Is Metabolic Flexibility Determined by Early Life Nutrition?

Amy Mae Norman

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Abstract

The environment during development has a strong influence on metabolism in later life. In particular, reduced nutrition in early development is associated with obesity in later adulthood. However, the causal mechanisms and processes underlying the phenomenon are not fully defined. The primary objective of this thesis was therefore to identify and investigate mechanisms underlying the above association. My experimental studies focussed on structure and function of skeletal muscle, regulation of gene transcription, and postnatal outcomes for prenatally undernourished rats relative to their controls. The outcome of the study is that undernourished rats follow a developmental pathway characterised by efficient energy use and storage together with capacity for metabolic flexibility that differs from dietary-induced obesity.

A well-established rat model of reduced maternal nutrition during pregnancy consistently produces offspring that become obese as adults. These offspring are metabolically distinct from the offspring of adequately nourished mothers and this thesis contributes to understanding their unique metabolic phenotype. My experiments investigated: (1) the capacity for metabolic flexibility from birth through to weaning and adulthood; (2) factors integral to pathways that regulate metabolic plasticity; and (3) interactions between the prenatal environment, varied postnatal environmental conditions, the outcomes for body composition and skeletal muscle metabolism.

My study demonstrated changes in skeletal muscle structure and metabolic function for offspring of undernourished mothers compared with their controls. The impact of prenatal undernutrition on postnatal skeletal muscle development became apparent as the offspring matured. In particular, mechanisms may include altered patterns of gene expression for mitochondrial biogenesis and calcium signalling pathways. The altered regulation of these genetic factors highlights mechanisms for the observed fibre type composition and oxidative capacity of skeletal muscle, even in a sedentary postnatal environment.

In sum, this thesis demonstrates that the consequences for adult rats of growth restriction *in utero* are substantially different from dietary obesity in control adult rats despite superficial similarity between the two conditions. This difference arises from

under-nourished foetuses following a developmental pathway that prepares them for survival under conditions where food may be scarce. The difference was recognised from the structure and function of muscles, retained insulin sensitivity, deposition and use of stored fat, and at the level of gene transcription. These results support the hypothesis that offspring of undernourished mothers have increased flexibility of energy storage and use by way of enhanced oxidative metabolism.

THE UNIVERSITY OF AUCKLAND
SCHOOL OF GRADUATE STUDIES

Co-Authorship Form

Graduate Centre
ClockTower – East Wing
22 Princes Street, Auckland
Phone: +64 9 373 7599 ext 81321
Fax: +64 9 373 7610
Email: postgraduate@auckland.ac.nz
www.postgrad.auckland.ac.nz

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CO-AUTHORS

Name	Nature of Contribution	Extent of Contribution (%)
J.L. Miles-Chan	Animal experiment, biochemical lab work	10
N.M. Thompson	Animal experiment, biochemical lab work	10
B.H. Breier	Study design, manuscript assistance	10
K. Huber	Study design, manuscript assistance	10

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- the PhD candidate was the lead author of the work and wrote the text.

Name	Signature	Date
Dr. Jennifer Miles-Chan		06-21-2011
Dr. Nichola Thompson		06-21-2011
Prof. Bernhard Breier		22. June 2011
Prof. Korinna Huber		06-21-2011

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Dedication

During the course of this PhD journey, ten additions were made to my *whānau*: Malachi (born weighing 0.80 kg), Charlotte (0.99 kg), Ava (3.29 kg), Ruby (1.98 kg), Schafer (2.44 kg), Lucia (3.52 kg), Christian (4.34 kg), Louie (2.32 kg), Heidi (4.35 kg) and Harrison (3.62 kg).

This thesis is dedicated to all of my nieces and nephews who are the most delightful people I have ever met.

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Abbreviations

ACC	Acetyl-CoA carboxylase
AD	*Ad libitum* fed
α MSH	α-melanocyte-stimulating hormone
AMPK	Adenosine 5'-monophosphate-activated protein kinase
ATP	Adenosine triphosphate
ATP Synth	ATP synthase
Calna1	Calcineurin
CaMK	$Ca^{2+/}$calmodulin-dependent protein kinases
cAMP	Cyclic adenosine monophosphate
CoA	Coenzyme A
COX	Cytochrome oxidase
C-peptide	Connecting peptide
Cpt-1	Carnitine palmitoyltransferase 1
DEXA	Dual energy x-ray absorptiometry
cDNA	copy Deoxyribose nucleic acid
DTNB	5', 5'-dithiobis(2-nitrobenzoate)
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethlene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide
Fboxo32	F-box only protein 32/ Atrogin1
FFA	Free fatty acid
FOXO1	Forkhead box protein O1
G6P	Glucose 6-phosphate
GLUT4	Type-4 glucose transporter
GSUP	Gastrocnemius superficial muscle
HDAC	Histone deacetylase
HDL	High density lipoprotein
HEPES	N-2-hydroxyethlypiperazine-N'-2-ethanesulfonic acid
IGF	Insulin-like growth factor
IMTG	Intramyocellular triglyceride
IUGR	Intrauterine growth restriction
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
MEF	Myocyte enhancer factor 2
MHC	Myosin heavy-chain
MRF	Myogenic regulatory factor
Mstn	Myostatin
mTOR	Mammalian target of rapamysin
MT-CO1	Mitocondrially encoded cytochrome c oxidase 1
Mt-DNA	mitochondrial DNA
Murf1	Muscle RING-finger protein-1
Myf5	Myogenic regulatory factor 5
Myog	Myogenin

Na2HPO4	Disodium hydrogen orthophosphate
NADH	Nicotinamide adenine dinucleotide
NaH2PO4	Sodium dihydrogen phosphate
NaN_3	Sodium azide
NFAT	Nuclear factor of activated T-cells
NRF	Nuclear respiratory factor 1
OXPHOS	Oxidative phosphorylation
PAX	Paired-homeobox transcription factor
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + TWEEN
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGC 1	Peroxisome proliferator γ coactivator 1
PI3 K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PPAR-α	Peroxisome proliferator activated receptor-α
PVN	Paraventricular nucleus
QPCR	Quantitative reverse transcriptase real-time PCR
RIA	Radioimmunoassay
RIN	RNA integrity number
SEM	Standard error of the mean
SOL	Soleus muscle
T_3	Triiodothyronine
T_4	Thyroxine
TCA	Tricaboxylic acid
Tfam	Mitochondrial transcription factor A
TG	Triglyceride
TGF β	Transforming growth factor-β
TRIS	Tris(hydroxymethyl)aminomethane
TSH	Thyroid stimulating hormone
TWEEN	Polyoxyehtylene (20) sorbitan monolaurate
UCP	Uncoupling protein

Chapter 1.

Introduction

During my lifetime, obesity rates have doubled. The main assumption underlying the extent and pace of increased obesity prevalence is that modern living is the culprit. Simply put, obesity is the accumulation of fat in adipose and other body tissues and is the result of an imbalance in energy intake versus energy expenditure. Modern living is proposed to be a hotbed of 'obesogenic' influences, replete with increased consumption of energy-dense foods and decreased participation in physical activity. The increased adiposity in itself is not the main cause for concern; it is the co-morbidities such as insulin resistance and heart disease that are responsible for the associated health burden. Therefore, the overall goal is to understand the mechanisms responsible for this health burden.

This thesis explores the metabolic response to both environmental scarcity and abundance. This introductory chapter outlines the body's ability to adapt to changes in nutrient supply and energy expenditure. I begin with a brief overview of the key concepts in metabolism and endocrinology followed by a description of the influence of the prenatal environment on postnatal metabolic profile. I will then describe the interaction between skeletal muscle and whole-body metabolism from initial myogenic development to the adaptive processes that occur at maturity. The consequences of either enhanced or impaired metabolic flexibility, as evidenced by skeletal muscle structure and function, are outlined. Lastly, this chapter will introduce the interrelationship between the prenatal environment, postnatal metabolism and the influence of 'lifestyle'.

1.1 Metabolic Flexibility

Metabolic flexibility is the term used to describe the ability to coordinate homeorhetic regulation of energy metabolism. Challenged with an irregular supply of nutrients, organisms have adapted to shift their energy metabolism relative to both the energy supply and demand (Storlien *et al.*, 2004). The irregular supply, or cyclical periods of fasting and feeding encountered on a daily basis are met with appropriate adjustments

of both substrate storage and utilisation (Storlien *et al.*, 2004). Metabolic flexibility encompasses the capacity to switch substrate utilisation between glucose and lipids in times of carbohydrate abundance and scarcity, respectively (Andres *et al.*, 1956). The ability to shift between different substrates for energy production is critical for survival and is the core of metabolic flexibility. Put simply, this whole body adaptation allows an organism to change energy fuel usage in a dynamic manner according to fuel availability (Kelley *et al.*, 2002). For example, in the case of a predominantly high-fat diet, lipids would be preferentially oxidised in the post-prandial phase. It is also the tissue sensitivity to hormones that influences which substrate is preferentially oxidized. For instance, insulin hormone is a pivotal endocrine hormone for directing carbohydrate and lipid metabolism in multiple tissues. Once stimulated, the substrate oxidation at the tissue level is under the influence of a host of intracellular complexes (Morris *et al.*, 2004). Ultimately, the intensity of metabolic demands on the organism controls the direction of energy utilisation and storage.

A metabolically inflexible state is of particular interest as it relates to an inability to cater to whole body energy requirements efficiently. Metabolic flexibility is characterised by insulin resistance and impaired regulation of glucose and fatty acid metabolism. The consequences of impaired energy regulation are likely to lead to poor metabolic health such as Type II diabetes and heart disease. Therefore, it is quite clear that the coordinated regulation of metabolic fuel selection is paramount for optimal energy homeostasis. Historically, insulin resistance was deemed to be primarily a disorder of carbohydrate metabolism (Sugden, 2007). The pathway described by Randle and colleagues has provided insight into the role of lipids in insulin resistance. Randle's 'glucose-fatty acid cycle' appropriately describes the coordinated regulation of metabolism. In particular, the Randle cycle is proposed to account for the reciprocal relationship between the two key substrates of metabolic flexibility, carbohydrates and lipids (Randle *et al.*, 1963). The use and storage of metabolic fuels are now widely accepted to form part of a larger metabolic system.

The key concepts of metabolic flexibility, or the converse inflexibility, are illustrated in Figure 1.1. In healthy, insulin-sensitive individuals, the postprandial state is an opportunity for increased lipolysis to be matched with increased lipid oxidation.

During fasting, the body relies on lipids to fuel energy demands primarily because fat stores represent the largest endogenous energy store. This balance seems to be deranged in the case of obese individuals, who have reduced lipid oxidation and increased carbohydrate utilisation (reviewed by (Thyfault *et al.*, 2006)). In obese, insulin-resistant individuals there is a decreased capacity for insulin-stimulated glucose oxidation and storage, whereas fatty acid uptake remains relatively unchanged (Kelley *et al.*, 1999). Lipid oxidation in this situation is thought to be blunted and certainly appears to compound the resistance to insulin. The ectopic storage of fat in peripheral tissues is one consequence of impaired lipid metabolism. One example is intramyocellular triglyceride (IMTG), which is correlated with insulin resistance in obese individuals (van Loon and Goodpaster, 2006).

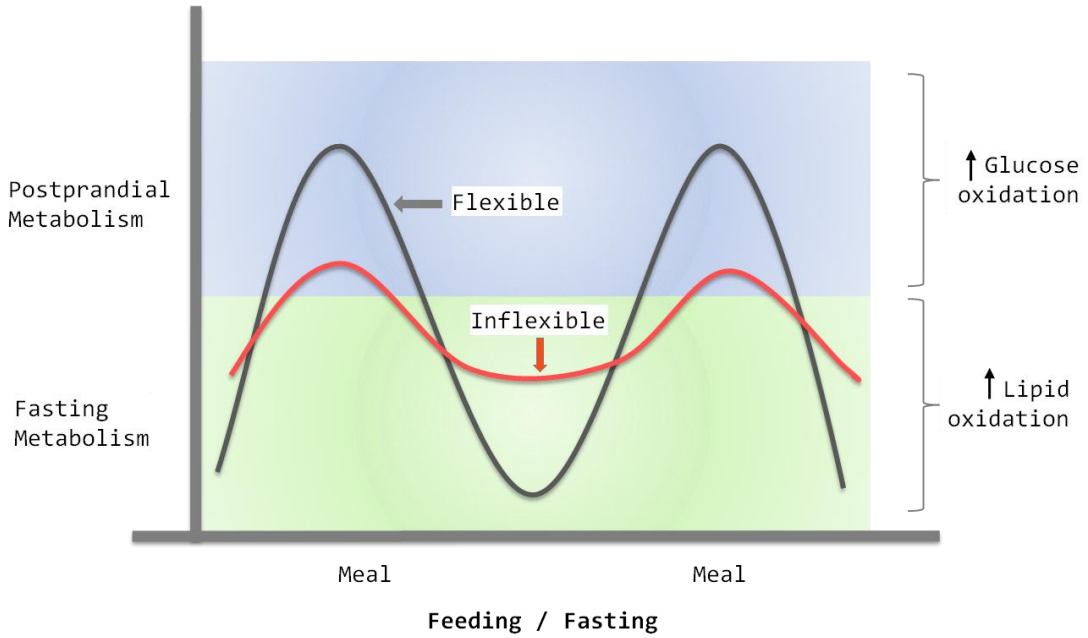

Figure 1.1 Profiles of metabolism during feeding and fasting.

The flexible profile exhibits cyclical increases in glucose oxidation at meal times with a clear switch to increases in lipid oxidation during fasting. The red inflexible line represents an example profile for an obese, insulin-resistant individual. Impaired metabolic flexibility in obesity and insulin resistance is marked by a blunted response to lipid oxidation during fasting conditions and insulin resistance during postprandial conditions.

Insulin stimulated glucose transport and insulin signalling in peripheral tissues is blunted in the postprandial phase for inflexible individuals. The figure above (Figure 1.1) also demonstrates the effect of being inflexible during the postprandial phase. Compounding this diminished insulin sensitivity, circulating glucose and insulin are further increased by reduced suppression of hepatic gluconeogenesis and glycogenolysis (Thyfault *et al.*, 2006). The consequence of uninhibited glucose supplies is a constantly elevated blood glucose concentration. Therefore, the metabolic state that results is marked by hyperglycaemia and hyperinsulinaemia, given that the individual has diminished ability to clear both insulin and glucose from circulation (Houmard *et al.*, 2002). Given the importance of maintaining metabolic flexibility for energy metabolism, this review will focus on the interconnected pathways of intermediary metabolism. A particular emphasis will be placed on peripheral pathways of skeletal muscle metabolism in response to nutrition and physical activity.

1.2 Optimal Intermediary Metabolism: What it means to be 'Flexible'

1.2.1 Key Factors of Oxidative Metabolism

Efficient utilisation of glucose after a meal and the appropriate storage of substrates are key characteristics of healthy carbohydrate and lipid metabolism. The metabolism of these two key nutrients is tightly coordinated by the major tissues of carbohydrate and lipid flux including pancreas, liver, adipose and skeletal muscle. Ideally, the response to a meal is rapid and begins with insulin secretion from pancreatic beta cells, followed by effective suppression of lipid mobilisation and then finally, activation of diverse metabolic pathways of tissue energy uptake and storage.

As described above, the driving force behind the storage of nutrients is the hormone insulin, produced by the pancreas. In response to an elevation of circulating glucose after a meal, insulin is released by the beta (β) cells in the pancreatic islets of Langerhans. Insulin is initially produced as a preprohormone that is then converted to proinsulin. Proinsulin is then cleaved prior to secretion with the removal of a connection peptide (C-peptide) forming the active form of insulin. Carbohydrates, amino acids and fatty acids are all nutrients whose transport and uptake are stimulated by insulin.

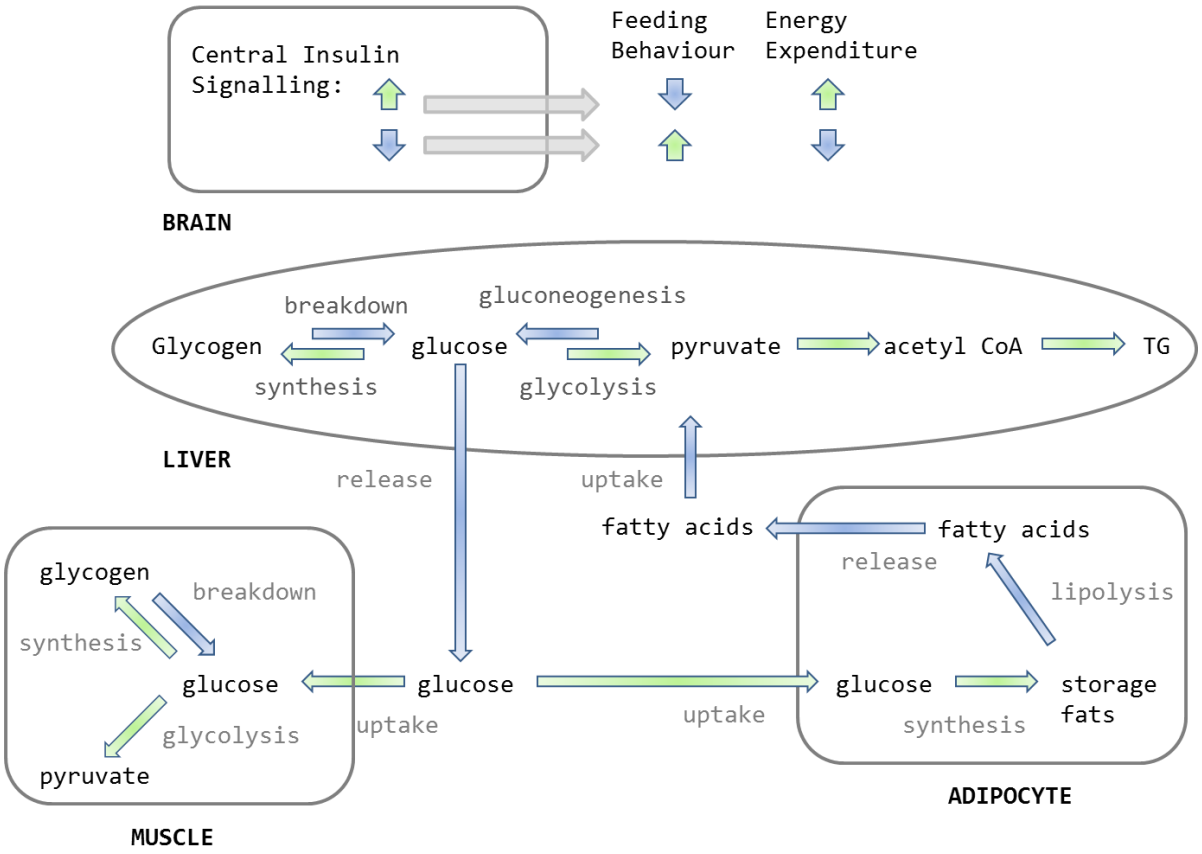

Figure 1.2 Overview of the factors that stimulate or inhibit uptake and storage of glucose.

In muscle and liver, glucose is primarily stored as glycogen, stimulated by insulin (green arrows). Insulin signalling in the brain inhibits (blue arrows) feeding behaviour and activates energy expenditure pathways. Release of lipids from fat or hepatic glucose is inhibited by insulin. TG, Triglycerides. Modified from Ashrafi, 2007.

Secondary to uptake, insulin plays a fundamental role in converting nutrients to their storage forms – glycogen, protein and lipids (Rhodes and White, 2002). Insulin also has a critical role in the suppression of fuel mobilization by inhibiting the process of glycogenolysis, or glycogen breakdown (Figure 1.2).

Figure 1.2 shows the tightly regulated process of glucose metabolism and how feeding behaviours and energy expenditure are regulated centrally. The critical circulating pool of glucose must be maintained by either: diet; gluconeogenesis where glucose is

synthesised during periods of fasting; or glycogenolysis whereby glucose is liberated from glycogen stores (Totora and Grabowski, 2000). Circulating glucose then has three important fates, the most important of which is uptake into tissues for use as energy. The other two fates account for two important energy storage strategies: as glycogen via glycogenesis or as fat via *de novo* lipogenesis. In terms of efficient energy storage, fats make up the body's main energy reservoir with protein following second at 20% of all stores. Carbohydrate constitutes less than 1% of energy stores, yet tissues such as the brain are dependent on the continuous supply of glucose (Frayn, 2003a).

Integration of fat, carbohydrate and, to a lesser extent, protein metabolism is essential for effective blood glucose regulation. The free fatty acid (FFA) pool comprises the balance between dietary FFA absorbed from the diet, FFA released from adipose tissue after lipolysis and FFA utilisation by peripheral tissues (Lodish *et al.*, 2000). Insulin drives FFA into storage as lipids (Figure 1.2). Insulin also stimulates the uptake of amino acids and serves as an anabolic factor for protein metabolism. Protein from the diet is broken down by digestive enzymes in the gut lumen and resulting amino acids are absorbed and directed to either the synthesis of proteins for growth and repair or, in severe cases, oxidation to produce adenosine triphosphate (ATP). The amino acid pool in circulation comprises the balance between protein break down, protein synthesis and the entry of amino acids into gluconeogenic pathways for glucose generation (Frayn, 2003a).

1.2.2 Key Concepts of Oxidative Metabolism

The purpose of this section is to provide a very brief overview of the metabolic pathways that convert substrates, mainly carbohydrates and lipids, into ATP, the major energy currency. Firstly, the storage of these two principal substrates will be introduced, with particular emphasis on pathways specific to skeletal muscle. Then, brief introduction to their fate in the TCA cycle will follow.

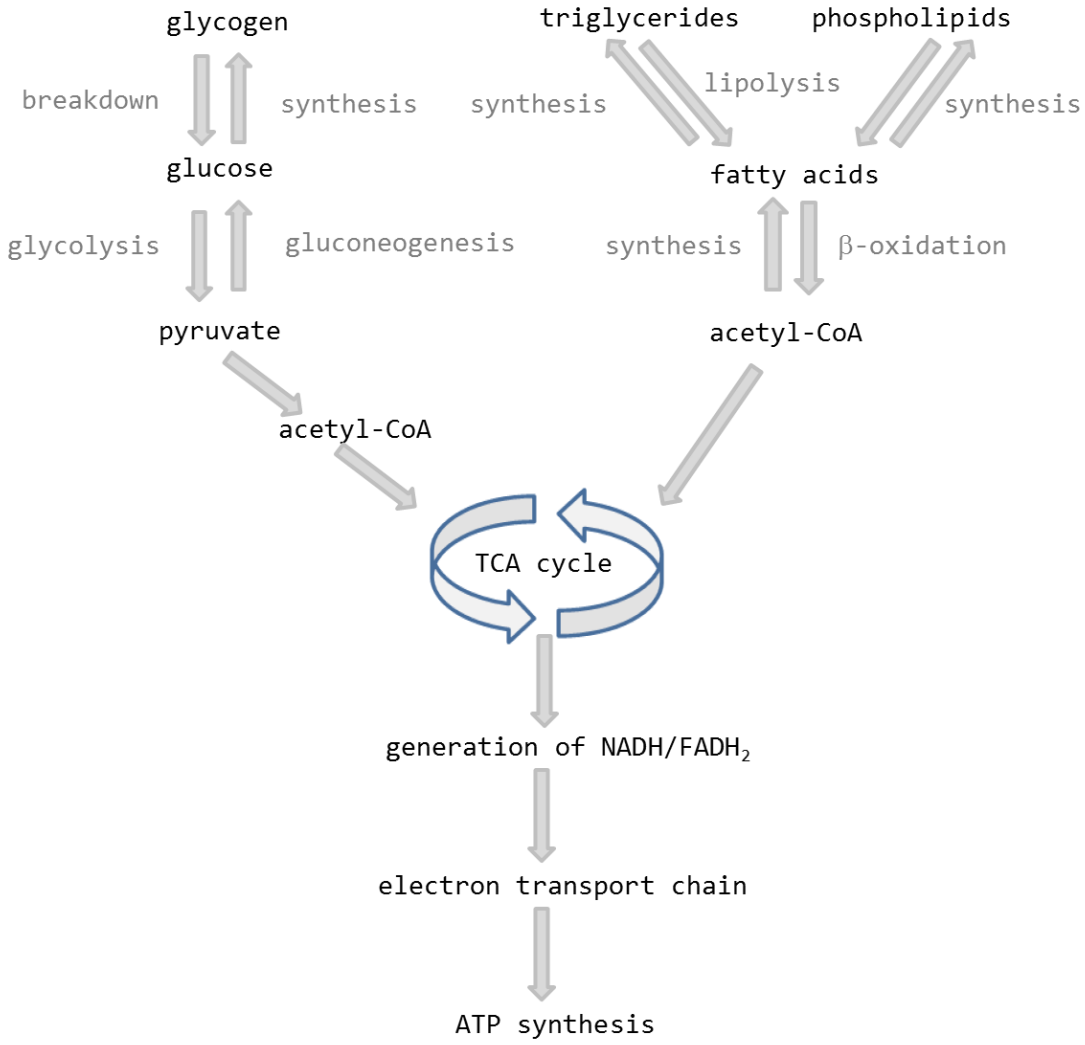

Figure 1.3 Overview of lipid and carbohydrate synthesis and breakdown pathways.

Pathways leading from the glucose and fatty acid substrates indicate progression to the tricarboxylic acid cycle (TCA), with subsequent generation of adenosine triphosphate (ATP). Pathways leading away from the substrates indicate energy storage. Modified from Ashrafi, 2007

Glycogen

Homeostatic regulation of blood glucose allows excess glucose to be taken up and converted into glycogen. Glycogen synthesis is maximally efficient during conditions of excess of glucose, favouring deposition or storage, and is largely regulated by hormones such as insulin. Insulin inhibits gluconeogenesis, endogenous glucose production, and acts to promote glucose uptake into tissues and storage (Frayn,

2003a). Conversely, periods of energy utilisation such as anaerobic exercise induce glycogenolysis, or the breakdown of glucose, in order to ensure fuel availability.

Glycogenolysis and Glycogen Synthesis

Skeletal muscle cells break down glycogen to glucose 6-phosphate (G6P), the initial substrate for glycolysis and subsequent TCA cycle driven ATP production for muscle contraction. In liver, this G6P can be released into the circulation after conversion to free glucose. Insulin negatively regulates gluconeogenesis via suppression of the transcription of genes for key gluconeogenic enzymes such as glucose-6-phosphatase (G-6-Pase) and PEPCK. The substrates of gluconeogenesis include lactate, gluconeogenic amino acids and glycerol (Barthel and Schmoll, 2003). Gluconeogenesis is primarily initiated by glucagon during starvation but can also be initiated by both glucocorticoids produced during stress and catecholamines during exercise. Pyruvate carboxylase (PC) is a mitochondrial enzyme that catalyses the first regulated step in gluconeogenesis (Hagopian et al., 2003). In addition, PC is also involved in lipogenesis, or lipid synthesis. Facilitating the export of acetyl group from mitochondria as citrate, PC is important for the cytoplasmic biosynthesis of fatty acids in adipose tissue (Jitrapakdee and Wallace, 1999).

Lipid synthesis and storage

The biosynthesis of lipid from carbohydrate or amino acid precursors in both adipose tissue and the liver is stimulated by insulin. Specifically, in response to raised caloric intake exceeding energy requirements, glycerol and fatty acids generated can be converted to triglycerides. These triglycerides are either stored or enter a further pathway to produce other lipids, such as lipoproteins, phospholipids, and cholesterol (Totora and Grabowski, 2000; Gibson and Harris, 2002).

Fatty Acid Oxidation

In the starved state, fatty acids are liberated from storage in response to hormonal factors such as glucagon, catecholamines and corticosteroids. Lipolysis breaks down the triglycerides in storage. The fatty acids are then transported to the mitochondria to be oxidized progressively via the TCA cycle. Firstly, the acyl group from fatty acids is transferred to carnitine in order to be transported into the mitochondrial space where β oxidation can take place. This process is catalyzed by carnitine palmitoyltransferase-1 (CPT-1), the rate limiting factor of β oxidation. CPT-1 is induced during fasting, most

likely via elevated levels of FFA that in turn activate the transcription factor Peroxisome proliferator activated receptor- gamma (PGC) (Gibson and Harris, 2002).

1.2.3 Oxidative Metabolism

The molecule participating most often in energy exchanges in living cells is ATP. The major source of ATP is the oxidation of glucose (Totora and Grabowski, 2000). For ATP generation, an elaborate set of enzyme-catalyzed reactions couple the metabolism of 1 molecule of glucose to the synthesis of as many as 36 molecules of ATP from 36 molecules of ADP (Lodish *et al.*, 2000). Cells can use the energy released during this reaction to power many otherwise energetically unfavourable processes, for instance the contraction of skeletal muscles (Lodish *et al.*, 2000).

Cellular respiration, or the complete oxidation of glucose to CO_2 and H_2O, integrates the process of glycolysis, acetyl coenzyme A (acetyl-coA) formation, the Krebs or tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain (*outlined in Figure* 1.3). In short, glucose is converted to pyruvate via the glycolytic pathway, with the net formation of two ATP and the net reduction of two NAD+ molecules to NADH. Prior to the initiation of the TCA cycle, pyruvate is decarboxylated to a two carbon fragment called acetyl. This conversion is facilitated by the mitochondrial pyruvate dehydrogenase, a multi-enzyme complex that results in net reduction of glucose as well as reduced glycogen, lactate, citric acid cycle intermediates, and amino acids that serve as glucose precursors (Berg *et al.*, 2002). The acetyl group attaches to coenzyme A with the resulting compound, acetyl-coA, ready to enter the TCA cycle. The complete oxidation of acetyl-CoA in the citric acid cycle is the final common path of oxidative metabolism as defined by the conversion of the reduced carbon in organic fuels to the fully oxidized carbon of CO_2. The carbons of glucose are joined by the carbons of fatty acids and most carbons of amino acids in the flux through acetyl-CoA. The successive conversions of the intermediates in the TCA cycle were known before the concept of a cycle was suggested by Hans Krebs (Gibson and Harris, 2002).

The reduction of pyruvate by the enzyme lactate dehydrogenase (LDH) produces lactate under anaerobic conditions. Lactate can be readily transported across the cell

membrane and via the blood stream to the liver or heart to be converted back to pyruvate or to glucose via hepatic gluconeogenesis. However, under aerobic conditions the energy yield is significantly increased and the pyruvate molecule can be completely oxidised to CO_2. The main purpose of the TCA cycle though is to remove two carbon atoms of the acetyl group as CO_2 and channel the electrons released from these oxidation reactions into the electron transport chain to produce ATP. Catalysed by citrate synthase, this begins when the acetyl group attached to coenzyme A breaks away and the two-carbon acetyl group attaches to a four-carbon molecule of oxaloacetate to form a six-carbon molecule called citrate. Citrate is particularly important as it is capable of being transported to the cytosol where it inhibits glycolysis and activates fatty acid synthesis. The citrate then goes through a series of chemical transformations, losing first one, then a second carboxyl group as CO_2. The relative expression of the citrate synthase enzyme relates well as an experimental measure of oxidative capacity in addition to mitochondrial content (Lodish *et al.*, 2000; Gibson and Harris, 2002).

Oxidative phosphorylation

As mentioned, most of the energy made available by the oxidative steps of the cycle is transferred as energy-rich electrons to NAD^+, forming NADH. For each acetyl group that enters the citric acid cycle, three molecules of NADH are produced. Electrons are also transferred to the electron acceptor flavin adenine dinucleotide (FAD), forming $FADH_2$ (Berg *et al.*, 2002). Then at the end of each cycle, oxaloacetate is regenerated and the cycle continues. The electron transport chain is embedded in the inner membrane of the mitochondria. The mitochondrion itself is visualised as a structure with a double membrane that houses both the respiratory chain and oxidative phosphorylation (OXPHOS) system (Gnaiger, 2009). The process of OXPHOS involves transfer of energy to ATP synthase via chemiosmosis and transport of protons down the electrochemical gradient across the membrane space. The respiratory chain is made up of a series of NADH dehydrogenases acting as the complex I, complex II or succinate dehydrogenase, complex III or bc1 complex, and complex IV or cytochrome c oxidase (COX) (Berg *et al.*, 2002). All the elements forming the respiratory chain act collectively to transfer electrons from NADH and FADH to molecular oxygen. The transfer of electrons is tightly coupled to the formation of a

proton gradient in the intermembrane space that is used by the ATP synthase to drive the synthesis of ATP (Gnaiger, 2009).

Oxidative Metabolism and Substrate Preference

Blunted oxidation of lipids in skeletal muscle is correlated with insulin resistance in obese individuals (van Loon and Goodpaster, 2006). Elevations in IMTG content have also been linked to the development of insulin resistance and Type 2 diabetes (Pan *et al.*, 1997; Shaw *et al.*, 2010). Recently, it has emerged that this seemingly aberrant storage does not directly confer insulin resistance but is more likely to indicate other bioactive lipid metabolites within muscle such as sphingolipids and diacylglycerol (DAG) (Coen *et al.*). Other schools of thought have suggested that IMTG content itself might actively prevent the build-up of these deleterious metabolites (Watt, 2009). In any case, increased IMTG content can be seen in inactive obese subjects (Coen *et al.*), and is also increased in Type I fibres of insulin-sensitive endurance athletes (Goodpaster *et al.*, 2001). The seemingly paradoxical finding of increased lipid stores in both obese and highly trained individuals is accounted for by the principle of metabolic flexibility. The inability to utilise the lipid stores marks the obese subject as having metabolic inflexibility, whereas the endurance athletes are considered flexible with the ability to burn the fuels stored efficiently (van Loon and Goodpaster, 2006).

A high oxidative capacity in muscle is a key feature of metabolic flexibility. As described, increases in the glucose concentration and subsequent insulin secretion, suppresses fatty acid release from adipose tissues (*see section* 1.2.1). This reduces the competition of fatty acids for oxidative utilisation and glucose becomes the major fuel. Under fasting conditions, decreased levels of plasma glucose and insulin induce an increase in fatty acid concentration concomitant with increased fatty acid oxidation. Several metabolites of fatty acid oxidation also play a role in suppressing glucose metabolism via the activity of glycolytic enzymes (Jeukendrup, 2002). Elevation of acetyl-CoA, citrate and glucose-6-phosphate suppresses the activity of pyruvate dehydrogenase (PDH), phosphofructokinase (PFK), and hexokinase 2 respectively. In contrast, insulin activates acetyl-CoA carboxylase (ACC) to suppress skeletal muscle fatty acid oxidation (Brownsey *et al.*, 2006). In skeletal muscle, the shift towards lipid utilisation is supported by a coordinated increase in the expression of genes such as

CPT-1 (Jeukendrup, 2002), uncoupling protein 3 (UCP3) (Dulloo *et al.*, 2001) and peroxisome proliferator-activated receptor gamma coactivator (PGC-1) (Pilegaard *et al.*, 2003; Puigserver and Spiegelman, 2003).

Mechanisms of Substrate Selection

The relative contribution of lipid and carbohydrate substrates to whole-body fuel utilisation is dependent on complex regulation at multiple levels. Substrate availability, tissue sensitivity to hormones, in addition to the activity and expression of a host of intracellular complexes, all exert influence on the substrate preferentially oxidized (Morris *et al.*, 2004). The flexibility to adjust energy flux through various catabolic and anabolic pathways in response to changing nutritional status is said to be coordinated via metabolic sensing mechanisms (Ashrafi, 2007). How nutrients and variations in energy status are detected, or 'sensed', is of considerable interest. Both the mammalian target of rapamysin (mTOR) and adenosine 5'-monophosphate-activated protein kinase (AMPK) are nutrient-sensitive downstream targets of insulin, by way of the AKT signalling pathway. Particularly responsive to skeletal muscle contraction, AMPK is often referred to as the "master switch" nutrient sensor (Hardie *et al.*, 2006). In skeletal muscle, AMPK is an important regulator of glucose uptake and stimulates β oxidation of fatty acids (Merrill *et al.*, 1997). In addition to modulating mitochondrial function, mTOR regulates protein synthesis and skeletal fibre size (Winder, 2001; Sakamoto and Goodyear, 2002; Assifi *et al.*, 2005; Sandri *et al.*, 2006).

The mammalian target of rapamycin (mTOR)

The mTOR is an evolutionarily conserved serine/threonine kinase belonging to the phosphatidylinositol 3-kinase kinase-related (PI3 K) protein family that is a nutrient sensitive downstream target of insulin. This target is a principal effector of protein synthesis and is also complexly influenced by nutritional factors and ATP levels (Hay and Sonenberg, 2004). Rapamycin-sensitive mTORC1, a complex formed in conjunction with other molecules, controls several pathways involved in protein synthesis coupled to nutrient availability and overall growth (Winder, 2001; Sakamoto and Goodyear, 2002; Assifi *et al.*, 2005).

Adenosine Monophosphate-Activated Protein Kinase (AMPK)

AMPK is a highly conserved protein kinase that plays a role in the regulation of many cellular processes. There has been considerable interest in AMPK as the master switch nutrient sensor (Hardie *et al.*, 2006). The energy sensor AMPK has influence over numerous cellular changes, that together down-regulate energy-consumptive pathways and up-regulate energy-generating pathways (Ashrafi, 2007). In skeletal muscle, AMPK is an important insulin-independent regulator of glucose uptake during exercise (Musi *et al.*, 2003; Roepstorff *et al.*, 2005) and is activated by decreases in ratios of ATP to AMP and Phosphocreatine to Creatine, key indicators of metabolic stress. AMPK is implicated in exercised-induced nutrient partitioning in skeletal muscle (Sakamoto and Goodyear, 2002). In particular, AMPK α 2 isoform is increased during exercise and α 1 isoform in the extreme case (Nakano *et al.*, 2006). Mice with inactivated AMPK would be expected to have greatly reduced glucose uptake, yet they have been shown to retain 60-70% of their ability to transport glucose, suggesting there is compensation (Mu *et al.*, 2001).

Importantly, AMPK is thought to coordinate the transition from catabolism or breakdown of energy stores to anabolism, the building of energy stores (Assifi *et al.*, 2005). Another role of AMPK comprises the inhibition of protein synthesis in skeletal muscle. Inhibition of the mTOR pathway in particular is suppressed by AMPK activation in skeletal muscle with consequences for protein synthesis (van Wessel *et al.*, 2010). In promotion of oxidative metabolism in skeletal muscle, AMPK is thought to stimulate Type I fibre gene expression through PGC-1 α signalling (van Wessel *et al.*, 2010). Importantly, AMPK activity is reduced in relation to fatty acid oxidation and pharmacological treatment with an insulin sensitizer Metformin leading to increased type-4 glucose transporter (GLUT4) expression in skeletal muscle and decreased fat depots (Liu *et al.*, 2006).

Peroxisome proliferator-activated receptor gamma coactivators (PCG-1)

The PGC-1 is an important regulator of energy metabolism and nutrient homeostasis that exerts powerful effects on gene transcription (Finck and Kelly, 2006). Recently described as the master regulator of mitochondrial biogenesis and energy expenditure (Fernandez-Marcos and Auwerx, 2011) two isoforms, PGC-1α and PGC-1β, are expressed broadly in brown and white fat, skeletal muscle, liver, heart, kidney, and the brain (Puigserver, 2005). Their metabolic functions appear to overlap and also both

isoforms appear to be involved in mitochondrial biogenesis (Handschin and Spiegelman, 2006). The α isoform, PGC-1α, is associated with hepatic gluconeogenesis via stimulation of PEPCK and glucose-6-phosphatase. In addition to this, PGC-1α has influence over fatty acid oxidation in the liver via regulatory effects on CPT-1 gene expression (Puigserver and Spiegelman, 2003).

PGC-1α acts as an energy status sensor (Fernandez-Marcos and Auwerx, 2011) and when activated by low energy status has influence on GLUT4 translocation to the membrane for glucose uptake (Al-Khalili *et al.*, 2005). When energy is abundant, PGC-1α is down-regulated by the insulin sensitive AKT-mediated signalling pathway (Southgate *et al.*, 2005) representing a feedback system. Fibre type determination in skeletal muscle has been reported to be under the regulation of PGC-1α (Lin *et al.*, 2002; Ling *et al.*, 2004). The transcriptional coactivator PGC-1α is thought to modulate adaptation to physical activity by way of a conversion of muscle fibres from Type II (fast twitch) to Type I (slow twitch) (Puigserver, 2005). The increase in PGC-1α observed in transgenic models suggests that PGC-1α is activated by way of calcium signalling cascades and is sufficient for the Type II to Type I fibre switch observed (Wu *et al.*, 2002). The consequences of this switch and a more detailed description of skeletal muscle fibre type will be detailed in section 1.3.2 *Muscle Morphology*.

Leptin

The traditional view of the adipocyte-derived hormone leptin was that it functioned primarily to prevent obesity via the regulation of food intake and thermogenesis. The consequences of absolute leptin deficiency were described originally by Zhang and colleagues (1994) and have since been extensively investigated (Zhang *et al.*, 1994). In particular, the ob/ob mouse that lacks the leptin gene and consequently the leptin hormone, has served as an important experimental model to study leptin action. Leptin deficient ob/ob animals are obese, hyperphagic, diabetic and exhibit reduced metabolic processes including decreased body temperature (Baile *et al.*, 2000). In addition, other studies have shown heterozygous leptin gene mutations lead to low circulating leptin concentrations paralleled by increased body adiposity (Havel, 2004). Exogenous leptin treatment is able to reverse the adiposity and increase metabolic rate in ob/ob mice (Pelleymounter *et al.*, 1995). Knockout models have demonstrated the effect of peripheral resistance to leptin when the signalling form of

the leptin receptor is deleted in db/db mice. The db/db mice are then unresponsive to any endogenous or exogenous leptin (Chen *et al.*, 1996). The knockout studies of both leptin and its receptor have provided a picture of a hormone fundamental to energy regulation.

Even though leptin has been touted as an anti-obesity, or lipostatic, hormone, we are reminded that "hormones do not evolve to prevent the consequences of their own deficiency" (Unger, 2000). It is more likely that the main biological role of leptin is that of a satiety signal and also a means to adapt to reduced energy availability (Unger, 2000; Unger and Orci, 2001). One study in particular showed that low leptin levels in mice set off the response to conserve energy in response to fasting (Ahima *et al.*, 1996). Since this pivotal study, researchers have learned that leptin's role in humans is important for stimulating food intake, decreasing energy expenditure and also suppression of the reproductive and other endocrine axes (Farooqi, 2011).

Centrally, leptin acts on energy balance via hypothalamic neurons, reducing energy intake and increasing energy expenditure (Campfield *et al.*, 1995; Ahima and Osei, 2004). The arcuate nucleus, a key target of appetite regulatory factors, contains populations of both appetite stimulating (orexigenic) and appetite depressing (anorexigenic) neurons (Baskin *et al.*, 2001). Leptin stimulates the pro-opiomelanocortin (POMC) neuronal pathway subsequently activating melanocyte-stimulating hormones (MSH), in particular α-MSH, which lead to increases in energy expenditure (Dardeno *et al.*, 2010). The neurological pathways coordinate appetite and modulate efferent signals to the periphery. For example, a rat study showed that POMC is subject to post-translational modification processes giving rise to the melanocortins, which then mediate an anorectic response (Li *et al.*, 2007).

Leptin inhibits orexigenic pathways that are mediated by neurons expressing the melanocortin antagonist Agouti-related peptide and Neuropeptide Y. Regulating the pathways associated with appetite is a feedback relationship that exists between leptin and the orexigenic fasting hormone ghrelin. Ghrelin's release is stimulated from the stomach during fasting periods and circulating concentrations are generally inversely proportional to calorie intake (Briggs and Andrews, 2010). The balance between these two energy-sensing hormones, leptin and ghrelin, represents an integral control mechanism for energy regulation. The interplay between insulin and

leptin has also been explored, especially given that levels of body fat correlate with insulin resistance and that weight loss can improve insulin sensitivity. A hypothetical adipoinsular axis has been proposed (Kieffer and Habener, 2000). Fat mass is thought to be involved in the modulation of glucose and insulin metabolism via circulating leptin (Ballantyne *et al.*, 2005). Leptin can induce acute changes to insulin, glucocorticoids and even noradrenalin which has led some to believe that leptin might be involved in sensing energy balance. Leptin is also acutely altered by feeding and fasting (Konturek *et al.*, 2004), which are notably influenced by insulin. Interestingly, insulin and leptin levels decrease before body mass is even affected after bariatric surgery (Ballantyne *et al.*, 2005). Therefore, the interplay between insulin and leptin is not a simple case, and the adipoinsular axis is thought to be dysregulated in those susceptible already to the development of diabetes (Kieffer and Habener, 2000).

Perhaps of more interest is the imprinting effect of leptin during early life that has recently been described in detail (Pico *et al.*, 2011). Rodent studies of leptin concentrations in the perinatal period indicate its role in the programming of postnatal appetite regulation (Breier *et al.*, 2001; Vickers *et al.*, 2001; Gorski *et al.*, 2006; Krechowec *et al.*, 2006; Vickers, 2007; Briana and Malamitsi-Puchner, 2009). In particular, early postnatal leptin treatment may program either a lean or obese phenotype, which also may depend on the nature of maternal nutrition during pregnancy (Breier *et al.*, 2001; Krechowec *et al.*, 2006; Vickers *et al.*, 2008). Furthermore, studies have also suggested the physiological importance of the timing of the postnatal surge in leptinaemia for the programming effect (Ahima *et al.*, 1998; Delahaye *et al.*, 2008). During the perinatal period the supply of leptin is maternally-derived and is present in the maternal milk (Casabiell *et al.*, 1997). Therefore, neonatal leptin concentrations are influenced by maternal nutritional status (Smith-Kirwin *et al.*, 1998; Delahaye *et al.*, 2008). The transition made from fetal to neonatal digestion is also marked by a switch from primarily-glucose to catabolism of lipids from milk and may be directly influenced by leptin (Pico *et al.*, 2011). The endogenous leptin production upon encountering the extrauterine environment becomes important later at weaning where pups then transition from an exclusive milk diet to a solid diet (Oliver *et al.*, 2001).

