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Low Birth Weight and Post-Natal Diet Induced Alterations in Skeletal Muscle Oxygen Consumption and Fiber Type Composition

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Abstract

Adverse *in utero* and postnatal conditions can increase susceptibility to metabolic syndrome (MS). Altered muscle respiration contributes to MS, but the effects of restricted oxygen and nutrients *in utero* on skeletal muscle mitochondria remain unknown. In this study guinea pig sows underwent uterine artery ablations mid-gestation, producing fetuses with low birth weight (LBW). Soleus muscle was collected near term or at four months of age, from LBW and control fetuses and offspring, where the offspring were fed either a Western Diet (WD) or a control diet (CD). Soleus muscles from LBW fetuses exhibit lower maximal respiration rates than normal birth weight (NBW) sham-surgery controls. Additionally, LBW/CD, NBW/WD and LBW/WD adult guinea pigs displayed reduced respiration compared with NBW/CD. Cultured C_2C_{12} cells were utilized to better understand independent effects of hypoxia and fatty acid saturation upon cellular respiration. Both chronic (5 days) hypoxia and palmitate (16:0) reduced respiration compared with normoxia.

Lay Summary

The environment in which we develop during pregnancy can determine certain aspects of our health as adults. Previous studies have identified a correlation between low birth weight, an outcome related to intrauterine growth restriction and the development of metabolic diseases such as type 2 diabetes and cardiovascular disease later in life. The present study aimed to identify a link between an environment low in oxygen and nutrients during pregnancy, which is observed in intrauterine growth restriction and metabolic disorders in adulthood. As there is a reduced skeletal muscle mass in cases of intrauterine growth restriction and skeletal muscle plays an important role in insulin sensing, an important contributor to metabolic health, it was chosen as the focus of this study. An intrauterine growth restricted fetal guinea pig model was used to determine a link between a poor environment during pregnancy and skeletal muscle oxygen consumption. A second study was undertaken where growth restricted animals were given either a control or Western diet to determine if a post-natal diet further affected skeletal muscle oxygen consumption. Finally, a cell culture model was used

to isolate the effects of hypoxia and the common saturated fatty acid palmitate, the mono unsaturated fatty acid palmitoleate on skeletal muscle oxygen consumption. Growth restricted animals had reduced skeletal muscle oxygen consumption; however, the addition of the Western diet did not further reduce oxygen consumption but did reduce oxygen consumption in normal birth weight animals. Similarly, hypoxic cultured cells had reduced oxygen consumption and the saturated fatty acid reduced oxygen consumption of only cells cultured under normal oxygen levels. The monounsaturated fatty acid had no effect on oxygen consumption. Discovering this link allows for further studies to be conducted with potential treatments.

Keywords

Skeletal Muscle, Intrauterine Growth Restriction, Low Birth Weight, Oxygen Consumption, C_2C_{12}, Insulin Resistance, Mitochondria, Western Diet

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List of Abbreviations

CS	Citrate Synthase
CVD	Cardiovascular disease
DOHaD	Developmental origins of health and disease
ETC	Electron transport chain
FA	Fatty acid
HIF1-α	Hypoxia inducible factor 1-alpha
IMM	Inner mitochondrial membrane
IUGR	Intrauterine growth restriction
LBW	Low birth weight
MEF	Myocyte enhancer factors
MHC	Myosin heavy chain
MHC	Myosin heavy chain
MRF	Myogenic regulatory factors
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NBW	Normal birth weight
OXPHOS	Oxidative phosphorylation
PDK1	Pyruvate dehydrogenase kinase 1

PGC-1α	PPAR-γ coactivator-1α
PI	Placental insufficiency
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SGA	Small for gestational age
SM	Skeletal muscle
TCA	Tricarboxylic acid
UAA	Uterine artery ablation
UCP	Uncoupling protein
WD	Western diet

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1 Introduction

1.1 Developmental Origins of Health and Disease

The *in utero* environment in which a eutherian mammal fetus develops can determine pre-dispositions to metabolic diseases later in life. The maternal uterine lining connects to the developing fetus' umbilical cord via the placenta. This organ regulates fetal gas exchange, macronutrient supply and carbon dioxide elimination. Oxygen concentration and macronutrient supply are two *in utero* environmental conditions that can affect immediate fetal and later life post-partum metabolism. In fact, the association between a poor *in utero* environment and later life development of metabolic diseases has recently emerged as a global health concern for humans (Hales and Barker, 2001). The notion that the *in utero* environment could play a role in adult-onset diseases was first entertained by David Barker. His hypothesis (deemed the Barker hypothesis) stated that low oxygen and nutrient transfer across the placenta can result in increased risk of metabolic syndrome, a cluster of risk factors for metabolic diseases such as cardiovascular disease (CVD), type 2 diabetes and non-alcoholic fatty liver disease (NAFLD), in later life (Barker, 2004). This hypothesis led to the investigation of *in utero* hypoxia and under nutrition as possible contributors to such diseases (De Boo and Harding, 2006).

Many in-depth human population studies, covering a diversity of race and socio-economic factors, now provide additional support for the advancement of research into developmental origins of health and disease (DOHaD) (Villar and Belizán, 1982; Strauss, 2000; De Boo and Harding, 2006). A poor *in utero* environment, resulting from poor maternal nutrition may lead to reduced oxygen and nutrient transfer across the placenta, priming the offspring with a metabolism that is prepared for a similar, nutrient deprived environment outside the womb (Glickman, Hanson and Beedle, 2007). Additionally, maternal and fetal genetic factors influence placental nutrient and oxygen transfer

(Valsamakis *et al.*, 2006). Decreased expression of *homeobox* genes *HLX1* and *ESX1L* (Murthi *et al.*, 2006; Murthi *et al.*, 2006) involved in embryonic proliferation, migration and invasion as well as increased placental a1-antichymotrypsin mRNA (a protease inhibitor involved in inflammation) have been linked to IUGR (Chelbi *et al.*, 2012). Independent of these factors, however a nutrient and oxygen deficient *in utero* environment is associated with a low birth weight, and is considered crucial to adult-onset metabolic disorders (Villar and Belizán, 1982; Chatelain, 2000; Cox and Marton, 2009).

Negative early-life environmental experiences, such as *in utero* hypoxia, can cause lasting epigenetic perturbations that may underly DOHaD observations (Hales and Barker, 2001; De Boo and Harding, 2006; Valsamakis *et al.*, 2006). These chemical modifications of the DNA resulting in alterations in gene expression and regulation are associated with an increased risk of later life metabolic disorders (Lal *et al.*, 2003; Gluckman *et al.*, 2005). Such disorders may not emerge until early adulthood, or until a second challenge, such as poor diet or even exercise, is imposed (Valsamakis *et al.*, 2006; Reyes *et al.*, 2015), suggesting that an *in utero* perturbation may impact gene expression both during development and after birth. It is not yet clear, however, whether *in utero* reprogramming events are reversible, and specific genes affected in a variety of tissues and animal models are topics of current research efforts (Zeng *et al.*, 2013; Iglesias-Platas *et al.*, 2014; Venhoranta *et al.*, 2014). Interventions such as N-acetylcysteine a precursor of the antioxidant glutathione, (Herrera *et al.*, 2017) folic acid, (Ding and Cui, 2018) and gene therapy (David, 2017) are currently being explored.

Although the mechanisms underlying the altered regulation of specific genes related to an adverse *in utero* environment are still being identified, the general pattern of low birth weight, prioritizing of blood flow to organs such as the brain, heart and adrenal glands, and resulting changes in expression of specific genes, is termed the “thrifty phenotype” (Neel, 1999; Hales and Barker, 2001). Thrifty phenotype offspring are not simply proportionally smaller, but are asymmetrically restricted in growth; they exhibit a larger

brain to liver mass ratio and reduced muscle mass (Hales and Barker, 2001). During human evolution, it is speculated that "thrifty genes" were selected for when food was scarce, and may have primed offspring for a post-natal life where nutrient availability was poor (Neel, 1999; Hales and Barker, 2001; Valsamakis *et al.*, 2006). Today, however, maternal and post-natal nutrient deficiency are rare in Western societies, and offspring that display a thrifty phenotype, often result from a placental insufficiency-induced oxygen deficient *in utero* environment (Godfrey and Robinson, 1994). The thrifty phenotype is assumed to increase the probability of *in utero* survival, but establishes a mismatch between the nutrient and oxygen 'poor' *in utero* environment, for which the fetus was primed, and the nutrient and oxygen rich environment experienced after birth. As a result, the offspring is born with a metabolic profile programmed to survive in an energy- and oxygen-deficient (hypoxia) environment, but is exposed to an environment with sufficient oxygen that is energy dense, especially when exposed to a Western Diet (WD). In these instances human post-partum growth rates above average (Boersma and Wit, 2013) are often observed (Bol *et al.*, 2009; Coupé *et al.*, 2009; Crume *et al.*, 2014). This rapid weight gain in infancy increases the risk of adult-onset obesity (Adair *et al.*, 2013). There is; however, a critical period of approximately 1000 days where the thrifty phenotype appears to remain plastic (Victora *et al.*, 2010) which provides a window for interventions.

1.2 Intrauterine Growth Restriction

Placental nutrient and oxygen transfer, genetic growth potential and the maternal environment all contribute to mammalian fetal development. Intrauterine growth restriction (IUGR) results in a pregnancy where one or more of these factors are negatively altered, creating a sub-optimal developmental environment. IUGR is a condition in which a fetus' growth is restricted and is associated with low birth weight (LBW) and in some cases, evidence of asymmetrical growth e.g. high brain to liver weight ratio (Mitchell, 2001; Bauer *et al.*, 2003; Rosenberg, 2008). Fetuses which fall below the 10^{th} percentile for gestational age and sex are deemed small for gestational age (SGA), an outcome related to IUGR. Of these births the majority of pathological cases– i.e. those related to adult onset metabolic disorders - involve low abdominal circumference but normal brain circumference (Peleg *et al.*, 1998) . A human birth weight threshold of 2,500g for IUGR, however, is a crude and somewhat arbitrary criterion that does not account for fetuses outside these parameters who have failed to reach their genetic growth potential and were perhaps exposed to a suboptimal *in utero* environment (Gale *et al.*, 2006; Mayer and Joseph, 2013; Melamed, 2014). Metabolic remodeling as a result of a sub-optimal *in utero* environment, and associated with later life metabolic disorders may therefore affect individuals across all birth weights (Kuzawa and Adair, 2003; Gluckman *et al.*, 2009) and there is emerging evidence of long-term metabolic dysfunction independent of birth weight (Moore and Davies, 2005; Walsh and McAuliffe, 2015). We must then consider additional parameters for classifying growth restriction, such as the mentioned brain and abdominal circumferences, blood inflammatory markers, differential microRNA expression, as well as ponderal index ($mass/height^3$), a measure of thinness, (Peleg, Kennedy and Hunter, 1998; Hales and Barker, 2001; Lausten-Thomsen *et al.*, 2014; Thamotharan *et al.*, 2017) in order to include those who are a normal birth weight, but may have undergone a reprogramming event due to poor *in utero* conditions. Low birth weight classifications alone fail to include offspring of average birth weight who may have been larger under more favorable *in utero* conditions.

A well characterized classification of a sub-optimal *in utero* environment is placental insufficiency (PI), which is an idiopathic condition characterized by a reduction in maternal blood flow through the placenta. The resultant hypoxia and poor nutrient exchange can lead to IUGR (Gagnon *et al.*, 1996; Trudinger and Giles, 1996). PI occurs when the placental fails to develop or implant properly, often resulting in a smaller surface area for nutrient and oxygen exchange (Salafia, 1997) leading to a hypoxic, nutrient-deprived *in utero* environment (Pardi *et al.*, 2002). Experimentally PI can be mimicked using partial ablation of the uterine artery in guinea pigs (Briscoe *et al.*, 2004; Turner and Trudinger, 2009). Larger animal models, such as sheep, which are more easily probed with oxygen sensors, are excellent models for studying changes in oxygen flow in IUGR (Gagnon *et al.*, 1996; Morrison, 2008; Thompson *et al.*, 2011). Other animals, such as swine and rats have also been studied to reveal differences in gene expression between control and IUGR offspring, which exhibited reduced insulin sensitivity (Kind *et al.*, 2003; Ying *et al.*, 2019). Moreover, culturing muscle precursor cells, such as C_2C_{12} mouse myoblast cells, under hypoxia can mimic the effects of PI on muscle metabolism (Regnault *et al.*, 2010; Yu *et al.*, 2011).

The pathological outcome associated with PI is IUGR, which can be categorized as either symmetrical or asymmetrical. Symmetrical IUGR is typically a result of maternal or fetal genetic abnormalities and results in fetuses which are proportionally small relative to the average fetus. In asymmetrical IUGR certain organs (brain, heart, adrenal gland) are disproportionately large (Peleg *et al.*, 1998). Asymmetrical IUGR may result from a number of causes including maternal hypertension, maternal under nutrition and, most commonly, placental insufficiency (Pollack and Divon, 1992; Monk and Moore, 2004; Valsamakis *et al.*, 2006). As a result of placental insufficiency, fetal blood flow to the heart, brain and adrenal glands increases, but blood flow to other organs including the liver, kidneys and skeletal muscle (SM) decreases. This pattern results in a small abdomen to head size, i.e. the asymmetrical phenotype. The reduced SM mass has also been linked to altered insulin sensitivity in adulthood (Phillips *et al.*, 1994; Desai *et al.*, 1996; Ozanne *et al.*, 2003).

1.3 Hypoxia-induced alterations in skeletal muscle metabolism

Independent of deficient nutrient transfer, chronic hypoxia *in utero* is associated with IUGR (Kajimura *et al.*, 2006; Rueda-Clausen *et al.*, 2011); pregnancies at high altitudes result in higher proportions of IUGR births in human populations that are not adapted to such environments (Murphy *et al.*, 1986; Cogswell and Yip, 1995; Semenza, 2000). Further studies in chick embryos isolate hypoxia's modulating role in IUGR, independent of the placenta (Giussani *et al.*, 2007; Mehta and Mehta, 2008; Tintu *et al.*, 2009).

In prolonged hypoxia, hypoxia inducible factor 1-alpha (HIF1-α) accumulates, leading to altered tissue metabolism. HIF1-α is a powerful transcriptional regulator of energy metabolism (Lee *et al.*, 2004; J. W. Kim *et al.*, 2006; Denko, 2008) and is degraded under normoxic conditions but allowed to dimerize with HIF1-β under cellular hypoxia (Wiener *et al.*, 1996; Ladoux and Frelin, 1997; Wenger *et al.*, 1997). Accumulation of HIF1-α attenuates glycolytic input to the tricarboxylic acid (TCA) cycle through increased transcription of pyruvate dehydrogenase kinase 1 (PDK1), which inactivates pyruvate dehydrogenase, halting the conversion of pyruvate to acetyl-CoA (Kim *et al.*, 2006).Overexpression of HIF1-α in otherwise normoxic pregnant mice led to IUGR and reduced placental weights compared to controls (Tal *et al.*, 2010), indicating hypoxia as a contributor to IUGR, independent of reduced nutrient transfer. HIF1 is also involved in up-regulating the transcription of glycolysis enzymes and glucose transporters (J. W. Kim *et al.*, 2006; Papandreou *et al.*, 2006; Aragonés *et al.*, 2008) facilitating anaerobic ATP production. Further supporting the observed transcriptional effects of HIF1, functional assays in human RCC4 cells show reduced oxygen consumption following normoxic recovery from chronic hypoxia (Soares *et al.*, 2005; Papandreou *et al.*, 2006). Yet another study has identified electron transport chain (ETC) complex I as a more specific target for inhibition under hypoxic conditions; HIF-1α induced downregulation of NDUFA4L2, a subunit of complex I, lowers oxygen consumption and suppresses complex I activity under hypoxia (Tello *et al.*, 2011).

