THE INTERACTION BETWEEN MATERNAL NUTRIENT RESTRICTION AND POSTNATAL NUTRIENT EXCESS IN AN OVINE MODEL

Phillip Steven Rhodes, BSc (hons).

A thesis submitted for the degree of Doctor of Philosophy

to

The University of Nottingham

December 2010

1. Abstract

Evidence from human and animal studies has highlighted the sensitivity of the developing fetus to environmental insults, such as maternal undernutrition, during gestation. These nutritional perturbations to the intrauterine milieu may engender a legacy of deleterious health consequences in adulthood. This thesis presents a series of studies which test the `mis-match' fetal programming theory; that is, whether a nutritionally poor diet prenatally interacts with a nutritionally excessive diet postnatally to overtly increase risk factors for adult disease. The effect of a maternal global energy restriction is contrasted against a maternal specific protein restriction, each fed during either early or late gestation. Adult offspring were subsequently exposed to an obesogenic environment (elevated feed with increased lipid content whilst restricting physical activity). Offspring metabolic flexibility and competence were assessed through routine blood samples throughout postnatal life and at 7, 18 and 24 months of age by glucose (GTT) and insulin (ITT) tolerance tests and body composition by dual energy x-ray absorptiometry. In general males appeared more susceptible to developmental programming than females at a number of timepoints. Furthermore, an increased first-phase or incremental area-under-the-insulin-response curve was observed in 1) offspring following maternal protein restriction in early gestation at 7 and 24 months of age, despite maternal protein restriction in late gestation significantly reducing birthweight and 2) in offspring exposed to maternal energy restriction during late gestation at 24 months of age in response to a GTT. Since, in both groups, the response of all offspring to an ITT (from a euglycemic baseline) was similar, infers that developmental programming in sheep followed by nutritional excess is first revealed as affecting either the pancreas (~insulin hypersecretion) or the liver (hepatic insulin resistance, reduced first-pass insulin metabolism). The studies illustrate the importance of habitual consumption of an `optimal' balanced diet through gestation on postnatal health, especially in light of the current obesity epidemic.

2. Related Publications

Gardner DS & **Rhodes PS**. (2009). Developmental Origins of Obesity: Programming of Food Intake or Physical Activity? *Early Nutrition Programming and Health Outcomes in Later Life: Obesity and Beyond* **646,** 83-93.

Rhodes PS, Craigon J, Gray C, Rhind SM, Loughna PT & Gardner DS. (2009). Adult-onset obesity reveals prenatal programming of glucose-insulin sensitivity in male sheep nutrient restricted during late gestation. *PLoS One* **4,** e7393.

Presentations at scientific conferences related to this thesis

Life Sciences 2007, Glasgow-

Maternal Undernutrition and Offspring Birth Weight, Growth and Body Composition in Sheep (Poster).

Institute of Clinical Research 2008, QMC Nottingham &

Fetal & Neonatal Physiological Society (FNPS) 2008, Maastricht

Prenatal diet and adult-onset obesity influence fat, protein and carbohydrate metabolism in adult sheep (Oral).

Nutrition Society 2008, Nottingham

Interactions between pre & postnatal diet on metabolic competence in sheep (Oral).

British Endocrine Society 2008, Harrogate

Prenatal diet and adult-onset obesity influence fat, protein and carbohydrate metabolism in adult sheep (Poster).

Physiological Society 2008, Cambridge

Prenatal diet and adult-onset obesity influence plasma amino acids in adult sheep (oral).

Funding received-

Awarded £150 Biochemical Society bursary

Given as a contribution towards attending the Lifesciences 2007 Conference

Awarded £500 Pfizer bursary

Given as a contribution towards the costs of attending FNPS 2008 conference

Awarded £1000 Physiological Society bursary

Given to pay for *in vivo* physiological training at the University of Bristol

Awarded £150 University of Nottingham student prize

Given by the graduate school in recognition of progress made throughout the PhD

3. Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the test.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:……..……

Date:……

4. Acknowledgements

My first acknowledgement goes to the staff of the School of Biosciences in the University of Nottingham. Not only did these people instil a passion for scientific investigation in me whilst completing my undergraduate studies, but they were also willing to support me with time and resources when times were hard throughout my postgraduate studies. Following from this, I would also like to thank the staff and postgraduate students at the School of Veterinary Medicine & Science, who gave me the opportunity to study for this PhD, and then supported me throughout. Particular mentions must go to Scott Hulme, Senior Research technician, for his technical support and Clint Gray, a fellow student and housemate who was always willing to help and go the extra mile, whatever the task entailed! Regarding the study itself, I need to acknowledge Dr Stewart Rhind, Carol Kyle and the other members of the Macauley institute who were involved in the development of the early stages of this research model, as well as for providing information, materials and data. A mention must go to all the people who kept nudging me along the way. On far too many occasions the end has not been insight during this study, I've had some great times, but it's the people who kept me going through the dark ages who really deserve a mention. My final, and most important "thank you" goes to Dr David Gardner and Dr Paul Loughna, my supervisors. Above being great scientists, they proved themselves to be very decent men, for thom I will always hold in the highest regard. I certainly would not be here now adding the final touches to this thesis without them, so thank you!

5. Table of contents

6. List of Tables and Figures

7. Commonly Used Abbreviations

	11βHSD2	**11-β Hydroxysteroid Dehydrogenase (type 2)**
A	**AA**	**Amino Acids**
	AUC	**Area Under the Curve**
B	**BCS**	**Body Condition Score**
	BMI	**Body Mass Index**
	BMP	**Bone Morphogenic Protein**
C	**CE**	**Control Energy**
	CP	**Control Protein**
D	**DEXA**	**Dual Energy X-Ray Absorptiometry**
E	**EDTA**	**ethylenediaminetetraacetic acid**
	ELISA	**Enzyme-linked immunosorbent assay**
F		
G	**G**	**Gender**
	GC-MS	**Gas Chromatography–Mass Spectrometry**
	G*Ti	**Gender*Time Interaction**
	GTT	**Glucose Tolerance Test**
	GLMM	**General Linear Mixed Model**
	GLMM-RM	**General Linear Mixed Model Repeated Measures**

H	**HDL**	**High Density Lipoprotein**
	HNF	**Hepatocyte Nuclear Facor**
	HPA axis	**Hypothalamic–pituitary-adrenal axis**
	hrs	**Hours**
I	**IGF-1**	**Insulin-like Growth Factor 1**
	I.M.	**Intramuscular**
	ITT	**Insulin Tolerance Test**
	IU	**International Units**
	I.V.	**Intravenous**
J		
K	**kg**	**kilogram**
	K_{ITT}	**Rate of glucose disappearance after ITT**
L	**LDL**	**Low Density Lipoprotein**
	LEE	**Low Energy Early**
	LEL	**Low Energy Late**
	LPE	**Low Protein Early**
	LPL	**Low Protein Late**
M	**ME**	**Metabolisable Energy**
	ml	**millilitre**
	$mmol.L^{-1}$	**millimoles per litre**
N	**NEFA**	**Non-Esterified Fatty Acid**
	ng.ml	**nanograms per millilitre**
O		

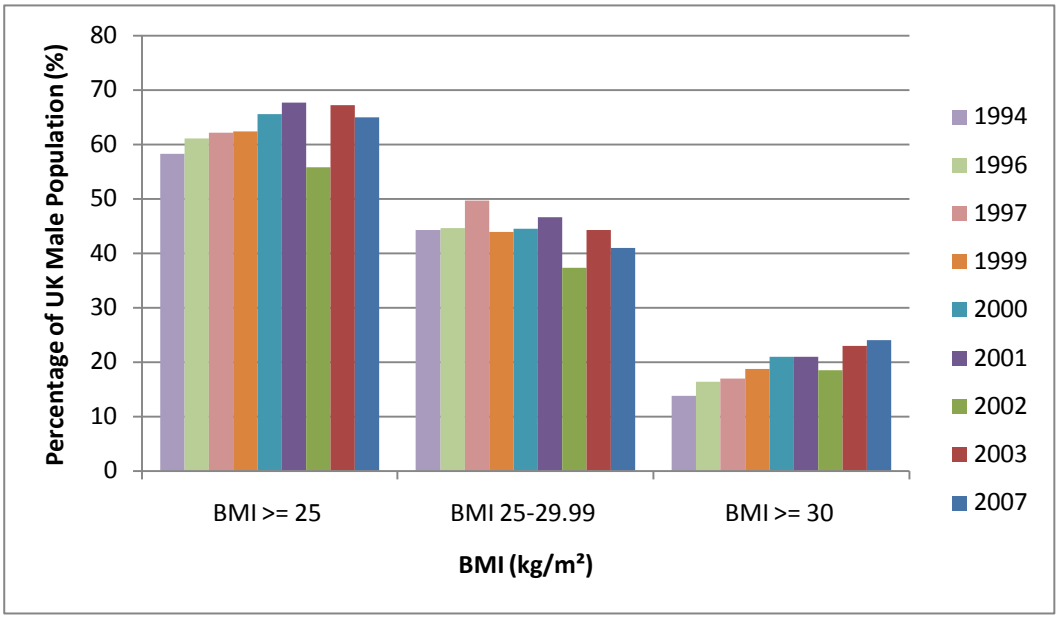
P	**Pdx1**	**Pancreatic and duodenal homeobox 1**
Q		
R	**RIA**	**Radio-Immuno Assay**
S	**S.E.D.**	**Standard Error of the Difference**
	S.E.M.	**Standard Error of the Mean**
T	***T***	**Treatment**
	TAG	**Triglyceride**
	Ti	**Time**
	T*Ti	**Treatment*Time Interaction**
	T*G	***Treatment*Gender Interaction***
	T*G*Ti	**Treatment*Gender*Time Interaction**
U	**UK**	**United Kingdom**
V	**VFA**	**Volatile Fatty Acids**
W		
XYZ	**yrs**	**years**

1. Literature Review

1.1 Global Health

Over the past 30 years the incidence of metabolic syndrome, and the components of it, have rapidly increased in prevalence within westernised populations. The metabolic syndrome is classically comprised of (to varying extents); obesity, glucose intolerance, insulin resistance (both constituents of type 2 diabetes), hypertension and dyslipidemia (Alberti *et al.*, 2006).

Obesity is defined as having a Body Mass Index (BMI) equal to or greater than 30kg/m^2. Figure 1.1 shows that in the UK alone between 1994 and 2007 there was a significant increase in the number of obese individuals, both males (13.8 to 24%) and females (17.3 to 24%). This data also shows that in England in 2007, 65% of men and 56% of women were either overweight (BMI 25-29.99) or obese (BMI >=30).



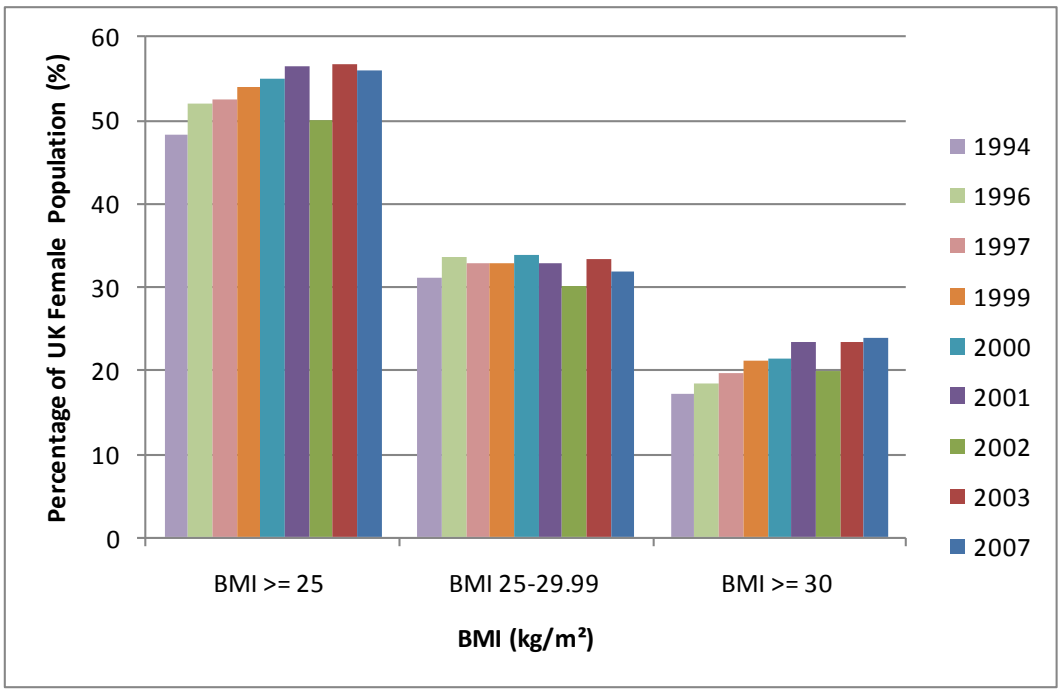


Figure 1.1 Change in BMI of the English population between 1994-2007

Graphs showing the change in population BMI in England for males (top) and females (bottom) between 1994 & 2007. Sample size per gender per year ranged between 3000-8000. All subjects were aged between 16-100yrs. Data obtained from (www.who.int/en, 2010)(accessed August 20th 2010).

The prevalence of overweight and obesity in the United States is even greater than in the UK. Table 1.1 shows that 1 in 3 U.S. adults are obese, with 2 in 3 being

overweight. The number of obese males in the U.S. has tripled between 1962-2006 from 10.7 to 32.4%, while the number of obese females has doubled in the same period of time.

	% of U.S. Males		% of U.S. Females	
Year	**>= 25**	**>=30**	**>= 25**	**>=30**
1960-1962	49.4	10.7	41.2	16.1
1995-1995	46	21	28	23
2003-2006	72.1	32.4	61.3	34.3

Table 1.1 Change in BMI of the United States population between 1960-2006

Data showing the change in BMI (kg/m^2) for males and females in the U.S. population between 1960 & 2006. Sample size per gender per year ranged between 2000-3000. All subjects were aged between 20-100yrs. Data obtained from (www.who.int/en, 2010)(accessed August 20th 2010).

Associated with the current global epidemic of obesity is the sharp rise in individuals diagnosed with type 2 diabetes. Wild *et al*., (2004) estimated that the number of people diagnosed with type 2 diabetes globally in the year 2000 was 171 million people, which is 2.8% of the world population, they expect this value to rise to 366 million by 2030 (4.4% of the global population) (Table 1.2). Their estimate is based on the global obesity rate remaining at that of the year 2000, with an aging, more urbanised global population.

	Year	**2000**	**2030**
Ranking	**Country**	**People with diabetes (millions)**	
1	India	31.7	79.4
2	China	20.8	42.3
3	U.S.	17.7	30.3

Table 1.2 Diabetes estimates for the year 2000 and 2030.

The 3 counties shown are estimated to have the highest current (2000) and future (2030) diabetes incidence globally (Wild *et al.*, 2004).

1.2 Obesity & Type 2 Diabetes

Insulin resistance develops as the insulin sensitive tissues become desensitised to its action and increasing quantities of insulin are required to elicit the same response. Insulin is an anabolic hormone released from the pancreatic beta cells of the endocrine pancreas (Islets of Langerhans). Glucose stimulated insulin secretion is the primary mechanism for postprandial glucose uptake, with the majority (90%) of the glucose being taken up by the skeletal muscle and the rest by the liver and adipose tissue. Insulin resistance is characterised by hyperinsulinemia and euglycaemia, when the pancreas is unable to produce sufficient insulin to maintain euglycaemia, possibly due to exhaustion, then hyperglycaemia prevails, which is a key pathology in type 2 diabetes. Other effects of insulin resistance include the reduced ability of insulin to inhibit lipolysis in adipose tissue and gluconeogenesis in the liver (Mlinar *et al.*, 2007). These effects exacerbate homeostatic dysregulation of plasma lipids and glucose in individuals with obesity and/or high glucose/fat intake.

The risk of developing insulin resistance can be accounted for by genetic and epigenetic factors, as well as lifestyle choices such as diet and physical activity levels, but some susceptibility is not accounted for by these factors. Insulin resistance is multifaceted and is generally, but not exclusively, associated with excessive lipid storage. Adipose tissue is the primary site for plasma lipid uptake and over the last 15 years much research has been conducted into the importance of

adipose tissue as an endocrine organ. Adipokines, cytokines secreted by adipose tissue, have been linked with the pathogenesis of insulin resistance. The adipokines concerned are tumor necrosis factor alpha (TNFα), adiponectin, interleukin-6 (IL6), resistin, leptin (Arner, 2005) and Retinol Binding Protein 4 (Wolf, 2007). The physiological locality of adipose tissue affects its functionality, specifically the endocrine activity of the tissue, with central/abdominal adipose tissue secreting higher levels of adipokines than peripheral tissue (Anghel & Wahli, 2007). Changes in adipokine secretion and sensitivity have been implicated in the pathogenesis of insulin resistance (Arner, 2005; Guilherme *et al.*, 2008), with Retinol Binding Protein 4 (RBP4) being associated with decreased PI3K signalling following insulin stimulation (Wolf, 2007). As well as increased adipokine secretion, adipocytes secrete increasing volumes of pro-inflammatory cytokines in the obese state. Factors such as IL6, TNFα and Monocyte Chemoattractant Protein-1 (MCP1) stimulate JNK and IκK signalling pathways, which are associated with insulin resistance (Kahn *et al.*, 2006; Guilherme *et al.*, 2008). It is not just the fat accretion in adipose tissue that affects systemic insulin resistance; ectopic intramuscular fat deposition (Mlinar *et al.*, 2007) and Non-Alcoholic Fatty Liver disease (Ciba & Widhalm, 2007; Fan *et al.*, 2007; Holland *et al.*, 2007; Parekh & Anania, 2007) have both been identified in the pathogenesis of insulin resistance. Therefore any inherent mechanisms that lead to the localisation of fatty acids in any of these compartments (ectopic deposition and visceral adipose tissue accumulation) would greatly increase the risk of the developing insulin resistance (Holland *et al.*, 2007).

1.3 Glucose Uptake & Metabolism

This sub chapter aims to give an overview of glucose uptake and metabolism. Further details can be found in texts related to metabolism and nutrition such as Frayn (2003) or McDonald *et al.,* (2002).

1.3.1 Overview of Respiration

Glucose is a 6-carbon (hexose) sugar molecule that is either unbound as a monosaccharide or linked to other hexose molecules to form larger, complex polysaccharides. It is a key metabolic substrate and is produced in photosynthesis whilst degraded in respiration resulting in ATP synthesis from ADP molecules. The initial anaerobic phase of respiration is glycolysis, where energy is produced throughout the conversion of glucose to 2 pyruvate molecules. The pyruvate molecules are then moved from the cytosol to the mitochondria to undergo oxidative decarboxylation producing acetyl-CoA. In the presence of oxygen, Acetyl-CoA enters the krebs cycle leading to the reduction of the electron transporters NAD^+ and FAD to NADH and $FADH_2$ respectively, as well as producing CO_2 and H_2O. The coenzymes NADH and $FADH_2$ then go through oxidative phosphorylation, generating ATP, CO_2 and H_2O.

1.3.2 Pancreatic Glucose Sensing

Polysaccharides found in food are digested to liberate monosaccharides which are then absorbed into the portal blood stream (covered in Chapter 1.7). The endocrine pancreas reacts to the stimulus of an elevated glucose level (hyperglycaemia) and

acts to return euglycaemia. The pancreas has both exocrine and endocrine functions. The endocrine pancreas accounts for approximately 2% of the pancreatic tissue mass, of which 60% are β cells of the Islets of Langerhans. Glucose is taken up into the β cells by Glut 2 glucose transporters, these transporters have a high K_m meaning that the higher the blood glucose concentration, the greater their affinity for glucose. Therefore the glucose uptake is proportionate to the plasma glucose concentration. The β cell metabolises the glucose to produce ATP. ATP sensitive K^+ channels are then closed, causing depolarisation, opening voltage sensitive Ca^{2+} channels. The influx of calcium ions leads to the exocytosis of insulin containing vesicles, thus depositing insulin into the hepatic portal vein.

1.3.3 HEPATIC ANABOLISM

The portal blood reaching the liver, which is hyperglycaemic and hyperinsulinaemic, stimulates glucose and insulin clearance by the hepatocytes. Glucose, as in the pancreas, enters the hepatocyte via Glut 2 transportation. The insulin initiates a switch from catabolism to anabolism, involving the inhibition of gluconeogenesis, which is a de novo glucose synthetic pathway generating glucose from non carbohydrate carbon backbones such as volatile fatty acids, lactate, amino acids and glycerol. Insulin also modulates the storage of glucose as glycogen (glycogenesis) by switching on glycogenic pathways and inhibiting glycogenolytic pathways. Glycogenolysis and gluconeogenesis in the liver are fundamental pathways involved in maintaining systemic euglycaemia in the fasted state and hence must be inhibited in the fed state to prevent prolonged hyperglycaemia. Although skeletal

muscle cells also store glucose as glycogen, skeletal muscle glycogen stores can not influence systemic levels due to the lack of glucose 6 phosphatase expression, which is a key hepatic enzyme allowing glucose to leave the hepatocyte and enter the circulation.

1.3.4 SKELETAL MUSCLE INSULIN STIMULATED GLUCOSE UPTAKE

In the fasted euglycaemic state, Glut 1 transporters are primarily responsible for basal glucose uptake in all cells. In the fed state however, the insulin and exercise sensitive glucose transporter, Glut 4, accounts for the mass glucose clearance from the blood into the skeletal muscle (~90%), and to a lesser extent the adipose tissue as well. Following the first pass of glucose and insulin through the liver, approximately 70% of the plasma insulin has been removed by the liver. The remaining insulin binds with the insulin receptor located in the plasma membrane of skeletal muscle and adipose cells. Stimulation of the insulin receptor leads to a downstream cascade of intracellular phosphorylation signalling effects. In brief, auto-phosphorylation of the insulin receptor (IR) causes the phosphorylation of insulin receptor substrate (IRS), consequently activating Phosphatidylinositol-3-kinase (PI3K). PI3K enables the phosphorylation of Phosphatidylinositol (4’5’) Bisphosphate (PIP_2) into PIP_3, which in turn binds to 3’-Phosphoinositide dependent kinase-1 (PDK1), thus activating it. Activated PDK1 is then able to phosphorylate Phospho-Kinase B (PKB, also called Akt), as well as Phospho-Kinase C (PKC). PKB then modulates the translocation of Glut 4 vesicles to the plasma membrane, facilitating insulin stimulated glucose uptake in these tissues. PKB also

phosphorylates Glycogen Synthase Kinase 3 β (GSK3β) causing its deactivation, thus preventing the GSK3β inhibition of glycogen synthase. Active glycogen synthase is then able to convert glucose into its stored form, glycogen. The plethora of anabolic effects insulin stimulates in skeletal muscle goes far beyond simple glucose uptake and storage, including inhibition of lipolysis, elevated protein and fatty acid synthesis, as well as increased cell survival/decreased apoptosis, as shown in Figure 1.2.

It is important to note that another major stimulator of glucose uptake in skeletal muscle is exercise, as modulated by AMPK activation, with many similar biochemical outcomes to insulin signalling, for AMPK signalling details see recent reviews by Hardie (2011b, a).

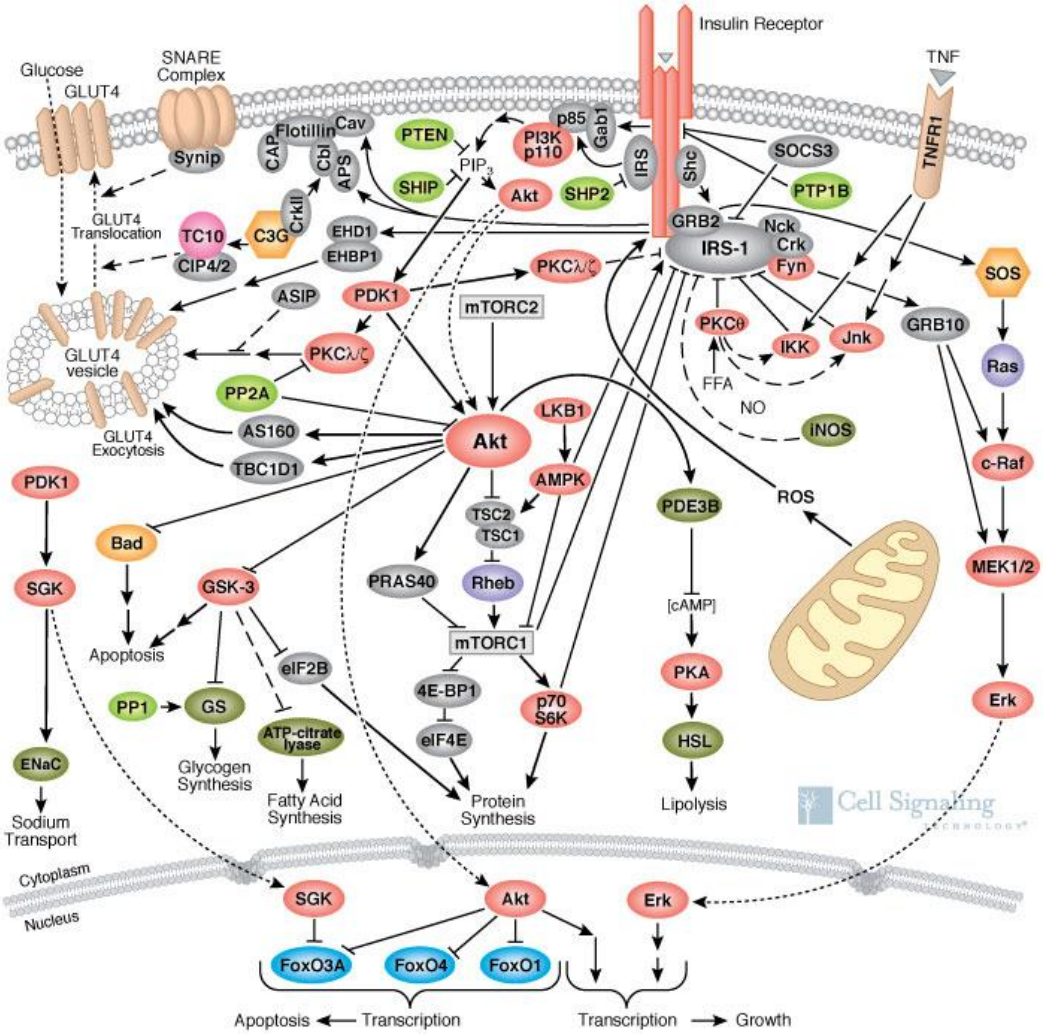


Figure 1.2 Insulin Stimulated Glucose Uptake in Skeletal Muscle

Diagram showing insulin signal transduction in skeletal muscle. Insulin stimulation causing GLUT4 translocation to the plasma membrane leading to glucose uptake. The insulin signal also initiates the downstream cascade resulting in, glycogen, protein and fatty acid synthesis, cell survival and inhibition of glycogenolysis, lipolysis, protein degradation and apoptosis. In brief, red (kinases), light green (phosphatases), dark green (enzymes), light blue (transcription factor), arrows (stimulatory effects), blunt ends (inhibitory effects) and dashed lines (translocation). The diagram and legend details were taken from www.cellsignalling.com (2010).

1.3.5 EMBRYONIC AND FETAL DEVELOPMENT

1.3.5.1 *Early Development*

Mammalian development starts with embryogenesis, which is initiated after fertilisation, the fusion of the male and female gametes, producing a zygote. The zygote then undergoes a period of rapid mitotic cell divisions, termed cleavage, producing the blastocyst, containing the inner cell mass, which goes on to form the embryo, and the trophoblast, which develops into the placenta. The blastocyst starts to form three distinctive germ layers, the endoderm, mesoderm and ectoderm (Twyman, 2001). Implantation occurs around this time, with the blastocyst imbedding into the uterine lining. The germ layers continue to develop, producing a multitude of different cell fates via organogenesis. The developing ectoderm's major product is the nervous system; the bones, cartilage, connective tissues, muscles and notochord develop from the mesoderm, while the endoderm forms the gastrointestinal, urinary and respiratory tracts.

1.3.5.2 *Skeletal Muscle Development*

Skeletal muscle develops as part of a process termed myogenesis during embryogenesis. In most mammals, (excluding rodents) myogenesis is completed prenatally, thus setting the myofibre number in skeletal muscle before birth (Brameld *et al.*, 1998). The myogenic lineage originates from the somite segments, which are derived from the dorsal mesoderm region of the developing embryo (Twyman, 2001). The newly formed somites then divide again creating the

sclerotome and the dermomyotome; of which it is the dermomyotome which segregates again to form the dermatome and the myotome (Christ & Brand-Saberi, 2002; Buckingham *et al.*, 2003). The production of Sonic hedgehog (Shh) from the notochord and floor plate stimulates the commitment of the myotome to two separate fates; epaxial myotome producing the musculature of the back, and the hypaxial myotome forming the limbs and ventral body muscle (Yun & Wold, 1996; Twyman, 2001; Buckingham *et al.*, 2003). Epithelial cells delaminate from the various myotome surfaces to form myogenic progenitor cells. Delamination is initiated and controlled by wnt (1, 2 & 4) secretions by the dorsal neural tube for epaxial myotome delamination and by the ectoderm region for hypaxial myotome delamination (Twyman, 2001).

Following delamination the myogenic progenitor cells migrate to their final destination, where they will eventually form part of the functional musculoskeletal apparatus (Christ & Brand-Saberi, 2002; Buckingham *et al.*, 2003). The hypaxial cells which are destined for the limb buds are promoted towards this region by c-met (via pax-3) and Lbx1 (Christ & Brand-Saberi, 2002; Buckingham *et al.*, 2003). The hypaxial cells are prevented from differentiating prematurely during their migration by the inhibitory effect of Bone Morphogenetic Protein 4 (BMP4) secreted from the lateral mesoderm (Twyman, 2001). Due to the variation in distance covered by migrating progenitor cells some cells are determined and even differentiated before others have finished proliferating.

Myogenic progenitor cells undergo hyperplasia (proliferation) during migration whilst under the control of several transcription factors. These include pax-3, c-met, Msx1 and Mox2, as well as SIX 1 and 4 (with cofactors Dach2 and Eya 2,4) (Yun & Wold, 1996; Buckingham *et al.*, 2003).

Following progenitor cell proliferation, determination occurs. Determination is the transition of myogenic progenitor cells into myoblasts, and thus the commitment to a myogenic fate. Determination results in the production of transcription factors that control the differentiation of the cells into myotubes. This developmental process is fundamentally controlled by Myogenic Determination factor (MyoD) and Myogenic factor 5 (Myf5) (Sassoon, 1993; Yun & Wold, 1996), which can be collectively referred to as the Determination Class Muscle Regulatory Factors (MRFs). MRFs are characterised by their basic Helix-Loop-Helix (bHLH) structure. They can either form homodimers or heterodimers with E proteins, which consequently bind to E boxes. E boxes are consensus sequences found within a major groove binding site on the promoter/enhancer region of responsive myogenic genes, with the sequence CANNTG (Yun & Wold, 1996; Langlands *et al.*, 1997; Berkes & Tapscott, 2005). Inhibitory mechanisms operate to regulate MRF-MRF/E-protein complex formation, and its binding to the E box, these include Twist and Id (Inhibitor of DNA binding) 1-4. Benezra *et al.,* (1990) postulated that Id could be expressed as a mechanism for controlling and preventing premature determination during precursor migration and proliferation. Ids bind to Determination Class MRFs

and E proteins with a high affinity, but due to their helix-loop-helix structure and hence lack of basic, amino terminal DNA binding region they are unable to bind to the E box DNA region (Yun & Wold, 1996; Langlands *et al.*, 1997).

Myf5 and MyoD are only expressed once the progenitor cell completes its migration to the limb bud (Tajbakhsh & Buckingham, 1994). This suggests that migration has to be completed for determination to occur. The potency of Myf5 and MyoD transcription factors can be seen by their ability to induce myogenic protein expression in non-muscle cells, *in vitro*, such as myogenic protein expression by fibroblasts (Davis *et al.*, 1987). Mutation to these genes shows dramatic effects on skeletal muscle cell development. The double null Myf5/MyoD mutant developed by Rudnicki *et al.,* (1993) shows the absence of these genes prevents the development of the myoblast population and hence muscle fibres. Consequently following migration these progenitor cells develop into non-myogenic fates, such as adipocytes (Tajbakhsh & Buckingham, 1994). MyoD and Myf5 appear to have very similar effects on gene expression. Single deletions of these genes by Kablar *et al.,* (1998) showed that functional muscle still develops in the embryos. In depth analysis of these embryos does however show some developmental abnormalities. Null Myf5 embryos have defects related to trunk skeletal muscle. Whereas null MyoD embryos show early limb and branchial arch muscle development is delayed. These deletions show the possible specific developmental mechanisms controlled by these two MRFs.

Myf5 and MyoD have the effect of turning on the genes which will control terminal differentiation of the myoblasts into myotubes. These genes include the Differentiation Class MRFs (myogenin/Myf4 & MRF4/Myf6) and the Myocyte Enhancer Factor 2 (MEF2) (Yun & Wold, 1996). These MRFs also activate genes causing the synthesis of proteins such as M-cadherin, myosin heavy and light chain, as well as creatine kinase (Berkes & Tapscott, 2005). Following myoblast proliferation the cell exits the cell cycle. This is a specialised and permanent G_0/G_1 arrest which prevents the myoblasts from proliferating and dividing further (Yun & Wold, 1996). The exit from the cell cycle is under the control of myogenin expression and the protein p21 (Andres & Walsh, 1996). The myoblast becomes a quiescent muscle cell and consequently undergoes terminal differentiation (Naya & Olson, 1999).

Differentiation can be defined as the expression of tissue specific genes, leading to specific protein synthesis giving a cell its tissue specific phenotype. Terminal differentiation is the transition from mononucleated myoblasts into myotubes containing functional contractile proteins, then finally into multinucleated myofibres (Krauss *et al.*, 2005). The MRF myogenin is required for terminal differentiation of myoblasts and it is aided downstream by the MEF2s (Yun & Wold, 1996). Null mutations of the myogenin gene in murine models lead to perinatal death. The musculature of these models contained significant levels of myoblasts, but depleted levels of myofibres, showing failure of the myoblasts to terminally

differentiate (Hasty *et al.*, 1993; Yun & Wold, 1996; Brameld *et al.*, 1998). Myogenin can therefore be shown to be crucial for the proper development of muscle *in* utero and its function is unique within the MRF family (Berkes & Tapscott, 2005).

MEF2s work by enhancing the efficiency of binding by MRFs, via the MCM1, Adamous, Deficiens, serum response factor (MADS) box (Yun & Wold, 1996; Pell & Schofield, 1999; Christ & Brand-Saberi, 2002). Yun & Wold (1996) proposed that this could be attributed to various interactions between MEF2 and the MRF-E complex (MRF-E protein heterodimer in association with an E box). In particular the binding of MRF-E heterodimer to MEF2 homodimers, whilst at the same time interacting with their respective DNA elements, E and MADS box (Pell & Schofield, 1999). This interaction stimulates improved expression of myogenic proteins containing the MRF consensus sequence in their enhancer region, specifically myogenin (Pell & Schofield, 1999). MEF2s cannot themselves initiate myogenic differentiation, in the same way as Myf5 and MyoD do in fibroblasts for instance, but they do significantly enhance it in the presence of the MRFs (Muntoni *et al.*, 2002).

The final MRF involved in myogenesis, is MRF4. Its expression fluctuates during embryonic development between the period of determination and terminal differentiation. The exact function of this transcription factor is hard to isolate. It has been shown to partially restore skeletal development in myogenin deficient mutants, whilst also facilitating skeletal development in Myf5/MyoD double null

mice (Berkes & Tapscott, 2005). Therefore MRF4 can be seen to be subservient to the actions of the determination (Myf5/MyoD) and differentiation (myogenin) specific MRFs (Berkes & Tapscott, 2005). The most published function of MRF4 is in the maturation of myotubes to myofibres, downstream of myogenin (models shown by (Yun & Wold, 1996; Brameld *et al.*, 1998; Pell & Schofield, 1999; Sartorelli & Caretti, 2005).

Reviews by Maltin *et al.,* (2001) and Du *et al.,* (2010) exquisitely highlight the effects of fetal programming in ruminants on *in utero* skeletal muscle development.

1.3.5.3 ***Adipose tissue development***

Adipose tissue can be characterised into two distinct populations, white and brown adipose tissue (WAT & BAT respectively). BAT is traditionally considered rare in humans postnatally, with it helping to regulate energy expenditure and thermogenesis during fetal and neonatal life, white adipose tissue is a storage site for triglycerides (Gesta *et al.*, 2007). Adipocytes are derived from the same pool of mesendermal cells as myoblasts, which develop into skeletal muscle tissue. Following the proliferation of these progenitor cells, there is commitment to becoming pre-adipocytes, under the stimulation of bone morphogenic protein 4 (BMP4) (Tang *et al.*, 2004). Once committed to the adipogenic lineage, pre-adipocytes undergo four phases of differentiation; growth arrest due to cell-cell contact, hormone stimulated mitotic clonal expansion, early and subsequent terminal differentiation (Otto & Lane, 2005; Gesta *et al.*, 2007; Lefterova & Lazar,

2009). The two major transcription factors regulating end stage differentiation are peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). Differentiated adipocytes are classified by their fatty acid synthetic potential, ability to uptake glucose following insulin stimulation and their production and secretion of adipokines such as leptin (Otto & Lane, 2005; Anghel & Wahli, 2007; Gesta *et al.*, 2007; Lefterova & Lazar, 2009). Postnatally, the adipose tissue mass can expand by hypertrophy (increase in cell volume, namely triglyceride accretion), cell division and commitment of adult mesenchymal stem cells into pre-adipocytes and consequently mature adipocytes (Otto & Lane, 2005; Anghel & Wahli, 2007; Gesta *et al.*, 2007; Lefterova & Lazar, 2009).

Reviews by Mostyn & Symonds (2009) and Taylor & Poston (2007) highlight the contribution intrauterine factors have on prenatal and postnatal adipose tissue function.

1.3.5.4 ***Hepatic development***

Approximately 78% of the adult liver is made from heptocytes (Motta, 1984). The hepatocytes have a heterogeneous function, termed metabolic zonation, whereby the nature of the vascular supply to the cell determines the relative anabolic or catabolic nature of the cell. Hyatt *et al.,* (2008) for instance state that the greater the oxygen and substrate supply the greater the gluconeogenic activity and the lower the glycolytic activity, and *vice versa* in cells with a low nutrient supply. While skeletal muscle and adipose tissue develop from the mesoderm, the liver originates

from the entoderm (Twyman, 2001). The key early molecular regulator of hepatogenesis appears to be Fibroblast Growth Factor (FGF), which the immature cardiac tissue secretes during development while the developing foregut is in close proximity (Serls *et al.*, 2005). Later commitment and differentiation to the hepatocyte lineage are invoked primarily by FGF, BMPs and Wnt signalling, with terminal differentiation being orchestrated by transcription factors such as the Hepatocyte Nuclear Factors (HNFs) (Si-Tayeb *et al.*, 2010). For a more comprehensive review of hepatogenesis see Si-Tayeb *et al.,* (2010). Hyatt et al., (2008) further detail the specific effect of modifying the intrauterine environment on hepatic development and function.

1.3.5.5 ***Pancreatic Development***

Several reviews have been published on the transcriptional regulation of pancreagenesis (Wilson *et al.*, 2003; Jensen, 2004; Servitja & Ferrer, 2004; Habener *et al.*, 2005), with specific interest in this area related to developing a method for generating glucose sensitive insulin secreting cells (β-like cells) from host progenitors as a treatment primarily for type 1 diabetes. The fetal pancreas develops from the endoderm germ layer, buddy off either side of the foregut and then recapitulating later in development.

The changes in structure (Figure 1.3) and key transcription factors (Figure 1.4), highlighted by murine knock out studies, has been reviewed and eloquently shown by Habener et al., (2005) and Servitja & Ferrer (2004), respectively.

Of the transcription factors involved, pancreatic duodenal homeobox gene-1 (Pdx-1) has been shown to be vital in the promotion of the β cell phenotype (Habener *et al.*, 2005).

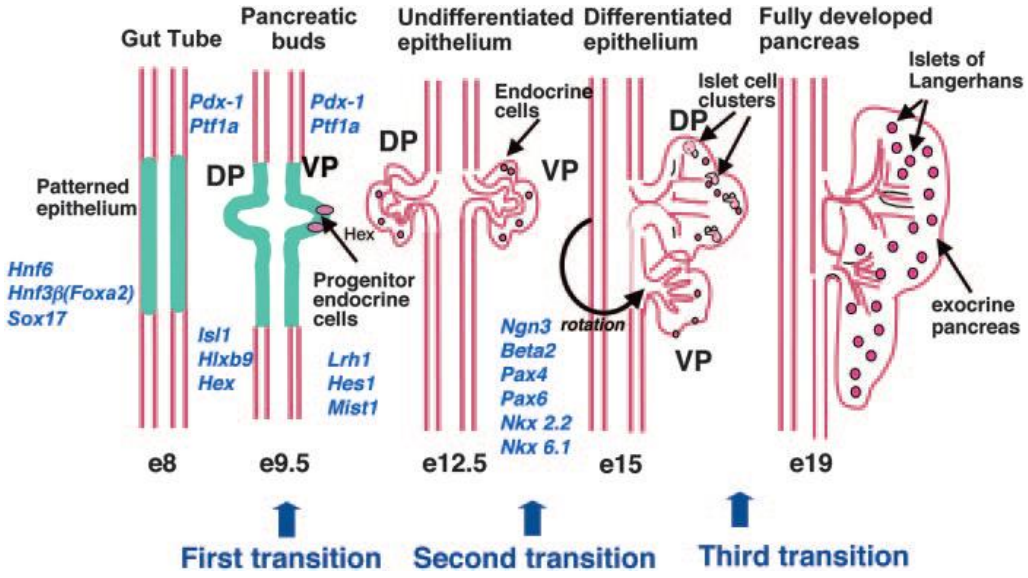


Figure 1.3 Diagram showing the major structural changes throughout pancreatic development
Diagram taken from Habener *et al.,* (2005) showing the major structural changes in the murine pancreas throughout pancreagenesis. Dorsal (DP) and Ventral (VP) buds. Embryonic days (e) throughout murine gestation.

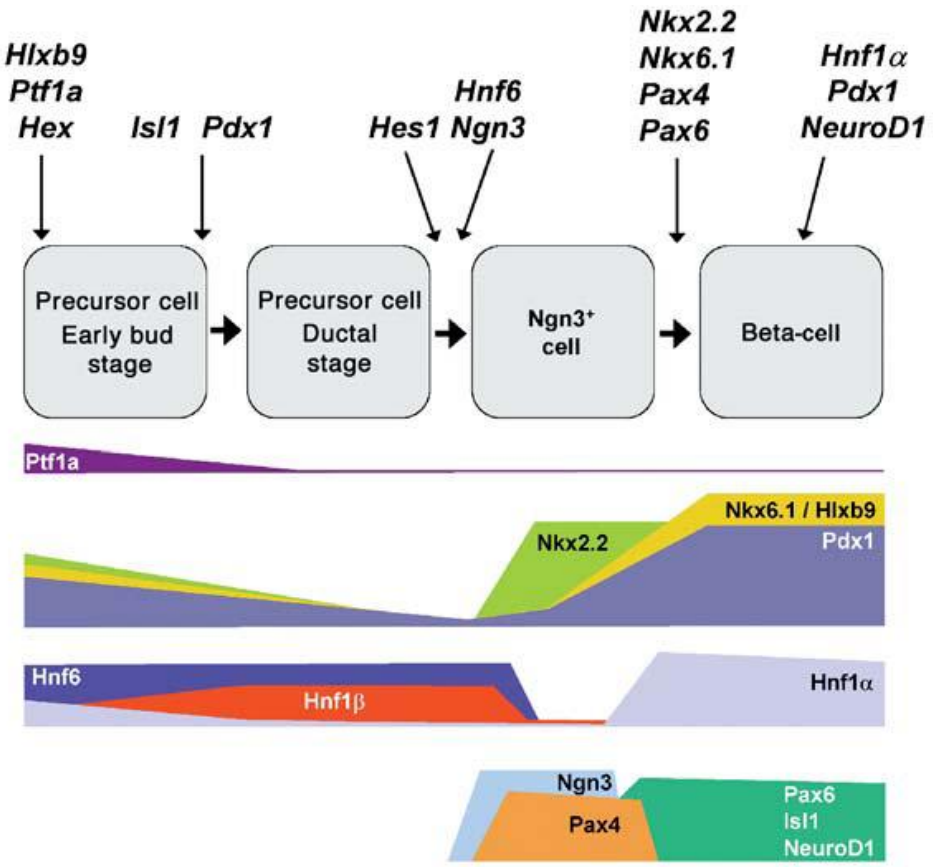


Figure 1.4 Transcription factor modulation of murine β cell development in the pancreas
Figure taken from Servitja & Ferrer's (2004) review. The figure shows the changes in pancreatic transcription factors, as elucidated through mouse gene knock out modeling, as part of β cell development within the Islets of Langerhans.

For further detail of the development of these organs, specifically following in utero restriction, then see (Dahri *et al.*, 1995; Fowden & Hill, 2001; Holemans *et al.*, 2003; Remacle *et al.*, 2007).

1.4 Fetal Programming

Since the early 1990's human epidemiological observations have pointed towards a link between birthweight and postnatal health status. The seminal work published by D.J. Barker & C.N. Hales pointed to a correlation between low birth weight and increased risk of cardiovascular disease and type 2 diabetes in later life. Their work led to the "Thrifty Phenotype Hypothesis", which put forward a "programming" effect of adversity in the fetal environment on postnatal metabolism, and consequently adult health (Hales & Barker, 1992, 2001). Low birth weight is now a well recognised surrogate marker of intrauterine growth restriction (Jensen *et al.*, 2007), although birthweight can also be affected by many other factors such as genetic lineage, epigenetic imprinting, endocrine function (both fetal and maternal) and gestational age at birth (Kuzawa, 2004).

A working hypothesis of fetal physiological programming put forward by Schwartz & Morrison (2005) (derived from (Lucas, 1991) stated "adjustments made during fetal life in response to adverse changes in the biological environment with permanent consequences that may have been advantageous in fetal life but confer disease after birth". This is the basis of theories such as the "Thrifty Phenotype Hypothesis", "Fetal Origins Hypothesis", "Predictive Adaptive Response" and "Mis-Match Theory". It puts forward the idea that the fetus adapts to its perceived plane of nutrition *in utero,* metabolically, biochemically and physiologically. As the maternal plane of nutrition would historically reflect the nutrient availability postnatally, it

would follow that adaptations to optimise fetal metabolism would be beneficial in postnatal life also. In pre-civilised times these preparatory mechanisms may have been positively selected for, as they would optimise postnatal chances of survival. Unfortunately in the rapidly developing world that we live in today the *in utero* milieu may not be representative of the postnatal environment. Therefore, the prenatal adaptations may be detrimental at a time of increased nutrient supply and longevity.

The incidence of fetal under nourishment, and the adaptations associated with it, followed by postnatal over nutrition is increasing globally. Fetal programming has been implied as a potential confounding factor behind the pending epidemic of metabolic syndrome in developing counties going through rapid economic growth such as India and China (Yajnik, 2004b, a). The traditional dietary intakes of the populations from these counties is rapidly undergoing `nutrition transition' to a more westernised dietary regiment (Prentice & Moore, 2005; Popkin, 2006; Prentice, 2006) Maternal malnourishment coupled with postnatal over nutrition could also be seen in offspring who have migrated from a nutrient deprived maternal lifestyle into a country where food is plentiful. Intrauterine growth restriction is also possible in developed countries; predominately caused by reduced placental blood flow leading to fetal nutrient restriction. Other risk factors include maternal age, maternal lifestyle, socio-economic background and medical factors

prior to or during pregnancy (Valero De Bernabe *et al.*, 2004; Gluckman *et al.*, 2008).

1.5 Fetal Programming Models

Following on from the initial human epidemiological evidence, the past 10 years have seen much research into elucidating the molecular mechanisms behind fetal programming. A degree of work has been conducted on human subjects, most of the studies have concentrated on humans with a birthweight in the bottom centile (Hovi *et al.*, 2007) or members of the Dutch famine cohort, who are the offspring (or subsequent progeny) of a large collection of Dutch families who were subjected to famine (<1000kcal/person/day) for 7 months during the winter of 1944-1945 at the end of World War II (Stein *et al.*, 2004). The limitations of human work have been met with the use of animal models, such as maternal nutrient restriction (total calorie or low protein), hypoxia, placental embolisation (reducing blood flow from the placenta to the fetus) and pharmacological intervention (elevation of fetal glucocorticoid levels) (Martin-Gronert & Ozanne, 2007). Various animal species have been used in this work from primates to large farm animals (sheep and pigs) to small lab animals (rats, mice, guinea pigs), with rats being the animal of choice for most researchers. Unfortunately no biological system (*in vivo, in vitro, ex vivo*) or species is totally reflective of the human condition, the choice of model will depend on the specific question being asked, as well as other non-biological considerations

such as resource availability (time, finance, equipment, expertise in working with a particular material or method).

