UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Academic Unit of Human Development and Health

The effect of early life nutrition on hepatic function

by

Lisa Jane Hollis

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that alterations in early life nutrition results in developmental adaptations that permanently change structure, physiology and metabolism to enable survival. In humans and animals environmental constraints during pre- and postnatal life are associated with altered metabolic disease risk in later life and the liver, a key organ in glucose and lipid metabolism, is implicated. Altered maternal nutrition is associated with changes in fetal liver blood flow, liver weight and gene expression. Glucocorticoid receptor (GR), Phosphoenolpyruvate carboxykinase (PEPCK), Glucose-6-Phosphatase (G6Pase) and the insulin receptor are key components in the gluconeogenic pathway which is implicated in disturbances in metabolic control. In this thesis I have examined the general hypothesis that early life undernutrition affects the liver gluconeogenic pathway in sheep.

Three cohorts of sheep were used; 2.5 year old adult, late gestation fetal and 4.5 year old adult. Each group had different nutritional exposure in early life. In the 2.5 year old adult study Welsh mountain ewes received either 100% or 50% of total nutrient requirements from days 1-31 of gestation, and 100% requirements thereafter. At 12-25 weeks of age offspring nutrition was restricted to reduce growth to 85% of the normal growth. In the fetal study 127 day gestational age (dGA) animals received 100% nutrition throughout gestation or either 50% or 40% of total requirements during days 1-31 (PI50, PI40, respectively), or 50% requirements from days 104 – 127 (L). The 4.5 year old adult study consisted of offspring from mothers with either high or low body condition scores (HBCS, LBCS) prior to, during pregnancy and suckling.

In 2.5yr old offspring early life nutrition had no effect on hepatic gluconeogenic mRNA expression. Male liver weight was less than females, however, GR and PEPCK mRNA levels were greater. The right liver lobe had greater GR and PEPCK mRNA expression, and twins had greater GR mRNA expression than singletons. A preliminary methylation investigation found no changes in GR methylation. In fetal sheep GR, PEPCK and G6Pase mRNA expression was lower in the PI40 and late gestation groups, and also in the right liver lobe compared to the left. Maternal LBCS increased the mRNA expression of the insulin receptor in offspring but had no effect on basal plasma metabolites or lobar gene expression.

In conclusion, the liver responded to nutrient restriction in both early and late gestation with a reduction in key gluconeogenic genes as well as differential expression between the fetal liver lobes. These effects did not persist into adult life and indeed, as with the lobar differences in gene expression patterns, appear even to switch direction over the life course. However, although these effects of maternal undernutrition on hepatic mRNA imply potential changes in gluconeogenic capacity, they may not be of physiological significance to offspring until they are challenged further.

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DECLARATION OF AUTHORSHIP

I, Lisa Jane Hollis

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The effect of early life nutrition on hepatic function

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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   Poore KR, **Hollis LJ**, Murray RJ, Warlow A, Brewin A, Fulford L, Cleal JK, Lillycrop KA, Burdge GC, Hanson MA, Green LR. Differential Pathways to Adult Metabolic Dysfunction following Poor Nutrition at Two Critical Developmental Periods in Sheep. *PLoS One.* (2014) Mar 6;9(3):e90994.

   Costello PM, **Hollis LJ**, Cripps RL, Bearpark N, Patel HP, Sayer AA, Cooper C, Hanson MA, Ozanne SE, Green LR. Lower maternal body condition during pregnancy affects skeletal muscle structure and glut-4 protein levels but not glucose tolerance in mature adult sheep. *Reprod Sci.* (2013) Oct;20(10):1144-55.

Signed:..

Date:...

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Abbreviations

11β-HSD2	11 beta hydroxysteroid dehydrogenase
AC	abdominal circumference
ACE	angiotensin-converting enzyme
ANOVA	analysis of variance
ATIR	angiotensin type 1 receptor
AUC	area under the curve
BCS	body condition score
BMI	body mass index
BPD	biparietal diameter
C	control
cDNA	complimentary DNA
CHD	coronary heart disease
CO_2	carbon dioxide
CpG	cytosine-phosphate-guanine
CRL	crown rump length
Ct	cycle threshold
CVD	cardiovascular disease
dGA	days gestational age
DLK/MEG	delta-like homolog 1/maternally expressed gene
DNA	deoxyribose nucleic acid

DOHaD	Developmental Origins of Health and Disease
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
FL	femur length
G	gauge
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G6PT	glucose-6-phosphate transporter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GH	growth hormone
GLUT	glucose transporter
GR	glucocorticoid receptor
HBCS	high body condition score
HDL	high density lipoprotein
HMTs	histone methyltransferase
HSP	heat shock protein
IGF	insulin-like growth factor
IR	insulin receptor
IUGR	intrauterine growth restriction
LBCS	low body condition score
LDL	low density lipoprotein
MECP	methyl CpG binding protein

miRNA	microRNA
MLP	maternal low protein
mRNA	messenger ribonucleic acid
msPCR	methylation sensitive PCR
ncRNA	non coding RNA
NEFA	non esterified fatty acid
PAR	predictive adaptive response
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvatecarboxykinase
PI	peri-implantation
RNA	ribonucleic acid
RPL-19	ribosomal protein L19
RPM	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	real time PCR
RVC	Royal Veterinary College
SEM	standard error of the mean
TAE	tris acetate EDTA
TAG	triacylglyceride
U	undernutrition
VLDL	very low density lipoprotein

Chapter 1. **Introduction**

1.1 Adult metabolic disease - the big problem

The World Health Organization has called obesity one of the greatest public health challenges of the 21st century. Levels of obesity have tripled in many European countries since the 1980s, and the number of those affected continues to rise at an alarming rate. In England there has been an increase in the proportion of adults that were obese between 1993 and 2012 from 13.2% to 24.4% in men and from 16.4% to 25.1% in women. The adults of the future are not immune to the problem either with school children aged 10/11 (school year 6), having obesity levels of 18.9% in 2012/13 compared to 17.5% in 2006/07 (Lifestyles statistics team, 2014). Obesity is a major public health problem due to its association with serious chronic diseases such as type 2 diabetes, hypertension (high blood pressure), and hyperlipidemia (high levels of fats in the blood that can lead to narrowing and blockages of blood vessels), which are major risk factors for cardiovascular disease and cardiovascular related mortality. This group of diseases is often referred to as the metabolic syndrome and is also being seen in the developing world making it a global health issue. Liver function is central to metabolic homeostasis and is the focus of this thesis.

1.2 Early origins of metabolic disease

1.2.1 Developmental Origins of Health and Disease

Early epidemiological observations discovered that ischemic heart disease is strongly correlated with both neonatal and post neonatal mortality and it was suggested that poor early life nutrition increases susceptibility to the effects of a later affluent diet (Barker & Osmond, 1986). This and further epidemiological studies led to the hypothesis that the risk of developing some chronic adult diseases is influenced not only by genetic and adult lifestyle factors but also by early life environmental factors (Gluckman & Hanson, 2004b). Clinical evidence and epidemiological studies have suggested a link between the metabolic syndrome, cardiovascular disease and the antenatal environment. Evidence now also exists for links to a wider range of diseases including mood disorders (Thompson *et al.*, 2001) and polycystic ovarian syndrome (Ibanez *et al.*, 2001). Studies have linked the size (or weight) at birth to the subsequent risk of developing insulin resistance and/or type 2 diabetes mellitus in middle age with both low birth weight and high birth weight babies at a higher risk. One such study was carried out on a cohort of people in Hertfordshire whose detailed birth and childhood health records were recorded from 1911 until 1948. Follow up data was obtained on mortality and morbidity from these records. The findings included increased incidence of death from cardiovascular disease in those of low birth weights. Small size at birth and during infancy was shown to be associated with increased risk of developing coronary heart disease and type 2 diabetes mellitus, the metabolic syndrome and insulin resistance and osteoporosis in adult life (Syddall *et al.*, 2005b). There is also a link between those who are small at birth and their postnatal weight gain. Therefore those at greatest risk of developing a symptom of the metabolic syndrome are those who are small or thin at birth who then go on to develop obesity postnatally (Eriksson *et al.*, 1999). It was discovered that people who developed coronary heart disease grew differently from others during fetal life and in childhood and this led to a developmental model for the disease (Robinson & Barker, 2002).

1.2.2 **Fetal life**

There are several ways in which the environment can influence development including nutrition, season of birth (Lawlor *et al.*, 2004;Doblhammer & Vaupel, 2001) and smoking (Power & Jefferis, 2002) (fig.1.1). A developing fetus receives all the nutrients from its mother either by direct contact with the uterine environment or via the yolk sac and placenta. Environmental factors such as the nutrient levels within the intrauterine environment have been shown to constrain fetal growth. Fetal environmental factors are also thought to induce change in metabolic (Cleal *et al.*, 2007a;Poore *et al.*, 2014;Poore *et al.*, 2005) and cardiovascular development (Hawkins *et al.*, 2000a;Burrage *et al.*, 2009;Cleal *et al.*, 2007a), often independent of direct effects on fetal growth. Malnutrition during pregnancy in animal models has been shown to lead to *in utero* growth retardation because of inadequate nutrient delivery to the fetus (Holemans *et al.*, 2003).

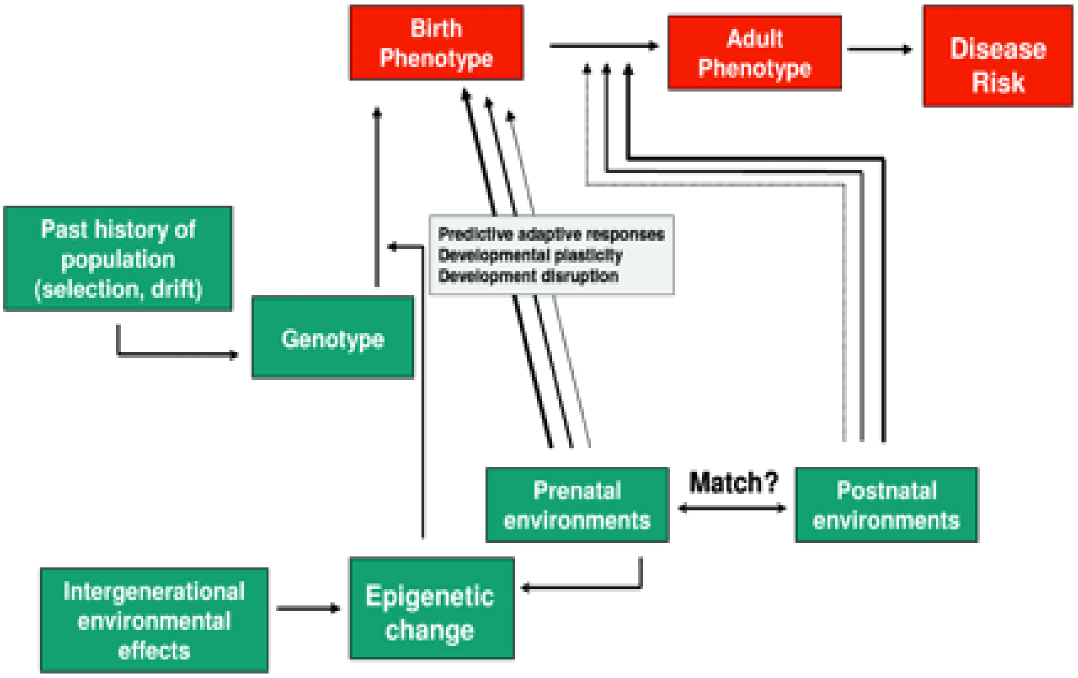


Figure 1.1 A general model of how intergenerational, genetic and environmental and pre and postnatal factors interact to change disease risk in adulthood. If pre and postnatal factors match then the organism is well prepared for the postnatal environment (Gluckman & Hanson, 2004b).

1.3 Predictive Adaptive Responses

Some responses made by the developing fetus may not have immediate survival or adaptive value but may be made to promote survival to reproductive age in a predicted postnatal environment. These have been named Predictive Adaptive Responses (PARs) and it is thought that disease occurs when the prediction *in utero* does not match the actual postnatal environment experienced (Gluckman *et al.*, 2005) . One example of a PAR is the coat of the meadow vole which is thicker at birth if the offspring is born at a time of decreasing day length, thus preparing the offspring *in utero* for winter (Gluckman & Hanson, 2004a). These PARs are believed to allow the developing animal to set its postnatal physiological phenotype to match its predicted postnatal environment to give optimal chance of survival to reproduce (Gluckman & Hanson, 2004b). Where a mismatch occurs between the predicted and actual postnatal environment it may lead to an increased risk of disease.

1.4 Key role of liver in metabolism

This section is a review of the metabolism and anatomy of the liver. The relevance of this background information to this thesis is detailed in section 1.7. Metabolism is the name given to the chemical processes occurring within a living cell or organism that are necessary for the maintenance of life. In metabolism some substances are broken down to provide energy for vital processes while other substances, necessary for life, are synthesized. The liver plays a key role in metabolism and has a number of functions in the body, including glycogen storage, glucose production, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. Specifically in glucose homeostasis the liver works in conjunction with muscle, pancreas and adipose tissues, and under the influence of insulin and glucagon, to regulate the storage and release of glucose into the circulation. The liver both stores and produces glucose and is therefore a key organ in the process.

1.4.1 Anatomy of the Liver

The liver is the largest internal organ in the human body and lies immediately below the diaphragm. It is supplied with blood through two major vessels: the hepatic artery (which supplies 20% of the blood) and the hepatic portal vein. The portal vein carries blood that has passed through the vessels around the intestinal tract. The liver therefore receives its main blood supply via a vein and this gives the liver a special role in metabolism (Frayn, 2003). The portal vein is short and is formed by the joining of veins from different parts of the intestinal tract including the stomach and spleen. These veins carry substances absorbed from the intestinal tract into the blood. Water soluble substances from the diet are first taken to the liver before entering the general circulation. The pancreatic veins also join the portal vein before it enters the liver and these carry blood containing insulin and glucagon. These hormones exert their effects initially on the liver before being diluted and entering the general circulation (Rappaport, 1980). Blood leaves the liver through hepatic veins which enter the inferior vena cava (the main blood vessel returning blood from the lower parts of the body to the heart). The other important system of vessels associated with the liver carry bile to the gall bladder. Bile contains the bile salts that are crucial to the digestion and absorption of fats in the diet. These vessels are also used as a route for excretion of organic compounds detoxified by the liver.

80% of the liver is composed of one cell type – the hepatocyte. Other cell types include the phagocytic Kupffer cells and endothelial cells. Hepatocytes are arranged in a very characteristic manner which appear in cross section as hexagonal units or lobules, each around 1mm across (fig.1.2). At each corner is a triad of three vessels: tiny branches of the portal vein, the hepatic artery and the bile duct. In the centre of the lobule is a branch of the hepatic vein that carries blood away. The hepatocytes radiate out from the central vein. Blood flows from the triads towards the central vein in small passages between the hepatocytes, the sinusoids; these are equivalent to the capillaries in other tissues and are lined by flat endothelial cells.

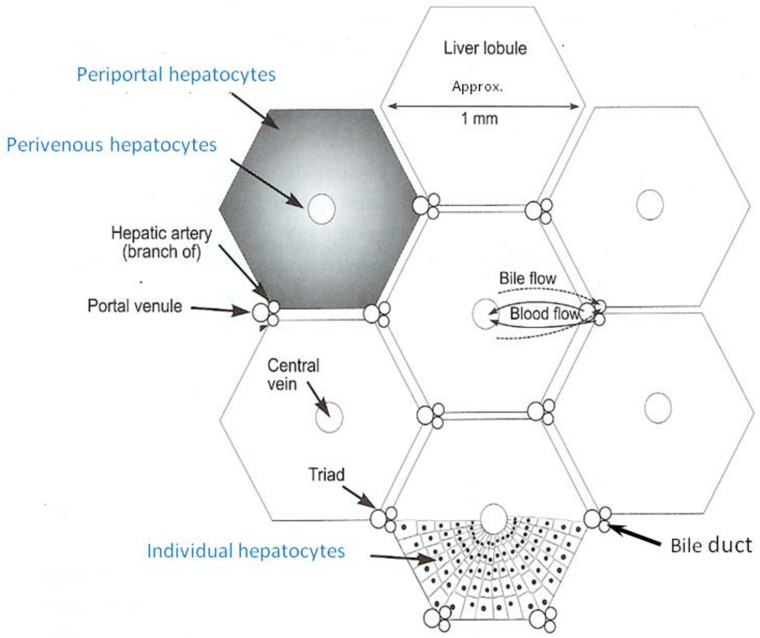


Figure 1.2 Arrangement of hepatocytes. In a cross section of the liver, the hepatocytes appear to radiate out from a central vein (modified from Frayn, 2003).

Blood in the sinusoids is in close contact with the hepatocytes. Bile formed in the hepatocytes passes out to the bile duct branch in the triad along the lines of hepatocytes by the bile canaliculi. Metabolic zonation of hepatic metabolism occurs within the liver whereby the arrangement of hepatocytes is related to the function. Periportal hepatocytes (hepatocytes on the outside of the lobule) are exposed to blood which has recently arrived at the liver in the portal vein and hepatic artery. These cells are well oxygenated and supplied with substrates and therefore oxidative metabolism predominates. Gluconeogenesis occurs mainly in these cells whereas glycolysis and ketone production takes place in the perivenous hepatocytes (those nearest the centre of each lobule).

1.4.2 Liver Metabolism

The liver is the first organ to receive nutrients obtained from the intestine after a meal and has a major role in carbohydrate storage and the later release of glucose, but also in fat and amino acid metabolism.

Carbohydrate metabolism in the liver

The major pathways of glucose metabolism and their hormonal regulation are shown in figure 1.3 below.

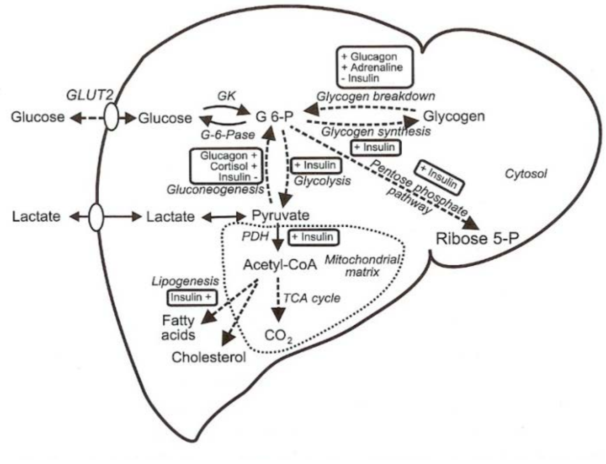


Figure 1.3 Outline of glucose metabolism and its hormonal regulation in the liver. Dashed arrows indicate multiple enzymatic steps. The dotted shape is the mitochondrial membrane. GLUT2 – hepatic glucose transporter, G 6-P – glucose 6-phosphate, LDH – lactate dehydrogenase, PDH – pyruvate dehydrogenase, Ribose 5-P – ribose 5-phosphate, TCA cycle – tricarboxylic acid (Krebs) cycle. + indicates stimulation, - inhibition. No detail of cholesterol and fatty acid synthesis is shown. (Frayn, 2003).

Fed conditions

Following a meal glucose is absorbed into the portal vein. The periportal hepatocytes are exposed to high concentrations of glucose. Liver cells have the GLUT-2 type of glucose transporter which is not responsive to insulin and has a high K_m for glucose so that it functions below saturation (Nordlie *et al.*, 1999). There are a high number of transporters and therefore the rate and direction of movement of glucose across the hepatocyte membrane are determined by the relative glucose concentrations inside and outside the cell. When inside the cell, glucose is phosphorylated to form glucose 6-phosphate by glucokinase. Like GLUT-2, this is unaffected in the short term by insulin. This results in glucose being taken up and phosphorylated by the hepatocyte quickly when the glucose concentration rises outside. Glucose 6-phosphate may enter the pathways of glycolysis or glycogen synthesis. Insulin and glucose both activate the storage of glucose as glycogen. They activate glycogen synthase to synthesise glycogen and glycogen phosphorylase to inhibit glycogen breakdown (Granner & Pilkis, 1990). Because insulin reaches the liver directly from the pancreas control of this regulation is accurate. Glucose 6-phosphate can also be metabolised via glycolysis to pyruvate in hepatocytes. Energy used by the liver itself for its many functions comes from the oxidation of amino acids and fatty acids and not glucose (Frayn, 2003).

Fasted conditions

When glucose is not readily available from a meal the body requires it from other sources namely glycogen and fat. Glycogen is stored in the liver and muscles and its breakdown is controlled by glycogen phosphorylase and the inhibition of glycogen synthase and is initiated by a change in hormones (Gaboriaud-Kolar & Skaltsounis, 2013). Glycogen phosphorylase is regulated by glucagon, adrenaline and noradrenaline (these catecholamines become important in stress situations). As the absorption of a meal is completed brain and muscle tissues still require a supply of glucose but the blood concentration will fall. This induces a change in the hormone balance of insulin and glucagon in favour of glucagon and the hepatic metabolism is directly affected by this change. Glycogen will be broken down to release carbohydrate stores in the liver into the blood. Glycogen is broken down to glucose 1-phosphate which is in equilibrium with glucose 6-phosphate. Glucose 6-phosphate cannot be converted back to glucose by glucokinase but can by glucose 6-phosphatase (Granner & Pilkis, 1990). Glucose 6-phosphatase is a membrane bound enzyme in the endoplasmic reticulum and is associated with subunits that act as transporters for the facilitated diffusion of glucose 6-phosphate and glucose (Hutton & O'Brien, 2009). Neither glucokinase nor glucose 6-phosphatase is regulated directly by hormonal signals and therefore the net flux between glucose and glucose 6-phosphate is determined by their relative concentrations.

Gluconeogenesis is another important function of the liver in glucose metabolism in fasted conditions. It is the synthesis of glucose from other precursors such as lactate, alanine and glycerol (fig.1.3) and, as it formed a major focus of this thesis is discussed in more detail in section 1.10.

Fat metabolism in the liver.

The liver has important roles in fat metabolism. The main pathways of fatty acid metabolism (oxidation and synthesis) in the liver and their hormonal regulation are shown in figure 1.4. In humans the overall rate of fatty acid synthesis from other molecules including glucose is small in comparison to dietary fatty acid intake. The liver may take up non esterified fatty acids (NEFAs) from the plasma and these fatty acids have two fates: oxidation or triacylglycerol formation.

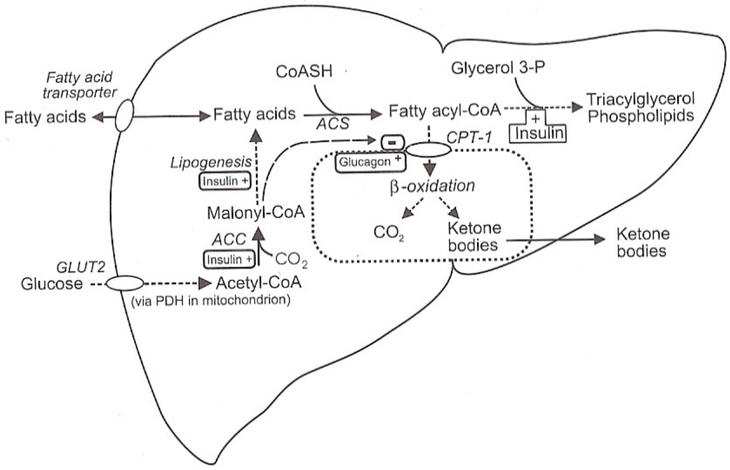


Figure 1.4 Outline of fatty acid metabolism in the liver. Fatty acids cross the hepatocytes membrane by a carrier mediated process. Inside the cell they are transported by specific binding proteins to the cytosol and activated by esterification to coenzyme-A (CoASH) by acyl-CoA synthase (ACS). Fatty acyl-CoA esters are converted to acyl-carnitine derivatives by carnitine-palmitoly transferase-1 (CPT-1) in order to enter the mitochondrion for oxidation in the tricarboxylic acid cycle (TCA). CPT-1 is inhibited by malonyl-CoA. Insulin inhibits fatty acid oxidation by increasing the concentration of malonyl-CoA and stimulating fatty acid esterification to form triaglycerol (Frayn, 2003).

Fatty acid oxidation

The liver may oxidise fatty acids by the mitochondrial β-oxidation pathway to produce energy for its own metabolic functions. An alternative β-oxidation pathway in peroxisomes operates to shorten very long chain fatty acids using different enzymes and, in rats, is upregulated by PPARα. In humans PPARα activation increases fatty acid oxidation in the mitochondria. As a fatty acid derivative is a natural ligand for PPARα this may be a method for increasing the oxidation when fatty acid supply is high. Gluconeogenesis is fuelled by the oxidation of fatty acids. The pathway of fatty acid oxidation diverges from glycerol lipid synthesis when acyl-CoA enters the mitochondrion for oxidation. The acyl group is transferred to carnitine by carnitine-palmitoyl transferase-1 (CPT-1) to enable transfer across the mitochondrial membrane. The activity of this enzyme is controlled by malonyl-CoA which provides a role between fat and carbohydrate metabolism (McGarry, 1998). During the oxidation of fatty acids in the liver ketone bodies (acetoacetate and 3-hydroxybutyrate) are produced and exported to the circulation. Ketogenesis is regulated by several steps but ketone body production is determined by the rate of fatty acid oxidation (Frayn, 2003).

Lipid Synthesis

The alternative route for fatty acid metabolism in the liver is esterification to form triacylglycerol which is stored in the hepatocytes. In addition lipids – glycerolipids (phospholipids and triacylglycerol) and cholesterol may be synthesized from non-lipid precursors such as glucose and amino acids via actyl-CoA. These pathways are stimulated by insulin in the short term. Therefore under conditions when glucose is in excess it is converted to lipids which, in addition to fatty acids taken up by the liver, are used for glycerolipid synthesis rather than oxidation. Malonyl-CoA coordinates glucose and fat oxidation by inhibiting fatty acid entry into the mitochondrion for oxidation. Fatty acids are also diverted from oxidation into glycerolipid synthesis. In fed conditions when insulin is raised malonyl-CoA will be high and fatty acid oxidation will be inhibited (Foster, 2012). Fatty acids will be diverted into esterification with glycerol 3-phosphase. So, the liver stores fatty acids as triacylglycerol in a fed state. Hepatic energy requirements will in these circumstances be met by amino acid oxidation. The triacylglycerol pool is dedicated to use by the liver (adipose stored triacylglycerol supplies the rest of the body) and is used as the substrate to release fat into the blood stream in the form of VLDL (very low density lipoprotein) (Frayn, 2003).

Amino acid metabolism.

Under normal circumstances in adult life the body does not continuously accumulate or lose protein. The rate of amino acid oxidation in the body must balance the rate of entry of dietary protein. The liver first receives dietary amino acids via the portal vein and is the only organ able to eliminate nitrogen from amino acids by synthesizing urea for secretion by the kidneys. Amino acids as well as providing half the hepatic energy requirements through oxidation also provide a substrate for the synthesis of glucose, fatty acids and ketone bodies. They also serve as precursors for hepatic synthesis of the proteins required by the liver and other proteins exported into the circulation such as albumin (Frayn, 2003).

1.5 The Fetal Liver

1.5.1 Liver Development

The liver develops around day 20 in sheep (term is 147-150 days) as an endodermal outpocketing of the foregut called the hepatic diverticulum. Its initial blood supply is primarily from the vitelline veins that drain blood from the yolk sac. Lobes appear in the sheep liver as early as the 21st day and by the 23rd day the liver is lobated deeply into left and right lobes with a small caudate lobe between them (Bryden *et al.*, 1972). The large cranial portion of the hepatic diverticulum gives rise to the hepatocytes and bile ducts, while the small caudal part becomes the gallbladder and its associated cystic duct (Blackburn, 2003). Bile secretion can be observed in humans at 12 weeks gestation and full maturity does not occur until up to two years after birth (Beath, 2003).

1.5.2 Fetal circulation

In the growing fetus, the fetal liver is the first organ to receive maternal nutrients. The umbilical vein provides a major source of blood to the liver which supplies nutrients to the growing fetus (fig.1.5). The umbilical vein enters the abdomen at the umbilicus, and passes upward along the free margin of the falciform ligament of the liver to the inferior surface of the liver. There it joins with the left branch of the portal vein. The ductus venosus carries blood from the left portal vein to the left hepatic vein and then to the inferior vena cava, allowing placental blood to bypass the liver. In cases of hypoxia more blood is shunted away from the liver by the ductus venosus to the heart and brain creating the “brain sparing” effect (Blackburn, 2003).

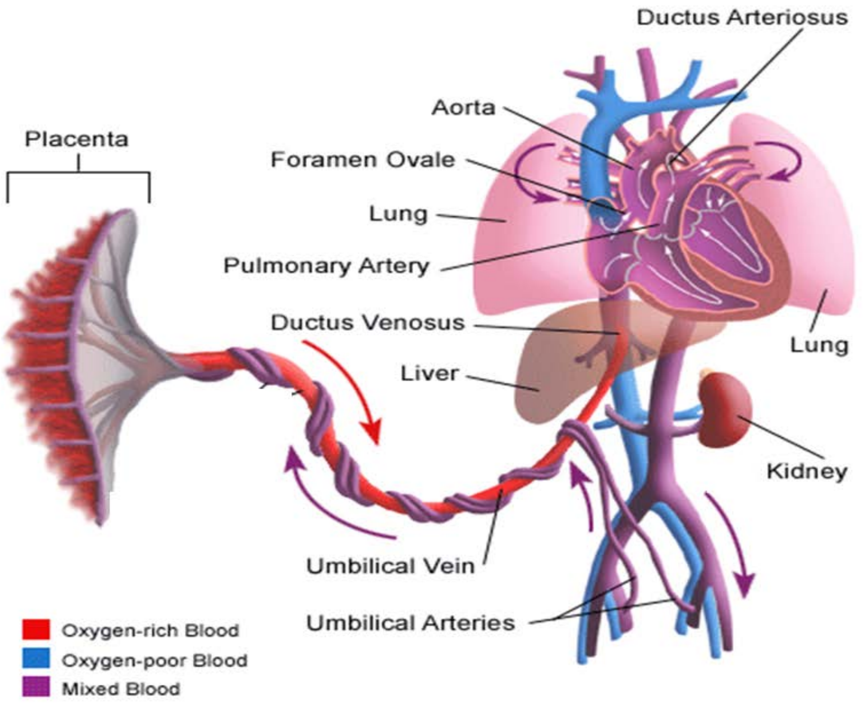


Figure 1.5 The fetal circulation. The fetal circulation showing umbilical venous supply to the fetal liver and the ductus venosus (modified from http://www.chw.org/display/PPF/DocID/23045/router.asp).

1.5.3 Liver lobe differences

The fetal liver has a unique blood supply that meets varying nutrient levels and stimuli to the left and right lobes (fig.1.5). Liver lobes can be seen in figure 1.6. The fetal left liver lobe receives a greater blood supply than the right liver lobe due to the differential blood supply between the left and right lobes derived from the umbilical vein.

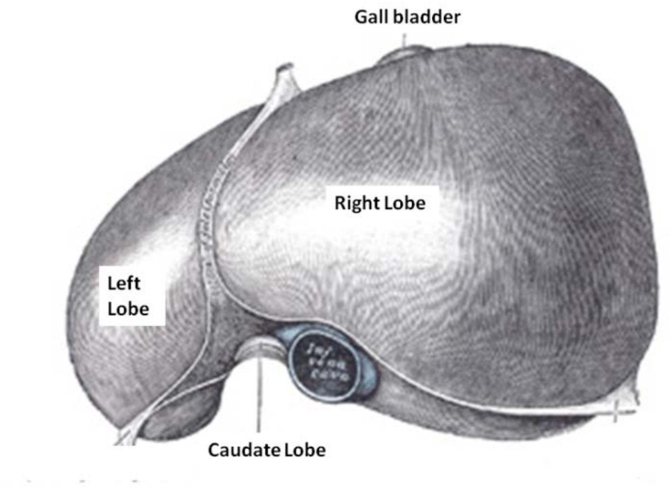


Figure 1.6 Superior view of the liver. Modified from Gray's Anatomy, 1918 (Wikipedia contributors, 2006).

Thus the left liver lobe should normally receive a greater supply of nutrients, oxygen, growth factors and hormones. Lobar differences in liver have been seen in studies on adult rats that looked at fibrinogen gene expression in the livers from offspring of dams fed a control (20% protein) or low protein diet (MLP: 8% protein) (Zhang & Byrne, 2000). Another study carried out on fetal baboons has shown differential gene expression between left and right liver lobes in 875 genes (Cox *et al.*, 2006).

1.6 Animal models and relevance to humans

It has been difficult to study the effects of early life nutrition and the onset of adult diseases in humans due to the length of time between fetal and adult life but human cohort studies have proved invaluable and include the Dutch famine cohort (Roseboom *et al.*, 2001), the Hertfordshire cohort (Syddall *et al.*, 2005a) and the Southampton Women's Survey which has recently studied a cohort of women before and during pregnancy and subsequent early childhood life (Inskip *et al.*, 2006).

Animal models are crucial to the study of the origins of disease because several factors can be controlled including dietary manipulation (global restriction, low protein, high fat, low carbohydrate etc.), genetic modification (gene knockout/knock in), surgical and pharmacological interventions (reviewed in Bertram & Hanson, 2001). Rodents are often preferred models because they have a relatively short gestation period (typically 21 days), have genome information available, are cheaper, and are easier to house and maintain than larger mammals. However in order to more closely associate with the human, sheep are often used due to their predisposition to singleton pregnancies and the fact that, like humans but unlike rodents, they are precocial developers – having the full complement of nephrons and cardiomyocytes by term. The ovine gestation period is longer than that of rodents at typically 147 days duration, but this is still shorter than the human gestation period of 280 days (40 weeks). The fetuses are also able to handle surgical intervention and the high rate of singletons helps comparison to humans as a well-defined model of fetal physiology.

1.7 Evidence from humans and animals of early origin of metabolic disease implicates the liver

1.7.1 Human

Initial observations in humans revealed that babies who had received glucocorticoid *in utero* were small at birth and that low birthweight is associated with metabolic disease in later life. People who were *in utero* during the Dutch Winter famine and therefore experienced severe undernutrition gestationally had decreased glucose tolerance and increased insulin at the age of 50 (de Rooij *et al.*, 2006). In India, there is an epidemic of type 2 diabetes thought to be associated with the undernutrition in fetal life and low birthweight and the increasing improvement in nutrition postnatally (Yajnik, 2004). Fetuses of mothers with lower body fat stores and those eating an unbalanced diet had a greater liver blood flow and shunted less blood away from the liver via the ductus venosus (Haugen *et al.*, 2005) and offspring with a greater fetal liver blood flow in the third trimester were shown to have a greater fat mass at birth and at 4 years of age (Godfrey *et al.*, 2012).

1.8 Animal models

1.8.1 Nutritional models

In rats a maternal low protein diet (MLP, 9% protein content, control was 18%) has been used, primarily throughout gestation, and has been found to cause smaller livers and lower liver enzyme activity in newborns compared with controls (Kang-Lee & Harper, 1975;Belfiore *et al.*, 2009a;Belfiore *et al.*, 2009b). A MLP diet throughout gestation has also been found to increase hepatic PEPCK activity in 21 day and 11 month old rats (Desai et al., 1997) and to increase hepatic glucose output and cause liver structural changes in 5 month old offspring (Burns *et al.*, 1997). A MLP diet fed for the peri-implantation period resulted in increased male fetal liver (20dGA) PEPCK mRNA expression (Kwong *et al.*, 2007). In sheep a global nutrient restriction is the method commonly used for nutrition reduction. Early gestation nutrient restriction (50% of requirements during 0 – 95 days) has been shown to lower the liver weights and hepatic mRNA expression of growth related genes in 3 year old male sheep when compared to control animals (Hyatt *et al.*, 2007b). Impaired glucose tolerance was also found in male sheep at 63 and 250 days of age following a period of 50% undernutrition during early to mid gestation days 28 – 78. These sheep were also found to be heavier than those fed a control diet and also had more back-fat (Ford *et al.*, 2007). Also a late gestation (110-term) 50% global nutrient restriction was applied to sheep and these were compared to a group of control animals who had received 100% of the nutrient requirement throughout gestation. At 1 year of age the late gestation animals were found to have impaired glucose tolerance (Gardner *et al.*, 2005).

Sheep studies in which a match or mismatch in nutrition was created between the early gestation and early postnatal periods showed that males had accelerated growth following a postnatal undernutrition challenge and increased blood pressure response but this was not the case if the challenge was also received gestationally (Cleal *et al.*, 2007a). Females were shown to grow slower, but were fatter and more glucose tolerant following postweaning undernutrition regardless of prenatal challenges (Poore *et al.*, 2007).

Thus it appears that the early and late gestation periods coupled with the intensity of the nutritional challenge and possible mismatch with the postnatal environment are critical to the development of key organs and processes in early life.

1.8.2 Glucocorticoid models

Prenatal exposure to corticosteroids has been associated with a reduction in birthweight (Reinisch *et al.*, 1978) and postnatal alterations in glucose homeostasis (Lindsay *et al.*, 1996). Adult rats from mothers who were exposed to glucocorticoids in late gestation were found to have increased hepatic GR and PEPCK mRNA expression (Nyirenda et al., 1998) and in sheep, increased activity of G6Pase was found in adults prenatally exposed to glucocorticoids (Sloboda *et al.*, 2005). Repeated doses of glucocorticoids resulted in increased 11β-HSD (11β- hydroxysteroid dehydrogenase) and CBG (corticosteroid-binding protein) mRNA expression in fetal sheep (Sloboda *et al.*, 2002).

1.9 Epigenetics & developmental origins of disease

It is clear from the evidence that stress (be it from the lack of oxygen, nutrition or glucocorticoid administration) and the severity and timing in early life influence the possible risk of adult disease. These changes may be occurring at the level of the DNA whereby epigenetic modifications alter the transcription of the genes without changing the genetic code. These changes are able to be passed on through cell division. This epigenome is set up in early life and is, during this time vulnerable to influence from the environment. Epigenetic processes are responsible for determining when and where specific genes are expressed. Therefore altered epigenetic regulation is a possible mechanism that could underpin the changes in gene expression seen in programmed offspring. The major epigenetic processes are DNA methylation, histone modification and non-coding RNAs which work to expose (allow) or shield (prevent) promoter regions to factors that control or direct transcription (reviewed in Relton & Davey, 2010).

1.9.1 DNA Methylation

Methylation (addition of a methyl group (CH_3)) in DNA takes place at the 5 position of cytosine within a CpG (Cytosine-p-Guanine) dinucleotide (the p denotes the intervening phosphate group) and is a common modification in mammalian genomes which constitutes a stable epigenetic mark that is transmitted through DNA replication and cell division. CpG dinucleotides are found clustered at the 5' ends of genes in regions known as CpG islands. Hypermethylation of these CpG islands is associated with transcriptional repression, while hypomethylation of CpG islands is associated with transcriptional activation. However, hypomethylation does not mean that the gene will be expressed just that it has the ability to be expressed (Tweedie *et al.*, 1997). DNA methylation is important for asymmetrical silencing of imprinted genes, X chromosome inactivation and for cell specification and tissue specific gene expression and is largely established during embryogenesis or in early postnatal life. Maternal and paternal genomes undergo extensive demethylation after fertilisation followed by global methylation *de novo* just prior to blastocyst implantation during which 70% of CpGs are methylated. Methylation of tissue specific genes occurs throughout development in gestation and early postnatal life and determines the developmental fates of differentiating cells. The methylation of CpG dinucleotides *de novo* is catalyzed by the DNA methyl transferases (Dnmt) 3a and 3b, and is maintained through mitosis by gene-specific methylation of hemi-methylated DNA by Dnmt1. However DNA methylation, once established, is essentially maintained throughout life (although hypomethylation can occur as a result of ageing) (reviewed by Lillycrop & Burdge, 2012).

1.9.2 Histone modification

The DNA in cells is packaged as chromatin of which the basic unit is a nucleosome comprising 147bp of DNA wrapped around a core of histone proteins (two copies of histone H2A, H2B, H3 and H4). Histone proteins contain two domains: a globular and an N terminal domain. Modifications to the N terminal tails of the histones (by acetylation, methylation, ubiquitination, and phosphorylation) lead to the recruitment of effector proteins which in turn induce an open or closed chromatin structure to bring about specific cellular processes. The combination of these proteins with the histone tails is known as the histone code (reviewed by Lillycrop & Burdge, 2012). Histone acetylation is associated with active chromatin states, while the methylation of lysines can either be an active or repressive mark depending on the specific lysine involved. Many families of histone-modifying enzymes have been identified, the so called 'writers of the code' and these include the histone acetyl transferases, and methyl transferases, while the 'erasers' include the deacetylases, and demethylases.

Crosstalk can occur between DNA methylation and histone modification. Methylated DNA is bound by Methyl CpG binding protein-2 (MeCP2) which can recruit both histone deacetylases (HDACs) (which remove acetyl groups from the histones), a signal of transcriptionally active chromatin, and histone methyl transferases (HMTs) such as Suv39 which methylates lysine 9 on H3, resulting in a closed chromatin structure and transcriptional silencing. Studies have shown that Dnmt1 can be recruited by a number of histone-modifying enzymes such as HDAC1 and HDAC2, and the histone methyl transferases SUV39 and EZH2 suggesting that chromatin structure may also determine DNA methylation status and that there is a reciprocal relationship between these two processes (Brenner & Fuks, 2007).

1.9.3 **Non-coding RNAs**

Epigenetic regulation has also been found to be under the influence of non-coding RNAs (ncRNAs). Non-coding RNAs can either act in cis or in trans. The cis-acting ncRNAs are the long/macro- ncRNAs (up to 100,000 nt) and the trans acting ncRNAs include the microRNAs (miRNAs) (~22 nt), which mostly target the 3' untranslated region of mRNAs for degradation (Siomi & Siomi, 2010). Recent studies have shown that the human miRNAs can also induce chromatin remodelling, suggesting that DNA methylation, histone modification and miRNAs may work in together to regulate gene expression (reviewed by Lillycrop & Burdge, 2012).

1.9.4 **Animal studies**

Several animal studies have demonstrated the epigenetic changes that occur in the young induced by either maternal behaviour or nutrition. Increased maternal care during the first week of life was found to reduce methylation in hippocampal exon 1(7) GR (Weaver *et al.*, 2004), and this effect was reversed in adult life following supplementation of L-methionine, a precursor to S-adenosyl-methionine that serves as the donor of methyl groups for DNA methylation, highlighting a potential reversal of programmed effects (Weaver *et al.*, 2005). Hepatic GR methylation was found to be lower in 34d rats from mothers fed a low protein diet throughout gestation; however, when this diet was supplemented with folic acid the methylation of GR did not differ from controls (Lillycrop *et al.*, 2005). In sheep, hypothalamic GR methylation was found to be decreased in 5 year old male and female sheep from mothers fed 50% of nutrient requirements periconceptionally (Begum *et al.*, 2013). The lack of genome information for the sheep makes data for this model rare.

1.9.5 **Methods for measuring DNA methylation**

There are various methods available to measure DNA methylation levels and these vary in cost, ease of use, sensitivity, scale and level of throughput. The methods range from simple, which measure average DNA methylation over a stretch of DNA, to methods which look at individual CpG methylation of the ‘whole genome’. The decision on which method is used will therefore depend on cost, level of detail and level of throughput required. The more simple methods are used for an initial look at DNA methylation of a known region of DNA. Methylation sensitive PCR is a simple semi-quantitative method which uses methylation sensitive restriction enzymes to differentiate between methylated and unmethylated sequences in DNA. Subsequent real time PCR will only amplify amplicons containing undigested methylated DNA, whilst digested unmethylated DNA is unable to be amplified. More sensitive and expensive quantitative methods are available which look at the methylation status of individual CpGs within a stretch of DNA, for example Pyrosequencing or Sequenom analysis. These methods are thought to be the ‘gold standard’ for measuring DNA methylation of specific CpGs within a short stretch of DNA. Pyrosequencing uses bisulfite converted DNA and PCR of the region of interest to introduce a C-T SNP (single nucleotide polymorphism) which depends on the methylation status of the CpG; methylated C remains a ‘C’, whereas unmethylated C becomes a ‘T’. This SNP is accurately quantified in sequencing by synthesis reaction. This involves taking a single strand of DNA and synthesising its complementary strand. The method detects the activity of DNA polymerase with chemiluminescence. The method allows sequencing of a strand of DNA one base pair at a time thus detecting what was added at each step. The Sequenom analysis uses bisulfite converted DNA which is then amplified, treated, reverse transcribed, digested then assessed on a CHIP array to give quantative methylation data including the ratio of methylated to unmethylated DNA.

Whole genome technology can determine the methylation status of the “whole genome”, on a larger scale but this can be expensive to use. For example, ChIP-ChIP can be performed in which antibodies (e.g. for methylated cytosine) or protein complexes (e.g. MBD2) are used to capture methylated DNA from sonicated chromatin, whilst the unmethylated DNA is washed away. The captured methylated DNA can then be applied to an array for samples to be identified. Alternatively, enriched methylated samples may be sequenced and then mapped (ChIP-Seq), for example using the Illumina Genome Analyzer and Solexa sequencing.

In this thesis I used the methylation sensitive PCR method to obtain preliminary methylation data.

1.10 Gluconeogenesis and candidate genes

1.10.1 Gluconeogenesis

One important function of the liver in glucose metabolism is gluconeogenesis (the synthesis of glucose from other precursors) (fig.1.7). The substrates for gluconeogenesis are lactate, alanine and glycerol. The gluconeogenic pathway is controlled by the rate of supply of the substrate and by hormonal regulation of the key enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose 1-6 bisphophatase and glucose 6 phosphatase (G6Pase). Gluconeogenesis is stimulated by glucagon and inhibited by insulin (Dashty, 2013). Stimulation by glucagon takes place as a result of the action of transporters in the uptake of substrates from the blood into the liver cell. The net result is that when glucagon is higher than insulin the liver will produce glucose 6-phosphate to export as glucose. Therefore the processes of gluconeogenesis and glycogenolysis will be active at the same time (Frayn, 2003).

