integral component in not only aging, but also age-related metabolic disease progression, by promoting epigenetic alterations in metabolism through miRNAs.[93] As such, future work investigating the epigenetic mechanisms underlying the persistent molecular alterations in skeletal muscle metabolism observed following an adverse *in utero* environment and the interaction with the postnatal environment is required.

4.5 Conclusions

The results of the present study provide further evidence for the importance of the *in utero* environment in modulating later-life disease risk. We also highlight that while young LBW offspring may outwardly appear healthy due to a non-obese, glucose tolerant phenotype, subtle changes at the level of the skeletal muscle represent a developing metabolically unhealthy phenotype. These persistent molecular alterations to the insulin signaling pathway and mitochondrial lipid metabolism following consumption of a postnatal WD or an adverse *in utero* environment are early markers of insulin resistance pathogenesis, increasing the risk of these offspring for developing insulin resistance and the components of MetS. Additionally, consumption of a postnatal WD following a

LBW outcome appears to exacerbate the metabolic disturbances initiated *in utero*, putting these offspring at greater risk for developing insulin resistance and other aspects of MetS.

Studies from other groups, as well as colleagues in our laboratory, have highlighted that other physiological parameters, including vascular and adipose development, of LBW offspring is unique in comparison to NBW offspring.[13,14,24-26] The mechanisms underlying metabolic disease in the LBW offspring in comparison to that induced by poor diet in adulthood may be different. In this study, an adverse *in utero* environment modulated a different set of enzymes and transcription factors regulating mitochondrial lipid oxidation than consumption of a postnatal WD. However, the divergent pathways converge upon similar stress-induced kinases and each induced alterations in the insulin signaling pathway, leading to a similar overall phenotype. Activation of these multiple pathways, when superimposed upon each other due to consumption of a poor postnatal diet following IUGR, may have the potential to further increase disease risk.

Of particular concern are recent studies highlighting that LBW offspring are unable to lose weight as efficiently as normal birth weight offspring when they are placed on a calorie restricted diet,[94] and that IUGR-induced deficiencies in PGC-1α appear resistant to exercise interventions targeted to improve symptoms associated with MetS.[95] These LBW offspring with a unique metabolic profile, while being at higher risk of developing components of MetS, may be resistant, or lack the plasticity to respond to current intervention practices, highlighting the need for LBW-specific therapeutic options. Additionally, current intervention strategies to combat metabolic disease are mostly targeted to overweight or obese individuals.[96,97] However, as observed in these

studies, as well as others,[72,74,98] these young LBW offspring are non-obese, highlighting the need for further investigation into therapeutic options that are not centered around obesity and its contribution to MetS pathogenesis. Therefore, it is imperative that further investigation into not only the molecular mechanisms underlying the developmental programming of metabolic disease be conducted, but also investigation into how we can translate this knowledge into LBW-specific therapeutic options designed for the unique metabolic profile associated with adverse growth *in utero*.

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APPENDIX I: Acylcarnitine Profile Short Hands

Acylcarnitine Short Hand	Common Name
C2	Acetyl Carnitine
C3	Propionyl Carnitine
C4	Isobutyryl Caritine
C5	Isovaleryl Carnitine
C6	Hexanoyl Carnitine
C8	Octanoyl Carnitine
C10	Decanoyl Carnitine
C12	Lauroyl Carnitine
C14	Myristoyl Carnitine
C16	Palmitoyl Carnitine
C18	Stearoyl Carnitine
C14:1	Tetradecenoyl Carnitine
C16:1	Palmitoleoyl Carnitine
C18:1	Oleyl Carnitine
C18:2	Linoleoyl Carnitine
C18:3	Octadecatrienoyl Carnitine
C20:4	Arachiodonoyl Carnitine
C22:5	Docosapentaenoyl Carnitine
C4-OH	3-Hydroxy-Butyryl Carnitine
C12-OH	3-Hydroxy-Dodecanoyl Carnitine
C14-OH	3-Hydroxy-Tetradecanoyl Carnitine
C16-OH	3-Hydroxy-Hexadecanoyl Carnitine
C18-OH	3-Hydroxy-Octadecanoyl Carnitine
C18:1-OH	3-Hydroxy-Octadecenoyl Carnitine

Appendix Table 1.1: Acylcarnitine Profile Short Hands.