Absence of leptin has been associated with reduced skeletal muscle mass in ob/ob rodent models (Trostler *et al.*, 1979). In a recent study of these ob/ob leptin deficient rodents, exogenous leptin treatment stimulated muscle growth and prevented atrophy (Sainz *et al.*, 2009). In this study, the reduced skeletal muscle mass was prevented by leptin inducing a down-regulation of the negative regulator of muscle growth, myostatin, in addition to oxidative fibre type promotion (Sainz *et al.*, 2009). Leptin has a direct effect on lipid metabolism stimulating both lipolysis and lipid oxidation (Ceddia, 2005) an effect which is regulated in part by AMPK activity in skeletal muscle (Muoio *et al.*, 1997). As described above, AMPK plays a major role in the regulation of fatty acid oxidation, while inhibiting fatty acid synthesis and enhancing the activity of CPT-1 and β-oxidation of fatty acids. The stimulation of muscle AMPK occurs downstream of leptin after direct interaction with its receptor in the muscle (Minokoshi *et al.*, 2002). In rodents, leptin plays a key role in nonshivering thermogenesis, which may be related to the AMPK signalling in oxidative muscles (Minokoshi *et al.*, 2002). Whether skeletal muscle can mediate nonshivering thermogenesis in mammals remains controversial (Golozoubova *et al.*, 2001; Silva, 2006; Pico *et al.*, 2011).

Thyroid hormones

The thyroid hormones have been described as primary denominators of energy and metabolic homeostasis (Havekes and Sauerwein, 2010). Controlled by central AMPK activity in the hypothalamus, thyroid hormones are fundamental for feedback regulation of appetite and energy expenditure (Dhillo, 2007; Duntas *et al.*, 2011). Thyroid hormones also have a direct effect on glucose and lipid metabolism via insulin regulation and glucose disposal in peripheral tissues (Duntas *et al.*, 2011). Two types of thyroid hormone are released from the thyroid gland: thyroxine (T_4) and triiodothyronine (T_3). Thyroid stimulating hormone (TSH) is released from the anterior pituitary and is the principal regulator of thyroid hormone production. Thyroid hormones are proposed to participate at the level of the mitochondrial energy transduction. Heat dissipation as the molecular basis of resting basal metabolism is proposed to be influenced by the relationship of thyroid hormones to uncoupling proteins in mitochondria. In particular, T_3 has been shown to influence thermogenesis by influencing transcription of uncoupling proteins such as UCP3 (Reitman *et al.*, 1999). Both hypothyroidism and hyperthyroidism have been associated with insulin

resistance in skeletal muscle (Havekes and Sauerwein, 2010). In these conditions of dysthyroidism, decreased glycogen synthesis and decreased muscle oxidative capacity have been reported (Peppa *et al.*, 2010).

1.3 Skeletal Muscle and Metabolic Flexibility

To a large extent, we are completely dependent on skeletal muscle; not only for locomotion and maintenance of posture, but also regulation of fuel utilisation. The metabolic property of healthy skeletal muscle is highly flexible and adapts to various physiological demands by shifting energy substrate metabolism. This flexibility is a testament to the finely tuned mechanisms of skeletal muscle development and the composition of the muscle in maturity. Inflexibility, or impaired responsiveness of skeletal muscle to substrate utilisation and storage, is closely associated with metabolic diseases (*see section* 1.1 *Metabolic Flexibility*). Homeostasis of adult musculature is dependent on the initial development or myogenesis, and subsequent regulation of muscle size (Guttridge, 2004). Structure and function of skeletal muscle is maintained in part by a network of signal transduction pathways that regulate transcription and post-transcriptional processes (Guttridge, 2004).

The long fibril cells of skeletal muscle, known as muscle fibres, are grouped together into fascicular bundles that collectively make up the muscle. The contractile units, the sarcomeres, are organised within the muscle fibre as myofibrils and are made up of long chains of actin fixed at Z stripes and interacting myosin proteins. The molecular basis of muscle contraction is dependent on energy in the form of ATP, which is dephosphorylated by ATPases to release ADP + P_i. In order to maintain the ATP requirement for contraction on demand, phosphocreatine serves as a 'buffer store' under the control of creatine kinase (Hochachka, 1994).

The major pathways for generation of ATP in muscle are illustrated in Figure 1.4. Skeletal muscle can use energy in the form of stored fuels, glycogen or TG, or it can use the likes of dietary glucose and fatty acids as substrates. As explained previously (*see section* 1.2.1), glucose uptake is mainly mediated by the insulin-sensitive glucose transporter GLUT4. Once transported into the cell this glucose may be used for glycogen synthesis or for glycolytic and oxidative catabolism serving as an energy substrate (*see section* 1.2.1 *Glycolysis).*

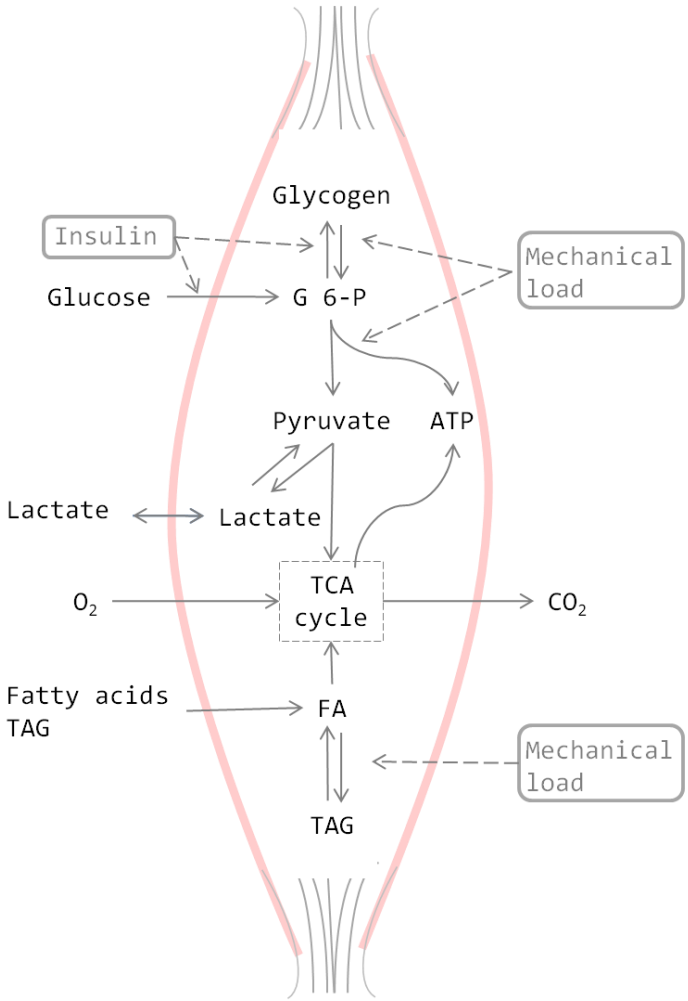

Figure 1.4 Generation of ATP in skeletal muscle.

Stored fuels glycogen and triacylglcerol (TAG) can be progressively broken down with substrates fed into the TCA cycle for production of adenosine triphosphate (ATP). Insulin stimulates glucose uptake to glucose-6-phosphate (G 6-P), which may be used for glycogen synthesis or metabolism via the pathway of glycolysis. Fatty acids (FA) are taken up particularly by oxidative fibres and breakdown is stimulated by mechanical load by contraction (modified from Frayn, 2003).

Skeletal Muscle Development

Skeletal muscle derives from the epithelial somites of the developing embryo. The complex series of signals driving the myogenic process is highly organised and dependent on timing of genetic programmes (Gilbert, 2000). Importantly, the intrinsic metabolic and contractile functions of skeletal muscle are laid down during myogenesis and will be firstly outlined in brief, followed by the key factors driving the process described in more detail. The process of myogenesis, visualised schematically

in Figure *1.5*, proceeds through four main steps of development including: determination at the level of the progenitor mesodermal cell, with commitment to myoblastic lineage; differentiation into a myotube; proliferation and final maturation and growth of the myofibre.

A unique set of transcriptional regulators called the myogenic regulatory factors (MRFs) are the key inducers, and as their name suggests, regulators of myogenesis. The MRFs initiate the various stages of muscle development with both timing of their expression and relative location of the signal (Bass *et al.*, 2000). Whether a myoblast continues dividing or differentiates is also dependent on these MRF signals. Then the balance between trophic and maturation pressures is under the control of insulin-like-growth-factor (IGF) or transforming growth factor-β (TGF-β), two factors that exert opposing effects. Proliferation is the next fundamental step in muscle development and is driven by two key signalling pathways: mitogen-activated protein (MAP) kinase and Akt/insulin signalling pathways. At the next key stage, proliferation, myostatin plays its famed role in negative regulation of muscle growth (Bass *et al.*, 2000).

Myostatin is a negative regulator of skeletal muscle mass in both early developmental and later postnatal periods. During skeletal muscle development, myostatin expression is restricted to the myotome region of the developing somites and also to the myogenic lineage at later stages of development and in the mature adult (Rios et al., 2002). Myostatin is synthesised as a precursor protein, which consists of an N-terminal propeptide domain that harbours the signal sequence and a C-terminal domain that forms a disulfide-linked dimer and functions as the active ligand (Thomas et al., 2000). Mutations to this propeptide region that may be involved in the folding and secretion of myostatin have been linked to increased muscle mass in the compact hypermuscular mouse (Bass et al., 2000).

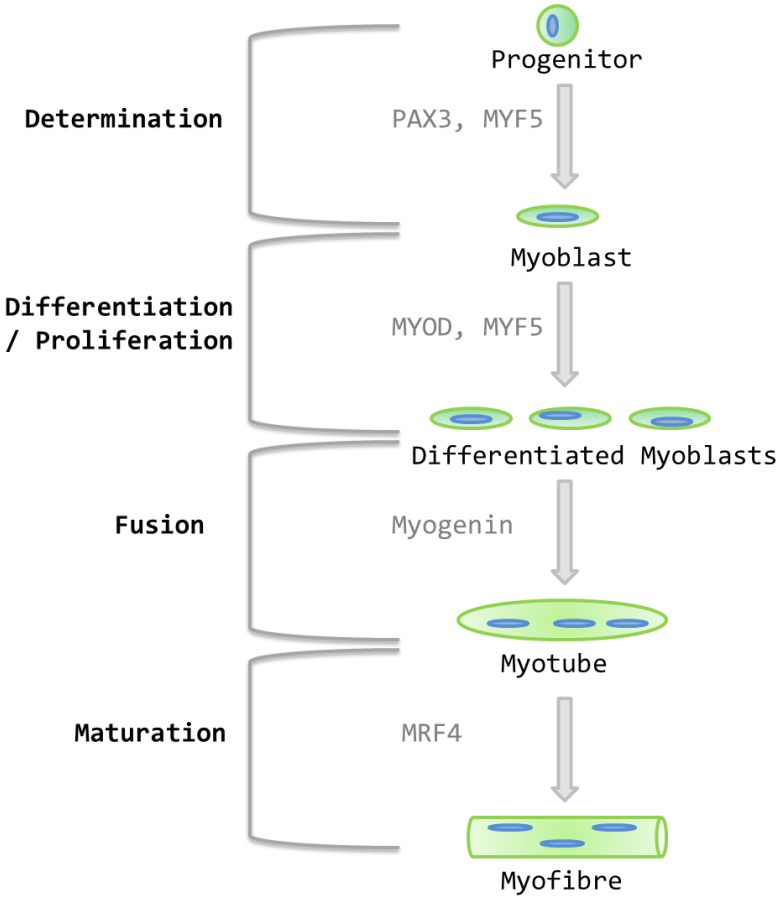

Figure 1.5 Factors driving the different stages of myogenesis.

During development, progenitor cells become committed to the skeletal muscle cell lineage to become myoblasts, which then differentiate and fuse to become myotubes. Multiple muscle-specific genes are expressed during the process that leads to maturation of myofibres, a few key factors are indicated on the figure including: paired-homeobox transcription factor 3 (PAX3), myogenic regulatory factor 5 (Myf5), MyoD, myogenin, myogenic regulatory factor4 (MRF4). (Modified from Bass et al., 2000).

The current view on the mechanisms for myostatin function is understood to involve up-regulation of p21, the cyclin-dependent kinase inhibitor, and signaling that ultimately leads to cell cycle arrest. The double-muscling phenomenon that occurs in myostatin deficient animals has been well documented. From these animals we learn that myostatin negatively regulates muscle growth by suppressing myoblast proliferation and ultimately cell cycle arrest. In these myostatin deficient animals the absence of negative regulation equals extended proliferation. Knockout myostatin mice have been reported to adapt their muscle fibre types and function in response to endurance training (Matsakas et al., 2010). This is interesting because myostatin null mice are able to adapt to exercise training in postnatal life and increase their oxidative

capacity in the absence of myostatin. As will be described later (*Section 1.4*), skeletal muscle can adapt to the demands of motility during postnatal development by altering fibre composition (Pette, 2002).

1.3.1 The Myogenic Program

Premyogenic factors

The first molecular markers that label myogenic precursor cells, myoblasts, in the dermomyotome structure are the paired-homeobox transcription factors Pax3 and Pax7. The expression of Pax3 and Pax7 is induced by signals from surrounding tissues (Otto *et al.*, 2006). These two factors deliver important instructions during the initial myogenesis. Pax3 expression is somewhat ubiquitous during organogenesis, but importantly, its absence leads to impaired muscle development, with death of progenitor cells in the somites (Buckingham, 2002). To some extent, Pax7 shows redundancy with Pax3 during primary myogenesis and studies have shown it is more important for postnatal myogenesis (Relaix *et al.*, 2005). However, the combined lack of Pax3 and Pax7 is deleterious at the primary myogenesis stage and deficient myoblasts are unable to enter the myogenic program (Relaix *et al.*, 2005; Bismuth and Relaix, 2010). The protein products of Pax3 and Pax7 are responsible for setting in motion the myogenic process, further carried out by the myogenic regulatory factors (MRFs).

Myogenic Regulatory factors (MRF)

The MRFs are a family of basic helix–loop–helix (bHLH) transcription factors whose role is to ensure overall muscle development. The characterised MRFs listed below are known for their ability to convert certain non-muscle cell lines, such as fibroblasts, into myoblasts or myotubes (Bass *et al.*, 2000). These MRFs were shown early on to activate transcription by heterodimerizing with E box proteins (Lassar et al., 1991). In response to Ca^{2+} signalling (*see section* 1.3.4 *Calcium Signalling*) the myocyte enhancer factor 2 (MEF2) is activated in the nucleus (Nakagawa *et al.*, 2005). The activation leads to transcription of target gene products MyoD and myogenin which play roles in muscle cell hypertrophy (Naidu *et al.*, 1995). Much of what we know about the control of skeletal muscle development comes from knockout or knockdown studies of these factors. In the absence of the MRFs, such as MyoD, Myf5, myogenin and Mrf4, myogenesis is inhibited (Bass *et al.*, 2000; Chanoine *et al.*, 2004; Guttridge,

2004; Bismuth and Relaix, 2010). Additionally, these regulators are able to induce myogenic differentiation when expressed in non-muscle cells (Pownall *et al.*, 2002). Researchers have also long been aware that the MRFs display a certain amount of redundancy, and, in some cases, loss of one member can be compensated for by up-regulation of another (Rudnicki *et al.*, 1992).

The first MRF, Myf5, is the key determination factor directing myogenic progression (Rudnicki *et al.*, 1992). Myf5 marks the initial migration of cells in the dermomyotome and is under the influence of both Pax3 and Sonic Hedgehog (Shh), produced by the notochord of the developing embryo (Gustafsson *et al.*, 2002). In this instance, Shh is a survival and proliferation factor responsible for the maintenance of MRF expression (Kruger *et al.*, 2001). In mice, Pax3 acts upstream of Myf5, and in the absence of either Pax3 or Myf5 body muscles remain unformed, demonstrating that these factors also act genetically upstream of MyoD (Buckingham, 2002). Further down the myogenic line, disruption mutations to either MyoD or Myf5, on their own, result in apparently normal muscle development, demonstrating a degree of genetic redundancy between these two family members (Bass *et al.*, 2000).

Primary myogenesis is under the control of myogenin where the committed myoblasts then divide and eventually fuse end-to-end to form a syncytium, called a myotube (Myer *et al.*, 1997). The first population of myotubes then can mature and acquire their own basal lamina, or basement membrane of the fibre. After this maturation, secondary myoblasts use the primary myotubes as a scaffold for their own formation (Bass *et al.*, 2000). However, some myoblasts do not fuse to form fibres and remain outside the sarcolemma, but within the basal lamina. These cells are known as satellite cells. A particular MRF, MRF-4, appears to have influence on the differentiation of mature myofibres that are, after this phase, fully functional skeletal muscle fibres (Patapoutian *et al.*, 1995). The terminal muscle cell differentiation program is eventually completed with the sequential expression of muscle-specific genes, including desmin, acetylcholine receptors, and specific isoforms of actin, myosin heavy chain (MHC), troponin, tropomyosin and creatine kinase leading to the assembly of the sarcomere, the mature myotube (Gilbert, 2000).

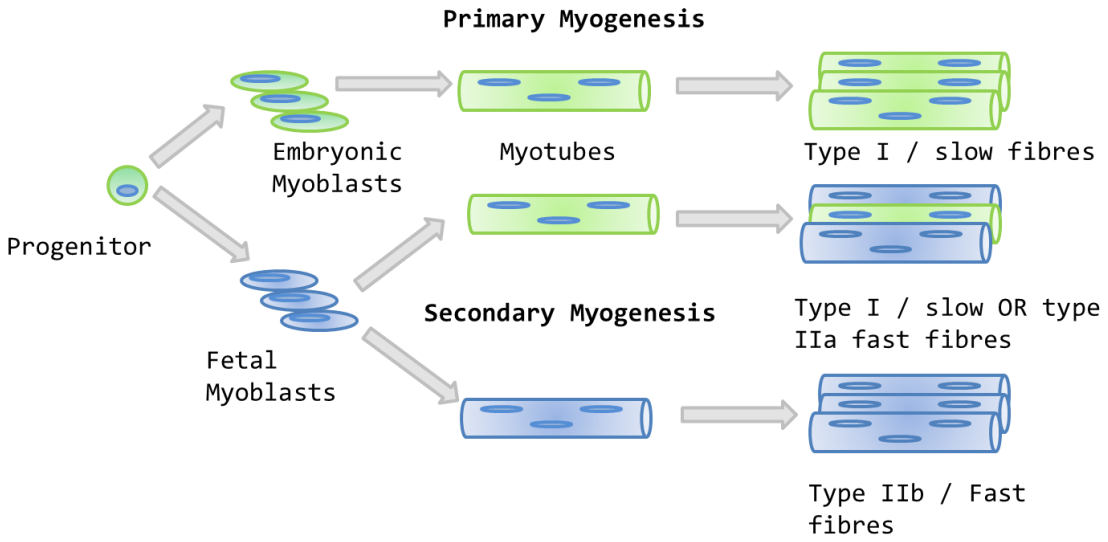

Figure 1.6 Primary and secondary myogenesis

Fibre type diversity in skeletal muscle is directed during primary and secondary myogenesis. Slow Type I fibres are indicated in green and fast Type II fibres are indicated in blue (Modified from Maltin 2008).

Muscle cells exhibit remarkable capacities to adapt to physiological demands such as growth and injury from environmental stimuli, the ability to adapt being attributable to satellite cells (Hawke and Garry, 2001). During hypertrophy of muscle, as well as in response to stretch, muscle fibres lengthen by sequential addition of sarcomeres at the ends of these fibres (Bass *et al.*, 2000). Specific muscle hypertrophy can be load-, or work-induced, which results in the increased local expression of IGF-I and an increase in insulin sensitivity. This is in association with increased glucose metabolism, amino acid transport, protein synthesis, and protein degradation.

1.3.2 Muscle Morphology.

Muscle fibres differ in their capacity for oxidative metabolism versus glycolytic metabolism. Importantly, fibre morphology and composition are important determinants of growth potential, endurance fitness, resistance to fatigue and adaptability to environmental stress (Fahey *et al.*, 2005b). Furthermore, the composition of skeletal muscle is an important element in effectively meeting the metabolic demands required for contraction and exertion. Myofibres display considerable differences in metabolic, contractile and endurance capabilities.

In simple terms, myofibres are categorised into two main phenotypic isoforms: Type I oxidative and Type II glycolytic fibres. However, these myofibres are not so simple with respect to their metabolic and contractile properties. In more detail, oxidative or red Type I fibres have a high content of myoglobin, which assists the diffusion of oxygen into the muscle and provide oxygen storage even though they are small. They have abundant mitochondria and are in close proximity to vascular system enhancing perfusion with oxygen and access to substrates. These fibres oxidise substrates and contract in a manner that is considered to be slow in comparison to the Type II fibres. Glycolytic myofibres in the Type II fibre category (type IIx and IIb) are white in colour as they are relatively lacking in myoglobin (Schiaffino *et al.*, 1989). The glycolytic myofibres also contain less oxidative proteins (Gibson and Harris, 2002). The main substrate of energy for these Type II fibres is derived from the intracellular glycogen stores by way of anaerobic glycolysis (*described in section* 1.2.1 *Glycolysis*).

A fibre type can also be identified by the MHC isoform it contains (Pette, 2002). The MHC contains the catalytic site for ATPase enzyme activity (Sivaramakrishnan and Burke, 1982) and the velocity of unloading in the Type II fibres is rapid. Therefore, contractile properties of Type II fibres are fast with Type IIb being the fastest twitch glycolytic fibre (Bottinelli *et al.*, 1994; Mallinson *et al.*, 2009). Fast twitch fibres can be further categorized into Type IIa, Type IIb and Type IIx fibres. Type IIa fibres are also known as intermediate fast-twitch fibres and can use both aerobic and anaerobic metabolism. Type IIb fibres principally utilise anaerobic metabolic processes for energy production. The fatigue resistance, or endurance, of the muscle is relative to the proportion of the oxidative Type I fibres that express a high proportion of oxidative enzymes (Mallinson *et al.*, 2009). In rat skeletal muscle, Type IIx fibres have moderate to strong oxidative properties by succinate dehydrogenase staining (Larsson *et al.*, 1995) and their speed of contraction is intermediate, somewhere between that of IIa and IIb fibres (Schiaffino and Reggiani, 2011). The range of skeletal muscle fibre types with some of their relevant metabolic characteristics is indicated in the figure below (Figure 1.7). It is important to remember that in human skeletal muscle, Type IIx fibres have the lowest oxidative capacity and that while the range of properties is remarkably similar amongst mammalian species, they are not all identical (Schiaffino and Reggiani, 2011). A predominance of Type IIb fibre type is associated with obesity and insulin resistance, both conditions of metabolic inflexibility (Storlien *et al.*, 2004).

While the expression of fibre-type-specific gene programs can be detected during embryonic myoblast development, the differentiation into subtypes occurs later during development. It is this plasticity that leaves skeletal muscle flexible in adulthood to modification in response to shifts in metabolic demands. It is generally accepted that the physiological response to contractile loading during exercise training is to promote changes within the population of fast-twitch fibres (i.e., Type IIb to IIa) and to a lesser extent changes from fast- to slow-twitch fibres (Gollnick *et al.*, 1972; Holloszy and Coyle, 1984). The sensitive nature of skeletal muscle to adaptive processes induced from training, hormonal status and disease state can all play a part in ultimately determining the mature phenotype (Punkt *et al.*, 2004).

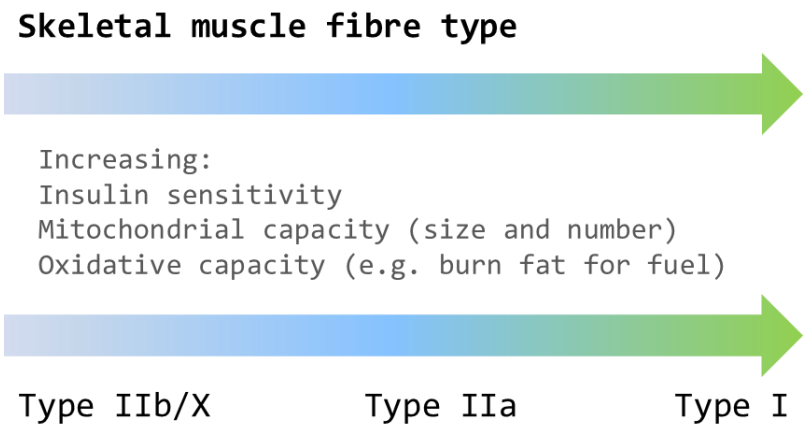

Figure 1.7 Range of skeletal muscle fibre type properties.

Scanning from left to right, the figure illustrates with the gradient of blue to green the increasing oxidative capacity of fibre type populations for rat skeletal muscle (Adapted from Storlein, 2004).

1.3.3 Determinants of Muscle Growth and Atrophy

While the body tries to conserve muscle to maintain locomotion, it is sometimes necessary to utilize protein stores as a glucogenic fuel source. During prolonged fasting and disease amino acids are mobilized for hepatic gluconeogenesis and energy production (Frayn, 2003a). An important physiological adaptation to fasting is control of muscle protein breakdown which can lead to vast muscle wasting. A similar rapid

atrophy of muscle is a common debilitating feature of many systemic diseases including diabetes and cancer or by disuse and/or nerve injuries (Gomes *et al.*, 2001). In muscle undergoing atrophy, a common set of transcription factors are responsible for directing the break down the muscle proteins (Cao *et al.*, 2005). The next section will outline the theory associated with the atrophy factor atrogin-1 and its upstream effector fork-head box O (FOXO).

Forkhead box O.

The FOXO transcription factors are a family that regulate a number of metabolic pathways and response systems (Maltin *et al.*, 2001). Insulin/Akt signaling inhibits FOXO by mediating its export from the nucleus (Nakae *et al.*, 2008) where it is unable to act on target genes, e.g. the insulin receptor and PGC1 α (Southgate *et al.*, 2005). Furthermore, FOXO1 may play a role in fibre type and the control of muscle differentiation. FOXO1 is abundant in the predominantly oxidative soleus muscle and ablation of FOXO1 leads to a reduction in Type I fibres (Kitamura *et al.*, 2007). FOXO1 also has a negative effect on myogenesis (Wu *et al.*, 2008) targeting the mTOR pathway. The active FOXO in the nucleus is thought to suppress mTOR signaling and subsequent feedback through the Insulin/AKT signaling pathway that promotes cell differentiation in muscle (Wu *et al.*, 2008). The expression of constitutively active FOXO1 has been shown to increase both the abundance myostatin transcripts in vitro (Allen and Unterman, 2007).

Atrogin-1

The ubiquitin ligase and atrophy-related gene atrogin-1 is also referred to as muscle atrophy F-box (MAFbx), or Fbox32 (Fbox-32). Atrogin1 is a major E3 ligase in muscle (Tacchi *et al.*, 2010), an ubiquitin ligase and an atrophy-related gene involved in proteolysis and ubiquination of contractile proteins (Nowell *et al.*, 2011). In rodents, atrogin-1 and the related muscle ring-finger (MuRF1) are markedly up-regulated during various forms of atrophy (Bodine *et al.*, 2001). Atrogin-1 is itself partially regulated in expression by FoxO transcription factors (Sandri *et al.*, 2004). When desphosphorylated, FOXO translocates to the nucleus and promotes atrogin-1 expression (Mammucari *et al.*, 2008). Reduction of atrogin-1 was thought to represent a potential mechanism for reducing proteolytic catabolism of contractile proteins (Gomes et al., 2000). However, it is still not certain whether atrogins influence protein

synthesis as recent research points to cross-talk between pathways leading to both growth and atrophy (Attaix and Baracos, 2010; Attaix *et al.*, 2012). The point of cross-talk between protein synthesis and proteolysis may be related to the insulin signaling pathway (Grunnet *et al.*, 2009). The Akt/insulin and AMPK signaling pathways are described above as important for regulating the rates of protein turnover in muscle. Within the pathways are the insulin receptor substrates 1 and 2 (IRS1 and IRS2), which are involved in both IGF1 and insulin signaling regulating metabolism. Potentially, these IRS factors represent the point of cross-talk for making or breaking muscle (Grunnet *et al.*, 2009).

1.3.4 Calcium Signalling Pathways

Central to the maintenance of muscle architecture and postnatal plasticity is the ability to adapt to demands. Adaptation comes about in skeletal muscle by changing protein synthesis/degradation ratios (as described above), fibre type composition and finally mitochondrial biogenesis (Handschin and Spiegelman, 2006). Changes in intracellular Ca^{2+} concentrations modulate the physiological activities of pathways regulating adaptability in skeletal muscle.

Calmodulin and Calmodulin kinases

Calmodulin is a ubiquitous Ca^{2+} binding protein involved in a variety of signalling pathways. Calmodulin, in the presence of Ca^{2+}, acts as the signal transducer activating a wide range of downstream targets including the phosphorylase kinase, Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Al-Shanti and Stewart, 2009). Calmodulin kinases play important roles in skeletal muscle function, CaMKI is multifunctional and CaMKII and CaMKIV are thought to play an important role in muscle hypertrophy (Fluck *et al.*, 2000; Wu *et al.*, 2002). The high energy requirement of skeletal muscle is met by CaMKIV signalling to transcription factors that modulate mitochondrial biogenesis and oxidative genes (Duguez *et al.*, 2002). For example, CaMKIV enhances the expression of mitochondrial COX-IV gene (Koulmann *et al.*, 2008) by way of PGC-1α (Handschin *et al.*, 2003). The downstream targets of Ca^{2+} signalling also include the MEF. The MEF2 transcription factor is normally present in the nucleus but is inhibited by the interaction with class II histone deacetylases (HDACs). The conformation of MEF2-HDAC is kept closed, inhibiting transcription until CaMK disrupts the complex via phosphorylation and export of HDAC from the

nucleus (McKinsey *et al.*, 2000). Via the MEF2 activation, the transduction of the Ca2+ signalling can have its downstream effect on the transcription of genes regulating Type I slow skeletal muscle fibres (Calvo *et al.*, 1999), particularly during initial development (Potthoff *et al.*, 2007).

Calcineurin

Calcineurin is a calcium-calmodulin-regulated serine threonine phosphatase involved in the regulation of differentiation of muscle and determination of fibre type, (Chin *et al.*, 1998; Friday *et al.*, 2000). Furthermore, there is much evidence that calcineurin is involved in the postnatal conversion of fibre type (da Costa *et al.*, 2007). During physical activity, the intracellular Ca^{2+} levels increase and calmodulin can bind to calcineurin leading to increased expression of oxidative proteins e.g. myoglobin and slow fibre genes (Naya *et al.*, 2000). It seems that calcineurin has more of an involvement in fibre type switching than muscle hypertrophy (Parsons *et al.*, 2004). Blocking calcineurin signal does not correlate with a down-regulation of hypertrophy necessarily (Bodine *et al.*, 2001), whereas other transgenic knockdowns of calcineurin did correlate with the down-regulation of the oxidative/slow fibres in multiple muscle types (Parsons *et al.*, 2004). Calcineurin appears to be more important for maintenance of slow fibres in adult muscle than during embryonic development (Oh *et al.*, 2005).

Nuclear factor of activated T-cells

Calcineurin-NFAT signalling is thought to act as a nerve activity sensor in skeletal muscle controlling nerve activity-dependent myosin switching (Tothova *et al.*, 2006). Early denervation experiments in neonates demonstrated an interference with the development of slow myosin, but not with abundance of fast myosins in rodent muscles (Butler-Browne *et al.*, 1982). This indicated that the downstream effectors of Ca^{2+} signalling pathway were intrinsic to the oxidative fibre type. Nuclear factor of activated T-cells (NFATs) are downstream targets of calcineurin and play a role in differentiation for myogenic tissues and mature myotubes. Four main isoforms exist for skeletal muscle and are activated by calcineurin, these are NFATc1, -c2, -c3 and –c4. In response to increased intracellular calcium, calcineurin dephosphorylates the NFAT proteins which are then translocated to the nucleus (Beals *et al.*, 1997; Crabtree and Olson, 2002; Michel *et al.*, 2004). Once inside the nucleus they act to potentiate NFAT-dependent gene expression by binding with DNA and interacting with the likes

of MEF2 (Wu *et al.*, 2001). The –c3 isoform of NFAT has been reported to be activated in myoblasts before their fusion to myotubes, thus during primary myogenesis (Abbott *et al.*, 1998). While the NFATc1 and -c2 isforms show nuclear translocation in mature myocytes it is also thought that they play a role in nerve-dependent fibre type specification in adult muscle (Tothova *et al.*, 2006). The –c2, -c3 and –c4 isoforms of NFAT have been shown to be involved in the regulation of the MHC-fast program (Calabria *et al.*, 2009). Transgenic experiments have highlighted NFAT-c1 as the key regulator of the MHC-slow promoter when constitutively activated (McCullagh *et al.*, 2004) or when pharmacologically inhibited (Oh *et al.*, 2005).

1.3.5 Metabolic Response to Exercise

The inconsistency of energy intake and expenditure is the primary drive for storing energy and can result in obesity (Jequier, 2002). Energy expenditure by regular physical activity not only acts to influence energy balance favourably, it is well established to be an effective means of moderating metabolic disturbances. The health benefits of physical activity are evident with dose-response relationships (Haskell *et al.*, 2007) and is widespread as a public health recommendation (Vuori, 2001). The load that a bout of physical activity places on the body is met with functional responses in the whole organism that act to maintain homeostasis. In order to affect physical activity, skeletal muscle has a variety of metabolic profiles that are also intrinsic to whole-body metabolic regulation (Schiaffino and Reggiani, 1996).

Exercise and metabolic flexibility

The link between exercise-training and increased insulin sensitivity is well established (Zierath, 2002). One of the proposed mechanisms for such an improvement is increased translocation of glucose transporter GLUT4 protein to the membrane after endurance exercise (Greiwe *et al.*, 1999). Studies have demonstrated insulin- and contraction-induced glucose uptake was increased in proportion to the elevation in GLUT4 protein (Host *et al.*, 1998). Enhancement of insulin signal transduction is thought to be at multiple steps of the insulin signalling pathway, with IRS-1 and 2, PI3-kinase, and AKT phosphorylation eliciting exercise-induced responses with subsequent improvements in metabolism (Tanner *et al.*, 2002). Distinctions in the partitioning and utilisation of fuels for energy metabolism have been identified

between physically active and obese sedentary subjects (van Baak, 1999; van Loon and Goodpaster, 2006). As discussed, AMP-activated protein kinase (AMPK) is an important sensor of cellular energy status, detecting the elevation of intracellular AMP concentrations. The AMPK system represents a signalling pathway for contraction-induced GLUT4 activation during exercise.

Habitual exercise training induces substantial metabolic and gene expression adaptations in skeletal muscle (Coffey and Hawley, 2007). Dependent on the level of intensity and duration, the relative contribution of glucose and fatty acid substrates to the energy demand varies during exercise. During mild to moderate aerobic exercise, plasma fatty acids make up the main energy substrate (van Loon *et al.*, 2001). If the intensity is increased, intramuscular stores such as IMTG and glycogen are recruited. In trained individuals, the metabolic response to submaximal exertion is to avoid complete depletion of glycogen energy stores, thereby delaying exhaustion. The preservation of energy stores during high-intensity exercise also increases the capacity for oxidative lipid metabolism in skeletal muscle (van Loon *et al.*, 2001).

The composition of skeletal muscle is further emphasized as an important determinant for metabolically efficient exercise. Fibre type and abundance of energy stores within the muscle also become essential factors and apparently malleable to training (Storlien *et al.*, 2004). As discussed, greater insulin sensitivity is clearly observed in highly oxidative Type I skeletal muscle compared with the more glycolytic muscle fibres. This is consistent with the finding that endurance athletes tend to have increased proportions of Type I myofibres (Putman, 2004). MHC content is altered by physical exercise and studies in humans have shown that MHC fibre composition transitions from a faster to a slower phenotype as follows: Type IIb → Type IIx/b → Type IIx → Type IIa / x → Type IIa → Type I / IIa → Type I (Pette, 2002). Skeletal muscle-specific PGC-1 α overexpression led to increased percentage of slow-twitch myofibres (Lin *et al.*, 2002). Endurance training led to increases in the abundance of skeletal muscle PGC-1 α protein expression in one study (Russell *et al.*, 2003) but others have shown it is not necessary for exercise-induced fibre type transformation (Zechner *et al.*, 2010). Instead, PGC-1α may influence the maintenance of slow-twitch, Type I fibres in an alternate pathway to its role in metabolic signalling pathways.

1.3.6 Mitochondrial Biogenesis

Skeletal muscle has a remarkable ability to adapt to environmental demands such as physical activity. Early studies by Holloszy (1967) demonstrated that endurance exercise training led to mitochondrial biogenesis in skeletal muscle (Holloszy, 1967). Mitochondrial biogenesis is a term that is used to describe up-regulation of mitochondrial mass and function. Concomitant with biogenesis is promotion of oxidative metabolic capacity. Mitochondrial content in skeletal muscle is important for efficient oxidative performance as well as metabolic health. Mitochondrial dysfunction in muscle is associated with muscle atrophy (Powers *et al.*, 2007) and poor metabolic health (Lowell and Shulman, 2005). As described previously, Ca^{2+}-dependent pathways are stimulated by nerve activity in skeletal muscle. Downstream of calcineurin is PGC-1α (Handschin *et al.*, 2003), which not only acts on fibre type pathways, but is a co-activator of nuclear respiratory factor (NRF1). NRF1 then also induces mitochondrial transcription factor A (TFAM) expression (Wu *et al.*, 1999), which in turn regulates mitochondrial DNA (mtDNA) transcription. Exercise elicits intracellular signals contributing mitochondrial biogenesis including via PGC-1 α (Leick *et al.*, 2010). Exercise-mediated mitochondrial biogenesis has implications for increased capacity to oxidise lipids for energy consumption helping to maintain fat balance, and thus energy balance.

1.4 Nutritional Control of Flexibility/Inflexibility

1.4.1 The Influence of Early Life Nutrition on Metabolic Flexibility

The instructions for successful development of a multicellular organism are predominantly derived from a highly coordinated genetic program (Amemiya-Kudo *et al.*, 2005); (Patel and Srinivasan, 2002). This developmental program is responsible for the connection of genotype and phenotype (Gilbert, 2000). However, the developing organism is sensitive to environmental changes that may leave it vulnerable to developmental disruptions. Therefore, a certain amount of plasticity is necessary in order to adjust to such environmental cues.

In humans, the extent to which a fetus will grow is determined primarily by environmental influences such as maternal endocrine status and placental function in addition to the fetal genome. Ultimately, the chief environmental factor affecting fetal growth is nutrient supply (Harding, 2003). It is well established that poor nutrient supply during gestation restricts fetal growth, with the essential organs such as the brain, heart, kidneys spared (Langley-Evans, 2006). Maternal constraint is a term commonly used to describe limitations of nutrient supply to support fetal growth. Maternal constraint encompasses factors such as maternal size, age, endocrine status, diet and nutrition in addition to parity, or the number of previous live births (Gluckman and Hanson, 2004). With respect to maternal size, the size of the offspring at birth is unrelated to the ovum donor, and is instead relative to the size of the birth mother (Brooks et al., 1995). These studies demonstrated that neither the maternal nor paternal genotype have influence over gestational growth. Maternal constraint is the fundamental determinant of fetal growth.

The early life environment has also been implicated as a major determinant of adult health. This idea is based on a number of epidemiological studies that have provided evidence for adverse environments apparent during initial fetal development may be manifested years later as adult disease. Research into these influences has led to what is frequently termed the fetal, or developmental, origins of adult disease hypothesis (Hales and Barker, 2001). The concept underlying this hypothesis is based on evidence for an inverse relationship between size at birth and the incidence of metabolic disorders and cardiovascular disease in later life (Barker and Osmond, 1986). Proposed mechanisms for these phenomena are widely debated, but a common theme emerging is based on environmental insults. These insults, acting at critical periods of development, are responsible for initiating a series of metabolic and physiological adaptations, made in the context of survival, commonly referred to as fetal programming (Reynolds and Phillips, 1998). The plasticity of the organism in response to stress may potentially be beneficial in the short term with respect to immediate survival. However, such adaptations made in response to an adverse or constrained intrauterine environment are thought to be detrimental in adult life (Singhal *et al.*, 2003). It is thought that the consequences of disproportionate growth extend well into the postnatal period and are evident as metabolic perturbations.

Thrifty phenotype and Predictive adaptive responses.

Gluckman and Hanson (2008) have proposed that the various forms of supply constraint operate to confer survival advantage, ensuring that the fetus develops a phenotype appropriate for the predicted postnatal environment. Such *predictive adaptive responses*, as they are referred to, are proposed to induce a phenotype capable of utilising postnatally available nutrients efficiently (Gluckman and Hanson, 2004). If the fetus adopts the appropriate developmental survival strategy and lives to reproduce, evolutionary advantage is achieved and the genome can then be passed on. For example, gestational nutrient deprivation is proposed to be responsible for the shift in postnatal energy balance to be in favour of fat storage (Ozanne and Hales, 1998). This is thought to be in anticipation of a low nutrient postnatal environment based on the sub-optimal conditions in the womb and is commonly referred to as the "thrifty phenotype" hypothesis (Hales and Barker, 2001). This hypothesis suggests that when the offspring, prepared for a low nutrient postnatal environment is instead exposed to the converse, such as the so-called Western Diet, the mismatch results in metabolic perturbations (Hales and Barker, 2001).

Human Epidemiological Evidence.

The relationship between early growth and subsequent development of adult disease is well established arising primarily from the epidemiology studies by Barker and Osmond (1986) that correlated poorer infant mortality rates with cardiovascular deaths. The parameters at birth that serve as indices of fetal growth restriction, such as size at birth, were also correlated to rates of coronary heart disease (Barker and Osmond, 1986) and metabolic perturbations such as insulin resistance (Stocker *et al.*, 2005). Such studies led to the proposal that poor nutrition results in either neonatal mortality or a phenotype that left the surviving offspring susceptible to non-communicable chronic adult disease. Hales and colleagues (Hales et al., 1991) studied men born in Hertfordshire, UK with evidence of retarded fetal growth and found increased rates of glucose intolerance corresponding to lower birth weights (Hales et al., 1991). Significantly greater waist/hip circumference ratios for any given body mass index is also observed in similar cohorts with those in the lowest third of birth weight displaying greater truncal fat than those in the highest third (Roseboom, 2001).

The Dutch hunger winter is one of the most commonly cited historical events that provide evidence to support these hypotheses. Furthermore, the hunger winter highlights that the timing of the nutritional deficit is critical for the postnatal outcomes (Harding, 2003). Individuals that were exposed to severe undernutrition during early pregnancy displayed increased adiposity, but the converse was observed if the exposure was during late pregnancy (Jackson *et al.*, 1996). Maternal undernutrition in late pregnancy in both rats and humans is associated with reduced birth weight and increased postnatal risk of metabolic disorders. However, maternal undernutrition around conception is associated with other metabolic risks including obesity and reduced birth weight in the second generation (Harding, 2003). These interesting observations indicate that parameters such as size at birth may underestimate the later-life consequences of exposure to an adverse intrauterine environment (Patel & Srinivasan, 2002). The mechanisms for the foundation of developmentally-induced metabolic disorders are thought to be intrinsically linked to regulation of homeostatic and metabolic factors, potentially independent of postnatal nutrition.

The nutritional manipulation of muscle development has long been of considerable interest in the agricultural industry with the interest of improving the quality of meat in farm animals. Size of muscle in the mature offspring is the first obvious indication that nutritional manipulation during development has had a lasting effect. In addition to overall growth, Gluckman and Hanson (2005) proposed that adaptations made in response to available nutrition during early development are more evident in the metabolic profiles of the offspring in later life (Gluckman *et al.*, 2005a). Such insights have influenced further investigation into skeletal muscle as a key tissue that is malleable to programming of metabolic disturbances in later life.

Early life nutrition, experienced either by maternal nutrition during pregnancy or by nutrition during infancy and childhood, is thought to be a factor influencing subsequent metabolic flexibility in adulthood. Being of lower priority in nutrient partitioning during development, skeletal muscle is particularly vulnerable to nutritional deficiencies (Zhu *et al.*, 2006). Therefore, assessing the mechanisms by which nutrition alters the development and morphology of skeletal muscle is paramount for unravelling the aetiology of obesity and metabolic syndrome.

Inadequate nutrition during gestation and lactation has a strong impact on muscle growth, fibre number, fibre size and fibre-type differentiation in IUGR (intrauterine growth restricted) offspring (Bedi *et al.*, 1982). Since skeletal muscle mass and physical activity regulate insulin sensitivity, such findings indicate that low birth weight influences eventual metabolic flexibility in adults. Poor fetal growth, as evident by reduced birth weight, is consistently linked with reduced height and reduced lean body mass in children and adults (Singhal *et al.*, 2003; Elia *et al.*, 2007). Birth weight is only a proxy measure indicating poor velocity of growth in *utero*, or IUGR. Jensen and colleagues reported alterations to the skeletal muscle composition of men and that small size at birth preceded alterations in metabolic flexibility (Jensen *et al.*, 2007). The authors were careful to note that it was only speculation that low birth weight indicated that a suboptimal environment was experienced *in utero* (Jensen *et al.*, 2007). A study by Laaksonen et al., (2003) identified vigorous leisure-time activity as prophylactic for metabolic syndrome development in adult men who were small at birth (Laaksonen *et al.*, 2003). However, the specific mechanisms by which this occurs in IUGR are not well understood.

In principle, enhanced capacity for energy expenditure would be advantageous if the predicted postnatal environment was to be nutrient-deprived. The predictions made in the context of a nutrient-poor environment may result in a phenotype that is not suited to a rich environment in the postnatal period (Gluckman *et al.*, 2005b). For example, organisms exposed during development to environmental cues that indicate nutrient availability alter their energy expenditure in later life accordingly. There are a number of studies that associate reduced nutrition events during development with the development of adult obesity (Woodall *et al.*, 1996a; Woodall *et al.*, 1996b; Shepherd *et al.*, 1997; Vickers *et al.*, 2000; Ong, 2006; Martin-Gronert and Ozanne, 2010). However, the development of obesity in IUGR offspring of undernourished mothers is different from diet-induced obesity, with respect to the underlying mechanisms (Thompson *et al.*, 2007). Furthermore, it was shown that these offspring have increased preference for running in a wheel versus lever pressing for a reinforcer treat when compared with control offspring (Miles *et al.*, 2009b). In addition, the muscle structure and function of these IUGR offspring was found to facilitate physical activity (Huber *et al.*, 2009) and their unique metabolism indicated that moderate exercise prevented obesity (Miles *et al.*, 2009a). Therefore, it could be said that the ability of

these offspring to react to metabolic challenges such as exercise may be related to the composition and local regulation of skeletal muscle.