1.3.1 Electron Transport Chain

A number of cellular processes, critical to energy metabolism, including angiogenesis (the formation of new blood vessels) glucose metabolism, and oxidative phosphorylation are regulated by hypoxia (Mu *et al.*, 2001; Pugh and Ratcliffe, 2003; Magalhães *et al.*, 2005). As oxygen is central to oxidative phosphorylation (OXPHOS), the role of hypoxia in IUGR has been chosen as the focus of this study. OXPHOS is an aerobic metabolic pathway occurring in the inner mitochondrial membrane. In this pathway exergonic reactions of the ETC, including the donating and accepting of electrons, are coupled with, and help drive endergonic reactions such as ATP production. The ETC is comprised of five main enzyme complexes and a variety of electron donors with oxygen as the terminal electron acceptor. The energy released by the donation of two electrons is used to transport two protons across the inner mitochondrial membrane, creating an electrochemical gradient which can drive the phosphorylation of ADP, producing ATP.

The five principal enzyme complexes involved in electron transport are NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V; Figure 1.3-1). Each enzyme complex is comprised of a great number of proteins that are encoded by both nuclear and mitochondrial DNA.

Complex I oxidizes NADH to NAD^+ while reducing coenzyme Q10, or ubiquinone, a coenzyme within the inner mitochondrial membrane. Complex II is the site of succinate and $FADH_2$ oxidation and is the secondary entry point of the electron transport chain. While complex II oxidizes succinate to fumarate and reduces ubiquinone, unlike complex I, the energy released by this reaction is insufficient to transfer of protons into the intermembrane space.

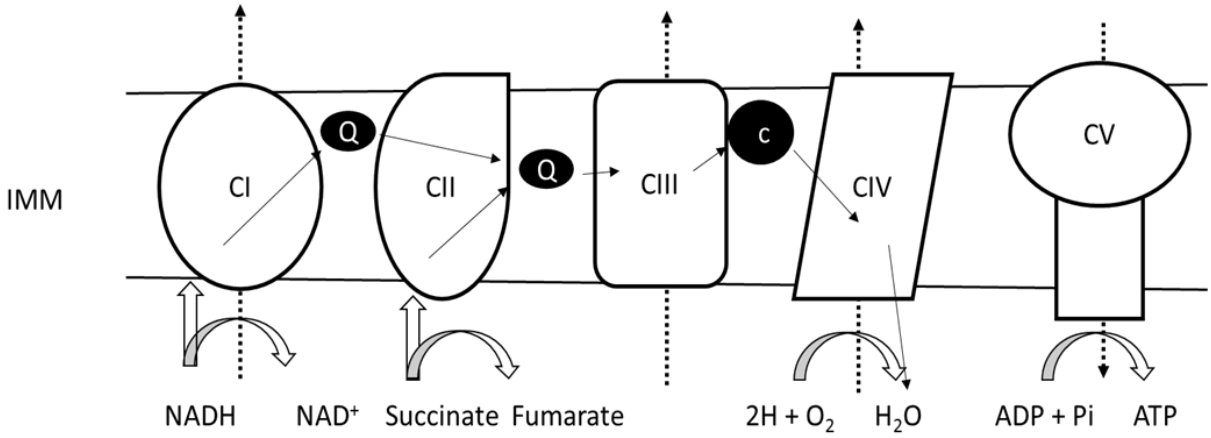

Figure 1.3-1 Overview of the Electron Transport Chain. Each of the embedded complexes are depicted within the inner mitochondrial membrane (IMM), and the dissociable carriers' coenzyme Q and cytochrome C are represented as (Q) and (c) respectively. Electron flow is indicated by solid arrows and proton pumping by dashed arrows.

Following entry into the ETC from either complex I or complex II, the electrons are transferred to complex III by ubiquinone. From complex III, cytochrome C transports the electrons to complex IV. When stimulated by sufficient concentrations of ADP and inorganic phosphate (P_i), complex V uses the proton gradient created by the ETC to generate ATP (Figure 1.3-1).

Under acute hypoxic conditions, glucose metabolism is altered through what is termed the Pasteur Effect. With less oxygen available to act as an electron acceptor at ETC complex IV, flux through the ETC, as well as ATP synthesis by complex V, slows. As a result, fewer NADH molecules are oxidized by complex I. The accumulated NADH would decrease glycolytic flux pyruvate (key TCA metabolite), but it can be oxidized by lactate dehydrogenase, while converting pyruvate to lactate. As a result, in acute hypoxia, glycolysis can continue at high rates without the accumulation of pyruvate, permitting some anaerobic ATP production by way of substrate level phosphorylation.

1.3.2 Myogenesis and adverse *in utero* environments

As previously mentioned, many fetal organ systems are impacted in situations of PI hypoxia. Skeletal muscle mass is greatly reduced in IUGR offspring (Padoan *et al.*, 2004; Larciprete *et al.*, 2005), as a result of hypoxic induced cardiac redistribution, suggesting that myogenesis likely occurs in a sub-optimal oxygen environment. Hypoxia is strongly linked to the IUGR *in utero* environment and may contribute to the reduction in overall terminal differentiation (Di Carlo *et al.*, 2004; Yun *et al.*, 2005) and decreased muscle mass thereby linking hypoxia to the thinness component of the thrifty phenotype.

Skeletal muscle development during embryogenesis can be affected by the *in utero* environment, and in adulthood. Successful myogenesis requires a precise coordination of spatial and temporal events. Myogenesis involves the synchronization of a number of embryonic and fetal progenitor cells specified by paired box transcription factors Pax3 and Pax7 respectively 63,64 which results in determination of myofibers. Following activation by Pax3/Pax7, fetal and embryonic muscle growth is orchestrated by the myogenic regulatory factors (MRF) myogenin, MyoD, Mrf4, and Myf5 which induce expression of genes which encode contractile proteins unique to muscle cells (Figure 1.3).

Pax 3 and Pax 7 specify embryonic progenitor cells resulting in satellite cells. These are later activated through the continued expression of Pax7 with some regulation by Myf5. Satellite cell proliferation begins with the expression of MyoD in addition to Pax7 and Myf5 regulation, resulting in myoblast formation. Early differentiation into myocytes is marked by declined Pax7 and Myf5 expression with increased myogenin expression and slowly increasing Mrf4 expression. Finally, the transition from myocytes to myofibers, or late differentiation, is regulated by minimal MyoD expression, tapering myogenin expression and maximal Mrf4 expression (Figure 1.3-3).

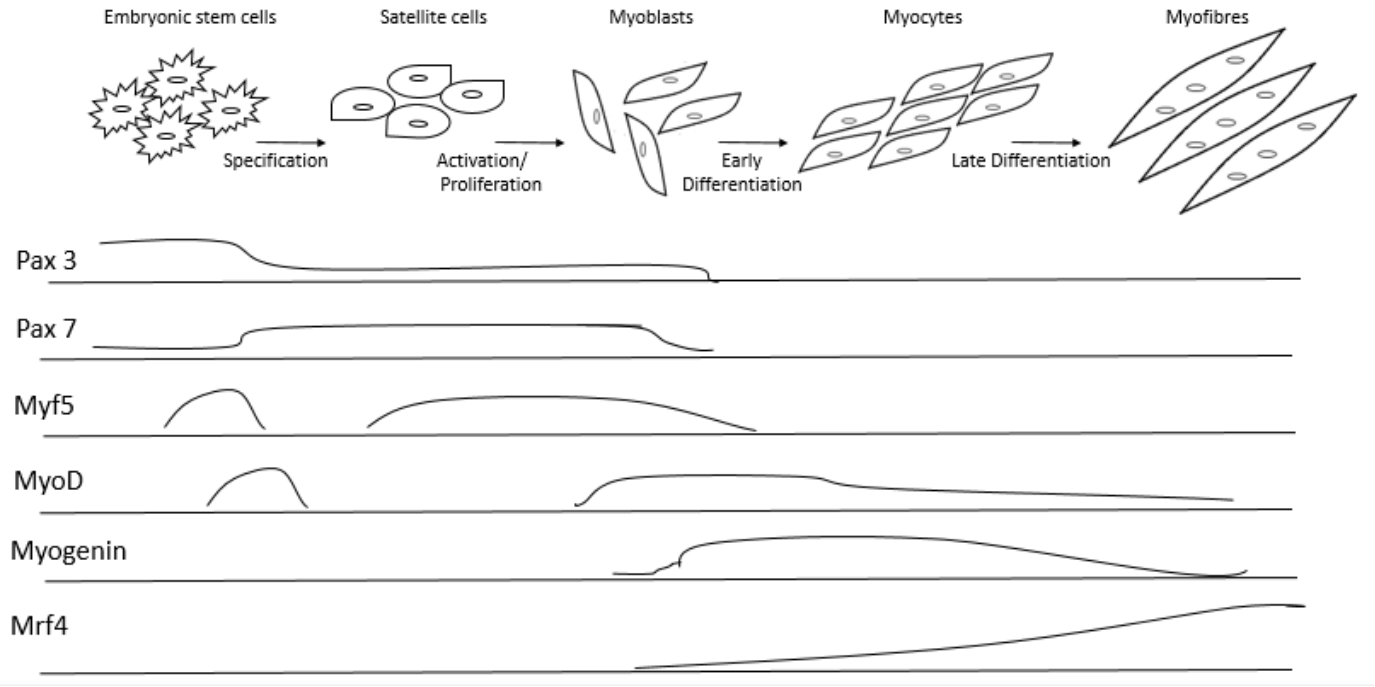

Figure 1.3-2 Embryonic Myogenesis. Determination and differentiation of muscle cells regulated by Pax3/Pax7and MRFs MyoD, Myogenin, Mrf4 and Myf5. Transcription factor expression is shown as area under the curve. Modified from Benzinger *et al*, 2012.

Downstream targets of myogenin and MyoD include the transcription factors known as myocyte enhancer factors (MEFs): Mef2A, Mef2B and Mef2C. Together, MRFs and MEFs activate the transcription of contractile and structural proteins found in myoblasts (Blais *et al.*, 2005). Under IUGR conditions expression of MEF2a decreases but MEF2d expression increases, with no change in MEF2c (Raychaudhuri *et al.*, 2008). Because MEF2 isoform binding with MyoD relates to GLUT 4 transcription (Raychaudhuri *et al.*, 2008; Zheng, Rollet and Pan, 2012) in addition to fiber type determination (Potthoff *et al.*, 2007), IUGR changes in MEF2 expression potentially contribute to increased risk of metabolic syndrome. Additionally, post-natal myogenesis is impaired in IUGR offspring (Wirfalt *et al.*, 2001; De Blasio *et al.*, 2007) and lean mass and muscle strength remains

decreased compared to controls (Aihie Sayer *et al.*, 2004; Inskip *et al.*, 2007; Ylihärsilä *et al.*, 2007).

Moreover, there have been extensive reports studying *in vitro* differentiation in the C_2C_{12} *Mus musculus* cell line under hypoxia (Yun, *et al.*, 2005; Cicchillitti *et al.*, 2012; Slot *et al.*, 2014). Although the cells are derived from adult satellite cells, *in vitro* differentiation closely mimics the *in utero* fetal differentiation process (Messina *et al.*, 2010). Important markers of differentiation and cell signaling, necessary for the initiation of determination and differentiation, are expressed similarly *in utero* and in this cell line (Burattini *et al.*, 2004) making this cell line a useful and tractable experimental model.

1.4 Skeletal Muscle Fiber Type Composition and adverse *in utero* environments

Fiber type composition determines contractile characteristics, oxidative function and metabolic capacity of skeletal muscle. There are two ways to classify fiber types: by their myosin ATPase type (affecting twitch speed) and their principle mode of generating ATP (oxidative, glycolytic or oxidative/glycolytic). Oxidative and glycolytic fibers express different isoforms of myosin heavy chain (MHC) and preferentially metabolize different substrates in order to produce ATP. Oxidative fibers have a higher concentration of mitochondria and use predominantly oxidative phosphorylation to yield ATP, whereas glycolytic fibers primarily use anaerobic glycolysis to yield ATP and have fewer mitochondria. Muscle fibers can be alternatively categorized as fast-twitch (Type I) or slow-twitch (Type II), referring to the speed of which myosin can hydrolyze ATP. Slow-twitch fibers are primarily oxidative and in humans are referred to as type Ia fibers, whereas Type II fibers can be either oxidative-glycolytic (Type IIa) or glycolytic (Type IIb).

1.4.1 Skeletal muscle fiber composition

Slow and fast muscle fibers originate during fetal development as populations of primary (type I MHC) and secondary (type IIa, IIb and IIx MHC) myotubes respectively. Fiber type fate is considered plastic; fiber type switching can occur with exercise or diet (Simoneau *et al.*, 1985; Schrauwen *et al.*, 2001; Handschin *et al.*, 2007). In disease states, for example individuals with type 2 diabetes and insulin resistance, there is a notable shift towards more glycolytic fibers (Tanner *et al.*, 2002; Ten Broek, Grefte and Von den Hoff, 2010; Tello *et al.*, 2011), which is associated with reduced muscle oxygen consumption (He *et al.*, 2001; Oberbach *et al.*, 2006). The mammalian target of rapamycin, peroxisome proliferator-activated receptor (PPAR)s and PPAR-γ coactivator-1α (PGC-1α) together regulate fiber type composition and, by extension oxidative capacity. PGC-1α increases insulin sensitivity by maintaining a larger number of functional mitochondria by inducing fiber type switching towards type I and IIa expression.

The importance of PPARs in normal fetal development is supported by the fact that PPARγ knock-out mice are growth-restricted at birth (Gokina *et al.*, 2013). Furthermore, the targeted expression of an activated form of PPARβ/δ in the skeletal muscles makes mice resistant to obesity by increasing the numbers of oxidative (Type I and IIa) muscle fibers (Slot *et al.*, 2014), while the selective ablation of PPARβ/δ induces obesity by reducing the oxidative capacity of the muscles (Schuler *et al.*, 2006). PPARβ/δ also directly and indirectly regulates genes involved in fatty acid transport and oxidation through binding to specific peroxisome proliferator response elements (PPREs) on these genes (Narravula and Colgan, 2001). Examples of these genes include Cluster of Differentiation 36 or Fatty acid translocase which is involved in fatty acid uptake (Schuler *et al.*, 2006; Jans *et al.*, 2012), carnitine palmitoyl transferase 1, involved in the mitochondrial transfer of long-chain fatty acids, and the uncoupling protein (UCP-1, -2 and -3) family (St-Pierre *et al.*, 2003; Cantó *et al.*, 2012). Altered expression of these genes directly correlates with insulin resistance, altered fatty acid oxidation, intramyocellular lipid accumulation, and mitochondrial damage (St-Pierre *et al.*, 2003; Schuler *et al.*, 2006; Jans *et al.*, 2012).