Fetal nutrient restriction has been shown to have deleterious effects on the musculoskeletal system and body composition. Low birth weight has been correlated with adult lean tissue mass and muscle strength (Yliharsila *et al.*, 2007). Reduced secondary skeletal muscle fibre number is a consistent interspecies (humans, sheep, pigs, rats & guinea pigs) response observed following *in utero* nutrient deprivation (Dwyer *et al.*, 1994; Dwyer *et al.*, 1995; Greenwood *et al.*, 2000; Maltin *et al.*, 2001; Brameld, 2004; Zhu *et al.*, 2004; Fahey *et al.*, 2005a; Quigley *et al.*, 2005; Zhu *et al.*, 2006; Daniel *et al.*, 2007; Jensen *et al.*, 2007; Mallinson *et al.*, 2007). These studies and others have also found a greater proportion of glycolytic muscle fibres (type IIB/IIX) at the expense of oxidative fibres (type I and IIA) in response to maternal nutrient restriction during early to mid gestation (Zhu *et al.*, 2006; Daniel *et al.*, 2007; Jensen *et al.*, 2007). Late gestation maternal nutrient restriction (Greenwood *et al.*, 1999) or maternal intrauterine constraint via twinning (McCoard *et al.*, 2000) cause reduced skeletal muscle hypertrophy, thus reduced muscle mass and birthweight , without causing changes in fibre number and type, (for review see Du *et al.,* (2010)). Oxidative fibres express more insulin sensitive glucose transporter (GLUT4) (Gaster *et al.*, 2000), so the combined effect of reduced fibre number and decreased proportion of oxidative fibres will reduce the mass of tissue capable of postprandial glucose uptake

(assuming compensatory hypertrophy does not occur). Maternal nutrient restriction/low birth weight has also been shown to reduce the expression of signalling proteins involved in the insulin intracellular signalling pathway in skeletal muscle in both humans and animal models. These proteins include Protein Kinase C zeta (PKCζ), GLUT4, Phosphoinositide 3-kinase isoforms (PI3K) p85α and p110β (p110β rat only) (Ozanne *et al.*, 2005; Vaag *et al.*, 2006). Along with these quantitative changes in protein expression from nucleic DNA, changes have also been observed in mitochondrial function in response to *in utero* nutrient restriction (Park *et al.*, 2003; Lee *et al.*, 2005), with reduced mitochondrial protein expression being associated with decreased aerobic capacity and consequently hyperglycemia. *In utero* nutrient restriction has also been shown to decrease pancreatic β-cell mass and insulin secretion in postnatal life (Blondeau *et al.*, 1999; Simmons *et al.*, 2001; Bertin *et al.*, 2002; Boujendar *et al.*, 2002; Limesand *et al.*, 2005; Styrud *et al.*, 2005; Limesand *et al.*, 2006), as well as increased gluconeogenesis in the liver (Burns *et al.*, 1997; Desai *et al.*, 1997) and decreased lipolysis (Martin-Gronert & Ozanne, 2007) and glucose uptake (Gardner *et al.*, 2005) in adipose tissue.

Increased adiposity and greater risk of obesity are common findings from *in utero* nutrient restriction observations, such as those made of the Dutch Hunger Famine cohort (Ravelli *et al.*, 1976; Ravelli & Belmont, 1979; Ravelli *et al.*, 1999, 2000). Increased ectopic intramuscular fat deposition (Lane *et al.*, 2001; Zhu *et al.*, 2006; Daniel *et al.*, 2007), non-alcoholic fatty liver disease (Choi *et al.*, 2007) and

increased abdominal adiposity (Guan *et al.*, 2005; Vaag *et al.*, 2006) have also been observed following maternal nutrient restriction. It appears that a key effect of intrauterine nutrient restriction, or other manipulations in maternal gestational diet, are changes in postnatal appetite, both in the preference for different foods and the quantity of foods consumed (Vickers *et al.*, 2000; Bellinger *et al.*, 2004; Bellinger & Langley-Evans, 2005; McMillen *et al.*, 2006; Bayol *et al.*, 2007; Erhuma *et al.*, 2007a; Sebert *et al.*, 2009). These studies are of relevance to the study design of this period of work, as we tried to elucidate whether changes in insulin resistance were associated with differences in appetite, lipid deposition and localisation. The propensity to thrive generated by *in utero* restriction, as outlined by the `Predictive Adaptive Response' theory of fetal programming, would suggest that deleterious consequences would follow should offspring be exposure to an abundant nutritional environment, especially when their requirement to undergo physical activity has been depleted.

1.6 Ovine Models of Fetal Programming

The ovine model has historically been well utilised in fetal physiological investigation, predominately due to the similarities between the lamb and human baby development. Although selective breeding and artificial insemination have caused sheep to increase the frequency of multiple fetal pregnancies, sheep generally give birth to singletons or twins like humans. A ewe will generally give birth to a ~4.5kg lamb following a 147 day pregnancy, while a human gives birth to a

~3.5kg baby with gestation lasting between 259–294 days. As well as the absolute fetal weight being comparable to the human new born, the maternal to fetal weight ratio is also akin. The nature of ovine singleton pregnancy not only makes it comparable to the human condition, but also facilitates fetal investigation, which would be harder in a species with a larger litter size such as the pig. Ovine fetal organogenesis is analogous to human development, with organs developing at similar rates and times during critical windows of gestation. Lambs are born precocial, like human babies, unlike the altrical rat offspring who undergo considerable postnatal development, in line with third trimester development in a human or sheep fetal tissues (Symonds & Budge, 2009).

Research started in the early 1950's to characterise maternal nutrient requirements in ewes (Phillipson, 1959; Thomson & Aitken, 1959). JJ Robinson and colleagues then developed the field further (detailed in Chapter 5), firstly by investigating nutrition requirements and then by looking at the effect of maternal nutrition on fetal development (Robinson & Forbes, 1967; Guada & Robinson, 1974; Robinson, 1977, 1980). Early intrauterine physiological examination of ovine development and metabolism has given a vital background into processes controlling fetal and placental growth and function (Fowden, 1979; Jenkin *et al.*, 1979; Fowden, 1980; Gow *et al.*, 1981; Brockman & Laarveld, 1986; Fowden *et al.*, 1986; Gu *et al.*, 1987; Fowden & Hay, 1988; Jones *et al.*, 1988; Challis *et al.*, 1989).

Following the release of Barker's seminal work (Barker & Osmond, 1986) linking early development with postnatal health, the area of ovine fetal research slowly changed to incorporate the postnatal consequences of intrauterine insults, such as undernutrition.

Below is a selected review of the literature, using the search terms "sheep" and "undernutrition" with the PubMed search engine. Of the 196 articles that were found, 26 were reviews, two of which were of significant relevance (Greenwood & Bell, 2003; Oliver *et al.*, 2007). As the specific area of interest for this review was postnatal health, all fetal physiology papers were removed, which left 40 original articles related to the offspring of undernourished ewes. Two articles (in grey below) have been added to the review, as they were not highlighted by the search, although they should fit with search terms, these articles are used for comparison in later chapters.

The one paper published prior to the Barker & Osmond (1986) paper highlighted in this search related to the parameters of interest is a paper by Lawlor & Hopkins (1981) who describe the effect of ewe undernutrition during pregnancy on ewe lactation, lamb growth and body composition.

Below, the remaining papers are characterised into groups, with the main effects highlighted.

- Muscle; early nutrient restriction (50%) causes a reduction in fast twitch fibres (Nordby *et al.*, 1987; Fahey *et al.*, 2005a; Daniel *et al.*, 2007)
- Glucose; elevated insulin response to a GTT in males following early and late energy restriction

  (Oliver *et al.*, 2002; Gardner *et al.*, 2005; Ford *et al.*, 2007; Poore *et al.*, 2007; Cripps *et al.*, 2008; Husted *et al.*, 2008; Rhodes *et al.*, 2009; Todd *et al.*, 2009; Smith *et al.*, 2010)
- Mitochondrial; increased UCP2 and VDAC in the lungs and adipose tissue following late gestation maternal energy restriction. Increased VO_2 max in 24 month old late gestation restricted offspring.

  (Mostyn *et al.*, 2003; Jorgensen *et al.*, 2009)
- Hypothalamic–pituitary–adrenal axis, cardiovascular and renal function; hypertension, cardiac hypertrophy, reduced renal clearance and endothelial dysfunction, greater mean arterial pressure, adrenocorticotropic hormone and cortisol responses, blunted baroreflex sensitivity during angiotensin II infusion.

  (Hawkins *et al.*, 2000; Oliver *et al.*, 2002; Bloomfield *et al.*, 2003a; Borwick *et al.*, 2003; Symonds *et al.*, 2003; Gardner *et al.*, 2004; Gopalakrishnan *et al.*, 2004; Khan *et al.*, 2005; Gardner *et al.*, 2006; Chadio *et al.*, 2007; Cleal *et al.*, 2007a; Williams *et al.*, 2007; Chan *et al.*, 2009; Kotsampasi *et al.*, 2009b; Torrens *et al.*, 2009; Long *et al.*, 2010; Poore *et al.*, 2010).
- Behaviour; reduced ewe/lamb bonding, increased emotional reactivity and changes in side preference

(Dwyer *et al.*, 2003; Erhard *et al.*, 2004; Hernandez *et al.*, 2009; Hernandez *et al.*, 2010)

- Fertility; reduced ovulation rate, reduced Sertoli cell number, changes in female offspring lactation performance, reduced progesterone secretion and fertility.

  (Rae *et al.*, 2002; Kotsampasi *et al.*, 2009a; Blair *et al.*, 2010; Long *et al.*, 2010)
- Immune; reduced immune response to vaccination

  (Eckersall *et al.*, 2008)
- Growth and the GH Axis; various reprogramming events in the prolactin-GH-IGF-1 axis.

  (Hyatt *et al.*, 2004; Cleal *et al.*, 2007b)
- Adrenal; decreased adrenal weight and reduced methylation of the proximal binding site for the IGF2/H19 gene.

  (Zhang *et al.*, 2010)
- Appetite; reduced energy intake when obese and decreased hypothalamic gene expression.

  (Sebert *et al.*, 2009)

The above show the wide ranging physiological effects that have been observed in offspring following ovine prenatal energy restriction. As individual studies utilised different critical windows of gestation without a consistent postnatal phenotype, it suggests that fetal programming is modulated differentially depending upon the gestational period that the restriction is imposed.

1.7 Ruminant Nutrition

Species such as humans, pigs and rats are monogastrics with one stomach, whereas some herbivorous species such as sheep and cows are termed ruminants, as they have four stomachs, the rumen being the largest and most important. Monogastric species, such as the omnivorous animals listed above, extract the majority of their nutrients from digestion of the food they consume. The digestion of food macronutrients into constituent components (polysaccharides to sugars, proteins to amino acids and lipids to fatty acids) is a result of mechanical and chemical degradation, with a relatively low level of nutrient production due to microbial digestion (in monogastrics). Food is ingested in the mouth and undergoes mechanical disruption by the teeth and chemical degradation by saliva. The bolus of food then enters the stomach via the oesophagus, where it is exposed to a highly acidic soup of digestive enzymes secreted from the stomach. The stomach contents then enter the small intestine (duodenum, jejunum and ileum), which is a far less acidic environment than the stomach where digestion is completed and nutrient absorption takes place. In the duodenum secretions from the duodenal glands (e.g. bicarbonate), exocrine pancreas (trypsin and chymotrypsin for proteolysis and lipase for lipolysis) and liver/gall bladder (bile salts for lipid emulsification and facilitation of lipolysis) are mixed with the chyme. The brush border of the small intestine also secretes locally acting enzymes, which complete the membraneous phase of digestion e.g. breakdown of oligosaccharides to monosaccharides or cleavage of individual amino acids from peptides. The jejunum and ileum have

many folds, with finger like projections (villi), themselves with smaller projections (microvilli) exponentially increasing the surface area available for nutrient absorption. Nutrients are absorbed into the brush border cells via diffusion, facilitated diffusion or co-transported (such as glucose and amino acids) with sodium. These nutrients are then rapidly transported from the cytoplasm into the portal blood to help maximise the concentration gradient between the lumen of the small intestine and intracellular concentration of the brush border cells. The portal blood flows to the liver via the pancreas and mixes with the endocrine pancreatic secretions. Upon first pass through the liver, remaining blood nutrients are either used for immediate energy provision (oxidation) or 'stored' as glycogen (carbohydrate), esterified into triglyceride and stored in the adipose tissue (NEFA), while amino acids are utilised in protein synthesis.

Ruminants have developed many ways of increasing the nutrient availability from their largely high insoluble fibre diets, for review see McDonald *et al.,* (2002). One of these mechanisms is rumination, which is a process the ruminant uses to move partially digested material from the rumen back into the mouth for further mastication, thus increasing mechanical (teeth) and chemical (salivary enzymes) degradation. Ruminants utilise microbial fermentation in a mutualistic symbiotic manner as a means of digesting complex carbohydrates, containing a high proportion of β-linked polysaccharides such as cellulose, that are otherwise undegradable by mammalian digestion. The microorganisms in the rumen include

various species of bacteria, protozoa and fungi. These organisms digest the fibrous material consumed by the ruminant (containing cellulose, starch, hemicelluloses, fructans and pectins for instance) by the use of extracellular enzymes to eventually produce simple sugars. These simple sugar products, along with any dietary intake, are then absorbed by the rumen microorganisms and converted into pyruvate. The pyruvate is then metabolised, producing volatile fatty acids (VFAs) such as acetate, propionate, butyrate, lactate, succinate and formate. Other products of rumen pyruvate metabolism are carbon dioxide and methane; these are subsequently removed by eructation. The VFAs are then absorbed directly across the wall of the ruminant stomachs, which is especially important in helping to maintain the pH of the rumen liquor.

Regarding ruminant protein uptake, the microorganisms are once again crucial (summarised in Figure 1.5). Microorganisms in the rumen hydrolyse degradable protein into peptides and then amino acids, these amino acids are then used to synthesize microbial proteins. Some amino acids may be deaminated into non-protein nitrogen, such as ammonia. Ammonia is also produced by the degradation of other non-protein nitrogen sources such as from the hydrolysis of urea. This ammonia can be re-utilised by the microbiota to produce microbial proteins, or it can be absorbed by the host either through the wall of the rumen or from the small intestine following passage through the abomasum. Once the ammonia is in the mammalian blood stream it is converted to urea in the liver. The urea can either be

incorporated into saliva and ingested or secreted by glands in the rumen (replenishing the rumen nitrogen content), finally, urea can be excreted out of the ruminant by the kidneys in urine. As microbes and non-degraded protein reach the abomasum and small intestine they then undergo proteolysis in line with monogastric digestion detailed above, with the amino acids being absorbed by the host.

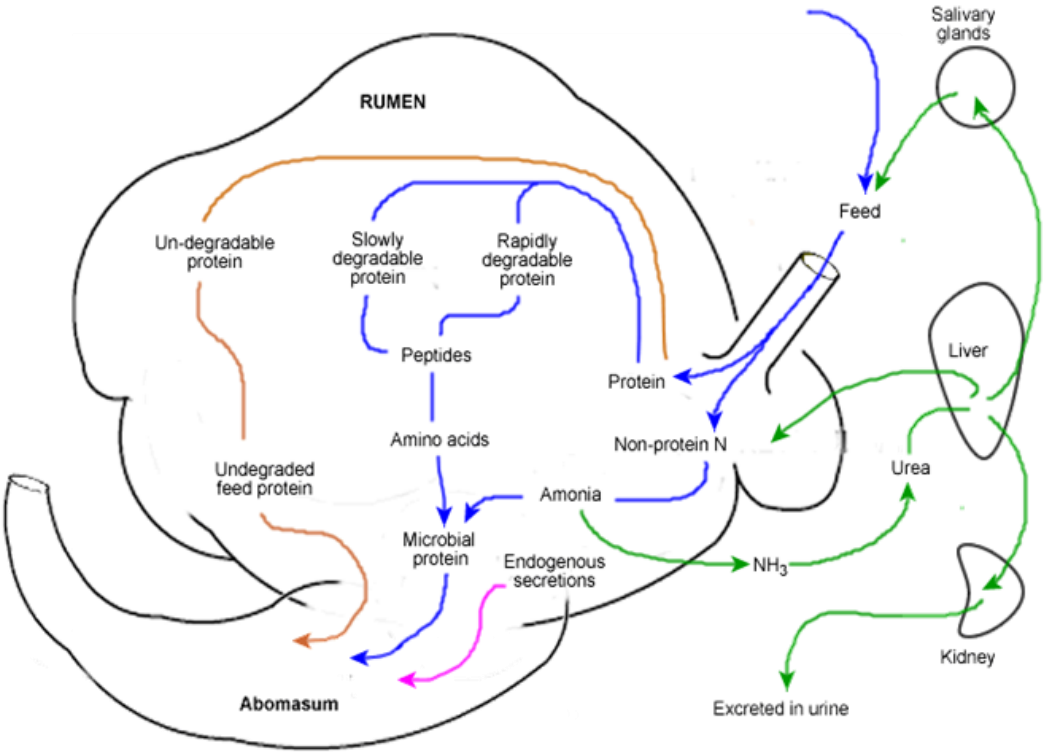


Figure 1.5 Diagram showing flow of nitrogenous compounds during ruminant digestion

The diagram shows the flow of nitrogen during ruminant feeding. With blue arrows representing digestible protein, orange arrows indigestible dietary protein, green host nitrogen cycling and pink host endogenous enzymatic secretions. The diagram is taken from (www.smallstock.info, 2010).

The microbial flora are also involved in the digestion of dietary lipids consumed by the ruminant. Triglycerides are hydrolysed in the rumen by bacterial lipases to form long chain non-esterified fatty acids. The majority of fatty acids consumed by ruminants are C_{18} polyunsaturated, either linoleic or linolenic. These free fatty acids are hydrogenated forming the C_{18} saturated fatty acid stearic acid. Unlike the short carbon chain volatile fatty acids produced by microorganism metabolism of pyruvate, these long chain fatty acids cannot be absorbed across the wall of the ruminant stomach, and so they pass to the small intestine for digestion by the hosts enzymes and absorption.

1.8 Aims & Hypothesis

1.8.1 AIM

The aim of this programme of work was to investigate the effect of *in utero* nutrient restriction (global, i.e. energy restriction or specific, i.e. protein restriction) at specific periods of gestation on fetal growth and development and subsequent adult male and female offspring glucose-insulin metabolism in the sheep. A further aim of the study was to challenge the programmed metabolic phenotype in the adult with acquired obesity.

1.8.2 HYPOTHESES

1) Programmed metabolic disease in the sheep is evident when nutrient restriction is unbalanced (i.e. low protein) rather than balanced (i.e. global energy restriction).

2) Maternal protein restriction during late (as oppose to early) gestation compromises fetal growth and development leading to low birth weight and early signs of risk factors for metabolic disease in the adult offspring.

3) Maternal nutritional programming of metabolic disease is sex-specific in the sheep.

4) Adult-onset obesity exacerbates metabolic dysfunction and reveals overt metabolic disease in the adult male and female offspring.

2. General Methods

2.1 Background

2.1.1 OVERVIEW

Two studies were set up at the Macaulay Institute in Scotland to investigate the effect of maternal nutrient restriction on fetal development and postnatal metabolism, immunity and behaviour. One study looked at the effect of restricted dietary energy supply, whereas the second looked at the effect of restricted dietary protein supply. Both studies investigated the effects of restriction during early or late gestation. Early gestation was deemed up to 65 days after conception and late gestation was from day 66 to term. The nutrient restriction periods were chosen to correspond with periods of either no or partial fetal luteinising and follicle stimulating hormone production (i.e. none up to day 65 and some thereafter) but also, partially, coincided with the phases of fetal muscle development, which is largely proliferative up to day 85 and differentiating (hypertrophic) thereafter (Fahey *et al.*, 2005b).

Although the designs for the two studies (Protein & Energy) are comparable and they ran in parallel, they are statistically treated as two separate studies as the low protein study was begun two months after the low energy study. We found this to have effects on the condition of the ewes entering each study, on average birth weights and also various other unquantifiable effects such as the quality of the pasture when the lambs were born, which meant that the pre-mating and postnatal

supplementation levels were different (Gootwine & Rozov, 2006).

2.1.2 ETHICS

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and were approved by the relevant local ethical review committees of the Macaulay Institute and the University of Nottingham.

2.2 Maternal Low Energy Study

2.2.1 STUDY DESIGN

1 month prior to mating, 87 Scottish blackface ewes were brought inside and barn housed. They were weighed and body condition scored (BCS) 12 times between 50 days prior and partition. Animals with a low BCS were given elevated feed rations (Green Keil; see 2.5.2.1, with *ad libitum* hay). Ewes were oestrus synchronised with a progesterone sponge (Cronolone, 30 mg; Intervet, Cambridge, UK) for 2 weeks. The following day ewes were mated naturally to Scottish blackface rams. Ewes were then assigned to one of three experimental groups; Control Energy (CE), whom received 100% live weight maintenance requirement (1M; (AFRC, 1993)) throughout gestation to term; Low Energy Late (LEL), which were fed 1M between day 0-65 and then 0.7M from day 66 to day 128 and then realigned with control (1M) (Table 2.1); Low Energy Early, which were fed 0.65M until day 65 of gestation and then switched to 1M until term (Table 2.1). On day 65 a group of control and LEE ewes were euthanised, and maternal, placental and fetal tissues were collected. The remaining LEE ewes were switched to control feed, whilst the LEL group were

switched from control to restricted feed rations. The nutrient restriction was gradually, over a period of 2 weeks, removed prior to term to mitigate any effect of the prenatal restriction on lactation and thus confound the results. Ewes were weighed and had their body condition score (BCS; (Russel *et al.*, 1969)) measured up to weaning. For overview see Figure 2.1.

2.2.2 DIETARY DESIGN

	Control				Restricted				
	Single		Twin		Single			Twin	
Gestation Period (days)	Energy Requirement (MJ)	Green Keil (g)	Energy Requirement (MJ)	Green Keil (g)	Energy Requirement (MJ)		Green Keil (g)	Energy Requirement (MJ)	Green Keil (g)
<65	8	600	8	600	LEE	5.2	330	5.2	330
65-70	8	600	8	600	LEL	5.6	370	5.6	370
70-80	8.3	620	8.5	640	LEL	5.8	380	5.9	400
80-90	9.1	700	9.6	750	LEL	6.3	430	6.7	470
90-100	9.8	760	10.6	840	LEL	6.8	480	7.4	540
100-110	10.6	840	11.6	930	LEL	7.4	540	8.1	600
110-120	11.3	900	12.7	1040	LEL	7.9	580	8.8	670
120-128	12	970	13.7	1130	LEL	8.5	640	9.6	750
128-birth	12.8#	1300	14.7#	1500	LEL	#Moved to control intake			

Table 2.1 Maternal Low Energy Study Dietary Rationing

The restricted treatment groups were fed a 65% ration of their live weight maintenance requirement until day 65 (LEE) and a 70% restriction until day 128 (LEL). The nutritional intake increased in late gestation to reflect the increased energetic demand of the ewe due to the developing fetus. #At day 128 all animals were put on 100% maintenance requirement. For the first two weeks postnatally all ewes were offered 1.5kg green keil (see 2.5.2.1) and access to hay *ad libitum*. Ewes were scanned on day 70 to assess litter size (singleton or twin), as nutritional requirement increases with litter size. All maternal diets were supplemented with 250g hay (1.7MJ).

2.3 Maternal Low Protein Study

2.3.1 STUDY DESIGN

In similarity to the Low Energy study, 74 Scottish Blackface ewes were brought into barn housing one month prior to mating to help improve and align their body condition. A progesterone sponge was inserted for 2 weeks and the ewes were mated to Scottish blackface rams the day after the sponge was removed. They were weighed and body condition scored (BCS) 12 times between 50 days prior and partition. The low protein study also consisted of three experimental groups; a control protein (CP) receiving 18% protein throughout pregnancy (see 2.5.2.2.1), a low protein early (LPE) group, receiving 9% protein (see 2.5.2.2.2) from day 0 (mating) to day 65 then 18% protein thereafter to term (approx. ~147 days), and a low protein late (LPL) group, receiving 18% protein from day 0-65 then 9% protein from day 65 to term. Ewes were weighed and had their body condition score (BCS; (Russel *et al.*, 1969)) recorded. For overview see Figure 2.1

2.3.2 DIETARY DESIGN

Due to the nature of ruminant protein nutrition it is difficult to differentiate between protein intake required for energy and non-energy uses, so it is difficult to calculate the exact maintenance protein requirement. 18% protein is a moderately high nutrient level in a normal ovine diet; therefore a 50% reduction (9% protein) is

expected to be sufficient to elicit changes in amino acid cycling in the sheep and reduce late gestation growth of the fetus (for more detail, see 5.2.).

2.4 Postnatal Methodology

2.4.1 Postnatal Methodology at the Macaulay Institute, Scotland (0-1.5yrs old)

Once born, offspring from both studies (low energy and low protein) were treated equally and as a single group at all stages post-natally. After birth, ewes were offered 1.5kg of feed, with *ad libitum* hay to reduce any possible effects of the gestational nutrient restriction on milk quality during lactation. All male lambs were castrated shortly after birth. When the lambs were approx 2 weeks old they were vaccinated, the ewes and lambs were then put to pasture and Green Keil (2.5.2.1) was fed at a rate of 1kg/ewe/day for 4 weeks. After this period feed was offered at 0.6kg pellets per head per day. Lambs were weaned aged 3 months. Liveweights and body condition scores of ewes and lambs were recorded at intervals of 3 weeks from birth, and blood samples (7ml) were collected every 3 weeks by jugular venepuncture to determine insulin concentration. 7 month old offspring underwent an insulin tolerance test and the following day a glucose tolerance test to assess insulin/glucose dynamics. The sheep were maintained on pasture with feed supplement until they were either euthanised or transported to Sutton Bonington Campus, University of Nottingham. Euthanasia was carried out using a lethal dose of sodium pentobarbital, followed by exsanguination. For overview see Figure 2.1.

2.4.2 POSTNATAL METHODOLOGY AT SUTTON BONINGTON CAMPUS, ENGLAND (1.5-2YRS OLD)

2.4.2.1 *Overview*

A cohort of 39 sheep at 1.5 years of age were moved to Sutton Bonington Campus for further *in vivo* investigation. On arrival all animals were randomly retagged, as well as having their body weight and condition score recorded and habituated to their new environment for 4 weeks. At this time, a further glucose tolerance test (GTT) was conducted on all sheep, this will be referred to as the "lean GTT" (see 2.4.2.2) and, shortly after, the sheep underwent body composition analysis by dual energy x-ray analysis (DEXA- see 2.4.2.3), referred to as "lean DEXA". Following these initial, lean investigations, the animals were exposed to an obesogenic environment (see 0), with the intention of increasing the animals' fat deposition. All animals were encouraged over a period of 6 months to put on 50% of their original weight prior to their lean GTT. At this point the GTT ("obese GTT") and DEXA ("obese DEXA") were repeated, individual appetite was recorded (see 2.4.2.5) and a further insulin tolerance test conducted (ITT- see 2.4.2.6) followed by an insulin stimulated skeletal muscle biopsy (see 2.4.2.7). A week later all animals were euthanised (see 2.4.2.8). For overview see Figure 2.1.

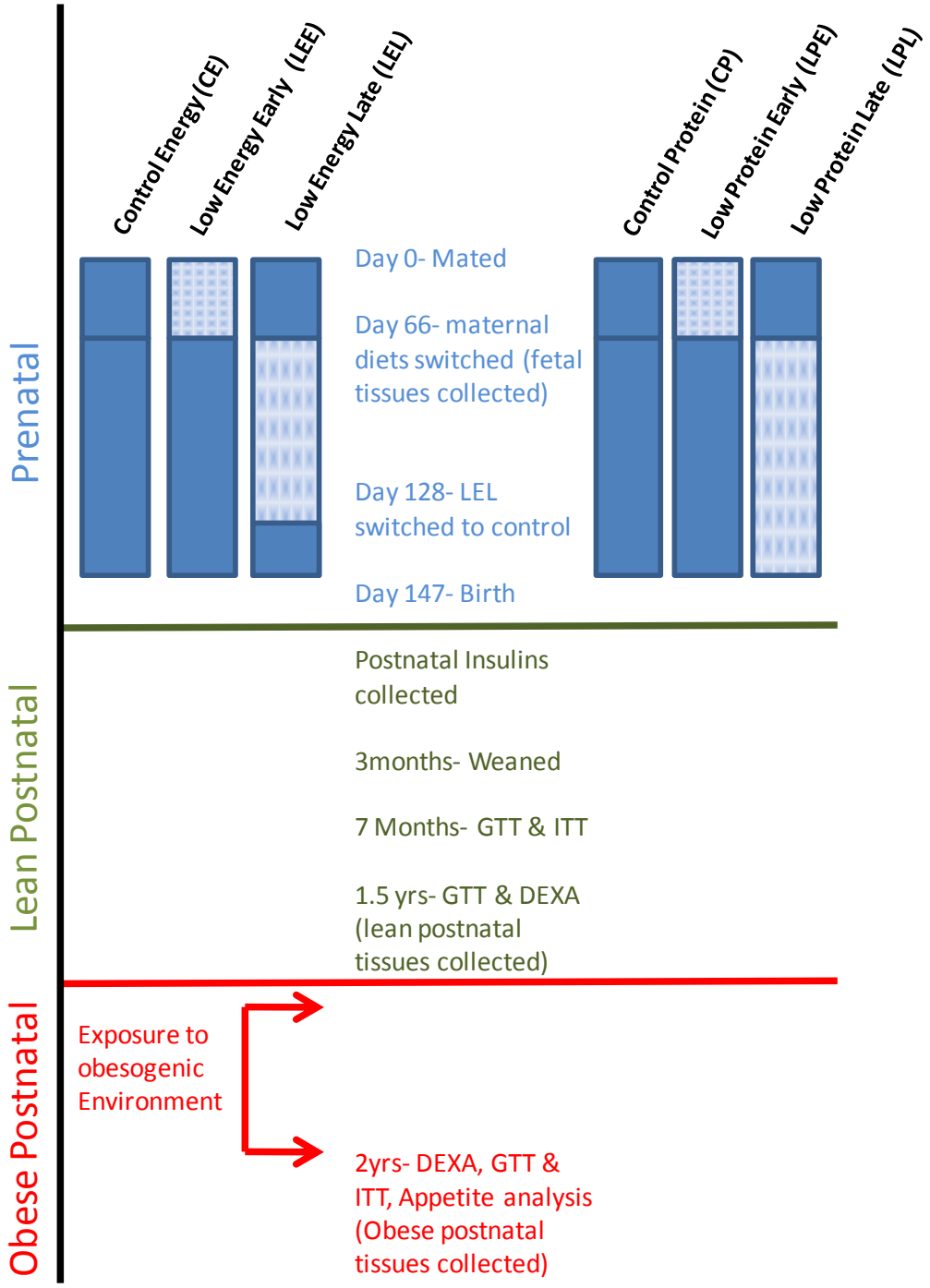


Figure 2.1 Overview of Prenatal & Postnatal Study Design

Figure showing the study design for both the Low Protein and Low Energy investigations, indicating the key interventions during both prenatal and postnatal life. GTT (Glucose Tolerance Test), ITT (Insulin Tolerance Test), DEXA (Dual Energy X-Ray Absorptiometry). Details of these methods are highlighted in the remainder of this chapter.

2.4.2.2 *Glucose Tolerance Test (GTT)*

2.4.2.2.1 GTT at 7 months of age

Animals had an intra-jugular cannula implanted under local anaesthesia (lignocaine) on the day prior to the GTT. Cannula were kept patent by flushing with 1% heparinised (25000 units/ml) saline (0.9% NaCl). Animals were fasted overnight (1600-0900). A 5ml baseline blood sample was collected from each animal 45, 30 and 15 minutes before the start of the GTT, and transferred into potassium EDTA tubes. A further baseline sample (2ml) was collected immediately prior to glucose infusion (40ml 50% w/v glucose solution). Samples continued to be collected at 15 minute intervals until 135 minutes post infusion. Following blood centrifugation, plasma was isolated and frozen at -20^{o}C until further analysis. All animals had cannulae removed and were returned to barn housing.

2.4.2.2.2 GTT at 1.5 & 2 Years of age

A similar protocol was conducted as described for the GTT at 7-months of age with a few exceptions; blood samples were collected at slightly different times but at intervals known to adequately characterise glucose tolerance in sheep (5 and 10 minutes baseline and subsequently at 10, 20, 30, 60, 90, 120 minutes post infusion). Secondly, the glucose infusion rate was given at 1ml 50% w/v glucose solution per kg body mass. This was done to overcome any confounding factors had the animals gained variable weight during exposure to the obesogenic environment.

2.4.2.3 *Dual Energy X-Ray Absorptiometry (DEXA)*

Animals were sedated (I.M. injection of 1.5 $mg.kg^{-1}$ Ketamine with 0.1 $mg.kg^{-1}$ Xylazine) and scanned in a transverse position using a Lunar DPX-L (fast-detail whole body smartscan). The scan lasted ~15 min after which animals were allowed to recover in a pen and returned to the barn. Regular phantom spine and internal validations showed the DXA to be >97% reproducible for estimations of body composition. In addition, in a separate experiment, DXA-determined fat and fat-free body composition was validated against whole carcass chemical analysis. Briefly, 14 half-carcasses were macerated (WolfKing macerator) and 250 g dried, homogenized and nitrogen content determined by a FlashEA1112 nitrogen analyzer (Thermo Scientific, UK). Percentage fat was determined by rapid soxhlet extraction using a Gerhardt Soxtherm (Wolflabs, York, UK). All procedures have been described previously in detail, (Daniel et al, 2007). There was a significant correlation between methods (Pearson correlation 0.92, $P<0.001$; Figure 2.2), but the DXA had a lower limit of fat detection in the sheep (at 4–5% body fat) and estimated ~75% less body fat than complete chemical extraction, as indicated by a Bland-Altman plot (Figure 2.2).

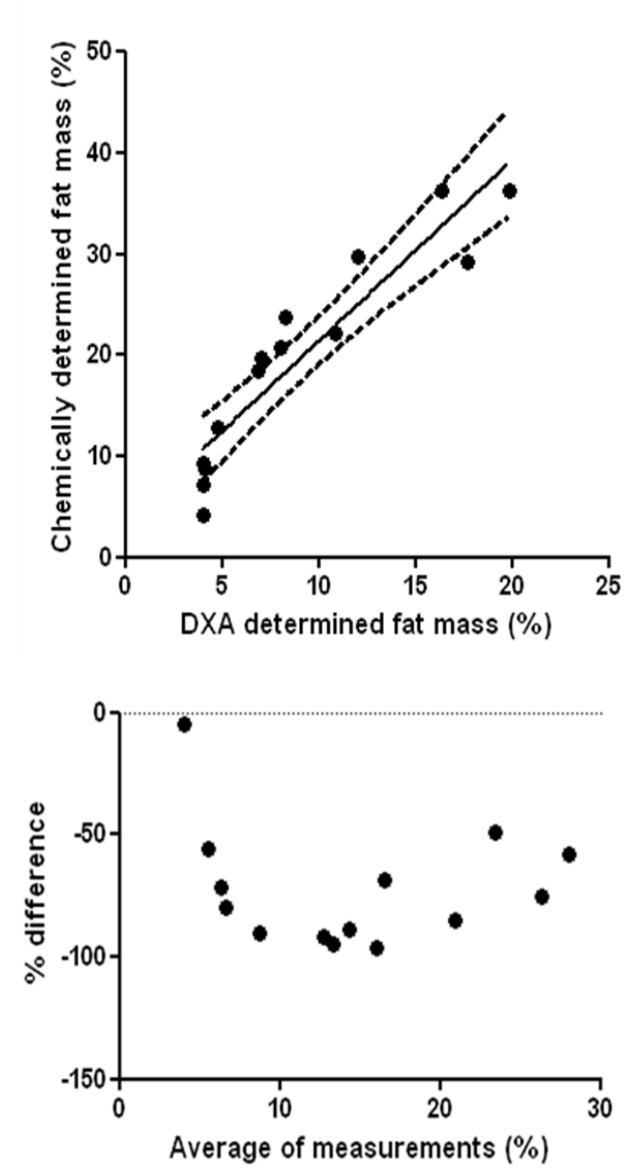


Figure 2.2 Validation of DXA-determined vs. chemically determined fat mass in sheep (top) with a representative Bland-Altman plot (bottom).

Top) 14 sheep of differing body composition as determined by DXA were euthanised and body composition determined by chemical analysis (see 2.4.2.3 for details). The two methods were significantly correlated (P<0.001, Pearson correlation; spline with 95% CI shown) with an equation for the line of y=1.79x+3.51.

Bottom) The Bland Altman plot illustrates the lower limit of detection for DXA in the sheep (4-5% fat) and that DXA vs. chemical analysis estimates ~75% less whole body fat.

2.4.2.4 ***Obesogenic Environment***

Animals had their dietary intake increased from maintenance feeding (1M) ~6-7 MJ/day to 1.5M through a combination of increased dietary intake and an increase in the percentage fat of the diet (from 3% to 6% oil- see 2.5.2.3). As well as increasing food intake, energy expenditure was minimised through barn housing the animals, thus reducing physical activity (Williams *et al.*, 2007), as well as potentially reducing the requirement for thermogenesis due to the increased shelter. All animals were weighed weekly in order to monitor their weight gain over this period, with the experimental end-point being a ~50% increase in weight between 1.5-2 years, which represents a theoretical change in BMI from ~22.5 to ~32.5 (as the animals do not gain height at this age). After this period of weight gain and throughout the repeated experiments the animals were considered to be 'obese'.

2.4.2.5 ***Appetite Studies***

Over a one week period, animals were individually housed and daily food intake of pelleted feed and hay nuts measured over a minimum consecutive 3 day period, during which no other experimental procedures were taking place. On a further day appetite was assessed; briefly, animals were fasted overnight (at least 12hrs), and feed reintroduced *ad libitum* over a 2 hour period, when total intake was recorded (Sebert *et al.*, 2008).

2.4.2.6 *Insulin Tolerance Test (ITT)*

2.4.2.6.1 ITT at 7 months of age

An ITT assesses whole body insulin-dependent glucose uptake from a euglycaemic baseline and correlates closely with the hyperinsulinaemic–euglycaemic clamp technique (Bonora *et al.*, 1989). The dose of insulin given is designed to reduce resting plasma glucose by 1–1.5 mmol.L^{-1} over a 15–16 min period and from this the K_{ITT}, which is the percentage decline in blood glucose concentration per minute (%min^{-1}) from 4 to 16 min relative to baseline (0 and 2 mins), is calculated (Norton *et al.*, 2007). The ITT was performed the day after the GTT following an overnight fast, allowing the cannula from the GTT to be reused. The cannula was flushed with heparinised saline 1% heparinised (25000 units/ml) saline (0.9% NaCl) to check the patency of the cannula. A 2ml baseline blood sample was collected in a potassium EDTA tube, as for all blood samples. Insulin was then infused (0.25 IU/kg; Novorapid, UK). Blood samples were then collected at 15 minute intervals post insulin infusion for 2 hrs post insulin infusion. Blood was centrifuged and plasma collected and frozen at -20°C.

2.4.2.6.2 ITT at 2 years of age

At 2 years of age, the sheep were infused with 0.75 IU/kg Insulin (Novorapid, UK) in order to reduce glucose by 1–1.5 mmol.L^{-1} and to guarantee sufficient glucose clearance in the short term (15 minutes) to allow for assessment of peripheral insulin sensitivity. The dosage was increased as aging and the onset of obesity were

expected to have a negative effect on insulin sensitivity. Sampling times were reduced to concentrate on the short-term effects (i.e. first phase insulin response); hence blood was taken at 2, 4, 6, 8, 10, 12, 14, 16 minutes post insulin infusion and K_{ITT} calculated as before.

2.4.2.7 ***Skeletal Muscle Biopsy***

At 20 minutes after the Insulin infusion from the ITT, a skeletal muscle biopsy was taken. Animals were individually restrained and an area of fleece was shorn off directly above the left *vastus lateralis*. Biopsies were performed using a Bergstrom muscle biopsy needle under local subcutaneous anaesthesia (lignocaine). The wound was sprayed with an antibiotic agent and sealed with skin clips. The isolated muscle samples were snap frozen in liquid nitrogen and stored at -80^{o}C for future analysis. All biopsies were performed by the same individual.

2.4.2.8 ***Euthanasia***

Animals were euthanised using electrocortical stunning followed by exsanguination. During the post mortem, organs (total brain, hippocampus, pituitary, perirenal fat, cardiac fat, omental fat, average kidney, average adrenal, liver, spleen, heart, septum, left ventricle, right ventricle, lungs, pancreas) were collected, weighed and snap frozen in liquid nitrogen prior to long term storage (-80^{o} C freezer).

2.5 Laboratory Investigation

2.5.1 BLOOD ANALYSIS

Plasma cortisol levels were investigated in maternal and fetal plasma at day 65. Plasma insulin concentration was assessed in postnatal bloods up to weaning. 7 month GTT plasma was analysed for glucose and insulin concentrations at all time points. ITT data (7 month and 2yr) was analysed for glucose, NEFA, glycerol and TAG. At 1.5yrs and 2yrs, GTT baseline samples were analysed for amino acid and leptin concentrations, while baseline and 120 minute samples were auto analysed (2.5.1.1) for electrolytes and gaseous values. All GTT time points at these ages were analysed for insulin, glucose, NEFA, glycerol and TAG.

2.5.1.1 *Auto-Analyser*

GTT samples were auto-analysed (ABL805-FLEX, Radiometer, Crawley, UK) for [glucose, lactate, $K^{+,}$ Na^{+}, Ca^{2+}, Cl^{-}], pH, *pv*CO_2, *pv*O_2, and HCO_3^{-}, as well as % haemoglobin (OSM3, Radiometer, Crawley, UK) and % haematocrit at baseline “pre-GTT” and 120 minutes after glucose infusion “post-GTT”.

2.5.1.2 *Randox Imola*

Plasma samples were thawed and analysed for [Glucose], [Urea], [Triglycerides (TAGs)], [Glycerol] and [Non-Esterified Fatty Acids (NEFAs)] (Randox RX Imola). In brief, the Randox RX Imola is a clinical chemistry auto-analyser performing automated pipetting, calibration and reading for clinical and non-clinical

photometric assays. A maximum of 150µl of sample is analysed in each assay with an intra and inter assay variance (%CV) <5%.

2.5.1.3 *Cortisol Radioimmuno Assay*

Plasma samples were assayed for cortisol by using a radioimmunoassay kit (Euro/DPC, Caernarfon, Gwynedd, UK) and following the recommended procedure. In brief, the kit contained cortisol antibody coated tubes, ^{125}I cortisol and cold cortisol standards. Suitable dilutions were made of standards to improve accuracy at the lower end of the standard scale; in total seven standards varying from 0-276 nmol.L^{-1} were used. Duplicate 25 µl plasma samples were incubated with ^{125}I cortisol in each tube for 45 minutes at 37°C, decanted and counted for 1 min/tube with a gamma counter. The detection limit, defined as the apparent concentration at 95% B/B0, was 5nmoles.L^{-1}. The intra- and inter-assay coefficients of variation were 5.6% and <10%.

2.5.1.4 *Insulin Elisa*

Plasma insulin levels were assessed with an ovine specific insulin ELISA (Mercodia AB). Samples and calibrators were all pipetted in duplicate. Two pooled plasma samples of varying insulin concentration were also pipetted in duplicate to assess inter-assay variation. GTT samples from time points 20 and 30 minutes were diluted 1:5 with the provided diluent, as undiluted samples were outside the range of the internal (per plate) standard curves (0-2.5µl/L). Plates were then read at 450nm

(Labsystems Multiskan Ascent plate reader plus Ascent software 2.6). A <15% inter- & intra-assay variation was accepted.

2.5.1.5 *Leptin Radioimmunoassay*

Plasma leptin concentrations ($ng.ml^{-1}$) were determined using a validated double-antibody radio-immuno assay (RIA) as previously reported (Delavaud *et al.*, 2000; Williams *et al.*, 2007). Samples were assayed in triplicate (200 ml) using a rabbit antiovine leptin primary antibody, iodinated ovine leptin and sheep anti-rabbit secondary antibody. The limit of leptin detection was 0.1 $ng.ml^{-1}$ and the intra- and inter assay coefficients of variation for the assay was typically <5%.

2.5.1.6 *Amino Acid Profile*

Amino acids were isolated from plasma and derivatised using the EZ:Faast^{TM} amino acid kit (Phenomenex, Macclesfield, UK), as previously described (Sinclair *et al.*, 2008). 25µl of each sample was combined with 20nmol (100µl) norvaline which acted as an internal standard. This solution was passed through the EZ:Faast^{TM} solid phase extraction absorbent which was washed with 200µl propanol. A solution of propanol and sodium hydroxide (200µl) was then used to remove the absorbent (and the amino acids retained on it) from the pipette tip. 50µl chloroform and 100µl isooctane were then added to the solution to derivatise the amino acids which were recovered in the upper organic layer, dried under a stream of nitrogen and the sample re-dissolved in 100µl isooctane:chloroform (80:20 v/v) prior to analysis using Gas Chromatography–Mass Spectrometry (GC-MS). For GC-MS analysis, 1µl of

the sample was injected in splitless mode (split closed for 10s) using an AS800 autosampler (PerkinElmer, Beaconsfield, UK). The injector of the GC8000 gas chromatograph (Fisons, Manchester UK) was maintained at 250°C, with an initial oven temperature of 90°C which was increased to 320°C at 20°C/min (transfer line from the oven to mass spectrometer, 300°C). Helium (12psi) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10m x 0.25mm ID). The MD800 mass spectrometer (Fisons) was operated in selected ion mode, recording ions at 101, 114, 116, 130, 144, 146, 155, 156, 158, 172, 180, 184, 243 and 244 seconds with a dwell time of 0.03s. Calibration was achieved by comparison of peak areas for the amino acids in standard and sample runs after adjustment for variation in the peak area of the internal standard. The method permitted the analysis of 18 amino acids, cysteine and arginine not being detected.

2.5.2 FEED COMPOSITION

2.5.2.1 *Green Keil*

Green Keil feed was used in the maternal low energy study during gestation, and by both the maternal low energy and protein studies during weaning. The dietary restriction was based on decreased feeding of Green Keil, as described in Table 2.1. 12.5 MJ ME/kg dry matter (DM), 16% crude protein/MJ ME (North Eastern Farmers, Aberdeen, UK).

2.5.2.2 *Maternal Protein Study*

2.5.2.2.1 Maternal 18% Protein Diet

10.6 MJ ME/kg Fresh Weight, 18 % crude protein/MJ ME (Harbro Ltd, Turriff, UK).

2.5.2.2.2 Maternal 9% Protein Diet

9.2 MJ ME/kg Fresh Weight, 9% crude protein/MJ ME (Harbro Ltd, Turriff, UK).

Both these diets were formulated by Dr Stewart Rhind (Macauley Research Institute) and Professor Emeritus John Robinson (Scottish Agricultural College). The higher protein content of the 18% diet comes from soya protein, while the lower protein content of the 9% diet comes from bran protein; therefore the 9% diet has a greater fibre composition, while sugar was used to try to balance the ME between the two diets.

2.5.2.3 *High Lipid diet*

Following the transportation of the offspring from the pastures of the Macauley Institute to the grounds at Sutton Bonington Campus, animals were acclimatised on a `standard' feed concentrate-

12.7 MJ ME/kg DM, 14% crude protein/MJ ME, 3% palm kernel oil (Manor Farm Feeds, East Leake, UK).

Following basal, lean evaluation, animals were exposed to an obesogenic diet, which regarding dietary intake involved more feed being offered (200%) and an increased lipid content to the feed-

13.3 MJ ME/kg DM, 14% crude protein/MJ ME, 6% palm kernel oil (Manor Farm Feeds, East Leake, UK).

2.5.3 STATISTICAL ANALYSIS

2.5.3.1 *Single determination continuous data*

Normally distributed data at a single time point e.g. maternal weight at mating or plasma glucose or baseline insulin for any 3 treatment groups (e.g. CP, LPE and LPL), were analysed by general linear mixed model (GLMM) with treatment, gender (male or female) and where appropriate fetal number (single or twin) as fixed effects. Non-normally distributed data, i.e. data from hormone assays, was converted logarithmically to produce log-normally distributed data. This data was then analysed as described above, with means presented as the back transformed values, so as not to present data in a logged format.

2.5.3.2 *Repeated measures analysis*

The main outcome i.e. the interaction of prenatal diet and postnatal environment (obesity) was assessed as a General Linear Mixed Model with Repeated Measures (GLMM-RM. i.e. data when lean and obese), therefore assessing time (becoming obese) as a fixed effect. *A priori* contrasts of interest to be examined were specifically time*treatment or time*treatment*gender interactions. Other effects of time within an individual animal, such as maternal changes during gestation, lamb insulin and responses to metabolic challenges were also investigated using GLMM-RM analysis in Genstat V13.

2.5.3.3 *Area under the curve*

Area under the curve data was subtracted from baseline and hence an area under the response curve (AUC) was generated using the trapezoid rule in (Graphpad Prism 5).