The number of muscle fibres is an important determinant of growth potential, endurance fitness, and adaptability to environmental stress (Fahey *et al.*, 2005a). Fibre numbers influence not only the size of muscle, strength and capacity for physical activity in later life (Maltin *et al.*, 2001) but also influence metabolic flexibility regulating glucose and lipid metabolism (Frayn, 2003a). The proportion of Type I myofibres in skeletal muscle is particularly important to the overall oxidative capacity of the tissue. A shift in fibre-type population, for instance from Type I slow oxidative to Type II fast glycolytic fibres, alters the activity of key intermediary metabolic enzymes (Pette *et al.*, 1980). It has been established that insulin sensitivity is positively correlated with the proportion of slow oxidative Type I fibres in skeletal muscle (Lillioja *et al.*, 1987). The converse is also evident with the percentage of Type I fibres being negatively correlated with percent body fat in human subjects (Helge *et al.*, 1999). As such, it is the ratio between the fibre types in skeletal muscle of Type 2 diabetic or obese individuals that is closely related to insulin resistance or metabolic inflexibility (Simoneau *et al.*, 1999). It remains to be established whether this type of metabolic adjustment is induced as a result of obesity, or is a causal factor for insulin-resistance.

As described above (*see section* 1.3.1 *Myogenesis*), rodent skeletal muscles develop at different times, with the total number of fibres in some rat muscles (e.g., soleus) being fixed at the time of birth, whereas in other muscles this occurs between birth and weaning (Wilson *et al.*, 1988). Studies into the gene expression of important factors influencing myogenesis have been performed in sheep to assess the state of muscle differentiation (Fahey *et al.*, 2005b). Such ontogeny studies into muscle cell differentiation during pregnancy identified that the ovine fetus is potentially sensitive to external factors such as maternal nutrition (Fahey *et al.*, 2005b). Therefore, the influence of early nutrition in the neonatal period also plays an important role in the development of the adult skeletal muscle phenotype. In addition, Zhu and colleagues have found distinct derangements in the mature skeletal muscle of sheep that were nutrient-restricted during development (Zhu *et al.*, 2004; Zhu *et al.*, 2006). The two

main features observed are increased proportion of Type IIb fibres, and increased IMTG content (Zhu *et al.*, 2006).

Further influence of nutrition on myogenesis is observed in genetically obese rats that have increased proportion of faster Type II fibres in comparison to genetically lean rats (Abou Mrad *et al.*, 1992). Additionally, Suwa and colleagues bred rats to have a higher percentage of Type II fast fibres and discovered that these rats were resistant to high-fat diet-induced obesity (Suwa *et al.*, 2002). The inference from these results is that the overall oxidative capacity in these rats has been increased in relation to the fibre composition of the muscle. Furthermore, studies by Wank and colleagues have demonstrated that newborn piglets classified as IUGR have accelerated skeletal-muscle development with precocious type II to type I conversion and, as a consequence, increased proportion and maturation of type I fibres (Wank *et al.*, 2000).

Studies in rats demonstrate reduced insulin sensitivity in adult offspring born to mothers exposed to a low-protein diet throughout gestation and lactation (Ozanne *et al.*, 2003; Fernandez-Twinn and Ozanne, 2006). This sub-optimal nutrition during development was associated also with reduced muscle mass (Ozanne *et al.*, 2003). In different rat model of maternal undernutrition by global nutrient restriction during gestation, the numbers of fibres in the skeletal muscle of offspring was unaffected, yet the number of nuclei was significantly decreased (Bayol *et al.*, 2004). Also in rats, Lane and colleagues have demonstrated altered skeletal muscle β-oxidation activity in juvenile IUGR offspring, with increased protein and mRNA levels of CPT1 and UCP3 (Lane *et al.*, 2001). As discussed, the expression of these lipid metabolising enzymes is regulated by PGC-1 α, a transcription factor that is also increased in their model of uteroplacental insufficiency during development (Lane *et al.*, 2003).

The long-term consequences of nutritionally-induced perturbations/alterations may be instrumental in the development of metabolic dysregulation. While programming factors induce adaptations in skeletal muscle, as evident in subsequent alterations to morphology and composition, there have been relatively few studies demonstrating this, and even fewer studies attempting to elucidate the mechanistic process. Importantly, skeletal muscle is the most influential tissue determining the overall state of metabolic flexibility. Its morphological composition and metabolic regulation is a pivotal factor influencing the ability to undertake metabolically-efficient exercise.

1.5 Scope of Thesis

Nā te moa i takahi te rātā - the tree has been trampled by the Moa.

This *whakatauki,* or proverb, describes how harm that occurs during development manifests later in life. In the case of the *Rātā* tree, its inability to grow straight is a telltale signal that it experienced an insult in its youth by a large Moa bird. The *whakatauki* speaks to the strong influence of environmental factors during development and how insults can leave indelible marks. An inherent resilience during development is also highlighted, in spite of such environmental challenges. For instance, in the context of the thesis presented here, an environment deprived of energy fuels could direct the developmental program toward a phenotype that is designed to maximise both deposition and utilisation of fuels.

Research in our laboratory has shown pregnant rats that are exposed to global calorie restriction give birth to offspring with intrauterine growth restriction (IUGR). The undernutrition experienced as a fetus consistently induces a unique obese phenotype in adulthood with maintained insulin sensitivity (Thompson *et al.*, 2007). This led to the proposal that prenatally-induced obesity is metabolically distinct from other forms of obesity, particularly high-fat diet-induced obesity (Thompson *et al.*, 2007). Obese offspring of undernourished dams not only have the capacity to store large amounts of fat compared with controls, but also have an enhanced ability to store glycogen in their skeletal muscle and liver. Another key finding is that they appear to have the capacity to utilise these energy stores efficiently. When given a prescribed amount of daily exercise in postnatal life, the prenatally-induced obesity was prevented in the offspring of undernourished dams (Miles et al., 2009a). Furthermore, these offspring showed a strong preference for running in a wheel for a food reward than offspring of adequately nourished dams (Miles *et al.*, 2009b).

Baldwin and Haddad (2010) recently described the prenatal undernourished rat model developed in our lab as generating offspring that are imprinted with a biochemical footprint favouring the economy of energy balance (Baldwin and Haddad, 2010). The capacity for metabolically flexible fuel turnover after the experience of prenatal undernourishment is the focus of my research. Metabolic flexibility is the body's capacity to adapt metabolism to changes in both energy demands and nutrient supply.

For example, in the fasted state, metabolically flexible individuals would be able to oxidise lipid fuel sources successfully. Additionally, in the fed state, carbohydrate stores are preferentially oxidised. In contrast, an *inflexible* individual cannot cope efficiently with the cyclical nature of feeding and fasting. As a consequence, fats are shunted into unhealthy storage and the reduced capacity to metabolise carbohydrates results in excess blood glucose, precipitating insulin resistance. To date, the pathways to metabolic flexibility, or the converse inflexible condition, have not been well understood. However, there is increasing evidence that the structure and function of skeletal muscle plays an important role in maintaining metabolic flexibility (Stump *et al.*, 2006).

The goal of my thesis was to investigate how metabolic flexibility is established from birth. I sought to uncover the mechanisms for the unique way IUGR offspring utilise and store metabolic fuels. The main objective was to determine the structure and function of specific skeletal muscle in these IUGR offspring. A further aim was to uncover the metabolic pathways responsive to both prenatal nutrition and postnatal physical activity. The specific aims were to:

- carry out developmental analysis of metabolic flexibility markers in IUGR offspring. In particular, I aimed to determine whether exposure to prenatal undernutrition was associated with the development of skeletal muscle structure and function that ensured metabolic flexibility.
- determine whether the prenatal undernutrition was associated with changes in gene expression of factors regulating the key structural and functional pathways that ultimately determine capacity for metabolic flexibility in skeletal muscle.
- assess the metabolic outcomes that result from the interaction between prenatal nutrition and postnatal environment conditions that could be termed "lifestyles".

Three experimental studies were designed in pursuit of the above aims. The first experimental chapter, Chapter 2, describes the ontogeny of skeletal muscle structure and function (Norman *et al.*, 2012). The ontogeny study details the generation of the IUGR model and assesses the postnatal development of offspring at three key developmental ages – birth, weaning and adulthood when growth has plateaued. Both

skeletal muscle fibre-type morphology and key enzyme activity that impacts on metabolic flexibility were investigated. I hypothesised that the plastic patterns of skeletal muscle development adapt to IUGR by the promotion of pathways favouring metabolic flexibility.

Chapter 3 continues testing the prenatally-induced metabolic flexibility hypothesis by assessing gene expression in skeletal muscle. I investigated factors fundamental to skeletal muscle plasticity and oxidative potential. A total of 17 target genes, measured by quantitative polymerase chain reaction (QPCR), were selected for this study based on their relevance to myogenic determination; fibre type transcription; mitochondrial biogenesis, and fibre growth/atrophy factors.

Chapter 4 compares interactions between the prenatal environment and varied postnatal "lifestyle" paradigms. It explores the hypothesis that postnatal lifestyle factors, in particular physical activity and diet, offer therapeutic potential to improve metabolic flexibility. Three different manipulations represent the alternate postnatal lifestyles. The first condition is a relatively sedentary style condition with an unrestricted *ad libitum* feeding regime and no access to physical activity. The second condition is one that provides more opportunity for physical activity with extra handling and environmental enrichment. The third lifestyle condition allows running-wheel exercise. These lifestyle factors are proposed to have a dose-like effect on the prevention of prenatally-induced obesity. Time-restricted feeding is common to both the second and the third lifestyle conditions where feeding is *ad libitum* but access is restricted to a 2 hour period each day.

Collectively, these studies were designed to demonstrate whether metabolic plasticity is directly influenced by an interaction of both prenatal and postnatal environmental conditions. Mechanistic studies serve to strengthen our understanding of the intricate relationship between early developmental influences and potential in later life.

Chapter 2.

Postnatal Development of Metabolic Flexibility and Enhanced Oxidative Capacity after Prenatal Undernutrition

2.1 Introduction

The daily challenge of cyclic feeding and fasting requires metabolic regulation that coordinates both energy supply and demand. Metabolic flexibility describes the ability to coordinate effectively the homeorhetic regulation of fatty acids, glucose metabolic flux as well as being associated with insulin sensitivity (Storlien *et al.*, 2004). In contrast, a metabolically inflexible state is characterised by insulin resistance and impaired fat oxidation in skeletal muscle. In the metabolically inflexible state, the ability of skeletal muscle to switch from fat oxidation to carbohydrate oxidation in the postprandial phase is blunted (Randle *et al.*, 1963; Corpeleijn *et al.*, 2008). The ability to utilise an abundant supply of metabolic fuels is also diminished and can result in obesity and related disorders. Diet-induced obesity further exacerbates the inflexible metabolic state due to excessive and non-physiological storage of fat. The pathways to a metabolically flexible state, or the converse inflexible condition, are not well understood. However, there is increasing evidence that the oxidative capacity of skeletal muscle plays an important role in maintaining metabolic flexibility (Phielix and Mensink, 2008).

The ability to respond to metabolic challenges, such as exercise, is linked to structural and functional properties of skeletal muscle (Pagel-Langenickel *et al.*, 2010). Nutrition alterations during early development have also been shown to drive changes in intrinsic metabolic properties of skeletal muscle. The developing fetus is sensitive to poor nutrition and may reduce or delay skeletal muscle growth in order to favour neuronal development (Zhu *et al.*, 2006). In particular, dietary influences during pregnancy and lactation are known to have a strong impact on skeletal muscle differentiation and development as quantified by fibre number, fibre size and fibre type (Bedi *et al.*, 1982). Previous work in this laboratory investigated the impact of prenatal undernutrition on adult rat skeletal muscle morphology (Huber et al., 2009).

The impact of prenatal undernutrition in that particular study manifested as altered fibre type composition in adults and may reflect a state of enhanced metabolic flexibility during adult life (Huber *et al.*, 2009).

The impact of prenatal undernutrition is long-lasting (Symonds *et al.*, 2007) but the underlying developmental path for the observed adult metabolic status remains unclear. The aim of this study was to examine the postnatal development of muscle structure and function that are linked to metabolic fuel use. I hypothesise that the plastic patterns of skeletal muscle development adapt to reduced prenatal nutrition through structural and functional changes that favour pathways of flexible fuel usage and storage. This chapter explores this hypothesis in prenatally-undernourished rats by examining muscle morphology, fibre composition and metabolic parameters at three developmentally important time points: birth, weaning and adulthood.

The time points were chosen to mark the beginning of postnatal life, the pre-puberty stage and the end of somatic growth, respectively. The soleus muscle and the gastrocnemius superficial (GSUP) muscle were chosen to investigate owing to their differing metabolic profiles. Both GSUP and soleus are ideal for this type of study because the distribution of muscle fibre types is unique. The GSUP is exclusively composed of type IIb fibres with a scattering of a few type IIx or IIa fibres (Maggs *et al.*, 2000; Shi *et al.*, 2008). Soleus muscle is mainly composed of type I and type IIa fibres. I hypothesize that prenatal nutrition will impact on structure and function of these two muscles, becoming evident in the postnatal lifetime and contributing to a metabolically flexible phenotype. This chapter will investigate nutritionally-induced changes to muscle tissue oxidative capacity and the long-term changes that could explain increased metabolic flexibility during postnatal life.

2.2 Materials and Methods

2.2.1 Experimental Design

The breeding protocol used in this study was identical to that previously described (Thompson *et al.*, 2007; Miles *et al.*, 2009a; Miles *et al.*, 2009b). Briefly, virgin Wistar rats (n = 9 per group) were time-mated and assigned to receive chow either *ad libitum* (AD) or at 30% of *ad libitum* intake (IUGR) throughout pregnancy. Maternal

undernutrition during pregnancy did not alter litter size, with an average litter size of 13 for both AD and IUGR.

From the day of birth until weaning, food was available *ad libitum* to all dams. IUGR offspring were cross-fostered to *ad libitum* fed mothers at day of birth to limit the nutritional influence to the prenatal period only. In both groups, litter size was adjusted to eight pups on the first postnatal day, to assure adequate and standardised nutrition until weaning (Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009a) Distinct groups of AD and IUGR offspring, each group containing pups that had descended from different mothers, were investigated at three developmental periods:

1. Neonatal, culled on the first postnatal day (n = 6 per group),
2. Weaning, culled on postnatal day 21 at weaning (n = 8 per group) and
3. Adult, culled on day 240 during adult life (n = 8 per group).

The first two groups, Neonatal and Weaning, included both male and female offspring. Sex-associated differences are most unlikely to occur at day 1 and 21 of life since onset of puberty in females occurs around day 30, as indicated by vaginal opening (Chen *et al.*, 2009). No sex differences in body mass, lean body mass, % body fat, plasma leptin and ghrelin levels were detected in male and female offspring of 50 % food-restricted rats mothers at ages of 1 day and 3 weeks (Desai *et al.*, 2005). Importantly, muscle fibre type composition is not affected by sex in AD and IUGR rat pups younger than 30 days (Lane *et al.*, 2003). Male litter-mate pairs in the Adult group were on a scheduled feeding regime of 2 hours (h) *ad libitum* access to food per day from 46 days (d) of age onwards. All procedures involving animals were carried out with the prior approval of the Animal Ethics Committee of the University of Auckland.

Sample collection of Adults was performed as previously described (Huber *et al.*, 2009; Miles *et al.*, 2009a). In short, blood was collected into heparinised tubes and stored on ice until centrifugation. Plasma was stored at -20 °C until analysis. Liver samples were snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis. Soleus and GSUP muscle from one leg of offspring at weaning were also snap-frozen and stored at -80 °C. The respective muscles from the other leg were prepared by

fixing onto cork plates. Fixed muscles were frozen in liquid N_2-cooled isopentane and stored at -80 °C to ensure tissue integrity for immunohistochemical studies. Due to size of soleus and GSUP samples in the Neonatal group, the entire hind limbs were snap-frozen.

2.2.2 Immunohistochemical and morphometrical studies

Skeletal muscle morphology and fibre type composition were analysed in offspring at weaning and at adulthood immunohistochemically as previously described (Huber *et al.*, 2009). For convenience, all buffers are listed in table format in Appendix I. Briefly, to examine fibre type in GSUP and soleus muscles, 6 µm slices were cut from the mid-belly region of frozen muscles across the fibre direction. Slow and fast MHC antibodies were used to detect type I fibres (MHC slow positive; MHCslow antibody, Novocastra Laboratories) and type IIa fibres (MHC fast positive; MHCfast (Type IIa), Alexis Biochemicals). Antibodies are also listed in Appendix II. Type IIb fibres of GSUP were detected as unstained fibres on slices with Type I/IIa co-staining. Immunodetection procedure involved washing frozen sections and blocking with blocking solution (filter sterilised 4 % goat serum, 0.5 % Triton X, 0.1 % NaN3 in 1x PBS). Primary antibody was incubated in blocking solution overnight in a humidified chamber at 4 °C. Slices were then incubated for 2 h with secondary antibody (anti mouse Cy3 labelled, Jackson Immuno Research Laboratories Inc) at RT, washed again with PBS and covered with covering medium (80 % glycerol in 1x PBS with 0.1 % NaN3, pH 7.0). Fluorescence signals were detected by a fluorescence microscope (Olympus) and 5 pictures/slice/staining were taken with 20x magnification using a digital camera. In each muscle, 700 - 850 fibres per animal were counted and type-classified fibres by Baerbel Leppich (Hannover, Germany) and were expressed as a percentage of total counted fibres. Size measurements of 50 randomly selected specifically immunostained fibres were made. In neonates, it was not possible to characterise respective fibre type composition.

2.2.3 Determination of Liver Glycogen Content and PKC ζ Protein Expression

Liver glycogen of offspring at weaning was determined according to Roehrig and Allred (Roehrig and Allred, 1974). For Western blotting, livers of all three groups

were ground under liquid N_2, and further homogenised on ice in pre-chilled homogenisation buffer (in mmol/L: 50 N-2-hydroxyethlypiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, 0.1 % Triton X-100, 4 Ethlene glycol tetraacetic acid (EGTA), 10 Ethylene diamine tetraacetic acid (EDTA), 100 β-glycerophosphate, 15 tetrasodium pyrophosphate, 5 sodium orthovanadate, 25 sodium fluoride, protease inhibitors (Roche, Mannheim, Germany)); at a ratio of 30 - 50 mg tissue powder/mL of buffer using an Eppendorf pestle and cup (Appendix I). Protein concentrations of the homogenates were measured according to Lowry method using a commercial kit to the manufacturer's specifications (BioRad protein quantification kit) and read on plate reader at A_{750} nm (Wallac 1420 Victor2 multiple reader and SOFTMAXPRO software).

Homogenates in loading buffer (50 mmol/L TrisHCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.1 % bromphenol blue, 2 % mercaptoethanol) were denatured by incubation at 95 °C for five minutes, separated by SDS gel electrophoresis with 40 µg of protein loaded per lane and transferred onto nitrocellulose membranes according to Laemmli (Laemmli, 1970). Detection of protein kinase C (PKC) ζ (sc-216; Santa Cruz Biotechnology Inc, Santa Cruz, CA) was performed after blocking the membranes in 10 % fat free milk/PBST for 3 - 4 h at RT. Membranes were incubated overnight at 4 °C with rat-specific primary antibody. Detection of the primary antibody was performed by incubation with Sigma anti-rabbit-HRP secondary antibody (A0545) for 1 h at RT followed by incubation with enhanced chemiluminescence detection substrate (SuperSignal® West Dura Extended Duration Substrate Kit). After band detection, expression was quantified by densitometry with Quantity One software (BioRad, Hercules, CA).

2.2.4 Muscle Assays

Tissue triglycerides were extracted as described by Chen & Nyomba (Chen and Nyomba, 2004) Briefly, Triglycerides were extracted from 30 mg frozen powdered muscle and liver samples with 2 ml of 2:1 chloroform methanol for 90 min. Methanol (0.4 ml) was then added, and the extract was vortexed for 30 s. After centrifugation at 1,100 g for 10 min, the supernatant was collected, mixed with 0.5 ml of 0.04% Calcium Chloride, and centrifuged at 550 g for 20 min. The upper phase was removed and the interface was washed three times with a mixture of chloroform (1.5 ml),

methanol (24 ml) and water (23.5 ml). The final wash was removed, and methanol (0.05 ml) was added to obtain one phase. The samples were dried at 60°C under nitrogen and re-dissolved in 0.05 ml of 3:2 tert-butyl alcohol-Triton X-100. Triglycerides were quantified by enzyme colorimetric assay in an automated bioanalyser (Roche/Hitachi GOD-PAP).

Liver triglycerides were assessed for all three developmental groups, whereas the triglycerides in muscle samples were extracted only for the Weaning and Adult groups. Liver and muscle glycogen contents for all three groups were measured as previously described (Roehrig and Allred, 1974; Thompson *et al.*, 2007). Briefly, glycogen storage was measured using an assay based on where a total of 0.05 – 0.1 g of tissue was weighed into polypropylene tubes and combined with 19 volumes (0.45 – 0.9 ml) of 10 mmol/L sodium acetate buffer (pH 4.6) to make a 1:20 solution. Samples were homogenized on ice using an Ultra Turrax homogenizer, and 0.5 ml of homogenate transferred to a 2 ml vessel containing 0.1 ml of amyloglucosidase (60 units/ml). The mixture was then incubated at 37°C in a water bath for 2 hours to digest the glycogen to free glucose. Samples were centrifuged for 5 minutes and analyzed for glucose concentration using an automated bioanalyser (Roche/Hitachi GOD-PAP, Roche Diagnostics, Penzberg, Germany) by the glucose oxidase method. Glycogen concentrations were expressed relative to tissue weight. All samples were analysed on a single assay in triplicate.

Activity of citrate synthase and lactate dehydrogenase (LDH) were assayed in mixed muscle homogenates for Neonatal cohort and in distinct soleus and GSUP tissues for the Adult and Weaning groups (Huber *et al.*, 2009). Briefly, muscle tissue was ground under liquid N_2 and homogenised on ice in a pre-chilled homogenisation buffer (50% glycerol, in mmol/L: 40 KCl, 2 EDTA, 25 Tris-HCl (pH 7.8) and 0.2% Triton-X) using an Eppendorf pestle and cup. Sample was centrifuged at 10,000 RPM for 10 minutes at 4°C and supernatant stored at -80 °C. Enzyme activity was then quantified by spectrophotometric assay according to Newsholme and Crabtree (Newsholme and Crabtree, 1986) (citrate synthase assay), and using a commercially available kit (Tox-7 toxicology assay kit, Sigma; LDH assay). The homogenates for citrate synthase assay were made up to 1 in 100 dilution in homogenisation buffer.

Volumes of master mix (200 µL) placed in wells of a 96 well plate (UV visible, flat bottomed plate) at room temperature, typically 25°C. Then 10 µL of undiluted sample and standards (Roche Pig Heart CS #10103381001) at appropriate concentrations were added followed by 5 minutes incubation. Reaction was 'started' by addition of 10 µl concentrated starter substrate using a multi-track pipette then read at A_{412} nm on the Wallac plate reader with the change in absorption per minute set up to read strips for 5 minutes with a 0.04 minute interval (kinetic). Values were then adjusted for dilution factors and corrected for protein (U / mg protein).

The Tox-7 toxicology assay kit for LDH activity used the same homogenates as the citrate synthase assay with GSUP homogenates diluted 1:600 and soleus diluted 1:100. Using a multichannel pipette, 25 µL of working mix, including dye, enzyme and substrates at a ratio of 1:2:2 respectively (see Appendix I) were added to wells containing samples and standards (Bovine LDH standard, not included in kit (Sigma #L-2526), and briefly mixed on an orbital shaker and then covered and incubated at room temperature for 5 to 20 minutes until colour was developed and subsequently terminated by adition of 40 µL of 2% HCL. The assay was then read at A_{490} nm using a microplate reader (Wallac 1420 Victor2 multiple reader) LDH levels determined following curve fitting and extrapolation using the on-board Workout software and expressed corrected for protein concentration (U/mg protein).

2.2.5 Plasma Metabolite and Hormone Assays

Plasma glucose and TG were measured by enzyme colorimetric assay using an automated bioanalyser (Roche/Hitachi 902; Roche Diagnostics, Penzberg, Germany). Total plasma leptin and C-peptide were measured by using commercially available coated tube RIA kits (RL-83K and RCP-21K, respectively, Linco, St Charles, MO, USA). Fasting plasma insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (l0-1124-01 Mercodia AB, Uppsala, Sweden; sensitivity equal to 0.07 µg/liter; intraassay variation, 1.2%; interassay variation, 3.6%). According to the manufacturer's directions, calibrators and samples were loaded onto a 96 well plate in volumes of 25 µl. During 2 hour incubation, insulin in sample reacted with peroxidase-conjugated anti-insulin antibodies. A wash step removed unbound antibody and the reaction was ultimately stopped with an acid solution. The

colourimetric endpoint was measured by spectrophotometry in Wallac plate reader at A_{450} nm.

2.2.6 Statistical Analyses

Data were analysed by Two-way ANOVA using GraphPad Prism (Version 5.00 for Windows; GraphPad Software, San Diego, CA). Differences between groups were compared by Bonferroni *post hoc* test. In situations where samples were from different tissue types, for example because Neonatal muscles could not be separated, statistical comparisons were made within each age group. Using the Kolmogorov-Smirnov test, I determined which of these data were compatible with a Gaussian distribution. The data that did not fit a normal distribution were subjected to non-parametric testing (Mann-Whitney U test) where indicated. Data are presented ± SEM, n = 6-10 animals per group. A value of $P < 0.05$ was considered significant.

2.3 Results

2.3.1 Postnatal Growth

In this study the effect of prenatal nutrition on body weight was evident across the three developmental time points. The body weights at the conclusion of studies are presented in Figure 2.1with the statistical analyses presented in Table 2.1. A significant interaction of prenatal nutrition and age by Two-way ANOVA demonstrated an altered trajectory of postnatal growth for the IUGR offspring of undernourished mothers (Table 2.1)

2.3.2 Measures of Lipid Metabolism

Plasma leptin levels showed a dynamic pattern in response to prenatal nutrition at the respective developmental ages (Figure 2.2). Overall, plasma leptin levels were not significantly influenced by prenatal nutrition, but were strongly affected by age ($P < 0.001$, Two-way ANOVA). At weaning and maturity, a developmental pattern for the AD offspring was observed to tend towards decreased leptin levels compared with the high levels measured at birth. Then, in comparison, the plasma concentrations of leptin in IUGR offspring show a unique trajectory across the three developmental ages., plasma leptin for IUGR offspring was initially much lower in the Neonatal sample and

then steadily increased in the Weaning and Adult samples (Table 2.2). The outcome of this experiment shows that prenatal nutrition significantly influences plasma levels and results in hyperleptinaemia for IUGR Adults ($P < 0.05$, Bonferroni *posthoc* test, Table 2.2).

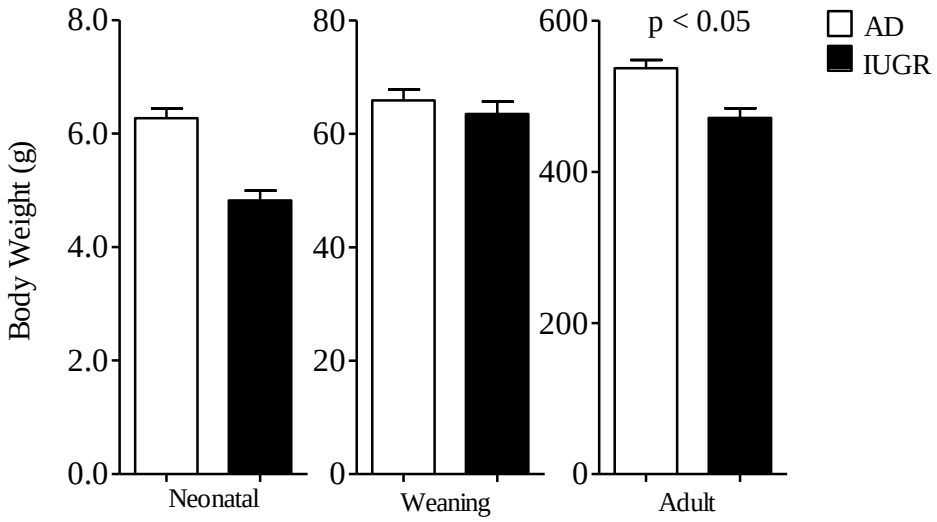

Figure 2.1 Effect of prenatal nutrition on body weight.

The body weight (g) for AD control offspring (white columns) and IUGR offspring (black bars) are shown with separate y axes for each age group: Neonatal, Weaning and Adult (values are mean ± SEM; *n* = 6-8). Data are analysed by Two-Way ANOVA with Bonferroni *posthoc* test. Significantly different results are indicated above the corresponding columns.

Prenatal Nutrition	**Age**	**Body Weight** (g)
AD	**Neonate**	6.23 ± 0.17
	Weaner	65.9 ± 1.92
	Adult	536.9 ± 10.8
IUGR	**Neonate**	4.83 ± 0.17
	Weaner	63.55 ± 2.20
	Adult	**471.24 ± 12.5***
Two way ANOVA		
Prenatal nutrition		**0.0460**
Age		**0.0001**
Interaction		**0.0427**

Table 2.1 Effect of prenatal nutrition on body weight.

The body weight for AD offspring and IUGR offspring at Neonatal, Weaning and Adult age points (values are mean ± SEM; n = 6-8). Data analyses by Two-way ANOVA with Bonferonni posthoc test (* $P < 0.05$) and all statistically significant results are indicated in bold.

Prenatal nutrition had no overall effect on plasma TG levels, whereas there is an overall significant effect of Age (by Two-way ANOVA). Plasma TG levels were comparable between AD and IUGR offspring at all three developmental time points with both showing a trend for decreasing concentrations from birth to maturity. Interestingly, the plasma TG levels in Neonatal IUGR offspring are highly variable in comparison to the five other experimental conditions.

2.3.3 Measures of Whole Body Glucose Metabolism

Plasma glucose, C-peptide and insulin levels

In Neonatal and Weaning animals, no significant effects of prenatal undernutrition on plasma glucose were observed (Table 2.3). However, age was a significant factor influencing plasma glucose levels, as it was for both plasma insulin and C-peptide levels. Plasma circulating insulin levels could not be measured for the Neonatal animals owing to low blood volumes. Nevertheless, as C-peptide serves as a marker of insulin secretion from the pancreas, C-peptide levels demonstrated the developmental pattern for insulin secretion across the three age points. While Two-way ANOVA did not detect a significant effect of prenatal undernutrition on either C-peptide or insulin measures, a significant interaction for plasma C-peptide demonstrated different insulin secretion trajectories in IUGR and AD offspring. Initially low, IUGR plasma C-peptide levels increased to levels higher than the AD offspring levels at the Adult stage. No significant differences were detected between AD and IUGR offspring at the different age groups by Bonferroni *posthoc* test.

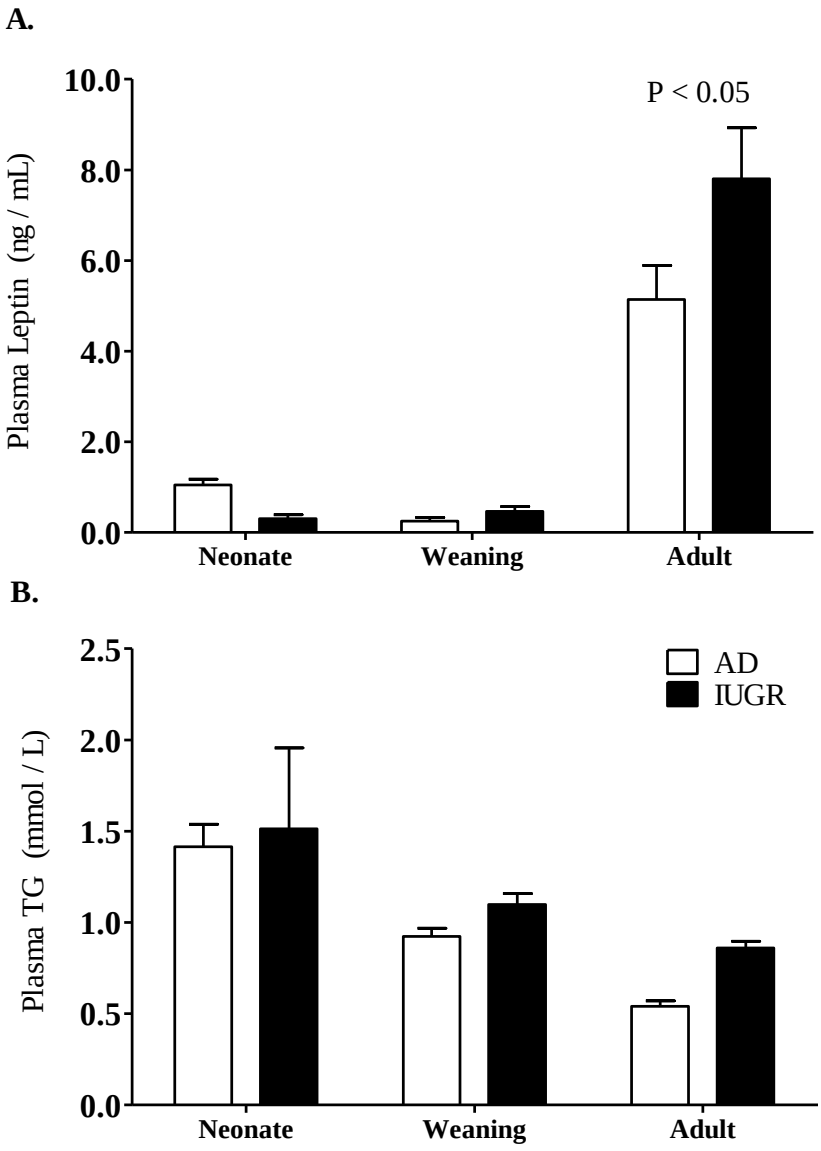

Figure 2.2 Effect of prenatal nutrition on plasma leptin and triglyceride levels.

The plasma leptin (A.) and TG (B.) concentrations for AD offspring and IUGR offspring at Neonatal, Weaning and Adult age points (values are mean ± SEM; n = 6-8). Data analyses by Two-way ANOVA are shown in **Table 2.2** while Bonferroni *posthoc* test results are indicated above the corresponding columns in the figure, were significantly different.

Prenatal Nutrition	Age	Plasma Leptin (ng / mL)	Plasma TG (mmol/L)
AD	**Neonate**	1.05 ± 0.12	1.42 ± 0.12
	Weaner	0.23 ± 0.08	0.92 ± 0.04
	Adult	5.14 ± 0.75	0.54 ± 0.03
IUGR	**Neonate**	0.30 ± 0.08	1.51 ± 0.44
	Weaner	0.46 ± 0.11	1.10 ± 0.06
	Adult	**7.80 ± 1.13***	0.86 ± 0.04
Two way ANOVA			
Prenatal nutrition		0.2685	0.1236
Age		**< 0.0001**	**0.0001**
Interaction		0.0708	0.7536

Table 2.2 Effect of prenatal nutrition on plasma leptin and triglyceride levels.

The plasma leptin concentrations for AD offspring and IUGR offspring at Neonatal, Weaning and Adult age points (values are mean ± SEM; n = 6-8). Data analyses by Two-way ANOVA with Bonferonni *posthoc* test (* $P < 0.05$) and all statistically significant results are indicated in bold.

Hepatic insulin signalling

Prenatal nutrition did not have a significant effect on the relative expression of the key insulin signalling protein in liver, PKC ζ (Table 2.4). However, the significant interaction detected indicated that IUGR and AD offspring have distinct patterns of PKC ζ expression across the age groups. *Posthoc* testing revealed that the expression of PKC ζ was significantly increased for IUGR offspring compared with AD offspring (Bonferroni *posthoc* test $P < 0.05$). The PKC ζ protein expression was paralleled by similar measures of hepatic glycogen storage. There was no effect of prenatal nutrition on the level of liver glycogen across the weaning and adult developmental time points. Measurement of glycogen in Neonatal livers was not possible due to an insufficient amount of tissue. There was also a pattern of increasing glycogen observed for IUGR offspring between the Weaning and Adult groups with a significant interaction of prenatal nutrition and postnatal age (Table 2.4). Bonferroni *posthoc* test showed that Adult glycogen was significantly elevated in the livers of the IUGR offspring compared with AD (*$P < 0.05$).

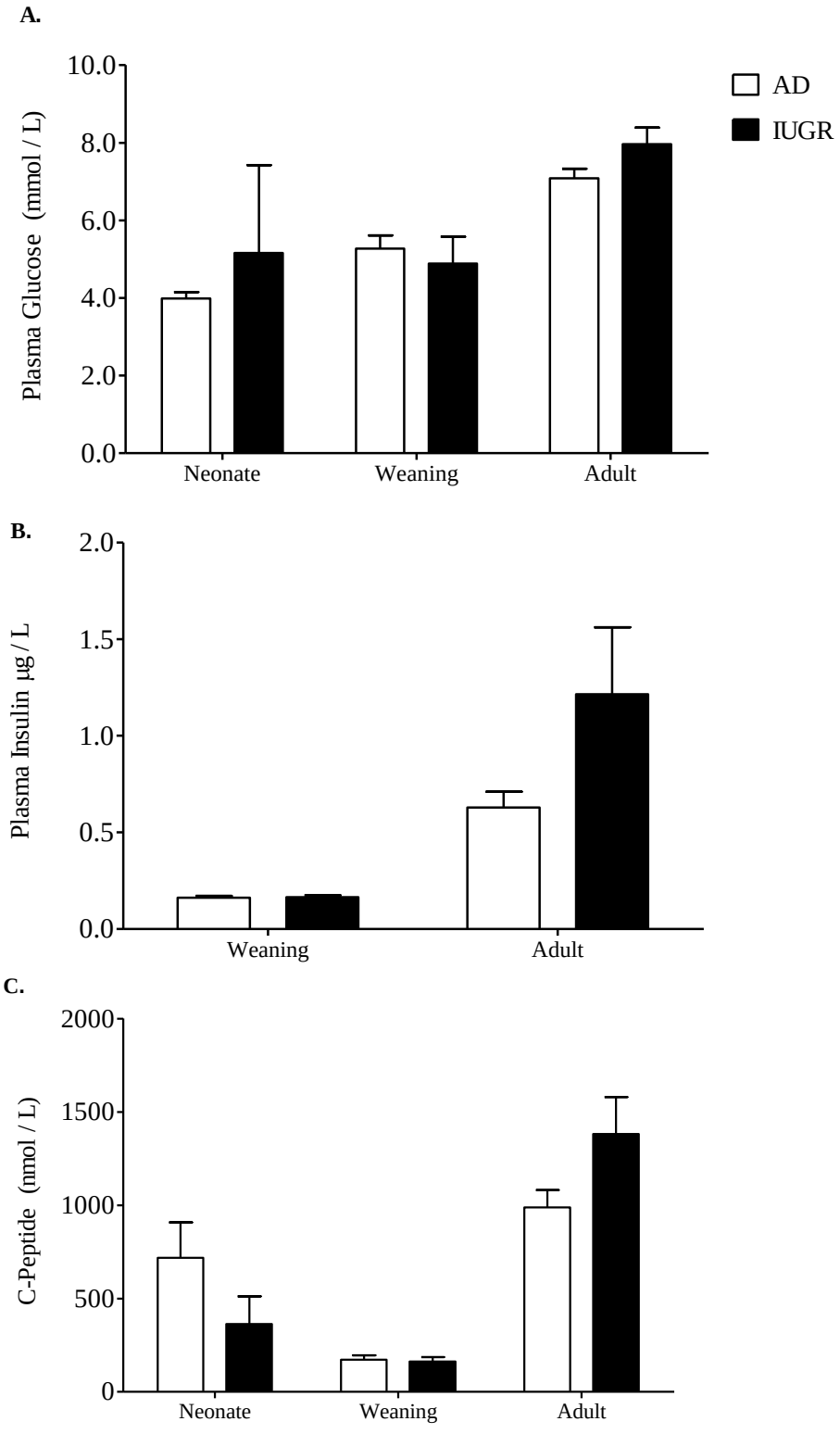

Figure 2.3 Effect of prenatal nutrition on measures of glucose metabolism.

The plasma glucose (A.), c-peptide (B.) and insulin (C.) concentration for AD offspring and IUGR offspring at Neonatal, Weaning and Adult groups (values are mean ± SEM; n = 6-8). Insulin could not be assessed in neonates owing to insufficient sample for measurement.

Prenatal Nutrition	**Age**	**Plasma Glucose** (mmol/L)	**Plasma C-Peptide** (nmol / L)	**Plasma Insulin** (ng / mL)
AD	**Neonate**	3.99 ± 0.17	716.9 ± 190.2	-
	Weaner	5.28 ± 0.34	172.7 ± 22.9	0.16 ± 0.01
	Adult	7.08 ± 0.25	981.8 ± 93.4	0.63 ± 0.08
IUGR	**Neonate**	5.16 ± 2.26	363.0 ± 148.2	-
	Weaner	4.88 ± 0.70	163.1 ± 22.7	0.17 ± 0.01
	Adult	7.96 ± 0.43	1381.5 ± 197.2	1.22 ± 0.35
Two Way ANOVA				
Prenatal nutrition		0.4394	0.9310	0.1506
Age		**0.0016**	**< 0.0001**	**0.0006**
Interaction		0.6237	**0.0399**	0.1540

Table 2.3 Effect of prenatal nutrition on measures of glucose metabolism.

The plasma glucose, c-peptide and insulin concentration for AD offspring and IUGR offspring at Neonatal, Weaning and Adult groups (values are mean ± SEM; n = 6-8). Insulin could not be measured in neonates owing to insufficient sample for measurement. Data analyses by Two-way ANOVA and all statistically significant results are indicated in bold.

2.3.4 Skeletal Muscle Structure and Metabolic Function

In both Neonatal and Weaning offspring, glycogen concentration was measured in whole leg muscle composed of mixed skeletal muscle. The dissected soleus and GSUP muscles of the Adult offspring were deemed to be incomparable to the mixed skeletal muscle samples. Therefore, two-way ANOVA analysis could be performed between the Neonatal and Weaning age groups only (Table 2.5). The soleus and GSUP muscles of the Adult age group were treated separately by Mann-Whitney U test (Figure 2.5). A significant effect of age was observed for the glycogen content of mixed muscle at birth and Weaning, but there were no significant differences in glycogen content between AD and IUGR offspring at these age points (Table 2.5). There were no apparent differences detected in soleus glycogen content between IUGR and AD Adult offspring (Figure 2.5, measured by Mann-Whitney U test). However, an increase in the glycogen content was observed in GSUP in the Adult IUGR offspring (Adult GSUP glycogen (mg/g tissue) AD 0.51 ± 0.04, IUGR 0.72 ± 0.04; $P < 0.01$, measured by Mann-Whitney U test).

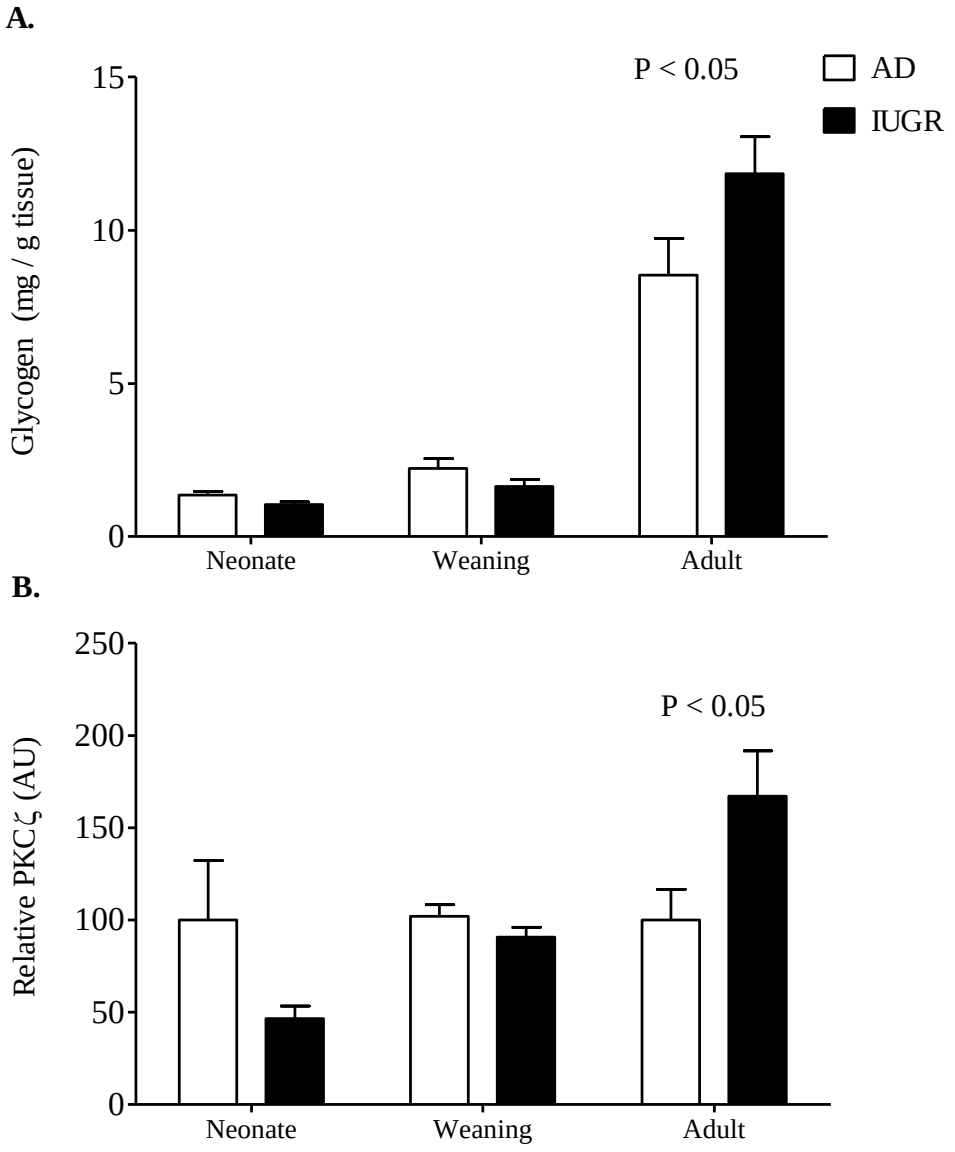

Figure 2.4 Effect of prenatal nutrition on hepatic insulin signalling and glycogen storage.