Studies of pig and sheep, which have similar muscle differentiation to humans, show that differentiation occurs near mid-gestation and fiber type ratios are set before birth. As fiber type determines oxidative capacity, the ratio of oxidative to glycolytic fibers is an important factor in predisposition to metabolic syndrome. In human studies, reduced PGC-1α mRNA in adult skeletal muscle has been linked to the development of insulin resistance and Type II diabetes, in conjunction with inactivity and nutrient excess (Handschin *et al.*, 2007; Holmstrom *et al.*, 2012; Kristensen *et al.*, 2014). Further, in IUGR rats, fetal PGC-1α promoter activation and expression is impaired, through epigenetic modifications (Zeng *et al.*, 2013). These changes are related to the development of insulin resistance later in postnatal life and intriguingly also in later generations, supportive of the concept of *in utero* inter-generational programmed PPAR modifications (Martins *et al.*, 2012; Shen *et al.*, 2018). These data suggest that adverse PGC-1α/PPAR control promotes altered muscle fiber determination and oxidative function, which may have inter-generational effects.

1.5 The Western Diet

1.5.1 Western Diet origin and composition and the development of metabolic disease

Metabolic disorders, many of which exhibit reduced skeletal muscle oxygen consumption and altered fiber type composition, are associated with long term consumption of the high-fat, high-sugar Western Diet (WD) in adults (Wirfalt *et al.*, 2001). Though diet has long been the focus of research in metabolic disorders, emerging data suggest the *in utero* environment plays a role as well (Valsamakis *et al.*, 2006; Nevin *et al.*, 2018). On an evolutionary time-scale, agriculture and industrialization are recent developments, which have caused extreme changes in the human diet over a short time span. Modern technology and urbanization have drastically altered not only nutrient composition but also food availability, changing quality and quantity of the human diet. Pre-agricultural human populations, or hunter-gatherers, did not have a consistent diet but rather one which varied by climate, location and ecological surroundings (Cordain *et al.*, 2000a). While their diets varied by location and food availability, many similarities among the diets of different pre-agricultural populations existed, which provide insight regarding the Western Diet, as it relates to development of chronic, metabolic disorders.

The World Health Organization recommends humans intake 10% of our daily energy from sugar (The World Health Organization, 2003); however in 2004 Canadians consumed, on average, 21% of calories from sugar (Health Canada, 2005). Although it is difficult to determine sugar consumption of early humans, it is estimated to have accounted for a much smaller percentage of total energy (Cordain *et al.*, 2000b; Brand-Miller *et al.*, 2002). Furthermore, there has been a marked shift in fatty acid consumption between pre- and post-industrial humans (Cordain *et al.*, 2000b). Additional changes in fatty acid composition between pre- and post-agricultural populations stem from meat sources. Wild mammals exhibit seasonal changes in fat storage, where excess energy is converted to triacylglycerides and mainly stored in adipocytes as saturated fatty acids

(SFAs) (Mercer, 1998). Fatty acids found in muscle and other tissues however are mainly polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) (Rule *et al.*, 2002) thus hunters eating skeletal muscle as meat would have had a diet rich in PUFAs and MUFAs for the majority of the year (Cordain and Watkins, 2002). In contrast, post-agricultural populations consume higher SFA content from meat for two main reasons: animal husbandry and technological advancements in corn harvesting. First, animal husbandry has allowed for slaughter at peak fatty acid storage year round compared to the seasonal peak fatty acid found in wild mammals (Whitaker, 1975). Second, animals are now kept in feed lots and fed grain (mostly corn) and exhibit "marbled meat", i.e. intramuscular adipose deposits resulting from excess triacylglycerols accumulation (Whitaker, 1975). The triacylglycerol in these deposits is comprised largely of SFA (Cordain *et al.*, 2005). Wild meat does not exhibit this "marbling" (Cordain and Watkins, 2002).

On an evolutionary time-scale, 5,000 to 10,000 years is a relatively short period in which to undergo such drastic environmental changes. Environmental pressures can either positively or negatively select for genetic traits, and a changing environment may be discordant with widely favorable genetic traits from a previous environmental pressure (Gould, 2002; Caldararo, 2003). The current human genome has been primed for the diet of our ancestors and is discordant to the present day "Western Diet" (Eaton *et al.*, 1988; Caldararo, 2003). Increased sugar consumption and a shift in the fatty acid composition from mainly PUFAs and MUFAs to SFAs are staples of the Western Diet (Cordain *et al.*, 2005).

An emerging body of evidence suggests that high fat, high sugar diets are closely linked to metabolic disorders (Hu *et al.*, 2001; Cordain *et al.*, 2005; Kuo *et al.*, 2008; Lottenberg *et al.*, 2012). High sugar diets increase blood glucose levels, leading to increased insulin secretion (Ludwig, 2002). Over time cells become insulin resistant, even compared to isocaloric diets with low sugar (Miller, 1994). Promotion of insulin resistance by a high sugar diet has adverse metabolic effects and is an early indicator for type II diabetes and cardiovascular disease (Ludwig, 2002; Cordain *et al.*, 2003). Additionally, following a 16 week high-fat high sugar diet, mice have shown decreased skeletal muscle citrate

synthase activity (a marker of functional mitochondrial abundance) and decreased oxidative fiber expression in conjunction with insulin resistance (Bonnard *et al.*, 2008), indicating an association between mitochondrial dysfunction, diet and development of metabolic disorders.

1.5.2 Mechanisms of sugar and saturated fats' negative impacts and increased risk of metabolic disease

Metabolic dysfunction with respect to FAs occurs due to insulin resistance, increased plasma cholesterol and adiposity (Hokanson and Austin, 1996; Turco *et al.*, 2014). Although the total amount of FAs consumed can contribute to metabolic perturbations, recent studies show that the FA quality (PUFA, MUFA or SFA) can also influence disease development (Hokanson and Austin, 1996; Turco *et al.*, 2014) likely because of their different biochemical properties.

Saturated fatty acids are classified as such because each carbon of the acyl chain is saturated with hydrogen molecules, and there are no double bonded carbons. Mono and polyunsaturated fatty acids are those with one or multiple double bonds, respectively. These double bonds create bends in the hydrocarbon chain and therefore unsaturated fats have less dense overall structures. Dietary intake of SFAs, particularly palmitate, the most abundant dietary SFA, increases total plasma cholesterol and decreases low density lipoprotein (LDL) receptor expression, thereby indirectly increasing plasma LDL levels (Chappell *et al.*, 1993; Rioux and Legrand, 2007). Plasma cholesterol, specifically in the form of LDL or VLDL are important risk factors in metabolic syndrome, CVD and type II diabetes (Hokanson and Austin, 1996). Intracerebroventricular injections of palmitate simulate insulin resistance in the brain (Posey *et al.*, 2009) and decrease PGC-1α expression in skeletal muscle (Coll *et al.*, 2008), providing further support for palmitate's association with development of metabolic disorders.

A recent review noted that adolescents in half of the 30 studied countries are ingesting more SFAs than recommended by World Health Organization, and failing to reach the recommended intake of PUFAs (Harika *et al.*, 2011). PUFAs are important in membrane fluidity but can influence lipogenesis by modulation of PPARα, similar to MUFAs (Lichtenstein and Ausman, 1994; Hannah, Ou and Luong, 2001; Assy and Nassar, 2009; X. Yang *et al.*, 2011). Studies demonstrate that SFA induced insulin resistance is associated with impaired glucose transport or phosphorylation (Holland *et al.*, 2011; Martins *et al.*, 2012). Skeletal muscle specific studies show reduced IRS (insulin receptor substrates)-1 tyrosine phosphorylation (Yu *et al.*, 2002) following SFA infusion. Tyrosine phosphorylation of IRS-1 leads to a phosphorylation cascade which results in increased glucose uptake. Conversely, dietary intake of MUFAs ameliorate insulin resistance in skeletal muscle (Yang *et al.*, 2011).

1.6 Thesis Rationale and Objectives

1.6.1 Rationale

Metabolic disorders are linked to poor *in utero* environments and can develop independent of post-natal diets (Barker, 2004; de Boo and Harding, 2006). A poor *in utero* environment, characterized by decreased oxygen and nutrient transfer across the placenta, is associated with LBW and IUGR outcomes (Byberg *et al.*, 2000; Pardi *et al.*, 2002; Burke *et al.*, 2006). These outcomes are observable phenotypes linked to an underlying physiological perturbation: a reprograming event (Neel, 1999; Barker, 2004). Fetal reprograming occurs in low oxygen and low nutrient environments, and consists primarily of a redirection of blood flow, sparing some organs (brain, heart and adrenals)(de Boo and Harding, 2006). Meanwhile, other organs and tissues such as the liver, kidneys and skeletal muscle experience a reduction in blood flow (Peleg, Kennedy and Hunter, 1998). This *in utero* environment is proposed to reprogram tissue gene

expression leading to the observed predisposition of IUGR offspring to metabolic disorders (Barker, 2004; de Boo and Harding, 2006).

In addition to their associations with a poor *in utero* environment, increased incidence of metabolic disorders such as insulin resistance and type II diabetes have been linked to the increased prevalence of a high-fat, high-sugar WD (Cordain *et al.*, 2005). Specifically, SFAs, and processed sugars, present in higher proportion in the WD compared to other diets (Cordain *et al.*, 2000a), have been identified as key components in the development of metabolic disorders (Holland *et al.*, 2011).

Underlying *in utero* reprogrammed changes in IUGR mitochondrial function and how these changes may interact with an adverse postnatal environment, such as a WD are currently ill-defined. Skeletal muscle biopsied from insulin resistant individuals have reduced rates of oxygen consumption (Gosker *et al.*, 2002; Mogensen *et al.*, 2007). Oxygen consumption may be altered due to the changes in fiber type composition because of differences in preferred metabolic pathways of the different fiber types. Oxidative fibers (type Ia, IIa and IIx) have a high density of mitochondria and capillaries and therefore respire at higher rates than their glycolytic (type IIb) counterparts (Lillioja *et al.*, 1987; Schiaffino and Reggiani, 2011). IUGR offspring show impaired mitochondrial function manifesting as reduced oxygen consumption in skeletal muscle attributed to reduced electron transport chain complex I activity, therefore skeletal muscle oxygen consumption is proposed as an early predictor for such metabolic diseases.

I therefore postulate that *in utero* hypoxia reprograming reduces oxygen consumption, and that a WD, or palmitate alone, will aggravate mitochondrial dysfunction whereas palmitoleate (a MUFA) will mitigate hypoxia-induced reductions in oxygen consumption.

1.6.2 Objectives and Hypotheses

My first objective was to determine the rate of oxygen consumption in LBW fetal guinea pig soleus muscle compared to normal birth weight (NBW) animals, *ex vivo*. I hypothesized that hypoxia (through placental insufficiency) will alter muscle oxygen consumption during development. I predicted that LBW animals would have reduced soleus oxygen consumption.

My second objective was to determine the effects of growth restriction on oxygen consumption rate and skeletal muscle fiber type composition in young adult LBW guinea pigs independently and in conjunction with a post-natal WD. I hypothesized that WD interacts with IUGR metabolic reprogramming and predicted that LBW/control diet-fed animals and NBW/western diet fed animals will exhibit increased glycolytic fiber ratios and reduced oxygen consumption while LBW/western diet-fed animals will have even further increases in glycolytic fiber ratios compared to NBW/control-diet fed animals as well as either treatment independently. I tested these two hypotheses using a whole-animal model of IUGR leading to a LBW outcome.

My final objective was to isolate hypoxia-specific effects of IUGR on skeletal muscle differentiation, metabolism and mitochondrial function and evaluate the effects of saturated fatty acids and monounsaturated fatty acids on oxygen consumption in a cell-based model system. It would be difficult to achieve this objective in a whole-animal model, so I used a skeletal muscle cell culture model where I could control oxygen as well as nutrient quantity and quality. It was postulated that hypoxia and fatty acid type alter oxidative metabolism through changes in fiber type. Specifically, I predicted that hypoxic cultured cells would consume less oxygen than normoxic cultured cells and that SFAs will exacerbate this while MUFAs would have a mitigating effect through changes in oxidative and glycolytic fibers.

2 Materials and Methods

2.1.1 Surgical Induction of Growth Restriction

All animal procedures were approved by the University of Western Ontario Animal Use Subcommittee in accordance with the Canadian Council on Animal Care guidelines. Pregnant Dunkin-Hartley guinea pigs (Charles River Laboratories, Wilmington, MA, USA) were housed with a 12/12 hour light/dark cycle and allowed access to standard guinea pig chow (LabDiet diet 5025: 27% protein, 13% fat, 60% carbohydrates) *ad libitium.* Fetal samples were obtained from sows that underwent uterine artery ablations (UAA) performed mid-term (approximately day 35 of a 68-day gestation) to induce IUGR by a veterinary technician. Following anesthesia (4-5% Isoflurane with 2L/min O_2 for induction, followed by 2.5-3% Isoflurane with 1L/min O_2 for maintenance), a subcutaneous injection of Robinul (Glycopyrrolate, 0.01mg/kg, Sandoz Can Inc., Montreal QC) was administered. To expose the bicornate uterus, a midline incision was made below the umbilicus and subsequently the UAA was performed by cauterization of 50% of uterine artery branches using an Aaron 2250 electrosurgical generator (Bovie Medical, Clearwater, FL)(Turner and Trudinger, 2009)

Guinea pig studies were divided into two experiments, one examined the direct impact of the *in utero* insult and a second focused upon the long-term effects of an adverse *in utero* environment combined with a second insult of a poor Western diet during a post-natal growth phase. In the first study, fetal soleus muscle was collected near term (approximately gestational day 65 of a 68-day term). These muscles were used for respirometry experiments and came from fetuses that were categorized as either control (fetuses weighing more than 90 grams) or IUGR (fetuses weighing less than 85 grams; Figure 2.1-1).

In the second study both UAA and sham-operated controls were carried to term and allowed to grow for four months, fed either a control or Western diet (see section 2.1.2,

below). Animals whose birth weight fell below the 25th percentile of all pups born were categorized as LBW and those whose birth weight fell between the 25th and the 75th percentile were categorized as normal birth weight (Thompson *et al.*, 2011). In accordance with previous data (Kind *et al.*, 2003; Briscoe *et al.*, 2004) and meeting these criteria, LBW animals were below 85g at birth and normal birth weight animals were above 90g at birth. Four months after birth soleus muscle was collected from these animals for respirometry and molecular analysis (further described in section 2.4)

2.1.2 Post-Natal Life and Feeding

Guinea pigs that carried to term were weaned at 15 days of age and housed in individual cages maintained at 20°C ± 2°C, 30-40% relative humidity with a 12-hour light/dark cycle. At weaning, offspring were randomly assigned to either a control diet (CD, 3.4 kcal/g; product number TD: 110240, Harlan Laboratories, Madison WI) or a Western diet (WD, 4.2 kcal/g; product number TD: 110239, Harlan Laboratories, Madison WI; Figure 2.1-1). The control diet was comprised of a high complex carbohydrate content, but low fructose and sucrose with the majority of fats being PUFA, and very low SFA content. In contrast, the WD has less overall carbohydrate composition, but higher fructose and sucrose content, and a larger percentage of SFA (Table 2.2-1). Once weaned, pups were then classified into four experimental groups: normal birth weight on a control diet (NBW/CD), low birth weight on a control diet (LBW/CD), normal birth weight on a Western diet (NBW/WD), and low birth weight on a Western diet (LBW/WD). Animals were maintained on these diets and sacrificed at ~150 days, corresponding to young adulthood (Gomez-Pinilla *et al.*, 2007).

