Low birth weight is associated with impaired skeletal and cardiac muscle energetics in adult mice

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Abstract

In utero undernutrition is associated with increased risk for insulin resistance, obesity, and cardiovascular disease during adult life. A common phenotype associated with low birth weight is reduced skeletal muscle mass. Given the central role of skeletal muscle in whole body metabolism, we hypothesized that predisposition to metabolic disease is, in part, due to low oxidative capacity and dysfunctional mitochondrial energetics in muscle. We used an experimental mouse model system of maternal undernutrition during late pregnancy to examine female offspring from undernourished dams (U) and control offspring from *ad libitum* fed dams (C). U have increased adiposity and decreased glucose tolerance compared to C. Strikingly, when U are put on a 4 week 40% calorie restricted diet they lose half as much weight as calorie restricted controls. Skeletal muscle mitochondria from U have decreased coupled and uncoupled respiration and increased maximal respiration compared to C. In permeabilized fiber preparations from mixed fiber type muscle, U have decreased mitochondrial content and decreased adenylate free leak respiration, fatty acid oxidative capacity, and state 3 respiratory capacity through complex I. Fiber maximal oxidative phosphorylation capacity does not differ between U and C.

We next aimed to determine if the impaired skeletal muscle energetics observed in U also exist in primary muscle cells derived from these mice. We measured oxidative and glycolytic capacities in primary myotubes from U and C using cellular bioenergetics. Myotubes from U have decreased resting respiration and increased glycolysis

compared to myotubes from C. There was no difference in myotube mitochondrial content. Findings suggest that undernutrition *in utero* causes a primary muscle defect.

Energetics in cardiac muscle were also examined. U have impaired cardiac muscle homogenate energetics, including decreased fatty acid oxidative capacity, decreased maximum oxidative phosphorylation rate, and decreased proton leak respiration. Additionally, we measured plasma acylcarnitine levels and found that short-chain acylcarnitines are increased in U. Overall, results reveal that *in utero* undernutrition alters metabolic physiology through a profound effect on skeletal muscle and cardiac muscle energetics. These effects may be mediated by epigenetic mechanisms which could be explored in future research.

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List of Abbreviations

ACC: acetyl-coA carboxylase

ADP: adenosine diphosphate

AMPK: AMP-activated protein kinase

ANOVA: analysis of variance

ANT: adenine nucleotide translocase

ATP: adenosine triphosphate

BM: basic medium

BMI: body mass index

BMR: basal metabolic rate

BSA: bovine serum albumin

C: control offspring from *ad libitum* fed dams

C-L: control offspring fed *ad libitum*

COX: cytochrome c oxidase

CPT I: carnitine palmitoyltransferase I

CR: calorie restriction

C-R: control offspring that were calorie restricted for 4 weeks during adulthood

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle's Medium

DNA: deoxyribonucleic acid

ECAR: extracellular acidification rate

ETF: electron transferring flavoprotein

F1: first filial generation

F2: second filial generation

F3: third filial generation

$FADH_2$: flavin adenine dinucleotide (reduced)

FBS: fetal bovine serum

FCCP: carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone

FFM: fat free mass

FTO: fat mass and obesity associated

GLUT4: glucose transporter 4

GR: glucocorticoid receptor

gWAT: gonadal white adipose tissue

IUGR: intrauterine growth restriction

LEPR: leptin receptor

L_N: adenylate free leak respiration

L_{omy}: oligomycin-induced leak respiration

LPL: lipoprotein lipase

MHC: myosin heavy chain

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

NAD: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide (reduced)

OCR: oxygen consumption rate

P_{CI}: submaximal state 3 respiratory capacity through complex I

P_{CI+CII}: maximum oxidative phosphorylation capacity

PCr: phosphocreatine

PDH: pyruvate dehydrogenase

Pdx-1: pancreatic and duodenal homeobox 1

P_{ETF}: maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity

PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α

P_{OXPHOS}: maximum oxidative phosphorylation

PPARα: peroxisome proliferator-activated receptor-α

rGAS: red *gastrocnemius*

RER: respiratory exchange ratio

RIPA buffer: radioimmunoprecipitation assay buffer

RNA: ribonucleic acid

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel ectrophoresis

SEM: standard error of the mean

T2DM: type 2 diabetes mellitus

TA: *tibialis anterior*

TCA cycle: tricarboxylic acid cycle

U: offspring from undernourished dams

UCP3: uncoupling protein 3

U-L: *in utero* undernourished offspring fed *ad libitum*

U-R: *in utero* undernourished offspring that were calorie restricted for 4 weeks during adulthood

Vinc: vinculin

wGAS: white *gastrocnemius*

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Chapter 1: General Introduction

Obesity, the condition of having an excessive accumulation of fat in the body, is a prevalent and expanding health threat in today's society. It is tightly associated with insulin resistance, which precedes and predicts the development of type 2 diabetes mellitus (T2DM) (1-3). The prevalence of obesity and T2DM has increased at an alarming rate over recent years and is predicted to continue to increase in the years to come. Since 1980, obesity worldwide has more than doubled. In 2014, more than 1.9 billion adults were overweight (39% of adults; Body mass index (BMI)≥25) and over 600 million were obese (13% of adults; BMI >30) (4). As of 2013, there were more than 382 million people living with diabetes (9% of adults), approximately 90% of whom were living with T2DM (5). Currently, it's projected that by 2030 obesity rates will double and the prevalence of T2DM will increase by over 50% (4, 5). In addition to T2DM, obesity represents a major risk factor for the development of cardiovascular disease, hypertension, dyslipidemias, some cancers, and mortality (as reviewed in (6)). Evidently, this presents a significant public health problem with global societal and economic impact.

Energy metabolism

Energy expenditure by the body is continuous and is supported by dietary energy intake. This energy is needed for a vast number of biological processes including

mechanical work such as contraction of skeletal muscle, repair and maintenance of cells and tissues, growth, and molecular transport. All of the cells of the body require energy to carry out their specific functions. The majority of energy consumed comes from the macronutrients: carbohydrates, lipids, and proteins. These macronutrients are digested to liberate their simpler molecules, sugars, fatty acids, and amino acids. Ultimately, these molecules are metabolized to generate ATP, often referred to as the energy currency of the cell. The dephosphorylation of ATP to ADP is a very spontaneous reaction that can be coupled to many endergonic reactions, thereby enabling their activity.

Glucose metabolism

Simple sugars, primarily glucose from dietary complex carbohydrates, are absorbed by the intestine and enter the bloodstream. Most insulin-stimulated peripheral glucose uptake takes place in skeletal muscle and adipose tissue, with 80-90% of post-prandial glucose uptake occurring in skeletal muscle (7, 8). Glucose is mainly taken up into these tissues by glucose transporter 4 (GLUT4), which moves to the plasma membrane in response to insulin (9-12). Glucose can be metabolized aerobically, using oxygen, or anaerobically, without oxygen.

Aerobic respiration of carbohydrate substrates occurs via the major metabolic pathways of glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. The TCA cycle and oxidative phosphorylation occur in mitochondria,

often described as the powerhouses of the cell, as they are the major site of energy conversion to ATP. Glucose is broken down into pyruvate by the glycolytic pathway, producing ATP and $NADH+H^+$. Pyruvate can then be converted to acetyl-CoA to produce ATP, $NADH+H^+$, and $FADH_2$ via the TCA cycle. Reducing equivalents associated with $NADH+H^+$ and $FADH_2$ are then transferred to the electron transport chain, located in the mitochondrial inner membrane, the main site of ATP production. Electrons are transferred through a series of macromolecular complexes, the respiratory chain complexes. Electrons are passed through oxidation-reduction reactions until they are accepted by molecular oxygen, which finally generates water. As the electrons are shuttled from complex to complex, protons are pumped out of the mitochondrial matrix by complexes I, III, and IV into the intermembrane space generating an electrochemical gradient, referred to as proton motive force. Protons can return to the matrix by ATP synthase, which uses the energy released to synthesize ATP from ADP. The fact that ATP synthase is estimated to produce roughly 70 kg of ATP daily, clearly demonstrates the importance of this process (13). However, the process of coupling proton extrusion to proton return through ATP synthase is not perfect and some protons leak back across the membrane through other proton conductance pathways. Proton leak from the intermembrane space to the matrix decreases the protonmotive force and generates heat instead of ATP, thus making the process inefficient. It is estimated that 20-25% of an individual's metabolic rate may be attributed to metabolic inefficiency, such as proton leak (14). This process requires activation of mitochondrial anion carrier protein function which can be catalyzed by adenine nucleotide translocase and the uncoupling proteins (15-17).

If insufficient oxygen is available, such as during vigorous exercise, muscle can produce ATP anaerobically. During anaerobic respiration, or lactic acid fermentation, pyruvate is reduced to lactate thereby generating NAD+, which can then be used to accept reducing equivalents in order to sustain glycolytic activity. However, anaerobic respiration is not nearly as efficient as aerobic respiration, releasing a much smaller yield of ATP.

Fatty acid metabolism

In the small intestine, fatty acids are emulsified with bile salts and converted back to triglycerides. Triglycerides are then packaged with phospholipids, cholesterol, and apolipoproteins to make chylomicrons which enter the bloodstream via the lymphatic system. Lipoprotein lipase degrades the triglyceride into fatty acids and glycerol which can then be taken up into tissues. Fatty acids are transported across the mitochondrial outer membrane by carnitine acyl transferases and then across the mitochondrial inner membrane by carnitine. Free fatty acids undergo β-oxidation in the matrix, where the fatty acids are broken down to generate acetyl-CoA and reducing equivalents. Acetyl-CoA can then enter the TCA cycle and the reducing equivalents can be used by the electron transport chain. Compared to the other macronutrients, carbohydrate and protein, fatty acids yield the most ATP per gram.

Amino acid metabolism

Proteins play many vital roles in the body and a continuous supply is needed for growth, repair, and other functions. Proteins are required for the formation of structural tissues, hormones, antibodies, enzymes, and transport molecules. The body does not store excess protein therefore if protein intake exceeds the body's requirement, amino acids are converted to other intermediates. Most amino acids can be converted in the liver to glucose or ketone bodies, which can then be used as a fuel source.

Energy storage

Living organisms require a continuous supply of energy to carry out their functions. However, food is usually consumed in the form of distinct meals periodically over the course of a day; therefore dietary energy must be metabolized and stored for its use between meals. Ultimately, carbohydrates, proteins, and fats are transformed *in vivo* to substrates that produce metabolically useful energy or are stored. Despite the fact that most of dietary energy is typically in the form of carbohydrates, the main form of energy storage in the body is triglycerides. A much smaller amount of energy is stored in the form of glycogen. The largest glycogen stores are found in liver and skeletal muscle while the majority of triglycerides are stored in adipose tissue. Glycogen stores in the liver can be quickly mobilized to provide the body with glucose. In muscle, glycogen is converted into glucose to meet the energy needs of the muscle itself.

Fatty acids are bound to glycerol to produce triglycerides, which are stored primarily in adipocytes. Stored triglycerides can be used for energy by tissues. Adipose triglyceride lipase initiates the hydrolysis of stored triglycerides, producing glycerol and nonesterified fatty acids. These fatty acids are then bound to albumin for transport to tissues such as skeletal muscle, which can then use fatty acids for respiration. The amount of energy stored in the body varies greatly between individuals and is dependent upon factors such as size, food intake and physical activity.

Obesity – an energy imbalance

As with all other isolated systems, the body must obey the first law of thermodynamics which states that energy can neither be created nor destroyed. Therefore, the total energy taken in must be equal to energy expended with any remaining energy being stored. Obesity is the result of an energy imbalance, where energy intake exceeds energy expenditure over a sustained period of time. In the long-term, this results in energy storage in the form of triglycerides in adipose tissue. This imbalance arises when the amount of energy consumed, from fats, carbohydrates, proteins and alcohol, exceeds the amount of energy expended in the normal maintenance of obligatory cellular processes and in physical activity (and growth, during youth and pregnancy). Mitochondria play a vital role in metabolism, linking the oxidation of energy supplying nutrients to the production of cellular ATP. As ATP is essential for most cellular processes, mitochondrial function plays an important role in metabolic health. The regulation of mitochondrial function is complex and is still poorly understood but it

involves adaptations such as protein post-translational modifications, supercomplex assembly, fission and fusion, mitophagy, and mitochondrial biogenesis (18-20). Mitochondrial dysfunction is implicated in many disease states, including obesity and T2DM (18, 21).

Basal metabolic rate

The largest contributor to whole body energy expenditure is the basal metabolic rate (BMR) (22). BMR is the amount of energy expended at rest, in the post-absorptive state at thermoneutrality. Additional contributors to whole body energy expenditure are attributed to physical activity, growth, cold-induced thermogenesis, and diet-induced thermogenesis (the increase in energy expenditure after meals for digestion, absorption, and fuel storage). Every organ of the body contributes to BMR, however, some organs such as the brain, kidney, and heart contribute a larger portion of BMR relative to their mass compared to others such as adipose tissue and skin (22). Lean body mass, primarily skeletal muscle, is known to be the best predictor of BMR (22, 23). Skeletal muscle comprises approximately 40% of the body mass in an adult human and although its metabolic rate per gram of tissue is relatively low, it greatly contributes to BMR due to its high fractional contribution to body mass (22, 23). Therefore, differences in skeletal muscle metabolism have potentially substantial implications in determining one's susceptibility to obesity. Furthermore, skeletal muscle is the largest insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose utilization (7).

Skeletal muscle

Skeletal muscle has the ability to adapt and respond to its environment and physiological challenges by changing its phenotype in terms of size, composition, and aerobic capacity, outcomes that are brought about by changes in gene expression, biochemical, and metabolic properties (24-26). As such, skeletal muscle can modify its functional characteristics to adapt to metabolic need. For example, with regular exercise training muscle can increase its size, mitochondrial content, and respiratory capacity and becomes more resistant to fatigue (27, 28). In contrast, a loss of skeletal muscle mass and function is associated with a sedentary lifestyle and immobilization (29-31). Skeletal muscle is made up of heterogeneous types of myofibers that differ in their metabolic characteristics. Muscle fibers are traditionally classified into two broad categories according to myosin heavy chain isoforms: type I and type II. Type I myofibers are very oxidative/aerobic, have high mitochondrial and capillary densities, have high myoglobin content, and are resistant to fatigue (32). In contrast, type II myofibers are more glycolytic, have low mitochondrial and capillary densities, have low myoglobin content, and are sensitive to fatigue (32). Type II fibers are now known to be heterogeneous with type IIa fibers being highly aerobic and type IIx fibers being glycolytic. Within humans, there is considerable variability in fiber type proportions (33, 34). Evidence is now emerging in support of the notion that differences in muscle fiber composition may play an important role in determining susceptibility to obesity (35-37). It has been shown that skeletal muscle of obese subjects has reduced oxidative

capacity, increased glycolytic capacity, and a relatively lower proportion of type I fibers (36, 38-40).

Skeletal muscle metabolism in obesity and insulin resistance

Disordered skeletal muscle metabolism is associated with the adverse metabolic complications of obesity and T2DM. Insulin resistance and metabolic dysfunction are prominent muscle abnormalities in these disease states. Insulin maintains glucose homeostasis by promoting glucose uptake in skeletal muscle and suppressing glucose production in the liver. Insulin resistance is defined as a reduced response of target tissues, such as skeletal muscle, adipose tissue and liver, to insulin and remains tightly associated with obesity. Generally, it is thought that as people become obese their tissues become resistant to the actions of insulin. Pancreatic β-cells respond by increasing their output of insulin but over time the β-cells fail to adequately compensate for insulin resistance. As a result, blood glucose levels rise and insulin resistance progresses to T2DM. However, it remains unclear how insulin resistance arises in obesity. Many factors have been shown to contribute to obesity associated insulin resistance and it is likely that it is the interplay between multiple factors that causes the pathophysiolgoy of insulin resistance. Two of these factors include ectopic fat storage and mitochondrial dysfunction.

Ectopic fat accumulation is the storage of triglycerides in non-adipose tissues, primarily skeletal muscle and liver. Ectopic fat deposition in these tissues can interfere with

cellular functions leading to organ dysfunction and is associated with insulin resistance (41-43). It has been suggested that as adipocytes accumulate triglycerides, they reach a limit to how much fat they can store and once this limit is exceeded, there is increased fat in circulation which then gets stored ectopically. Insulin resistance is closely associated with fat deposition in liver and muscle (44-47). If the supply of lipids exceeds the oxidative capacity of these tissues, lipids are stored intracellulary. In skeletal muscle, free fatty acids are taken up by the mitochondria and then broken down by β-oxidation. If there is an oversupply of free fatty acids and/or an impairment in mitochondrial β-oxidation, long-chain acyl-CoA accumulates and is broken down to intermediates like diacylglycerol and ceramide. These lipid intermediates impair insulin signaling leading to decreased GLUT4 regulated glucose transport (41, 43, 48). Interestingly, although accumulation of intramyocellular lipids is associated with insulin resistance and T2DM, it does not necessitate development of these conditions. For example, endurance trained athletes who are insulin sensitive have increased intramyocellular lipid content (49, 50). In these athletes, the increased intramyocellular lipid is likely an adaptive response, providing a readily available energy source. This is combined with an increased capacity to oxidize fatty acids. In contrast, in an insulin resistant or diabetic state, the increased intramyocellular lipid content is accompanied by decreased fatty acid oxidative capacity resulting in accumulation of deleterious lipid intermediates.

Observations that fatty acid oxidation is impaired in insulin resistance and obesity led to the hypothesis that mitochondrial dysfunction may be the cause of insulin resistance

(51, 52). A decreased fatty acid oxidative capacity could cause the increased accumulation of intramyocellular lipids, impairing insulin action on glucose metabolism. In support of this, decreased mitochondrial function has been reported in insulin resistant offspring of T2DM patients and is associated with insulin resistance, T2DM, and obesity (53-57). The idea that mitochondrial dysfunction causes insulin resistance is however, highly debated. It may be that mitochondrial dysfunction is the result of an increased amount of fatty acids and fatty acid intermediates. Further investigations are required to determine whether mitochondrial dysfunction is a cause or consequence of insulin resistance.

Factors contributing to obesity

Obesity is a multi-factorial condition, in which environmental and genetic factors have contributory roles. Genome-wide association studies have been able to identify many genomic loci associated with obesity, and the genetic contributions are most often polygenic. The genetic contribution to body weight has been estimated at 40-70% heritability (58-61). Monogenetic forms of obesity include Bardet-Biedl syndrome and Prader-Willi syndrome, and there are other known mutations in specific genes that cause obesity such as mutations in genes encoding leptin and the leptin receptor (62-65). Genome-wide association studies have identified numerous polymorphisms associated with BMI and obesity, including single nucleotide polymorphisms in genes such as fat mass and obesity-associated gene (*FTO*), lipoprotein lipase (*LPL*), and the leptin receptor (*LEPR*) (66-69). However, the overall effect size remains small, can only

explain a very small portion of the variance in BMI, and has not been able to account for the recent dramatic rise in prevalence of obesity. Therefore, the biological basis of an individual's susceptibility to obesity remains poorly understood. There is also an increasing recognition of the importance of environmental factors to the development of obesity. For example, an environment that encourages excessive food intake and discourages physical activity promotes weight gain (70). Complicating things further, is the interaction between genetic and environmental factors.

Intrauterine growth restriction

Early life environmental factors, such as maternal food restriction, contribute to the development of metabolic diseases in offspring (71). Intrauterine growth restriction (IUGR) is one environmental perturbation that has been linked to the development of obesity and T2DM. The idea that prenatal events may be important in determining risk for adult disease was first reported by David Barker who made a landmark observation that birth weight is inversely correlated with the risk of coronary heart disease in adulthood (72). The birth records of 16000 men and women who were born in Hertfordshire, England between 1911 and 1930 were examined. Death from coronary heart disease was associated with low birth weight, with the rates falling progressively between individuals with a birth weight less than 2500g and individuals with a birth weight of 4310g.

Low birth weight is defined by the World Health Organization as weight at birth less than 2500 g (73). 15.5% of all babies are born with low birth weight, representing over 20 million infants worldwide (73). While the incidence of low birth weight is greater in developing countries, it remains a significant problem in developed countries as well. In North America, 7.7% of infants are low birth weight (73). Low birth weight may be a result of preterm birth or poor fetal growth. Poor fetal substrate supply can be due to poor maternal energy intake (insufficient intake of a specific micro- or macronutrient, or reduced total calories), placental insufficiency, maternal smoking, pregnancy at high altitude, or high maternal levels of stress hormones (e.g., cortisol). Interestingly, infants who are born with a high birth weight are also susceptible to metabolic disease (74). Studies have shown that there is a U-shaped correlation between birth weight and obesity with a higher prevalence of obesity for low birth weight and high birth weight (75, 76). Here the focus will be on low birth weight as a result of poor fetal substrate supply.

After Barker's initial observation, subsequent epidemiological studies showed a strong correlation between *in utero* undernutrition, low birth weight, and risk of adult cardiovascular disease, impaired glucose tolerance, T2DM, and obesity (77-81). One well-studied example of the importance of adequate fetal nutrition is epidemiological data from the Dutch Hunger Winter. During this short-term famine (1944-1945), the daily nutritional intake was reduced to approximately 400-1000 calories. Adults whose mothers were exposed to the famine during pregnancy had low birth weight and had impaired glucose tolerance and predisposition to T2DM (78). These studies gave rise to

the developmental origins of adult disease hypothesis. This hypothesis states that adverse influences early in development may result in physiological adaptations that increase susceptibility to adult disease. The increased risk of adult obesity, insulin resistance, and T2DM has been suggested to be due to a thrifty phenotype programmed *in utero* (82). Thus, when the nutrients provided to a fetus are limited, the fetus adapts to this environment through physiological and metabolic changes that enhance its survival under these conditions. However, if the fetus is born into an environment where nutrients are abundant, the adaptations made *in utero* may become a disadvantage (83). Thus, disparities between the predicted environment and the actual environment into which the fetus is born may result in an increased disease risk.

Animal models of IUGR

Research based on numerous animal models of IUGR has provided extensive support for the findings from human epidemiogolical studies and has substantially advanced our understanding of the negative impact of a suboptimal *in utero* environment. The most commonly used animal models of IUGR are maternal caloric or protein restriction and induction of uteroplacental insufficiency. These models have shown that a suboptimal *in utero* environment has deleterious consequences for adult health, with effects in many organs and tissues including skeletal muscle, heart, pancreas, liver, blood, and the brain. Studies in sheep, pigs, rats and mice have shown that IUGR results in offspring with reduced circulating levels of insulin-like growth factor 1, reduced insulin secretion *in vivo*, decreased β-cell mass and proliferation, impaired glucose

tolerance, increased adiposity, increased susceptibility to ischemia/reperfusion injury, increased susceptibility to hypertension and cardiac dysfunction, decreased mitochondrial DNA content and oxygen consumption in isolated mitochondria from liver, increased hepatic glucose production, decreased neuronal development, amongst a myriad of other effects (84-98). Thus, while not inclusive, this list highlights the profound effect of maternal undernutrition on offspring health.

IUGR: effects on skeletal muscle

A common phenotype in IUGR humans and animals is reduced lean mass (87, 99-101). As muscle is a key determinant of whole body metabolism and insulin sensitivity, reductions in muscle mass and/or function may be especially important (7, 22, 23). In addition to reduced lean mass, it has been shown that people born with low birth weight have altered skeletal muscle fiber composition, glucose uptake, insulin signalling, and oxidative capacity. Low birth weight is associated with a shift towards more type II glycolytic fibers, which is associated with skeletal muscle insulin resistance (102). When challenged with a hyperinsulinemic-euglycemic clamp, a measure of tissue insulin sensitivity, those with low birth weight have decreased glucose uptake consistent with impaired insulin sensitivity (103). People with low birth weight have also been shown to have reduced muscle glucose uptake after local insulin infusions and decreased expression of insulin signalling proteins and GLUT4 in skeletal muscle (104-106). In more rigorously controlled animal models of low birth weight, many of these same skeletal muscle alterations have also been observed. In IUGR

animals, skeletal muscle has reduced mass, decreased GLUT4 expression, decreased glycogen content, decreased insulin-stimulated glucose uptake, decreased oxidative capacity, and increased lipid accumulation (87, 107-111). While there is considerable evidence supporting the negative effects of *in utero* undernutrition on skeletal muscle, studies examining muscle energetics are limited. Furthermore, the exact mechanisms mediating these effects are unknown.

It has been hypothesized that mitochondrial programming may be a key adaptation made by an IUGR fetus to promote survival in a nutrient-restricted environment (112). As reviewed above, mitochondria play a key metabolic role and are responsible for oxidizing energy substrates to support ATP synthesis, which can then be used to drive a very wide range of energy-demanding reactions in cells. Thus, mitochondrial dysfunction may be a link between *in utero* nutrition and health and disease in adult life. IUGR has been associated with decreased skeletal muscle mitochondria DNA content and decreased expression levels of genes involved in mitochondrial biogenesis and function (93, 113, 114). Impaired mitochondrial function has been observed in animal models of IUGR with adult offspring having decreased mitochondrial oxygen consumption and decreased ATP production (93, 109). However, detailed energetics in isolated mitochondria have not been assessed nor have mitochondrial energetics been analyzed *in situ*. Therefore, the effects of maternal undernutrition on skeletal muscle mitochondrial energetics remain to be elucidated.

IUGR: effects on the heart

Low birth weight and IUGR are associated with an increased risk for cardiovascular disease in adulthood (71, 77, 115-119). The heart is a highly oxidative tissue that requires constant production of ATP for its proper function with fatty acids being the major source of energy substrate. Many cardiac diseases and heart failure are associated with altered metabolism in the heart, including a general decrease in oxidative capacity and the down-regulation of enzymes of fatty acid oxidation (120-125). In humans, IUGR is associated with changes in cardiac morphology, premature stiffening of carotid arteries, impaired cardiac function, and elevated blood pressure (126-128). In animal models, IUGR is associated with the development of adult hypertension, vascular dysfunction, and increased myocardial lipid content (129-131). IUGR rats have an increased susceptibility to ischemia/reperfusion that is associated with a mismatch between myocardial glycolysis and glucose oxidation rates (132). Studies examining the metabolic effects of IUGR on the heart remain very limited. Given the high energy requirements of the heart, IUGR may be associated with cardiac metabolic alterations that have negative effects in adulthood.

Catch-up growth

In addition to low birth weight, rapid postnatal weight gain, or catch-up growth, is associated with increased susceptibility to obesity and diabetes. Clinically, catch-up growth in low birth weight individuals is associated with adverse effects on insulin sensitivity, obesity, and cardiac function (133-139). Experimentally, it has been shown

that if IUGR offspring continue on a nutrient restricted diet after birth, thus preventing catch-up growth, they do not develop obesity or glucose intolerance (140, 141). This supports the idea that a mismatch between the predicted and actual environment that the offspring are exposed to increases susceptibility to disease. This mismatch concept is further highlighted when comparing the Dutch Hunger Winter, a relatively short famine, to the Leningrad famine which lasted over 800 days. During the Leningrad famine, fetuses were exposed to calorie restriction but they also experienced undernutrition during infancy. Unlike the children who experienced fetal caloric restriction during the Dutch Hunger Winter, those who survived the Leningrad famine did not have increased rates of insulin resistance, dyslipidemia, or hypertension (142, 143). Therefore, children born during the Leningrad famine may have developed thrifty mechanisms *in utero* for times of limited nutrient availability which became advantageous when born into a food restricted environment. Thus, adaptations made *in utero* were well matched to their extra-uterine life and were protective. In contrast, children born during the Dutch Hunger winter may have developed similar thrifty mechanisms that became disadvantageous in life where nutrition was abundant.

Intergenerational transmission

There is evidence to suggest that adverse events during pregnancy can be transmissible across generations. Early life programming effects have been shown to affect not only the offspring of the pregnancy in which the adverse event occurred (F1), but also the next generation (F2) even when F1 females had been well-nourished since weaning. In

a longitudinal study of data from the British National Child Development Study, authors found a positive association between babies' birth weight and parental birth weight, suggesting that birth weight is passed on to the subsequent generation (144, 145). Experimental studies have shown intergenerational effects on birth weight, glucose metabolism, and blood pressure. In a rat model, a maternal low protein diet raised blood pressure in F1 and F2 offspring (145). In a separate study, maternal low protein diet in rats reduced insulin secretion in the F1 offspring and insulin resistance in the F2 offspring (146). Furthermore, this study also examined glucose metabolism in the grand offspring (F3) and found that they had altered glucose homeostasis (146). In a mouse model of maternal undernutrition during late pregnancy, the F1 and F2 offspring had low birth weight, impaired glucose tolerance, and obesity (88). Together, these studies demonstrate intergenerational transmission of the effects of maternal undernutrition during pregnancy. While epigenetic mechanisms are hypothesized, the exact mechanisms underlying the developmental programming of metabolism remain unknown.

Epigenetics: A possible mechanism?

The fetal adaptations to undernutrition that produce the long-term outcomes of IUGR are not fully understood. The increased susceptibility to adult disease may arise, at least in part, from epigenetic mediated alterations in gene expression (82, 147). Epigenetic modification refers to modifications of DNA that result in differential gene expression without altering the DNA sequence itself. These modifications include DNA

methylation, genomic imprinting, and chromatin modifications such as post-translational modification of histones. These epigenetic modifications alter the binding of transcription factors to specific promoters and/or alter chromatin conformation which modulates gene expression. Thus, epigenetic modifications of the fetal genome based on maternal environmental cues may reset the metabolic state of the fetus to produce phenotypes in the offspring that are best suited for the predicted environment and that are maintained into adulthood. Evidence indicates that environmental factors acting during critical developmental periods can alter the epigenome. For example, in the mouse, the level of methyl donors in the maternal diet has been shown to alter DNA methylation in the offspring (148).

Human data that link maternal undernutrition to epigenetic changes are limited. In one study, whole blood genomic DNA was analyzed in adults who were *in utero* during the Dutch Hunger Winter, a period of famine, compared to their unexposed same-sex sibling. Adults who were *in utero* during the famine, and thus were undernourished, showed hypomethylation of the insulin-like growth factor II gene, a maternally imprinted gene that is a key factor in mammalian growth (149). Modifications to the methylation status of genes produce stable alterations in gene expression and represent a potential mechanism by which early life nutrition may influence susceptibility to metabolic disease in adulthood (147).