APPENDIX II: Ethics Approval

AUP Number: 2010-229
PI Name: Regnault, Timothy
AUP Title: In Utero Origins Of Adult Insulin Resistance
Approval Date: 09/08/2014
Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "In Utero Origins Of Adult Insulin Resistance" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2010-229::5

1 This AUP number must be indicated when ordering animals for this project.
2 Animals for other projects may not be ordered under this AUP number.
3 Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

APPENDIX III: Curriculum Vitae

Curriculum Vitae

Name: Kristyn Dunlop

University Educational Background:

Sept 2013 -present	Masters of Science, Physiology and Pharmacology, Western University, London, Ontario, Canada
Sept 2009-Nov 2013	Honours Bachelor of Science, Physiology and Human Biology, Trinity College, University of Toronto, Toronto, Ontario, Canada

Honours, Scholarships & Awards:

2014-2015	Ontario Graduate Scholarship Value: $15, 000
May 2014	Paul Harding Research Day Poster Presentation Award Second place, Graduate student category Department of Obstetrics and Gynaecology Annual Paul Harding Research Day, Western University, Value: $100
Nov 2013	Physiology & Pharmacology Annual Research Day Poster Presentation Award Second place, Reproductive Biology and Endocrinology category Department of Physiology & Pharmacology Annual Research Day, Western University, Value: $100
2013-2014	Obstetrics and Gynaecology Graduate Scholarship Department of Obstetrics and Gynaecology, Western University, Value: $15, 500

Related Work Experience:

2014-2015	Physiology 4700 Teaching Assistant
2013-2014	Physiology 3120 Teaching Assistant

Publications, Presentations, Abstracts:

April 2015	Talks on Fridays (TOFs); Lawson Research Institute Seminar Series, Oral Presentation **K. Dunlop** Altered Skeletal Muscle Metabolism in Young Male Low Birth Weight Offspring in Conjunction with Postnatal High-Fat Feeding London, Ontario, Canada

March 2015

Society for Reproductive Investigation Meeting, 2015; Abstract & Oral Presentation
K. Dunlop, O. Sarr, T.Y. Lee, T.R.H. Regnault
Young, low birth weight, male guinea pig offspring are at risk for developing markers of insulin resistance and mitochondrial overload, independent of postnatal diet
San Francisco, California, USA

February 2015

Canadian Perinatal Research Meeting, 2015; Abstract & Oral Presentation
K. Dunlop, O. Sarr, T.Y. Lee, T.R.H. Regnault
Independent of a high-energy/high-refined carbohydrate postnatal diet, young low birth weight male guinea pigs are at risk for developing markers of insulin resistance and mitochondrial overload
Montebello, Quebec, Canada

January 2015

Altered Fetal Skeletal Muscle Nutrient Metabolism Following An Adverse *In Utero* Environment and the Modulation of Later Life Insulin Sensitivity
K. Dunlop, M. Cedrone, J.F. Staples, T.R.H. Regnault
Published in Nutrients (Nutrients. 2015 Feb 12;7(2):1202-1216.)

October 2014

Rho-Kinase Mediates Right Ventricular Systolic Dysfunction in Rats with Chronic Neonatal Pulmonary Hypertension
K. Gosal, **K. Dunlop,** R. Dhaliwal, J. Ivanovska, C. Kantores, J.F. Desjardins, K.A. Connelly, P.J. McNamara, A. Jain, R. P. Jankov
Published in American Journal of Respiratory Cell and Molecular Biology (Am J Respir Cell Mol Biol (October 22, 2014) doi: 10.1165/rcmb.2014-0201OC)

May 2014

Paul Harding Research Day at Western University; Abstract & Poster
K. Dunlop, O. Sarr, L. Zhao, T.R.H. Regnault
Low birth weight induces mitochondrial overload and markers of insulin resistance in young guinea pig offspring, independent of diet.
London, Ontario, Canada

May 2014

Southern Ontario Reproductive Biology Conference; Abstract & Oral Presentation
K. Dunlop, O. Sarr, L. Zhao, T.Y. Lee, T.R.H. Regnault
Sex-specific differences in markers of altered muscle insulin sensitivity following an adverse *in utero* environment and postnatal Western diet.
Toronto, Ontario, Canada

April 2014

London Health Research Day; Abstract & Poster
K. Dunlop, A. Blake, L. Zhao, O. Sarr, T.R.H. Regnault
Indicators of insulin resistance and mitochondrial overload in young low birth weight male offspring independent of postnatal control or Western diet.
London, Ontario, Canada

April 2014

Talks on Fridays (TOFs); Lawson Research Institute Seminar Series, Oral Presentation
M. Cedrone and **K. Dunlop**
Adverse skeletal muscle function & metabolism in offspring of an adverse *in utero* environment following a high fat/high sugar postnatal diet.
London, Ontario, Canada

April 2014

Therapeutic Hypercapnia Prevents Inhaled Nitric Oxide-Induced Right Ventricular Systolic Dysfunction in Juvenile Rats
K. Dunlop, K. Gosal, C. Kantores, J. Ivanovska, R. Dhaliwal, J.F. Desjardins, K.A. Connelly, A. Jain, P.J. McNamara, R.P. Jankov
Published in Free Radical Biology & Medicine; (Free Radic Biol Med. 2014 Apr;69:35-49.doi:10.1016/j.freeradbiomed.2014.01.008. Epub 2014 Jan 11.)

Nov 2013

Physiology and Pharmacology Research Day at Western University; Abstract & Poster
K. Dunlop, A. Blake, L. Zhao, O. Sarr, T.R.H. Regnault
Indicators of mitochondrial overload and insulin resistance in adult male guinea pigs secondary to low birth weight and postnatal high-fat feeding.
London, Ontario, Canada