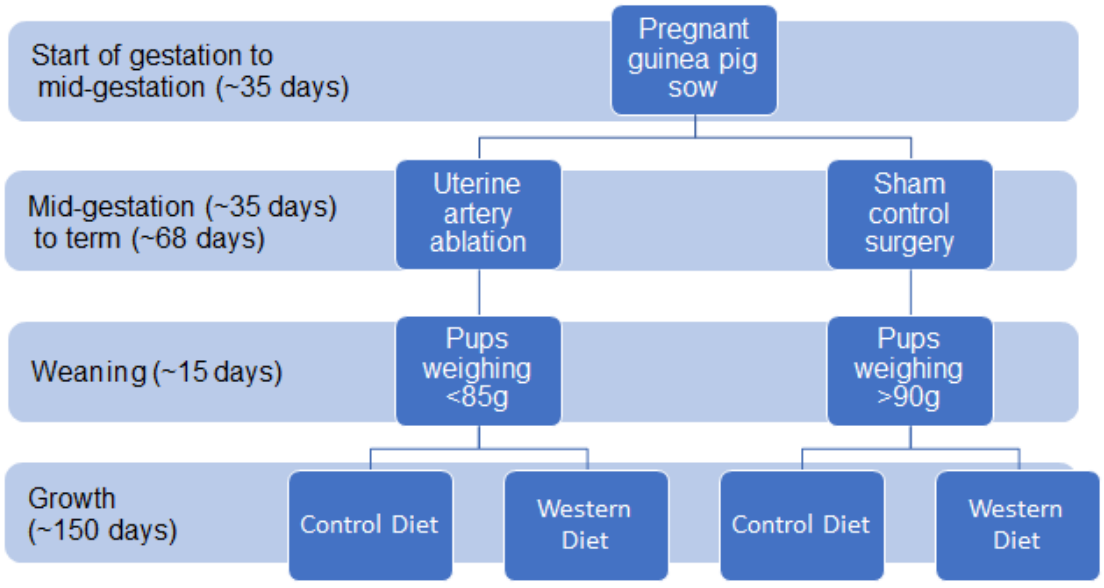

Figure 2.1-1 Overview of Guinea Pig animal model for uterine artery ablation and post-natal diet. Uterine artery ablations (UAA) or sham control surgeries were performed on pregnant sows. Pups born from UAA sows weighing less than 85g were considered LBW, weaned and then given either a control or Western diet. Pups born from sham surgeries weighing more than 90g were considered NBW, weaned and given either a control or Western diet. Animals were sacrificed and soleus muscle was collected at day 150.

Table 1.6-1 Guinea Pig Diet Comparison (percent calories)

Nutritional information	Control Diet	Western Diet
Calories	3.4 Kcal/g	4.2 Kcal/g
Protein	22%	21%
Carbohydrates	60%	33%
Sucrose	11%	22%
Fructose	-	7.6%
Fat	18%	46%
Saturated fatty acids	2.7%	32.2%
Monounsaturated fatty acids	4.32%	11.96%
Polyunsaturated fatty acids	10.98%	1.84%

2.1.3 Tissue Collection

In the first study, which compared control and IUGR fetuses, pregnant sows near term (gestational day 65) were sacrificed by CO_2 asphyxiation following an overnight fast (Greulich *et al.*, 2011) and fetuses were removed. In the second study of post-natal diet effects on LBW and NBW offspring, animals born to both UAA and sham operated sows were sacrificed at postnatal day 150, corresponding to young adulthood (Briscoe *et al.*, 2004), by CO_2 asphyxiation following an overnight fast (Greulich *et al.*, 2011). From every animal (fetus and offspring) one soleus muscle was removed and used immediately for respirometry (described in section 2.2, below). and the contralateral muscle, flash frozen in liquid nitrogen for CS activity. In addition, for the 150-day old NBW and LBW animals only, soleus muscle was also collected and frozen for fiber typing mRNA determinations and ETC immunoblot analysis (described in sections 2.3 and 2.4 below).

2.2 Soleus Respirometry

The soleus muscle dissected from control and IUGR fetuses, as well as 150 day old NBW and LBW animals was stored on ice in BIOPS buffer (50mM K^+-MES, 20mM taurine, 0.5mM dithiothreitol, 6.56mM $MgCl_2$, 5.77mM ATP, 15mM phosphocreatine, 20mM imidazole pH 7.1 adjusted with 5N KOH at 0°C, containing a Ca-EGTA buffer (2.77mM CaK_2EGTA + 7.2mM K_2EGTA; 0.1μM free calcium) until permeabilized. The soleus muscle tissue was kept ice-cold and teased apart using sharp forceps to isolate muscle fiber bundles 3-4mm long and 1mm in diameter, exposing a fairly large surface area. The fibers were incubated in BIOPS supplemented with saponin (50μg/ml) for 30 minutes (optimal for skeletal muscle; Kuznetsov et al., 2008), permeabilizing the cells by binding to cholesterol in the sarcolemma, thereby allowing access of oxidative substrates and ADP to the mitochondria without further disrupting the tissue. In contrast to sarcolemma, mitochondrial membranes contain little cholesterol, so are relatively unaffected by saponin incubation. Following permeabilization, tissue was washed 3 times in MiR05 buffer (110mM sucrose, 60mM K-lactobionate, 0.5mM EGTA, 3mM $MgCl_2$, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES adjusted to pH 7.1 with KOH at 37°C; and 1g/litre BSA fatty acid free) for 2 minutes.

Rates of oxygen consumption, with oxidative substrates and ADP (state 3) and following ATP synthesis (state 4), were determined using the Oxygraph-2k (Oroboros), with Clark-type O_2 electrodes in airtight 2mL chambers at 37°C. Glutamate (10mM final concentration) and pyruvate (5mM) were injected into the chamber along with malate (2mM) as substrates for respiration through ETC complex I. In separate incubations succinate (10mM) was solely used as an ETC complex II substrate, with rotenone (0.5μM) added to inhibit ETC complex I arising from endogenous substrate metabolism. Once respiration rate with only substrates stabilized, ADP (2mM) was added, stimulating state 3 (maximal physiological) respiration. After recording stable state 3 rates oligomycin (5mM) was injected into the Oxygraph chamber to inhibit complex V activity, allowing estimation of state 4 respiration.

2.3 Citrate Synthase Activity

Soleus muscle from control and IUGR fetuses, as well as 150 day old NBW and LBW animals was dissected and frozen in liquid N_2 and tissue stored at -80°C. Samples were then homogenized in radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-HCl, NP-40 1%, Na-deoxycholate 0.25%, 1mM EDTA, 150mM NaCl, 50mM NAF, 1mM NaV, 25mM β-glycerophosphate, pH 7.4) with protease and phosphatase inhibitors. One aliquot was used for western blots (see section 2.4) and another frozen at -80°C until it was used for citrate synthase (CS) activity. Thawed homogenate was sonicated then centrifuged at 2000*g* for 5 min at 4°C.

The CS reaction was followed in 96-well plates in triplicate by measuring absorbance at 412nm using a spectrophotometer. Wells were loaded with 220µl reaction buffer (consisting of 25mM HEPES, 0.1% Triton X-100 and 2mM EDTA) 30µl of 0.15mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 30µl of 0.15mM acetyl CoA and 3µl of homogenate (Eigentler, Draxl and Wiethüchter, 2012). The reaction was started by adding 30µl of 0.33mM oxaloacetate. Blank rates were also recorded in a well which contained homogenate but to which buffer was added instead of oxaloacetate. Absorbance was recorded for 5 minutes at 37°C and maximal activity was calculated from the slope of absorbance vs. time using a minimum of 7 consecutive absorbance readings during the 5 minutes (readings taken every 1 second), after subtracting rates from blank wells. Data were expressed as enzymatic activity per mg soleus muscle protein.

2.4 Molecular Analysis

Frozen soleus muscle from 150-day old animals NBW and LBW animals only were used for the following mRNA and immunoblotting procedures.

2.4.1 Rt-qPCR for Myosin Heavy Chain isoform expression

Guinea pig soleus was powdered using a mortar and pestle in liquid N_2, homogenized using a disposable homogenization pestle and stored in Trizol reagent (Invitrogen, Burlington ON) at -80°C until use. Total RNA was extracted using the Trizol procedure; samples were thawed and incubated at room temperature for 5 minutes, 200µL of chloroform was added to the solution and shaken vigorously by hand for 15 seconds. Samples were left at room temperature for 2 minutes and then centrifuged for 15 minutes at 12,000*g* at 4°C separating the aqueous phase (containing RNA), an interphase and a phenol-chloroform phase. The aqueous layer was transferred to a new RNAase-free tube and 500µL of isopropanol was added to the solution which was incubated at room temperature for 10 minutes. Following incubation, samples were centrifuged for 10 minutes at 12,000*g* at 4°C, precipitating RNA into a pellet. Samples were then washed twice with 100µl of 75% ethanol, centrifuging at 7,500*g* for 5 minutes after each wash. Samples were stored at -80°C until use.

The yield of isolated RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo-Scientific) and quality was measured using the A260/A280 ratio (≥1.8). Isolated RNA (4µg) was incubated with deoxyribonuclease (Invitrogen) to remove any contaminating DNA and a recombinant ribonuclease inhibitor (Invitrogen) at 37°C for 30 minutes. Samples were then incubated at 37°C for two hours with murine leukemia virus reverse transcriptase (Invitrogen) to produce cDNA with the use of random primers. Real-time quantitative PCR (qPCR) was performed using SoFast EvaGreen Supermix

(Invitrogen) and the BioRad CFX384 Real-Time PCR Detection System at denature temperature of 95°C, annealing temperature of 59.5°C and elongation temperature of 72°C for 40 cycles. A total volume of 8µl in each well contained 3µl of cDNA, 0.08µl primer mix, 4µl SYBR Green and the remaining volume was filled with autoclaved water. Primer sets directed against guinea pig specific myosin heavy chain genes were generated using the NCBI Primer-BLAST tool based on predicted *Cavia porcellus* sequences (Table 2.4-1). The data from qPCR was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using β-actin as the internal control gene.

Table 2.4-1 Guinea Pig Myosin Heavy Chain primer pairs.

Primers	NCBI accession #	Strand	Sequence (5'➡3')
Myh7 (type 1A)	XM_003474485.2	Forward	CAGGAGTGCAGGAATGCTGA
		Reverse	GTTATGCAGGTGCCAGGGAT
Myh4 (type 2B)	XM_005002092.1	Forward	GTACACCTAACCTGCAGCCA
		Reverse	GTTGTCGTTCCTCACGGTCT
Myh2 (type 2A)	XM_005002092.1	Forward	CCTTTGAAGAGCGACACTGG
		Reverse	TCAGCATGAACTGGTAGGCG
Myh1 (type 2x)	XM_003466219.1	Forward	TCATCCCTACAGGCAAGGTC
		Reverse	AGAGGCCCGAGTAGGTGTAG

2.4.2 Immunoblotting of Electron Transport Chain Complex Subunits

Protein concentrations of homogenized soleus (described in section 2.3) were determined using BCA protein concentration kits (Pierce Biotechnology, Rockford IL). Samples were then prepared using NuPAGE LDS sample buffer (Invitrogen) and NuPAGE Sample Reducing Agent (Invitrogen). Approximately 15µl of soleus extracts (20µg of protein) were separated on NuPAGE 4-12% Bis-Tris gels and subsequently washed in TBST (137mM NaCl, 2.7mM KCl, 19mM Tris base, 0.1% Tween 20) 3 times for 10 minutes. Membranes were then incubated in TBST supplemented with the MitoProfile Total OXPHOS Rodent Antibody Cocktail (Complex I subunit NADH dehydrogenase [ubiquinone] 1 beta subcomplex 8, NDUFB8; Complex II Iron-sulfur protein subunit; Complex III Core protein 2; Complex IV subunit I; Complex V alpha subunit) at a 1:1000 dilution (ab110413, host: mouse; Mitosciences, Eugene, OR). Membranes were then washed three times with TBST for 15 minutes and then incubated for one hour in TBST supplemented with a donkey anti-mouse horseradish peroxidase secondary antibody. To probe for the housekeeping protein (voltage dependent anion-selective

channel protein 1, VDAC1), membranes were prepared in a similar manner as described above with primary antibody at a 1:5000 dilution (ab14734, host: mouse; Invitrogen). Immunoreactive bands were detected with enhanced chemiluminescence and imaged with a VersaDoc Imaging System (Bio-Rad, Mississauga, ON). Densitometry was performed with ImageLab Software (Bio-Rad).

2.5 Hypoxic C_2C_{12} Cells and Fatty Acid Supplementation

C_2C_{12} mouse myoblast cells were cultured to test the third hypothesis; that hypoxia and fatty acid type alter oxidative metabolism. Cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin until cells reached 70-80% confluence (~ 48 hours) at 20.9% O_2 (5% CO_2 with N_2 balance). Once cells reached confluence, growth media was replaced with differentiation media (DMEM supplemented with 5% horse serum and 1% penicillin/streptomycin) inducing myoblast differentiation. The cells were then allowed to differentiate at 37°C at either 1% (to simulate *in utero* hypoxia) or 5% O_2 (to simulate typical *in utero* conditions) for five days of differentiation. Following differentiation, a subset of cells was incubated at 20.9% O_2 for 48 hours (recovery; Figure 2.2-1). During recovery, a further subset of cells was exposed to 0.5mM palmitate (saturated fatty acid; 16:0), 0.5mM palmitoleate (mono-unsaturated fatty acid; 16:1) 10% fatty acid free BSA (vehicle used to conjugate fatty acids) was used as a control for 24 hours (figure 2.2-2).

2.5.1 Time points for collection

Untreated whole cells were collected prior to differentiation (day 0; cells cultured in growth media at 20.9% O_2), after 5 days of differentiation (day 5; cells cultured in differentiation media at either 5% O_2 or 1% O_2) and following 48 hours of recovery (day 7; cells differentiated at either 5% O_2 or 1% O_2 for 5 days, then transferred to 20.9% O_2 for 2 days). Cells treated with fatty acids or BSA were collected at days 0, 5 and following 24hrs of fatty acid exposure (day 6).