Animal models are increasing our understanding of the underlying mechanisms of IUGR. Epigenetic modifications that affect glucose metabolism have been described in

IUGR pancreas, liver, and muscle. Pancreatic and duodenal homeobox 1 (Pdx-1) is a transcription factor that plays an important role in β-cell development and function. Expression of the *Pdx-1* promoter is decreased in IUGR and promotes the development of diabetes in adulthood. It has been shown that islets isolated from IUGR fetuses have decreased histone acetylation at the proximal promoter of *Pdx-1* which is associated with decreased *Pdx-1* expression and defective glucose homeostasis (150). In another study, maternal protein restriction in rats led to decreased methylation of genes for the glucocorticoid receptor (GR) and peroxisome proliferator-activated receptor alpha (PPARα) in the liver of the offspring after weaning (151). This was associated with greater mRNA expression of GR and PPARα, both of which are involved in glucose and lipid metabolism (151). In IUGR, skeletal muscle becomes insulin-resistant and glucose transport is reduced. It has been shown that IUGR is associated with alterations in transcription factor binding to the GLUT4 promoter which was associated with silencing histone modifications and reduced *glut4* gene expression (107). Together, these results support the idea that alterations in the maternal diet can induce epigenetic changes that are associated with altered gene expression.

While there is growing evidence for the role of epigenetics in metabolic programming, a better understanding of these mechanisms is needed. In the future, epigenetic markers such as methylation may be able to serve as biomarkers to identify individuals with increased susceptibility to disease in adulthood. Ultimately, this may allow prevention of disease by nutritional or pharmacological interventions. In animal models, some

adverse effects of suboptimal nutrition *in utero* have been shown to be prevented or reversed by nutritional interventions (152, 153).

Obesity treatment

Currently, treatment for obesity includes lifestyle modifications, a limited extent of pharmacotherapy, and weight-loss surgery. To lose weight, energy expenditure must exceed energy intake for a prolonged period of time. Alternatively, some excess fat can be surgically removed. Current efforts to treat obesity with lifestyle modifications, including dietary changes and increased physical activity, while effective, have not been able to provide a long-term solution to the obesity epidemic. Very few pharmacological options are currently approved for the treatment of obesity, and those that are have small effects. Weight-loss surgery is the most effective long-term treatment for severe obesity, resulting in significant weight loss and reduction in obesity associated co-morbidities (154, 155). Weight loss surgery is an important option for obesity treatment, but it is an invasive life-altering procedure that is associated with serious risks and side effects. Therefore, there remains a great need for obesity research to further understand mechanisms that cause and control obesity to improve treatment and prevention.

Project Objectives and Hypotheses

Objective 1: To elucidate metabolic alterations that contribute to altered muscle energy metabolism in mice of undernourished dams.

Hypothesis: Maternal undernutrition in the third term of pregnancy in mice predisposes offspring to obesity and diabetes, and this predisposition is related in part to low oxidative capacity and dysfunctional mitochondrial energetics in skeletal muscle.

Objective 2: To determine if the impaired skeletal muscle energetics observed in low birth weight mice also exist in primary muscle cells derived from these mice.

Hypothesis: Undernutrition *in utero* in mice causes a primary muscle defect that is associated with increased susceptibility to metabolic disease in adulthood.

Objective 3: To characterize the effect of exposure to maternal undernutrition in mice on mitochondrial energetics in the adult heart.

Hypothesis: The increased susceptibility to metabolic disease observed in low birth weight mice is due in part to low oxidative capacity and dysfunctional energetics in cardiac muscle.

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Chapter 2

Low birth weight is associated with adiposity, impaired skeletal muscle energetics, and weight loss resistance in mice

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Statement of Manuscript Status and Contributions

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Brittany Beauchamp designed experiments, performed and/or assisted with all experiments, prepared all figures and analyzed all data except for the gene expression analysis, interpreted all data, wrote the manuscript, and revised the manuscript.

Sujoy Ghosh analyzed, interpreted, and prepared figures for the gene expression data and edited the manuscript.

Michael W. Dysart assisted significantly with animal work (feeding and weighing), indirect calorimetry experiments, and some of the histological analyses.

Georges N. Kanaan assisted significantly with high resolution respirometry experiments.

Alphonse Chu performed gene expression analysis and edited the manuscript.

Alexandre Blais interpreted the gene expression analysis data and edited the manuscript.

Karunanithi Rajamanickam performed and analyzed MRI data.

Eve C. Tsai interpreted MRI results and edited the manuscript.

Mary-Elizabeth Patti interpreted data and edited the manuscript.

Mary-Ellen Harper designed experiments, interpreted data, assisted with writing the manuscript and revised the manuscript.

Contributions statement from manuscript: Conceived and/or designed the work (BB, MEH); performed experiments (BB, MD, GNK, AC, KR); analyzed or interpreting results (BB, SG, MD, AC, AB, KR, ECT, MEP, MEH); wrote the manuscript (BB, MEH). All authors revised the manuscript and approved the final version.

Abstract

Background: *In utero* undernutrition is associated with obesity and insulin resistance, although its effects on skeletal muscle remain poorly defined. Therefore, in the current study we explored the effects of *in utero* food restriction on muscle energy metabolism in mice.

Methods: We used an experimental mouse model system of maternal undernutrition during late pregnancy to examine offspring from undernourished dams (U) and control offspring from *ad libitum* fed dams (C). Weight loss of 10 wk old offspring on a 4 wk 40% calorie restricted diet was also followed. Experimental approaches included bioenergetic analyses in isolated mitochondria, intact (permeabilized) muscle and at the whole body level.

Results: U have increased adiposity and decreased glucose tolerance compared to C. Strikingly, when U are put on a 40% calorie restricted diet they lose half as much weight as calorie restricted controls. Mitochondria from muscle overall from U had decreased coupled (state 3) and uncoupled (state 4) respiration and increased maximal respiration compared to C. Mitochondrial yield was lower in U than C. In permeabilized fiber preparations from mixed fiber type muscle U had decreased mitochondrial content and decreased adenylate free leak respiration, fatty acid oxidative capacity, and state 3 respiratory capacity through complex I. Fiber maximal oxidative phosphorylation capacity did not differ between U and C but was decreased with calorie restriction.

Conclusions: Our results reveal that *in utero* undernutrition alters metabolic physiology through a profound effect on skeletal muscle energetics and blunts response to a hypocaloric diet in adulthood. We propose that mitochondrial dysfunction links undernutrition *in utero* with metabolic disease in adulthood.

Introduction

Obesity risk in adulthood can be influenced by events during intrauterine life. The developmental programming hypothesis holds that adverse influences during critical periods in development permanently alter tissue structure and function, which in turn increases disease risk. Hales and Barker demonstrated a strong association of low birth weight with cardiovascular disease and type 2 diabetes mellitus (T2DM) risk and hypothesized that this resulted from the offspring developing a thrifty phenotype *in utero* in anticipation of life with limited food (1, 2). Epidemiological studies in humans and animal models show that during the prenatal period, it is crucial to achieve optimal nutrition as both low and high birth weights are associated with risk of metabolic disease (3). In the current study, we have used a mouse model of low birth weight generated through 50% food restriction of mouse dams during the third week of gestation (4). Initial studies using this mouse model reported that offspring of undernourished pregnancies develop progressive, severe glucose intolerance by 6 months of age, beta cell dysfunction, and increased lipogenic gene expression and adipocyte size (4, 5).

Disordered skeletal muscle metabolism is associated with the adverse metabolic complications of obesity and T2DM, and has not been investigated in this model of obesity. Skeletal muscle in obese individuals exhibits reduced oxidative capacity, increased glycolysis, mitochondrial dysfunction, and a shift in fiber type distribution towards more glycolytic fibers (6-10). Healthy individuals with low birth weight have

been shown to have abnormalities in muscle including decreased mass, reduced oxidative capacity, increased glycolytic capacity, and a lower proportion of oxidative type I fibers (11-13). It is well known that muscle is highly adaptable and responds to environmental and physiological challenges by changing its size, composition, and aerobic capacity (14, 15). Therefore, we hypothesized that the increased susceptibility to obesity and glucose intolerance in low birth weight mice is due in part to dysfunctional muscle mitochondrial energetics. This is supported by observations in rats showing that a low protein diet during pregnancy is associated with decreased mitochondrial DNA content in muscle of offspring (16) and that growth restriction by bilateral uterine artery ligation in late gestation causes decreased ADP-stimulated respiration in muscle mitochondria (17). No studies to date have examined muscle mitochondrial energetics in animals having low birth weight as a result of maternal food restriction and none have assessed the response of the adult offspring to a hypocaloric diet. Although links are well established between low birth weight and increased susceptibility to obesity and T2DM, the mechanisms by which maternal food restriction alters the long-term metabolic health of offspring remain to be fully understood.

Materials and Methods

Animals

All experiments were performed according to the principles and guidelines of the Canadian Council of Animal Care and the study was approved by the Animal Care Committee of the University of Ottawa. Animals were housed with controlled temperature, humidity, and light-dark cycle (0600h – 1800h). Virgin female ICR mice (Harlan, Indianapolis, IN; age 6-8 wk) were paired with male ICR mice (Harlan; age 6-8 wk). Pregnancies were dated by vaginal plug (day 0.5) and pregnant mice were housed individually with *ad libitum* access to standard rodent chow (T.2018, Harlan Teklad, Indianapolis, IN, USA). On day 12.5 of pregnancy, dams were randomly assigned to either a control or an undernutrition group. Dams in the undernutrition group were 50% food restricted from days 12.5 to 18.5, calculated based on the food intake of gestational day matched controls. At birth, all mothers were given *ad libitum* access to chow. On post-natal day 1, litters were equalized to eight, with additional pups being removed randomly from the litter. Pups were weaned at age three wk and mice were housed in groups of 3-4 from age 3 to 10 wk. At age 8 wk, mice were separated into individual cages and at age 10 wk mice were randomly assigned to either a 40% calorie restricted group (D01092702: Research Diets) or an *ad libitum* control group. The calorie restricted diet was specially formulated to ensure that the nutrient intake is normal despite decreased energy intake; it is thus used to study the effects of calorie restriction rather than food restriction. Therefore, at 14 wk of age 4 groups of mice

were studied: *in utero* undernourished offspring fed *ad libitum* postnatally (U-L), *in utero* undernourished offspring that were calorie restricted for 4 wk during adulthood (U-R), control offspring fed *ad libitum* (C-L), and control offspring that were calorie restricted for 4 wk during adulthood (C-R). Calorie restricted mice were fed daily at 16:00. Female mice were used for all determinations. All end point determinations were performed with mice from at least four different litters per group, with no more than two mice obtained from the same litter. All mice were fasted overnight prior to sacrifice to reduce any acute starvation response differences between the groups.

Indirect calorimetry and activity

At ages 10 and 14 wk, randomly selected mice from each group were placed in a customized 4-chamber open-circuit indirect calorimeter (Columbus Instruments, Columbus, OH), as described by Boily *et al* (18). Chambers were configured with dual axis (X, Y) detection of motion using infrared photocells (Columbus Instruments; Columbus, OH) to measure activity. Measurements were conducted over 24h after 2h of acclimatization. The respiratory exchange ratio (RER) was calculated as VCO_2/VO_2. Mouse activity was calculated as total number of laser beam breaks.

Glucose tolerance testing

Oral glucose tolerance tests were performed on mice fasted for 6h. Glucose (2 mg/g body weight) was given by gavage at time 0. Saphenous vein blood was collected at 0,

15, 30, 60 and 120 min and glucose concentration was determined by a glucometer (Bayer, Canada).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) of 14 wk old mice was performed as in Du *et al* (19) with modifications detailed below. 4 mice at a time were euthanized with sodium pentobarbital and then imaged. Coronal spin-echo T1-weighted sequences were obtained from head to anus with a Siemens 3-T MRI system using a standard human head coil. Adipose tissue volume was calculated from the spin-echo MR images with volume segmentation for adipose tissue. Axial and coronal images were obtained for each mouse and the average of these images was calculated.

Histology

At 14 wk of age, the *soleus* and *tibialis anterior* (TA) muscles were isolated for histology. Sections were stained for myosin heavy chain (MHC) isoforms and cytochrome c oxidase (COX) activity (20, 21). Analyses were performed using ImageJ software (NIH).

Muscle homogenate

Quadriceps muscles were dissected and immediately flash frozen in liquid N_2. Muscle was homogenized on ice in a lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 50 mM

NaCl, 250 mM sucrose, 2% β-mercaptoethanol, 50 mM NaF, 5 mM NaPP, 1mM Na_3VO_4, and protease inhibitors) and spun at 14 000 g for 20 min at 4°C. Protein content was measured using a bicinchoninic acid assay and samples were stored at -80°C.

Western blotting

Muscle homogenate and isolated mitochondria were subjected to standard Western blotting procedures. For additional information, including antibodies used see Appendix A. Band intensity was quantified by density analysis using Image J (NIH) and normalized to complex IV for mitochondrial samples or α-tubulin for muscle homogenate samples.

Mitochondrial isolation

Mitochondria from pooled forelimb, hindlimb, and pectoral muscles were isolated using the method described by Chappell and Perry (22) with modifications published previously (23). For additional details, see Appendix A. Mitochondria were kept on ice and used for bioenergetic determinations within 2h. Protein concentration was measured using the Bradford assay.

Mitochondrial bioenergetics

Isolated mitochondria were studied using the Seahorse XF24 Analyzer (Seahorse Bioscience Inc., USA) using a method adapted from (24), as described previously (25). Oxygen consumption rate (OCR) was determined in quintuplicate under state 2 conditions (10 mM pyruvate and 2 mM malate) prior to sequential additions of ADP (0.1 mM), oligomycin (2.5 µg/ml), FCCP (8 µM), and antimycin A (4 µM) to assess state 3, state 4_O, maximal, and non-mitochondrial respiration, respectively. OCR was corrected by subtracting non-mitochondrial values (after addition of antimycin A).

High resolution respirometry

In a separate cohort of mice, the white and red *gastrocnemius* (wGAS and rGAS respectively) were removed and fibers were permeabilized with 50 µg/ml saponin. Respiration was determined in duplicate and at 37°C in MIRO5 (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 0.1% BSA, 60 mM lactobionic acid; pH 7.1) using the Oxygraph-2k (Oroboros, Austria). Malate (2 mM) and octanoyl carnitine (200 µM) were added to determine adenylate free leak respiration (L_N). ADP + Mg^{2+} (5 mM) were subsequently added to determine maximal electron flow through electron-transferring flavoprotein (ETF) and fatty acid oxidative capacity (P_{ETF}). Submaximal state 3 respiratory capacity through complex I (P_{CI}) was determined following the addition of pyruvate (5 mM) and glutamate (10 mM). Succinate (10 mM) and ADP + Mg^{2+} (5 mM) were then added to determine maximum oxidative phosphorylation capacity (P_{CI+CII}). Cytochrome c (10 µM) was added to test the integrity of the mitochondrial outer membrane. Only preparations in

which addition of cytochrome c caused <10% increase in respiration were included. Antimycin A (2.5 μM) was added to inhibit complex III and terminate respiration to determine nonmitochondrial oxygen consumption. All values were corrected for residual oxygen consumption.

Statistical analyses

All measures were analyzed using GraphPad Prism, version 5.0 (La Jolla, CA, USA). Data between groups at 10 wk of age were compared using an unpaired Student's *t*-test. Body weight measurements over time and glucose tolerance tests were analyzed using two-way repeated measures ANOVA with Bonferroni post-hoc tests. All data between groups at 14 wk of age were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. Values are given as mean ± SEM. $P<0.05$ was considered significant.

Results

Low birth weight offspring have decreased glucose tolerance

Body weights of offspring from undernourished dams (U) were 27% lower on post-natal day 1 compared to those of offspring from control dams (C) (Figure 2.1A). The body weight of U was similar to C by three wk of age and their growth curves and food intake afterwards did not differ significantly (Figure 2.1B and Figure S2.1A, respectively). U had decreased glucose tolerance at 10 wk of age (Figure 2.1C), which became progressively worse by 14 wk (Figure S2.1B). Tail and femur lengths were not different between U and C, indicating no difference in linear growth. There was also no difference in liver or heart weights (Table S2.1; 10 wk and Table S2.2; 14 wk).

In utero undernutrition is associated with increased adiposity and blunted weight loss in response to calorie restriction

Given our interest in weight loss response to hypocaloric diets (21, 26), we studied whole body and skeletal muscle metabolism in offspring in both *ad libitum* and calorie restricted states. At 10 wk of age, mice were put on a 40% calorie restriction diet for 4 wk. With calorie restriction, U lost significantly less weight than controls losing 15% and 26% of their initial body weight respectively (Figure 2.1D). The absolute value of the weight lost was ~50% less in U than C. Body fat volume was quantified using MRI (Figure 2.1E). Body fat percent was greater in *ad libitum* and calorie restricted U

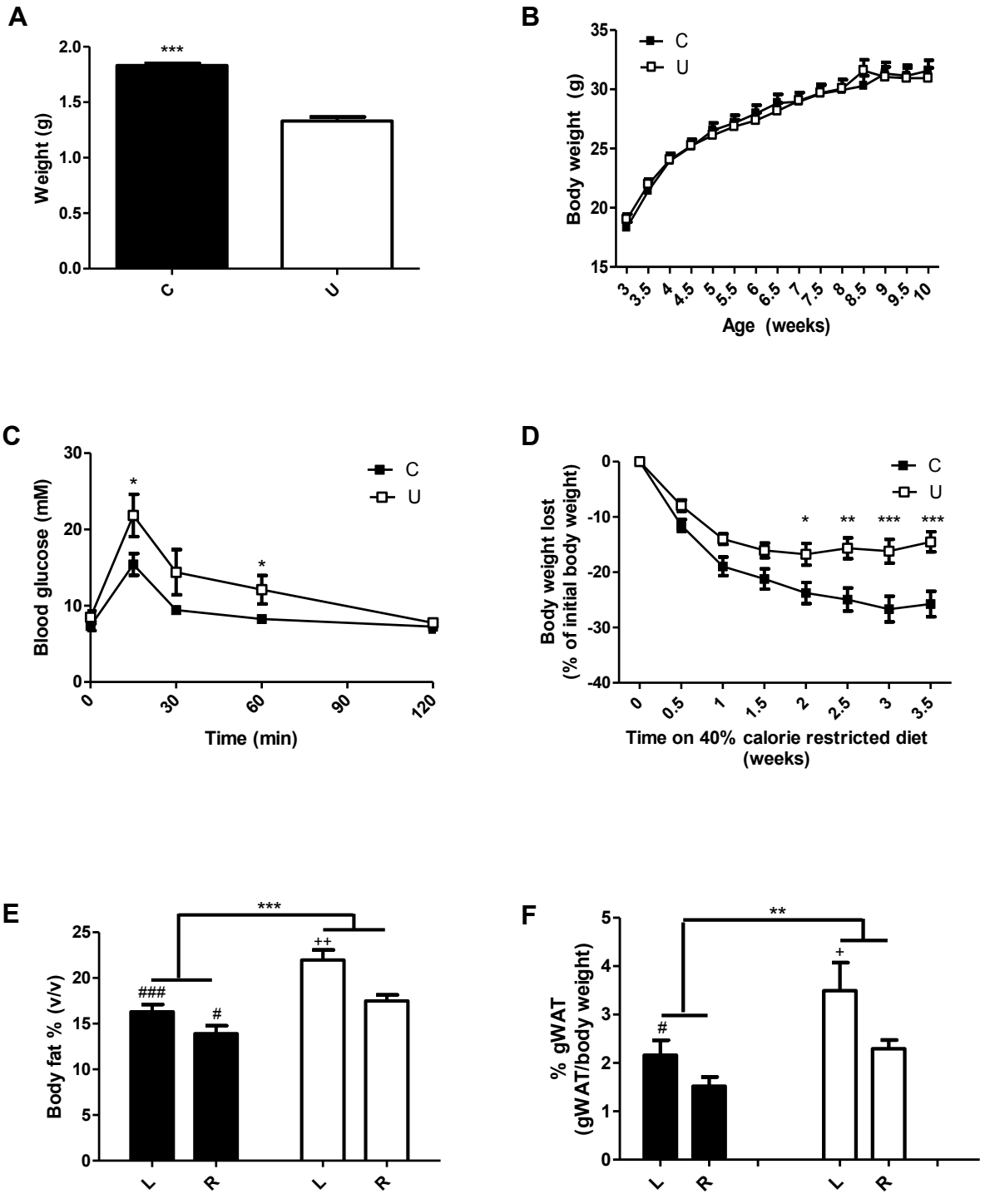

A
***
Weight (g)
2.0
1.5
1.0
0.5
0.0
C
U
B
C
U
Body weight (g)
35
30
25
20
15
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
Age (weeks)
C
C
U
Blood glucose (mM)
30
20
10
0
*
*
0
30
60
90
120
Time (min)
D
C
U
Body weight lost
(% of initial body weight)
0
-10
-20
-30
-40
*
**
***
***
0
0.5
1
1.5
2
2.5
3
3.5
Time on 40% calorie restricted diet
(weeks)
E
***
Body fat % (v/v)
25
20
15
10
5
0
###
#
++
L
R
L
R
F
**
% gWAT
(gWAT/body weight)
5
4
3
2
1
0
#
+
L
R
L
R

Figure 2.1. *In utero* undernutrition is associated with low birth weight, decreased glucose tolerance, decreased weight loss in response to calorie restriction and increased adiposity. A) Weight of the offspring at 1 day of age. Offspring from 7 dams per group were weighed. Student's *t*-test, *** = $p<0.0001$, n=50-87. B) Bi-weekly body weight of offspring. Two way-repeated measures ANOVA, not significant, n=34. C) Blood glucose concentrations before and for 2 h after oral glucose administration (2 mg/g body weight). Oral glucose tolerance test was performed at age 10 wk. Two-way repeated measures ANOVA with Bonferroni post-hoc test, * = $p<0.05$, n=6-8. D) Change in bodyweight on a 40% calorie restricted diet started at 10 wk of age. Values are presented as percent of body weight prior to caloric restriction. Two-way repeated measures ANOVA with Bonferroni post-hoc test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, n=11. E and F) Increased adiposity in 14 wk old offspring fed *ad libitum* (L) and after a 4 week 40% calorie restriction (R). Quantification of adipose tissue volume from spin-echo MR images with volume segmentation for total adipose tissue, n=4-5 (E), and amount of gonadal white adipose tissue (gWAT) expressed as a percentage of total body weight (F), n=8. Two-way ANOVA with Bonferroni post-hoc test, ** = $p<0.01$, *** = $p<0.001$, # = $p<0.05$ (C vs. U), ### = $p<0.001$ (C vs. U), + = $p<0.05$ (L vs. R), ++ = $p<0.01$ (L vs. R). Black = C (control offspring), white = U (*in utero* undernourished offspring). Values are mean ± SEM.

compared to *ad libitum* and calorie restricted C. Correspondingly, fat free mass was decreased in U compared to C. Moreover, U had greater gonadal white adipose tissue (gWAT) weights at 10 and 14 wk of age compared to C (Figure S2.1C and 2.1F respectively). Differences in body composition were not due to differences in heart or liver weight as indicated above (Table S2.2) and there were no noticeable differences in organ size at time of sacrifice or on MRI images.

In utero undernutrition decreases metabolic rate in the dark phase

Indirect calorimetry and physical activity monitoring were conducted in the dark phase, when mice are awake and active, and the light phase, when mice typically sleep and are less active. At 10 wk of age, there was no significant difference in oxygen consumption, RER, or activity (Figure S2.2). At 14 wk of age, oxygen consumption per mouse was decreased in U in the dark phase, including a significant decrease in U-R vs. C-R (Figure 2.2B). Oxygen consumption per mouse was not different in the light phase (Figure 2.2A). Given the differences in adiposity and that adipose tissue is metabolically much less active than other tissues such as muscle, oxygen consumption was also normalized to fat free mass (FFM). Results expressed per gram of FFM showed the same significant differences (Figure 2.2C, 2.2D). Thus, data suggest that U have a decreased metabolic rate in the dark phase compared to C, particularly when mice are calorie restricted. No differences were observed in RER suggesting that U and C metabolize similar relative amounts of carbohydrates and fats (Figure 2.2E, 2.2F).

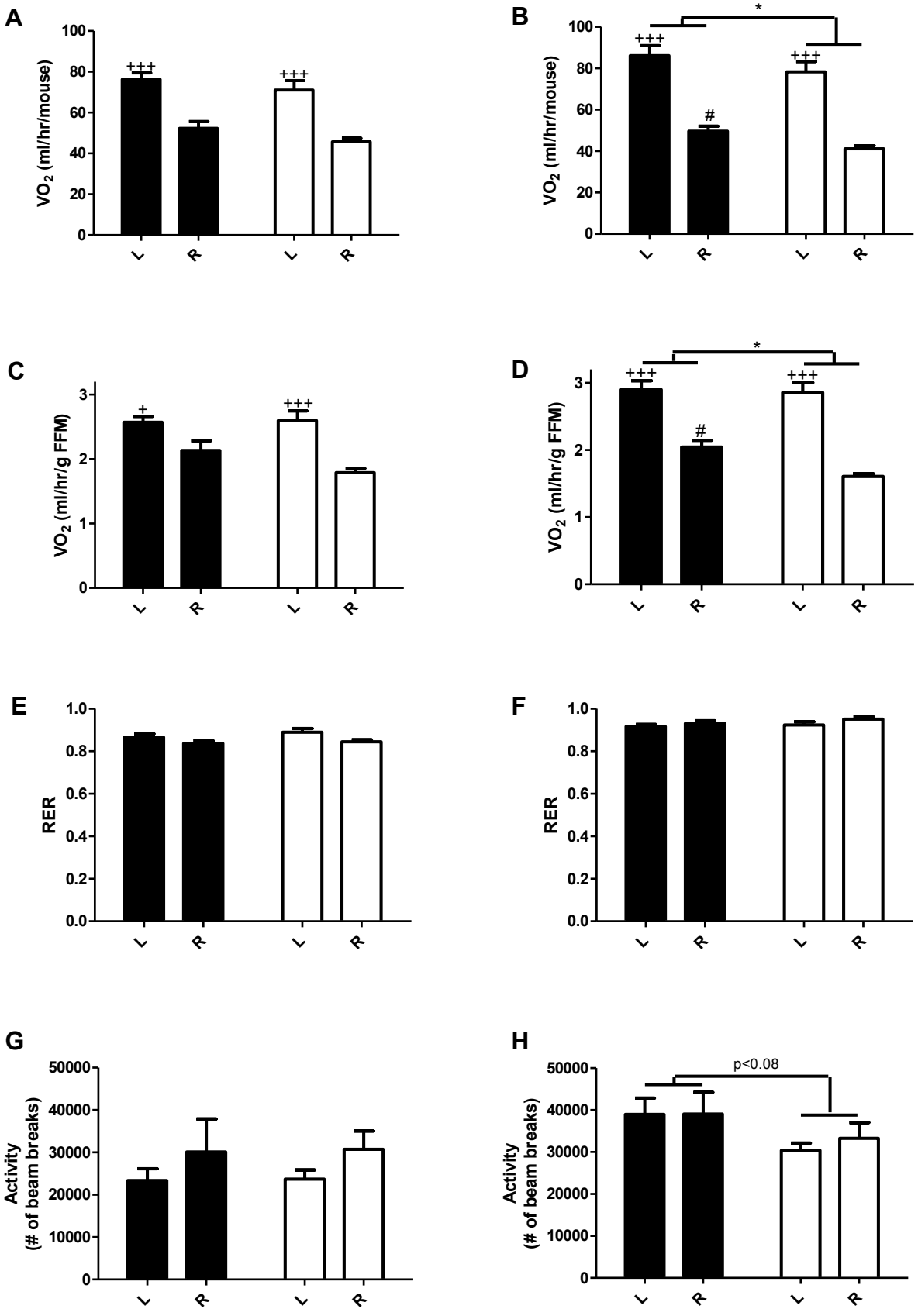

A
B
C
D
E
F
G
H
VO2 (ml/hr/mouse)
VO2 (ml/hr/g FFM)
RER
Activity (# of beam breaks)
L
R
+++
+
#
*
p<0.08

Figure 2.2. *In utero* undernutrition decreases metabolic rate and activity in the dark phase. Data for 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Data were collected for 24h and averaged over each of the light (Left; A, C, E, G) and dark phases (Right; B, D, F, H) of the day (lights on 6:00-18:00). A-D) Whole body oxygen consumption by indirect calorimetry expressed per mouse (A, B) and per gram of fat free mass (FFM) (C, D). E, F) Respiratory exchange ratio (RER), calculated as VCO_2/VO_2. G, H) Activity expressed as the sum of beam breaks in the x and y direction. Values are mean ± SEM, n=8. Two-way ANOVA with Bonferroni post-hoc test,* = $p<0.05$, # = $p<0.05$ (C vs. U), + = $p<0.05$ (L vs. R), +++ = $p<0.001$ (L vs. R). Black = C (control offspring), white = U (*in utero* undernourished offspring).

There was a trend for decreased physical activity in U in the dark phase (Figure 2.2H). Physical activity was not different in the light phase (Figure 2.2G).