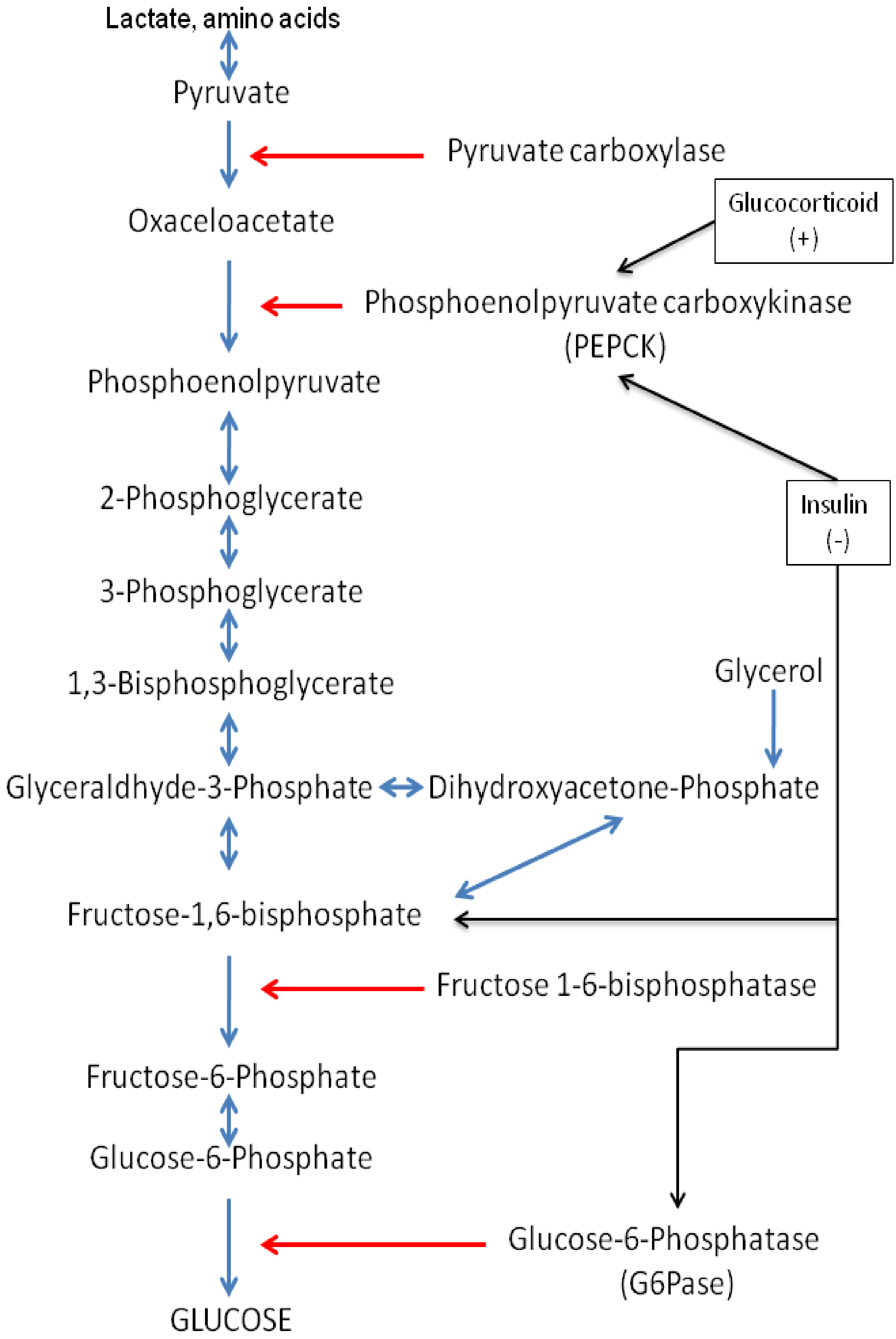


Figure 1.7 **Gluconeogenic pathway.** Including action of glucocorticoid and insulin on pathway. Red arrows indicate enzymatic action.

1.10.2 **Candidate genes**

Glucocorticoid Receptor (GR)

Glucocorticoids are essential steroid hormones that are secreted by the adrenal cortex and affect multiple organ systems. They act to regulate the transcription of their target genes through the GR. The GR is a nuclear steroid receptor whose endogenous ligand is the adrenal hormone cortisol. There are three known isoforms GRα, GRβ and GRγ. GRα modulates the expression of glucocorticoid responsive genes, GRβ does not bind glucocorticoid and is therefore inactive and the function of GRγ is unknown (Rosmond, 2002). GRα was investigated in this thesis. The enzymes 11β-hydroxysteroid dehydrogenases (11βHSDs) interconvert active and inactive glucocorticoids. There are two known isozymes, 11βHSD1 and 11βHSD2. 11βHSD1 is found in the liver and adipose tissue and acts in the opposite way to 11βHSD2 in that it converts cortisol from inactive cortisone (Wake & Walker, 2004). These enzymes act as regulators for the receptors.

In the absence of glucocorticoids the GR is sequestered in the cytoplasm by a protein complex that includes heat shock protein 70 and HSP90. When glucocorticoids are present they cross the plasma membrane, bind to the GR which enables dissociation from the protein complex and translocation to the nucleus. Within the nucleus the ligand bound GR binds to DNA as a monomer or dimer to palindromic glucocorticoid response elements and modulates transcription. The GR can activate and repress transcription of hundreds of genes including some of those involved in gluconeogenesis and amino acid metabolism (Phuc *et al.*, 2005).

Glucocorticoid in gluconeogenesis

Glucocorticoid affects the intermediary metabolism which includes glucose, fatty acids, and amino acids. Cortisol is the most important glucocorticoid and regulates gluconeogenesis by acting on PEPCK and G6Pase. Glucocorticoids enhance transcription of these two genes whilst insulin represses basal and hormone induced expression. PEPCK is the first enzyme in the gluconeogenic pathway and G6Pase is the last. To exert these actions, glucocorticoids diffuse through the plasma membrane and bind to cytosolic receptors. The hormone bound receptor translocates to the nucleus and occupies the specific response elements. Response elements are specific DNA sequences which are docking sites for cognate transcription factors. The ligand bound GR binds the sequence as a dimer. PEPCK is controlled at the level of its transcription and has a glucocorticoid response unit which consists of two response elements and three accessory factor elements (AF1, AF2 and AF3) and a cAMP response element. The ligand bound GR binds to Glucocorticoid Response Element (GRE) 1 and GRE2 to enable transcription. Insulin binds to AF2 of the GRU and triggers dissociation of the transcription initiation complex.

G6Pase is located in the endoplasmic reticulum and is a multicomponent system with a catalytic unit and transporters for G6P and glucose. The occupancy of three positive elements of ligand bound GR and binding of other cofactors are essential of G6Pase by glucocorticoids. Insulin acts on a separate hormone response unit within the gene (Yabaluri & Bashyam, 2010).

Animal studies

Several animal studies have examined the effect of maternal nutrition on hepatic GR expression in the offspring. Maternal nutrient restriction of 50% of the nutritional requirement during days 28 – 77 of pregnancy in sheep (the time of rapid placentation) increased GR expression in liver, lung and kidney in the offspring (Whorwood *et al.*, 2001). In rats maternal behaviour such as pup licking, grooming and arched back nursing in the first week of life has been shown to increase the expression of the GR in the brain of the offspring in both cross fostered and their own pups (Weaver *et al.*, 2004). This gene is of particular interest because alterations in its expression are associated with disturbances in cardiovascular and metabolic control in animals and humans. In adults, GR activity is important for regulation of blood pressure.

Unbalanced maternal nutrition affects the methylation status and expression of the GR gene in the liver of rat offspring post weaning. Dams fed a reduced protein diet throughout pregnancy produced offspring that had lower methylation and increased expression of GR than those from dams fed a control diet. Interestingly when the maternal diet was supplemented with folic acid the methylation and expression differences were not observed (Lillycrop *et al.*, 2005). Maternal overfeeding postnatally of rats (by way of reduced litter size) has also been shown to upregulate the GR mRNA expression in the adult rat adipose tissue (Boullu-Ciocca *et al.*, 2005).

PEPCK

Phosphoenolpyruvate carboxykinase is an enzyme in the lyase family and thought to be the rate limiting enzyme in the metabolic pathway of gluconeogenesis as it is the first enzyme in the pathway and converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. It is found in two forms PEPCK-C (cytosolic) and PEPCK-M (mitochondrial). PEPCK-C is found in a number of mammalian tissues with the highest expression found in liver, kidney cortex and adipose tissue and has been shown to participate in lipid metabolism as part of glyceroneogenesis (a process to generate 3-phosphoglycerol from pyruvate) and cataplerosis (removal of citric acid anions generated from the degradation of amino acids). Little is known about PEPCK-M but there is an equal activity of the two isoforms in humans, very little PEPCK-C in the mouse and only PEPCK-M in adult birds. It is thought that PEPCK-M is involved in gluconeogenesis from lactate. In the liver, glucagon and glucocorticoids increase the transcription of PEPCK-C increasing the rate of gluconeogenesis and glyceroneogenesis in the fasted state. This results in increased hepatic glucose output and triglyceride recycling (Chakravarty *et al.*, 2005). In this thesis PEPCK-C was the form studied.

Animal studies

Several animal studies have examined the effect of the maternal environment on targeted hepatic PEPCK expression in offspring. Fetal rats from mothers fed a low protein diet in later pregnancy were found to have increased PEPCK activity, but not when the diet was administered in early pregnancy (Franko *et al.*, 2009). Glucocorticoid exposure in late gestation was found to increase the mRNA expression and activity of PEPCK in adult rats compared to controls (Nyirenda *et al.*, 1998). Fetal sheep (136dGA) were found to have increased PEPCK mRNA expression following a periconception and preimplantation period of maternal undernutrition (Lie *et al.*, 2014). PEPCK mRNA expression was also found to be increased following late gestation chronic hypoglycaemia in 130dGA fetal sheep (Rozance *et al.*, 2008) and also with placental insufficiency IUGT fetal sheep at 134dGA (Thorn *et al.*, 2009).

G6Pase

Glucose-6-phosphatase is found in the liver and kidney and catalyses the final step in the gluconeogenic pathway, allowing for the release of glucose into the bloodstream. It is thought to exist as a multi component integral membrane system. Two subunits form a system, G6Pase and G6Pase Transporter and both are associated with the endoplasmic reticulum membrane. G6PT translocates glucose-6-phosphate from the cytoplasm into the lumen where G6Pase hydrolyses the G6P into glucose and phosphate. G6Pase transporter only transports G6P in the presence of G6Pase so together they regulate gluconeogenesis (Chou *et al.*, 2002).

Animal studies

Fetal rats from mothers fed a low protein diet in later pregnancy were found to have increased G6Pase activity, but not when the diet was administered in early pregnancy (Franko *et al.*, 2009) and rat pups from mothers fed MLP diet throughout gestation were also found to have lower activity of G6Pase (Kang-Lee & Harper, 1975). However, glucocorticoid exposure in late gestation was found to have no effect on the mRNA expression or activity of G6Pase in adult rats compared to controls (Nyirenda *et al.*, 1998).

Insulin receptor

The insulin receptor is located in the plasma membrane and is activated by insulin, insulin like growth factor I and IGF II. It is expressed in the main insulin target tissues of adipose, liver and muscle, but is also found in brain, heart and kidney. It is most expressed in adipose tissue, with liver having 30%, and muscle 10% the levels of adipose. The insulin receptor exists in two isoforms IR-A and IR-B. IR-A has a high affinity for IGFII and IR-B has a role in metabolic insulin action. IR-B is the type most expressed in liver and was the isoform examined in this thesis. The insulin receptor consists of two subunits (α and β) held together by disulfide bonds. Ligand binding to the α unit stimulates a conformational change which brings the β units together and leads to changes which facilitates recruitment of insulin receptor substrate proteins (IRS). IRS binding leads to increased GLUT4 (a glucose transporter) on muscle and adipose membranes. (The liver has the GLUT2 glucose transporter which has a low affinity and allows glucose to equilibrate across the membrane without the need of insulin (Thorens, 1992)). Insulin activates hexokinase in the liver which phosphorylates glucose, trapping it in the hepatocyte. It also activates phosphofructokinase and glycogen synthase to enable the synthesis into glycogen for storage. It also inhibits PEPCK and G6Pase therefore stopping the production and release of glucose into the blood stream (Belfiore *et al.*, 2009a).

Animal studies

Animal studies have investigated the hepatic insulin receptor and the effect of maternal environment on its expression in the offspring. Male rats from MLP mothers were found to have increased insulin receptor numbers compared to controls (Ozanne *et al.*, 1996). Fetal rats (22dGA) were found to have increased insulin receptor mRNA expression following MLP diet throughout gestation (Nusken *et al.*, 2011).

1.11 Hypotheses

When I commenced my PhD there was good evidence from human studies (Haugen *et al.*, 2005;Kiserud & Acharya, 2004) that the fetal liver blood flow was capable of adapting to an altered maternal environment, including nutrition, and it was therefore possible that such adaptations might underpin offspring liver metabolism, adiposity and later metabolic health. At the same time, there was also mounting evidence from across the Developmental Origins of Health and Disease field from human and animal studies (Jackson *et al.*, 1996;Kwong *et al.*, 2007;Cleal *et al.*, 2007a;Poore *et al.*, 2007) to suggest that there were critical windows in development, both pre- and postnatally, during which adaptations by the developing offspring physiology could be made. Furthermore a concept had been developed that a mismatch in ‘environments’ (e.g. nutrition) between windows of development might be critical in setting up an individual’s risk of disease. In this thesis I pursued these concepts in the sheep model in relation to the liver gluconeogenic pathway (fig.1.8).

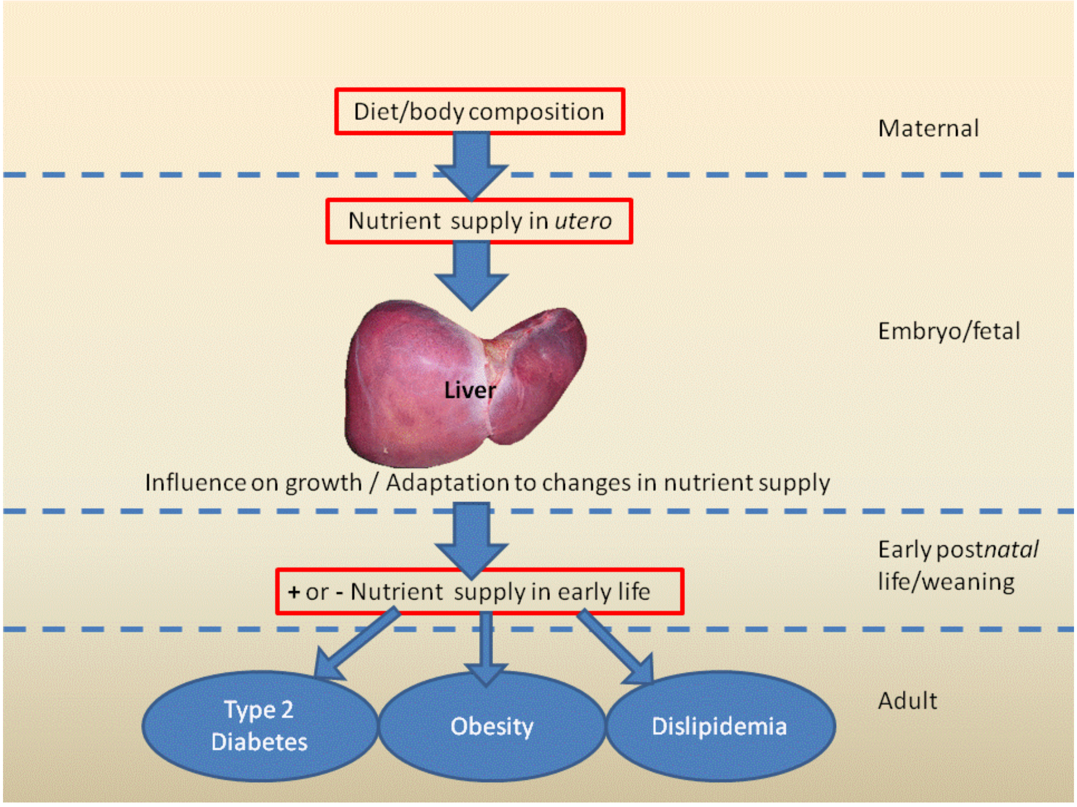


Figure 1.8 Liver as a metabolic target organ, its early life nutritional influences and potential outcomes in adulthood.

1.11.1 Overarching hypothesis

The experiments reported in this thesis have examined the following general hypothesis:

> **Early life undernutrition affects the liver gluconeogenic pathway in sheep.**

1.11.2 Chapter hypotheses:

In order to investigate the mRNA expression in the liver I needed to correct the measurements obtained to allow for experimental variations. Initial investigations with a commonly used housekeeping gene, 18S rRNA, showed that 18S was too abundant and therefore unsuitable for use as a control gene.

Therefore in Chapter 3 I hypothesized that:

The single control gene 18S will not be suitable as a housekeeping gene in sheep liver.

> *In this chapter I established a novel housekeeping gene approach in sheep liver to avoid reliance on a single, highly expressed control gene. The investigation resulted in the development of a kit to enable determining the most stably expressed genes for use as control and the finding that β-actin, GAPDH and RPL-19 were the most suitable housekeeping genes to use in sheep liver.*

Following the findings that the postnatal dietary challenge resulted in metabolic changes in a 2.5 year old sheep cohort, and the new conceptual frameworks of predictive adaptive responses, I investigated if these changes came about through alterations in liver metabolism.

Therefore in Chapter 4 I hypothesized that:

Restricted postnatal nutrition will increase the expression of the hepatic genes involved in gluconeogenesis in adult sheep depending on nutrition received in early gestation.

> *In this chapter I have investigated the expression of key genes involved in gluconeogenesis and following a period of fetal undernutrition combined (or not) with a postnatal period of nutrition challenge, along with investigations into the effect of sex, lobe and offspring number. No differences were found as a*

> *result of dietary challenges; however sex and lobe differences were discovered.*

It was possible that the lack of effect of dietary challenges in Chapter 4 was due to the timing or intensity of the challenge – a more intense challenge in early gestation or a late gestation challenge might lead to changes in hepatic gluconeogenic pathway.

Therefore in Chapter 5 I hypothesized that:

More severely restricted early or late gestation nutrition will increase the expression of the hepatic genes involved in gluconeogenesis in a lobe-dependent manner in the fetus.

> *In this chapter I have investigated the expression of the key genes involved in gluconeogenesis following an early or late gestation nutrient challenge, with increased intensity of the nutritional challenge in early gestation. These challenges were indeed found to have an effect on fetal hepatic gene expression; however, there were no sex differences and only lobe differences in GR and G6Pase mRNA expression.*

Thus a more intense challenge, or one touching upon a later critical window, affected fetal hepatic gluconeogenic gene mRNA expression, but it was not known if this would continue to manifest in adult life. Thus I investigated adults which had experienced a nutritional challenge which changed body composition throughout the whole of gestation.

Therefore in Chapter 6 I hypothesized that:

Maternal body condition before, during pregnancy and suckling affects hepatic metabolism in 4.5 year old male offspring.

> *In this chapter I have investigated the effect of a whole gestation and suckling nutrient challenge on hepatic function in adult male sheep, including metabolic marker measurements before and during a glucose tolerance test prior and subsequent liver*

> *gluconeogenic pathway analysis. I found no differences in basal metabolic marker levels such as cortisol, cholesterol or urea, but did find that the NEFA levels were slower to reach minimum levels in the challenged group. The insulin receptor mRNA was higher in the dietary challenged group, but no other differences in hepatic gene expression were discovered.*

Following the availability of some DNA sequence for the GR promoter region in sheep I decided to investigate the adult sheep whose hepatic mRNA expression had been investigated in Chapter 4. There had been no effect of diet on the hepatic mRNA expression of the genes investigated, but there was still a possibility that methylation changes existed since metabolic alterations had previously been discovered in these animals (Poore *et al.*, 2007).

Therefore in Chapter 7 I hypothesized that:

Restricted early life nutrition either pre or postnatally will reduce the DNA methylation in the hepatic GR in adult sheep.

> *In this chapter I have investigated for the first time possible epigenetic changes in the GR promoter region of the sheep. No differences in GR promoter methylation were found in the region looked at in relation to diet, sex, lobe or offspring number.*

Chapter 2. **General Methods**

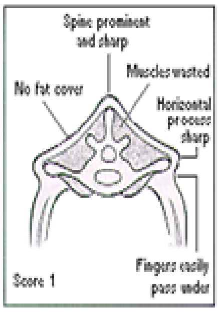
All procedures contained in this thesis were approved by the Home Office and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.1 Sheep

Welsh Mountain sheep were used as they are small, adapted to harsh highland environments which may influence how they respond to undernutrition. They were adopted by this research group 20 years ago because they produce a higher proportion of singleton fetuses compared to other breeds (e.g. Clun Forest). This breed has been used in many studies to characterise the responses to undernutrition (Hawkins *et al.*, 2000b;Poore *et al.*, 2007;Cleal *et al.*, 2007b;Cripps *et al.*, 2008;Burrage *et al.*, 2009;Braddick *et al.*, 2011;Costello *et al.*, 2013).

2.1.1 Body Condition Score (BCS)

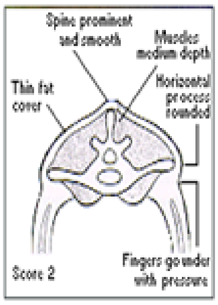
Ewes in the following studies were body condition scored as a means of assessing the degree of fatness of the animal (Russel, 1984). This was carried out by one or two people at the Royal Veterinary College for the studies concerned and the two individuals standardized their conditioning to one another and checked randomly. Briefly, the ewe is palpated in the 3rd lumbar region, on and around the spine immediately behind the last rib and above the kidneys. The degree of sharpness (thin) or roundness (fat) of the spinous processes of the lumbar vertebrate is estimated and so is the prominence and degree of fat cover of the transverse processes. The extent of the muscular and fatty tissues below the transverse processes is judged by the ease with which the fingers pass under the ends of these bones. In addition, the fullness of the eye-muscle area and its degree of fat cover in the angle between the spinous and transverse processes is estimated. The animal is then awarded a score between 0-5, with 0 being extremely emaciated and 5 being very fat (fig. 2.1).



SCORE 1

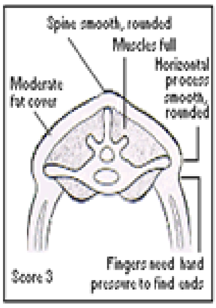
The vertical and horizontal processes are prominent and sharp. The fingers can be pushed easily below the horizontals and each process can be felt.

The loin muscle is thin and with no fat cover.



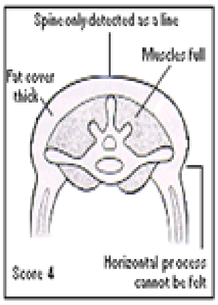
SCORE 2

The vertical processes are prominent but smooth, individual processes being felt only as corrugations. The horizontal processes are smooth and rounded, but it is still possible to press the fingers under. The loin muscle is of moderate depth but with little fat cover.



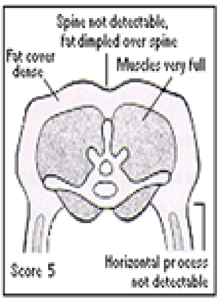
SCORE 3

The vertical processes are smooth and rounded; the bone is only felt with pressure. The horizontal processes are also smooth and well covered; hard pressure with the fingers is needed to find the ends. The loin muscle is full, with a moderate fat cover.



SCORE 4

The vertical processes are only felt as a line; the ends of the horizontal processes cannot be felt. The loin muscles are full and have a thick covering of fat.



SCORE 5

The vertical processes cannot be detected even with pressure; there is a dimple in the fat layers where the processes should be. The horizontal processes cannot be detected. The loin muscles are very full and covered with very thick fat

Figure 2.1 Diagrammatic representation of Body Condition Scoring system. (Adapted from DEFRA Pb No. 1875). Score zero is not shown as it refers to animals that are emaciated to the point of death with no palpable muscle or fatty tissue.

2.1.2 Fat and muscle depth measurement

In all adult sheep (Chapters 4 and 6) ultrasound images of the 3rd lumbar region were collected by John Thompson (RVC) using a real-time ultrasound unit (Aloka SSD 210 DX11, BCF Technology, Livingstone, UK) with a 7.5 MHz linear array transducer. After freezing the digitised image on the screen, subcutaneous fat and muscle depth were measured using the internal callipers of the machine (fig.2.2).

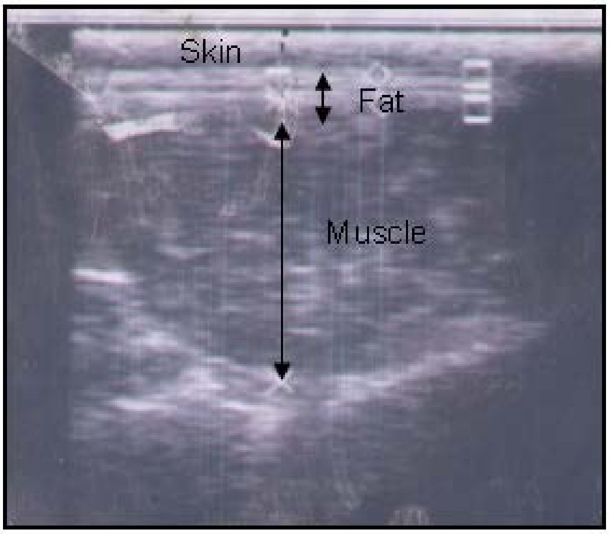


Figure 2.2 An ultrasonic image of the 3rd lumbar region of a sheep.

2.2 Laboratory Methods

2.2.1 RNA extraction

Total RNA was extracted from sheep liver, using TRI REAGENT (Sigma, USA). Frozen tissue (stored at -80°C) was cut using a razor blade on a cooled granite surface placed on dry ice. The recommended amount of tissue (liver, 50 – 70mg) was placed in 1ml of TRI REAGENT and homogenized at high speed until no visible fragments remained. 0.2ml of chloroform was added to the homogenate, mixed by vortex and left to stand at room temperature for 2 – 15 mins, and then centrifuged at 4°C for 15 minutes at 12000 x g. The supernatant was removed and placed into a labelled eppendorf tube. To this tube 0.5ml of isopropanol was added, mixed and allowed to stand at room temperature for 5 - 10 mins. The tRNA mix was then centrifuged at 4°C for 10 mins at 12000 x g. The supernatant was then discarded and the remaining pellet was washed in 1ml of 75% ethanol and centrifuged at 4°C for 5 mins at 7500 x g. The wash was then discarded and the pellet allowed to air dry at room temperature for 5 – 10 mins. 50µl of ddH_20 was added to reconstitute the RNA and then heated at 60°C for 10 mins, mixed again, analysed for RNA yield and quality (see below) and then stored at -80°C.

2.2.2 Determination of RNA yield and quality

A spectrophotometer set at 260 nm and 280 nm was used to determine the quantity and quality of RNA in each sample, after calibration using 500µl H_2O in a quartz cuvette of 1 cm path length. 5µl of sample RNA was added to 495µl ultra-pure H_2O in a second quartz cuvette and the absorbance at 260nm (peak absorbance of RNA) and 280nm (peak absorbance of protein) was recorded. The purity was estimated using the ratio: A_{260}/A_{280} (a good result is 1.7 or above). The yield of total RNA was determined at 260nm, where 1 absorbance unit (A_{260}) equals 40 µg of single stranded RNA/ml:

$$\mu g/\mu l = [(OD_{260} \text{ X } 40\ \mu g/ml/OD\ unit) \text{ X } 0.5\ ml] / 5\ \mu l$$

$$= OD_{260} \text{ X } 4.$$

The purity of the RNA was also determined using gel electrophoresis (fig.2.3). 50ml agarose gel was made (500mg agarose (1%), 50ml 1 X Tris-Acetate-EDTA (TAE) buffer, 1.5µl ethidium bromide) and 10µl of the following mix was added to each gel well: 5µl ultra-pure water, 2µl deionised formamide, 1µl Northern loading buffer, 2µl sample RNA. The gel was set to run for 30 minutes at 90V and then removed and photographed (UV light).

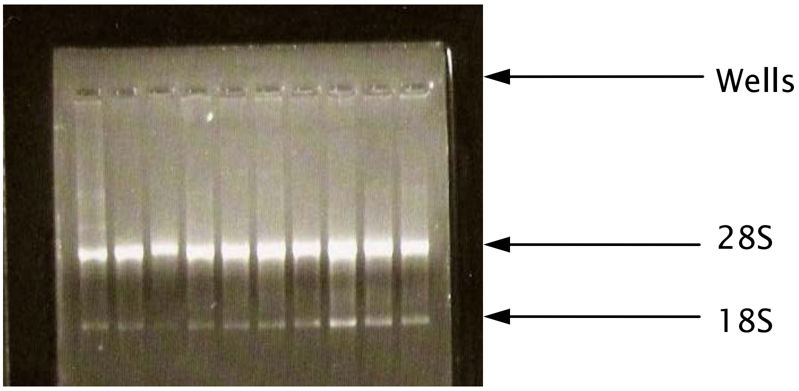


Figure 2.3 An agarose gel stained with ethidium bromide. The gel shows the 28s and 18s ribosomal RNA bands from an ovine liver sample. The 28s rRNA band is twice as intense as the 18s rRNA band, indicating that the RNA is intact.

2.2.3 Reverse transcription

The RNA was reverse transcribed into cDNA for the polymerase chain reaction (PCR). 2μl of each RNA sample (0.5μg/μl) was added to 1μl of random primers (0.5μg/μl, Promega, UK) and 12μl of ultra-pure water. This solution was heated to 70°C for 5 minutes using a PCR machine (Hybaid Ltd, UK) and then immediately cooled on ice. A master mix was made containing the following reagents (Promega, UK) per RNA sample: 5μl M-MLV reverse transcriptase 5 X buffer, 1.25μl PCR nucleotide mix, 0.625μl recombinant RNAsin ribonuclease inhibitor (40u/μl), 1μl M-MLV reverse transcriptase (200u/μl) and 2.125μl ultra-pure water. 10μl of master mix was added to each RNA sample and heated to 37°C for 1 hour, 42°C for 10 minutes and 75°C for 10 minutes to stop the reaction.

2.2.4 Real-time PCR

GR, PEPCK, G6Pase and insulin receptor mRNA levels were analysed in sheep liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7500 Sequence Detection System, USA) which uses a comparative cycle of threshold fluorescence method (Heid *et al.*, 1996). The cDNA acts as a template which is amplified during the PCR reaction, by primers annealing to the strands of

DNA (separated by heating) and then being extended by polymerase enzymes at 37°C. The Taqman PCR method (fig.2.4) involves a probe containing a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end which suppresses the reporter dye. During polymerization the primers extend by the addition of nucleotides and when the polymerase reaches the probe it cleaves the probe separating the reporter dye increasing the fluorescent signal released. The accumulation of PCR products within every PCR cycle is detected by the increase in fluorescence (ΔRn). The relative amount of cDNA (representative of RNA) is characterized by the number of cycles of PCR (Ct) it takes for the fluorescence (ΔRn) to exceed a defined fixed threshold (fig.2.5). The higher the starting amount of cDNA the sooner it reaches the threshold level and the lower its Ct value.

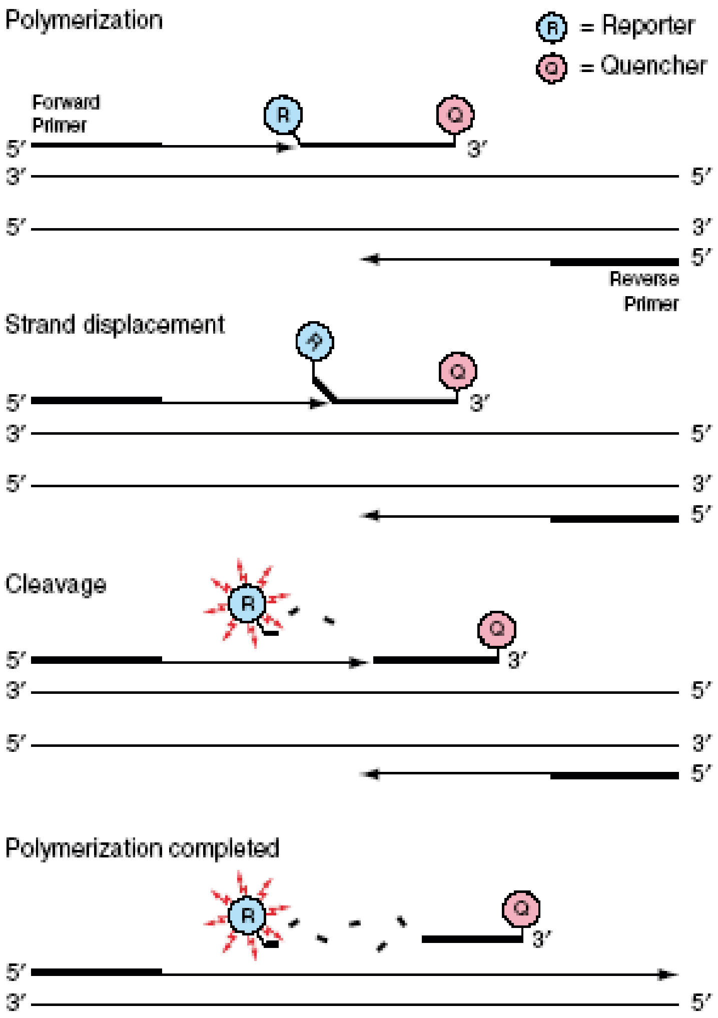


Figure 2.4 The Taqman real-time PCR reaction. (From the Taqman Universal Master Mix Protocol).

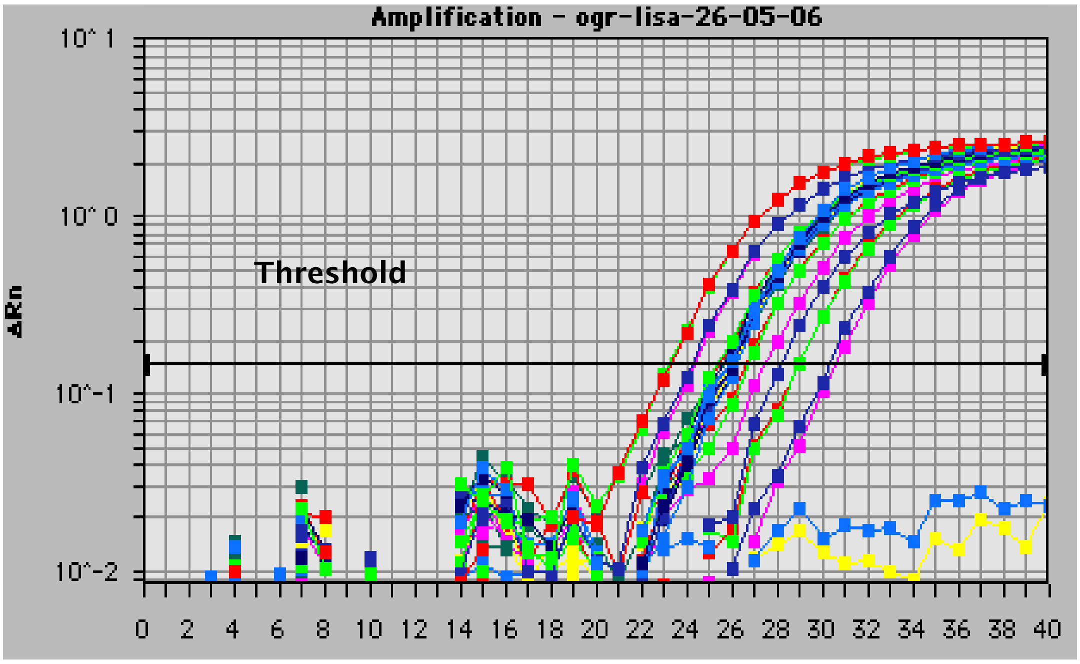


Figure 2.5 Example of an amplification curve for cDNA. cDNA was transcribed from mRNA in the ovine adult liver. Cycle number (Ct) is along the x-axis and the increase in fluorescence (ΔRn) which represents relative amount of cDNA is on the *y*-axis. The black line represents the threshold value.

Primers and probes were designed using Primer Express Software (Applied Biosystems, USA) (table 2.1) and preliminary experiments were carried out to determine the optimum concentration of each primer and probe (the lowest Ct and highest Δ Rn). 2.0µl of cDNA were used in each reaction containing universal master mix, 1.5µl forward and reverse primers (of 10µM concentration) and 1.5µl of the probe (of 5µM concentration). Each sample was analysed in duplicate for gene expression. One sample of liver RNA was used to produce a standard curve (fig.2.6). The highest standard equals 1µg of RNA and was given the relative RNA concentration of 100; this was double diluted to produce the standard curve. The standard curve shows the log of the relative RNA against the Ct, as there is an exponential change in fluorescence. The relative RNA concentration for all samples was calculated from this curve and was expressed relative to the appropriate housekeeping genes.

Primer /Probe	Sequence	Accession no.	Concentration (mM)
GR Forward Primer	ACTGCCCCAAGTGAAAACAGA	S44554	10
GR Reverse Primer	GCCCAGTTTCTCCTGCTTAATTAC		10
GR TaqMan Probe	AGAAGATTTTATCGAACTCTGCACCCCTGG		5
PEPCK Forward Primer	GATTGGCATCGAGCTGACAGA	EF062862	10
PEPCK Reverse Primer	CGCCCATCCTCGTCATG		10
PEPCK TaqMan Probe	TCGCCCTACGTGGTGACCAGCA		5
G6Pase Forward Primer	TGGAGTCTTTTCAGGCATTGC	EF062861	10
G6Pase Reverse Primer	CTTGAGACTGGCATTGTAGATGCT		10
G6Pase TaqMan Probe	TTGCTGAGACTTTCCGCCACATCCA		5
Ins Rec Forward Primer	ACCGCCAAGGGCAAGAC	AJ844652	10
Ins Rec Reverse Primer	AGCACCGCTCCACAAACTG		10
Ins Rec TaqMan Probe	AACTGCCCTGCCACTGTCATCAACG		5

Table 2.1 Primers and probes. The RNA sequence, accession number and concentration of primers and probes (Eurogentec S.A., Belgium) used in the real-time PCR reaction for the amplification of GR, PEPCK, G6Pase and Insulin receptor cDNA.

Figure 2.6 The standard curve for GR mRNA. The slope of the line needs to be close to -3.3 and R^2 close to 1. Ct, cycle number.

2.3 Power calculations

When setting up a study, a power calculation is used to calculate the appropriate sample size needed to detect a statistically significant difference of a given magnitude (Yuen, 2001). A power calculation was performed using SigmaStat for windows 3.5 (2006) to calculate the required sample size and power.

The primary outcome was taken to be the level of glucocorticoid receptor expression in the lobes of the liver. A difference of approximately 1.27 mRNA/geometric mean of housekeeping genes from preliminary data (in this study) on the left and right lobes of female liver was previously observed as significant. The average standard deviation was 0.68. Therefore, to achieve a power of 80% at a significance level of 0.05, 6 animals are required per group.

2.4 Data analysis

Specific analysis strategies are described at the start of each results chapter. All results were expressed as mean ± standard error of the mean.

Statistical analyses were performed with SPSS (version 21, SPSS Inc., USA), and GraphPad Prism (version 6, Graphpad Software Inc., USA).

All data were checked for normal distribution by histogram prior to analysis. Data not normally distributed was transformed by the natural logarithm and subsequently checked for normal distribution (fig.2.7).

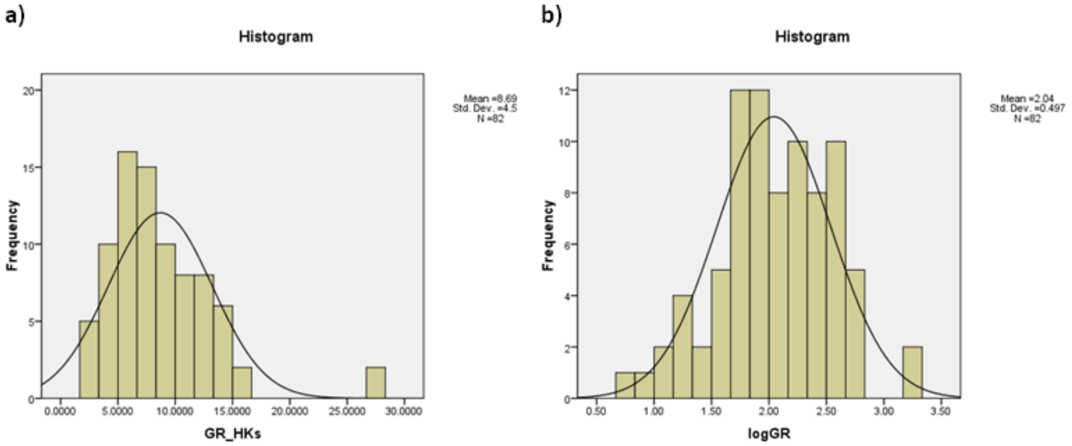


Figure 2.7 Histograms showing example data prior to (a) and after logarithmic correction to obtain normal distribution (b).

Differences between group means were analysed by students unpaired t test (2 tailed) (comparisons between two groups) when simple comparisons of two sets of single figures were involved.

More complex comparisons necessitate the use of analysis of variance (ANOVA) where the explained (between group, the whole data set) variability is compared to the unexplained (within group, in each individual group) variability. For comparisons of more than one experimental group with a control group a one way ANOVA is used. Where two factors may affect one outcome a two way ANOVA determines both the main effects of each factor and detects interactions between the factors.

For each animal two liver lobes were analysed and therefore the presence of a statistically significant response was investigated using *repeated* measure ANOVA since a standard ANOVA assumes that each observation is independent of the last. These between subjects tests will show if different groups have a different response (Matthews *et al.*, 1990). Bonferroni *post-hoc* tests were performed where appropriate.

Correlation analysis was used to test for significant relationships between data (e.g. gene expression versus liver blood flow). The Pearson correlation coefficient is calculated (R). The R^2 value ranges from 0 – 1 and is the fraction of the variance in the two variables that is “shared”. Regression analysis fits a line to the data as an estimate of the relationship between the two variables. The slope and its direction (positive or negative) indicate the strength of the relationship.

Significance was accepted when $p < 0.05$, however ‘trends’ were discussed when $0.5 < p < 0.1$.

Chapter 3. **Development of a reliable method in determining appropriate housekeeping genes in sheep liver.**

3.1 **Introduction**

During the preparation of cDNA for real-time PCR analysis there is significant potential for small errors to accumulate. For example, differences in sample size, RNA extraction efficiency, pippetting accuracy and reverse transcription will all add variability to samples. Normalising real-time PCR to the expression of an internal control or housekeeping gene is a practical strategy to adjust for these potential problems. It has previously been common practice to normalise mRNA to the expression of a single housekeeping gene thought to be stably expressed in all cell types. Indeed previous molecular studies from our group in the sheep kidney (Cleal, 2005), have used 18S as the internal control so at the start of my PhD I decided to use this to normalise the results for each gene of interest. 18S is part of the ribosomal RNA and is a component of the small ribosomal subunit (40S). The ribosome is responsible for the synthesis of proteins in cells and is found in all organisms. It serves to convert the instructions found in messenger RNA (mRNA, which itself is made from instructions in DNA) into the chains of amino-acids that make up proteins and thus one of the basic components of all mammalian cells (Moore & Steitz, 2002) and was therefore deemed suitable as a housekeeping gene. However it is now more widely appreciated that housekeeping genes may not be stably expressed in all tissues (Bemeur *et al.*, 2004;Bas *et al.*, 2004;Pombo-Suarez *et al.*, 2008) and may change according to location within the tissue, gender and stressor (Derks *et al.*, 2008;Wagner & Roizman, 1969;Tanaka *et al.*, 1975). This is important because using a variably expressed housekeeping gene for normalisation will add large unpredictable errors to the analysis and so normalising to a single, randomly selected housekeeping gene e.g. 18S, could be problematic. Therefore for accurate gene quantisation, it is essential to normalise real-time PCR data to a fixed reference that is not affected by the experimental conditions.

There has been an increase in research into identifying the most stable housekeeping gene or genes for normalisation (Vorachek *et al.*, 2013;Silver *et al.*, 2006;Silver *et al.*, 2008). Gene normalising kits typically assess six or twelve of the most commonly used normalising (housekeeping) genes and determine the best genes for use in a particular sample set. GeNorm$_{TM}$ is one such system which selects the best candidate reference gene for each individual experimental scenario. Until now, this system has not been available for use in the sheep model. **The purpose of this chapter was to assess the validity of traditional approaches to housekeeping genes and to validate a robust normalization approach for sheep tissues.**

3.2 Hypothesis

The single control gene 18S will not be suitable as a housekeeping gene in sheep liver.

The aim of this chapter is to determine the best housekeeping genes against which to normalise the expression of genes involved in hepatic metabolism in sheep liver.

3.3 Methods

3.3.1 Summary

In this chapter samples from male and female adult sheep (analysed in Chapter 4) were employed. Three cohorts of sheep were assessed in this thesis: 2 adult and 1 fetal. Housekeeping gene selection using the geNorm$_{TM}$ method was carried out on sheep samples for the first time. Real-time PCR was used (with SYBR green) to measure mRNA expression of the normalising genes from the twelve gene geNorm$_{TM}$ kit. SYBR green is inexpensive and relatively simple to use as it can be used for any gene of interest in a PCR reaction, it is not specifically designed for the gene as is the case for Taqman probes (which have to be designed for each gene of interest and can be expensive). Samples from the left and right liver lobes of male and female sheep (including twins and singletons) from all four treatment groups were used in the assessment.

3.3.2 Initial 18S investigations

It was initially planned to use a single control or housekeeping gene as per previous studies (Cleal, 2005). The RT-PCR was carried out using Taqman primers and probe (18S rRNA control kit, Eurogentec, Belgium) (see 2.2.4 for methods). This kit is designed to detect human, rat, mouse or rabbit but had been used in sheep studies successfully. However a suitable standard curve could not be obtained in sheep liver (fig. 3.1) which resulted in further investigations using different master mixes, dilutions of cDNA (1 in 10, 100 and 1000) and also with a different dilution of RNA prior to reverse transcription (0.5µg/µl – usual is 1µg/µl).

3.3.3 Analysis of housekeeping genes in sheep using geNorm$_{TM}$

geNorm$_{TM}$ is a software package designed to establish the most stably expressed housekeeping genes for any particular model. Following measurement of a number of candidate housekeeping genes in approximately 10 samples by real-time PCR (with SYBR green), the user inputs the data into geNorm$_{TM}$. The software then carries out analysis of the relative ratio of expression of each housekeeping gene. Thus the candidate housekeeping genes are ranked in order of stability.

The software also provides useful information about the optimal number of housekeeping genes that need to be averaged in order to achieve the best normalisation strategy. No licence is required when used for academic purposes. Until the work in this thesis no kit was available for use with sheep tissue. We worked with Primer Design Ltd. to develop a kit for use in the sheep. A copy of the geNorm$_{TM}$ optimisation kit handbook can be found in Appendix 1.

3.3.3.1 geNormTM kit

The geNorm$_{TM}$ kit was supplied with the following genes that have been historically used as housekeeping genes for analysis:

Ovis aries 18S Ribosomal (18S) rRNA

Ovis aries tyrosine 3-monooxygenase (YWHAZ) mRNA

Ovis aries acetyl-CoA carboxylase (aCoA) mRNA.

Ovis aries ribosomal protein S26 (RPS26) mRNA

Ovis aries beta-2 microglobulin (B2M), mRNA

Ovis aries malate dehydrogenase (MDH1) mRNA

Ovis aries ribosomal protein S2 (RPS2) mRNA

Ovis aries ribosomal protein L19 gene (RPL19) mRNA

Ovis aries H(+)-transporting ATP synthase (ATPsynth), mRNA

Ovis aries glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA

Ovis aries cytochrome P4501A1 (CYP1A1) mRNA

Ovis aries beta actin (β-actin), mRNA

The kit supplied is now commercially available and as such no sequences are made available from the manufacturer.

3.3.4 Real-time PCR using SYBR green

Housekeeping gene mRNA expression were measured in sheep liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7500 Sequence Detection System, USA) for methods which uses a comparative cycle of threshold fluorescence method (Heid *et al.*, 1996). The DNA is amplified during the PCR reaction, by primers annealing to the strands of DNA (separated by heating) and then being extended by polymerase enzymes at 37°C. The newly made double stranded DNA is detected by use of SYBR Green I, a commonly used fluorescent DNA binding dye. It binds to all double-stranded DNA. The accumulation of PCR products within every PCR cycle is detected by the increase in fluorescence (ΔRn). The relative amount of DNA is characterized by the number of cycles of PCR (Ct) it takes for the fluorescence (ΔRn) to exceed a defined fixed threshold (figure 2.6). The higher the starting amount of cDNA the sooner it reaches the threshold level and the lower its Ct value.