2.5.3.4 *Power calculations*

All data are presented as predicted means from the above models with either standard error of the treatment mean (S.E.M) or standard error of the difference (S.E.D.) between comparisons, as appropriate, used to represent the variance. S.E.D. values are greater than S.E.M. values and therefore reflect the degree of variance more accurately. S.E.M. values are generated through SPSS (v16), whereas S.E.D. values were obtained through Genstat (v13). The S.E.D. or S.E.M. presented graphically is the variance associated with the significant effect demonstrated by the graph, for instance the treatment S.E.D. or the treatment*time interactive S.E.D. As an example of how to interpret S.E.D. values, for generalised linear mixed model (GLMM) statistical comparisons in the low energy study we had n=20 animals and therefore our total degrees of freedom (df) was 19 with 14 residual df ($df_{treatment}$,2; df_{sex},1; $df_{treatment*sex}$,2); thus, the 95% confidence interval for probability testing was ±2.14 the S.E.D. for that comparison. Thus a difference between two means of greater than 2.14 multiplied by the S.E.D. equates to a significant difference. The study was designed to test the hypothesis that maternal undernutrition at a specific stage of gestation would influence offspring metabolic

function and this would be exacerbated when they became obese. In relation to the low protein study, for example, we had 90% power of being able to detect an increase in peak insulin (one-sided test) of 3.6 $ng.ml^{-1}$, in a time*treatment*gender interaction design assuming a minimum of 3 males and 3 females per treatment group measured when lean and obese, with significance (α) set at 0.05 and an average (within-treatment) residual mean square (SD^2) for insulin of 1.2 $ng.ml^{-1}$ (derived from the general linear model). Effects were deemed significant when $P<0.05$, but $P=0.06$-0.09 were also highlighted to indicate that the effects were close to the significance boundary, i.e. an insignificant trend.

2.5.3.5 ***Analysis of amino acid concentrations***

Baseline plasma was analysed for amino acid concentration before each GTT test. Given the complex, non-independent interrelationships between amino acid pathways, simple univariate RM-GLM analysis for individual amino acids was not suitable to describe the changes in plasma concentration of multiple amino acids with obesity in sheep. Therefore the multivariate analysis principal component (PC) procedure was used to describe the change in amino acid levels with obesity. PC analysis indicated that most of the variation in amino acid concentrations between the lean and obese state could be explained by three principal components which explained >95% of the variation in amino acid concentrations. Scores were assigned for each of these components and were incorporated into a generalised linear regression model to identify whether the variation in amino acid concentration defined by each PC significantly explained the variation in the independent variate

e.g. resting [urea]. The latent vector loadings (which indicate the relative importance of the individual amino acids to a particular PC) for any PC that contributed significantly to a linear model were examined to determine which amino acids were most influential in defining the PC. All PC analyses were conducted using Genstat v13.

3. Low Energy Study – conception to 18 months of age

3.1 SUMMARY

This chapter focuses on the effect of maternal energy restriction during early or late gestation on the ewe, the fetus, the weaned lamb and its development up to 1.5 yrs of age. A global energy restriction, 65% maintenance requirements, was applied from conception to day 65 of gestation (Low Energy Early; LEE) or a 70% restriction from day 66 of gestation through to day 128 (Low Energy Late; LEL) together with a control group fed 100% maintenance requirements throughout gestation (Control Energy; CE). Ewes undergoing nutrient restriction were significantly lighter during the restriction period compared to controls, while the body condition score of LEE ewes was significantly lower during early gestation compared to control mothers (P<0.001). No effect of diet was observed on maternal or fetal cortisol or post mortem tissue weights at day 65 of gestation. At birth, as expected, singletons were heavier than twins, while LEE offspring were significantly heavier than control (P<0.001). Singletons were born with a higher baseline insulin than twins (96 *vs.* 0.58ng.ml^{-1} respectively. P=0.042), with the values aligning at 3 months of age. Following a glucose tolerance test at 7 months of age, female LEE and male LEL animals had the greatest peak insulin response to the glucose bolus, but no treatment effects were observed following an insulin tolerance test. At 1.5yrs, males were significantly heavier than females but undernourished groups (LEE &

LEL) had significantly lighter livers than controls (Liver- CE 907.2; LEE 833.1 & LEL 780.8 g. SED 47.04. *P*=0.040).

3.2 Introduction

Extensive research has been conducted over the last 60 years into the nutrient requirements of ewes during pregnancy (Blaxter, 1957; Robinson, 1977, 1980; Wu *et al.*, 2006). Initially, short-term pregnancy outcomes of economic importance were investigated such as better nutrition for reducing lamb mortality. As scientific understanding progressed and agricultural production pressure increased, farmers looked to maximise breeding performance, with birth weight being a key performance marker of the quality of ewe nutrition. With high fetal number (for example twinning) being a desirable selection trait (doubling ewe productivity), the research broadened to encompass the demands of ewes carrying multiple fetuses, which has additional nutrient demands in late gestation. Better formulation of sheep diets enabled various aspects of the pregnant ewe diet to be manipulated such as macro and micro-nutrient content to energy density.

In the late 1980s evidence began to accumulate in humans that the prenatal environment can have long term postnatal health implications (Barker & Osmond, 1986) and led to renewed interest in ewe nutrition, as the sheep was seen as an ideal model for the effects of changes in diet on gestational environment given the similarities between ovine and human fetal development and maturation (Wallace

et al., 2005). Thus the developing ovine fetus became the model of choice for many developmental physiologists investigating perturbations in the intrauterine milieu. No longer was the outcome simply production markers (agricultural scientists) or post mortem products of organogenesis (developmental physiologists), but now disturbances in postnatal cardiovascular and metabolic function were investigated (Gardner *et al.*, 2005; Cleal *et al.*, 2007a; Poore *et al.*, 2007; Sinclair *et al.*, 2007; Williams *et al.*, 2007; Cripps *et al.*, 2008; Chan *et al.*, 2009).

The capacity for fetal growth is governed by the permutation of fetal genotype (the combination of maternal and paternal genetics resulting in the fetuses `drive to thrive'), maternal genotype (the ewe's ability to physiologically provide for the fetus, e.g. her ability to gain and deliver substrates to the fetus) and finally the external environment (for example; maternal stress, fetal number, ewe nutrition and oxygenation) (Gootwine *et al.*, 2007). As the fetus undergoes organogenesis, different organs develop at different times throughout gestation. Organogenesis involves, to varying extents, cells proliferating (multiplying or hyperplasia), committing to a cell fate i.e. differentiating (and thus developing functionality) and finally undergoing an increase in cell size (hypertrophy, further growth of existing cells). It is therefore understandable that intrauterine perturbations at certain times during gestation will impact upon the development of different tissues in different ways dependent upon the particular phase of the growth process that particular tissue is undergoing.

Many different models have been used in sheep to generate *in utero* growth retardation, from surgical and pharmacological to environmental insults such as nutritional modifications, stress and hypoxia. These insults can be chronic or acute, with acute models aiming to impact upon a specific period of organogenesis for an individual or multiple organ system. The model of choice for the present intrauterine growth restriction study was maternal global dietary restriction due to the vast knowledge from the agricultural nutritionists, the relatively low invasive nature of the study and the fact that it reflected our model of nutritional transition (i.e. from prenatal restriction to postnatal excess). Two restriction periods were chosen, one during early and one during mid-late gestation, with the intention of impacting before (early restriction) and during (mid-late restriction) skeletal muscle fibre differentiation (Fahey *et al.*, 2005b) and thus potentially affecting muscle insulin sensitivity later in life.

Cortisol is a hormone secreted by the ewe in response to stress, whether nutritional or emotional. One of the postulated mechanisms for maternal under nutrition leading to modifications in post natal health is via elevated fetal cortisol exposure (Gardner *et al.*, 2006; Jaquiery *et al.*, 2006; Chadio *et al.*, 2007). The amount of maternal cortisol reaching the developing fetus is dependent upon the quantity secreted by the dam and the amount that is actually transferred from the maternal blood supply to the fetus by the placenta, since bioactive cortisol can be inactivated by placental 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) (Clarke *et al.*,

2002). One of the widespread effects of cortisol is that it rapidly induces maturation of proliferating cells during organogenesis; hence excessive cortisol reaching the fetus could cause premature maturation of organs, thus permanently restricting their function in post natal life (Fowden & Forhead, 2009). It has also been hypothesised that *in utero* exposure of the fetus to cortisol `programmes' the fetus's hypothalamic-pituitary-adrenal function, which influences physiological function of the offspring in later life (Jaquiery *et al.*, 2006; Bloomfield *et al.*, 2007; De Blasio *et al.*, 2007a; Seckl & Holmes, 2007).

Insulin is a well-described regulator of glucose homeostasis, as well as being an anabolic hormone. The function of insulin as a growth promoter was first implicated in 1953 (Salter & Best, 1953). Since then the role of each of the somatotrophic factors (Growth Hormone, Insulin, Insulin-like Factors 1 & 2) and their interplay during fetal and neonatal development has been elucidated (Ogilvy-Stuart, 1995; Ogilvy-Stuart *et al.*, 1998; Murphy *et al.*, 2006; Saenger *et al.*, 2007). Following intrauterine growth restriction, catch up growth is often observed, for which insulin has been inferred as a key regulator (De Blasio *et al.*, 2007b; Fowden & Forhead, 2009). Catch up growth following prenatal growth restriction results in greater adiposity, and hence reduced lean mass accretion in comparison to "normal" growth. This period of catch up growth in small for gestational age babies has been implicated in the development of adult onset metabolic dysregulation, amongst other deleterious sequelae (Dulloo, 2008). Preterm (Singhal *et al.*, 2003) and small

for gestational age births (Soto *et al.*, 2003), associated with early catch up growth, have been implicated in insulin resistance in humans. While glucose tolerance and insulin resistance was predicted by catch up growth in a porcine model of low birthweight (Poore & Fowden, 2002, 2004)

The initial focus of this study was therefore to investigate the impact of a modest, nutritional challenge during early or mid-late gestation on postnatal glucose homeostasis. When food is digested, it is broken down into absorbable units, in the case of carbohydrates the absorbable units are simple sugars, of which glucose is one. Glucose is an important metabolic substrate, whose dysregulation can have dire consequences. As glucose levels rise postprandially, insulin is secreted by the pancreas; in this state of hyperglycaemia (high blood glucose) and hyperinsulinemia (high blood insulin) the majority of excess blood glucose is cleared by skeletal muscle tissue (insulin stimulated glucose uptake) and by the liver (glucose stimulated glucose uptake) (Huang & Czech, 2007; Larance *et al.*, 2008; Zaid *et al.*, 2008) and deposited (stored) as glycogen or oxidised to yield ATP. There is a growing evidence base to suggest that under nutrition during ovine gestation causes glucose intolerance and insulin resistance in the offspring, shown during a glucose tolerance test as a reduced ability to regain euglycaemia or by the hyper secretion of insulin in order to reattain euglycaemia (Gardner *et al.*, 2005; Ford *et al.*, 2007; Cripps *et al.*, 2008). Todd et al., (2009) observed slightly conflicting results in comparison to the above mentioned studies, where by early gestational maternal

undernutrition in sheep resulted in postnatal glucose intolerance (increased Glucose AUC and decreased insulin AUC) in female sheep following a GTT. Thus, it was hypothesised that a deleterious effect of maternal nutrient restriction would be observed postnatally as effects on offspring glucose homeostasis. Effects have been observed following early (Poore *et al.*, 2007), mid (Ford *et al.*, 2007) and late (Gardner *et al.*, 2005) gestational nutrient restriction (50% global nutrient restriction). Restricted body condition score and low maternal nutrient intake prior to and throughout gestation has also been shown to effect glucose tolerance (Cripps *et al.*, 2008).

Due to the importance of skeletal muscle in insulin stimulated glucose uptake and that skeletal muscle volume development is a late gestational event, then it was postulated that late gestation would hold the more critical window for development for glucose homeostasis in the adult offspring. During this late period, skeletal muscle fibres differentiate and undernutrition during this period has been shown to reduce skeletal muscle mass (Fahey *et al.*, 2005a). As the hypothesis relates to a reduction in the capacity of the skeletal muscle tissue to uptake glucose in the insulin stimulated state, it was envisaged that a difference in glucose clearance would be shown via a glucose tolerance test (hyperglycaemia + hyperinsulinemia) as well as an insulin tolerance test (euglycaemia + hyperinsulinemia).

3.3 Materials and Methods

See Chapter 2.2 for details of dietary design. In brief, Scottish blackface ewes were oestrus synchronised and mated to Scottish blackface rams. Ewes were fed either maintenance requirements throughout gestation, from day 0-term (Control Energy; CE) or they were allocated to a restriction protocol. The early restriction animals were fed 65% of CE feed rations from day 0- 65 gestation (Low Energy Early; LEE). On day 65 a proportion of CE and LEE animals were euthanised for collection of fetal tissues and blood (fetal and maternal cortisol was analysed- described in 2.5.1.3). On day 66 the remaining LEE animals were switched to 100% maintenance requirements, while the late restriction group (Low Energy Late; LEL) was switched from 100% feed rations to 70%. LEE animals remained on 70% energy requirements until day 128, when they were aligned with CE and LEE animals on 100% feed until term. Maternal weight and body condition score (BCS) were recorded frequently throughout gestation. Males were castrated shortly after birth and all offspring were weaning aged 3 months. Lamb weights, BCS and blood were collected every 3 weeks postnatally. Blood was analysed for plasma insulin (see Chapter 2.5.1.4). At 7 months old offspring were cannulated, and a glucose tolerance test (GTT- see Chapter 2.4.2.2.1) and insulin tolerance test (ITT- see Chapter 2.4.2.6.1) were performed. Aged ~16 months, animals were either euthansied or transported to Sutton Bonington Campus, University of Nottingham for further investigation.

3.4 Results

3.4.1 MATERNAL & FETAL DATA

3.4.1.1 *Ewe Weight*

As expected, the ewes' weight increased significantly as gestation progressed ($P<0.001$. Figure 3.1). As an average throughout gestation, ewes bearing singletons were significantly lighter than ewes carrying twins ($P=0.025$. singletons 60.41 *vs*. twins 63.77 kg, SED 0.44). A significant interaction between fetal number*time ($P<0.001$) was seen, with twin bearing ewes accruing more weight in late pregnancy than ewes carrying a single fetus. A significant treatment*time interaction ($P<0.001$) was observed on maternal body weight, with LEE ewes being lighter during early gestation and LEL being lighter in late pregnancy compared to controls (Figure 3.1).

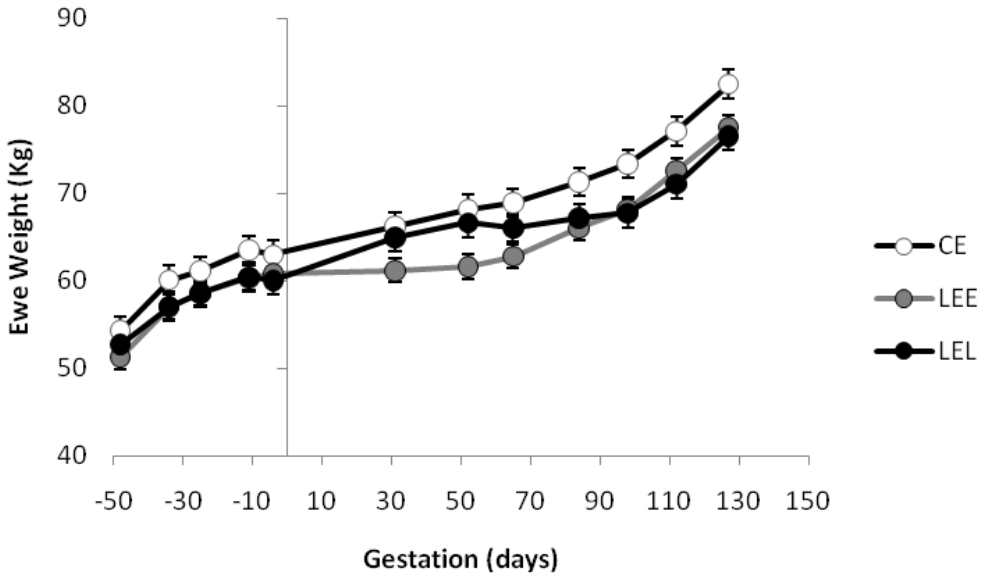


Figure 3.1 Low energy study ewe weights throughout gestation

Errors shown are ±1 SEM. Treatment groups are CE (Control Energy), LEE (Low Energy Early) and LEL (Low Energy Late).

3.4.1.2 *Ewe Body Condition Score (BCS)*

A significant effect of time was observed on ewe body condition score (BCS) during gestation (P<0.001), with BCS rising until day 84 and then declining rapidly during late pregnancy (Figure 3.2). Maternal nutrient restriction had a significant effect on BCS (P=0.002), with a treatment*time interaction showing that at the end of the early gestational nutrient restriction (day 65 LEE), the LEE ewe had a significantly lower BCS than control ewes. Fetal number had no effect on maternal BCS.

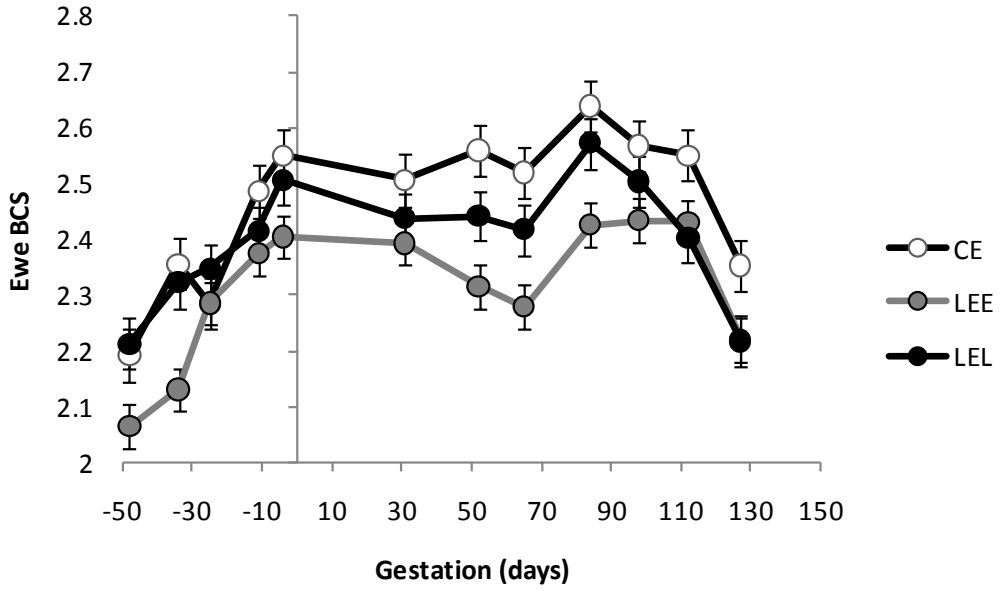


Figure 3.2 Ewe body condition scores following nutrient energy restriction

BCS (Body Condition Score) is judged on an arbitrary scale between 1- emaciated and 5-severely overweight. Errors shown are ±1 SEM. Treatment groups are CE (Control Energy), LEE (Low Energy Early) and LEL (Low Energy Late).

3.4.1.3 *Maternal & Fetal Cortisol*

At day 65 there was no significant effect of maternal nutrient restriction on fetal or maternal plasma cortisol levels (fetal control 51.08 vs. low energy 59.45 $ng.ml^{-1}$, SED 6.426; maternal control 53.19 *vs*. low energy 80.88 SED 34.61).

3.4.1.4 *Fetal Organ Weights*

A significant effect of gender (male 108.8 vs. female 98.3g, SED 5.1; *P*=0.030) was observed on fetal weight at day 65 gestation. No effect of treatment (CE 100.7 vs. LEE 106.4g. SED 4.5) or fetal number (singleton 103.4 vs. twin 103.7g. SED 5.6) was observed. No effects (treatment, gender, fetal number) were observed on heart

(grand mean 965.1mg, SE 49.3), kidney (grand mean 1292mg, SE 62.3), lungs (grand mean 5160mg, SE 252.5) or liver weight (grand mean 7274mg, SE 253.5), but a significant treatment effect was shown on brain weight (CE 3.458 vs. LEE 3.747g. SED 0.13; *P*=0.038.). No effects of maternal energy restriction, gender or fetal number were observed once data were expressed relative to fetal weight, indicating proportionate (allometric) changes to fetal and/or organ growth.

3.4.2 BIRTH WEIGHT AND POSTNATAL GROWTH

3.4.2.1 *Birth Weight*

Maternal nutrient restriction had a significant effect on birth weight (*P*<0.001), with LEE offspring being significantly heavier than control (CE 4.44; LEE 4.75; LEL 4.49 kg. SED 0.07), while female but not male LEL offspring were also heavier than controls. As expected, singletons were born heavier than twins (4.99kg *vs*. 4.13kg. SED 0.06), while no effect of gender was shown on birth weight. A treatment*gender*fetal number interaction was observed (*P*=0.023, Figure 3.3), with male LEE and female LEL singletons being significantly heavier than control. The derivative interactions were also found to be significant (*P*<0.01).

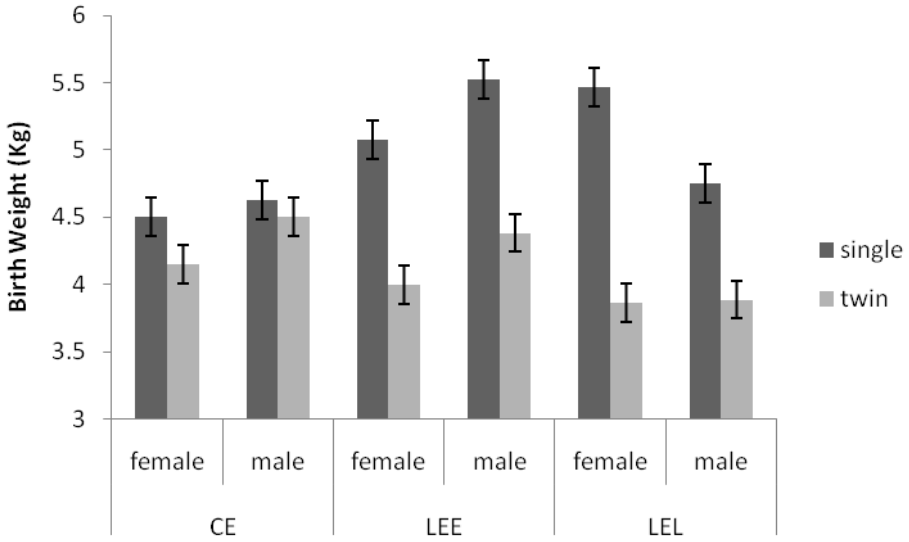


Figure 3.3 Offspring birth weights following maternal nutrient energy restriction

Predicted means are shown with the average SED for the treatment*gender*fetal number interaction (P=0.023). Treatment*fetal number (*P*<0.001), treatment*gender (*P*<0.001), fetal number*gender (P=0.010) interactions were also observed. Treatment groups are CE (Control Energy), LEE (Low Energy Early) and LEL (Low Energy Late).

3.4.2.2 ***Weaning Weight***

At weaning, aged 3 months, the treatment*gender*fetal number interaction, and all derivative interactions, were still significant (all *P*<0.01. Figure 3.4). The strongest effects on weaning weight were fetal number (*P*<0.001, F stat 201.5), with twins generally being lighter than singletons (except CE females), and gender (*P*<0.001, F stat 53.43), where males were heavier than females, with the reverse being shown in control singletons.

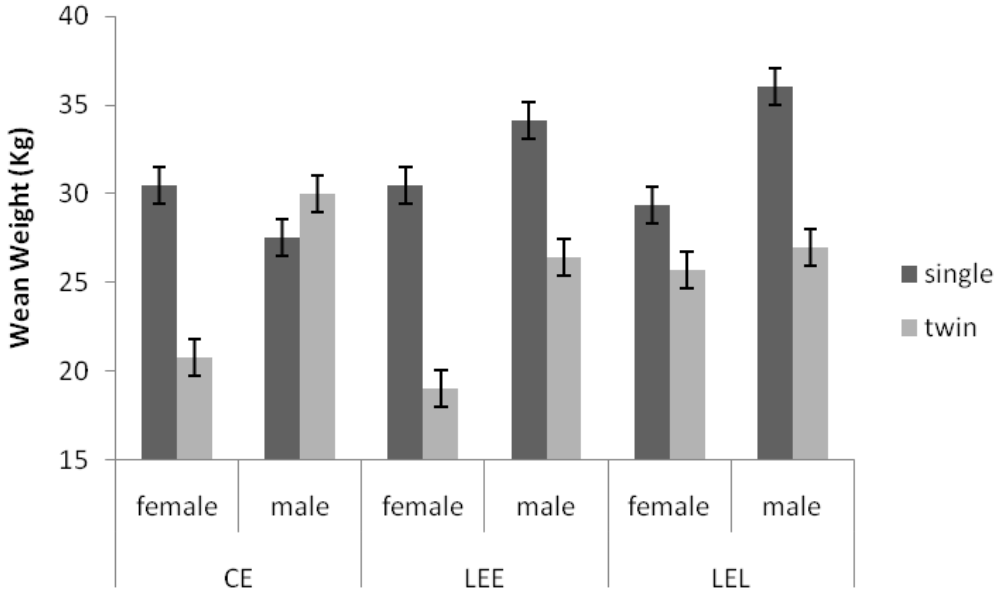


Figure 3.4 Weaning weight for offspring following maternal nutrient energy restriction
Data shown are predicted means ±1 average SED for the treatment*gender*fetal number interaction. Treatment groups are CE (Control Energy), LEE (Low Energy Early) and LEL (Low Energy Late).

3.4.2.3 *Lamb Insulin*

Lamb plasma insulin levels were recorded at regular intervals from birth to 6 months of age. Lamb plasma insulin was significantly affected by time ($P<0.001$), with insulin levels declining after birth but then starting to rise again after 3 months of age. Values were not influenced by prenatal treatment or gender, but fetal number did have a significant effect ($P=0.042$), and a trend was observed with time (time*fetal number, $P=0.059$), with twin insulin at birth being lower than that of singletons, but values aligned by 3 months of age (Figure 3.5).

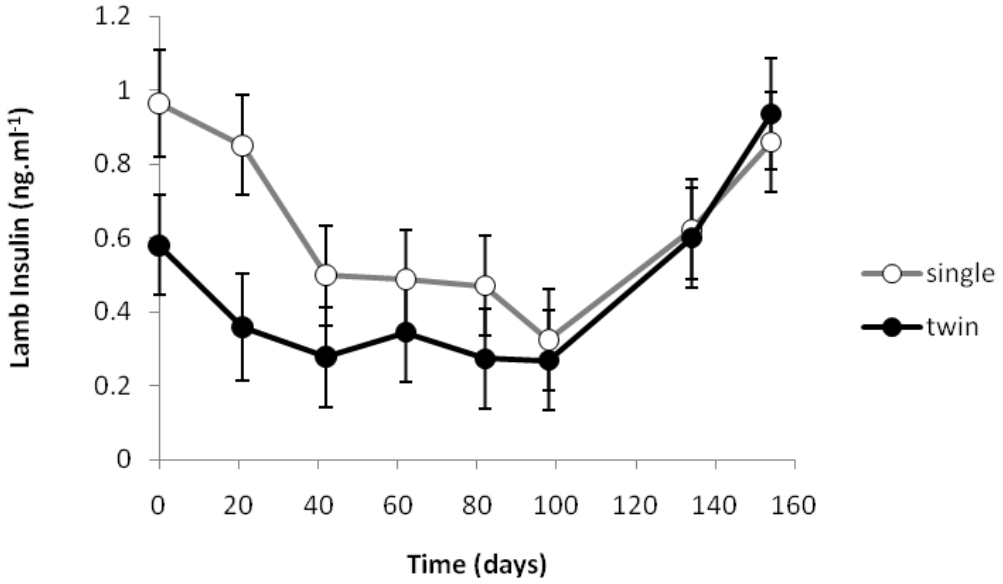


Figure 3.5 Lamb insulin from birth to 5.5 months.

Values shown are back-transformed predicted means, following log transformation, using a generalized linear mixed model with standard errors (±1 SEM).

3.4.3 RESPONSE TO A GLUCOSE TOLERANCE TEST (GTT) AGED 7MONTHS

Following the administration of an intra-jugular glucose bolus, the plasma insulin level significantly elevated (P<0.001). A treatment*gender*time interaction was also observed (P<0.001) as shown in Figure 3.6, with female LEE having a significantly greater response to the glucose challenge than control females. Male LEL animals were also observed to have a higher insulin response to a glucose bolus in comparison to all offspring. A sex*time interaction was also seen (P=0.040) with male offspring having a greater peak insulin response compared to females.

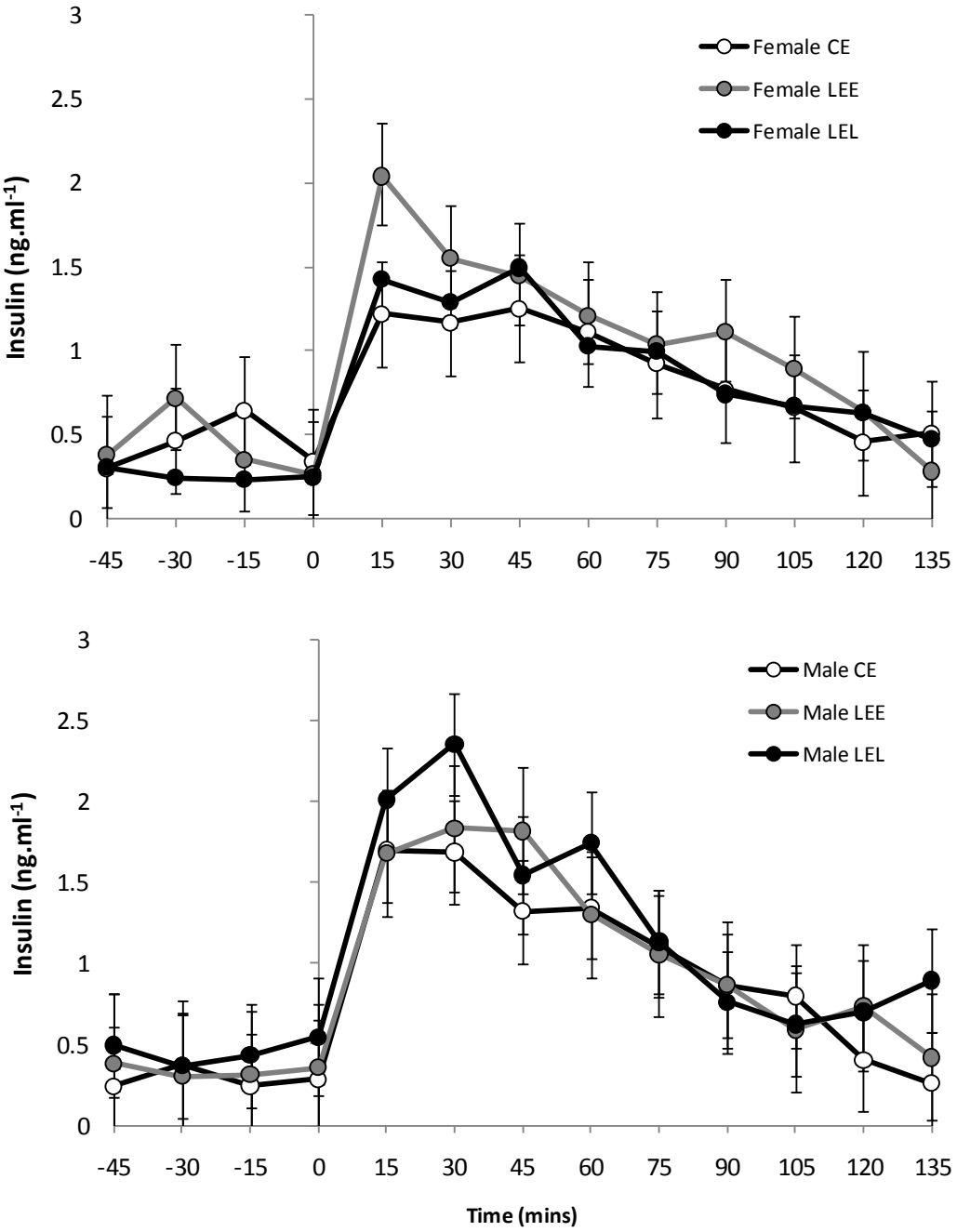


Figure 3.6 The effect of a glucose bolus on plasma insulin levels in offspring aged 7 months from nutrient restricted mothers.

Values are back-transformed predicted means following generalized linear mixed model analysis, errors are ±1 standard error. Treatment groups are CE (control), LEE (Low Energy Early) and LEL (Low Energy Late). Time (P<0.001) and treatment*time*gender (P<0.001) were seen to have a significant effect.

3.4.4 RESPONSE TO AN INSULIN TOLERANCE TEST (ITT) AGED 7 MONTHS

Aged 7 months, glucose rapidly declined in response to an ITT with no significant effect of prenatal treatment on glucose K_{ITT} (CE, 3.00±0.33; LEE, 2.83±0.37; LEL, 2.57±0.27 %/min) or AUC (CE, -6.10±0.60; LEE, -4.46±0.55; LEL, -4.44±0.49 mmoles decrease in 2h (Figure 3.7)). NEFA and glycerol exhibited a similar profile in response to the insulin infusion, with an immediate decline but shortly after a rapid increase in plasma appearance, peaking approximately one hour post infusion. The triglyceride response to the ITT showed no consistent pattern (Figure 3.7).

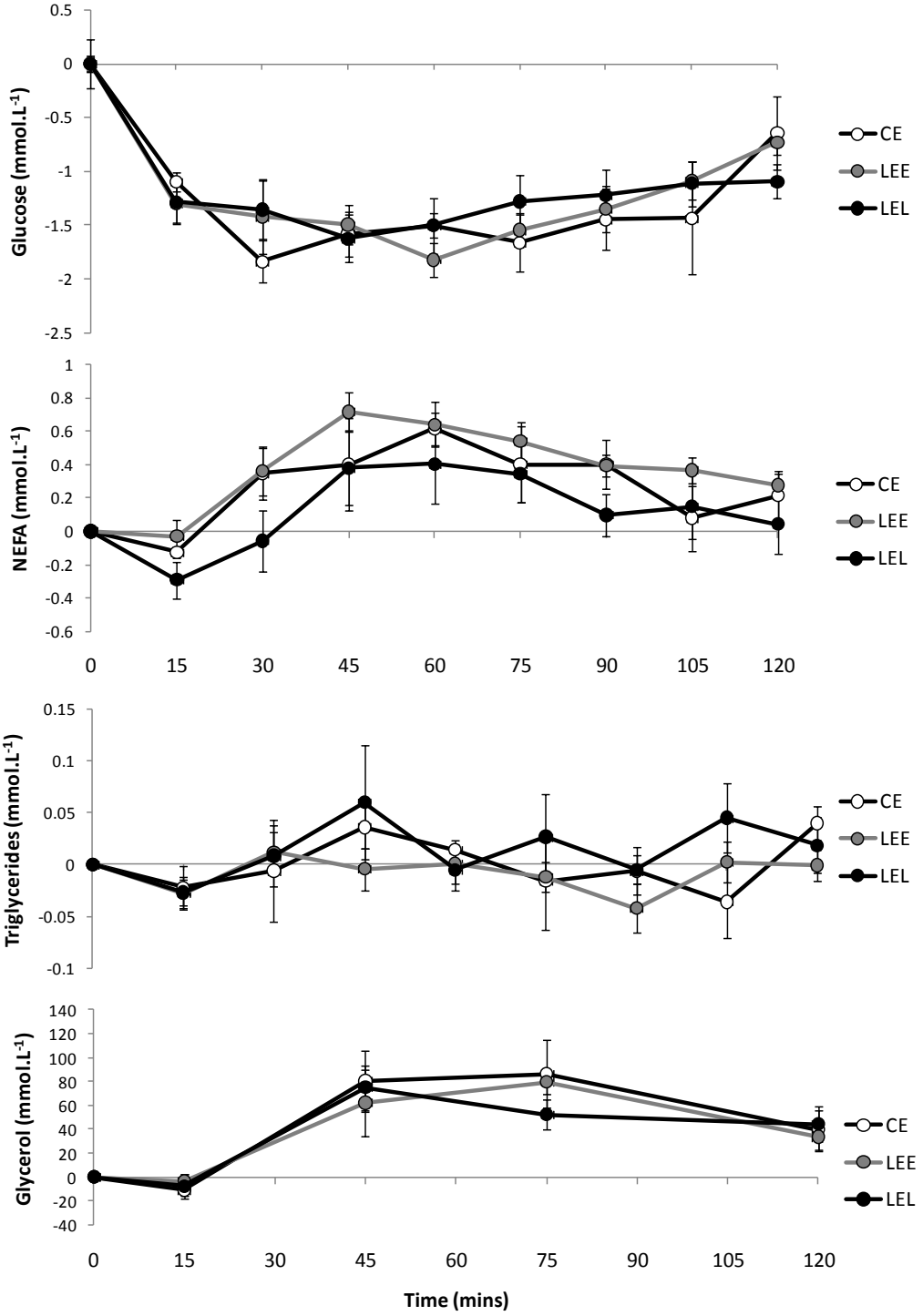


Figure 3.7 Plasma metabolite response to an I.V. insulin bolus in offspring aged 7 months old. Data shown are means ±1 SEM. Groups are CE (Control), LEE (Low Energy Early) and LEL (Low Energy Late).

3.4.5 ORGAN WEIGHTS

Aged 1.5yrs, body weight was significantly affected by gender (*P*=0.002), with males being heavier than females (male 56.64 & female 50.64 kg. SED 1.674), although body condition score was not affected by gender. Body weight and body condition score were not affected by maternal treatment or fetal number. Grand means for post mortem tissues are shown in Table 3.1. Liver weight was significantly affected by maternal treatment (CE 907.2; LEE 833.1 & LEL 780.8 g. SED 47.04. *P*=0.040), while a trend was observed regarding the effect of maternal treatment on average ovary weight (CE 676.8; LEE 485.3 & LEL 511.4 mg. SED 93.75; *P*=0.058.), in both cases treatment groups had less ovarian mass than control animals. Both of these effects became stronger when organ weights were expressed relative to body weight (ovary, *P*=0.016, CE 13.25; LEE 9.64 & LEL 10.18g g/kg, SED 1.368) and (liver, *P*=0.017, CE 17.95; LEE 15.49 & LEL 14.64 g/kg. SED 0.7988). When organ weights were expressed relative to body weight, a gender effect was also observed on kidney (male 1.25 & female 1.42 g/kg. SED 0.08; *P*=0.033,) and average adrenal mass (male 34.97 & female 54.74 mg/kg. SED 8.843).

	Absolute weight	Relative weight (g/kg)
Body weight (kg)	53.64±0.837	
Liver (g.kg)	840.4±19.2*	15.7±0.33*
Heart (g.kg)	259.2±6.7	4.84±0.12
Average Adrenal (mg.kg)	2.39±0.24	†44.86±4.421#
Kidney (g.kg)	71.3±2.2	1.33±0.04#
Spleen (g.kg)	270.7±17.54	5.08±0.32
Average Ovary (mg.kg)	557.8±38.3	†11.02±0.56*

Table 3.1 Post mortem grand means for low energy study animals aged 1.5yr.

Values shown are grand predicted means. Relative weight is organ weight (g)/body weight (kg). †Adrenal and average ovary relative weights are expressed as mg/kg. *Maternal treatment or #Gender had a significant effect (P<0.005).

3.5 Discussion

In this study few effects of a modest, maternal energy restriction were observed in the young, lean offspring. Although the dietary restriction was lower compared to several other studies (0.65 maintenance *vs*. 0.50 maintenance; as used by (Gardner *et al.*, 2005; Cleal *et al.*, 2007b; Daniel *et al.*, 2007; Ford *et al.*, 2007), both the early and late nutrient restriction elicited a significant reduction in maternal body weight and/or maternal BCS.

No significant difference was observed as a result of the nutritional challenge on maternal or fetal cortisol levels, in contrast to previous studies reporting either an increase (Bloomfield *et al.*, 2003a; Bloomfield *et al.*, 2003b) or decrease (Bispham *et al.*, 2003). However, spot samples of plasma cortisol are probably not reflective of overall HPA axis functionality across the maternal-placental-fetal compartments. For a better understanding of the effect of maternal cortisol levels on fetal tissues using this malnourishment model, more invasive research needs to be conducted measuring transplacental flux of cortisol across the placenta together with analyses of placental 11βHSD2 activity and expression; furthermore, the sensitivity of fetal tissues to cortisol (through GR expression analyses) would be required as well, outcomes beyond the remit of our study. Thus, whilst the current study illustrates a lack of effect of maternal nutrition on maternal and fetal plasma cortisol levels it is inconclusive with regard to the potential programming role of cortisol in this experimental paradigm.

When tissues were collected on day 65 of gestation, no effect of treatment or fetal number was observed on any organ weight measured once tissue weights were expressed as a proportion of total fetal weight. Fetal weight was however significantly affected by gender, with males being found to be heavier than females. This is interesting and argues for a fundamental difference between the early development of males and females regardless of secondary sex hormone production and exposure. The lack of effect of maternal nutrient restriction on fetal

weight is unsurprising given the weight of the fetus at this time *vs.* weight of the dam (100g vs. ~70kg).

These observations regarding total fetal weight were not maintained through to birth. No effect of gender was observed on birthweight, while singletons were found to be significantly heavier than twins, suggesting that the birth weight of a twin may be limited during late pregnancy. This observation is in line with the evidence base, which states that fetal growth is slow during the early stages of gestation, and then rapidly increases half way through gestation, with fetal growth being exponential in the later stages of pregnancy (Gootwine *et al.*, 2007). With this in mind, a logical hypothesis would be that maternal global energy restriction during early pregnancy would have no effect on birthweight, while the same nutritional challenge in later pregnancy would decrease birthweight compared to control. Data from this study suggests that compensatory mechanisms must exist to counteract the maternal nutrient restriction, as both early (males and females) and late (females only) nutrient restricted ewes gave birth to offspring with significantly increased birthweight. When contrasted to other similar ovine studies where a 50% global energy restriction was performed during early pregnancy, no significant effect of birthweight was generally observed (Gilbert *et al.*, 2005; Cleal *et al.*, 2007b; Daniel *et al.*, 2007; Ford *et al.*, 2007; Poore *et al.*, 2007) with Gardner *et al.,* (2005) showing a significant decrease in birthweight. One possible compensatory mechanism is placental hypertrophy. Although placental weight was not measured

in this study (as lambs went to term), it has been observed that nutrient restriction during early to mid gestation causes placental growth restriction and thus restricts fetal nutrient supply. When the nutrient restriction is alleviated, compensatory placental hypertrophy occurs, increasing fetal nutrient supply beyond control. The consequence of this is even greater fetal growth in late gestation, resulting in increased birth weight compared to control (Kelly, 1992). Regarding the importance of altered birth weight in fetal programming models, Harding's (2001) review of the literature lead her to conclude that an over-importance has been given to modifications in birthweight as a prerequisite for fetal programming. She suggests that perturbations in fetal growth trajectories (the process) are far more relevant to postnatal health than birthweight (the endpoint) following *in utero* growth retardation via maternal nutrient restriction. This suggests that *in utero* catch up growth causing IUGR fetuses to reflect or overtake control birth weights, could be as dangerous in post natal life as being born under weight.

Maternal treatment had no effect on plasma insulin levels at birth or throughout weaning. Plasma insulin was observed to be significantly lower in twins compared to singletons, which fits with the observation that twins were lighter than singletons, as fetal insulin is an important *in utero* growth (anabolic) hormone in the later stages of gestation (Fowden *et al.*, 1989). Research suggests that when a fetus is exposed to hypoinsulinaemic conditions during late pregnancy (by fetal pancreatectomy), it is growth that is restricted, while tissue development and

differentiation remains normal (Fowden *et al.*, 1998). Hence, once the twins in this study become euinsulinaemic, due to elevated glucose or amino acid supply, then their growth rate should start to recover in line with singleton offspring.

LEE female offspring were observed to have a significantly higher insulin response to a glucose bolus than other female offspring, while male LEL offspring had the greatest insulin response out of all the offspring. While the GTT elicited these treatment effects, there was no observed treatment effect during the ITT. As glucose uptake during a GTT is both glucose stimulated (liver & pancreas) and insulin stimulated (skeletal muscle & adipose tissue), while during an ITT, glucose uptake is purely insulin stimulated in peripheral tissues, the data would suggest that the observed differences in glucose handling by LEL males and LEE females is probably due to central (hepatic) differences although it is not possible to clarify this hypothesis in the current program of work.

At weaning and at 1.5 yrs, males were significantly heavier than females, as expected (despite being gonadectomised). Females that were exposed to nutrient restriction prenatally had significantly lighter ovaries than control females. Although ovarian tissue has not been investigated, it is possible that the smaller ovaries would reflect a reduced reproductive function and link the prenatal environment to postnatal fertility, as reviewed by Gardner *et al., (2008)*. Interestingly, livers of offspring (mainly LEL) from nutrient restricted ewes were lighter at post mortem aged 1.5 yrs when compared to control animals. This finding helps to support the

postulation above that the differences in glucose homeostasis observed in offspring are perhaps due to prenatally-induced modifications in hepatic functionality. In many respects, these hypothesised differences should be 'revealed' or become more obvious when challenged with obesity and therefore in the following chapter, the offspring from 1.5 to 2 years of age were reared in an 'obesogenic' environment to test this hypothesis.

4. Low Energy Study – 1.5 to 2 years of age

4.1 SUMMARY

Offspring from global nutrient restricted ewes as described in Chapter 3 were further analysed aged 1.5 yrs ("lean") and reassessed at 2 yrs old ("obese") following a 6 month exposure to an obesogenic environment. This comprised access to 150% maintenance feed requirements, on a diet containing twice the lipid content of standard feed (6%) with reduced physical activity (barn housing). Animals gained approximately 50% of their starting weight (~50kg to 75kg). Lean mass significantly decreased, while percentage fat mass doubled (~15 to 30%). No major effects of time*treatment interactions were observed on body composition, daily food intake, appetite or response to an insulin tolerance test. Weight gain caused baseline glucose, total amino acid, urea, glycerol, non-esterified fatty acid (NEFA) and low density lipoprotein concentration to decrease, while triglyceride (TAG), leptin, total cholesterol, high density lipoprotein all increased. Following a glucose tolerance test plasma glucose and insulin increased compared to basal levels, while NEFA, glycerol and TAG decreased. The peak insulin response and area under the curve were significantly greater in obese male LEL offspring, compared to obese control offspring (time*treatment*gender interaction, $P<0.05$). The onset of obesity blunted the metabolic response to a glucose bolus in most of the blood biochemical factors measured. There were no interesting differences in organ weights at post mortem.

4.2 Introduction

It is estimated that, at present, every 3-4 out of 10 adults worldwide are obese, defined as a body mass index of ≥30 kg/m^2 (International Obesity Taskforce; http://www.iotf.org/database/index.asp) which represents a huge economic burden (Allender & Rayner, 2007). The early (fetal and neonatal) developmental environment has been proposed as a key sensitive period for increasing susceptibility to adult-onset obesity (Dietz, 1994). Programming of body composition and non-communicable disease (for which obesity is a significant risk factor) has been identified by the Grand Challenges Global Partnership as a research priority in order to mitigate the rising rise of diabetes and cardiovascular disease (Daar *et al.*, 2007).

To date, several laboratory animal concept studies have shown proof-of-principle with regard to the developmental 'programming' of adult health, for review see (Armitage *et al.*, 2004; McMillen & Robinson, 2005). Most of these studies have used the laboratory rodent in which marked reductions or increases in macronutrient provision during gestation may or may not reduce birth weight, and when coupled with marked hypercaloric nutrition postnatally e.g. of fat or simple sugars, then a 'programmed' disadvantageous phenotype emerges (Khan *et al.*, 2004; Ozanne & Hales, 2004). It is important, however, when extrapolating such interesting results to the human that the marked differences in energy partitioning, allometry, metabolic rate and life history traits between laboratory and larger

animals are taken into account (Gillooly *et al.*, 2001; Demetrius, 2004; Phelan & Rose, 2005). Therefore, to conduct similar nutritional and metabolic studies in larger animal models is imperative and to date the sheep and pig has been widely used.

In pigs, it has been shown that reduced birth weight enhances fat deposition which impacts upon glucose tolerance in these animals (Poore & Fowden, 2002). In the sheep, historically used as the animal model of choice for fetal developmental physiological studies because of the similarities in fetal physiology, endocrinological maturity and weight at birth to the human fetus (McMillen & Robinson, 2005), prenatal undernutrition has been shown to have a number of physiological consequences on the adult offspring dependent upon the stage of gestation the nutritional insult occurs (Daniel *et al.*, 2007; Gardner *et al.*, 2007). However, in these large animal studies the experimental endpoint has invariably been to characterise a given phenotypic consequence, and have been conducted when the animals have been raised in an environment appropriate for that species (e.g. for sheep, on pasture for 1-3 years; (Gopalakrishnan *et al.*, 2004; Gardner *et al.*, 2005)). Thus, invariably, at the time of study these animals are relatively lean and are physically active on a daily basis making the results of limited applicability to the current human condition in the Western World. In addition, given the increased 'metabolic reserve' of larger animals i.e. their greater ability to accommodate periods of undernutrition and their relatively slower metabolism, compared with laboratory

rodents (Gillooly *et al.*, 2001), it is all the more remarkable that developmental programming has been shown at all in larger animals. Clearly, therefore, early environmental 'metabolic imprinting' of a thrifty phenotype, i.e. after maternal undernutrition (Hales & Barker, 2001) has the potential to have an enormous impact in the context of the nutrition transition in the Western world (Popkin, 2006). In an experimental context, it is important to challenge the nutrition-transition paradigm i.e. the interaction between the pre- and postnatal nutritional environments, as has been done often in laboratory studies (Khan *et al.*, 2004; Ozanne & Hales, 2004), but much less so in larger animal work.