Blunted or absent response to calorie restriction in muscle fiber types and gene transcriptome in U compared to C offspring

The *soleus* and TA muscles were selected for histological analyses as they are very different in fiber type composition. Fiber type proportions were classified based on MHC isoforms. In order of most oxidative to least oxidative (*i.e.,* most glycolytic), mouse muscle contains type I, IIa, IIx, and IIb fibers (27). The *soleus* is highly oxidative, comprising almost exclusively type I and type IIa fibers. In contrast, the TA is more glycolytic containing a mix of fiber types. There were no differences in fiber type in *soleus* between U-L and C-L or between U-R and C-R (Figure S2.4A-2.4C). Interestingly, in the more glycolytic TA, U and C responded differently to caloric restriction (Figure 2.3A-2.3E). In U, calorie restriction did not alter fiber type proportions. In contrast, calorie restriction in C resulted in increased types I and IIb fibers and decreased type IIa fibers. Overall, there was a small but significant increase in the proportion of type IIa fibers in U (5.8% increase U-L vs C-L, 7.6% increase U-R vs C-R). Given these effects, microarray analyses were performed to measure the content of gene transcripts in the TA. Despite robust effects of calorie restriction on the expression of genes involved in skeletal muscle development and oxidative phosphorylation in control mice, gene expression was not drastically different between U and C (Figure S2.3 A). We also compared genes that were differentially expressed with calorie restriction in the two

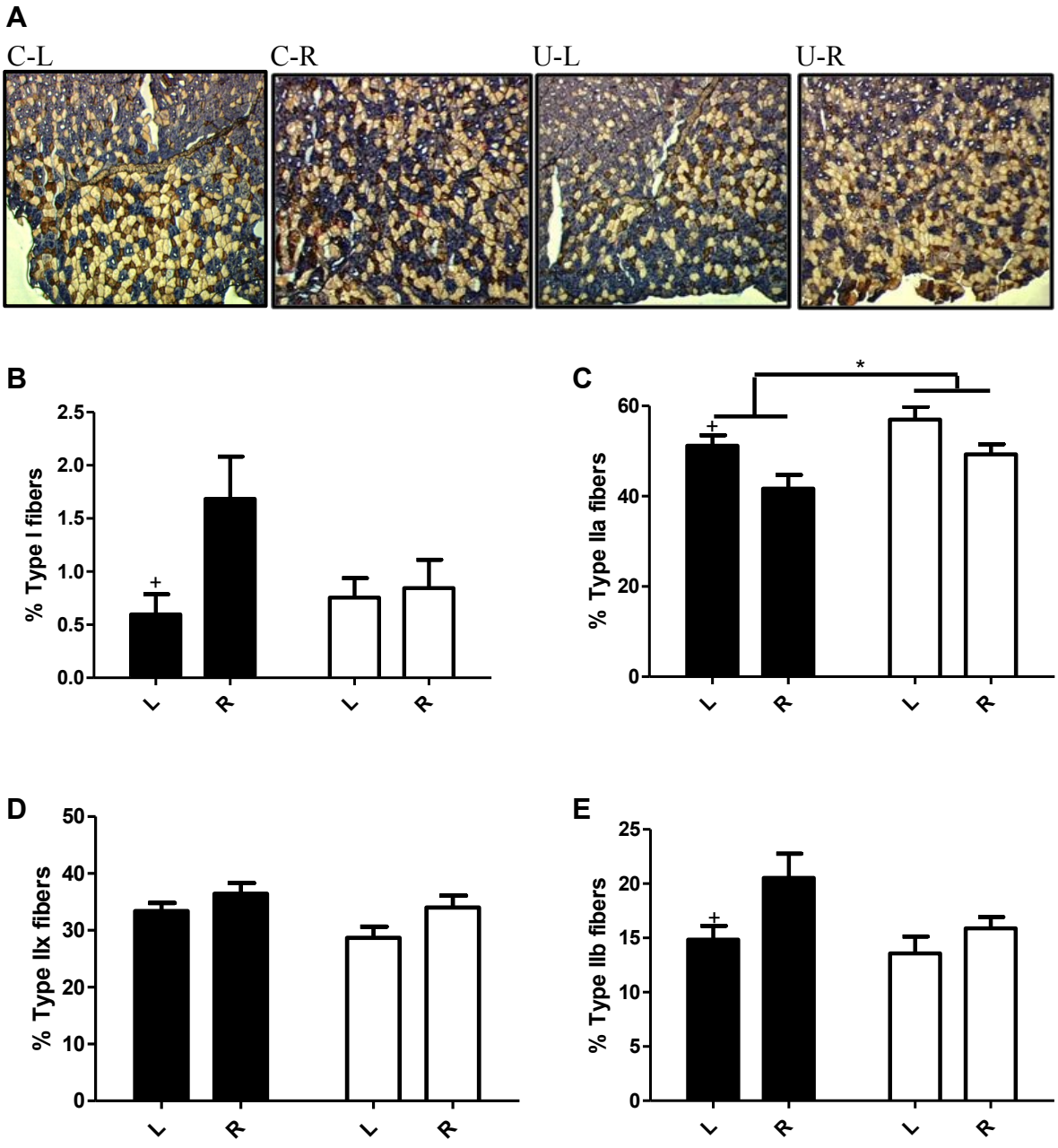
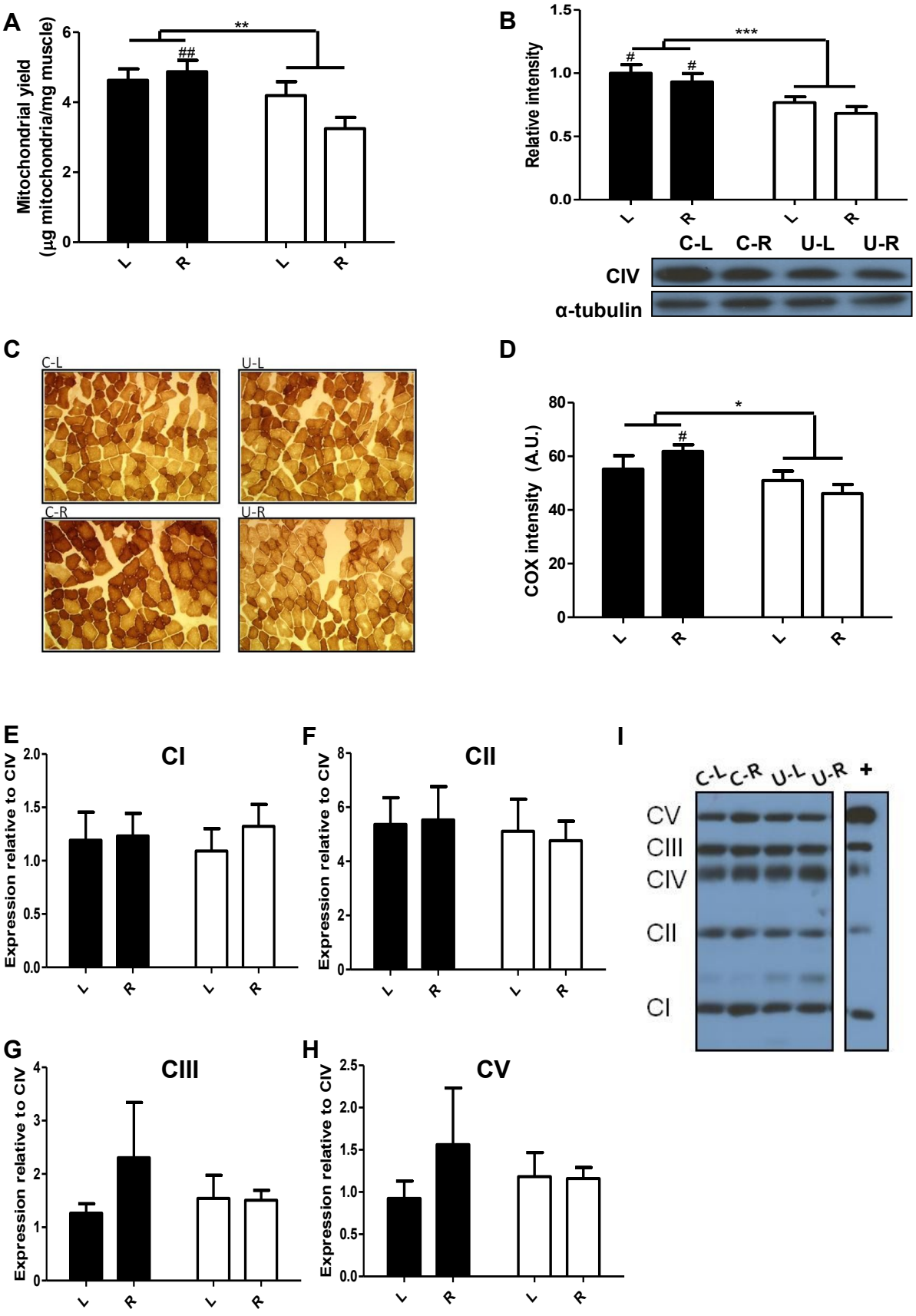

A
C-L
C-R
U-L
U-R
B
% Type I fibers
2.5
2.0
1.5
1.0
0.5
0.0
+
L
R
L
R
C
% Type IIa fibers
60
40
20
0
*
+
L
R
L
R
D
% Type IIx fibers
50
40
30
20
10
0
L
R
L
R
E
% Type IIb fibers
25
20
15
10
5
0
+
L
R
L
R

Figure 2.3. Changes in fiber type in response to calorie restriction are eliminated with *in utero* undernutrition. Data for 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Fiber type proportions in the *tibialis anterior*. Muscle sections were stained based on myosin heavy chain expression for type I fibers (red; B), type IIa fibers (blue; C) and type IIb fibers (brown; D). Unstained fibers were counted as type IIx fibers (E). Proportions were calculated as the percent of the total number of fibers with an average of 988±38 fibers counted per image. Representative images are shown in A. Values are mean ± SEM, n=8. Two-way ANOVA with Bonferroni post-hoc test, * = $p<0.05$, + = $p<0.05$ (L vs. R). Black = C (control offspring), white = U (*in utero* undernourished offspring).

groups. Overall, results show that with calorie restriction far fewer genes are differentially expressed in U (U-R vs U-L; 1010) compared to C (C-R vs C-L; 2267) (Figure S2.3B).

In utero undernutrition results in reduced skeletal muscle mitochondrial content

As mitochondria play a key role in energy metabolism, the above-described differences in weight loss and whole body metabolism could be due in part to differences in mitochondrial content. Crude mitochondrial yield, calculated as total mitochondrial protein isolated from pooled skeletal muscle per unit wet weight of the pooled skeletal muscle, was decreased in U compared to C (Figure 2.4A). With calorie restriction, mitochondrial content was decreased in U-R compared to C-R. Given the approximate nature of this assessment of mitochondrial content, we also measured complex IV (cytochrome c oxidase) protein, in quadriceps muscle. Levels were significantly decreased in U compared to C (Figure 2.4B). To further assess mitochondrial content, sections of the *soleus* and TA were stained for COX activity. In the *soleus*, COX activity did not differ between U and C (Figure S2.4D-2.4E). In the TA, COX activity was decreased in U compared to C (Figure 2.4C, 2.4D). Altogether findings indicate that mitochondrial content is decreased overall in muscle, as indicated by crude mitochondrial yield of pooled skeletal muscle, but COX analysis of specific muscles indicates that mitochondrial content is decreased in mixed and low oxidative muscle groups while not in the highly oxidative *soleus* muscle in offspring of undernourished dams.


A
Mitochondrial yield (μg mitochondria/mg muscle)
**
##
L
R
B
Relative intensity
***
#
C-L
C-R
U-L
U-R
CIV
α-tubulin
C
C-L
U-L
C-R
U-R
D
COX intensity (A.U.)
*
E
CI
Expression relative to CIV
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CII
I
C-L
C-R
U-L
U-R
+
CV
CIII
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CI
G
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Figure 2.4. The effect of *in utero* undernutrition on skeletal muscle mitochondrial content from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). A) Crude mitochondrial yield of pooled skeletal muscle, n=6. B) Protein expression of mitochondrial complex IV (CIV) in *quadriceps* muscle homogenate, representative Western blot on bottom and quantification above, n=8. C,D) *Tibialis anterior* muscle sections were stained for cytochrome c oxidase (COX) activity, representative images (C) and quantification (D). Fiber intensity was calculated for an average of 213 ± 14 fibers per mouse, n=5. E-I) Protein expression of mitochondrial complexes I, II, III, and V relative to complex IV in isolated mitochondria, representative Western blot (I) and quantification (E-H), n=6. Values are mean ± SEM. Two-way ANOVA with Bonferroni post-hoc test, *=$p<0.05$, ** = $p<0.01$, *** = $p<0.001$, # = $p<0.05$ (C vs. U), ## = $p<0.01$ (C vs. U). Black = C (control offspring), white = U (*in utero* undernourished offspring).

In utero undernutrition alters mitochondrial bioenergetics in skeletal muscle

Bioenergetic determinations were conducted on mitochondria from pooled forelimb, hindlimb, and pectoral muscles. State 2 (Figure 2.5A) and state 4_O (Figure 2.5C) respiration rates were decreased in U with a trend for a decrease in state 3 respiration (Figure 2.5B). Intriguingly, FCCP-induced maximal respiration was higher in mitochondria from U vs C (Figure 2.5D). More specifically, in mitochondria from the *ad libitum* fed U mice, state 2 and state 4_O respiration were decreased while maximal respiration was increased compared to C (U-L vs. C-L). Interestingly, in the calorie restricted state there were no significant differences in mitochondrial oxygen consumption (U-R vs. C-R). Given these differences, we assessed the relative proportion of the mitochondrial protein complexes in mitochondria. The relative protein expression of complexes I, II, III, and V compared to complex IV did not differ between the groups (Figure 2.4E-2.4I). Since state 4_O respiration was decreased in U, we also assessed protein levels of adenine nucleotide translocase (ANT) and uncoupling protein 3 (UCP3). There was no difference in ANT or UCP3 protein expression in mitochondria (Figure 2.5E and 2.5F, respectively).

In utero undernutrition alters energetics in permeabilized fibers from white *gastrocnemius* but not red *gastrocnemius*

To further assess energetic differences in muscle, we performed high resolution respirometry on saponin permeabilized muscle fibers of the more glycolytic wGAS and

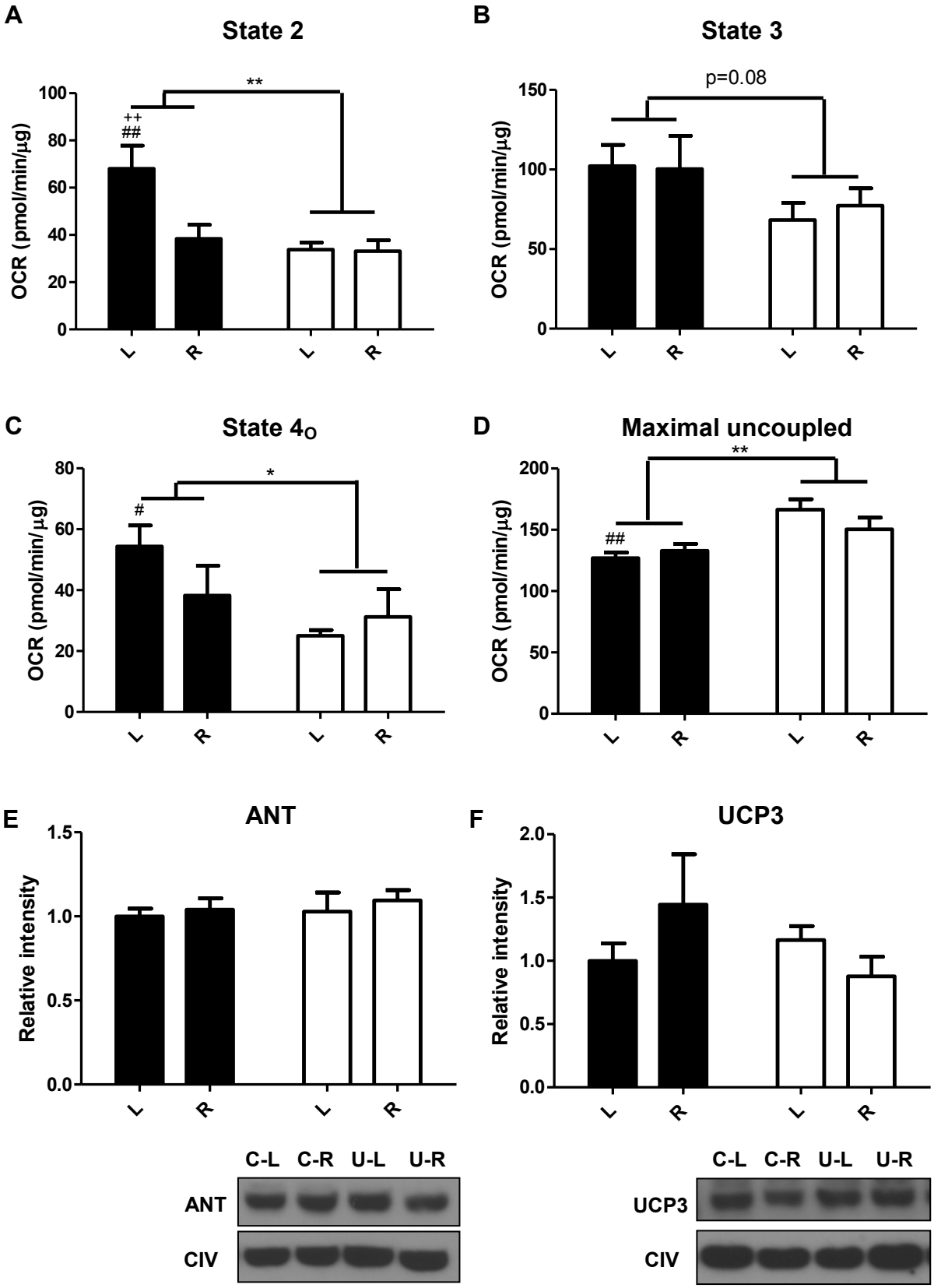
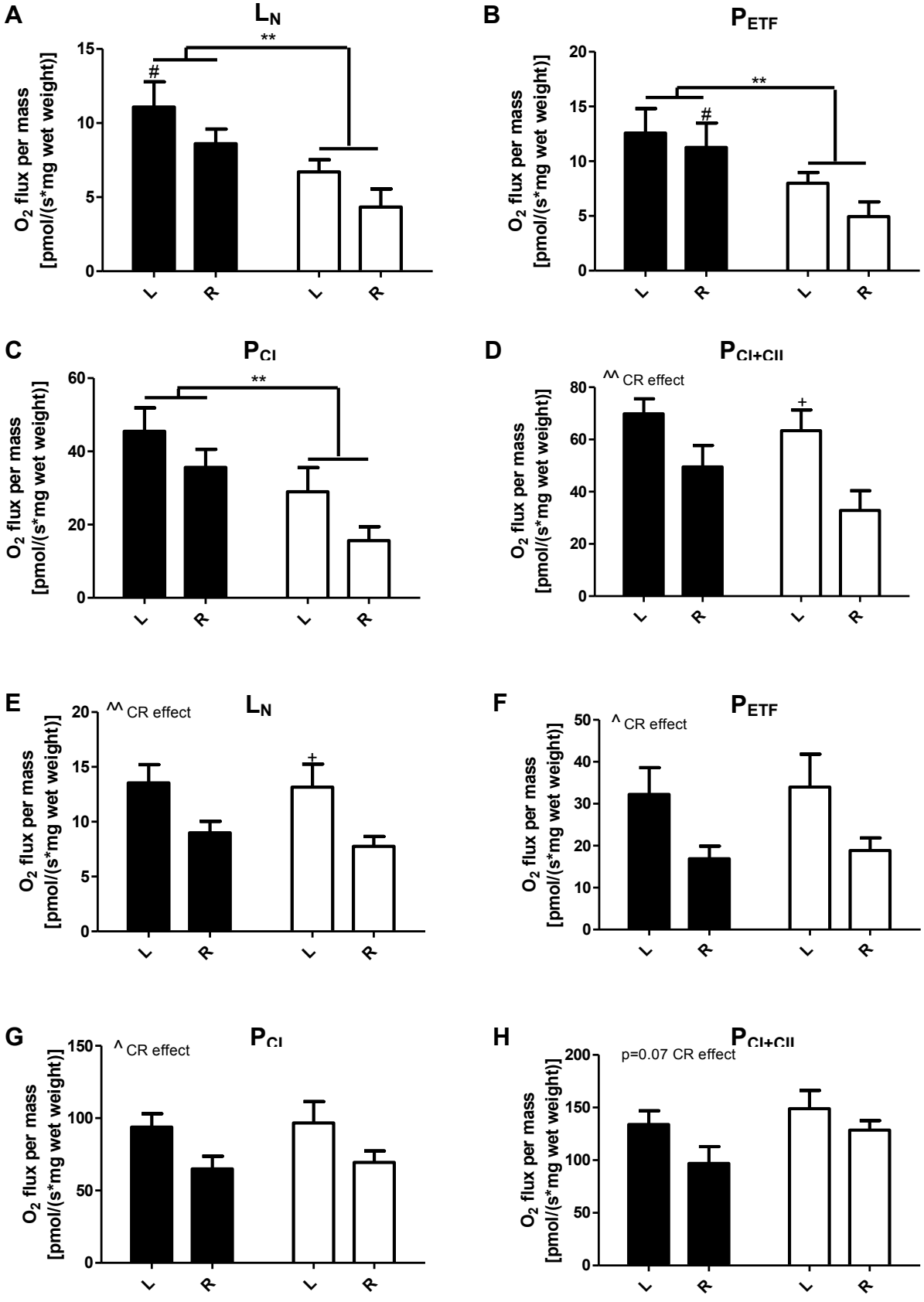

A
State 2
OCR (pmol/min/µg)
**
++
##
L
R
B
State 3
p=0.08
C
State 4O
*
#
D
Maximal uncoupled
**
##
E
ANT
Relative intensity
C-L C-R U-L U-R
ANT
CIV
F
UCP3
Relative intensity
C-L C-R U-L U-R
UCP3
CIV

Figure 2.5. *In utero* undernutrition alters mitochondrial bioenergetics in skeletal muscle from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Data are shown for State 2 (A), State 3 (B), State 4_O (C), and Maximal respiration (D). OCR = Oxygen consumption rate. E,F) Protein expression of ANT (adenine nucleotide translocase; E) and UCP3 (uncoupling protein 3; F) in isolated mitochondria, representative Western blots (top) and quantification (bottom). Complex IV was used as a loading control. Values are mean ± SEM, n=6. Two-way ANOVA with Bonferroni post-hoc test,* = $p<0.05$, ** = $p<0.01$, # = $p<0.05$ (C vs. U), ## = $p<0.01$ (C vs. U), ++ = $p<0.01$ (L vs. R). Black = C (control offspring), white = U (*in utero* undernourished offspring).

the more oxidative rGAS. wGAS fibers from U had decreased adenylate free leak respiration (Figure 2.6A), fatty acid oxidative capacity (Figure 2.6B), and state 3 respiratory capacity through complex I (Figure 2.6C). Maximal oxidative phosphorylation capacity did not differ between U and C but was significantly decreased with calorie restriction (Figure 2.6D). In contrast, respiration in fibers from rGAS was not altered in U (Figure 2.6E-2.6H). However, calorie restriction significantly decreased adenylate free leak respiration (Figure 2.6E), fatty acid oxidative capacity (Figure 2.6F), and state 3 respiratory capacity through complex I (Figure 2.6G) with a trend for a decrease in maximal oxidative phosphorylation capacity ($p=0.07$; Figure 6H). Similar differences between U and C were also measured in wGAS and rGAS at 10 wk of age (Figure S2.5).


A
L_N
**
#
O_2 flux per mass [pmol/(s*mg wet weight)]
15
10
5
0
L
R
L
R
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P_{ETF}
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#
20
15
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+
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E
L_N
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+
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15
10
5
0
F
P_{ETF}
^ CR effect
50
40
30
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G
P_{CI}
^ CR effect
150
100
50
0
H
P_{CI+CII}
p=0.07 CR effect
200
150
100
50
0

Figure 2.6. *In utero* undernutrition alters energetics in permeabilized fibers from white *gastrocnemius* (wGAS) but not red *gastrocnemius* (rGAS). O_2 flux in permeabilized fibers from wGAS (A-D) and rGAS (E-H) from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Data are shown for adenylate free leak respiration (L_N; A, E), maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity (P_{ETF}; B, F), submaximal state 3 respiratory capacity through complex I ($P_{CI;}$ C, G), and maximum oxidative phosphorylation capacity (P_{CI+CII}; D, H). Values are mean ± SEM, n=6-8, *=p<0.05, **=p<0.01, #=p<0.05 (C vs. U), +=p<0.05 (L vs. R), ^=p<0.05 (calorie restriction (CR) effect), ^^p<0.01 (CR effect). Black = C (control offspring), white = U (*in utero* undernourished offspring).

Discussion

Although there is an increasing appreciation of the role of *in utero* nutrition in modifying the susceptibility for metabolic disease, its effects on skeletal muscle have remained largely unexplored. *In utero* undernutrition has been linked to obesity and insulin resistance in animal models and is associated with metabolic disease in humans (3). In the current study, we used a mouse model of maternal undernutrition to show that low birth weight is associated with impaired skeletal muscle bioenergetics. Most notable is our demonstration of a negative impact of maternal undernutrition on skeletal muscle mitochondrial energetics and the capacity for weight loss. We provide evidence that maternal undernutrition alters muscle physiology (*e.g.,* fiber content) and down-regulates mitochondrial content and energetics, as studied in two experimental systems (*i.e.,* isolated mitochondria and permeabilized muscle fibers). Interestingly, these alterations were seen in predominantly mixed muscles (*quadriceps*, TA, wGAS), but not in highly oxidative muscles (*soleus* and rGAS).

The developmental programming hypothesis proposes that food restriction *in utero* causes metabolic adaptations to maintain energy homeostasis that favour survival in an environment with limited nutrient supply, resulting in a thrifty phenotype. However, with post-natal nutrient excess, these adaptations made *in utero* become detrimental, increasing susceptibility to obesity and glucose intolerance (1). Here we extend this hypothesis and demonstrate that low birth weight offspring when calorie restricted in adulthood, are resistant to weight loss due apparently to the thrifty metabolic

mechanisms programmed *in utero.* This study has used a global food restriction *in utero* and therefore we cannot exclude the possibility that our observations are largely a result of the reduction of a specific macro or micronutrient.

A key finding was that in response to a 4 wk 40% calorie restriction, U lost 50% less weight than C. This is particularly interesting given documented weight loss variation in diet-adherent obese women in a clinical weight loss program (21, 26, 28). The latter studies showed that obese diet-resistant subjects (i.e., in the lowest quintile for rate of weight loss) had decreased 'energy-wasting' mitochondrial proton leak respiration, decreased UCP3 mRNA content, down-regulation of genes involved in oxidative phosphorylation and glucose and fatty acid metabolism, and a decrease in oxidative muscle fibers in skeletal muscle compared to diet-sensitive subjects (21, 26). Consistent with this, pathway enrichment analysis of blood gene expression profiles showed down-regulation of the oxidative phosphorylation pathway in the obese diet-resistant subjects *prior to the hypocaloric diet program* (28). While many studies have examined the variation in weight gain, very few studies have focused on weight loss. Therefore, the latter human studies show that mitochondrial energetics are more efficient and fiber composition is less 'oxidative' in muscle of diet-resistant women compared to diet-sensitive women. The findings from the current study in which we document diet resistance in a murine epigenetic model are analogous in many ways, and thus are consistent with the possibility that environmental factors and epigenetic mechanisms may contribute, at least in part, to the clinical phenomenon of weight loss resistance.

Skeletal muscle accounts for ~20% of resting metabolic rate in humans and its mass and oxidative capacity are positively related to resting energy expenditure (29, 30). Given that muscle is an important determinant of whole body metabolism and insulin sensitivity, reductions in skeletal muscle mass are especially important. We found that offspring of undernourished dams (U) have reduced lean body mass and increased fat mass, as calculated from MRI. This alteration in body composition is a common phenotype associated with low birth weight in both humans and animal models (4, 31-34). This reduction in muscle mass may disrupt systemic metabolism and contribute to the decreased glucose tolerance and adult disease risk in this model. Metabolic rate was decreased in U in the dark phase after calorie restriction, as calculated from indirect calorimetry results compared to C. This decrease in metabolic rate during calorie restriction supports our observations of variation in weight loss and could explain, in part, why U lose less weight under this condition. There was a trend towards decreased physical activity in U in the dark phase, which may contribute to the decrease in metabolic rate. However, physical activity was not significantly different after calorie restriction. These findings suggest that with caloric restriction, U have an increased ability to conserve energy.

We were intrigued by the dramatic differences in rate of weight loss between U and C and therefore examined mitochondrial content in skeletal muscle. Overall, our findings show that mitochondrial content is decreased in U as assessed by mitochondrial yield from pooled skeletal muscle. More specifically, we identified that mitochondrial content is decreased in mixed fiber muscles as assessed by COX activity staining in TA and

complex IV (COX) protein expression in *quadriceps* muscle but remains unchanged in the highly oxidative *soleus* as assessed by COX activity staining. To further assess differences, mitochondrial energetics were assessed in isolated mitochondria and permeabilized muscle fibers. *In utero* undernutrition altered mitochondrial energetics with mitochondria from U having decreased state 2, state 3 (trend), and state 4_O respiration rates but an increased maximal respiration rate. These differences were not due to decreased expression of UCP3 or ANT or to a decrease in the relative proportion of the mitochondrial complexes but may be a result of their increased activity. Respiration in fibers from wGAS complemented and extended these results and showed that U have decreased adenylate free (*i.e.,* proton leak) respiration, fatty acid oxidative capacity, and complex I driven state 3 respiration. Maximal oxidative phosphorylation however was not different in permeabilized fiber preparations. Surprisingly, our results indicate that U have decreased muscle mitochondrial content while isolated mitochondria have increased maximal respiratory capacity. Given that muscle mitochondrial content is decreased, it seems that although skeletal muscle from U has a high capacity for oxygen consumption, under resting conditions metabolism is decreased. Therefore, we have shown that *in utero* undernutrition results in not only a decrease in mitochondrial content but also decreased respiration in muscle fibers and decreased respiration at the mitochondrial level. Taken together, these findings explain, at least in part, the dramatically blunted weight loss with calorie restriction in U. Given the central role that muscle plays in whole body metabolism, we suggest that muscle mitochondrial dysfunction caused by *in utero* undernutrition is largely responsible for resistance to weight loss and increased susceptibility to obesity and glucose intolerance

in adulthood. However, the impact of *in utero* undernutrition on other highly metabolic tissues, such as liver, may potentially contribute to the metabolic phenotype of these mice. This is beyond the scope of this paper but will be addressed in future studies.

Interestingly, *in utero* undernutrition did not affect respiration in fibers from rGAS, although calorie restriction had a more profound effect in rGAS compared to wGAS. These data demonstrate that alterations in metabolism that occur with *in utero* undernutrition are partly dependent on skeletal muscle type. Further supporting this, *in utero* undernutrition resulted in an increase in the proportion of type IIa fibers in the TA and eliminated the change in fiber type proportions induced by calorie restriction in this muscle, whereas there was no effect of *in utero* undernutrition on fiber type proportions in *soleus*. Similarly, COX activity was decreased in the TA of U, but not in the *soleus*. The increase in type IIa fibers in this mouse model is similar to the fiber type shift towards more glycolytic fibers and a lower oxidative capacity observed in muscle from obese individuals with T2DM (6, 9, 10). Our results are also interesting in light of our previous finding that weight loss resistant obese patients have a greater proportion of glycolytic fibers than weight loss sensitive obese patients (21). Taken together, results indicate that *in utero* undernutrition causes metabolic dysfunction in more glycolytic muscle, such as wGAS and TA, with minimal effects in more oxidative muscle, such as rGAS and *soleus*. Gene expression analysis of the TA showed that despite robust effects of calorie restriction on the expression of genes involved in skeletal muscle development and oxidative phosphorylation in control mice, gene expression was not drastically different between U and C, *especially under conditions of caloric restriction*

(Figure S2.3). Overall, it is apparent that calorie restriction has less of an effect on the transcriptome in U than C muscle. This is intriguing in light of the decreased weight loss and lack of change in fiber type induced by calorie restriction in U compared to C.