3.3.4.1 geNorm$_{TM}$ Analysis

The ct values obtained from real time PCR were transformed into delta-ct values by subtracting the highest ct value from all other ct values for each gene measured (the highest ct value will have a delta-ct of 0 and all others will be less than 0). The equation $2_{(\text{-delta ct})}$ was then applied to all data resulting in the data being expressed relative to the expression of the least expressed gene. These data are then entered into the geNorm$_{TM}$ software and processed to show the least stably expressed gene (that is the gene that has the most variation within the sample set). The least stable gene is then removed and the analysis is repeated with the remaining genes. The software then produces results charts detailing the most stable genes and the optimum number of normalisation genes required.

3.3.4.2 geNorm$_{TM}$ Output Charts.

Pairwise Variation Chart: Number of reference genes for optimal normalisation. This chart gives guidance for the optimum number of genes to use for normalisation. The chart generated indicates the levels of variation in average reference gene stability beginning with the first two most stable genes with the sequential addition of the next most stable genes. This is termed the "pairwise variation V". A V score of <0.15 is recommended by Primer Design Ltd.

Expression Stability Chart: The most stably expressed reference genes. The chart generated indicates the average expression stability value M of reference genes at each step during stepwise exclusion of the least stable expressed reference gene. Starting from the least stable gene at the left, the genes are ranked according to increasing expression stability, ending with the two most stable genes on the right.

3.3.4.3 Data analysis

Once suitable housekeeping genes were decided upon for fetal and adult liver (see below), the geometric mean of these genes was calculated according to the following formula: $GM_{\bar{y}} = \sqrt[n]{y_1 y_2 y_3 \cdots y_n}$. This mean was used to normalise each gene of interest in the relevant results chapters (Vandesompele *et al.*, 2002).

3.4 **Results**

Initial studies were carried out on male and female adult sheep liver using 18S as the chosen housekeeping gene.

Measuring the 18S gene using real time PCR (methods are previously described in chapter 2) was problematic in a suitable standard curve with a gradient of -2.009 and R^2 value of 0.6987 (ideally should be -3.3 and R^2=1.000) was obtained (fig.3.1.a). Repeat attempts were made with cDNA dilutions (1 in 10, 1 in 100 (fig.3.1b) and 1 in 1000 (not shown)), two master mixes (Eurogentec and Primer Design) (fig.3.1b) and also with RNA dilution of 0.5µg/µl prior to producing the cDNA (not shown) but no improvement in the standard curve was achieved (Eurogentec 1 in 10 dilution: -0.02655 R^2 = 0.3398, 1 in 100 dilution: -0.03681, R^2 = 0.4696, Primer Design 1 in 10 dilution: -0.02399, R^2 = 0.5195, 1 in 100 dilution: -0.02853, R^2 = 0.5569).

Figure 3.1 The standard curves for 18S mRNA (a) and 18S diluted (b). Standard curves for 18S using a set of 7 standards (a) and diluted standards (1 in 10 and 1 in 100) (b). Ct = Threshold cycle E = Eurogentec master mix, P = Primer design master mix, 10 = 1 in 10 dilution, 100 = 1 in 100 dilution.

3.4.1 Adult sheep liver housekeeping (control) gene selection (geNorm$_{TM}$)

All twelve genes from the geNorm$_{TM}$ kit were measured in 11 male 2.5 yr old sheep liver samples (including twins and singletons and from all dietary groups as detailed in chapter 4). One plate containing four gene reactions (YWHAZ, RPS26, RPS2 and ATPsynth) did not give adequate results for geNorm$_{TM}$ analysis (possibly due to experimental error). It was then decided that because the data already obtained from the remaining 8 genes was sufficient to select adequate housekeeping genes for normalisation that YWHAZ, RPS26, RPS2 and ATPsynth should not be repeated. The high relative abundance of 18S within the samples was immediately obvious from the low Ct values obtained in samples (e.g. fig.3.2). Thus while 18S may have previously been shown to be stable in some experiments, its high abundance means that the reagents are used up quickly and variations between samples may not be detected.

The pairwise variation chart (fig.3.3) suggested that the optimum number of genes to use is 4 however this is both time consuming and costly. The use of 3 genes gives a V score of 0.121 (which is still below the recommended value of 0.15). I therefore decided to use three genes for normalising my genes of interest. β-actin, 18s, GAPDH, B2M, MDH1, RPL-19 and CYP1A1 had normalisation factors below the 1.5 cut off recommended (Primer Design Ltd.) for use as a HK gene (table 3.1). β-actin, GAPDH, RPL19 and 18s were the most stable genes in adult sheep liver (fig 3.4). The three genes selected were: β-Actin, RPL19 and GAPDH. 18S from this kit gave a stable result, but I avoided using it due to the problems encountered in my initial investigations and the abundance shown here.

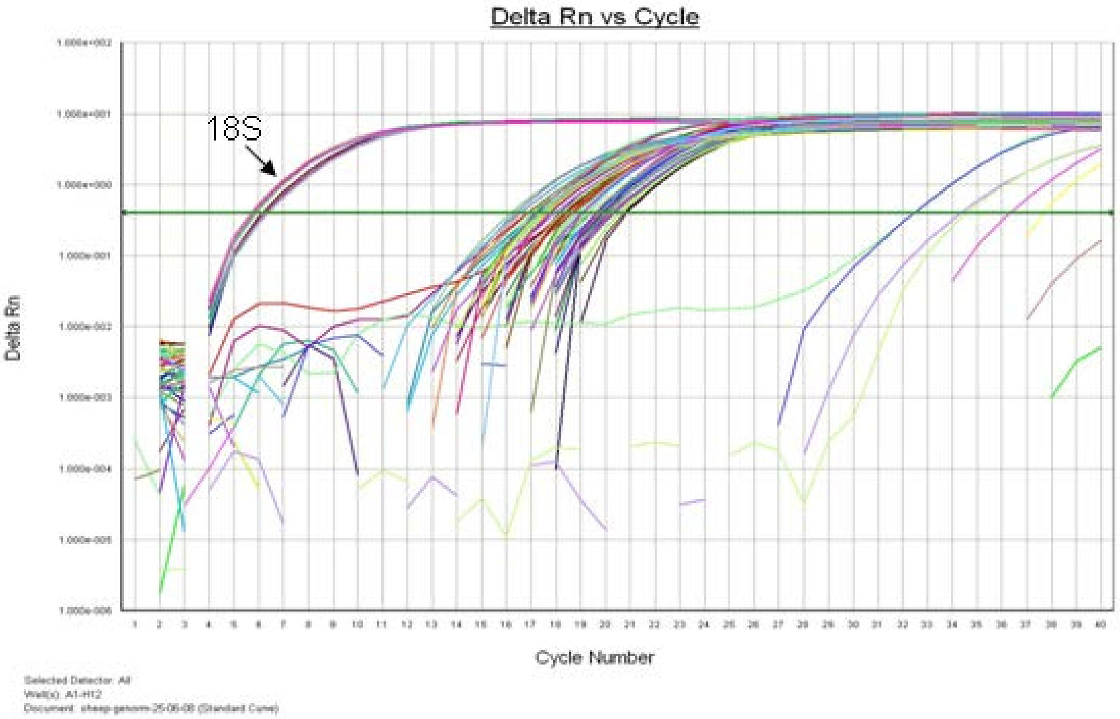


Figure 3.2 Gene expression graph obtained from real time PCR. Housekeeping gene mRNA expression in 11 samples of fetal sheep liver showing the early expression and therefore abundance of the 18S mRNA. Other mRNA gene expression shown is B2M, GAPDH and β-actin.

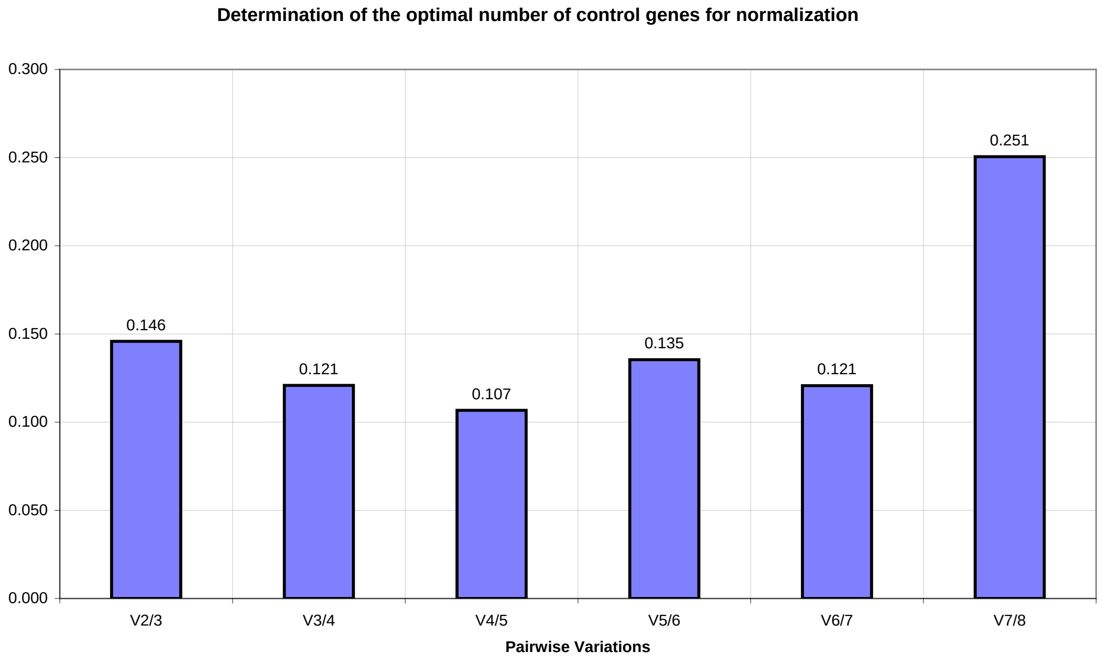


Figure 3.3 Pairwise variation chart of housekeeping genes in adult sheep liver.

Sample no.	β-Actin	18S	GAPDH	b2m	acoa	mdh1	RPL-19	cyp1a1	Normalisation Factor
L1419	1.00 E+00	7.40 E-01	8.83 E-01	4.02 E-01	1.07 E-01	8.83 E-01	1.00 E+00	3.46 E-01	**1.3391**
L1629	5.64 E-01	8.35 E-01	6.83 E-01	4.16 E-01	2.17 E-02	5.49 E-01	8.89 E-01	1.29 E-01	**0.9702**
L1570	4.10 E-01	9.66 E-01	5.40 E-01	3.56 E-01	1.86 E-02	2.33 E-01	7.79 E-01	1.57 E-01	**0.7998**
L1475	7.27 E-01	8.47 E-01	1.00 E+00	6.97 E-01	4.79 E-02	3.66 E-01	9.97 E-01	1.00 E+00	**1.4734**
L1423	2.25 E-01	8.65 E-01	4.60 E-01	2.49 E-01	5.37 E-02	1.00 E+00	8.89 E-01	4.73 E-01	**0.9857**
L1560	4.09 E-01	8.74 E-01	6.83 E-01	2.50 E-01	1.00 E+00	3.87 E-01	5.86 E-01	2.56 E-01	**0.8576**
L1459	4.48 E-01	7.74 E-01	3.00 E-01	2.72 E-01	4.37 E-01	2.97 E-01	6.53 E-01	1.64 E-01	**0.7049**
L1451	3.75 E-01	7.47 E-01	4.70 E-01	6.16 E-01	1.43 E-02	1.70 E-01	9.04 E-01	4.52 E-01	**0.9160**
L1584	7.14 E-01	1.00 E+00	7.85 E-01	7.27 E-01	2.35 E-01	2.75 E-01	9.83 E-01	2.34 E-01	**1.1390**
L1543	5.09 E-01	8.59 E-01	8.59 E-01	1.00 E+00	1.38 E-01	4.31 E-01	9.59 E-01	4.15 E-01	**1.2974**
L1563	3.62 E-01	7.71 E-01	5.99 E-01	3.51 E-01	1.09 E-01	2.39 E-01	8.65 E-01	1.95 E-01	**0.8098**
M< 1.5	**0.882**	**0.820**	**0.800**	**0.952**	**2.062**	**1.094**	**0.779**	**1.097**	

Table 3.1 Housekeeping gene measurement for adult sheep liver. (-delta ct) values for 11 samples using eight housekeeping genes. Samples are left lobe liver cDNA samples from 2.5 year old male sheep across all dietary groups and include both twins and singletons.

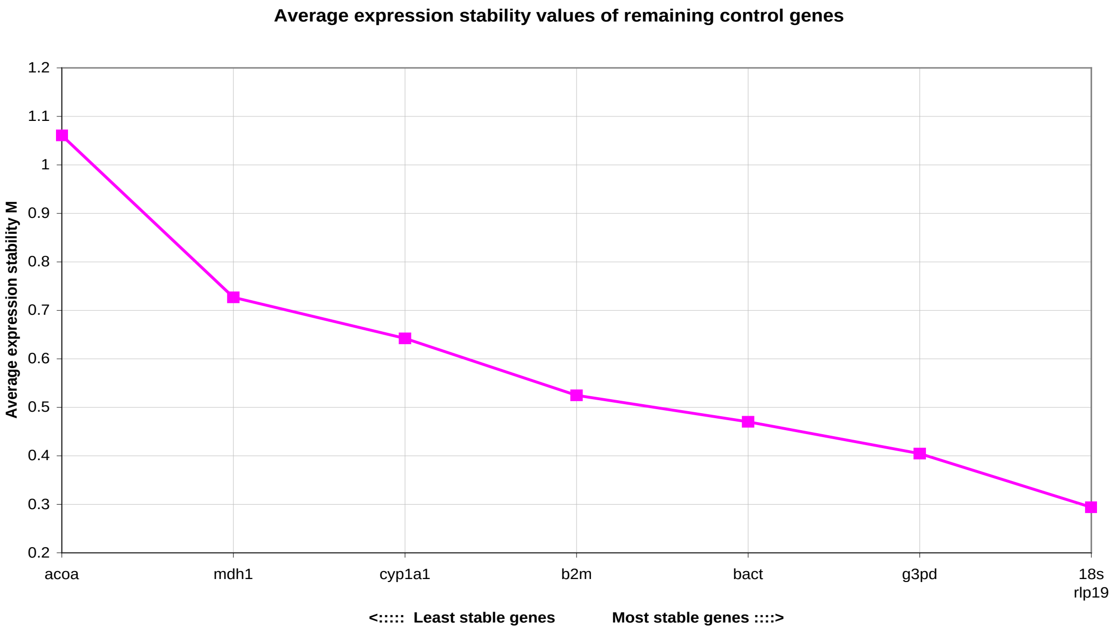


Figure 3.4 Expression stability chart for adult sheep liver. Average expression stability value M of reference genes at each step during stepwise inclusion of the least stable expressed reference gene. Starting from the least stable gene at the left the genes are ranked according to increasing expression stability ending with the two most stable genes on the right. Here 18S and RPL-19 are the two most stable genes.

3.4.2 Fetal sheep liver housekeeping selection (geNorm$_{TM}$)

11 fetal liver samples (from left and right liver lobes) were analysed using the geNorm$_{TM}$ kit. From the pairwise variation chart (fig. 3.5) the optimum number of genes to use is 7 however this is both time consuming and costly. The use of 3 genes gives a V score of 0.114 (which is still below the recommended value of 0.15). I decided to use three genes for normalising my genes of interest. Table 3.2 shows that all the genes measured resulted in a normalisation factor of <0.15 indicating that they may all be suitable for use as housekeeping genes. Figure 3.6 shows that β-actin; GAPDH, RPL19 and 18S appear to be the most stable genes to use for normalisation in fetal liver samples and so these were selected. As before, 18S from this kit appeared to give a stable result I decided not to use it due to the early expression seen in the real-time PCR analysis (fig.3.2).

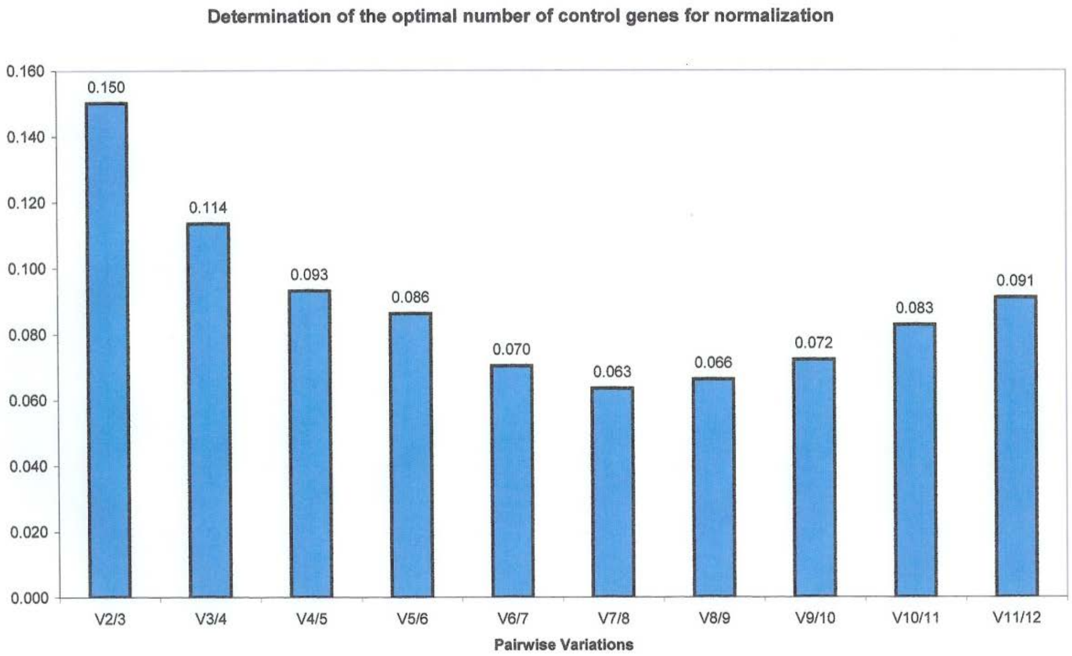


Figure 3.5 Fetal sheep pairwise variation chart. The "pairwise variation" (V) - levels of variation in average reference gene stability in 11 fetal sheep liver samples with the sequential addition of each reference gene to the equation. Starting with the two most stably expressed genes on the left with the inclusion of a 3rd, 4th and 5th gene etc. moving to the right. A V score of < 0.15 is recommended.

Sample no.	STPSyn	acoa	RPL-19	RPS2	18S	GAPDH	b2m	β-Actin	cyp1a1	RPS26	mdh1	YWHAZ	Normalisation Factor
L1884	5.74 E-01	1.00 E-01	3.83 E-01	5.60 E-01	7.34 E-01	5.82 E-01	5.85 E-01	4.98 E-01	7.39 E-01	3.67 E-01	6.96 E-01	6.90 E-01	1.1119
L1886	5.34 E-01	2.74 E-01	1.00 E+00	3.39 E-01	8.83 E-01	5.78 E-01	1.00 E+00	6.23 E-01	2.87 E-01	6.84 E-01	6.15 E-01	5.23 E-01	1.0494
L3031	1.00 E+00	2.78 E-01	7.74 E-01	6.68 E-01	9.48 E-01	6.07 E-01	8.87 E-01	7.82 E-01	7.07 E-01	6.38 E-01	5.78 E-01	5.34 E-01	1.2487
L3034	3.82 E-01	5.73 E-01	3.25 E-01	6.24 E-01	6.90 E-01	2.52 E-01	1.48 E-01	3.73 E-01	1.00 E+00	5.93 E-01	7.08 E-01	4.59 E-01	0.8530
L3043	8.17 E-01	8.37 E-01	5.09 E-01	6.56 E-01	6.76 E-01	3.99 E-01	1.56 E-01	2.91 E-01	3.07 E-01	3.16 E-01	5.02 E-01	1.00 E+00	0.8891
L3051	3.57 E-01	2.97 E-01	4.14 E-01	5.05 E-01	6.91 E-01	2.31 E-01	5.96 E-01	2.41 E-01	2.35 E-01	3.14 E-01	1.64 E-01	2.61 E-01	0.6138
R1885	3.07 E-01	5.70 E-01	4.04 E-01	4.42 E-01	6.98 E-01	6.18 E-01	3.31 E-01	5.61 E-01	4.54 E-01	3.56 E-01	1.00 E+00	5.19 E-01	0.9218
R1971	7.47 E-01	1.88 E-01	8.91 E-01	9.84 E-01	9.92 E-01	7.06 E-01	3.78 E-01	6.29 E-01	3.69 E-01	8.75 E-01	7.15 E-01	9.05 E-01	1.1849
R1980	6.03 E-01	2.14 E-01	4.69 E-01	1.00 E+00	9.69 E-01	7.65 E-01	7.80 E-01	8.77 E-01	4.71 E-01	4.34 E-01	6.56 E-01	7.33 E-01	1.1510
R3047	3.54 E-01	5.91 E-01	4.52 E-01	5.49 E-01	6.21 E-01	3.02 E-01	4.41 E-01	5.33 E-01	3.81 E-01	6.74 E-01	7.62 E-01	4.45 E-01	0.9179
R3064	5.46 E-01	2.16 E-01	8.53 E-01	9.35 E-01	1.00 E+00	1.00 E+00	7.99 E-01	1.00 E+00	3.37 E-01	1.00 E+00	8.41 E-01	4.97 E-01	1.2777
M < 1.5	0.713	1.184	0.715	0.715	0.594	0.712	1.052	0.677	0.869	0.736	0.779	0.718	

Table 3.2 Housekeeping gene measurement for fetal sheep liver. (-delta ct) values for eleven samples using eight housekeeping genes. Data is expressed relative to the expression of the least expressed gene. An M value of < 1.5 is deemed to produce an adequate normalising gene. Samples are left lobe liver cDNA samples from 2.5 year old male sheep across all dietary groups and include both twins and singletons.

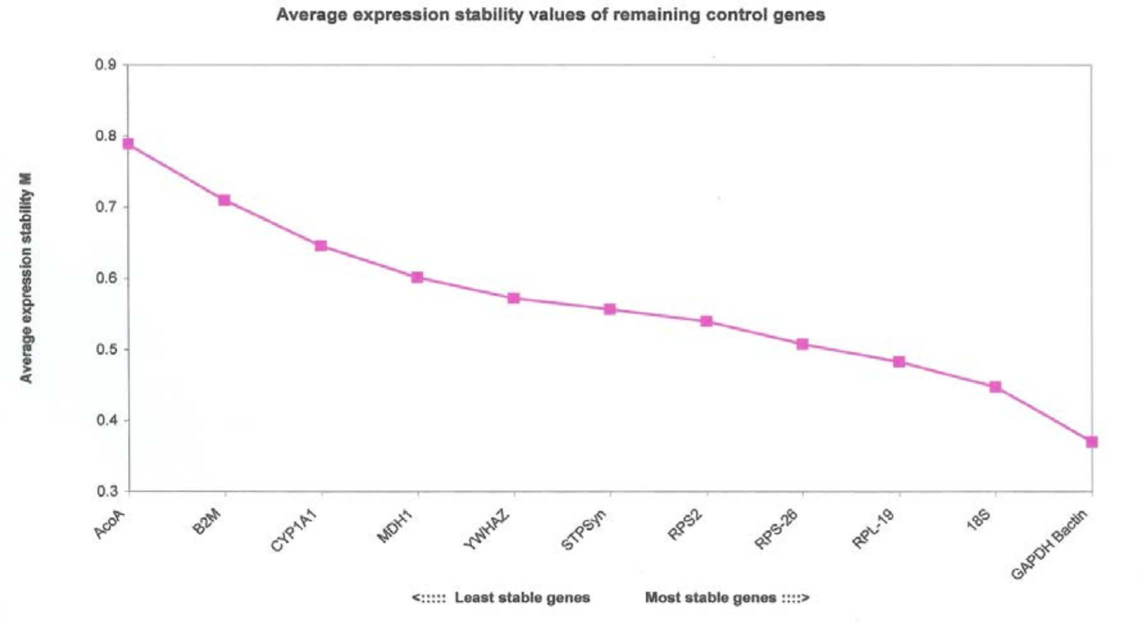


Figure 3.6 Fetal sheep expression stability chart. The chart indicates the average expression stability value M of reference genes in 11 fetal sheep liver samples at each step during stepwise inclusion of the least stable expressed reference gene. Starting from the least stable gene at the left the genes are ranked according to increasing expression stability ending with the two most stable genes on the right.

3.4.3 Selection of housekeeping genes for adult male sheep study in Chapter 6

The results obtained in both the adult and fetal geNorm$_{TM}$ analyses showed that GAPDH, β-actin & RPL-19 were the three most suitable genes. In Chapter 6 of this thesis the studies involve samples from the same liver regions and the same breed of sheep as described in the rest of the thesis and therefore no further geNorm$_{TM}$ selection of housekeeping genes was carried out.

3.5 Discussion

3.5.1 Summary

In this chapter the suitability of 18S as a single control gene was investigated and was shown not to be effective as a housekeeping or normalising factor in sheep liver. Further investigations were carried out with the development of a geNorm$_{TM}$ kit specifically for use the sheep animal model and showed that normalisation of sheep liver samples could be obtained using the geometric mean of three housekeeping genes. The three identified in this chapter for subsequent use in this thesis were β-actin, GAPDH and RPL-19.

β-actin

β-actin is a nonmuscle cytoskeletal actin and is a highly conserved protein that is involved in cell motility, structure and integrity (reviewed in Ruan & Lai, 2007).

RPL-19

RPL-19 is ribosomal protein L-19 and is one of the 34 different proteins that stabilises the RNA matrix within the in the 50S subunit in the ribosome complex (Berg *et al.*, 2002).

GAPDH

GAPDH or Glyceraldehyde 3-phosphate dehydrogenase is a multifunction protein with roles involved in energy production, apoptosis and a variety of critical nuclear pathways (reviewed in Sirover, 2005).

3.5.2 18S was not suitable due to high abundance in sheep liver

18S ribosomal RNA is one of the most commonly used housekeeping genes and was historically used in Northern blots. This was because it is expressed at relatively high levels in all cells and made ideal positive control if the gene of interest was switched off (Huggett et al., 2005). However, too much of this or any housekeeping gene makes variations difficult to detect worse still, should the housekeeping gene be influenced by the experimental condition then completely different results may be obtained. The results in this study found that although 18S was stably expressed in sheep liver it was hugely abundant. The over-abundance of 18S has also been found when investigating housekeeping genes in bovine blood (Robinson *et al.*, 2007). The over-abundance makes 18S an unsuitable housekeeper since the housekeeping gene and the target gene (gene of interest) should have similar ranges of expression in the samples to be analyzed (Cappelli *et al.*, 2008).

3.5.3 The combination of three housekeeping genes was found to be best for normalisation in adult and fetal sheep liver

Historically many studies have normalised their results to a single gene of interest however, variations due to tissue type (Craythorn *et al.*, 2009), disease (Boujedidi *et al.*, 2012) or diet (Janovick-Guretzky et al., 2007) have been discovered making the choice and reliability of a single housekeeper more difficult. The geNorm$_{TM}$ kit used to assess the right housekeeping genes was a novel kit in sheep and was developed between our group and Primer Design Ltd. in direct response to my thesis needs. Its results recommended the use of the combination of the three most stably expressed genes as the most reliable way to normalise for any experimental variations. This has also been suggested in other studies involving different species and tissues as a solid normalisation method (Riedel *et al.*, 2014;Bonnet *et al.*, 2013).

3.6 Conclusions

The development of the geNorm$_{\text{TM}}$ kit for use in sheep tissue has shown that use of the single housekeeping gene 18S is not the best option for sheep liver. A combination of three genes (β-actin, GAPDH and RPL-19) was discovered to be the optimum method for correcting for environmental conditions for mRNA gene expression in fetal and adult sheep liver and this was the basis of normalising the mRNA gene expression within this thesis.

Chapter 4. **Effect of postnatal undernutrition on adult sheep hepatic gluconeogenic gene expression following an early gestation nutritional challenge**

4.1 Introduction

The increase in obesity and type 2 diabetes is a major concern to human health around the world. Previous studies in humans and animals have demonstrated that the effect of early developmental changes/alterations in nutrient supply on postnatal metabolic control seems to depend on the period of gestation and early postnatal life in which the alteration occurs. The mechanisms regulating such metabolic profiles are likely to include adaptations in metabolic tissues such as liver, adipose and muscle that are developing during the time that the challenge is imposed (Sayer *et al.*, 2004;Poore & Fowden, 2004). Increased insulin resistance is associated with the prevalence of type 2 diabetes and occurs within the muscles, liver and adipose tissues. The liver is a major endocrine organ and responsible for producing glucose in times of fasting. Because glucose levels are elevated in type 2 diabetes (Kuzuya *et al.*, 2002) and the liver is a key organ in the supply of glucose during fasting I decided to focus on the liver as a possible candidate for the cause of these metabolic disorders in later life.

During early development the fetus is susceptible to environmental cues indicative of the future environment and makes a predictive adaptive response in the form of epigenetic changes. This can affect a single organ (i.e. the liver) or whole system. If the gestational environment does not match the reality in later postnatal life then there is a possibility for metabolic disease to occur. The greater the mismatch the greater the disease risk. Recent sheep studies in which a match or mismatch in nutrition was created between the early gestation and early postnatal periods showed that males had accelerated growth following a postnatal undernutrition challenge and increased blood pressure response but this was not the case if the challenge was also received gestationally (Cleal *et al.*, 2007a). Females grow slower, are fatter, have improved insulin sensitivity and are more glucose tolerant following postweaning undernutrition regardless of prenatal challenges (Poore *et al.*, 2007). In contrast, female sheep have been shown to have increased feed intake, body weight, lower insulin sensitivity and increased hepatic lipid and glycogen following a gestational period of undernutrition only (George *et al.*, 2012). To date investigations have suggested that the metabolic changes caused by insulin sensitivity and glucose tolerance may be attributed to adaptations in adipose (Poore & Fowden, 2004;Ford *et al.*, 2007) and muscle (De Blasio *et al.*, 2012), but the role of the liver has not been examined.

This chapter investigated the effect of matching or greater mismatch in the pre/post nutrition on the gluconeogenic gene expression in the liver (fig.4.1).

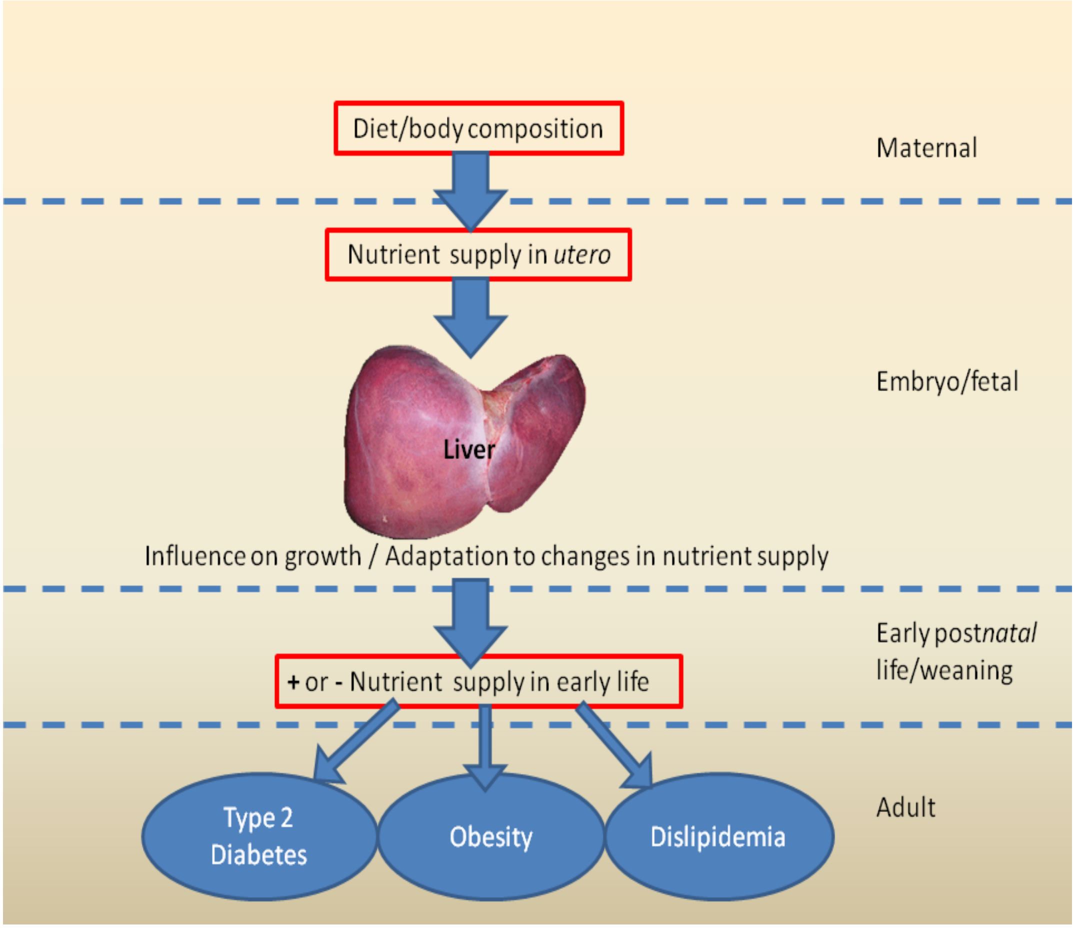


Figure 4.1 Liver as a metabolic target organ, its early life nutritional influences and potential outcomes in adulthood.

One key role of the liver in carbohydrate metabolism is the metabolic pathway of gluconeogenesis (figs.1.7 & 4.2) that results in the generation of glucose from non carbohydrate sources such as lactate and amino acids. It takes place during periods of fasting and starvation and although this occurs primarily in the liver it is also found to occur in the kidneys. Glucocorticoids are known to increase PEPCK gene transcription (Granner & Pilkis, 1990) which in turn increases the production of glucose via the gluconeogenic pathway. Insulin (secreted when there is an excess of glucose in the bloodstream) inhibits the pathway by stopping the expression of key genes (figs.1.7 & 4.2) to help regulate glucose metabolism.

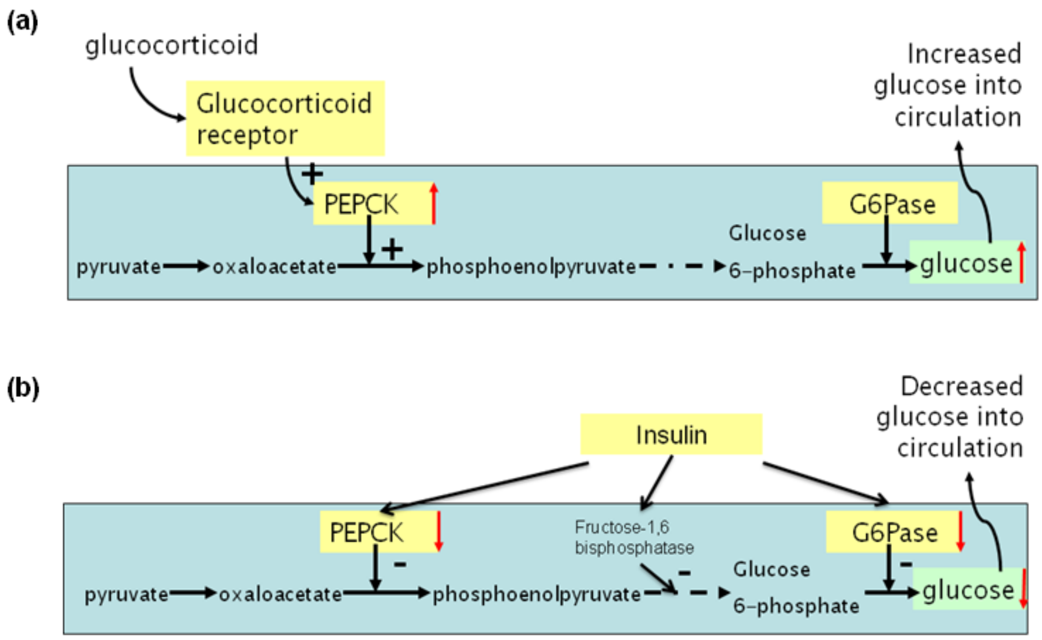


Figure 4.2 Simplified gluconeogenic pathway. Gluconeogenic pathway showing (a) increased glucose and (b) decreased glucose into the circulation from hepatic gluconeogenesis.

In the sheep liver lobes appear as early as the 21st day of gestation and by the 23rd day of gestation left and right lobes are formed with a small caudate lobe between them (Bryden *et al*., 1972), thus it is quite likely that this period of gestation constitutes a critical window in development which may be affected by changes in nutrient supply. The enzymes necessary for gluconeogenesis are present early in gestation with the activity of PEPCK comparable to that in mature animals detected at 45 days gestation. Glucose-6-phosphatase and fructose-1,6-diphosphatase, two of the four key gluconeogenic enzymes, appear in significant amounts between 100 and 120 days gestation (Stevenson *et al.*, 1976). Previous studies have shown that glucose production in the fetus occurs mainly in the face of maternal starvation (Lemons *et al.*, 1986;Leury *et al.*, 1990). In late gestation fetal sheep liver weight was reduced and the hepatic enzymes PEPCK and G6Pase were found to be increased following maternal fasting (Lemons *et al.*, 1986). A 50% nutrient restriction during days 1-95 of gestation resulted in reduced liver weight in adult male sheep with reduced hepatic expression of several genes including prolactin, IGF-II receptor and growth hormone (Hyatt *et al.*, 2007b). Burns *et al.* showed that the livers of adult offspring of rat dams fed a low protein diet (8% protein content) during pregnancy and lactation (MLP) had increased gluconeogenesis producing more glucose from lactate than the controls (20% protein content). In control livers, glucose produced from lactate in the periportal region of the lobule was taken up by the most distal perivenous cells; this distal perivenous uptake was greatly diminished in maternal low protein livers. This accounted for a major part of the increased glucose output of MLP livers. Hepatic lobular volume in MLP livers was twice that of controls indicating that MLP livers had half the normal number of lobules (Burns *et al.*, 1997). Adult rat studies have also shown that the gene expression of fibrinogen and GR following a low protein (8% protein content) maternal diet throughout pregnancy and lactation was lower in the left liver lobe compared to the right (Zhang & Byrne, 2000).

Thus there is reason to believe that there are critical windows of susceptibility in gestation. Current concepts highlight the importance of the prenatal environment with the postnatal interaction, but the effects of this on gluconeogenic genes in the sheep liver are not yet known.

4.2 **Hypothesis**

Restricted postnatal nutrition will increase the expression of the hepatic genes involved in gluconeogenesis in adult sheep depending on nutrition received in early gestation.

The aim of this chapter is to determine if there is an effect of pre and/or postnatal nutrient challenge in the liver in a large animal model such as the sheep. This chapter will investigate the effect of poor early life nutrition on some of the genes involved in gluconeogenesis GR, PEPCK, G6Pase and the insulin receptor in adult sheep liver.

4.3 Methods

4.3.1 Summary

In this chapter a gestational and/or post weaned life dietary challenge is employed with the aim of inducing changes to the liver metabolism in adult life. All procedures contained in this chapter were approved by the Home Office and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. There were four groups of animals: one group had reduced nutrition in the 1-31 days of gestation, another had reduced nutrition after weaning from their mothers (12-25 weeks), and one group experienced both periods of undernutrition whereas the final control group received full nutrient requirements throughout. Real-time PCR was used to measure mRNA expression of the gluconeogenic genes in the liver. Samples from the left and right liver lobes of 2.5 year old male and female sheep (including twins and singletons) were investigated.

4.3.2 Ewes

Ewes in their second or third parity (n = 88) were bought in by The Royal Veterinary College (North Mymms, Hertfordshire) from known suppliers, double ear tagged and identichipped for identification and group housed in open barns on straw. All were vaccinated against Chlamydia, toxoplasmosis and clostridia disease, and routine vaccinations were performed as necessary. Routine anthelmintic treatments were administered every six - eight weeks and foot care was performed as necessary. The health and welfare of the animals was checked throughout the ewes study and any signs of ill-health or disease were reported to a veterinary surgeon who advised treatment according to standard veterinary practice. All routine and prescribed treatments were recorded in health records. Prior to study enrolment they were condition scored and fed as necessary to maintain them at the required score of 2.5 to 3.0.

Body Condition Score (BCS)

All ewes in the study were body condition scored by Professor D. Noakes and John Thompson (RVC) as a means of assessing the degree of fatness of the animal (see 2.1.1). The ewes had a score of approximately 3 at the time of mating.

4.3.3 **Mating**

The oestrous cycle of groups of 3-4 ewes of uniform good BCS (~3.0) were synchronised by withdrawing vaginal medroxyprogesterone acetate impregnated sponges (Veramix, Upjohn Ltd, Crawley, UK) 12 days after insertion. One of 3 raddled Welsh Mountain rams was introduced at this time for three days. Day 0 of gestation was taken as the first date at which an obvious raddle mark was observed. Pregnancy was confirmed by measuring plasma progesterone concentrations at day 16 of gestation using an enzyme immunoassay kit (Ridgeway Science Ltd, Avington, UK), animals with low progesterone (< 2ng/ml (Bassett *et al.*, 1969)) were deemed not pregnant and removed from the study. On Study Day 60 of gestation each ewe was scanned for pregnancy using ultrasound and any remaining non-pregnant ewes were withdrawn from the study.

4.3.4 **Maternal Dietary Manipulation**

Ewes were individually housed on straw, from 7 days before conception to 37 days of gestation thereafter being group housed on straw with animals at a similar gestational age. They were allowed to acclimatize to a complete pelleted diet (P316-RVC EXP Sheep Nuts GJW Titmus, Hertfordshire, UK; Appendix 2.1) for the 7 days before conception and were fed once a day (early morning). During tupping the animals had group access to the same complete diet. The diet, consisted of barley, wheat, micronized full fat soya, grass meal, molasses, chopped straw, calcium carbonate, dicalcium phosphate salt and sheep vitamin/mineral supplement. It provided 10.8MJ (kg dry matter)$^{-1}$(metabolisable energy) and 16.69g crude protein (100g dry matter)$^{-1}$. As fed, the pelleted diet was 89.205% dry matter. Rations were calculated according to body weight at enrolment and adjusted according to gestational age as per standard guidelines for pregnant sheep (AFRC Technical Committee, 1993). Water was provided *ad libitum* at all times.

Before conception ewes were randomly assigned to a control group or a dietary restricted group (fig.4.3) and identified using a coloured ear tag. From 1-31 days of gestation (dGA), ewes received either 100% (control; group C, *n* = 41) or 50% of global nutritional requirements (50% reduction in standard pellet ration; group U, *n* = 47), and 100% of requirements for the remainder of gestation. All nutritional manipulations were carried out by RVC technicians. Maternal body weight and BCS were measured weekly and any animal that lost more than 15% of its original body weight whilst on the 50% restricted diet was offered diet *ad libitum* from the next day. Animals were at least Condition Score 2.5 before starting the restricted diet and any animal that dropped below this score prior to starting was withdrawn from the study.

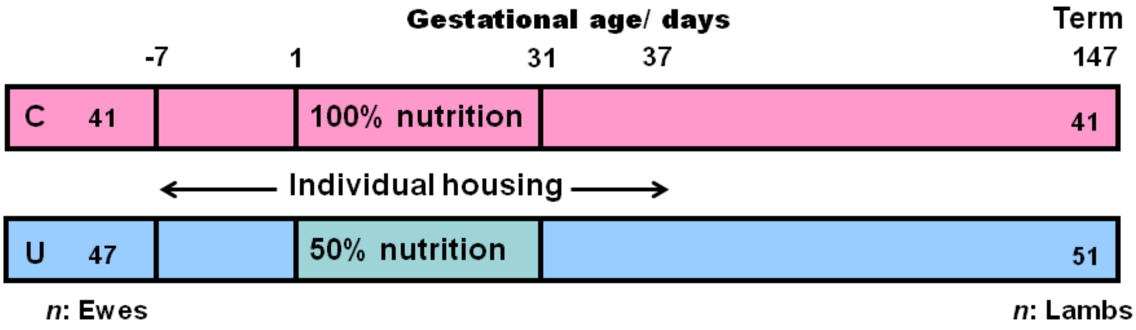


Figure 4.3 Maternal nutritional protocol. Maternal nutritional protocol from before conception to term (147 days). C = control, U = undernutrition, *n* = number.

4.3.5 Lambs

The lambs were delivered at term and allowed to suckle naturally until weaning at 12 weeks of age (C, *n* = 41 and U, *n* = 51 lambs). There were approximately equal numbers of males and females and the ratio of singletons to twins was

2:3. All ewes lambed in individual pens and returned to the group housing when the lambs were 1 week old. Each lamb was identified with a uniquely numbered ear tag and a microchip. At birth, all lambs were weighed. Lambs were weighed again at 4, 8 and 12 weeks of age and every week thereafter. Post-weaning lamb diet consisted of free access to water and hay (7-9MJ/kgDM, 85% dry matter) and each morning and afternoon a ration of creep pellets were provided to the group calculated according to the average group body weight (AFRC Technical Committee, 1993). Creep pellets provide 12.22MJ (kg dry matter)$^{-1}$ (metabolisable energy), 20.76g crude protein (100g dry matter)$^{-1}$ and contain 86.7% dry matter (Prestige Lamb Pellets + Decox, BOCM Pauls Ltd, UK) (Appendix 2.2).

Postnatal nutrient restriction

Between 12 and 25 weeks of age, lambs were grouped with approximately 10 lambs of similar body weight and treatment group and were fed as above (CC, n = 21 and UC, n = 19) or at an intake level that reduced body weight to 85% of their target weight (Appendix 2.4), as predicted from each animal's individual growth trajectory between birth and 12 weeks of age, by removing the pelleted diet but maintaining free access to hay (CU, n = 17 and UU, n = 21) (fig.4.4). All lambs were weighed weekly and the restricted lambs had their feed adjusted so as to keep them on the desired trajectory. Each group contained approximately equal numbers of males and females and the ratio of singleton to twin lambs was 2:3, twin lambs were divided so that one was assigned to low nutrient and one to the control diet.

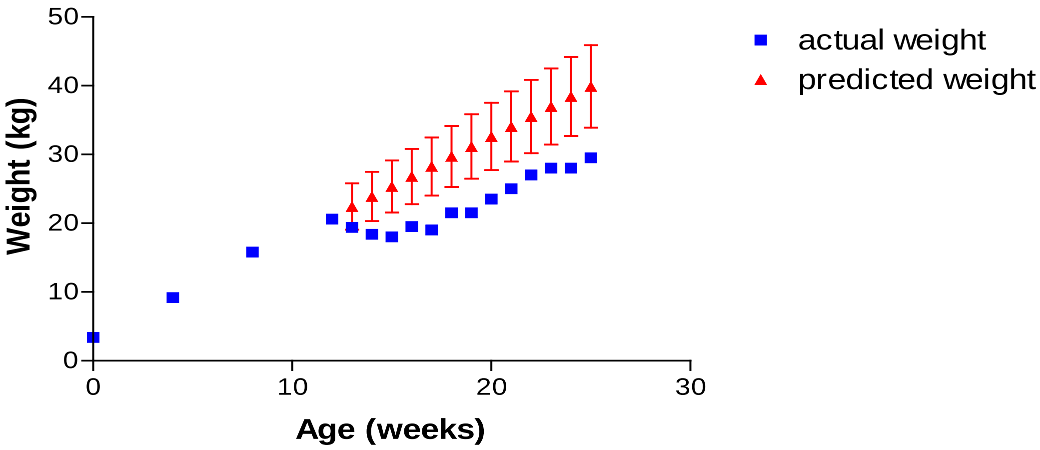


Figure 4.4 An example of one lamb's growth trajectory (blue).