Consequently, we have previously developed a model of juvenile-onset obesity after prenatal undernutrition in sheep to test the predicted low-high/thin-fat deleterious phenotype. We find, as expected, that juvenile-onset obesity *per se* is metabolically disadvantageous but the pre-postnatal nutritional interactions are not always so clear cut or indeed evident (Williams *et al.*, 2007; Sebert *et al.*, 2009; Sharkey *et al.*, 2009). To the best of our knowledge, no study to date in larger animal models has directly tested an interaction between prenatal undernutrition followed by a regime of postnatal nutritional excess adopted as an adult. Adult obesity is known to influence the function of multiple organs, encapsulated in the term 'metabolic inflexibility' (Storlien *et al.*, 2004) in which animals have a reduced ability to switch substrate-level oxidation between carbohydrate and lipid sources (Koves *et al.*, 2008). Epidemiological evidence from the Dutch Hunger Winter

Famine suggests that specific periods of gestation may impact upon different organ systems in adult life to induce a metabolic phenotype that leads to obesity (Painter *et al.*, 2005). Thus, in this study, we aim to test the hypotheses that prenatal global nutrient restriction in sheep will interact with postnatal obesity to influence metabolic flexibility in the adult offspring and the response will be dependent on the gestational stage at which the nutritional insult occurred. Metabolic flexibility will be assessed in the lean and obese state (body composition determined by dual-energy X-ray absorptiometry) by measurement of carbohydrate, fat and the protein metabolite response to a glucose tolerance test.

4.3 Materials and Methods

In brief, offspring were transported from the Macaulay Institute and acclimatised at Sutton Bonington Campus. Following acclimatisation a further glucose tolerance test (GTT) was conducted on all sheep, this will be referred to as the "lean GTT" (see 2.4.2.2) and, shortly after, the sheep underwent body composition analysis by dual energy x-ray analysis (DEXA- see 2.4.2.3), referred to as "lean DEXA". Following these initial investigations, when lean, the animals were exposed to an obesogenic environment (see 0); with the intention of increasing the animals' fat deposition. All animals were encouraged over a period of 6 months to put on 50% of their original weight prior to their lean GTT. At this point the GTT was repeated ("obese GTT") and DEXA ("obese DEXA"), individual appetite was recorded (see 2.4.2.5) and a further insulin tolerance test conducted (ITT- see 2.4.2.6) followed by an insulin

stimulated skeletal muscle biopsy (see 2.4.2.7). A week later all animals were euthanized (see 2.4.2.8).

4.4 Results

4.4.1 Body Composition, Weight Gain & Appetite

4.4.1.1 *DEXA body composition*

In 'lean' animals aged 1.5yrs i.e. before exposure to the obesogenic environment, there were no effects of the prenatal environment on any aspect of body composition (Table 4.1). However, significant effects of gender were observed, males had higher % body fat (17.48 *vs.* 13.54%, s.e.d. 1.60) and greater absolute fat mass, (9.88 *vs.* 6.27 kg, s.e.d. 0.98), lean mass (46.53 *vs.* 40.02 kg s.e.d. 2.54) and bone mineral content (1400 *vs.* 1170 kg s.e.d. 60) than females (Table 4.1).

		Experimental Group				Statistics		
	Gender	**CE**	**LEE**	**LEL**	**s.e.d**	***T***	***G***	***T*G***
Fat (%)	male	16.93	15.95	19.57				
	female	13.75	13.67	13.2	2.76	NS	0.02	NS
Fat mass (kg)	male	8.94	9.17	11.54				
	female	5.97	6.65	6.18	1.69	NS	0.002	NS
Lean mass (kg)	male	44.31	48.11	47.18				
	female	37.57	42.37	40.12	4.39	NS	0.02	NS
BMD ($g.kg^3$)	male	1.1	1.12	1.11				
	female	1.09	1.06	1.09	0.03	NS	NS	NS
BMC (g)	male	1368	1398	1436				
	female	1121	1211	1193	118	NS	0.006	NS

Table 4.1 Body composition of prenatally undernourished sheep when lean at 1.5 years of age. Data are predicted means with the average standard error of the difference (s.e.d with 14 degrees of freedom in all cases) for the comparison. CE, Controls (n=6); LEE, Low Energy Early (n=6), LEL, Low Energy Late (n=7). BMD- Bone Mineral Density. BMC- Bone Mineral Content. T, main effect of treatment; G, main effect of gender; T*G, interaction between treatment and gender.

4.4.1.2 ***Weight gain***

During the 6 month exposure to the obesogenic environment, all animals put on significant mass (Figure 4.1). When considering the change in weight with time, all measured variables significantly increased ($P<0.001$ all cases) except lean body mass which significantly decreased. For most comparisons, there were no significant time*treatment or time*treatment*gender interactions for any aspect of the change in body composition after prolonged exposure to an obesogenic environment (i.e. Table 4.1 *vs.* Table 4.2). However, some effects were noted; there was a trend ($P=0.08$) for LEL to put on less fat mass (effect size, -1616g s.e.d. 1189) and the increase in BMD was greater in LEE females (effect size, 0.12 s.e.d. 0.03) *vs.* control groups.

However, the previously observed gender effects remained; obese males had higher % body fat (32.67 *vs.* 30.27%, s.e.d. 1.85) and greater absolute fat mass, (21.38 *vs.* 16.70 kg, s.e.d. 1.48), lean mass (44.06 *vs.* 38.32 kg s.e.d. 2.1.76) and bone mineral content (1746 *vs.* 1500 g s.e.d. 72) than females (Table 4.2).

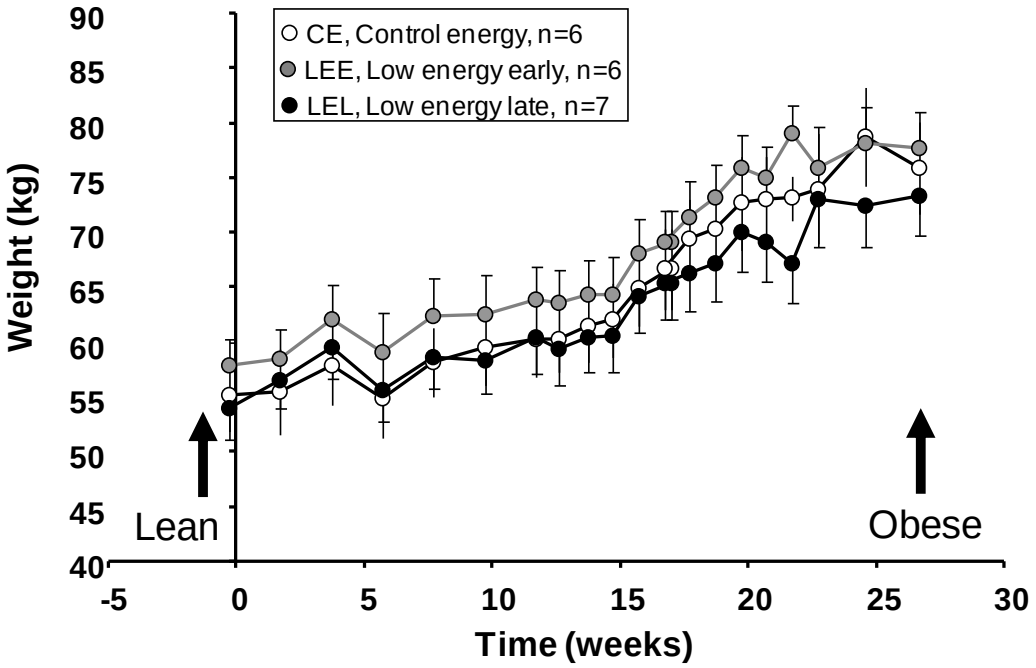


Figure 4.1 Weight gain in sheep reared in an 'obesogenic' environment.

Data are Means±SEM. Sheep were group housed in a barn and fed at 1.5M for a period of 6-7 months to achieve a specified weight gain (see 2.4.2.4). Baseline studies were conducted when animals were designated as 'lean' and were repeated when 'obese'. There were no differences in weight gain between treatment groups.

		Experimental Group				Statistics		
	Gender	**CE**	**LEE**	**LEL**	**s.e.d**	***T***	***G***	***T*G***
Fat (%)	male	31.73	32.02	34.27	3.19	NS	NS	NS
	female	32.95	29.93	27.93				
Fat mass (kg)	male	21.16	20.82	22.15	2.55	NS	0.01	NS
	female	17.05	17.66	15.4				
Lean mass (kg)	male	45.42	44.15	42.6	3.04	NS	0.09	NS
	female	34.77	41.11	39.05				
BMD ($g.kg^3$)	male	1.21	1.14	1.14	0.03	0.09	NS	NS
	female	1.15	1.16	1.13				
BMC (g)	male	1832	1710	1695	124	NS	0.01	NS
	female	1450	1599	1450				

Table 4.2 Body composition of prenatally undernourished sheep when obese at 2 years of age.

Data are predicted means with the average standard error of the difference (s.e.d with 14 degrees of freedom in all cases) for the comparison. CE, Controls (n=6); LEE, Low Energy Early (n=6), LEL, Low Energy Late (n=7). BMD- Bone Mineral Density. BMC- Bone Mineral Content. T, main effect of treatment; G, main effect of gender; T*G, interaction between treatment and gender.

4.4.1.3 *Appetite*

Individual food intake (over 24h) and appetite (over 2h) were assessed. There was no effect of treatment or gender on daily food intake (CE, 20.0; LEE, 21.3; LEL, 19.2 MJ/day s.e.d. 1.80) or appetite (i.e. 2h intake for CE, 13.6; LEE, 13.3; LEL, 13.8 MJ/day s.e.d. 1.73).

4.4.2 GTT & ITT BLOOD ANALYSIS

4.4.2.1 *Insulin*

4.4.2.1.1 Baseline

Fasted plasma insulin concentrations were not different between dietary groups (CE, 0.29; LEE, 0.37; LEL, 0.35 $ng.ml^{-1}$ s.e.d. 0.09) or gender (male, 0.30 vs. 0.38 $ng.ml^{-1}$ s.e.d. 0.07). Fasted plasma insulin when obese remained at a similar level as when lean (CE, 0.38; LEE, 0.42; LEL, 0.43 $mmol.L^{-1}$ s.e.d. 0.09) and was similar between sexes (male, 0.35 *vs.* female, 0.47 $mmol.L^{-1}$ s.e.d. 0.07).

4.4.2.1.2 Response to a Glucose Bolus (GTT)

As expected, following the intra-jugular glucose injection, the plasma insulin level rose significantly, peaking around 20 minutes after the bolus was administered. In lean animals there were no effects of treatment, gender or treatment*gender interactions on insulin sensitivity (area under the insulin response curve - see Figure 4.2). In obese animals the insulin AUC was significantly greater than in lean animals (*P*<0.001. lean 137.3; obese 318.8. SED 21.61). A time*treatment*gender interaction (*P*=0.04) was observed for the insulin AUC (Figure 4.2. CP lean 151.3 *vs.* obese 231.3, LPE lean 129.3 *vs.* obese 423.3, LPL lean 131.3 *vs.* obese 302.0. SED 58.36); as well as the peak plasma insulin response being significantly greater in male LEL relative to both CE and LEE males (Figure 4.3). These interactions show that obese male LEL offspring had a greater insulin response (AUC and peak insulin) in relation to lean values from the same animal or values from other male offspring in the study.

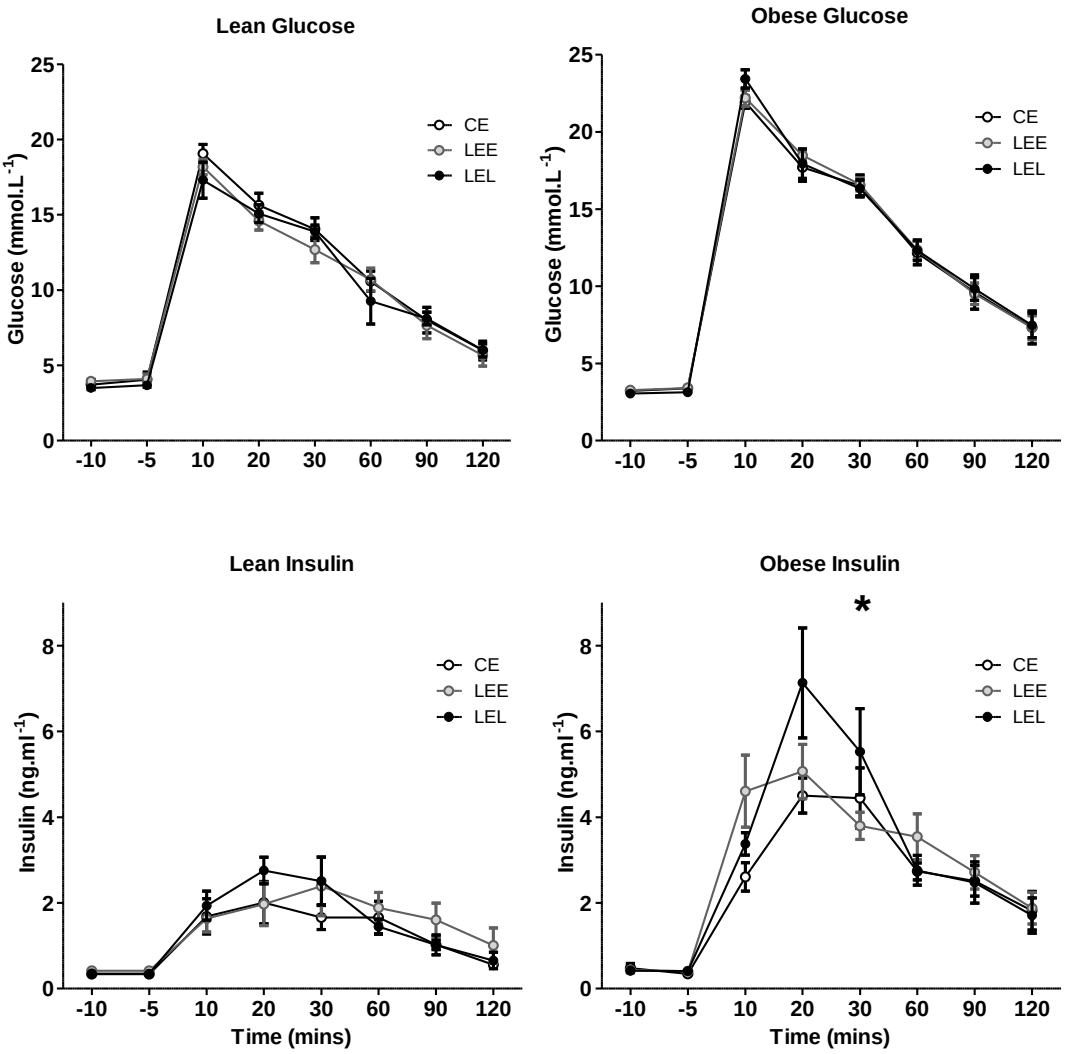


Figure 4.2 The response of prenatally energy restricted adult sheep to a glucose tolerance test when lean and obese.

Data are Mean ± SEM. Glucose was administered I.V. (0.5g.kg-1) and blood samples collected for measurement of plasma glucose and insulin (by ELISA, see 2.5.1.4). CE, Controls (n=6); LEE, Low Energy Early (n=7), LEL, Low Energy Late (n=7). Statistics are *, $P<0.05$ LEL vs. CE and LEE for peak insulin concentration.

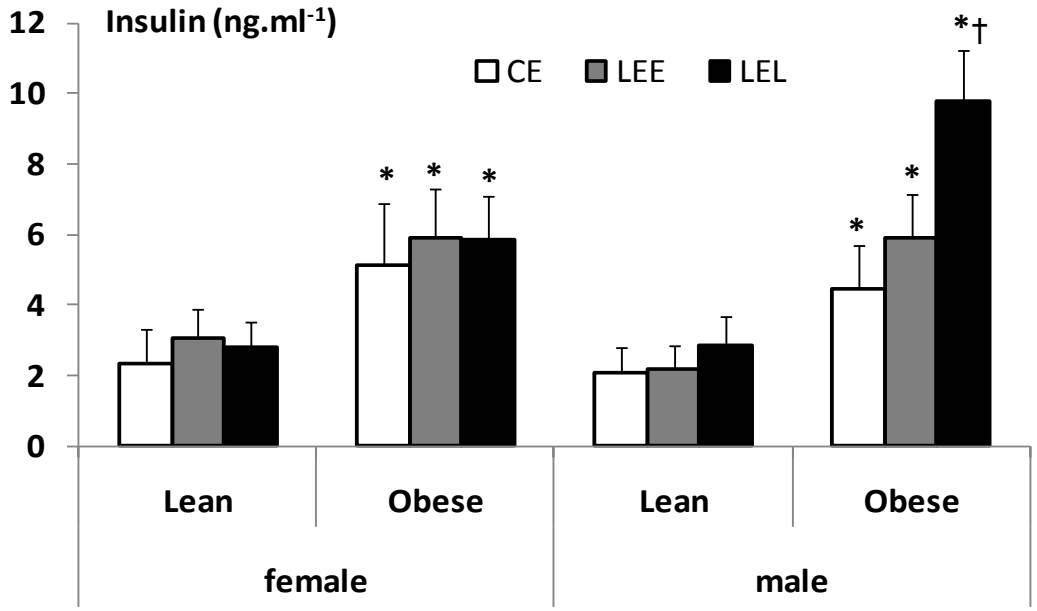


Figure 4.3 Peak plasma insulin in lean and obese male and female prenatally nutrient restricted adult sheep during a glucose tolerance test.

Data are predicted means and SEM. CE, Controls (n=6); LEE, Low Energy Early (n=7), LEL, Low Energy Late (n=7). Data were analysed by repeated measures general linear model revealing a significant effect of time (*; P<0.001 all cases) or a significant interaction (P<0.05) between time*treatment*gender (†).

4.4.2.2 *Glucose*

4.4.2.2.1 Baseline

When lean, fasted plasma glucose concentrations were similar between dietary groups (CE, 3.65; LEE, 3.95; LEL, 3.70 mmol.L^{-1} s.e.d. 0.49) and gender (male, 3.91 *vs.* female, 3.61 mmol.L^{-1} s.e.d. 0.40). On becoming overweight, fasted plasma glucose concentrations significantly reduced (P<0.01) relative to when lean but remained similar between dietary groups (CE, 3.28; LEE, 3.29; LEL, 3.08 mmol.L^{-1} s.e.d. 0.17) and gender (male, 3.12 *vs.* female, 3.31 mmol.L^{-1} s.e.d. 0.14).

4.4.2.2.2 Response to a Glucose Bolus (GTT)

Following the administration of the glucose bolus intravenously, the plasma glucose level rose significantly in both lean and obese animals, peaking around 10 minutes post injection. In lean and obese animals, there were no treatment, gender or treatment*gender interactions on glucose tolerance (area under the glucose response curve). There was however a significant increase in the glucose AUC in the obese state compared to lean (Figure 4.2. P<0.001. lean 742.2 *vs.* obese 1105.0. SED 47.41).

4.4.2.2.3 Response to an Insulin Bolus (ITT)

Following the I.V. insulin injection, the plasma glucose level rose slightly but then decreased as expected (Figure 4.4). The rate glucose was removed from the blood following the insulin bolus (K_{itt}) was not affected by prenatal treatment, however it was significantly affected by sex (male 2.47±0.24 female 3.30±0.28 mmol.L^{-1}; P=0.041,), reflected in the glucose AUC (male 4.483±0.581 female 6.556±0.662 mmol.L^{-1}; P=0.034,).

4.4.2.3 *Insulin:Glucose Ratio*

A insulin:glucose ratio was calculated by dividing the Insulin AUC in response to a GTT by the Glucose AUC in lean offspring and comparing it to the equivalent ratio for obese offspring. The intention of this was to evaluate the confounding effect of administering differing glucose qualities in lean *vs.* obese offspring. A significant effect of time (P<0.001. lean 0.1702; obese 0.3129; SED 0.0332) was observed, with

obese offspring being observed to have a greater insulin response to the glucose load following the development of obesity. No treatment or sex effects were observed.

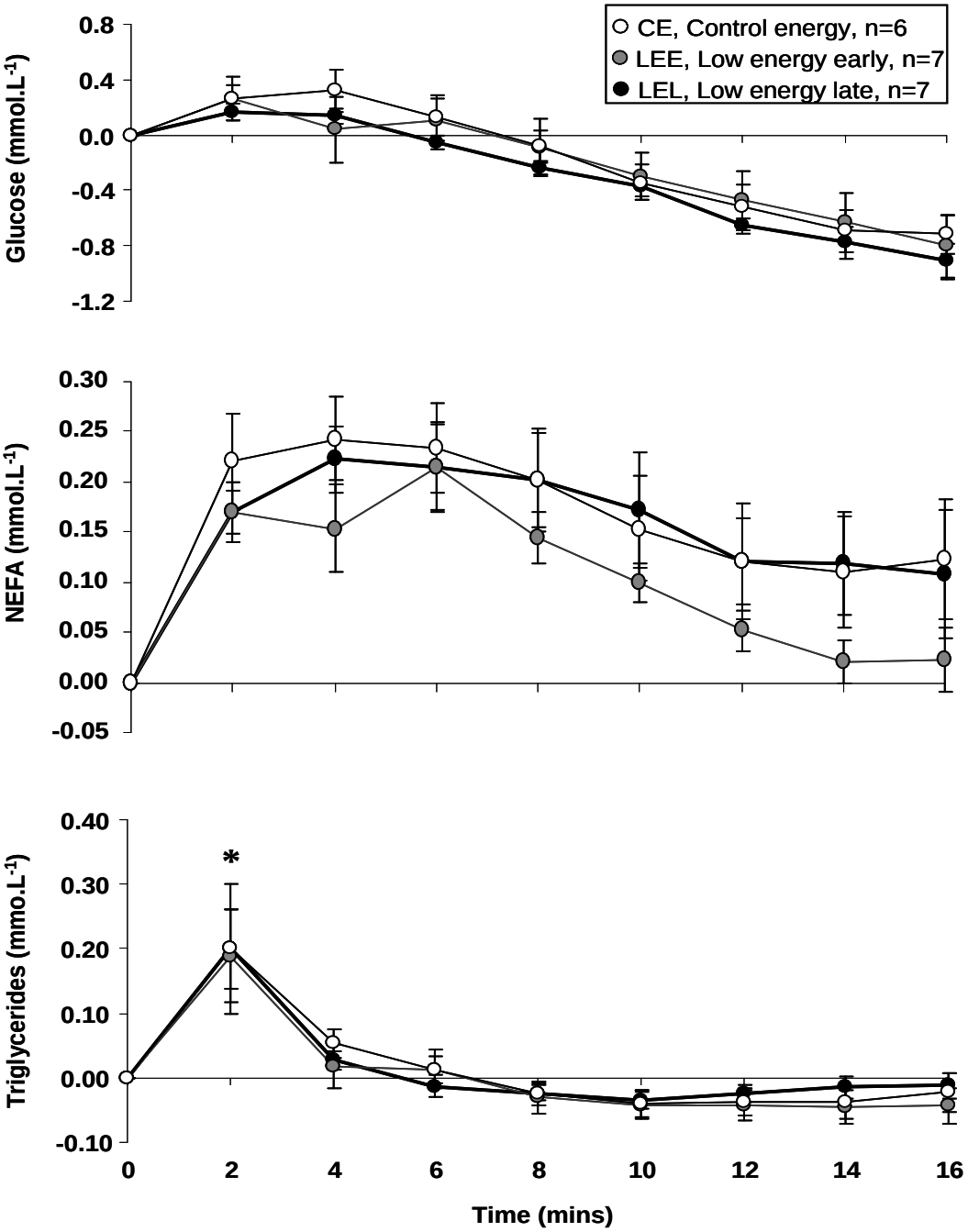


Figure 4.4 The glucose, NEFA and triglyceride response of prenatally nutrient restricted adult sheep to an insulin tolerance test when obese.

Data are Mean±SEM. Insulin (0.75IU.kg^{-1}) was injected I.V. at time zero. Statistics are *, P<0.05, relative to baseline.

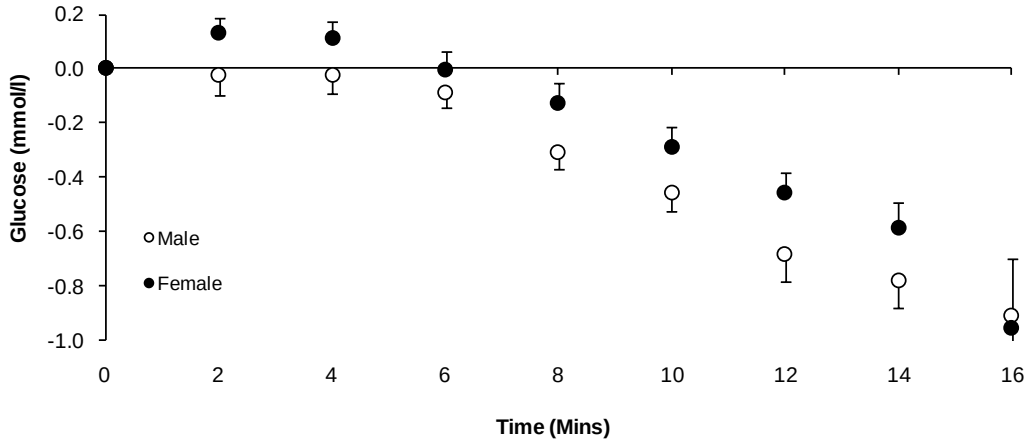


Figure 4.5 Graph showing the effect of sex on glucose clearance following an ITT aged 2yrs.
Graph showing the predicted means and 1±SEM for the sex effect on glucose clearance following an insulin bolus (ITT). Males had a significantly greater glucose clearance than females, both in respect to the K_{ITT} (*P*=0.041) and glucose AUC (*P*=0.034).

4.4.2.4 ***Lipid Metabolism***

In lean and obese animals in this study, very few treatment, gender or treatment*gender effects were shown on the various components of lipid metabolism that were assessed, except lean baseline glycerol was reduced in LEL vs. control and obese baseline cholesterol was less in LEE vs. control (Table 4.3). The development of obesity significantly elevated baseline plasma triglyceride, total cholesterol, HDL, and leptin levels, as well as significantly reducing glycerol and NEFA levels, but LDL levels were unaffected (Table 4.3). A treatment*time interaction was observed for baseline glycerol, (LEL baseline decreased less than control with the onset of obesity).

		Experimental Group				Statistics		
	Time	**CE**	**LEE**	**LEL**	**s.e.d**	***T***	***Ti***	***T*Ti***
Triglyceride	lean	0.16	0.15	0.14				
($mmol.L^{-1}$)	obese	0.22	0.23	0.22	0.02	NS	<0.001	NS
Glycerol	lean	110	88	69				
($\mu mol.L^{-1}$)	obese	70	66	71	10	0.04	0.006	0.03
NEFA	lean	0.99	0.88	0.84				
($mmol.L^{-1}$)	obese	0.73	0.65	0.65	0.11	NS	<0.001	NS
Total cholesterol	lean	1.44	1.33	1.42				
($mmol.L^{-1}$)	obese	2.52	1.75	2.31	0.21	0.04	<0.001	0.08
HDL	lean	0.66	0.63	0.66				
($mmol.L^{-1}$)	obese	1.43	0.99	1.36	0.14	0.08	<0.001	0.09
LDL	lean	0.25	0.23	0.23				
($mmol.L^{-1}$)	obese	0.31	0.21	0.25	0.04	NS	NS	NS
Leptin	lean	4.10	3.90	4.13				
($ng.ml^{-1}$)	obese	12.63	10.99	10.36	2.08	NS	<0.001	NS

Table 4.3 The effect of adult-onset obesity on lipid metabolites in sheep.

Data are predicted means with the average standard error of the difference (s.e.d with 14 degrees of freedom in all cases) for the comparison. CE, Controls (n=6); LEE, Low Energy Early (n=6), LEL, Low Energy Late (n=7). NEFA, non-esterified fatty acids, HDL – High density lipoprotein. LDL – Low density lipoprotein. T, main effect of treatment; Ti, main effect of time (i.e. becoming obese); T*Ti, interaction between treatment and time.

Following an injection of glucose plasma NEFA, TAG and glycerol concentrations declined ($P<0.01$) in both lean and obese animals. The area under the curve (AUC) for this response, was not affected by treatment, but the plasma NEFA AUC was significantly lower in the obese relative to lean state in both males and females but, for triglyceride, the AUC was significantly greater in obese females relative to all other groups (Figure 4.6).

Figure 4.6 The lipid metabolite response to a glucose tolerance test in male and female lean and obese sheep.

Data are Mean±SEM for areas under the glucose response curve (respective AUC units). Statistics are *, $P<0.05$, lean vs. obese or †, for a time*gender interaction.

During the ITT, under hyperinsulinemic, euglycaemic conditions plasma NEFA increased immediately and was sustained ($P< 0.05$), whereas plasma TAG only increased transiently (Figure 4.4). Plasma glycerol, measured at three time points during the ITT (0, 8 and 16mins), matched the NEFA profile i.e. a sustained increase was observed. There were no significant treatment, gender or treatment*gender interactions observed for the TAG, NEFA or glycerol response to the ITT.

4.4.2.5 ***Protein Metabolism***

As an index of lean mass metabolism, the fasted plasma concentrations of amino acids and urea in the lean and obese state were measured. In general, plasma amino acid concentrations and urea were significantly lower in the obese relative to the lean state, (8191 *vs.* 5289 (s.e.d. 639) nmoles.ml^{-1}; urea 8.59 *vs.* 5.70 (s.e.d. 0.42) mmol.L^{-1}), with no effect of prenatal diet or gender or any interactions on this response. Given the multiple levels of interaction between individual amino acids a multivariate analysis tool was employed, principal components analysis, to identify any components that may explain the majority of the variation in amino acids with obesity. Three principal components were identified that accounted for >95% of the variation in amino acid concentrations between groups from the lean to obese phenotype; PC1 accounted for 85.6% of the variation, PC2, 8.1% and PC3, 2.4%. For PC1, the greatest latent vector loadings (i.e. indicating those amino acids contributing most to the variation) were the reductions in the plasma concentration of glycine (0.9660), alanine (0.1437), valine (0.0887) and glutamic acid (0.0687) and the increases in the plasma concentration of serine (-0.1518). The respective

changes in the absolute plasma concentration for these amino acids are given in Figure 4.7. Using the principal component scores for PC1, as independent variates in a general linear regression model indicated that the amino acid variation reflected by PC1 was significantly associated with the decline in plasma [urea] concentration. There was no effect of prenatal treatment or gender on these effects.

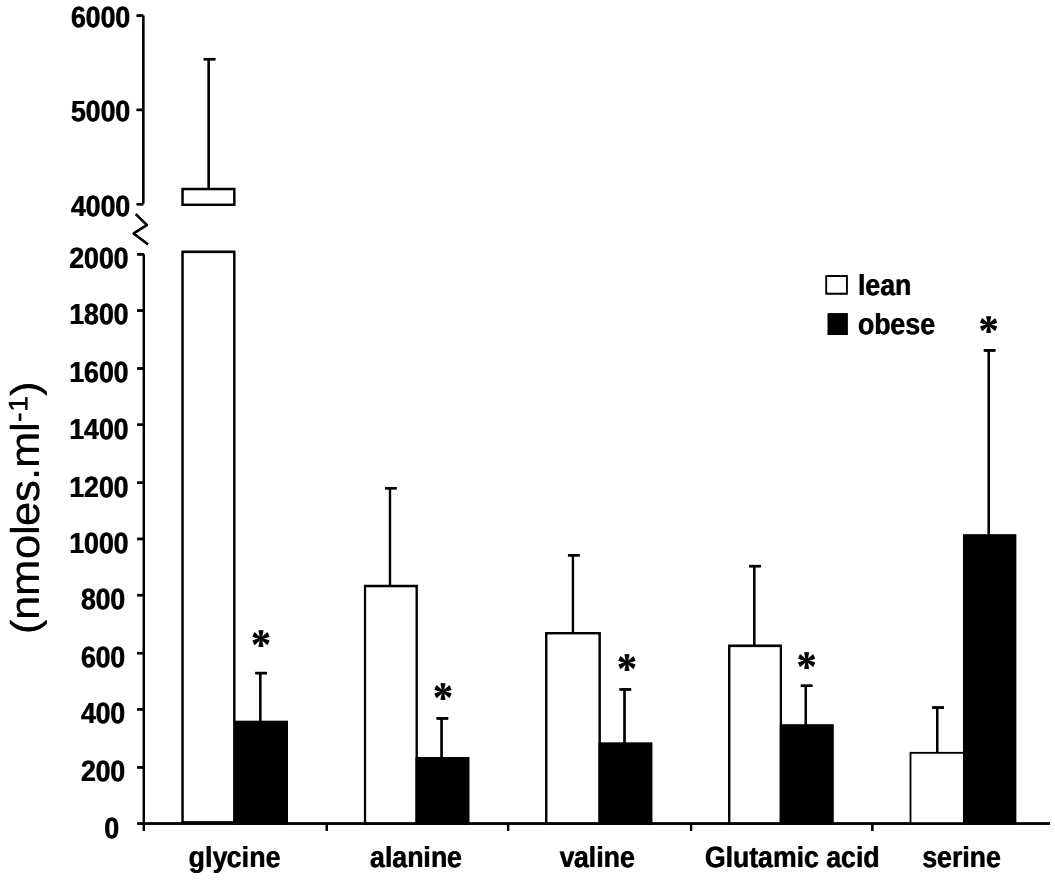


Figure 4.7 Amino acids contributing most to the variation in concentration with adult-onset obesity.

Data are Mean±SEM for amino acid concentrations. Statistics are *, *P*<0.01, lean *vs.* obese.

4.4.2.6 *Electrolytes and Haemodynamics*

4.4.2.6.1 Baseline

Fasted blood biochemistry when lean and obese are given in Table 4.4. With obesity, animals became more alkalotic as reflected in increases in blood pH, p_vO_2 and acid-base excess and a decrease in P_vCO_2. In addition, blood electrolytes also changed significantly; Ca^{2+} increased, whilst Na^+ and K^+ declined (Table 4.4). No time* treatment effects were observed.

	Baseline concentration		Statistics	
	Lean	Obese	*s.e.d*	*P*
pH	7.38	7.43	0.01	0.07
P_vCO_2 (mmHg)	47.22	41.71	1.93	0.01
HCO^-_3 ($mmol.L^{-1}$)	25.38	26.37	0.81	ns
P_vO_2 (mmHg)	35.31	51.94	4.95	0.01
ABE ($mEq.L^{-1}$)	2.47	3.06	0.78	ns
Na^+ ($mmol.L^{-1}$)	143	140	0.41	0.001
K^+ ($mmol.L^{-1}$)	4.26	4.03	0.07	0.01
Ca^{2+} ($mmol.L^{-1}$)	1.16	1.21	0.01	0.008
Cl^- ($mmol.L^{-1}$)	104	104	0.9	ns

Table 4.4 Baseline blood biochemistry in lean and obese adult sheep.

The change in resting blood biochemistry from a lean to obese state as measured on an ABL-800Flex (Radiometer Ltd, UK). Data are Grand Means with standard error of the difference and the df for the comparison of 14. P, for effect of time (i.e. onset of obesity). ns, not significant.

4.4.2.6.2 Response to a Glucose Bolus (GTT)

Changes in metabolic flexibility were indicated from a comparison of the delta change in lipid metabolites during the lean or obese GTT, for example, the blunting of the increase in plasma NEFA (Figure 4.4). Therefore, the delta change (i.e. values at 120mins minus values at 0mins) in blood biochemistry was considered to mark metabolic flexibility: again, no effect of sex and little effect of prenatal diet (treatment) influenced this response (Table 4.5). However, the delta change in blood biochemistry during a GTT changed with obesity (i.e. a significant effect of time was indicated); the changes in pH, P_vCO_2, P_vO_2, ABE, were all blunted (Table 4.5). Haemoglobin (Hb) concentrations and haematocrit (Hct) were similar in all groups when measured as obese animals (Hb: CE, 14.0±0.7; LEE, 15.3±0.6; LEL, 12.9±0.6 $g.dL^{-1}$; Hct: CE, 36.5±2.2; LEE, 36.4±1.9; LEL, 37.1±1.9 %).

		Experimental Group			Statistics			
	Time	**CE**	**LEE**	**LEL**	**s.e.d**	***Tr***	***Time***	***Tr*Time***
pH	lean	0.06	0.07	0.06				
	obese	-0.01	0.02	0.01	0.03	NS	0.01	NS
pCO_2	lean	-7.73	-5.58	6.00				
(mmHg)	obese	0.45	-1.17	-1.17	4.31	NS	0.02	NS
pO_2	lean	9.48	6.91	8.45				
(mmHg)	obese	-14.65	-1.98	1.42	9.17	NS	0.02	NS
ABE	lean	0.36	2.30	1.90				
($meQ.L^{-1}$)	obese	-1.35	1.60	-0.01	1.32	NS	0.04	NS
K^+	lean	-0.46	-0.43	-0.22				
($mmol.L^{-1}$)	obese	-0.30	-0.32	-0.12	0.20	NS	NS	NS
Na^+	lean	-1.00	-0.85	-1.85				
($mmol.L^{-1}$)	obese	1.83	-1.42	-1.42	1.13	0.06	NS	NS
Ca^{2+}	lean	-0.02	-0.02	-0.01				
($mmol.L^{-1}$	obese	-0.03	-0.04	-0.01	0.02	NS	NS	NS
Cl^-	lean	0.83	-0.85	0.00				
($mmol.L^{-1}$)	obese	-0.16	-0.57	-0.42	1.22	NS	NS	NS

Table 4.5 The delta change in metabolites during a GTT when lean or obese.

Data are predicted means with the average standard error of the difference (s.e.d with 14 degrees of freedom in all cases) for the comparison. CE, Controls (n=6); LEE, Low Energy Early (n=6), LEL, Low Energy Late (n=7). Tr, main effect of treatment; Time, main effect of time (i.e. becoming obese); Tr*Time, interaction between treatment and time.

4.4.3 ORGAN WEIGHTS

There were no treatment effects on absolute or relative wet organ weights at 2 years of age. Absolute weights were greater in male relative to female offspring, as expected, but when expressed in relative terms, few effects were observed with the exception of the brain, which was significantly larger in females than in their male counterparts (Table 4.6).

	Absolute weights		*P*	Relative weights (g/kg)		*P*
	Male	Female		Male	Female	
Body weight (kg)	82.2±01.5	66.5±1.2	***	-	-	-
Brain (g)	103±2	95±4	ns	1.26±0.03	1.44±0.06	**
†Pituitary (g)	0.81±0.12	0.65±0.08	ns	9.71±1.27	9.87±1.07	ns
Perirenal Fat (kg)	2.88±0.30	2.39±0.30	ns	33.5±2.7	37.1±2.8	ns
Pericardial Fat (g)	219±15	161±9	*	2.53±0.11	2.56±0.13	ns
Omental Fat (kg)	3.88±0.30	2.88±0.18	ns	2.53±0.11	2.56±0.13	ns
Total kidney (g)	150±8	117±3	***	1.82±0.07	1.76±0.03	ns
†Total adrenal (g)	5.39±0.80	3.64±0.45	ns	66.4±11.2	54.7±6.7	ns
Liver (g)	697±89	627±20	ns	8.55±1.11	9.42±0.21	ns
Pancreas (g)	70.7±5.8	61.8±4.1	*	0.86±0.07	0.93±0.06	ns
Spleen (g)	121±9	100±9	ns	1.48±0.12	1.49±0.12	ns
Lungs (g)	594±26	552±43	ns	7.25±0.39	8.28±0.59	ns
Heart (g)	316±9	271±8	***	3.86±0.14	4.07±0.10	ns
Septum (mm)	18.3±0.99	14.4±0.36	***	-	-	-
Left Ventricle (mm)	17.6±0.89	15.6±1.0	ns	-	-	-
Right Ventricle (mm)	7.42±0.52	6.71±0.60	ns	-	-	-

Table 4.6 Post Mortem organ weights in male and female sheep.

Body and organ wet weights of male (n=11) and female (n=9) offspring at post mortem. Data are Grand Means ±1 SEM. Statistics are *, **, ***, $P<0.05$, $P<0.01$ or $P<0.001$ respectively for male vs. female. ns, not significant. †, relative adrenal and pituitary weight expressed as $(g.kg^{-1}) \times 10^3$.

4.5 Discussion

We have shown that global nutrient restriction of the pregnant ewe from mid-late gestation leads specifically to a greater early-phase (first 20 mins) increase in plasma insulin in response to an intravenous glucose bolus in the adult offspring. The response was sex-specific (greater in males > females) and only observed when the adult offspring were obese. In addition, for this study we broadly assessed metabolic health in the adult offspring when lean and the deterioration in metabolic health when they became obese. Obesity was clearly detrimental to metabolic health in many respects (lipoprotein and cholesterol profile, insulin sensitivity) but, contrary to our hypothesis, we found little evidence of greater deterioration of health in those offspring prenatally undernourished by global energy deficit. Taken together, the current study provides evidence to suggest that prenatal energy deficit has a sex-specific effect on adult glucose-insulin sensitivity when, and only when, excess calories are consumed; if the offspring remain lean and physically active through adolescence and early adulthood then good metabolic health prevails. The extent to which metabolic deterioration in programmed offspring may by exacerbated by older age cannot be determined by this study but clearly, prevention of excess weight gain through controlled food intake and lots of habitual physical activity can be inferred.

Pregnant women that suffered the Dutch Hunger Winter Famine during WWII, with the severe nutrient deficiency that it entailed gave birth to babies that were on

average only ~300g less than contemporaneous women that were un-exposed (Roseboom *et al.*, 2001). The middle-aged adult offspring, however, had elevated plasma lipids, obesity and an increased incidence of coronary heart disease if exposed during early gestation (Roseboom *et al.*, 2000), whereas those exposed during late gestation had reduced glucose tolerance (Painter *et al.*, 2005). The current study broadly concurs with these epidemiological observations and suggests the liver as the likely candidate for reduced whole body glucose tolerance (e.g. blunted first pass insulin metabolism and insulin-induced suppression of hepatic glucose output). This is inferred from our results indicating greater early (0-20mins) plasma insulin concentrations in LEL (exposed to undernutrition during late gestation) relative to control animals in response to the GTT, but no difference in their response to an I.V. ITT (i.e. as a measure of insulin-dependent peripheral glucose uptake). In particular, we noted a clear treatment*gender interaction in the response to the GTT when obese, with male offspring mounting a much greater first phase insulin response relative to females. Given that no such interaction was observed during the ITT, as previously shown by Poore *et al*., (2007), suggests that in offspring undernourished during late gestation the major sites of peripheral glucose uptake (skeletal muscle and adipose tissue) are not overtly resistant to insulin action *per se*. Rather, when hyperglycaemia and hyperinsulinaemia co-exist, such as postprandially (or, for example, after a GTT), differences in other insulin-sensitive tissues (pancreas and liver) that are not activated during the ITT but are

during the GTT must account for the observed significant treatment*gender interaction.

In terms of the developmental programming paradigm the sex-specificity of many diverse programmed sequalae has been the source of much debate, but in general, it would appear that the male offspring are particularly vulnerable. For example, such a sex-specific effect on glucose-insulin metabolism has also been observed in the male offspring from mothers that were methyl-deficient around the time of conception (Sinclair *et al.*, 2007) or placentally restricted during gestation, in similarity to the current study (Owens *et al.*, 2007). Males also appear more susceptible with respect to the programming of function in alternative organs e.g. the heart and kidney (McMullen & Langley-Evans, 2005; Elmes *et al.*, 2008). In the present study the males were castrates, therefore mitigating any effect of postnatal reproductive hormones. Thus, the sex-specific differences may relate to either female reproductive hormones (Ojeda *et al.*, 2007; Grigore *et al.*, 2008) or a delayed effect pertaining to sex-specific differences in the intrauterine response to nutrient deficit *per se* (Hattersley & Tooke, 1999). From the present results and taking into account other studies it is still not clear what mechanism may underlie the sex-specificity of developmental programming, although recent work would suggest there is differential sensitivity to epigenetic programming and altered methylation of key genes could underpin many of the sex-specific responses (Lillycrop *et al.*,

2005; Sinclair *et al.*, 2007); the results, however, do emphasise the importance of including sex as a factor in the design of developmental programming experiments.

We have previously shown that late gestation undernutrition can marginally reduce glucose tolerance in *lean* adult sheep (Gardner *et al.*, 2005) in contrast to the current study, where reduced glucose only became apparent with significant obesity. The reason for this difference is likely due to the milder nutritional challenge employed over late gestation and birth in the current study (i.e. a 35% reduction in the current study *vs*. 50% in the previous study (Gardner *et al.*, 2005)). In addition, it is important to reiterate that ewes in the current study were also gradually realimented from day 128 to term to avoid any potential confounding by carry-over effects of prenatal undernutrition into lactation (Hammond *et al.*, 1976). This can be considerable and likely affects many previous studies in this area; in the current study, that singles and twins grew at equivalent rates during lactation suggests that the lactational ability of ewes in our study was not hampered by prenatal undernutrition. Our study therefore uniquely illustrates that isolated mid-late gestation global undernutrition can have specific long-term effects on glucose-insulin dynamics in the adult male offspring when they become overweight.

In the ruminant in the resting state (euglycaemia), glucose clearance is likely orchestrated by Glut4-mediated insulin-dependent transfer into skeletal muscle and adipose tissue. During hyperglycaemia, clearance is additionally coupled to mass-

action passive glucose transfer into pancreatic and hepatic cells (via Glut2, which has a high K_m for glucose (Sasaki, 2002)). Thus, in contrast to our previous observation in which a reduction in adipose Glut4 was associated with the decline in insulin sensitivity (Gardner *et al.*, 2005), in the current study it would appear that the key 'programmed' effect is on either hepatic insulin resistance i.e. decreased first-pass hepatic insulin extraction, which is approximately 50% in the sheep under basal conditions, (Brockman & Bergman, 1975) or insensitivity to insulin-induced blunting of hepatic glucose output (via gluconeogenesis or glycogenolysis) and/or pancreatic dysfunction e.g. insulin hypersecretion (Gower *et al.*, 2002). As yet, we are unable to discern a pancreatic from hepatic effect, although the available evidence from other animal models that have attempted to address this conundrum suggests a combination of both (Kim *et al.*, 2007). Certainly, it would appear that at a systemic level potential changes in skeletal muscle fibre type (Daniel *et al.*, 2007) and/or skeletal muscle insulin signalling protein expression (Ozanne *et al.*, 2005) are not important in mediating the differences in early insulin secretion in the current study.

From literature in Man and in animal models it is apparent that following a deficit in growth, rapid weight loss or undernutrition there is a period of preferential fat accretion or 'catch-up fat' (Dulloo, 2008). This paradigm is akin to the original 'thrifty phenotype' hypothesis (Hales & Barker, 2001), the later 'mis-match' (Gluckman & Hanson, 2004) or alternative thin-fat (Popkin, 2006) hypotheses. Here,

nutritional insufficiency leading to deficits in weight and/or growth (particularly during the key developmental periods of gestation and lactation) when coupled with postnatal nutritional excess increase the risk of adult metabolic disease (Sayer *et al.*, 2004; Singhal & Lucas, 2004; Barker *et al.*, 2005) and actually reduce lifespan in male mice (Ozanne & Hales, 2004). Hence, in the current study we specifically designed the experiments, using a relevant large animal model, to test this hypothesis. Our results largely agree with the null hypothesis in that few time*treatment interactions were observed; prenatal undernutrition did not make the adults more susceptible to greater adipose tissue gain *per se*. There are a number of reasons that may go some way to explaining this variance with the prevailing literature. First, when measured in a lean condition the animals had been in their habitual environment for 1.5 years, were relatively active and eating a low energy dense diet, in stark contrast to laboratory species (Vickers *et al.*, 2000) and much epidemiological data in Man. The animals were still relatively young (1.5 years) when sheep may live up to 12 years, however, catch-up fat has been observed from a young age (Dulloo, 2008). Alternatively, 30% global nutrient restriction of the pregnant sheep could be considered a relatively milder challenge when compared to, for example, 50% protein restriction in the rat (Armitage *et al.*, 2004; McMillen & Robinson, 2005). The long-term consequences of prenatal programming may therefore be relatively harder to elucidate in larger animal models in comparison to small because of species-specific allometry and the metabolic 'buffer' offered by the larger body surface area:volume ratio of the sheep

(Gardner *et al.*, 2007). Nevertheless, with time, common programmed end-points have been shown to develop in larger animal species (Dodic *et al.*, 2001; Gopalakrishnan *et al.*, 2004; Cleal *et al.*, 2007a).