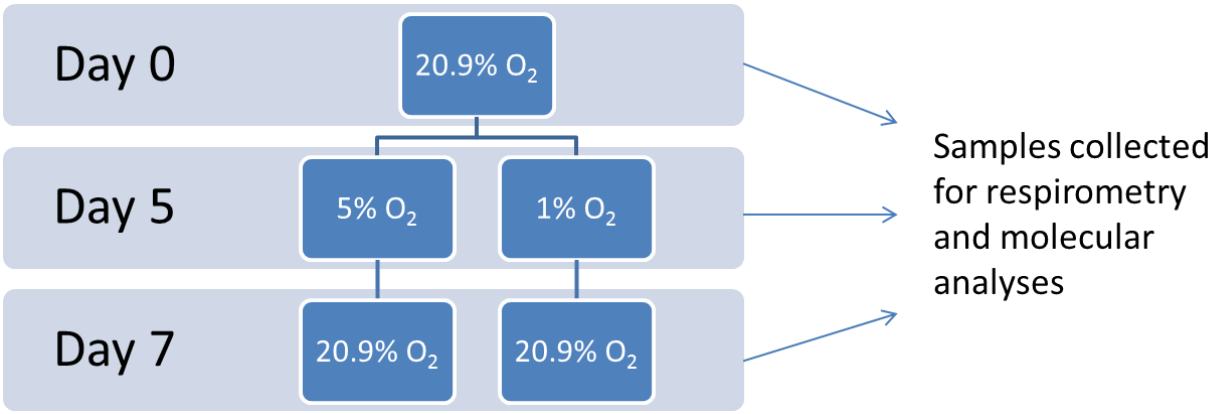

Figure 2.5-1 Diagram of cell culture experiments. C_2C_{12} mouse myoblasts were cultured at 20.9% oxygen until they reached ~ 80% confluence. Cells were then differentiated under either 5% O_2 (simulating normal *in utero* oxygen levels) or 1% O_2 (simulating oxygen under IUGR conditions) for 5 days. Following differentiation, cells were placed into recovery conditions for 48 hours (20.9% O_2).

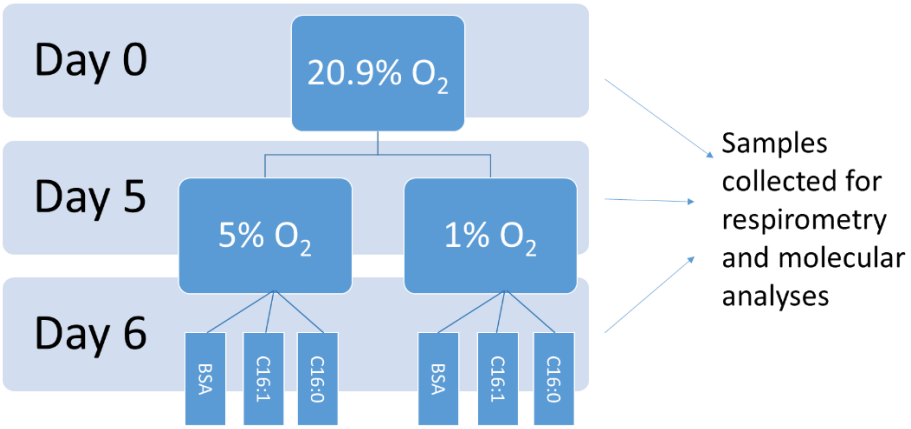

Figure 2.5-2 Diagram of hypoxic cells treated with fatty acids. C_2C_{12} mouse myoblast were cultured at 20.9% oxygen until they reached ~ 80% confluence. Cells were then differentiated under 5% O_2 (simulating normal *in utero* oxygen levels) or 1% O_2 (simulating oxygen under IUGR conditions) for 5 days. Following differentiation, 0.5mM palmitate (saturated fatty acid; 16:0), 0.5mM palmitoleate (mono-unsaturated fatty acid; 16:1) or 0.5mM 20% BSA (vehicle used to conjugate fatty acids) was added to the differentiation media for 24 hours at 20.9% O_2.

2.5.2 Rate of Oxygen Consumption in Cell Culture

Whole cells were collected for respirometry in a standard buffer (130mM NaCl, 5mM KCl, 0.5mM $MgCl_2$, 25mM HEPES; pH 7.4) and supplemented with 10mM glucose as metabolic substrate. Rates of O_2 consumption were measured using the Oroboros Oxygraph-2k. These rates were calculated at 50µM O_2 (equivalent to equilibration at 5% O_2) and 10µM O_2 (1% O_2) and expressed per 10^6 viable cells. Cell counts and viability were determined using a heamocytometer and trypan blue, as previously described (Yun et al., 2005).

2.5.3 Extraction and Quantification of Mitochondrial DNA from Cultured Cells

Mitochondrial DNA was extracted to determine if any changes in oxygen consumption rates in hypoxia were related to mtDNA content. C_2C_{12} Cells were collected in phosphate buffer saline solution (PBS) and pelleted at 10,000*g* for 5 minutes. The PBS supernatant was removed with a pipette and the pellet was stored at -80°C. The pellets were re-suspended in 200µL TNES (50mM Tris-HCl pH 8.0, 100mM EDTA pH 8.0, 100mM NaCl, 500mg/mL Proteinase K, 1% SDS) and heated at 55°C for 4 hours. Each sample was supplemented with 500µL phenol/chloroform/isoamyl alcohol (25:24:1) and repeatedly inverted by hand for 1 minute. Samples were then centrifuged at 10,000*g* for 3 minutes separating the sample into an upper aqueous phase, an interphase containing DNA, and a lower phenol/chloroform phase. The aqueous phase and interphase were transferred to a fresh tube. Next, DNA was extracted with chloroform following the same steps as with the phenol/chloroform/isoamyl alcohol extraction, again collecting the aqueous phase and interphase. 50µL of 3M $C_2H_3NaO_2$ and 1mL of 100% ethanol were added to the samples which were then inverted for 30 seconds, precipitating the DNA. The supernatant was removed and 500µL of 70% ethanol was added. Samples were inverted for 30 seconds and centrifuged at 12,000*g* for 10 minutes, pelleting the DNA. The ethanol was removed and the pellet was re-suspended in 200µL H_2O and stored at -80°C until use for qPCR.

The yield of isolated DNA was determined using the NanoDrop 2000 spectrophotometer (Thermo-Scientific) and quality was measured using the A260/A280 ratio (≥1.8). Real-time quantitative PCR was performed as described in section 2.4 Primer sets directed against mouse specific mitochondrial D-loop genes were generated using the NCBI Primer-BLAST tool based on *Mus musculus* sequences (Table 2.2.1). The data from qPCR was analyzed using the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen, 2001) using β-actin as the internal control gene.

Table 2.5-1 Mitochondrial DNA mouse primer pairs.

Primers	NCBI accession #	Strand	Sequence (5'➡3')
mt D-loop	KM114847.1	Forward	TGCGTTATCGCCCATACGTT
		Reverse	GCGTCTAGACTGTGTGCTGT

2.6 Statistics

GraphPad Prism 5 was used for all statistical analyses. Guinea pig weight and thinness data are presented in box whisker plots, representing the median, 25th and 75th quartiles and min/max values. An unpaired Student's t-test was used to compare the means of fetal respiration of control and IUGR animals and an f-test was used to ensure equal variance. Means compared between NBW/CD, NBW/WD, LBW/CD and LBW/WD for soleus respiration, mitoprofile protein expression, myosin heavy chain mRNA expression and citrate synthase activity were analysed using a two-way ANOVA with a Bonferroni post-hoc test. A two-way ANOVA with Bonferroni post-hoc test was used to compare mean oxygen consumption of C_2C_{12} mouse myoblast cells cultured under 5% or 1% oxygen and following reoxygenation (day 5 and day 7). Mean oxygen consumption of C_2C_{12} mouse myoblast cultured under 5% and 1% oxygen and whose media was supplemented with BSA, palmitate or palmitoleate was compared using a two-way ANOVA with a Bonferroni post-hoc test. A p value of less than 0.05 was considered significant. The Bartlett's test was used to measure equal variance for all grouped data, MHC2, MHC4, MHC1 and MHC7 data did not have equal variance.

3 Results

3.1 Growth Restriction Induced Alterations in Fetal Soleus Muscle Respiration

Fetal weights were recorded at collection which occurred at approximately gestational day 65 (term 68 days). Fetuses arising from UAA surgeries and weighing less than 85 grams were categorized as IUGR and those born from sham surgeries weighing more than 90 grams were categorized as controls. IUGR fetuses had a 28% lower birth weight (Figure 3.3-1; $p<0.001$).

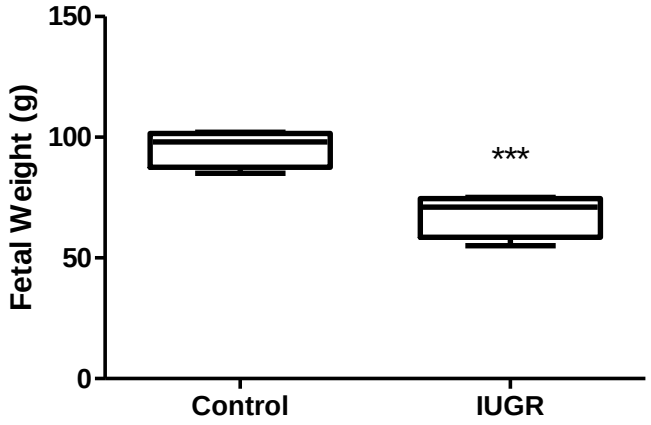

Figure 3.1-1 Fetal body weights at collection. Body weights of fetuses from UAA pregnancies weighing less than 85g (IUGR) compared to fetuses born from sham surgeries weighing more than 90g (control). The boxes represent the 25th and 75th percentiles, the bars indicate min/max values and the line indicated the median (Control n=9, IUGR n=6; df=13). *** indicates significance of $p<0.001$ between groups as determined by an unpaired Student's t-test**.**

Fetal soleus was collected and permeabilized using saponin in order to measure respiration states. IUGR soleus had 24% lower state 3 respiration with pyruvate as substrate ($p<0.05$), but there were no significant differences with succinate or glutamate (Figure 3.1-2). There were no significant changes in state 4 respiration between IUGR and control animals.

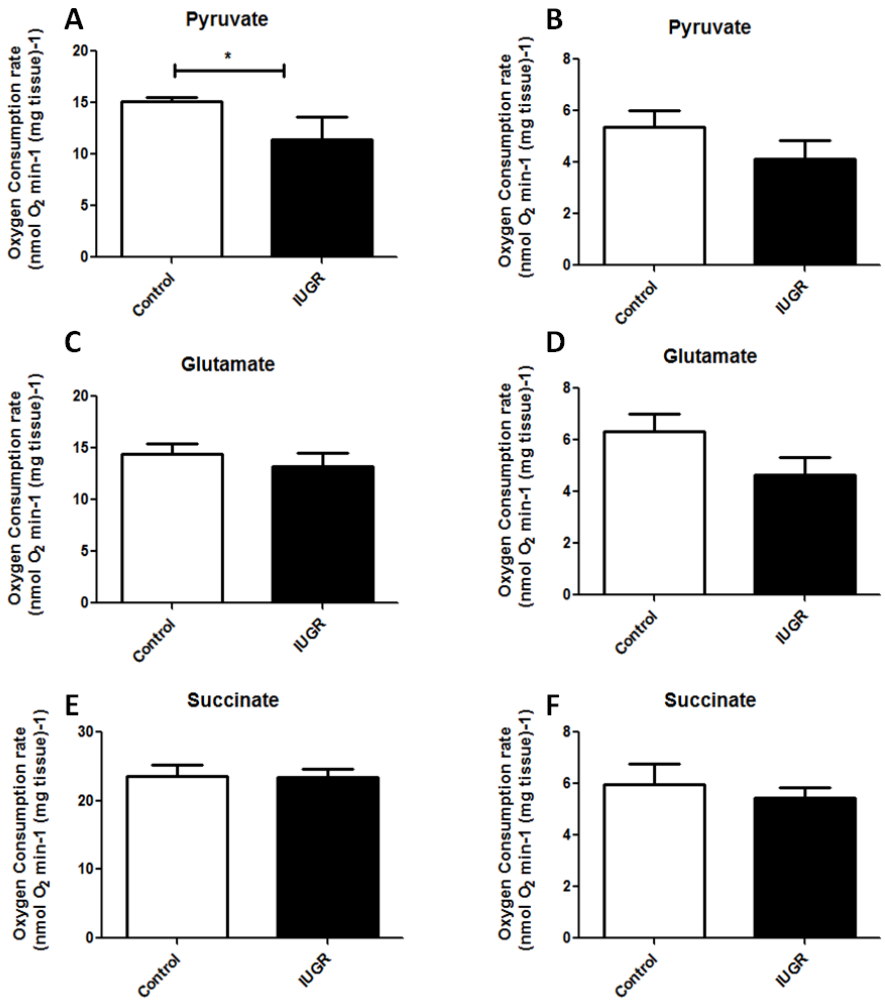

Figure 3.1-2 State 3 (A, C, E) and State 4 (B, D, F) respiration of fetal Guinea Pig soleus muscle with either (A, B) pyruvate, (C, D) glutamate or (E, F) succinate as oxidative substrate. Rate of oxygen consumption was measured by closed cell respirometry using the Oxygraph-2K at 37°C in MiR05 buffer. Tissue was permeabilized with 0.9 mM saponin in BIOPS for 20 minutes followed by three 2 minute washes in MiR05. State 3 data displays rate of oxygen consumption subsequent to the addition of (A) 10mM pyruvate and malate 5mM ADP, (B) 30mM glutamate and malate 5mM ADP or (C) 0.5µM rotenone, 30mM succinate and ADP. State 4 data displays rate of oxygen consumption following complex V inhibition by 2.5µM oligomycin. Data presented as means ± SEM, $p<0.05$ between IUGR and Control state 3 reported from an unpaired Student's t-test (Control n=9, IUGR n=6; df=13).

3.2 Low Birth Weight and Western Diet Effects on Soleus Muscle Respiration

3.2.1 Birth Weights and Thinness

Animals at 150 days old were used for soleus respirometry and molecular analysis. Animals resulting from the UAA surgeries and weighing less than 85 grams at birth were considered Low Birth Weight animals. Animals resulting from sham controls weighing more than 90 grams were categorized as Normal Birth Weight animals. Female offspring of UAA mothers display a 31% lower birth weight compared to sham control animals (Figure 3.2-1A; $p<0.001$), and male offspring of ablated mothers display a 28% lower birth weight compared to controls (Figure 3.2-1B; $p<0.001$). To further characterize the LBW phenotype and its association with the pathophysiology of IUGR, I calculated a measure of thinness (body mass/crown-rump length) and found that LBW animals were 25% thinner (Figure 3.2-2; $p<0.001$).