At 25 weeks of age nutrient restricted lambs were treated with an anti-coxidia agent (Vecoxan, 2ml/kg) and all lambs were returned to larger group housing on straw with ad libitum hay (7-9MJ/kgDM, 85% dry matter), water and creep pellets (~ 0.5kg each). At approximately 32 weeks of age, lambs were transferred onto a standard ration of an adult complete pelleted diet (1kg each, Ewbol 18 nuts, BOCM Pauls LTD, Loughborough, UK, Appendix 2.3), according to body weight, as per standard guidelines (AFRC Technical Committee, 1993) plus *ad libitum* hay and water. The nutritional protocol of all the lambs is shown in figure 4.5.

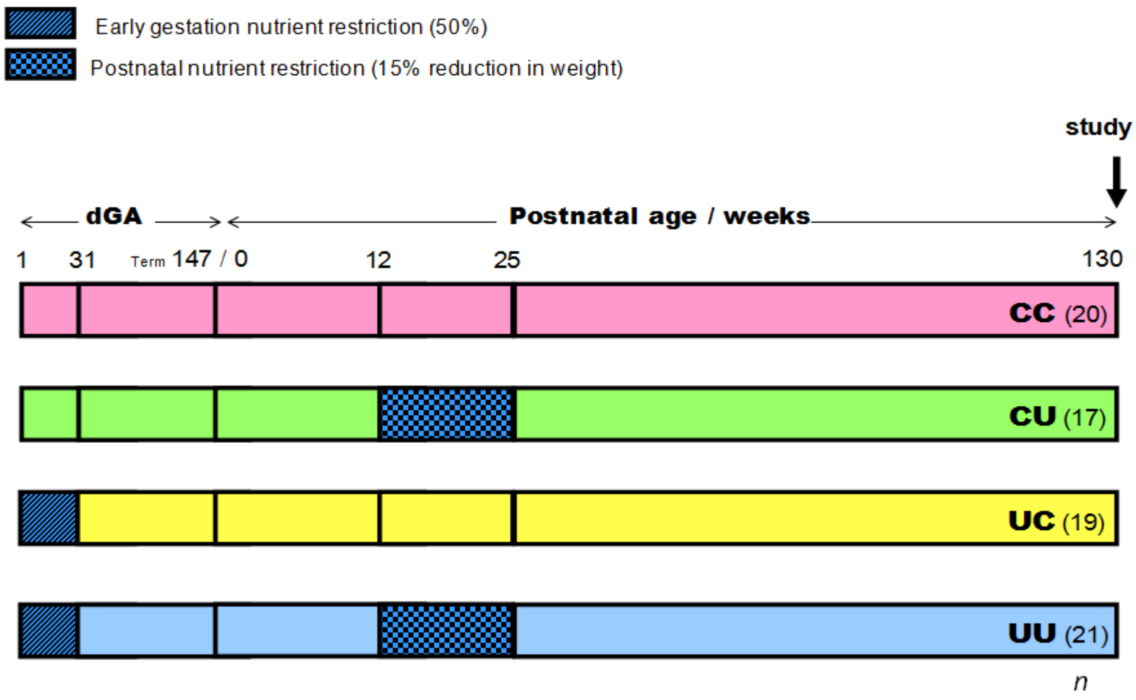


Figure 4.5 Lamb nutritional protocol. Blue boxes represent period of under nutrition and 100% nutrient requirements were fed at all other times. CC = control, CU = postnatal undernutrition, UC = prenatal undernutrition UU = pre and postnatal undernutrition, Group numbers represent both single and twin offspring, *n* = number of lambs (shown in brackets).

4.3.6 Adult post-mortem

Prior to the post-mortem, drapes, trays and surgical instruments were sterilised by autoclave. A pack of instruments was also cold sterilized using Novasapa for the initial sterile dissection of the abdomen.

At 2.5 years old lambs were killed by an overdose of barbiturate (Pentobarbitone Sodium Ph. Eur 0.8 l/kg i.v., 200mg/ml, Animalcare Ltd, York, UK). The animal was secured to the post mortem table and a large square was cut in the abdomen. Drapes covered the animal leaving the area of interest exposed and under sterile conditions the organs were removed according to the post-mortem sheet (Appendix 2.5). The organs were weighed in sterile trays and the required regions were dissected and processed according to the post-mortem sheet. Samples were fast frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis.

4.3.7 RNA extraction

Total RNA was extracted from the left and right lobes of 2.5 year old sheep liver using the methods previously described (see 2.2.1). RNA yield and quality was determined as previously described (see 2.2.2).

4.3.8 Reverse transcription

The RNA was reverse transcribed into cDNA for the polymerase chain reaction (PCR) (see 2.2.3).

4.3.9 Real-time PCR

Target gene mRNA levels were analysed in 2.5 year old adult liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7700 and 7500 Sequence Detection System, USA) (see 2.2.4).

Primers and probes (table 2.1) were designed using Primer Express Software (Applied Biosystems, USA) and preliminary experiments were carried out to determine the optimum concentration of each primer and probe (the lowest Ct and highest Δ Rn) as described in Chapter 2 (2.2.4). One sample of liver RNA (Male, Twin, Left lobe, diet group: DR) was used to produce a standard curve (see figure 4.6). The highest standard equals 1μg of RNA and was given the relative RNA concentration of 100; this was double diluted to produce the standard curve. The standard curve shows the log of the relative RNA against the Ct, as there is an exponential change in fluorescence. The relative RNA concentration for all samples was calculated from this curve and was expressed relative to the mean of housekeeping genes β-actin, GAPDH and RPL-19.

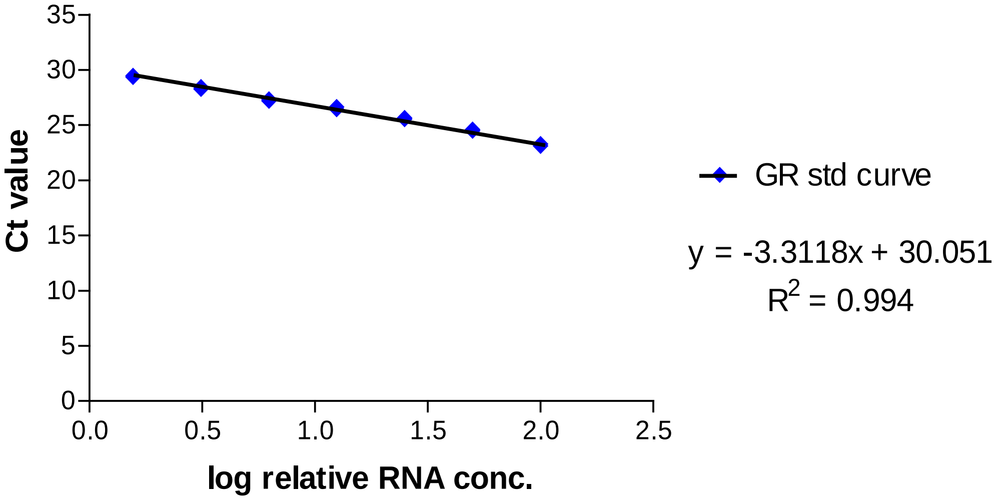


Figure 4.6 **The standard curve for GR mRNA.**

4.3.10 Analysis strategy and statistics

For a general description of the analysis strategy, the power calculations and statistics packages used see 2.3.

All mRNA expression data were normalized using the geometric mean of three housekeeping genes (β-actin, GAPDH and RPL-19).

All data were checked for normal distribution by histogram prior to analysis. Data not normally distributed was transformed by the natural logarithm and subsequently checked for normal distribution.

Differences in liver weight were analysed by univariate ANOVA with between subject factors of diet, sex and offspring.

Differences in lobar gene expression were analysed by repeated measures ANOVA with between subject factors of sex, offspring and diet.

Significance was accepted when $p < 0.05$, however ‘trends’ are discussed when $0.5 < p < 0.1$.

4.4 Results

All *P* values can be found in Appendix 2.6.

4.4.1 Body weight, fat, muscle and metabolic markers

Early gestation diet had no effect on birth weight or size. From 12 weeks female sheep were lighter than male sheep but at 2.5 years of age females had more fat and muscle relative to their body weight and twins more muscle depth than singletons (data not shown) (Poore *et al.*, 2007). Basal glucose was found to be lower in males who had undernutrition in early gestation. Glucose area under the curve (AUC) was found to be lower in females from the postnatal undernutrition group. There were no differences in basal insulin or insulin AUC between males and females or the dietary groups (data not shown) (Poore *et al.*, 2007).

4.4.2 Liver weight

Liver as a percentage of body weight was assessed and found that this was lower in males than females (fig.4.7b). There was no effect of pre- or postnatal diet restriction or offspring number.

a)

Figure 4.7 Liver weight. Liver weight as a percentage of body weight in a) dietary groups CC = control, *n* = 22, CU = postnatal undernutrition, *n* = 14, UC = prenatal undernutrition *n* = 17 UU = pre and postnatal undernutrition *n* = 22, b) sex, male (*n* = 39) and female (*n* = 36) of adult sheep. Values are mean ± SEM. $^*P < 0.05$.

4.4.3 Hepatic Glucocorticoid Receptor mRNA expression

At 2.5 years of age adult male sheep had higher liver GR mRNA expression than females (fig.4.8b). The right lobe had a higher expression of GR than the left (fig.4.8c). This was the case for both males and females when assessed separately (fig.4.9). Singleton offspring were found to have a lower GR mRNA expression than twins (fig.4.8d). There was no effect of diet (fig.4.8a).

Figure 4.8 Hepatic Glucocorticoid Receptor mRNA expression. GR mRNA expression in a) dietary group (CC = control *n* = 22, CU = postnatal undernutrition *n* = 14, UC = prenatal undernutrition *n* = 17, UU = pre- and postnatal undernutrition *n* = 22), b) sex (male *n* = 39, female *n* = 36), c) lobe (left *n* = 75, right *n* = 75) and d) offspring (singleton *n* = 28, twin *n* = 47) in adult sheep liver. Values are mean ± SEM. *P<0.05, **P < 0.001.

Figure 4.9 Hepatic Glucocorticoid Receptor mRNA expression between the sexes and in lobes. GR mRNA expression in adult male (M *n* = 39) and female (F *n* = 36) sheep left (L) and right (R) liver lobes. Values are mean ± SEM. *P <0.05.

4.4.4 Hepatic PEPCK mRNA expression

At 2.5 years of age adult male sheep had higher liver PEPCK mRNA expression than females (P < 0.001, fig.4.10b) and the left lobe had a higher expression of PEPCK than the right (P < 0.001, fig.4.10c). There was no effect of diet (fig 4.10a) or offspring (fig.4.10d). However, female twins were found to have a higher expression of PEPCK mRNA than singletons (fig 4.10d).

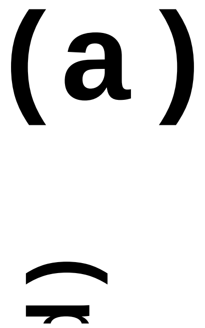


Figure 4.10 Hepatic PEPCK mRNA expression. PEPCK mRNA expression in a) dietary group (CC = control *n* = 22, CU = postnatal undernutrition *n* = 14, UC = prenatal undernutrition *n* = 17, UU = pre- and postnatal undernutrition *n* = 22), b) sex (male *n* = 39, female *n* = 36), c) lobe (left *n* = 75, right *n* = 75) and d) offspring (singleton *n* = 28, twin *n* = 47) in adult sheep liver. Values are mean ± SEM. *P<0.05, **P < 0.001.

Figure 4.11 Hepatic PEPCK mRNA expression between sexes and offspring number. PEPCK mRNA expression in adult male and female sheep singleton and twin sheep liver (MS = male singleton *n* = 28, MT = male twin *n* = 47, FS = female singleton *n* = 10, FT = female twin *n* = 26). Values are mean ± SEM. *P<0.05.

4.4.5 Hepatic G6Pase mRNA expression

At 2.5 years of age no differences were seen in G6Pase mRNA expression as a result of nutrient restriction, between the lobes of the liver, the sexes, or due to offspring type (fig.4.12).

a)

Figure 4.12 Hepatic G6Pase mRNA expression. G6Pase mRNA expression in a) dietary group (CC = control *n* = 22, CU = postnatal undernutrition *n* = 14, UC = prenatal undernutrition *n* = 17, UU = pre- and postnatal undernutrition *n* = 22), b) sex (male *n* = 39, female *n* = 36), c) lobe (left *n* = 75, right *n* = 75) and d) offspring (singleton *n* = 28, twin *n* = 47) in adult sheep liver. Values are mean ± SEM.

4.4.6 **Hepatic Insulin Receptor mRNA expression**

At 2.5 years of age no differences were seen in insulin receptor mRNA expression as a result of nutrient restriction, between the lobes of the liver, the sexes, or due to offspring type (fig. 4.13).

a)

b)

Figure 4.13 Hepatic Insulin Receptor mRNA expression. Insulin receptor mRNA expression in a) dietary group (CC = control *n* = 22, CU = postnatal undernutrition *n* = 14, UC = prenatal undernutrition *n* = 17, UU = pre- and postnatal undernutrition *n* = 22), b) sex (male *n* = 39, female *n* = 36), c) lobe (left *n* = 75, right *n* = 75) and d) offspring (singleton *n* = 28, twin *n* = 47) in adult sheep liver. Values are mean ± SEM.

4.5 Discussion

4.5.1 Summary

In this chapter the effect of postnatal undernutrition on adult sheep hepatic gluconeogenic gene expression following an early gestation nutritional challenge was investigated as a possibility for the mechanistic basis within the liver for the dysregulation of glucose homeostasis associated in adult disease. The results show that there was no effect of maternal dietary restriction in early gestation and/or dietary restriction in early postnatal life on the liver weight of the adult sheep. Dietary restriction also had no effect on the expression of the glucocorticoid receptor, PEPCK, G6Pase and the insulin receptor. However, liver weight was lower in males than females. Males had a higher mRNA expression of GR and PEPCK than females. There was a higher expression of GR in the right lobe than the left, whereas PEPCK expression was lower in the right lobe when compared to the left. GR mRNA was found to be higher in twins than singletons but there was no difference in liver weight. The significance of these findings is discussed below.

4.5.2 Pre and postnatal undernutrition had no effect on liver weight

Early gestation and/or postnatal periods of undernutrition had no effect on liver weight in adult sheep. Previous studies have found that liver weight was lower in adult male sheep following a longer period of early gestation nutrient restriction for 1 - 95 days (Hyatt *et al.*, 2007b). In the present study the period of undernutrition was significantly shorter (1 - 31 days). Liver lobes appear as early as the 21st day of gestation and by the 23rd day of gestation left and right lobes are formed with a small caudate lobe between them (Bryden *et al.*, 1972), therefore, the timing of the gestational nutritional challenge applied here (1 -31 days) did cover the period that the liver is initially forming but perhaps the increased length of challenge (to 95 days) has a stronger influence as it is covering a longer period of liver growth. The present observation of no effect of postnatal undernutrition on liver weight after a period of normal refeeding agrees with other studies in sheep (Kabbali *et al.*, 1992a;Kabbali *et al.*, 1992b;Wester *et al.*, 1995). This suggests that the liver is capable of recovering in size following periods of undernutrition if indeed; it was at all affected in the first instance, which we did not measure. We can only speculate that the liver weight was reduced during the postnatal nutritional challenge (of 15% weight reduction) during weeks 12 – 25. If the liver was affected in size and recovered following the period of refeeding this does not mean that the function of the cells recovered in the same way and therefore that the metabolic response of the organ was not altered. Indeed, following a period of catch up growth post weaning, the offspring of undernourished rat dams were found to have increased glycogen stores in their livers but not as a result of changes in gluconeogenesis (Thompson *et al.*, 2007). The changes appear to be as a result of an increase in adipocyte size in subcutaneous and retroperitoneal adipose tissue even though actual adipose tissue weights showed no differences when compared to controls. The increased adipocyte size was accompanied by a reduction in glucose uptake and insulin response which is thought to then shift glucose preferentially to liver and muscle (Thompson *et al.*, 2014).

4.5.3 Pre and postnatal undernutrition had no effect on hepatic gluconeogenic gene expression

There were no differences in mRNA expression of GR, PEPCK, G6Pase or the insulin receptor as a result of early gestation and/or postnatal nutrient restriction in the livers of adult sheep. Other studies in neonatal sheep have found increased hepatic glucocorticoid receptor in *utero* following a nutrient restriction during days 28 – 77 of gestation (Whorwood *et al.*, 2001), but no differences in PEPCK, insulin receptor gene expression or G6Pase activity in 30 day old sheep following a gestational undernutrition period (115 days – term) (Hyatt *et al.*, 2008). In rats, hepatic GR, (Bertram *et al.*, 2001) PEPCK and G6Pase were found to be raised in adults following undernutrition in *utero (Liu et al., 2009)*. The nutritional challenges applied in the rat studies consisted of a 9% protein content applied throughout pregnancy and lactation. Obviously, the rat is a small animal model with multiple offspring, different digestion and metabolism compared to the large animal sheep model used in this study, the nutrient restriction used also differed in that a low protein diet was administered throughout pregnancy therefore covering all possible developmental windows whereas the sheep received a 50% global nutrient restriction during a restricted timeframe during pregnancy and then postnatally. The postnatal challenge was seen to increase insulin sensitivity in female adult sheep (Poore *et al.*, 2005) but this challenge had no effect on the hepatic gluconeogenic mRNA expression. Very few studies have looked at the effect of postnatal challenges following prenatal undernutrition and those that have have investigated postnatal high fat diets (Johnsen *et al.*, 2013;Hou *et al.*, 2014;Khanal *et al.*, 2014;Kongsted *et al.*, 2013).

This highlights the possibility that the window of gestation targeted in this present study was not critical in priming / perturbing liver gluconeogenic pathways, AND /OR that the intensity of the prenatal challenge was not great enough.

4.5.4 Female livers were heavier than males

In the present study male livers weighed less than female livers as a percentage of body weight. Very few other studies have looked at both sexes in adult sheep and therefore it is difficult to put our observations in context. However, one study found no difference in liver/bodyweight between the sexes regardless of peri implantation and early gestation nutrient restriction (-61 - +30dGA) (Jaquiery *et al.*, 2012).

4.5.5 Males had greater hepatic gluconeogenic gene expression

Male sheep, regardless of diet, had a greater mRNA expression of the glucocorticoid receptor and PEPCK than females. Increased GR and PEPCK indicate that there is a possibility of increased gluconeogenesis and therefore suggest an increase in glucose in the circulation. Due to lack of sheep antibodies protein expression was not measured, but these mRNA data do suggest that males have increased gluconeogenesis capacity than females. In this model we have previously shown that females have better overall glucose tolerance to a glucose load compared to males, and improved insulin sensitivity (Poore *et al.*, 2007;Poore *et al.*, 2014). Increased insulin sensitivity could result in increased inhibition by insulin on the hepatic gluconeogenic pathway and could therefore be contributing to the lower expression of these genes in females. However, there were no differences between the sexes in the expression of G6Pase or the insulin receptor. Many sheep studies contain males only (Hyatt *et al.*, 2007b) so it is often difficult to compare differences between the sexes and perhaps possible effects that are only seen in one of the sexes may be missed. The studies that have been carried out on both sexes indicate that differences in metabolic responses do exist between the sexes, the mechanisms of which require further consideration.

4.5.6 Lobe differences in hepatic gluconeogenic gene expression

GR mRNA expression was found to be higher in the right liver lobe when compared to the left, regardless of diet. The left lobe was found to have a greater expression of PEPCK mRNA. This suggests that the metabolic function between the liver lobes may be different and that the cells may have slightly different functions according to their location within the organ. In adult rat offspring from mothers fed a low protein diet throughout gestation hepatic GR mRNA expression was lower in the right compared left liver lobe. In contrast to the present study, no difference between the lobes in GR mRNA was observed following normal nutrition throughout pregnancy and lactation (Zhang & Byrne, 2000). As previously discussed in Chapter 1 (section 1.5), there are fundamental differences between rat and sheep models but in addition the rat maternal low protein diet was administered throughout gestation and therefore likely to have covered a critical time point affecting liver lobe development. The global nutrient restriction in this study covered the time point at which the fetal liver lobes initially differentiate in gestation but this may not be the critical time point to affect lobar hepatic GR, PEPCK, G6Pase or IR expression through nutrient restriction. **Dietary influences on**

lobar gene expression may emerge with more profound restriction of diet or if it is imposed later in fetal development when the blood flow to the left and right developing liver lobes is more mature.

4.5.7 Offspring number only affected PEPCK expression in females

There was no difference in liver weights (as a percentage of body weight) between twins and singletons, or in the hepatic GR, G6Pase or insulin receptor gene expression. However, in female twins PEPCK mRNA expression was found to be higher than singletons. Hepatic PEPCK mRNA has also been shown to be higher in twin fetal sheep at 136dGA (Lie *et al.*, 2014). Previous studies in these sheep have showed that female twins had increased insulin sensitivity (Poore *et al.*, 2007) which may explain why PEPCK was found to be higher in this study as PEPCK is regulated by insulin. The increase in PEPCK suggests that female twins may have a raised gluconeogenic capability and therefore produce more glucose into the circulation to enable efficient catch up growth. They therefore, may be at risk of metabolic disorders such as type 2 diabetes and obesity in later life.

4.6 Conclusions

In this chapter it was thought that early and or postnatal nutrient restriction may have an effect on the sheep liver (fig.4.14). It was found that in this adult sheep model there were no effects of postnatal undernutrition on adult sheep hepatic gluconeogenic gene expression or liver weight following an early gestation nutritional challenge and therefore that a mismatch between the pre- and postnatal environments did not have an effect. These findings did not support my initial hypothesis that restricted postnatal nutrition will increase the expression of the hepatic genes involved in gluconeogenesis in adult sheep depending on nutrition received in early gestation. In addition the dietary mismatch effects on sex specific improved insulin sensitivity and glucose tolerance previously observed in this model (Poore *et al.*, 2007) were not mirrored by effects on hepatic gluconeogenic gene alterations.

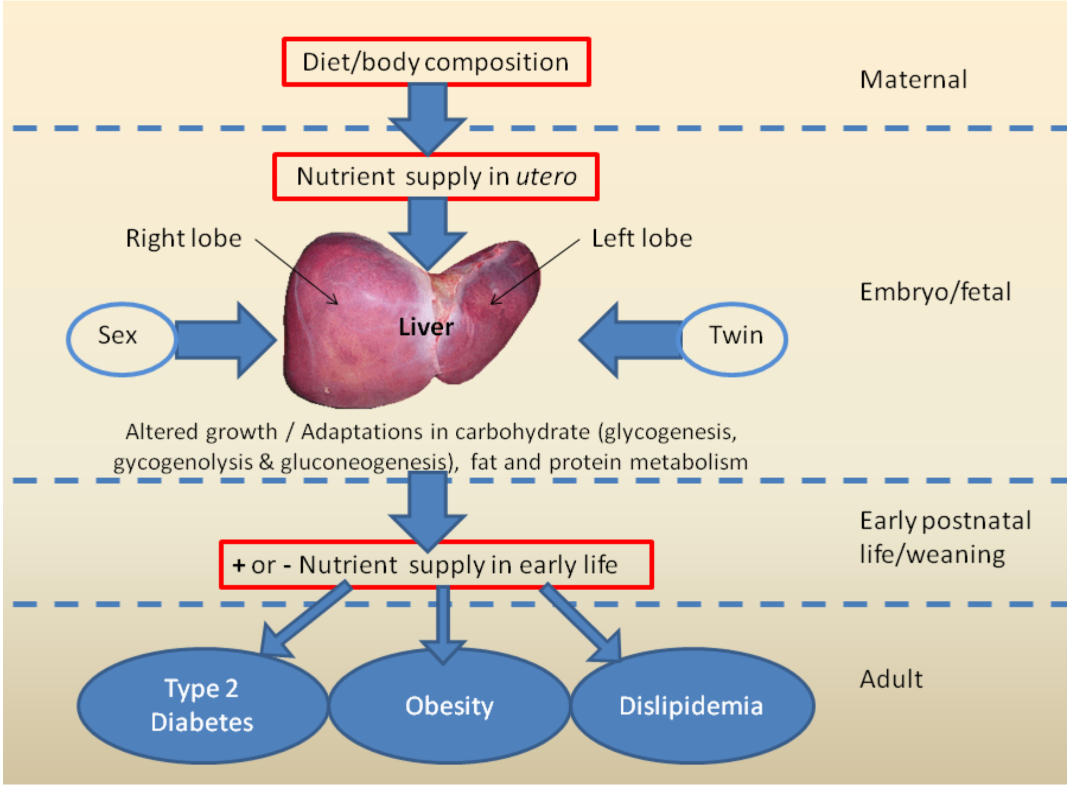


Figure 4.14 Possible influences on liver. Nutrient, sex, lobe and offspring number thought to have influences on the liver.

The intensity and length of the nutritional challenge may not have been sufficient to see changes in hepatic gluconeogenic gene expression. A harsher challenge in early, late or perhaps across the whole gestation length may produce changes in this pathway and these will be investigated further in this thesis.

Chapter 5. **Hepatic gluconeogenic gene expression in fetal sheep following early or late gestation undernutrition**

5.1 Introduction

In Chapter 4 the two major observations were firstly, of a lack of effect of diet restriction (during early gestation or post-weaning) on adult hepatic gluconeogenic gene expression and second, that there were differences in the expression of GR and PEPCK mRNA between the left and right lobes of the liver. These results were regardless of any periods of undernutrition during early gestation or early postnatal life. This chapter considers further these findings in the late gestation fetus model. The fetus is capable of adapting its cardiovascular system and metabolism in response to intrauterine nutrition challenges (Vonnahme *et al.*, 2003;Gentili *et al.*, 2009;Dong *et al.*, 2005). Current concepts suggest that these adaptations could be important in determining disease risk in later life and fetal liver adaptations could be part of this mechanism.

The fetal liver does not perform the same function of a postnatal liver as the fetus is not consuming meals but instead receiving its nutrition from its mother via the umbilical vein. It may therefore be considered that the differences between the lobes seen in Chapter 4 are influenced during fetal development of the liver as the fetal circulation is fundamentally different to that in postnatal life. During fetal development the left lobe of the liver is supplied with blood containing more oxygen than the right lobe (see 1.5.2 and fig.5.1).

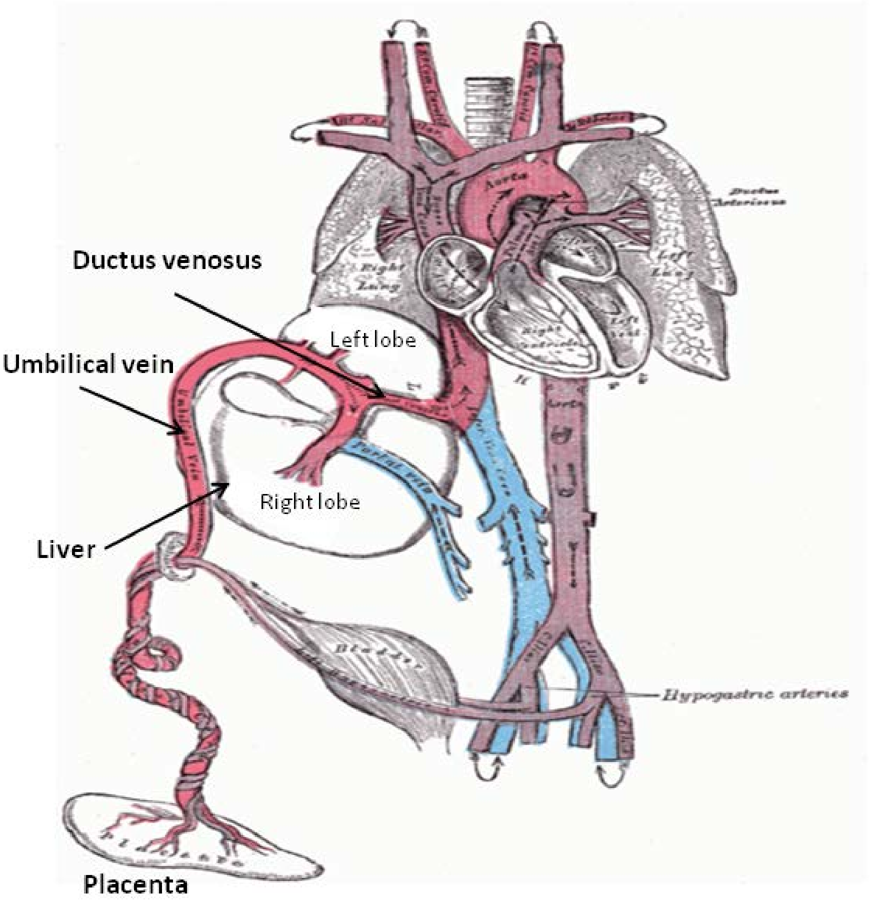


Figure 5.1 Fetal circulation. Modified from Gray's Anatomy, 1918 (Wikipedia contributors, 2007).

Propranolol extraction in vivo by the left lobe of the late gestation fetal sheep was found to be greater than by the right lobe (Ring *et al.*, 1998). Any change in fetal liver perfusion could alter hepatic development, with long-term consequences for the risk of metabolic disease. Synthetic activity and cell function are oxygen dependent and therefore I expected that there would be differences in gene expression as a result of this fetal circulation in the liver lobes in fetal sheep. This has been seen in the livers of fetal baboons whereby 872 genes were differentially expressed in the left and right lobes (Cox *et al.*, 2006). Changes in nutrient supply to the liver lobes from the mother may alter the expression of genes within each of the main lobes in fetal sheep and maybe linked to disease in later life (fig.5.2). **The effect of lobes on the mRNA expression of hepatic gluconeogenic genes in fetal sheep liver lobes has not yet been investigated.**

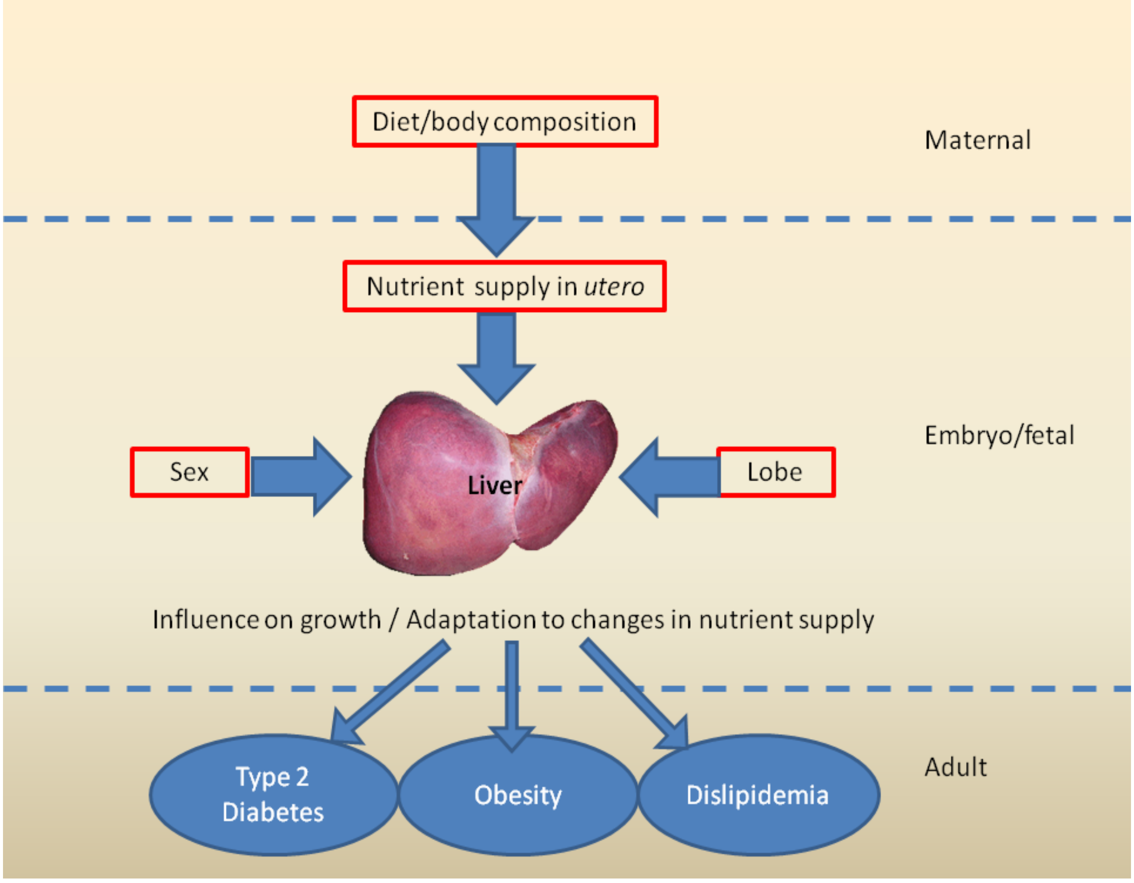


Figure 5.2 Liver as a metabolic target organ in fetal life, its nutritional influences and potential outcomes in adulthood.

The liver develops around day 20 in sheep as an endodermal outpocketing of the foregut called the hepatic diverticulum. Its initial blood supply is primarily from the vitelline veins that drain blood from the yolk sac. Lobes appear in the sheep liver as early as the 21st day and by the 23rd day left and right lobes are formed with a small caudate lobe between them (Bryden *et al.*, 1972) and so may be affected by adaptations to the early life nutrient supply. The enzymes necessary for gluconeogenesis are present in early sheep gestation with the activity of PEPCK comparable to that in mature animals detected at 45 days gestation. Other enzyme levels are low until later in gestation (100+ days) (Stevenson *et al.*, 1976). Previous studies have shown that glucose production in the fetus occurs mainly in the face of maternal starvation (Lemons *et al.*, 1986;Leury *et al.*, 1990). It has been shown that the fetus makes adaptations in response to the timing and intensity of the challenge in gestation. An early gestation (0-70 days) 15% reduction in nutrition resulted in an altered cardiovascular system (Hawkins *et al.*, 2000a) and increased blood pressure postnatally (Hawkins *et al.*, 2000b). Insulin secretion (Oliver *et al.*, 2001) and elevated glucose and lactate (Oliver *et al.*, 2005) was found to be increased in fetal sheep from mothers who had undernutrition from -61 to +30 days of pregnancy.

A mid gestation 50% nutrient restriction resulted in increased body weight, fat and dysregulated glucose uptake in male offspring (Ford *et al.*, 2007) and an early to mid gestation 50% nutrition resulted in increased cardiac IGF expression and enlarged left ventricle in fetal sheep (Dong *et al.*, 2005). In late gestation fetal sheep liver weight was reduced and the hepatic enzymes PEPCK and G6Pase were found to be increased during maternal fasting (Lemons *et al.*, 1986). Offspring from ewes fed a 50% nutrient requirement diet during late gestation showed glucose intolerance and increased adipose tissue (Gardner *et al.*, 2005). Burns *et al.* showed that the livers of adult offspring of rat dams fed a low protein diet (8% protein content) during pregnancy and lactation (MLP) had increased gluconeogenesis producing more glucose from lactate than the controls (20% protein content). In control livers, glucose produced from lactate in the periportal region of the lobule was taken up by the most distal perivenous cells; this distal perivenous uptake was greatly diminished in maternal low protein (MLP) livers. This accounted for a major part of the increased glucose output of MLP livers. Hepatic lobular volume in MLP livers was twice that of controls indicating that MLP livers had half the normal number of lobules (Burns *et al.*, 1997). Adult rat studies have also shown that the gene expression of fibrinogen and GR following a low protein (8% protein content) maternal diet throughout pregnancy and lactation was lower in the left liver lobe compared to the right (Zhang & Byrne, 2000).

These studies have shown that the gluconeogenic pathway has shown to be influenced by undernutrition in gestation. The lack of effect of the early gestation nutritional challenge in Chapter 4 may be caused by the lack of gluconeogenic enzymes present at that time. Other studies have shown that early gestational challenges have had an effect on the gluconeogenic pathway in later life and therefore the nutritional challenges may be influencing liver structure even if the enzymes themselves are not mature. **The effect of timing and intensity of maternal undernutrition in gestation on the mRNA expression of hepatic gluconeogenic genes in fetal sheep liver has not yet been investigated.**

5.2 Hypothesis

Restricted early or late gestation nutrition will increase the expression of the hepatic genes involved in gluconeogenesis in a lobe-dependent manner in the fetus.

The aim of this chapter is to determine if there is an effect of an early (with an added increased intensity) or late gestation nutrient challenge on the fetal sheep liver genes involved in gluconeogenesis (GR, PEPCK, G6Pase and the Insulin receptor).

5.3 Methods

5.3.1 Summary

Two studies were carried out in this chapter to investigate the effect of maternal undernutrition on the fetus during critical peri implantation (PI) and late gestation (L) windows. In study 2 the intensity of the peri implantational challenge was increased and the late gestation challenge was added. Control groups received full nutrient requirements throughout. No comparisons between the studies took place due to the different sheep seasons. Real-time PCR was used to measure mRNA expression of the hepatic gluconeogenic genes (GR, PEPCK, G6Pase and Insulin receptor). Samples from the left and right liver lobes of 127dGA male and female sheep were investigated.

All procedures contained in this chapter were approved by the Home Office and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

5.3.2 Ewes

Welsh Mountain ewes in their 1st parity (Study 1 n = 48, Study 2 n = 52) were brought in and maintained according to normal sheep husbandry at the Royal Veterinary College (RVC) (North Mymms, Hertfordshire). Prior to study enrolment the ewes were kept at grass. All ewes in the study were weighed and body condition scored over the 3rd lumbar vertebra as a means of assessing the fatness of the animal (see 2.1.1). Muscle and fat depth were measured from ultrasound (Aloka SSD 210 DX11, BCF Technology, Livingstone, UK) images of the 3rd lumbar region, using the internal callipers of the ultrasound machine (see 2.1.2).

5.3.3 Mating

Ewes with a condition score of 2.5 or above were enrolled over the sheep breeding season (Oct-Feb) in batches of 3-4. The ewes had a progesterone sponge inserted vaginally to synchronize oestrous, the progesterone sponge was removed at -2dGA and a raddled ram was introduced. Day 0 of gestation was taken as the first date at which an obvious raddle mark was observed.

5.3.4 Maternal Dietary Manipulation

Ewes were housed on straw and nutritional requirements were calculated according to ewe start weight (measured at -16dGA) and adjusted for fetal gestational age according to Agriculture and Food Research Council (AFRC) nutritional guidelines 1993, (RUMNUT (Ruminant Nutrition) version 5 for sheep software A T Chamberlain, Hampshire).

In study 1 ewes were fed either 100% nutrient requirements (C1, *n* = 9) or a restricted diet at peri-implantation using a complete pellet diet (see Appendix 3.1) (PI50, *n* = 10, 50%, 1-31dGA (term ~147)). In study 2 ewes were fed either 100% nutrient requirements (C2, *n* = 8) or a restricted diet at peri-implantation (PI40, *n* = 9, 40%, 1-31dGA (term ~147)) or in late gestation (L, *n* = 6, 50%, 104dGA – post-mortem) (fig.5.3). At all other times animals were fed 100% of the nutrient requirements. Water was provided *ad libitum* at all times.

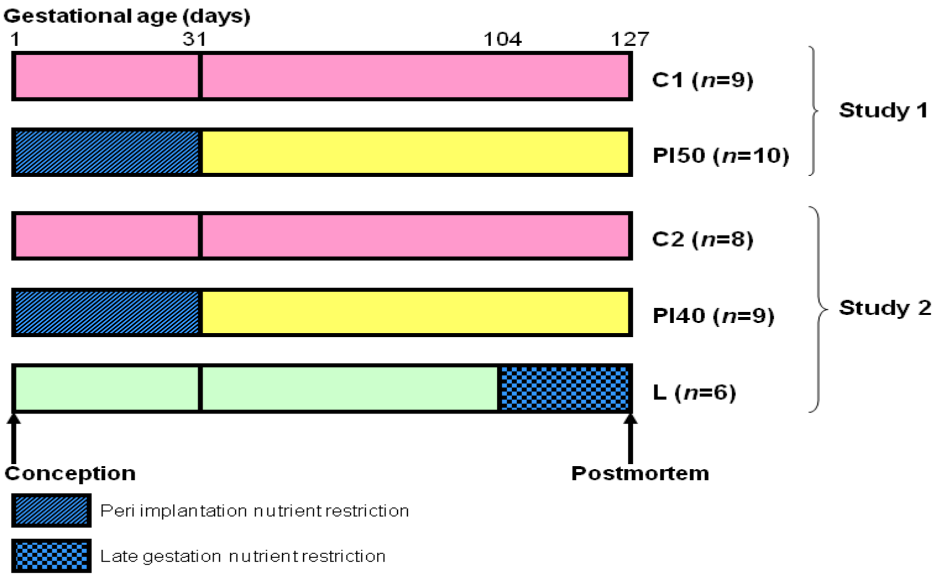


Figure 5.3 Maternal nutritional protocol. Maternal nutritional protocol; blue boxes show periods of undernutrition, for studies 1 and 2. C1 and C2, control groups; PI50 and PI40, peri-implantation restriction to 50% and 40% control, respectively; and L, late gestation restriction to 50% of controls from before conception to post-mortem (127 days) (where term is 147d), *n* = number.

5.3.5 Maternal monitoring

Pregnancy was confirmed using a progesterone assay (ELISA, Ridgeway Science, UK) on plasma collected directly from the jugular vein into a chilled lithium heparin blood collection tubes at 18dGA. Ewes were ultrasound scanned at ~55dGA and any ewes that were empty or carrying twins were removed from the study.

5.3.6 Fetal post-mortem

All ewes and fetuses were killed with an overdose of sodium pentobarbitone to the ewe intravenously at 127dGA. The abdomen was opened and the uterus removed at the cervix. Under sterile conditions the uterus was opened and the fetus removed and the umbilical cord cut. The organs were dissected, weighed in sterile trays and the required regions were dissected and processed according to the post-mortem sheet (Appendix 3.2). Samples were fast frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

5.3.7 RNA extraction, Reverse transcription & Real-time PCR

Total RNA was extracted, yield, quality and purity were determined from the left and right lobes of fetal lamb liver using the methods previously described (see 2.2.1). The RNA was reverse transcribed into cDNA for the polymerase chain reaction (see 2.2.3). mRNA levels were analysed in 127dGA fetal liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7500 Sequence Detection System, USA). Primers and probes as previously designed for use in the adult sheep in Chapter 4 were used (see table 2.1). One sample of liver RNA (Male, right lobe) was used to produce a standard curve (fig.5.4). The highest standard equals 1μg of RNA and was given the relative RNA concentration of 100; this was double diluted to produce the standard curve. The standard curve shows the log of the relative RNA against the Ct, as there is an exponential change in fluorescence. The relative RNA concentration for all samples was calculated from the standard curve and was expressed relative to the housekeeping genes β-Actin, RPL-19 and GAPDH as these had been previously determined in fetal ovine liver tissue (see Chapter 3).

Ct value

Figure 5.4 The standard curve for GR mRNA in fetal sheep.

5.3.8 Analysis strategy and statistics

For a general description of the analysis strategy, the power calculations and statistics packages used see Chapter 2.

All mRNA expression data were normalized using the geometric mean of three housekeeping genes (β-actin, GAPDH and RLP-19).

All data were checked for normal distribution by histogram prior to analysis. Data not normally distributed was transformed by the natural logarithm and subsequently checked for normal distribution.

Differences in liver weight were analysed by univariate ANOVA with between subject factors of diet, sex and offspring.

Differences in lobar gene expression were analysed by repeated measures ANOVA with between subject factors of sex, offspring and diet.

Significance was accepted when $p < 0.05$, however 'trends' are discussed when $0.5 < p < 0.1$.

5.4 Results

All *P* values can be found in Appendix 3.3.

5.4.1 Fetal weight and metabolic markers

Early or late gestation undernutrition had no effect on fetal weight. There were no differences in fetal basal glucose, cortisol, insulin, ACTH or catecholamines between any of the diet groups or sexes (data not shown) (Burrage *et al.*, 2009).

5.4.2 Liver weight

There was no difference in liver weight as a percentage of body weight between dietary groups (fig.5.5a and c) or male and female (fig.5.5b and d) 127dGA fetuses in either study.

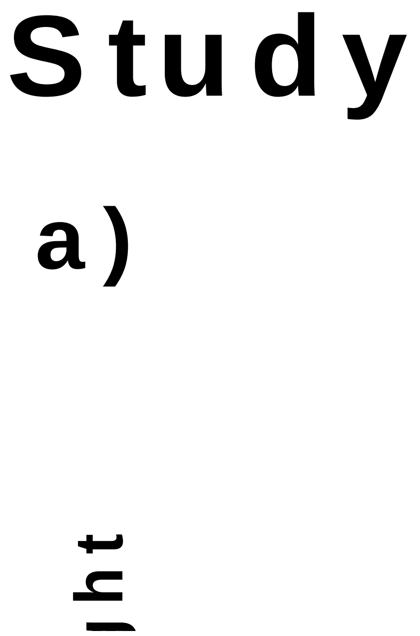


Figure 5.5 Liver weight. Liver weight as a percentage of body weight in dietary groups and male and female fetal sheep (127dGA) Study 1 dietary groups (a) Control = 100% nutrient requirement *n* = 9, PI50 = 50% nutrient requirement *n* = 10, study 1 sex (b): male *n* = 8, female *n* = 11, study 2 dietary groups (c): Control *n* = 7, PI40 = 40% nutrient requirement *n* = 9, Late = 50% nutrient requirement *n* = 6, study 2 sex (d) male *n* = 13, female *n* = 9. Values are mean ± SEM.

5.4.3 **Hepatic Glucocorticoid Receptor mRNA expression**

In study 1 there was no effect of diet, sex or lobe on the expression of GR mRNA (fig.5.6 a, c and e), but within the right lobe GR mRNA was found to be lower in the peri implantation nutrient restriction group (PI50) when compared to controls (fig.5.6g).

In study 2 GR mRNA expression was lower in both the early (PI40) and late gestation nutrient restricted groups compared to controls (fig.5.6b). There was no effect of sex on GR mRNA expression (fig 5.6d). However lobe differences were seen with a lower GR mRNA expression seen in the right lobe (fig 5.6f). Both PI40 and late gestation dietary groups were found to have a lower GR MRNA expression than controls in the left lobe (fig.5.6h) but only PI40 was found to be lower than controls in the right lobe.