The level of liver glycogen (A.) and PKC z (B.) for AD control offspring (white columns) and IUGR offspring (black columns) are shown (values are mean ± SEM; *n* = 6-8). Data analyses by Two-way ANOVA are shown in Table 2.4 while Bonferroni *posthoc* test results are indicated above the corresponding columns, where significantly different.

Prenatal Nutrition	**Age**	**Liver Glycogen** (mg/g tissue)	**PKC ζ** (Arbitrary Units)
AD	**Neonate**	-	100.0 ± 32.2
	Weaner	2.22 ± 0.32	100 ± 7.0
	Adult	8.53 ± 1.21	100.0 ± 16.4
IUGR	**Neonate**	-	43.5 ± 8.6
	Weaner	1.63 ± 0.23	90.71 ± 5.2
	Adult	**11.84 ± 1.21***	**167.03 ± 24.5***
Two way ANOVA			
Prenatal nutrition		0.2155	0.9538
Age		**< 0.0001**	**0.0096**
Interaction		**0.0240**	**0.0080**

Table 2.4 Effect of prenatal nutrition on hepatic insulin signalling and glycogen storage.

Liver glycogen and PKC ζ protein levels for AD offspring and IUGR offspring at Neonatal, Weaning and Adult age points (values are mean ± SEM; n = 6-8). Data analyses by Two-way ANOVA with Bonferonni *posthoc* for AD and IUGR comparisons (* P < 0.05). All statistically significant results are indicated in bold.

The TG levels could not be assessed in Neonate muscles due to the insufficient amount of samples. The TG content was assessed in mixed skeletal muscle of Weaning offspring and the dissected soleus and GSUP muscles of Adult AD and prenatally undernourished IUGR offspring. Given that the Weaning tissue was comprised of mixed tissue, it could not be compared with the dissected Adult tissues. Therefore, the comparisons could only be made within tissue type by Mann-Whitney U non-parametric test. All samples had similar TG content between AD and IUGR offspring (Weaning muscle TG (mmol/mg tissue) AD 0.12 ± 0.02; IUGR 0.11 ± 0.02 P = 0.86, Mann-Whitney U test; Adult soleus TG (mmol/mg tissue): AD 5.33 ± 1.18, IUGR 5.06 ± 1.14 P = 0.87, Mann-Whitney U test); GSUP TG (mmol/mg tissue): AD 1.61 ± 0.34, IUGR 1.33 ± 0.30 P = 0.56 Mann-Whitney U test).

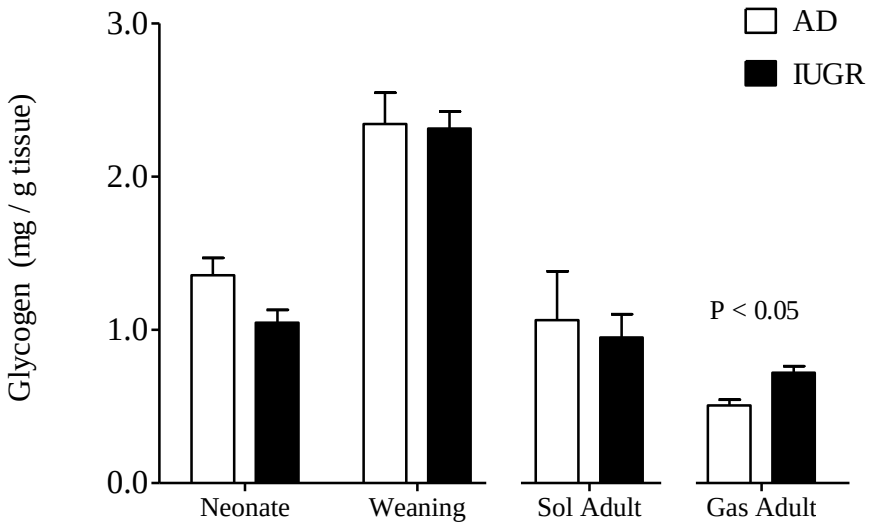

Figure 2.5 Muscle glycogen levels in Adult *m. Soleus* and *m. Gastrocnemius*.

Skeletal muscle glycogen (mg / g tissue) for AD offspring (white columns) and IUGR offspring (black columns) at birth, Weaning and in the dissected soleus (SOL) and GSUP (GAS) of Adults. Two-way ANOVA comparisons are between the Neonate and Weaning groups only, while Mann Whitney U comparisons are made within group for the dissected muscles. Statistical significance is indicated above the corresponding columns.

Prenatal Nutrition	**Age**	**Glycogen** (mg/g tissue)
AD	**Neonatal**	1.14 ± 0.11
	Weaning	2.34 ± 0.20
IUGR	**Neonatal**	1.05 ± 0.08
	Weaning	2.31 ± 0.11
Two Way ANOVA		
Prenatal nutrition		0.2099
Age		**P<0.0001**
Interaction		0.3000

Table 2.5 Effect of prenatal nutrition on muscle glycogen at Neonatal and Weaning ages.

The level of glycogen (m / g tissue) for mixed leg muscle samples for AD offspring and IUGR offspring at Neonatal and Weaning age points. Values are shown as means ± SEM (n = 6 - 8). Data analysed by Two-way ANOVA

Muscle structure

Accurate analysis of the neonate skeletal muscles was not possible due to the loss of sample integrity and inconsistent immunohistochemical staining response. However, immunohistochemical staining for analysis of soleus and GSUP was performed at both Weaning and at Adult ages. Figure **2.6** and Figure **2.7** visually represent the morphology and fibre type composition of soleus and GSUP muscles when stained for the diferent myosin types. The Type I/IIa myosin antibody co-staining for Type IIb determination in GSUP muscle sections is shown in Figure **2.7**.

Table 2.6 and Table 2.7 show the results of the quantification of all fibre types detected and fibre size measurements for the two muscles. With respect to soleus muscle, there were no differences detected in the Type I or IIa fibre percentages per area in IUGR offspring compared with AD offspring (Two-way ANOVA). The significant interaction for Type I fibre percentage in soleus indicated that the IUGR offspring were differentially affected by prenatal nutrition over time, starting out with lower number of Type I fibres in the Weaning IUGR offspring and increasing with age at a different rate to AD offspring. Overall, the proportion of Type I/IIa fibres in the soleus muscle change significantly between the two ages with Type I fibres increasing inversely to the proportion of Type IIa fibre percentage ($P < 0.0001$). In particular, Type IIa fibres of the soleus were influenced by prenatal nutrition ($P < 0.05$) and Two-way ANOVA also demonstrated a significant interaction, differentially influencing the size of these fibres across the developmental age groups for IUGR offspring (Table 2.6). *Posthoc* comparisons by Bonferroni test showed that the sizes of both Type I and IIa fibres in soleus muscle were significantly less for IUGR compared with AD offspring ($*P < 0.05$).

There were no differences in the percentages of GSUP Type I or IIb muscle fibres observed. However, there was a significant effect of prenatal nutrition on the percentage of Type IIa fibres in GSUP of IUGR offspring (Table 2.7). At adult age, significant differences in Type IIb fibre size of GSUP was demonstrated between IUGR and AD offspring (Bonferroni *posthoc* test $P < 0.05$). The percentage of these Type IIb fibres was not significantly influenced by age, remaining relatively constant over the two developmental age points. All other fibre types measured were significantly affected by age, i.e. these animals grew larger.

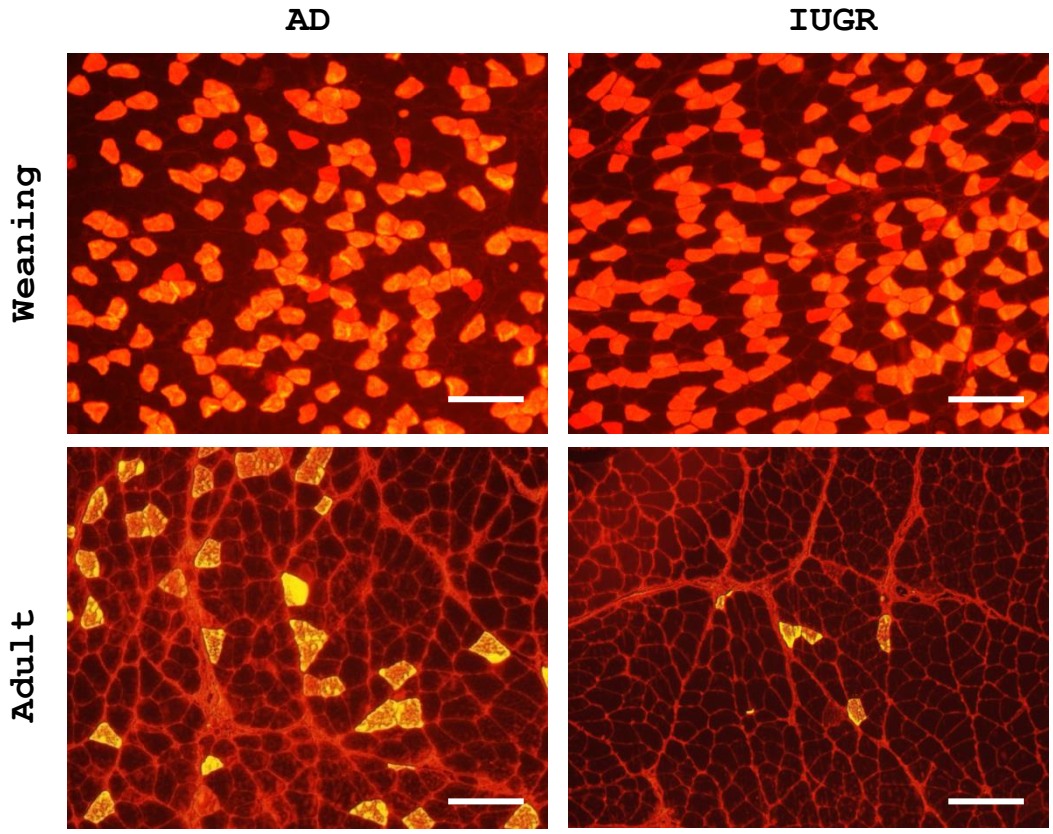

Figure 2.6 Fibre type composition of *M. Soleus*.

Type IIa fibres for d21 Weaning offspring (top row) and d250 Adult offspring for both AD (left column) and IUGR (right column) groups. Sections of muscle were stained immunohistochemically using an antibody against MHC Type IIa, unstained fibres are Type I fibres. White scale bar indicates 100 µm.

	Age	Type I %	Type IIa %	Type I size (um^2)	Type IIa size (um^2)
AD	Weaner	64.12 ± 1.66	46.38 ± 3.56	1040 ± 22.90	752.1 ± 23.88
	Adult	92.70 ± 1.62	7.30 ± 1.62	2287 ± 109.8	2110 ± 101.4
IUGR	Weaner	60.76 ± 2.21	44.04 ± 1.64	1067 ± 46.73	667.1 ± 27.07
	Adult	97.24 ± 0.93	2.65 ± 0.96	**1971 ± 89.64***	**1570 ± 163.8***
Two way ANOVA					
Prenatal Nutrition		0.6891	0.0713	0.1585	**0.0068**
Age		**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Interaction		**0.0196**	0.5358	0.0893	**0.0399**

Table 2.6 The effect of prenatal nutrition on *M. soleus* morphology.

Structure of soleus fibre types and fibre size measured in AD and IUGR offspring at Weaning and adulthood. Values are given as means ± SEM using Two way ANOVA and Bonferoni *posthoc* test * = P < 0.05; *n*=8). Fibre size refers to the measured size (um^2) of specifically immunostained fibres for either myosin type I or myosin type IIa; (%) refers to percentage of type I or II fibres/totally counted fibres.

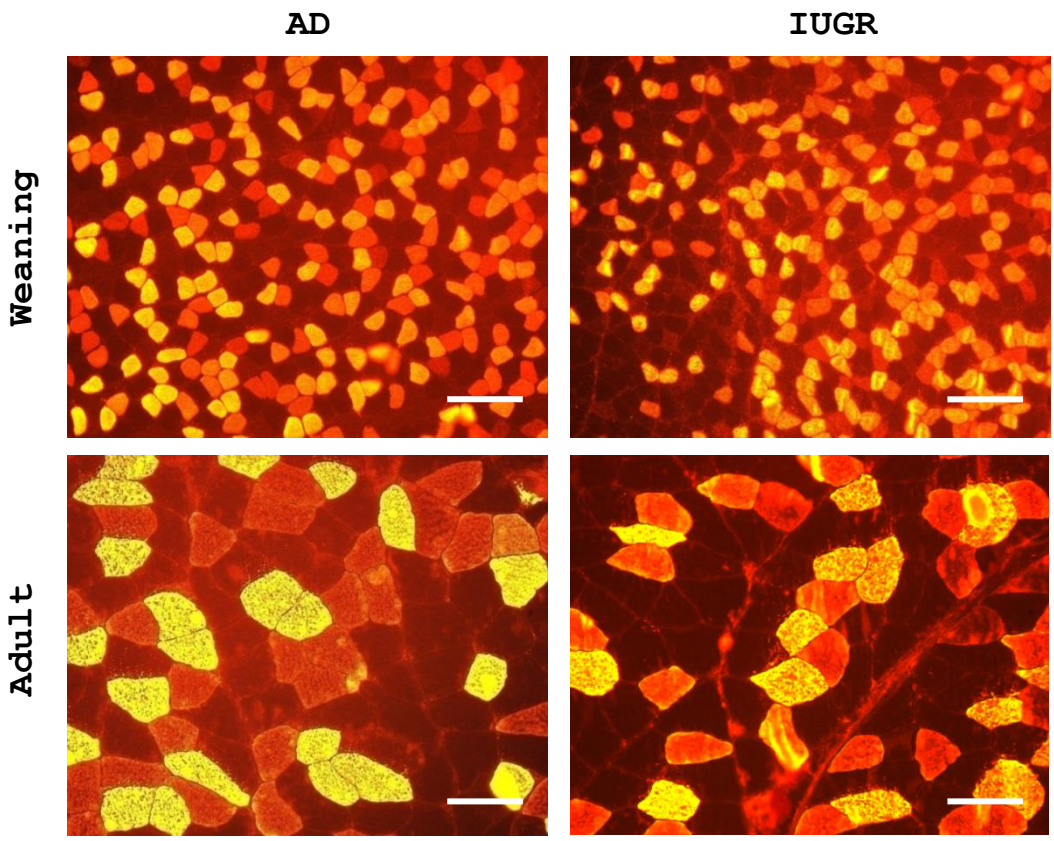

Figure 2.7 Fibre type composition of *M. Gastrocnemius superficial*.

Type IIa fibres for d21 Weaning offspring (top row) and d250 Adult offspring (bottom row) of both AD (left column) and IUGR (right column). Sections of muscle were stained immunohistochemically using antibodies against MHC Type I (yellow) and MHC Type IIa (orange), unstained fibres are Type IIB fibres. White scale bar indicates 100 µm.

	Age	Type I %	Type IIa %	Type IIb %	Type I size (um^2)	Type IIa size (um^2)	Type IIb size (um^2)
AD	Weaning	13.0 ± 0.9	53.8 ± 1.5	41.9 ± 3.6	653.8 ± 84.9	555.2 ± 53.6	801.0 ± 36.1
	Adult	26.0 ± 2.1	34.9 ± 3.1	48.5 ± 2.4	3840 ± 230.9	3701 ± 310.2	4705 ± 399.1
IUGR	Weaning	13.0 ± 0.9	48.3 ± 2.5	48.3 ± 3.8	771.8 ± 74.8	595.4 ± 83.6	819.0 ± 48.5
	Adult	22.5 ± 1.2	29.4 ± 1.3	43.3 ± 1.1	3692 ± 319.1	2981 ± 252.6	**3680 ± 338.9***
Two way ANOVA							
Prenatal Nutrition		0.2402	**0.0270**	0.8316	0.9509	0.1583	0.1173
Age		**< 0.0001**	**< 0.0001**	0.7804	**< 0.0001**	**< 0.0001**	**< 0.0001**
Interaction		0.2446	0.9881	0.0560	0.5916	0.1167	0.1054

Table 2.7 The effect of prenatal nutrition on *M. gastrocnemius* (GSUP) morphology.

Structure of GSUP fibre types and fibre size measured in AD and IUGR offspring at Weaning and adulthood. Values are given as means ± SEM using Two way ANOVA and Bonferoni *posthoc* test * = $P < 0.05$; n=8). Fibre size refers to the measured size (um^2) of specifically immunostained fibres for either myosin type I, type IIa, or myosin type IIb; (%) refers to percentage of type I or II fibres/totally counted fibres.

Markers of oxidative and glycolytic metabolism in muscle

Homogenates of combined leg muscle tissue were used for the Neonatal animals and could not be directly compared with the dissected muscles of the soleus or GSUP at Weaning or Adult ages. There was no significant influence of prenatal undernutrition on these oxidative or glycolytic markers in skeletal muscle respectively at this age (Figure 2.8). Mann-whitney U non parametric test showed no significant effect of prenatal nutrition on oxidative enzyme citrate synthase levels in mixed Neonatal muscle (citrate synthase (U / mg protein) (AD = 9.1 ± 0.3; IUGR = 9.0 ± 0.3; P = 0.80, n = 8) nor any difference between AD and IUGR offspring glycolytic enzyme lactate dehydrogenase (LDH) levels in mixed muscle (AD = 3.1 ± 0.2; IUGR = 2.9 ± 0.1 P = 0.49, n = 8). In addition, no effect of prenatal nutrition was observed on these key enzymes in either soleus or GSUP at Weaning (Table 2.8). However, in Adult soleus, IUGR offspring had significantly decreased LDH activity (Bonferroni *posthoc* test $P < 0.05$). The GSUP of the older offspring showed no significant effect of either prenatal undernutrition or age on these two marker enzymes.

2.4 Discussion

The results show an altered trajectory for glucose and lipid metabolism in the offspring of undernourished mothers. This altered trajectory was evident across a range of experimental measures for the life course for IUGR offspring in comparison to their AD controls. In particular, prenatal undernutrition led to structural and functional changes in both the liver and skeletal muscle. The results show that IUGR offspring had: (1) increased glycogen storage; (2) increased proportions and higher surface to volume ratios of oxidative skeletal muscle fibres; (3) decreased glycolytic enzyme function; and (4) increased plasma leptin concentrations compared with AD offspring. These data support an altered metabolic fuel utilisation capacity, particularly in the skeletal muscle of Adult IUGR offspring.

Postnatal growth is the primary objective for both adequately and undernourished offspring. In spite of their early nutritional insult, the IUGR offspring still achieve 100-fold growth in body size. As described in Chapter 1, the IUGR condition induces sparing of the vital organs such as brain and heart, with compromised growth of skeletal muscle as a result.

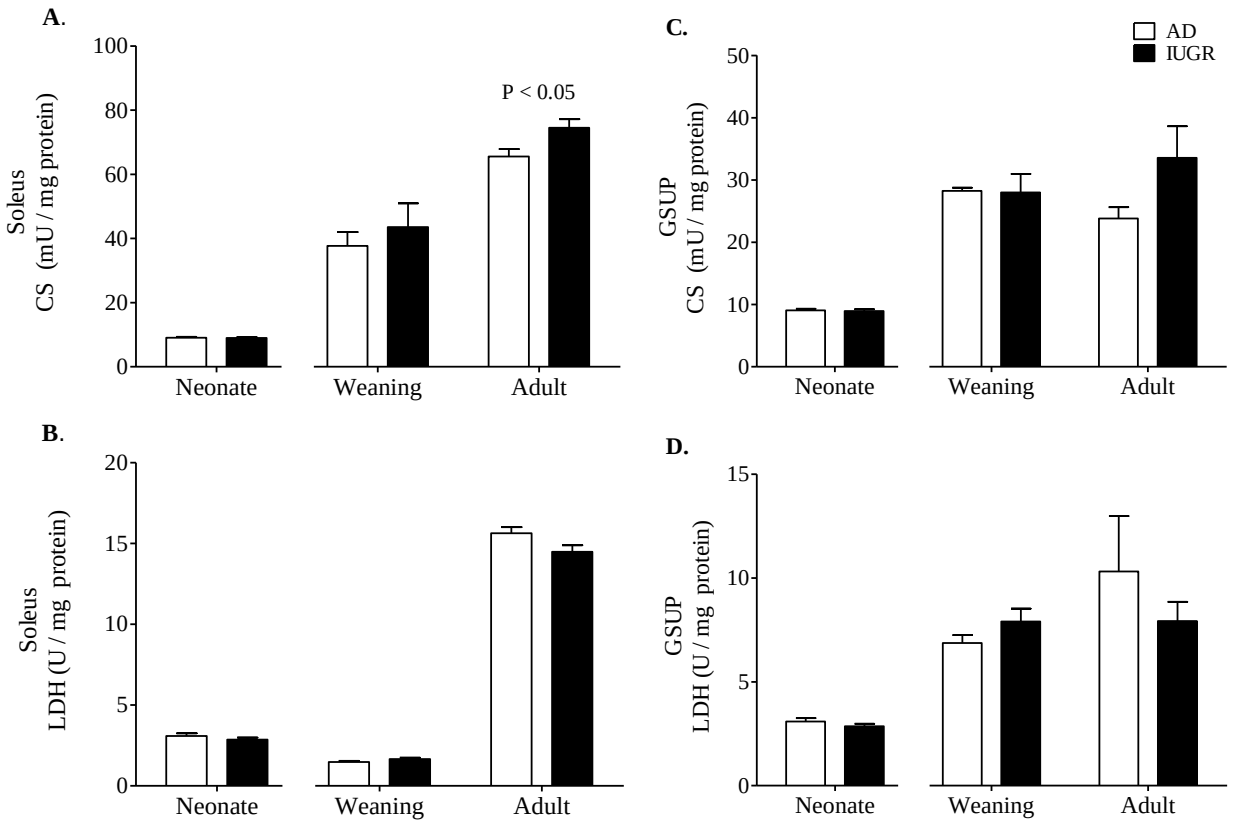

Figure 2.8 Effect of prenatal nutrition on muscle oxidative and glycolytic enzymes.

Citrate synthase and lactate dehydrogenase (LDH) levels (U / mg protein) from dissected soleus (figures A. and B. respectively) and gastrocnemius (figures C. and D.) of Weaner and Adult offspring are represented (means ± SEM using Two way ANOVA and Bonferroni *posthoc* test indicated above the columns $P < 0.05$; $n = 8$).

		M. *soleus*		**M. *gastrocnemius***	
	Age	**CS**	**LDH**	**CS**	**LDH**
AD	Weaning	37.7 ± 4.3	1.5 ± 0.1	28.2 ± 0.5	6.9 ± 0.4
	Adult	65.6 ± 2.3	15.6 ± 0.4	23.8 ± 1.8	10.3 ± 2.3
IUGR	Weaning	43.6 ± 7.4	1.7 ± 0.1	28.0 ± 3.0	7.9 ± 0.6
	Adult	74.5 ± 2.7	**14.5 ± 0.4***	33.6 ± 5.1	7.9 ± 0.9
Two way ANOVA					
Prenatal nutrition		0.0752	0.1851	0.1853	0.7035
Age		**P<0.0001**	**P<0.0001**	0.8693	0.3342
Interaction		0.7025	0.0663	0.1661	0.3422

Table 2.8 Effect of prenatal nutrition on oxidative and glycolytic enzymes in Weaning and adult muscles.

Citrate synthase and lactate dehydrogenase (LDH) levels (U / mg protein) from dissected soleus and gastrocnemius of Weaner and Adult offspring are represented (means ± SEM using Two way ANOVA and Bonferoni *posthoc* test * = $P < 0.05$; $n = 8$). All statistical significance is indicated in bold.

Independent of the experimental model employed, IUGR has been shown to be detrimental to growth and development with the effects lasting into adulthood. The results also show that the biological effects on metabolic fuel utilisation are detectable later during the postnatal period. From the data, it seems that prenatal alterations to development become physiologically apparent after the animal has grown 100-fold in size and reach adulthood. While the influence of prenatal nutrition

I will interpret the results from this study in light of the existing literature, also with consideration for methodological variations. Using an IUGR rat model with maternal food restriction at 30 % of *ad libitum* provided important insight into long-term consequences for the later metabolic status of offspring. The broader context of this experimental setting also has implications for physical activity capacity. IUGR offspring of undernourished mothers consistently develop obesity, hyperinsulinaemia and hypertriglyceridaemia. However, they have been shown to be insulin sensitive throughout adult life (Thompson *et al.*, 2007). Prenatally undernourished offspring had maintained insulin sensitivity. They also had increased glycogen stores as adults, much like earlier studies where these stores can facilitate fuelling of skeletal muscle (Huber *et al.*, 2009). The considerable muscle glycogen stores supported the increased running behaviour observed in IUGR offspring compared with AD controls (Miles *et al.*, 2009b). The capacity to adjust fuel utilisation to fuel availability for efficient exercise agrees with the thrifty phenotype concept proposed by Hales and Barker (Hales and Barker, 2001). According to that concept, nutrient restriction during fetal development may induce adaptations in preparation for a nutrient-poor postnatal environment. The adaptations observed in offspring of undernourished mothers might suit an environment that necessitates the ability to travel greater distances to obtain food (*described further in* Chapter 4).

2.4.1 Glucose Metabolism

This study has demonstrated a diverging developmental pattern of glucose metabolism for prenatally undernourished IUGR and AD offspring. The IUGR offspring exhibit a nutrient sparing phenotype in the neonatal period with diminished insulin secretion and insulin action in the liver. It is apparent that glucose metabolism may be acutely influenced by the reduced substrate supply experienced during fetal development. Looking at the trend that continues from birth in these IUGR animals, the development

of a thrifty phenotype begins with a picture of diminished insulin responsiveness. The Neonatal IUGR offspring have lower insulin secretion and hepatic signalling measures (PKC ζ) relative to AD offspring. Then, as the animals reach the older developmental time points, the measures of glucose metabolism increase at a sharper rate for IUGR than for AD offspring. Interestingly, plasma glucose levels remain relatively constant throughout these three developmental age points, suggesting a successful adaptive outcome of energy metabolism during fetal development.

The large increase in relative PKC ζ in liver from weaning to adulthood may explain, at least in part, the significantly higher hepatic glycogen levels in IUGR offspring. The increased levels of hepatic PKC ζ protein findings suggest IUGR offspring have more efficient insulin action and further support a thrifty metabolism, leaning towards enhanced energy storage (Summermatter *et al.*, 2008). In the perinatal period, metabolic energy would have been scarce in these Neonatal IUGR offspring. Interestingly, by weaning, muscle glycogen stores in prenatally undernourished offspring increased to levels observed in AD control offspring, indicating the onset of increased capacity to store energy. Furthermore, one of the main strategies of preserving protein stores might be to maintain insulin sensitivity (Stannard and Johnson, 2004)

Effective storage of energy is a key feature of metabolic flexibility (Storlien *et al.*, 2004). However, it is unclear if this increased energy storage is derived from a higher energy intake or a decreased energy expenditure of IUGR offspring during the suckling period. In adult offspring of undernourished mothers, indications of increased metabolic flexibility included elevations in plasma insulin and C-peptide concentrations in addition to significantly higher expression of hepatic PKC ζ. The IUGR animals have enhanced glycogen storage in the GSUP, a muscle where energy is mainly produced by the process of glycolysis. The pattern of glycogen storage over time in IUGR offspring is consistent with the developmental trajectory for other measures of energy storage and metabolism.

2.4.2 Lipid Metabolism

Neonatal catch up growth was evident in the offspring of undernourished mothers as their weight became comparable to AD offspring at weaning age. While the plasma

concentrations of TG appear to be elevated for the IUGR offspring at each of the developmental ages, the significant effect of age on TG is equal for both AD and IUGR offspring. It is unclear why plasma TG levels in these Neonatal IUGR offspring are so highly variable in comparison to AD offspring. Perhaps the variation in TG levels reflects variable patterns of feeding amongst these offspring in the neonatal period. Given that all of the neonates were cross-fostered at birth, it is unlikely that the variation in IUGR pups' TG levels is related to the process of cross-fostering. It is possible that measurement error is a contributing factor to the high variation, considering that such small samples were available. However, the overall trend that emerges is TG concentrations decreasing from higher levels in neonates to reduced levels in adulthood. While TG is an important fuel source, elevated plasma levels are also a marker of risk for atherogenesis and cardiovascular disease (Hokanson and Austin, 1996). As the TG levels cannot be distinguished between AD and IUGR offspring, it is likely that there is no difference in risk of atherogenesis between the two groups.

Given the dynamic relationship of whole body and tissue metabolism, adaptive changes in response to maternal nutrition are likely to continue beyond the prenatal period. With increasing age, TG levels remain equal to AD offspring levels whereas leptin levels increase for IUGR offspring. The trajectory of these two plasma measures over time indicates a thrifty phenotype for enhanced energy storage in the offspring of undernourished dams.

In IUGR neonates, the lower plasma leptin levels did not change between birth and Weaning timepoints. This is in contrast to the trajectory of plasma leptin levels observed in AD offspring, which were reduced at weaning compared with birth. As leptin is strongly correlated with birth weight in rats and humans (Desai *et al.*, 2005; Jahan *et al.*, 2009), the altered trajectory of leptin levels for offspring of undernourished mothers is not surprising. The trajectory for AD offspring, with plasma leptin concentrations decreasing between birth and weaning, is possibly due to a reduction in fat accretion/deposition at this critical developmental shift in nutrition. This period of time marks the change in diet from milk to solid food. However, the lack of such a decline in plasma leptin for IUGR offspring suggests increased fat accretion during this postnatal phase compared with AD offspring.

As described in Chapter 1 of this thesis, leptin is a key determinant of energy expenditure. Therefore, the low levels of leptin at birth may indicate a lower metabolic rate or a lower level of energy available for storage as lipids. In newborn rats, circulating concentrations of leptin are derived from brown adipose tissue, which is present at higher levels in the perinatal period (Oliver *et al.*, 2001). The main function of brown adipose tissue in neonatal mammals is thermogenesis, and consequently it is a major determinant of basal metabolic rate during early life. However, whilst a lower basal metabolic rate has already been suggested in adult offspring of undernourished mothers (Huber *et al.*, 2009), this is our first indication that basal metabolic rate may also be altered during the perinatal period.

The delayed and reduced leptin secretion observed for Neonatal IUGR offspring has been shown in previous studies to drive key endocrine, phenotypic and functional changes that manifest during later life (Bouret *et al.*, 2004; Delahaye *et al.*, 2008). Indeed, hypoleptinaemia, during the critical early neonatal period, was hypothesised as the trigger for irreversible obesity development in a sedentary postnatal environment (Vickers *et al.*, 2005). Neonatal leptin treatment has been used to prevent the development of obesity and hyperphagia in adult offspring (Vickers *et al.*, 2005, 2008). It has also been proposed that leptin may play a role in the regulation of skeletal muscle mass and muscle fibre differentiation, as discussed below.

2.4.3 Developmental Changes of Muscle Morphology and Oxidative Capacity

In this study, muscle morphology and function assessed by immunohistochemical, biochemical and enzymatic analyses demonstrated the influence of maternal undernutrition from weaning to adulthood. Changes in fibre size and fibre-type composition in the IUGR offspring were becoming apparent at weaning and then fully developed by adulthood. As mentioned above, adult IUGR offspring have increased proportions of Type I and Type IIa fibres in soleus muscle compared with AD offspring (Huber *et al.*, 2009). I have now shown that with advancing age, fibre proportions and size in both GSUP and soleus muscles are differentially affected, depending on their prenatal experience. In particular, this study has discovered IUGR offspring have a significant reduction in the size of Type I and IIa in soleus and Type IIb fibres in GSUP muscle. Changes to fibre morphology in the soleus muscle of IUGR offspring paralleled a significant reduction in LDH glycolytic enzyme activity.

Potentially, the fibre morphology and enzyme activity in soleus of IUGR offspring reflects a shift to a more oxidative metabolic profile.

The capacity of skeletal muscle to adapt its cellular regulation of fuel utilisation to nutrient supply and demand is critical for the maintenance of metabolic health. The essential underlying metabolic features of skeletal muscle are influenced by multiple factors including structure, fibre type composition, fibre size, mitochondrial density, innervation and capillary density (Hochachka, 1994). The morphological and functional properties of muscle are determined during embryonic development and during the fetal and the early postnatal periods. It has been shown previously in both IUGR piglets and IUGR rodents that dietary influences, including calorie or protein restriction during the prenatal period, can change muscle development in terms of fibre number and fibre type composition (Sayer and Cooper, 2005; Bauer *et al.*, 2006). In rats, skeletal muscle develops at distinct time points during both the prenatal and the early postnatal periods. While the majority of different muscle tissues in rats are developed during the early postnatal period, between birth and weaning, the *number* of fibres in some muscles, particularly the soleus, are fixed at the time of birth (Wilson *et al.*, 1988). The significant effect of prenatal nutrition on the *size* of Type IIa fibres in IUGR soleus, combined with a significant interaction between prenatal nutrition and age, demonstrates the dynamic changes in muscle metabolism with age.

The Neonatal and Weaning groups included both male and female offspring. Sex-associated differences are most unlikely to occur at day 1 and 21 of life since onset of puberty in females occurs around day 30, as indicated by vaginal opening (Chen *et al.*, 2009). No sex differences in body mass, lean body mass, % body fat, plasma leptin and ghrelin levels were detected in male and female offspring of 50 % food-restricted rats mothers at ages of 1 day and 3 weeks (Desai *et al.*, 2005). Importantly, muscle fibre type composition is not affected by sex in AD and IUGR rat pups younger than 30 days (Lane *et al.*, 2003). By adulthood, IUGR soleus morphology was characterised by a shift to more Type I fibres and a reduction in Type IIa fibre size (Huber *et al.*, 2009). Type IIa fibre size was significantly reduced by prenatal undernutrition in IUGR offspring of this experiment. Whilst investigation of Neonatal muscle morphology was not possible, the significant interaction of age and prenatal nutrition

for Type IIa fibres size indicates that this was a dynamic developmental pattern, potentially initiated in the early postnatal period.

The suggestion from the specific reduction in Type IIa fibre size is that the growth and development of secondary fibres and progenitors of Type II fibres may already be reduced during early fetal development in the offspring of undernourished mothers. Although the underlying mechanism requires further investigation, this is supported by the significant decrease observed by *posthoc* test in GSUP Type IIb fibres of Adult IUGR offspring. This decrease in fibre size appears to have occurred in a muscle-specific manner during early postnatal development and may facilitate the exchange of metabolic fuels more effectively. Given that these fibres have strong oxidative properties, the smaller Type IIa fibres in soleus will have a greater surface area for gas exchange with the surrounding capillaries (Nakatani *et al.*, 2000; Lunde *et al.*, 2007). Furthermore, Type IIa fibres of GSUP may have undergone inhibited differentiation, as indicated by reduced percentage (effect of prenatal nutrition $P<0.027$), whereas their postnatal growth/size was not affected. The postnatal growth of Type IIb fibres in this GSUP muscle was inhibited but compensated for as reflected by equal glycolytic and oxidative enzyme levels.

Prenatally-induced pathways may initiate characteristic changes in morphological features of muscle during the post-weaning period. As mentioned above, soleus and GSUP muscles have proportions of myofibres that are differentially responsive to energy demands. The soleus muscle represents an endurance muscle owing to its anatomical function of facilitating standing and locomotion. The GSUP is also in high demand during locomotion, commonly during sprinting but stores more glycogen for rapid conversion to glucose 6-phosphate. For the GSUP muscle, the structural features appeared to have no functional consequences for oxidative and glycolytic capacity of muscle metabolism at either weaning or adulthood. No significant influence of age or prenatal nutrition was detected for either citrate synthase or LDH enzyme activity. Perhaps this reflects a capacity to maintain flexible metabolism in spite of the significantly reduced Type IIa fibre proportions and reduced Type IIb fibre size in GSUP muscle. Furthermore, the case for increased flexibility in GSUP muscle of IUGR offspring is supported by increased glycogen storage in these animals, despite smaller proportions of Type IIa fibres.

Coupled with the work of others, these data suggest that adaptive processes leading to increased metabolic flexibility may be initiated by changes in plasma leptin levels during critical periods of development (Krechowec *et al.*, 2006; Vickers, 2007; Vickers *et al.*, 2008; Briana and Malamitsi-Puchner, 2009). For example, the role leptin plays in regulation of skeletal muscle development has been illustrated by studies in pig primary cell cultures, where leptin promoted proliferation and inhibited differentiation of myoblasts (Yu *et al.*, 2008). Additional studies have demonstrated exogenous leptin treatment in leptin-deficient ob/ob model stimulated muscle growth and prevented atrophy (Sainz et al., 2009). In the present study, the age-dependent trajectory of plasma leptin concentration was different between IUGR and AD offspring. Therefore, these altered patterns of circulating leptin may underlie the observed changes in muscle fibre size and composition in offspring of undernourished mothers.

Selective atrophy of skeletal muscle fibres is known to occur via the FOXO signalling pathway under conditions that remove FOXO inhibition of atrogenes responsible for atrophy and wasting of skeletal muscle (Sandri *et al.*, 2004). Upstream of FOXO is the PGC-1 α protein that was previously shown to be decreased in soleus of adult offspring of undernourished mothers (Huber *et al.*, 2009). Decreased PGC-1 α could impact on signalling pathways via a diminished FOXO inhibition of atrogenes (Sandri *et al.*, 2006). Fasting (Li and Goldberg, 1976) and high glucocorticoids (Fowden and Forhead, 2004), both experienced by the IUGR fetus, may influence the selective atrophy of type II glycolytic fibres in adults, potentially via FOXO-dependent pathways (Sandri *et al.*, 2004). Furthermore, leptin treatment in leptin deficient mice has been shown to inhibit FOXO atrophy processes (Sainz *et al.*, 2009). Therefore, it is tempting to speculate that the lower plasma leptin concentrations observed in neonatal offspring of undernourished rats might drive an early myogenic response to prenatal undernutrition.

The present study suggests that prenatal undernutrition sets in train both structural and functional changes in muscle. However, the biological consequences of these structural changes may only manifest much later, potentially with adverse outcomes if the postnatal environment is sedentary with abundant nutrition to saturate energy storage capacities.

2.5 Summary and Conclusions

This study describes the apparent plasticity of skeletal muscle during the postnatal period in offspring of undernourished mothers. The results of this study propose novel pathways for the dynamic development of metabolic flexibility in postnatal life. Earlier studies showed that features of metabolic flexibility were observed even when IUGR offspring were obese as a result of exposure to a postnatal sedentary environment (Thompson *et al.,* 2007; Miles *et al.,* 2009a; Miles *et al.,* 2009b). The research identified that, in IUGR rat offspring, higher metabolic flexibility is based on the enhanced capacity to store energy in times of excess as glycogen and fat in physiological depots. While the metabolic profile showed changes commencing on the first day of postnatal life, changes to muscle structure that facilitate effective utilisation of metabolic fuels become apparent much later. Leptin has an influence on both the regulation of morphological as well as metabolic features of skeletal muscle. From this study, leptin seems to be a key endocrine driver for differential susceptibility to muscle fibre atrophy. Further investigations into the pathways responsible for stimulating Type I fibre expression and selective atrophy of secondary fibres are warranted. Type I and IIa fibre sizes and LDH activity are decreased in Adult soleus muscle and support the idea that IUGR offspring have a higher skeletal muscle oxidative capacity. Higher oxidative capacity in skeletal muscle indicates that stored energy can be used efficiently through catabolic pathways in times of scarcity or in times of high energy need. In a clinical setting, knowledge of the metabolic conditions imposed during prenatal development will inform appropriate postnatal lifestyle adaptations. Stimulating these muscles by postnatal exercise was an efficient intervention that prevented obesity development in IUGR offspring (Miles *et al.,* 2009a). In principle, the high level of skeletal muscle plasticity in response to environmental stimuli, could be the reason exercise is able to correct metabolic disturbances induced by prenatal nutrition.

Chapter 3.

Muscle Development and Potential for Plasticity after Prenatal Undernutrition

3.1 Introduction

A range of structural and functional studies in the previous chapter confirmed prenatal experience had an influence on postnatal skeletal muscle phenotype. This led to the question, what are the specific mechanisms responsible for skeletal muscle development in IUGR offspring of undernourished dams? I am specifically asking the question, do the phenotypic changes seen in the IUGR offspring relate to changes in gene regulation. My aim was to identify mechanisms that drive the differential trajectory of both the red/slow/oxidative soleus and the white/fast/glycolytic GSUP muscles in offspring of undernourished dams. The three key developmental time points of birth, weaning and adulthood were chosen to examine molecular markers of metabolic flexibility. The rationale for selecting these key time points was to (1) align with the previous chapter's whole of life approach and (2) assess whether the phenotypic changes of IUGR offspring compared with AD animals are driven by an underlying and cumulative developmental program.

Quantitative real-time reverse transcription polymerase chain reaction (QPCR) is regarded as a robust gene detection and quantification system. The QPCR method was selected based on its ability to determine mRNA expression of key elements in signalling pathways for skeletal muscle morphology and metabolism (Figure 3.1). Firstly, I examined the effect of prenatal undernutrition on the myogenic developmental program. The myogenic developmental program is responsible for establishing morphology in early life with structural consequences in later life. Secondly, I assessed transcriptional control of oxidative capacity and consequences for metabolism in later life. A total of 18 target genes were selected for this study, based on their relevance to **myogenic determination** (myogenic regulatory factors, MRF); **fibre type differentiation** (calcium signalling); **mitochondrial biogenesis**; fibre growth (**Insulin-AKT signalling**) and atrophy factors (Forkhead box protein O1, **FOXO signalling**). The targets are outlined in Table 3.1 and Figure 3.1.

Rationale for selection of gene targets

The ratio of slow Type I and fast Type II fibres in skeletal muscle is the result of both developmental instructions and nerve activity-dependent plasticity. The initial approach investigated the **developmental programme** for skeletal muscle fibre type composition. Myogenic determination is most evident in the perinatal and early postnatal period. Therefore, the strategy was to quantify expression of MRF genes for Neonatal and Weaning muscle samples only. These factors are fundamental in the terminal differentiation of myoblasts (1.3.1 *The Myogenic Program*). Both myogenin (Myog) and myogenic regulatory factor 5 (Myf5) play a fundamental role in myogenesis, directing skeletal muscle progenitor cells to either an oxidative or glycolytic programme (Muroya *et al.*, 2002). Myostatin (Mstn) is a negative regulator of skeletal muscle mass in both early developmental and later postnatal periods. The gene Mstn has more recently been linked with fibre type switching properties, specifically with respect to glycolytic type II fibres (Hennebry *et al.*, 2009).

Pathway	Gene	Gene Name
Myogenic Regulatory Factors (MRF)	Myf5 Myog Mstn	Myogenic regulatory factor 5 Myogenin Myostatin
Ca^{2+} signalling	Calna1 Camk1 NFATc1	Calcineurin Calcium/calmodulin dependent protein kinase 1 Nuclear factor of activated T-cells cytoplasmic 1
Mitochondrial Biogenesis	PPARα PGC1α Tfam ATP Synth NRF UCP3 MT-CO1 COX4i1	Peroxisome proliferator-activated receptor α PPAR- coactivator α Transcription factor A ATP synthase Nuclear respiratory factor 1 Uncoupling protein 3 Mitocondrially encoded cytochrome c oxidase 1 Cytochrome C oxidase 4
Insulin AKT signalling	mTOR AMPK	Mammalian target of rapamycin F'adenosine monophosphate-activated protein kinase
FOXO signalling	FOXO1 Fboxo32	Forkhead box protein O1 F-box only protein 32/ Atrogin1

Table 3.1 The five key groups for interrelated mRNA targets of muscle development and postnatal plasticity.

The gene acronyms/initialisations are listed alongside their full names and are grouped by pathway related to myogenesis, metabolism, plasticity and oxidative potential.

Finally, I investigated the negative feedback to the AKT/insulin signalling pathway, **FOXO transcription activity**. This pathway modulates expression of genes involved in apoptosis, glucose metabolism, cell differentiation and oxidative stress. Metabolic stress as a result of fasting is resisted by the FOXO transcription factors, modulated by Silent mating type information regulators (Sirtuins) in a mechanism designed to enhance somatic protection. Ultimately, FOXO and its downstream target atrogin can influence insulin signalling metabolism (Nakae *et al.*, 2008) and also potentially suppress protein synthesis in skeletal muscle (Gomes *et al.*, 2001).

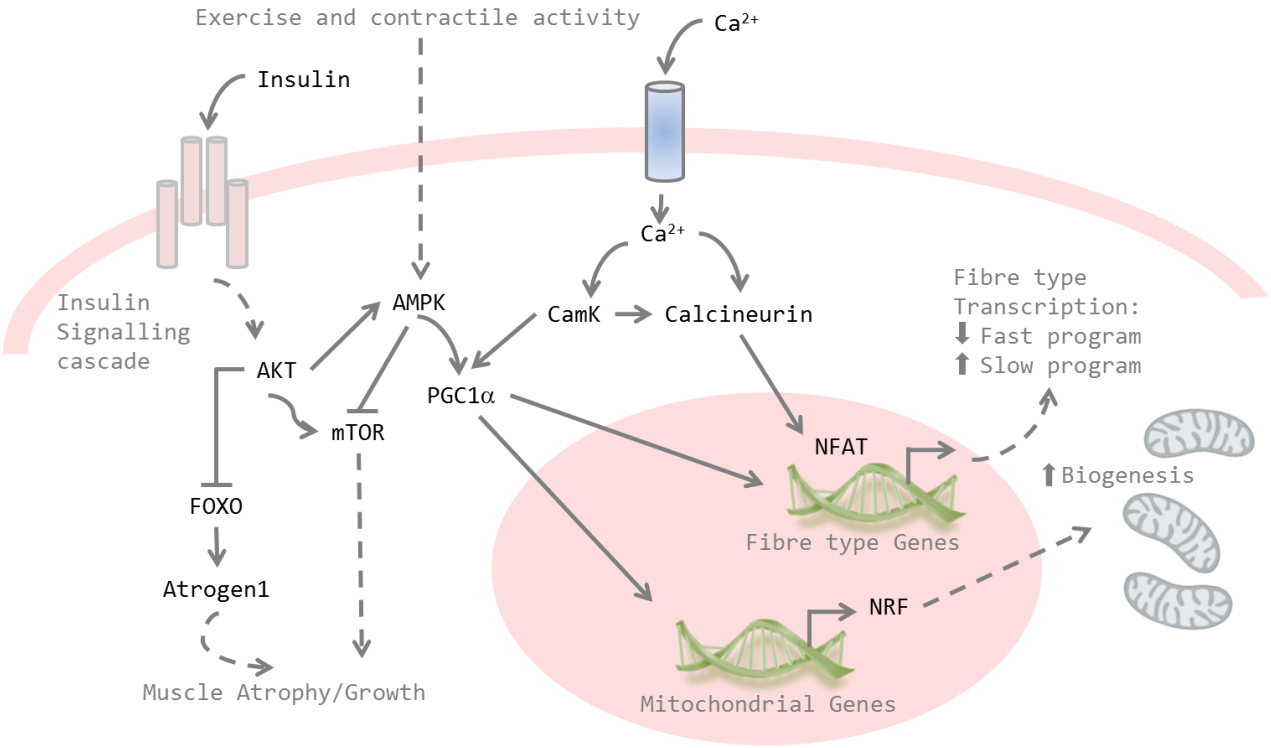

Figure 3.1 Factors that affect skeletal muscle phenotype.