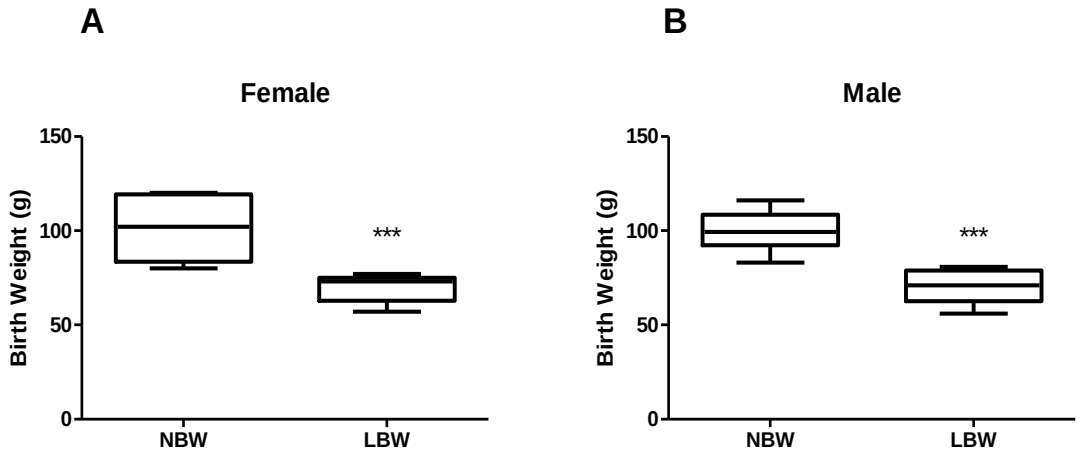

Figure 3.2-1 Birth weights of animals used for respirometry and molecular analyses. Birth weights of animals born from ablation pregnancies weighing less than 85g (LBW) compared to animals born form sham surgeries weighing more than 90g (NBW). The boxes represent the 25th and 75th percentiles, the bars indicate min/max values and the line indicated the median. *** indicates significance of $p<0.001$ between groups as determined by an unpaired Student's t-test (NBW females n=8, LBW females n=7, NBW males n=8, LBW males n=5; df-A=13, df-B=11).

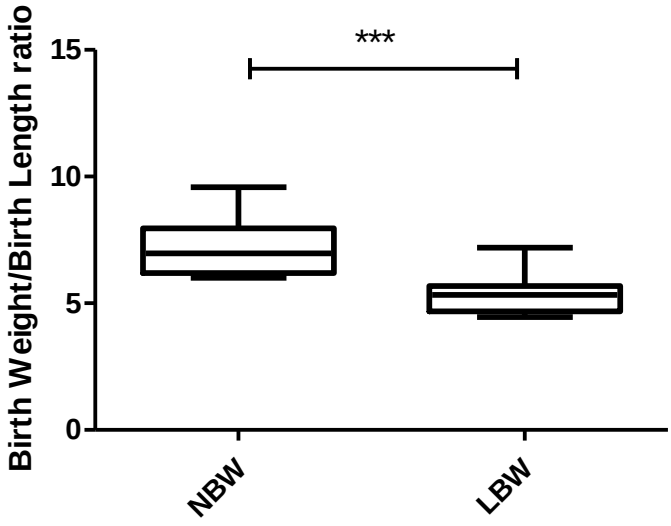

Figure 3.2-2 Birth weight to birth length ratio of 150-day old guinea pigs. Birth weight/birth length ratio at birth for animals used in 150-days old studies. Animals born from sham surgeries weighing more than 90g are considered NBW and those born from ablation pregnancies weighing less than 85g are considered LBW. The boxes represent the 25th and 75th percentiles, the bars indicate min/max values and the line indicated the median. *** indicates significance of $p<0.001$ between groups as determined by an unpaired Student's t-test (NBW n=14, LBW n=12; df=24).

3.2.2 Soleus Muscle Oxygen Consumption of Young Adult Guinea Pigs

Soleus muscle from young adult animals was collected at approximately 150 days of age and prepared for respiration in the same way as the fetal muscle. Two-way ANOVA indicated strong interaction effects for state 3 respiration with pyruvate and glutamate, indicating that the effect of birth weight on respiration depended on post-natal diet. ($p<0.001$ for both; Figure 3.2-3). State 3 respiration was over 2-fold higher in all NBW/CD, males and females combined compared to treatment groups (NBW/WD, LBW/CD and LBW/WD) with pyruvate as a metabolic substrate ($p<0.001$; Figure 3.2-3). Similarly, there was a reduction in state 3 respiration of over 45% in all treatment groups with glutamate ($p<0.001$; Figure 3.2-3) when compared to NBW/CD animals. There was no significant effect of treatment on state 3 respiration with succinate as substrate. Interestingly, LBW/WD group did not suffer any additional reductions in oxygen consumption when compared to NBW/WD and LBW/CD animals.

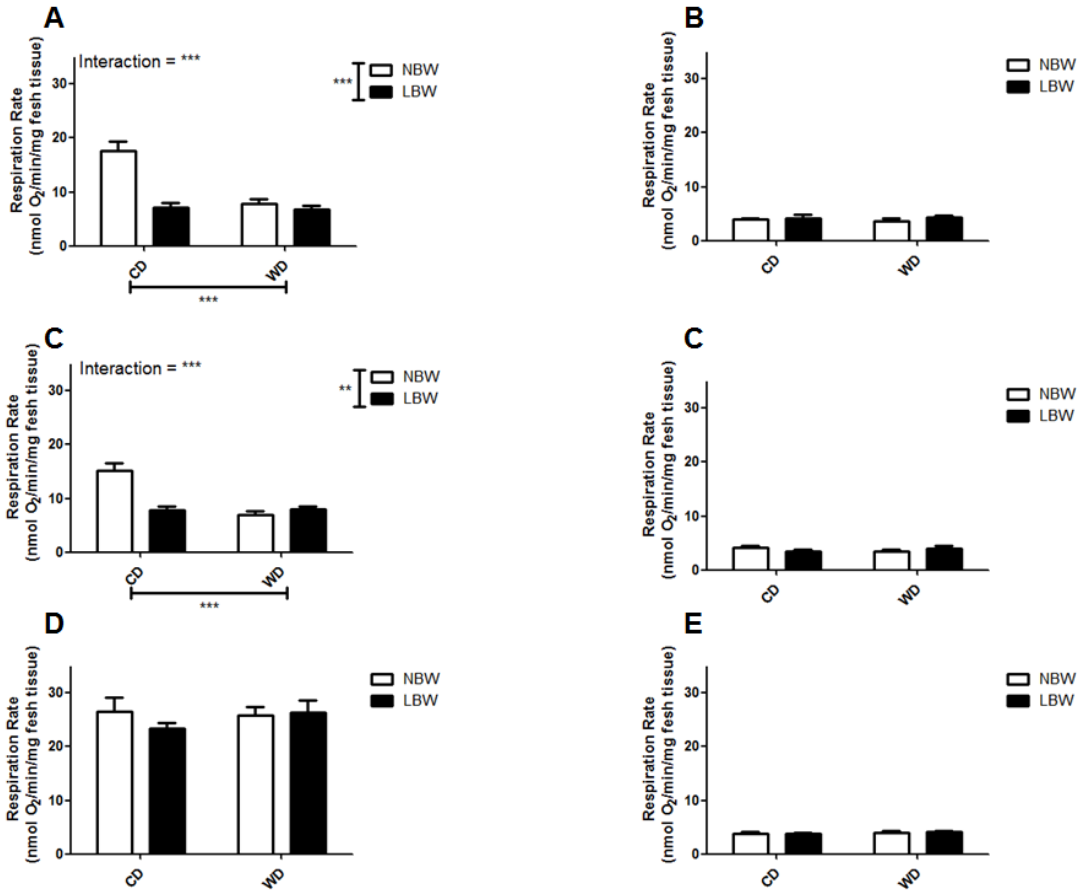

Figure 3.2-3 State 3 (A,C, E) and State 4 (B, D, E) respiration of permeabilized soleus from 150-day old Guinea Pig with (A, B) pyruvate, (C, D) glutamate and (D, E) succinate as oxidative substrate. Rate of oxygen consumption was measured by closed cell respirometry using the Oxygraph-2K at 37°C in MiR05 buffer. Tissue was permeabilized with 0.9mM saponin in BIOPS for 20 minutes followed by three 2 minute washes in MiR05. State 3 data displays rate of oxygen consumption subsequent to the addition of (A) 10mM pyruvate and 5mM ADP, (C) 30mM glutamate and 5mM ADP or (E) 0.5µM rotenone, 30mM succinate and ADP. State 4 data displays rate of oxygen consumption following ATPase inhibition by 2.5µM oligomycin. Data presented as means ± SEM (NBW/CD n=9, NBW/DW n=8, LBW/CD n=6, LBW/WD n=6; df=28). Connecting lines indicate significance between those groups as determined by a 2-way ANOVA and Bonferroni post-test (**p<0.01; ***p<0.001).

3.2.3 ETC Protein Expression and Citrate Synthase Activity

Relative protein expression of subunits from ETC complexes were analyzed by western immunoblotting. Nuclear subunits NDUFB8, SDH8, UQCRC3 and ATP5A from Complexes I, II, III and V respectively as well as mitochondrially encoded subunit MTCO1 of complex IV were targeted using the rodent MitoProfile cocktail antibody. Complexes II, IV and V did not change across groups (Figure 3.2-4). Protein expression of complex III were decreased in LBW animals by 49% and this effect did not depend on post-natal diet ($p<0.001$; Figure 3.3-4). Two-way ANOVA indicated an interaction effect on the expression of Complex I ($p<0.05$). NBW/WD, LBW/CD and LBW/WD having decreased protein expression by 42%, 43% and 39%, respectively compared to NBW/CD animals.

Citrate synthase activity, commonly used to measure functional mitochondrial content, was measured by a spectrophotometric assay. No significant differences were found between treatment groups when compared to NBW/CD animals, indicating similar mitochondrial content in the soleus muscle of 150-day old offspring across treatments; however, the interaction effect neared significance (Figure 3.2-5; $p=0.051$).

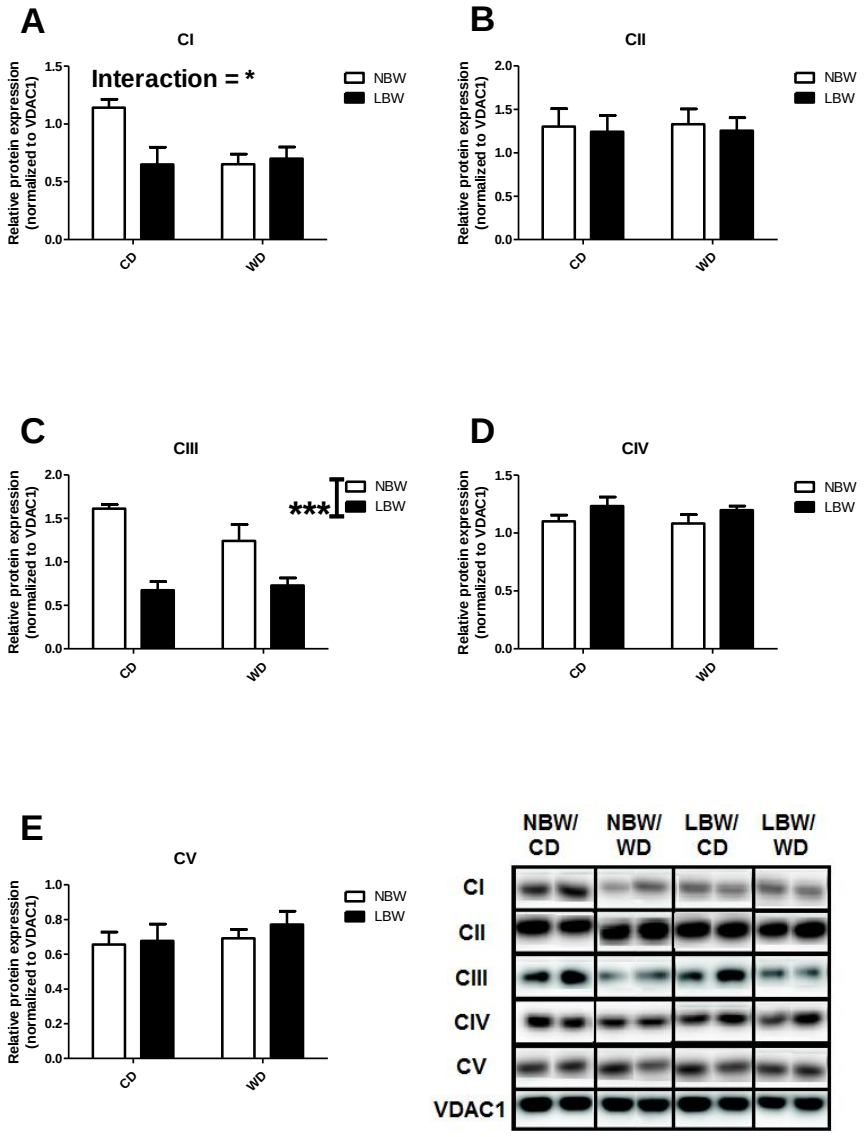

Figure 3.2-4 Relative protein expression of subunits of electron transport chain complex I subunit NDUFB8 (A), complex II subunit SDH8(B), complex III subunit UQCRC2 (C), complex IV subunit MTCO1 (D), complex V subunit ATP5A (E) in NBW or LBW 150-day old Guinea Pigs fed either a Control or Western Diet. Protein levels from ETC complexes 1-5 were measured using Western blot analysis, normalized to VDAC1, and outer mitochondrial membrane protein as a loading control. Representative blots displayed on the lower right panel. Data presented as means ± SEM, 2-way ANOVA and Bonferroni post-test determined significance (*$p<0.05$; ***$p<0.001$; n=5 for each experimental group; df=19).

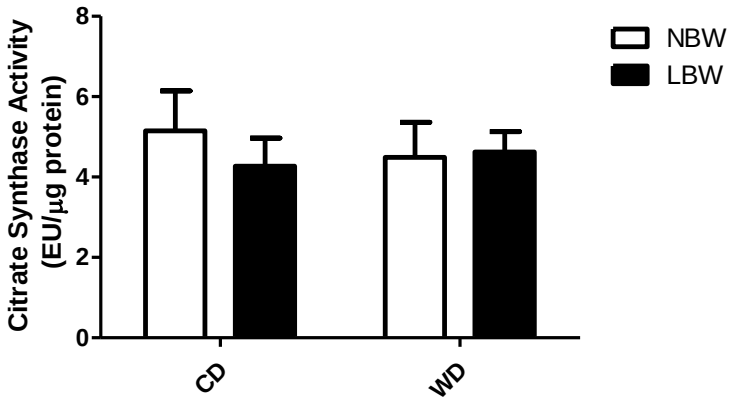

Figure 3.2-5 Citrate synthase activity in the 4-month-old Guinea Pig Soleus. Soleus muscle from guinea pigs or normal birth weight (NBW) or low birth weight (LBW) fed a control diet (CD) or western diet (WD) at 4 months of age measured using citrate synthase activity assay (NBW/CD n=8, NBW/WD n=6, LBW/CD n=4, LBW/WD n=3, df=23)). Data presented as means ± SEM compared by a two-way ANOVA with Bonferroni post-hoc test.

3.2.4 Fiber Type Compositions

Soleus myosin heavy chain isoform mRNA expression was determined using qRT-PCR. Relative mRNA expression of MHC7 (type Ia; slow oxidative fiber) shows an interaction effect ($p<0.05$), with the LBW/WD diet animals having increased expression relative to other groups (Figure 3.2-6 A). Expression of MHC2 (type IIa; fast oxidative) also has a strong interaction effect ($p<0.001$), a birth weight effect ($p<0.01$) and a diet effect ($p<0.01$); expression being high in LBW/WD animals and very low in LBW/CD as well as NBW/WD animals (Figure 3.2-6B). MHC1 (type IIx; fast oxidative) expression is significantly higher in LBW animals ($p<0.001$) however there was no diet effect (Figure 3.2-6C) and similarly, LBW animals had higher MHC4 (type IIB; glycolytic fibers) expression ($p<0.05$) without diet effect (Figure 3.2-6D).