It has been shown in the same model of *in utero* undernutrition that the prevention of rapid catch-up growth in early life prevents the development of obesity and glucose intolerance in adulthood (35). In a study of low birth weight rats that were undernourished throughout gestation, exercise in adulthood was shown to eliminate prenatally induced obesity (36). These findings combined with our results suggest that, in low birth weight offspring, calorie restriction in early life is more effective than in adulthood to oppose obesity. Moreover, a physical activity intervention in adulthood may be more effective than calorie restriction to reduce obesity associated with low birth weight. This finding could be particularly important when designing weight loss programs for obese diet-resistant adults. Further research into the effectiveness of these interventions and their timing, and the combination of calorie restriction and physical activity is warranted.

In summary, our results show that *in utero* undernutrition impacts skeletal muscle energetics by altering mitochondrial content and oxidative functions. This is supported by several observations. First, adult mice from undernourished pregnancies have increased adiposity and when challenged with calorie restriction, have a dramatically decreased weight loss. Secondly, skeletal muscle mitochondrial content is decreased in mixed fiber muscles with *in utero* undernutrition. Thirdly, *in utero* undernutrition

resulted in decreased respiration in both isolated mitochondria and permeabilized muscle fibers. Taken together, results suggest that the decreased mitochondrial content and activity in mixed fiber muscles contribute to increased adiposity and impaired glucose tolerance and, when faced with dietary energy deficit there is an apparent heightened metabolic efficiency. Overall, findings suggest that low birth weight offspring may have developed a protective mechanism *in utero* for species survival in times when energy supply is restricted. As low birth weight is a significant risk factor for obesity and T2DM, understanding how it alters skeletal muscle is important for both prevention and therapy.

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Chapter 3

Altered oxidative and glycolytic capacities in primary muscle cells of low birth weight mice

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Statement of Manuscript Status and Contributions

The manuscript "Altered oxidative and glycolytic capacities in primary muscle cells of low birth weight mice" is under preparation for submission to Molecular Metabolism Journal. The specific contributions of each author are listed below.

Brittany Beauchamp designed experiments, performed and/or assisted with all experiments, prepared all figures, analyzed and interpreted all data, and wrote the manuscript.

Olivier Charette performed some of the bioenergetic determinations, prepared some of the samples for Western blots, and assisted with analysis and interpretation of data.

Kijoo Kim performed some of the bioenergetic determinations, prepared some of the samples for Western blots, and assisted with data analysis.

Mary-Elizabeth Patti interpreted data and edited the manuscript.

Mary-Ellen Harper designed experiments, interpreted data, and assisted with writing the manuscript.

Abstract

Objective: *In utero* undernutrition is associated with an increased risk for obesity and insulin resistance in adulthood. Low birth weight offspring have been shown to have alterations in skeletal muscle mass and function. Here we hypothesized that primary muscle cells of low birth weight offspring exhibit cell autonomous metabolic defects.

Methods: In differentiated myotubes from *in utero* undernourished mice (U) and control mice (C), we assessed mitochondrial bioenergetics, as well as oxidative and glycolytic capacities, and mitochondrial content.

Results: Myotubes from U have decreased resting respiration, impaired fatty acid oxidation characteristics and increased glycolysis compared to myotubes from C. There was no difference in myotube mitochondrial content.

Conclusions: We provide the first evidence that myotubes established from satellite cells of *in utero* undernourished mice have marked impairments in oxidative metabolism and enhanced glycolytic characteristics. Findings are consistent with the conclusion that susceptibility to metabolic disease in adulthood can be caused by primary muscle defects that are programmed *in utero*.

Introduction

Suboptimal early life nutrition can increase risk for the development of disease in adulthood. Human epidemiological studies and research examinig animal models have shown that low birth weight, as a result of maternal undernutrition, is associated with increased risk of metabolic disease, such as type 2 diabetes mellitus (Reviewed in (1)). It has been hypothesized that permanent alterations in tissue structure and function can be induced by perinatal events and thereby increase disease risk (2, 3). However, how a suboptimal *in utero* environment leads to adult disease has not been fully elucidated.

Skeletal muscle is a remarkably adaptable tissue that modifies its structure and function in response to need (4, 5). Skeletal muscle is a key determinant of systemic metabolism and insulin sensitivity and therefore alterations in its function may contribute to adult disease risk (6-8). A common physiological phenotype in low birth weight offspring is alterations in skeletal muscle mass and function. Low birth weight has been associated with reduced muscle mass and a shift toward more glycolytic muscle fibers (9-14). Skeletal muscle dysfunction is also strongly associated with obesity and insulin resistance (15-19). We have recently used a mouse model of low birth weight generated through 50% food restriction during the third week of pregnancy, originally described in (12). It has been shown that these low birth weight offspring develop glucose intolerance and increased adiposity in adulthood, similar to findings in human populations (12, 14). We recently showed that the low birth weight

offspring have decreased skeletal muscle mitochondrial content in mixed fiber muscles, decreased respiration in isolated skeletal muscle mitochondria, and decreased fatty acid supported respiration in permeablized muscle fibers (14). Intriguingly, low birth weight offspring had a blunted weight loss response to a hypocaloric diet in adulthood (14). Furthermore, prenatally undernourished mice have been shown to have reduced myogenic stem cell frequency and reduced regenerative capacity (20).

Therefore, in the current study we aimed to determine if there are primary, cell autonomous, defects in metabolic functions in cells derived from muscle progenitors (i.e., satellite cells). We hypothesized that the previously documented increased susceptibility to obesity and glucose intolerance is due in part to a primary muscle defect that is programmed *in utero*.

Materials and Methods

Animals

All procedures involving the use of animals were performed according to the principles and guidelines of the Canadian Council of Animal Care and the study was approved by the Animal Care Committee of the University of Ottawa. Mice were housed in a facility with controlled temperature, humidity, and light-dark cycle (0600h – 1800h). Virgin female ICR mice (Harlan, Indianapolis, IN, USA; age 6-8 weeks) were paired with male ICR mice (Harlan; age 6-8 weeks). Pregnancies were dated by vaginal plug (day 0.5) and pregnant mice were housed individually with *ad libitum* access to standard rodent chow (T.2018, Harlan Teklad, Indianapolis, IN, USA). On day 12.5 of pregnancy, dams were randomly assigned to either a control or undernutrition group. In the undernutrition group, food was restricted to 50% that of gestational day-matched controls for the remainder of pregnancy. After delivery, mothers were given *ad libitum* access to chow and 24 hours after birth, litters were equalized to eight. We studied two groups of female mice, *in utero* undernourished offpring (U) and control offspring (C).

Isolation of mouse primary cells

Primary muscle cells were isolated from the *quadriceps* of U and C at 3 weeks old. Cells were isolated as previously described (21).

Cell culture

Isolated primary muscle cells were grown on Matrigel coated flasks in low glucose (5.5 mmol/L) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% FBS, 1X antibiotic-antimycotic, 2.5µg/ml gentamycin and 2.5ng/ml basic fibroblast growth factor. For experiments, when cells reached approximately 90% confluency, they were differentiated in low glucose DMEM supplemented with 2% FBS, 1X antibiotic-antimycotic, and 2.5µg/ml gentamycin. Differentiation was verified by immunofluorescence staining (Desmin and DAPI) as described previously (22).

Functional analyses of cellular metabolic characteristics

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at 37°C using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience Inc., North Billerica, MA). Cells were plated at 20 000 cells per well on Matrigel coated Seahorse plates.

Cellular bioenergetics

Myotubes were provided with an unbuffered assay medium (HCO_3-free DMEM with 5mM glucose, 4mM glutamine, 1mM pyruvate, pH 7.4) and incubated at 37°C in a non CO_2 incubator for 30 min before being placed in the Extracellular Flux Analyzer for OCR measurement. Resting measurements were taken before sequential additions of

oligomycin (1μg/ml), FCCP (1μM), and antimycin A (1μM) plus rotenone (40uM) to determine state 4, maximal, and non-mitochondrial OCR, respectively. All measurements were obtained in quintuplicate over 2 min measurement periods, with 2 min mix and 2 min incubation periods between measurements. Values were corrected to cellular protein content determined using a Bradford assay.

Myotube fatty acid oxidation assay

Myotubes were incubated overnight in a substrate-limited medium (glucose- and glutamine- free DMEM, supplemented with 0.5mM glucose, 1mM glutamine, 0.5mM carnitine and 1% (v/v) FBS; pH 7.4). 45 minutes prior to the experiment, cells were provided with a fatty acid oxidation assay medium (111mM NaCl, 4.7mM KCl, 2mM $MgSO_4$, 1.2mM Na_3PO_4, 2.5mM glucose, 0.5mM carnitine and 5mM HEPES; pH 7.4) and incubated at 37˚C at ambient CO_2. 40μM etomoxir or vehicle control was added 15 minutes prior to the experiment. 75μM Palmitate:BSA or BSA control was added immediately prior to the experiment. OCR measurements were taken at rest before sequential additions of oligomycin (2.5μg/ml), FCCP (1μM), and antimycin A (1μM) and rotenone (40uM), which then allowed determinations of state 4, maximal, and non-mitochondrial OCR respectively. All measurements were obtained in quintuplicate over 2 min measurement periods, with 2 min mix and 2 min incubation periods between measurements. Values were corrected to protein content determined using a Bradford assay. Exogenous fatty acid use calculated as rate with Palmitate:BSA (no

etomoxir) minus rate with BSA control (no etomoxir). Endogenous fatty acid use calculated as rate with BSA control (no etomoxir) minus rate with BSA and etomoxir.

Myotube glycolysis assay

60 minutes prior to the experiment, myotubes were incubated at 37˚C at ambient CO_2 in a glycolysis stress assay medium (DMEM without glucose and glutamine, supplemented with 143mM NaCl, 0.5% phenol red and 2mM glutamine; pH 7.4). ECAR was measured at baseline followed by sequential injections of glucose (10mM), oligomycin (2.5µg/mL), and 2-deoxy-D-glucose (100mM) to measure glycolysis, maximal glycolytic capacity, and non-glycolytic acidification respectively. Measurements were obtained in quintuplicate over 2 min measurement periods with 2 min mix and 2 min incubation periods between measurements with the exception of basal rates that were measured for 4 min. Values were corrected to protein content determined using a Bradford assay.

Western blotting

Myotubes were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM NaF, 0.2 mM Na_3VO_4, protease inhibitor cocktail (Roche, Mississauga, ON); pH 7.6). Protein content was measured using a bicinchoninic acid assay and samples were stored at -80°C. Samples were subjected to reducing SDS-PAGE. Proteins were electroblotted onto nitrocellulose

membranes and stained with Ponceau *S*. After blocking for 1 h at room temperature in 5% (w/v H_2O) skim milk powder, incubation in primary antibody was overnight at 4°C. The following primary antibodies were used at the indicated dilutions: MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam; 1:800), AMPK alpha (2532, Cell Signaling; 1:1000), pAMPK alpha (T172) (2531, Cell Signaling, 1:1000), acetyl-CoA carboxylase (3662, Cell Signaling, 1:500), p-acetyl-CoA carboxylase (S79) (3661, Cell Signaling; 1:500), PDH E1-alpha (456600, Life Technologies; 1:1000), phospho-PDH E1-alpha type 1 (Ser293) (ABS204, EMD Millipore; 1:5000). Following 3 x 10 min washes with TBS + 0.1% Tween-20, membranes were incubated in the appropriate horseradish peroxidise-conjugated secondary antibody diluted in 5% skim milk at room temperature for 1h. Bands were visualized using enhanced chemiluminescence. Band intensity was quantified by density analysis using Image J (NIH) and normalized to α-tubulin or Ponceau *S* stained membrane, as indicated.

Statistical analyses

All measures were analyzed using GraphPad Prism (La Jolla, CA, USA). Differences between groups were analyzed using a two-tailed Student's *t*-test. Values are reported as mean ± SEM and $p<0.05$ was considered significant.

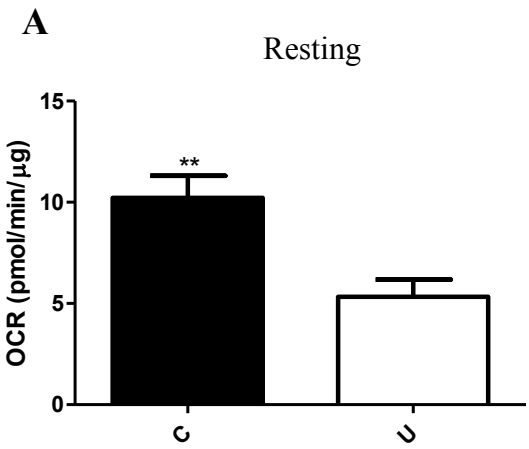
Results

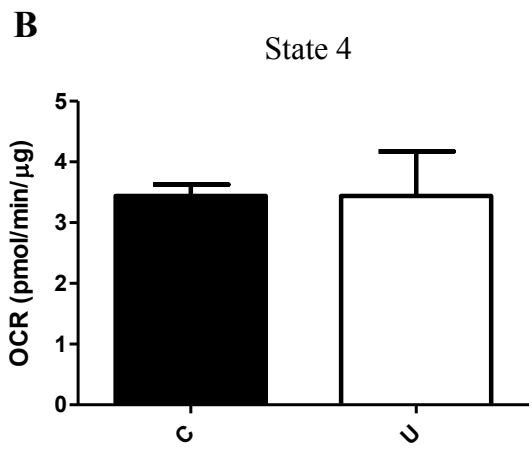
Lower respiration rates in myotubes from *in utero* undernourished mice

To assess overall bioenergetics characteristics of myotubes from U and C, mitochondrial OCR was measured under resting, oligomycin-induced state 4, and maximal uncoupled (FCCP-induced) conditions. Respiration under resting conditions was 48% lower in U (Figure 3.1A). There was no difference in respiration under state 4 or maximal uncoupled conditions (Figure 3.1B, 3.1C). To further assess metabolic characteristics of the cells, we measured fatty acid oxidation and glycolysis.

Dysfunctional fatty acid oxidation in myotubes from *in utero* undernourished mice

To measure fatty acid oxidation, cells were first incubated overnight in a substrate-limited medium, which was then changed to a fatty acid oxidation assay medium and treated with 75 μM palmitate. OCR in the presence of palmitate was measured under resting, state 4, and maximal uncoupled conditions. Respiration under resting (Figure 3.2A) and maximal uncoupled conditions (Figure 3.2C) was lower in U compared to C, with a trend for lower state 4 respiration (Figure 3.2B). Cells were also treated with etomoxir to allow for determination of fatty acid oxidation due to endogenous fatty acid use or exogenous fatty acid (palmitate) use. Etomoxir inhibits carnitine palmitoyltransferase I (CPT-I), an enzyme required for the formation and transport of


A
Resting
OCR (pmol/min/µg)
15
10
5
0
**
C
U


B
State 4
OCR (pmol/min/µg)
5
4
3
2
1
0
C
U

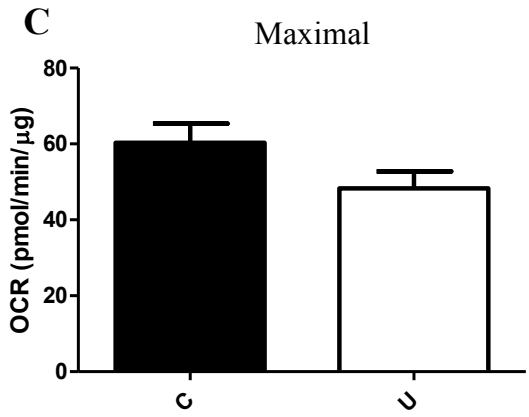

C
Maximal
OCR (pmol/min/µg)
80
60
40
20
0
C
U

Figure 3.1. Metabolic characterization of myotubes from U and C mice. Data are shown for resting (A), state 4 (B), and maximal (C) oxygen consumption rate (OCR) in myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars). Values are mean ± SEM, n=5-6. Student's *t*-test, ** = $p<0.01$.

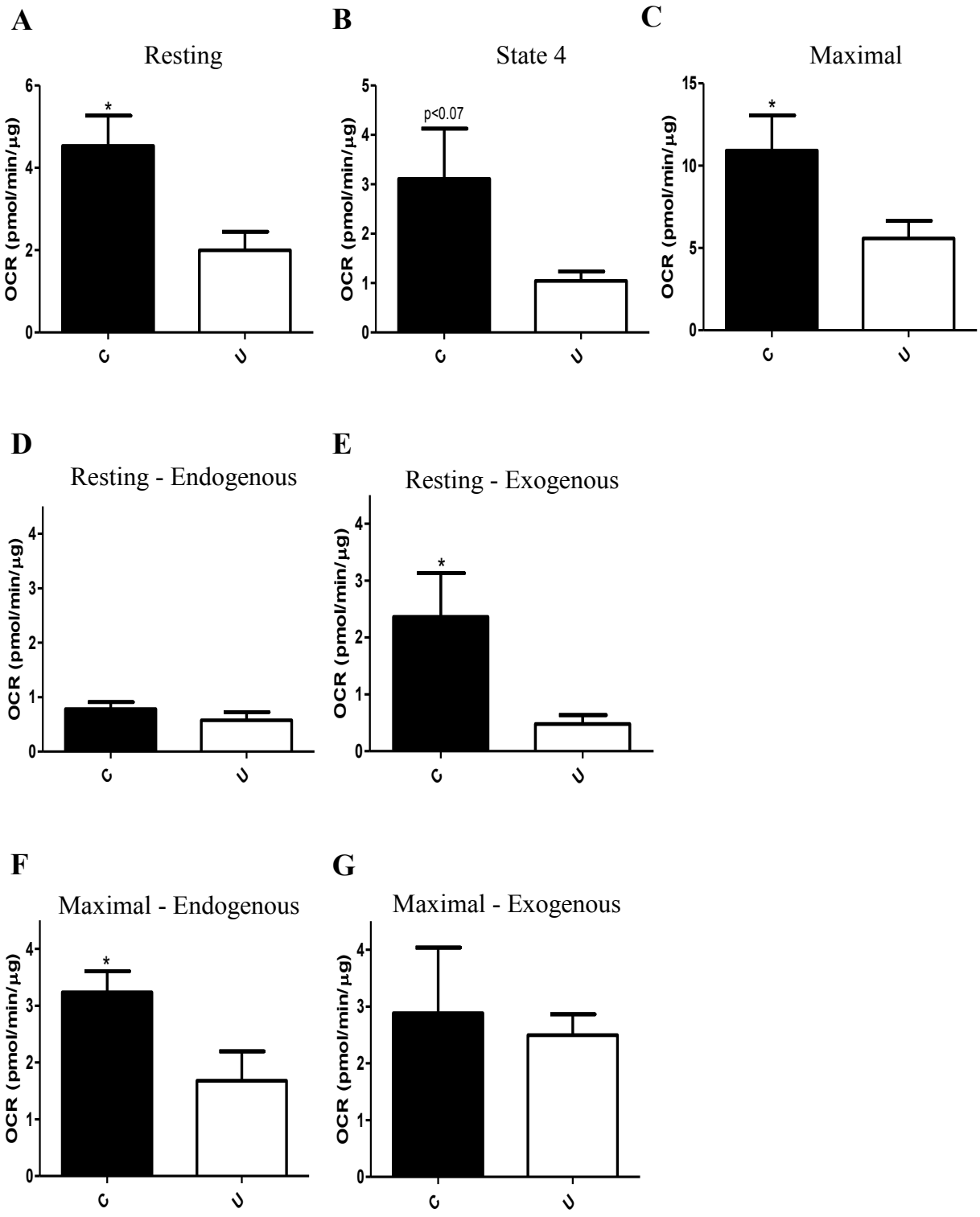

A
Resting
OCR (pmol/min/µg)
*
C
U
B
State 4
p<0.07
C
Maximal
15
10
5
D
Resting - Endogenous
E
Resting - Exogenous
F
Maximal - Endogenous
G
Maximal - Exogenous

Figure 3.2. Dysfunctional fatty acid oxidation in myotubes from *in utero* undernourished mice. Oxygen consumption rate (OCR) was measured in myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars) in the presence of 75 uM palmitate. Resting (A), state 4 (B), and maximal (C) OCR were measured. Resting respiration due to utilization of endogenous fatty acids (D), resting respiration due to utilization of exogenous fatty acids (E), maximal respiration due to utilization of endogenous fatty acids (F), and maximal respiration due to utilization of exogenous fatty acids (G) were determined. For determination of endogenous fatty acid use, OCR +etomoxir (40 μM) was subtracted from OCR –etomoxir. Values are mean ± SEM, n=7. Student's *t*-test, * = $p<0.05$.

long chain acyl carnitines from the cytosol into the mitochondria (23). Under resting conditions, there is no difference in the rates of endogenous fatty acid oxidation between U and C (Figure 3.2D) but exogenous fatty acid oxidation is decreased in U (roughly 20% of C; Figure 3.2E). Under maximal respiration conditions (*i.e.,* in the presence of FCCP), endogenous fatty acid oxidation in U is only half of that in C and there is no difference in exogenous fatty acid oxidation (Figure 3.2F, 3.2G).

Enhanced glycolysis in myotubes from *in utero* undernourished mice

For the glycolysis assay, cells were incubated in glucose-free media for 1 hour and ECAR was measured after the sequential addition of glucose, oligomycin, and 2-deoxy-D-glucose to assess glycolysis, maximal glycolytic capacity, and non-glycolytic acidification, respectively. Myotubes from U had an approximately doubled glycolytic activity in response to the addition of glucose and had a similarly increased maximal glycolytic capacity compared to C (Figure 3.3A, 3.3B). Non-glycolytic acidification was not significantly different between U and C (Figure 3.3C).

Mitochondrial content is unaltered, but there is lower AMPK phosphorylation in myotubes from *in utero* undernourished mice

Given the metabolic differences observed, we assessed protein levels of mitochondrial complexes I-V as a marker of mitochondrial content. There was no difference in the protein levels of any of these mitochondrial complexes between U and C (Figure 3.4).

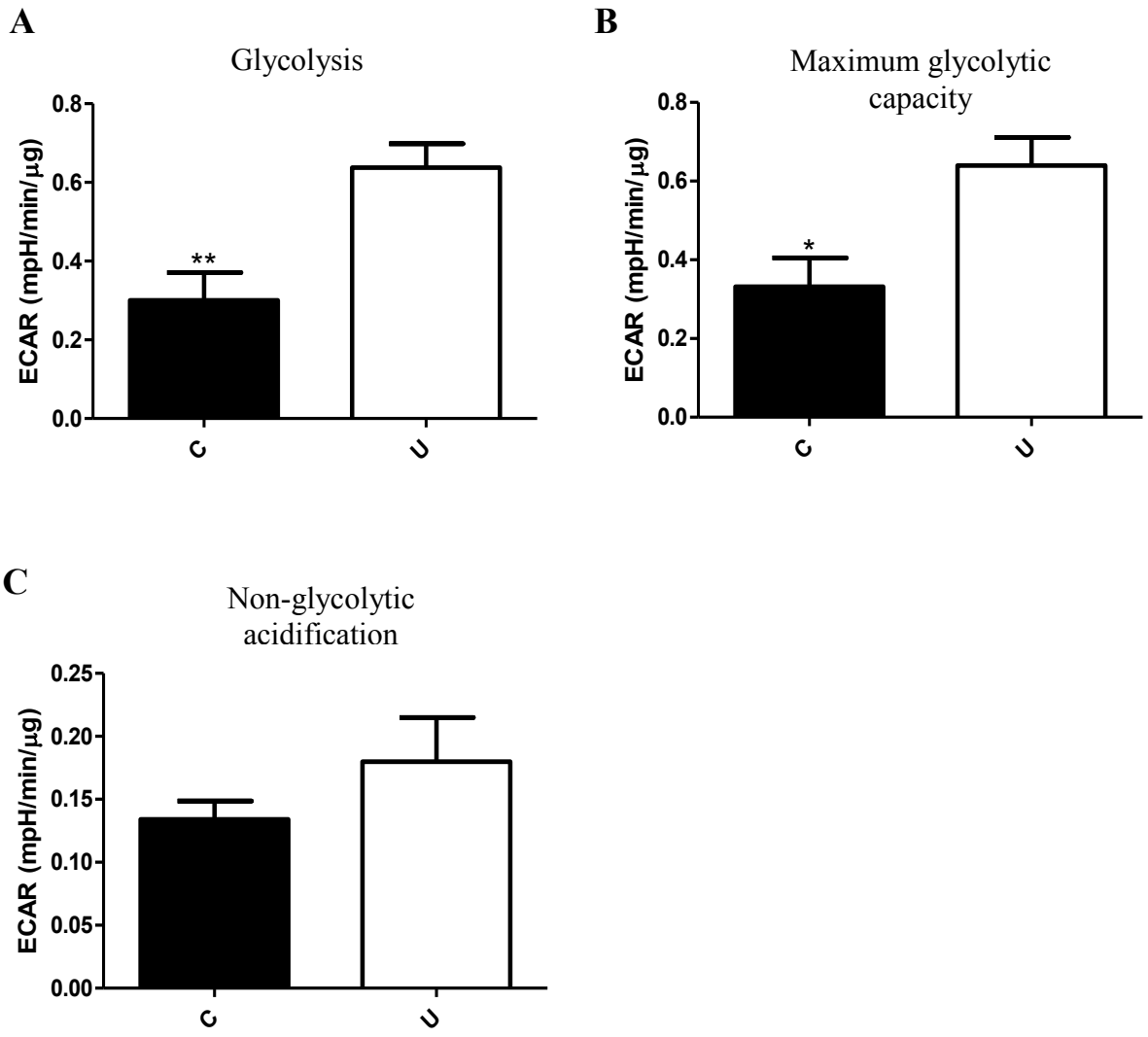

A
Glycolysis
ECAR (mpH/min/µg)
0.8
0.6
0.4
0.2
0.0
**
C
U
B
Maximum glycolytic capacity
ECAR (mpH/min/µg)
0.8
0.6
0.4
0.2
0.0
*
C
U
C
Non-glycolytic acidification
ECAR (mpH/min/µg)
0.25
0.20
0.15
0.10
0.05
0.00
C
U

Figure 3.3. Enhanced glycolysis in myotubes from *in utero* undernourished mice. Extracellular acidification rate (ECAR) was measured to determine glycolysis (A), maximum glycolytic capacity (B), and non-glycolytic acidification (C) in myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars) in the presence of 10 mM glucose. Values are mean ± SEM, n=7. Student's *t*-test, * = $p<0.05$, ** = $p<0.01$.

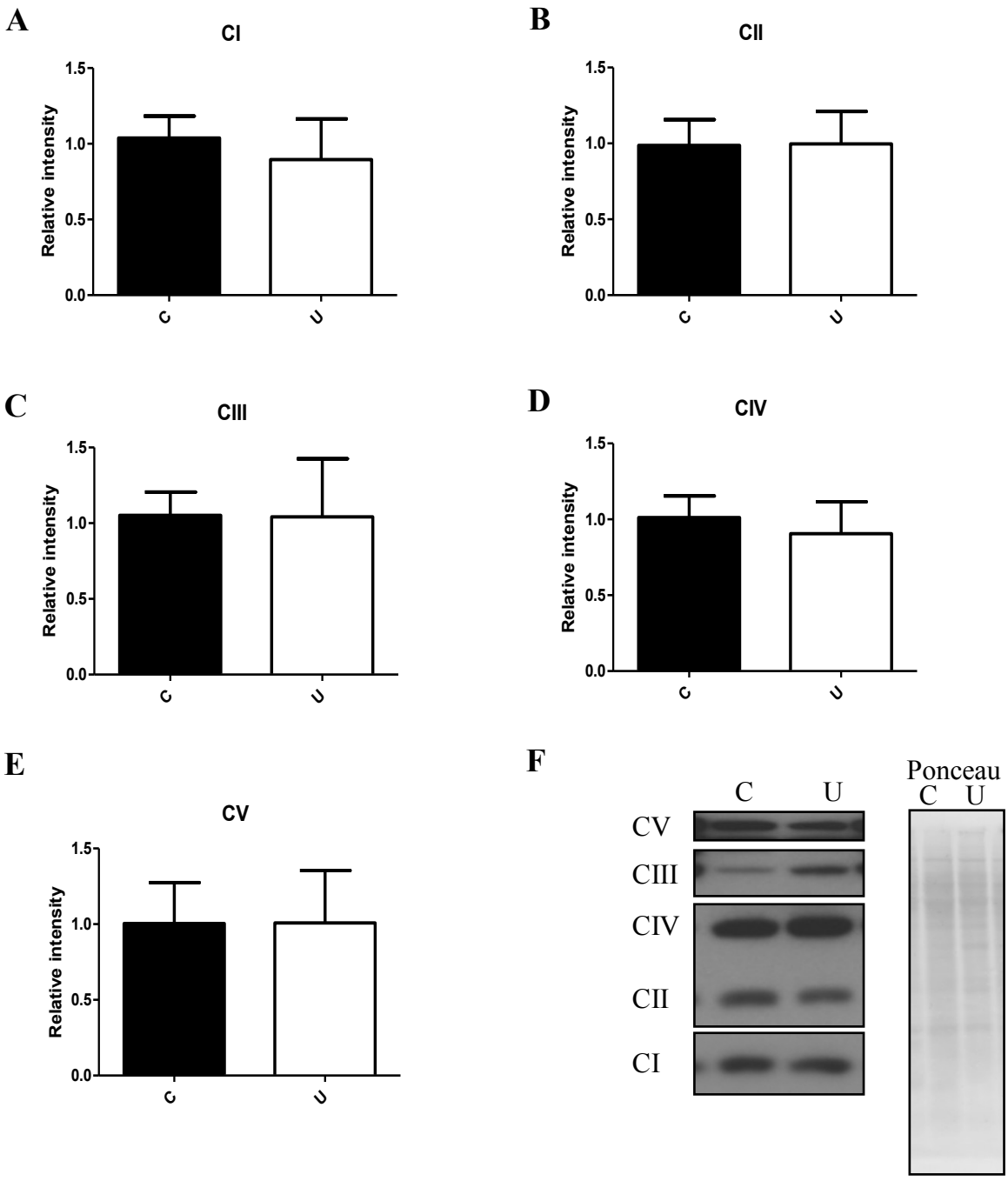

A
CI
Relative intensity
1.5
1.0
0.5
0.0
C
U
B
CII
Relative intensity
1.5
1.0
0.5
0.0
C
U
C
CIII
Relative intensity
1.5
1.0
0.5
0.0
C
U
D
CIV
Relative intensity
1.5
1.0
0.5
0.0
C
U
E
CV
Relative intensity
1.5
1.0
0.5
0.0
C
U
F
C
U
CV
CIII
CIV
CII
CI
Ponceau
C
U

Figure 3.4. Mitochondrial content is not altered in myotubes from *in utero* undernourished mice. Protein expression of mitochondrial complexes normalized to Ponceau *S* stained membrane in lysed myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars). Quantification of complex I (CI; A), II (CII; B), III (CIII; C), IV (CIV; D), and V (CV; E) expression and representative Western blot (F).Values are mean ± SEM, n=7.