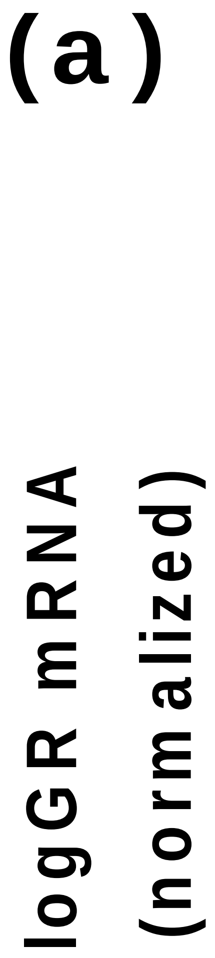


Figure 5.6 Hepatic Glucocorticoid Receptor mRNA expression. GR mRNA expression in 127dGA fetal sheep. Study 1: Dietary groups (a) Control = 100% nutrient requirement *n* = 9, PI50 = peri implantation 50% nutrient requirement *n* = 10, Sex (c) male *n* = 8, female *n* = 11, Lobe (e) *n* = 19 and Diet & Lobe (g) C L = control group left lobe *n* = 9, PI150 L = peri implantation 50% nutrient requirement left lobe *n* = 10, C R = control group right lobe *n* = 8, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 10. Study 2: Dietary groups (b) Control *n* = 7, PI40 = 40% nutrient requirement *n* = 9, La = late *n* = 6, (d) sex, male *n* = 13, female *n* = 9, (f) lobe, *n* = 22, (h) C L = control group left lobe *n* = 7, PI140 L = peri implantation 40% nutrient requirement left lobe *n* = 9, C R = control group right lobe *n* = 7, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 9, La *n* = 6. Values are mean ± SEM. * P< 0.05 ** P<0.001.

5.4.4 **Hepatic PEPCK mRNA expression**

In study 1 there was no effect of diet, sex or lobe on the expression of PEPCK mRNA (fig.5.7a, c and e) but within the right lobe PEPCK mRNA was found to be lower in the peri implantation nutrient restriction group (PI50) when compared to controls (fig.5.7g).

In study 2 PEPCK mRNA expression was lower in both the peri implantation (PI40) and late gestation nutrient restricted groups compared to controls (fig.5.7b). There was no effect of sex or lobe on PEPCK mRNA expression (fig.5.7d & f). Both PI40 and late gestation dietary groups were found to have a lower PEPCK mRNA expression than controls in the left lobe but only PI40 was found to be lower than controls in the right lobe (fig.5.7h).

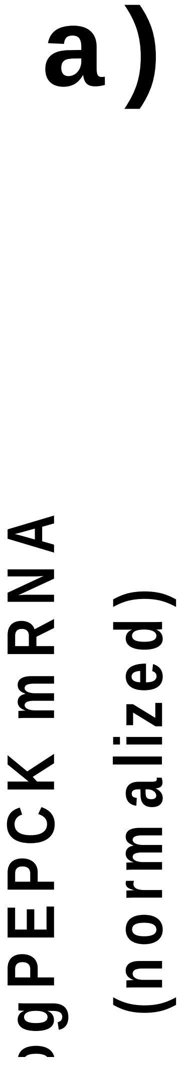


Figure 5.7 Hepatic PEPCK mRNA expression. PEPCK mRNA expression in 127dGA fetal sheep. Study 1: Dietary groups (a) Control = 100% nutrient requirement *n* = 9, PI50 = peri implantation 50% nutrient requirement *n* = 10, Sex (c) male *n* = 8, female *n* = 11, Lobe (e) *n* = 19 and Diet & Lobe (g) C L = control group left lobe *n* = 9, PI150 L = peri implantation 50% nutrient requirement left lobe *n* = 10, C R = control group right lobe *n* = 8, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 10. Study 2: Dietary groups (b) Control *n* = 7, PI40 = 40% nutrient requirement *n* = 9, La = late *n* = 6, (d) sex, male *n* = 13, female *n* = 9, (f) lobe, *n* = 22, (h) C L = control group left lobe *n* = 7, PI140 L = peri implantation 40% nutrient requirement left lobe *n* = 9, C R = control group right lobe *n* = 7, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 9, La *n* = 6. Values are mean ± SEM. * P< 0.05 ** P<0.001.

5.4.5 **Hepatic G6Pase mRNA expression**

In study 1 there was no effect of diet, sex or lobe on the expression of G6Pase mRNA (fig.5.8a, c and e) but within the right lobe G6Pase mRNA was found to be lower in the peri implantation nutrient restriction group (PI50) when compared to controls (fig.5.8g).

In study 2 G6Pase mRNA expression was lower in both the peri implantation (PI40) and late gestation nutrient restricted groups compared to controls (fig.5.8b). There was no difference between the sexes in G6Pase mRNA expression (fig.5.8d). The right lobe was found to have a lower G6Pase mRNA expression than the left lobe (fig.5.8g). Both PI40 and late gestation dietary groups were found to have a lower PEPCK mRNA expression than controls in the left lobe but there was no difference between the dietary groups in the right lobe (fig.5.8h).

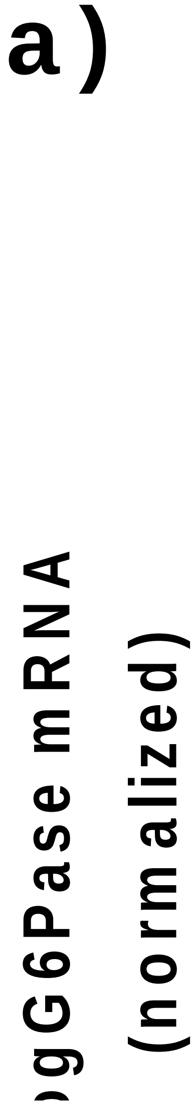


Figure 5.8 Hepatic G6Pase mRNA expression. G6Pase mRNA expression in 127dGA fetal sheep. Study 1: Dietary groups (a) Control = 100% nutrient requirement *n* = 9, PI50 = peri implantation 50% nutrient requirement *n* = 10, Sex (c) male *n* = 8, female *n* = 11, Lobe (e) *n* = 19 and Diet & Lobe (g) C L = control group left lobe *n* = 9, PI150 L = peri implantation 50% nutrient requirement left lobe *n* = 10, C R = control group right lobe *n* = 8, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 10. Study 2: Dietary groups (b) Control *n* = 7, PI40 = 40% nutrient requirement *n* = 9, La = late *n* = 6, (d) sex, male *n* = 13, female *n* = 9, (f) lobe, *n* = 22, (h) C L = control group left lobe *n* = 7, PI140 L = peri implantation 40% nutrient requirement left lobe *n* = 9, C R = control group right lobe *n* = 7, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 9, La *n* = 6. Values are mean ± SEM. * P< 0.05.

5.4.6 Hepatic Insulin Receptor mRNA expression

In study 1 insulin receptor mRNA expression was found to be higher in the peri implantation nutrient restriction group compared to controls (fig.5.9a) there was no effect of sex or lobe on the expression of insulin receptor mRNA (fig.5.9c and e), but there was a trend for the right lobe insulin receptor mRNA to be higher in the peri implantation nutrient restriction group when compared to controls (P=0.084) (fig.5.9g).

In study 2 there were no dietary differences in insulin receptor mRNA expression (fig.5.9b) nor between the sexes (fig.5.9d) or lobes (fig.5.9f) or in the dietary groups within the individual liver lobe (fig.5.9h).

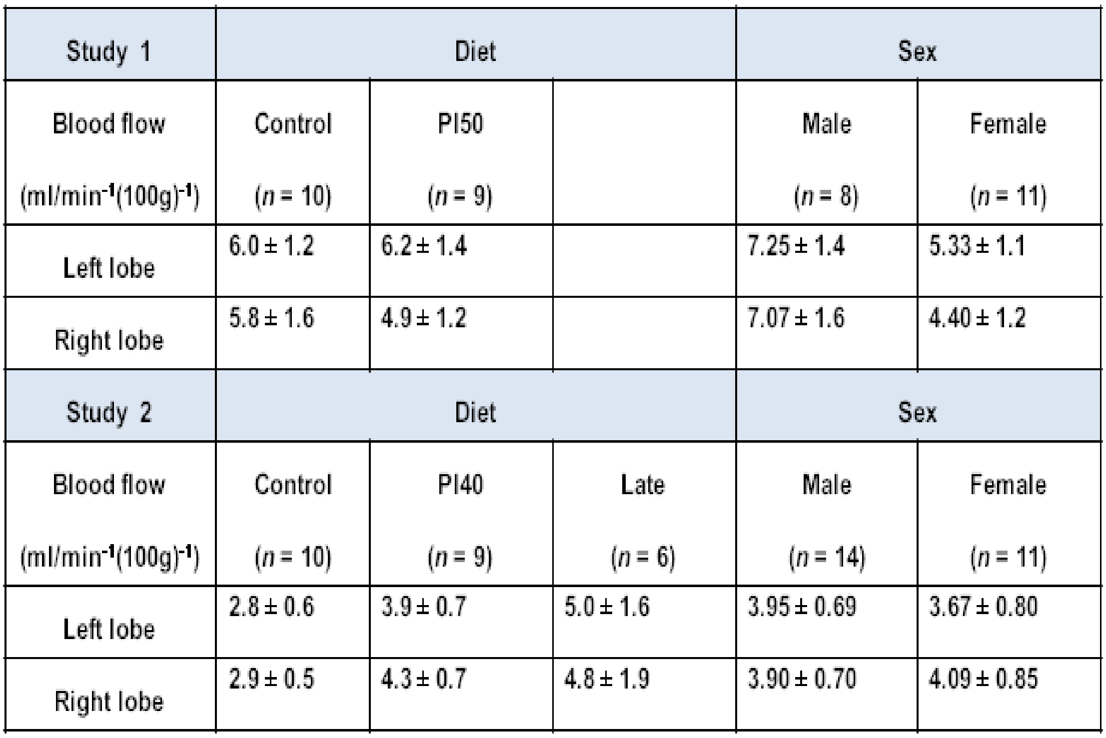
a)

gInsrec mRNA
(normalized)

Figure 5.9 Hepatic Insulin Receptor mRNA expression. . Insulin receptor mRNA expression in 127dGA fetal sheep. Study 1: Dietary groups (a) Control = 100% nutrient requirement *n* = 9, PI50 = peri implantation 50% nutrient requirement *n* = 10, Sex (c) male *n* = 8, female *n* = 11, Lobe (e) *n* = 19 and Diet & Lobe (g) C L = control group left lobe *n* = 9, PI150 L = peri implantation 50% nutrient requirement left lobe *n* = 10, C R = control group right lobe *n* = 8, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 10. Study 2: Dietary groups (b) Control *n* = 7, PI40 = 40% nutrient requirement *n* = 9, La = late *n* = 6, (d) sex, male *n* = 13, female *n* = 9, (f) lobe, *n* = 22, (h) C L = control group left lobe *n* = 7, PI140 L = peri implantation 40% nutrient requirement left lobe *n* = 9, C R = control group right lobe *n* = 7, PI150 R = peri implantation 50% nutrient requirement left lobe *n* = 9, La *n* = 6. Values are mean ± SEM. * P< 0.05.

5.4.7 Fetal liver blood flow and gene expression

Total fetal blood flow to the left and right lobes of the liver in the 127dGA sheep fetus were previously reported (Burrage, 2006) (table 5.1). In all fetuses there was no difference in blood flow to the lobes regardless of the dietary challenges or their sex. Fetal lobar liver blood flow was correlated to the mRNA gene expression with no significant correlation found (see Appendix 3.4).



Study 1	Diet			Sex	
Blood flow (ml/min^{-1}(100g)$^{-1}$)	Control (*n* = 10)	PI50 (*n* = 9)		Male (*n* = 8)	Female (*n* = 11)
Left lobe	6.0 ± 1.2	6.2 ± 1.4		7.25 ± 1.4	5.33 ± 1.1
Right lobe	5.8 ± 1.6	4.9 ± 1.2		7.07 ± 1.6	4.40 ± 1.2
Study 2	**Diet**			**Sex**	
Blood flow (ml/min^{-1}(100g)$^{-1}$)	Control (*n* = 10)	PI40 (*n* = 9)	Late (*n* = 6)	Male (*n* = 14)	Female (*n* = 11)
Left lobe	2.8 ± 0.6	3.9 ± 0.7	5.0 ± 1.6	3.95 ± 0.69	3.67 ± 0.80
Right lobe	2.9 ± 0.5	4.3 ± 0.7	4.8 ± 1.9	3.90 ± 0.70	4.09 ± 0.85

Table 5.1 Total blood flow to the liver lobes of fetal sheep. Liver blood flow to the left and right fetal liver lobes for studies 1 and 2 by dietary groups and sex (Control = 100% of nutrient requirements, PI50 = peri implantation, 50% nutrient requirements, PI40 = peri implantation, 40% nutrient requirements and Late = late gestation, 50% of nutrient requirements). Values are mean ± SEM. *n* = number. (Burrage *et al.*, 2009).

5.5 Discussion

5.5.1 Summary

In Chapter 4 it was found that diet had no effect on the expression of gluconeogenic genes but that lobe and sex differences did exist in adult sheep. This Chapter examined the intensity and timing of a nutritional challenge on the fetus. Early gestational nutrient challenges (during days 1-31 of gestation) of 50% and 40% of requirements were employed as was a late gestational challenge (during days 104 – 127) of 50%. There was no effect of maternal dietary restriction in early or late gestation on fetal sheep liver weight at 127dGA nor was there any effect of sex. Early gestation (50%) dietary restriction was shown to increase mRNA expression of the insulin receptor, and reduced mRNA expression of the glucocorticoid receptor, PEPCK and G6Pase genes in the right but not the left fetal liver lobe. A more intense dietary restriction (40%) in early gestation and a late gestation undernutrition reduced the mRNA expression of GR, PEPCK and G6Pase, but had no effect on the insulin receptor. For GR and PEPCK, the impact of late gestation undernutrition was predominantly in the left lobe. The significance of these findings is discussed below.

5.5.2 Gestational nutrient restriction had no effect on liver weight

The results from both studies 1 and 2 showed that neither the early gestation nor late gestation periods of undernutrition had an effect on liver weight. In previous studies 78dGA fetal liver weight as % body weight was higher following 50% nutrient restriction during 28 – 78 dGA (Vonnahme *et al.*, 2003). Lower fetal liver weights (compared to controls) were found in 135dGA sheep following 60% nutrient requirement diet from 64 - 135dGA (Reed *et al.*, 2007) and also when a more severe nutrient restriction of 25% nutrient requirements during late gestation (100-124dGA) was administered (Bauer *et al.*, 1995) and also when subjected to placental restriction (Kind *et al.*, 1995). In rats liver weight is reduced in fetuses from maternal low protein diet throughout gestation and liver structure is altered (Ramadan *et al.*, 2013). These studies found that liver weight was affected at a timepoint in gestation further along than the study here and for a longer duration. No difference in fetal liver weight was found in sheep that experienced a 60% nutrient requirement challenge during days 28 – 80 (Hyatt *et al.*, 2004) suggesting that the severity of the restriction and duration/timepoint at which the restriction is experienced affects the outcome in liver weight. It appears that timepoint and duration at which the nutritional challenge was administered in this study may have been reason why the nutritional challenge had no effect on the liver weight.

5.5.3 Early and late undernutrition altered hepatic gluconeogenic gene expression

Early gestation nutrient restriction of 40% of requirements reduced GR, PEPCK and G6Pase mRNA expression in the livers of fetal sheep. Early gestation nutrient restriction of 50% of requirements was found to increase the mRNA expression of the insulin receptor. This does not fit with my initial hypothesis that undernutrition would lead to an increase in the expression of genes involved in gluconeogenesis as a mechanism for increasing supply of glucose from non dietary sources in the face of reduced maternal nutrition. However, it may be that in the case of the less severe nutritional challenge in study 1 the increased expression of the insulin receptor is a response to restrict the growth of the fetus to enable survival in the face of reduced nutrition. When the nutrition is reduced further as in the case of study 2 the fetus responds with a harsher response in reducing the expression of the gluconeogenic genes themselves. Gluconeogenesis is thought to become active in late gestation in the sheep fetus (Fowden *et al.*, 1998) and therefore because the expression of the gluconeogenic genes was found to be lower in fetal sheep from peri implantation nutrient restricted mothers the point of post-mortem may be too soon (at 127 days) in this case. Other studies in 20dGA rats have found increased hepatic GR mRNA expression due to maternal low protein diets (MLP) throughout gestation (Bertram *et al.*, 2001). PEPCK mRNA expression was found to be increased in 20dGA rats following a peri implantation low protein diet (Kwong *et al.*, 2007). Hepatic PEPCK and G6Pase enzyme activity has been found to be increased 20dGA rats following late gestation MLP diet (Franko *et al.*, 2009). Hepatic PEPCK mRNA expression has also been found to be higher in 165dGA (term 180 days) fetal baboons following reduced maternal nutrition (70% requirements) from early gestation (Nijland *et al.*, 2010). In neonatal sheep hepatic GR mRNA increased following a 50% nutrient reduction during days 28 – 77 of gestation (Whorwood *et al.*, 2001). PEPCK and G6Pase mRNA expression in the liver was increased in a placental insufficiency fetal sheep model at 134dGA (Thorn *et al.*, 2009). However, one study does agree with the findings here; hepatic PEPCK and insulin receptor mRNA expression were found to be lower in 138dGA fetal sheep following a peri implantation nutrient restriction (Lie *et al.*, 2014). In study 2, when the intensity of the early gestation undernutrition was greater (40% of requirements) the effect on gluconeogenic genes was apparent in both left and right liver lobes.

5.5.4 **No differences between the sexes**

No differences between the sexes were found in the liver weights, blood flow, or mRNA expression of any of the genes examined. Thus it appears that the sex differences are not seen until adult life (as discovered in Chapter 4), though what triggers these is unknown. Possible theories may be that hormonal changes at puberty whereby insulin sensitivity drops, fat increases and a major shift in hormone production occurs (Goran & Gower, 2001) contribute to the gender differences seen in insulin sensitivity in adult life. Indeed, elevated secretion of cortisol and/or testosterone induces insulin resistance in muscle. This also seems to be the case for low testosterone concentrations in men. In addition, cortisol increases hepatic gluconeogenesis. Cortisol and testosterone have "permissive" effects on adipose lipolysis and therefore amplify lipolytic stimulation; FFA, cortisol, and testosterone thus have powerful combined effects, resulting in insulin resistance and increased hepatic gluconeogenesis (Bjorntorp, 1991). Testosterone deficiency in male rats was seen to elevate fasting blood glucose levels (Xia *et al.*, 2013) and low testosterone levels in men is associated with type 2 diabetes (Grossmann, 2011). In contrast, estrogen was seen to depress gluconeogenesis in trout (Washburn *et al.*, 1993).

5.5.5 Lobe differences in hepatic gluconeogenic gene expression

In study 2 but not study 1, the right liver lobe was found to have a lower mRNA expression of GR, and G6Pase mRNA than the left. The left and right lobe differences existed regardless of any periods of nutrient restriction. This is in keeping with the findings in adult sheep (Chapter 4) whereby the expression of GR and PEPCK hepatic genes was shown to differ between the left and right lobes.

The differential lobe expression in study 2 suggests that the metabolic function between the liver lobes may be different in that perhaps one produces or stores more glucose as one lobe may be more active in gluconeogenesis as shown by the difference in genes expressed here. In this case it would suggest that the right lobe is less gluconeogenic than the left but without further investigation this cannot be confirmed. In keeping with this chapter, differential gene expression has been seen between the lobes of fetal baboons (Cox *et al.*, 2006) and fetal mice (Chianale *et al.*, 1988) and is thought to exist due to different microenvironments. The left and right lobes are perfused by vascular systems carrying different concentrations of oxygen and nutrients (Chianale *et al.*, 1988) and these are thought to influence the gene expression and possible function. The umbilical vein delivers blood equally to both fetal liver lobes (even though anatomically that does not appear to be the case) (Edelstone *et al.*, 1978) and agrees with the finding in this study where no difference in blood flow to the lobes was seen. However, the right lobe is additionally supplied with poorly oxygenated blood from the portal vein (Edelstone *et al.*, 1980) which reduces the oxygen delivery to the right lobe and may explain differences in gene expression. Indeed, the right lobe was seen to appear less well oxygenated during isolated perfusion of fetal sheep liver (Ring *et al.*, 1998). This suggests that the metabolic function between the liver lobes may be different and that the cells may have slightly different functions according to their location within the organ. The global nutrient restriction in these studies covered the time point in gestation at which the fetal liver lobes initially differentiate, but this may not be the critical time point to affect lobar hepatic GR, PEPCK, G6Pase or IR expression through nutrient restriction. Differences in lobar gene expression may originate later in fetal development when the blood flow to the left and right developing liver lobes is very different to the adult circulation. The reason for a lack of lobar difference in gene expression in Study 1 is hard to explain and may simply be due to a different cohort of sheep used in a different season from those used in study 2 or that the difference is influenced by an unknown external factor not accounted for such as differing winter temperatures between the two cohorts. Colder temperatures have been shown to increase liver growth and alter gene expression in sheep (Hyatt *et al.*, 2008) but this cannot be confirmed as an explanation for the differing results seen between the two studies here as it is something that is unknown.

5.6 **Conclusions**

Overall the data in this chapter suggest that as hypothesized the late gestation fetal hepatic gluconeogenic pathway is affected by early nutrition challenges and more immediate challenges in late gestation (fig.5.10). A less severe nutrient challenge (as seen in study 1) appears to influence the hepatic insulin pathway as a possible mechanism to restrict fetal growth by inhibiting gluconeogenesis whereas a more intense restriction (as seen in study 2) sees an even harsher response in the actual reduction of the gluconeogenic genes themselves. Moreover the data suggest that the impact is more uniform (i.e. in both lobes) with a more severe peri-implantation challenge. In adult offspring (Chapter 4) which were exposed to a similar period and intensity of undernutrition during early gestation as used in study 1, there was no such effect on hepatic gluconeogenic genes. It may be that any differences affected during fetal life had been ‘adjusted’ for by mature adulthood. It is possible that epigenetic marks laid down by early challenges may remain into adult life with later implications particularly under conditions of further stress. The nutrition challenge in question in the current study was of moderate intensity and for a reasonably small window in early gestation.

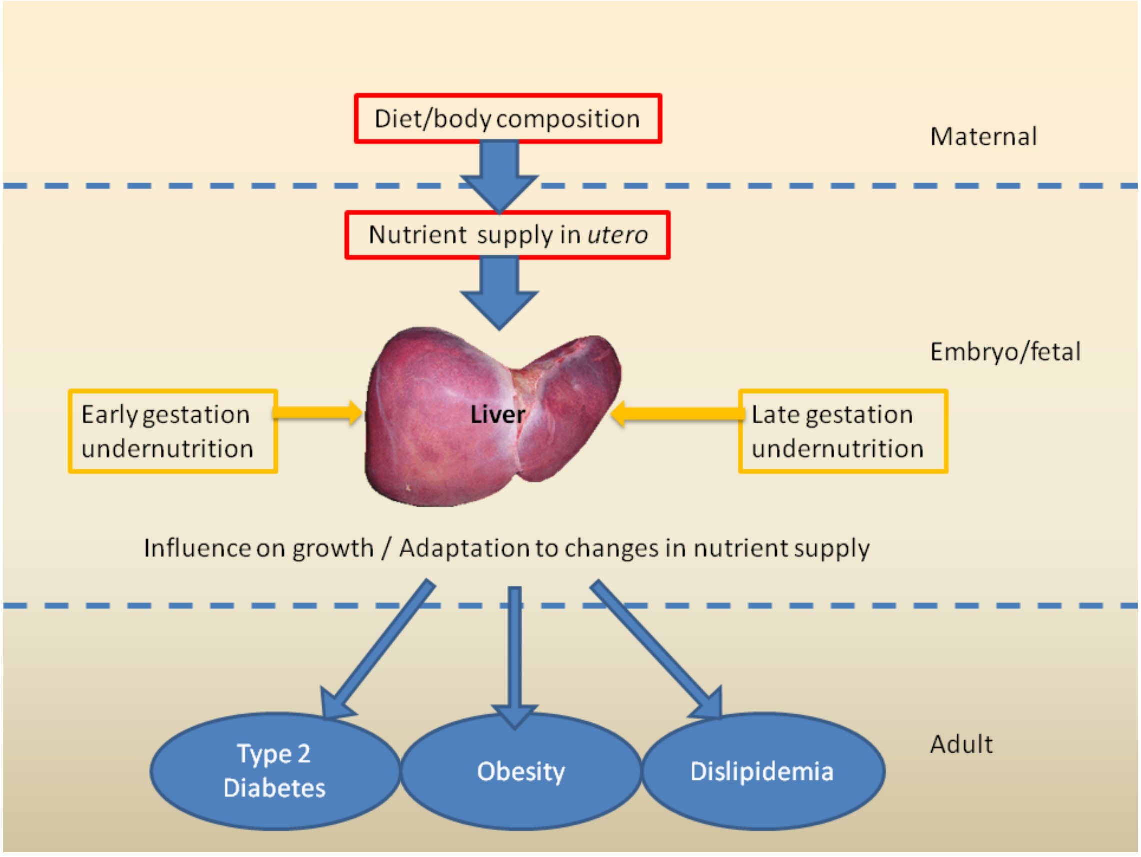


Figure 5.10 Undernutrition influences on fetal liver. Undernutrition in early and late gestation was shown to influence fetal liver gene expression.

Indeed we do not know if the late gestation undernutrition challenge used in this current chapter would have resulted in altered hepatic gluconeogenic gene expression in adulthood. This concept is pursued further in Chapter 6, in which the effect of an undernutrition challenge which spans the whole of gestation is examined.

Chapter 6. **Effect of early life undernutrition throughout gestation and weaning on the liver of 4.5 year old male sheep**

6.1 **Introduction**

The risk of metabolic disease including obesity and type 2 diabetes has been shown to be increased due to poor nutrition in early life (Jackson *et al.*, 1996;Roseboom *et al.*, 2011). Previous studies have shown that changes in metabolic response can be altered by nutrient restriction during fetal development. Human studies have found an increase in plasma glucose, insulin and triacylglycerides was associated with a low maternal body mass index (Mi *et al.*, 2000). It has been demonstrated that blood flow to the liver can be altered in response to environmental conditions such as the thinness of the mother or poor nutrition. Haugen *et al.* discovered, in humans, that fetuses of slimmer mothers with lower body fat stores and those eating an unbalanced diet had greater liver blood flow and shunted less blood away from the liver through the ductus venosus at 36 weeks (Haugen *et al.*, 2005) and it is thought that the change in nutrient supply to the liver may affect the expression of genes and may therefore influence its function in later life (fig.6.1). In Chapter 4 the effect of undernutrition in early gestation with or without an accompanying postnatal challenge was investigated and not found to induce any changes in hepatic mRNA gene expression compared to controls, this was thought to perhaps be due to the severity and/or the timing of the challenge. In Chapter 5 an early and a late gestation challenge was employed and was found to induce changes in hepatic mRNA gene expression of GR, PEPCK and G6Pase in fetal sheep. An opportunity arose to study a group of adult male sheep that had experienced poor nutrition throughout gestation and weaning via the control of their mother's body condition score. Metabolic markers had previously been studied in these sheep at 1.5 years of age and those from low body condition scored mothers were found to have increased fasting glycemia, glucose intolerance and impaired insulin secretory response when compared to those from mothers with a higher body condition score (Cripps *et al.*, 2008).

Metabolic markers in blood plasma that we are able to measure include: cortisol, cholesterol, high density lipoprotein, low density lipoprotein, triacylglyceride, nonesterifed fatty acid, and urea.

Cortisol

Cortisol is a glucocorticoid steroid hormone, produced in the adrenal glands and released in response to stress. Its primary functions are to increase blood sugar through gluconeogenesis, suppress the immune system, and aid with fat, protein, and carbohydrate metabolisms (Frayn, 2003).

Cholesterol

Cholesterol is a lipid synthesised by the liver and is vital for normal functioning of the body as it builds and maintains membranes. Hyperlipidemia increases the risk of serious health conditions such as heart disease. Cholesterol is carried in the blood by proteins; these two combined are known as lipoproteins. There are harmful and protective lipoproteins known as HDL and LDL, (or 'good' and 'bad' cholesterol) (Frayn, 2003).

High-density lipoprotein (HDL)

HDL carries cholesterol from the cells to the liver, where it is either broken down or removed as waste hence the name "good cholesterol" (Frayn, 2003).

Low-density lipoprotein (LDL)

LDL carries cholesterol from the liver to the cells that need it. Excess cholesterol builds up in artery walls causing arterial disease hence the name "bad cholesterol" (Frayn, 2003).

Triacylglyceride (TAG)

Triacylglyceride is glyceride in which the glycerol is esterified with three fatty acids. Unlike other molecules, triacylglyceride (also known as neutral fat) can pass through the cell membrane freely, due to its non-polar characteristic which doesn't react with the phospholipids bilayer membrane. Triacylglycerides, play an important role in metabolism as energy sources and transporters of dietary fat (Frayn, 2003).

Non esterified fatty acid (NEFA)

NEFA or free fatty acids are fundamental units in the structure of lipids in membranes and lipoproteins. They are molecules released from triacylglycerides which are transported in the blood bound to albumin. They provide an important source of energy for the heart and skeletal muscle. Plasma NEFA concentrations are increased in obesity and type 2 diabetes. Insulin resistance leads to impaired NEFA uptake and a rise in plasma NEFA and triglyceride concentrations (Frayn, 2003).

Urea

Urea is a by-product of protein metabolism that is formed in the liver. Because urea contains ammonia, which is toxic to the body, it must be quickly filtered from the blood by the kidneys and excreted in the urine (Frayn, 2003).

The effect of undernutrition on metabolic markers have been studied in sheep and early to mid gestation (days 28 – 78) undernutrition in the ewe resulted in dysregulated glucose uptake in young male sheep. Sheep that experienced nutrient restricted during gestation had a greater area under the curve for glucose following an intravenous glucose tolerance test indicating glucose intolerance (Ford *et al.*, 2007). This was also seen in sheep that experienced nutrient restricted following restriction at days 0 – 30 and 110 - term (Gardner *et al.*, 2005). Gene expression of growth factors such as insulin-like growth factor I (IGF-I) and hepatocyte growth factor (HGF) were also seen in 30 day old sheep following maternal nutrient restriction in late gestation (Hyatt *et al.*, 2007a).

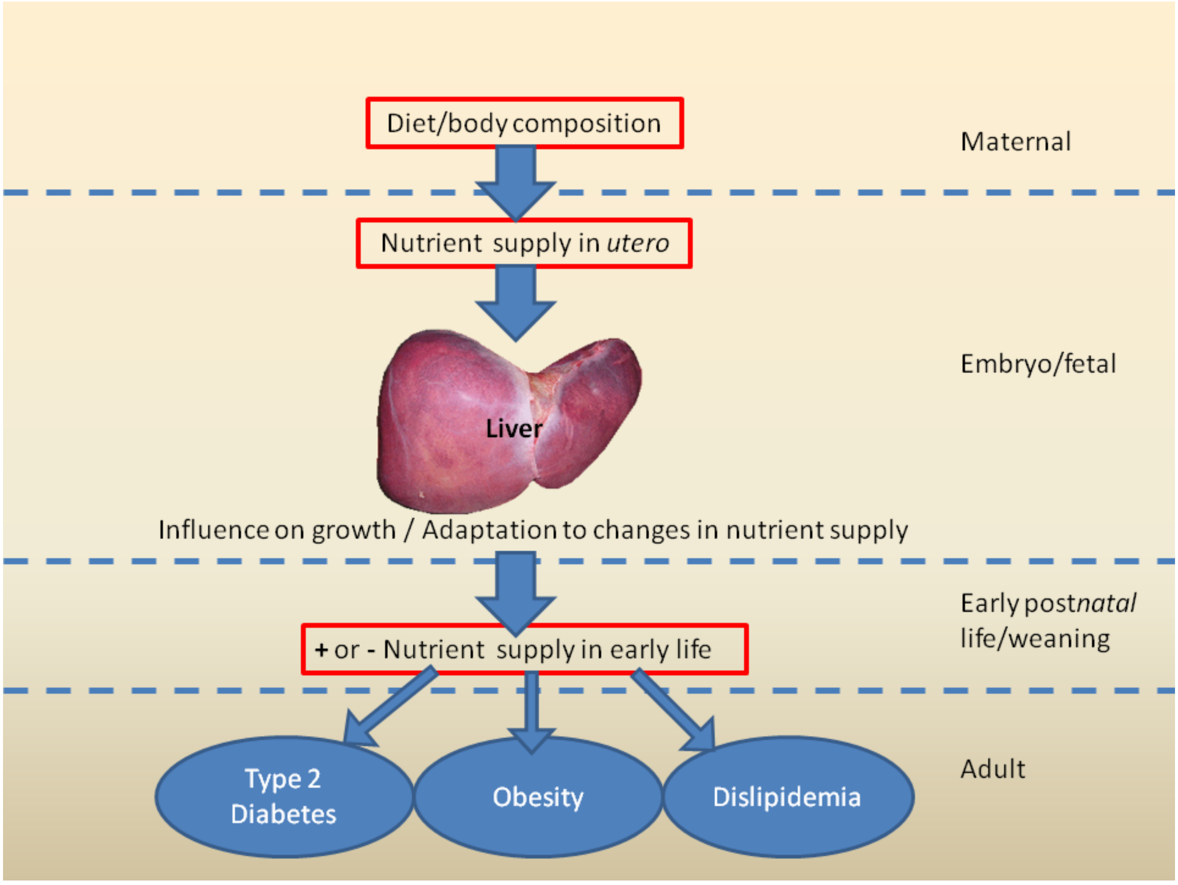


Figure 6.1 Liver as a metabolic target organ, its early life nutritional influences and potential outcomes in adulthood.

It was decided to investigate this as a model for whole gestation and weaning nutrient restriction (fig.6.1) and to see if the effects, seen in the previous fetal study, from undernutrition in early and late gestation would be seen in adulthood. Moreover, it enabled direct comparative studies to take place against the 1.5 year investigations and build a picture of any developmental changes in these sheep during their lifespan.

There is reason to believe that there are critical windows of susceptibility in gestation. This study investigates the maternal body condition status prior to, during pregnancy and during weaning (therefore covering all possible windows) the effects of which are not yet known on hepatic metabolism of 4.5 year old male sheep.

6.2 **Hypothesis**

The maternal body condition before, during pregnancy and suckling affects hepatic metabolism in 4.5 year old male offspring.

The aim of this chapter is to determine if there is an effect of whole gestation and postnatal nutrient challenge in the liver in a large animal model such as the sheep. This chapter will investigate the effect of poor early life nutrition on some of the hepatic metabolic markers and also genes involved in gluconeogenesis (GR, G6Pase, PEPCK and the Insulin receptor) in adult sheep liver.

This work was carried out in collaboration with the University of Cambridge.

6.3 Methods

6.3.1 Summary

In this chapter a gestational and/or postnatal life dietary challenge is employed with the aim of inducing changes to the liver metabolism in adult life. All procedures contained in this chapter were approved by the Home Office and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. There were two groups of animals: one group were from mothers who had their body condition maintained at 3 or above and another who had their body condition maintained at BCS 2 prior to, during pregnancy and suckling (Cripps *et al.*, 2008). The length of time to maintain BCS prior to pregnancy was variable with some ewes requiring a longer duration of dietary adjustment to meet the body condition score than others. The process was carried out over several weeks. Hepatic metabolic markers were measured at baseline to determine if there were any differences as a result of the altered nutritional status during gestation and also in response to an intravenous glucose tolerance test. Real-time PCR was used to measure mRNA expression of the gluconeogenic genes in the liver. Samples from the left and right liver lobes of 4.5 year old male sheep were investigated.

6.3.2 Ewes

Sixty 2nd parity Welsh Mountain ewes were housed individually on straw and established at a BCS of 2 (n=30) (LBCS) or equal or greater than 3 (n=30) (HBCS) by adjusting the daily ration of complete pelleted diet (Charnwood Milling Co. Ltd, Suffolk, UK, Appendix 4.1). As fed it provided 9.57 MJkg^{-1} metabolisable energy and 14.75g crude protein per 100g. Uneaten food was measured daily allowing accurate measurement of energy intake.

6.3.3 Body condition score

Body Condition Score (BCS) is a system used to estimate the amount of muscle and fat coverage in the third lumbar region and is measured on a scale of 1-5 (see 2.1.1). In this study, BCS was measured using 0.25 increments. Increments along this scale were used in this study as measurements were taken by the same sheep husbandry technician on each occasion.

6.3.4 Mating

Once the designated stable body condition had been reached, oestrous was synchronised by removal of a vaginal medroxyprogesterone acetate-impregnated sponge (inserted for 14 days. Veramix, Pharmacia and Upjohn, UK) 48h before mating. Ewes were mated in batches by one of twin rams (random allocation). Twin pregnancies were removed from the study after ultrasound scanning at 50 days gestation.

6.3.5 Maternal nutrition

Gestational increases in energy intake were applied to both groups according to AFRC guidelines (AFRC, 1993). To compensate for the increase in diet ration, the pelleted feed was switched to a higher energy formula at 91 days of gestation (Charnwood Milling Co. Ltd, Suffolk, UK) which as fed provided 10.94MJkg^{-1} metabolisable energy and 14.56g crude protein per 100g. This allowed the ewes to consume enough of the pelleted food to give the desired energy intake. Ewe body weights and BCS were measured weekly and daily pelleted food ration was adjusted accordingly. To achieve the lower body condition the LBCS group were fed approximately 57% of that given to the HBCS group.

6.3.6 Fat and muscle depth

At –5, 50, 99 and 142 days gestation fat and muscle depth were measured by ultrasound of the spine in the third lumbar region (Aloka SSD 210 DX11, BCF Technology, Livingstone, UK).

6.3.7 **Lambs**

The ewes were allowed to deliver spontaneously and female offspring were removed from the study after the numbers of females and males were recorded. Ewes and male offspring were housed in two groups according to the maternal BCS, namely HBCS (n = 17) or LBCS (n = 12). The lambs were weaned at 12 weeks and given free access to hay and water and a standardised ration (AFRC, 1993) of creep pellets. As fed, these provide 10.59 MJ/Kg metabolisable energy and 18g crude protein per 100g (Prestige Lamb Pellets + Decox, BOCM Pauls Ltd., Loughborough, UK, Appendix 1.2). From 32 weeks of age, free access to hay and water was supplemented instead with adult pelleted feed, which as fed provided 10.38MJkg^{-1} and 18g crude protein per 100g (Ewbol 18, BOCM Pauls Ltd, Loughborough, UK, Appendix 1.3).

6.3.8 **Experimental procedure**

At 4.5 years of age, the adult male offspring (HBCS (n = 14) or LBCS (n = 10)) were acclimatised to metabolic carts for five days. After an overnight fast a radiopaque FEP IV catheter (14G x 140mm, Abbott Laboratories Ltd., Maidenhead, UK) was placed in the jugular vein via a small incision in the skin under local anaesthesia (2ml Lignol s/c, 2% w/v Arnolds Veterinary Products Ltd, Shrewsbury, UK). It was then sutured in place. Patency was confirmed by withdrawing blood using a syringe and a heparinised extension catheter was attached. (Heparinised saline (100U/ml. Heparin sodium: Leo Pharmaceuticals, UK; Saline 0.9 %: 3S-Healthcare, UK)). Extension catheters were made from lengths of polyvinyl tubing (I.D. 2.0mm, O.D. 3.0mm, Portex Ltd., Hythe, Kent, UK). A 14-gauge blunt (VetTech, UK) was inserted into each end of the catheter and a male/male luer connector (Sims Portex Ltd., UK) fitted to allow connection to the vascular catheter and a 3-way tap (Vygon, UK).

Sterile technique was used and catheters were sterilized using IMS (Industrial methylated spirit BP 70%, Adams Healthcare, Leeds, UK). Suture and incision sites were sprayed with oxytetracycline hydrochloride (Terramycin, Pfizer, UK). Surgifix netting (Colorline surgifix, size 7, FRA, Italy) was placed over the neck and incision site and the extension catheter secured to the back of the sheep by tying into wool. Long acting antibiotic (1ml/10kg, Betamox LA, Amoxycillin, Norbrook Laboratories Ltd., UK) was given intramuscularly at the time of catheterisation.

Blood sampling

Blood sampling during an intravenous glucose tolerance test (IVGTT) followed the overnight fast, catheter insertion and a two hour recovery process. Samples (8 ml) were collected before (-30, -15, -7, 0 min) and after (5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 210 min) an iv glucose (40% w/v, Arnolds Veterinary Products Ltd, Shrewsbury, UK) bolus (0.5g/kg body weight) with appropriate volume replacement. Whole blood was collected and placed immediately in chilled fluoride, EDTA and LiHep (Teklab Ltd, Durham, UK) plasma collection tubes, centrifuged, the plasma frozen in aliquots and stored initially at - 20°C then transferred to - 80°C. Food was returned on completion of the procedure.

Post-operative care

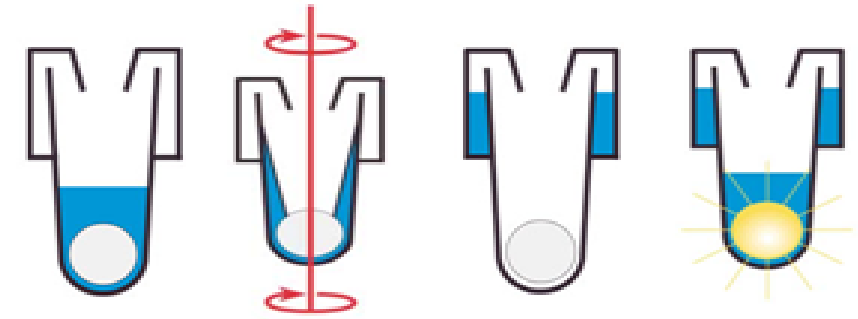
Patency of catheters was maintained by a continuous infusion (syringe pump PHD2000, Harvard Apparatus Ltd., Kent, UK) of saline (100U/ml at 1ml/h) and catheters were flushed with saline each morning.

6.3.9 Biochemical analysis

Plasma was analysed by autoanalyser using commercial kits as follows: β-hydroxybutyrate measured on lithium heparin plasma (Analox instruments GM-7 Micro-Stat), high density lipoprotein (Dade-Behring), low density lipoprotein (calculated using the Friedwald formula [LDL=cholesterol - (triacylglyceride/2.2 + HDL]), total cholesterol (Dade-Behring) (Rautela & Liedtke, 1978), urea (urease enzyme assay (Dade-Behring) and nonesterified fatty acids (NEFA; Alpha Laboratories Ltd, Eastleigh, UK) These analyses were measured as part of routine assays carried out at the National Health Services, Clinical Biochemistry Department, Addenbrookes Hospital, Cambridge on the Dade-Behring Dimension RXL analyser and at the Institute of Human Nutrition, University of Southampton, Southampton (Konelab 20 autoanalyzer).

Cortisol

Cortisol was measured (in duplicate) in EDTA plasma using an Immulite analyser (DPC, UK). A summary of the analysis process within the Immulite analyser is shown in figure 5.1. Cortisol (µg/dl) was measured in 10µl of plasma by a solid-phase, competitive chemiluminescent enzyme immunoassay, with an incubation cycle of 30 minutes. The use of this system with sheep plasma was validated by: comparing a set of results with those of the same samples analysed using a radioimmunoassay developed specifically for sheep plasma; assaying a known sheep sample under various dilutions to show linearity; and spiking a sheep sample with a known concentration of human cortisol to show that the expected results were achieved, with no interference due to sheep plasma. The range of the assay was 0.42 to 1.70ng/ml and the intraassay coefficient was 5.7%.



Sample and reagent are automatically pipetted into the Test Unit, which is then incubated at 37°C with intermittent agitation.

Following incubation, the Test Unit is spun at high speed about its vertical axis. Reaction fluid is forced up and completely captured in the sump chamber.

A series of washes efficiently removes unbound material from the bead and inner tube.

Chemiluminescent substrate is added to the Test Unit. Light emission is read with a high sensitivity photon counter.

Figure 6.2 **A summary of the Immulite analyzer analysis process.**

6.3.10 Triacylglyceride analysis by gas chromatography

Sample preparation

Plasma samples must undergo processing to extract their lipid content before they can be analysed for their fatty acid composition by gas chromatography. The lipid content can then be separated into various polar and non-polar lipid fractions and the resulting lipids must be saponified and methylated prior to analysis by gas chromatography (fig. 6.3).

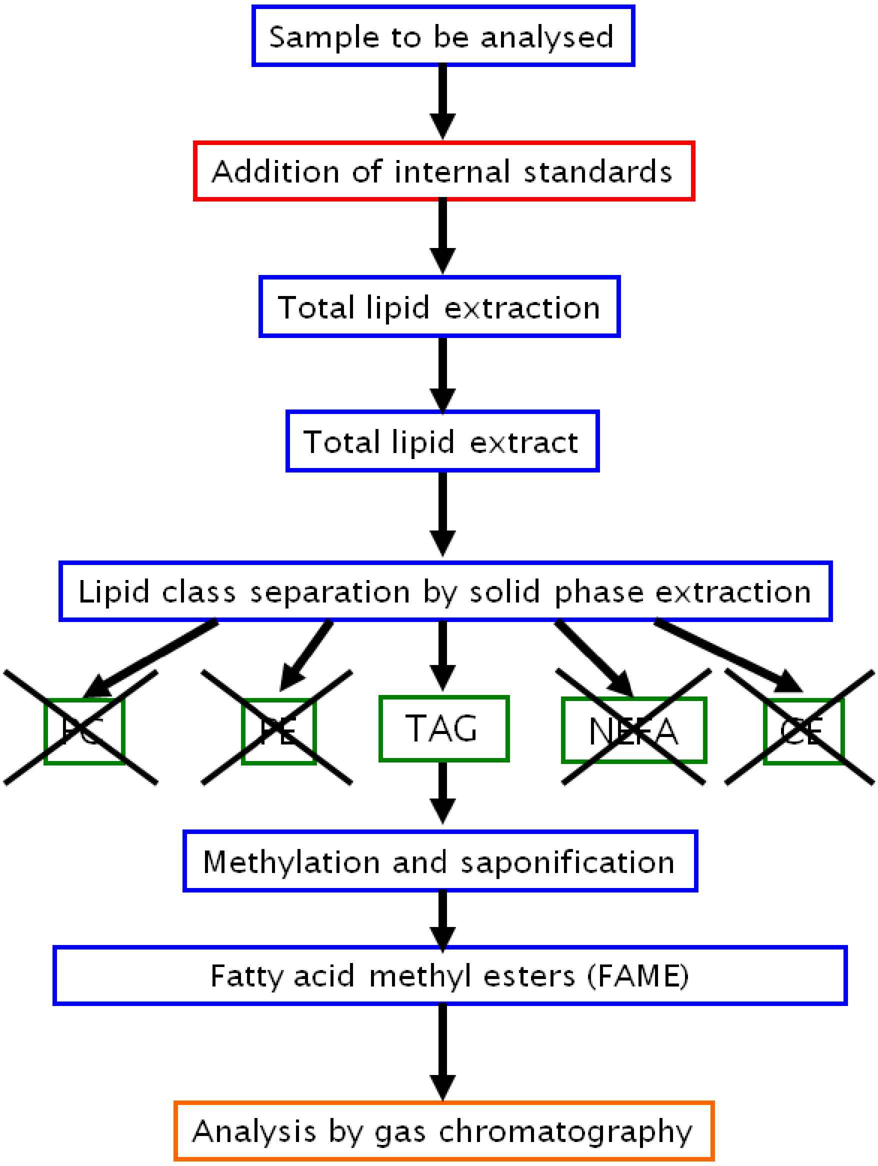


Figure 6.3 Flow chart of methodologies used in preparation of samples for analysis by gas chromatography. (PC, phosphotidylcholine; PE, phosphotidylethanolamine; TAG, triacylglyceride; NEFA, non-esterified fatty acid; CE, cholesteryl ester). **X** indicates elutant discarded.

Chemicals used for preparation of samples for gas chromatography were obtained from Fisher (chloroform, sodium chloride, methanol, hexane, and toluene), Sigma-Aldrich (butylated hydroxytoluene [BHT], glacial acetic acid, sulphuric acid, potassium bicarbonate, potassium carbonate) and BDH Lab Supplies (ethyl acetate).