The calcineurin-NFATc1 signalling pathway is activated by Ca2+ or slow fibre nerve activity, raising intracellular [Ca2+]. NFATc1 is activated and translocates to the nucleus of the myotube where it binds to slow fibre program promoters. The Insulin/Akt pathway regulates muscle hypertrophy and activates downstream effectors such as mTOR to stimulate protein synthesis and inhibits muscle atrophic pathways FOXO. Upstream, exercise and contractile activity influence AMPK directing PGC1 α activity. PGC1 α is a dominant regulator of mitochondrial content, acting upstream of NRF, and PGC1 α also influences muscle fibre type plasticity via NFATc1. Broken lines indicate some intermediate steps have been omitted. Helices and mitochondrial clipart faithfully excerpted from published sources in the public domain, 2011.

I hypothesised that the plastic patterns of skeletal muscle development adapt to suboptimal nutrition during development by making structural and functional changes, ultimately favouring pathways of flexible fuel usage and storage. Using QPCR, factors fundamental to skeletal muscle plasticity and oxidative potential were selected and measured in the skeletal muscle tissues. Mixed muscle samples were assessed at a Neonatal age, at Weaning (22 days) and dissected soleus and GSUP muscles at an adult age (150 days) for both AD control offspring and IUGR offspring of dams undernourished during pregnancy. The two distinct muscles soleus and GSUP muscles were chosen to investigate at weaning and adult ages. The two muscles serve distinct physiological purposes and have proportions of myofibres that are differentially responsive to energy demands.

Identification of differences in gene expression provides clues to the mechanisms that underlie the unique morphology and metabolism of skeletal muscle, after exposure to maternal undernutrition during pregnancy. The present study is the first to investigate the influence of nutritional parameters during pregnancy on postnatal calcium signalling in offspring. I asked whether enhanced calcium signalling induce the oxidative postnatal phenotype in the offspring of dams undernourished during pregnancy.

3.2 Methods.

3.2.1 Study Design

The study design used for this experiment is fully described in Chapter 2, *section* 2.2. Specifically, the Neonatal and Weaning animals are the same as Chapter 2. However, the samples for the Adults group were taken at 150 days of age from a subset of AD and IUGR male rats, kept exclusively for metabolic measurements. The 150 d end point for studies was selected as appropriate based on *post-hoc* analysis of endocrine measures from previous studies (Miles *et al.*, 2009a). At this age, plasma concentrations of endocrine measures demonstrated the key features of the IUGR model (Huber *et al.*, 2009; Miles *et al.*, 2009a). The Adult offspring were housed four littermates per cage and fed standard chow *ad libitum* throughout the experiment. All animals were generated using the same methodology outlined in Chapter 2 with virgin

Wistar rats (n = 9 per group) time-mated and assigned to receive chow (Teklad 18% protein, 5% fat diet, Oxon, UK) either *ad libitum* (AD) or at 30% of *ad libitum* intake (IUGR) throughout pregnancy. In both groups, litter size was adjusted to eight pups on the first postnatal day, to assure adequate and standardised nutrition until weaning (Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009a). Distinct groups of AD and IUGR offspring (each group containing pups that had descended from different mothers) were investigated at three developmental periods:

1. Neonatal, culled on the first postnatal day (n = 6 per group),
2. Weaning, culled on postnatal day 21 at weaning (n = 8 per group) and
3. Adult, culled on day 150 during adult life (n = 8 per group).

All procedures involving animals were carried out with the prior approval of the Animal Ethics Committee of the University of Auckland. Sample collection of Adults was performed as previously described (Huber *et al.*, 2009; Miles *et al.*, 2009a). In short, soleus and GSUP muscle from one leg of Adult offspring were snap-frozen and stored at -80 °C. Mixed soleus and gastrocnemius muscles were snap-frozen at weaning. Due to size of soleus and GSUP samples in the Neonatal group, the entire hind limbs were snap-frozen, also termed mixed muscle. Selected gene targets were then assessed in appropriate samples – MRF genes in neonatal tissues; MRF and Ca^{2+} signalling genes in weaning tissues; Ca^{2+} signalling, mitochondrial, insulin signalling and FOXO signalling genes in adult tissues.

3.2.2 RNA Extraction and Quality Control

Total RNA was isolated from snap frozen tissues using a combination of TRIzol® extraction (Invitrogen) and silica-gel-based membrane filtration (Invitrogen purelink RNA mini kit). To achieve an average yield of 5 – 10 μg of total RNA for each sample, approximately 30 mg of mixed and soleus muscle were processed. Tissue samples were first ground to powder in a mortar and pestle under liquid nitrogen and then transferred into 15 ml polypropylene tubes sitting on dry ice. After evaporation of liquid nitrogen, 1 ml of TRIzol® reagent was added.

Tissue samples were homogenised firstly using an Eppendorf pestle and cup and secondly by passing through a 22 gauge needle 20 times using a 1 ml syringe. Samples were then transferred to ultra-clear RNase free 1.5 ml eppendorf tubes (Ambion) and incubated at RT for 5 min. Skeletal muscle tissue samples were centrifuged at 12,000 rpm for 2 min at RT to separate the lipid layer. An ultra-fine 1 ml insulin syringe was used to transfer the lower TRIzol layer to a new 1.5 ml eppendorf tube. 400 µl of chloroform was then added to samples which were mixed by vortexing for 15 s and incubated for 5 min at RT. Immediately after homogenisation, an additional 200 µl of chloroform was added immediately; samples were mixed by vortexing and incubated for a further 5 minutes at RT. After chloroform incubation, all samples were centrifuged at 12,000 rpm for 15 min at 4^{o}C. After centrifugation the upper aqueous phase was transferred to a fresh 1.5 ml eppendorf tube. One volume of 70 % ethanol (in DEPC-treated water) was then added and samples were mixed immediately by pipetting.

Up to 700 µl of sample was loaded onto Invitrogen PureLink mini columns which were then centrifuged at 12,000 rpm for 15 s at RT. If sample volume exceeded 700 µl successive aliquots were applied to the same column as above. Flow-through was discarded after each centrifugation. 700 µl of wash buffer 1 was added to each column and centrifuged at 12,000 rpm for 15 s at RT. Columns were then transferred to a fresh collection tube. 500 µl of wash buffer 2 was added and columns were centrifuged again at 12,000 rpm for 15 s at RT. Flow-through was discarded and this wash was repeated with a 2 minute centrifugation time followed by a final 1 min centrifugation. The columns were transferred to a new 1.5 ml collection tube and RNA was eluted with 30 µl of RNase free water. The columns were incubated with eluant for 1 min at RT then centrifuged at 12,000 rpm for 2 min at RT.

To obtain a higher total RNA concentration, the first elution was reapplied to the column which was spun again at 12,000 rpm for 1 min at RT. After secondary elution two 1 µl aliquots were removed for quality control procedures detailed below in section 4.2.5. Total RNA samples were subsequently stored at -80^{o}C until further analysis.

3.2.3 Quantification of RNA samples

All RNA samples were subjected to rigorous tests for purity and integrity prior to use in real time QPCR experiments. The concentration and purity of all RNA samples was initially quantified by measuring sample absorbance at 260 nm and 280 nm using Nanodrop spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies). For use in microarray experiments all RNA samples were required to have an A_{260}/A_{280} ratio within a range of 1.9-2.1, an A_{260}/A_{230} ratio greater than 1.9 and a total RNA concentration no less than 0.5 µg/µl. RNA sample integrity and purity was further tested using an Agilent 2100 Bioanalyzer (Agilent Technologies) as follows. Briefly, 1 µl aliquots of each RNA sample were diluted to a concentration of 500 ng/µl, heat denatured for 2 minutes at 70^{o}C, and loaded onto a gel-filled 12-well RNA nano chip (RNA 6000 Nano LabChip® Kit). The RNA nano chip was vortexed for 1 min at 2400 rpm then loaded into the Agilent 2100 Bioanalyzer and analysed. For each sample an electropherogram trace and an RNA integrity number (RIN) was generated. The RIN number provides an unambiguous assessment of RNA integrity that allows RNA quality to be standardised cross multiple samples (Schroeder *et al.*, 2006). RIN values ranged from 1 to 10 with 10 indicating completely intact RNA and 1 indicating complete degradation (Schroeder *et al.*, 2006). All RNA samples were required to have a RIN number greater than 8.9 and demonstrate no signs of genomic DNA contamination on either the electropherogram trace or gel-like image.

3.2.4 Preparation of cDNA

The cDNA was synthesised from 5 µg of total RNA using the SuperScript™ VILO synthesis system for RT-PCR (Invitrogen). The cDNA synthesis was undertaken according to the manufacturer's instructions. Briefly, 1.5 µg of total RNA, in a volume up to 10 µl, was combined with 4 µl of 5x reaction mix, 2 µl 10x enzyme mix in a 96 well plate (labcon PCR). Sample volumes were made up to 20 µl with DEPC-treated water. This reaction mixture was gently mixed, spun down by brief centrifugation, and incubated at 42 ^{o}C for 60 min using the GENEAMP 9700 PCR system. The reaction was terminated by incubating at 85 ^{o}C for 5 min and then placing samples on ice for at least 1 min. After a brief centrifugation and transfer to 1.5 ml RNase-free tube, cDNA was stored at -20 ^{o}C until further use.

3.2.5 TaqMan® Gene Expression Assays

All QPCR assays were carried out using inventoried TaqMan® Gene Expression Assays from Applied Biosystems (ABI, USA). Pre-formulated expression assays consisted of a single 20x mix containing 1 pair of unlabelled gene specific PCR primers (each primer at a final concentration of 900 nM) and 1 FAM™ dye-labelled TaqMan® minor groove- binder probe (250 nM final concentration). The following rat specific assays were used in the QPCR experiments detailed in this chapter:

Myog (ID Rn00567418_m1), *Myf5* (ID Rn01502778_m1), *Mstn* (ID Rn01437895_m1), *Calna1* (ID Rn00566855_m1), *Camk1* (ID Rn00593272_m1), *PGC1a* (ID Rn00580241_m1), *PPARA* (ID Rn00566193_m1), *Tfam* (ID Rn00580051_m1), *ATPsynth* (ID Rn00821491_g1), *NRF1* (ID Rn01455958_m1), *MTCO1* (ID Rn03296721_s1), *COX4i1* (ID Rn00567950_m1), *UCP3* (ID Rn00565874_m1), *mTOR* (ID Rn00571541_m1), *AMPK* (ID Rn00576935_m1), *FOXO1* (ID Rn01494868_m1), *Fboxo32* (ID Rn00591730_m1).

Assays with the suffix _m1 are designed over exon-exon boundaries. Assays with the _g1 are also designed over exon-exon boundaries but have the potential to detect both the functional transcript and non-transcribed pseudogenes, in the presence of high-levels of contaminating genomic DNA. Similarly, _s1 indicates an assay whose probes and primers are designed within a single exon also with the possibility of genomic DNA detection.

Separate from the inventoried primer/probe mixes, one further primer/probe mix for NFATc1 was custom designed and analysed using Primer Express software (Applied Biosystems) with assistance from Dr Lara Cullen. Primer sequences were:

Forward Primer: 5' CCAGTACACCAGCTCTGCTATTGT
Reverse Primer: 5' CCCAGGTCCAGAGTGCTATCA
MGB Probe: 5' CAGCCATCAACGCC

[National Center for Biotechnology Information (NCBI) Reference Sequence XM_001058445]

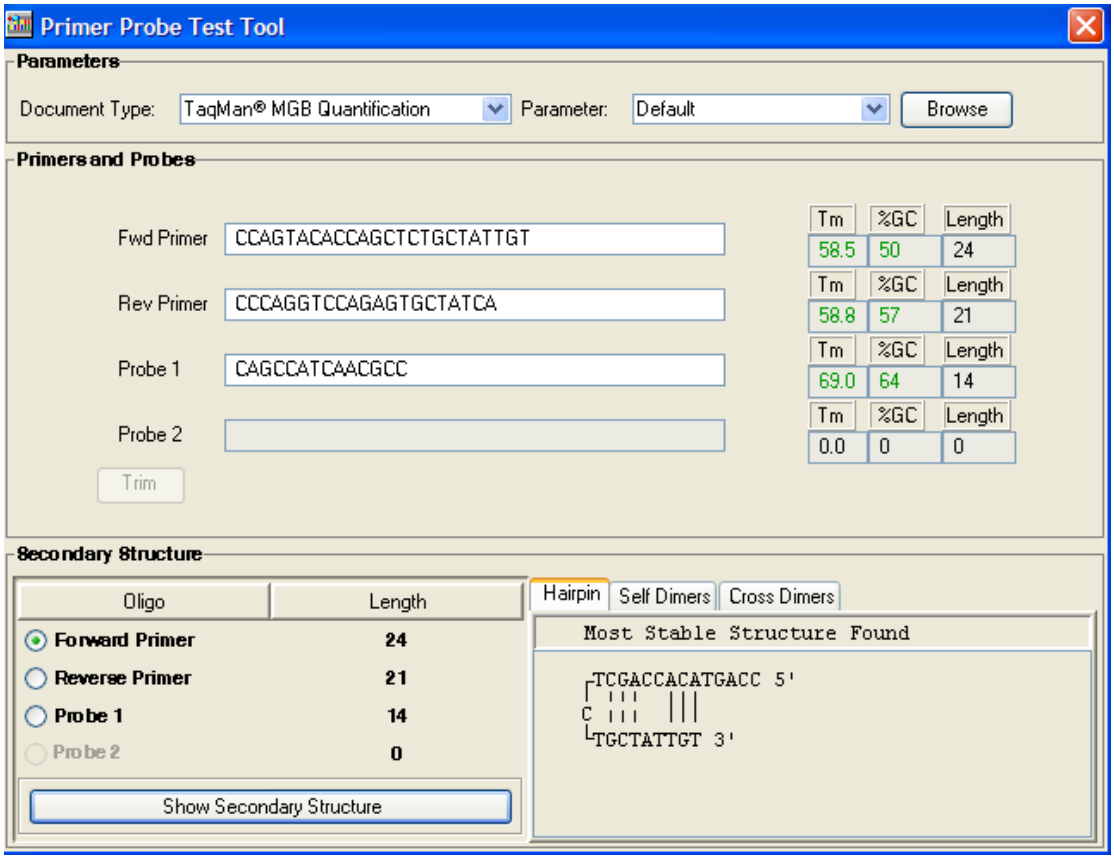

Figure 3.2 Screenshot of Primer Probe Test Tool for NFATc1 primer probe design.

Levels of cDNA were normalised to eukaryotic 18S rRNA expression from TaqMan® Ribosomal RNA Control 18s Reagent kit (Applied Biosystems). The 18s gene was selected as the endogenous reference on the basis of prior studies demonstrating that its expression was constant and not differentially affected by experimental manipulations (Miles *et al.*, 2009a). A 5x mix was made combining the primers and probe, aliquoted and frozen until required.

Assay protocol and QPCR cycling conditions

All QPCR reactions were singleplex reactions with a total volume of 10 µl. All reactions were set up in ABI Prism™ 384-well clear optical reaction plates (Applied Biosystems) and loaded using EpMotion automated pipetting system for accurate loading (Eppendorf). Within each plate well 3 µl of cDNA template, amounting to an input amount of approximately 30 ng of total RNA, was combined with 7 µl of real-time RT-PCR reaction mix (Table 3.2).

Component	Volume
TaqMan® Gene Expression Assay (20x)	0.5 μl
Invitrogen Express PCR Master Mix	5 μl
Nuclease free H_2O	1.5 μl

Component	Volume
18s TaqMan® Gene Expression Assay (5x)	2 μl
Invitrogen Express PCR Master Mix	5 μl

Table 3.2 Components and volumes of master mixes per well in QPCR reaction.

Step	Activation	Taq Enzyme Activation	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/Extend
Time	2 min	10 min	15 sec	1 min
Temp	50 ^{o}C	95 ^{o}C	95 ^{o}C	60 ^{o}C

Table 3.3 QPCR cycle conditions.

All samples were assayed in triplicate. Also in triplicate were no template control reactions, loaded with RNAse free H_2O. After loading wells, 384-well plates were sealed with an ABI Prism™ optical adhesive cover. Experimental plates were run under standard universal thermal cycling conditions (
Table 3.3) in an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). Levels of cDNA were quantified relative to the standard curve generated from a reference sample and normalised to 18s gene expression.

3.2.6 Statistical Analysis

In the instances where tissues were comparable between animals of different prenatal nutrition and different age, Type two ANOVA with Bonferroni *posthoc* test was performed. When dissected soleus and GSUP samples were not comparable with mixed muscle samples, statistical significance of difference measures were estimated by Mann-Whitney U non-parametric test. Analyses were performed using GraphPad prism (Version 5.00 for Windows; GraphPad Software, San Diego, CA). Data are presented + SEM, n = 6-8 animals per group. A value of $P < 0.05$ was considered significant.

3.3 Results

3.3.1 Effects of Prenatal Undernutrition on Target Gene Expression

The levels of MRF transcripts in group Neonatal and Weaning animals

The levels of the IUGR mRNA are presented relative to the respective AD mRNA expression and demonstrate the age-specific effects of prenatal undernutrition on gene expression within the skeletal muscle of offspring Note that each of the tables detail information about: gene name and description; relative expression of gene target normalised for the 18s reference gene; and ANOVA results. The relative expression listed on the table is for the offspring of dams undernourished during pregnancy, labelled 'IUGR'. The offspring of ad libitum fed dams, labelled 'AD', are consistently taken to be 1.0 with arbitrary units for comparison to the IUGR offspring. In the mixed leg muscles of day 2 Neonatal offspring and day 22 Weaning offspring, there were no differences in the relative expression of Mstn, Myf5 or Myog mRNA expression between the IUGR and AD groups.

The levels of calcium signalling factors in Weaning and Adult animals

The mRNA levels of NFATc1 in IUGR mixed skeletal muscle tissue of Weaning group were not significantly different from AD controls Analysed by Mann-Whitney U non-parametric test (Figure **3.4** and Table **3.5**). The effect of prenatal undernutrition on this important transcription factor is evident at an older age in the Adult group (Bonferroni *posthoc* test $P < 0.05$). Prenatal undernutrition significantly increased the relative expression of NFATc1 mRNA in the soleus tissue of chow-fed Adult IUGR offspring. The upstream factor, Calna1, was also significantly increased in IUGR offspring of Adult group ($P < 0.05$). No differences were detected in the expression of Camk1 of soleus muscle. In the GSUP skeletal muscle of IUGR Adult offspring, prenatal undernutrition had no effect on the relative mRNA expression of any calcium signalling target genes.

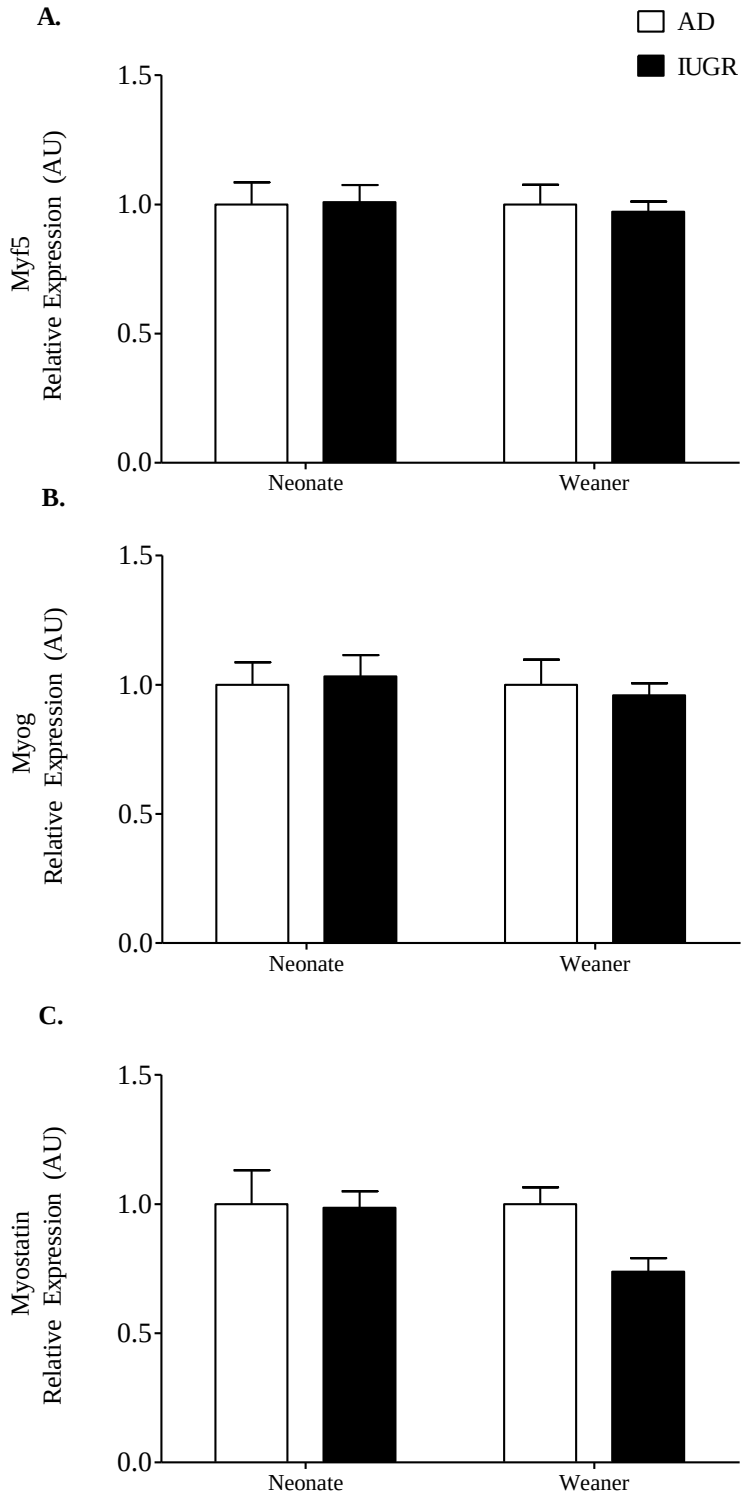

Figure 3.3 Relative expression levels of gene targets in Neonatal and Weaning skeletal muscle by QPCR.

The relative expression of gene targets myogenic regulatory factor 5 (Myf5), myogenin (Myog) and myostatin (Mstn) in non-specific mixed leg muscles for IUGR offspring compared with AD offspring. AD offspring gene expression set to an arbitrary 1.0. Gene targets measured at Neonatal and Weaning age points (values are mean ± SEM; n = 6-8).

Prenatal Nutrition	Age	Myf5	Myog	Mstn
AD	**Neonatal**	1.0 ± 0.09	1.0 ± 0.09	1.0 ± 0.13
	Weaning	1.0 ± 0.08	1.0 ± 0.10	1.0 ± 0.07
IUGR	**Neonatal**	1.01 ± 0.07	1.03 ± 0.08	0.99 ± 0.06
	Weaning	0.97 ± 0.04	0.96 ± 0.05	0.74 ± 0.05
Two way ANOVA				
Prenatal nutrition		0.8944	0.9587	0.1270
Age		0.8047	0.6708	0.1687
Interaction		0.8047	0.6708	0.1687

Table 3.4 Relative expression levels of gene targets in Neonatal and Weaning skeletal muscle by QPCR.

The relative expression of gene targets myogenic regulatory factor 5 (Myf5), myogenin (Myog) and myostatin (Mstn) in non-specific mixed leg muscles for IUGR offspring compared with AD offspring. AD offspring gene expression set to an arbitrary 1.0. Gene targets measured at Neonatal and Weaning age points (values are mean ± SEM; n = 6-8).

FOXO atrophy pathway factors in Weaning and Adult animals

There was no significant effect of prenatal nutrition on the levels of atrophy gene targets in either Weaning or Adult offspring. Comparisons between AD and IUGR offspring were made in the mixed skeletal muscle sample containing both soleus and GSUP muscles at Weaning. The levels of Fboxo32 mRNA showed no significant difference between the AD and IUGR at that time (Figure 3.4). Furthermore, the dissected soleus (Table 3.6, Figure 3.5) and GSUP (Table 3.7, Figure 3.6) muscles at the Adult stage had highly variable errors around the mean for IUGR offspring, particularly for Fboxo32. The FOXO levels were not changed by prenatal nutrition in either soleus or GSUP tissue for the Adult group.

The levels of transcripts controlling mitochondrial content/biogenesis in Adult animals

The levels of PPARA, ATP synth, MT-CO1, COX4i1, UCP3 mRNA were all unchanged in both dissected soleus (Table 3.6, Figure 3.5) and GSUP (Table 3.7, Figure 3.6) of Adult IUGR offspring compared with AD controls. The level of NRF1 was differentially affected by prenatal nutrition in the Adult soleus muscle only (Mann-Whitney U test, $P < 0.05$) (Table 3.6), with significantly increased mRNA expression of IUGR relative to AD offspring ($P < 0.05$).

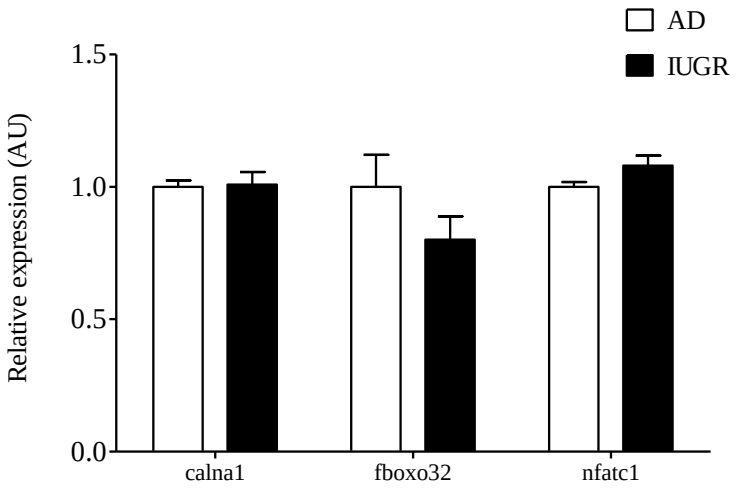

Figure 3.4 Weaning expression of calcium and atrophy genes in mixed skeletal muscle.

Relative expression levels of gene targets calcineurin (calna1), atrogin (fboxo32) and nuclear factor of activated T cells 1 (NFATc1) in Weaning skeletal muscle measured by QPCR. All results were measured in mixed skeletal muscle samples of Weaning rats at 22 d age (n = 8). Results for each assay, relative expression of IUGR group compared with AD group (AD group expression is set to an arbitrary 1.0 value).

Prenatal Nutrition	Calna1	Fboxo32	Nfatc1
AD	1.0 ± 0.02	1.0 ± 0.12	1.0 ± 0.02
IUGR	1.09 ± 0.05	0.8 ± 0.09	1.08 ± 0.04
MWU Test Results	0.8665	0.3969	0.1206

Table 3.5 Relative expression levels of gene targets in mixed Weaning muscle measured by QPCR.

Relative expression levels of gene targets calcineurin (calna1), atrogin (fboxo32) and nuclear factor of activated T cells 1 (NFATc1) in Weaning skeletal muscle measured by QPCR. All results were measured in mixed skeletal muscle samples of Weaning rats at 22 d age (n = 8). The relative expression of gene targets for IUGR offspring compared to AD offspring, with AD offspring gene expression set to an arbitrary 1.0 and analysed by Mann-Whitney U (MWU) non-parametric test).

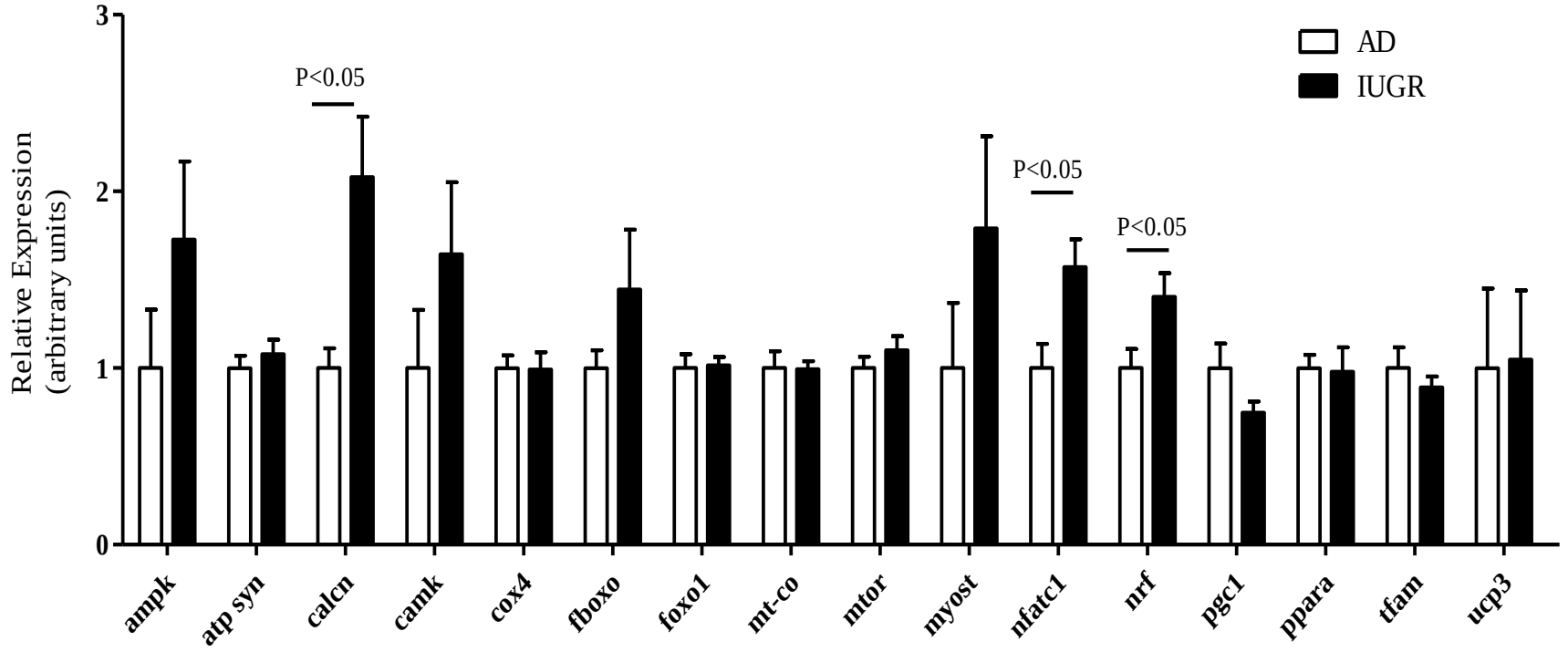

Figure 3.5 Relative expression levels of selected gene targets in Adult soleus muscle measured by QPCR.

All results were measured in soleus muscle samples of postnatal rats at 150 d age (n = 6). The relative expression of gene targets for IUGR offspring compared with AD offspring, with AD offspring gene expression set to an arbitrary 1.0 and analysed by Mann-Whitney U non-parametric test). The significant up-regulation of Calcn, NFATc1 and NRF gene expression for IUGR compared with AD ($P < 0.05$)

F'adenosine monophosphate-activated protein kinase (AMPK); ATP synthase (ATP Synth); Calcineurin (Calna1); Calcium/calmodulin dependent protein kinase type 1 (camk); Cytochrome C oxidase 4 (COX4); Forkhead box protein O1(FOXO); F-box only protein 32/ Atrogin1(FOXO1); Mitocondrially encoded cytochrome c oxidase 1(MT-CO); Mammalian target of rapamycin (mTOR); Myostatin (Mstn); Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1); Nuclear respiratory factor 1(NRF1); Peroxisome proliferator-activated receptor α (PPARα); PPAR- coactivator α (PGC1 α); Transcription factor A (Tfam); Uncoupling protein 3 (UCP3).

A.

Prenatal Nutrition	AMPK	ATP Syn	Calna1	CamK	Cox4	Fboxo32
AD	1.0 ± 0.33	1.0 ± 0.07	1.0 ± 0.11	1.0 ± 0.33	1.0 ± 0.07	1.0 ± 0.10
IUGR	1.73 ± 0.44	1.08 ± 0.08	2.08 ± 0.34	1.64 ± 0.41	0.99 ± 0.10	1.45 ± 0.34
MWU Test Results	0.0704	0.6991	**0.0131**	0.2667	0.9458	0.2356

B.

Prenatal Nutrition	FOXO1	Mt-CO	mTOR	Myostatin	Nfatc1	NRF1
AD	1.0 ± 0.08	1.0 ± 0.06	1.0 ± 0.06	1.0 ± 0.37	1.0 ± 0.14	1.0 ± 0.11
IUGR	1.02 ± 0.05	1.10 ± 0.08	1.10 ± 0.08	1.79 ± 0.52	1.57 ± 0.16	1.40 ± 0.13
MWU Test Results	0.865	0.3494	0.3494	0.0975	**0.0206**	**0.0422**

C.

Prenatal Nutrition	PGC1	PPara	TFam	UCP3
AD	1.0 ± 0.14	1.0 ± 0.08	1.0 ± 0.12	1.0 ± 0.45
IUGR	0.75 ± 0.06	0.98 ± 0.14	0.89 ± 0.06	1.05 ± 0.39
MWU Test Results	0.1299	0.9006	0.4245	0.4023

Table 3.6 Relative expression levels of selected gene targets in Adult soleus muscle measured by QPCR.

All results were measured in soleus muscle samples of postnatal rats at 150 d age (n = 6). The relative expression of gene targets for IUGR offspring compared with AD offspring, with AD offspring gene expression set to an arbitrary 1.0 and analysed by Mann-Whitney U (MWU) non-parametric test).

A. F'adenosine monophosphate-activated protein kinase (AMPK); ATP synthase (ATP Synth); Calcineurin (Calna1); Calcium/calmodulin dependent protein kinase type 1 (camk); Cytochrome C oxidase 4 (COX4); F-box only protein 32/ Atrogin1(FboxO32); **B.** Forkhead box protein O1(FOXO); Mitocondrially encoded cytochrome c oxidase 1(MT-CO); Mammalian target of rapamycin (mTOR); Myostatin (Mstn); Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1); Nuclear respiratory factor 1(NRF1); **C.** Peroxisome proliferator-activated receptor α (PPARα); PPAR- coactivator α (PGC1 α); Transcription factor A (Tfam); Uncoupling protein 3 (UCP3).

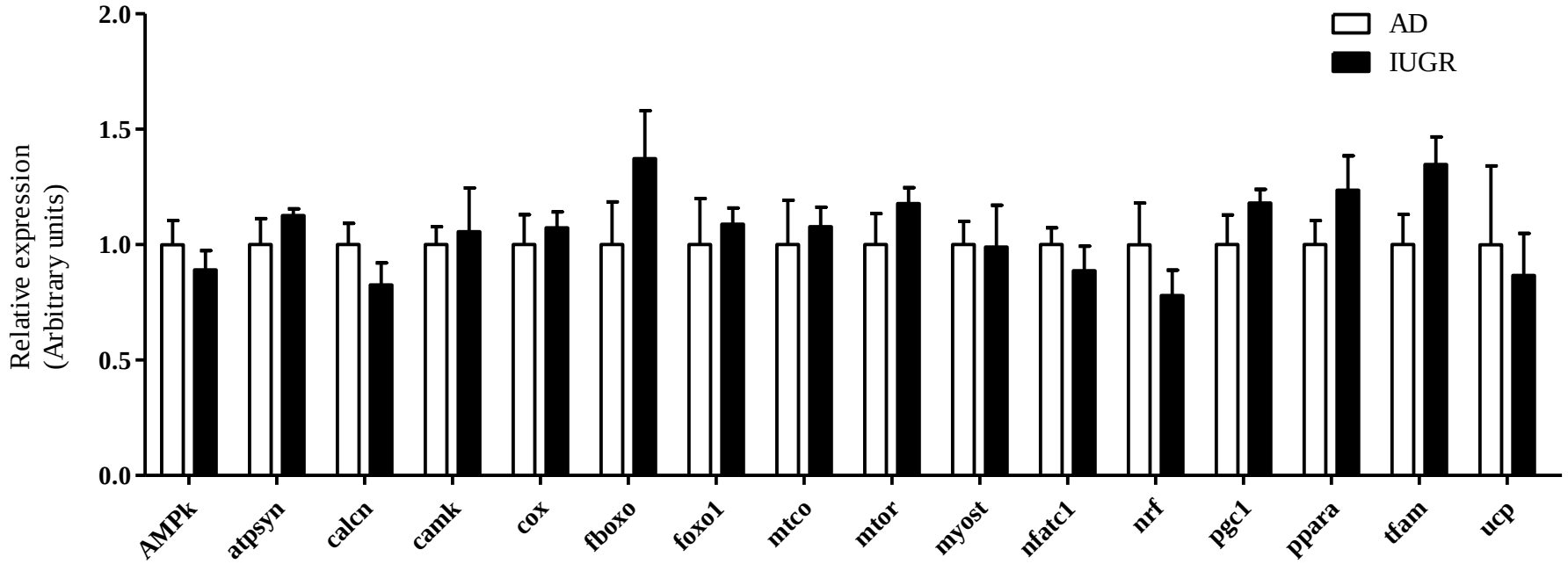

Figure 3.6 Relative expression levels of selected gene targets in Adult GSUP muscle measured by QPCR.

All results were measured in GSUP muscle samples of postnatal rats at 150 d age (n = 6). Samples are normalised for expression of 18s and the relative expression of gene targets for IUGR offspring compared with AD offspring, with AD offspring gene expression set to an arbitrary 1.0 and analysed by Mann-Whitney U test with $P > 0.05$ in every instance.

F'adenosine monophosphate-activated protein kinase (AMPK); ATP synthase (ATP Synth); Calcineurin (Calna1); Calcium/calmodulin dependent protein kinase type 1(camk); Cytochrome C oxidase 4 (COX4); Forkhead box protein O1(FOXO); F-box only protein 32/ Atrogin1(FOXO1); Mitocondrially encoded cytochrome c oxidase 1(MT-CO); Mammalian target of rapamycin (mTOR); Myostatin (Mstn); Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1); Nuclear respiratory factor 1(NRF1); Peroxisome proliferator-activated receptor α (PPARα); PPAR- coactivator α (PGC1 α); Transcription factor A (Tfam); Uncoupling protein 3 (UCP3).

A.

Prenatal Nutrition	AMPK	ATP Syn	Calna1	CamK	Cox4	Fboxo32
AD	1.0 ± 0.10	1.0 ± 0.11	1.0 ± 0.09	1.0 ± 0.08	1.0 ± 0.13	1.0 ± 0.19
IUGR	0.89 ± 0.08	1.13 ± 0.03	0.83 ± 0.10	1.06 ± 0.19	1.07 ± 0.07	1.37 ± 0.21
MWU Test Results	0.2187	0.304	0.2186	0.7909	0.6243	0.2097

B.

Prenatal Nutrition	FOXO1	Mt-CO	mTOR	Myostatin	Nfatc1	NRF1
AD	1.0 ± 0.20	1.0 ± 0.19	1.0 ± 0.14	1.0 ± 0.10	1.0 ± 0.07	1.0 ± 0.18
IUGR	1.09 ± 0.07	1.08 ± 0.08	1.18 ± 0.07	0.99 ± 0.18	0.89 ± 0.11	0.78 ± 0.11
MWU Test Results	0.6829	0.7176	0.2696	0.9633	0.3939	0.323

C.

Prenatal Nutrition	PGC1	PPara	TFam	UCP3
AD	1.0 ± 0.13	1.0 ± 0.10	1.0 ± 0.13	1.0 ± 0.34
IUGR	1.18 ± 0.06	1.24 ± 0.15	1.35 ± 0.12	0.87 ± 0.18
MWU Test Results	0.2331	0.2223	0.0789	0.7372

Table 3.7 Relative expression levels of selected gene targets in Adult GSUP muscle measured by QPCR.

All results were measured in GSUP muscle samples of postnatal rats at 150 d age (n = 6). The relative expression of gene targets for IUGR offspring compared with AD offspring, with AD offspring gene expression set to an arbitrary 1.0 and analysed by Mann-Whitney U (MWU) non-parametric test).

A. F'adenosine monophosphate-activated protein kinase (AMPK); ATP synthase (ATP Synth); Calcineurin (Calna1); Calcium/calmodulin dependent protein kinase type 1 (camk); Cytochrome C oxidase 4 (COX4); F-box only protein 32/ Atrogin1(FboxO32); **B.** Forkhead box protein O1(FOXO); Mitocondrially encoded cytochrome c oxidase 1 (MT-CO); Mammalian target of rapamycin (mTOR); Myostatin (Mstn); Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1); Nuclear respiratory factor 1(NRF1); **C.** Peroxisome proliferator-activated receptor α (PPARα); PPAR- coactivator α (PGC1 α); Transcription factor A (Tfam); Uncoupling protein 3 (UCP3).

Insulin/AKT protein synthesis pathway factors in Adult animals

Prenatal undernutrition did not have a significant effect on the mRNA levels of mTOR or AMPK in either of the soleus (Table 3.6, Figure 3.5) or GSUP (Table 3.7, Figure 3.6) muscles of Adult group. In each case, for each tissue, the Mann-Whitney U non-parametric test gives results that are $P > 0.05$).

3.4 Discussion

This study examined the influence of prenatal undernutrition on skeletal muscle growth and metabolic function over time. The study follows on from Chapter 2 that examined the unique morphological and functional features of skeletal muscle development in IUGR offspring. The novel findings of this chapter suggest a mechanism for the influence of nutrition on skeletal muscle development. In particular, maternal undernutrition during pregnancy induced changes in gene expression patterns that affect skeletal muscle fibre differentiation and mitochondrial biogenesis; but not transcriptional factors influencing muscle fibre growth or atrophy.

The results of the study are represented as relative gene expressions, whereby IUGR factors are essentially ratios of the AD controls. The ability to detect statistical difference between ratios is difficult using ANOVA. In the case of the neonate and weaner age groups, no differences were observed. It may be that there are simply no detectable differences in the gene expression at these early ages. Or, it may be that the mixed tissue, i.e. both GSUP and soleus muscles, made it difficult to detect the specific effect of nutrition on gene expression. The two soleus and GSUP muscles could be investigated separately in the 150 d Adults. The two muscles serve specific physiological purposes and have proportions of myofibres that are differentially responsive to energy demands. In particular, the results of this study provide evidence for enhanced calcium signalling pathway and mitochondrial biogenesis in soleus muscle of IUGR offspring compared with AD controls. As will be discussed below, this has implications for nutritional influence of postnatal fibre differentiation and plasticity. Given that the soleus muscle has more Type I fibres than GSUP muscle, it

was not surprising that the main differences in calcium signalling were seen in the soleus and not GSUP.

The results of this study will be discussed in the context of the literature below. The five areas integral to establishing morphology and metabolism will be outlined in detail, including: myogenic determination; calcium signalling for fibre type differentiation; mitochondrial biogenesis; insulin stimulated growth; and FOXO stimulated atrophy. Of the five areas assessed, Calcium signalling pathways showed significant differences in mRNA expression in muscle from offspring of undernourished mothers compared with controls. Measures of insulin signalling or atrophic FOXO signalling pathways showed little or no effect of prenatal undernutrition.

Overall, the results of this study provide additional support for the notion that prenatal undernutrition during pregnancy may lead to increased morphological and physiological plasticity of skeletal muscle in the later postnatal period. For the first time, calcium signalling may be linked to changes in Adult offspring soleus muscle, as a consequence of prenatal undernutrition. Therefore, both NFATc1 and calcineurin are implicated as potential mechanistic factors determining the unique oxidative phenotype observed in soleus muscle of Adult offspring from undernourished mothers.

3.4.1 The Myogenic Regulatory Factors that Govern Muscle Determination

Because maternal undernutrition altered the postnatal skeletal muscle phenotype of the IUGR offspring, I tested whether undernutrition could also alter the expression of Myogenin, Myf5 and myostatin mRNA. Initially the hypothesis was that reduced levels of myostatin mRNA in muscle of neonate and weaner IUGR offspring might represent a compensatory mechanism to maintain postnatal muscle mass. However, no significant effect of prenatal nutrition on myostatin across Neonatal and Weaning time periods was apparent. As discussed above, it may be that the differences could not be detected by ANOVA across the two developmental time points. There were no differences in mRNA expression of myogenin or Myf5 MRFs in the mixed muscle samples of IUGR offspring compared with controls at either birth or weaning. The

absence of any observable change in these MRFs suggests that offspring of undernourished dams have the potential to form and retain Type I fibres. In particular, Myf5 is known to play an important role in the survival and commitment of the myoblast phenotype (Pownall *et al.*, 2002). If there were fewer determined myoblasts directed to the secondary myogenesis of Type II fibres, Myf5 would be expected to be reduced. However, the results of this study did not show any effect of treatment on Myf5 mRNA expression levels.