In this study we uniquely measured the development of obesity in sheep by repeated DXA which has a very low internal coefficient of variation and is very accurate for tissue weights from 500g to 90kg. However, we sought to further assess DXA against chemically determined body composition. Whilst there was good correlation between the two methods, chemical extraction of tissue for fat increased the apparent % fat determined by 75%. This is likely due to the Soxhlet method extracting absolutely all lipids available in that tissue (extracellular, intracellular, and membraneous) rather than those that are simply metabolically available. Thus, the Soxhlet method is useful for determining fat composition of food to be eaten (as all fat will be extracted intestinally) but is perhaps less relevant in metabolic studies whereas DXA is a more appropriate method to determine body composition for assessing effects of adiposity on metabolism *in vivo*. Indeed, the value of 30% whole body fat mass in the current study determined by DXA concur with levels achieved previously by us after juvenile-onset obesity (Sebert *et al.*, 2009). Adult onset obesity in the current study was associated with a significant decrement of lean mass (~2-3kgs), contrary to the often-reported increase with obesity, presumably as a consequence of increased weight-bearing (Wells *et al.*, 2006). Previous work in sheep, however, has also reported a reduction in carcass

protein content with adult-onset obesity induced by overconsumption of feed (Bergman *et al.*, 1989). The similar responses observed in that study and the current one, together with the reductions in plasma amino acid and urea concentrations, suggest reduced protein turnover in obese sheep, perhaps due to the reduced activity of the sheep within our 'obesogenic' environment. In Man, increased protein catabolism has been observed with obesity, related to the resistance of glucose and protein metabolism to insulin and the increased hepatic supply of glucogenic amino acids (Chevalier *et al.*, 2006). However, the fact that no overt peripheral insulin insensitivity was observed and the majority of the change in plasma amino acids could be attributed to only those few amino acids originally present in the highest concentrations in the plasma suggests, by the law of parsimony, that the increased dietary fat incorporation in the current study (from 3 to 6%) reduced rumen microflora protein synthesis and thus overall amino acid availability; the net effect being reduced protein turnover, and reduced measured amino acid and urea concentrations. Taken together with the changes in fat metabolism it would appear that obesity in sheep engendered by 7-8months exposure to an 'obesogenic environment' created by increasing food and fat intake and reducing physical activity has put the sheep into a state of decreased protein turnover (likely through reduced protein synthesis) with net lipid deposition and decreased oxidation (as reflected in reduced plasma NEFA and glycerol) (Bergman *et al.*, 1989).

In addition, obesity in sheep was characterised by a decline in the individuals' capacity to transition between metabolic substrates under conditions of rapidly changing fuel supply, here recreated after a glucose tolerance test. The obese individual, in response to elevated blood glucose, responded with markedly blunted excursions in plasma metabolites and biochemistry. Such a reduction in 'metabolic flexibility', for example a reduced transition from fatty acid efflux to storage in response to a carbohydrate rich meal (Storlien *et al.*, 2004) has recently been characterised in obese individuals and is thought to contribute to Metabolic Syndrome. We observed no overall effect of the prenatal diet on this response. However, for LEL, that had a greater plasma insulin response to high glucose coupled with reduced metabolic flexibility from being obese, then over time (i.e. longer exposure to the obesogenic environment), overt peripheral insulin resistance would perhaps be more likely to develop and be more pronounced in those sheep undernourished during late gestation (Galgani *et al.*, 2008).

In our study, there is potential that the effect is confounded by the study design in two ways; first, the increase in weight with obesity necessitated an increase in delivered glucose (administered v/w) relative to the first or lean GTT, therefore precipitating an overall greater response when obese. In defence, we highlight the fact that the gain in body weight was equivalent between treatment groups and that statistical adjustment of the insulin response to GTT for the weight gain (included as a co-variate) and thus the increased delivery of glucose, actually

increased the F-statistic for the treatment*gender interaction, indicating a specific effect of the mismatch between pre- and post-natal diet on first phase insulin response in male offspring. The increased insulin to glucose AUC ratio also supports the fact that the obese offspring produced a greater insulin response than when lean. Secondly, we acknowledge that following prior consultation with a statistician our best option for testing the hypothesis that prenatal undernutrition interacts deleteriously with postnatal nutritional excess was to use a repeated measures design. Such a design offers greater precision in testing for potential time*treatment*gender interactions as within animal variation is generally less than between animal variation. Furthermore, if no interactions are present (e.g. with time) then the study can more precisely determine potential main effects as the second measurement acts as a replicate of the first (i.e. the benefits of a factorial design); such experimental designs are therefore encouraged by the National Centre for the Reduction, Refinement and/or Replacement of animals in research (NC3Rs) (Festing *et al.*, 2004). Nevertheless, we are unable to control for an overall effect of time (being confounded with acquisition of obesity). However, we suggest that a 6-month period in an adult animal that may live for up to 12-15 years is not a significant length of time and that a doubling of fat-mass over that period is by far the over-riding effect. In addition, all treatment groups were randomly group housed together over this period, thus any effect of time *per se* would be expected to act equally on each individual sheep; given that our longitudinal design increased our sensitivity to observe time*treatment*gender

interactions and we only observed such an effect of time (i.e. obesity) in one treatment*gender combination (male LEL) suggests that male LEL specifically differ with respect to groups in their response to adult-onset obesity.

In conclusion, global nutrient restriction of sheep had little delayed 'programming' effect on carbohydrate, fat and/or protein metabolism in the adult offspring when they were lean and physically active. However, when coupled with an obesogenic environment, those male sheep that experienced a poor diet during late gestation exhibited evidence of reduced insulin sensitivity.

5. Low Protein Study – conception to 18 months of age

5.1 SUMMARY

This chapter describes the effects of a modest, isocalorific, maternal protein restriction (18% *vs*. 9%) from conception to day 65 gestation (Low Protein Early; LPE) or from day 66 to term (Low Protein Late; LPL). The low protein diet had no effect on maternal weight in either treatment group in comparison to control, although LPE ewes had a significantly lower body condition score during trimester one and two than control ewes. Maternal dietary regime had no effect on maternal and fetal cortisol levels, or fetal weight at day 65 gestation; at this time, however, males were heavier than females (P=0.002). At term, singletons were heavier than twins, as expected, but LPL singletons were growth restricted (~500g. P<0.001); an effect still evident at weaning (3 months of age). Furthermore, at term, LPL offspring had significantly lower basal plasma insulin that persisted for ~2 weeks. In the adolescent offspring at 7 months of age and in response to a glucose bolus, the peak insulin (P=0.024) and incremental area under the curve (P=0.048) were greater in LPE offspring than control. However, there was no treatment effect following an insulin tolerance test at the same age and all offspring were morphologically similar at *post mortem*.

5.2 Introduction

The fact that total ruminant nitrogen uptake cannot be simply quantified from fed sources makes ruminal protein nutrition research less clear cut than monogastric nutritional research. Samuel Brody was one of the first animal scientists to calculate ovine protein requirements (Brody, 1945). He calculated the protein requirement of a non-pregnant ewe to be 3.6g digestible crude protein per kg $W^{0.73}$ (where W is the sheep's mass), which equates roughly to 60g digestible crude protein for a 50kg ewe. The first estimates for pregnant ewes were published in 1959 (Phillipson, 1959; Thomson & Aitken, 1959), where digestible crude protein requirement for a pregnant ewe was calculated to be twice that of a non pregnant ewe. As the knowledge of nutrition improved, these estimated requirements were subsequently reconfigured. The seminal work of JJ Robinson and colleagues has greatly advanced the field of ovine nutrition, in particular of pregnant ewes and developing ovine fetuses (Robinson & Forbes, 1966, 1967; Guada & Robinson, 1974; Robinson, 1977, 1980). Robinson & Forbes (1967) recalculated the nitrogen requirement of a pregnant ewe nearing parturition as 77g of digestible crude protein for a 68kg ewe, rather than 120g (Phillipson, 1959; Thomson & Aitken, 1959). This difference was mainly attributed to the nitrogen retention rate being recalculated as 62g protein for a 68kg ewe, which is higher than previous estimates by Klosterman *et al.*, (1951). Robinson highlighted the importance of metabolisable energy supply when calculating ewe protein requirements during pregnancy. As energy intake increases,

the digestibility of crude protein decreases, which is important as during pregnancy both metabolisable energy and protein requirements elevate. As pregnancy progresses the nitrogen retention from the ewe increases without a subsequent increase in urinary nitrogen output, which infers an increase in nitrogen utilisation (Robinson & Forbes, 1967). McNeill *et al.,* (1997) observed a significant correlation between maternal protein intake and nitrogen accretion in twin bearing ewes in late gestation, although the efficiency at which absorbed nitrogen was deposited was elevated with protein restriction. Amino acid utilisation during pregnancy will depend on the stage of gestation as well as the energy supply. During early to mid gestation there is no additional protein requirement of the pregnant ewe over that of the non-pregnant ewe (Robinson & Forbes, 1967), with placental development accounting for a high proportion of the increased tissue accretion at this time. As the ewe enters the later stages of gestation, not only will the fetus have a high demand for amino acids to support its exponential growth rate, but it will also utilise amino acids alongside glucose as a fuel for oxidative metabolism (Robinson & Symonds, 1995). The interplay between protein and energy nutrition during pregnancy, especially nearing term, is why it is crucial to try to maintain an isoenergetic diet when investigating protein requirement, as reductions in energy supply will repartition amino acids towards oxidative catabolism rather than fetal protein synthesis.

The daily flux of metabolic substrates is influenced by hormonal status and glucocorticoids help mediate this process (Dallman *et al.*, 1993). Glucocorticoids have long been thought to act as intermediary factors that transcribe the developmental programming sequalae of maternal nutrient restriction and there is much evidence in animal models (Gardner *et al.*, 1998; Phillips *et al.*, 1998) to suggest just such a role. In sheep the change in maternal cortisol with undernutrition appears age-dependant, being elevated with late gestation undernutrition (Bloomfield *et al.*, 2003a) and reduced with early to mid gestation undernutrition (Bispham *et al.*, 2003). Interestingly, periconceptional undernutrition has been shown to markedly activate fetal HPA axis activity in late gestation leading to preterm birth in sheep (Bloomfield *et al.*, 2003b). Thus in this study we sought to broadly characterise the effects of maternal protein undernutrition on maternal and fetal plasma cortisol.

The effect of protein malnourishment on *in utero* fetal development and postnatal production characteristics of ovine offspring has been studied extensively, as detailed above. Until the current study, little evidence was known about the effect of restricted protein nutrition during specific periods of gestation on ovine postnatal health factors. The low protein model of fetal programming is an extensively used research model in rats, with the advantage over some models that the combined process (maternal protein malnourishment) and end point (IUGR fetuses) are frequently observed in the human populous (Symonds *et al.*, 2009). The rat low

protein model was established by Snoeck *et al.,* (1990) and Dahri *et al.,* (1991), and has been further developed and extensively characterised since (Langley-Evans *et al.*, 1996; Fernandez-Twinn *et al.*, 2003; Bellinger & Langley-Evans, 2005; Fernandez-Twinn *et al.*, 2005; Erhuma *et al.*, 2007b; Mallinson *et al.*, 2007; Harrison & Langley-Evans, 2009).

As previously mentioned, the low protein model offers a more mechanistic insight into fetal programming versus global energy restriction (Symonds *et al.*, 2009); with this in mind, the sheep has many physiological benefits when investigating human *in utero* development and postnatal health (reviewed by Luther *et al.,* (2005) and Wallace *et al.,* (2005)). The level of protein restriction in this study (9% *vs.* 18%) was chosen on an agricultural basis, rather than as a replication of the protein levels utilised during rat studies (Harrison & Langley-Evans, 2009). A crude protein level of 18% more than meets adequacy for pregnant ewes, but 9% dietary crude protein was selected to represent the minimal protein availability that a sheep would naturally be exposed to if it was to consume a relatively high tannin diet. Tannins are polyphenolic compounds found in high concentrations in many plant species, including heathers (*Calluna vulgaris*). When ruminants consume tannins, the tannins form strong complexes with protein molecules in the rumen, thus reducing nitrogen uptake, although tannins may also reduce ruminant protein uptake by inhibiting enzymes due to tannin-protein (enzyme) complex formation (Frutos *et al.*, 2004). In similarity and to provide a dietary contrast, two periods of gestation were

chosen to feed the low protein diet, 0-65d gestation (Low Protein Early; LPE) and 66-120d gestation (Low Protein Late; LPL).

5.3 Materials and methods

See Chapter 2.3 for details of dietary design. In brief, Scottish blackface ewes were oestrus synchronised and mated to Scottish blackface rams. Ewes were fed either maintenance protein requirements (18% protein) throughout gestation, from day 0-term (Control Protein; CP) or they were allocated to a restriction protocol. The early restriction animals were fed 50% of CP protein intake (9% protein) from day 0- 65 gestation (Low Protein Early; LPE). On day 65 a proportion of CP and LPE animals were euthanised for collection of fetal tissues and blood (fetal and maternal cortisol was analysed- described in 2.5.1.3). On day 66 the remaining LPE animals were switched to the 18% protein diet, while the late restriction group (Low Protein Late; LPL) was switched from 18% protein feed to 9% and they remained on this until term. Maternal weight and body condition score (BCS) were recorded frequently throughout gestation. Males were castrated shortly after birth and all offspring were weaning aged 3 months. Lamb weights, BCS and blood were collected every 3 weeks postnatally. Blood was analysed for plasma insulin (see Chapter 2.5.1.4). At 7 months old offspring were cannulated, and a glucose tolerance test (GTT- see Chapter 2.4.2.2.1) and insulin tolerance test (ITT- see Chapter 2.4.2.6.1) were

performed. Aged ~16 months, animals were either euthanised or transported to Sutton Bonington Campus, University of Nottingham for further investigation.

5.4 Results

5.4.1 MATERNAL & FETAL DATA

5.4.1.1 *Ewe Weight*

Maternal weight was significantly affected by the time of gestation ($P<0.001$), with the ewe continually accruing body mass throughout pregnancy (Figure 5.1). Maternal weight was also influenced by the number of fetuses the ewe was carrying as pregnancy progressed (fetal number*time $P<0.001$), with the disparity between single and twin bearing ewes increasing, such that at the last time point (gestation day 124) twin bearing ewes weighed ~8kg more than singleton bearing ewes.

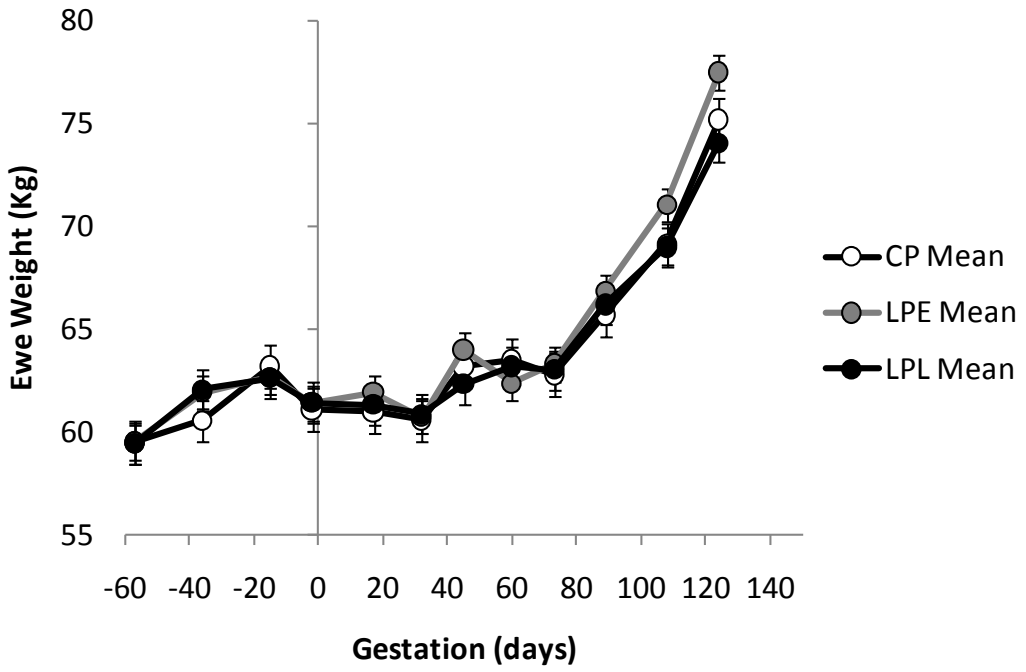


Figure 5.1 Ewe weight throughout gestation

Weights shown are means ±1 SEM. Treatment groups are CP (Control Protein), LPE (Low Protein Early) & LPL (Low Protein Late). A significant effect of time (P<0.001) and fetal number*time (P<0.001) was observed.

5.4.1.2 *Ewe Body Condition Score (BCS)*

Ewe body condition score (BCS) changed significantly with the stage of gestation (P<0.001), with a dramatic decline in maternal BCS in the later stages of pregnancy (Figure 5.2). A treatment*time interaction was also observed (P=0.013), with LPE ewes having a lower BCS compared, in particular, to LPL during the first and second trimester of pregnancy.

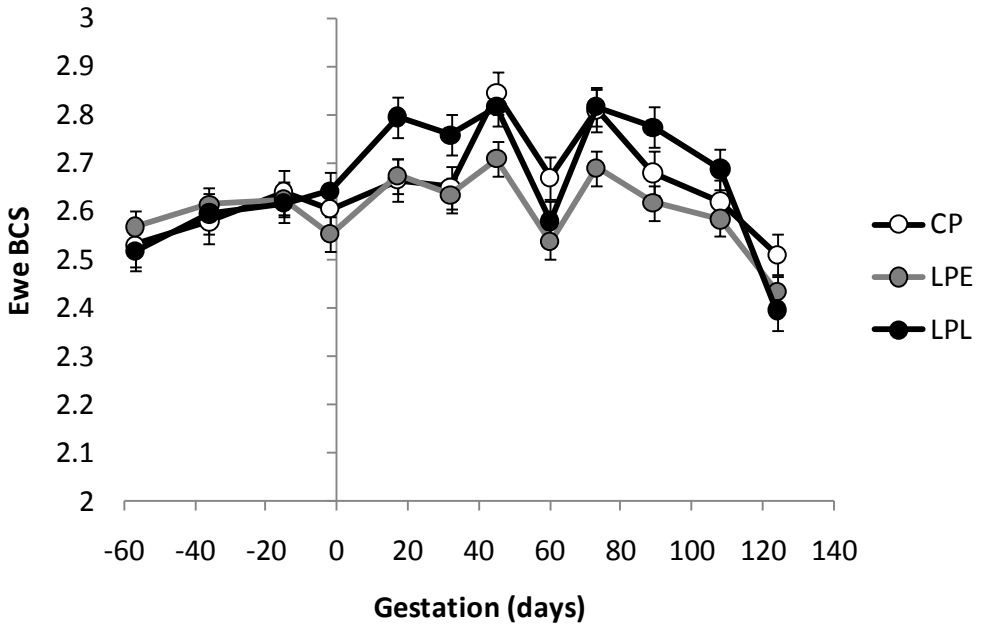


Figure 5.2 Ewe Body Condition Score for Maternal Low Protein Study.
BCS (Body Condition Score) is judged on an arbitrary scale between 1- emaciated and 5-severely overweight. Errors shown are ±1 SEM. Treatment groups are CP (Control Protein), LPE (Low Protein Early) and LPL (Low Protein Late).

5.4.1.3 *Maternal & Fetal Cortisol*

At day 65 there was no significant effect of maternal nutrient restriction on fetal or maternal plasma cortisol levels (fetal control 63.52 vs. low protein 65.07ng.ml^{-1}; maternal control 63.81 *vs*. low protein 57.92 ng.ml^{-1} SED 16.39).

5.4.1.4 *Fetal Organ Weights at day 65 gestation*

The gender of the fetus had a significant effect on the weight of the fetus (male 110.8 & female 92.6g, SED 5.1; *P*=0.002.), but maternal protein restriction had no effect (CP 100.6 & LPE 102.8g, SED 5.103). A sex effect on heart (male 1.07 & female

0.89g, SED 0.08) and liver weight (male 8.72 & female 7.11, SED 0.51) was observed, although both these effects were removed when these organs were expressed relative to fetal weight. No effect of gender or treatment was seen on brain weight (grand mean 3.466g, SE 0.05), kidney weight (grand mean 1.59g SE 0.06) or lung weight (grand mean 5.19g SE 0.34), although brain:body weight ratio was significantly (*P*=0.006) influenced by gender (male 0.032 & female 0.037 g/kg, SED 0.001).

5.4.2 BIRTH WEIGHT & POSTNATAL GROWTH

5.4.2.1 *Birth Weight*

A significant effect (*P*<0.001) of treatment and fetal number was observed on birth weight; LPL singletons were significantly lighter than control singletons, while maternal protein restriction elevated the birth weight of LPE twins compared to control (Figure 5.3). Gender had no main or interactive effect on birth weight.

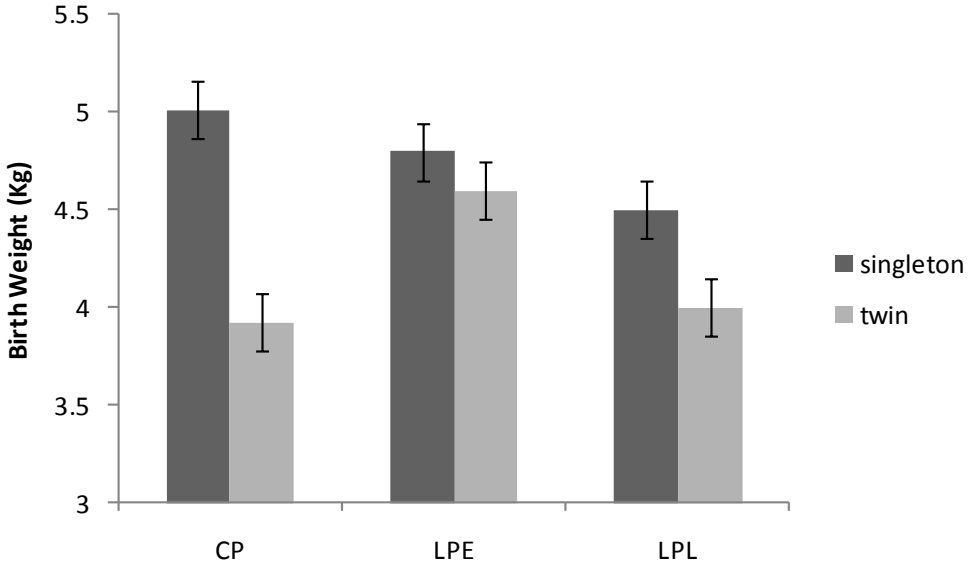


Figure 5.3 Graph showing the effect of maternal protein restriction and fetal number on birth weight.

Values shown are predicted ±1 SED. SED is the average SED for the interaction between treatment and fetal number. Treatment groups are CP (Control Protein), LPE (Low Protein Early) and LPL (Low Protein Late). A significant effect of fetal number and treatment group was observed on birth weight (P<0.001).

5.4.2.2 ***Weaning Weight***

Weaning weight, like birth weight, was significantly influenced by maternal treatment (P=0.008) and fetal number (P<0.001) (Figure 5.4), but at this time there was no effect of gender. LPE and LPL singleton offspring were significantly lighter than controls, while singletons were still observed to be higher than twins.

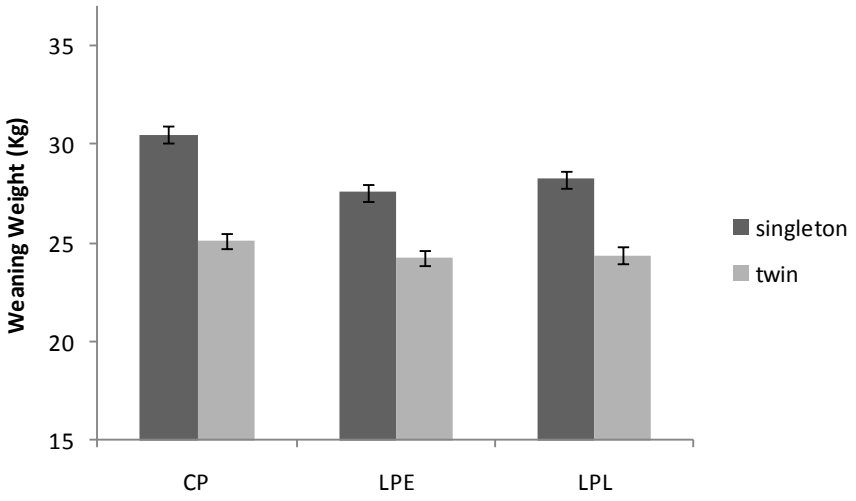


Figure 5.4 A graph showing the effect of maternal protein restriction and fetal number on weaning weight.
Values shown are predicted means ±1 SED. Treatment groups are CP (Control Protein), LPE (Low Protein Early) & LPL (Low Protein Late).

5.4.2.3 ***Lamb Insulin***

Lamb plasma insulin levels changed significantly over the lambs' first ~5 months of postnatal life (P<0.001, Figure 5.5). A significant interaction between treatment*time (P=0.009) was also observed, although this seems to be derived from the significant treatment effect on lamb insulin levels at birth (P=0.007), with LPL plasma insulin being significantly lower than both control and LPE offspring.

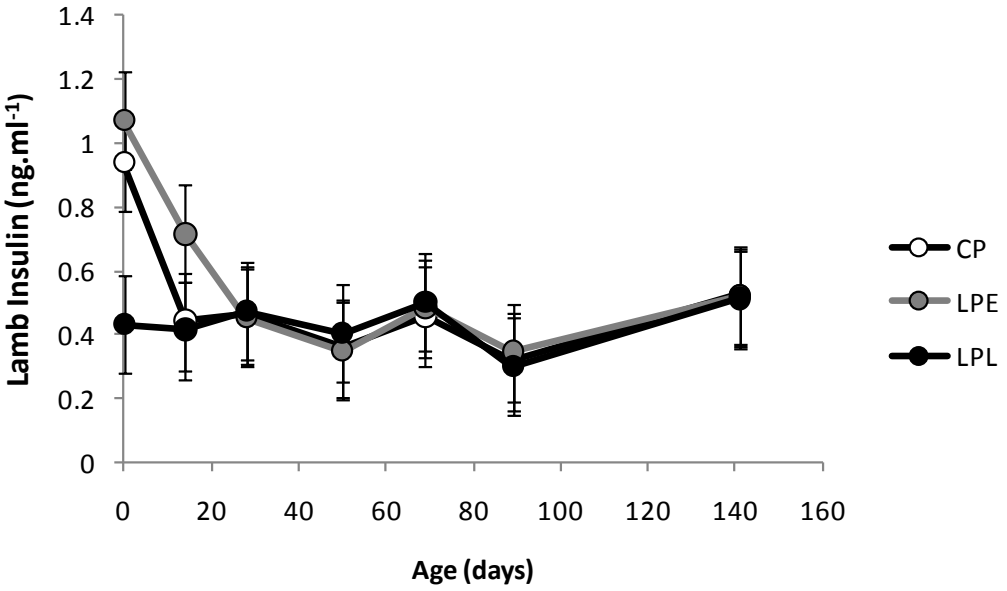


Figure 5.5 Graph showing the effect of maternal nutrition on plasma insulin concentration. Values are predicted means ±1 SED. Error bars shown are the average SED for treatment & fetal number. Treatment groups are CP (Control), LPE (Low Protein Early) and LPL (Low Protein Late).

5.4.3 RESPONSE TO A GLUCOSE TOLERANCE TEST (GTT) AGED 7 MONTHS

5.4.3.1 *Plasma Glucose*

As expected, plasma glucose was significantly elevated following the administration of an I.V. glucose bolus ($P<0.001$, Figure 5.6). No effect of maternal treatment, gender or fetal number was observed on baseline glucose or the incremental glucose AUC.

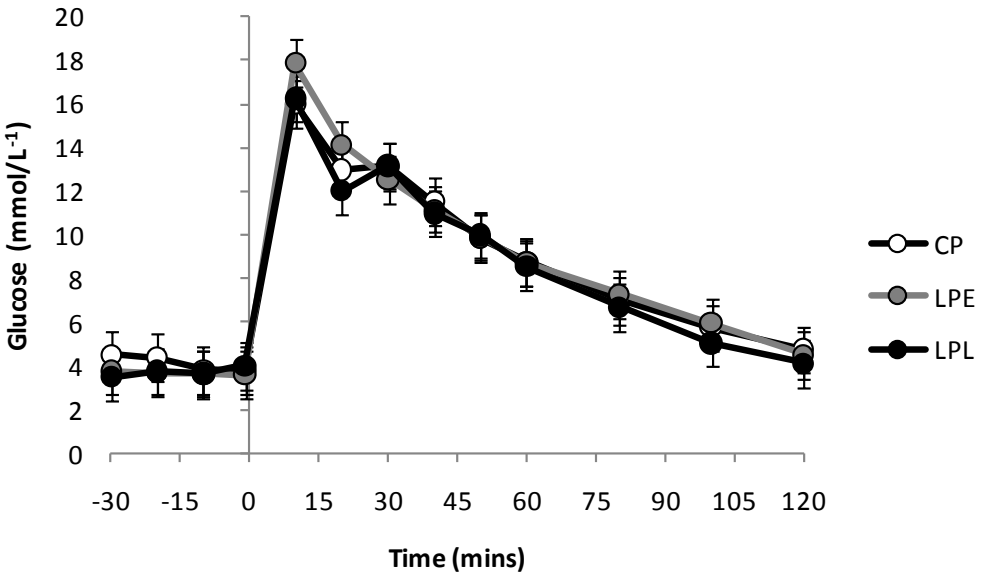


Figure 5.6 A graph showing the effect of maternal protein restriction on plasma glucose levels following a GTT aged 7 months.

Values shown are predicted means following generalized linear mixed model analysis. Error bars are ±1 SED (SED is the average SED for the time*treatment interaction). Treatment groups are CP (Control Protein), LPE (Low Protein Early) and LPL (Low Protein Late).

5.4.3.2 *Insulin*

Following the injection of the glucose bolus, plasma insulin rose significantly (P<0.001, Figure 5.7). A significant effect of treatment was observed on the insulin AUC (P=0.048) and peak insulin response (P=0.024, Figure 5.7) with LPE animals having a greater peak insulin response and therefore a greater AUC in response to a glucose bolus compared to control and LPL animals.

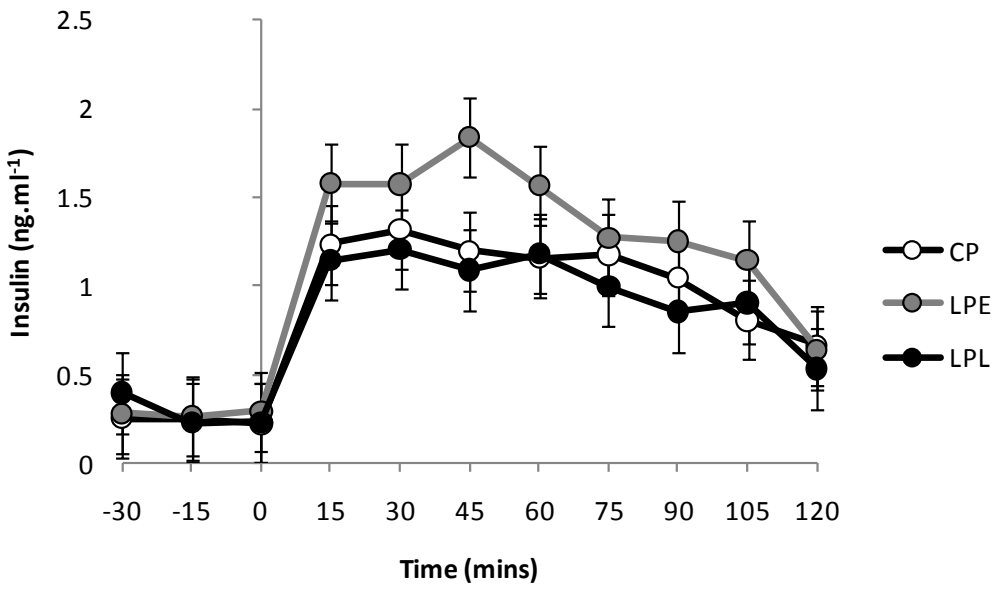


Figure 5.7 A graph showing the effect of maternal protein restriction on the plasma insulin response to a GTT aged 7 months.

Values shown are back transformed predicted means from generalized linear mixed model analysis. Error bars are average SED for the treatment*time interaction. Treatment groups are CP (Control Protein), LPE (Low Protein Early) and LPL (Low Protein Late).

5.4.4 RESPONSE TO AN INSULIN TOLERANCE TEST (ITT) AGED 7 MONTHS

Aged 7 months old, there was no effect of prenatal treatment on glucose K_{ITT} (CP, 2.80±0.51; LPE, 3.56±0.49; LPL, 3.70±0.21 %/min) or AUC (CP, -5.35±0.45; LPE, -6.38±0.45; LPL, -5.78±0.32 mmoles decrease in 2h (Figure 5.8). Whilst NEFA and glycerol increased similarly during the challenge, the plasma triglyceride response tended to reflect the change in NEFA and glycerol, with LPL offspring having a greater initial plasma clearance followed by a restricted elevation after 30mins.

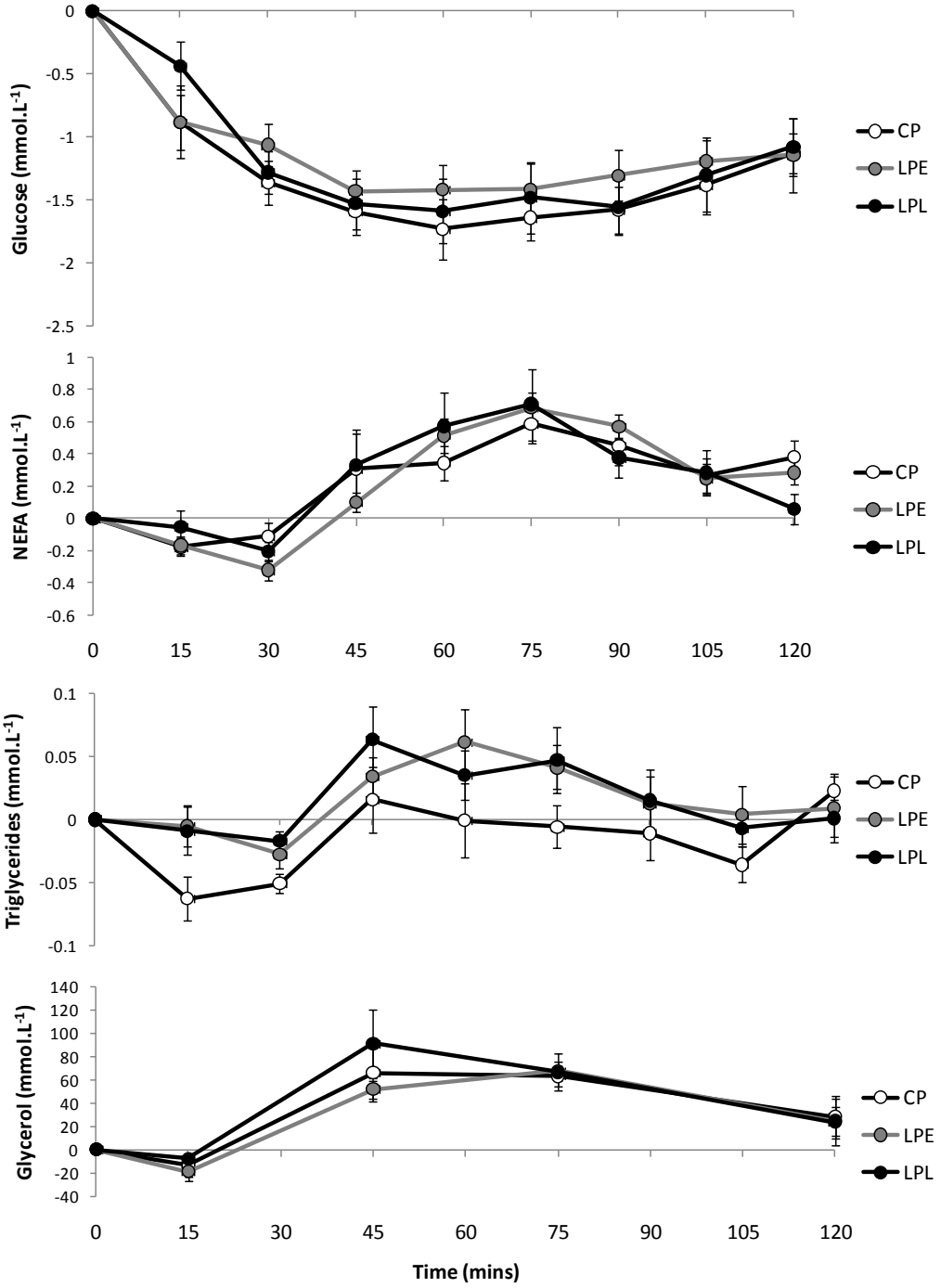


Figure 5.8 Plasma metabolite response to an insulin bolus aged 7 months old.

Data shown are means ±1 SEM. Groups are CP (Control), LPE (Low Protein Early) and LPL (Low Protein Late).

5.4.5 ORGAN WEIGHTS

No effect of maternal treatment, gender or fetal number was observed on any organ weight (Table 5.1), with the exception of the liver (*P*=0.021), which was significantly influenced by gender (male 816.1 & female 751.2 g. SED 26.77). Males had larger livers, but not when expressed relative to body weight.

	Absolute weight	Relative weight (g/kg)
Body weight (kg)	48.6±0.8	
Liver (g)	783.7±13.4[#]	16.15±0.26
Heart (g)	241.0±9.0	4.96±0.17
Average Adrenal (g)	1.98±0.25	[†]40.28±4.58
Kidney (g)	64.92±1.96	1.34±0.03
Spleen (g)	232.5±25.0	4.76±0.49
Average Ovary (mg)	606±23	[†]12.76±0.47

Table 5.1 Post mortem values for 1yr old offspring from the low protein study

Values shown are grand predicted means. Relative weight is organ weight (g)/body weight (kg). †Adrenal and average ovary relative weights are expressed as mg/kg. [#]Gender had a significant effect (*P*<0.005).

5.5 Discussion

In this Chapter it has been shown that a maternal low protein diet in sheep does not affect maternal weight or body condition in early gestation, or fetal weight utilising our experimental protocol. Thus maternal and fetal cortisol levels were also unaffected. However, the dietary regimen clearly impacted fetal growth in late gestation with singleton LPL being growth restricted at term and at weaning. Nevertheless, of interest, whilst the growth restricted LPL offspring had significantly lower basal plasma insulin at term when glucose-insulin metabolism was challenged at 7 months of age, it was the period of early protein restriction that appeared to have most affect i.e. increased peak insulin and incremental area under the curve. This was observed despite no obvious effect on peripheral insulin-mediated glucose uptake, as reflected in an ITT, but all offspring were morphologically similar at *post mortem*.

Throughout gestation neither of the two maternal protein restriction periods had any significant effect on ewe weight. As pregnant ewe protein requirements are calculated to be the same as non-pregnant ewe's until mid gestation (Robinson & Forbes, 1967), no significant effect of the LPE diet was expected to be observed on ewe weight and BCS. With the elevated protein requirements of the pregnant ewe in late gestation, a significant drop in ewe weight might have been expected, but

none was observed, although twin-bearing ewes were significantly heavier than singleton bearing mothers, as expected (Gootwine *et al.*, 2007). As fetuses, there was no effect of the treatment on overall weight or on their early organ development, which is again unsurprising given their weight relative to that of the ewe, although male fetuses were heavier than females indicating, as described previously for the low energy study that there is something fundamentally different about the early development of male and female embryos. However, at term, LPE and control offspring birth weights were not significantly different, while LPL offspring were significantly lighter, an effect which carried through to weaning. When this observation is viewed alongside the ewe weight data, it supports the fact that the dietary restriction was not supra-physiological (as occurs in many developmental programming studies) as maternal weight and BCS were maintained relative to control, but clearly illustrates that the diets were having knock-on effects on the fetal environment, reflective of a deficit in substrate for fetal protein accretion. The reduction in birthweight of LPL offspring suggests that dietary protein supply was not sufficient to maintain birthweight, even though ewe weight was preserved. This could be due to maternal protein demands being met at the expense of feto-placental compartment and therefore the ewe not mobilising her lean tissue reserves to continue to support the placenta and developing fetal tissues, as suggested by McNeill et al., (1997), where maternal dietary nitrogen retention was negative following exposure to a low protein diet in late gestation. Ovine placental nutrient transfer is readily restricted in response to undernutrition,

via reduced placental size, changes in morphology and expression/functionality of transporter proteins, as reviewed by Fowden *et al.*, (2006), which could be a potential mechanism behind the reduced birthweight. It has been shown in rats that placental amino acid transfer is reduced in late gestation following maternal consumption of a low protein diet (Jansson *et al.*, 2006). The reduction in birthweight independent of a reduction in maternal weight could suggest an indirect effect of undernutrition, for instance increased fetal exposure to cortisol via increased maternal production or decreased placental inactivation by 11βHSD2 (Whorwood *et al.*, 2001). Indeed, maternal undernutrition in late gestation has been shown to increase maternal and fetal cortisol (Edwards & McMillen, 2001). Since, plasma insulin levels are significantly lower in LPL offspring at birth compared to control, it is entirely plausible that low birthweight in this group is due to low nutrient supply (specially amino acids), leading to hypoinsulinaemia and a resultant decrease in the anabolic effects of insulin, consequently restricting birthweight (Fowden *et al.*, 1989).

At day 65 of gestation, no effect of dietary restriction was observed on maternal or fetal plasma cortisol level. Glucocorticoids have, from early in the genesis of the programming of health and disease hypothesis, been thought to act as intermediary factors that transcribe the developmental programming sequalae of maternal undernutrition, from the early rodent studies (Gardner *et al.*, 1998) to later large animal models (Challis *et al.*, 2000; Edwards *et al.*, 2001; Bloomfield *et al.*, 2004).

However, a causal relationship has proved difficult to ascertain as there are two HPA axes to consider (maternal and fetal) and the fetal HPA axis gradually develops increased function with gestation (Challis & Brooks, 1989). Furthermore, the placenta can contribute in some species to fetal HPA axis development and function (Florio *et al.*, 2002), and acts as a control point for maternal glucocorticoid entry into the fetal compartment through the expression and activity of the two 11ß-hydroxysteroid dehydrogenase isoforms 1 & 2 (Seckl, 1997). Consequently the initial response of maternal-fetal HPA axes to maternal undernutrition has only been partially described and appears gestational age dependent. Thus early to mid gestation global undernutrition reduces maternal resting plasma cortisol levels (Bispham *et al.*, 2003) whereas late gestation undernutrition appears to elevate them (Edwards & McMillen, 2001). In the current study we have shown that specific protein undernutrition within an isoenergetic diet has no effect on maternal or fetal plasma cortisol levels at day 65, and therefore longer-term sequalae of feeding these diets are unlikely to be due to gross changes in maternal and/or fetal HPA axis function.

In the adolescent offspring, we assessed glucose-insulin metabolism through a combination of a GTT and ITT to assess whole body (GTT) and specifically peripheral (ITT) glucose uptake or disappearance from the circulation. Interestingly, whilst LPL were of lower birth weight it was the LPE offspring that appeared to display early signs of metabolic dysfunction such as a significantly higher insulin response to the

GTT and increased AUC. All animals were of a similar weight at this time (except male female differences) and thus one caveat, that an equal dose of glucose was given to all animals, can be disregarded as contributing to the greater response in LPE. To our knowledge, this is the first time postnatal glucose homeostasis has been evaluated in sheep following maternal protein restriction in sheep and in this chapter the adolescent (~7 months of age) offspring were assessed. The adult offspring were also re-assessed at 1.5 years when lean and 2 years of age (when obese) and much of the discussion as to the effects of a maternal low protein diet on offspring glucose-insulin metabolism is in these chapters. In brief, and in relation to the relatively young offspring, it is of interest that the offspring who showed the perturbation in glucose handling (LPE) were born with a normal birthweight, hence `fetal programming' of glucose handling was observed independent of a reduction in birthweight. Birthweight and intrauterine growth restriction were originally seen as key markers of disturbances in the fetal milieu (Frankel *et al.*, 1996; Rich-Edwards *et al.*, 1997), but a growing evidence base suggests that many offspring born with normal birth weights may still be influenced by the consequences of `fetal programming'. It is now apparent that whilst birth weight is a useful 'indicator' of prenatal development for large scale epidemiological studies it is not overly important in the context of subtle physiological changes as a result of developmental programming. For example, Sinclair *et al.,* (2007) observed substantial effects on glucose handling and cardiovascular independent of a

significant reduction in birthweight following ovine exposure to a methyl deficient environment during the periconceptional period.

The GTT data suggests that LPE offspring have got a heightened insulin response to a glucose load. The ITT data suggests that insulin stimulated glucose uptake pathways (skeletal muscle and adipose tissue glucose uptake via GLUT4) are not affected by maternal low protein. When these two findings are combined, it infers central (pancreatic & hepatic) dysregulation rather than peripheral (skeletal muscle & adipose tissue) dysfunction. Central dysregulation could be due to pancreatic β cell hypersecretion or reduced hepatic insulin clearance. A vast amount of research has been conducted into the endocrine pancreas following maternal low protein diet (reviewed in (Holemans *et al.*, 2003). Snoeck *et al.,* (1990) observed reduced pancreatic β cell proliferation, size and vascularisation following exposure to a maternal low protein diet. These observations were supported by Petrik *et al.,* (1999), who as well as observing decreased proliferation of β cells, also showed increased β cell apoptosis. Hepatic dysregulation has also been observed in the offspring from maternally protein restricted rats, with reduced glucose uptake by glucokinase (Burns *et al.*, 1997). Male offspring have been observed to have a higher blood insulin concentration and reduced expression of hepatic glucose homeostatic enzymes (Desai *et al.*, 1997). In general, it seems to be the male offspring that exhibit a more detrimental phenotype following maternal low protein nutrition in rats (Petry *et al.*, 2001; Sugden & Holness, 2002; Ozanne *et al.*, 2003),

although this is not the case in all studies (Fernandez-Twinn *et al.*, 2005; Chamson-Reig *et al.*, 2006; Chamson-Reig *et al.*, 2009) or at this time point (7 months) in this study.

In addition, in response to either high glucose and high insulin, or high insulin alone there were no apparent differences in adipose tissue sensitivity to insulin, reflected as similar excursions in plasma NEFA and glycerol in response to the challenges. Furthermore, the animals were all proportionate, despite being different at birth and weaning (LPL) illustrating that in large animal species, much of the early, neonatal differences can be overcome with sufficient time allowance to restore body sizes back to normal, known as 'regression to the mean'. This is also the case with twinned animals, who were born with a lower birthweight, but postnatally appear to be relatively unaffected by this intrauterine restriction. Thus, in the context of developmental programming it is important to firstly observe the programmed animals at later ages and witness if age reveals any aspect of programming and secondly, to challenge the programmed phenotype with an insult known to be bad for physiological health. Chapter 6 therefore follows these animals to 1.5 years and exposes them to an obesogenic environment to encourage significant weight gain and thus 'reveal' greater metabolic dysfunction, than when the animals were lean.

6. Low Protein Study – 1.5 to 2 years of age

6.1 SUMMARY

In this chapter the interaction between maternal protein restriction during early (conception- day 65 gestation; LPE) or late (day 66 -term; LPL) gestation and adult exposure to an obesogenic environment in post pubertal (1.5 yrs) offspring was examined. When 'lean' aged 1.5 yrs, there was no effect of treatment on body composition as assessed by DXA. After a 6-month period of excessive nutrient consumption on a higher lipid diet, combined with reduced physical activity, all offspring went from a healthy body condition score (BCS) of 2.5 to an overweight BCS of 4.5 with no marked differences between groups on the amount of fat deposited internally. Over this period, females generally had less lean mass than males, with males across all groups having a greater daily intake than females. There was a small difference in food intake, with LPE offspring having a lower daily food intake (and appetite) than control offspring. Of interest, when obese LPE males (but not females) had an increased baseline insulin and a markedly greater (P=0.009 for treatment*time*gender interaction) peak insulin and area under the insulin response curve than control offspring following a glucose tolerance test. In addition, a treatment*gender interaction was observed in baseline glucose in lean offspring, with LPE males and LPL females having an elevated baseline glucose. With respect to protein metabolism then we saw a general decrease in plasma urea and total

amino acid concentrations with the onset of obesity. Metabolic flexibility also decreased with obesity, reflected as decreased excursions of plasma metabolites and electrolytes compared to when lean. In similarity to the adolescent data (Chapter 5), we saw no treatment effect on insulin-mediated glucose uptake during an ITT which was corroborated by no differences in the expression profile of genes in the insulin-signalling pathway in muscle as assessed by microarray. However, the majority (87%) of differentially-expressed genes were in the LPE group, which were predominantly down-regulated and associated with proteosomal activity and ubiquitination.

6.2 Introduction

To the best of our knowledge, this is the first time that offspring from ewes' protein malnourished during early or late gestation have been assessed for metabolic health before and after exposure to an obesogenic environment. As deleterious effects of maternal low protein during early gestation have been described in this model (Chapter 5), it was hypothesised that these effects would be exacerbated by rapid weight gain, leading to a worsening of glucose homeostasis following a glucose or insulin challenge.

As the names of fetal programming theories ("Thrifty Phenotype Hypothesis", "Fetal Origins Hypothesis", "Predictive Adaptive Response" and "Mis-Match Theory", to

name but a few) change with time and the growing evidence base, the overriding principle remains unchanged - that prenatal insults affect fetal development, invoke a fetal adaptive response which either becomes disadvantageous later in life (thrifty phenotype) or advantageous (predictive-adaptive response) if exposed to a similar insult post-natally. The greater the 'mis-match' (fetal-postnatal transition) the greater the adverse health problems later in life (mismatch hypothesis). Thus, in Chapter 5 it was observed that LPL offspring were low birth weight, then it follows that when these offspring are exposed to a hypercaloric, obesogenic environment they will have a reduced functional capacity to adapt and exhibit greater evidence of physiological dysfunction. This Chapter challenges that hypothesis.