We also assessed protein levels of AMP-activated protein kinase (AMPK), pyruvate dehydrogenase (PDH), acetyl-CoA carboxylase (ACC), and the phosphorylated forms of these proteins. AMPK protein levels were not different between U and C, however pAMPK was lower in U (Figure 3.5A, 3.5B). We did not detect any significant differences in PDH, pPDH, ACC, or pACC (Figure 3.5C-3.5F).

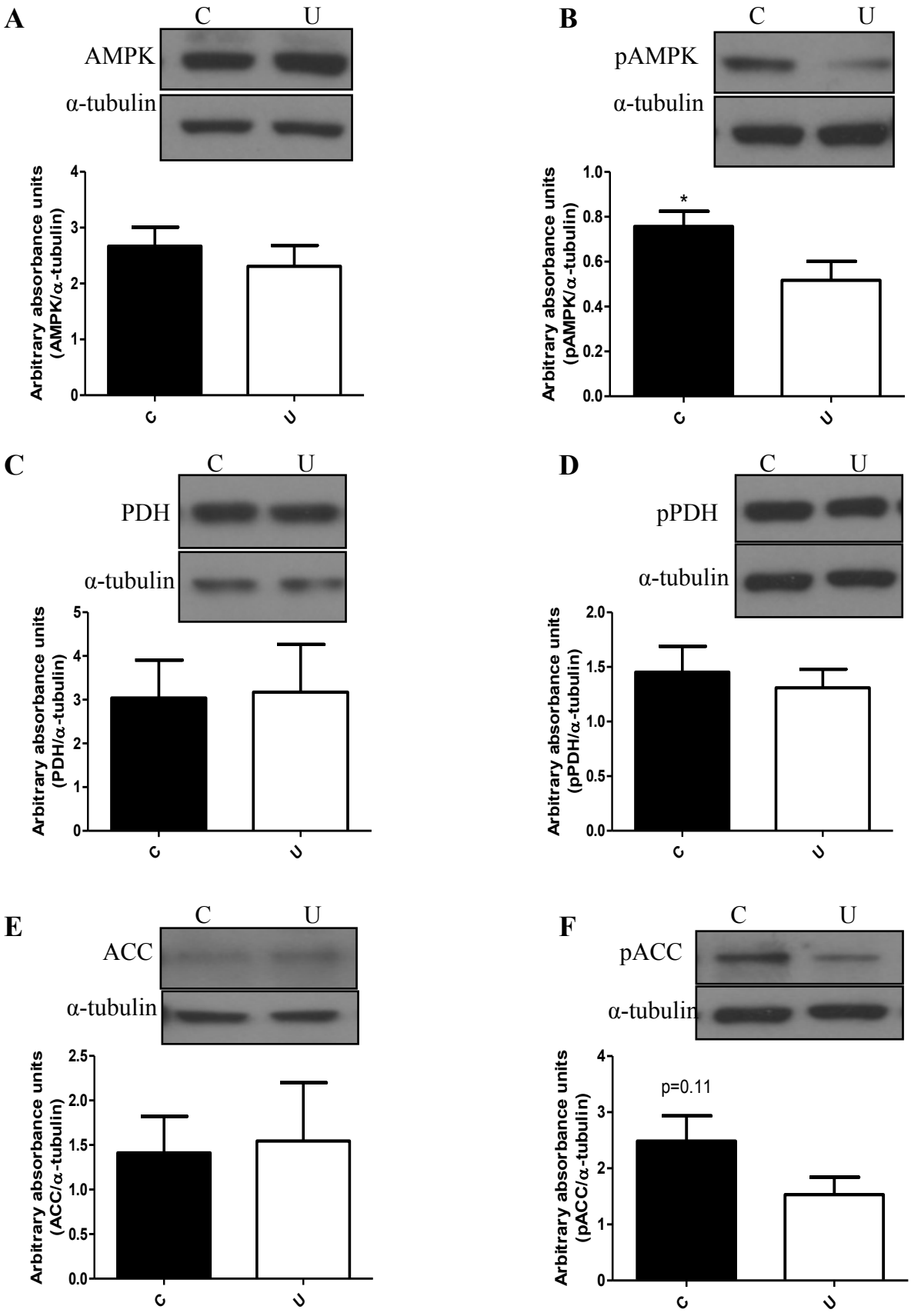

A
C
U
AMPK
α-tubulin
Arbitrary absorbance units (AMPK/α-tubulin)
B
pAMPK
α-tubulin
Arbitrary absorbance units (pAMPK/α-tubulin)
*
C
PDH
α-tubulin
Arbitrary absorbance units (PDH/α-tubulin)
D
pPDH
α-tubulin
Arbitrary absorbance units (pPDH/α-tubulin)
E
ACC
α-tubulin
Arbitrary absorbance units (ACC/α-tubulin)
F
pACC
α-tubulin
Arbitrary absorbance units (pACC/α-tubulin)
p=0.11

Figure 3.5. Impaired oxidative metabolism in myotubes of *in utero* undernourished mice is associated with lower levels of AMPK phosphorylation. Protein expression of AMPK (AMP-activated protein kinase; A), pAMPK (phosphorylated AMPK; B), PDH (pyruvate dehydrogenase; C), pPDH (phosphorylated PDH; D), ACC (acetyl-CoA carboxylase; E), and pACC (phosphorylated ACC; F) normalized to α-tubulin loading control in lysed myotubes from *in utero* undernourished mice (U; white bars) and control mice (C; black bars). Representative Western blots are shown above and quantification is shown below. Values are mean ± SEM, n=7. Student's *t*-test, * = $p<0.05$.

Discussion

In this study, we provide the first evidence that functional characteristics of primary muscle cells established from satellite cells of low birth weight mice have 'programmed' metabolic defects. We and others have previously reported on the increased risk of metabolic disease, including obesity and type 2 diabetes, associated with low birth weight (1, 12, 14). However the degree to which these phenotypic outcomes were dependent upon effects to differentiated cells and developed systems in the embryo versus effects on stem cells was unknown. Here we show that myotubes derived from muscle satellite cells of U have decreased resting respiration and increased glycolysis. Thus, previous observations of impaired oxidative metabolism in human populations and in mouse models of low birth weight are likely associated with cell autonomous mechanisms (13, 14, 24).

Previously, we have shown that adult mouse offspring that were undernourished *in utero* have decreased skeletal muscle mitochondrial respiration and decreased respiration in permeabilized muscle fibers (14). Our current findings confirm these previous observations and extend them by positing that the decreased respiration in skeletal muscle is a primary muscle defect. Studies have repeatedly shown that skeletal muscle oxidative activity is decreased in individuals with obesity and type 2 diabetes (16, 17, 25, 26). Interestingly, similar observations of decreased oxidative capacity combined with an increased glycolytic capacity have been found in humans with obesity and type 2 diabetes (27, 28). Furthermore, skeletal muscle from individuals

with obesity and type 2 diabetes has been shown to have a decreased proportion of slow oxidative fibers and an increase in the proportion of fast glycolytic fibers (16, 29-32). Moreover, our group noted that obese women in the lowest quintile for the rate of weight loss in an intensively supervised clinical weight loss program had significantly higher proportions of glycolytic fibers than age- and initial body weight-matched controls (33). Therefore, a common metabolic alteration associated with obesity, insulin resistance and diet resistance is an increase in glycolytic capacity and a decrease in oxidative capacity, which is also present in myotubes established from low birth weight mice.

Here we have demonstrated lower resting respiration in myotubes derived from muscle satellite cells of U, compared to C. When myotubes were then forced to use palmitate as a substrate, respiration in myotubes from U was lower compared to C when cells were analyzed under resting metabolic conditions and when mitochondrial oxidative phosphorylation was maximally uncoupled. Thus myotubes from U have dysfunctional fatty acid oxidation. We also assessed the contribution of exogenous and endogenous fatty acid oxidation to further understand the observed differences. Results are consistent with the conclusion that the dysfunctional fatty acid oxidation under resting conditions in myotubes from U is due to an impaired capacity to use exogenous fatty acid. In contrast, the dysfunctional fatty acid oxidation under maximally uncoupled conditions in myotubes from U is due to a decrease in endogenous fatty acid use. This suggests that, although myotubes from U have the

ability to use similar levels of exogenous fatty acids, under resting conditions they are using less.

In skeletal muscle, fatty acid oxidation is largely dependent on the rate at which fatty acyl coenzyme A is transported into the mitochondria by CPT-I. ACC activity decreases fatty acid oxidation by inhibiting CPT-I via increased malonyl-CoA concentrations. ACC is inactivated by its phosphorylation which is catalyzed by kinases, such as AMPK (34). We did not observe a significant difference in ACC or pACC protein levels, although there was a trend for decreased pACC. The observed differences may be due in part to other mechanisms of CPT-I regulation or alterations in other enzymes of β-oxidation. Further studies are needed to determine the specific defect in fatty acid oxidation in U. This is of interest given that impaired fatty acid oxidative capacity has been shown to be associated with obesity and insulin resistance (18, 19, 35). Lipid oxidation has also been shown to be reduced in myotubes from type 2 diabetic patients (36). It was suggested that the reduced complete palmitate oxidation in diabetic muscle observed *in vivo* may be of genetic origin (36). Our results demonstrate that the fatty acid oxidation defects observed in myotubes from low birth weight mice are not of genetic origin but may be of environmental or epigenetic origin. Overall, findings suggest that myotubes established from U express inherited 'programmed' defects in fatty acid metabolism which may contribute to the development of obesity and insulin resistance in adulthood. This study was limited to the effect of a specific fatty acid, palmitate. Palmitate was chosen since it is the most abundant dietary saturated fatty acid and has

been shown to impair insulin signaling in muscle cells (37). However, other fatty acids may have different effects on metabolism.

We hypothesized that the observed dysfunctional respiration in myotubes may be due to decreased mitochondrial content. We previously found decreased mitochondrial content in muscle of adult U compared to C mice (14). In contrast, in the primary cells studied herein, there were no differences in mitochondrial content between U and C myotubes. Therefore, findings suggest that the observed energetic differences in the myotubes are due to altered mitochondrial function rather than content. We also assessed protein levels of AMPK, an important regulator of cellular energy metabolism and pAMPK, its activated form. AMPK levels were not different in myotubes from U compared to C but pAMPK was significantly lower. Therefore, overall there is a decrease in activated AMPK in myotubes from U compared to C. AMPK has insulin-sensitizing effects and, amongst other functions, stimulates glucose uptake in skeletal muscle (38). There is evidence suggesting that AMPK is dysregulated in animals and humans with obesity and type 2 diabetes (39, 40). Thus, a decrease in activated AMPK may contribute to the metabolic differences observed in the myotubes derived from U compared to C. Future studies will investigate mechanistic aspects further. We also assessed protein levels of PDH and its inactive form, pPDH. PDH catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA, which can then be used for respiration. We did not detect differences in protein levels of PDH or pPDH in myotubes from U compared to C. It is possible that the observed oxidative and glycolytic differences are due to a difference in the activity of one or more of the mitochondrial complexes and/or

their post-translational modification. Overall, the nutritional intervention in this study was *in utero*, which suggests that the functional changes may be mediated by epigenetic molecular events that modulate metabolic gene expression. Future studies should investigate potential epigenetic mechanisms.

In summary, we show that muscle satellite cell-derived myotubes from low birth weight mice have inherited, non-genetic, defects in the metabolism of glucose and fatty acid that are consistent with an insulin-resistant phenotype. We have shown that myotubes established from U have substantially impaired fatty acid oxidation and enhanced glycolysis. These findings are consistent with the hypothesis that mitochondrial dysfunction in muscle of U may contribute to their increased susceptibility to obesity and insulin resistance. Overall, the altered muscle metabolism may be a compensatory mechanism programmed *in utero* to handle times of limited nutrient availability that becomes detrimental in adult life when nutrients are abundant. Thus, our findings suggest that undernutrition *in utero* causes a primary muscle defect that increases susceptibility to metabolic disease in adulthood.

Acknowledgements

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Chapter 4

Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring

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Statement of Manuscript Status and Contributions

The manuscript "Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring" has been accepted for publication in Bioscience Reports. The specific contributions of each author are listed below.

Brittany Beauchamp designed experiments, performed and/or assisted with all experiments, prepared all figures, analyzed and interpreted all data, wrote the manuscript, and revised the manuscript.

A. Brianne Thrush assisted significantly with high resolution respirometry experiments, citrate synthase activity assays, and Western blots and edited the manuscript.

Jessica Quizi performed citrate synthase activity assays and some Western blots.

Ghadi Antoun assisted with high resolution respirometry experiments.

Nathan McIntosh performed and wrote the methods for the acylcarnitine determinations.

Osama Y. Al-Dirbashi assisted with interpretation of the acylcarnitine data and edited the manuscript.

Mary-Elizabeth Patti assisted with interpretation of data and edited the manuscript.

Mary-Ellen Harper designed experiments, interpreted data, and assisted with writing and revising the manuscript.

Contributions statement from manuscript: Conceived and/or designed the work (BB, MEH); performed experiments (BB, ABT, JQ, GA, NM); analyzed and/or interpreted results (BB, ABT, JQ, NM, OYA, MEP, MEH); wrote the manuscript (BB, MEH). All authors revised the manuscript and approved the final version. All authors revised the article, approved the final version to be published, and agreed to be accountable for the work.

Abstract

Intrauterine growth restriction is associated with an increased risk of developing obesity, insulin resistance, and cardiovascular disease. However its effect on energetics in heart remains unknown. In this study, we examined respiration in cardiac muscle and liver from adult mice that were undernourished *in utero*. We report that *in utero* undernutrition is associated with impaired cardiac muscle energetics, including decreased fatty acid oxidative capacity, decreased maximum oxidative phosphorylation rate, and decreased proton leak respiration. No differences in oxidative characteristics were detected in liver. We also measured plasma acylcarnitine levels and found that short-chain acylcarnitines are increased with *in utero* undernutrition. Results reveal the negative impact of suboptimal maternal nutrition on adult offspring cardiac energy metabolism, which may have lifelong implications for cardiovascular function and disease risk.

Introduction

The developmental programming hypothesis suggests that adverse influences during critical periods in development permanently alter tissue structure and function, which may have persistent consequences for the long-term health of the offspring (1). Epidemiological studies in humans and animal models have shown that intrauterine growth restriction (IUGR) is associated with an increased risk of developing obesity, insulin resistance, and cardiovascular disease in the offspring (as reviewed in (2)). IUGR fetuses show a significant decline in cardiac systolic function and children affected by IUGR have been found to have premature stiffening of carotid arteries and elevated blood pressure (3-5). In rodent models, IUGR offspring are more susceptible to the development of hypertension and cardiac dysfunction in adulthood (6, 7). Although there is a strong association between IUGR and cardiovascular disease, the effect of exposure to maternal undernutrition on energetics in the heart has not been investigated.

Previous research in mice has shown that 50% food restriction during late pregnancy leads to IUGR, low birth weight and offspring that develop progressive, severe glucose intolerance and beta cell dysfunction (8, 9). Using this model, we previously showed that these low birth weight offspring have increased adiposity, decreased skeletal muscle energetics in mixed muscle and a blunted weight loss response to a hypocaloric diet in adulthood (9). Using adult mice from this subsequent study, we have investigated the effects of *in utero* undernutrition on energetics in the heart and liver.

We also report analysis of plasma acylcarnitines, which have previously been shown to be biomarkers of metabolic and/or cardiovascular risk (10-14).

The heart is an organ with a high energy requirement, turning over ~30 kg of ATP daily in humans (15). Thus, it requires high oxygen uptake to synthesize sufficient ATP by oxidative phosphorylation for proper function. Cardiac diseases are associated with changes in myocardial energy metabolism. A general decrease in oxidative capacity and down-regulation of enzymes of fatty acid oxidation in cardiac muscle have been reported in different models of heart failure (16-21). Mitochondria in cardiac muscle of individuals with type 2 diabetes mellitus (T2DM) have a decreased capacity for fatty acid-supported respiration (22). Additionally, in a mouse model of obesity and diabetes, mitochondria have reduced oxidative capacity (23). We therefore hypothesized that low birth weight induced by maternal undernutrition during late pregnancy leads to long-term impairment of cardiac energy metabolism in offspring.

Materials and Methods

Animals

All procedures involving the use of animals were performed according to the principles and guidelines of the Canadian Council of Animal Care and the study was approved by the Animal Care Committee of the University of Ottawa. Mice were housed in a facility with controlled temperature, humidity, and light-dark cycle (0600h – 1800h). Virgin female ICR mice (Harlan, Indianapolis, IN, USA; age 6-8 weeks) were paired with male ICR mice (Harlan; age 6-8 weeks). Pregnancies were dated by vaginal plug (day 0.5) and pregnant mice were housed individually with *ad libitum* access to standard rodent chow (T.2018, Harlan Teklad, Indianapolis, IN, USA). On day 12.5 of pregnancy, dams were randomly assigned to either a control or undernutrition group. In the undernutrition group, food was restricted to 50% that of gestational day matched controls for the remainder of pregnancy. After delivery, mothers were given *ad libitum* access to chow and 24 hours after birth, litters were equalized to eight. Pups were weaned at three weeks. At 10 weeks, these offspring were randomly assigned to either a 40% calorie restricted group (D01092702: Research Diets) or an *ad libitum* control group. At 14 weeks of age, 4 groups of mice were studied: *in utero* undernourished offspring fed *ad libitum* postnatally (U-L); *in utero* undernourished offspring that were calorie restricted for 4 weeks during adulthood (U-R); control offspring fed *ad libitum* (C-L); and control offspring that were calorie restricted for 4 weeks during adulthood (C-R). Female mice were used for all determinations, since preliminary work showed a

stronger phenotype in female than male offspring. All endpoint determinations were performed with mice from at least four different litters per group, with no more than two mice from the same litter.

Tissue collection

Blood was collected by cardiac puncture. Plasma was stored at -80°C for future analyses. Heart and liver were removed immediately after sacrifice. Myocardial tissue was taken from the left ventricle at the apex of the heart. Approximately 3-5 mg of tissue was used for high resolution respirometry and the remainder was flash-frozen in liquid N_2 for Western blot determinations.

High resolution respirometry

3-5 mg of tissue was homogenized in MIRO5 (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 0.1% BSA, 60 mM lactobionic acid; pH 7.1) using the PBI-Shredder SG3 (Oroboros, Austria). This mechanical homogenization system applies reproducible force to the tissue yielding standardized preparations. Respiration was determined at 37°C in MIRO5 using the Oxygraph-2k (Oroboros, Austria). Malate (2 mM) and octanoyl carnitine (1 mM) were added to determine adenylate free leak respiration. ADP + Mg^{2+} (5 mM) were subsequently added to determine maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity. Glutamate (10 mM) and succinate (10

mM) were then added to determine maximum oxidative phosphorylation capacity. Oligomycin (2 μg/ml) was added to determine oligomycin-induced leak respiration. Antimycin A (2.5 μM) was added to inhibit complex III and terminate respiration to determine nonmitochondrial oxygen consumption. All values were corrected for residual oxygen consumption. The oxygen consumption (pmol/(s*mg)) was expressed relative to citrate synthase activity (μmol/min/mg).

Citrate synthase activity

Citrate synthase activity was determined in homogenized tissue according to the method of Srere (24).

Western blotting and sample preparation

Left ventricular cardiac muscle was homogenized on ice in a lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 2% β-mercaptoethanol, 50 mM NaF, 5 mM NaPP, 1mM Na_3VO_4, and protease inhibitors) and spun at 14 000 g for 20 min at 4°C. Protein content was measured using a bicinchoninic acid assay and samples were stored at -80°C. Samples were subjected to reducing SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and stained with Ponceau S. After blocking for 1 h at room temperature in 5% skim milk, incubation in primary antibody was overnight at 4°C. The following primary antibodies were used at the indicated dilutions: ANT (N-19, SC-9200, Santa Cruz Biotechnology; 1:1000), UCP3 (ab3477,

Abcam; 1:1000), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam; 1:800), vinculin (ab129002, Abcam; 1:10000). Following 3 x 10 min washes with TBS + 0.1% Tween-20, membranes were incubated in the appropriate horseradish peroxidise-conjugated secondary antibody diluted in 5% skim milk at room temperature for 1h. Bands were visualized using enhanced chemiluminescence. Band intensity was quantified by density analysis using Image J (NIH) and normalized to vinculin.

Plasma acylcarnitine measurement

A 3.2 mm punch from a dried plasma sample was collected on Whatman 903™ filter paper card and put into the designated well of a 96-well plate. Samples were collected from 14 week old mice. Samples were prepared at the Newborn Screening Ontario laboratory based on methods described by Turgeon *et al* (25) with minor modification. Briefly, target analytes were extracted from the dried plasma samples using a methanolic solution containing isotope-labelled internal standards. Samples were evaporated under nitrogen followed by derivatization using butanolic-HCl at 60 °C for 20 min. Excess reagent was evaporated to dryness using nitrogen and the residue was reconstituted with 80% acetonitrile solution. A 10 µL portion of sample was injected into an MS/MS system consisting of a Waters TQ Detector, Waters 1525µ Binary HPLC Pump, and a Waters 2777C Sample Manager (Waters, Milford, MA, USA). MS/MS analysis of target analytes was achieved using a combination of multiple reaction

monitoring and precursor ion scans, with the electrospray ionization source being operated in the positive ion mode.

Statistical analyses

All measures were analyzed using GraphPad Prism, version 5.0 (La Jolla, CA, USA). Differences between groups were analyzed using a Student's *t*-test or two-way ANOVA followed by Bonferroni post hoc tests, as appropriate. Values are reported as mean ± SEM. $P<0.05$ was considered significant.

Results

Body weight 24 h after birth was 26% reduced in U compared to C (Figure S4.1). As expected, based on previously published work, there was no difference in linear growth as assessed by tail and femur length at age 14 weeks (Table 4.1). Body weight between C-L and U-L was not different (Table 4.1). This indicates that U experience catch-up growth, which has been shown to be required for the development of the phenotype in this model (26). Additionally, there was no difference in the weight of the heart or liver (Table 4.1). U had increased gonadal white adipose tissue (gWAT; Table 4.1).

In utero undernutrition decreases energetics in heart homogenate

Respiration in left ventricular cardiac muscle homogenate from 14 week old mice was assessed under different conditions using high resolution respirometry and normalized to citrate synthase activity, a marker of mitochondrial content. Adenylate free leak respiration (Figure 4.1A), fatty acid oxidative capacity (Figure 4.1B), maximum oxidative phosphorylation capacity (Figure 4.1C), and oligomycin-induced leak respiration (Figure 4.1D) were reduced in U compared to C. Calorie restriction also decreased adenylate free leak respiration, fatty acid oxidative capacity, and maximum oxidative phosphorylation capacity (Figure 4.1). Similar differences between U and C were also observed at 10 weeks of age (Figure S4.2). There was no difference in citrate synthase activity between groups.

Table 4.1. Mouse characteristics at age 14 weeks

	C-L	C-R	U-L	U-R
Body mass (g)	31.96 ± 1.34^{++}	24.78 ± 0.53^{a}	33.81 ± 2.61^{+}	28.03 ± 0.65
Tail length (cm)	9.3 ± 0.1	9.3 ± 0.1	9.4 ± 0.1	9.2 ± 0.1
Femur length (cm)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Heart (g)	0.138 ± 0.005	0.123 ± 0.003	0.123 ± 0.007	0.120 ± 0.004
Liver (g)	1.235 ± 0.042	1.307 ± 0.049	1.164 ± 0.091	1.219 ± 0.039
gWAT (g)	0.572 ± 0.068$^{\#}$	0.375 ± 0.019^{a}	1.169 ± 0.285	0.708 ± 0.053

C = control offspring, U = *in utero* undernourished offspring, L = fed *ad libitum*, R = after a 4 wk 40% calorie restriction. gWAT = gonadal white adipose tissue. Values are mean ± SEM, n=8-9. Two-way ANOVA with Bonferroni post-hoc test, ^{a} = $p<0.1$ (C vs. U), $^{\#}$ = $p<0.05$ (C vs. U), $^{+}$ = $p<0.05$ (L vs. R), $^{++}$ = $p<0.01$ (L vs. R).

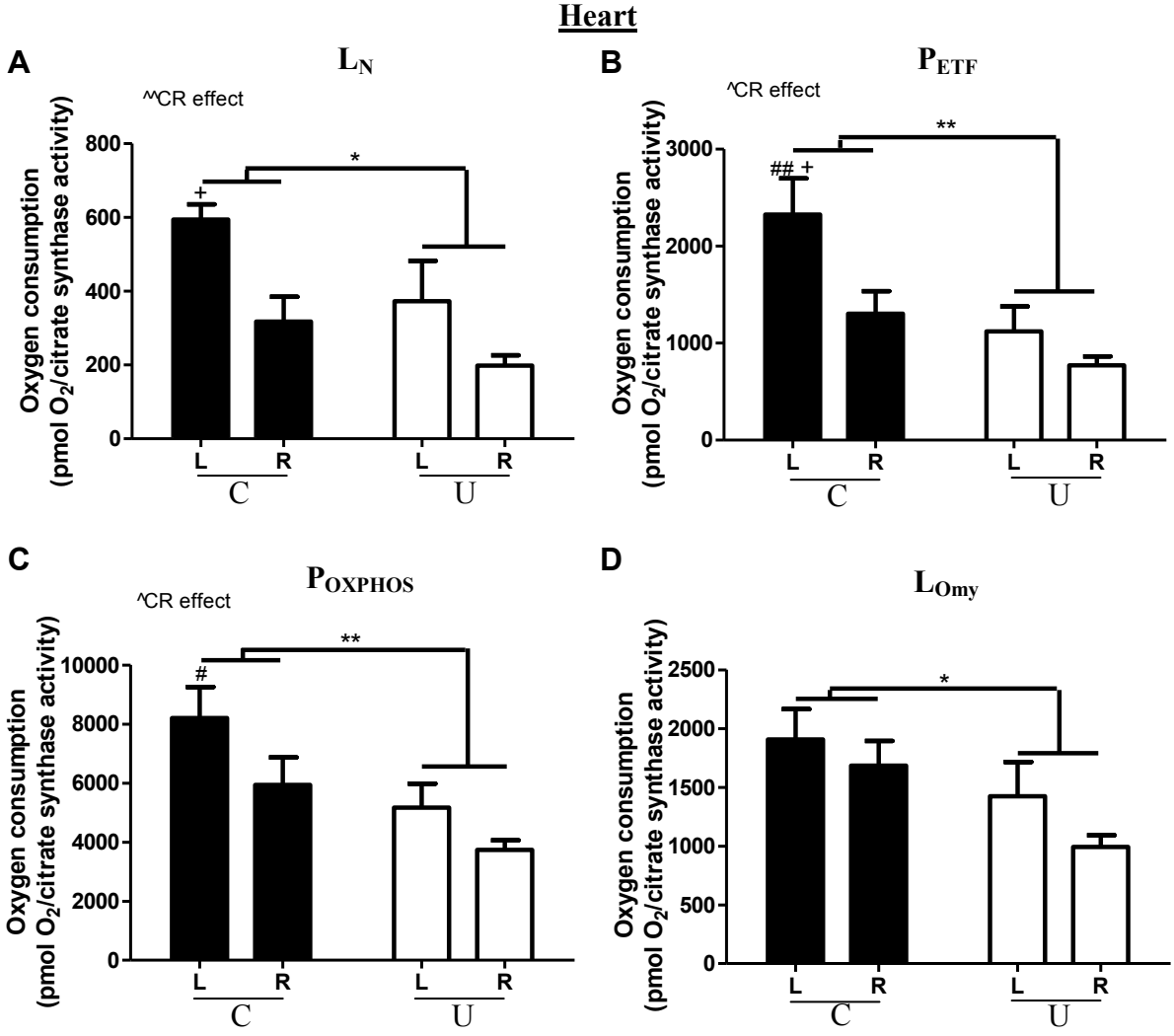
Heart
A
L_N
^^CR effect
Oxygen consumption (pmol O_2/citrate synthase activity)
800
600
400
200
0
*
+
L
R
L
R
C
U
B
P_{ETF}
^CR effect
3000
2000
1000
0
**
+
C
U
C
P_{OXPHOS}
^CR effect
10000
8000
6000
4000
2000
0
**
#
C
U
D
L_{Omy}
2500
2000
1500
1000
500
0
*
C
U

Figure 4.1. *In utero* undernutrition alters energetics in heart homogenate. O_2 flux in heart homogenate from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Values are expressed relative to citrate synthase activity. Data are shown for adenylate free leak respiration (L_N; A), maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity (P_{ETF}; B), maximum oxidative phosphorylation capacity (P_{OXPHOS}; C), and oligomycin-induced leak respiration (L_{omy}; D). Values are mean ± SEM, n=6-8, *=p<0.05, **=p<0.01, #=p<0.05 (C vs. U), ##=p<0.01 (C vs. U), +=p<0.05 (L vs. R), ^=p<0.05 (calorie restriction (CR) effect), ^^p<0.01 (CR effect). Black = C (control offspring), white = U (*in utero* undernourished offspring).

In utero undernutrition does not alter energetics in liver homogenate

In contrast to the effects of *in utero* undernutrition on energetics in heart, adenylate free leak respiration (Figure 4.2A), fatty acid oxidative capacity (Figure 4.2B), maximum oxidative phosphorylation capacity (Figure 4.2C), and oligomycin-induced leak respiration (Figure 4.2D) did not differ between U and C in liver homogenate. Calorie restriction also did not have a significant effect in the liver (Figure 4.2). Citrate synthase activity in liver was not different between the groups. Therefore, under the conditions assessed there were no effects on energetics in liver tissue.