The data show unchanged myostatin mRNA expression in muscle of prenatally undernourished offspring. The findings of this study do not differ drastically from other studies assessing prenatal nutritional effects on postnatal myogenesis, one reporting an inconsistent increase in Weaning myostatin expression (Bayol *et al.*, 2004). Maternal nutrition in a study by Bayol et al., (2004) was reduced to either 40% or 50% of control nutrition during gestation followed by a rehabilitation diet during the lactation period. The weanling IUGR pups were also segregated into heaviest and lightest groups. The lightest pups from the more severely undernourished group (40%) had increased myostatin mRNA levels compared with the lightest pups from the 50% undernourished group, as determined by absolute quantification of mRNA levels and SyBR Green methodology. Additional markers of skeletal muscle proliferation and differentiation were unchanged for all comparisons (MyoD and M-cadherin) in that particular study. The authors suggested that these data may not provide strong evidence for nutritional regulation of postnatal growth and conclude that there does not appear to be a linear correlation between undernutrition and myogenic markers (Bayol *et al.*, 2004). The muscle tissue used in Bayol et al. was sampled from the gastrocnemius muscle of the weanling rats where they also report no change in muscle fibre populations.

In a different model of maternal nutrient restriction during pregnancy, no differences in the MRF expression at both the mRNA and protein levels were observed in the muscles of 30 day old offspring (Cabeço *et al.*, 2012). The study by Cabeço and colleagues compared levels of myogenin and MyoD in the soleus and extensor digitalis longus (EDL) muscle of normal-protein offspring versus low-protein offspring (Cabeço *et al.*, 2012). Interestingly, with the low-protein model, but otherwise identical methodology, the researchers also found a reduction in cross-

sectional size of soleus Type I fibres at both 30 and 112 days of age. Fibre proportions for low-protein offspring were only altered in the fast twitch, glycolytic EDL muscle with an increase in Type IIb fibres compared with controls at 30 days only (Cabeço et al., 2012). No changes were observed at either age for soleus muscle fibre type proportions in that study. The authors were intrigued that neither MyoD nor myogenin were affected by the level of maternal protein intake. They suggested that these MRFs might be involved in the differentiation and maintenance of muscle phenotype, especially as EDL MyoD protein levels were increased overall (Cabeço et al., 2012). While the rationale was to assess only these MRFs during the Neonatal and Weaning periods, it would be interesting to understand whether they are still playing a role in myogenic differentiation in adulthood.

The calcium signalling factors that control fibre type selection/differentiation

This is the first study to show an effect of maternal undernutrition during pregnancy on calcium signalling pathways in skeletal muscles of offspring during postnatal life. Calcium signalling pathways are intrinsically linked with the oxidative Type I fibre programme in skeletal muscle. In the previous chapter, Chapter 2, I described how Adult soleus muscle in offspring of undernourished mothers had reduced Type I fibre size and an altered trajectory of fibre proportions from weaning to adulthood, trending towards increased proportions of Type I oxidative fibres. The next step was to investigate the role of calcium signalling on muscle fibre differentiation after maternal undernutrition as other studies have shown an effect of maternal undernutrition on Type I slow fibres in offspring (Maltin *et al.*, 2001; Bayol *et al.*, 2004; Fahey *et al.*, 2005a; Mallinson *et al.*, 2007). However, this is the first study to investigate Ca^{2+} signalling pathways as a potential mechanism for determining postnatal fibre type composition. Importantly, there was significantly increased mRNA expression of two key factors in the pathway, Calna1 and NFATc1 in Adult IUGR soleus. The enhanced mRNA expression of calcium signalling factors add further evidence to previously published observations that indicated increased oxidative capacity of muscle after prenatal undernutrition (Miles *et al.*, 2009a; Miles *et al.*, 2009b).

Calcineurin is a serine, threonine protein phosphatase, which is regulated by Ca^{2+} calmodulin. When activated, calcineurin dephosphorylates the transcription factors of the NFAT family, whereupon they are transported into the nucleus to activate the

slow-fibre transcription program (Beals *et al.*, 1997; Hogan *et al.*, 2003). NFATc1 is a specific determinant of slow muscle gene expression (Calabria *et al.*, 2009). Specifically, calcineurin-mediated signal transduction via NFATc1 has been implicated in fast-twitch to slow-twitch skeletal muscle fibre type transformation (Chin *et al.*, 1998; McCullagh *et al.*, 2004; Bassel-Duby and Olson, 2006; Mu *et al.*, 2007). It is understood that once inside the nucleus, the NFATc1 collaborates with MEF2 and other transcriptional regulatory proteins to induce the slow-fibre-specific gene transcription (Chin *et al.*, 1998). Friday and colleagues have also demonstrated that calcineurin is a downstream effector of calcium in regulating myogenic commitment to differentiation. However, they contest, with emphasis, whether NFATc1 is required for differentiation in myogenesis (Friday *et al.*, 2000).

The possibility of fibre type transformation in postnatal life is the subject of much interest and research, given the application to preventing or improving muscle and metabolic disease such as impaired lipid oxidation and insulin resistance. In particular, the focus is on improving oxidative potential and metabolic flexibility for therapeutic gain. Chapter 1 of this thesis reviews the rationale for attempts made to alter the skeletal muscle composition. Postnatal myogenesis results from a set of complex processes that mimic initial development. Further described in Chapter 2 of this thesis, rodent skeletal muscle is plastic during the first weeks after birth. After birth the embryonic and Neonatal myosins are typically reduced with subsequent up-regulation of fast Type II fibres (Whalen *et al.*, 1984; Schiaffino, 2010).

Chapter 1 of this thesis describes how the calcineurin/NFATc1 pathway plays an important role in determining muscle fibre type composition (Shen *et al.*, 2010). The calcineurin/NFAT pathway is induced by elevated Ca^{2+} concentrations in the muscle cell due to increased neuronal activation, for example by muscle contraction during exercise. Calcium signalling has been described in studies where calcineurin and NFATc1 are constitutively activated by transgenic manipulations resulting in a shift of skeletal muscle fibre type composition to Type I fibres (Naya *et al.*, 2000). In line with the transgenic studies, the results of this study show maintained Type I fibres of IUGR soleus muscle. It is likely that prenatal undernutrition impacted on the developmental programme resulting in the maintenance of the Type I fibre development and potentially a reduced development of Type II fibres. Schiaffino (2010) highlights the

importance of making a clear distinction between the embryonic programme and the established adult phenotype. Manipulations of the latter adult programme can be met with a change in fibre type population, but this is mainly in the case of regenerating muscle. Schiaffino proposes that perhaps what we see is not a Type II to Type I fibre (fast-to-slow) conversion but rather a block of the normal Type I to Type II fibre (slow-to-fast) switch that occurs after birth in fast muscles (Schiaffino, 2010). Therefore, the data of this study can provide support for an altered transcriptional programme as a result of prenatal nutrition.

Other researchers report transformations of fibre type in mature muscle fibres induced by calcinueruin inhibition (Serrano *et al.*, 2001), or by electrical stimulation (Liu *et al.*, 2001). The present study presents the idea that prenatal nutrition has the ability to affect the postnatal plasticity of skeletal muscle. The biological consequences of the postnatal conditions facing the Adult animals from this study are outlined in the following chapter (Chapter 4). The most important feature to note is that these Adult animals were maintained under sedentary conditions. Nonetheless, the Ca^{2+} pathway factors were up-regulated under these sedentary conditions. The offspring of undernourished mothers expressed a higher level of both Calcineurin and NFATc1. There were no differences in CAMK1 mRNA expression between IUGR and AD controls, but an up-regulation in an alternate isoform is possible, e.g. CAMK4, that is known to enhance the function of MEF2 in skeletal muscle (Wu *et al.*, 2001; Wu *et al.*, 2002).

3.4.2 Pathways of mitochondrial biogenesis influencing oxidative potential

The main outcome of alterations to fibre morphology observed in Chapter 2 might be enhanced oxidative potential. Therefore, it is not surprising that the results of this chapter suggest altered mitochondrial biogenesis in IUGR offspring compared with AD rats. The trajectory of measures for structure and function, fibre type composition and citrate synthase respectively, all pointed towards an increase in oxidative potential for IUGR offspring muscles. Adding support to the hypothesis is increased NRF1 mRNA expression in soleus muscle from Adult offspring of undernourished mothers.

The relative expressions of these mitochondrial factors were not investigated at the weaning age in the present study. The rationale was based on the finding (Chapter 2) that no prenatal effect was observed on the citrate synthase enzyme in either soleus or GSUP at weaning. It is also evident that other researchers have found no changes in skeletal muscle mitochondrial biogenesis at weaning age due to prenatal nutrient deficiency (Laker *et al.*, 2012). The IUGR rat offspring in the Laker study were generated via maternal uterine ligation and were subjected to exercise at weaning. The researchers found no influence of reduced prenatal nutrition on mitochondrial biogenesis factor PGC1 α and, additionally, found no influence of treadmill exercise on Tfam or Cyt C protein levels in gastrocnemius muscle (Laker et al., 2012). The results of this study do show increased NRF1 mRNA expression for Adult IUGR offspring in soleus muscle compared with AD offspring. As shown in Figure 3.7, NRF1 functions as a transcription factor that activates the expression of key metabolic genes regulating nucleus-located genes required for stimulating mitochondrial biogenesis (Seebacher et al., 2010; Patti et al., 2003).

One transcription factor upstream of NRF1, PGC-1 α, is unaltered in this study. PGC-1α activates the expression of Tfam, another regulatory factor that was unaltered in this study. Through the co-activation of NRF-1-mediated transcription, Tfam subsequently translocates to the mitochondrion and directly acts on mitochondrial DNA to influence oxidative phosphorylation (Puigserver et al., 2009). It is interesting that NRF1 is significantly increased at the transcriptional level without any apparent effect of prenatal nutrition on either PGC-1 α or Tfam. Previous reports showed an effect of prenatal undernutrition leading to a down-regulation of PGC-1 α protein in IUGR skeletal muscle (Huber *et al.*, 2009). However, there is no evidence in this model of IUGR that the reduction of PGC-1 α was related to insulin insensitivity (Thompson et al., 2007). Perhaps the non-linear relationship between NRF1 and PGC-1 α noted in this chapter indicates that alternative pathways are acting to preserve and enhance metabolic flexibility in these IUGR offspring.

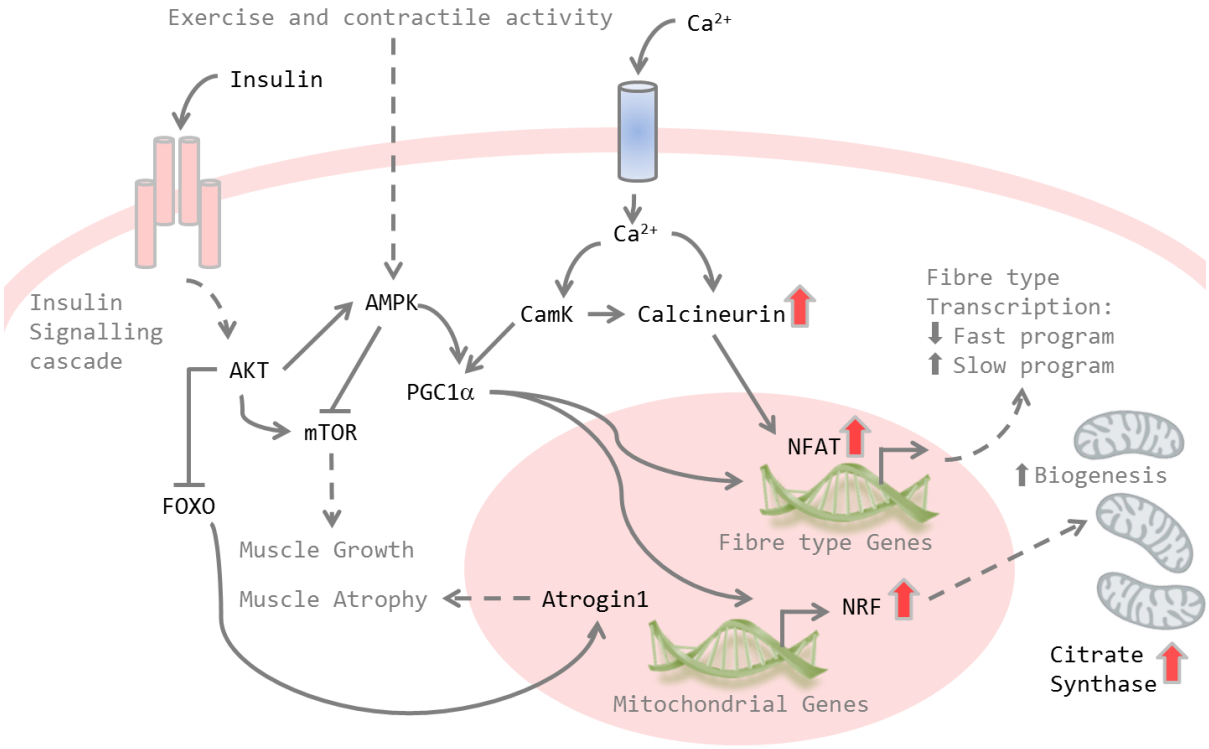

Figure 3.7 Overview of the factors that affect skeletal muscle phenotype.

Repeated here to assist consideration of results discussed in the text, the interrelated pathways proposed to be affected by prenatal undernutrition. The calcineurin-NFATc1 signalling pathway is activated by Ca2+ or slow fibre nerve activity, raising intracellular [Ca2+]. NFATc1 is activated and translocates to the nucleus of the myotube where it binds to slow fibre program promoters. The Insulin/Akt pathway regulates muscle hypertrophy and activates downstream effectors such as mTOR to stimulate protein synthesis and inhibits muscle atrophic pathways FOXO. Upstream, exercise and contractile activity influence AMPK directing PGC1 α activity. PGC1 α is a dominant regulator of mitochondrial content, acting upstream of NRF, and PGC1 α also influences muscle fibre type plasticity via NFATc1. Citrate synthase enzyme activity is a proxy measure that indicates oxidative metabolism (increased in soleus muscle of IUGR offspring compared with AD controls *see Chapter 2).* Red arrows indicate the effect of IUGR on relative mRNA expression measured in the present study. Broken lines indicate some intermediate steps have been omitted. Helices and mitochondrial clipart faithfully excerpted from published sources in the public domain, 2011.

The role that mitochondrial factors play in influencing oxidative capacity has generated conflicting reports in the literature. For example, animals lacking PGC-1 α have been reported to be exercise intolerant and with reduced expression of key mitochondrial proteins (Uguccioni and Hood, 2011). Whereas, alternative studies have shown that PGC-1 α deficiency does not limit mitochondrial biogenesis and that adaptation to an exercise programme was possible (Leick *et al.*, 2008). More recently, researchers studying the influence of prenatal undernutrition and postnatal exercise effects on PGC-1 α noted a 20% reduction in PGC1 α protein levels in early adulthood. They went on to suggest that additional metabolic stress such as exercise, aging or high-fat feeding was needed to 'unmask' the programmed effect on mitochondrial biogenesis (Laker et al., 2012). While exercise was not a factor investigated in the present study, the capacity to engage in exercise is of considerable interest. Markers of mitochondrial content, indicating oxidative capacity, were largely unchanged at the mRNA level by prenatal undernutrition.

Furthermore, atrophy (and atrophy inhibiting) pathways do not appear to be changed as a result of prenatal undernutrition, although there is increased variation of fboxo32/Atrogin mRNA in the soleus of IUGR Adults. As described in Chapter 2 of this thesis, an increase in the factors responsible for actively reducing Type II fibre size in IUGR muscle was anticipated. However, there is no significant difference between IUGR and AD muscle mRNA expression of atrogenic factors. While there were indications that mitochondrial content might be influenced by prenatal undernutrition, for instance with the proxy citrate synthase measures, we do not see this clearly, at least not at the mRNA level. The phenotype consistently observed in offspring of undernourished dams may be further explained by other pathways that were not investigated in the present studies. Furthermore, there may be alternate mechanisms evident at the protein level.

While it is possible that the Type I fibres in the muscles of the IUGR offspring are specifically protected from atrophy at weaning, further studies would be needed to confirm this. Given that the data for the atrophy factor fboxo32 do not show significant difference between offspring of either level of maternal nutrition, it could be possible that fboxo32 plays a minimal role in preserving muscle after a nutritional

insult. It could be argued equally plausibly that fboxo32 was not responsible for the initiation of Type IIa fibre atrophy in this model given that there are no differences between IUGR and AD offspring mRNA expression.

Data presented in Chapter 2 of this thesis suggest that a decrease in fibre size may be the first step in the development of muscle morphology characteristic of a metabolically flexible status. At least two potential mechanisms can be proposed to attempt to explain shifts in fibre type composition. Firstly, fibre type conversion from Type IIa to Type I fibres may be increased. This shifting in fibre type is a known consequence of endurance training; however, rats in the present study were not exposed to endurance exercise. Secondly, and perhaps more likely, a change in apoptotic rate may explain a selective decrease in muscle fibre size, which may later lead to an increase in Type IIa fibre numbers in IUGR offspring. Selective atrophy of skeletal muscle fibres is known to occur via the FOXO signalling pathway under conditions that remove FOXO inhibition of atrogenes responsible for atrophy and wasting of skeletal muscle (Sandri *et al.,* 2004). Upstream of FOXO is the PGC-1 α protein that was previously shown to be decreased in soleus of adult offspring of undernourished mothers (Huber *et al.,* 2009). Decreased PGC-1 α could impact on signalling pathways via a diminished FOXO inhibition of atrogenes (Sandri *et al.,* 2006). However, this study did not show any significant effect of prenatal nutrition on PGC-1 α of either muscle. Control of both myogenic and atrophy processes in skeletal muscle are not limited to modulation by gene transcription. Post-transcriptional events must also be considered (Jackson, 2000). Following that, the demands placed on the IUGR offspring of undernourished dams may be met by a constitutively higher expression of factors key to the calcineurin/NFAT pathway.

Methodological considerations.

It is important to recognise that many of the experimental results have failed to falsify the null hypothesis that no differences in the mRNA levels exist between IUGR and AD offspring. The variation in the data is relatively high compared with the differences between the variables. This variation arose from several sources. First, the cohort of Weaning rats sampled in my study included both male and female rats, and, second, the muscle samples at birth and weaning contained both gastrocnemius and soleus muscle tissues. In addition, some factors alter with sex and age. For example,

myostatin is known to decrease with age in rodents and exhibits sexual dimorphism, with males being lower than females (McMahon *et al.*, 2003; McFarlane *et al.*, 2005). The combination of: (1) only being able to sample mixed muscles; and (2) the short timeframe from birth to weaning meant that the experiment lacked the statistical power required to detect any effects of prenatal nutrition and their interactions. As acknowledged earlier, the difficulties in showing statistical differences between ratios by ANOVA mean that larger sample sizes would also be needed. Further study would require careful design to overcome the challenges experienced in this study. Any further attempt to continue such work would very likely benefit from parallel experiments at, for example, the protein level to test whether processes at the RNA and protein levels were consistent with each other and with similar experiments elsewhere.

3.5 Summary and Conclusions

Prenatal undernutrition has the potential to alter pathways that influence skeletal muscle development, plasticity and oxidative potential. I now report enhanced pathways known to influence the slow Type I fibre type programme at a transcriptional level for offspring of undernourished rats in adulthood. In particular, at the transcriptional level, the mRNA expression of factors in the calcium signalling pathway is up-regulated by maternal undernourishment during pregnancy. Both NFATc1 and calcineurin are physiologically activated by exercise and I have shown these factors are enhanced even in a sedentary environment. In addition, there were indications of enhanced mitochondrial biogenesis at the mRNA level in soleus muscle of IUGR compared with AD offspring. The alterations to NRF1 mRNA expression may relate to the proposed enhanced oxidative metabolism of IUGR offspring and warrants further investigation. There were no significant differences detected in Neonatal or Weaning skeletal muscle because of detectability issues together with inability to separate muscle types. In future studies it would be necessary to enhance the methodological approach with protein measures, particularly for the MRFs and myostatin. The present study provides further evidence that skeletal muscle compensates for poor prenatal nutrition to enhance chances of survival in postnatal life.

Chapter 4.

Effects of Prenatal Undernutrition and Postnatal Physical Activity on Metabolic Flexibility

4.1 Introduction

Over the last century there has been a dramatic change in the physical environment, especially with respect to food availability and physical activity. Cultural shifts, particularly in developed countries with the so-called western lifestyle have led to physical activity being engineered out of daily life (Booth and Shanely, 2004). Relative inactivity, in combination with increased consumption of energy-dense food is typical of the western lifestyle. The metabolic consequences of the western lifestyle are acknowledged to lead to obesity. At a basic level, obesity is the result of an energy balance tipped to higher energy intake and reduced energy expenditure (Jequier, 2002). Physical activity can influence energy balance favourably and is also an effective means of improving metabolic health (Fogelholm, 2006). However, physical activity is multidimensional and a complex variable to measure (Andersen *et al.*, 2009). That is, the 'dose-response' relationship between physical activity and health can vary greatly from individual to individual. Therefore, it is imperative to dissect the molecular benefits of lifestyle behaviours such as physical activity in order to promote metabolic health.

Susceptibility to obesogenic influences can be dependent on multiple factors, including both genetics and environment. Studies in this laboratory and others have demonstrated that maternal undernutrition during pregnancy, a model of intrauterine growth restriction (IUGR), influences metabolism in offspring (Woodall *et al.*, 1996a; Breier *et al.*, 2004). The nutritional deprivation experienced in the womb leaves these IUGR offspring of undernourished dams with increased risk of developing postnatal obesity (Thompson *et al.*, 2007). Initially, work in this field explored the idea that the IUGR offspring were 'programmed' to be inactive as a consequence of their undernourished state during gestation. The IUGR offspring were deemed 'couch potatoes' as they exhibited both hyperphagia and apparently reduced locomotor

behaviour in preliminary investigations (Vickers *et al.*, 2000; Breier *et al.*, 2001). Subsequent to that research, Miles et al. (2009) used robust theory-based quantitative methods of behaviour analysis to show an almost converse effect of prenatal under-nutrition on postnatal choice for running in a wheel in IUGR offspring (Miles *et al.*, 2009b). In the Miles (2009) studies, the IUGR offspring showed increased preference for running in a wheel rather than lever pressing for food rewards when compared with the AD offspring. Furthermore, the metabolic benefits of engaging in this 'moderate physical activity' became apparent when the specific prenatally-induced obesity was prevented in these offspring of undernourished dams. Therefore, the results of these studies have led to the hypothesis that offspring of undernourished mothers are willing to engage in exercise and are able to gain metabolic benefits from moderate daily exercise. This chapter builds on the findings of the research and explores the hypothesis that an active postnatal lifestyle offers potential to improve metabolic flexibility and increase fuel utilisation in the IUGR offspring of undernourished dams. The particular postnatal lifestyle factors explored here are physical activity and feeding regime.

As discussed in Chapter 2 (Norman *et al.*, 2012), a thrifty metabolism might mean IUGR offspring of undernourished dams have increased capacity to store fat and glycogen, and may also have adequate flexibility to utilise these stores. In prenatally-induced obese rats, fat deposition has already been shown to be restricted to physiological fat depots and, importantly, insulin sensitivity was conserved (Thompson *et al.*, 2007). The IUGR offspring show hyperinsulinaemia and hyperglycaemia, yet the hyperinsulinaemic-euglycaemic clamp demonstrated they had maintained peripheral insulin sensitivity. This suggests a unique metabolic setting for offspring of undernourished dams: a metabolic setting that has an enhanced ability to oxidize carbohydrate and lipid stores. As discussed in Chapters 2 and 3, IUGR offspring are proposed to have the additional advantage of a skeletal muscle physiology that supports metabolic flexibility. In this context, the thrifty phenotype enables offspring who experienced nutritional deprivation during fetal development to be capable of going long distances for food in later postnatal life. A thrifty metabolism might imply effective energy storage for later utilisation during endurance style physical activity. Therefore, I ask the question: what are the effects of postnatal lifestyle on metabolic flexibility for IUGR offspring?

The main aim of the research was to assess the impact of postnatal lifestyle manipulations on metabolic outcomes for IUGR offspring of undernourished dams. The present study was designed to compare interactions between the prenatal environment and varied postnatal "lifestyle" paradigms. Three different postnatal manipulations represent the alternate "lifestyles". The first is a **Sedentary** style condition with confinement to home cages with littermates and an unrestricted *ad libitum* feeding regime. For both the second and third lifestyle conditions, a restricted timed-feeding regime was employed in which access to food was confined to a 2-hour period each day. The second postnatal condition is **Non-exercised**, but unlike the sedentary condition, non-exercised animals were challenged by environmental enrichment, providing a small amount of physical activity associated with exploring the behavioural cage and lever pressing. The third postnatal group, the **Exercised** group, were also able to explore the behavioural cages, but gained additional exercise in a running in a wheel.

4.2 Methods.

4.2.1 Experimental Design

Rat offspring were bred as in Chapter 2, in accordance with previously published experimental procedures (Woodall *et al.*, 1996a; Woodall *et al.*, 1996b; Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009b). At Weaning (22 d) male IUGR rats, the offspring of undernourished dams, and male AD rats were housed in pairs with *ad libitum* access to water and chow (Teklad: 18% protein, 5% fat diet, Oxon, UK). All rats were exposed to a 12-hr light:dark cycle with lights on at 0600 h. At 46 d of age, male offspring were further divided into six experimental groups: AD Sedentary (n = 12), AD Non-exercised (n = 10), AD Exercised (n = 10), IUGR Sedentary (n = 12), IUGR Non-exercised (n = 10) and IUGR Exercised (n = 10).

Scheduled feeding was established for AD and IUGR Non-exercised and Exercised groups, in which food access was limited to two hours per day during the light-phase for all animals to ensure standardisation of feeding patterns for these four groups. The remaining AD and IUGR Sedentary groups were given 24 h unrestricted *ad libitum* food access.

In summary, the experimental groups are listed below:

1. AD offspring of ad libitum fed mothers,
    a. sedentary postnatal experience
    b. Non-exercised postnatal experience
    c. Exercised postnatal experience
2. IUGR offspring of undernourished mothers,
    a. sedentary postnatal experience
    b. Non-exercised postnatal experience
    c. Exercised postnatal experience

From 60 d of age, both exercised and non-exercised groups were individually placed into operant chambers (Med Associates Inc., St. Albans, VT, USA, Model ENV-007) with a running wheel for 1 h per day. The running wheel was controlled by a brake system in such a way as to ensure each animal ran (on average) the same distance each day (56 ± 0.6 m). Controlled voluntary exercise as opposed to forced, treadmill-type, exercise was employed to exclude any possible influence of hormonal or metabolic stress responses. This daily exercise programme was determined through a validation study (using different animals) in which AD and IUGR animals (*n* per group = 10) were given free access to a running wheel for 2 hours a day over the course of several weeks. Under these conditions, all rats voluntarily ran at least 56 m per daily session. The exercise sessions were conducted during the light phase as voluntary wheel running during this phase has been shown to be far more stable within and between sessions than during the dark phase (Eikelboom and Lattanzio, 2003; Lattanzio and Eikelboom, 2003; Belke and McLaughlin, 2005). For the Non-exercised animals, the brake was fixed in place so that no wheel running could take place. They had also access to the operant chambers for 1 h per day but exact quantity of locomotory activity could not be determined in this group.

Body weights and food intake of all animals were measured daily throughout the study. Food intake was calculated as kcal/g (BW) and included any calories consumed via the sugar pellets in the behavioural chambers. At 150 d of age, all animals were placed under halothane anaesthesia to enable dual energy x-ray absorptiometry (DEXA) scanning (Lunar Prodigy, GE Medical Systems, USA). Afterwards, rats were fasted overnight, killed by decapitation under halothane anaesthesia, and trunk blood

was collected. Body length (nose-anus), retroperitoneal fat pad weight, liver weight, soleus and gastrocnemius skeletal muscles weight were recorded. Importantly, the last bout of exercise occurred approximately 18 hours prior to tissue and plasma collection, so that the analyses in the present study represent the metabolic conditions during the post-exercise recovery phase.

The 150 d end point for studies was selected as appropriate based on *post-hoc* analysis of endocrine measures from the previous studies (Miles *et al.*, 2009a). At this age, plasma concentrations of endocrine measures demonstrated the key features of the IUGR model (Huber *et al.*, 2009; Miles *et al.*, 2009a). All blood samples were taken into heparinised tubes and centrifuged at 4°C to separate plasma. Plasma was stored at -20°C until analysis. Livers were snap-frozen in liquid nitrogen and stored at -80°C for future analysis. All procedures involving animals were approved by the University of Auckland Animal Ethics Committee.

4.2.2 Apparatus

Apparatus setup included ten conventional operant chambers for rats (Med Associates Inc., St. Albans, VT, USA, Model ENV-007) with dimensions 305 mm x 241 mm x 292 mm situated in sound- and light-attenuating shells. A house light provided ambient light, and a fan provided ventilation. Access to a 356 mm diameter running wheel was provided throughout the session through a raised guillotine door on the back wall. The distance run in the wheel was measured during each of the exercise sessions in terms of centimetres run in either direction. All experimental events were controlled remotely by an IBM-compatible PC running MED-PC© software (Med Associates Inc., St. Albans, VT, USA) and timing of each experimental event was recorded at a resolution of 10 ms.

4.2.3 Scheduled feeding and the Behavioural Tests

Scheduled feeding was established in all offspring of the Non-exercised and Exercised groups. Scheduled feeding meant that these animals had unrestricted food access limited to a two hour period per day. The rationale was to ensure food pellets in the experimental chambers were effective reinforcers during the behaviour tests. Behavioural sessions for AD and IUGR Exercised and Non-exercised groups were

conducted daily between 0900 h and 1200 h, immediately followed by 2 hours of *ad libitum* feeding.

4.2.4 Oral Glucose Tolerance Test

Following an overnight fast a fasting blood sample was taken (150 µl) from Sedentary animals only. Rats were then orally gavaged with 50% glucose solution (3g glucose /kg body weight) and 100 µl blood samples were collected at 15, 30, 45, 60, 90, 120 minutes post-gavage. All blood samples were collected via tail vein bleeding into 0.5 ml microfuge tubes containing heparinised saline. Microtubes were then centrifuged for 5 mins at 12,000 g and plasma pipetted into fresh tubes. All samples were stored at -20ºC until further analysis. Insulin from plasma was measured by ELISA kit according to the manufacturer's instructions (Mercodia ultra-sensitive Rat Insulin Elisa). Plasma glucose was measured by enzyme colorimetric assay using an automated bioanalyser (Roche/Hitachi 902; Roche Diagnostics, Penzberg, Germany).

4.2.5 Plasma and Tissue Collection

Sample collection of Adults was performed as previously described in Chapter 2 and 3 of this thesis (Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009a). In short, blood was collected into heparinised tubes and stored on ice until centrifugation. Plasma was stored at -20 °C until analysis. Soleus and superficial gastrocnemius (GSUP) muscle from one leg of offspring were also snap-frozen and stored at -80 °C. The respective muscles from the other leg were prepared by fixing onto cork plates. Fixed muscles were frozen in liquid N_2-cooled isopentane and stored at -80 °C to ensure tissue integrity for immunohistochemical studies.

4.2.6 Immunohistochemical and Morphometrical Studies

Skeletal muscle morphology and fibre type composition was analysed in offspring immunohistochemically as previously described in Chapter 2 (Huber *et al.*, 2009). Briefly, to examine fibre type in GSUP and soleus muscles, 6 µm slices were cut from the mid-belly region of frozen muscles across the fibre direction. Slow and fast MHC antibodies were used to detect type I fibres (MHC slow positive; MHCslow antibody, Novocastra Laboratories) and type IIa fibres (MHC fast positive; MHCfast (Type IIa), Alexis Biochemicals). Antibodies are also listed in Appendix II. Type IIb fibres of

GSUP were detected as unstained fibres on slices with Type I/IIa co-staining. Immunodetection procedure involved washing frozen sections and blocking with blocking solution (filter sterilised 4 % goat serum, 0.5 % Triton X, 0.1 % NaN3 in 1x PBS). Primary antibody was incubated in blocking solution overnight in a humidified chamber at 4 °C. Slices were then incubated for 2 h with secondary antibody (anti mouse Cy3 labelled, Jackson Immuno Research Laboratories Inc) at RT, washed again with PBS and covered with covering medium (80 % glycerol in 1x PBS with 0.1 % NaN3, pH 7.0). Fluorescence signals were detected by a fluorescence microscope (Olympus) and 5 pictures/slice/staining were taken with 20x magnification using a digital camera. In each muscle, 700 - 850 fibres per animal were counted, type-classified by Baerbel Leppich (Hannover, Germany) and were expressed as a percentage of total counted fibres. Size measurements of 50 randomly selected specifically immunostained fibres were made.

4.2.7 Plasma Metabolite and Hormone Assays

Plasma glucose, FFA, urea, glycerol, low density lipoproteins (LDL) and high density lipoproteins (HDL) were measured by enzyme colorimetric assay using an automated bioanalyser (Roche/Hitachi 902; Roche Diagnostics, Penzberg, Germany). Total plasma T_3 and T_4 were measured by a commercially available coated tube radioimmuno assay according to the manufacturer's protocol (Siemens Count-a-Coat, sensitivity: 0.1nmol/L # TKT31; 3.2 nmol/L # TKT41).

4.2.8 Muscle Assays

The muscle triglycerides (TG) for the Non-exercised and Exercised groups only, and glycogen contents for all was measured as previously described (see Chapter 2). Tissue triglycerides were extracted as described by Chen & Nyomba (Chen and Nyomba, 2004). Briefly, triglycerides were extracted from 30 mg frozen powdered muscle and liver samples with 2 ml of 2:1 chloroform methanol for 90 min. Methanol (0.4 ml) was then added, and the extract was vortexed for 30 s. After centrifugation at 1,100 g for 10 min, the supernatant was collected, mixed with 0.5 ml of 0.04% Calcium Chloride, and centrifuged at 550 g for 20 min. The upper phase was removed and the interface was washed three times with a mixture of chloroform (1.5 ml), methanol (24 ml) and water (23.5 ml). The final wash was removed, and methanol

(0.05 ml) was added to obtain one phase. The samples were dried at 60°C under nitrogen and re-dissolved in 0.05 ml of 3:2 tert-butyl alcohol-Triton X-100. Triglycerides were quantified by enzyme colorimetric assay in an automated bioanalyser (Roche/Hitachi GOD-PAP).

A total of 0.05 – 0.1 g of muscle tissue was weighed into polypropylene tubes and combined with 19 volumes (0.45 – 0.9 ml) of 10 mmol/L sodium acetate buffer (pH 4.6) to make a 1:20 solution. Samples were homogenized on ice using an Ultra Turrax homogenizer, and 0.5 ml of homogenate transferred to a 2 ml vessel containing 0.1 ml of amyloglucosidase (60 units/ml). The mixture was then incubated at 37 °C in a water bath for 2 hours to digest the glycogen to free glucose. Samples were centrifuged for 5 minutes and analyzed for glucose concentration using an automated bioanalyser (Roche/Hitachi GOD-PAP, Roche Diagnostics, Penzberg, Germany) by the glucose oxidase method. Glycogen concentrations were expressed relative to tissue weight. All samples were analysed on a single assay.

4.3 Results

4.3.1 Whole organism measures

Body Size, Composition and Growth Rates

The results of this study indicate that postnatal environmental conditions had a strong impact on body composition. The growth trajectory observed for offspring of undernourished dams in this study shows a reduced velocity of postnatal growth, especially for the Sedentary group (Figure 4.1). Throughout the study, the IUGR offspring were generally lighter than their AD counterparts. Figure 4.1 shows that both Prenatal nutrition and Postnatal lifestyle had a significant effect on body weight Comparisons of area under the curve showed a significant effect of both pre- and postnatal nutrition on body weight (Two-way ANOVA: prenatal nutrition ($P < 0.0001$); postnatal lifestyle ($P < 0.0001$)). *Posthoc* comparisons by Bonferroni test within group showed that prenatal nutrition produced significantly lighter offspring for the Sedentary and Non-exercised groups ($P < 0.01$).

Prenatal	Postnatal	**Day 22 (g)**	**Day 37 (g)**	**Day 49 (g)**	**Day 61 (g)**	**Day 71 (g)**	**Day 100 (g)**	**Day 121 (g)**	**Day 131 (g)**
AD	Sedentary	63.3 ± 1.0	170.4 ± 6.5	274.9 ± 8.0	355.3 ± 13.6	423.5 ± 10.3	503.1 ± 14.3	550.0 ± 17.9	570.5 ± 18.8
	Non-exercised	63.9 ± 1.2	168.0 ± 4.8	201.2 ± 3.5	277.5 ± 3.6	322.2 ± 5.4	383.3 ± 6.8	416.8 ± 7.7	430.0 ± 6.9
	Exercised	63.7 ± 1.0	174.0 ± 3.0	196.9 ± 4.3	263.8 ± 6.0	307.2 ± 8.2	367.8 ± 9.7	397.6 ± 8.7	412.9 ± 9.1
IUGR	Sedentary	**50.9 ± 1.8***	**144.6 ± 5.5***	**235.4 ± 7.4***	**320.5 ± 8.0***	**372.5 ± 9.3***	**460.6 ± 11.7***	**508.1 ± 14.2***	**526.7 ± 13.6***
	Non-exercised	**51.8 ± 2.8***	**134.7 ± 8.8***	**172.5 ± 7.8***	**237.3 ± 10.8***	**285.1 ± 14.2***	345.2 ± 12.9	374.1 ± 12.1	390.7 ± 12.0
	Exercised	**50.9 ± 1.5***	**140.6 ± 5.7***	175.4 ± 4.5	238.7 ± 5.2	283.1 ± 6.4	340.4 ± 7.5	370.0 ± 8.1	384.0 ± 9.07
Two Way ANOVA									
	Prenatal	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0008**	**0.0001**	**0.0001**	**0.0001**
	Postnatal	0.8958	0.5133	**0.0001**	**0.0001**	0.0862	**0.0001**	**0.0001**	**0.0001**
	Interaction	0.9729	0.7489	0.3644	0.7152	**0.0050**	0.7885	0.8059	0.8416

Table 4.1 The effect of prenatal nutrition and postnatal lifestyle on body weight.

The body weight for AD offspring and IUGR offspring in the Sedentary, Non-exercised and Exercised groups (values are mean ± SEM; *n* = 10-12).Significant treatment or interaction effects are indicated in bold (assessed by Two-way ANOVA). *Posthoc* comparisons by Bonferroni test within group showed that there were significant differences AD vs. IUGR indicated by bold * ($P < 0.05$).

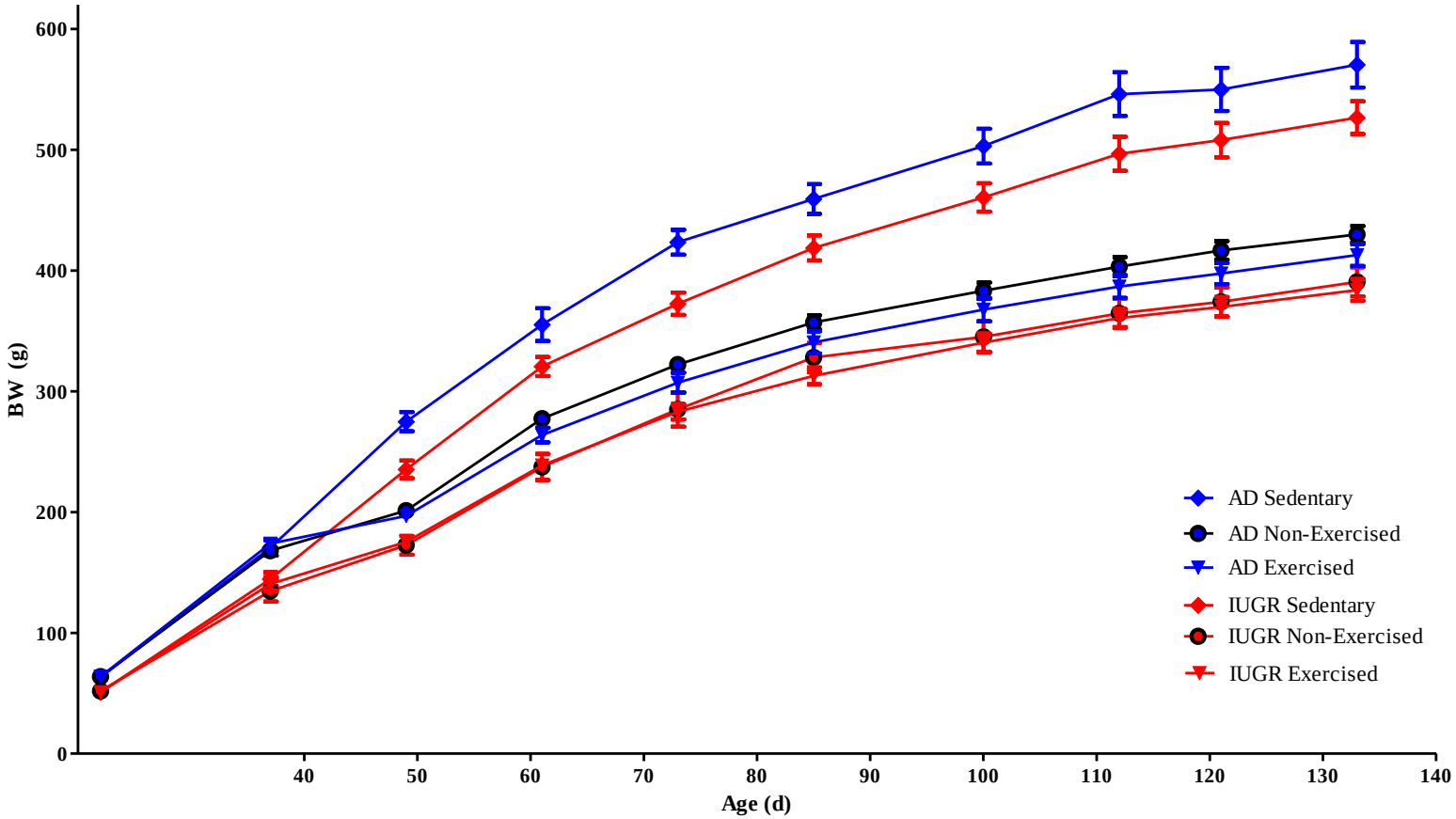

Figure 4.1 The effect of prenatal nutrition and postnatal lifestyle on body weight.

The body weight for AD offspring (blue symbols) and IUGR offspring (red symbols) in the Sedentary (diamonds), Non-exercised (circles) and Exercised (triangles) groups (values are mean ± SEM; n = 10-12). Area under the curve analysis (Two-way ANOVA: prenatal nutrition ($P < 0.0001$); postnatal lifestyle ($P < 0.0001$) *Posthoc* Bonferroni test, AD vs. IUGR offspring in the Sedentary and Non-exercised groups ($P < 0.01$).

4.3.2 Food Intake

Over the course of the experiment, food intake across the experimental groups was highly variable (Figure **4.2** and Table 4.2). Initially, the animals adjusted to their assigned feeding regime at around 58 days and then after the onset of the exercise, the influence on food intake behaviour became apparent. The influence of prenatal nutrition was evident from day 79 onward (Two-way ANOVA $P < 0.01$). Postnatal conditions had significant influence on food intake from the day 79 - 81 period until the day 121 – 123, where an interaction between pre- and postnatal conditions was evident. The *posthoc* Bonferroni analysis showed variability across the postnatal lifestyle conditions for within group comparisons, AD vs. IUGR offspring. Offspring of the Sedentary group showed the first sign of AD and IUGR differences in food intake at the day 79 – 81 time period. Then, at the day 100 – 103 time period, there is a significant *posthoc* difference between AD and IUGR offspring of the Exercised group (Table 4.2). Finally, there is a significant difference between AD and IUGR offspring for food intake in both Non-exercised and Exercised postnatal groups (Table 4.1). Area under the curve analysis showed that there was no overall effect of prenatal nutrition or postnatal lifestyle (Two-way ANOVA $P = 0.7841$) Postnatal lifestyle ($P = 0.3853$) on food intake over time with an interaction of $P = 0.7198$.

Furthermore, the Non-exercised and Exercised animals demonstrated no differences in the number of sugar pellets consumed in the behavioural chambers (number of pellets: AD Non-exercised: 65.79 ± 1.3; IUGR Non-exercised: 65.58 ± 1.4; AD Exercised: 66.72 ± 0.3; IUGR Exercised: 66.52 ± 0.68 (ANOVA effect of prenatal nutrition $P = 0.8450$; effect of exercise $P = 0.3687$; interaction $P = 0.9969$. $n = 10$).