In the commonly studied maternal low protein rat model, perturbations in fetal growth and postnatal performance are suggested to occur through alterations in the pattern of amino acid supply to the fetus (Jackson *et al.*, 2002). For example, research suggested that one consequence of the 8% maternal low protein diet is that it lowers maternal and fetal plasma taurine levels (Reusens *et al.*, 1995). Cherif et al., (1998) fed pregnant rats the 8% low protein diet, but also offered the dams a 2.5% aqueous taurine solution and found that supplemented offspring had normal insulin secretion. Similarly, supplementation of glycine to an alternative low protein diet prevented the development of higher blood pressure in the offspring (Jackson *et al.*, 2002).

Thus, increased delivery of specific amino acids to low protein fed dams can prevent the programmed phenotype from developing, implying that the pattern of protein intake during pregnancy is critical with respect to later physiological function. Indeed, it may be critical with respect to longer term gene expression. Jaenisch & Bird (2003) stated "Cells of a multicellular organism are genetically homogeneous but structurally and functionally heterogeneous owing to the differential expression of genes" thus environmental cues can modify the mechanisms controlling this tissue specific gene expression regulation, or 'epigenetics'. Of interest to this study is how perturbations in fetal nutrient supply can cause lifelong changes in gene expression and even potentially transgenerational implications (Harrison & Langley-Evans, 2009). One of the mechanisms for epigenetic regulation is gene silencing by increased methylation. DNA methylation involves the addition of a methyl group (-CH_3) to the CpG dinucleotides found in the 5' gene promoter region (Bird, 2002), thus preventing the binding of RNA polymerase and silencing the gene. The methylated cytosine residues then facilitate the binding of CpG binding protein (MeCP)-2 which allows the binding of histone modification complexes (Fuks *et al.*, 2003). These complexes cause the deacetylation and methylation of histones, which cause the histones to form a closed chromatin structure, thus preventing gene expression (Fuks *et al.*, 2003). It then follows that hyper-methylated DNA is generally silenced, while hypo-methylated DNA is transcribable. DNA methylation is an important factor during organogenesis and cellular differentiation; hence a

supply of maternal dietary methyl donors is vital to fetal developmental and postnatal health (Oligny, 2001; Reik *et al.*, 2001). Dietary sources of methyl donors include folic acid (vitamin B_{12}), choline and the amino acid methionine (Niculescu & Zeisel, 2002). Homocysteine is methylated to form methionine via methyl donation from choline or folate, the methionine goes on to form S-adenosylmethionine which in turn donates the methyl group to macromolecules such as DNA (Niculescu & Zeisel, 2002), see Figure 6.1 (Maloney *et al.*, 2007). It therefore follows that maternal dietary restriction of folate, choline and methionine will cause fetal DNA hypo-methylation, as reviewed by Burdge *et al.,* (2007).

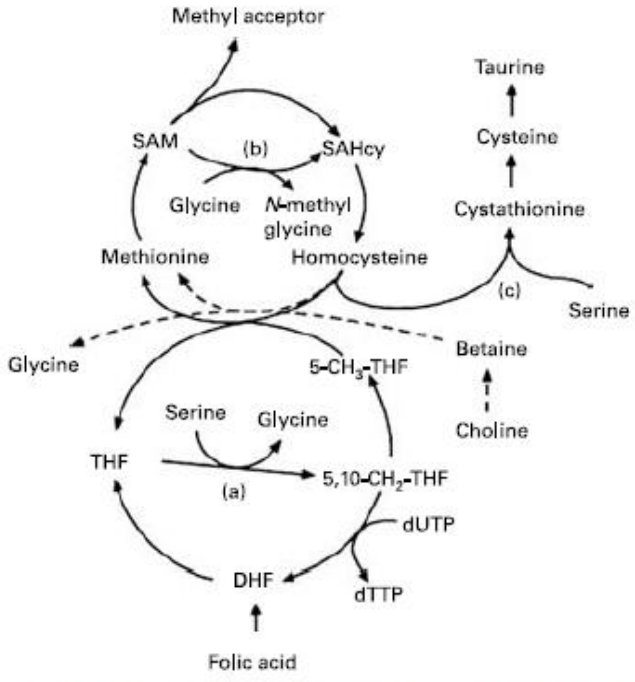


Figure 6.1 Diagram showing the interplay between methyl donors prior to DNA methylation

SAM, S-adenosyl methionine; SAHcy, S-adenosylhomocysteine; 5-CH3-THF, methyl tetrahyrofolate; 5,10-CH2-THF, methylene tetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate. (a) serine hydroxymethyl transferase; (b) glycine N-methyl transferase; (c) cystathionine synthase. Diagram and legend taken from Maloney *et al.,* (2007).

Petrie *et al.,* (2002) observed elevated homocysteine in the pregnant rat following low protein feeding, while Andersson *et al.,* (1992) found that maternal homocysteine is reduced by as much as 50% during pregnancy in healthy women, with homocysteine returning to non-pregnant levels shortly after parturition. Andersson *et al.,* (1992) hypothesised that the reduction in homocysteine was due to the high fetal requirement of methionine. Lillycrop *et al.,* (2005) showed that the deleterious effects of the maternal low protein diet in the rat could be overcome by folate supplementation, suggesting a mechanistic role for methyl deficiency in the developmental programming of disease induced by a maternal low protein diet. Indeed, ewes rendered methyl deficient around the time of conception produced offspring with higher blood pressure, reduced insulin sensitivity and altered immune function at 2 years of age (Sinclair *et al.*, 2007).

It was postulated that a maternal low protein diet in sheep would influence offspring physiological function, functions that became worse when the animals were metabolically stressed with obesity. It was hypothesised that maternal protein restriction would have effects on organogenesis, especially in a tissue with a demonstrated high plasticity following changes in the plane of maternal nutrition, such as the skeletal muscle. Furthermore, given that there is much information on the sensitivity of skeletal muscle to intrauterine growth restriction (Wigmore & Stickland, 1983; Handel & Stickland, 1987; Dwyer *et al.*, 1994; Dwyer *et al.*, 1995; Greenwood *et al.*, 2000; Maltin *et al.*, 2001; Brameld, 2004; Zhu *et al.*, 2004; Fahey

et al., 2005a; Zhu *et al.*, 2006; Daniel *et al.*, 2007; Mallinson *et al.*, 2007; Du *et al.*, 2010), reflected as changes in fibre type and fibre number and that skeletal muscle is the primary site for insulin-mediated glucose uptake, hence it was postulated that the expected changes in skeletal muscle morphology would persist into adult life and cause deleterious effects on glucose homeostasis in both the hyperglycaemic and hyperinsulinemic state. In order to obtain a global assessment of gene expression changes in this tissue type a X-species affymetric microarray was employed, as recently demonstrated in the horse (Graham *et al.*, 2009).

6.3 Materials & Methods

6.3.1 General Methods

In brief, offspring were transported from the Macaulay Institute and acclimatised at the Sutton Bonington Campus. Following acclimatisation a further glucose tolerance test (GTT) was conducted on all sheep, this will be referred to as the "lean GTT" (see 2.4.2.2) and, shortly after, the sheep underwent body composition analysis by dual energy x-ray analysis (DEXA- see 2.4.2.3), referred to as "lean DEXA". Following these initial, lean investigations, the animals were exposed to an obesogenic environment (see 0), with the intention of increasing the animals' fat deposition. All animals were encouraged over a period of 6 months to put on 50% of their original weight prior to their lean GTT. At this point the GTT ("obese GTT") and DEXA ("obese DEXA") were repeated, individual appetite was recorded (see 2.4.2.5) and a

further insulin tolerance test conducted (ITT- see 2.4.2.6) followed by an insulin stimulated skeletal muscle biopsy (see 2.4.2.7). A week later all animals were euthanised (see 2.4.2.8).

6.3.2 MICRO ARRAY

6.3.2.1 *DNA Isolation*

DNA was isolation using a Qiagen DNeasy Tissue & Blood Kit (Qiagen; Crawley, UK). In brief, 2.5g of frozen liver was ground under liquid nitrogen. The sample was placed in a micro centrifuge tube, 180µl of ATL buffer was added, followed by 20µl of Proteinase K. The solution was then vortexed and incubated for 3 hours at 56^{o}C with occasional vortexing. 200µl of AL buffer and 200µl of 100% ethanol were then added to the lysed solution. The lysate was then pippetted into a DNeasy spin column with collection tube. This was centrifuged for 1 minute at 6,000g and the collection tube and its contents were discarded, and replaced with an empty tube. 500µl of AW1 buffer was added to the column containing the DNA, this was then centrifuged for 1 minute at 6,000g, and the collection tube with contents were discarded and replaced again. 500µl of AW2 buffer was added to the column and centrifuged for 3 minutes at 20,000g. The collection tube and contents were discarded once again. The column was carefully placed into another collection tube. 100µl of AE buffer was incubated in the column for 1 minute at room temperature and then centrifuged at 6,000g for 1 minute. A further 50µl of AE buffer was incubated and centrifuged through the column, as above. The collection tube

containing the DNA eluted in 150µl of AE was then analysed for quality and quantity by use of spectrophotometry (Nanodrop, Thermo Fisher).

6.3.2.2 ***RNA Isolation***

0.75g of Vastus lateralis muscle was homogenised (Polytron) over ice in 7.5ml TRIreagent (Sigma) and then centrifuged (10 minutes, 12,000g at 4^{o}C). The supernatant was isolated, allowed to stand for 5 minutes and 1.5ml chloroform was added. Samples were shook vigorously for 15 seconds, allowed to stand for 10 minutes and then centrifuged (15 minutes, 12,000g at 4^{o}C). The upper, colourless aqueous phase was decanted off and mixed 1:1 with isopropanol (Sigma). Samples were mixed by inversion, stood for 10 minutes and then centrifuged (10 minutes, 12,000g at 4^{o}C). The supernatant was discarded, the remaining RNA pellet was washed with 7.5ml 75% ethanol (Fisher Scientific), vortexed and then centrifuged (5 minutes, 7,500g at 5^{o}C). The supernatant was pippetted off, allowing the RNA pellet to dry. The RNA pellet was then dissolved in 50µl DEPC-treated water, whilst heating to 55^{o}C for 10 minutes. Sample RNA quantity was then verified by spectrophotometry (Nanodrop, Thermo Fisher); this also gave a crude assessment of sample quality prior to capillary electrophoresis (Agilent Technologies).

6.3.2.3 ***Affymetrix Profiling***

X-species affymetrix investigation was used to investigate up or down regulation of mRNA from offspring skeletal muscle (Vastus lateralis). The microarray was performed by the Nottingham Arabidopsis Stock Centre's (NASC; Sutton Bonington Campus, University of Nottingham) microarray service. X-species Affymetrical analysis involves the hybridisation of genomic DNA from the species of interest, in this case Scottish blackface sheep, onto an Affymetric array from a heterologous species, in this case the GeneChip Human Genome U133 Plus 2.0 Array (9 samples) and GeneChip Human Genome ST Array (4 samples) (Affymetrix; High Wycombe, UK) as recently described by Graham *et al.,* (2009) using an equine cross hybridisation. In brief, the genomic DNA hybridisation is used to develop a filter for normalisation, highlighting all the genes where the ovine DNA sequence is sufficiently homologous to the human DNA to allow analysis of mRNA regulation. Fluorescently labelled Scottish blackface mRNA was then pippetted into the GeneChip Human Genome Array (either U133 Plus 2.0 or ST), following the hybridisation of homologous mRNA, it was subsequently washed. The fluorescent intensity for each gene was then recorded and compared between treatment groups. mRNA was analysed from 4 animals at random per treatment group (i.e. 4xCP, 4xLPE, 4xLPL).

6.3.2.4 ***Statistical analysis of microarray data***

Two genechip platforms were used in the study, one for n=9 samples and one for n=3 further samples (to increase study power). Therefore, prior to analysis, all data from both genechip platforms were combined (n=12 genechips) by mapping the genetic probes to gene symbols and subsequently normalized by using an Empirical Bayes cross-study method (Johnson *et al.*, 2007). In addition, all equivalent data (i.e. from the n=9 U133 arrays only) were used as an alternative cross-checking input for the statistical analyses, as it contained replicates for all groups and therefore did not require further genechip normalization and reduction of results to only those overlapping genes from both platforms. Density and quantile-quantile plots before and after the cross-study normalization, indicated that the combined data, when normalised were suitable for formal statistical analysis and the results for a parametric and non-parametric normalization were very similar, but for the purposes of clarity only the equivalent geneset (U-133) are presented.

For the statistical analysis of datasets, an empirical Bayes moderated t-statistic (eBayes) was used, where genes are sorted by q-value significance score adjusted for multiple testing according to the Benjamini-Hochberg method. All statistical analysis of microarray data were performed in a blinded fashion by Enrico Glaab, School of Computer science and are publicly available at http://www.arraymining.net/.

6.4 Results

6.4.1 WEIGHT GAIN, BODY COMPOSITION & APPETITE

6.4.1.1 *Dual Energy X-Ray Absorptiometry*

At 1.5 years of age body weights were similar in each treatment group (CP, 55.0±2.0 *vs.* LPE, 56.7±1.8 and LPL, 55.3±1.9 kg) and between males and females (57.5±1.6 *vs.* 53.8±1.4 kg, respectively). At this time, body composition was also similar between treatment groups (Table 6.1), but bone mineral density was significantly different between females *vs.* males (Female, 1.12, Male, 1.063 SED 0.02).

		Experimental Group				**Statistics**		
	Gender	**CP**	**LPE**	**LPL**	**s.e.d**	***T***	***G***	***T*G***
Fat (%)	male	15.4	14.3	8.9				
	female	13.6	13.8	16.1	3.29	NS	NS	NS
Fat mass	male	7.8	7.4	4.2				
(kg)	female	6.1	6.4	7.9	1.85	NS	NS	NS
Lean mass	male	43.6	44.1	43.7				
(kg)	female	42.1	40.3	40.1	3.47	NS	NS	NS
BMD ($g.kg^3$)	male	1.088	1.04	1.06				
	female	1.13	1.116	1.115	0.04	NS	0.016	NS
BMC (g)	male	1265	1171	1241				
	female	1338	1177	1186	87.77	NS	NS	NS

Table 6.1 Maternal Low Protein Study Offspring Body Composition for lean animals.
Data are estimated marginal means ±1 SEM. CP, Control Protein (n=6); LPE, Low Protein Early (n=7), LPL, Low Protein Late (n=6). BMD (Bone Mineral Density) & BMC (Bone Mineral Content).

Considering the change in weight with time, there were no effects of treatment or gender on the gain in weight (all animals gained weight at ~107 g.day^{-1} for the first 29 weeks) moving from a body condition score of ~2.5 to 4.5 arbitrary units.

After the period of weight gain, body composition was similar between groups (Table 6.2), with the exception of bone mineral density (CP, 1.183, LPE, 1.133, LPL, 1.151, SED 0.018) and bone mineral content which was significantly less in prenatally protein restricted groups (CP, 1.687, LPE, 1.594, LPL, 1.489kg, SED 0.061). In addition, females now had significantly less lean mass (female, 39.425 *vs.* male, 45.83kg SED 2.06) and bone mineral content (female, 1532 *vs.* male, 1648, SED 49.85) than male offspring (Table 6.2).

		Experimental Group				**Statistics**		
	Gender	**CP**	**LPE**	**LPL**	**s.e.d**	***T***	***G***	***T*G***
Fat (%)	male	28.73	28.80	17.55				
	female	30.40	29.60	29.63	4.15	NS	NS	NS
Fat mass (kg)	male	18.12	10.42	10.02				
	female	17.84	16.75	16.24	4.01	NS	NS	NS
Lean mass (kg)	male	45.05	45.12	47.31				
	female	40.03	39.81	38.43	3.51	NS	0.008	NS
BMD (g.kg^3)	male	1.20	1.12	1.15				
	female	1.17	1.15	1.16	0.03	0.028	0.028	NS
BMC (g)	male	1761	1648	1534				
	female	1612	1540	1443	85.7	0.004	0.037	NS

Table 6.2 Maternal Low Protein Study Body Composition in obese animals.

Data are estimated marginal means±SEM. CP, Control Protein (n=6); LPE, Low Protein Early (n=7), LPL, Low Protein Late (n=6). BMD (Bone Mineral Density) & BMC (Bone Mineral Content).

The delta change in body composition was investigated using a repeated measures REML analysis to assess the additional main effect of time, together with the previous main effects of treatment and gender with possible interactions (Table 6.1 *vs*. Table 6.2). Time, i.e. the exposure to the obesogenic environment, had a significant effect ($P<0.001$) on all measured variables excluding lean mass. However, the effect was clearly sex-specific with females putting on more fat mass ($P=0.051$), losing more lean mass ($P=0.030$) whilst male offspring gained a modest amount of lean mass, and thus the percentage fat mass also elevated more in females ($P=0.048$). Bone mineral density also increased less in the female, relative to male ($P=0.005$), offspring (Table 6.1 vs Table 6.2).

6.4.1.2 ***Appetite***

Over a 3-4 day period, food intake was assessed each day. There was a significant effect ($P=0.03$) of treatment on daily food intake, with LPE eating significantly less (20.7±0.5 MJ/day) than either CP (22.2±0.5 MJ/day) or LPL (21.5±0.5 MJ/day), but this effect was not reflected during an acute 2h-intake study (CP, 12.4±0.9; LPE, 13.2±0.8; LPL, 12.6±0.8 MJ/day). In addition, males tended (F,4.11; $P=0.06$) to eat more than females (22.1±0.4 vs. 20.9±0.4 MJ/day, respectively). Appetite, however, was not influenced by prenatal diet or gender with all animals eating on average 13.2±0.8 MJ in the first 2 hours after 24h fasting.

6.4.2 GTT & ITT BLOOD ANALYSIS

6.4.2.1 *Insulin*

6.4.2.1.1 Baseline

Lean fasted plasma insulin concentrations were similar for all dietary groups (pooled estimate, 0.39±0.07 ng.ml^{-1}). In obese animals, fasted plasma insulin was significantly elevated (F, 7.12; *P*=0.009) in LPE males (0.61±0.08 ng.ml^{-1}) *vs.* other males (pooled estimate, 0.24±0.07 ng.ml^{-1}).

6.4.2.1.2 Response to a Glucose Bolus (GTT)

There were no main effects, treatment or gender, or interactive effects on insulin sensitivity (area under the insulin response curve) when the animals were lean (Figure 6.4). The peak insulin response and insulin AUC were significantly greater in all obese *vs*. lean animals (both *P*<0.001 Figure 6.4). For both the peak insulin response and insulin AUC in the obese animals there was a significant time*treatment interaction (*P*=0.010 & *P*=0.008, respectively). These significant effects were generated by the elevated insulin response to glucose in the LPE obese offspring. A time*treatment*gender effect was also observed for the Insulin AUC, this is mainly attributed to the greater insulin response between lean and obese LPE males compared to all other respective groups.

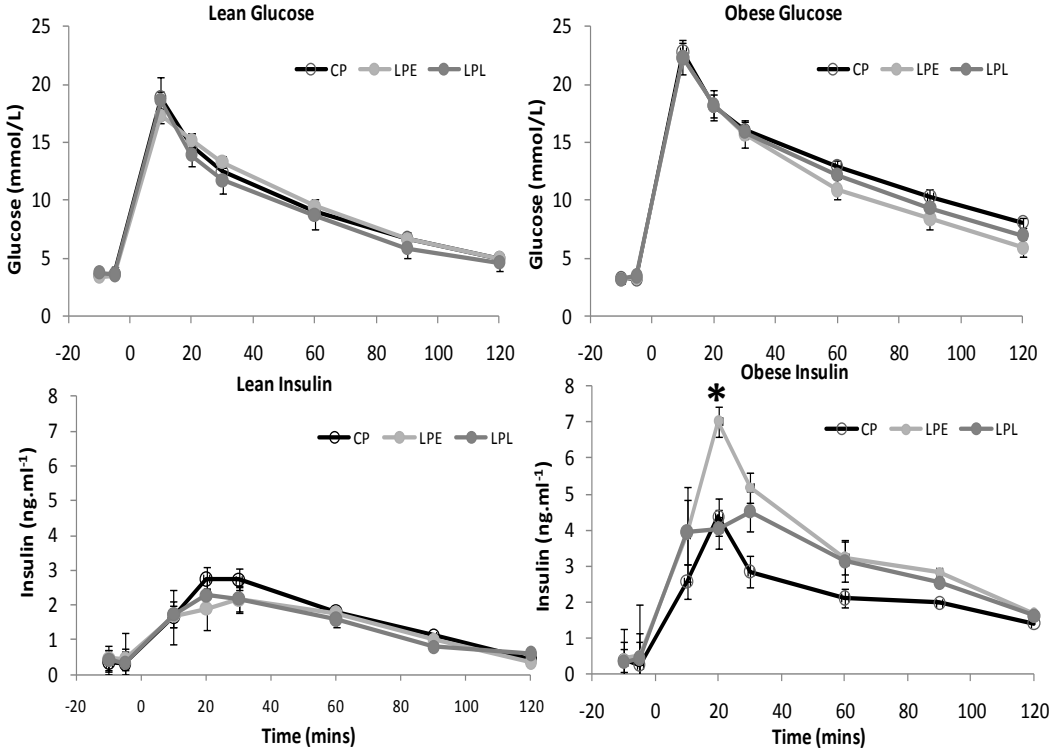


Figure 6.2 The response of prenatally protein restricted adult sheep to a glucose tolerance test when lean and obese.

Data are Mean ±1 SEM. Glucose was administered I.V. (0.5g.kg-1) and blood samples collected for measurement of plasma glucose and insulin (by ELISA, 2.5.1.4). *indicates significant time*treatment interaction on insulin response (P=0.010) and AUC (P=0.008). CP, Control protein (n=6); LPE, Low Protein Early (n=7), LPL, Low Protein Late (n=6).

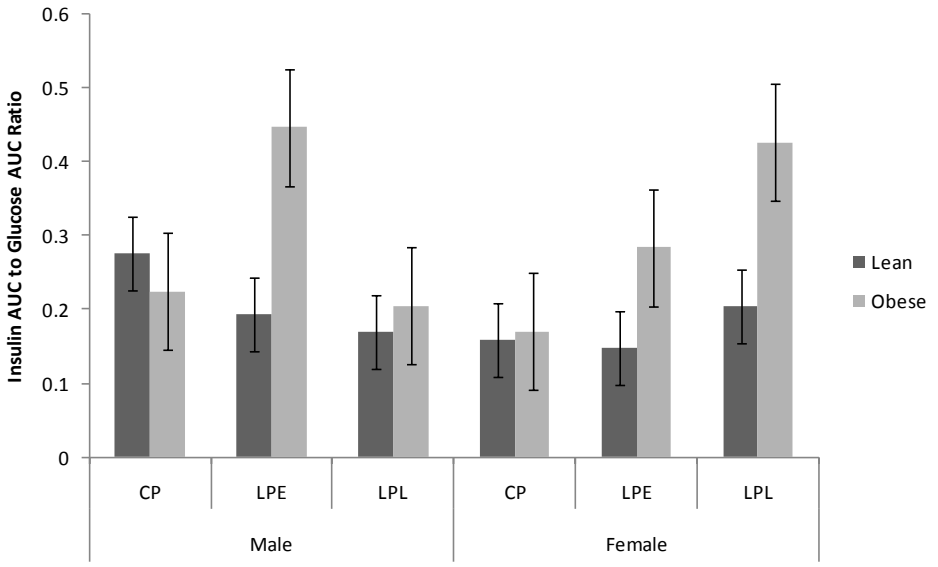


Figure 6.3 GTT Insulin AUC to Glucose AUC Ratio

Figure showing the Insulin AUC to Glucose AUC ratio in lean and obese offspring. Error bars are ±1 SED. Treatment Groups are CP (Control Protein), LPE (Low Protein Early) and LPL (Low Protein Late). A significant effect of time, i.e. the development of obesity, was observed (P<0.001), while a time*treatment effect (P=0.01) and treatment*sex (P=0.054) trend were observed seen.

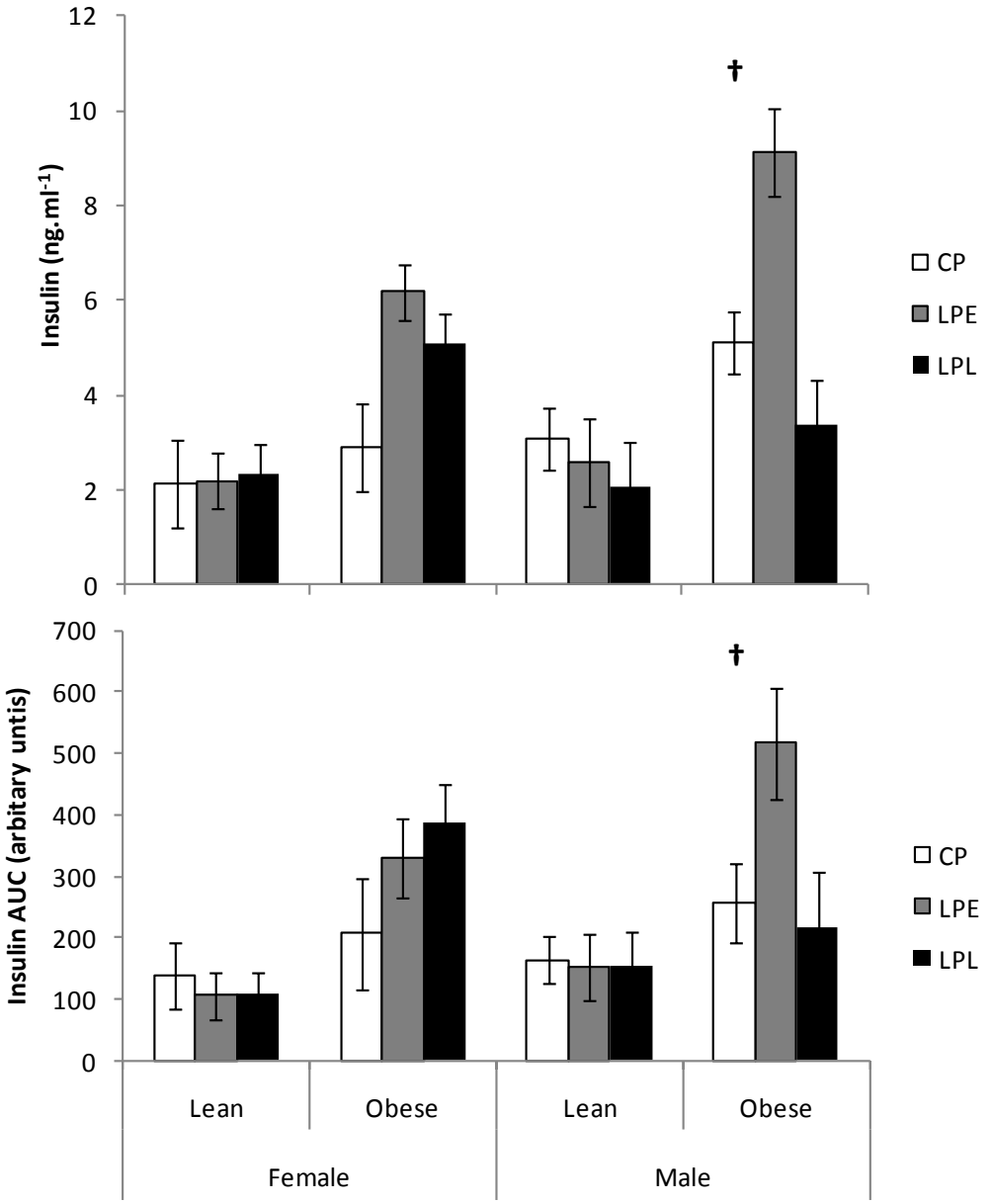


Figure 6.4 Plasma insulin peak response (top) and AUC (bottom) in lean and obese male and female prenatally protein restricted adult sheep during a glucose tolerance test.

Data are predicted means ±1 SEM. CP, Control Protein (n=6); LPE, Low Protein Early (n=7), LPL, Low Protein Late (n=6). Data were analysed by repeated measures linear mixed model revealing a significant effect of time (Lean *vs.* obese; *P*<0.001 all cases), treatment*time (Peak Insulin P=0.010 & Insulin AUC P=0.008) and an insulin AUC treatment*time*gender interaction (P=0.015).

6.4.2.2 *Glucose*

6.4.2.2.1 Baseline

A treatment*gender interaction was observed on baseline plasma glucose in lean animals (*P*=0.009). Male LPE animals had a higher baseline glucose than their female counterparts (male 4.00±.029 *vs*. female 3.22±.018 mmol/L), this effect was reversed in LPL animals (male 3.23±.029 vs. female 3.88±0.20 mmol/L, CP male 3.61±0.20; female 3.53±0.29 mmol/L). In obese animals, fasted plasma glucose concentrations were similar, irrespective of treatment group or gender (pooled estimate, 3.19±0.14 $mmol.L^{-1}$). In addition, fasted plasma glucose was significantly lower in obese animals compared to lean (*P*=0.020).

6.4.2.2.2 Response to a Glucose Bolus (GTT)

After administration of I.V. glucose, plasma glucose rose to a similar extent in all groups (Figure 6.2). No effect of prenatal treatment or gender was observed on lean or obese glucose tolerance (area under the glucose response curve), but a significant effect of time was observed (lean 742.2 vs. obese 1105.0 arbitrary AUC units; SED 47.4 *P*<0.001).

6.4.2.2.3 Response to an Insulin Bolus (ITT)

Following the administration of the insulin bolus, the blood glucose level initially elevated, but then declined (Figure 6.5), the rate and extent of decline was similar between groups (pooled estimate, K_{ITT}, 3.36±0.45; AUC, -5.84±0.68 mmoles in 2h).

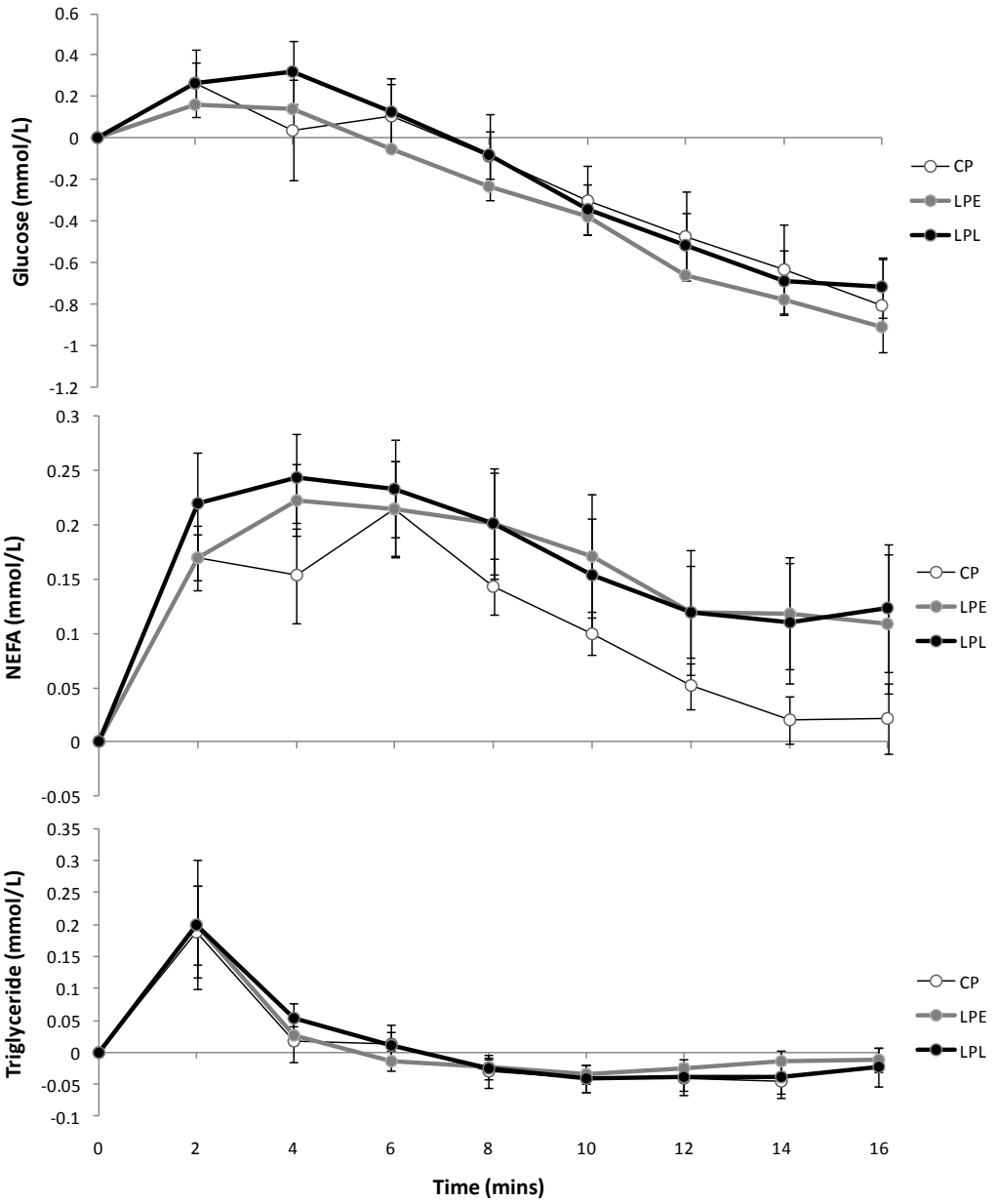


Figure 6.5 The glucose, NEFA and triglyceride response of prenatally nutrient restricted adult sheep to an insulin tolerance test when obese.

Data are Mean ±1 SEM. Insulin (0.75IU.kg^{-1}) was injected I.V. at time zero. Statistics are *, P<0.05, relative to baseline. CP- Control Protein, LPE- Low Protein Early, LPL- Low Protein Late.

6.4.2.3 *Insulin AUC : Glucose AUC Ratio*

A insulin:glucose ratio was calculated by dividing the Insulin AUC in response to a GTT by the Glucose AUC in lean offspring and comparing it to the equivalent ratio for obese offspring. The intention of this was to evaluate the confounding effect of administering differing glucose qualities in lean *vs.* obese offspring. A significant effect of time, i.e. the development of obesity, was observed ($P<0.001$), with a greater insulin to glucose response being observed in obese animals compared to control. A time*treatment effect ($P=0.01$) and treatment*sex ($P=0.054$) trend was observed (Figure 6.3). These observations relate to a greater insulin response in all obese LPE and female LPL offspring.

6.4.2.4 *Lipid Metabolism*

6.4.2.4.1 Baseline

Error! Reference source not found. shows the observed treatment effects on components of lipid metabolism in lean and obese offspring. The only treatment effects observed were in lean animals; Glycerol (LPE > Control, $P=0.029$) and LDL (LPE < Control, $P=0.050$). An effect of gender was observed on lean plasma baseline NEFA (Female 0.7232 *vs*. Male 0.9871 mmol/L; SED 0.1205. $P=0.041$). A time*treatment*gender effect was shown on NEFA, ($P=0.021$), which is attributed to a shift in male LPE offspring with the onset of obesity (lean 1.37 vs. obese 0.6375 mmol/L, SED 0.1721). The development of obesity significantly elevated baseline

plasma triglyceride, total cholesterol, HDL, and leptin levels, as well as significantly reducing glycerol and NEFA levels, LDL levels were unaffected (**Error! Reference source not found.**). A treatment*time interaction was observed for baseline total cholesterol (*P*=0.036) and HDL (*P*=0.020), both showing the same trend, male controls > treatment groups and female controls < treatment groups (total cholesterol CP female 1.23 & male 1.563, LPE female 1.51 & male 1.175, LPL female 1.623 & male 1.165 mmol/L SED 0.20) (HDL CP female 0.53 & male 0.765, LPE female 0.746 & male 0.58, LPL female 0.73 & male 0.54 SED 0.1018). A time*gender effect (*P*=0.004) was observed for plasma baseline LDL levels with lean male LDL baseline being lower than female, but then obese male being far greater than the female baseline (female lean 0.2388 *vs*. obese 0.2467, male lean 0.2042 *vs*. obese 0.2908 SED 0.02383).

		Experimental Group				Statistics		
	Time	**CP**	**LPE**	**LPL**	**s.e.d**	***T***	***Ti***	***T*Ti***
Triglyceride ($mmol.L^{-1}$)	lean	0.11	0.15	0.12				
	obese	0.20	0.23	0.23	0.027	NS	*P*<0.001	NS
Glycerol ($\mu mol.L^{-1}$)	lean	65.49	97.1	64.52		P=0.029*		
	obese	69.13	58.15	56.08	11.24		P=0.026	P=0.056
NEFA ($mmol.L^{-1}$)	lean	0.84	1.05	0.68				
	obese	0.63	0.68	0.71	0.12	NS	P=0.056	NS
Total cholesterol ($mmol.L^{-1}$)	lean	1.40	1.34	1.39				
	obese	2.46	2.04	2.15	0.19	NS	*P*<0.001	P=0.037
HDL ($mmol.L^{-1}$)	lean	0.65	0.66	0.64				
	obese	1.44	1.18	1.17	0.11	NS	*P*<0.001	P=0.021
LDL ($mmol.L^{-1}$)	lean	0.23	0.19	0.24		P=0.050*		
	obese	0.28	0.26	0.30	0.03		P=0.004	NS
Leptin ($ng.ml^{-1}$)	lean	3.40	4.23	3.88				
	obese	11.48	11.93	7.32	2.84	NS	*P*<0.001	NS

Table 6.3 The effect of adult-onset obesity on lipid metabolites in sheep.

Data are predicted means with the average standard error of the difference (s.e.d with 13 degrees of freedom in all cases) for the comparison. CP, Controls (n=6); LPE, Low Protein Early (n=6), LPL, Low Protein Late (n=7). NEFA, non-esterified fatty acids, HDL – High density lipoprotein. LDL – Low density lipoprotein. T, main effect of treatment; Ti, main effect of time (i.e. becoming obese); T*Ti, interaction between treatment and time. Treatment effects observed for plasma glycerol and LDL were seen in lean animals only.

6.4.2.4.2 Response to a Glucose Bolus (GTT)

Components of lipid metabolism were also measured during the GTT (Figure 6.6). A treatment*gender*time interaction was observed in NEFA levels following the GTT (*P*=0.045; Figure 6.6), showing a greater response in lean LPE males to a GTT.

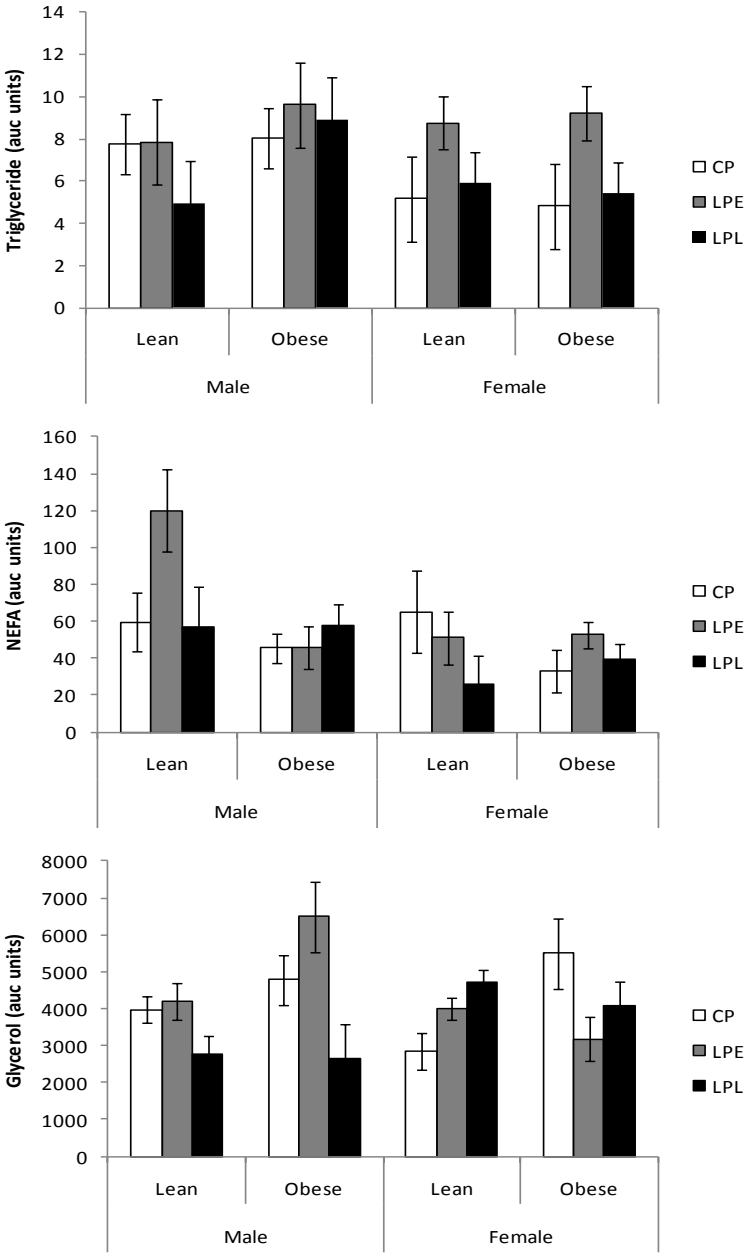


Figure 6.6 The lipid metabolite response to a glucose tolerance test in male and female lean and obese sheep.

Data are Mean ±1 SEM for areas under the glucose response curve (respective AUC units). Treatment groups are CP (Control Protein); LPE (Low Protein Early) & LPL (Low Protein Late).

6.4.2.4.3 Response to an Insulin Bolus (ITT)

Following the intra-jugular insulin injection, plasma NEFA level elevated and almost returned to baseline after the 16 min recording period (Figure 6.5). Plasma triglyceride levels peaked around 2 mins after the insulin bolus was injected, then dropped back to baseline levels by 16 minutes (Figure 6.5). Neither the NEFA or TAG response to the insulin (K_{ITT}) or the AUC were affected by gender or prenatal environment.

6.4.2.5 *Protein Metabolism*

As a crude measure of protein metabolism, plasma amino acid concentrations and urea were measured. No effects of maternal treatment or gender were observed, but a significant effect ($P<0.001$) was seen with the development of obesity, with total plasma amino acid and urea concentrations decreasing (total amino acid- lean 9210 *vs*. obese 6282 nmoles.ml^{-1}, SED 1113; urea- lean 8.805 *vs*. obese 5.155 mmol.L^{-1}, SED 0.4). Due to the interrelationship between the various amino acids, principle components analysis (PCA) was employed to draw out the amino acids which account for the majority of the variation between the lean and obese state. PC1 accounted for 79% of the variation, PC2 12% and PC3 5%, which in total accounted for >95% of the variability in amino acid concentrations between the lean and obese states. PC1 was then run as a variate within regression analysis and indicated a strong treatment*time interaction ($P<0.001$; Figure 6.6); with the average predicted means being (CP lean 4.169±0.4247 to obese -5.140±0.4247; LPE

lean 4.936±0.4103 to obese -6.020±0.4103; LPL lean 7.396±0.4247 to obese -6.567±0.4460). The graph represents these means +/- 95% confidence interval of the mean and illustrates the main effect of becoming obese (e.g. time, PC1)

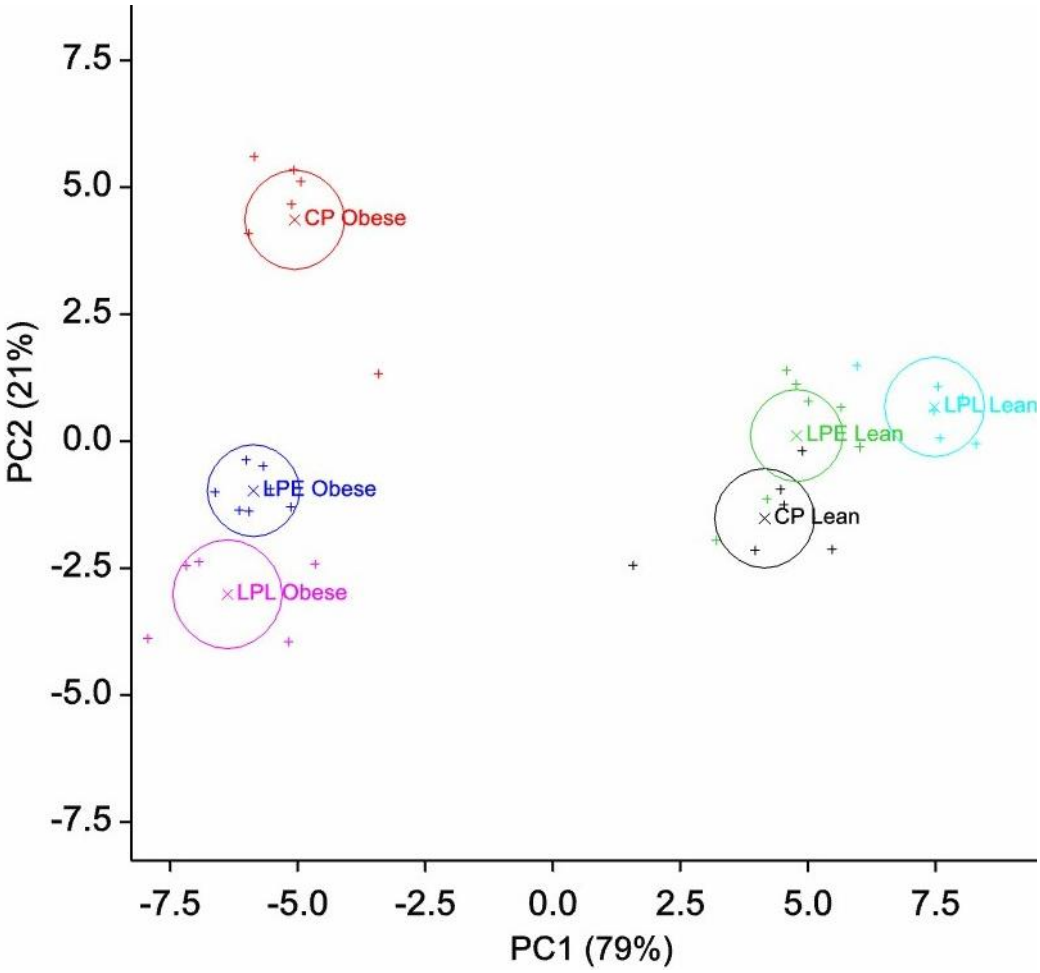


Figure 6.7 Graph showing the interaction between time (lean or obese) and maternal treatment on the amino acid population.

PC1 and PC2 are arbitrary values generated by principle components analysis to account for the variation across the entirety of the amino acids analysed. PC1 accounts for 79% and PC2 21% of the variation. Treatment, time and treatment*time all significantly (P<0.001) affected the PC1 value. Circles around treatment groups represent 95% confidence intervals. Treatment groups are CP (Control Protein); LPE (Low Protein Early) & LPL (Low Protein Late).

6.4.2.6 *Electrolytes & Haemodynamics*

6.4.2.6.1 Baseline

Fasted blood biochemistry when lean and obese are given in Table 6.4. With obesity, animals became more alkalotic as reflected in increases in blood pH, p_vO_2 and acid-base excess and a decrease in P_vCO_2. In addition, blood electrolytes also changed significantly; Ca^{2+} increased, whilst Na^+ and K^+ declined (Table 6.4). Apart from the effect of obesity, a significant effect of gender (*P*=0.031) was observed on lean P_vO_2 (female 47.24 vs. male 42.47 mmHg, SED 1.954) as was a treatment*gender interaction effect for lean K^+ (CP male 4.2 & female 4.3; LPE male 4.3 & female 4.4; LPL male 4.5 & female 3.9, mmol.l^{-1}, SED 0.2). Finally there was a significant interaction between the onset of obesity with treatment on P_vCO_2 (CP, lean 41.65 & obese 40.81; LPE lean 51.67 & obese 40.87; LPL lean 46.46 & obese 45.01mmol.L-1. SED 3.61).

	Baseline concentration		Statistics	
	Lean	Obese	*s.e.d*	*P*
pH	7.396	7.424	0.02058	NS
P_vCO_2 (mmHg)	46.59	42.23	1.731	0.014
HCO^-_3 ($mmol.L^{-1}$)	26.14	26.4	0.8881	NS
P_vO_2 (mmHg)	37.17	44.85	0.9845	<0.001
ABE ($mEq.L^{-1}$)	3.174	2.783	0.9623	NS
Na^+ ($mmol.L^{-1}$)	143.6	140.9	0.4635	<0.001
K^+ ($mmol.L^{-1}$)	4.246	4.038	0.07009	0.005
Ca^{2+} ($mmol.L^{-1}$)	1.171	1.232	0.0165	0.004
Cl^- ($mmol.L^{-1}$)	104.5	103.3	0.8914	NS

Table 6.4 Baseline blood biochemistry in lean and obese adult sheep.

The change in resting blood biochemistry from a lean to obese state as measured on an ABL-800Flex (Radiometer Ltd, UK). Data are Grand Means with standard error of the difference and the *df* for the comparison of 13. *P*, for effect of time (i.e. onset of obesity). NS, not significant.