The fatty acid composition was assessed using an aliquot of plasma which was weighed prior to analysis. TAG standard (15:0, Sigma-Aldrich) was added. Lipid was extracted; samples were mixed with 5ml chloroform:methanol (2:1 v/v containing 50mg/L of the anti-oxidant BHT) and 1ml 1M NaCl in a glass screw-cap test tube. Test tubes were vortexed thoroughly and centrifuged at 2,000 rpm for 10 min in order to separate the lower solvent-lipid phase from the upper aqueous phase. The lower phase containing the lipid was collected, dried down at 40°C under nitrogen gas and redissolved in 1ml chloroform. Individual lipid classes were separated by solid phase extraction using NH_2-bonded silica cartridges. The total lipid extract (dissolved in 1ml chloroform) was applied to a NH_2-bonded silica column. The lipid fractions were then eluted by the addition of solvent mixtures of varying polarity and acidity. 2ml chloroform was used to elute CE and TAG from the column. Then 2ml of chloroform:methanol 60:40(v/v) was used to elute PC from the column and this was discarded. The CE and TAG phase that had been collected was dried down at 40°C under nitrogen gas and re-dissolved 1ml in hexane. A new NH_2-bonded silica cartridge column was pre-treated with hexane washes before applying the combined CE and TAG fraction. CE was then eluted using 2ml of hexane and then discarded followed by TAG using 2ml hexane:chloroform:ethyl acetate (100:5:5 v/v/v). The separated lipid classes were saponified and methylated in a simultaneous reaction involving the addition of 0.5ml toluene and 1ml of a methanol:sulphuric acid mixture (2% H_2SO_4 v/v) and incubation at 50°C for 2 hrs. Samples were then neutralised with a solution containing 0.25M potassium bicarbonate ($KHCO_3$) and 0.5M potassium carbonate (K_2CO_3). The fatty acid methyl esters (FAME) produced were recovered using hexane. FAME were separated and analysed by gas chromatography on a HP6890 Hewlett Packard GC System with a BPX-70 column (Agilent Technologies). Samples were run under the following conditions: initial temperature: 115°C; hold 2 min; increase temperature at 10°C/min to 200°C; hold 10 min; increase temperature at 60°C/min to 245°C; hold 4 min.

Calibration and analysis

Identification of FAME peaks in gas chromatograms was achieved by comparing the retention time of the peaks of interest with the known retention time of FAME standards. To ensure identification of a wide range of FAME, standards were purchased (Sigma-Aldrich, Nuchek) to enable identification and calibration of the FAME within samples of interest:

- Saturates – 12:0, 14:0, 16:0, 18:0, 20:0, 22:0
- Monounsaturates – 16:1n-7, 18:1n-9, 20:1n-9, 24:1n-9
- n-6 polyunsaturates – 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:5n-6
- n-3 polyunsaturates – 18:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3

6.3.11 **Post-mortem**

Prior to the post-mortem, drapes, trays and surgical instruments were sterilised by autoclave. A pack of instruments was also cold sterilized using Novasapa for the initial sterile dissection of the abdomen.

At 4.5 years old lambs were killed by an overdose of barbiturate (Pentobarbitone Sodium Ph. Eur 0.8 ml/kg i.v., 200mg/ml, Animalcare Ltd, York, UK) and CRL, AC, FL, BPD and shoulder height (SH) were measured. The animal was secured to the post-mortem table and a large square was cut in the abdomen. Drapes covered the animal leaving the area of interest exposed and under sterile conditions the organs were removed according to the post-mortem sheet (Appendix 4.4). The organs were weighed in sterile trays and the required regions were dissected and processed according to the post-mortem sheet. Samples were fast frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis.

6.3.12 RNA extraction

Total RNA was extracted from the left and right lobes of 4.5 year old sheep liver using the methods previously described (see 2.2.1). RNA yield and quality was determined as previously described (see 2.2.2).

6.3.13 Reverse transcription

The RNA was reverse transcribed into cDNA for the polymerase chain reaction (PCR) (see 2.2.3).

6.3.14 Real-time PCR

Target gene mRNA levels were analysed in 4 year old adult liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7700 and 7500 Sequence Detection System, USA)(see 2.2.4). Primers and probes (table 2.1) were used as per the previous chapters. One sample of liver RNA (Right lobe, diet group: LBCS) was used to produce a standard curve (fig.6.4). The highest standard equals 1μg of RNA and was given the relative RNA concentration of 100; this was double diluted to produce the standard curve. The standard curve shows the log of the relative RNA against the Ct, as there is an exponential change in fluorescence. The relative RNA concentration for all samples was calculated from this curve and was expressed relative to the geometric mean of housekeeping genes β-actin, GAPDH and RPL-19.

Ct value

Figure 6.4 The standard curve for G6Pase mRNA.

6.3.15 Analysis strategy and statistics

For a general description of the analysis strategy, the power calculations and statistics packages used see Chapter 2.

Area under the curve was calculated from baseline plasma levels prior to the glucose bolus being administered (using Graphpad Prism software).

Time to maximum response was used to analyse TAGs and NEFAs in this thesis. The maximum response was defined as the lowest value obtained during the challenge.

All mRNA expression data were normalized using the geometric mean of three housekeeping genes (β-actin, GAPDH and RPL-19).

All data were checked for normal distribution by histogram prior to analysis. Data not normally distributed were transformed by the natural logarithm and subsequently checked for normal distribution.

Differences in liver weight were analysed by unpaired student's t-test.

Differences in lobar gene expression were analysed by repeated measures ANOVA with the between subject factor of group.

Significance was accepted when $p < 0.05$, however 'trends' are discussed when $0.5 < p < 0.1$.

Acknowledgements

George Hamilton-Baker (student) assisted on some of the work carried out in this chapter. He prepared the liver tissue, carried out RNA extraction and RT-PCR on the glucocorticoid receptor. He also prepared the TAG samples to the point of lipid extraction (fig.6.3).

6.4 Results

All *P* values can be found in Appendix 4.5.

6.4.1 Birth weight, body weight, fat and muscle

There was no difference in birth weight, postnatal growth body weight, weaning to young adulthood body weight or young adulthood to mature adulthood bodyweight. There was no difference in bodyweight, body condition score, back fat depth at 4.5 years of age but muscle depth was found to be greater in offspring from mothers of a low body condition score compared to those from mothers of a high body condition score (data not shown) (Costello *et al.*, 2013).

6.4.2 Liver weight

At 4.5 years old there was no difference in liver weight as a percentage of body weight of male adult offspring born to mothers of a low or high body condition score prior to, during pregnancy and weaning (fig. 6.5).

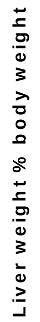


Figure 6.5 Liver weight. Liver weight as a percentage of body weight in male sheep at 4.5 years of age born to ewes of high body condition score (HBCS *n* = 14) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. Values are mean ± SEM.

6.4.3 Baseline fasting metabolic markers

At 4.5 years old there was no difference in the baseline cortisol (fig.6.6a), cholesterol (fig.6.6b), HDL (fig.6.6c), LDL (fig.6.6d), TAG (fig.6.6e), NEFA (fig.6.6f) and urea levels in blood plasma (fig.6.6g) following an overnight fast in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14) or low body condition score (LBCS *n* = 10) prior to, during pregnancy and weaning.

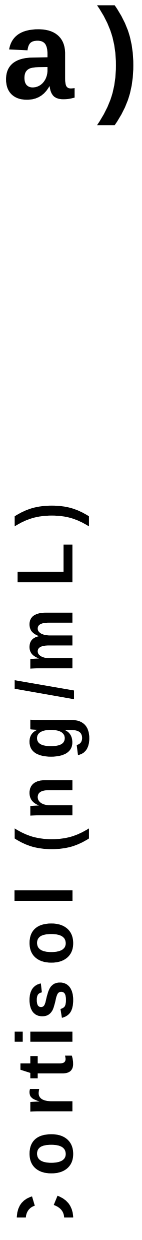


Figure 6.6 Baseline metabolic markers. Basal a) cortisol, b) cholesterol, c) HDL, d) LDL, e) TAG, f) NEFA and g) urea levels following an overnight fast in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. Values are mean ± SEM.

6.4.4 Insulin response to IVGTT

The insulin response was measured in blood plasma of 4.5 year old adult male sheep from mothers who had either a high body condition score (HBCS) or a low body condition score (LBCS) at set time points during an intravenous glucose tolerance test (IVGTT). The insulin was seen to rise on administration of the glucose bolus and then fall over time. There were no differences in response between the two groups of sheep as previously reported by Costello (Costello *et al.*, 2013) (fig. 6.7a & b).

a)

Figure 6.7 Insulin response of adult sheep during IVGTT. (a) Insulin levels at time points during an intravenous glucose tolerance test following an overnight fast in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. A glucose bolus was administered at time point 0. (b) The area under the curve shown in (a) was calculated from baseline plasma levels prior to the glucose bolus. Values are mean ± SEM.

6.4.5 Triacylglyceride response to IVGTT

Triacylglycerides were measured in blood plasma at set time points during an intravenous glucose tolerance test (IVGTT) and no difference in response (fig. 6.8a & b) or the time taken to reach the minimum level (table 6.1) between the two groups were found.

a)

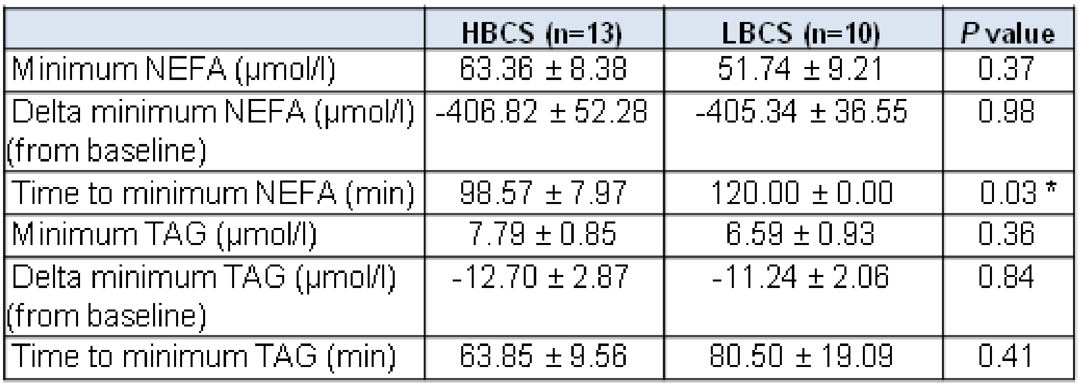
Figure 6.8 Triglyceride response during IVGTT. (a) Triglyceride (TAG) levels at time points during an intravenous glucose tolerance test following an overnight fast in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14)) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. A glucose bolus was administered at time point 0. (b) The area under the curve shown in (a) was calculated from baseline plasma levels prior to the glucose bolus. Values are mean ± SEM.

6.4.6 Non Esterified Fatty Acids response to IVGTT

NEFAs were measured in blood plasma at set time points during an intravenous glucose tolerance test (IVGTT) and no difference in the overall response was found (fig.6.9a & b). However the time to reach the LBCS group took longer to reach the minimum levels than the HBCS group (table 6.1).

a)

Figure 6.9 NEFA response during IVGTT. NEFA levels at time points during an intravenous glucose tolerance test following an overnight fast in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14)) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. A glucose bolus was administered at time point 0. (b) The area under the curve shown in (a) was calculated from baseline plasma levels prior to the glucose bolus. Values are mean ± SEM.



	HBCS (n=13)	LBCS (n=10)	*P* value
Minimum NEFA (µmol/l)	63.36 ± 8.38	51.74 ± 9.21	0.37
Delta minimum NEFA (µmol/l) (from baseline)	-406.82 ± 52.28	-405.34 ± 36.55	0.98
Time to minimum NEFA (min)	98.57 ± 7.97	120.00 ± 0.00	0.03 *
Minimum TAG (µmol/l)	7.79 ± 0.85	6.59 ± 0.93	0.36
Delta minimum TAG (µmol/l) (from baseline)	-12.70 ± 2.87	-11.24 ± 2.06	0.84
Time to minimum TAG (min)	63.85 ± 9.56	80.50 ± 19.09	0.41

Table 6.1 Minimum plasma non-esterified fatty acid (NEFA) and (TAG) levels. Minimum plasma NEFA and TAG levels reached during an IVGTT in 4.5 year old adult male sheep born to mothers of high body condition score (HBCS *n* = 14) or low body condition score (LBCS *n* = 10) condition score prior to, during pregnancy and weaning. Values are mean ± SEM. * P<0.05.

6.4.7 β-hydroxybutyrate

The plasma was also analysed for β–hydroxybutyrate at timepoints -15, 30, 90 and 210 of the IVGTT but no valid results were obtained due to measurements reading below the analyser standard. Further analysis of this was not carried out.

6.4.8 Hepatic Glucocorticoid Receptor mRNA expression

Adult male sheep showed no differences in glucocorticoid mRNA expression between the two body condition scored groups (fig. 6.10a) or the left and right liver lobes (fig. 6.10b).

a)

Figure 6.10 Hepatic Glucocorticoid Receptor mRNA expression. GR mRNA expression in livers of 4.5 year old adult male sheep born to mothers of high body condition score or low body condition score, condition score prior to, during pregnancy and weaning in (a) group (HBCS = High body condition score, *n* = 14, LBCS = Low body condition score, *n* = 10) and (b) lobe (*n* = 24). Values are mean ± SEM.

6.4.9 **Hepatic PEPCK mRNA expression**

There were no differences in PEPCK mRNA expression between the left and right liver lobes (fig.6.11a) or as a result of the body condition score of their mother (fig.6.11b).

a)

Figure 6.11 Hepatic PEPCK mRNA expression. PEPCK mRNA expression in livers of 4.5 year old adult male sheep born to mothers of high body condition score or low body condition score, condition score prior to, during pregnancy and weaning in (a) group (HBCS = High body condition score, *n* = 14, LBCS = Low body condition score, *n* = 10) and (b) lobe (*n* = 24). Values are mean ± SEM.

6.4.10 Hepatic G6Pase mRNA expression

There were no differences in G6Pase mRNA expression between the left and right liver lobes (fig.6.12a) or as a result of the body condition score of their mother (fig.6.12b).

a)

Figure 6.12 Hepatic G6Pase mRNA expression. G6Pase mRNA expression in livers of 4.5 year old adult male sheep born to mothers of high body condition score or low body condition score, condition score prior to, during pregnancy and weaning in (a) group (HBCS = High body condition score, *n* = 14, LBCS = Low body condition score, *n* = 10) and (b) lobe (*n* = 24). Values are mean ± SEM.

6.4.11 Hepatic Insulin Receptor mRNA expression

Adult male sheep showed no differences in insulin receptor mRNA expression between the left and right liver lobes (fig.6.13a) but did show a difference in expression between the offspring of high and low body condition scored ewes (fig.6.13b).

Figure 6.13 Hepatic Insulin Receptor mRNA expression. Insulin receptor (InsRec) mRNA expression in livers of 4.5 year old adult male sheep born to mothers of high body condition score or low body condition score, condition score prior to, during pregnancy and weaning in (a) group (HBCS = High body condition score, *n* = 14, LBCS = Low body condition score, *n* = 10) and (b) lobe (*n* = 24). Values are mean ± SEM. * P<0.05.

6.5 Discussion

6.5.1 Summary

In this chapter the effect of whole gestation undernutrition on adult sheep liver metabolic markers and hepatic gluconeogenic gene expression was investigated as a possibility for the mechanistic basis within the liver for the dysregulation of glucose homeostasis associated in adult disease. This chapter investigated the effect of maternal body composition prior to, during and after pregnancy when the lamb is still suckling on the adult male offspring at 4.5 years of age. This cohort of male sheep from mothers with either a high (HBCS) or low body condition score (LBCS) were previously studied at 1.5 years of age and those from the LBCS mothers were found to have increased fasting glucose, poorer glucose tolerance and reduced insulin secretory response (Cripps *et al.*, 2008). These sheep were studied in this chapter at 4.5 years of age to determine if this impaired glucose metabolism persisted in later life and was associated with changes in both whole body and liver metabolisms.

The results showed that there were no liver weight differences between male 4.5 year old sheep whose mothers had a low body condition score and those from mothers who had a high body condition score. There were no differences in basal cholesterol, HDL, LDL, urea or cortisol levels in the plasma of 4.5 year old male sheep from either HBCS or LBCS mothers. The basal cholesterol, HDL, urea and cortisol results for these sheep at 4.5 years of age are similar to the levels previously reported in these sheep and agree with the previous findings of no differences between the groups (Cripps *et al.*, 2008). These data show that there has been no effect on these metabolites with age.

Responses to the glucose tolerance test showed no differences between the groups in triacylglyceride or nonesterifed fatty acid response although the LBCS group did take longer to reach its minimum level. The body condition of the mother was shown increase the mRNA expression of the hepatic insulin receptor in offspring of LBCS mothers but no effects of diet were seen in hepatic GR, PEPCK or G6Pase mRNA expression. There were no differences in mRNA expression of these genes seen in the lobes of the liver. The significance of these findings is discussed below.

6.5.2 Maternal undernutrition had no effect on liver weight

Low maternal body condition (inducing whole gestation undernutrition on the fetus) had no effect on liver weight in adult male sheep. Previous studies have found that liver weight was lower in adult male sheep following a period of early gestation nutrient restriction for 1 - 95 days (Hyatt *et al.*, 2007b). The nutrient restriction applied was a global reduction of 50% but no data is available regarding the body condition score of the mother so it is difficult to compare the severities of the challenge. In the present study the period of undernutrition covered this period and longer and was therefore thought to be able influence the developing liver throughout gestation. The present observation of no effect of postnatal undernutrition on liver weight after a period of normal refeeding agrees with other studies in sheep (Wester *et al.*, 1995;Kabbali *et al.*, 1992a;Kabbali *et al.*, 1992b). This suggests that the liver is capable of recovering in size following periods of undernutrition if indeed, it was at all affected in the first instance, which we did not measure. We can only speculate that the liver weight was reduced during the gestational and weaning nutritional challenge (via the nutrition to the mother to maintain the required body score).

6.5.3 Baseline metabolic markers were not affected by maternal body condition

Low maternal body condition had no effect on the baseline metabolic markers of cortisol, cholesterol, HDL, LDL, NEFA, TAG or urea in adult male sheep. There was no change in these markers compared to the measurements taken at 1.5 years of age (Cripps *et al.*, 2008). Baseline levels of NEFA and urea were not found to differ in 7 month old lambs from mothers who were fed 50% nutrition in late gestation compared to controls (Kiani *et al.*, 2011) However, late gestation undernutrition (50% of requirements) resulted in increased triacylglyceride and NEFA in the blood plasma in female adult sheep (Hou *et al.*, 2014). Male sheep were not studied in this case and so it is difficult to make a direct comparison with the study here.

6.5.4 TAG & NEFA levels were not affected by maternal body condition in response to IVGTT

There was no difference in TAG and NEFA levels in response to the IVGTT but the time to reach a minimum level was delayed in offspring of LBCS mothers. NEFA levels were found to be increased in 19-wk-old lambs from mothers fed 50% nutrition requirements in late gestation (Husted et al., 2007).

6.5.5 Maternal undernutrition increased insulin receptor mRNA expression

Insulin receptor mRNA expression was found to be higher in the liver of the LBCS group sheep compared to HBCS. No other mRNA expression was found to vary between sheep from the BCS groups or between the lobes. In the previous study on adult sheep which had an early gestation and/or a postnatal challenge there were no differences seen in mRNA expression of hepatic genes as a result of the nutrient challenge (Chapter 4) however, the fetal sheep in study 2 (Chapter 5) were also found to have increased insulin receptor mRNA expression as a result of the nutrient challenge (which was 50% nutrition during days 1-31 of gestation). Fetal rats from maternal low protein fed dams were also found to have increased hepatic insulin receptor (Nusken *et al.*, 2011).

6.5.6 No lobar differences in hepatic gene expression

No differences were seen in hepatic lobe mRNA expression. This is in contrast to the study in Chapter 4 whereby the adult sheep were seen to have lobar differences in GR PEPCK but not G6Pase or insulin receptor mRNA expression. The sheep in Chapter 4 were 2.5 years of age compared to the 4 year olds in this study. This study only looked at male sheep. Perhaps the lobe differences become less apparent as the animal ages.

Dietary influences on lobar gene expression may emerge with more profound restriction of diet throughout gestation.

6.6 Conclusions

In this chapter it was thought whole gestation and early life nutrient restriction in the guise of the body condition status of the mother may have an effect on sheep liver (fig.6.14).

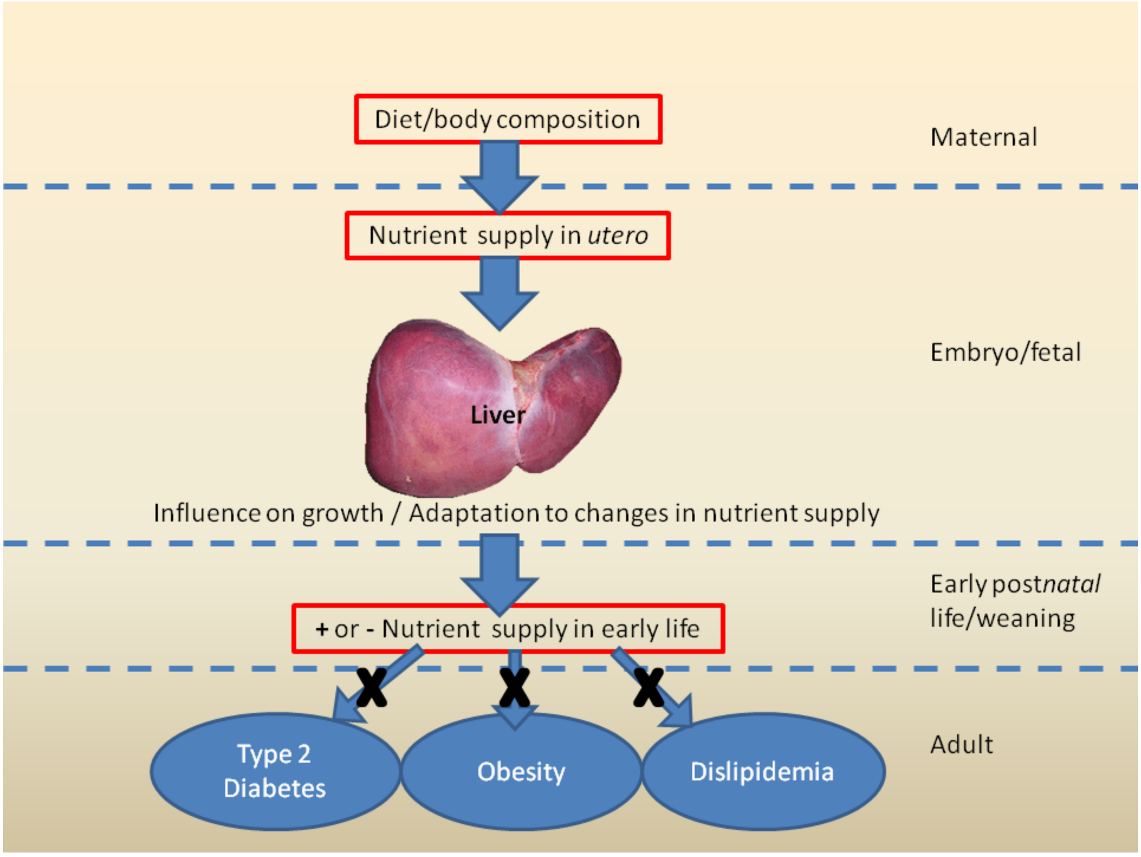


Figure 6.14 Possible nutrition influences on liver. Maternal body composition was not found to affect the liver in a way as to influence the risk of adult disease.

It was found that in this adult sheep model there were no effects of the body condition score on adult sheep liver weight, blood plasma metabolic markers or the hepatic gluconeogenic gene expression of GR, PEPCK or G6Pase. However the insulin receptor mRNA was found to be increased in the livers of sheep from the LBCS group.

These findings did not support the initial hypothesis that the maternal body condition before, during pregnancy and suckling would affect hepatic metabolism in 4.5 year old male offspring.

The intensity of the nutritional challenge (estimated to be approximately 57% of nutrient requirements) may not have been sufficient to see changes in hepatic metabolism. A harsher challenge may produce changes in this pathway but this was not able to be investigated further.

Chapter 7. **Impact of early life nutrient restriction on methylation of the hepatic glucocorticoid receptor in adult sheep.**

7.1 Introduction

In Chapter 4 the effect of postnatal undernutrition following an early gestation nutritional challenge on adult sheep hepatic gluconeogenic gene expression was investigated to explore the mechanistic basis within the liver for the dysregulation of glucose homeostasis associated in adult disease. However while the results showed no such effects of early life nutrient restriction on the hepatic GR mRNA expression, mRNA expression of GR was higher in males than females, in the right than the left lobe and in twin than in singleton offspring. In this final experimental chapter of the thesis I investigated a potential epigenetic basis for the differences observed in GR mRNA levels. In addition, despite the lack of effect of early life diet on GR mRNA levels, it was still possible that methylation changes had been affected by changes in the early life environment. The impact of any such effect might only become manifest in later life, potentially after an additional challenge. Indeed previous observations in these sheep (Poore *et al.*, 2007) showed effects of the early developmental environment on later life glucose metabolism.

7.1.1 Epigenetics & developmental origins of disease

Epigenetics and the evidence for developmental origins of disease were previously described in section 1.7. Briefly, epigenetic changes alter the transcription of a gene without altering the genetic code itself, are able to be passed on through cell division and can be influenced by the environment prior to, during and after pregnancy including maternal behaviour and nutrition. The major epigenetic processes as previously described (1.7.1-3) are DNA methylation, histone modification and non-coding RNAs which work to expose or shield promoter regions to / from factors that control transcription.

The epigenome is set up in early development, during the window in which environmental constraints such as maternal diet are able to influence developmental trajectories. Altered epigenetic regulation is therefore one mechanism which could underpin the persistent changes in gene expression identified in offspring. It is therefore important to understand the nature of epigenetic modifications in order to recognise how early life constraints may affect certain metabolic pathways in later life. Epigenetic modifications are known to allow (or prevent) the exposure of the regulatory promoter regions to factors which control and direct transcription. Thus epigenetic changes which expose these regions allow transcription to occur if the correct regulatory complexes are available, whilst those which shield these regions inhibit transcription by preventing regulatory proteins from binding. This in turn influences the expression of the particular gene, either increasing or decreasing expression which may in turn lead to alterations in protein expression, the functions of cells and eventually the whole organism, resulting in physiological changes.

7.1.2 Animal studies

Several animal studies have demonstrated the epigenetic changes that occur in the young by either maternal behaviour or nutrition. Increased maternal care during the first week of life was found to reduce methylation in hippocampal exon 1(7) GR (Weaver *et al.*, 2004). This effect was reversed in adult life following supplementation with L-methionine, a precursor to S-adenosyl-methionine that serves as the donor of methyl groups for DNA methylation highlighting a potential reversal of programmed effects (Weaver *et al.*, 2005). Hepatic GR methylation was found to be lower in 34 day old rats from mother fed a low protein diet throughout gestation however, when this diet was supplemented with folic acid the methylation of GR did not differ from controls (Lillycrop *et al.*, 2005).

The sheep genome has not been sequenced fully to date, although several initiatives have been started by the International Sheep Genomics Consortium (http://www.sheephapmap.org/) and The U.S. Sheep/Goat Genome Project (http://www.animalgenome.org/sheep/). This makes gene expression analysis challenging in sheep, and poses even more of a problem for epigenetic analysis where information on the promoter regions of genes is required. In sheep, hypothalamic GR methylation was found to be decreased in fetal sheep from mothers fed a periconception diet giving 50% of nutrient requirements (Stevens *et al.*, 2010). This was also seen in a further study in 5 year old adult sheep (Begum *et al.*, 2013). The lack of genome information for the sheep makes data for this model rare, and even in Begum *et al*'s study they used the human sequence for hypothalamic GR.

7.1.3 **Methods for measuring DNA methylation**

As discussed in 1.7.5 there are various methods available to measure DNA methylation levels and these vary in cost, ease of use, sensitivity, scale and level of throughput. The methods range from simple, which measure average DNA methylation over a stretch of DNA, to methods which look at individual CpG methylation of the 'whole genome'. Methylation sensitive PCR is a simple semi-quantitative method which uses methylation sensitive restriction enzymes to differentiate between methylated and unmethylated sequences in DNA. Subsequent real time PCR will only amplify amplicons containing undigested methylated DNA, whilst digested unmethylated DNA is unable to be amplified. More sensitive and expensive quantitative methods are available which look at the methylation status of individual CpGs within a stretch of DNA, for example Pyrosequencing or Sequenom analysis. In this thesis I used methylation sensitive PCR in order to provide initial first-step information on possible epigenetic changes occurring within the hepatic GR promoter region.

There is reason to believe that methylation changes exist within the GR promoter following critical windows of susceptibility in gestation. Current concepts highlight the importance of the prenatal environment with the postnatal interaction. The effects of which are not yet known on GR methylation in the sheep liver.

7.1.4 **Hypothesis**

Restricted postnatal nutrition will reduce the methylation of the hepatic GR in adult sheep depending on nutrition received in early gestation.

The aim of this chapter is to determine if methylation differences could be detected in the adult sheep hepatic GR to explain for the sex, lobe and offspring differences in mRNA expression seen in Chapter 4 (males had a higher expression than females, the right lobe had a higher expression than left and twins had a higher expression than singletons). This chapter will investigate methylation sensitive PCR for the first time in a newly available partial ovine DNA sequence.

7.2 Methods

7.2.1 Summary

In this chapter DNA methylation within the GR promoter region was investigated using methylation-sensitive PCR on sheep liver from the cohort previously assessed in Chapter 4 (see 4.2 for details). Partial DNA sequences for the GR were received very late into my studentship and therefore time and funding were somewhat restricted. However, PCR was used to amplify DNA extracted from adult left and right lobe liver and treated with methylation-sensitive restriction enzymes (AciI and HpaII). GR primers were designed to amplify a region across two CpG islands within the GR promoter region, based on a partial ovine sequence determined by express sequence tag mapping to the bovine sequence (with thanks to Allan Sheppard, AgResearch, NZ) . This work was carried out prior to access to the pyrosequencing methodology.

7.2.2 DNA sequence

The partial DNA sequence for GR was obtained from Allan Sheppard, AgResearch, NZ and had been express sequence tag mapped against the bovine GR. Analysis was made to determine the regions of CpG concentration – possible CpG islands (see Appendix 5.1), enzyme cut sites were located, and primer locations designed so that the GR primer covered enzyme cut sites and the control region contained none. All primer and enzyme cut site locations can be found on the sequence data shown in Appendix.5.2.

7.2.3 DNA extraction

DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega). 50mg of snap frozen tissue was crushed and incubated with 275µl digestion solution at 55ºC for 16 hours. Samples were centrifuged at 13000g for 3 minutes and the supernatant removed to a new tube. 250µl of lysis buffer was added, mixed then transferred to a minicolumn assembly and centrifuged at

13000g for 3 minutes. Waste liquid was discarded from the collection tube. 650µl of wash solution was added to the minicolumn centrifuged again at 13000g for 1 minute. Liquid from the collection tube was discarded and the wash was repeated a further three times. 250µl of heated nuclease free water was added and allowed to stand for two minutes at room temperature. This was centrifuged again at 13000g for 1 minute. The minicolumn was removed (the eluted DNA secured and labelled) and placed into another tube, 250µl of nuclease free water was added and the process repeated to obtain a second DNA sample. The DNA was analysed for quality and quantity and stored at -20ºC.

7.2.4 DNA Spectrophotometry

Quality and quantity of genomic DNA was checked on the spectrophotometer. All DNA had a 260/280 ratio of ~1.7. Genomic DNA was stored at -20ºC.

7.2.5 DNA gel

The DNA was then analysed on a 1.5% agarose gel and then stored at -20ºC.

7.2.6 Treatment with enzymes

All DNA was cut with the methylation sensitive restriction endonucleases AciI (5'-C▼CGC) (New England Biolabs, UK) and HpaII (C▼CGG) (Promega, UK). 400ng genomic DNA was incubated with 0.5µl of each enzyme at 37ºC for 24 hours in a total volume of 50µl. Upon completion the DNA was placed on a heat block at 65 ºC for 20 mins. The DNA was then analysed for digestion on a 1.5% agarose gel and then stored for later use at -20ºC.

7.2.7 Real-time PCR

Glucocorticoid receptor DNA methylation levels were analysed in 2.5 year old adult sheep liver using real-time PCR (Taqman® Applied Biosystems ABL Prism 7500 Sequence Detection System, USA) for methods which uses a comparative cycle of threshold fluorescence method (Heid *et al.*, 1996). The DNA is amplified during the PCR reaction, by primers annealing to the strands of DNA (separated by heating) and then being extended by polymerase enzymes. The newly made double stranded DNA is detected by use of SYBR Green I, a commonly used fluorescent DNA binding dye. It binds to all double-stranded DNA. The accumulation of PCR products within every PCR cycle is detected by the increase in fluorescence (ΔRn). The relative amount of DNA is characterized by the number of cycles of PCR (Ct) it takes for ΔRn to exceed a defined fixed threshold (figure 2.6). The higher the starting amount of cDNA the sooner it reaches the threshold level and the lower its Ct value.

Primers were designed using Primer Express Software (Applied Biosystems, USA) see table 7.1. 6μl of DNA were used in each reaction containing 27.5μl universal master mix with SYBR green, 2μl forward and reverse primers (of 10μM concentration) (run in duplicate).

Primer	Sequence	Concentration (μM)
GR Forward Primer	CAACCTTTCCCCGAGTCACA	10
GR Reverse Primer	CAATGCGTTGCTCACCATTT	10
GR Control Forward Primer	CATGTGGGATCTGGTTTCTTGA	10
GR Control Reverse Primer	CACCTGAGTACTCCTTCCTAAAAAAATT	10

Table 7.1 Primers for methylation sensitive PCR. The concentration and DNA sequence of the primers (Eurogentec S.A., Belgium) used in the methylation sensitive real-time PCR reaction for the amplification of GR.

Many gradient experiments across the temperatures 50 - 65ºC were carried out (Applied Biosystems Step One Plus, USA) to determine the optimum PCR conditions for the GR and control primers. The final cycle and temperature details used are shown in table 7.2.

One sample of uncut liver DNA (male, right lobe, diet group: UU) was used to produce a standard curve (see figure 3.5). The highest standard equals 1μg of DNA and was given the relative DNA concentration of 100; this was double diluted to produce the standard curve. The standard curve shows the log of the relative DNA against the Ct, as there is an exponential change in fluorescence. The relative DNA concentration for all samples was calculated from this curve and was expressed relative to the control region.

Ct value

Figure 7.1 The standard curve for GR control DNA.

GR THERMAL CYCLER PROFILE

Stage	Repetitions	Temperature	Time	Ramp Rate
1	1	95.0 °C	10:00	100
2	40	95.0 °C	00:30	100
		58.0 °C	01:00	100
		72.0 °C	01:00	100
3 (Dissociation)	1	95.0 °C	00:15	Auto
		60.0 °C	01:00	Auto
		95.0 °C	00:15	Auto
		60.0 °C	00:15	Auto

CONTROL THERMAL CYCLER PROFILE

Stage	Repetitions	Temperature	Time	Ramp Rate
1	**1**	**95.0 °C**	**10:00**	**100**
2	**40**	**95.0 °C**	**00:30**	**100**
		65.0 °C	**01:00**	**100**
		72.0 °C	**01:00**	**100**
3 (Dissociation)	**1**	**95.0 °C**	**00:15**	**Auto**
		60.0 °C	**01:00**	**Auto**
		95.0 °C	**00:15**	**Auto**
		60.0 °C	**00:15**	**Auto**

Table 7.2 PCR cycle details for GR and Control genes.

7.2.8 Analysis strategy and statistics

For a general description of the analysis strategy, the power calculations and statistics packages used see section 2.3.

All DNA expression data were normalized to the control region DNA expression.

All data were checked for normal distribution by histogram prior to analysis. Data not normally distributed was transformed by the natural logarithm and subsequently checked for normal distribution.

Differences in lobar gene expression were analysed by repeated measures ANOVA with between subject factors of sex, offspring and diet.

Significance was accepted when $p < 0.05$, however ‘trends’ are discussed when $0.5 < p < 0.1$.

7.3 **Results**

All *P* values can be found in Appendix 5.3.

7.3.1 **Glucocorticoid receptor methylation**

There were no differences in hepatic GR methylation between the lobes (fig.7.2a), sexes (fig.7.2b), dietary groups (fig. 7.2c) or between singletons and twins (fig.7.2d) in 2.5 year old sheep.

These data were also correlated to the GR mRNA expression (in Chapter 4) but no significant correlation was found (see Appendix 5.4).

Figure 7.2 Hepatic Glucocorticoid Receptor methylation in adult sheep. Liver GR methylation in a) dietary group (CC = control *n* = 22, CU = postnatal undernutrition *n* = 14, UC = early gestation undernutrition *n* = 17, UU = early gestation and postnatal undernutrition *n* = 22), b) sex (male *n* = 39, female *n* = 36), c) lobe (Left *n* = 75, Right *n* = 75) and d) offspring (singleton *n* = 28, twin *n* = 47). Values are mean ± SEM.

7.4 Discussion

7.4.1 Summary

The availability of partial GR DNA sequences (from Allan Sheppard, AgResearch, NZ) was an exciting development for this thesis as mRNA expression differences had been found and it was a great opportunity to investigate if these changes were as a result of methylation alterations, or indeed if epigenetic changes were detected which might lead to later expression changes. No investigations into the methylation status of sheep genes had been attempted previously in our laboratory due to lack of genome information, so the data in this chapter was a significant step forward. I did not detect any methylation differences in the region between two GR CpG islands. The significance of these findings is discussed below.

7.4.2 Diet, sex, lobe or offspring number were not found to alter the hepatic GR methylation in adult sheep liver

Despite lobe and sex mRNA expression differences seen in chapter 4 no methylation differences in the hepatic GR promoter region could be detected in the area examined using methylation sensitive PCR. The field of epigenetics and its application to sheep is very new due to limited sequence availability, but judging by the literature and some results coming out in other genes such as insulin growth factor 2, hepatic PEPCK-C, angiotensin-converting enzyme, angiotensin type 1 receptor (Poore *et al.*, 2014;Lie *et al.*, 2014;Zhang *et al.*, 2013) there are developments ongoing all the time. However, to date no data exists on the effects of lobe or sex on hepatic GR methylation. It could be that the lobar and sex mRNA expression differences seen in chapter 4 are not driven by methylation changes at all and that their regulation is driven by other factors such as adult regional blood flow and nutrient levels or, in the case of sex by fluctuating hormone levels in adults. Alternatively, as a partial sequence was obtained it is possible that there are indeed methylation differences in other CpG areas within this promoter region for which we do not yet have the sequence data.

In this study there was no effect on the methylation status of the hepatic GR promoter by postnatal dietary challenge (regardless of the challenge in gestation) and this is in accordance with the lack a dietary effect on GR mRNA expression detected in Chapter 4. Other more recent methylation studies in our laboratory have shown increases in methylation within the imprinting control region of IGF2R in left liver lobe from 2.5 year-old male sheep undernourished in early gestation, but not in females (Poore *et al.*, 2014). Early gestation undernutrition had no effect on methylation at the IGF2/H19 or DLK/MEG3 (delta-like homolog 1/ maternally expressed gene 3) loci in adult liver in either sex but post-weaning undernutrition significantly reduced hepatic DLK/MEG3 methylation at CpG dinucleotides 1, 2, 3, and 4 (Poore *et al.*, 2014). Although these results were obtained in the same sheep model as my studies, the genes of interest were different and the results were obtained by Pyrosequencing which is a more accurate method than methylation sensitive PCR. This method was not available to me at the time of this thesis study. However in agreement with my data, PEPCK methylation measured by combined bisulphite restriction assay (COBRA) (using a bovine sequence) was not affected by undernutrition in early gestation nor offspring number in fetal sheep liver even though the mRNA expression was decreased (138dGA) (Lie *et al.*, 2014). In contrast, 131dGA fetal sheep from undernourished ewes (-60 - +30d) were found to have decreased methylation in the hypothalamic GR (sequences mapped from human and bovine genome information) (Stevens *et al.*, 2010) and increased GR mRNA expression and that these changes persisted until adulthood (Begum *et al.*, 2013). In rats, hepatic GR methylation was found to be reduced and mRNA expression higher in 34 day old offspring from mothers fed a low protein diet throughout gestation, and this effect was prevented if the diet was supplemented with folic acid (Lillycrop *et al.*, 2005). Obviously the rat model is different to the sheep (multiple offspring, post natal development in the young and different protein content dietary challenge, full genome sequence availability) and so therefore it is difficult to compare the results found in that model with the sheep. These initial findings do not however support the idea that early life undernutrition altered the methylation of the hepatic GR promoter. This appears to support the lack of effect on the GR mRNA expression found. However, certainty on this matter would require more rigorous methylation analysis in a larger portion of the promoter region.

7.5 Conclusions

Early life undernutrition was found to have no effect on hepatic GR methylation in this study, nor were any methylation differences found according to sex, offspring number or liver lobe. However, this does not necessarily mean that the expression differences between lobes and sexes seen in chapter 4 were not driven by DNA methylation changes since in this chapter I have used a short sequence of the GR promoter region; further work may be possible when more sequence becomes available. In addition, improved detection methods are now more readily accessible so in the future a more accurate analysis is also a possibility.

Chapter 8. **General Discussion**

8.1 Summary

This thesis investigated the effect of early life undernutrition on hepatic metabolism in sheep. The studies looked at different severities of undernutrition applied in fetal and postnatal life on the effect of hepatic metabolism in fetal and adult sheep. A summary of each chapter's findings follows:

8.1.1 Chapter 3

Following the initial laboratory investigations into mRNA expression in genes of the sheep liver it became apparent that the usual housekeeping gene 18S may not be suitable for use in the liver tissue. Chapter 3 therefore investigated the suitability of 18S as a single control gene for use in sheep liver mRNA assessments. This led to the development in conjunction with Primer Design Ltd of a geNorm$_{TM}$ housekeeping assessment kit specifically for use in sheep and showed that normalisation of sheep liver samples could be obtained using the geometric mean of three housekeeping genes, β-actin, GAPDH and RPL-19.

8.1.2 Chapter 4

Chapter 4 investigated the effect of postnatal undernutrition on adult sheep hepatic gluconeogenic gene expression following an early gestation nutritional challenge. The results showed that there was no effect of maternal dietary restriction in early gestation and/or dietary restriction in early postnatal life on the liver weight of the adult sheep. Dietary restriction also had no effect on the expression of the hepatic GR, PEPCK, G6Pase or the insulin receptor. However, liver weight (as a percentage of body weight) was found to be lower in males than females and males had a higher mRNA expression of GR and

PEPCK than females. Hepatic lobar gene expression differences were discovered with a higher mRNA expression of GR in the right lobe than the left, and lower PEPCK mRNA expression in the right lobe than the left. Increased offspring number was found to cause increased hepatic GR mRNA expression but no difference in liver weight.

8.1.3 **Chapter 5**

The discovery in Chapter 4 that periods of gestational and postnatal undernutrition had no effect on the mRNA expression of hepatic gluconeogenic genes was thought to be due perhaps to the duration and/or intensity of the challenges. Therefore, Chapter 5 investigated the effect of an increased intensity and the addition of a late gestational nutrition challenge on the sheep fetus. Early gestational nutrient challenges (during days 1-31 of gestation) of 50% and 40% of requirements were employed as was a late gestational challenge (during days 104 – 127) of 50%. There was no effect of maternal dietary restriction in early or late gestation on fetal sheep liver weight (as a percentage of bodyweight) at 127dGA nor was there any effect of sex. Early gestation (50% of requirements) dietary restriction was shown to increase mRNA expression of the insulin receptor, and reduce mRNA expression of the glucocorticoid receptor, PEPCK and G6Pase genes in the right but not the left fetal liver lobe.

A more intense dietary restriction (40%) in early gestation reduced the mRNA expression of GR, PEPCK and G6Pase but not the insulin receptor. This was also seen following the late gestation challenge of 50% nutrition requirements. The dietary challenges were found to affect the individual lobes of the liver with a reduction in GR, PEPCK and G6Pase seen in the left lobe as a result of the early (40%) and late (50%) challenges. The right lobe was less affected with GR and PEPCK being reduced as a result of the early gestation (40%) challenge only

8.1.4 **Chapter 6**

Chapter 6 investigated the effect of whole gestation and suckling undernutrition on adult male sheep offspring liver metabolic markers and hepatic gluconeogenic gene expression. Previously at 1.5 years of age these male offspring of low body condition score (LBCS) mothers were found to have increased fasting glucose, poorer glucose tolerance and reduced insulin secretory response (Cripps *et al.*, 2008). The sheep were studied in this chapter at 4.5 years of age to determine if this impaired glucose metabolism persisted in later life and was associated with changes in both whole body and liver metabolism.

The results showed that there were no liver weight (as a percentage of bodyweight) differences between male 4.5 year old sheep from LBCS mothers and those from HBCS mothers. There were no differences in basal cholesterol, HDL, LDL, urea or cortisol levels in the plasma of 4.5 year old male sheep from either HBCS or LBCS mothers. The basal cholesterol, HDL, urea and cortisol results for these sheep at 4.5 years of age are similar to the levels previously reported and agreed with the previous finding of no differences between the groups (Cripps *et al.*, 2008). These data show that there has been no effect on these metabolites with age. Responses to the glucose tolerance test showed no differences between the groups in triacylglyceride or nonesterifed fatty acid response although the LBCS group did take longer to reach its minimum level. Hepatic insulin receptor mRNA expression was increased in the offspring of LBCS mothers, but no effects of diet were seen on hepatic GR, PEPCK or G6Pase mRNA expression. There were no differences in mRNA expression of these genes seen between the lobes of the liver.

8.1.5 **Chapter 7**

In Chapter 7 I investigated the methylation status in a region of the GR promoter in the adult sheep liver previously studied in Chapter 4. The availability of partial GR DNA sequences at this point in my thesis was an exciting development since no investigations into the methylation status of sheep genes had been attempted previously in our laboratory (or indeed very few others) due to lack of genome information. I hypothesized that although there were no dietary effects on gene expression in adult sheep (Chapter 4) there could still be methylation changes which might lead to future expression changes in a perturbed postnatal environment. However, no methylation alterations were found as a result of diet, sex, and lobe or offspring number.

8.2 Early life nutrient environment alters liver metabolism

In this thesis different nutrient restriction timings were used (fig.8.1): early gestation (1 -31 days: 50% nutrient requirements), (1-31 days: 40% nutrient requirements), late gestation (104 – 127 days: 50% nutrient requirements), postnatal (12 – 25 weeks: growth controlled to 85 % of the normal growth) (Chapter 4) and nutrient restriction throughout pregnancy and weaning (Chapter 6). The liver develops as early as 21-23 days of gestation (Bryden *et al*., 1972), therefore the timing of the early gestational nutritional challenge applied (1 -31 days) covered the period that the liver is initially forming. The late gestation challenge covers a period of fetal hepatic growth whereas the restricted diet applied in Chapter 6 covered the whole of gestation and therefore a much longer period of liver growth and development. Adult sheep of 2.5 years (Chapter 4) and 4.5 years (Chapter 6) and fetal sheep of 127dGA (Chapter 5) were studied.