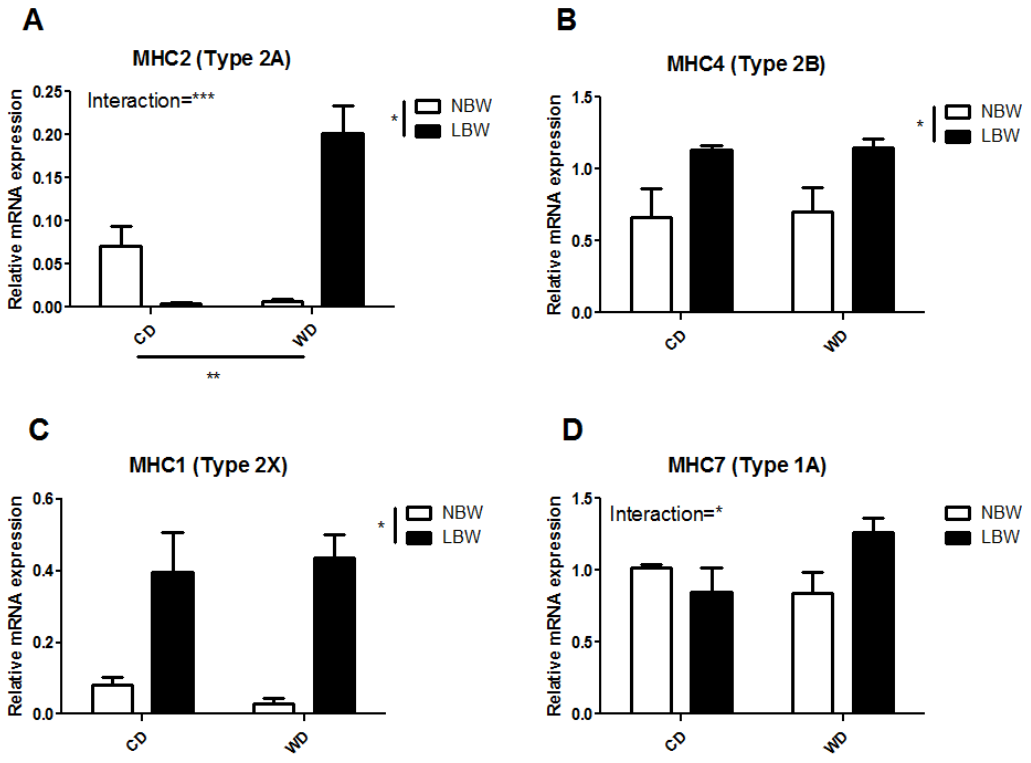

Figure 3.2-6 Relative mRNA expression of myosin heavy chain genes (MHC) in NBW or LBW 4 month old Guinea Pigs fed a Control or Western Diet (normalized to βactin). Fast oxidative fibers, types 2A and 2X, express MHC2 (A) and MHC1(C) respectively, fast glycolytic fibers, type 2B, express MHC4 (B), and slow oxidative fibers, type 1A, express MHC7 (B), (NBW/CD n=7, NBW/WD n=6, LBW/CD n=5, LBW/WD n=5, df=22). Data presented as means ± SEM (significance determined by a two-way ANOVA with Bonferroni post-hoc test,*p<0.05; ***p<0.001).

3.3 Hypoxia and Fatty Acid Induced Alterations in Mitochondrial Function in C_2C_{12} Cells

3.3.1 Hypoxia reduced oxygen consumption which persisted after normoxic recovery

Rates of oxygen consumption were measured using the Oxygraph-2k, within the airtight chamber at 50µM O_2 (5% O_2). Five days of differentiation under 1% oxygen decreased respiration rates by 67% when compared to cells cultured under 5% oxygen ($p<0.001$; figure 3.3-1A). Depressed rates of oxygen consumption were not ameliorated after two days of normoxic recovery ($p<0.001$; 3.3-1A). Similar results were seen when oxygen consumption was measured with 10µM O_2 within the chamber ($p<0.001$; figure 3.3-1B), and again recovery failed to alleviate the reduction in respiration rate ($p<0.001$; figure 3-6B).

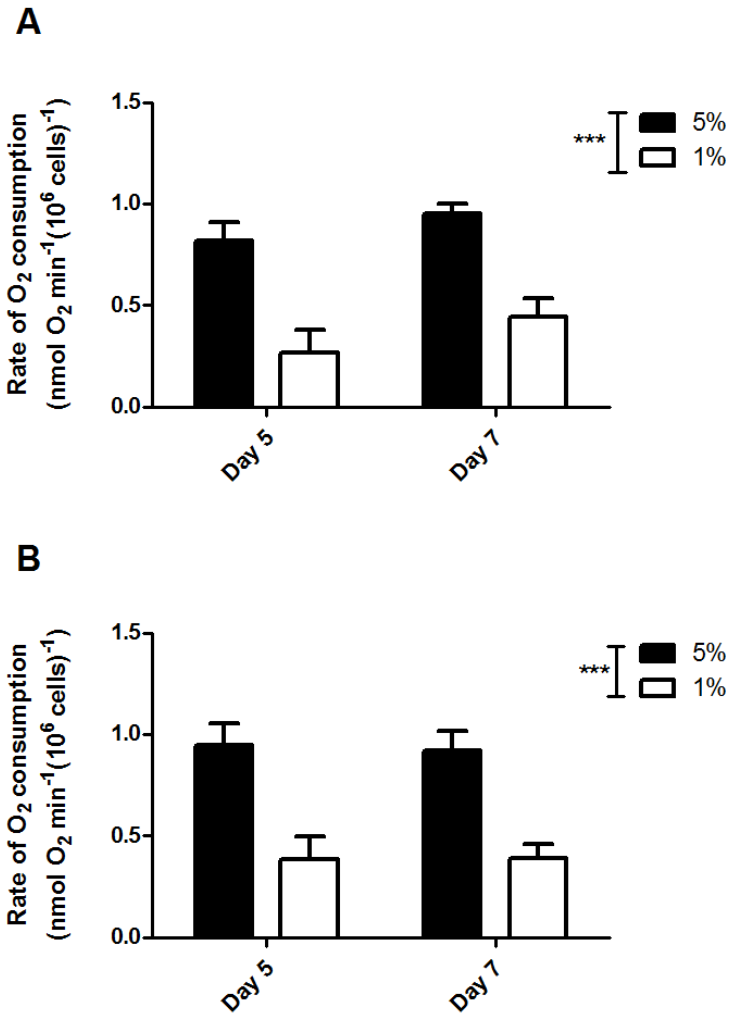

Figure 3.3-1 Rate of oxygen consumption in C_2C_{12} cells cultured under hypoxia using the Oxygraph-2k. Respiration rates measured at (A) 50µM (PO_2 38mmHg) oxygen in the chamber after 5 days of differentiation and after 48 hours recovery (i.e. Day 7) and at (B) 10µM (PO_2 7.6mmHg)oxygen in the chamber after 5 days and after 2 days recovery (i.e. Day 7). Data are presented as means ± SEM, significance determined by a two-way ANOVA with a Bonferroni post-hoc test***$p<0.001$ (n=6 for cells cultured in 5% O_2, n=5 for cells cultured in 1% O_2; df=21).

After 5 days of differentiation at either 5% or 1% O_2 cells were exposed to different fatty acids for 24-hours at 20.9% O_2, and the effects on oxygen consumption were measured. Exposure to 0.5mM saturated fatty acid (palmitate, C16:0) during recovery reduced the rate of oxygen consumption by 47% in 5% O_2 cultured cells when compared to BSA controls ($p<0.05$) at 50µM O_2 within the chamber. By contrast palmitate had no significant effect on cells differentiated at 1% (Figure 3.3-2A). Addition of 0.5mM monounsaturated fatty acid (palmitoleate; C16:1) had no effect on oxygen consumption of either 5% or 1% cultured cells. Similar results were observed with 10µM O_2 within the chamber; oxygen consumption was reduced by 60% with the addition of palmitate, on 5% cultured cells only (Figure 3.3-2B).

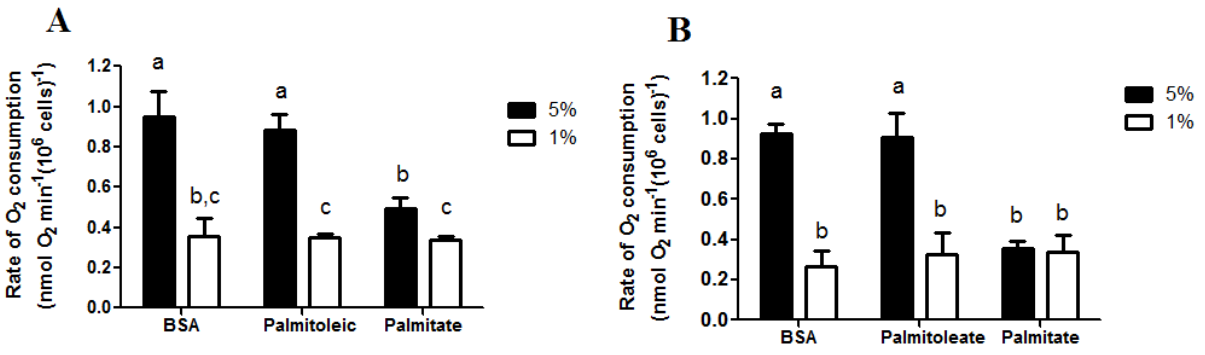

Figure 3.3-2 Rates of oxygen consumption of cells cultured in 5% and 1% O_2 after 24 hours 0.5mM fatty acid exposure (SFA palmitate C16:0, UFA palmitoleate C16:1 or BSA control) at 20.9% O_2. Rates were measured with 50µM (PO_2 38mmHg; A) and 10uM (PO_2 7.6mmHg; B) within the Oxygraph chamber. Measurements taken using the Oxygraph-2k with Clark-type electrodes at 37°C in a 10µM glucose solution in standard buffer. Data analyzed using a two-way ANOVA with a Bonferroni post-test. Data are presented as means ± SEM groups with different letters are significant at the 95% level (n= 6 for all cells cultured at 5% O_2, n=5 for all cells cultured at 1% O_2; df=32).

3.3.2 Mitochondrial DNA Was Not Altered by Hypoxia

Mitochondrial DNA was extracted from C_2C_{12} cells at days 2 and 5 of differentiation and after recovery (day 7). Using primers specific to the mitochondrial DNA D-Loop sequence, quantitative PCR was used to determine mtDNA content. Relative to β-actin mRNA expression, there was no significant difference in mtDNA content at any of the time points measured between the hypoxic and normoxic cultured cells (Figure 3.3-2)

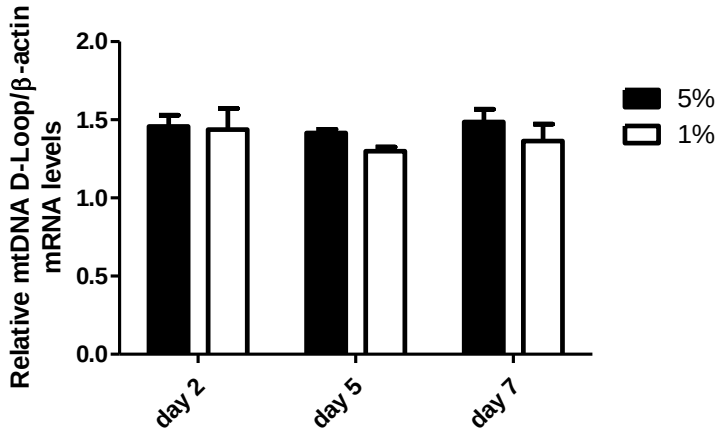

Figure 3.3-3 Mitochondrial DNA unaltered after 2 and 5 days of differentiation or after recovery in hypoxic cultured cells. Mitochondrial DNA was extracted using liquid-liquid phase separation and subsequently amplified using qRT-PCR and normalized to β-actin mRNA expression. Data were analyzed using a two-way ANOVA and a Bonferroni post-test (n=6 for all groups; df=35). Data presented at means ± SEM

4 Discussion

4.1 IUGR is Associated with Altered Soleus Oxygen Consumption

The metabolic alterations in peripheral tissue, such as skeletal muscle, resulting from low oxygen and nutrient supply to the fetus are thought to underly the relationship between IUGR and later-onset metabolic disorders (Arbeille *et al.*, 1995; Pardi *et al.*, 2002; de Boo and Harding, 2006). In this study I present data that support the contention that fetal soleus muscle, an oxidative muscle, is negatively altered following uterine artery ablation. My data suggests that growth restriction following UAA leads to immediate disruption of mitochondrial metabolism in the soleus muscle, which is indicative of an *in utero* reprogramming event. While there is a significant reduction in pyruvate-fueled state 3 respiration in fetal soleus (figure 3.1-2) a reduction in glutamate oxidation (figure 3.1-2) which only becomes evident at 4 months of age (figure 3.2-3). Before glutamate can be metabolized in the TCA cycle and enter OXPHOS as NADH it must be converted to α-ketoglutarate by glutamate dehydrogenase (GDH). An adaptive response of low glutamate conversion to α-ketoglutarate in IUGR pregnancies may account for this observation as glutamate is present in larger quantities in circulation of IUGR pregnancies, however; GDH expression is significantly lower in IUGR placentae and liver (Jozwik *et al.*, 2009). I speculate GDH expression may be altered in the soleus however further work would be needed to better understand this relationship.

4.2 IUGR and Diet Independently Reduce Soleus Oxygen Consumption

Rates of mitochondrial oxygen consumption for 4-month-old animals were measured in permeabilized soleus muscle as it is comprised of mainly oxidative fiber types and thus produces most of its ATP by the aerobic process of OXPHOS. Both LBW and WD treatments reduced maximal (state 3) oxygen consumption when pyruvate and glutamate were used as metabolic substrates (Figure 3.3-3) but these effects were not additive as no further reductions in oxygen consumption were noted in the LBW/WD group. Additionally, no changes in oxygen consumption across any treatments were noted following succinate administration, suggesting a loss of function at complex I, where electrons from NADH (downstream product of pyruvate and glutamate) enter the ETC but maintained function of complex III (where electrons from succinate enter the ETC). Furthermore, I found no differences in citrate synthase activity, suggesting a similar number of functional mitochondria among treatment groups. Taken together, these data suggest reduced oxygen consumption in IUGR and WD can be attributed to impaired complex I activity rather than a reduction in mitochondrial number. This is supported in the present study by reduced protein expression of mitochondrial subunit NDUFB8 in all treatment groups (Figure 3.2-4A). A recent study of rat heart tissue however saw no changes in NDUFB8 expression in IUGR groups (Beauchamp *et al.*, 2015) while still noting a decrease in state 3 respiration. This discrepancy may be explained by comparing *in utero* blood flow, while the heart is spared, skeletal muscle exhibits a marked decrease in blood flow (Phillips *et al.*, 1994; Hales and Barker, 2001). Reduced NDUFB8 could therefore be an example of persistent epigenetic reprogramming target as a consequence of a poor *in utero* environment. Previous studies have determined that complex I proteins are in fact the target of epigenetic modification, for example mtDNA protein MT-ND6 is differentially methylated in NAFLD (Pirola *et al.*, 2013) and that microRNA miR-663 alters the expression of complex I protein NDUFAF1 (Carden *et al.*, 2017).