6.4.2.6.2 Response to a Glucose Bolus (GTT)

Metabolic flexibility was assessed by measurement of blood metabolic markers, before and after a metabolic challenge such as glucose (GTT) or insulin (ITT) bolus. When blood biochemistry was investigated in response to a GTT, no effect of sex was observed, with the only variate affected by treatment being lean blood Ca^{2+} concentration (Table 6.5). However, the delta change in blood biochemistry during a GTT changed with obesity (i.e. a significant effect of time was indicated) i.e. the changes in pH, P_vCO_2, P_vO_2, ABE, and Na^+ were all blunted (Table 6.5). Haemoglobin (Hb) concentrations and haematocrit (Hct) were similar in all groups when measured as obese animals (Hb: CP 13.1±0.9; LPE 15.8±0.9; LPL 14.3±0.9$g.dL^{-1}$; Hct: CP 35.9; LPE 40.2; LPL 38.6 %. SEM 2.7).

		Experimental Group			Statistics			
	Time	**CP**	**LPE**	**LPL**	**s.e.d**	***T***	***Ti***	***T*Ti***
pH	lean	0.02	0.12	0.08				
	obese	0.00	0.02	0.04	0.037	NS	0.009	NS
P_vCO_2	lean	-0.10	-10.92	-6.61				
(mmHg)	obese	-1.73	-0.41	-4.51	3.726	NS	0.042	0.045
P_vO2	lean	-0.10	-10.92	-6.61				
(mmHg)	obese	-1.73	-0.41	-4.51	3.726	NS	0.042	0.045
ABE	lean	1.44	3.35	1.41				
($meQ.L^{-1}$)	obese	-0.75	1.58	0.20	1.307	NS	0.011	NS
K^+	lean	-0.35	-0.47	-0.40				
($mmol.L^{-1}$)	obese	-0.06	-0.68	-0.48	0.176	NS	NS	NS
Na^+	lean	-0.75	-2.35	-1.00				
($mmol.L^{-1}$)	obese	-1.63	0.95	0.13	1.078	NS	0.035	0.007
Ca^{2+}	lean	0.01	-0.03	-0.04				
($mmol.L^{-1}$	obese	0.01	-0.06	-0.07	0.026	P=0.017	NS	NS
Cl^-	lean	-0.88	-1.00	1.13				
($mmol.L^{-1}$)	obese	-0.25	0.05	0.88	1.231	NS	NS	NS

Table 6.5 The delta change in metabolites during a GTT when lean or obese.

Data are predicted means with the average standard error of the difference (s.e.d with 13 degrees of freedom in all cases) for the comparison. CP, Controls (n=6); LPE, Low Energy Early (n=7), LPL, Low Energy Late (n=6). T, main effect of treatment; Ti, main effect of time (i.e. becoming obese); T*Ti, interaction between treatment and time.

6.4.3 ORGAN WEIGHTS

Organ absolute weight and organ:body weight ratios are presented in (Table 6.6). Although male offspring were significantly heavier than female, males had a significantly heavier brain, total kidney mass, liver, lungs and larger right ventricle wall. All of these effects were removed when organ weight/size were expressed as a ratio of body weight, except the lungs, which were still heavier in males than

females. Per unit of body weight, females had more omental fat than males. Treatment effects were also observed on left ventricular size, which was significantly larger in LPE (CP, 15.4±0.8; LPE 18.0±0.7; LPL 15.3±0.8 mm, *P*=0.038) who had more pericardial fat (CP, 196.15±18.83; LPE 262.63±18.19; LPL 172.19±18.83 g, *P*=0.011), even when adjusted to body weight (CP, 2.69; LPE 3.41; LPL 2.39 g SEM 0.26, *P*=0.044). A treatment effect was also observed on the pituitary weight (CP, 11; LPE, 9; LPL, 8 $g.kg^{-1}$ $\times 10^3$ SEM 1, *P*=0.022). A treatment*gender interaction was also observed on pancreatic tissue weight (CP male 0.94±0.07, female 1.02±0.1; LPE male 0.79±0.10, female 1.02±0.06; LPL male 1.18±0.10, female 0.86±0.07).

	Absolute weights		*P*	Relative weights (g/kg)		*P*
	Male	Female		Male	Female	
Body weight (kg)	78.2±2.8	68.6±3.2	*	-	-	-
Brain (g)	107±3	91±3	**	1.37±0.06	1.26±0.05	NS
†Hippocampus (g)	3.95±1.78	2.87±1.55	NS	56±28	41±25	*
†Pituitary (g)	0.67±0.06	0.75±0.05	NS	9±1	11±1	*
Perirenal Fat (kg)	2.083±0.26	2.527±0.23	NS	26.71±3.64	35.66±3.17	NS
Pericardial Fat (g)	220.9±16.2	199.7±14.1	NS	2.82±0.23	2.84±0.20	NS
Omental Fat (kg)	2.62±0.35	3.31±0.30	NS	33.68±4.30	46.71±3.74	*
Total kidney (g)	156±8	126±7	**	2.03±0.12	1.77±0.10	NS
†Total adrenal (g)	4.06±0.41	4.51±0.35	NS	53±7	65±6	NS
Liver (g)	789±35	662±31	*	10.26±0.45	9.34±0.39	NS
Pancreas (g)	74.5±4.3	68.2±3.8	NS	0.97±0.05	0.97±0.05	NS
Spleen (g)	103±7	89±6	NS	1.360.08	1.25±0.07	NS
Lungs (g)	651±47	471±41	*	8.44±0.60	6.64±0.52	*
Heart (g)	306±28	286±24	NS	3.96±0.33	4.03±0.29	NS
Septum (mm)	17.8±1.0	17.7±0.9	NS	-	-	-
Left Ventricle (mm)	16.1±0.7	16.3±0.6	NS	-	-	-
Right Ventricle (mm)	8.3±0.4	6.9±0.3	*	-	-	-

Table 6.6 Post Mortem organ weights in male and female sheep.

Body and organ wet weights of male (n=11) and female (n=9) offspring at *post mortem*. Data are Grand Means±SEM. Statistics are *, **, ***, $P<0.05$, $P<0.01$ or $P<0.001$ respectively for male *vs.* female. NS, not significant. †, relative hippocampal, adrenal and pituitary weight expressed as $(g.kg^{-1})\times10^{3}$.

6.4.4 MICROARRAY RESULTS

The independent and unbiased microarray data analysis suggested that of the top 100 differentially expressed genes that satisfied our *a priori* statistical criteria, then three main outcomes were evident; 1) that the LPE group displayed by far the largest proportion of differentially expressed genes (87%, Figure 6.7), 2) that these differentially expressed genes in LPE were largely down-regulated relative to other groups, as indicated by the heat-map (Figure 6.8) and 3) that the only pathway or related gene-set that was significantly down-regulated, according to KEGG pathway criteria was 'ubiquitin-mediated proteolysis', with the other pathways influenced (determined by geneset enrichment analysis; GSEA) being all associated with ubiquitination, intracellular proteosomal activity and protein degradation or TGF-beta signaling. Importantly, in relation to our original hypothesis we saw no significant effects on pathways, or indeed individual genes, in the insulin-signalling pathway.

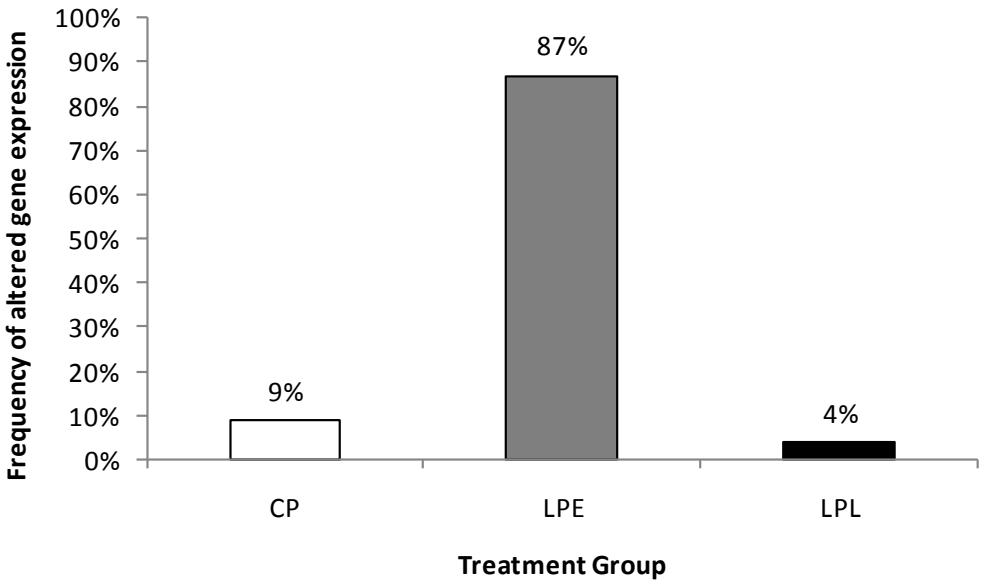


Figure 6.8 Graph showing the effect of maternal protein restriction on differential regulation of mRNA transcription.

The graph shows the quantity of differentially regulated genes by maternal treatment groups using X-Species hybridisation with the GeneChip Human Genome U133 Plus 2.0 Array. Treatment groups are CP; Control Protein, LPE; Low Protein Early, LPL; Low Protein Late.

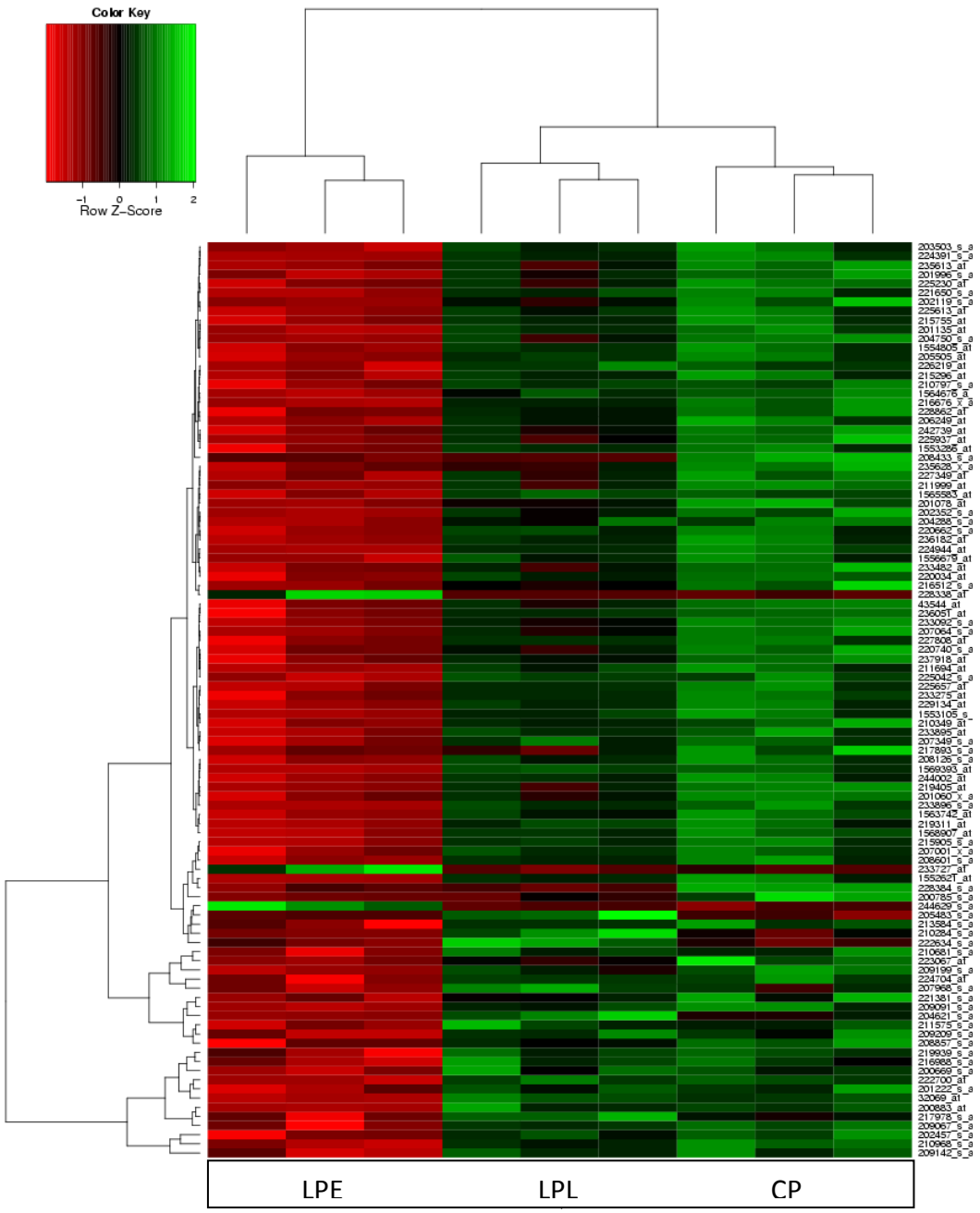


Figure 6.9 Heat map showing the up or down regulation of gene transcription following maternal protein restriction.

Heat map showing differential regulation of mRNA transcription from the skeletal muscle (Vastus lateralis) of obese offspring following maternal protein malnourishment. Treatment groups are CP; Control Protein, LPE; Low Protein Early, LPL; Low Protein Late, n=3 in all groups.

6.5 Discussion

Aged 1.5 yrs and lean, maternal protein restriction had no effect on offspring body mass or body composition. With significant weight gain (essentially of fat), there was also no effect of maternal diet on the amount or distribution of the increase in fat mass but bone mineral content and density significantly reduced in offspring exposed to low protein during early or late gestation. The effects of maternal insults, such as low vitamin D status have been reported as a cause of reduced bone mineral content in children aged 9 yrs (Javaid *et al.*, 2006). Mehta *et al.,* (2002) also observed reduced bone area and mineral content in adult rat offspring following exposure to maternal low protein diet. Our observation that a reduction in bone mineral content and density relative to control was only observed after exposure to an obesogenic environment would fit with the mis-match theory of fetal programming, similar to the sequelae leading to the deleterious effects seen on cardiovascular function in sheep (Cleal *et al.*, 2007a). It would appear that the protein restriction *in utero* altered underlying physiology that was only 'revealed' once the offspring were challenged with exposure to an obesogenic milieu, albeit relatively short-term – emphasising the significance of prenatal programming and the importance of maternal consumption of a balanced diet.

This study has once again revealed significant sex-specificity in response to alternation in maternal diet; effects only apparent after a nutritional transition in

adult life, with female offspring gaining more fat mass, losing more lean mass, thus increasing the percentage fat, as well as female bones increasing their bone mineral density to a lesser extent than males exposed to the same environmental conditions. Mendez *et al.,* (2005) found that women seem to be particularly susceptible to obesity following the recent global nutritional transition (shifts in quality and quantity of food intake and reductions in physical activity). It is possible that one cause underlying this sex effect in female offspring in this study is that they were observed to consume more food in a 24hr period than male offspring, although their acute 2hr consumption was comparable to males. Although this could be an artefact of the appetite testing protocol, as female humans have been observed to eat more following exposure to a stressful situation (Epel *et al.*, 2001); it is possible that the female offspring found the appetite testing regimen (e.g. individual housing etc) particularly stressful, and hence they consumed more than males.

Once again, as discussed in Chapter 4, the levels of glucose and insulin response following the GTT were greater in obese animals compared to lean. This effect is attributed to both the biological effects of obesity (e.g. increased adipose deposition), as well as the study design. The glucose was administered per unit of body weight, so a larger glucose bolus was given to the obese animals compared to lean, which will result in a greater physiological response to retain euglycemia. The insulin to glucose AUC ratio helps to support the fact that we are observing a true

biological effect rather than a consequence of a confounding variable, as the obese ratio was significantly greater than the lean, and the treatment effects were also reflected in the LPE offspring. One other significant consequence of the onset of obesity was the reduced `metabolic flexibility' (Storlien *et al.*, 2004), as assessed by the shift in metabolic and physiological parameters following a change in fuel supply, in this case a glucose bolus.

Few effects of prenatal protein restriction were observed when the adult offspring were lean, although male LPE offspring had a significantly lower resting glucose and LDL concentration, with a significantly higher glycerol compared to control. With the transition to obesity, however, male LPE offspring had the greatest decrease in plasma NEFA concentration, a high baseline plasma insulin concentration and a markedly greater insulin response (both peak and AUC) following a glucose challenge (hyperglycaemia and hyperinsulinemia). These effects occurred without change to plasma glucose dynamics over the course of the challenge. It is of interest that the elevated insulin response to a GTT in 7 month old LPE male offspring was not as evident at 18 months, but had returned following significant fat mass accretion in adult life. One potential explanation may be that the response is linked to growth, in that at 7 months the offspring are going through a relatively high growth phase, at least in comparison to aged 18-24 months and again with significant fat accretion, there is a substantial redistribution/reallocation of energy. Growth hormone, IGF and other components of the growth hormone axis are

known to have negative consequences on insulin action and subsequently, glucose homeostasis (as reviewed by Holt *et al.,* (2003)). It is possible that aged 7 months the animals were in a dynamic phase of their development, with a particularly active GH-IGF system causing elevating insulin resistance. One could only assume that this effect is only seen in the LPE offspring as the stimulated GH-IGF axis may exacerbate an underlying condition generated by the fetal protein restriction during early gestation. Aged 18 months the animals are deemed adult, having completed puberty, as well as reaching their genetic/epigenetic growth potential. In this more stable endocrine environment it would appear that offspring were more able to cope with the hyperglycaemia and subsequent hyperinsulinemia following a GTT. Aged 24 months, and once again undergoing a metabolically dynamic response to environmental conditions, then the disadvantageous phenotype reappeared.

At this time, and in similarity to 7 months of age, the elevated insulin peak and AUC following a glucose challenge in LPE were observed independently from any significant effect on glucose disposal i.e. inferred from no difference in either the glucose curve after the GTT and glucose disposal rate after the ITT. As previously described in earlier Chapters, this suggests a deregulation of hepatic glucose handling (increased hepatic portal vein appearance of insulin coupled with decreased hepatic insulin removal). Work in the late 1980s aimed to elucidate the mechanisms involved in elevated insulin resistance in the obese state in sheep

(McCann *et al.*, 1986; McCann *et al.*, 1987; Bergman *et al.*, 1989; McCann *et al.*, 1989). This work not only pointed to increased pancreatic insulin secretion (McCann *et al.*, 1989), but also to reduced peripheral insulin receptor number/function, rather than peripheral post receptor malfunction (Bergman *et al.*, 1989). The obesity model by this group in the later publication was an adult onset, chronic (one year), high fat/isocalorific model leading to a doubling of body mass (lean~50 to obese~100kg) during a relatively static growth phase. Whilst there are many similarities between this model and the second phase of the current study i.e. the phase of fat mass accretion, two major differences are key: firstly, the level of obesity we produced in the model described here is half that of the Bergman model and secondly, Bergmann observed elevated basal plasma glucose presumably through increased hepatic gluconeogenesis. In the present study, a significant decrease in basal plasma glucose was observed in obese relative to lean offspring. An acute (5 day) high fat/high calorific feeding study in healthy human subjects, without a predisposition to insulin resistance, resulted in hepatic insulin resitance rather than periphereal (Brons *et al.*, 2009). Kim *et al.,* (2007) investigated changes in glucose homeostasis following obesity induced by a high-fat diet in dogs, and concluded that hyperinsulinemia was as a result of a combined increase in pancreatic insulin secretion and reduced first pass insulin clearance by the liver. They suggest that reduced hepatic insulin clearance is an adaptive mechanism to help prevent β cell exhaustion. Aged 2 yrs old, the LPE male and all LPL offspring had reduced pancreatic tissue weight at post mortem. Bonner-Weir (Bonner-Weir,

2000a, b) explains that changes in postnatal β-cell mass are due to 4 mechanisms, 1) apoptosis, 2) neogenesis/differentiation of precursor cells, 3) proliferation and/or 4) hypertrophy of existing cells. These may all operate albeit at different rates under different physiological environments and at different postnatal ages. Thus it is difficult to conclude in the current dataset which of these mechanisms may underpin the differences we observe in pancreatic mass, and even whether the differences in pancreatic mass relate to a reduction in β cell mass. Reductions in β cell mass and function postnatally are a consistent observation following maternal low protein diet in the rat model (Holemans *et al.*, 2003). One potential hypothesis linking the lower pancreatic mass with the increased insulin response to a glucose challenge is that if LPE offspring are rendered less able to increase β cell mass or have increased apoptosis within pancreatic tissue, then development of an alternative strategy to preserve insulin and prevent β-cell exhaustion (such as reduced first pass hepatic insulin clearance) would seem a logical adaptation in order to maintain the longer term health of the animal, especially in a ruminant where the liver is central to the production of new glucose.

As well as differences in LPE pancreatic tissue mass at post mortem aged 2yrs, differences were also observed in LPE hearts (increased pericardial fat mass and left ventricular width). Left ventricular hypertrophy has also been observed following prenatal energy restriction in combination with postnatal exposure to an obesogenic environment (Chan *et al.*, 2009). Chan's study did not observe major

physiological effects, but cardiac lipid infiltration was observed, which may fit with our finding that pericardial mass was increased. Although in our study cardiovascular function was not assessed, these remodelling events (e.g. left ventricular hypertrophy) have been observed in obese humans with cardiovascular disease (Powell *et al.*, 2006; Abel *et al.*, 2008). Finally, prenatal protein restriction also significantly limited the size of the pituitary in obese offspring compared to control. However, given the effect size in this instance, the difference may reflect sampling variation and further confirmation in other studies would provide more convincing evidence of a specific effect of prenatal protein restriction on pituitary growth.

In terms of the overall effect of programming on markers of protein metabolism then whilst lean, the adult offspring are relatively comparable despite a maternal low protein diet (Figure 6.6). Once offspring develop obesity, a definite downward shift in the amino acid profiles of all offspring occurs (toward lower total plasma amino acid) but in those that were maternally protein restricted, the downward shift separates them dimensionally from control offspring, in effect, the low protein exposed groups exhibit less intraspecific variation with obesity relative to controls.

Although the intention was to investigate the specific amino acid profile of the offspring following maternal protein malnourishment, it has become clear that a snap shot approach (a single blood sample) would not give a true reflection of the

amino acid metabolism due to the high complexity of the interplay between various amino acids. The reduction in lean mass in female offspring, with modest lean mass accretion in male offspring, paralleled with the depleted blood total amino acid and urea concentration suggests a reduction in protein metabolism, especially in female offspring. A recent review on the effect of obesity on protein metabolism (Guillet *et al.*, 2011) still fails to draw a firm conclusion as to the effect of fat mass accretion on protein synthesis and degradation, and without specific stable isotope measures of the rates of flux of essential amino acids into and out of the free amino acid pool it is difficult to draw firm conclusions as to the present results on protein metabolism with obesity. As discussed previously in Chapter 4, the elevated lipid content of the diet (6%) could be having an inhibitory effect on microbial protein synthesis, thus reducing host protein availability, amino acid absorption and hence plasma amino acid concentration. With decreased availability of amino acids, protein synthetic rate will be reduced and therefore in order to maintain skeletal muscle mass, it would follow that protein degradation must also be down-regulated to maintain relatively increased amino acid availability for synthesis.

Hence, the microarray data from skeletal muscle in a fasted state are interesting; all analyses suggest that there is a relative decrease in ubiquitination in the LPE animals, and assuming *ceteris paribus*, this finding would suggest decreased protein degradation in these animals. The ubiquitin proteasome system is crucial in maintaining cytosolic health by degradation of non-functioning proteins and well as

removing rapid action proteins such as transcription factors (Goldberg, 2003; Varshavsky, 2005). This finding fits with the theory above that protein degradation might be reduced in the obese state. It would be of interest to know whether the reduction in ubiquitination transcripts was evident in lean animals, as protein degradation is a vital intracellular control mechanism, and thus it follows that a reduction could have deleterious effects on long term health. Nevertheless, it is clear that a global snapshot of transcriptomics indicates early protein restriction permanently altering the expression of genes to a much greater extent than a later and longer period of protein restriction.

In hindsight, in view of the physiological evidence from this study and in relation to physiological investigations of glucose homeostasis in the obese state, the liver would appear to have been a better target organ for the microarray analysis instead of the skeletal muscle. Our hypothesis was that the maternal diet would illicit detrimental consequences on the skeletal muscle organogenesis of the offspring, with a prevailing legacy in postnatal life. It was therefore postulated that glucose homeostatic mechanisms would be modified, with the skeletal muscle contributing towards this physiological effect. There is one caveat to acknowledge in regard to the X-species microarray; it is possible that not all ovine genes mapped with a human homolog. Thus these genes are 'missing' and are unavailable for analysis, and some may be important in the context of our globally developmentally programmed state. A `percent present' analysis revealed that 10% of genes bound,

whereas a human mRNA sample on the human chip would normally bind 35-55% of genes. Furthermore, two array platforms (U133 Plus and ST) were used and thus a cross study normalisation process was performed. Ultimately data are only presented from the full (U133) dataset, but in defence, the major effects noted and represented as the top 100 differentially expressed genes were very similar between the U133 Plus chip and the combined data from the U133 Plus and the ST array.

7. Summary, conclusions and future directions

When comparing the maternal low energy study with the maternal low protein study, one of the most obvious contrasting effects is that energy restriction in late gestation, while protein restriction in early gestation has similar effects on the obese adult offspring i.e. an increased insulin response following a glucose challenge. This may suggest that if there is a uniform mechanism then it is not dependent on gestational age, but clearly is sex-specific; in both studies it was the male offspring most affected by the gestational insult. However, one could consider that a deficit in energy (and concomitantly of protein) in late gestation when fetal hypertrophic growth is highest is most likely to exert an effect whereas, an imbalance in the pattern of nutrients (but not overall energy quantity) when fetal rate-specific growth is highest is most likely to impact early hyperplastic growth. The data would appear to support this latter hypothesis.

Although a number of modifications may be observed in tissues of these offspring, the organs which appear to have the most physiological relevance regarding glucose-insulin (intermediary metabolic) homeostasis are the liver and pancreas rather than the peripheral sites of primarily oxidation or deposition e.g. skeletal muscle and adipose tissue. The liver and pancreas especially most susceptible to physiological dysadaptation when challenged with the nutritional transition aged 2 yrs, in both LPE and LEL males, which is in accord with the mis-match theory of fetal programming. Further work clarifying this effect is indicated but was beyond the

remit of the current program of work, as intra-abdominal catheterisation and hepatic biopsy are indicated.

Pancreatic C-peptide is used as an index of pancreatic insulin secretion rate (Faber *et al.*, 1981). C-peptide is secreted in equimolar concentrations to insulin, but unlike insulin which gets cleared from the blood by the liver (~70% on first pass), C-peptide passes through the liver into the post-hepatic circulation (Figure 7.1). The relationship between post hepatic plasma insulin and c-peptide concentrations can therefore be used to elucidate which organ contributes most to the homeostatic dysregulation.

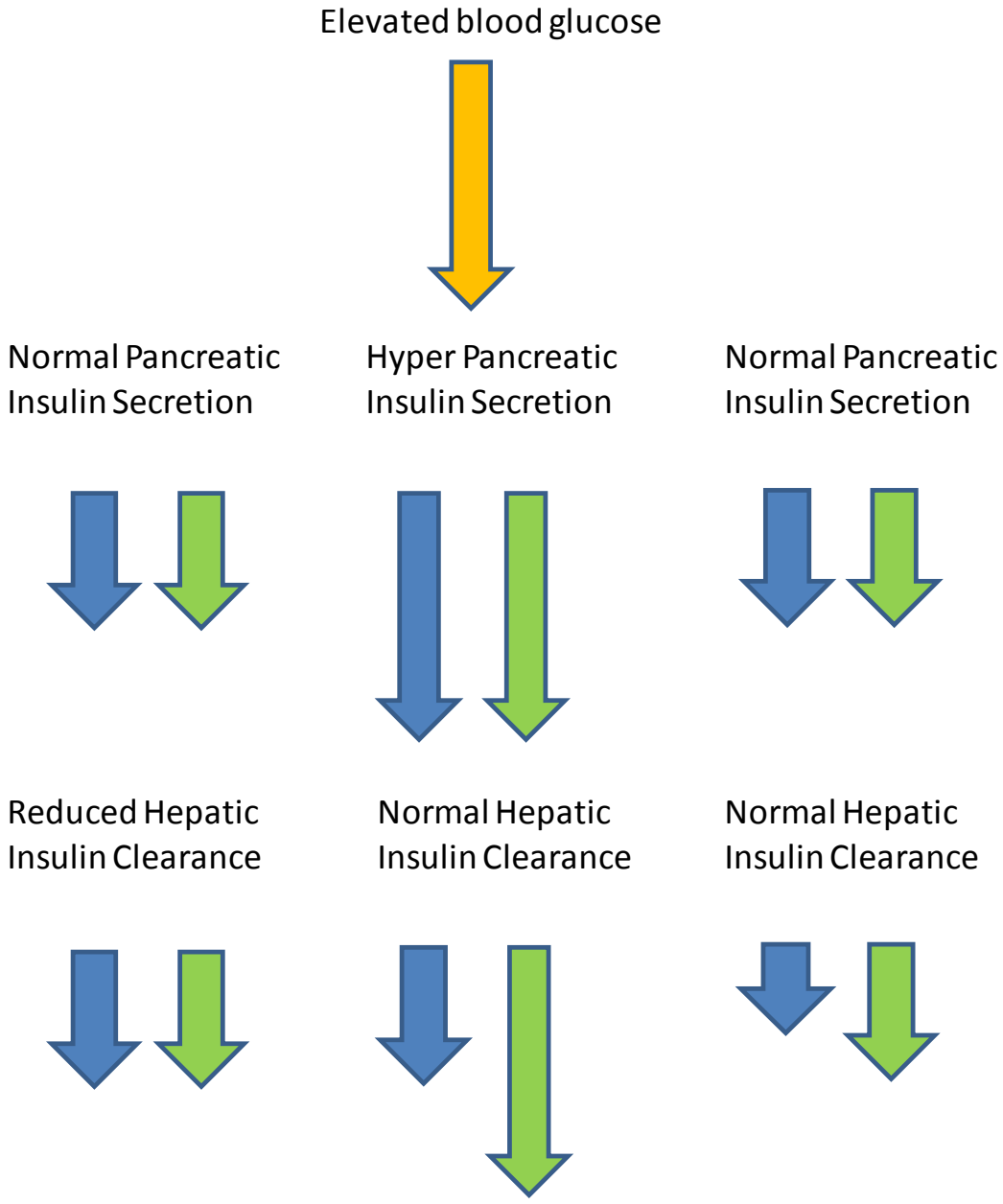


Figure 7.1 Impact of pancreatic and hepatic dysregulation on post hepatic blood glucose concentration.

Blue arrows represent insulin, while green arrows represent c-peptide. The diagram demonstrates that pancreatic hypersecretion of insulin and reduced hepatic insulin clearance result in the same plasma insulin concentration following a glucose challenge.

During the course of this study we partially developed an ovine specific assay for measurement of plasma C-peptide levels with a view to characterising the response post-GTT. As C-peptide has a low physiological function, its homology is poorly conserved between species. Of the commercially available c-peptide kits the pig has the closest homology to the ovine amino acid sequence (Peterson *et al.*, 1972), but even a porcine RIA kit (LINCO Research; Missouri, USA) was not capable of producing a concentration curve using ovine plasma. As a consequence of this, a primary antibody was produced against a synthesised c-peptide sequence, in the hope of developing an ovine specific c-peptide RIA, but as yet this has not yielded any results. In future studies, a means of negating the requirement for c-peptide levels, as well as gaining a true quantitative value for hepatic insulin extraction, would be cannulation of the hepatic portal vein and hepatic vein. This method, as used by Kim *et al*., (2007), would allow for the measurement of insulin and glucose leaving the pancreas and entering the liver (hepatic portal vein), as well as insulin and glucose levels leaving the liver (hepatic vein), thus a measurement of liver function would be gained regarding glucose homeostasis.

The models of maternal undernutrition (energy and protein) described here in sheep generally have less of an effect physiologically than previously observed in small laboratory animals, as well as other ovine models utilising a greater nutritional deficient, for example, the well published 50% energy restriction model compared to the 35% restriction used in this model. The general lack of treatment specific findings from this large animal, moderate restriction study demonstrate the

requirement to use large animal models (Roberts *et al.*, 2009), especially in a fetal programming setting (Reynolds *et al.*, 2010) when attempting to translate conclusions from small animals to the human condition. Small animal models (rats, mice, guinea pigs) have many advantages (low cost, lifespan, availability of molecular resources, monogastric omnivores, similarity in placentation) and disadvantages (high fetal number, differences in fetal and neonatal development) compared to farm animal models such as the sheep. Small animal models can magnify the effects of nutritional insults due to the lesser metabolic reserve of these animals compared to large animal models. The greater physiological relevance of the nutrient restriction shown in these studies, despite exposure to only a short-term obesogenic environment, highlights just how sensitive the fetal milieu is to environmental challenges. These effects are especially interesting as fetal programming was observed independently of birth weight (LPE), suggesting that subtle maternal insults, resulting in offspring with a normal birth weight, can have lifelong consequences in postnatal life, without any clear early prognostic marker. This emphasises the importance of clear-cut advice to pregnant women to consume what is currently considered to be an 'optimal' diet during pregnancy i.e. one in which micronutrient and macronutrient needs are adequately met in order to support the growth of the products of conception.

Here, the programming effects observed in late gestation are restricted to the fetal environment rather than the neonatal developmental period as lactational performance was preserved in the LEL offspring (and relative growth rates at this

time were similar between groups). This could be another reason behind the limited physiological effects observed following late gestation restriction in comparison to other studies of this kind (Gardner *et al.*, 2005; Cripps *et al.*, 2008), as well as in the Dutch Hunger Famine cohort (Ravelli *et al.*, 1998), hence why cross-fostering is sometimes utilised in rat studies (Desai *et al.*, 1996).

Chapters 4 and 6 show the consistent phenotype observed following exposure to the prescribed obesogenic environment postnatally. This model of ovine obesity was developed over ~6 months in line with the human transition to obesity (higher energy intake), especially lipid content coinciding with reduced physical activity (Caballero, 2007). The transition caused decreased plasma glucose, NEFA, glycerol, and LDL concentrations, while increasing plasma TAG, leptin, HDL, total cholesterol. Another consistent observation was the decreased ability of the offspring to switch from one fuel source to another (metabolic inflexibility (Storlien *et al.*, 2004; Galgani *et al.*, 2008)), observed following a glucose challenge.

Further work using tissues from these studies will hopefully elucidate further mechanistic insight into the causes behind the observed deleterious effects of fetal programming, as well as highlighting molecular irregularities which could have been involved in further metabolic dysfunction had the offspring aged further. The tissues of interest will be the liver and pancreas as implicated by the physiological data. Indeed, in further work on this model carried out by post-docs in the lab it has become clear that hepatic insulin resistance is present in LPE (from targeted qPCR

of genes in the insulin signalling pathway with associated down-regulation of protein abundance). Other potential laboratory investigations should include β cell mass quantification and characterisation (e.g. immunohistochemistry for insulin), skeletal muscle characterisation (fibre typing and intramyocellular lipid infiltration) and hepatic ectopic lipid quantification. Given the importance of epigenetics to the developmental programming paradigm, then analysis of specific gene methylation status (e.g. of promoter regions) would also be inferred.

Since my studies ended, further investigations are indeed being carried out in order to clarify the extent of change in the fetal amino acid environment in LPE offspring at the endstage of gestational protein restriction (day 65 gestation). This work will hopefully identify a pattern of amino acids that may be important with respect to programming the adult liver phenotype we have characterised here and will serve as a basis to conduct further, mechanistically-orientated hypothesis-driven research such as specific amino acid deficits and add-back together with observations on fetal pancreatic and liver development.

The concept that a change in the diet of a mother may impact the metabolism of the offspring many years later, and potentially into further generations, is now firmly accepted, despite much robust criticism in the early years of the fetal origins hypothesis. There is now a Developmental Origins of Health and Disease learned society with its own world congress and journal, all dedicated to elucidating how the early environment may lead to and account for much of non-communicable

disease in today's societies. The whole area has reinvigorated, and rightly so, a research focus on various aspects of the pregnant mothers environment and the neonatal to childhood transition. These issues will all become more important in time as the demography of pregnancy shifts toward older, primiparous mothers, of whom a greater percentage will require assisted reproductive techniques and who are generally of high body mass index at conception – a phenotype increasingly prevalent in the children as well.

Non-communicable diseases like hypertension and insulin resistance are undeniably multi-faceted and developmental programming will only begin to explain a part of their induction; however, merely explaining a small proportion of a very large population (35-40% people have hypertension (www.who.int, 2009), estimated 300 million with T2D (Wild *et al.*, 2004)) will mean that many people will benefit from the research in this area.

The present research in this thesis and many other models illustrate that adult-onset obesity can only be deleterious to health. A state of permanent moderate obesity is simply not compatible with longevity in species in which the excess adipose is purely used as an excess energy store rather than for any functional purpose e.g. insulation against extreme cold. In addition, the preponderance of physical inactivity in modern life *vs.* the amount likely experienced as we evolved is leading to a physiological and metabolic conflict from which it is extremely hard to extricate ourselves (Booth *et al.*, 2008).

8. Bibliography

Abel ED, Litwin SE & Sweeney G. (2008). Cardiac remodeling in obesity. *Physiological reviews* **88,** 389-419.

AFRC. (1993). *Energy and protein requirements of ruminants. An advisory manual prepared by the AFRC technical committee on responses to nutrients.* CAB international, Oxford.

Alberti KG, Zimmet P & Shaw J. (2006). Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med* **23,** 469-480.

Allender S & Rayner M. (2007). The burden of overweight and obesity-related ill health in the UK. *Obes Rev* **8,** 467-473.

Andersson A, Hultberg B, Brattstrom L & Isaksson A. (1992). Decreased serum homocysteine in pregnancy. *Eur J Clin Chem Clin Biochem* **30,** 377-379.

Andres V & Walsh K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J Cell Biol* **132,** 657-666.

Anghel SI & Wahli W. (2007). Fat poetry: a kingdom for PPARgamma. *Cell Res* **17,** 486-511.

Armitage JA, Khan IY, Taylor PD, Nathanielsz PW & Poston L. (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol* **561,** 355-377.

Arner P. (2005). Insulin resistance in type 2 diabetes -- role of the adipokines. *Current molecular medicine* **5,** 333-339.

Barker DJ & Osmond C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* **1,** 1077-1081.

Barker DJ, Osmond C, Forsen TJ, Kajantie E & Eriksson JG. (2005). Trajectories of growth among children who have coronary events as adults. *N Engl J Med* **353,** 1802-1809.

Bayol SA, Farrington SJ & Stickland NC. (2007). A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a

greater propensity for obesity in rat offspring. *The British journal of nutrition* **98,** 843-851.

Bellinger L & Langley-Evans SC. (2005). Fetal programming of appetite by exposure to a maternal low-protein diet in the rat. *Clin Sci (Lond)* **109,** 413-420.

Bellinger L, Lilley C & Langley-Evans SC. (2004). Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. *The British journal of nutrition* **92,** 513-520.

Benezra R, Davis RL, Lockshon D, Turner DL & Weintraub H. (1990). The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61,** 49-59.

Bergman EN, Reulein SS & Corlett RE. (1989). Effects of obesity on insulin sensitivity and responsiveness in sheep. *Am J Physiol Endocrinol Metab* **257,** E772-781.

Berkes CA & Tapscott SJ. (2005). MyoD and the transcriptional control of myogenesis. *Seminars in cell & developmental biology* **16,** 585-595.

Bertin E, Gangnerau MN, Bellon G, Bailbe D, Arbelot De Vacqueur A & Portha B. (2002). Development of beta-cell mass in fetuses of rats deprived of protein and/or energy in last trimester of pregnancy. *Am J Physiol Regul Integr Comp Physiol* **283,** R623-630.

Bird A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev* **16,** 6-21.

Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T & Symonds ME. (2003). Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* **144,** 3575-3585.

Blair HT, Jenkinson CM, Peterson SW, Kenyon PR, van der Linden DS, Davenport LC, Mackenzie DD, Morris ST & Firth EC. (2010). Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring. *J Anim Sci* **88,** E40-50.

Blaxter KL. (1957). The effects of defective nutrition during pregnancy in farm livestock. *The Proceedings of the Nutrition Society* **16,** 52-58.

Blondeau B, Garofano A, Czernichow P & Breant B. (1999). Age-dependent inability of the endocrine pancreas to adapt to pregnancy: a long-term consequence of perinatal malnutrition in the rat. *Endocrinology* **140,** 4208-4213.

Bloomfield FH, Oliver MH, Giannoulias CD, Gluckman PD, Harding JE & Challis JR. (2003a). Brief undernutrition in late-gestation sheep programs the hypothalamic-pituitary-adrenal axis in adult offspring. *Endocrinology* **144,** 2933-2940.

Bloomfield FH, Oliver MH & Harding JE. (2007). Effects of twinning, birth size, and postnatal growth on glucose tolerance and hypothalamic-pituitary-adrenal function in postpubertal sheep. *Am J Physiol Endocrinol Metab* **292,** E231-237.

Bloomfield FH, Oliver MH, Hawkins P, Campbell M, Phillips DJ, Gluckman PD, Challis JR & Harding JE. (2003b). A periconceptional nutritional origin for noninfectious preterm birth. *Science* **300,** 606.

Bloomfield FH, Oliver MH, Hawkins P, Holloway AC, Campbell M, Gluckman PD, Harding JE & Challis JR. (2004). Periconceptional undernutrition in sheep accelerates maturation of the fetal hypothalamic-pituitary-adrenal axis in late gestation. *Endocrinology* **145,** 4278-4285.

Bonner-Weir S. (2000a). Islet growth and development in the adult. *Journal of molecular endocrinology* **24,** 297-302.

Bonner-Weir S. (2000b). Perspective: Postnatal pancreatic beta cell growth. *Endocrinology* **141,** 1926-1929.

Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, Corgnati A & Muggeo M. (1989). Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. *J Clin Endocrinol Metab* **68,** 374-378.

Booth FW, Laye MJ, Lees SJ, Rector RS & Thyfault JP. (2008). Reduced physical activity and risk of chronic disease: the biology behind the consequences. *European journal of applied physiology* **102,** 381-390.

Borwick SC, Rae MT, Brooks J, McNeilly AS, Racey PA & Rhind SM. (2003). Undernutrition of ewe lambs in utero and in early post-natal life does not affect hypothalamic-pituitary function in adulthood. *Anim Reprod Sci* **77,** 61-70.

Boujendar S, Reusens B, Merezak S, Ahn MT, Arany E, Hill D & Remacle C. (2002). Taurine supplementation to a low protein diet during foetal and early postnatal life restores a normal proliferation and apoptosis of rat pancreatic islets. *Diabetologia* **45,** 856-866.

Brameld JM. (2004). The influence of undernutrition on skeletal muscle development. *British Journal of Nutrition* **91,** 327-328.

Brameld JM, Buttery PJ, Dawson JM & Harper JMM. (1998). Nutritional and hormonal control of skeletal-muscle cell growth and differentiation. *Proceedings of the Nutrition Society* **57,** 207-217.

Brockman RP & Bergman EN. (1975). Quantitative aspects of insulin secretion and its hepatic and renal removal in sheep. *The American journal of physiology* **229,** 1338-1343.

Brockman RP & Laarveld B. (1986). Effect of insulin on gluconeogenesis and the metabolism of lactate in sheep. *Canadian journal of physiology and pharmacology* **64,** 1055-1059.

Brody S. (1945). Bioenergetics and Growth. . Reinhold Publishing Corp., New York: .

Brons C, Jensen CB, Storgaard H, Hiscock NJ, White A, Appel JS, Jacobsen S, Nilsson E, Larsen CM, Astrup A, Quistorff B & Vaag A. (2009). Impact of short-term high-fat feeding on glucose and insulin metabolism in young healthy men. *J Physiol* **587,** 2387-2397.

Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D & Relaix F. (2003). The formation of skeletal muscle: from somite to limb. *Journal of Anatomy* **202,** 59-68.

Burdge GC, Hanson MA, Slater-Jefferies JL & Lillycrop KA. (2007). Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *The British journal of nutrition* **97,** 1036-1046.

Burns SP, Desai M, Cohen RD, Hales CN, Iles RA, Germain JP, Going TC & Bailey RA. (1997). Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *J Clin Invest* **100,** 1768-1774.

Caballero B. (2007). The global epidemic of obesity: an overview. *Epidemiol Rev* **29,** 1-5.

Chadio SE, Kotsampasi B, Papadomichelakis G, Deligeorgis S, Kalogiannis D, Menegatos I & Zervas G. (2007). Impact of maternal undernutrition on the hypothalamic-pituitary-adrenal axis responsiveness in sheep at different ages postnatal. *J Endocrinol* **192,** 495-503.

Challis J, Sloboda D, Matthews S, Holloway A, Alfaidy N, Howe D, Fraser M & Newnham J. (2000). Fetal hypothalamic-pituitary adrenal (HPA) development and activation as a determinant of the timing of birth, and of postnatal disease. *Endocrine research* **26,** 489-504.

Challis JR & Brooks AN. (1989). Maturation and activation of hypothalamic-pituitary adrenal function in fetal sheep. *Endocr Rev* **10,** 182-204.

Challis JR, Fraher L, Oosterhuis J, White SE & Bocking AD. (1989). Fetal and maternal endocrine responses to prolonged reductions in uterine blood flow in pregnant sheep. *American journal of obstetrics and gynecology* **160,** 926-932.

Chamson-Reig A, Thyssen SM, Arany E & Hill DJ. (2006). Altered pancreatic morphology in the offspring of pregnant rats given reduced dietary protein is time and gender specific. *J Endocrinol* **191,** 83-92.

Chamson-Reig A, Thyssen SM, Hill DJ & Arany E. (2009). Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. *Experimental biology and medicine (Maywood, NJ* **234,** 1425-1436.

Chan LL, Sebert SP, Hyatt MA, Stephenson T, Budge H, Symonds ME & Gardner DS. (2009). Effect of maternal nutrient restriction from early to midgestation on cardiac function and metabolism after adolescent-onset obesity. *Am J Physiol Regul Integr Comp Physiol* **296,** R1455-1463.

Cherif H, Reusens B, Ahn MT, Hoet JJ & Remacle C. (1998). Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. *J Endocrinol* **159,** 341-348.

Chevalier S, Burgess SC, Malloy CR, Gougeon R, Marliss EB & Morais JA. (2006). The greater contribution of gluconeogenesis to glucose production in obesity is related to increased whole-body protein catabolism. *Diabetes* **55,** 675-681.

Choi GY, Tosh DN, Garg A, Mansano R, Ross MG & Desai M. (2007). Gender-specific programmed hepatic lipid dysregulation in intrauterine growth-restricted offspring. *American journal of obstetrics and gynecology* **196,** 477 e471-477.

Christ B & Brand-Saberi B. (2002). Limb muscle development. *The International journal of developmental biology* **46,** 905-914.

Ciba I & Widhalm K. (2007). The association between non-alcoholic fatty liver disease and insulin resistance in 20 obese children and adolescents. *Acta Paediatr* **96,** 109-112.

Clarke KA, Ward JW, Forhead AJ, Giussani DA & Fowden AL. (2002). Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. *J Endocrinol* **172,** 527-534.

Cleal JK, Poore KR, Boullin JP, Khan O, Chau R, Hambidge O, Torrens C, Newman JP, Poston L, Noakes DE, Hanson MA & Green LR. (2007a). Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 9529-9533.

Cleal JK, Poore KR, Newman JP, Noakes DE, Hanson MA & Green LR. (2007b). The effect of maternal undernutrition in early gestation on gestation length and fetal and postnatal growth in sheep. *Pediatric research* **62,** 422-427.

Cripps RL, Green LR, Thompson J, Martin-Gronert MS, Monk M, Sheldon I, Hanson MA, Hales CND & Ozanne SE. (2008). The effect of maternal body condition score before and during pregnancy on the glucose tolerance of adult sheep offspring. *Reproductive sciences (Thousand Oaks, Calif*.

Daar AS, Singer PA, Persad DL, Pramming SK, Matthews DR, Beaglehole R, Bernstein A, Borysiewicz LK, Colagiuri S, Ganguly N, Glass RI, Finegood DT, Koplan J, Nabel EG, Sarna G, Sarrafzadegan N, Smith R, Yach D & Bell J. (2007). Grand challenges in chronic non-communicable diseases. *Nature* **450,** 494-496.

Dahri S, Reusens B, Remacle C & Hoet JJ. (1995). Nutritional influences on pancreatic development and potential links with non-insulin-dependent diabetes. *The Proceedings of the Nutrition Society* **54,** 345-356.

Dahri S, Snoeck A, Reusens-Billen B, Remacle C & Hoet JJ. (1991). Islet function in offspring of mothers on low-protein diet during gestation. *Diabetes* **40 Suppl 2,** 115-120.

Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA & Smith M. (1993). Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Front Neuroendocrinol* **14,** 303-347.

Daniel ZC, Brameld JM, Craigon J, Scollan ND & Buttery PJ. (2007). Effect of maternal dietary restriction during pregnancy on lamb carcass characteristics and muscle fiber composition. *J Anim Sci* **85,** 1565-1576.

Davis RL, Weintraub H & Lassar AB. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51,** 987-1000.

De Blasio MJ, Dodic M, Jefferies AJ, Moritz KM, Wintour EM & Owens JA. (2007a). Maternal exposure to dexamethasone or cortisol in early pregnancy differentially alters insulin secretion and glucose homeostasis in adult male sheep offspring. *Am J Physiol Endocrinol Metab* **293,** E75-82.