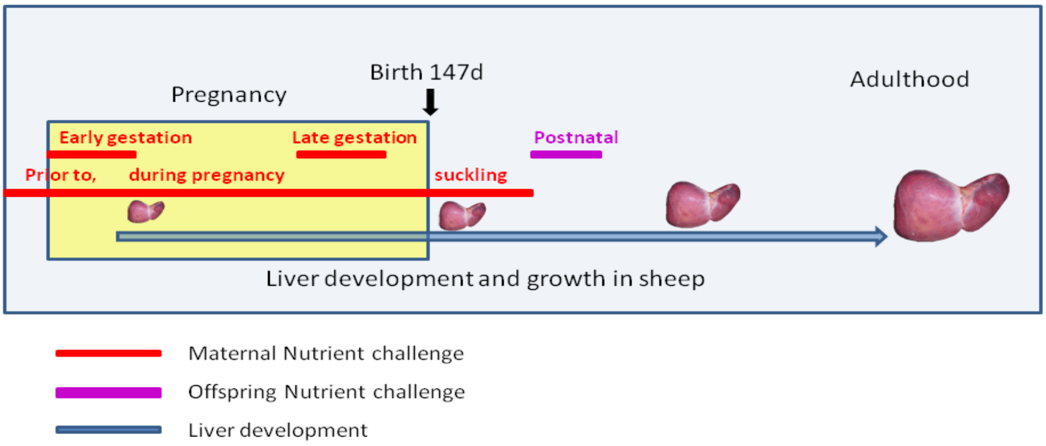


Figure 8.1 **Nutritional challenge timeline.**

Early gestation challenge

In this study, early gestation nutrient restriction was found to increase mRNA expression of the insulin receptor, and reduce GR, PEPCK and G6Pase mRNA expression (key genes in the gluconeogenic pathway) in fetal sheep depending on the severity of challenge used. This did not fit with the hypothesis that undernutrition would lead to an increase in the expression of genes involved in gluconeogenesis as a mechanism for increasing supply of glucose from non-dietary sources in the face of reduced nutrition. Indeed in other studies, PEPCK mRNA expression had been found to be increased in 20dGA rats following a peri implantation low protein diet (Kwong *et al.*, 2007). Hepatic PEPCK mRNA expression has also been found to be higher in 165dGA (term 180 days) fetal baboons following reduced maternal nutrition (70% requirements) from early gestation (Nijland *et al.*, 2010) and in neonatal sheep hepatic GR mRNA increased following a 50% nutrient reduction during days 28 – 77 of gestation (Whorwood *et al.*, 2001). This latter challenge has a longer duration in early – mid gestation than my study and begins (28dGA) just as my challenge was ending (31dGA). However, one study did agree with the findings in this thesis that hepatic PEPCK mRNA expression was lower in 138dGA fetal sheep following a peri-implantation nutrient restriction (Lie *et al.*, 2014).

My observations and those of Li *et al.* suggest that the reduced expression of genes in the gluconeogenic pathway in the liver is a possible mechanism to limit fetal growth in the face of reduced nutrient supply. However, in humans early gestation undernutrition, as a result of the Dutch Winter famine in 1944 - 1945, was found to result in a heavier birthweight with a more atherogenic lipid profile and higher risk of coronary heart disease in adult life (Roseboom *et al.*, 2000;Roseboom *et al.*, 2001). People who experienced undernutrition in late gestation were not affected in the same way and therefore, this suggests that the early time point of gestation is more critical to other developing fetal systems such as the coronary system. Although this study did not examine other organs, early gestation nutrient restriction was found to increase the myofibre and capillary densities in fetal sheep muscle (Costello *et al.*, 2008) and adipocyte size in cows (Long *et al.*, 2012) which shows that early gestation undernutrition can also affect other developing organs involved in glucose homeostasis and therefore may increase the risk of type 2 diabetes in later life.

In Chapter 4, the 50% undernutrition challenge between 1-31dGA was similar to that investigated in Chapter 5 but unlike Chapter 5 had no effect on adult hepatic gluconeogenic mRNA levels. This suggests that the changes elicited in the fetal liver were not sustained to adulthood, however, other studies on the 2.5 year old adult sheep used in this thesis found that early gestation undernutrition resulted in cardiac hypertrophy and altered cardiovascular function (Cleal *et al.*, 2007a) and increased expression of both LPL and GR mRNA in adipose tissue (Poore *et al.*, 2014) in male sheep. Whether any changes in liver metabolism would have been seen in adults if the challenge had been more intense (longer or more severe) was unknown and this idea was investigated in Chapter 6 (see discussion, 'Whole gestation challenge').

Late gestation challenge

In this study late gestation undernutrition was found to reduce the hepatic mRNA expression of GR, PEPCK and G6Pase in fetal sheep. As with the early gestation undernutrition, the reduced expression of genes in the gluconeogenic pathway in the liver is a possible mechanism to limit fetal growth in the face of reduced nutrient supply (even though we did not detect altered body weight). In humans late gestation undernutrition was found, as a result of the Dutch Winter famine in 1944 -1945, to result in a lighter birthweight with a reduced glucose tolerance in adult life (Roseboom *et al.*, 2001;de Rooij *et al.*, 2006;Ravelli *et al.*, 1998), suggesting that late gestation undernutrition can influence size at birth. The adult study (in Chapter 4) did not have a late gestation challenge so the effects, if any, could not be investigated in adult sheep. Other studies have reported that glucose intolerance was evident in 1 year old sheep that had a late gestation nutrient restriction (from days 110 to term (147 days)) (Gardner *et al.*, 2005) and in fetal rats from mothers fed a low protein diet in late pregnancy both hepatic PEPCK and G6Pase activity were found to be increased (Franko *et al.*, 2009).

These findings along with those from the Dutch Winter famine suggest that this time point of late gestation is critical to the glucose metabolic systems developing around that time. In other organs, late gestation undernutrition was found to increase the myofibre and capillary densities and increase mRNA expression of the insulin receptor, glucose transporter GLUT4 and IGF-1R in fetal sheep muscle (Costello *et al.*, 2008) and altered fat deposition patterns in lambs (Nielsen *et al.*, 2013) which shows that late gestation undernutrition can also affect other developing organs involved in glucose homeostasis and therefore may still increase the risk of type 2 diabetes in later life even though the cause is less likely to be driven by the liver.

Whole gestation challenge

In this study a low maternal body condition score prior to, during pregnancy and postnatally during suckling was employed as a whole of gestation challenge to the offspring and was found to alter hepatic glucose metabolism in the adult male offspring. They were found to have increased fasting glycemia, glucose intolerance and impaired insulin secretory response when compared to those from mothers with a higher body condition score (Cripps *et al.*, 2008). Therefore in view of these findings and the impact of diet in Chapter 5 on the mRNA expression of GR, PEPCK and G6Pase, it seemed important to determine if such a gestation-long challenge, spanning important windows of development, would impact on the hepatic metabolism in adulthood. However no differences in basal plasma cortisol, urea, cholesterol, HDL or LDL were found in these sheep at 4.5 years of age and in the main, hepatic mRNA gene expression was not greatly altered (the exception being the insulin receptor which was increased in offspring from LBCS mothers). In humans low birthweight has been used as an indicator of poor gestational nutrition and has been linked to the development of type 2 diabetes in later life and as such whole gestation undernutrition data is rare. Much of the Dutch Winter famine data is split into early, mid and late gestation studies which is why animal studies have been used to study effects of whole gestation undernutrition much more reliably. The rat model is one that has been used frequently to investigate the effects of a whole gestation length nutritional challenge using both under- and over- nutritional models and many studies have looked at various organs and biological systems in response to it, the liver being just one. In fact, 20dGA and 6 day old rats have increased hepatic GR mRNA expression due to maternal low protein diets (MLP) throughout gestation (Bertram *et al.*, 2001;Lillycrop *et al.*, 2005). Studies in larger animal models have shown that PEPCK and G6Pase mRNA expression in the liver was increased in a placental insufficiency fetal sheep model at 134dGA (Thorn *et al.*, 2009). Although this study did not examine other organs, whole gestation nutrient restriction has been found to result in reduced skeletal myofibre density in the 4.5 year old adult sheep studied in this thesis (Costello *et al.*, 2013) and was also found to alter the structural and enzymatic properties of skeletal muscle in rats (da Silva *et al.*, 2014).

In recent years early life overnutrition has also been associated with metabolic disease in later life but sheep studies are few and results are varied with some claiming that there is no effect on birth weight, fat or metabolic markers (Peel *et al.*, 2012) and others detailing that overnutrition reduced subcutaneous fat deposition (Khanal *et al.*, 2014) but research into this area in the larger animal model is ongoing. However, there have been more studies carried out in rats and whole gestation overnutrition studies have been found to increase the body fat in offspring and increase insulin resistance. Metabolic abnormalities associated with this maternal obesogenic study also include hepatic steatosis, mild hyperinsulemia and abnormal lipogenic gene signature in the liver (Borengasser *et al.*, 2014;Borengasser *et al.*, 2011;Rector *et al.*, 2010). Possible thoughts for the underlying causes for this include alterations to the hepatic mitochondrial genes which are involved in hepatic fatty acid oxidation. One of these, SIRT3, and mitochondrial protein content have been found to be reduced in rats from obese mothers indicating a reduction in fatty acid oxidation and a possible mechanism to adult obesity and its associated issues including insulin resistance (Borengasser *et al.*, 2011;Rector *et al.*, 2010).

Postnatal challenge

There were no effects of the postnatal diet seen in hepatic gene expression in adult sheep despite the 2.5 year old female sheep having improved glucose sensitivity when assessed previously (Poore *et al.*, 2007). Restricted postnatal diet has also been seen to reduce the plasma concentrations of glucose, urea, and insulin in young lambs (Greenwood *et al.*, 2002) and a low protein postnatal diet has been shown to reduce the mitochondrial DNA content of both liver and skeletal muscle in rats suggesting changes that may influence insulin resistance in later life (Park *et al.*, 2003). Studies are also looking at the effects of postnatal overnutrition and was found to cause hyperlipidemia in adult sheep (Kongsted *et al.*, 2013) and increase small adipocyte numbers in rats (Claycombe *et al.*, 2013).

Overall my findings in this thesis suggest that it is not just the intensity of the challenge as seen in the fetal study (Chapter 5), (where the increased intensity of the challenge in early gestation (to 40% requirements) resulted in more gluconeogenic gene expression changes), but the timing also that is critical.

Effect of nutritional challenges on liver weight

Liver weights (as a percentage of bodyweight) were found not to be different in response to early life nutrient restriction. Previous studies have found that liver weight was lower in adult male sheep following a longer period of early gestation nutrient restriction for 1 - 95 days (Hyatt *et al.*, 2007b) but in fetal sheep 78dGA fetal liver weight (as % body weight) was higher following 50% nutrient restriction during 28 – 78 dGA (Vonnahme *et al.*, 2003). Lower fetal liver weights (compared to controls) were found in 135dGA sheep following 60% nutrient requirement diet from 64 - 135dGA (Reed *et al.*, 2007) and also when nutrition was restricted to 25% of nutrient requirements during late gestation (100-124dGA) was administered (Bauer *et al.*, 1995). This was also discovered when the fetuses were subjected to placental restriction (Kind *et al.*, 1995). In rats liver weight is reduced in fetuses from maternal low protein diet throughout gestation and liver structure is altered (Ramadan *et al.*, 2013). These studies found that liver weight was affected at a timepoint in gestation later than the study here and for a longer duration. No difference in fetal liver weight was found in sheep that experienced a 60% nutrient requirement challenge during days 28 – 80 (Hyatt *et al.*, 2004) suggesting that the severity of the restriction and duration/timepoint at which the restriction is experienced affects the outcome in liver weight.

Effect of nutritional challenges on liver lobes

A previous study into the effects of maternal undernutrition in the male adult rat offspring (Zhang & Byrne, 2000) found that changes in expression of GR mRNA occurred in the lobes of the liver as a result of maternal diet. The nutritional challenge was not found to affect the mRNA expression of hepatic genes in the lobes of adult sheep as reported in Chapters 4 and 6. However rats as small rodents develop differently to sheep. They develop in large litters compared to single or twin offspring in sheep. They also are not as highly developed at birth unlike sheep. The rat model used a dietary undernutrition consisting of a 9% protein content (Langley-Evans et al., 1994) compared to normal (18%) whereas the dietary restriction used in the adult sheep studies was a global nutrient restriction of 50%. It may be that the nutritional challenge employed here was not harsh enough to create an adaptation response in the sheep and this was indeed seen in Chapter 5. In fetal sheep, in this thesis the effect of undernutrition in early and late gestation was found to lower the expression of GR in the left and right lobes. Early gestation nutrition reduction (during days 1-31) in the form of the more severe challenge (40% of requirements) was found to reduce the mRNA expression of PEPCK in both left and right lobes. The left lobe only was found have a lower G6Pase mRNA expression as a result of early and late challenges. This shows that the lobes are responding to their individual environments. In the fetus a major source of liver blood flow is the part of umbilical venous blood flow that does not bypass the liver via the ductus venosus. This blood is well oxygenated and rich in glucose from the maternal circulation. The right lobe receives less well oxygenated blood via the portal vein and this imbalance in nutrients supplied to the lobes is thought to influence the lobar differences.

From previous studies there appear to be two critical windows affecting hepatic size - early gestation when the liver is developing and late gestation when it is growing and preparing for postnatal life.

8.3 Gross anatomy of the liver influences gene expression

As well as the response to undernutrition, as discussed above, the gross anatomy of the liver itself also influences gene expression in 127dGA fetal (Chapter 5) and adult sheep aged 2.5 years (Chapter 4), but not in 4.5 year old adult sheep (Chapter 6). In fetal sheep the mRNA expression of GR and PEPCK was decreased in the right lobe and in adults GR was increased and PEPCK decreased in the right lobe. Mid gestation fetal baboons (90 day of 180 day term) differentially expressed more than 800 genes which suggests that the lobes have different functions (Cox *et al.*, 2006) and in fetal sheep IGR1 and IGF1r mRNA levels were differentially expressed in the two hepatic lobes. Furthermore, the relative expression in the two lobes was altered by both IUGR and intra-amniotic IGF-1 treatment (Darp *et al.*, 2010). It is thought that the different fetal circulation to the liver whereby the right lobe receives less well oxygenated blood influences the differences in gene expression.

8.4 Sex and liver metabolism

This study to date has shown that sex does alter liver metabolism in adult sheep aged 2.5 years but not in fetal sheep aged 127dGA. Sex effects could not be studied in the 4.5 year old sheep study as that study consisted of male offspring only. Female adult sheep (from the cohort assessed in Chapter 4 and 7) aged 1.5 years and at 2.5 years of age that experienced poor early postnatal growth during weeks 12 – 25 had increased insulin sensitivity and an increase in fat but this was unaffected in males when investigated previously (Poore *et al.*, 2005). 20dGA fetal rats were found to have sex specific alterations in response to a peri implantation MLP diet; males had increased PEPCK mRNA expression and females had increased 11β-HSD2 mRNA (Kwong *et al.*, 2007). Male adult sheep were found to have a higher hepatic mRNA expression of GR than females at 2.5 years of age and this was not affected by periods of undernutrition in early life. The assessment of hepatic GR mRNA expression in late gestational fetal sheep did not result in any differences between males and females. Possible explanations for differences between fetuses and adults in sex effects include hormonal changes during puberty or ageing. Indeed, cortisol (a ligand of the GR receptor) has been shown to be increased in men in response to a stressor compared to women (Kirschbaum *et al.*, 1992;Zimmer *et al.*, 2003;Dixon *et al.*, 2004) which may explain for the increase in hepatic GR receptor seen in the sheep in this study. Other hormone differences include the obvious, circulating sex hormones. Females, (whose main sex hormone is estrogen) differ with respect to distribution of adipose tissues compared to males (whose main sex hormone is testosterone) until menopause whereby the distribution of fat becomes similar due to the then lowered levels of estrogen. There appears to be no difference in the estrogen receptor levels between the sexes in the liver (Dickson & Eisenfeld, 1979). Adult females are also known to have higher levels of thyroid hormones than males (Hollowell *et al.*, 2002) and thyroid hormones are known to play a role in hepatic lipid homeostasis by increasing the expression of LDL receptors on hepatocytes (Ness *et al.*, 1998) and increasing the activity of lipid lowering hepatic enzymes (Taylor *et al.*, 1997). Although studies have looked at liver metabolism few have studied both sexes with studies in males often the preferred option.

My finding of increased GR mRNA expression in adult males suggests that their liver metabolism may be more sensitive to the modulatory actions of glucocorticoids and, while not investigated directly in this thesis, this could impact on numerous physiological systems including the gluconeogenic pathways during stress conditions.

8.5 Offspring number and liver metabolism

There was no difference in liver weights (as a percentage of body weight) between twins and singletons, or in the hepatic GR, G6Pase or insulin receptor gene expression. This thesis was only able to study multiple offspring number in adults (Chapter 4). However, one finding in female twins was that PEPCK mRNA expression was found to be higher than singletons but this was not seen in males. Hepatic PEPCK mRNA has also been shown to be higher in twin fetal sheep at 136dGA (Lie *et al.*, 2014). The increase in PEPCK suggests that female twins may have a raised gluconeogenic capability and therefore produce more glucose into the circulation which means that they may be at risk of metabolic disorders such as type 2 diabetes and obesity. Coupled with increased insulin sensitivity the excess glucose may be being stored as fat which may contribute to the increase in adipose tissue seen in females.

8.6 No methylation changes seen

This was the first time we had been able to investigate any possible methylation changes in sheep within our laboratory and therefore this was a really exciting development. There was no effect of diet, sex, lobe or offspring number on the GR methylation levels of 2.5 year old adult sheep that could be detected within the partial sequence we obtained. This does not mean that the nutritional environment experienced by these sheep in early life did not alter the methylation status of the genes in question, just that any methylation changes were not detected in the region investigated. As the DNA sequence obtained was incomplete it is possible the methylation changes have occurred in a CpG region not yet identified. The lack of sheep genome makes fully investigating any methylation changes in the hepatic gluconeogenic genes difficult although another group has recently investigated sheep PEPCK in the liver (Lie *et al.*, 2014) so advancements in this area are progressing.

8.7 Conclusions

The experiments reported in this thesis have examined the following general hypothesis:

- **Early life nutrition affects liver metabolism in sheep.**

The experiments carried out in this thesis do support this as it was found that the liver responded to nutrient restriction in both early and late gestation with a reduction in key gluconeogenic enzymes in late gestation as well as differential expression between the fetal liver lobes (fig.8.2). These effects did not persist into adult life and indeed, as with the lobar differences in gene expression patterns, appear even to switch direction over the life course. However, although these effects of maternal undernutrition on hepatic mRNA imply potential changes in gluconeogenic capacity, they may not be of physiological significance to offspring until they are challenged further e.g. by over- or under-abundant postnatal nutrition.

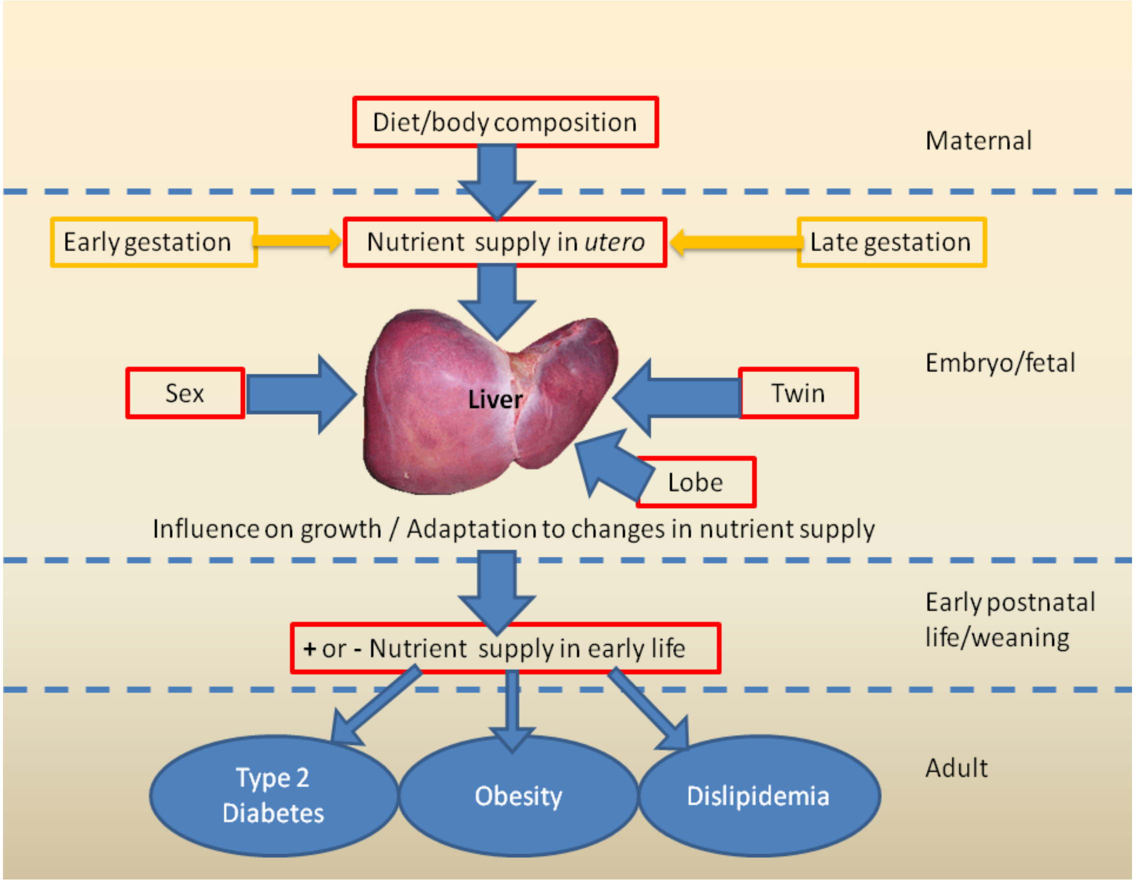


Figure 8.2 Nutritional protocol.

8.8 Limitations of the study

This thesis examined the hypothesis in three cohorts of sheep, two adult and one fetal. These provided a substantial resource of material tackling issues of challenge timing, intensity and age of offspring. However there was no opportunity to modify the intensity or timings of the nutritional challenges as these had already been designed and implemented prior to the commencement of my PhD. This also meant that except in Chapter 6, there was no opportunity to conduct in *vivo* experiments on liver metabolism in the 2.5 year old cohort and fetal cohorts (Chapters 4 and 5). It was not possible to examine potential sex differences in all cohorts as the 4.5 year old adult study contained only males. Twins were only able to be studied in the 2.5 year old sheep as there were no twins in the fetal or 4.5 year old cohort of sheep. A major limitation on the plan of my studies was the lack of sheep genome information which limited the pallet of genes which could be investigated.

This also limited the scope of the investigations into possible methylation changes associated with any differences in hepatic gene expression.

8.9 Future work

There are several areas which are beyond the scope of this thesis but which would be interesting to investigate in light of its results:

1) Dietary model – It would be interesting to determine the effect of a more intense nutrient restriction for a longer period in early or late gestation coupled with a postnatal nutrient surplus on hepatic metabolism in the adult. Indeed, several other studies have started to examine the effect of postnatal obesity and the mismatch to gestational undernutrition in sheep (Hou *et al.*, 2014;Johnsen *et al.*, 2013;Khanal *et al.*, 2014).

2) Starvation challenge – It would also be interesting to investigate the postnatal hepatic metabolic response (and the recovery from) to starvation following early or late gestation periods of undernutrition as this gives more information on the physiological response than simply baseline measurements.

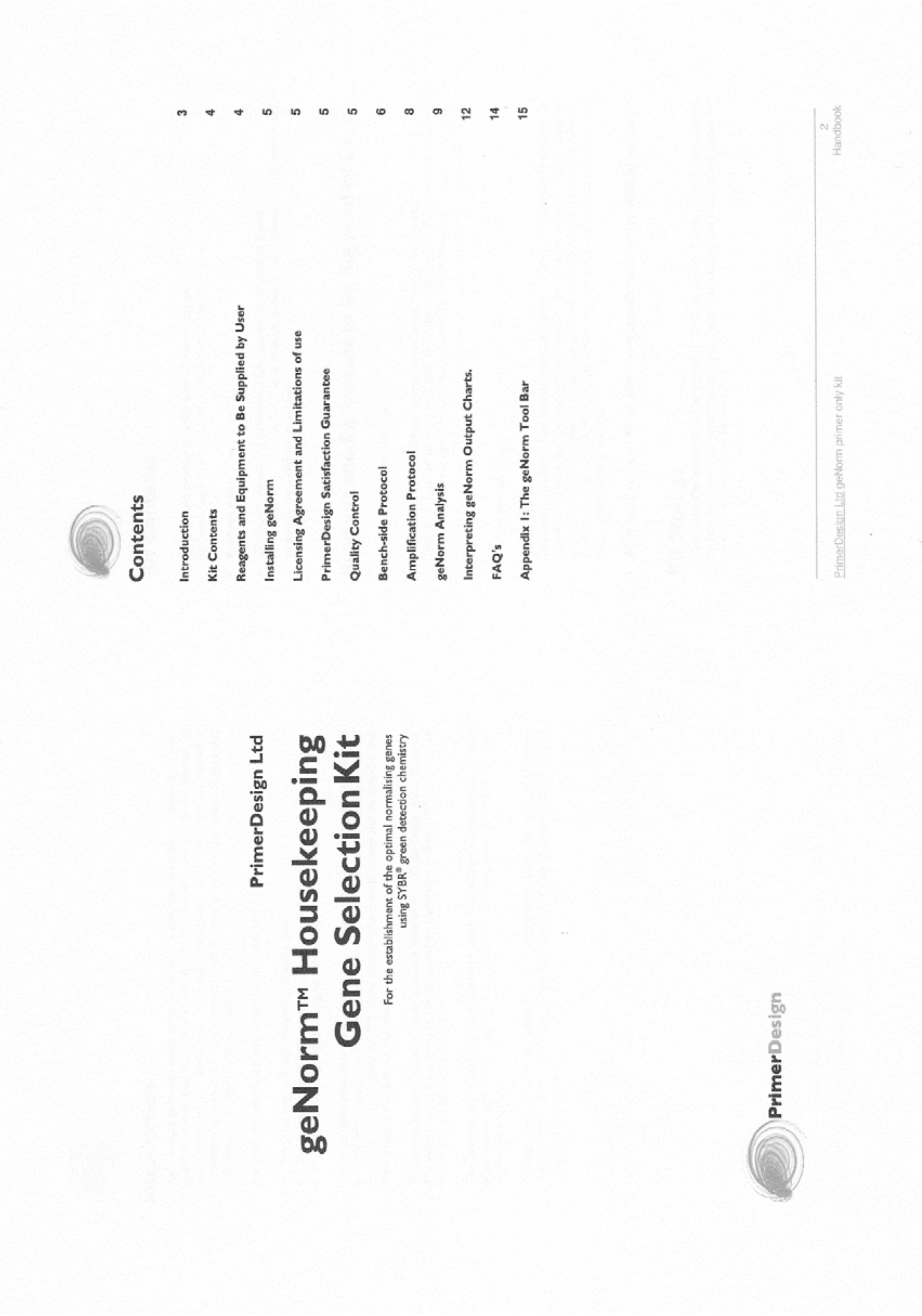
3) Molecular mechanisms - Further analysis of the thesis cohorts could be to determine if the changes in hepatic gluconeogenic gene mRNA expression can be seen at the protein level by way of western blotting perhaps using bovine antibodies. Also, to investigate the enzyme activity of PEPCK and G6Pase to see if the increase/decrease seen in mRNA expression had been translated into enzyme activity. Other genes could be investigated such as 11β-HSD1 which regulates the access of glucocorticoid to the receptor and therefore may in turn, influence the regulation of the gluconeogenic pathway.

4) Epigenetic mechanisms – Further analysis of the thesis cohorts could be to determine if the changes in hepatic GR do occur, as even though GR mRNA expression may or may not be altered it is still possible that

epigenetic changes exist on the genes. The assessment carried out in this study looked at a large section of the GR promoter and could not detect possible changes at the level of individual CpGs. There could also be changes in other intergenic regions or in genes relating to GR. Ultimately, when more DNA sequence becomes available further epigenetic investigations into the GR, PEPCK, G6Pase and insulin receptor methylation status in these animals may be possible.

Appendices

Appendix 1: geNorm Handbook



PrimerDesign Ltd

geNorm™ Housekeeping Gene Selection Kit

For the establishment of the optimal normalising genes
using SYBR® green detection chemistry

PrimerDesign

Contents

PrimerDesign Ltd geNorm primer only kit

2
Handbook

Introduction

For accurate gene expression measurements, it is essential to normalise results from your quantitative real-time PCR experiments to a fixed reference; one that is not affected by your experimental conditions. Although normalising to a constitutively expressed housekeeping gene is the most common method, there is no universal reference gene that is constant in all experimental situations.

For example, reference gene expression levels may vary by:

- The tissue origin of your sample e.g. brain vs. heart.
- The disease origin of your sample e.g. carcinoma vs. healthy tissue.
- Your experimental parameters e.g. stimulated cells vs. non-stimulated.

If your selected reference gene expression is variable, then normalising to this gene will severely limit the accuracy and sensitivity of your assay. Therefore, it is necessary to find the ideal reference genes for YOUR particular experimental system and to establish that these genes are indeed not regulated.

The PrimerDesign geNorm kits each contain a panel of 6 or 12 candidate reference genes. The expression of these genes can be measured by quantitative real-time PCR and the data analysed by the geNorm software.

The analysis will reveal the best reference gene for accurate normalisation in your experimental system by ranking the candidate reference genes according to their expression stability.

Having used geNorm to select the best normalising genes for your experiment, PrimerDesign can provide ready to use high quality normalising gene detection kits for your next experiments.

Kit Contents

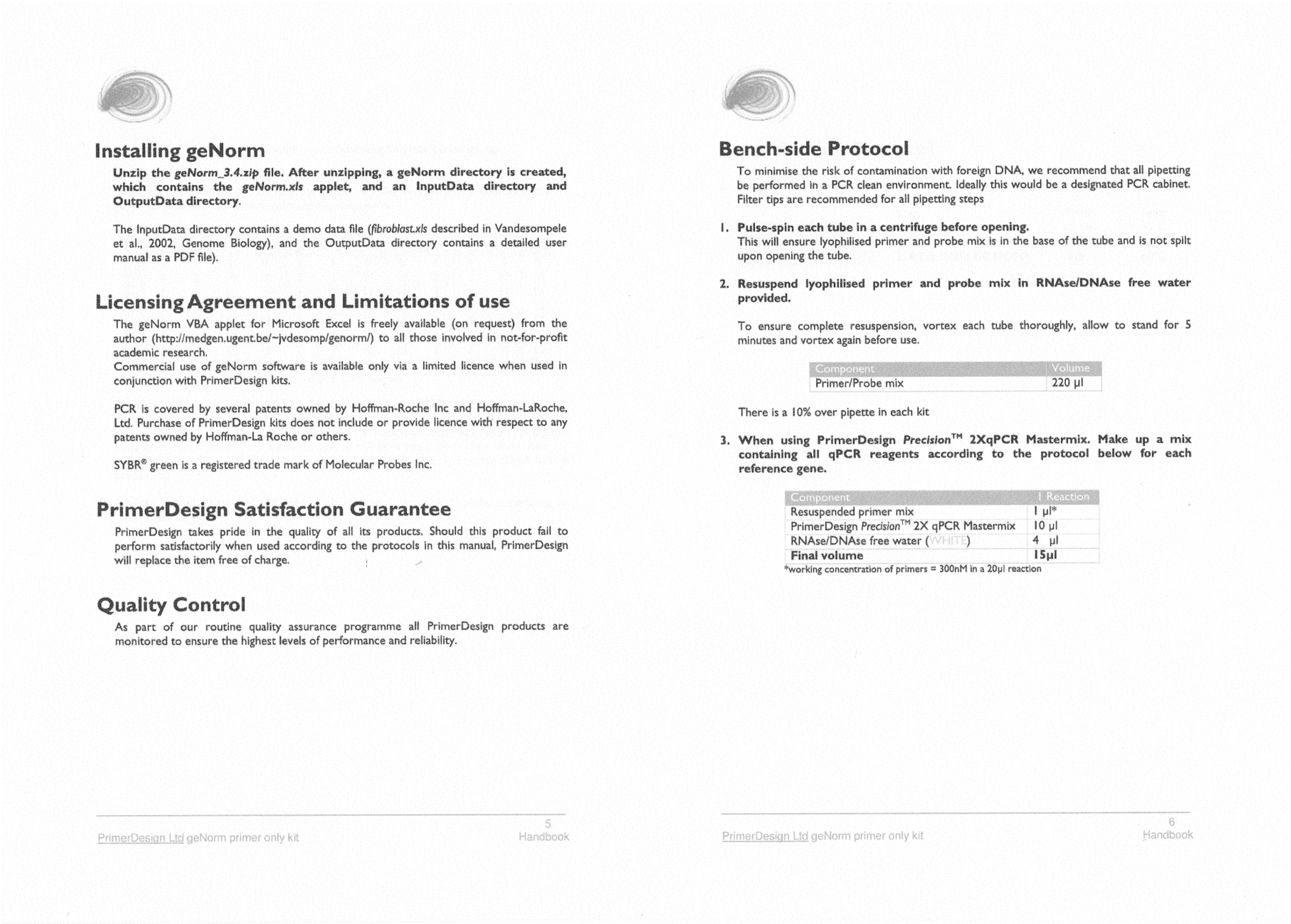
- **Lyophilised primer sets for 6 or 12 reference genes**
  Sufficient reagents are supplied for 200 20µl reactions per gene.
- **RNAse/DNAse free water (WHITE)**
- **CD-ROM containing geNorm VBA applet for Microsoft Excel**
  Also included on the CD-ROM is a detailed geNorm user manual in PDF format and annotated Excel files for data input.

Reagents and Equipment to Be Supplied by User

- **Real-Time PCR instrument**
- **Mastermix or mastermix components containing SYBR® green**
  This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*™ Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Sample cDNA**
  The quality of cDNA will directly affect the quality of data generated using this kit. 10 cDNA samples is the minimum recommended for geNorm analysis. When assessing expression stability in different groups (e.g. diseased vs. healthy) make sure that you use the same number of samples from each group in order to avoid bias towards the group with the largest number of samples
- **PC running windows XP or 2000 and Microsoft Excel version 2000, XP or 2003**

Kit Storage

This PrimerDesign kit should be stored at -20°C on arrival. Freeze/thawing cycles should be kept to a minimum once resuspended. Under these conditions reagents are stable for six months from date of resuspension.



Installing geNorm

Unzip the *geNorm_3.4.zip* file. After unzipping, a geNorm directory is created, which contains the *geNorm.xls* applet, and an InputData directory and OutputData directory.

The InputData directory contains a demo data file (*fibroblast.xls* described in Vandesompele et al., 2002, Genome Biology), and the OutputData directory contains a detailed user manual as a PDF file).

Licensing Agreement and Limitations of use

The geNorm VBA applet for Microsoft Excel is freely available (on request) from the author (http://medgen.ugent.be/~jvdesomp/genorm/) to all those involved in not-for-profit academic research.
Commercial use of geNorm software is available only via a limited licence when used in conjunction with PrimerDesign kits.

PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of PrimerDesign kits does not include or provide licence with respect to any patents owned by Hoffman-La Roche or others.

SYBR® green is a registered trade mark of Molecular Probes Inc.

PrimerDesign Satisfaction Guarantee

PrimerDesign takes pride in the quality of all its products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, PrimerDesign will replace the item free of charge.

Quality Control

As part of our routine quality assurance programme all PrimerDesign products are monitored to ensure the highest levels of performance and reliability.

Bench-side Protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend lyophilised primer and probe mix in RNAse/DNAse free water provided.**

   To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Primer/Probe mix	220 µl

There is a 10% over pipette in each kit

3. **When using PrimerDesign *Precision*™ 2XqPCR Mastermix. Make up a mix containing all qPCR reagents according to the protocol below for each reference gene.**

Component	1 Reaction
Resuspended primer mix	1 µl*
PrimerDesign *Precision*™ 2X qPCR Mastermix	10 µl
RNAse/DNAse free water (WHITE)	4 µl
Final volume	**15µl**

*working concentration of primers = 300nM in a 20µl reaction

4. **Pipette 15µl of the mix into each well according to your plate set up.**

All samples for each reference must be run on the same plate. However, different reference genes may be run on separate plates. If using multiple plates, pour all plates on the same occasion. Run all data points in duplicate wells.

	Reference Genes											
	ACTB				UBC				GAPDH			
cDNA samples	1	1	9	9	1	1	9	9	1	1	9	9
	2	2	10	10	2	2	10	10	2	2	10	10
	3	3	11	11	3	3	11	11	3	3	11	11
	4	4	12	12	4	4	12	12	4	4	12	12
	5	5	13	13	5	5	13	13	5	5	13	13
	6	6	14	14	6	6	14	14	6	6	14	14
	7	7	15	15	7	7	15	15	7	7	15	15
	8	8	Water	Water	8	8	Water	Water	8	8	Water	Water

Example plate layout for a geNorm analysis using 15 cDNA samples.

5. **Prepare 66µl (6 gene kit) or 132µl (12 gene kit) of cDNA for each sample at a concentration of 5ng/µl in RNAse/DNAse free water.**
These suggested volumes include an additional 10% more than required to allow for pipette calibration inaccuracies. If the concentration of cDNA is not known, then dilute your RT reactions 1:10 (10µl of RT and 90µl of water). Ensure you have high quality cDNA before proceeding.

6. **Pipette 5µl of diluted cDNA into each well of your 96-well plate according to your plate layout .**
The final volume in each well is 20µl.

Amplification Protocol

1. **Amplification conditions using PrimerDesign *Precision*™ 2X qPCR Mastermix.**

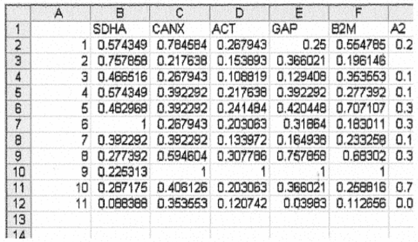
	Step	Time	Temp
Cycling x50	Enzyme activation	10min	95°C
	Denaturation	15s	95°C
	DATA COLLECTION*	60s	60°C

*Fluorogenic data should be collect during this step through the SYBR® green channel.

geNorm Analysis

1. **Transform CT values into relative quantification data using the deltaCT method.**
   Before Ct values from the quantitative real-time PCR are input into geNorm, all CT values from must be transformed into relative quantification data.
   - To achieve this **subtract the highest Ct value from all other Ct values for each gene measured**. Hence each Ct value has been transformed in to a "delta CT" value, with the highest deltaCT value as 0. All other values are less than 0.
   - Then for each data point **apply the equation** $2^{(\text{-delta Ct})}$. Hence all data is expressed relative to the expression of the least expressed gene.

2. **Prepare input file for geNorm analysis.**
   Data should be laid out in Microsoft Excel with the first column containing the sample names and the first row containing the gene names. The first cell of the first row and column (cell A1) should be empty.
   OTHER EMPTY CELLS ARE NOT ALLOWED.



	A	B	C	D	E	F	
1		SDHA	CANX	ACT	GAP	B2M	A2
2	1	0.574349	0.784584	0.267943	0.25	0.554785	0.2
3	2	0.757858	0.217638	0.153893	0.366021	0.196146	
4	3	0.466516	0.267943	0.108819	0.129408	0.353553	0.1
5	4	0.574349	0.392292	0.217638	0.392292	0.277392	0.1
6	5	0.482968	0.392292	0.241484	0.420448	0.707107	0.3
7	6	1	0.267943	0.203063	0.31864	0.183011	0.3
8	7	0.392292	0.392292	0.133972	0.164938	0.233258	0.1
9	8	0.277392	0.594604	0.307786	0.757858	0.68302	0.3
10	9	0.225313	1	1	1	1	
11	10	0.287175	0.406126	0.203063	0.366021	0.258816	0.7
12	11	0.088388	0.353553	0.120742	0.03983	0.112656	0.0
13							
14							

3. **Save the file in the InputData directory.**
   This folder will be found in the geNorm directory created upon geNorm installation.

4. **Close all running instances of Microsoft Excel.**

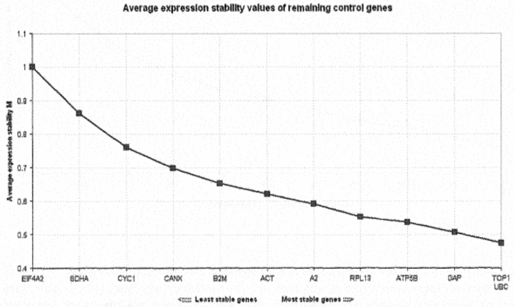
5. **Start up the geNorm applet (in Excel: *Open File*, or double click on the *geNorm.xls* file).**

6. **Enable macros when prompted.**

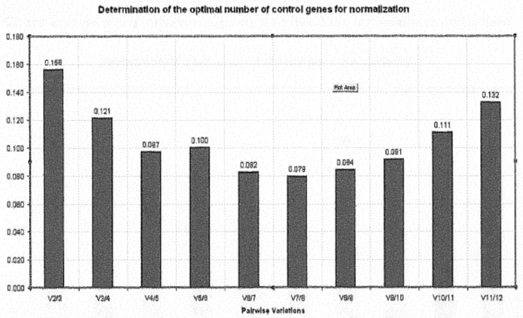
7. **Load the Input file.**
   This is achieved by clicking Load input data icon in the tool bar.

8. **Click on the "Calculate" button.**
   After a pause the screen will show the first stage of the geNorm analysis. The least stably expressed gene marked red will subsequently be removed and the analysis will repeat with the remaining genes. This process is repeated automatically in the following steps.

9. **Click the automated analysis icon.**
   After clicking 'Automated analysis icon', the first geNorm chart is generated. Automated analysis is recommended although manual analysis can be performed if required (see detailed geNorm manual PDF.)

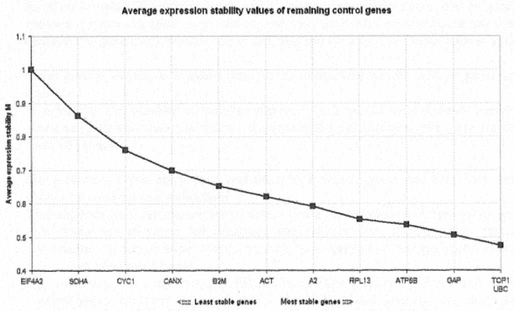


10. **Click the automated analysis icon a second time.**
    After clicking 'Automated analysis icon' again, a second geNorm chart is generated.



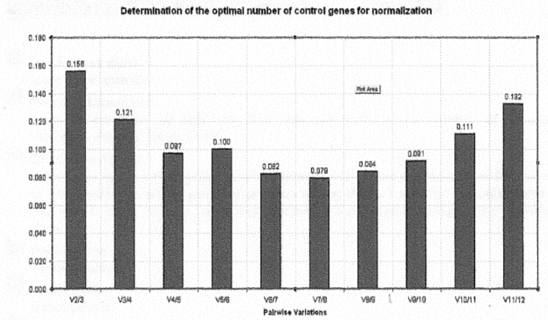
Interpreting geNorm Output Charts.

1. Chart 1: Which are the most stably expressed reference genes in my system?



The chart generated indicates the average expression stability value M of reference genes at each step during stepwise exclusion of the least stable expressed reference gene. Starting from the **least stable gene at the left**, the genes are ranked according to increasing expression stability, ending with the two **most stable genes on the right**. In this example TOP1 and UBC are the two most stable genes.

2. Chart 2: How many reference genes do I need for optimal normalisation?



The second chart illustrates the levels of variation in average reference gene stability with the sequential addition of each reference gene to the equation (for calculation of the normalization factor). Starting with the two most stably expressed genes on the left, with the inclusion of a 3rd, 4th 5th gene etc. moving to the right.
This measure is known as the "pairwise variation *V*". We recommend a *V* score of below 0.15 as the ideal for your system. e.g. If a *V* score of 0.25 is achieved using two genes but a *V* score of 0.14 is achieved with three reference genes, then the average of the top three genes in your system would be the optimal normalisation factor for your future experiments. In the example above, the two most stable genes would give high quality data, but three would be ideal.

Note: Please bear in mind that the proposed 0.15 value must not be taken as a too strict cut-off. The second graph is only intended to be guidance for determination of the optimal number of reference genes. Sometimes, the observed trend (of changing *V* values when using additional genes) can be equally informative. However, simply using the 3 best reference genes (and ignoring this second graph) is in most cases a valid normalisation strategy and results in much more accurate and reliable normalisation compared to the use of only one single non-validated reference gene.

FAQ's

Q1: When I load geNorm, **the menu bar is not visible**
A1: Close all open instances of Microsoft Excel and reload geNorm

Q2: What should I do if I have an **empty cell?**
A2: Remove sample OR remove gene which contains an empty value, and recalculate. To remove the sample, click on the empty cell, and then click on the *Delete row* button. To remove the gene, click on the empty cell, and the click on the *Delete column* button.

Q3: **How many samples** should I analyze to determine which control genes are most stable?
A3: In principle, any number of samples higher than 2 would be sufficient. However, the more samples you use, the more reliable are the conclusions. We propose to use at least 10 samples.

Q4: **Do I always have to retest** and determine which genes are the most stable, and should be used for normalisation?
A4: This depends on your experimental setup. Once you have determined which genes and how many are required for accurate and reliable normalisation, you can use this information for future experiments, as long as no significant changes in the experimental setup have been introduced.
e.g. once you have determined that *ACTB*, *GAPDH* and *YWHAZ* are the most stable control genes for short term cultured human fibroblasts, you can use these genes for normalization of all future fibroblast samples, as long as you keep the culture conditions, harvesting procedures, etc. identical.

Q5: Whom should I **contact** if I have **further questions?**
A5: Technical support is available from PrimerDesign for all of our geNorm products provided the kit have been used according to the protocols presented in this manual. A geNorm discussion group is also available for those wishing to learn more about this approach. http://groups.yahoo.com/group/genorm

Q6: When using **replicated tubes** in the same run, should I **first average** the Ct values and then transform the data for input into geNorm, or vice versa?
A6: Either approach is satisfactory, although we recommend transforming the arithmetic mean of the replicate Ct values.

Q7: What should I do if my **negative control** well produces an amplification plot?
A7: If the signal is very late e.g. CT>35, and there is a >8CT value difference between the negative control and all the cDNA sample results, then ignore the no template signal and proceed with the analysis. It is likely due to a very low level of cross contamination during the set up of the plate. If a strong signal similar to the cDNA samples is obtained then analysis cannot be performed. One of your reagents has likely become significantly contaminated during your experiment set up.

Appendix 1: The geNorm Tool Bar

Load input data
loads Excel data file

Manual data input
provides possibility to type the data manually; indicate the number of samples and reference genes to be analysed

Criteria settings
adjusts the expression stability threshold below which genes are included in the calculation of a normalization factor; genes (expression values) that are used for normalization are displayed in black, while genes in grey (inactive) are not used to calculate the normalization factors

Delete row
remove sample

Insert row
insert sample

Delete column
remove gene

Insert column
insert gene

Show matrix
displays the pairwise variation *V* values for each gene with all other genes; click *Return* to leave the matrix view

Print/Save report

Save input data

Automated analysis
automatic ranking of control genes according to their expression stability (chart 1) and determination of optimal number of control genes (chart 2)

101% **Zoom**
user-adjustable zoom level, e.g. allows to view all genes and/or samples by highlighting the cells of interest, and selecting 'fit to selection'

Clear screen

About geNorm
brief information on contact address, method, input file, menu icons, and link to the most recent manual on the web

Exit geNorm
quit the application

Appendix 2: 2.5 year old sheep study

Appendix 2.1 Maternal diet

P316 – RVC EXP Sheep Nuts (G J W TITMUS New Mill, Hamer Lane, Wheathampsted, Hertfordshire): a complementary feeding stuff for feeding to sheep should be fed diluted.