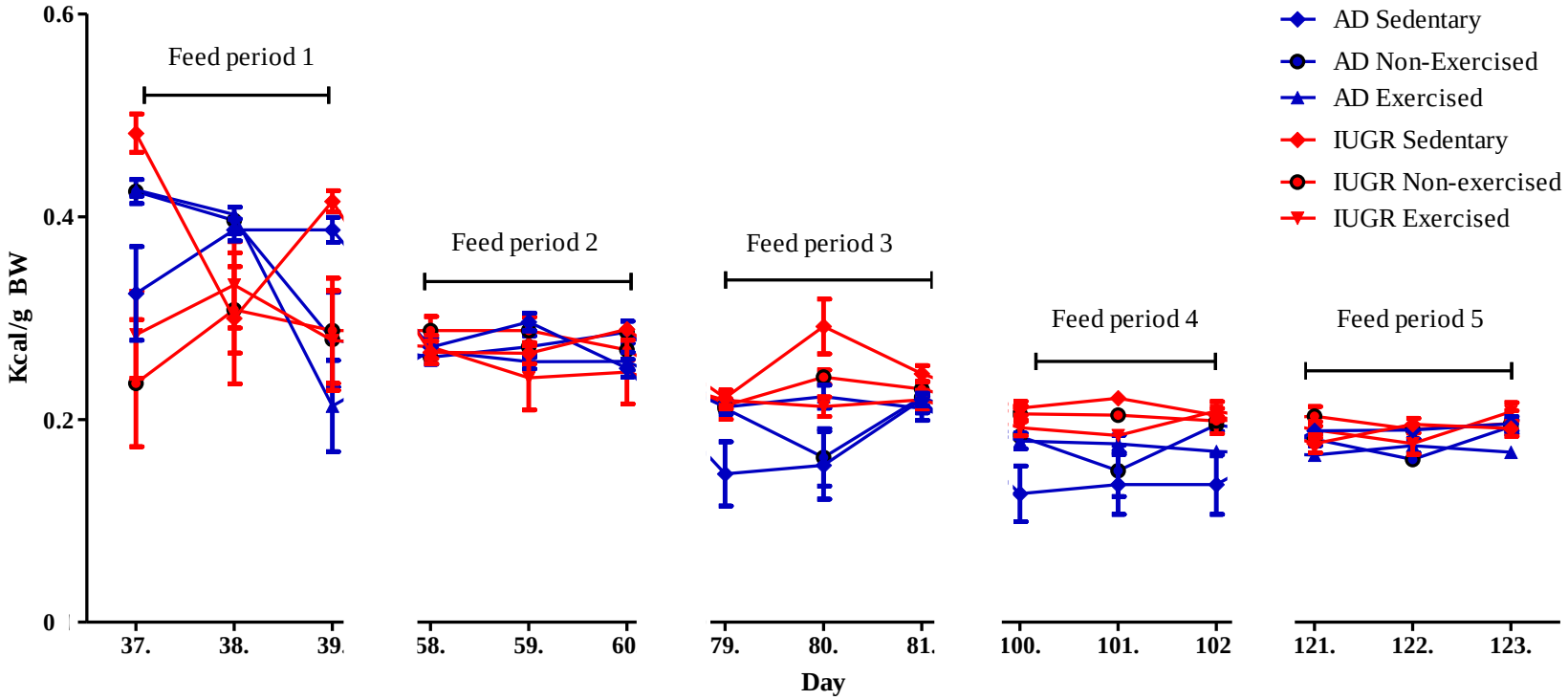

Figure 4.2 Representative figure for food intake measurement periods

Food intake for AD offspring (blue symbols) and IUGR offspring (red symbols) in the Sedentary Adults (diamonds), Non-exercised (circles) and Exercised (triangles) groups (values are mean ± SEM; *n* = 10-12). Food intake is average food per cage/body weight of individuals for days 37-39 where Exercised and Non-exercised animals were adjusting to the scheduled feeding; days 58-60; day79-80 after day 60, the Exercised group were exposed to the wheel for voluntary running sessions; at day100-102 these animals are all approaching adulthood; day 121-123 (indicated by black columns). Data analysis by Two-Way ANOVA is detailed in Table 4.2.

Prenatal	Postnatal	Day 37-39 (Kcal/g BW)	Day 58-60 (Kcal/g BW)	Day79-81 (Kcal/g BW)	Day100-102 (Kcal/g BW)	Day 121-123 (Kcal/g BW)
AD	Sedentary	0.37 ± 0.02	0.27 ± 0.01	0.22 ± 0.01	0.20 ± 0.004	0.19 ± 0.004
	Non-exercised	0.37 ± 0.02	0.27 ± 0.01	0.21 ± 0.01	0.19 ± 0.004	0.18 ± 0.004
	Exercised	0.35 ± 0.02	0.26 ± 0.01	0.22 ± 0.01	0.17 ± 0.003	0.17 ± 0.003
IUGR	Sedentary	**0.45 ± 0.01***	0.27 ± 0.01	**0.25 ± 0.01***	0.21 ± 0.003	0.19 ± 0.004
	Non-exercised	**0.29 ± 0.03***	0.28 ± 0.01	0.23 ± 0.01	0.20 ± 0.01	**0.20 ± 0.01***
	Exercised	0.30 ± 0.03	0.27 ± 0.01	0.22 ± 0.01	**0.20 ± 0.01***	**0.19 ± 0.01***
Two Way ANOVA						
	Prenatal Nutrition	0.3879	0.1347	**0.0078**	**0.0001**	**0.0008**
	Postnatal	**0.0001**	0.2477	**0.0057**	**0.0001**	0.0862
	Interaction	**0.0002**	0.5913	0.1501	0.6243	**0.0050**

Table 4.2 Food Intake (average food per cage/body weight of individuals)

The food intake for AD and IUGR offspring in the Sedentary, Non-exercised and Exercised groups (values are mean ± SEM; *n* = 10-12). Food consumed measured in Kcal/g BW averaged over days 37 – 39; d 58 – 60; d 79 – 81; d 100 – 102; d 121 - 123. Significant treatment or interaction effects are indicated in bold (assessed by Two-way ANOVA). *Posthoc* comparisons by Bonferroni test within group showed that there were significant differences AD vs. IUGR indicated by bold * ($P < 0.05$).

4.3.3 Physical Activity in Exercised Offspring

With the increasing number of sessions in the running wheel, IUGR Exercised rats completed more turns at a faster rate than the AD Exercised rats (Figure 4.3). Using non-linear regression in the Graphpad Prism software package, comparison of curve fits between AD and IUGR found the speed of running per five-session blocks changed in a statistically significant manner for the AD and IUGR rats. The mean speed of running across successive five-session blocks (Figure 4.3A) was significantly different in trajectory between AD Exercised and IUGR Exercised ($P < 0.001$) and had R^2 values of (AD) = 0.81 and (IUGR) = 0.91. The IUGR rats ran faster than the AD rats at the start of testing, but this difference progressively reduced across sessions until running rates were similar after a total of 85 sessions. Figure 4.3B shows the difference between paired AD and IUGR offspring decreased significantly over time with an R^2 of 0.71 ($P < 0.001$).

4.3.4 Internal physical structure

Organ weight and DEXA body composition

Two-way ANOVA test showed that the livers and brains were not significantly different in weight, relative to overall bodyweight, between the AD and IUGR offspring (Table 4.3). Also at the experimental end point, offspring in the IUGR group were not significantly different to the AD controls significantly lighter ($P < 0.05$) and shorter ($P < 0.05$), but there were no significant effects of prenatal nutrition on fat or lean mass at 150 d (Table 4.4). Bonferroni *posthoc* test showed there was no effect of prenatal nutrition on fat or lean mass for animals in the Non-exercised or Exercised postnatal groups at 150 d. However, Bonferroni *posthoc* test did reveal that Sedentary postnatal lifestyle led to a significant decrease in body mass for IUGR offspring of undernourished dams compared with Sedentary AD control offspring Table 4.4.

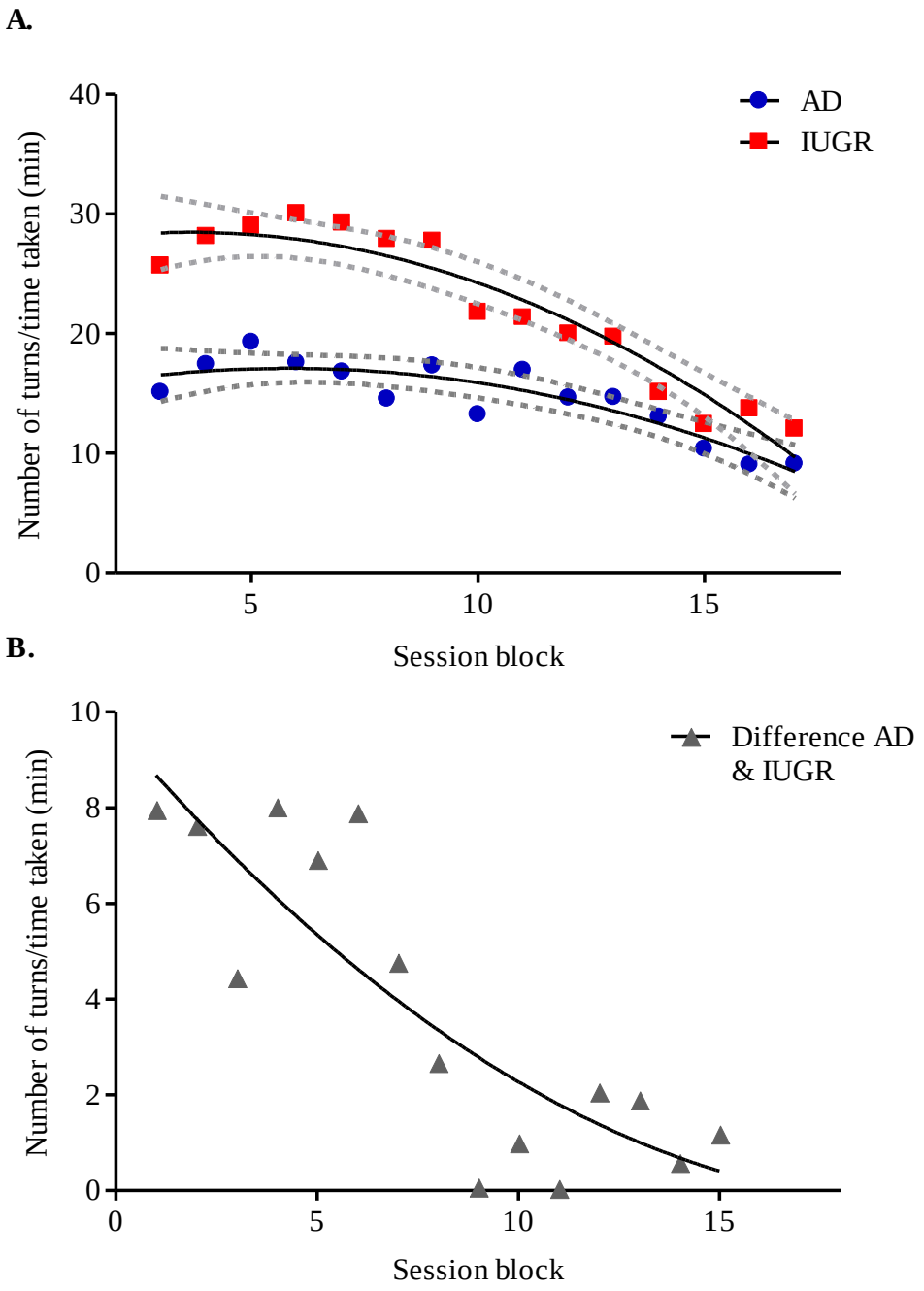

Figure 4.3 Mean speed of running for AD and IUGR Exercised offspring

Average wheel turns/average time (min) taken spent running per session. **A.)** Shown on the X axis are the mean results for every 5 sessions (session block) for AD Exercised (blue *n*=10) and IUGR Exercised groups (red *n*=9) with 95% confidence intervals (in grey broken lines). Also shown (in black) are the nonlinear regressions lines. R^2 values of (AD) = 0.81 and (IUGR) = 0.91. **B.**) Trend for average physical activity difference between paired AD and IUGR animals (grey triangles). Non-linear 2^{nd} order polynomial regression with 95% confidence intervals shows how speed decreased per session block. R^2 value for the fitted line = 0.72.

Prenatal	Postnatal	Liver (% BW)	Brain (% BW)
AD	Sedentary	2.3 ± 0.12	0.39 ± 0.02
	Non-exercised	2.8 ± 0.07	0.48 ± 0.01
	Exercised	2.7 ± 0.06	0.50 ± 0.01
IUGR	Sedentary	2.5 ± 0.09	0.38 ± 0.02
	Non-exercised	2.9 ± 0.07	0.58 ± 0.08
	Exercised	2.9 ± 0.11	0.51 ± 0.01
Two Way ANOVA			
	Prenatal Nutrition	0.1041	0.3277
	Postnatal	**0.0001**	**0.0036**
	Interaction	0.7973	0.3277

Table 4.3 The effect of prenatal nutrition and postnatal environment on vital organ size.

The liver and brain weights for AD vs. IUGR in the Sedentary, Non-exercised and Exercised groups taken at dissection (values are expressed relative to bodyweight (BW) and written mean ± SEM; *n* = 10-12). Two-way ANOVA results are presented beneath the values and where significantly different, are indicated by bold where (P < 0.05). No significant differences were detected in *post hoc* analysis.

Prenatal	Postnatal	Total Mass (kg)	%Fat	%Lean	Fat:Lean	BMD
AD	Sedentary	0.586 ± 0.021	32.60 ± 2.38	65.06 ± 2.3	0.51 ± 0.06	0.205 ± 0.002
	Non-exercised	0.424 ± 0.006	17.09 ± 1.67	80.03 ± 1.6	0.23 ± 0.03	0.208 ± 0.005
	Exercised	0.402 ± 0.008	18.62 ± 1.74	78.56 ± 1.7	0.21 ± 0.03	0.208 ± 0.005
IUGR	Sedentary	**0.532 ± 0.018***	31.55 ± 2.78	66.31 ± 2.7	0.48 ± 0.06	0.197 ± 0.003
	Non-exercised	0.381 ± 0.012	16.91 ± 1.41	83.29 ± 3.6	0.20 ± 0.02	0.202 ± 0.005
	Exercised	0.376 ± 0.009	19.83 ± 2.09	77.31 ± 2.1	0.26 ± 0.04	0.207 ± 0.006
Two way ANOVA						
Prenatal Nutrition		**0.0006**	0.9976	0.5847	0.9258	0.2175
Postnatal		**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	0.3620
Interaction		0.5836	0.8636	0.6583	0.8596	0.7117

Table 4.4 Total Mass & body composition by DEXA at 150 d age.

The body composition for AD vs. IUGR in the Sedentary, Non-exercised and Exercised groups (values are mean ± SEM; *n* = 10-12). Two-way ANOVA results are presented beneath the values and Bonferroni *posthoc* test, where significant, are indicated by bold with asterisk (*P < 0.05).

The DEXA studies revealed that both fat mass and lean mass were markedly affected by postnatal lifestyle (Table 4.4). The main effects pertaining to postnatal conditions were, that at 150 d, Sedentary animals were heavier ($P < 0.0001$) and fatter ($P < 0.001$) compared with Exercised and Non-exercised offspring. However, Bonferroni *posthoc* analysis did not reveal any difference in percentage fat between IUGR and AD groups as measured by DEXA. There was no significant influence of pre- or postnatal conditions on bone mineral density (BMD) (Table 4.4).

4.3.5 Skeletal Muscle Structure and Metabolism

The tissue weights as a percentage of body weight or the levels of triglyceride (TG) or glycogen (GLY) were not significantly affected by prenatal nutrition. Only the weight of soleus muscle was influenced significantly by postnatal conditions ($P < 0.05$), and the levels of TG could not be measured in the Sedentary group. Within group comparisons by Bonferroni *post hoc* test showed no differences within these postnatal groups. The main effect of prenatal nutrition on structural change was not observed in the soleus of lean IUGR Non-exercised animals at 150 d.

		M. *soleus*			**M. *gastrocnemius superficial***		
Prenatal	**Postnatal**	**% BW**	**TG**	**GLY**	**% BW**	**TG**	**GLY**
AD	Sedentary	0.05 ± 0.002	-	2.6 ± 0.3	0.55 ± 0.03	-	3.8 ± 0.2
	Non-Exercise	0.05 ± 0.002	0.15 ± 0.03	2.9 ± 0.1	0.58 ± 0.01	0.07 ± 0.02	3.6 ± 0.2
	Exercised	0.06 ± 0.002	0.11 ± 0.01	3.1 ± 0.3	0.61 ± 0.01	0.03 ± 0.01	3.8 ± 0.3
IUGR	Sedentary	0.05 ± 0.002	-	2.7 ± 0.3	0.57 ± 0.02	-	3.6 ± 0.6
	Non-Exercise	0.06 ± 0.003	0.16 ± 0.03	2.7 ± 0.2	0.60 ± 0.01	0.04 ± 0.01	3.5 ± 0.2
	Exercised	0.06 ± 0.002	0.13 ± 0.02	2.9 ± 0.3	0.60 ± 0.02	0.05 ± 0.01	3.2 ± 0.1
Two way ANOVA							
Prenatal Nutrition		0.0959	0.5596	0.5991	0.3844	0.7216	0.2160
Postnatal		**0.0497**	0.1227	0.3777	0.0661	0.2356	0.6230
Interaction		0.2672	0.7939	0.8778	0.4088	0.1116	0.7650

Table 4.5 Skeletal Muscle Weights and levels of TG & Glycogen

The tissue weights (as a percentage of body weight) or the levels of triglyceride (TG) or glycogen (GLY) (values are mean ± SEM; n = 10-12 and assessed with Two-way ANOVA). Soleus muscle was influenced significantly by postnatal conditions ($P < 0.05$), Bonferroni *post hoc* test showed no differences within these postnatal groups.

There were also no significant differences in the morphology or composition of fibres in IUGR Non-Exercised compared with AD Non-Exercised offspring at this stage (Table 4.6). Additionally, there were no significant differences observed in the structural analysis of IUGR Exercised skeletal muscle (Table 4.6) in comparison to muscle of AD Exercised offspring. However, the data show differences in skeletal muscle structure between IUGR Sedentary and AD Sedentary animals at day 150. As lean mass was reduced for Sedentary offspring (Table 4.5), it is not surprising that the soleus and GSUP muscle mass were reduced significantly in these animals at 150 d. The data show that the whole soleus muscles of Sedentary group were smaller in size and correlated with smaller fibre sizes. Both Type I and Type IIa fibre sizes were reduced in comparison to Non-exercised and Exercised groups (Table 4.6) (Two-way ANOVA $P < 0.05$). Furthermore, the proportion of Type IIa fibres was significantly reduced in IUGR Sedentary offspring soleus compared with AD Sedentary offspring (Bonferroni *posthoc* test $P < 0.05$).

4.3.6 Plasma Endocrine and Metabolic Measures

Overall, the Sedentary offspring are significantly different from the Non-exercised and Exercised groups with respect to plasma FFA concentrations, as evident by the significant interaction ANOVA result (Table 4.7). The Bonferroni *posthoc* test showed that IUGR Sedentary offspring had significantly reduced concentrations of plasma FFA ($P < 0.05$). A similar pattern was observed in the plasma glycerol levels (Table 4.7) where a significant postnatal effect and interaction with ANOVA test, was in part accounted for by the marked elevation in AD Sedentary plasma glycerol levels. However, the IUGR Sedentary offspring concentrations of plasma glycerol were aligned more closely to those of the Non-exercised and Exercised groups, accounting for the significant interaction ($P < 0.05$). Bonferroni *posthoc* test showed a significant difference between AD and IUGR offspring glycerol levels in the Sedentary postnatal group. Plasma HDL and LDL levels were similarly affected by postnatal conditions, but not by prenatal nutrition (Table 4.7).

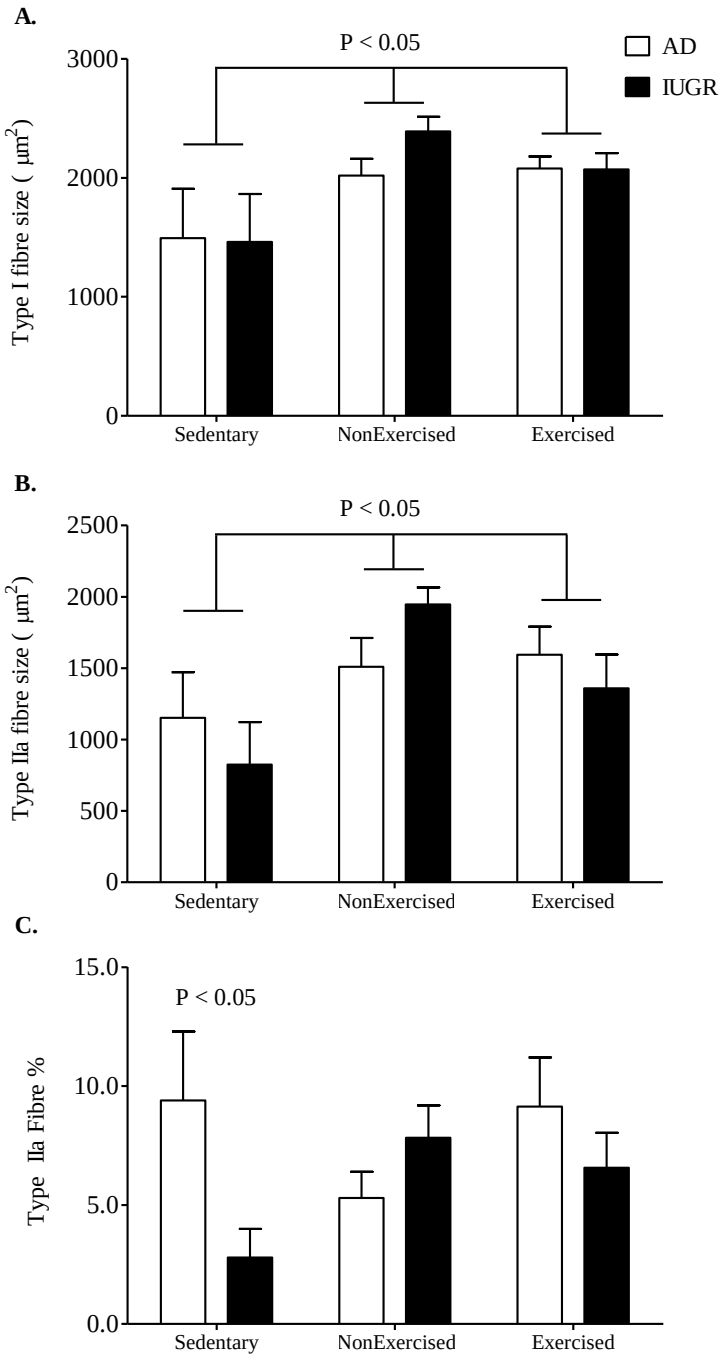

Figure 4.4 Postnatal lifestyle effects on structure of *M. soleus*.

The effect of prenatal undernutrition and postnatal conditions on skeletal muscle structure in 150 d rats (values are mean ± SEM, n = 10 – 12 per group, assessed by Type two ANOVA). A) Type I fibre size (μm^2), indicates a significant influence of postnatal conditions with ($P < 0.05$) marked above the columns. B) Type IIa fibre size (μm^2), a significant influence of postnatal conditions indicated (Type-two ANOVA $P < 0.05$) above the columns. C) Percentage of Type IIa fibres, Bonferroni *posthoc* test identifies a significant influence of prenatal nutrition for Sedentary animals indicated by ($P < 0.05$).

Prenatal	Postnatal	M. *soleus*		
		Type I fibre size (μm^2)	**Type IIa fibre size (μm^2)**	**Type IIa %**
AD	Sedentary	1493 ± 415	1152 ± 319	11.2 ± 2.8
	Non-Exercised	2020 ± 140	1510 ± 202	5.3 ± 1.1
	Exercised	2079 ± 101	1595 ± 196	9.1 ± 2.1
IUGR	Sedentary	1462 ± 404	824 ± 298	**3.2 ± 1.1***
	Non-Exercised	2392 ± 123	1945 ± 121	7.8 ± 1.4
	Exercised	2071 ± 137	1359 ± 236	6.6 ± 1.5
Two way ANOVA				
Prenatal Nutrition		0.6003	0.8272	0.0601
Postnatal		**0.0151**	**0.0103**	0.6987
Interaction		0.6831	0.2255	**0.0165**

Table 4.6 Soleus Muscle Composition and Morphology

Structure of soleus fibre types and fibre size measured in offspring at Weaning and adulthood for control offspring of adequately nourished mothers (AD) and offspring of undernourished mothers (IUGR). Values are given as means ± SEM. Two way ANOVA table and Bonferroni *posthoc* test * = $P < 0.05$; *n*=8. Fibre size refers to the measured size (μm^2) of specifically immunostained fibres for either myosin type I or myosin type IIa; (%) refers to percentage of type I or II fibres/totally counted fibres.

Sedentary animals were prone to hyperleptinaemia ($P < 0.05$) (Table 4.8) in addition to elevated cholesterol LDL ($P < 0.01$) and HDL ($P < 0.01$) although there were no differential effects for prenatal nutrition or in the ratio of these two cholesterols (Table 4.8). Plasma glucose levels were reduced in Sedentary offspring compared with Non-Exercised or Exercised offspring ($P < 0.05$). The two way ANOVA analysis with Bonferroni *post-hoc* test revealed a significant effect of prenatal nutrition in the Sedentary group only ($P < 0.05$). Bonferroni *posthoc* test within group (AD vs. IUGR) did not show any within group differences. The plasma concentrations of thyroid hormones were significantly affected by postnatal lifestyle: T_3 ($P < 0.05$) and T_4 ($P < 0.01$) and circulating levels of T_3 and T_4 were lowest in Sedentary offspring and highest for AD Exercised offspring (Table 4.8). The ratio of circulating T_3 to T_4 levels was not significantly affected either by prenatal nutrition or postnatal lifestyle (data not shown).

Prenatal	Postnatal	Urea (mmol/l)	CK (mol/l)	Glycerol (mmol/l)	FFA (mmol/l)	TG (mmol/l)	LDL (U/L)	HDL (U/L)	HDL/LDL
AD	Sedentary	6.0 ± 0.3	7.5 ± 0.7	0.20 ± 0.02	1.6 ± 0.5	0.9 ± 0.15	0.36 ± 0.04	1.9 ± 0.10	5.6 ± 0.6
	Non-exercised	5.7 ± 0.2	7.5 ± 0.8	0.13 ± 0.01	1.1 ± 0.2	0.8 ± 0.06	0.19 ± 0.02	1.4 ± 0.06	7.4 ± 0.6
	Exercised	6.3 ± 0.5	7.7 ± 1.1	0.14 ± 0.01	1.4 ± 0.1	0.8 ± 0.08	0.23 ± 0.03	1.4 ± 0.09	7.0 ± 0.9
IUGR	Sedentary	6.9 ± 0.2	6.4 ± 1.3	**0.15 ± 0.01***	0.5 ± 0.1	0.8 ± 0.07	0.26 ± 0.03	1.7 ± 0.16	6.6 ± 0.6
	Non-exercised	5.7 ± 0.3	6.9 ± 0.9	0.13 ± 0.01	1.2 ± 0.2	0.9 ± 0.09	0.23 ± 0.04	1.6 ± 0.05	10.2 ± 3.0
	Exercised	5.3 ± 0.2	8.1 ± 0.7	0.16 ± 0.01	1.5 ± 0.2	0.8 ± 0.08	0.19 ± 0.01	1.4 ± 0.05	7.7 ± 0.7
Two Way ANOVA									
	Prenatal Nutrition	0.9262	0.5977	0.1354	0.1141	0.6705	0.1592	0.7021	0.6439
	Postnatal	0.1060	0.5735	**0.0006**	0.1925	0.8248	**0.0052**	**0.0004**	**0.0143**
	Interaction	**0.0306**	0.7551	**0.0229**	**0.0277**	0.5451	0.0944	0.054	0.9741

Table 4.7 Plasma markers of obesity and lipid metabolism.

All results were measured in heparinised plasma of male rats at 150 d of age, none of which are significantly affected by prenatal nutrition (values are mean ± SEM; n = 10-12 and assessed with Two-way ANOVA). The plasma levels of creatine kinase (CK) or triglyceride (TG) are not significantly influenced by either pre- or postnatal conditions). Within group comparisons with Bonferroni *post hoc* test for glycerol and free fatty acids (FFA) in Sedentary group; AD vs. IUGR: * ($P < 0.05$)

Prenatal	Postnatal	Insulin (mg/ml)	C-peptide (pmol/l)	Glucose (mmol/l)	Leptin (ng/ml)	T3 (nm/ml)	T4 (nm/ml)
AD	**Sedentary**	0.82 ± 0.6	841.2 ± 257.8	7.6 ± 0.2	6.2 ± 1.9	52.8 ± 3.6	52.6 ± 3.3
	Non-exercised	0.99 ± 0.3	655.6 ± 102.4	9.1 ± 0.4	3.7 ± 0.4	53.8 ± 2.6	60.3 ± 2.8
	Exercised	1.45 ± 0.1	703.7 ± 129.2	8.8 ± 0.6	4.4 ± 0.8	61.6 ± 2.9	65.6 ± 2.7
IUGR	**Sedentary**	0.50 ± 0.1	818.2 ± 141.9	7.8 ± 0.6	7.2 ± 1.4	48.2 ± 4.7	54.3 ± 6.6
	Non-exercised	1.35 ± 0.5	797.5 ± 117.5	9.4 ± 0.5	3.6 ± 0.5	56.6 ± 3.3	57.5 ± 2.6
	Exercised	0.85 ± 0.2	698.2 ± 119.9	8.9 ± 0.4	5.6 ± 1.1	58.0 ± 4.1	65.6 ± 3.6
Two way ANOVA							
Prenatal Nutrition		0.5984	0.7457	0.6439	0.4338	0.2019	0.8919
Postnatal		0.4657	0.6748	**0.0143**	**0.0265**	**0.0466**	**0.0068**
Interaction		0.4546	0.8008	0.9741	0.7394	0.9811	0.8164

Table 4.8 Plasma Hormones & Metabolites

The plasma levels of insulin and C-peptide are not significantly influenced by either Pre- or postnatal conditions (values are mean ± SEM; *n* = 10-12). Plasma leptin, triiodothyronine (T_3) and thyroxine (T_4) in the AD and IUGR offspring of the Sedentary, Non-exercised and Exercised groups did not show any effect of prenatal nutrition on plasma measures. Whereas, postnatal conditions significantly influenced leptin, T3 and T4 (Two-way ANOVA: postnatal lifestyle (all $P < 0.05$). No significant differences were detected using *posthoc* analysis.

Neither insulin plasma concentrations nor C-peptide, the indicator of insulin secretion, were affected by prenatal nutrition or exercise (Table 4.8). Additionally the IUGR and AD Sedentary animals were not significantly different in their response to oral glucose tolerance test (OGTT) (Figure 4.5). Presented are the plasma glucose (Figure 4.5A) and plasma insulin levels (Figure 4.5B) over time and the area under the curve (AUC) comparisons (Figure 4.5C and D). In addition, the insulin:glucose ratio over time was assessed and AUC calculations confirmed no difference in the glucose tolerance of between IUGR Sedentary and AD Sedentary offspring (student's t test AD: 46.31 ± 7.617; IUGR 43.73 ± 7.493 ($P = 0.8145$) n = 6 per group).

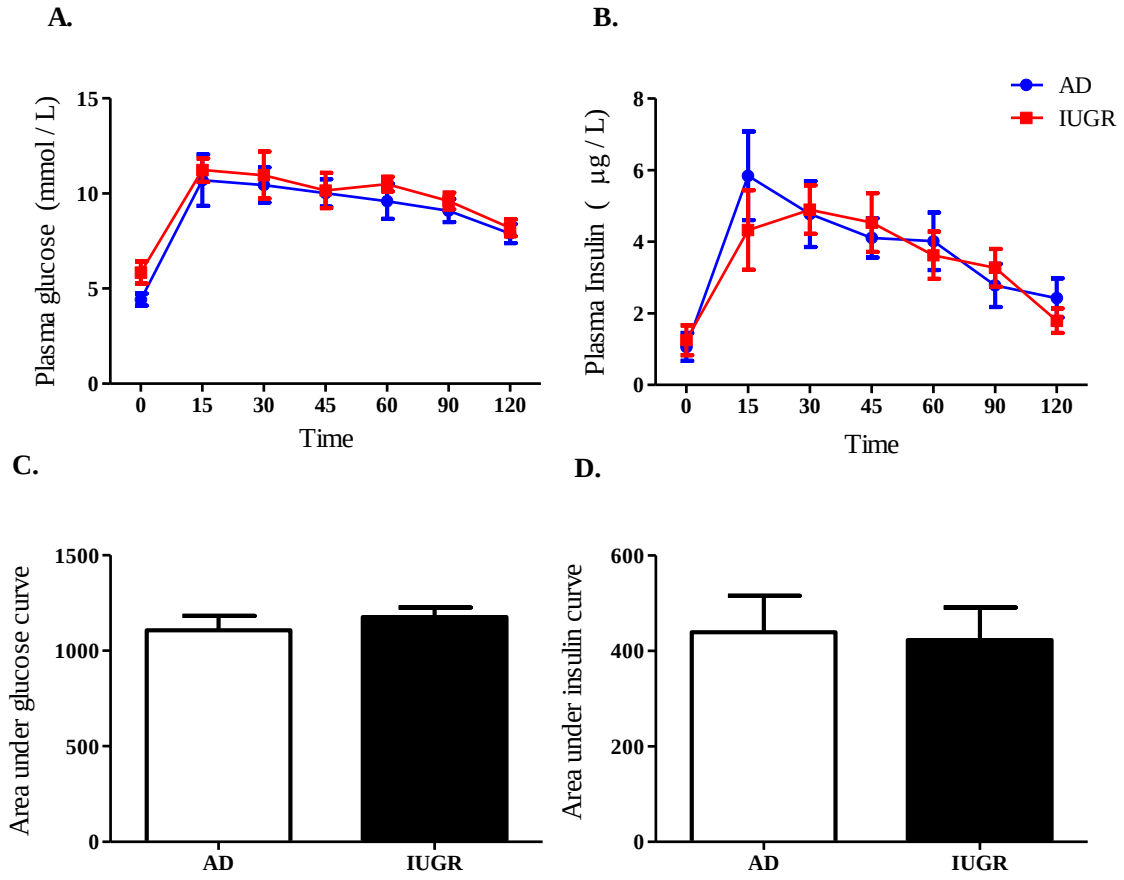

Figure 4.5 The effect of prenatal nutrition on glucose tolerance in Sedentary animals.

Figures A and B show the curves for glucose and insulin in plasma over time for AD and IUGR offspring after OGTT. Figures C and D show the 'area under the curve' comparisons between AD and IUGR for C) glucose and D) insulin. There is no significant effect of prenatal nutrition on measures of glucose tolerance in this study.

4.4 Discussion

This study demonstrates the complex interplay between pre- and postnatal environments. The aim of the study was to examine how three postnatal environmental conditions, akin to 'lifestyles', impacted on metabolic plasticity of IUGR offspring from undernourished dams. The three lifestyles of Sedentary, Non-exercised, and Exercised, all reinforced the legacy of prenatal nutrition. In particular, the sedentary lifestyle demonstrates the modifiable nature of skeletal muscle in postnatal life. Postnatal treatment initially permits a superficial recovery from IUGR but ultimately does not change the underlying thrifty phenotype.. Following weaning, all offspring in this study rapidly adjusted to their prescribed postnatal lifestyle. Structural, functional and metabolic adjustments were evident in Sedentary offspring, particularly for the Sedentary offspring of undernourished mothers. The inactive Sedentary offspring maintained on the unrestricted *ad libitum* feeding regime had increased adiposity, but intriguingly also showed the largest effect of prenatal nutrition on skeletal muscle fibre characteristics. This is further evidence that the obese phenotype seen in offspring of undernourished dams is metabolically distinct from diet-induced-obesity. The IUGR animals of this study are qualitatively different from their AD controls because IUGR rats initially produced much more activity and presumably habituated to the running wheel.

4.4.1 Postnatal Lifestyle and Body Composition and Physical Activity

It was surprising that the Exercised postnatal lifestyle induced no differences in fat or lean mass between offspring from both levels of prenatal nutrition. The data derived from DEXA scanning did not show any differences in body composition between AD controls and IUGR offspring of undernourished mothers. The impact of postnatal lifestyle is evident with a gradient of 'fatness' from the Sedentary offspring having the most fat mass to the Exercised offspring having the least. Previous studies have shown an effect of exercise on fat mass, with the offspring of undernourished dams being the most sensitive to this effect (Miles *et al.*, 2009a). However, at 150 d in the present study, this effect of exercise on body composition may not yet be realised in IUGR Exercised animals. Earlier studies showed that offspring of undernourished dams do show an increased preference for running in the wheel over pressing a lever (Miles *et al.*, 2009b). In the present study, the IUGR Exercised animals also show increased

running behaviour (Figure 4.3). While the methodology employed in the earlier studies allowed quantification of choice behaviour, the information available from this study only shows the speed to complete the wheel turns. However, these data do indicate that maternal nutrition during pregnancy influenced exercise behaviour and provide further support to the earlier studies by Miles et al. (2009).

4.4.2 Postnatal Lifestyle and Fibre Type Composition

The results of this study show that postnatal environment had a strong impact on muscle plasticity and oxidative potential in offspring of undernourished dams. Evaluation of skeletal muscle structure and function at 150 d suggested that increased adiposity was related to the induction of an oxidative phenotype. My study confirms the reduction in the proportion of Type IIa fibres previously observed in soleus of 150 d offspring in the IUGR group, but only when they were exposed to a Sedentary lifestyle condition. Previous studies have demonstrated a significant difference in the fibre types between IUGR and AD offspring of the same age (Huber et al., 2009). There were no fibre type differences observed between the IUGR and AD offspring assigned to the Non-exercised or Exercised postnatal lifestyles. This is interesting, as previous studies in this lab had shown a significant shift towards oxidative fibre types for 250 d offspring of undernourished dams, regardless of whether they were exposed to either Non-exercised or Exercised postnatal lifestyles (Huber *et al.*, 2009). The key differences between the present and previous studies are both age of offspring at point of tissue collection, and level of adiposity. In particular, the fat mass of Sedentary offspring at 150 d closely resembled the fat mass of Non-exercised offspring at 250 d. Therefore, it is tempting to speculate that offspring of undernourished dams, with sedentary postnatal lifestyle as a compounding factor, are provoked to induce an oxidative phenotype in their skeletal muscle at a younger age.

I suggest that survival in a sedentary postnatal environment puts pressure on IUGR offspring to adapt early and induce a plastic developmental programme. The earlier induction of a postnatal plastic programme for these IUGR offspring might be a mechanism of metabolic adaptation to the likely postnatal sedentary environment. However, increased adiposity cannot be held culpable in the case of the IUGR Exercised offspring at 250 d. Figure 4.6 illustrates that the IUGR Exercised animals

from the 2009 Huber et al. study were not obese at 250 d, yet still expressed the increased oxidative phenotype. It may be that oxidative phenotype is programmed to be expressed in offspring of undernourished dams in the long-term and that obesity accelerates this development. An exception is seen in the case for IUGR Exercised who experience long-term moderate daily exercise and may not need to change their muscle structure for successful adaptation to their specific environment.

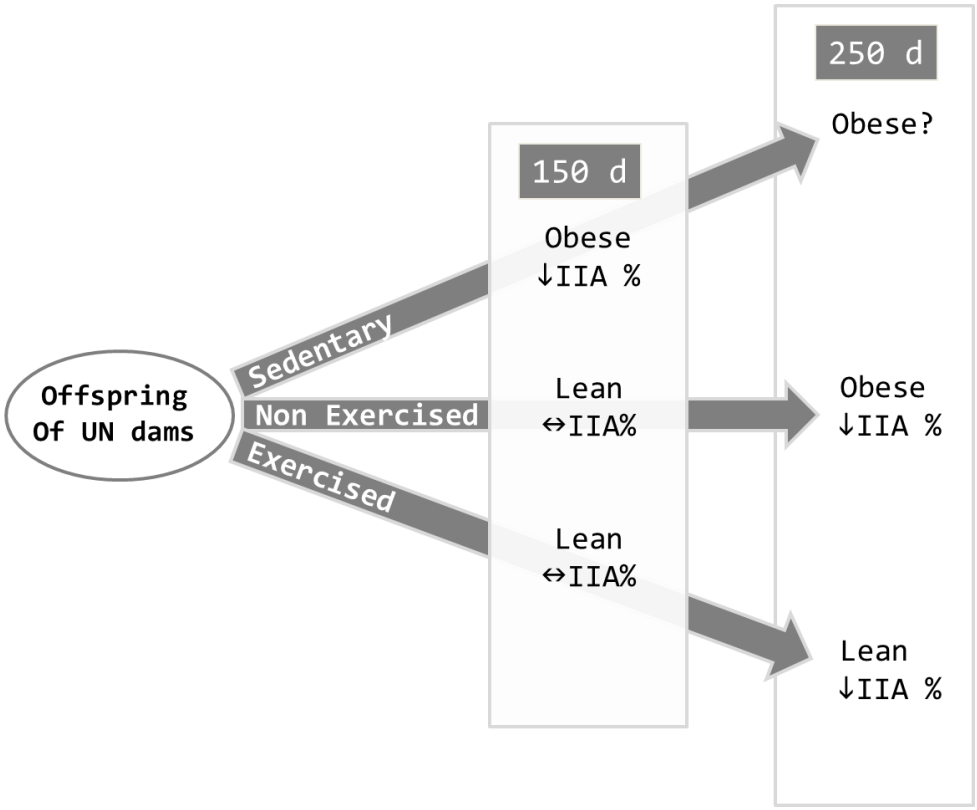

Figure 4.6 Postnatal pathways to obesity and fibre type composition

With increasing age and adiposity, Type IIa fibre changes in soleus muscle are observed for IUGR offspring of undernourished (UN) dams. The first and second columns represent changes in fibre type with corresponding lifestyle evident at 150 d and 250 d, respectively.

The modifiable nature of skeletal muscle in relation to prenatal nutrition is acknowledged in the literature (Pette, 2002; Buckley *et al.*, 2005; Quigley *et al.*, 2005; Sayer and Cooper, 2005; Zhu *et al.*, 2006; Mallinson *et al.*, 2007; Al-Shanti and Stewart, 2009). Additional emphasis is on how postnatal lifestyle factors might influence skeletal muscle fibre type characteristics (Bedi *et al.*, 1982; Maltin *et al.*, 2001; Bayol *et al.*, 2005; Du *et al.*, 2009). In particular, postnatal lifestyles that promote obesity, such as high-fat diet and physical inactivity, are known to impact on

skeletal muscle metabolism (Kelley, 2005). With a high-fat diet/physically inactive lifestyle, increased metabolic demands are placed on skeletal muscle, especially in relation to the increased body mass (Kelley, 2005). Recently, the genetically obese ob/ob mouse was reported to express both smaller fibre size, particularly for Type II fibres and higher Type I fibre proportions (Kemp *et al.*, 2009). The authors proposed that concomitant expression of increased transitional/hybrid fibres indicates that obesity must induce structural remodelling of muscle rather than a deviation from the developmental programme (Kemp *et al.*, 2009). The physiological benefits of smaller fibres, as outlined in Chapter 2 of this thesis, are primarily increased surface area for exchange of nutrients. Other examples of smaller fibres resulting from postnatal lifestyle have been reported. In line with the present study, sedentary behaviour (with *ad libitum* feeding) was found to correlate with reduced fibre size when compared with exercised counterparts (Maxwell *et al.*, 1992). This study employs alternate Type II fibre terminology, but nonetheless intensive exercise led to increases in the size of Type II fibres overall (10 weeks of daily 40-50 min treadmill running at a speed of 1.6 miles/ h, approximately 2.5 Km/h, with a gradient of 15%). For this study, I did not anticipate a large increase in muscle fibre size for Exercised offspring given that they are not engaged in long-term muscle force training (Goldspink and Ward, 1979). Furthermore, the amount of exercise (56 m/d) for Exercised rats is not expected to be intensive enough to evoke fibre size changes.

The Sedentary animals were also the subjects for the transcription study in Chapter 3 in which there was an increased mRNA expression for the calcium signalling factors calcineurin and NFATc1 in IUGR Sedentary soleus. The calcium signalling pathway is normally influenced by exercise-stimulated neuronal signalling. Perhaps the 'trained'-like features of IUGR Sedentary group skeletal muscle gives them the capacity to utilise fuel sources efficiently. The results suggest that IUGR Sedentary offspring implement their postnatal oxidative phenotype in advance of other offspring of undernourished dams. Does this mean that the sedentary lifestyle incites an earlier implementation of the postnatal plasticity programme – Type I fibre/oxidative promotion – specifically for IUGR offspring? Perhaps the additional burden of physical inactivity and unrestricted feeding regime provokes an earlier impetus to induce a more oxidative phenotype? The potential benefits to the shift in fibre type composition towards a slower phenotype would likely improve metabolic flexibility

and include improved insulin binding capacity, insulin stimulated glucose uptake, and an increased capacity for FFA uptake and oxidation (Kelley *et al.*, 1999). However, it is difficult to confirm whether this phenotypic change has consequences for muscle strength. Does a reduction in Type IIa fibres result in less force potential for these offspring of undernourished dams? Study of force and contractile properties would need to be studied by electrical stimulation of in situ muscle preparations (Barclay, 1988). The study was also unable to confirm whether these plastic processes are underway for Non-exercised or Exercised animals. There are no prenatally-induced differences for skeletal muscle structure and function in day 150 Non-exercised or Exercised animals. However, the exercise inherent in these more active lifestyles, and in combination with a set meal style feeding regime, clearly promotes an efficient metabolism compared with sedentary lifestyle.

4.4.3 Postnatal Lifestyle and Metabolic Measures

With respect to whole body metabolism, the differences in plasma cholesterols LDL and HDL, indicate a clear effect of postnatal lifestyle. Furthermore, they indicate that the fibre type changes observed facilitate participate in oxidative fuel metabolism. In particular, the IUGR Sedentary offspring have lower glycerol concentrations, with no significant differences in plasma FFA levels indicating equal ability to break down circulating fats for energy production. The IUGR Sedentary rats also have maintained glycerol plasma concentration reflecting potential lipolysis from adipose tissues. The statistical interaction observed for the measure of glycerol plasma concentrations indicates that the process of lipolysis in adipose tissue has a different trajectory for IUGR Sedentary animals compared with Non-exercised and Exercised groups. Perhaps sedentary lifestyle has a differential effect on lipolysis between IUGR and AD offspring, as the concentrations of glycerol are increased in AD Sedentary offspring and decreased in IUGR Sedentary offspring. Miles et al., 2009 described the effect of moderate daily exercise on hepatic lipid metabolism, noting that the rats of undernourished dams had decreased lipogenesis at 250 d. Furthermore, it was noted that the soleus muscle had reduced protein levels of CPT1a and PGC1 α indicating less oxidation of lipids in comparison with glucose oxidation (Huber *et al.*, 2009). So while there are indications that IUGR might have the enhanced capacity to oxidise lipids, the data do not show whether they do so. It would be of considerable interest to

investigate metabolism and oxidative phenotype in other skeletal muscle tissues of offspring from undernourished dams.

There were no differences observed in the glycogen levels of soleus or GSUP muscles for AD or IUGR offspring in the present study. Nor was there any effect of postnatal lifestyle on this fuel store. However, previous work showed increased glycogen storage in muscle of the offspring in this IUGR model, indicating that glucose is directed to storage, particularly under sedentary conditions (Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009a). Furthermore, the OGTT results of the present study confirm that glucose metabolism in the IUGR Sedentary offspring is maintained, in spite of their sedentary environmental conditions. With respect to protein metabolism, slightly elevated urea plasma concentrations may indicate some protein catabolism for IUGR Sedentary. However, the maintained levels of creatine kinase confirm that the muscle protein catabolism is minimal. This observation suggests that the hyperinsulinenaemia we see in IUGR offspring is working to compensate for protein catabolism (Wolfe, 2000).