De Blasio MJ, Gatford KL, McMillen IC, Robinson JS & Owens JA. (2007b). Placental restriction of fetal growth increases insulin action, growth, and adiposity in the young lamb. *Endocrinology* **148,** 1350-1358.

Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A & Kann G. (2000). Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *Journal of Endocrinology* **165,** 519-526.

Demetrius L. (2004). Caloric restriction, metabolic rate, and entropy. *The journals of gerontology* **59,** B902-915.

Desai M, Byrne CD, Meeran K, Martenz ND, Bloom SR & Hales CN. (1997). Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. *The American journal of physiology* **273,** G899-904.

Desai M, Crowther NJ, Lucas A & Hales CN. (1996). Organ-selective growth in the offspring of protein-restricted mothers. *The British journal of nutrition* **76,** 591-603.

Dietz WH. (1994). Critical periods in childhood for the development of obesity. *Am J Clin Nutr* **59,** 955-959.

Dodic M, Samuel C, Moritz K, Wintour EM, Morgan J, Grigg L & Wong J. (2001). Impaired cardiac functional reserve and left ventricular hypertrophy in adult sheep after prenatal dexamethasone exposure. *Circulation research* **89,** 623-629.

Du M, Tong J, Zhao J, Underwood KR, Zhu M, Ford SP & Nathanielsz PW. (2010). Fetal programming of skeletal muscle development in ruminant animals. *Journal of Animal Science* **88,** E51-E60.

Dulloo AG. (2008). Thrifty energy metabolism in catch-up growth trajectories to insulin and leptin resistance. *Best practice & research* **22,** 155-171.

Dwyer CM, Lawrence AB, Bishop SC & Lewis M. (2003). Ewe-lamb bonding behaviours at birth are affected by maternal undernutrition in pregnancy. *The British journal of nutrition* **89,** 123-136.

Dwyer CM, Madgwick AJ, Ward SS & Stickland NC. (1995). Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. *Reproduction, fertility, and development* **7,** 1285-1292.

Dwyer CM, Stickland NC & Fletcher JM. (1994). The influence of maternal nutrition on muscle fiber number development in the porcine fetus and on subsequent postnatal growth. *J Anim Sci* **72,** 911-917.

Eckersall PD, Lawson FP, Kyle CE, Waterston M, Bence L, Stear MJ & Rhind SM. (2008). Maternal undernutrition and the ovine acute phase response to vaccination. *BMC veterinary research* **4,** 1.

Edwards LJ & McMillen IC. (2001). Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation. *J Physiol* **533,** 561-570.

Edwards LJ, Symonds ME, Warnes KE, Owens JA, Butler TG, Jurisevic A & McMillen IC. (2001). Responses of the fetal pituitary-adrenal axis to acute and chronic hypoglycemia during late gestation in the sheep. *Endocrinology* **142,** 1778-1785.

Elmes MJ, Haase A, Gardner DS & Langley-Evans SC. (2008). Sex differences in sensitivity to beta-adrenergic agonist isoproterenol in the isolated adult rat heart following prenatal protein restriction. *The British journal of nutrition***,** 1-10.

Epel E, Lapidus R, McEwen B & Brownell K. (2001). Stress may add bite to appetite in women: a laboratory study of stress-induced cortisol and eating behavior. *Psychoneuroendocrinology* **26,** 37-49.

Erhard HW, Boissy A, Rae MT & Rhind SM. (2004). Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. *Behav Brain Res* **151,** 25-35.

Erhuma A, Bellinger L, Langley-Evans SC & Bennett AJ. (2007a). Prenatal exposure to undernutrition and programming of responses to high-fat feeding in the rat. *The British journal of nutrition* **98,** 517-524.

Erhuma A, Salter AM, Sculley DV, Langley-Evans SC & Bennett AJ. (2007b). Prenatal exposure to a low-protein diet programs disordered regulation of lipid metabolism in the aging rat. *Am J Physiol Endocrinol Metab* **292,** E1702-1714.

Faber OK, Christensen K, Kehlet H, Madsbad S & Binder C. (1981). Decreased insulin removal contributes to hyperinsulinemia in obesity. *J Clin Endocrinol Metab* **53,** 618-621.

Fahey AJ, Brameld JM, Parr T & Buttery PJ. (2005a). The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. *J Anim Sci* **83,** 2564-2571.

Fahey AJ, Brameld JM, Parr T & Buttery PJ. (2005b). Ontogeny of factors associated with proliferation and differentiation of muscle in the ovine fetus. *J Anim Sci* **83,** 2330-2338.

Fan JG, Li F, Cai XB, Peng YD, Ao QH & Gao Y. (2007). Effects of nonalcoholic fatty liver disease on the development of metabolic disorders. *Journal of gastroenterology and hepatology* **22,** 1086-1091.

Fernandez-Twinn DS, Ozanne SE, Ekizoglou S, Doherty C, James L, Gusterson B & Hales CN. (2003). The maternal endocrine environment in the low-protein model of intra-uterine growth restriction. *The British journal of nutrition* **90,** 815-822.

Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN & Ozanne SE. (2005). Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *Am J Physiol Regulatory Integrative Comp Physiol* **288,** R368-373.

Festing MFW, Overend P, Das RG, Borja MC & Berdov M. (2004). *The design of animal experiments: reducing the use of animals in research through better experimental design*. Royal Society of Medicine Press, London.

Florio P, Severi FM, Ciarmela P, Fiore G, Calonaci G, Merola A, De Felice C, Palumbo M & Petraglia F. (2002). Placental stress factors and maternal-fetal adaptive response: the corticotropin-releasing factor family. *Endocrine* **19,** 91-102.

Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H & Nathanielsz PW. (2007). Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *J Anim Sci* **85,** 1285-1294.

Fowden AL. (1979). Factors affecting insulin release in the sheep fetus [proceedings]. *J Physiol* **293,** 51P-52P.

Fowden AL. (1980). Effects of adrenaline and amino acids on the release of insulin in the sheep fetus. *J Endocrinol* **87,** 113-121.

Fowden AL & Forhead AJ. (2009). Endocrine regulation of feto-placental growth. *Hormone research* **72,** 257-265.

Fowden AL & Hay WW, Jr. (1988). The effects of pancreatectomy on the rates of glucose utilization, oxidation and production in the sheep fetus. *Quarterly journal of experimental physiology (Cambridge, England)* **73,** 973-984.

Fowden AL & Hill DJ. (2001). Intra-uterine programming of the endocrine pancreas. *Br Med Bull* **60,** 123-142.

Fowden AL, Hughes P & Comline RS. (1989). The effects of insulin on the growth rate of the sheep fetus during late gestation. *Quarterly journal of experimental physiology (Cambridge, England)* **74,** 703-714.

Fowden AL, Li J & Forhead AJ. (1998). Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? *The Proceedings of the Nutrition Society* **57,** 113-122.

Fowden AL, Mao XZ & Comline RS. (1986). Effects of pancreatectomy on the growth and metabolite concentrations of the sheep fetus. *J Endocrinol* **110,** 225-231.

Fowden AL, Ward JW, Wooding FP, Forhead AJ & Constancia M. (2006). Programming placental nutrient transport capacity. *J Physiol* **572,** 5-15.

Frankel S, Elwood P, Sweetnam P, Yarnell J & Smith GD. (1996). Birthweight, body-mass index in middle age, and incident coronary heart disease. *Lancet* **348,** 1478-1480.

Frayn K. (2003). *Metabolic Regulation: A Human Prespective*. Blackwell Publishing, Oxford.

Frutos P, Hervas G, Giraldez FJ & Mantecon AR. (2004). Review. Tannins and ruminant nutrition. *Spanish Journal of Argricultural Research* **2,** 191-202.

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP & Kouzarides T. (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *The Journal of biological chemistry* **278,** 4035-4040.

Galgani JE, Moro C & Ravussin E. (2008). Metabolic flexibility and insulin resistance. *Am J Physiol Endocrinol Metab* **295,** E1009-1017.

Gardner DS, Bell RC & Symonds ME. (2007). Fetal mechanisms that lead to later hypertension. *Current drug targets* **8,** 894-905.

Gardner DS, Jackson AA & Langley-Evans SC. (1998). The effect of prenatal diet and glucocorticoids on growth and systolic blood pressure in the rat. *The Proceedings of the Nutrition Society* **57,** 235-240.

Gardner DS, Lea RG & Sinclair KD. (2008). Developmental programming of reproduction and fertility: what is the evidence? *Animal* **2,** 1128-1134.

Gardner DS, Pearce S, Dandrea J, Walker R, Ramsay MM, Stephenson T & Symonds ME. (2004). Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep. *Hypertension* **43,** 1290-1296.

Gardner DS, Tingey K, Van Bon BW, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T & Symonds ME. (2005). Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *Am J Physiol Regul Integr Comp Physiol* **289,** R947-954.

Gardner DS, Van Bon BW, Dandrea J, Goddard PJ, May SF, Wilson V, Stephenson T & Symonds ME. (2006). Effect of periconceptional undernutrition and gender on hypothalamic-pituitary-adrenal axis function in young adult sheep. *J Endocrinol* **190,** 203-212.

Gaster M, Handberg A, Beck-Nielsen H & Schroder HD. (2000). Glucose transporter expression in human skeletal muscle fibers. *Am J Physiol Endocrinol Metab* **279,** E529-538.

Gesta S, Tseng YH & Kahn CR. (2007). Developmental origin of fat: tracking obesity to its source. *Cell* **131,** 242-256.

Gilbert JS, Lang AL, Grant AR & Nijland MJ. (2005). Maternal nutrient restriction in sheep: hypertension and decreased nephron number in offspring at 9 months of age. *Journal of Physiology-London* **565,** 137-147.

Gillooly JF, Brown JH, West GB, Savage VM & Charnov EL. (2001). Effects of size and temperature on metabolic rate. *Science* **293,** 2248-2251.

Gluckman PD & Hanson MA. (2004). Living with the past: evolution, development, and patterns of disease. *Science* **305,** 1733-1736.

Gluckman PD, Hanson MA, Cooper C & Thornburg KL. (2008). Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* **359,** 61-73.

Goldberg AL. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* **426,** 895-899.

Gootwine E & Rozov A. (2006). Seasonal effects on birth weight of lambs born to prolific ewes maintained under intensive management. *Livestock Science* **105,** 277-283.

Gootwine E, Spencer TE & Bazer FW. (2007). Litter-size-dependent intrauterine growth restriction in sheep. *Animal* **1,** 547-564.

Gopalakrishnan GS, Gardner DS, Rhind SM, Rae MT, Kyle CE, Brooks AN, Walker RM, Ramsay MM, Keisler DH, Stephenson T & Symonds ME. (2004). Programming of adult cardiovascular function after early maternal undernutrition in sheep. *Am J Physiol Regul Integr Comp Physiol* **287,** R12-20.

Gow CB, McDowell GH & Annison EF. (1981). Control of gluconeogenesis in the lactating sheep. *Australian journal of biological sciences* **34,** 469-478.

Gower BA, Granger WM, Franklin F, Shewchuk RM & Goran MI. (2002). Contribution of insulin secretion and clearance to glucose-induced insulin concentration in african-american and caucasian children. *J Clin Endocrinol Metab* **87,** 2218-2224.

Graham NS, Clutterbuck AL, James N, Lea RG, Mobasheri A, Broadley MR & May ST. (2009). Equine transcriptome quantification using human GeneChip arrays can be improved using genomic DNA hybridisation and probe selection. *Vet J* **186,** 323-327.

Greenwood PL & Bell AW. (2003). Consequences of intra-uterine growth retardation for postnatal growth, metabolism and pathophysiology. *Reproduction (Cambridge, England) Supplement* **61,** 195-206.

Greenwood PL, Hunt AS, Hermanson JW & Bell AW. (2000). Effects of birth weight and postnatal nutrition on neonatal sheep: II. Skeletal muscle growth and development. *J Anim Sci* **78,** 50-61.

Greenwood PL, Slepetis RM, Hermanson JW & Bell AW. (1999). Intrauterine growth retardation is associated with reduced cell cycle activity, but not myofibre number, in ovine fetal muscle. *Reproduction, fertility, and development* **11,** 281-291.

Grigore D, Ojeda NB & Alexander BT. (2008). Sex differences in the fetal programming of hypertension. *Gend Med* **5 Suppl A,** S121-132.

Gu W, Jones CT & Harding JE. (1987). Metabolism of glucose by fetus and placenta of sheep. The effects of normal fluctuations in uterine blood flow. *Journal of developmental physiology* **9,** 369-389.

Guada JA & Robinson JJ. (1974). Effect of undernutrition on nitrogen metabolism in the pregnant ewe. *The Proceedings of the Nutrition Society* **33,** 84A-85A.

Guan H, Arany E, van Beek JP, Chamson-Reig A, Thyssen S, Hill DJ & Yang K. (2005). Adipose tissue gene expression profiling reveals distinct molecular pathways that define visceral adiposity in offspring of maternal protein-restricted rats. *Am J Physiol Endocrinol Metab* **288,** E663-673.

Guilherme A, Virbasius JV, Puri V & Czech MP. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* **9,** 367-377.

Guillet C, Masgrau A & Boirie Y. (2011). Is protein metabolism changed with obesity? *Current opinion in clinical nutrition and metabolic care*.

Habener JF, Kemp DM & Thomas MK. (2005). Minireview: transcriptional regulation in pancreatic development. *Endocrinology* **146,** 1025-1034.

Hales CN & Barker DJ. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35,** 595-601.

Hales CN & Barker DJ. (2001). The thrifty phenotype hypothesis. *Br Med Bull* **60,** 5-20.

Hammond JJ, Mason IL & Robinson TJ. (1976). *Hammonds Farm Animals*. Edward Arnold, London.

Handel SE & Stickland NC. (1987). The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight. *J Anat* **152,** 107-119.

Hardie DG. (2011a). Energy sensing by the AMP-activated protein kinase and its effects on muscle metabolism. *The Proceedings of the Nutrition Society* **70,** 92-99.

Hardie DG. (2011b). Sensing of energy and nutrients by AMP-activated protein kinase. *Am J Clin Nutr* **93,** 891S-896S.

Harding JE. (2001). The nutritional basis of the fetal origins of adult disease. *Int J Epidemiol* **30,** 15-23.

Harrison M & Langley-Evans SC. (2009). Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. *British Journal of Nutrition* **101,** 1020-1030.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN & Klein WH. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364,** 501-506.

Hattersley AT & Tooke JE. (1999). The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease. *Lancet* **353,** 1789-1792.

Hawkins P, Steyn C, McGarrigle HH, Calder NA, Saito T, Stratford LL, Noakes DE & Hansona MA. (2000). Cardiovascular and hypothalamic-pituitary-adrenal axis development in late gestation fetal sheep and young lambs following modest maternal nutrient restriction in early gestation. *Reproduction, fertility, and development* **12,** 443-456.

Hernandez CE, Harding JE, Oliver MH, Bloomfield FH, Held SD & Matthews LR. (2009). Effects of litter size, sex and periconceptional ewe nutrition on side preference and cognitive flexibility in the offspring. *Behav Brain Res* **204,** 82-87.

Hernandez CE, Matthews LR, Oliver MH, Bloomfield FH & Harding JE. (2010). Effects of sex, litter size and periconceptional ewe nutrition on offspring behavioural and physiological response to isolation. *Physiol Behav* **101,** 588-594.

Holemans K, Aerts L & Van Assche FA. (2003). Lifetime consequences of abnormal fetal pancreatic development. *J Physiol* **547,** 11-20.

Holland WL, Knotts TA, Chavez JA, Wang LP, Hoehn KL & Summers SA. (2007). Lipid mediators of insulin resistance. *Nutrition reviews* **65,** S39-46.

Holt RI, Simpson HL & Sonksen PH. (2003). The role of the growth hormone-insulin-like growth factor axis in glucose homeostasis. *Diabet Med* **20,** 3-15.

Hovi P, Andersson S, Eriksson JG, Jarvenpaa AL, Strang-Karlsson S, Makitie O & Kajantie E. (2007). Glucose regulation in young adults with very low birth weight. *N Engl J Med* **356,** 2053-2063.

Huang S & Czech MP. (2007). The GLUT4 glucose transporter. *Cell Metab* **5,** 237-252.

Husted SM, Nielsen MO, Blache D & Ingvartsen KL. (2008). Glucose homeostasis and metabolic adaptation in the pregnant and lactating sheep are affected by the level of nutrition previously provided during her late fetal life. *Domest Anim Endocrinol* **34,** 419-431.

Hyatt MA, Budge H & Symonds ME. (2008). Early developmental influences on hepatic organogenesis. *Organogenesis* **4,** 170-175.

Hyatt MA, Walker DA, Stephenson T & Symonds ME. (2004). Ontogeny and nutritional manipulation of the hepatic prolactin-growth hormone-insulin-like growth factor axis in the ovine fetus and in neonate and juvenile sheep. *The Proceedings of the Nutrition Society* **63,** 127-135.

Jackson AA, Dunn RL, Marchand MC & Langley-Evans SC. (2002). Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine. *Clin Sci (Lond)* **103,** 633-639.

Jaenisch R & Bird A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics* **33 Suppl,** 245-254.

Jansson N, Pettersson J, Haafiz A, Ericsson A, Palmberg I, Tranberg M, Ganapathy V, Powell TL & Jansson T. (2006). Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *J Physiol* **576,** 935-946.

Jaquiery AL, Oliver MH, Bloomfield FH, Connor KL, Challis JR & Harding JE. (2006). Fetal exposure to excess glucocorticoid is unlikely to explain the effects of periconceptional undernutrition in sheep. *J Physiol* **572,** 109-118.

Javaid MK, Crozier SR, Harvey NC, Gale CR, Dennison EM, Boucher BJ, Arden NK, Godfrey KM, Cooper C & Grp PAHS. (2006). Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. *Lancet* **367,** 36-43.

Jenkin G, McMillen IC & Thorburn GD. (1979). The development of fetal hypothalamic-pituitary gonadal adrenal function. *Contrib Gynecol Obstet* **5,** 58-90.

Jensen CB, Storgaard H, Madsbad S, Richter EA & Vaag AA. (2007). Altered skeletal muscle fiber composition and size precede whole-body insulin resistance in young men with low birth weight. *J Clin Endocrinol Metab* **92,** 1530-1534.

Jensen J. (2004). Gene regulatory factors in pancreatic development. *Dev Dyn* **229,** 176-200.

Johnson WE, Li C & Rabinovic A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8,** 118-127.

Jones CT, Gu W, Harding JE, Price DA & Parer JT. (1988). Studies on the growth of the fetal sheep. Effects of surgical reduction in placental size, or experimental manipulation of uterine blood flow on plasma sulphation

promoting activity and on the concentration of insulin-like growth factors I and II. *Journal of developmental physiology* **10,** 179-189.

Jorgensen W, Gam C, Andersen JL, Schjerling P, Scheibye-Knudsen M, Mortensen OH, Grunnet N, Nielsen MO & Quistorff B. (2009). Changed mitochondrial function by pre- and/or postpartum diet alterations in sheep. *Am J Physiol Endocrinol Metab* **297,** E1349-1357.

Kablar B, Asakura A, Krastel K, Ying C, May LL, Goldhamer DJ & al e. (1998). MyoD and Myf5 define the specification of musculature of distinct embryonic origin. *Biochem Cell Biol* **76,** 1079-1091.

Kahn SE, Hull RL & Utzschneider KM. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444,** 840-846.

Kelly RW. (1992). Nutrition and placental development. *Nutr Soc Aust* **17,** 203-211.

Khan I, Dekou V, Hanson M, Poston L & Taylor P. (2004). Predictive adaptive responses to maternal high-fat diet prevent endothelial dysfunction but not hypertension in adult rat offspring. *Circulation* **110,** 1097-1102.

Khan OA, Torrens C, Noakes DE, Poston L, Hanson MA, Green LR & Ohri SK. (2005). Effects of pre-natal and early post-natal undernutrition on adult internal thoracic artery function. *Eur J Cardiothorac Surg* **28,** 811-815.

Kim SP, Ellmerer M, Kirkman EL & Bergman RN. (2007). Beta-cell "rest" accompanies reduced first-pass hepatic insulin extraction in the insulin-resistant, fat-fed canine model. *Am J Physiol Endocrinol Metab* **292,** E1581-1589.

Klosterman EW, Buchanan ML, Bolin DW & Bolin FM. (1951). Levels and sources of protein in rations for pregnant ewes. *J Anim Sci* **10,** 257-265.

Kotsampasi B, Balaskas C, Papadomichelakis G & Chadio SE. (2009a). Reduced Sertoli cell number and altered pituitary responsiveness in male lambs undernourished in utero. *Anim Reprod Sci* **114,** 135-147.

Kotsampasi B, Chadio S, Papadomichelakis G, Deligeorgis S, Kalogiannis D, Menegatos I & Zervas G. (2009b). Effects of maternal undernutrition on the hypothalamic-pituitary-gonadal axis function in female sheep offspring. *Reprod Domest Anim* **44,** 677-684.

Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD & Muoio DM. (2008). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* **7,** 45-56.

Krauss RS, Cole F, Gaio U, Takaesu G, Zhang W & Kang JS. (2005). Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. *Journal of cell science* **118,** 2355-2362.

Kuzawa CW. (2004). Modeling fetal adaptation to nutrient restriction: testing the fetal origins hypothesis with a supply-demand model. *J Nutr* **134,** 194-200.

Lane RH, Kelley DE, Ritov VH, Tsirka AE & Gruetzmacher EM. (2001). Altered expression and function of mitochondrial beta-oxidation enzymes in juvenile intrauterine-growth-retarded rat skeletal muscle. *Pediatric research* **50,** 83-90.

Langlands K, Yin X, Anand G & Prochownik EV. (1997). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *The Journal of biological chemistry* **272,** 19785-19793.

Langley-Evans SC, Gardner DS & Jackson AA. (1996). Maternal protein restriction influences the programming of the rat hypothalamic-pituitary-adrenal axis. *J Nutr* **126,** 1578-1585.

Larance M, Ramm G & James DE. (2008). The GLUT4 code. *Mol Endocrinol* **22,** 226-233.

Lawlor MJ & Hopkins SP. (1981). The influence of perinatal undernutrition of twin-bearing ewes on milk yields and lamb performance and the effects of postnatal nutrition on live weight gain and carcass composition. *The British journal of nutrition* **45,** 579-586.

Lee YY, Park KS, Pak YK & Lee HK. (2005). The role of mitochondrial DNA in the development of type 2 diabetes caused by fetal malnutrition. *The Journal of Nutritional Biochemistry* **16,** 195-204.

Lefterova MI & Lazar MA. (2009). New developments in adipogenesis. *Trends in endocrinology and metabolism: TEM* **20,** 107-114.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA & Burdge GC. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr* **135,** 1382-1386.

Limesand SW, Jensen J, Hutton JC & Hay WW, Jr. (2005). Diminished beta-cell replication contributes to reduced beta-cell mass in fetal sheep with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol* **288,** R1297-1305.

Limesand SW, Rozance PJ, Zerbe GO, Hutton JC & Hay WW, Jr. (2006). Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. *Endocrinology* **147,** 1488-1497.

Long NM, Nijland MJ, Nathanielsz PW & Ford SP. (2010). The effect of early to mid-gestational nutrient restriction on female offspring fertility and hypothalamic-pituitary-adrenal axis response to stress. *Journal of Animal Science* **88,** 2029-2037.

Lucas A. (1991). Programming by early nutrition in man. *Ciba Foundation symposium* **156,** 38-50; discussion 50-35.

Luther JS, Redmer DA, Reynolds LP & Wallace JM. (2005). Nutritional paradigms of ovine fetal growth restriction: implications for human pregnancy. *Human fertility (Cambridge, England)* **8,** 179-187.

Mallinson JE, Sculley DV, Craigon J, Plant R, Langley-Evans SC & Brameld JM. (2007). Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. *The British journal of nutrition* **98,** 1-8.

Maloney CA, Hay SM & Rees WD. (2007). Folate deficiency during pregnancy impacts on methyl metabolism without affecting global DNA methylation in the rat fetus. *The British journal of nutrition* **97,** 1090-1098.

Maltin CA, Delday MI, Sinclair KD, Steven J & Sneddon AA. (2001). Impact of manipulations of myogenesis in utero on the performance of adult skeletal muscle. *Reproduction* **122,** 359-374.

Martin-Gronert MS & Ozanne SE. (2007). Experimental IUGR and later diabetes. *Journal of internal medicine* **261,** 437-452.

McCann JP, Bergman EN & Reimers TJ. (1989). Effects of obesity and ovarian steroids on insulin secretion and removal in sheep. *Am J Physiol Endocrinol Metab* **256,** E116-128.

McCann JP, Reimers TJ & Bergman EN. (1987). Glucose-dose dependent characteristics of insulin secretion in obese and lean sheep. *Endocrinology* **121,** 553-560.

McCann JP, Ullmann MB, Temple MR, Reimers TJ & Bergman EN. (1986). Insulin and glucose responses to glucose injection in fed and fasted obese and lean sheep. *J Nutr* **116,** 1287-1297.

McCoard SA, McNabb WC, Peterson SW, McCutcheon SN & Harris PM. (2000). Muscle growth, cell number, type and morphometry in single and twin fetal lambs during mid to late gestation. *REPRODUCTION FERTILITY AND DEVELOPMENT* **12,** 319-327.

McDonald P, Edwards RA, Greenhalgh JFD & Morgan CA. (2002). Animal Nutrition. 6 edn, pp. 708. Prentice Hall, London, England.

McMillen IC, Edwards LJ, Duffield J & Muhlhausler BS. (2006). Regulation of leptin synthesis and secretion before birth: implications for the early programming of adult obesity. *Reproduction* **131,** 415-427.

McMillen IC & Robinson JS. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological reviews* **85,** 571-633.

McMullen S & Langley-Evans SC. (2005). Sex-specific effects of prenatal low-protein and carbenoxolone exposure on renal angiotensin receptor expression in rats. *Hypertension* **46,** 1374-1380.

McNeill DM, Slepetis R, Ehrhardt RA, Smith DM & Bell AW. (1997). Protein requirements of sheep in late pregnancy: partitioning of nitrogen between gravid uterus and maternal tissues. *J Anim Sci* **75,** 809-816.

Mehta G, Roach HI, Langley-Evans S, Taylor P, Reading I, Oreffo ROC, Aihie-Sayer A, Clarke NMP & Cooper C. (2002). Intrauterine exposure to a maternal low protein diet reduces adult bone mass and alters growth plate morphology in rats. *Calcified Tissue International* **71,** 493-498.

Mendez MA, Monteiro CA & Popkin BM. (2005). Overweight exceeds underweight among women in most developing countries. *Am J Clin Nutr* **81,** 714-721.

Mlinar B, Marc J, Janez A & Pfeifer M. (2007). Molecular mechanisms of insulin resistance and associated diseases. *Clinica Chimica Acta* **375,** 20-35.

Mostyn A & Symonds ME. (2009). Early programming of adipose tissue function: a large-animal perspective. *The Proceedings of the Nutrition Society* **68,** 393-400.

Mostyn A, Wilson V, Dandrea J, Yakubu DP, Budge H, Alves-Guerra MC, Pecqueur C, Miroux B, Symonds ME & Stephenson T. (2003). Ontogeny and nutritional manipulation of mitochondrial protein abundance in adipose tissue and the lungs of postnatal sheep. *The British journal of nutrition* **90,** 323-328.

Motta PM. (1984). The three-dimensional microanatomy of the liver. *Arch Histol Jpn* **47,** 1-30.

Muntoni F, Brown S, Sewry C & Patel K. (2002). Muscle development genes: their relevance in neuromuscular disorders. *Neuromuscular Disorders* **12,** 438-446.

Murphy VE, Smith R, Giles WB & Clifton VL. (2006). Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. *Endocr Rev* **27,** 141-169.

Naya FJ & Olson E. (1999). MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Current Opinion in Cell Biology* **11,** 683-688.

Niculescu MD & Zeisel SH. (2002). Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J Nutr* **132,** 2333S-2335S.

Nordby DJ, Field RA, Riley ML & Kercher CJ. (1987). Effects of maternal undernutrition during early pregnancy on growth, muscle cellularity, fiber type and carcass composition in lambs. *J Anim Sci* **64,** 1419-1427.

Norton L, Parr T, Bardsley RG, Ye H & Tsintzas K. (2007). Characterization of GLUT4 and calpain expression in healthy human skeletal muscle during fasting and refeeding. *Acta physiologica (Oxford, England)* **189,** 233-240.

Ogilvy-Stuart AL. (1995). Endocrinology of the neonate. *Br J Hosp Med* **54,** 207-211; quiz 212-203.

Ogilvy-Stuart AL, Hands SJ, Adcock CJ, Holly JM, Matthews DR, Mohamed-Ali V, Yudkin JS, Wilkinson AR & Dunger DB. (1998). Insulin, insulin-like growth factor I (IGF-I), IGF-binding protein-1, growth hormone, and feeding in the newborn. *J Clin Endocrinol Metab* **83,** 3550-3557.

Ojeda NB, Grigore D, Robertson EB & Alexander BT. (2007). Estrogen protects against increased blood pressure in postpubertal female growth restricted offspring. *Hypertension* **50,** 679-685.

Oligny LL. (2001). Human molecular embryogenesis: an overview. *Pediatr Dev Pathol* **4,** 324-343.

Oliver MH, Breier BH, Gluckman PD & Harding JE. (2002). Birth weight rather than maternal nutrition influences glucose tolerance, blood pressure, and IGF-I levels in sheep. *Pediatric research* **52,** 516-524.

Oliver MH, Jaquiery AL, Bloomfield FH & Harding JE. (2007). The effects of maternal nutrition around the time of conception on the health of the offspring. *Society of Reproduction and Fertility supplement* **64,** 397-410.

Otto TC & Lane MD. (2005). Adipose development: from stem cell to adipocyte. *Crit Rev Biochem Mol Biol* **40,** 229-242.

Owens JA, Thavaneswaran P, De Blasio MJ, McMillen IC, Robinson JS & Gatford KL. (2007). Sex-specific effects of placental restriction on components of the metabolic syndrome in young adult sheep. *Am J Physiol Endocrinol Metab* **292,** E1879-1889.

Ozanne SE & Hales CN. (2004). Lifespan: catch-up growth and obesity in male mice. *Nature* **427,** 411-412.

Ozanne SE, Jensen CB, Tingey KJ, Storgaard H, Madsbad S & Vaag AA. (2005). Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia* **48,** 547-552.

Ozanne SE, Olsen GS, Hansen LL, Tingey KJ, Nave BT, Wang CL, Hartil K, Petry CJ, Buckley AJ & Mosthaf-Seedorf L. (2003). Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol* **177,** 235-241.

Painter RC, Roseboom TJ & Bleker OP. (2005). Prenatal exposure to the Dutch famine and disease in later life: an overview. *Reproductive toxicology (Elmsford, NY* **20,** 345-352.

Parekh S & Anania FA. (2007). Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease. *Gastroenterology* **132,** 2191-2207.

Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee KU, Pak YK & Lee HK. (2003). Fetal and early postnatal protein malnutrition cause long-term changes in rat liver and muscle mitochondria. *J Nutr* **133,** 3085-3090.

Pell JM & Schofield PN. (1999). Molecular control of muscle development: specification, determination and differentiation in the amniote embryo. *Seminars in Neonatology* **4,** 79-91.

Peterson JD, Nehrlich S, Oyer PE & Steiner DF. (1972). Determination of the amino acid sequence of the monkey, sheep, and dog proinsulin C-peptides by a semi-micro Edman degradation procedure. *The Journal of biological chemistry* **247,** 4866-4871.

Petrie L, Duthie SJ, Rees WD & McConnell JM. (2002). Serum concentrations of homocysteine are elevated during early pregnancy in rodent models of fetal programming. *The British journal of nutrition* **88,** 471-477.

Petrik J, Reusens B, Arany E, Remacle C, Coelho C, Hoet JJ & Hill DJ. (1999). A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology* **140,** 4861-4873.

Petry CJ, Dorling MW, Pawlak DB, Ozanne SE & Hales CN. (2001). Diabetes in old male offspring of rat dams fed a reduced protein diet. *International journal of experimental diabetes research* **2,** 139-143.

Phelan JP & Rose MR. (2005). Why dietary restriction substantially increases longevity in animal models but won't in humans. *Ageing Res Rev* **4,** 339-350.

Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ & Walker BR. (1998). Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab* **83,** 757-760.

Phillipson AT. (1959). *Scientific Principles of Feeding Farm Livestock*. Farmer and Stock-Breeder Publications Ltd., London.

Poore KR, Boullin JP, Cleal JK, Newman JP, Noakes DE, Hanson MA & Green LR. (2010). Sex- and age-specific effects of nutrition in early gestation and early postnatal life on hypothalamo-pituitary-adrenal axis and sympathoadrenal function in adult sheep. *J Physiol* **588,** 2219-2237.

Poore KR, Cleal JK, Newman JP, Boullin JP, Noakes DE, Hanson MA & Green LR. (2007). Nutritional challenges during development induce sex-specific changes in glucose homeostasis in the adult sheep. *Am J Physiol Endocrinol Metab* **292,** E32-39.

Poore KR & Fowden AL. (2002). The effect of birth weight on glucose tolerance in pigs at 3 and 12 months of age. *Diabetologia* **45,** 1247-1254.

Poore KR & Fowden AL. (2004). Insulin sensitivity in juvenile and adult Large White pigs of low and high birthweight. *Diabetologia* **47,** 340-348.

Popkin BM. (2006). Global nutrition dynamics: the world is shifting rapidly toward a diet linked with noncommunicable diseases. *Am J Clin Nutr* **84,** 289-298.

Powell BD, Redfield MM, Bybee KA, Freeman WK & Rihal CS. (2006). Association of obesity with left ventricular remodeling and diastolic dysfunction in patients without coronary artery disease. *Am J Cardiol* **98,** 116-120.

Prentice AM. (2006). The emerging epidemic of obesity in developing countries. *Int J Epidemiol* **35,** 93-99.

Prentice AM & Moore SE. (2005). Early programming of adult diseases in resource poor countries. *Archives of disease in childhood* **90,** 429-432.

Quigley SP, Kleemann DO, Kakar MA, Owens JA, Nattrass GS, Maddocks S & Walker SK. (2005). Myogenesis in sheep is altered by maternal feed intake during the peri-conception period. *Animal Reproduction Science* **87,** 241-251.

Rae MT, Kyle CE, Miller DW, Hammond AJ, Brooks AN & Rhind SM. (2002). The effects of undernutrition, in utero, on reproductive function in adult male and female sheep. *Anim Reprod Sci* **72,** 63-71.

Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN & Bleker OP. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351,** 173-177.

Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ & Bleker OP. (1999). Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* **70,** 811-816.

Ravelli AC, van der Meulen JH, Osmond C, Barker DJ & Bleker OP. (2000). Infant feeding and adult glucose tolerance, lipid profile, blood pressure, and obesity. *Archives of disease in childhood* **82,** 248-252.

Ravelli GP & Belmont L. (1979). Obesity in nineteen-year-old men: family size and birth order associations. *Am J Epidemiol* **109,** 66-70.

Ravelli GP, Stein ZA & Susser MW. (1976). Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* **295,** 349-353.

Reik W, Dean W & Walter J. (2001). Epigenetic reprogramming in mammalian development. *Science* **293,** 1089-1093.

Remacle C, Dumortier O, Bol V, Goosse K, Romanus P, Theys N, Bouckenooghe T & Reusens B. (2007). Intrauterine programming of the endocrine pancreas. *Diabetes, obesity & metabolism* **9 Suppl 2,** 196-209.

Reusens B, Dahri S, Snoeck A, Bennistaleb N, Remacle C & Hoet JJ. (1995). Long-term consequences of diabetes and its complications may have a fetal origin: Experimental and epidemiological evidence. *Diabetes* **35,** 187-198 297.

Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Hammer CJ, Maddock Carlin KR, Grazul-Bilska AT & Redmer DA. (2010). Developmental programming: the concept, large animal models, and the key role of uteroplacental vascular development. *J Anim Sci* **88,** E61-72.

Rhodes P, Craigon J, Gray C, Rhind SM, Loughna PT & Gardner DS. (2009). Adult-onset obesity reveals prenatal programming of glucose-insulin sensitivity in male sheep nutrient restricted during late gestation. *PLoS One* **4,** e7393.

Rich-Edwards JW, Stampfer MJ, Manson JE, Rosner B, Hankinson SE, Colditz GA, Willett WC & Hennekens CH. (1997). Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ* **315,** 396-400.

Roberts RM, Smith GW, Bazer FW, Cibelli J, Seidel GE, Jr., Bauman DE, Reynolds LP & Ireland JJ. (2009). Research priorities. Farm animal research in crisis. *Science* **324,** 468-469.

Robinson JJ. (1977). The influence of maternal nutrition on ovine foetal growth. *The Proceedings of the Nutrition Society* **36,** 9-16.

Robinson JJ. (1980). Energy requirements of ewes during late pregnancy and early lactation. *Vet Rec* **106,** 282-284.

Robinson JJ & Forbes TJ. (1966). A study of the protein requirements of the mature breeding ewe. Maintenance requirement of the non-pregnant ewe. *The British journal of nutrition* **20,** 263-272.

Robinson JJ & Forbes TJ. (1967). A study of the protein requirements of the mature breeding ewe. 2. Protein utilization in the pregnant ewe. *The British journal of nutrition* **21,** 879-891.

Robinson JJ & Symonds ME. (1995). Whole-body fuel selection: 'reproduction'. *The Proceedings of the Nutrition Society* **54,** 283-299.

Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, Schroeder-Tanka JM, van Montfrans GA, Michels RP & Bleker OP. (2000). Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart (British Cardiac Society)* **84,** 595-598.

Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ & Bleker OP. (2001). Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Twin Res* **4,** 293-298.

Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH & Jaenisch R. (1993). MyoD or Myf5 is required for the formation of skeletal muscle *Cell* **75,** 1351-1359.

Russel AJF, Doney JM & Gunn RG. (1969). Subjective assessment of body fat in live sheep. *Journal of Agricultural Sciences* **72,** 451-454.

Saenger P, Czernichow P, Hughes I & Reiter EO. (2007). Small for gestational age: short stature and beyond. *Endocr Rev* **28,** 219-251.

Salter J & Best CH. (1953). Insulin as a growth hormone. *British medical journal* **2,** 353-356.

Sartorelli V & Caretti G. (2005). Mechanisms underlying the transcriptional regulation of skeletal myogenesis. *Current Opinion in Genetics & Development* **15,** 528-535.

Sasaki S-i. (2002). Mechanism of insulin action on glucose metabolism in ruminants. *Animal Science Journal* **73,** 423-433.

Sassoon DA. (1993). Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. *Dev Biol* **156,** 11-23.

Sayer AA, Syddall HE, Dennison EM, Gilbody HJ, Duggleby SL, Cooper C, Barker DJ & Phillips DI. (2004). Birth weight, weight at 1 y of age, and body composition in older men: findings from the Hertfordshire Cohort Study. *Am J Clin Nutr* **80,** 199-203.

Schwartz J & Morrison JL. (2005). Impact and mechanisms of fetal physiological programming. *Am J Physiol Regul Integr Comp Physiol* **288,** R11-15.

Sebert SP, Hyatt MA, Chan LL, Patel N, Bell RC, Keisler D, Stephenson T, Budge H, Symonds ME & Gardner DS. (2008). Maternal nutrient restriction between early-to-mid gestation and its impact upon appetite regulation following juvenile obesity. *Endocrinology*.

Sebert SP, Hyatt MA, Chan LL, Patel N, Bell RC, Keisler D, Stephenson T, Budge H, Symonds ME & Gardner DS. (2009). Maternal nutrient restriction between early and midgestation and its impact upon appetite regulation after juvenile obesity. *Endocrinology* **150,** 634-641.

Seckl JR. (1997). Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase type 2, and the early life origins of adult disease. *Steroids* **62,** 89-94.

Seckl JR & Holmes MC. (2007). Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. *Nature clinical practice* **3,** 479-488.

Serls AE, Doherty S, Parvatiyar P, Wells JM & Deutsch GH. (2005). Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* **132,** 35-47.

Servitja JM & Ferrer J. (2004). Transcriptional networks controlling pancreatic development and beta cell function. *Diabetologia* **47,** 597-613.

Sharkey D, Gardner DS, Fainberg HP, Sebert S, Bos P, Wilson V, Bell R, Symonds ME & Budge H. (2009). Maternal nutrient restriction during pregnancy differentially alters the unfolded protein response in adipose and renal tissue of obese juvenile offspring. *Faseb J* **23,** 1314-1324.

Si-Tayeb K, Lemaigre FP & Duncan SA. (2010). Organogenesis and development of the liver. *Developmental cell* **18,** 175-189.

Simmons RA, Templeton LJ & Gertz SJ. (2001). Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* **50,** 2279-2286.

Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG & Young LE. (2007). DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 19351-19356.

Sinclair KD, Lunn LA, Kwong WY, Wonnacott K, Linforth RST & Craigon J. (2008). Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. *Reproductive Biomedicine Online* **16,** 859-868.

Singhal A, Fewtrell M, Cole TJ & Lucas A. (2003). Low nutrient intake and early growth for later insulin resistance in adolescents born preterm. *Lancet* **361,** 1089-1097.

Singhal A & Lucas A. (2004). Early origins of cardiovascular disease: is there a unifying hypothesis? *Lancet* **363,** 1642-1645.

Smith NA, McAuliffe FM, Quinn K, Lonergan P & Evans AC. (2010). The negative effects of a short period of maternal undernutrition at conception on the glucose-insulin system of offspring in sheep. *Anim Reprod Sci* **121,** 94-100.

Snoeck A, Remacle C, Reusens B & Hoet JJ. (1990). Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biology of the neonate* **57,** 107-118.

Soto N, Bazaes RA, Pena V, Salazar T, Avila A, Iniguez G, Ong KK, Dunger DB & Mericq MV. (2003). Insulin sensitivity and secretion are related to catch-up growth in small-for-gestational-age infants at age 1 year: results from a prospective cohort. *J Clin Endocrinol Metab* **88,** 3645-3650.

Stein AD, Zybert PA, van de Bor M & Lumey LH. (2004). Intrauterine famine exposure and body proportions at birth: the Dutch Hunger Winter. *Int J Epidemiol* **33,** 831-836.

Storlien L, Oakes ND & Kelley DE. (2004). Metabolic flexibility. *The Proceedings of the Nutrition Society* **63,** 363-368.

Styrud J, Eriksson UJ, Grill V & Swenne I. (2005). Experimental intrauterine growth retardation in the rat causes a reduction of pancreatic B-cell mass, which persists into adulthood. *Biology of the neonate* **88,** 122-128.

Sugden MC & Holness MJ. (2002). Gender-specific programming of insulin secretion and action. *J Endocrinol* **175,** 757-767.

Symonds ME & Budge H. (2009). Nutritional models of the developmental programming of adult health and disease. *The Proceedings of the Nutrition Society* **68,** 173-178.

Symonds ME, Gopalakrishnan G, Bispham J, Pearce S, Dandrea J, Mostyn A, Ramsay MM & Stephenson T. (2003). Maternal nutrient restriction during placental growth, programming of fetal adiposity and juvenile blood pressure control. *Archives of physiology and biochemistry* **111,** 45-52.

Symonds ME, Sebert SP, Hyatt MA & Budge H. (2009). Nutritional programming of the metabolic syndrome. *Nat Rev Endocrinol* **5,** 604-610.

Tajbakhsh S & Buckingham ME. (1994). Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 747-751.

Tang QQ, Otto TC & Lane MD. (2004). Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 9607-9611.

Taylor PD & Poston L. (2007). Developmental programming of obesity in mammals. *Exp Physiol* **92,** 287-298.

Thomson W & Aitken FC. (1959). Diet in relation to reproduction and the viability of the young. Part II. Sheep. . *Commonw Bur Anim Nutr, Tech Commun* **20,** pp. 13-15.

Todd SE, Oliver MH, Jaquiery AL, Bloomfield FH & Harding JE. (2009). Periconceptional Undernutrition of Ewes Impairs Glucose Tolerance in Their Adult Offspring. *Pediatric research* **65,** 409-413.

Torrens C, Snelling TH, Chau R, Shanmuganathan M, Cleal JK, Poore KR, Noakes DE, Poston L, Hanson MA & Green LR. (2009). Effects of pre- and periconceptional undernutrition on arterial function in adult female sheep are vascular bed dependent. *Exp Physiol* **94,** 1024-1033.

Twyman RM. (2001). *Developmental Biology*. Bios Scientific Publishers Ltd, Oxford.

Vaag A, Jensen CB, Poulsen P, Brons C, Pilgaard K, Grunnet L, Vielwerth S & Alibegovic A. (2006). Metabolic aspects of insulin resistance in individuals born small for gestational age. *Hormone research* **65 Suppl 3,** 137-143.

Valero De Bernabe J, Soriano T, Albaladejo R, Juarranz M, Calle ME, Martinez D & Dominguez-Rojas V. (2004). Risk factors for low birth weight: a review. *European journal of obstetrics, gynecology, and reproductive biology* **116,** 3-15.

Varshavsky A. (2005). Regulated protein degradation. *Trends Biochem Sci* **30,** 283-286.

Vickers MH, Breier BH, Cutfield WS, Hofman PL & Gluckman PD. (2000). Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab* **279,** E83-87.

Wallace JM, Regnault TR, Limesand SW, Hay WW, Jr. & Anthony RV. (2005). Investigating the causes of low birth weight in contrasting ovine paradigms. *J Physiol* **565,** 19-26.

Wells JC, Fewtrell MS, Williams JE, Haroun D, Lawson MS & Cole TJ. (2006). Body composition in normal weight, overweight and obese children: matched case-control analyses of total and regional tissue masses, and body

composition trends in relation to relative weight. *International journal of obesity (2005)* **30,** 1506-1513.

Whorwood CB, Firth KM, Budge H & Symonds ME. (2001). Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. *Endocrinology* **142,** 2854-2864.

Wigmore PM & Stickland NC. (1983). Muscle development in large and small pig fetuses. *J Anat* **137 (Pt 2),** 235-245.

Wild S, Roglic G, Green A, Sicree R & King H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* **27,** 1047-1053.

Williams PJ, Kurlak LO, Perkins AC, Budge H, Stephenson T, Keisler D, Symonds ME & Gardner DS. (2007). Hypertension and impaired renal function accompany juvenile obesity: the effect of prenatal diet. *Kidney international* **72,** 279-289.

Wilson ME, Scheel D & German MS. (2003). Gene expression cascades in pancreatic development. *Mech Dev* **120,** 65-80.

Wolf G. (2007). Serum retinol-binding protein: a link between obesity, insulin resistance, and type 2 diabetes. *Nutrition reviews* **65,** 251-256.

Wu G, Bazer FW, Wallace JM & Spencer TE. (2006). Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* **84,** 2316-2337.

www.cellsignalling.com. (2010). Insulin Receptor Signalling, ed. Goodyear PL.

www.iotf.org/database/index.asp. (2009). International Obesity Taskforce.

www.smallstock.info. (2010). Protein digestion in the rumen. NR International (Livestock Production Programme; Department for International Development).

www.who.int. (2009). The global burden of disease: 2004 update In *Global health risks: mortality and burden of disease attributable to selected major risks* Geneva

www.who.int/en. (2010). Population BMI. World Health Organisation.

Yajnik CS. (2004a). Early life origins of insulin resistance and type 2 diabetes in India and other Asian countries. *J Nutr* **134,** 205-210.

Yajnik CS. (2004b). Obesity epidemic in India: intrauterine origins? *The Proceedings of the Nutrition Society* **63,** 387-396.

Yliharsila H, Kajantie E, Osmond C, Forsen T, Barker DJ & Eriksson JG. (2007). Birth size, adult body composition and muscle strength in later life. *International journal of obesity (2005)* **31,** 1392-1399.

Yun K & Wold B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Current Opinion in Cell Biology* **8,** 877-889.

Zaid H, Antonescu CN, Randhawa VK & Klip A. (2008). Insulin action on glucose transporters through molecular switches, tracks and tethers. *The Biochemical journal* **413,** 201-215.

Zhang S, Rattanatray L, MacLaughlin SM, Cropley JE, Suter CM, Molloy L, Kleemann D, Walker SK, Muhlhausler BS, Morrison JL & McMillen IC. (2010). Periconceptional undernutrition in normal and overweight ewes leads to increased adrenal growth and epigenetic changes in adrenal IGF2/H19 gene in offspring. *Faseb J* **24,** 2772-2782.

Zhu MJ, Ford SP, Means WJ, Hess BW, Nathanielsz PW & Du M. (2006). Maternal nutrient restriction affects properties of skeletal muscle in offspring. *J Physiol* **575,** 241-250.

Zhu MJ, Ford SP, Nathanielsz PW & Du M. (2004). Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. *Biol Reprod* **71,** 1968-1973.

9. Appendices

9.1 Appendix I: Rhodes *et al.,* (2009)

Rhodes PS, Craigon J, Gray C, Rhind SM, Loughna PT & Gardner DS. (2009). Adult-onset obesity reveals prenatal programming of glucose-insulin sensitivity in male sheep nutrient restricted during late gestation. *PLoS One* **4,** e7393.

9.2 Appendix II: Gardner & Rhodes (2009)

Gardner DS & Rhodes PS. (2009). Developmental Origins of Obesity: Programming of food intake or physical activity. *Early Nutrition Programming and Health Outcomes in Later Life - Obesity and beyond Advances in Experimental Biology and Medicine* **646:,** 83-94.