In utero undernutrition does not alter mitochondrial content in the heart

Although respiration values were normalized to citrate synthase activity, the decreased heart energetics in U could be due in part to differences in the amount of one or more of the mitochondrial complexes. Therefore, we assessed protein levels of mitochondrial complexes I-V. Protein expression of representative proteins for the five complexes did not differ between U and C (Figure 4.3). Given the observed difference in leak dependent respiration, we also measured protein levels of adenine nucleotide translocase (ANT; Figure 4.4A) and uncoupling protein 3 (UCP3; Figure 4.4B). There were no differences in the expression of these proteins.

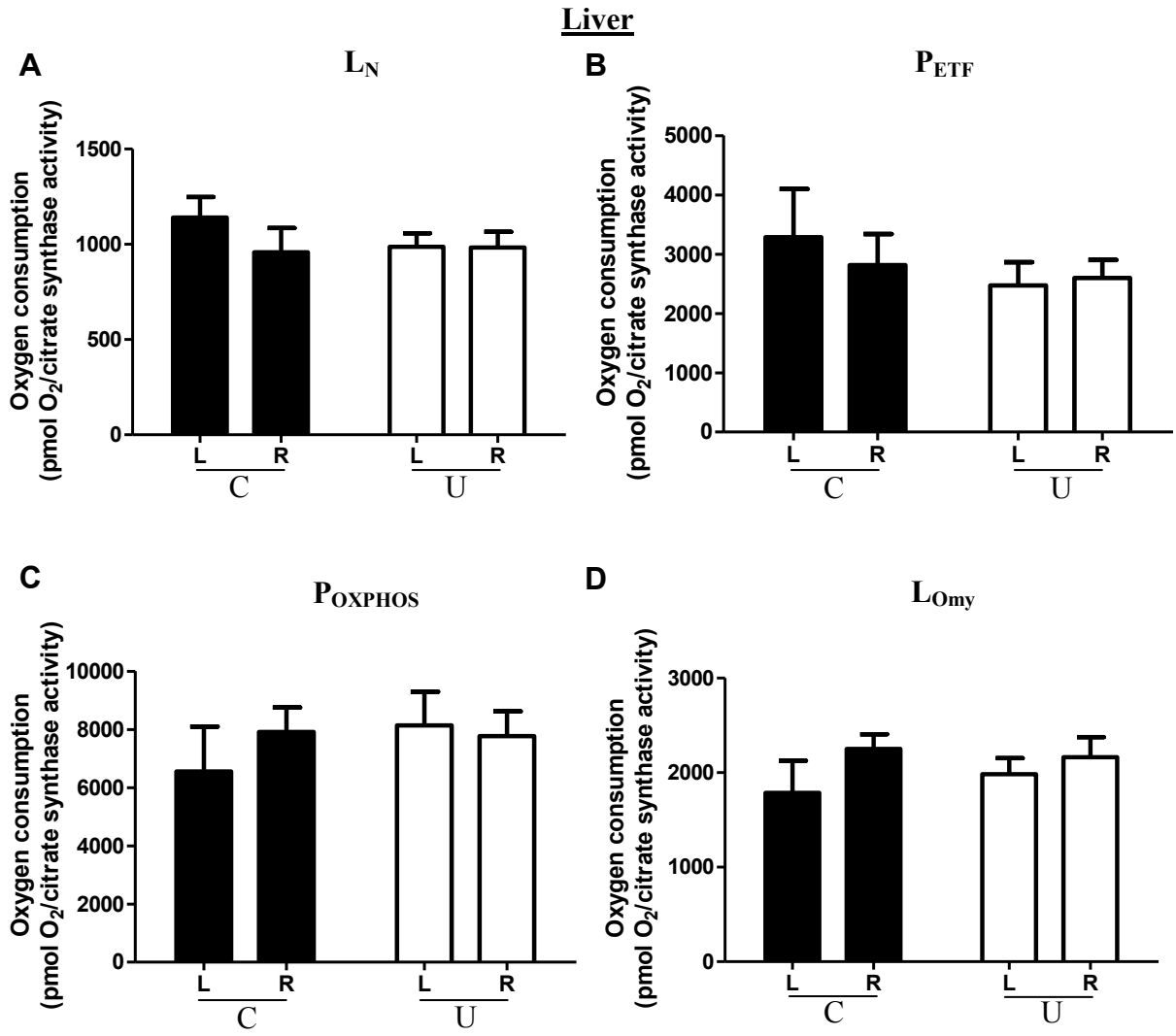
Liver
A
L_N
Oxygen consumption (pmol O_2/citrate synthase activity)
1500
1000
500
0
L
R
L
R
C
U
B
P_{ETF}
Oxygen consumption (pmol O_2/citrate synthase activity)
5000
4000
3000
2000
1000
0
L
R
L
R
C
U
C
P_{OXPHOS}
Oxygen consumption (pmol O_2/citrate synthase activity)
10000
8000
6000
4000
2000
0
L
R
L
R
C
U
D
L_{Omy}
Oxygen consumption (pmol O_2/citrate synthase activity)
3000
2000
1000
0
L
R
L
R
C
U

Figure 4.2. *In utero* undernutrition does not alter energetics in liver homogenate. O_2 flux in liver homogenate from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Values are expressed relative to citrate synthase activity. Data are shown for adenylate free leak respiration (L_N; A), maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity (P_{ETF}; B), maximum oxidative phosphorylation capacity (P_{OXPHOS}; C), and oligomycin-induced leak respiration (L_{omy}; D). Values are mean ± SEM, n=6-8. Black = C (control offspring), white = U (*in utero* undernourished offspring).

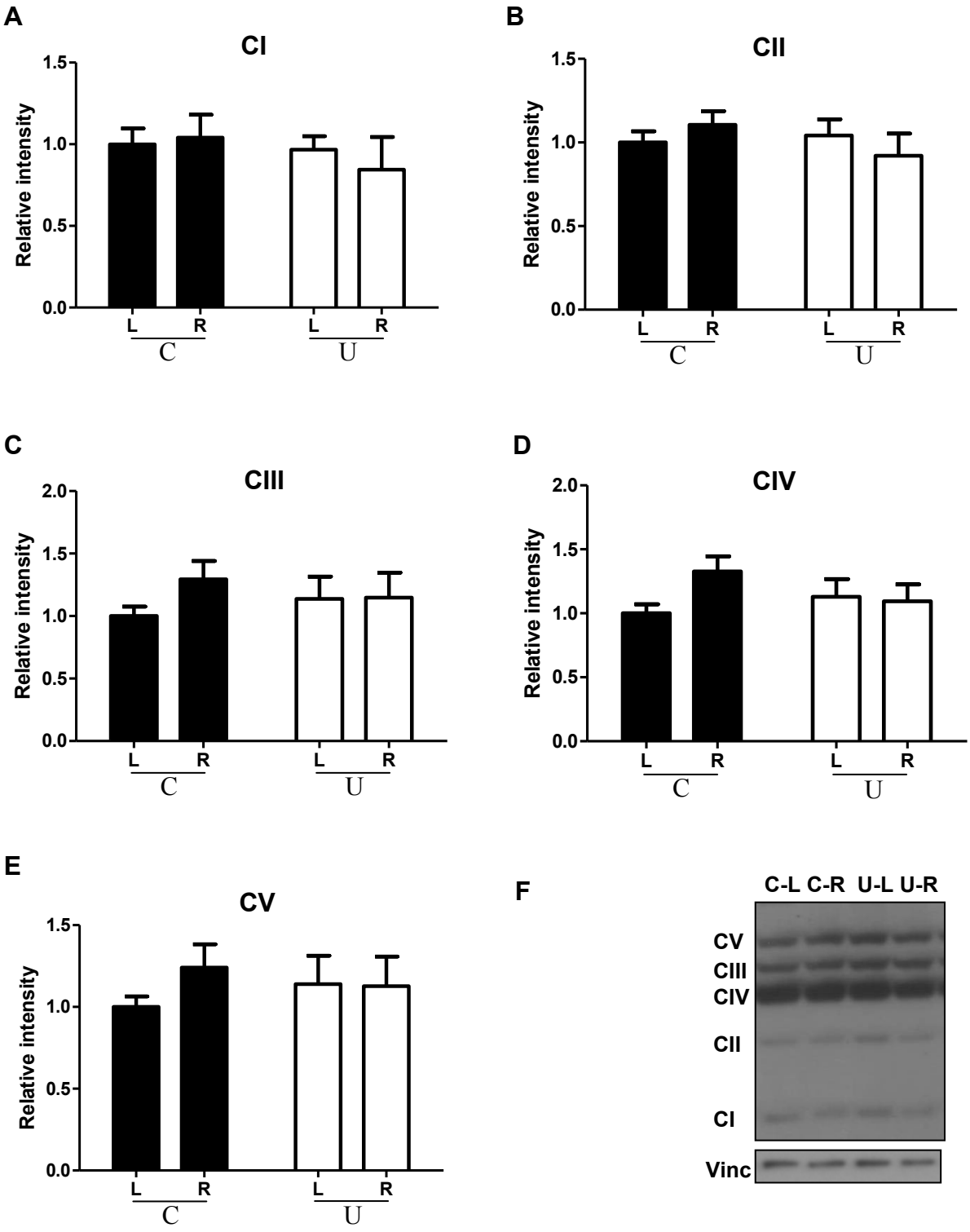
A
CI
Relative intensity
1.5
1.0
0.5
0.0
L R L R
C U
B
CII
Relative intensity
1.5
1.0
0.5
0.0
L R L R
C U
C
CIII
Relative intensity
2.0
1.5
1.0
0.5
0.0
L R L R
C U
D
CIV
Relative intensity
2.0
1.5
1.0
0.5
0.0
L R L R
C U
E
CV
Relative intensity
1.5
1.0
0.5
0.0
L R L R
C U
F
C-L C-R U-L U-R
CV
CIII
CIV
CII
CI
Vinc

Figure 4.3. *In utero* undernutrition does not alter mitochondrial content in the heart. Protein expression of mitochondrial complexes normalized to vinculin (Vinc) loading control in heart homogenate from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Quantification of complex I (CI; A), II (CII; B), III (CIII; C), IV (CIV; D), and V (CV; E) expression and representative Western blot (F).Values are mean ± SEM, n=8. Black = C (control offspring), white = U (*in utero* undernourished offspring).

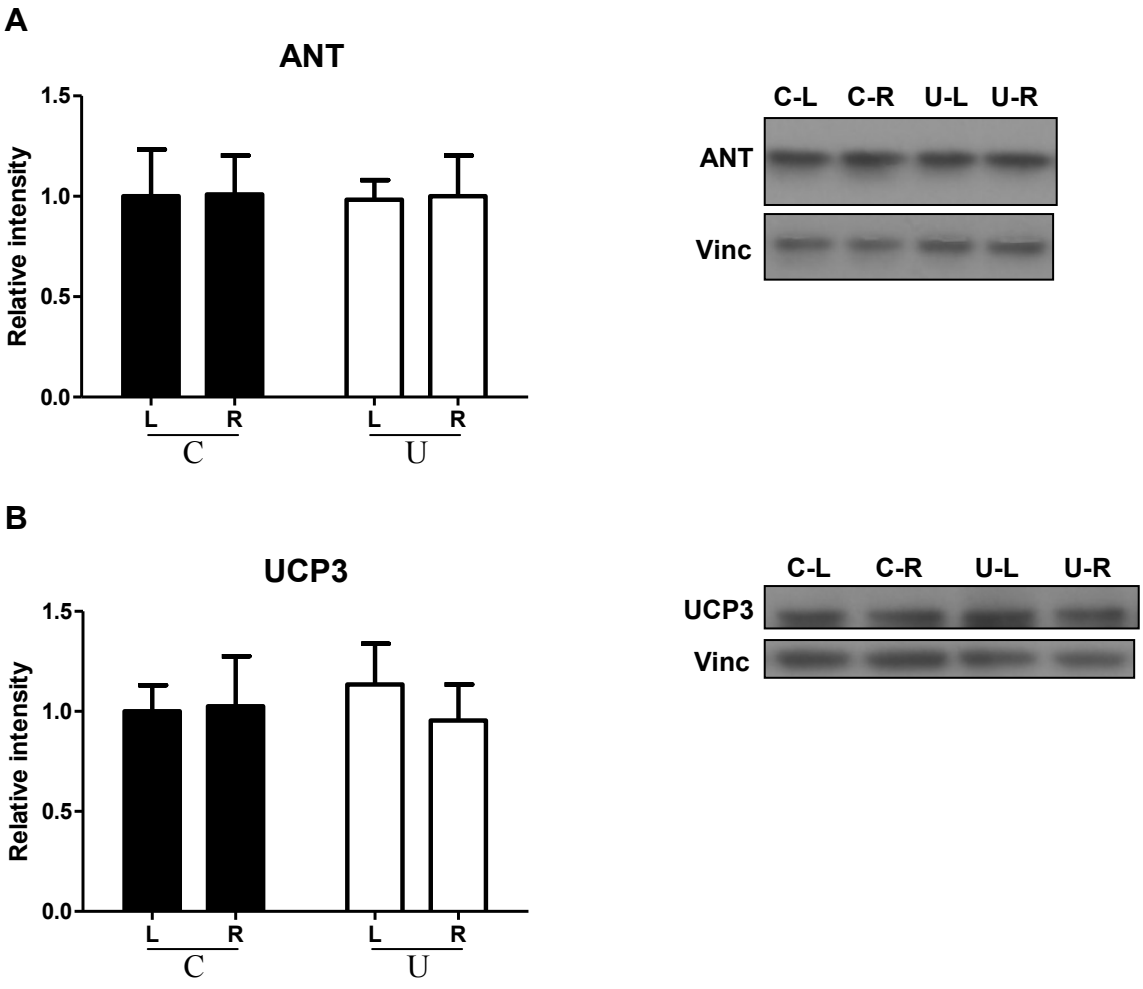
A
ANT
Relative intensity
1.5
1.0
0.5
0.0
L
R
L
R
C
U
C-L
C-R
U-L
U-R
ANT
Vinc
B
UCP3
Relative intensity
1.5
1.0
0.5
0.0
L
R
L
R
C
U
C-L
C-R
U-L
U-R
UCP3
Vinc

Figure 4.4. *In utero* undernutrition does not alter expression of adenine nucleotide translocase (ANT) or uncoupling protein 3 (UCP3). Protein expression of ANT (A) and UCP3 (B) normalized to vinculin (Vinc) loading control in heart homogenate from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). Quantification, left, and representative Western blot, right. Values are mean ± SEM, n=8. Black = C (control offspring), white = U (*in utero* undernourished offspring).

Plasma short-chain acylcarnitines are increased with *in utero* undernutrition

Impairments in fatty acid oxidation can result in the accumulation of intermediary metabolites, such as acylcarnitines. Given the current finding of decreased fatty acid oxidation in cardiac muscle and our previous finding of decreased fatty acid oxidation in skeletal muscle (9), we measured plasma acylcarnitines using tandem mass spectrometry. Under *ad libitum* fed conditions, U had increased acetylcarnitine (Figure 4.5A) and short-chain acylcarnitines (C3-C5; Figure 4.5B) compared to *ad libitum* fed controls (U-L vs. C-L). There was no difference in medium- (C6-C12; Figure 4.5C) or long-chain (>C12; Figure 4.5D) acylcarnitines between U-L and C-L. Interestingly, in U, calorie restriction significantly decreased acetylcarnitine, short-, medium-, and long-chain acylcarnitines (U-L vs. U-R; Figure 4.5A-D). In C, there was no significant effect of calorie restriction on acylcarnitine levels. However, there was a trend for decreased acetylcarnitine (C-L vs. C-R; Figure 4.5). Complete results of the plasma acylcarnitine analysis are provided in Table S4.1.

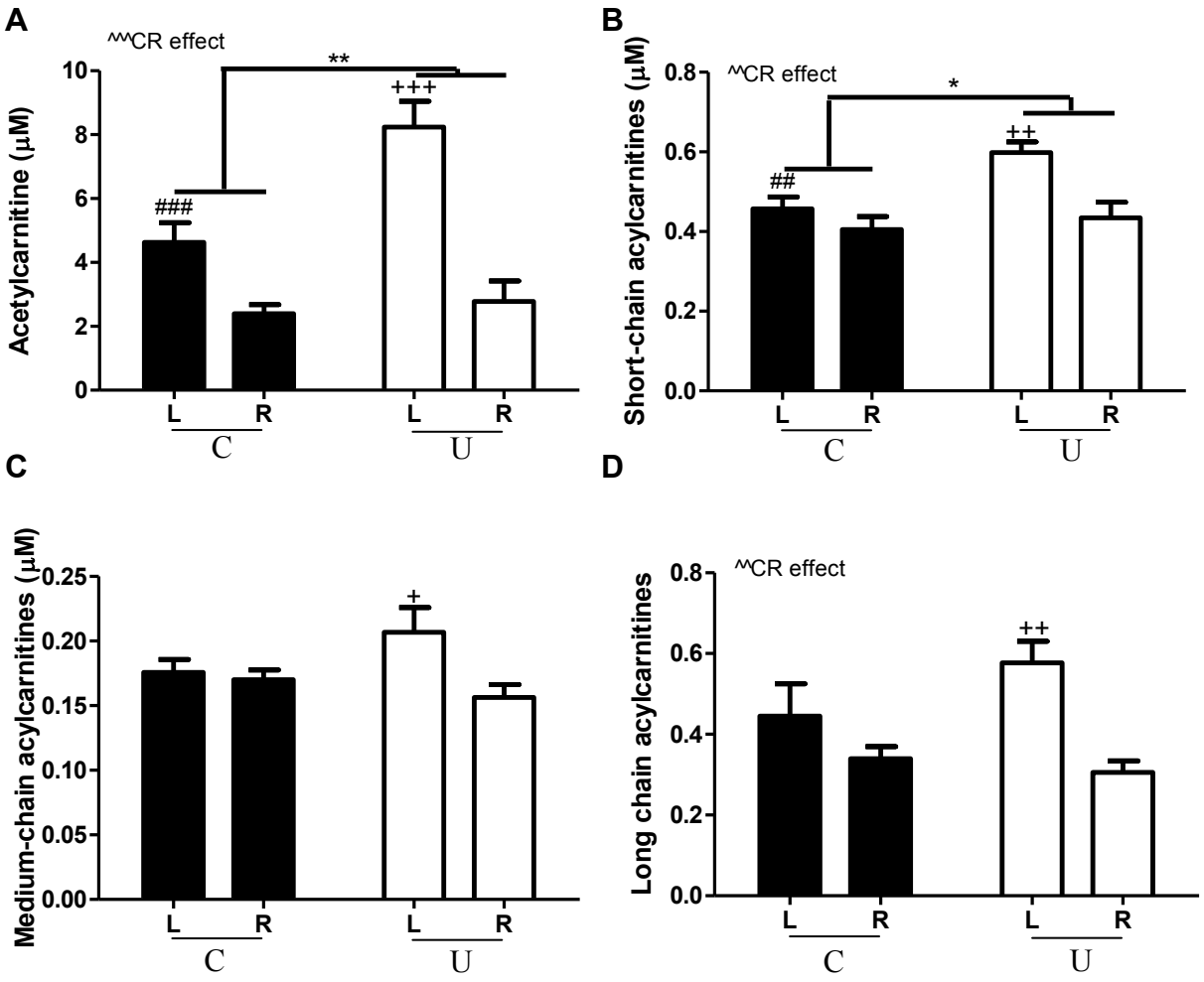
A
^^CR effect
**
+++
###
Acetylcarnitine (μM)
10
8
6
4
2
0
L
R
L
R
C
U
B
^^CR effect
*
++
##
Short-chain acylcarnitines (μM)
0.8
0.6
0.4
0.2
0.0
L
R
L
R
C
U
C
+
Medium-chain acylcarnitines (μM)
0.25
0.20
0.15
0.10
0.05
0.00
L
R
L
R
C
U
D
^^CR effect
++
Long chain acylcarnitines
0.8
0.6
0.4
0.2
0.0
L
R
L
R
C
U

Figure 4.5. Plasma short-chain acylcarnitines are increased with *in utero* undernutrition. Acylcarnitines measured in plasma from 14 wk old mice fed *ad libitum* (L) and after a 4 wk 40% calorie restriction (R). A) Acetylcarnitine; B) Sum of short-chain acylcarnitines (C3-C5); C) Sum of medium-chain acylcarnitines (C6-C12); D) Sum of long-chain acylcarnitines (>C12). Values are mean ± SEM, n=7-8, *=$p<0.05$, **=$p<0.01$, ##=$p<0.01$ (C vs. U), ###=$p<0.001$ (C vs. U), +=$p<0.05$ (L vs. R), ++=$p<0.01$ (L vs. R), +++=$p<0.001$ (L vs. R). Black = C (control offspring), white = U (*in utero* undernourished offspring).

Discussion

Here we show that *in utero* undernutrition in mice results in impaired cardiac muscle energetics without altering mitochondrial content and that this is associated with increased plasma short-chain acylcarnitines. While many studies have shown an association between IUGR and cardiovascular disease, the effects of *in utero* undernutrition on heart energetics have not been characterized. Given the strong association between IUGR and cardiovascular disease, our data demonstrate that maternal food restriction alters metabolism in the heart, which influences the cardiovascular health and disease risk in offspring.

Mitochondrial respiration in heart homogenate was decreased in U compared to C. Protein expression of mitochondrial complexes I-V in the heart was not different between U and C, indicating that the reduced respiration was not due to a decrease in mitochondrial content or the expression of one of the respiratory chain complexes. The observed differences in respiration may be due to differences in the activity of one or more of these complexes. Proton leak requires the activation of mitochondrial anion carrier protein function, which can be catalysed by ANT and UCP3 (27-29). The decrease in proton leak respiration measured in U does not appear to be due to differences in protein expression of ANT or UCP3 but may be due to differences in their activity or post-translational modification. Therefore future research should investigate possible changes in the composition of mitochondrial super-complexes and in protein post-translational modifications (e.g., phosphorylation and/or glutathionylation of

respiratory chain and other mitochondrial proteins). We also cannot exclude the possibility that differences are in part due to structural changes in the mitochondria that may make them more susceptible to damage during sample preparation.

Our finding of decreased respiration in cardiac tissue in this mouse model is consistent with the altered energetics reported in heart tissue of other rodent models of diabetes and in patients with diabetes. It has been shown that mitochondria from diabetic human heart have an impaired capacity to oxidize palmitoyl-carnitine (22) and in a mouse model of obesity and type 2 diabetes mitochondria were shown to have decreased oxidative capacity (23). Furthermore, there is a decrease in the expression of fatty acid oxidation genes and enzymes and a decrease in myocardial energy production in heart failure patients and in animal models of heart failure (16-18, 20, 21). Therefore, our results suggest that the decreased oxidative capacity in the heart of *in utero* undernourished offspring may contribute to their increased risk of cardiovascular and other metabolic diseases.

We have shown that respiration in heart tissue is decreased at 10 weeks of age and remains decreased at 14 weeks of age; however future work is needed to determine the time course of these metabolic derangements. Studies have shown that IUGR fetuses and neonates show changes in cardiac morphology and function (3, 4, 30-32), which, combined with the results presented here, suggest that IUGR may be associated with cardiac metabolic adaptations *in utero* that become detrimental in later life. This study was limited to the left ventricle. This region of the heart was chosen due to its relatively

high rate of oxygen consumption (approximately twice that of the right ventricle) and because the left ventricle has been most frequently studied in clinical and animal models (33).Thus it remains to be determined whether there are impaired energetics in other regions of the heart.

We also studied energetics in liver tissue but did not detect any differences between U and C under the conditions tested. Previously, in the same model, IUGR had no effect on hepatic insulin resistance (8). However, our finding was surprising given that others have found decreased mitochondrial DNA content and decreased oxygen consumption in isolated mitochondria from the liver of IUGR rats and piglets (34-36). This discrepancy may be due to differences in the animal and model of IUGR used. Previous studies were also limited to the use of isolated mitochondria. The *in situ* approach we have used allows the analysis of mitochondrial function within an integrated cellular system, preserving interactions with other cell components that are important for metabolic channeling and intracellular energy transfer (37-39). We also cannot exclude the possibility that effects in the liver may not be observed until later in life.

Our findings demonstrate that calorie restriction had a significant effect on cardiac muscle energetics, decreasing adenylate free leak respiration, fatty acid oxidative capacity, and maximum oxidative phosphorylation capacity. Cardiac respiration was also reduced in U, demonstrating that the heart is a tissue that responds to negative energy balance at the whole body level. Interestingly, we observed a significant

decrease in adenylate free leak respiration and fatty acid oxidative capacity with calorie restriction in C (C-L vs C-R), but not in U (although there appears to be a similar trend).

Overall, we have shown that U have decreased fatty acid-supported respiration in left ventricular cardiac muscle. Previously, we found that fatty acid-supported respiration was decreased in permeabilized muscle fibers from white *gastrocnemius* of U mice (9). Interestingly, in individuals with T2DM high-energy phosphate metabolism is impaired in both cardiac and skeletal muscle. In cardiac muscle, individuals with T2DM have a decreased phosphocreatine (PCr)/ATP ratio and in skeletal muscle, PCr loss was faster during exercise and PCr recovery was slower after exercise (40). Our findings in heart and muscle suggest that U have an impaired ability for fatty acid oxidation, which may be linked to an increase in incomplete fatty acid oxidation. Consistent with this hypothesis, we found that short-chain acylcarnitines are increased in *ad libitum* fed U compared to *ad libitum* fed C. Calorie restriction decreased short-chain acylcarnitines in U but not in C, and normalized acylcarnitine levels to that of the calorie restricted controls. Interestingly, higher levels of short-chain acylcarnitines have been reported in patients with T2DM and in patients with metabolic syndrome (12, 41). It has been suggested that insulin resistance may be linked to incomplete fatty acid beta oxidation and the resulting increase in acylcarnitines (42). In primary myotubes, the reduction of short-chain acylcarnitine and acetylcarnitine formation was shown to protect against palmitate-induced insulin resistance (42). Taken together, we suggest that the decrease in fatty acid-supported respiration seen in U may result in an increase in incomplete fatty acid oxidation and the subsequent increase in short-chain acylcarnitines, which

may be linked to the development of insulin resistance in this model. This idea will be explored further in future research.

In summary, we have shown that *in utero* undernutrition results in dysfunctional cardiac muscle energetics. Furthermore, *in utero* undernutrition increases plasma short-chain acylcarnitines. These findings support the hypothesis that *in utero* undernutrition is associated with a maladaptive programming process that has negative effects on the heart. A better understanding of mechanisms mediating the cardiovascular effects of maternal nutrition on adult offspring should aid in the design of interventions aimed to prevent these detrimental effects and improve health. We conclude that *in utero* undernutrition in late pregnancy alters energetics in the heart, which could have lifelong implications for cardiovascular function.

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Chapter 5: General Discussion

IUGR, which occurs when the supply of oxygen and nutrients to the growing fetus is inadequate, is associated with the development of obesity, insulin resistance, and cardiovascular disease in adulthood (1-8). The goal of this doctoral research was to assess the impact of IUGR on characteristics of energy metabolism in skeletal and cardiac muscle. Given the central role of skeletal muscle in whole body metabolism and insulin sensitivity we aimed to elucidate the metabolic alterations that contribute to altered muscle energy in mice of undernourished dams (9-11). Secondly, we aimed to determine if the impaired skeletal muscle energetics observed in low birth weight mice also exist in primary muscle cells derived from these mice. Lastly, given the increased susceptibility to cardiovascular disease in low birth weight offspring, we aimed to characterize mitochondrial energetics in cardiac muscle of mice exposed to maternal undernutrition. To study these aims we used a mouse model of IUGR generated through 50% food restriction of mouse dams during the third week of gestation. We studied female adult offspring from undernourished dams and control adult offspring from *ad libitum* fed dams. We also examined the effect of a metabolic challenge, a four week 40% calorie restriction, in these adult offspring.

Consistent with previous studies, adult offspring exposed to *in utero* undernutrition during late pregnancy had increased adiposity and decreased glucose tolerance compared to controls (12, 13). Studies presented in this thesis have revealed important new insights into the pathogenesis of low birth weight associated metabolic disease.

Specifically, they have identified defects in skeletal and cardiac muscle energetics. The main findings include:

1) *In utero* undernutrition is associated with decreased skeletal muscle mitochondrial content in mixed fiber muscles, decreased respiration in both isolated skeletal muscle mitochondria and permeablized muscle fibers and a blunted weight loss response to a hypocaloric diet in adulthood.
2) Myotubes established from satellite cells of *in utero* undernourished mice have marked impairments in oxidative metabolism and enhanced glycolytic characteristics, suggesting a programmed primary muscle defect.
3) *In utero* undernutrition is associated with decreased cardiac muscle energetics and increased plasma short-chain acylcarnitines.

The developmental programming hypothesis proposes that adverse conditions during periods of early life development increase susceptibility to adult disease (14). Thus, food restriction *in utero* may cause metabolic adaptations to maintain energy homeostasis that favour survival in an environment with limited nutrient supply, resulting in a thrifty phenotype (14, 15). However, with post natal nutrient excess, these adaptations made *in utero* become detrimental, increasing susceptibility to metabolic disease. Results presented herein align with this hypothesis with skeletal muscle of maternally undernourished mice having reduced mitochondrial content and decreased respiration in isolated mitochondria and permeabilized muscle fibers. Specifically, skeletal muscle mitochondria from *in utero* undernourished mice have decreased coupled and uncoupled respiration and permeabilized fibers from mixed

fiber type muscle (wGAS) have decreased adenylate free leak respiration, fatty acid oxidative capacity, and state 3 respiratory capacity through complex I. Thus, results suggest that with *in utero* undernutrition, muscle is programmed to be more efficient which persists into adult life, contributing to increased susceptibility to metabolic disease. Furthermore, we extend this hypothesis and demonstrate that low birth weight offspring, when calorie restricted in adulthood, are resistant to weight loss due apparently to the thrifty metabolic mechanisms programmed *in utero.*

Primary muscle cells were isolated from *in utero* undernourished offspring to determine if the impaired skeletal muscle energetics observed in low birth weight mice also exist in primary muscle cells derived from these mice. Myotubes from *in utero* undernourished mice were shown to have dysfunctional fatty acid oxidation and enhanced glycolysis. These findings are consistent with the decreased respiration in skeletal muscle fibers and isolated skeletal muscle mitochondria from *in utero* undernourished mice. Altogether, findings are consistent with the decreased oxidative activity and increased glycolytic capacity observed in individuals with obesity and T2DM (16-19). Thus, findings suggest that susceptibility to metabolic disease in adulthood is caused in part by primary muscle defects that are programmed *in utero*.