Other than complex I, complex III protein expression was the only other complex affected by my treatments. Where complex I protein expression was reduced by both

treatments, complex III protein expression was only reduced in the LBW group (Figure 3.2-4 C). This reduction in complex III expression does not seem to have a functional consequence in terms of oxygen consumption, as LBW animals do not exhibit a further decrease in oxygen consumption compared to WD animals (Figure 2.3-3), and there was no difference in state 3 rates with succinate as a substrate. Together, these results suggest that the reduction of cytochrome *c* by reduction of coenzyme Q by complex III is not a rate limiting step in SM oxidative phosphorylation.

4.3 Soleus Myosin Heavy Chain Expression Altered by Birth Weight and Diet

In conjunction with changes in oxygen consumption as a result of LBW expression of myosin heavy chain isoforms indicates a change in skeletal muscle fiber types. In the Guinea pig, MHC 7 is present in oxidative type 1 fibers, MHC 2 in oxidative type IIa fibers, MHC 1 in oxidative type IIx fibers and MHC 4 in glycolytic type IIb fibers (Tonge *et al.*, 2010). At the mRNA level, I observed an increase in MHC 4 expression which alone would support my hypothesis that a poor *in utero* environment alters fiber type composition but is not consistent with the mRNA expression pattern of the other MHC isoforms. If IUGR were to favor a glycolytic fiber type, it could be expected to see an increase in MHC 7 or MHC 2 in LBW/WD animals. The fact this was not the case could be explained by noting my data is at the mRNA level and these results could therefore a compensatory mechanism in the event that translation of these messages is downregulated. Furthermore, mRNA expression of MHC isoforms does not support the results of the citrate synthase activity assay, which suggests similar functional mitochondrial number across all groups. If the fiber types differ between groups as the mRNA would suggest, mitochondrial activity would be expected to differ between groups as well given that mitochondrial density is dictated by fiber type. Studies in rabbits and chicks found differences in CS activity between muscle groups considered to be of different fiber types (Howlett and Willis, 1998; Hakamata *et al.*, 2018) however a

study in highly trained athletes suggests that CS activity does not change with MHC isoform expression (Ørtenblad *et al.*, 2018) therefore CS activity alone cannot be used as a good marker for fiber type composition.

Many studies have shown that a high-fat diet increases protein expression of glycolytic fiber compared to oxidative fibers (Tanner *et al.*, 2002; Ten Broek *et al.*, 2010). While my mRNA data do not agree with these observations, we speculate that translational and post-translational regulation may determine fiber types composition for both diet and IUGR treatments. Further studies identifying protein expression of the MHC isoforms are needed to determine if fiber type is truly altered or if there is only a change in mitochondrial function rather than number as my CS assay would suggest (figure 3.2-5).

4.4 Hypoxic Regulation of C_2C_{12} Oxygen Consumption

Hypoxia has been identified as a contributing factor influencing placental insufficiency and growth restriction (Genbacev *et al.*, 1997; Thornburg and Louey, 2005; Ream *et al.*, 2008; Yinon *et al.*, 2008; Tal *et al.*, 2010). Oxygen homeostasis is critical to cellular function as oxygen availability plays a key role in oxidative phosphorylation, the process responsible for producing the majority of ATP. HIF-1α degradation is regulated by cellular oxygen concentration (Wang *et al.*, 1995) and alters metabolic gene expression. Under normoxic conditions, HIF-1 α is degraded by hydroxylation of two proline residues in the oxygen-dependent degradation domain, which does not occur in hypoxia (Wang *et al.*, 1995). When HIF-1 α is not degraded it dimerizes with HIF-1β which transcriptionally activates anaerobic, glycolytic enzymes phosphoglycerate kinase 1 and pyruvate kinase (PDK1; Kim *et al.*, 2006). Increased availability of HIF-1α is associated with increased activation of PDK1 (Kim *et al.*, 2006), a kinase that reduces pyruvate entry into the TCA cycle by inhibitory phosphorylation of pyruvate dehydrogenase. By inhibiting pyruvate entry into the TCA cycle, there is a decrease in NADH and FADH production when oxidizing carbohydrates and a corresponding decrease in ATP

production via OXPHOS, unless increased FA oxidation can compensate. Low cellular oxygen availability therefore results in increased anaerobic ATP through glycolysis and decreased aerobic ATP production from OXPHOS. The persistent reduction in rates of oxygen consumption seen in figure 3.4-1 during recovery, when provided with glucose as a substrate, is indicative of a reprograming event; after the hypoxic insult is removed, its decreased glucose oxidation capacity is still present. These data suggest a hypoxic *in utero* environment may result in permanently altered skeletal muscle metabolism.

4.5 Impact of Fatty Acids on Oxygen Consumption of Hypoxic Cultured Cells

To further assess the effects of hypoxia *in utero*, a second insult of saturated fatty acid palmitate was added to the media of cells recovering from hypoxia, I used palmitate as it is a prevalent SFA found in the WD. The saturated fatty acid reduces the oxygen consumption of normoxic cultured cells by 47% compared to BSA controls, whereas it had no effect on hypoxic cultured cells when given glucose as a substrate. SFAs are associated with decreased oxidative metabolism in heathy skeletal muscle (Gao *et al.*, 2011), consistent with results in Figure 3.3-2. Furthermore, a rat study found that palmitate promotes oxidative stress and apoptosis in hepatocytes indicating that dietary SFAs may alter oxidative metabolism by altering either reactive oxygen species production of detoxification (Egnatchik *et al.*, 2014). Of interest, however; is the lack of further decrease in oxygen consumption in the group treated with both hypoxia and SFA, which may indicate that hypoxia reduces OXPHOS to minimal functional levels. Conversely, palmitoleate a monounsaturated fatty acid which has the same number of carbons as palmitoleate and is necessary for cellular structure and function did not have any inhibitory or stimulatory effects on oxygen consumption of either normoxic or hypoxic cultured cells. By contrast palmitoleate has been found to increase oxygen consumption in white adipocytes (Cruz *et al.*, 2018).

4.6 Potential Limitations

All studies have limitations and must be acknowledged. First, the animal and cell culture models present limitations of their own. The C_2C_{12} mouse myoblast model, while excellent for the isolation of hypoxic effects during myoblast differentiation, is limited in a few ways. First, the cell line is an adult cell line but it was used to simulate *in utero* differentiation. I used these cells because methods concerning hypoxic differentiation and fatty acid treatments are well established. Second, the C_2C_{12} cell line does not permit studies of longevity. Cell viability declines past 7 days following differentiation therefore long-term effects of hypoxia on skeletal muscle differentiation could not be determined in the present study. Furthermore, oxygen tensions representing physiological normoxia (5%, 38mmHg) and hypoxia (1%, 7.6mmHg) were chosen based on experimental umbilical cord data (Wilkening and Meschia, 1992; Regnault *et al.*, 2007). Oxygen tensions in skeletal muscle is variable, a study found the mean PO_2 of 4mmHg in intracellular guinea pig muscle (Whalen and Nair, 1967).

Finally, this study is limited in its determination of skeletal muscle fiber type composition as mRNA expression was measured. Protein expression would have provided a clearer understanding of the soleus muscle fiber type as it is defined by the isoform of MHC protein expressed (Tanner *et al.*, 2002; Ten Broek *et al.*, 2010). Unfortunately, I was unable to successfully bind available MHC antibodies to cross-react with my tissue samples. Comparing MHC isoform protein expression with my CS activity and oxygen consumption would present a clearer idea of whether and how muscle fiber type composition is affected by IUGR and post-natal diet.

4.7 Future Studies

In this study I present evidence which supports that both an adverse *in utero* environment and hypoxic specific insults negatively affect skeletal muscle oxygen consumption. Additionally, I provide evidence that the postnatal diet, one high in fats also impairs muscle oxidative function. Specifically, I have identified that both a reduction in complex I protein expression and activity are related to both birthweight and diet. With that identified, the next logical progression is to identify and implement potential rescue treatments.

As previously mentioned, fiber type determination was unclear and inconsistent with my CS activity assay. Future studies should investigate the protein expression of MHC isoforms to identify if there is a marked shift in SM fiber type composition. Furthermore, previous studies have found a shift towards a more glycolytic fiber type in animals with IR and type 2 diabetes, thus it would be valuable to determine in a longitudinal study if similar findings are observed with my model, as my 4-month-old animals do not yet exhibit signs of such metabolic disorders (data not shown).

In my cell culture studies, I found that the SFA palmitate reduced oxygen consumption in normoxic cells and that the MUFA palmitoleate failed to rescue oxygen consumption in hypoxic cells. Treatments with PUFAs should be explored as they have been identified as potentially beneficial to metabolism in other studies. Also, molecular analyses of specific enzymes responsible for the observed decrease in oxygen consumption as a response to SFA exposure would facilitate the identification of potential treatment targets such as antioxidants.

4.8 Conclusions

In summary the results support my first hypothesis; SM oxygen consumption is decreased in LBW fetal guinea pigs compared to NBW animals (figure 3.2-1). Interestingly, reduced oxygen consumption in LBW fetal guinea pigs is observed following pyruvate administration but not glutamate both of which are reduced in 4-month-old animals (figure 3.2-2) and both metabolized by complex I of the ETC. The discordance in glutamate metabolism between fetal and 4-month-old animals may be explained by an adaptive response of lower GDH expression in low oxygen conditions (Jozwik *et al.*, 2009). The observed decrease in NDUF8 expression further supports these findings (3.2-4). Furthermore, it is of note that succinate metabolism was unaffected by birth-weight (Figure 3.2-1), suggesting complex II function remains intact in UAA animals, which is consistent in 4-month-old animals (Figure 3.2-2). Together, these results support an *in utero* reprograming event in response to PI resulting in decreased expression and activity of complex I and reduced oxidative capacity. Similar overall reductions in skeletal muscle oxidation have been linked to insulin resistance and type 2 diabetes (He *et al.*, 2001; Selak *et al.*, 2003). These findings may be a physiological contributor to the observed correlation between low birth weight and later-onset metabolic disorders such as insulin resistance and type 2 diabetes (Simmons *et al.*, 2001; Barker, 2004; Valsamakis *et al.*, 2006).

When measuring soleus oxygen consumption, no further reduction in rate of oxygen consumption was observed when comparing LBW animals on a control diet to LBW animals on a Western diet. Perhaps the more intriguing result is that the Western diet had a similar effect to the LBW further emphasizing that this physiological response of reduced oxygen consumption plays an important role in uncovering the link between IUGR and metabolic disorders, given that the Western diet is a primary contributor in the development of IR, type 2 diabetes and other metabolic diseases (Wirfalt *et al.*, 2001; James *et al.*, 2004; Cordain *et al.*, 2005). Given the common outcome of reduced oxygen consumption in both LBW and WD groups, both are forms of oxidative stress which I found to target complex I (figure 3.2-4).

In contrast to my postulation, I did not observe a clear link between fiber type composition and hypoxia (figure 3.3-4), however; fiber type was studied at the messenger level and not as protein expression. While an increased proportion of glycolytic fibers is associated with obesity and type 2 diabetes (He *et al.*, 2001; Tanner *et al.*, 2002) it was not supported at the mRNA level following a WD or in LBW animals. Moreover, CS activity did not differ between these groups (figure 3.2-5) further supporting that these animals do not differ in fiber type composition. However, these fiber type results should be confirmed by protein analysis in order to rule out a possible correlation between LBW and fiber type composition.

Lastly, the hypoxia-specific cell culture experiments supported the hypotheses: cells cultured under hypoxic conditions had lower rates of oxygen consumption even following re-oxygenation (figure 3.4-2) suggesting that hypoxic exposure during differentiation leads to persistent reductions in oxygen consumption, within the time period studied. As predicted, the addition of saturated fatty acid palmitate reduced oxygen consumption in normoxic cultured cells but had no further negative effects on hypoxic cultured cells (figure 3.4-3) which is consistent with the data obtained from the guinea pig offspring studies. It appears that both the hypoxic insult and diet insult produce similar effects, but interestingly are not additive, suggesting perhaps a lower threshold for oxygen consumption in skeletal muscle is reached with either insult. Unlike the saturated fatty acid treatment, monounsaturated fatty acids did not yield the anticipated results: palmitoleate did not significantly affect oxygen consumption (figure 3.4-3). These results suggest that a diet rich in monounsaturated fatty acids will not mitigate the reduction in oxygen consumption resulting from hypoxia during differentiation and that other treatments should be explored.

All together the results of the above described studies suggest that hypoxia *in utero* is a potential mechanism for predisposition to MS with similar but not additive effects of a WD. This study identified that IUGR and a post-natal WD both independently reduce skeletal muscle oxygen consumption which may contribute to the observed link between an adverse *in utero* environment and the development of adult-onset metabolic disorders.

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Appendix

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.

Curriculum Vitae

Name: Megan Cedrone

Post-secondary Education and Degrees:
Western University
London Ontario, Canada
2008-2012 B.Sc.

The University of Western Ontario
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2012-2019 M.Sc. Candidate

Related Work Experience:
Teaching Assistant
The University of Western Ontario
2012-2014

Let's Talk Science Classroom Leader,
The University of Western Ontario Chapter
2013-2014

Junior High Teacher
Montessori Academy of London
2018- present

Research Presentations:

Cedrone M., Staples J.F., Regnault, T.R. Intrauterine growth restriction and post-natal diet induced alterations in oxidative capacity. **Poster presentation** at the *Aspen/Snowmass Perinatal Biology Symposium* August 2013, ***Aspen Colorado***.

Cedrone, M., Staples, J.F., Regnault, T.R. Low birth weight and postnatal diet induced alterations in skeletal muscle oxidative capacity. **Poster presentation** at the *London Health Research Day* March 2014, ***London Ontario***.

Cedrone, M., Dunlop, K., Staples, J.F., Regnault, T.R. Altered skeletal muscle function & metabolism in offspring of an adverse in utero environment following a high fat/high sugar postnatal diet. **Oral Presentation** at the *Lawson Health Research Talks on Fridays* April 2014, ***London Ontario***.

Cedrone, M., Staples, J.F., Regnault, T.R. Low birth weight and postnatal diet induced alterations in skeletal muscle oxidative capacity. **Poster presentation** at the *Southern Ontario Reproductive Biology Research Day* May 2014, ***Toronto Ontario***.

Publications:

Dunlop, K., **Cedrone, M**., Staples, J. F., & Regnault, T. R. (2015). Altered fetal skeletal muscle nutrient metabolism following an adverse in utero environment and the modulation of later life insulin sensitivity. Nutrients, 7(2), 1202–1216. doi:10.3390/nu7021202