In the present study, thyroid function was not differentially affected by prenatal nutrition; instead, there was a significant effect of postnatal lifestyle on circulating total T_3 and T_4 levels. The Exercised offspring in this study have the highest levels of both T_3 and T_4 which might indicate increased basal metabolic status in these offspring (Jones and Huether, 2006). Thyroid hormone has been described as the primary denominator of energy homeostasis and hyperthyroidism is linked to increased energy expenditure (Havekes and Sauerwein, 2010). Thyroid hormones are proposed to participate at the level of the mitochondrial energy transduction. In particular, T_3 has been shown to influence thermogenesis by influencing transcription of uncoupling proteins such as UCP3 (Reitman *et al.*, 1999). In addition, increased thyroid hormones might also indicate these offspring are in a state of glycogenolysis (Dimitriadis *et al.*, 1988). However, there is no significant effect of exercise on any glycogen measures in the present study. Thyroid hormones have a renowned effect on skeletal muscle fibre type (Simonides and van Hardeveld, 2008), yet here there were no corresponding fibre type differences for Exercised offspring.

The plasma levels of leptin were significantly increased for IUGR offspring of undernourished dams in this study and were highest in IUGR Sedentary group. As

discussed in Chapter 2 of this thesis, leptin may play a role in the regulation of skeletal muscle mass and muscle fibre differentiation. Increased plasma leptin measures for the IUGR Sedentary group corresponds with the reduction in Type IIa fibre proportion compared with AD offspring. Although there is no significant effect of postnatal lifestyle on plasma leptin levels, these levels in Sedentary offspring more closely resemble those of the 250 d old Non-exercised and Exercised offspring (Huber *et al.*, 2009; Miles *et al.*, 2009b). Therefore, it is tempting to speculate that the levels of leptin in the IUGR Sedentary offspring might be the trigger for the initiation of the phenotype in postnatal life. Leptin exerts its metabolic function partly via activation of AMPK resulting in increased FFA oxidation (Minokoshi *et al.*, 2002). The IUGR Sedentary offspring tend to have increased AMPK mRNA expression (see Chapter 3) concomitant with the lowest circulating FFA levels. Thus a picture emerges of the unique way offspring of undernourished dams might manage homeostasis in postnatal life – leptin in relation to increased fat mass promotes skeletal muscle oxidative metabolism, perhaps also promoting insulin sensitivity.

4.4.4 Postnatal Lifestyle and Influence of Diurnal Food Intake

I acknowledge that in this study the difference in feeding regime would have influenced the metabolic outcomes between the different lifestyle conditions. Although the composition of the diet was identical, the access to the food as well as the timing of intake was distinct between the Sedentary offspring versus the Non-exercised and Exercised postnatal groups. Rats are naturally nocturnal and consume their food during the dark phase. Wells and colleagues recently reported that food consumption during the light phase had a significant temporal effect on the activity of the somatotropic axis and induced an increased susceptibility to diet induced obesity (Glad *et al.*, 2011). It has been suggested that reverse feeding induces 'phase inversion' of clock genes that are responsive to temporal cues such as light and nutrition (Damiola *et al.*, 2000; Schibler, 2000). The Sedentary animals of the present study were able to eat at their own leisure during the whole 24 h period. However, the Non-exercised and Exercised offspring were instead restricted to 2 h feeding during the light phase only. The rationale for restricting these subjects to 2 h was to address limitations associated with the behaviour chambers. It was necessary to restrict feeding for these studies to ensure that the food pellets used in the behavioural tests were

effective reinforcers. Furthermore, it was necessary to establish this regime so as not to interfere with the voluntary nature of exercise. The initial variation in food intake was then stabilised before exercise was introduced. The lack of differences at day 58 – 60 indicates that the scheduled feeding, in itself, did not lead to a reduction in calorie intake in comparison to the 24 h *ad libitum* feeding.

High variances are apparent for the IUGR Non-exercised and Exercised groups. However, variances overall appear to diminish as all of the animals grow and their calorie intakes converge over time. It is only when exercise is introduced that we see the strong variation in food intake. Given the nature of the staggered feeding measurement approach, it was not possible to gain information about the immediate impact of the exercise regime on food intake. There were no differences in the pellet consumption between these Non-exercised or Exercised groups during their times in the behavioural cages and the minimal calorie intake derived from pellets was included in the total food intake measures. Overall, the Sedentary animals consumed more food per gram body weight than Non-exercised or Exercised offspring. While a further analysis of scheduled feeding in the sedentary condition would be of interest, this study was not initially designed to include this factor. Further studies would be needed to conclude whether the timing of the feeding had influence on clock genes or metabolism.

4.4.5 The Impact of Postnatal Lifestyles

The results presented in this chapter speak to how offspring of undernourished dams cope in postnatal life. Although lifestyle can cause many of the same changes in both rats and humans, care should be taken when interpreting the experimental results (Young and Kirkland, 2007). As with all experimental models directed at the study of metabolism and disease processes, it would be premature to conclude that these results are directly applicable to the human condition. IUGR, in the sense of this rodent model, is a term strictly used to describe delayed growth velocity *in utero*. While IUGR certainly affects human babies (Figueras and Gardosi, 2011), it remains difficult to understand the pathophysiology or to predict the consequences of the IUGR condition. One overall aim of the research is to aid in our theoretical understanding about the potential for plasticity during postnatal life and the intricate relationship with early life experiences. This study provides an important perspective on metabolic

health and the influence of environment. The IUGR model has the potential to extend our understanding about susceptibility to diseases of modern living. As described earlier, modern living has induced a powerful shift in nutrition and physical activity. Aided by the absence of chronic infectious disease as the dominant selective advantage, it could be that a genetic shift in responses to diet and exercise is responsible for the prevalence of metabolic disorders (Roberts and Barnard, 2005). Therefore, it is interesting to consider the results of this study in the context of well-described theoretical paradigms. The concept of metabolic thrift, or energy conservation, is a common theme between two popular schools of thought – the thrifty *genotype* and the thrifty *phenotype* hypotheses.

Neels' (1962) thrifty genotype hypothesis postulates that the rapid rise in Type 2 diabetes and metabolic syndrome in modern times can be attributed to the hunter-gather era origins of our metabolism (Neel, 1962). According to the thrifty genotype, our ancestors would have developed an energy-conserving genotype to cope with sporadic periods of 'feast' and the more common periods of 'famine'. The maladaptive response to modern living comes into effect during sustained 'feast', such as a freely available Western diet. The alternative 'thrifty phenotype' hypothesis accounts for the more immediate influence of environmental factors on the metabolism of an organism (Hales and Barker, 1992). These two hypotheses are not mutually exclusive, necessarily. Thought to be initiated via epigenetic mechanisms, that is, for example the methylation of genes in response to nutritional cues, the thrifty phenotype hypothesis proposes a rationale for the life course trajectory of individuals being plastic during early life. For example, children born in the late summer to autumn generally live longer than those born in the spring (Doblhammer and Vaupel, 2001). The proposed mechanism is that the fetus developing in late winter to early spring faces a less nutritionally abundant environment, whereas the second and third trimesters generate exponential growth, with most growth occurring around harvest time (Doblhammer and Vaupel, 2001). So, the thrifty phenotype arising from epigenetic effects within years and across them can be evident in human populations. Chapter 2 of this thesis described how offspring exposed to poor nutrition *in utero* have a metabolic flexibility that potentially sets them up for physical activity in postnatal life. The predicted postnatal environment is one in which the individual must forage over a larger area than is normally required to obtain sufficient food.

Accordingly, the postnatal phenotype, perhaps manifested as increased oxidative fibre type and enhanced energy storage, is an adaptive mechanism that appears to be protective even in the sedentary lifestyle. In addition, glycogen conservation is a feature typical to offspring of undernourished dams, although there were no glycogen differences in the 150 d old rats of the current study. As described in Chapter 1 of this thesis, efficient substrate storage and utilisation is a key feature of metabolic flexibility. Food procurement is no longer as taxing as it was for our ancestors. Physical activity is touted as the core physiological regulator of thrifty genes, promoting metabolic flexibility (Cordain *et al.,* 1998). Therefore, the feast-famine and activity-rest cycling that our metabolic genes were accustomed to expect, are not necessarily invoked in modern living (Chakravarthy and Booth, 2004).

4.4.6 Physical Activity in Human Studies of IUGR

A study by Laaksonen and colleagues (2003) determined that vigorous leisure time activity is a key measure for preventing metabolic disturbances in adult men who were small at birth (Laaksonen *et al.,* 2003). An alternate study published more recently suggested small size at birth was associated with less involvement in exercise for adults (Kajantie *et al.,* 2010). Such studies indicate that lifestyle factors may have more impact on adult metabolism than size at birth in adulthood. Several studies have also assessed the impact of small size at birth and physical activity in youths (Hallal *et al.,* 2006; Mattocks *et al.,* 2008; Andersen *et al.,* 2009) reviewed recently by (Ridgway *et al.,* 2010b). While the research to assess physical activity had different approaches, the overall combined analysis suggested that birth weight may not be an important biological determinant of habitual physical activity or sedentary behaviour in youth (Ridgway et al., 2010b). Others have found the converse, with both low and high birth weights being associated with a lower probability of being physically active during leisure time (Andersen *et al.,* 2009). The apparent discrepancy between the findings of the two studies may relate to differences in the analytic strategy and as the authors note "...it can be difficult to disentangle the influence of birth weight from that of postnatal growth" (Ridgway *et al.,* 2010a). While the present study did not investigate choice behaviour, we know from Miles et al., (2009) that offspring of undernourished rat dams do show an increased preference for exercise in the running wheel. In the

present study, IUGR Exercised offspring would complete their allocated wheel turns at a faster pace than their AD Exercised counterparts.

4.5 Summary and Conclusions

The thrifty offspring of undernourished dams have begun to reveal the unique way they regulate homeostasis in postnatal life. Both sedentary behaviour and habitual exercise over time have the ability to invoke specific skeletal muscle adaptations in the IUGR offspring. The findings from this study suggest that the combination of prenatal undernutrition and postnatal sedentary conditions contribute to an oxidative phenotype of skeletal muscle. The decreased lipolysis of fatty acids from adipose tissue and enhanced capacity to oxidise fuel sources in Sedentary IUGR animals is particularly interesting. Given that skeletal muscles have very individual structural and functional properties, perhaps muscles other than the ones assessed in this study are able to utilise these free fatty acids as fuel sources? Therefore, a more detailed study of many different muscles of offspring from undernourished dams would be useful to determine muscle-specific metabolic conditions. Further studies are also be needed to confirm the specific effect that leptin has on skeletal muscle oxidative potential and insulin sensitivity. The main findings of the present research highlight the importance of postnatal nutrition and physical activity on metabolic health.

Chapter 5.

Final Discussion

Overview

The general aim of this thesis was to identify molecular mechanisms responsible for the postnatal development of metabolic flexibility in offspring of undernourished mothers. My research followed a novel path of inquiry that began with describing features of metabolic flexibility in the Neonatal, Weaning and Adult offspring of mothers undernourished throughout the entire pregnancy. The main discovery was that structural and functional aspects of skeletal muscle metabolism were altered for prenatally undernourished offspring but did not always become detectable until adulthood. Following on from this key finding, I investigated potential mechanisms responsible for induction of oxidative metabolism at the transcriptional level. The expression of genes, integral to enhanced oxidative metabolism, could be interpreted and correlated to the observable features of metabolic flexibility development. Lastly, a final study was designed to compare interactions between the prenatal environment and varied postnatal lifestyle paradigms on outcomes for body composition and skeletal muscle metabolism.

Previous studies of these offspring have indicated that metabolic flexibility, or the flexible storage and utilisation of energy sources, is enhanced as a consequence of poor maternal nutrition. The overall focus of this work was on the development of flexible metabolism in offspring, specifically the postnatal plasticity of skeletal muscle. I hypothesised that offspring of undernourished mothers would adapt to reduced prenatal nutrition through structural and functional changes in skeletal muscle that favour pathways of flexible fuel usage and storage.

Metabolic regulation controls the flow of nutrients or substrates along metabolic pathways, according to the body's needs (Frayn, 2003b). Metabolic flexibility is achieved by balancing substrate utilisation and substrate storage in line with energy intake and energy output requirements. Maintenance of metabolic flexibility is also dependent on a fine-tuned sensitivity to environmental factors. Non-responsiveness to environmental cues might be responsible for an imbalance in the flow of energy for insulin resistant individuals, whether they be rats or humans. The ultimate outcome of

metabolic *inflexibility* is an inability to use energy efficiently, which can have a large impact on health. Environmental challenges during development can determine the metabolic profile of the individual throughout life and across the levels of gene transcription, biochemical processes, efficiency of energy use and even behaviour.

Poor nutrition experienced *in utero* can also have long-lasting consequences for metabolic health. It is becoming apparent that the offspring of undernourished mothers in this rat model have a metabolically distinct physiology from offspring of adequately nourished mothers. The IUGR condition experienced during fetal development has evidently set these rats up to store and utilise energy efficiently. The metabolic consequences of the poor nutritional environment during development manifest as an obese phenotype when adequate nutrition is encountered in adulthood. However, in contrast to diet-induced obesity, the obesity observed in offspring of undernourished mothers is characterised by maintained insulin sensitivity and increased adiposity confined to physiological fat depots.

5.1 Development of Metabolic Flexibility

Independent of the experimental model employed, compromised growth leading to IUGR has profound influence on skeletal muscle development. If, for instance, the environmental insult encountered is reduced nutrition, energy investment in skeletal muscle growth is limited so as to favour vital organs like the heart and the brain (Zhu *et al.*, 2006). In addition, nutrition during gestation and lactation is known to have a strong impact on skeletal muscle composition; as quantified by fibre number, fibre size and fibre type differentiation (Bedi *et al.*, 1982).

In the first experimental study of this thesis, the metabolic status of IUGR offspring from undernourished dams was investigated at three important time points – birth, weaning and later adulthood. I chose two distinct muscles to investigate at both weaning and adult ages – the soleus and the gastrocnemius superficial. Soleus and gastrocnemius muscles are located adjacent to one another in the leg yet serve distinct physiological purposes in relation to their anatomical function. In particular, the two muscles have proportions of myofibres that are differentially responsive to their respective energy demands. The soleus muscle represents an endurance muscle owing to its anatomical function of facilitating standing and locomotion. The highly oxidative

soleus muscle is made up of haemoglobin-rich Type I fibres. Gastrocnemius muscle is also in high demand during locomotion yet stores more glycogen for rapid conversion to glucose 6-phosphate in order to achieve short bursts of activity. This muscle is made up of a mixture of glycolytic fibres Type IIa/b/x fibres and Type I oxidative fibres. Therefore, given that soleus and gastrocnemius muscle have unique metabolic functions it was important to ask how do these two different, yet closely associated, muscles develop in relation to prenatal nutritional experience?

Overall, the investigations for the thesis represent a broad overview of metabolic status in offspring of undernourished dams compared with the offspring of *ad libitum* fed dams. The measures of metabolic status encompassed: glucose metabolism and insulin sensitivity; fat storage and indications of lipid metabolism; and muscle structure and function. At all ages, metabolic status indicated similar insulin sensitivity in both offspring groups despite the development of adiposity in offspring of undernourished dams. Changes in soleus structure were apparent across the three developmental age groups, with a significant effect of prenatal nutrition on Type IIa fibre size in IUGR offspring of undernourished mothers compared with AD controls. The difference in progression through to adult skeletal muscle fibre morphology was evident with increased proportions of oxidative fibres in soleus muscle for offspring of undernourished mothers. Gastrocnemius muscle also showed alterations as a consequence of prenatal undernutrition, with a significant decrease in Type IIa fibre proportions in IUGR offspring compared with AD offspring. A significant decrease in the size of these Type IIa fibres was observed for the Adult IUGR offspring at a time when storage of glycogen is increased and no effect on the oxidative or glycolytic enzymes is observed. My thoughts on the consequences of GSUP muscle changes will be discussed below.

The observations of potentially enhanced metabolic flexibility in IUGR offspring are expressed even when these offspring of undernourished mothers become obese as a result of exposure to a postnatal sedentary environment. The combination of enhanced storage of glycogen and fat in physiological depots and the skeletal muscle structure and functional studies support the hypothesis that the IUGR offspring develop enhanced metabolic flexibility. That is to say, potential for higher oxidative capacity

of skeletal muscle indicates that stored energy can be used effectively through activation of catabolic pathways in times of energy requirement.

After describing the prenatally-induced progression towards altered fibre type composition and morphology, I investigated potential molecular mechanisms associated with the IUGR condition. Transcriptional control of skeletal muscle development was primarily investigated as a mechanism for the enhanced oxidative phenotype observed in offspring of undernourished rat dams. The hypothesis tested whether postnatal developmental pathways would be different between IUGR offspring and controls. Differences in the key factors for the calcium signalling pathway influencing fibre type plasticity and also the NRF1 factor influencing mitochondrial biogenesis were consistent with the hypothesis.

The transcriptional control of myogenic regulatory factors, assessed from birth to weaning, did not reveal any potential mechanisms for the developing phenotype associated with the IUGR condition. The trajectory of these factors from birth to weaning did not demonstrate the hypothesized alterations to the initial myogenic response to prenatal undernutrition. It is important to acknowledge the small number of samples available to measure, particularly in light of the lack of reduction of myostatin for IUGR offspring at weaning. The data available from my study can be used for future study power calculations. For instance, an estimate of the group size needed to detect a significant difference in Neonatal myostatin mRNA levels with a representative standard deviation of 0.2 can be made using GraphPad StatMate v2.0. Using this program, a sample size of 18 in each group is calculated to have an 80% power to detect a difference between means of 0.19 with a significance level of 0.05. Given the need to detect such small differences between the means with mRNA expression studies, a higher sample size would be needed.

In the gene expression study, the case for altered calcium signalling factors in Adult soleus muscle is stronger. In Adult offspring of undernourished dams, significant increases in calcineurin and NFATc1 gene expression were recorded in comparison to control offspring soleus muscle. The calcineurin-NFAT pathways are known to influence the oxidative fibre type programme (Chin *et al.*, 1998; Parsons *et al.*, 2004; da Costa *et al.*, 2007; Calabria *et al.*, 2009) and are up-regulated in the soleus muscle

of IUGR offspring. The postnatal plasticity observed and the relationship to calcium signalling in skeletal muscle has not previously been explored in the research field of developmental origins of adult health and disease. These novel findings indicate that influences on fibre type switching are potentially enhanced in the later postnatal period and also correlate with previous findings of soleus structure and function after prenatal undernutrition (Huber *et al.*, 2009; Miles *et al.*, 2009a). The NFATc1 and calcineurin factors are both physiologically activated by exercise and, for the first time, I show these factors are up-regulated for offspring of undernourished dams even in a sedentary environment.

I have also shown that the mitochondrial biogenesis stimulating factor, NRF1, was altered at the transcriptional level in the soleus muscle of IUGR offspring. In order to meet the energy requirements of higher proportions of oxidative Type I fibres, one might expect there to be changes in mitochondria or oxidative phosphorylation, relative to the metabolic characteristics observed in offspring of undernourished rat dams. Proxy measures such as citrate synthase enzyme activity led to the hypothesis that mitochondrial plasticity might be altered by prenatal nutrition, at least in adulthood. However, at the transcriptional level, the only significant difference detected between IUGR and AD offspring was NRF1. The NRF1 lies both down- and upstream of factors in the mitochondrial influencing pathway that were not significantly affected by prenatal nutrition. More extensive investigation of this pathway and surrounding factors is needed in order to interpret this result in the context of enhanced metabolic flexibility. In terms of the potential implications to human studies, the NRF1 factor has been inversely related to impaired insulin signalling and patients with Type 2 Diabetes (Patti et al., 2003). A nearly 30 % decrease in NRF1 expression was found in diabetic subjects and also found to be to inversely correlated with fasting glucose (Patti et al., 2003). In that same study, PGC-1 α was also found to be reduced in line with impaired glucose metabolism.

It is important to note that changes in gene expression alone are not evidence for functional differences. Further investigations into the pathways responsible for stimulating mitochondrial biogenesis at the protein level are warranted. In addition, it is possible that methodology utilising fresh tissue samples might provide more accurate information about myogenic regulation and mitochondrial status. The tissues

for the transcriptional studies discussed above were derived from rats at 150 d of age which were exposed to a sedentary postnatal environment. Researchers investigating a low-protein model of maternal undernutrition during pregnancy also found few differences in mitochondrial biogenesis at an early age, suggesting that additional stress such as ageing or high-fat diet is needed for the differences to become apparent (Cabeço et al., 2012). It could be that, perhaps at 150 d of age or younger, there is insufficient aging or dietary stress on the IUGR animals, masking any strong differences between the offspring on mitochondrial factors.

One of the present study's strengths was the use of the DEXA scanner to estimate the body composition of the rats at 150 days of age more accurately. The body composition measures at 150 d could subsequently be compared with the cohort of rats from the first experimental study at 250 d. The structural and morphological features of skeletal muscle were assessed at the 150 d age for the sedentary group as well as for the Non-exercised and Exercised groups, the more physically active cohorts. The composition of oxidative or glycolytic fibres in skeletal muscle is the result of both developmental instructions and activity-dependent plasticity. The way in which the three distinct postnatal lifestyle conditions impacted on IUGR offspring's metabolism was quite surprising. The sedentary environment induced increased adiposity but also triggered an earlier oxidative fibre type composition. Obesity is traditionally thought to encourage a less oxidative and metabolically inflexible phenotype (de Beaudrap *et al.*, 2006; Stump *et al.*, 2006; Phielix and Mensink, 2008). However, in the case of prenatally-induced obesity, the sedentary group at 150 d of age displayed an oxidative skeletal muscle phenotype. At this same developmental time point, neither the Non-exercised nor Exercised groups, with significantly less fat mass in comparison to their sedentary counterparts, displayed the oxidative phenotype. The older cohort of 250 d Non-exercised and Exercised offspring of undernourished mothers were already shown in the first study of this thesis to have reduced Type IIa fibre proportions (Huber *et al.*, 2009). Therefore, the unique skeletal muscle structure of the more active offspring of undernourished mothers does not become apparent until they are much older.

As mentioned above, the second study of this thesis investigated the gene expression of calcium signalling pathway factors, up-regulated for 150 d old Sedentary offspring

of undernourished dams. It would be of considerable interest to investigate NFATc1 and calcineurin expression in the muscles of the older subjects across the range of postnatal environmental conditions. However, in light of the NFATc1 and calcineurin up-regulation that has the potential to influence fibre type in postnatal life, it is tempting to speculate that 150 d marks the beginning of the 'post-weaning' induction for fibre type conversion to oxidative fibre type.

One potential trade-off involved with promotion of pathways for oxidative metabolism and reduced muscle fibre size is that force capacity and strength of skeletal muscle might be compromised. However, recent research has indicated that electrical stimulation of soleus muscle showed no difference in the maximal titanic contraction between prenatally undernourished or controls (K Huber, personal communication, 2009). Further investigations into the activity of satellite cells will be of particular interest with respect to maintenance of muscle mass and regeneration in this model. An additional transcription factor, Serum Response Factor (SRF), is related to muscle growth and force (Charvet *et al.*, 2006) and might provide information on the overall strength of muscles for offspring of undernourished rat dams. Further characterisation of skeletal muscle force and contraction might be achieved by incorporating resistance training regimes in the experimental design. A similar approach investigating muscle performance is needed to confirm the findings that the muscles of prenatally undernourished rats appear to be like the muscles of an exercise-trained rat, even though the postnatal environment is sedentary for the current study.

The combined studies of this thesis show features of metabolic flexibility that are revealed with both increasing age and degree of obesity. Increasing concentrations of plasma leptin might be the trigger for postnatal induction of these metabolic features, in particular for the skeletal muscle phenotype observed in offspring of undernourished mothers. The combination of prenatal undernutrition and postnatal sedentary lifestyle conditions contributed to the muscle phenotype that might be expected of exercise-trained muscle. Both sedentary behaviour and habitual exercise over time have the ability to invoke specific skeletal muscle adaptations in this model of prenatal undernutrition. It is interesting that structural and functional features of skeletal muscle in the sedentary offspring of undernourished rat dams are not too dissimilar to those of the genetically obese ob/ob mouse, with both reduced Type IIa

fibre proportions and size (Kemp *et al.*, 2009). The more oxidative fibre type composition in the obese state is described to have potential benefits for insulin-stimulated glucose uptake, and a lower tension cost during muscular activity (Kemp *et al.*, 2009). In addition, the known effect of leptin on FFA oxidation, by way of AMPK (Minokoshi *et al.*, 2002), might play a pivotal role in directing the utilisation of lipid fuel sources. The offspring of undernourished rat dams that experience a sedentary postnatal environment tend to the lowest circulating FFA concentrations. In the adult offspring of undernourished mothers, the question arises: does a decreased degree of lipolysis from adipose tissue concomitant with enhanced capacity to oxidise fuels indicate a state of economical energy storage and utilisation? To further characterise the fatty acid fuel utilisation, additional studies of various different muscles of offspring from undernourished dams would be useful to determine muscle-specific metabolic conditions. In addition, further studies would further reveal the mechanisms for the specific effect leptin has on skeletal muscle oxidative potential and insulin sensitivity.

Figure 5.1 below illustrates the developmental trajectory for the offspring of undernourished mothers from birth until adulthood. One of the main driving factors behind the research is that these offspring exhibit a phenotype superficially comparable to diet-induced obesity, yet their underlying metabolism is different. The pathway incited by prenatal nutrition is directed towards storage of energy and altered potential to utilise these stores, particularly in the case of adult skeletal muscle. Overall, the parallel studies of my thesis show agreement across different levels of structure and function (Figure 5.1). The prenatal undernutrition has generated a life path that converts to a thrifty phenotype that is healthy even when overweight. At the other end of the nutritional spectrum is the dietary obese organism that is insulin-insensitive and. In sum, this thesis can explain the issue of nutrition as a continuum in which development can prepare the organism for: (1) food shortage; and (2) adequate nutrition; but (2) fails to respond appropriately to situations that cause the diseases of excess nutrition i.e. high fat diet and reduced physical activity. In humans, the diseases of excess can paradoxically occur in impoverished populations.

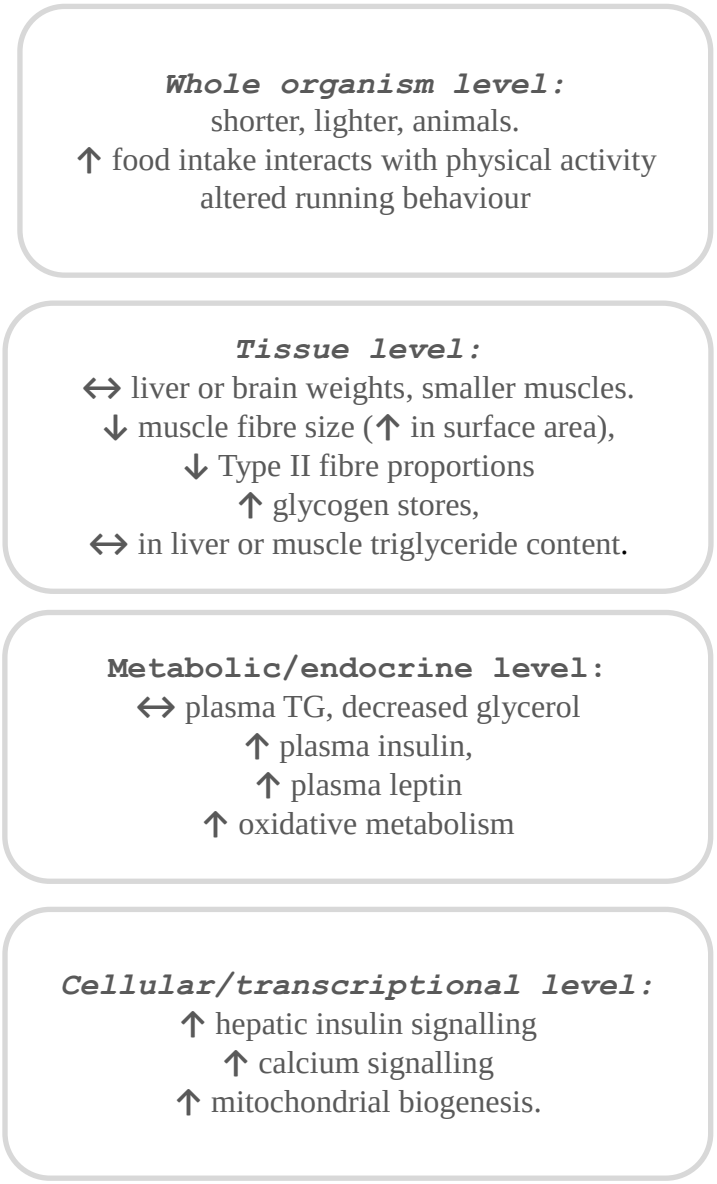

Figure 5.1 The key features of endurance phenotype in IUGR offspring of undernourished mothers.

Environmental challenges during development can determine the metabolic profile of the individual throughout life and across the levels of gene transcription, biochemical processes, efficiency of energy use and even behaviour. The figure demonstrates the effect of prenatal undernutrition on the experimental rat, at the level of: the whole organism, tissue, metabolism and at transcription.

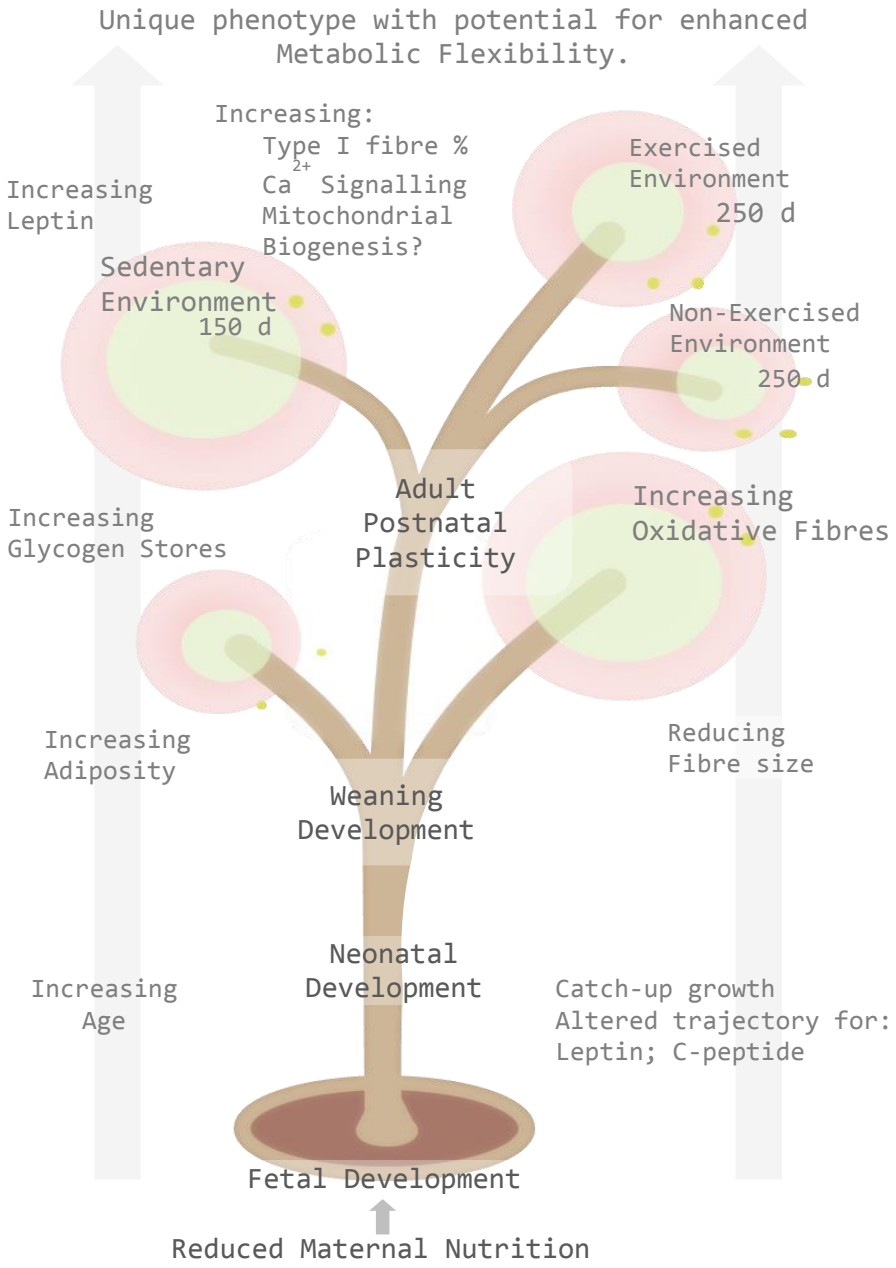

Figure 5.2 Pathways towards metabolic flexibility development in offspring of under-nourished mothers.

Extending the metaphor of the *rata* tree (*Section 1.5, Scope of Thesis*) the impact of prenatal undernutrition is illustrated conceptually with branches highlighting progressive development of key structural and functional adaptations. An altered developmental trajectory is emerging during fetal and neonatal growth and becoming apparent with increasing age. The features unique to IUGR offspring show a tendency towards metabolic flexibility, demonstrating an inherent resilience. As indicated on the figure, with increasing age, leptin concentrations increase in parallel with increases in glycogen/adipose tissue abundance. At the weaning branch point, the impact of prenatal nutrition is apparent by way of changes to muscle morphology. Then the adult branches show age- and activity-dependent induction of the skeletal muscle structural and functional features for enhanced oxidative metabolism.

It can be said that these IUGR offspring of undernourished mothers display characteristics that align with the Thrifty Phenotype hypothesis (Hales & Barker, 1992). Perhaps it may be more appropriate to describe the metabolism exhibited by offspring of undernourished dams as economical, with spare energy stored and readily available to be utilised when required. The spare energy may be derived from the abundant lipid or carbohydrate stores, considering the spare energy is not from increased dietary intake compared with controls. The key difference between the terms 'economical' and 'thrifty' is that the former might be more appropriate for a phenotype that is metabolically flexible even under sedentary conditions. An economical phenotype was induced at birth in the IUGR offspring and progressed along an altered developmental trajectory towards adulthood. The key features become fully apparent at a later adult stage (Thompson *et al.*, 2007; Huber *et al.*, 2009; Miles *et al.*, 2009a; Miles *et al.*, 2009b; Norman *et al.*, 2012) or maybe earlier if induced by postnatal environment.

5.2 Future Directions

The main findings of this research highlight the importance of both prenatal and postnatal nutrition and physical activity on metabolic health. As highlighted in the discussion above, in order to test my hypothesis that prenatal nutrition can impact postnatal metabolic flexibility, it will be necessary to measure oxidative metabolism directly. First of all, we would then be able to measure multiple time points, and with large number of subjects to increase the power of the studies. Second, it would be ideal to measure whole body oxidative metabolism to confirm speculations about fuel utilisation followed by *in vivo* measures of isolated tissue oxidative metabolism. Third, there remain unanswered questions about the mechanisms of the latent induction of skeletal muscle changes in the offspring of undernourished mothers.

Recent studies suggest the involvement of epigenetic process and their ability to cause long-term changes in metabolic regulation (Simmons, 2007). The hypothesis is that epigenetic processes might be responsible for the induction of these pathways long after the initial nutritional deficit encountered during fetal development and will need to be thoroughly tested. For example, epigenetic modulation of the expression of the

insulin-like growth factor -1 (IGF-1) gene has been proposed as a key candidate for the long-term regulation of organogenesis and functional changes under conditions of reduced substrate supply during fetal development. This hypothesis is especially attractive because the GLUT4 was found to be repressed by epigenetic mechanisms in IUGR offspring, offering long-term strategies that could explain permanent changes in fuel utilisation during later life (Raychaudhuri *et al.*, 2008). Furthermore, MyoD and MEF, additional muscle-specific transcription factors that drive myogenesis, that bind to the GLUT4 promotor regions were found to be altered in a different model of IUGR offspring (Raychaudhuri *et al.*, 2008). These novel strategies require further investigation to determine the extent to which muscle fibre type can be modified by specific epigenetic modulation of muscle precursor cells (Baar, 2010).

Nutrition Transition and the Impact of Lifestyle Modification

Nutrition transition is a broad term used to describe the progressive shift in dietary and physical activity patterns for humans in recent history (Chopra *et al.*, 2002). Diets high in saturated fats and sugars are often termed, collectively, the "Western diet." The combination of Western diet and reduced physical activity is commonly observed in modern societies. Such a combination is further associated with high levels of chronic non-communicable diseases such as obesity and Type 2 diabetes. A recent transition to the Western diet from a traditional lifestyle is thought to predispose indigenous populations to these chronic 'lifestyle diseases' (O'Dea, 1992). Indigenous populations are thought to be particularly vulnerable to chronic lifestyle diseases owing to a presumed dominance of thrifty genes (Yu and Zinman, 2007). In *Aotearoa*, New Zealand, the indigenous Maori population have recently (in some cases, as recent as three generations) transitioned from a traditional lifestyle with a diet consisting of primarily *kaimoana* (seafood), *tuna* (eel) and *kumara* (sweet potato). In conjunction with the reduced activity associated with the food procurement practices and urbanization, rates of chronic non-communicable disease have risen (Anderson *et al.*, 2006). However, a renaissance of traditional food practices has shown positive results at the community level. A case study that compared the practice of catching and consuming *tuna* to those eating a Western diet found indications of differences in the health and wellbeing of participants (Nixon-Benton, 2010). The consumption of eel was associated with significant health outcomes, potentially in relation to the omega

fatty acid content also measured in the eel (Nixon-Benton, 2010). While it is difficult to extrapolate these results to whole populations, the impact of positive cultural and traditional food practices are likely to give rise to a shift in the focus from health 'risk' to 'potential'. Perhaps the change in attitude towards eating and exercising, in association with cultural adaptation to present day conditions with a traditional or cultural perspective, may aid in the modification of populations to reduce the impact of metabolic disease.

The body has a large capacity to store energy in the form of fat and the resulting obesity is known to be potentially detrimental for metabolic health. The co-morbidities associated with obesity can include insulin resistance and cardiovascular disease and are evident in the state of metabolic *inflexibility*. Consequences of metabolic derangement, like insulin resistance etc. are increasing in incidence as populations get fatter. To a large extent, the increase in fatness might be explained by the abundance of energy-dense food in combination with reduced physical activity.

Metabolic flexibility can be quantified by measures of the change in respiratory quotient (respiratory exchange ratio) from the fasted state to the insulin-stimulated state (Meex *et al.*, 2011). For example, the respiratory quotient might be measured using a sophisticated apparatus such as the comprehensive lab animal monitoring system (CLAMS) (Columbus Instruments, Ohio, United States of America). Considerations for the influence of postnatal diet and physical activity using an inbuilt running wheel on metabolic flexibility could be standardised across subjects using such a CLAMS system. The indirect calorimetry of gas exchange would be able to determine the energy consumed/utilised/expended. It would be interesting to investigate long-term physical activity in the offspring of undernourished mothers. With considerations for the commonly observed exercise-induced anorexia in rats, the subjects of my studies were subjected to a standardised 56 m of moderate daily exercise. However, it would be of interest to investigate a broader range of activity intensity. A monitoring cage system would reinforce the voluntary nature of the exercise with a particular emphasis on access during the active nocturnal phase. Furthermore, the CLAMS system might enable studies to assess whether the timing of the feeding during the light phase had any significant influence on clock genes or metabolism. One of the main hypotheses driving my research is that nutrient

deprivation during development entrains altered skeletal muscle physiology adapted to endurance physical activity. Therefore, it may be necessary to further consider the neurological control of food seeking behaviours. The studies by Miles et al. demonstrated the choice behaviour of these IUGR offspring tending to favour the opportunity to run in a wheel (Miles et al., 2009). Therefore, it would be of considerable interest to investigate physical activity in relation to chronobiology (Carneiro et al., 2009). The broader implications of this style of study might be used to understand the physiological and health impacts of altered circadian rhythms associated with shift work (Carneiro and Araujo, 2009; Glad *et al.*, 2011).

Respirometry studies of intact and permeabilised muscle fibres could provide important information about mitochondrial respiratory function in offspring of undernourished mothers. Measures of substrate flux in isolated muscle and perhaps even in adipose tissues would possibly confirm the speculations discussed above about enhanced oxidative metabolism. High-resolution methodologies like the Oroboros system (Oroboros Instruments, Innsbruck, Austria) are able to measure oxidative phosphorylation (oxphos) flux in whole fibres and provide information that is more closely related to in vivo conditions (Scheibye-Knudsen and Quistorff, 2009). Using oxygraph methodologies, I could evaluate oxphos flux in individual muscle fibres and compare this to metabolic status between experimental offspring. Using this methodology could potentially add weight to the argument that the IUGR offspring of this model have enhanced oxidative metabolism.

To explore the changes seen in mitochondrial biogenesis further, it will also be necessary to assess protein content of the markers measured in the current study at the transcriptional mRNA level. Furthermore, recent studies suggest the involvement of epigenetic process and their ability to cause long-term changes in metabolic regulation (Chmurzynska, 2010). The epigenetic changes represent a potential mechanism behind the altered trajectory of developmental pathways induced by inadequate nutrition during fetal development. Therefore, further studies might reveal whether changes in promoter methylation patterns are the molecular mechanism responsible for the postnatal changes in gene expression observed.

Concluding remarks

The work for this thesis began by first asking the question whether metabolic flexibility was influenced by prenatal nutrition. The combined results of my studies revealed support for the main hypothesis that prenatal undernutrition induces a phenotype favouring energy storage and oxidative metabolism in postnatal life by altered skeletal muscle structure and function. From the skeletal muscle structure and function data I learned that the IUGR offspring diverge from AD controls at some time point after weaning. The experience *in utero* had life-long effects on the biology of IUGR offspring many of which develop over the whole life course. The experimental timeframe selected was not able to capture the specific mechanisms for the delayed response to prenatal undernutrition observed. However, it is possible that the economical phenotype we observe in IUGR offspring is related to both energy storage and oxidative capacity in adult skeletal muscle. Further studies into the expression of key factors for muscle determination/differentiation/growth and mitochondrial biogenesis might consider a focus at transcriptional and protein levels. The ontogeny of metabolic flexibility in the IUGR offspring might also be fully revealed by investigating respiratory exchange quotient and epigenetic factors.

Appendix I

I.1 Buffers and Solutions

I.1.1 Homogenisation Buffer:

Reagent	Volume
HEPES, pH 7.4	50 mmol/L
0.1% TRTON X-100	10 µl/10 ml
EGTA	4 mmol/L
EDTA	10 mmol/L
β-glycerophosphate	100 mmol/L
Sodium orthovanadate	5 mmol/L
Tetrasodium pyrophosphate	15 mmol/L
Sodium Fluoride	25 mmol/L
Complete Mini – EDTA free Protease Inhibitors (Roche Diagnostics)	1 tablets in 10 ml

I.1.2 Laemmli's Loading Buffer (10 X SDS Buffer):

Reagent	Volume
20 % Sodium dodecyl sulfate	6.25 ml
Dithiothreitol	770mg
Glycerol	1.75 ml
2.5M Tris pH 6.8	1.2 ml
0.5M EDTA	0.8 ml
Bromophenol blue	A few bits

I.1.3 Running Buffer (5x)

In a final volume of 1 Litre at pH of 8.3 -8.6

Reagent	Volume
Tris HCL	14.5 g
Glycine	72 g
Sodium dodecyl sulfate	5g

I.1.4 Transfer Buffer

Reagent	Volume
2.5mM Tris HCL	6.06 g
192 mM Glycine	28.8 g
Methanol	400 ml
MilliQ water	1600 ml

I.1.5 Phosphate Buffered Saline (PBS) Wash Buffer

Reagent	**Volume**
Sodium dihydrous phosphate monohydrate	0.4 g
Di sodium hydrogen phosphate anhydrous	2.75 g
Sodium chloride	17.85 g
TWEEN -20	2ml

I.1.6 Blocking Buffer

Reagent	**Volume**
Dry fat free milk	5g
Phosphate buffered Saline + Tween 20	100 ml

I.2 Enzyme Assays

I.2.1 Enzyme Assay Homogenisation Buffer:

Reagent	**Volume**
Glycerol	50%
KCl	40 mM
EDTA	2 mM
Tris-HCl, pH 7.8	25 mM
TRITON X-100	0.2 %

I.2.2 Citrate Synthase Assay Master Mix:

Reagent	**Volume**
Tris-HCl, pH 8.0	50%
Acetyl-CoA	0.1 mmol/L
DTNB	0.2 mmol/L

I.3 Immunohistochemsitry

I.3.1 PBS Blocking Solution

Reagent	**Volume**
Goat serum	2 ml
TRITON X-100	250 µl
1 x PBS + 0.1 % NaN_3	48 ml

I.3.2 PBS-Covering Medium (pH 7.0)

Reagent	**Volume**
PBS + 0.1 % NaN_3	10 ml
Glycerol	40 ml

Appendix II

II.1 Antibodies

The PKC ζ primary antibody used in the western blot studies was polyclonal raised in rabbits and diluted to the stated concentration in 5% milk blocking buffer. The secondary antibody was anti-rabbit HRP-conjugated IgG raised in goat. The primary antibodies for the immunohistochemistry (myosin Type I and IIa) were diluted as indicated in PBS blocking buffer. The secondary antibody was detected using TRITC fluorescence and was also diluted in PBS blocking buffer.

II.2 Antibody Concentrations:

Primary Antibody	**Host**	**Dilution**	**Supplier**
Anti-PKC ζ	Rabbit	Liver 1:3000	Santa Cruz Biotechnology (sc-216)
Myosin Type I	Mouse	1:100	Novocastra Laboratories (NCL-MHCs)
Myosin Type IIa	Mouse	1:100	Alexis Biochemicals (A4.74)
Secondary Antibody	**Host**	**Dilution**	**Supplier**
Anti-PKC ζ (anti-rabbit HRP conjugated IgG)	Goat	Liver 1:20000	Sigma (A5045)
Cy3 Red (TRITC)	Mouse	1:500	Jackson ImmunoResearch Laboratories Inc (115-165-146)

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