Energetics in the heart were assessed in a cardiac muscle homogenate. *In utero* undernourished mice have decreased cardiac muscle respiration under all conditions assessed. Specifically, there were decreases in adenylate free leak respiration, fatty acid oxidative capacity, maximum oxidative phosphorylation capacity, and oligomycin-

induced leak respiration. These findings are consistent with the decreased respiration in cardiac tissue reported in adults with obesity and T2DM and the decreased cardiac energy production associated with heart failure (20-24). Thus, our results demonstrate that maternal undernutrition alters metabolism in the heart, which may contribute to the increased risk of cardiovascular and other metabolic diseases in the offspring.

While there are many studies examining susceptibility to weight gain, very few have examined variation in rate of weight loss, particularly where this concerns non-behavioural mechanisms. Human studies have documented weight loss variation in diet-adherent obese women in a clinical weight loss program and have shown that mitochondrial energetics are more efficient in muscle of diet-resistant women compared to diet-sensitive women (25, 26). As our findings are analogous in many ways, this suggests the possibility that *in utero* environmental factors may contribute to the clinical phenomenon of weight loss resistance. Ultimately, if we can identify specific mechanisms that underlie inter-individual variation in response to a hypocaloric diet, we may be able to develop pharmacological treatment options and tailored treatment programs for weight loss. Further studies of the mechanistic aspects are warranted. It should be noted that our study was limited to a 4 week calorie restriction. It is possible that IUGR offspring have a slower response to calorie restriction and that if the calorie restriction was carried out further, weight loss and muscle energetics would normalize to those of controls. Further study is required to examine the effects of a longer term calorie restriction.

It is not clear if the effects of maternal undernutrition presented here are due to reductions in a specific macronutrient and/or micronutrient. Maternal micronutrient deficiency has been shown to predispose offspring to insulin resistance and metabolic disease in later life (27-31). Maternal zinc, magnesium, or chromium restriction decreases offspring birth weight, increases fat mass, decreases lean body mass, and impairs glucose tolerance (27-31). As these effects are consistent with our observations, this may be an important and interesting area of future study. If key dietary components could be identified that are causing the negative effects of IUGR we may be able to develop appropriate preventative strategies. In cases of IUGR due to poor maternal nutrition, a conceptually simple method of prevention is to have the mother consume adequate food and or a higher quality diet during pregnancy. However, this may be difficult due to economical, social, and/or mental health issues. If we could identify a specific dietary component that, when lacking, causes some or all of the negative effects of IUGR, providing a supplement may be a cost-effective way to reduce low birth weight. Interestingly, studies have shown that supplementing the maternal diet with folic acid, the synthetic form of folate, prevents some of the negative effects of low birth weight in the liver. Folate is a water soluble B vitamin that plays an important role in one-carbon transfer and, amongst other functions, is linked to DNA methylation (32). It has been shown that maternal folic acid supplementation reverses the hepatic gene expression changes in IUGR pigs and prevents epigenetic modification of hepatic gene expression in IUGR rats (33-35). It would be interesting to examine if supplementation with folic acid also prevents the negative impact of IUGR on skeletal and cardiac muscle.

Prevention of the negative consequences of IUGR may also be possible in early life. Catch-up growth, a period of accelerated growth after slowed development, is associated with higher susceptibility to metabolic diseases (36-43). Experimentally, the prevention of catch-up growth has been shown to inhibit the development of obesity and glucose intolerance, as discussed in chapter 1 (42, 43). However, the impact of preventing catch-up growth on skeletal muscle and heart in IUGR offspring has not been studied. Thus, it would be of interest to determine if the prevention of catch-up growth also prevents the alterations in skeletal and cardiac muscle that we have observed.

Exercise is well known to have beneficial effects on skeletal muscle and whole body metabolism. With endurance training, exercise increases cardiac output, increases lean body mass, decreases body fat, increases muscle mitochondrial content, increases expression of GLUT4, increases insulin sensitivity, and increases the activity of oxidative enzymes in mitochondria (44-46). All of these benefits of endurance exercise are the opposite of what occurs in IUGR offspring. Therefore, endurance training of IUGR offspring may be able to reverse some of the negative impacts of a suboptimal nutrition *in utero*. In a study of low birth weight rats that were undernourished *in utero*, obesity in adulthood was prevented by exercise (47). However, the impact of exercise on skeletal muscle metabolism in low birth weight offspring remains to be determined. We found that IUGR offspring have a blunted response to calorie restriction in adulthood, consistent with the hypothesis that thrifty mechanisms are programmed *in*

utero in preparation for future times characterized by limited nutrient availability. It may be possible that another intervention, such as endurance exercise, which the offspring were not exposed to *in utero*, may be more effective than calorie restriction to reduce obesity and skeletal and cardiac muscle dysfunction associated with low birth weight. This could have important clinical implications when treating people with low birth weight and in designing weight loss programs.

The molecular mechanisms mediating the effects of *in utero* undernutrition on increased susceptibility to metabolic disease in adulthood remain unknown. Some of these effects are transmissible across generations, suggesting that heritable changes in gene expression occur with *in utero* undernutrition. Experimental studies have shown intergenerational transmission of obesity and altered glucose metabolism associated with low birth weight (48-50). It is hypothesized that epigenetic mechanisms are, at least in part, responsible. Epigenetic mechanisms refer to modifications of DNA that result in changes in gene expression without altering the DNA sequence itself. A potential mechanism that can cause long-term changes in gene expression is altered DNA methylation. This is a common epigenetic modification and is associated with stable variations in gene expression. Furthermore, DNA methylation patterns are largely established *in utero*. Therefore, the fetal environment may alter DNA methylation causing changes in gene expression that are sustained into adulthood. Promoter methylation modulates transcription factor binding, with increased DNA methylation silencing gene transcription (51). The maternal environment is known to impact the methylation status and gene expression in multiple tissues (52-55). In

skeletal muscle, it has been shown that IUGR rats have increased methylation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis (54). Accordingly, this was associated with a reduction in PGC-1α transcription activity, mitochondrial content, and protein level of components of the insulin signaling pathway (54). However, additional studies are required to fully elucidate the impact of maternal undernutrition on the methylation status of skeletal muscle.

Our findings have revelaed important new insights into the effects of maternal undernutrion on adult offspring health. However, translating these findings to humans has its limitations. While there are many similarities between mice and humans, differences such as the developmental timing of organs and tissues will need to be considered. Our study was mainly limited to skeletal muscle, heart, and liver and therefore does not examine effects in other tissues nor does it consider the potentially important ways these tissues influence each other. These results are also limited in that they can not explain the mechanism repsonsible for the energetic differences observed in these mice which is an interesting area of future research. Therefore, additional research into the impact of *in utero* undernutrition on offspring health is required to have a better understanding of how our findings may be translated to the human population.

Finally, it is important to also remember that metabolic programming of offspring is characterized by a 'u-shaped' risk curve (56, 57). Indeed, over-nutrition prior to and

during pregnancy is also associated with increased risk of metabolic diseases in offspring (58-60). While undernutrition during pregnancy remains a problem in many parts of the world, obesity prior to and during pregnancy is prevalent in Western and economically-developed societies. This is associated with increased prevalence of obesity and T2DM in humans (58). In animal models, maternal high fat feeding results in offspring with increased body fat, increased cardiovascular disease, impaired glucose tolerance and decreased β cell function (61-66). Interestingly, children born of mothers following bariatric surgery, compared to children born of the same mothers prior to the surgery have decreased prevalence of obesity, hypertension, and dyslipidemia (67, 68). Moreover, bisulfite sequencing of whole blood DNA revealed differential methylation in glucoregulatory genes in children born after maternal bariatric surgery compared to siblings born before maternal surgery (69). These studies are consistent with the idea that the maternal diet induces epigenetic changes in the offspring. Furthermore, they demonstrate that maternal gastric bypass surgery has health benefits for the offspring. Maternal exercise has also been shown to be beneficial for offspring health. Maternal exercise before and during pregnancy has been shown to prevent some of the effects of a maternal high fat diet on offspring, including improved glucose tolerance (61). It is evident that the maternal environment plays an important role in offspring health, however the mechanistic details remain to be elucidated. Clearly, more research also is needed into metabolic programming by maternal overnutrition.

In conclusion, the findings from this doctoral research show that *in utero* undernutrition is associated in mice with alterations in skeletal and cardiac muscle

energetics. These findings are consistent with the idea that low birth weight offspring may develop a protective mechanism *in utero* for species survival in times when energy supply is restricted. However in an environment characterized by the abundant availability of highly palatable food and a decreased need for physical activity, such adaptive mechanisms become detrimental, increasing the risk for metabolic diseases including obesity, type 2 diabetes and cardiovascular disease.

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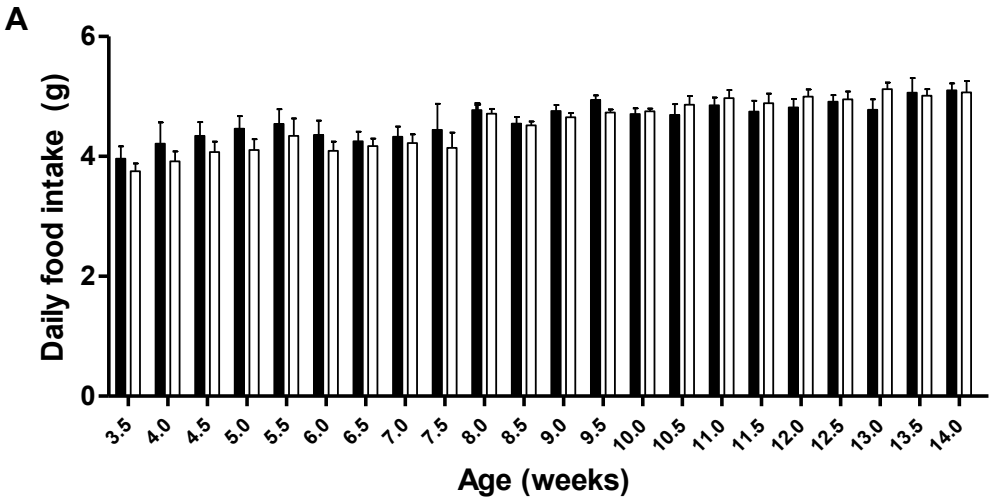
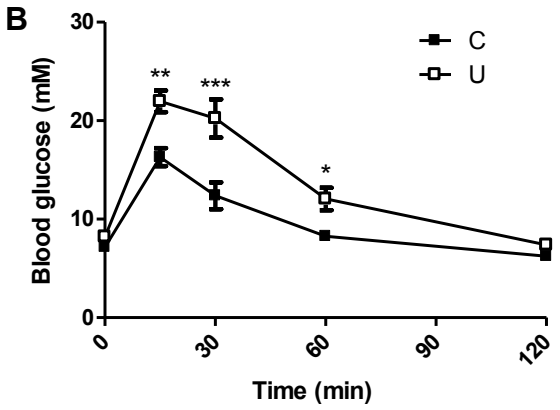
Appendix A: Supplementary materials for Chapter 2

This appendix contains supplementary materials for Chapter 2. This material has been published in the manuscript "Low birth weight is associated with adiposity, impaired skeletal muscle energetics and weight loss resistance in mice" (Beauchamp *et al*., *Int J Obes*. 2015 Apr;39(4):702-11).

Supplemental figures





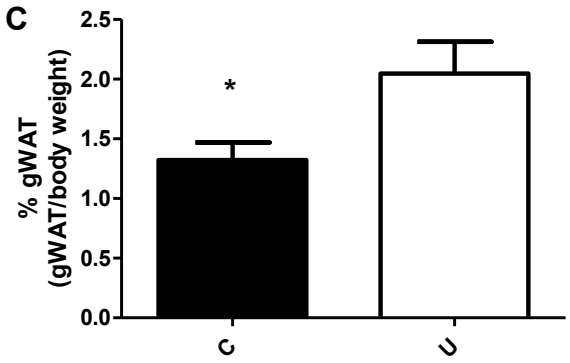


Figure S2.1. *In utero* undernutrition does not alter daily food intake but is associated with decreased glucose tolerance. A) Average daily food intake calculated from bi-weekly measurements of food consumption from 3.5 -14 wk of age. Mice were housed in groups of 3-4 until 8 wk of age and individually thereafter. Values are for 31-33 mice per group until 10 wk of age, and 10-13 mice per group thereafter. B) Oral glucose tolerance test was performed at age 14 weeks. Values are mean ± SEM n=6-8. Two-way repeated measures ANOVA with Bonferroni post-hoc test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. C) Amount of gonadal white adipose tissue (gWAT) expressed as a percentage of total body weight at age 10 weeks. Values are mean ± SEM n=10-13, Student's *t*-test, * = $p<0.05$. Black = C (control offspring), white = U (*in utero* undernourished offspring).

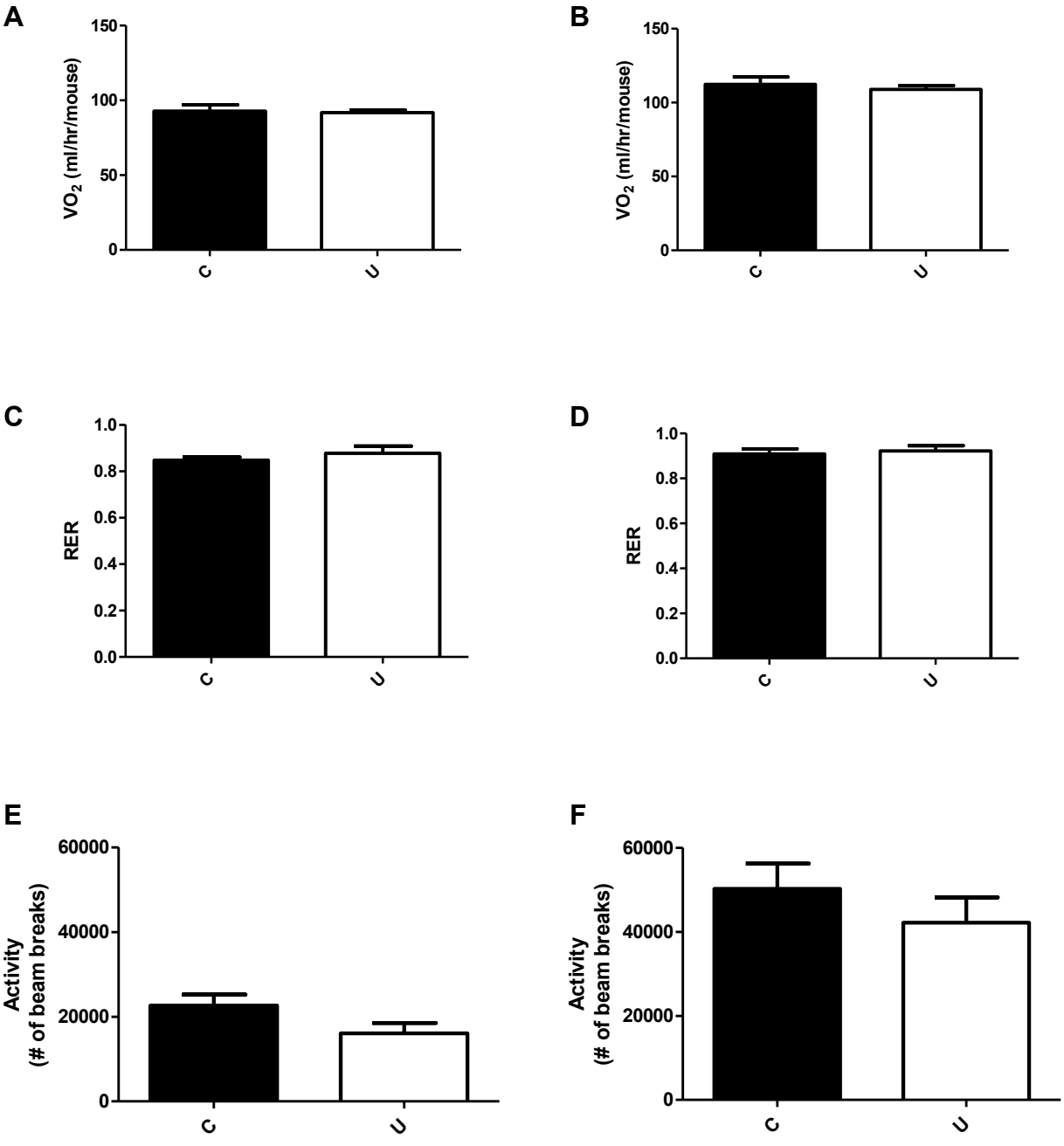
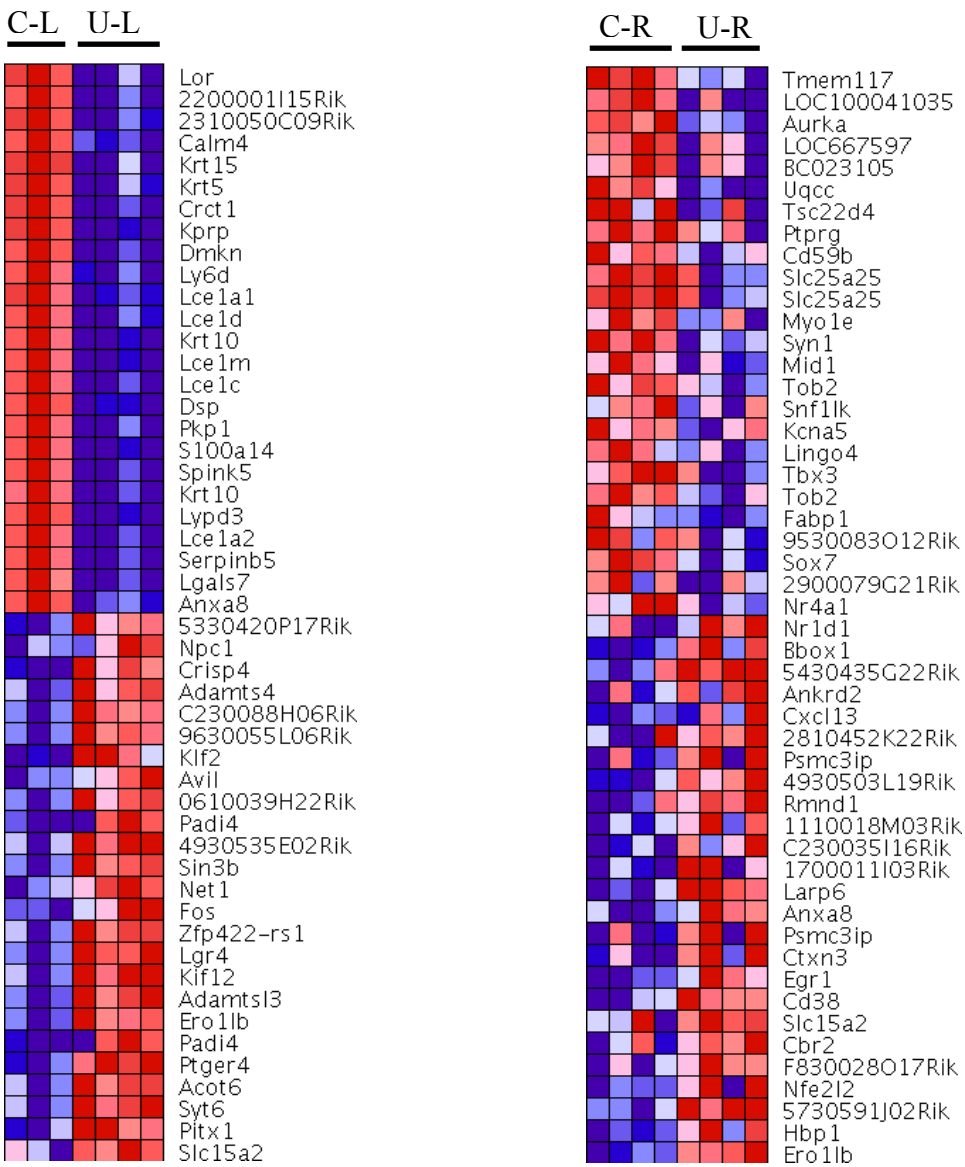

A
150
100
50
0
VO2 (ml/hr/mouse)
C
U
B
150
100
50
0
VO2 (ml/hr/mouse)
C
U
C
1.0
0.8
0.6
0.4
0.2
0.0
RER
C
U
D
1.0
0.8
0.6
0.4
0.2
0.0
RER
C
U
E
60000
40000
20000
0
Activity
(# of beam breaks)
C
U
F
60000
40000
20000
0
Activity
(# of beam breaks)
C
U

Figure S2.2. *In utero* undernutrition does not affect metabolic rate, respiratory exchange ratio (RER), or activity at 10 wks of age. Data were collected for 24h and averaged over each of the light (Left; A, C, E) and dark phases (Right; B, D, F) of the day (lights on 6:00-18:00). A,B) Whole body oxygen consumption by indirect calorimetry expressed per mouse. C,D) RER calculated as VCO_2/VO_2. E, F) Activity expressed as the sum of beam breaks in the x and y direction. Values are mean ± SEM, n=8-9. Black = C (control offspring), white = U (*in utero* undernourished offspring).

A


C-L
U-L
Lor
2200001I15Rik
2310050C09Rik
Calm4
Krt15
Krt5
Crct1
Kprp
Dmkn
Ly6d
Lce1a1
Lce1d
Krt10
Lce1m
Lce1c
Dsp
Pkp1
S100a14
Spink5
Krt10
Lypd3
Lce1a2
Serpinb5
Lgals7
Anxa8
5330420P17Rik
Npc1
Crisp4
Adamts4
C230088H06Rik
9630055L06Rik
Klf2
Avil
0610039H22Rik
Padi4
4930535E02Rik
Sin3b
Net1
Fos
Zfp422-rs1
Lgr4
Kif12
Adamtsl3
Ero1lb
Padi4
Ptger4
Acot6
Syt6
Pitx1
Slc15a2
C-R
U-R
Tmem117
LOC100041035
Aurka
LOC667597
BC023105
Uqcc
Tsc22d4
Ptprg
Cd59b
Slc25a25
Slc25a25
Myo1e
Syn1
Mid1
Tob2
Snf1lk
Kcna5
Lingo4
Tbx3
Tob2
Fabp1
9530083O12Rik
Sox7
2900079G21Rik
Nr4a1
Nr1d1
Bbox1
5430435G22Rik
Ankrd2
Cxcl13
2810452K22Rik
Psmc3ip
4930503L19Rik
Rmnd1
1110018M03Rik
C230035I16Rik
1700011I03Rik
Larp6
Anxa8
Psmc3ip
Ctxn3
Egr1
Cd38
Slc15a2
Cbr2
F830028O17Rik
Nfe2l2
5730591J02Rik
Hbp1
Ero1lb

B

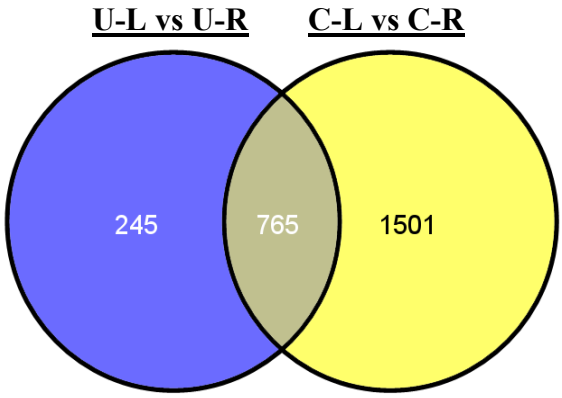

U-L vs U-R
C-L vs C-R
245
765
1501

Figure S2.3. Effects of *in utero* undernutrition on gene expression. Microarray analysis of tibialis anterior gene expression using the Agilent Technologies Whole Mouse Genome Microarray Kit 4x44K. 3-4 samples were run per group with 2 mice pooled per sample. A) Heatmaps were constructed by comparing the per-sample expression of genes with the top 25 and bottom 25 □□value scores for *ad libitum* (L) fed mice (left) and calorie restricted mice (R) (right). Lower levels of expression are represented in shades of blue and higher expression in red. B) Venn diagram of differentially expressed genes with calorie restriction in C (control offspring; C-L vs C-R) and U (in utero undernourished offspring; U-L vs U-R). Probes were filtered for $p \leq 0.005$ and abs. log ratio ≥ 2-fold.

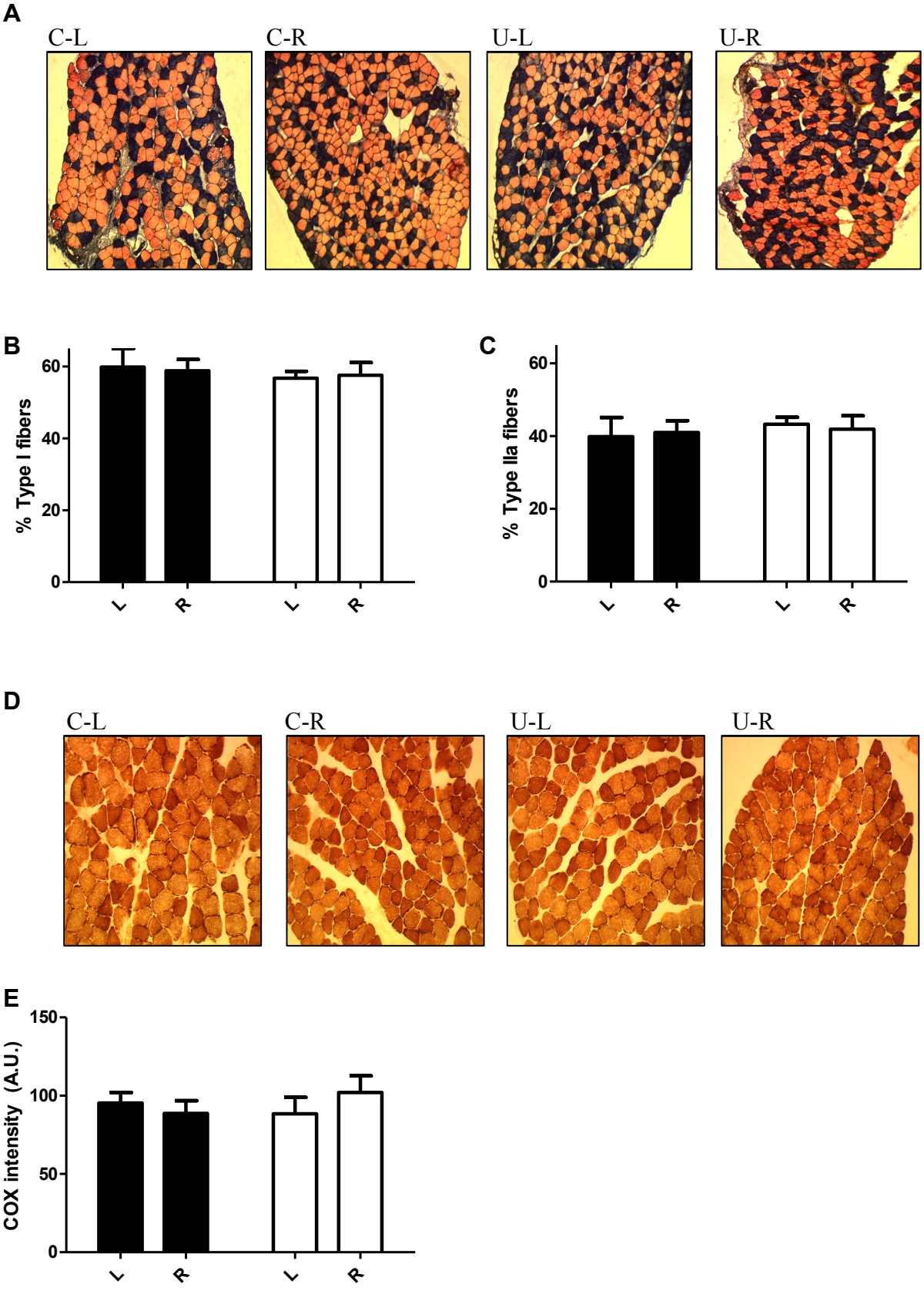

A
C-L
C-R
U-L
U-R
B
% Type I fibers
60
40
20
0
L
R
L
R
C
% Type IIa fibers
60
40
20
0
L
R
L
R
D
C-L
C-R
U-L
U-R
E
COX intensity (A.U.)
150
100
50
0
L
R
L
R

Figure S2.4. *In utero* undernutrition does not alter fiber type proportions or cytochrome c oxidase (COX) activity in the *soleus*. A-C) *Soleus* sections were stained based on myosin heavy chain expression for type I fibers (red; B) and type IIa fibers (blue; C). Proportions were calculated as the percent of the total number of fibers with an average of 271±17 fibers counted per mouse, n=8. Representative images are shown in A. D,E) *Soleus* sections were stained for COX activity, representative images (D) and quantification (E). Fiber intensity was calculated for an average of 165±23 fibers per mouse, n=4. Values are mean ± SEM. Black = C (control offspring), white = U (*in utero* undernourished offspring).

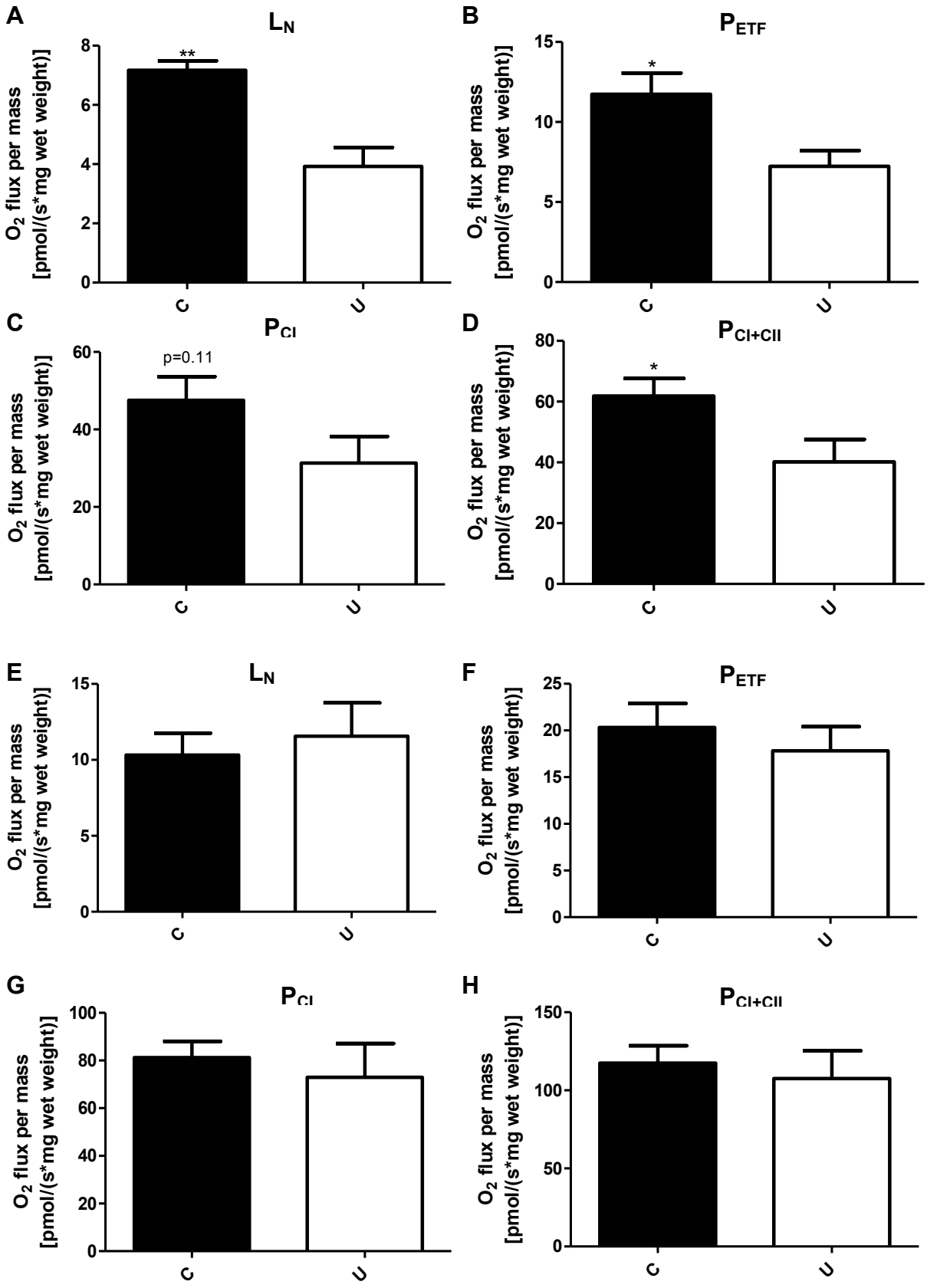

A
L_N
**
O_2 flux per mass [pmol/(s*mg wet weight)]
0
2
4
6
8
C
U
B
P_{ETF}
*
0
5
10
15
C
U
C
P_{CI}
p=0.11
0
20
40
60
C
U
D
P_{CI+CII}
*
0
20
40
60
80
C
U
E
L_N
0
5
10
15
C
U
F
P_{ETF}
0
5
10
15
20
25
C
U
G
P_{CI}
0
20
40
60
80
100
C
U
H
P_{CI+CII}
0
50
100
150
C
U

Figure S2.5. *In utero* undernutrition alters energetics in permeabilized fibers from white *gastrocnemius* (wGAS) but does not alter energetics in red *gastrocnemius* (rGAS)**.** O_2 flux in permeabilized fibers from wGAS (A-D) and rGAS (E-H) at 10 wks of age. Data are shown for adenylate free leak respiration (L_N; A, E), maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity (P_{ETF}; B, F), submaximal state 3 respiratory capacity through complex I ($P_{CI;}$ C, G), and maximum oxidative phosphorylation capacity (P_{CI+CII}; D, H). Values are mean ± SEM, n=6-8. Student's *t*-test, * =p<0.05, **=p<0.01. = C (control offspring), white = U (*in utero* undernourished offspring).

Supplemental Tables

Table S2.1. Mouse characteristics at 10 wks of age

	C Mean ± SEM	U Mean ± SEM
Body weight (g)	28.89 ± 1.34	29.88 ± 1.95
Tail length (cm)	8.9 ± 0.2	8.9 ± 0.2
Femur length (cm)	1.7 ± 0.1	1.7 ± 0.1
Heart (g)	0.130 ± 0.010	0.141 ± 0.008
Liver (g)	1.074 ± 0.051	1.120 ± 0.086

Values are mean ± SEM, n=10-13.

Table S2.2. Mouse characteristics at 14 wks of age

	C-L	U-L	C-R	U-R
Body weight (g)	$33.79 \pm 2.06^{+++}$	$31.98 \pm 1.33^{+}$	24.67 ± 1.05^{a}	27.70 ± 0.99
Tail length (cm)	9.2 ± 0.2	9.4 ± 0.2	9.0 ± 0.1	9.0 ± 0.1
Femur length (cm)	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Heart (g)	$0.147 \pm 0.006^{+}$	$0.142 \pm 0.005^{+}$	0.121 ± 0.006	0.123 ± 0.005
Liver (g)	1.302 ± 0.105	1.183 ± 0.057	1.173 ± 0.072	1.169 ± 0.062

Values are mean ± SEM, n=10-13. Two-way ANOVA with Bonferroni post-hoc test, ^{a} = $p<0.1$ (C vs. U), $^{+}$ = $p<0.05$ (L vs. R), $^{+++}$ = $p<0.001$ (L vs. R).

Supplemental Experimental Procedures

Microarray analysis of gene expression

At 14 weeks of age, the *tibialis anterior* was dissected for RNA extraction. Total RNA was isolated with Trizol Reagent (Invitrogen) and DNAse I treated with the Absolutely RNA Mini-Prep Kit (Stratagene) following the manufacturer's protocol. RNA quantity and quality were assessed with the NanoDrop ND-1000. Integrity of the RNA was determined by agarose gel electrophoresis.

Gene Expression Profiling was performed as described previously using the One-Color Microarray Gene Expression Platform from Agilent Technologies (1). Briefly, 200 ng of RNA was labelled and used for each microarray following the manufacturer's protocol for the One Color Quick Amp Labeling Kit (Agilent). The labeled RNA was hybridized at 65ºC for about 17 hours to the 4x44k Whole Mouse Genome Oligonucleotide microarray (Design ID #014868). After washing the slides following the recommended protocol, the slides were scanned with the Agilent DNA Microarray Scanner at a resolution of 5 μm with extended dynamic range (XDR). After scanning, the intensities of the spots were extracted via the program Feature Extraction Version 10.5.1.1 (Agilent Technologies). Array data was log-transformed (base 2) and subjected to quantile normalization via the BRB-Array Tools developed by Dr. Richard Simon and the BRB-Array Tools Development Team. Intensities were thresholded to a minimum value of 10. Genes with 95th percentile of intensities less than 50 were excluded from further analysis. Differences in gene expression between treatments were expressed as log ratios. Statistical significance of differential gene expression was ascertained by a

regularized t-test implemented in the CyberT software (2). For each gene, a signed π-value significance score was calculated by combining its signed log ratio and nominal p-value, as previously reported (3). Genes were ranked in order of their signed π-values. Heatmaps were constructed in the Genepattern software (4) by comparing the per-sample expression of genes with the top 25 and bottom 25 π-value scores. The microarray data used for the above analysis can be downloaded from the Gene Expression Omnibus database under the accession number GSE53520.

Western Blotting

Muscle homogenate and isolated mitochondria were subjected to reducing SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and stained with ponceau. After blocking for 1 h at room temperature in 5% skim milk, incubation in primary antibody was overnight at 4°C. The following primary antibodies were used at the indicated dilutions: ANT (N-19, SC-9200, Santa Cruz Biotechnology; 1:1000), UCP3 (ab3477, Abcam; 1:1000), COX IV [20E8C12] (ab14744, Abcam; 1:1000), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam; 1:800), α-tubulin (T9026, Sigma-Aldrich; 1:5000). Following 3 x 10 min washes with TBS + 0.1% Tween-20, incubation in the appropriate horseradish peroxidise-conjugated secondary antibody diluted in 5% skim milk was at room temperature for 1h. Bands were visualized using enhanced chemiluminescence.

Mitochondrial isolation

Skeletal muscle mitochondria were isolated using the method described by Chappell and Perry (5) with modifications published previously (6). All steps were performed at 4°C or on ice. Briefly, forelimb, hindlimb, and pectoral muscles were removed and immediately placed in basic medium (BM; 140 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, and 1 mM EGTA, 10 mM pyruvate, 2mM malate; pH 7.0). Muscle was minced with razor blades on a Teflon board and placed in homogenization medium (BM + 1mM ATP, 1U subtilisin A, 1% w/v defatted BSA). Tissue was homogenized using a glass/Teflon Potter-Elvehjem tissue grinder followed by centrifugation at 800 g for 9 min. The supernatant was centrifuged at 12 000 g for 9 min and the pellet obtained was resuspended in BM and incubated on ice for 5 min to allow myofibrillar repolymerization. The sample was then centrifuged at 800 g for 9 min. The supernatant was centrifuged at 12 000 g to obtain a mitochondrial pellet that was resuspended in incubation medium (IM; 70 mM sucrose, 220 mM mannitol, 1 mM EGTA, 2 mM HEPES, 10 mM pyruvate, 2 mM malate; pH 7.2).

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Appendix B: Supplementary materials for Chapter 4

This appendix contains supplementary materials for Chapter 4. This material has been published in the manuscript "Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring" (Beauchamp *et al*., *Biosci Rep*. 2015 Apr 10).

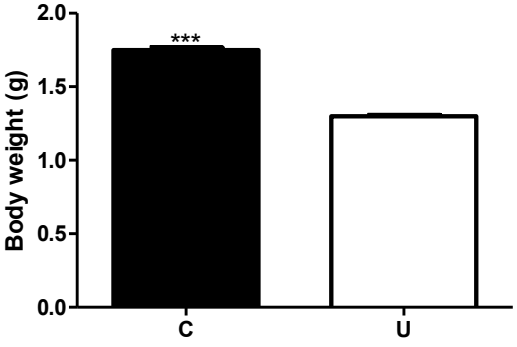
2.0
1.5
1.0
0.5
0.0
Body weight (g)
***
C
U

Figure S4.1. *In utero* undernutrition causes low birth weight. Weight of the offspring at 1 day of age. Student's *t*-test, ***=p<0.0001. Values are mean ± SEM, n=32. Black = C (control offspring), white = U (*in utero* undernourished offspring).

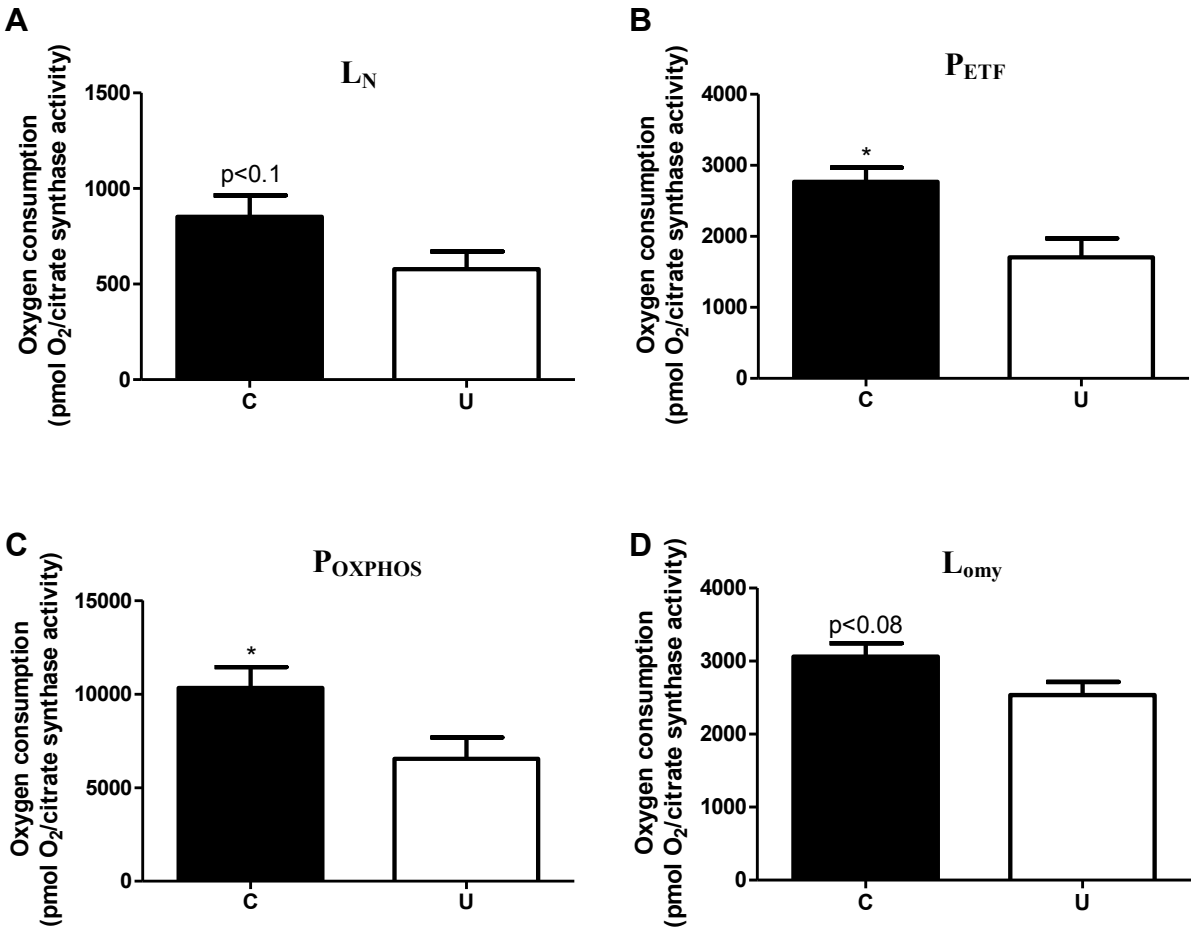
A
L_N
Oxygen consumption (pmol O_2/citrate synthase activity)
1500
1000
500
0
p<0.1
C
U
B
P_{ETF}
Oxygen consumption (pmol O_2/citrate synthase activity)
4000
3000
2000
1000
0
*
C
U
C
P_{OXPHOS}
Oxygen consumption (pmol O_2/citrate synthase activity)
15000
10000
5000
0
*
C
U
D
L_{omy}
Oxygen consumption (pmol O_2/citrate synthase activity)
4000
3000
2000
1000
0
p<0.08
C
U

Figure S4.2. *In utero* undernutrition alters energetics in heart homogenate. O_2 flux in heart homogenate from 10 wk old mice. Values are expressed relative to citrate synthase activity. Data are shown for adenylate free leak respiration (L_N; A), maximal electron flow through electron-transferring flavoprotein and fatty acid oxidative capacity (P_{ETF}; B), maximum oxidative phosphorylation capacity (P_{OXPHOS}; C), and oligomycin-induced leak respiration (L_{omy}; D). Values are mean ± SEM, n=5, *=$p<0.05$. Black = C (control offspring), white = U (*in utero* undernourished offspring).

Table S4.1. Plasma acylcarnitine levels

Acylcarnitine	Plasma level (μM; mean ±SEM)			
	C-L	U-L	C-R	U-R
Acetylcarnitine	5.116±0.617	8.236±0.808	2.397±0.285	2.779±0.642
Propionylcarnitine	0.104±0.016	0.137±0.015	0.078±0.010	0.102±0.010
Malanoylcarnitine	0.012±0.001	0.018±0.003	0.010±0.001	0.011±0.002
Butyrylcarnitine and/or isobutyrylcarnitine	0.131±0.019	0.146±0.014	0.130±0.018	0.119±0.019
3-hydroxy-butyrylcarnitine	0.132±0.012	0.169±0.018	0.095±0.012	0.098±0.011
Methylmalanoylcarnitine and/or succinylcarnitine	0.019±0.001	0.029±0.004	0.024±0.003	0.035±0.007
Isovarylcarnitine and/or 2-methylbutyryl	0.044±0.005	0.047±0.004	0.026±0.006	0.029±0.006
Tiglylcarnitine	0.007±0.002	0.007±0.002	0.007±0.002	0.006±0.002
Hydroxyisovalerylcarnitine and/or 2-methyl-3-hydroxybutyrylcarnitine	0.021±0.002	0.024±0.005	0.016±0.003	0.014±0.005
Glutarylcarnitine	0.017±0.002	0.022±0.002	0.019±0.002	0.020±0.003
Hexanoylcarnitine	0.024±0.005	0.026±0.005	0.013±0.002	0.012±0.002
3-methylglutarylcarnitine	0.031±0.001	0.039±0.005	0.029±0.001	0.029±0.001
Octanoylcarnitine	0.007±0.002	0.013±0.003	0.011±0.003	0.009±0.002
Octenoylcarnitine	0.021±0.002	0.021±0.002	0.022±0.004	0.022±0.003
Decanylcarnitine	0.010±0.002	0.011±0.003	0.009±0.003	0.007±0.002
Decenoylcarnitine	0.019±0.002	0.021±0.003	0.017±0.002	0.015±0.002
Dodecanoylcarnitine	0.021±0.004	0.021±0.003	0.015±0.002	0.015±0.002
Dodecenoylcarnitine	0.011±0.001	0.014±0.004	0.012±0.003	0.010±0.003
3-hydroxydodecenoylcarnitine	0.027±0.004	0.028±0.003	0.029±0.003	0.022±0.003
3-hydroxydodecanoylcarnitine	0.010±0.003	0.013±0.002	0.012±0.003	0.014±0.002
Myristoylcarnitine	0.042±0.005	0.054±0.008	0.028±0.004	0.019±0.005
Tetradecenoylcarnitine	0.042±0.012	0.044±0.006	0.023±0.005	0.019±0.004
3-hydroxytetradecenoylcarnitine	0.021±0.004	0.024±0.004	0.018±0.004	0.014±0.003
Tetradecadienoylcarnitine	0.016±0.006	0.021±0.003	0.013±0.003	0.004±0.002
3-hydroxytetradecanoylcarnitine	0.021±0.004	0.026±0.003	0.023±0.002	0.026±0.006
Palmitoylcarnitine	0.126±0.019	0.155±0.014	0.096±0.008	0.089±0.006
3-hydroxyhexadecenoylcarnitine	0.011±0.003	0.013±0.003	0.009±0.002	0.010±0.002
3-	0.009±0.003	0.008±0.001	0.006±0.001	0.009±0.001

hydroxyhexadecanoylcarnitine				
Octadecanoylcarnitine	0.045±0.006	0.048±0.004	0.036±0.006	0.028±0.004
Octadecenoylcarnitine	0.119±0.023	0.158±0.025	0.073±0.016	0.066±0.011
3-hydroxyoctadecadienoylcarnitine	0.009±0.003	0.017±0.002	0.008±0.001	0.011±0.001
3-hydroxyoctadecanoylcarnitine	0.014±0.001	0.008±0.002	0.009±0.002	0.009±0.003

C = control offspring, U = *in utero* undernourished offspring, L = fed *ad libitum*, R = after a 4 wk 40% calorie restriction.

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Chapter 2: Low birth weight is associated with adiposity, impaired skeletal muscle energetics, and weight loss resistance in mice

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Chapter 4: Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring

This research was originally published in *Bioscience Reports*, published by the Biochemical Society. Brittany Beauchamp, A. Brianne Thrush, Jessica Quizi, Ghadi Antoun, Nathan McIntosh, Osama Y. Al-Dirbashi, Mary-Elizabeth Patti, Mary-Ellen Harper, Undernutrition during pregnancy in mice leads to dysfunctional cardiac muscle respiration in adult offspring, *Biosci Rep*. 2015 Apr 10. ©the Biochemical Society.

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Curriculum Vitae

Brittany Laura Beauchamp

EDUCATION

Doctor of Philosophy in Biochemistry, Faculty of Medicine, University of Ottawa. Thesis topic: Epigenetics of skeletal muscle metabolism in obesity and type 2 diabetes mellitus.	Sept. 2009-Present
Honours Bachelor of Science, Specialization in Biochemistry, University of Ottawa. Thesis topic: Whole body energetics and myotube characteristics as determinants of weight loss success.	Sept. 2006-Apr. 2009
Honours Bachelor of Science, Dietetics and Human Nutrition, McGill University, Montreal (transferred to University of Ottawa).	Sept. 2004-Apr. 2006

ACADEMIC AWARDS AND ACHIEVEMENTS

Ethan Sims Young Investigator Award finalist and recipient of travel award, Annual Scientific Meeting of the Obesity Society.	2013
Natural Sciences and Engineering Research Council of Canada Alexander Graham Bell Canada Graduate Scholarship (Value $105 000)	2011-2014
University of Ottawa Excellence Scholarship	2009-2014
CIHR Training Program in Neurodegenerative Lipidomics Conference Travel Award to attend Ottawa Institute for Systems Biology Symposium, Mont Tremblant, QC, Canada	2013
Biochemistry Graduate Student Seminar Day, 2nd place seminar	2013
Young Investigator Award, Society for Free Radical Biology and Medicine	2012
Michael Smith Foreign Study Supplement for 3 month research period at Harvard Medical School	2012
Biochemistry Graduate Student Poster Day, 3rd place poster	2012
Maternal Infant Child and Youth Research Network Research Trainee Recognition Award	2011
Ontario Graduate Scholarship	2009-2011
Biochemistry Graduate Student Poster Day, 1st place poster	2010

Calian Academic Excellence Scholarship	2004-2010
Dean's Honour list, University of Ottawa	2006-2009
Natural Sciences and Engineering Research Council of Canada Summer Research Award	2008
University of Ottawa Admission Scholarship	2007
Dean's Honour list, McGill University	2004-2006
McGill University Buchanan Academic Scholarship	2004-2006

ADDITIONAL ACADEMIC EXPERIENCE

Critical Reading of Medical Literature, training course, Health Canada, Ottawa, ON, Canada.	Jan. 2014
Visiting researcher at the Joslin Diabetes Center, Harvard Medical School funded by the Michael Smith Foreign Study Supplement Award, Boston, MA, USA.	Sept. 2012 – Dec. 2012
Canadian Institutes of Health Research Training Program in Neurodegenerative Lipidomics – Summer School in Systems Biology of Neurodegenerative Disease, Ottawa, ON, Canada	June 2012
Methods for Systematic Reviews and Meta-Analyses, training course, Health Canada, Ottawa, ON, Canada.	May 2009

TEACHING EXPERIENCE

Teaching assistant: BCH 3120, Intermediary Metabolism, University of Ottawa.	Jan. 2009-Jan. 2014
Professor of Biology, SCI 5952, College Preparation Biology, Algonquin College.	Sept. 2011-Jan. 2012
Guest lecture for Nutritional Determinants of Health (HSS 2342), University of Ottawa. "Natural Health Products".	Mar. 2012
Teaching assistant: BCH 3170, Molecular Biology, University of Ottawa.	Sept.-Dec. 2011

OTHER RELEVANT EXPERIENCE

Science and Research Evaluator, Marketed Health Products Directorate, Health Canada.	Nov. 2008 – Present
Graduate student representative on the Equity, Diversity and Gender Committee, Faculty of Medicine, University of Ottawa.	Sept. 2013 – Dec. 2014
Associate Editor, University of Ottawa Journal of Medicine	Sept. 2013 - Aug. 2014
Child and family support volunteer, Roger's House, pediatric palliative care facility.	Sept. 2006 - Dec. 2014

PUBLICATIONS

Ogrel S, Robitaille K, **Beauchamp B**, Nass T, McIntosh N, Fisher L, Screaton R, Harper ME, Chakraborty P, Al-Dirbashi O. Determination of cellular adenosine nucleotides using liquid chromatography electrospray ionization mass spectrometry. Manuscript under review at *Clinical Biochemistry*.

Amatullah H, Shan Y, **Beauchamp B**, Gali P, Maron-Gutierrez T, Zhou D, Tsang J, Yin J, Mei S, Murthy S, Mak T, Szeto V, Rocco P, Kuebler W, Marshall J, Grinstein S, Stewart D, Harper ME, Liaw P, Liles C, dos Santos C. Targeted deletion of DJ-1 reveals its fundamental role in regulation of autophagic bacterial clearance and outcomes in experimental sepsis. Manuscript submitted to *The Journal of Clinical Investigation*.

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Lebrun M, DiMuzio J, **Beauchamp B**, Reid S, Hogan V. Evaluating the health literacy burden of Canada's public advisories: a comparative effectiveness study on clarity and readability. *Drug Saf*. 2013 Dec;36(12):1179-87.

Aguer C, Pasqua M, Thrush AB, Moffat C, McBurney M, Jardine K, Zhang R, **Beauchamp B**, Dent R, McPherson R, Harper ME. Increased proton leak and SOD2 expression in myotubes from obese non-diabetic subjects with a family history of type 2 diabetes. *Biochim Biophys Acta*. 2013 Oct; 1832(10):1624-33.

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PRESENTATIONS

Peer-Reviewed Abstracts

Beauchamp B, Ghosh S, Dysart M, Kanaan G, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. Low birth weight is associated with adiposity, weight loss resistance and alterations in skeletal muscle energetics and H2O2 production. Canadian Oxidative Stress Consortium, Ottawa, ON, Canada, 2014 (Poster Presentation).

Thrush B, Antoun G, **Beauchamp B**, Boushel R, Doucet E, Imbeault P, Mauger JF, McPherson R, Dent R, Harper ME. Skeletal muscle mitochondrial respiration and ROS production is increased in obese diet sensitive compared to obese diet resistant women. Canadian Oxidative Stress Consortium, Ottawa, ON, Canada, 2014 (Oral Presentation).

Kanaan G, **Beauchamp B**, Harper ME. In utero undernutrition in mice lowers mitochondrial reactive oxygen species emission in skeletal muscle fibers of adult offspring. Canadian Oxidative Stress Consortium, Ottawa, ON, Canada, 2014 (Poster Presentation).

Beauchamp B, Dysart M, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. In utero undernutrition programs metabolic adaptations in skeletal muscle associated with obesity and weight loss resistance in adult mice. Annual Scientific Meeting of The Obesity Society, Atlanta, GA, USA, 2013 (Oral Presentation). Ethan Sims Young Investigator Award finalist, recipient of a travel award.

Beauchamp B, Dysart M, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. In utero undernutrition results in altered metabolism and resistance to weight loss. Ottawa Institute for Systems Biology Symposium, Mont Tremblant, QC, Canada, 2013 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. Resistance to weight loss is associated with altered skeletal muscle mitochondrial energetics in low birth weight mice. Keystone Symposia: Diabetes – New Insights into Mechanisms of Disease and its Treatment, Keystone, Colorado, USA, 2013 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. Epigenetic alterations in skeletal muscle metabolism are associated with weight loss

resistance. Society for Free Radical Research International 16th Biennial Meeting, London, UK, 2012 **(**Oral Presentation). Recipient of a Young Investigator Award.

Harper ME, **Beauchamp B**, Mailloux R, Zhang R, McPherson R, Dent R. Variable mitochondrial oxidative phosphorylation efficiency and its implications for obesity. Mitochondrial Physiology FEBS Workshop: Mitochondrial Biochemistry, Physiology and Pathology, Cambridge, UK, 2012 (Oral Presentation).

Lebrun M, **Beauchamp B**, DiMuzio J, Reid S, Hogan V, Turner C. Evaluating the effectiveness of Health Canada's public advisories: a health literacy study. 12th International Society of Pharmacovigilance Annual Meeting, Cancun, Mexico, 2012 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. Altered skeletal muscle metabolism and resistance to weight loss in a diet-induced epigenetic mouse model. Mitochondrial Dynamics: From Mechanism to Disease, Sardinia, Italy, 2011 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. Epigenetic Programming and Resistance to Weight Loss**.** 2nd National Obesity Summit, Montreal, QC, Canada, 2011 (Poster Presentation). Recipient of the Maternal Infant Child and Youth Research Network Research Trainee Recognition Award.

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. The Modulation of Skeletal Muscle Metabolism by Diet-induced Epigenetic Reprogramming. Keystone Symposia on Type 2 Diabetes, Insulin Resistance, and Metabolic Dysfunction, Keystone, Colorado, USA, 2011 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. Epigenetic Programming of Skeletal Muscle Energetics. 2nd Canadian Obesity Student Meeting, Ottawa, ON, Canada, 2010 (Poster Presentation).

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. Epigenetic reprogramming and the pathogenesis of obesity. Molecular Function & Imaging Symposium, Ottawa, ON, Canada, 2010 (Poster Presentation).

Additional Presentations

Beauchamp B, Dysart M, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. Effects of in utero undernutrition on skeletal muscle mitochondrial content in adulthood. BMI Graduate Student Seminar Day, University of Ottawa, Ottawa, ON, 2013 (Oral Presentation). Recipient of award for 2nd place.

Dysart M, **Beauchamp B**, Harper ME. Resistance to weight loss is associated with altered skeletal muscle mitochondrial energetics in low birth weight mice. Undergraduate Research Opportunity Program Symposium, University of Ottawa, Ottawa, ON, Canada, 2013 (Poster Presentation).

Beauchamp B and Salloum Z. Structural characterization of WDR5 in complex with RbBP5 Peptide Mutant D376N. 2012 China Canada Systems Biology Symposium and 19th Methods in Protein Structure Analysis, Ottawa ON, Canada, 2012 (Poster Presentation). Recipient of award for best poster, CIHR Training Program in Neurodegenerative Lipidomics.

Beauchamp B, Ghosh S, Chu A, Blais A, Rajamanickam K, Tsai E, Patti ME, Harper ME. Altered skeletal muscle metabolism is associated with weight loss resistance in mouse offspring from undernourished dams. BMI Graduate Student Poster Day, University of Ottawa, Ottawa, ON, 2012 (Poster Presentation). Recipient of award for 3rd place.

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. The modulation of skeletal muscle metabolism by diet-induced epigenetic programming. BMI Graduate Student Seminar Day, University of Ottawa, Ottawa, ON, Canada, 2011

Beauchamp B, Ghosh S, Chu A, Blais A, Patti ME, McPherson R, Dent R, Harper ME. Epigenetic Programming of Skeletal Muscle Energetics. BMI Graduate Student Poster Day, University of Ottawa, Ottawa, ON, 2010 (Poster Presentation). Recipient of award for 1st place.