Protein	14.75%
Fibre	16.80%
MER	10.725 MJ/kg dry matter
Vitamin D3	2000 iu/kg
Vitamin E	10mg/kg (alpha tocoperal)
Oil (A)	2.50%
Ash	8.20%
Vitamin A	10 000 iu/kg

Raw Materials:

Treated straw, Grass, Hipro soya E, Micronized wheat, Wheat, Barley, Full fat soya, Molasses, Limestone, Calcium carbonate, Dicalcium phosphate salt, Sheep vitamin/mineral supplement.

89.205 % dry matter

Appendix 2.2 Ewbol prestige lamb pellets plus decox

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feeding stuff for feeding, with forage, to growing and fattening lambs up to 70% of dry matter.

Oil	4.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	12.00 %	Vitamin E-alpha tocopherol	30 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.30 mg/kg
Moisture	13.80 %	MER	12.22 MJ/kgDM

Contains the following ingredients in descending order by weight:

Barley, Sunflower ext, Copr exp, Soyabean hulls, Molasses, Malt culms, Peas, Palm kernel exp, Brazilian toasted soya, Bakery by-product, Calcium carbonate, Low glucosinolate rape seed ext, Salt, Vegetable oils.

This medicated feedstuff:

Contains DECCOX (V.m 13997/4009) at a concentration of 100mg/kg DECOQUINATE BP as directed by the veterinary surgeon.

86 % dry matter

Appendix 2.3 Ewbol 18 nuts

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feeding stuff for feeding, with forage, to ewes up to 70% of dietary dry matter intake.

In the preparation of this diet precautions have been taken to minimise the potential risk for those sheep prone to copper toxicity. However no guarantee can be given due to the known differences in both breed and management systems. This feedstuff is not recommended for feeding to commercially milked ewes.

Oil	5.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	9.90 %	Vitamin E-alpha tocopherol	150 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.60 mg/kg
Moisture	13.80 %	MER	10.38 MJ/kg
Magnesium	0.53 %		as fed

This product contains 7.1g of cal mag in 1.42 kgs of feed. Animals should not be fed more than 15g cal mag per day.

Contains the following ingredients in descending order by weight:

Sunflower ext, Malt culms, Wheatfeed, Wheat, Palm kernel exp, Low glucosinolate rape seed ext, Molasses, Cocoa hulls, Maize gluten feed, Beans, Cat feed, Calcium carbonate, Vegetable oils, Salt, Magnesium oxide.

This product contains Addarome to optimise dry matter intake.

This product contains Selenomathionine for enhanced health.

86.5 % dry matter

Appendix 2.4: Calculation of 50% restricted diet based on Bodyweight.

Bodyweight (Kg)	**Complete Sheep Diet / Day (kg)**
30	0.220
31	0.225
32	0.230
33	0.235
34	0.240
35	0.245
36	0.250
37	0.255
38	0.260
39	0.265
40	0.270
41	0.275
42	0.280
43	0.285
44	0.290
45	0.295
46	0.300
47	0.305
48	0.310
49	0.315
50	0.320
51	0.325
52	0.330
53	0.335

Bodyweight (Kg)	**Complete Sheep Diet / Day (kg)**
54	0.340
55	0.345
56	0.350
57	0.355
58	0.360
59	0.365
60	0.370
61	0.375
62	0.380
63	0.385
64	0.390
65	0.395

Appendix 2.5: Adult sheep (2.5yrs) Post-mortem sheet

General Information		
DATE		**Investigator**
Animal ID		**Start time:**
Group **Sex**		**Completion without brain:**
Age		**Completion with brain:**

Measurements			
Body Weight	kg	Biparietal Diameter	cm
Crown-Rump Length	cm	Femur length	cm
Abdominal Circumference	cm	Shoulder height	mm

Tissue Collection								
			Weight	***Tray***	***Fast Freeze***	***Slow Freeze***	***Fix***	***other***
ORGANS								
1	**Pancreas**	*1*						**10ml HCl on ice**
2		*2*					*	
3		*3*			*			
4	**Adrenal**	*Left*			*			
5	**Peri-renal fat**				*		*	
6	**Kidney**	*Left*					*	**perfusion fix**
7	**Renal artery**	*Left*					*	
8	**Mesenteric artery**							**myography**
9	**Adrenal**	*Right*				*	*	
10	**Kidney**	*Right*				*		**myography**
11		*Rt cortex*			*			
12		*Rt medulla*			*			
13	**Renal artery**	*Right*			*			
14	**Liver**	*whole*						
15		*Right*			*			
16		*Left*			*			
17	**Lung**	*Right*			*			
18		*Left*			*			
19	**Heart**	*whole*						
20		*Right atrium*			*			
21		*Left atrium*			*			
22		*Rt ventricle*			* *			**myography**
23		*Lft ventricle*			* *			
24	**Aorta**				*		*	
25	**Skeletal Muscle**	*Soleus*			*			
26	**(back leg)**	*Gastrocnemius*						
		Medial			*			
27	**Brain**	without stem						
		or cerebellum						
28		*brain stem X5*				*		
29		*front cortex*			*			
30	**Hypothalamus**	*block*				*		
31	**Pituitary**	*stalk up*				*		
32	**Hippocampus**				*			

Tissues fixed in formalin for 48 hr at room temperature. If not taken to be blocked immediately, transfer to 70% EtOH

Pancreas - collect ~ 1 g into 180 mmol HCl, on ice, mince tissue with scissors then sonicate for 30 sec - leave at 4 °C O/N

Liver - remove gall bladder, place shiny side down, gall bladder side up: left lobe is on your left, right lobe on your right

Heart - freeze piece of papillary muscle complex from each atrium and ventricle, as well as block for molecular biology

Brain stem - cut into 5 slices, starting at rostral end, freeze rostral edge downwards, largest = number 1

Appendix 2.6: 2.5 year old sheep results

Adult Sheep liver weight (2.5yrs)

Source	Sig.
Sex	.039
Offspring	.425
Diet	.601
Sex * Offspring	.197
Sex * Diet	.897
Offspring * Diet	.943
Sex * Offspring * Diet	.175

Adult Sheep GR mRNA expression (2.5yrs)

Source	Sig.
GRlobe	.000
GRlobe * Sex	.005
GRlobe * Offspring	.802
GRlobe * Diet	.352
GRlobe * Sex * Offspring	.886
GRlobe * Sex * Diet	.263
GRlobe * Offspring * Diet	.331
GRlobe * Sex * Offspring * Diet	.081

Source	Sig.
Sex	.000
Offspring	.027
Diet	.744
Sex * Offspring	.233
Sex * Diet	.641
Offspring * Diet	.010
Sex * Offspring * Diet	.804

Left Lobe & Sex

Source	Sig.
Sex	.001

Right Lobe & Sex

Source	Sig.
Sex	.000

Adult PEPCK mRNA expression (2.5yrs)

Source	Sig.
PEPCKLobe	.014
PEPCKLobe * Sex	.614
PEPCKLobe * Diet	.681
PEPCKLobe * Offspring	.216
PEPCKLobe * Sex * Diet	.247
PEPCKLobe * Sex * Offspring	.065
PEPCKLobe * Diet * Offspring	.575
PEPCKLobe * Sex * Diet * Offspring	.275

Source	Sig.
Sex	.000
Diet	.563
Offspring	.379
Sex * Diet	.316
Sex * Offspring	.005
Diet * Offspring	.574
Sex * Diet * Offspring	.052

Adult G6Pase mRNA expression (2.5yrs)

Source	Sig.
G6Paselobe	.687
G6Paselobe * Sex	.199
G6Paselobe * Diet	.634
G6Paselobe * Offspring	.081
G6Paselobe * Sex * Diet	.981
G6Paselobe * Sex * Offspring	.653
G6Paselobe * Diet * Offspring	.989
G6Paselobe * Sex * Diet * Offspring	.749

Source	Sig.
Sex	.103
Diet	.981
Offspring	.154
Sex * Diet	.980
Sex * Offspring	.085
Diet * Offspring	.596
Sex * Diet * Offspring	.329

Adult Insulin Receptor mRNA expression (2.5yrs)

Source	Sig.
IRlobe	.513
IRlobe * Sex	.724
IRlobe * Diet	.451
IRlobe * Offspring	.290
IRlobe * Sex * Diet	.612
IRlobe * Sex * Offspring	.909
IRlobe * Diet * Offspring	.590
IRlobe * Sex * Diet * Offspring	.967

Source	Sig.
Sex	.655
Diet	.756
Offspring	.786
Sex * Diet	.732
Sex * Offspring	.820
Diet * Offspring	.531
Sex * Diet * Offspring	.102

Appendix 3: Fetal sheep study

Appendix 3.1 Fetal study nutrient requirements

Nutrient	As fed (%)	Nutrient	As fed
Protein	14.750	Metabolisable energy	9.57 MJ/kg
Fibre	16.653	Selenium	200.037 mg/kg
Starch	15.200	Zinc	65.610 mg/kg
Ash	8.175	Magnesium	65.050 mg/kg
OIL (A)	2.493	Iron	39.500 mg/kg
Rumen degradable protein	1.375	Vitamin E	10.000 mg/kg
Lysine	0.766	Iodine	4.086 mg/kg
Sodium	0.692	Cobalt	2.033 mg/kg
Salt	0.691	Copper	1.500 mg/kg
Calcium	0.688	Molybdenum	0.053 mg/kg
Threonine	0.615	Vitamin A	10000 iu/kg
Phosphorus	0.313	Vitamin D3	2000 iu/kg
Methionine	0.224		
Magnesium	0.150		
Chloride	0.086		

Nutritional composition of diet (P316, Charnwood Milling Co. Ltd, Suffolk, UK) fed to ewes at RVC and Southampton.

Appendix 3.2 Fetal study post-mortem sheet

Organ	**Part sampled**	**Treatment**			
		Fast freeze	**Slow freeze**	**Fix**	**Other**
Cotyledons	A B C D	* * * *			
Peri-renal fat		*			
Adrenal	left		½	½	
Kidney	left			*	Perfusion fix
Adrenal	right				
Kidney	right cortex right medulla	bottom ½ bottom ½			
Liver	left lobe right lobe	* *			
Heart	left ventricle right ventricle	* *			
Thoracic aorta					

Lung	left	*			
	right	*			
Skeletal muscle	right soleus	½	½		
	right FDL	½	½		
Brain	front cortex	*			
	hypothalamus		*		
	pituitary		*		
	hippocampus	*			

Treatment of samples taken from fetuses. Fixed tissues were fixed in formalin overnight; frozen tissues were stored at -80ºC.

Appendix 3.3: Fetal sheep results

Fetal Sheep GR mRNA expression Study 1

Source	Sig.
GRlobe	.436
GRlobe * Diet_overall	.017
GRlobe * Sex	.125
GRlobe * Diet_overall * Sex	.385

Source	Sig.
Diet_overall	.383
Sex	.995
Diet_overall * Sex	.879

Left lobe

Source	Sig.
Diet_overall	.531

Right Lobe

Source	Sig.
Diet_overall	.009

Fetal Sheep PEPCK mRNA expression Study 1

Source	Sig.
PEPCKlobe	.256
PEPCKlobe * Diet_overall	.113
PEPCKlobe * Sex	.616
PEPCKlobe * Diet_overall * Sex	.768

Source	Sig.
Diet_overall	.666
Sex	.688
Diet_overall * Sex	.670

Fetal sheep G6Pase mRNA expression Study 1

Source	Sig.
G6Pase	.178
G6Pase * Diet_overall	.734
G6Pase * Sex	.738
G6Pase * Diet_overall * Sex	.148

Source	Sig.
Diet_overall	.166
Sex	.431
Diet_overall * Sex	.481

Fetal Sheep Insulin Receptor mRNA expression Study 1

Source	Sig.
Insreclobe	.865
Insreclobe * Diet_overall	.715
Insreclobe * Sex	.885
Insreclobe * Diet_overall * Sex	.619

Source	Sig.
Diet_overall	.025
Sex	.223
Diet_overall * Sex	.483

Fetal Sheep GR mRNA expression Study 2

Source	Sig.
GRlobe	.050
GRlobe * Diet_overall	.216
GRlobe * Sex	.104
GRlobe * Diet_overall * Sex	.970

Source	Sig.
Diet_overall	.001
Sex	.588
Diet_overall * Sex	.461

Test	(I) Diet_overall	(J) Diet_overall	Sig
LSD	Control	Late gestation restriction	.022
		PI	.000
	Late gestation restriction	Control	.022
		PI	.041
	PI	Control	.000
		Late gestation restriction	.041

Test	(I) Diet_overall	(J) Diet_overall	Sig
Bonferroni	Control	Late gestation restriction	.067
		PI	.000
	Late gestation restriction	Control	.067
		PI	.124
	PI	Control	.000
		Late gestation restriction	.124

Fetal Sheep PEPCK mRNA expression Study 2

Source	Sig.
PEPCKlobe	.473
PEPCKlobe * Diet_overall	.938
PEPCKlobe * Sex	.469
PEPCKlobe * Diet_overall * Sex	.419

Source	Sig.
Diet_overall	.008
Sex	.719
Diet_overall * Sex	.484

Test	(I) Diet_overall	(J) Diet_overall	Sig
LSD	Control	Late gestation restriction	.024
		PI	.003
	Late gestation restriction	Control	.024
		PI	.431
	PI	Control	.003
		Late gestation restriction	.431

Test	(I) Diet_overall	(J) Diet_overall	Sig
Bonferroni	Control	Late gestation restriction	.071
		PI	.008
	Late gestation restriction	Control	.071
		PI	1.000
	PI	Control	.008
		Late gestation restriction	1.000

Fetal Sheep G6Pase mRNA expression Study 2

Source	Sig.
G6Paselobe	.006
G6Paselobe * Diet_overall	.644
G6Paselobe * Sex	.122
G6Paselobe * Diet_overall * Sex	.949

Source	Sig.
Diet_overall	.083
Sex	.261
Diet_overall * Sex	.320

Test	(I) Diet_overall	(J) Diet_overall	Sig
LSD	Control	Late gestation restriction	.035
		PI	.034
	Late gestation restriction	Control	.035
		PI	.884
	PI	Control	.034
		Late gestation restriction	.884

Test	(I) Diet_overall	(J) Diet_overall	Sig
Bonferroni	Control	Late gestation restriction	.104
		PI	.101
	Late gestation restriction	Control	.104
		PI	1.000
	PI	Control	.101
		Late gestation restriction	1.000

Fetal Sheep Insulin Receptor mRNA expression Study 2

Source	Sig.
Insreclobe	.105
Insreclobe * Diet_overall	.336
Insreclobe * Sex	.347
Insreclobe * Diet_overall * Sex	.247

Source	Sig.
Diet_overall	.675
Sex	.501
Diet_overall * Sex	.090

Appendix 3.4 Lobar Blood flow and gene expression

Study 1 Correlations

No significance

Study 2 Correlations

No significance

Appendix 4: 4.5 year old sheep study

Appendix 4.1 Maternal diet

P316 – RVC EXP Sheep Nuts (G J W TITMUS New Mill, Hamer Lane, Wheathampsted, Hertfordshire): a complementary feeding stuff for feeding to sheep should be fed diluted.

Protein	14.75%
Fibre	16.80%
MER	10.725 MJ/kg dry matter
Vitamin D3	2000 iu/kg
Vitamin E	10mg/kg (alpha tocoperal)
Oil (A)	2.50%
Ash	8.20%
Vitamin A	10 000 iu/kg

Raw Materials:

Treated straw, Grass, Hipro soya E, Micronized wheat, Wheat, Barley, Full fat soya, Molasses, Limestone, Calcium carbonate, Dicalcium phosphate salt, Sheep vitamin/mineral supplement.

89.205 % dry matter

Appendix 4.2 Ewbol prestige lamb pellets plus decox

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feeding stuff for feeding, with forage, to growing and fattening lambs up to 70% of dry matter.

Oil	4.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	12.00 %	Vitamin E-alpha tocopherol	30 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.30 mg/kg
Moisture	13.80 %	MER	12.22 MJ/kgDM

Contains the following ingredients in descending order by weight:

Barley, Sunflower ext, Copr exp, Soyabean hulls, Molasses, Malt culms, Peas, Palm kernel exp, Brazilian toasted soya, Bakery by-product, Calcium carbonate, Low glucosinolate rape seed ext, Salt, Vegetable oils.

This medicated feedstuff:

Contains DECCOX (V.m 13997/4009) at a concentration of 100mg/kg DECOQUINATE BP as directed by the veterinary surgeon.86 % dry matter

Appendix 4.3 Ewbol 18 nuts

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feeding stuff for feeding, with forage, to ewes up to 70% of dietary dry matter intake.

In the preparation of this diet precautions have been taken to minimise the potential risk for those sheep prone to copper toxicity. However no guarantee can be given due to the known differences in both breed and management systems. This feedstuff is not recommended for feeding to commercially milked ewes.

Oil	5.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	9.90 %	Vitamin E-alpha tocopherol	150 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.60 mg/kg
Moisture	13.80 %	MER	10.38 MJ/kg
Magnesium	0.53 %		as fed

This product contains 7.1g of cal mag in 1.42 kgs of feed. Animals should not be fed more than 15g cal mag per day.

Contains the following ingredients in descending order by weight:

Sunflower ext, Malt culms, Wheatfeed, Wheat, Palm kernel exp, Low glucosinolate rape seed ext, Molasses, Cocoa hulls, Maize gluten feed, Beans, Cat feed, Calcium carbonate, Vegetable oils, Salt, Magnesium oxide.

This product contains Addarome to optimise dry matter intake.

This product contains Selenomathionine for enhanced health.

86.5 % dry matter

Appendix 4.4: 4.5 year old adult sheep post-mortem sheet

DATE:		**Investigators**
Animal ID:		**Start time:**
Group : High/Low **Sex: Male**		**Completion time:**
Age:		

Measurements			
Body Weight	kg	Biparietal Diameter	cm
Crown-Rump Length	cm	Femur length	cm
Abdominal Circumference	cm	Shoulder height	cm

Tissue Collection

			Weight	***Tray***	***Circumference***	***Fast Freeze***	***Slow Freeze***	***other***
ORGANS								
1	**Pancreas**	*1*						**10ml HCl on ice**
2		*2*				*		
3	**Peri-renal fat**					*		
4	**Back fat**					*		
5	**Abdominal fat**					*		
6	**Adrenal**	*Right*					*	
7	**Kidney**	*Right*						
8		*Rt cortex*				*		
9		*Rt medulla*				*		
10	**Adrenal**	*Left*				*		
11	**Kidney**	*Left*						
12	**Liver**	*whole*						
13		*Right lobe*				*		
14		*Left lobe*				*		
15		*quadrate lobe*				*		
16		*caudate lobe*				*		
17	**Lung**	*Whole*						
18		*Right*				*		
19		*Left*				*		
20	**Heart**	*whole*						
21		*LV*				*		
22		*RV*				*		
23	**Skeletal Muscle**	*Soleus*				*	*	
24	**(back leg)**	*Gastrocnemius medial*				*	*	
25		*Vastus lateralis*				*	*	

Pancreas - collect ~ 1 g into 10ml 180 mmol HCl, on ice, mince tissue with scissors then sonicate for 30 sec - leave at 4 °C O/N

Liver - remove gall bladder, place shiny side down, gall bladder side up: left lobe is on your left, right lobe on your right

Appendix 4.5: 4.5 year old adult sheep results

Adult Sheep (4.5 yr) GR mRNA expression

Source	Sig.
GRlobe	.680
GRlobe * Group	.351

Source	Sig.
Group	.666

Adult Sheep (4.5 yr) PEPCK mRNA expression

Source	Sig.
PEPCKlobe	.935
PEPCK lobe* Group	.504

Source	Sig.
Group	.546

Adult Sheep (4.5 yr) G6Pase mRNA expression

Source	Sig.
G6Paselobe	.549
G6Paselobe * Group	.035

Source	Sig.
Group	.761

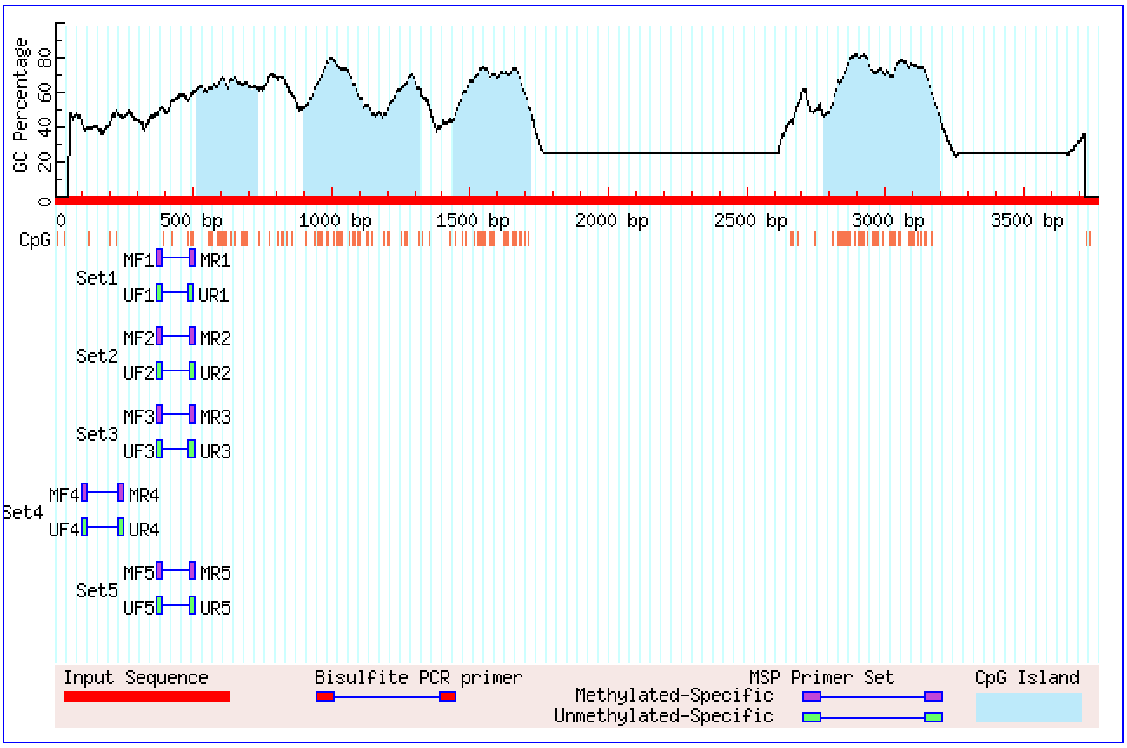
Adult Sheep (4.5 yr) Insulin Receptor mRNA expression

Source	Sig.
InsReclobe	.349
InsReclobe * Group	.575

Source	Sig.
Group	.048

Appendix 5: Methylation study 2.5 year sheep hepatic GR

Appendix 5.1 GR CpG Islands



Appendix 5.2: Sheep Hepatic GR sequence

OA_GR_upstream OAR5:56261196-56275165 No definition line found reverse compliment

60 bases per line:

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNTATGGGCAAAAATCTTAATAAAATTTCTAGATGAGTAAGAAA
ACTTCAGAGTAGGACACGACTGAAGTGACTTAGCAGTAGCAGTAGGACGTGTTACT
GGTATTTAGGGTTTTCCCTCCTCTCTCCCACTGCAAGGCAGTCAAGCCTAACTTTCT
GCATGTGCTTCAAAGATATGAGTTTGAGCCGACCATTTACTTCTCTCCTTCCACACG
CTGCTTCCTCCATCTGCTCTCAGGTGCAACTCATAGGAGCACTCACTTCCTCCAAC
CACTGAGCTTTCTCAGTCCTTGCCAGACAACTTTCCTTTAGCACGGGTAGTTCCTG
CAAGTCTATCTCTTCCCTGGAGCTGCCAAATGACTGGCTTGCCTTCCTTTGCCTCCA
AAATGCTTCCCCTGTCCAACCATAGCTTCCCACCTCCACTGGCGCCACCCCAGAGC
AAATCTGTGTGTTTCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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TTTTTGCCAAACTCTTGTACCCACAGTCCGCCTACAGCTACAATTCAATTATGTTTG

GCAAAAGCAGATCATATTTGGTTAATATTCAGTTCAGCAAAGAAGTCACTCATGCC
ACTGAAGTTCAAACATGAATGTTGATGACTTTTTTTTTNTTTTACATTAATGAGACA
AGATAGAAAAGTAAAACAAACAAAGATGTATTGTTTGGANNNNNNNNNNNNNNNN
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GGTCCCCAAGCAGGTGCAATTGGCTCCAGCACTCATGAATTGCCACCCCTGTGCTG
GAATTGTCAGGCTACCCATTGCACTATCTGGGGAAGGAGTGAAAAGAGGCAATAAT
GATTGTCAATAAAACTAATAATTACTTTTGTTGTGGTGAACTAACAATGAAAAATTA
TACACACCCCTCCTTGTTTCCCAAGCTTAGTGTTTAAAAACTCCTTTTGTACACACC
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GTTACAATTATTCAGTTTGCTCTAATGTCTGCAAACACTAAAAAAACATTAGNNNN
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TTAGGTAGCTTATGATGAAACCATTGAAAGCATCATGAGAATCAGCAAAACACCAG
GGATGTCCCTAACAATTTAGCATTTCTCAGTTCATGTGGGAATGTTAATTCCAATTA
CAAAAAGAAGTACCAAAATGGAATTCATTATGATTAAGAACTTGCTGTTCTAAATGT
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNTGGTAAACAACAGTAAAGGTGGAAATGA
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CAAAAGCTTTAGACCCTCATCAAAGGATTAACAACTAAATGTTCAGTCATAAATTCT
AGGTTTTAAAGCTTCAAATTTACAACTATAATTTTNNNNNNNNNNNNNNNNNNNNNN
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GGGCTTAATTGATGATTTGGAGGAGCATTCTGGCAGTGAGGCCTGGATTTTTTCTT
TTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNTTTTTAAAAAATTTTAAATATTTATTTATCTGGCTGTGCTGGGTCTTAGTTG
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TAATGTGCTACCAAGGTAAAGTACAGAACACTATAGGATCAAACAGTAATGGGTCT
TTTTTTGTTGTTTAATTTTTTTAGGAAGGAGTACTCAGGTGTTTAAACATAAGTCAA
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CG	CpG locations
CCGC	AciI cut site
CCGG	HpaII cut site
CAAC	Primer locations

Appendix 5.3: GR Methylation Results

Adult Sheep Methylation

Source	Sig.
Lobe	.941
Lobe * Sex	.288
Lobe * Diet	.856
Lobe * Offspring	.221
Lobe * Sex * Diet	.592
Lobe * Sex * Offspring	.399
Lobe * Diet * Offspring	.075
Lobe * Sex * Diet * Offspring	.905

Source	Sig.
Sex	.339
Diet	.267
Offspring	.086
Sex * Diet	.279
Sex * Offspring	.807
Diet * Offspring	.175
Sex * Diet * Offspring	.099

Appendix 5.4: MsPCR GR correlated to GR mRNA expression

No significance

Lo

Appendix 6: Published work

Original Article

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Lower Maternal Body Condition During Pregnancy Affects Skeletal Muscle Structure and Glut-4 Protein Levels But Not Glucose Tolerance in Mature Adult Sheep

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Abstract

Suboptimal maternal nutrition and body composition are implicated in metabolic disease risk in adult offspring. We hypothesized that modest disruption of glucose homeostasis previously observed in young adult sheep offspring from ewes of a lower body condition score (BCS) would deteriorate with age, due to changes in skeletal muscle structure and insulin signaling mechanisms. Ewes were fed to achieve a lower (LBCS, n = 10) or higher (HBCS, n = 14) BCS before and during pregnancy. Baseline plasma glucose, glucose tolerance and basal glucose uptake into isolated muscle strips were similar in male offspring at 210 ± 4 weeks. Vastus total myofiber density (HBCS, 343 ± 15; LBCS, 294 ± 14 fibers/mm^2, $P < .05$) and fast myofiber density (HBCS, 226 ± 10; LBCS 194 ± 10 fibers/mm^2, $P < .05$), capillary to myofiber ratio (HBCS, 1.5 ± 0.1; LBCS 1.2 ± 0.1 capillary:myofiber, $P < .05$) were lower in LBCS offspring. Vastus protein levels of Akt1 were lower (83% ± 7% of HBCS, $P < .05$), and total glucose transporter 4 was increased (157% ± 6% of HBCS, $P < .001$) in LBCS offspring, Despite the reduction in total myofiber density in LBCS offspring, glucose tolerance was normal in mature adult life. However, such adaptations may lead to complications in metabolic control in an overabundant postnatal nutrient environment.

Keywords
glucose uptake, myofibers, type 2 diabetes, maternal body condition, skeletal muscle

Introduction

Recent UK guidelines highlight the importance of maternal weight in influencing the health of the offspring, including the risk of adult obesity and metabolic dysfunction (eg, glucose intolerance and insulin resistance).[1] Indeed the offspring of low-body-weight or low-body-mass index mothers have been shown to be at increased risk of insulin resistance in studies worldwide.[2-4] It is clear that the trajectory of risk of such noncommunicable diseases commences early in life and then increases with age.[5-7] We previously reported increased fasting glycemia, mild glucose intolerance, and impaired initial insulin secretion in young male adult offspring of lower body condition score (LBCS) ewes, independent of any change in birth weight.[8] The aim of this study was to determine in the same animals whether this effect worsened with age and whether it was mediated by changes in skeletal muscle structure and insulin signaling mechanisms.

In this study, we focused on skeletal muscle as this is the primary tissue for glucose disposal postprandially, and changes in skeletal myofiber composition and capillary density are linked with type 2 diabetes[9] and obesity.[10] We have previously shown in sheep that either early or late gestation undernutrition reduced total myofiber and capillary density in the late gestation fetus (no effect on body weight) and that the effect of the

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late gestation maternal undernutrition was predominantly on the density of the slow-twitch myofibers.[11] In sheep, a 50% global nutrient restriction in the periconceptional period (−18-6 days gestation [dGA]) similarly reduced myofiber number in the mid-gestation fetus (no effect on fetal weight)[12] and a 50% maternal undernutrition from 28 to 70 dGA decreased total myofiber number and increased fast myosin type IIb isoform levels in 8-month-old offspring (live weight was increased).[13] In the present study, we investigated the effect of a change in maternal body condition before and throughout gestation to reflect the situation in humans that women who are lean during pregnancy are generally also lean before entering pregnancy. Previously in rodents a 40% global restriction throughout gestation reduced birth weight and reduced myofiber number in the neonatal guinea pig.[14] Such changes in muscle mass and composition may contribute to later insulin resistance and glucose intolerance, but to our knowledge this has not yet been determined together in 1 animal model.

Insulin resistance in muscle is one of the earliest identifiable abnormality in prediabetic patients.[15] Low birth weight has been associated with reduced expression of protein kinase C zeta (PKCζ), the p85α and p110β subunits of phosphoinositol-3-kinase, and glucose transporter 4 (GLUT-4) in skeletal muscle of young Danish men,[16] which could be a precursor to longer term altered glucose handling. Recently we found that GLUT-4 and insulin receptor messenger RNA (mRNA) levels were increased in fetal sheep skeletal muscle following late gestation maternal undernutrition and that these effects were accompanied by a decrease in slow-twitch myofiber density.[11] The GLUT-4 protein levels were reduced in young adult sheep perirenal fat following late gestation maternal undernutrition, and this was associated with impaired glucose tolerance (no effect on birth weight or weight at 1 year).[17] The content of GLUT-4 was lower in skeletal muscle of 8-month-old lambs following a 50% nutrient restriction from 28 to 70 dGA.[13] A low-protein diet during pregnancy and lactation led to insulin resistance in skeletal muscle in 15-month-old rat offspring (no effect on weight) and was associated with reduced expression of PKCζ in soleus muscle.[18]

In a range of species including humans, offspring phenotype can be influenced by the developmental environment even within the normal range.[19] We hypothesized that the effect of changes within the normal range maternal body condition on glucose tolerance of their offspring would worsen in mature adulthood. Therefore, in sheep we determined the effect of a higher or lower maternal body condition on mature adult offspring response to a glucose load in vivo. To investigate mechanisms contributing to glucose tolerance, we measured the uptake of glucose into isolated skeletal muscle strips, skeletal muscle morphology, and the expression of insulin signaling proteins in skeletal muscle and fat.

Materials and Methods

All procedures were carried out with local ethics approval and in accordance with the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986.

Animals and Study Design

The BCS is a system used to estimate the amount of muscle and fat coverage in the third lumbar region and is measured on a scale of 1 to 5 (*emaciated* to *obese*).[20] The study design and animal husbandry are described in full by Cripps et al[8]. Briefly, second-parity welsh Mountain ewes were housed individually on straw and established at a BCS of 2 (LBCS) or greater than or equal to 3 (higher BCS [HBCS]) by adjusting the daily ration of a complete pelleted diet (Charnwood Milling Co Ltd, Suffolk, UK). Ewes were mated and maintained at the desired BCS by adjusting daily rations and gestational increases in ration were applied according to standard guidelines.[21] To achieve the LBCS, animals were fed a daily energy intake of ∼57% the HBCS group. The ewes were allowed to deliver spontaneously, and female offspring were removed from the study. From birth, ewes and intact male offspring were housed in 2 groups according to the maternal BCS and the ewes were fed 1.25 times (birth to 2 weeks) and 1.5 times (3-12 weeks) their term diet ration in order to maintain their BCS during the lactation period[8] (at 10 weeks: LBCS ewes, 42.3 ± 1.3 kg/1.7 ± 0.0 BCS vs HBCS ewes, 55.2 ± 1.8 kg/3.1 ± 0.1 BCS. $P < .001$). At 12 weeks the lambs were weaned, housed as 1 group and allowed creep pelleted diet (as fed providing 10.59 MJ/kg and 18 g crude protein per 100 g. Prestige lamb pellets (Detox, BOCM Pauls Ltd, UK), hay, and water were provided ad libitum until 24 weeks. Twenty-four-week-old male offspring were fed a ration of creep pelleted diet (0.75 kg/sheep/day) with hay and water ad libitum. From 32 weeks until 1.5 years of age, male offspring were housed as 1 group and fed standard pelleted diet (0.5-1.0 kg/sheep per d. As fed providing 10.38 MJ/kg and 18 g crude protein per 100 g. Ewbol 18, BOCM Pauls Ltd., UK) with hay and water ad libitum.

An initial study was conducted at 1.5 years[8] (17 LBCS and 19 HBCS intact rams). Between 1.5-and 4-year-old (life expectancy of sheep is approximately 10-12 years), animals were kept on grass (as 1 group) from May to October or indoors (November to April) with access to standard pelleted diet (0.9-1.2 kg/sheep per d, ewbol 18 following assessment of flock weight and BCS) with hay and water ad libitum. There were no differences between the groups at 2 years of age in body weight (HBCS, 82.9 ± 1.4; LBCS, 84.1 ± 2.0 kg $P = .6$) and BCS (HBCS, 2.5 ± 0.1; LBCS, 2.5 ± 0.1 $P = 1.0$), or at 3 years of age in body weight (HBCS, 85.7 ± 1.6; LBCS, 87.6 ± 1.1 kg $P = .4$) and BCS (HBCS, 2.8 ± 0.1; LBCS, 3.0 ± 0.1 $P = .3$). After 1.5 years, 5 rams died due to either infection or fighting accidents (rams were intact). At 210 ± 4 weeks in 10 LBCS and 14 HBCS intact male singleton offspring, we measured body weight, BCS by palpitation, and fat and muscle depth by ultrasound scanning.[22]

Glucose Tolerance Test

At 210 ± 4 weeks, the mature intact adult male offspring were acclimatized to metabolic carts for 5 days with daily pelleted ration and ad libitum access to hay. Pelleted food was given

27 hours before, and hay was removed 19 hours before, the administration of the glucose bolus. Sheep were given ad libitum access to water throughout the protocol. A temporary intravenous (iv) catheter (Radiopaque FEP 14G × 140 mm; Abbott Laboratories Ltd, Maidenhead, UK) was placed in the jugular vein via a small incision in the skin under local anesthesia (2 mL Lignol, Arnolds Veterinary Products Ltd, Shrewsbury, UK). A 2-hour recovery period was allowed prior to the iv glucose tolerance test (GTT). The blood samples (7 mL) were collected before (−30, −15, 0 minutes) and after (5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 210 minutes) an iv glucose bolus (0.5 g/kg body weight over 2 minutes) onto chilled EDTA/fluoride tubes (Teklab Ltd, Durham, UK). These were centrifuged, and the plasma were frozen in aliquots and stored at −80°C. Once the procedure was completed, animals were given a standard pelleted ration and hay was returned.

Postmortem

The last pelleted feed was given 27 hours before, and hay was removed 19 hours before, the postmortem. Water was provided ad libitum. Animals were killed with an overdose of sodium pentobarbitone (iv 145 mg/kg). Body weight, crown-rump length, abdominal circumference, biparietal diameter, femur length, and shoulder height were recorded. Under sterile conditions major organs were weighed. A 1-g sample of pancreas was placed into 10 mL of 180 mmol HCl on ice and the tissue minced with scissors before being placed in a sonication bath for 30 seconds. The sample was then left overnight at 4°C, centrifuged at 1500 *g* for 20 minutes and the supernatant collected and frozen at −80°C.

Gastrocnemius and soleus muscle weights and mid-belly circumferences were recorded. Mid-belly muscle samples of the vastus lateralis and soleus muscles were removed immediately after death and were frozen by immersion into freezing isopentane (fibers in a vertical orientation, for histology) and liquid nitrogen (for molecular biology). All samples were stored at −80°C.

Glucose Uptake Studies

The uptake of glucose into isolated muscle strips was measured using techniques adapted from reference [18]. Briefly, small strips (~50 mg) were taken from the vastus, soleus, and gastrocnemius muscles and placed in chilled Tyrode solution (137 mmol/L NaCl, 5 mmol/L glucose, 5 mmol/L KCl, 12 mmol/L $NaHCO_3$, 1 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mmol/L pyruvate, 38 mmol/L mannitol, and 0.1% bovine serum albumin [BSA], pH 7.4) until commencement of the uptake experiment. All incubations were then carried out at 38°C in a shaking water bath. The strips were placed in 5 mL of warmed Tyrode solution for 10 minutes and then incubated for a further 20 minutes in an identical medium in the presence or absence of insulin (16 nmol/L, Novo Nordisk, UK). The strips were blotted on filter paper and incubated for 10 minutes in 3 mL Tyrodes solution supplemented with 8 mmol/L [^{3}H]methyl glucose (437 μCi/mmol) and 32 mmol/L [^{14}C]mannitol (8 μCi/mmol; GE Healthcare, UK). After incubation, the muscle strips were blotted on filter paper to remove any residual radioactive buffer and then frozen in liquid nitrogen and stored at −80°C. Frozen muscle strips were homogenized in 400 μL of water and centrifuged at 10 000 *g* for 5 minutes. Supernatant ^{3}H and ^{14}C activity was determined simultaneously by liquid scintillation counting (Tri-carb 2100TR, PerkinElmer, UK). Briefly, 300 μL was put into a scintillation vial along with 6 mL of Optiphase "Hisafe" II scintillation fluid (PerkinElmer, Massachusetts) and each vial run on the counter for 5 minutes. Protein levels were measured using Coomassie blue protein assay and glucose uptake corrected for the amount of protein. Extracellular space was corrected by subtracting mannitol levels.

Immunohistochemistry

All chemicals were from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Primary antibodies were mouse antiskeletal fast myosin antibody, clone MY32 (to positively indentify fast myofibers, cat no. M4276) and polyclonal rabbit antihuman von Willebrand factor (to positively identify capillary endothelial cells. DakoCytomation, cat no. A0082). In brief, 10 μm transverse cryosections from the midbelly of the skeletal muscles were fixed in water-free acetone at room temperature for 15 minutes, and endogenous peroxidase activity was inhibited by incubation in 0.5% hydrogen peroxide in methanol for 30 minutes. Nonspecific protein interactions were blocked with Dulbecco Modified Eagle Medium (DMEM) containing 20% fetal calf serum and 1% BSA for 30 minutes and then incubated with either antiskeletal fast myosin antibody (1:100) or antihuman von Willebrand factor antibody (1:300) at 4°C overnight. All antibodies were diluted in Tris-buffered saline (TBS). After rinsing with TBS, the sections were incubated for 30 minutes with biotinylated antimouse (1:400) or antirabbit (1:400) antibody. Sections were washed and treated for 15 minutes with streptavidin biotin–peroxidase complex (1 + 1:200) and then for 10 minutes in amino ethyl carbazole. Finally, sections were counterstained with Mayers hematoxylin and baked with crystal mount (AbD Serotec, Kidlington, UK) before being mounted in Pertex (Surgipath, Peterborough, UK). A negative control section was processed simultaneously (methodology as above, replacing the primary antibody with TBS).

Capillaries were hard to identify in the frozen sections and, therefore, these were reembedded in paraffin to allow thinner sections to be cut. In brief, muscle cryosections (4 μm) were reembedded in paraffin and then deparaffinized in clearene (2 × 5 minutes) and rehydrated through graded alcohols (5 minutes in each) to 70%. Endogenous peroxidase activity was inhibited by incubation with 0.5% hydrogen peroxide in methanol for 10 minutes. Slides were covered with working pronase solution (DakoCytomation, Denmark) and incubated at room temperature for 10 minutes. Sections were then washed in TBS (3 washes × 2 minutes), immersed in avidin solution for 20 minutes, rinsed in TBS, immersed in biotin solution for 20 minutes, and rinsed in

TBS. Sections were incubated in DMEM containing 20% calf serum and 1% BSA culture medium for 20 minutes to block nonspecific protein interactions. Following this, anti-human von Willebrand factor antibody (1:300) was applied and the sections were incubated at 4°C overnight. After rinsing with TBS, sections were incubated with biotinylated anti-rabbit (1:400) antibody for 30 minutes. Sections were washed and treated with streptavidin biotin–peroxidase complex (1 + 1:200) for 30 minutes and then with diaminobenzidine for 5 minutes. Sections were rinsed in TBS and washed in running tap water (5 minutes). Finally, sections were counterstained with Mayers hematoxylin and dehydrated through graded alcohols, cleared in clearene and mounted in Pertex (Surgipath, Peterborough, UK).

Image Analysis

The myofiber density and size in the sections was assessed using a photomicroscope (Zeiss, Axioskop II) and the KS-400 image analyzing system (Image Associates, Bicester, UK). Five microscopic images (validated as good representation of overall myofiber density, with <2% error), with ×40 objective, were selected randomly from each section and imported into the KS-400 image analyzing program. In each of these fields of view the total fascicular area was calculated, and using a nonbiased counting frame, all myofibers, fast-twitch myofibers (identified as the red positively stained fibers) and slow myofibers (negatively stained white fibers, Figure 1A) were counted, and their density expressed as the number of fibers per mm^2 fascicle. The average cross-sectional area (CSA) of the myofibers was defined by manually drawing around 41 individual fast-twitch and 24 slow-twitch fibers, with the cursor. Obvious deviations from a true cross section were excluded from the analysis.

Capillaries were counted within the fascicular area of 6 random fields (validated as good representation of overall capillary density, with <2.4% error) with ×40 objective (Figure 1B) and capillary density was expressed as capillary number per mm^2 fascicle. The numbers of myofibers from the same fields were counted and capillary–fiber ratio was calculated.

All measurements were made by one observer and the intraobserver variability tested by reproducing the counts from the same section, at different times. The intraobserver variability was less than 5.6% for all variables.

Western Blotting

These were performed as previously stated.[8] In brief, total protein was extracted from vastus muscle and abdominal fat. The cleared protein lysates from each animal were standardized to a final concentration of 1 mg/mL in Laemmli sample buffer and equal amounts of protein for each animal (20 μg) were loaded onto 10% sodium dodecyl sulfate polyacramide gels for separation by electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride membrane in singleton and Western blotting was carried out. The primary antibodies used in this study were to insulin receptor β subunit (InsR, 1:200), PI3-kinase p85α regulatory subunit (p85, 1:1000, Upstate Biotech, New York), PKCζ (1:200), insulin-like growth factor-I receptor β-subunit (IGF-IRβ, 1:200), GLUT-4 (1:5000, Abcam, Cambridge, UK) and Akt1 (1:2000). All antibodies were rabbit polyclonal, except Akt1 (monoclonal mouse) and were obtained from Santa Cruz Biotechnologies (Santa Cruz, California) unless stated otherwise. Autoradiographs of Western blots were imaged and the optical densities of the immunoreactive protein bands were measured. Linearity of signal was confirmed by the inclusion of 20 and 10 μg of a pooled sample on each gel. Positive controls (where available) were also included on each gel.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was extracted from liver using TRI Reagent (Sigma-Aldrich (Dorset, UK). Quality and quantity of the RNA was assessed by spectrophotometry (A260 nm/A280 nm), and the integrity of the RNA was checked by formaldehyde gel electrophoresis, staining with ethidium bromide and visualization of intact 18S and 28S ribosomal RNA bands under ultraviolet light. Total RNA from each sample was reverse transcribed using standard protocols with random primers, RNase inhibitors and reverse transcriptase (Promega, UK). The 3 most stable housekeeping genes were determined using an ovine gene normalizing (geNorm$_{TM}$) kit developed from liver samples from our laboratory (Primer Design Ltd, UK) to be β-actin, RPL-19, and glyceraldehyde-3-phosphate dehydrogenase. Housekeeping gene expression was measured using SYBR Green, and primers/probes were supplied in kit form (Primer Design, UK www.primerdesign.co.uk). Then real-time polymerase chain reaction (ABI Prism 7700 Sequence Detection System, Applied Biosystems, Paisley, UK) was used to evaluate mRNA levels of InsR, glucocorticoid receptor (GR), phosphoenolpyruvate carboxykinase (PEPCK), and glucose 6-phosphatase (G6Pase) using primers and probes (Eurogentec, UK) designed (Primer Express Software, Applied Biosystems, Paisley, UK) with reference to published sequences (Table 1). The InsR, GR, PEPCK, and G6Pase were expressed relative to the geometric mean of the 3 housekeeping genes.

Biochemical Analysis

Plasma was analyzed by autoanalyzer using commercial kits as follows: glucose (hexokinase-6-phosphate dehydrogenase method [Dade-Behring]), high-density lipoprotein (Dade-Behring), low-density lipoprotein (calculated using the Friedwald formula), total cholesterol (Dade-Behring), total serum protein (Biuret reaction [Dade-Behring]), urea (urease enzyme assay [Dade-Behring]), and nonesterified fatty acid (NEFA; Alpha Laboratories Ltd, Eastleigh, UK). These analyses were measured as part of routine assays carried out at the National Health Services, Clinical Biochemistry Department, Addenbrookes Hospital, Cambridge (Dade-Behring Dimension RXL analyzer) and at the Institute of Human Nutrition, University of Southampton, Southampton (Konelab 20 autoanalyzer).

Plasma triacylglyceride (TAG) was measured as described previously in full.[23] In brief, total lipids were extracted and

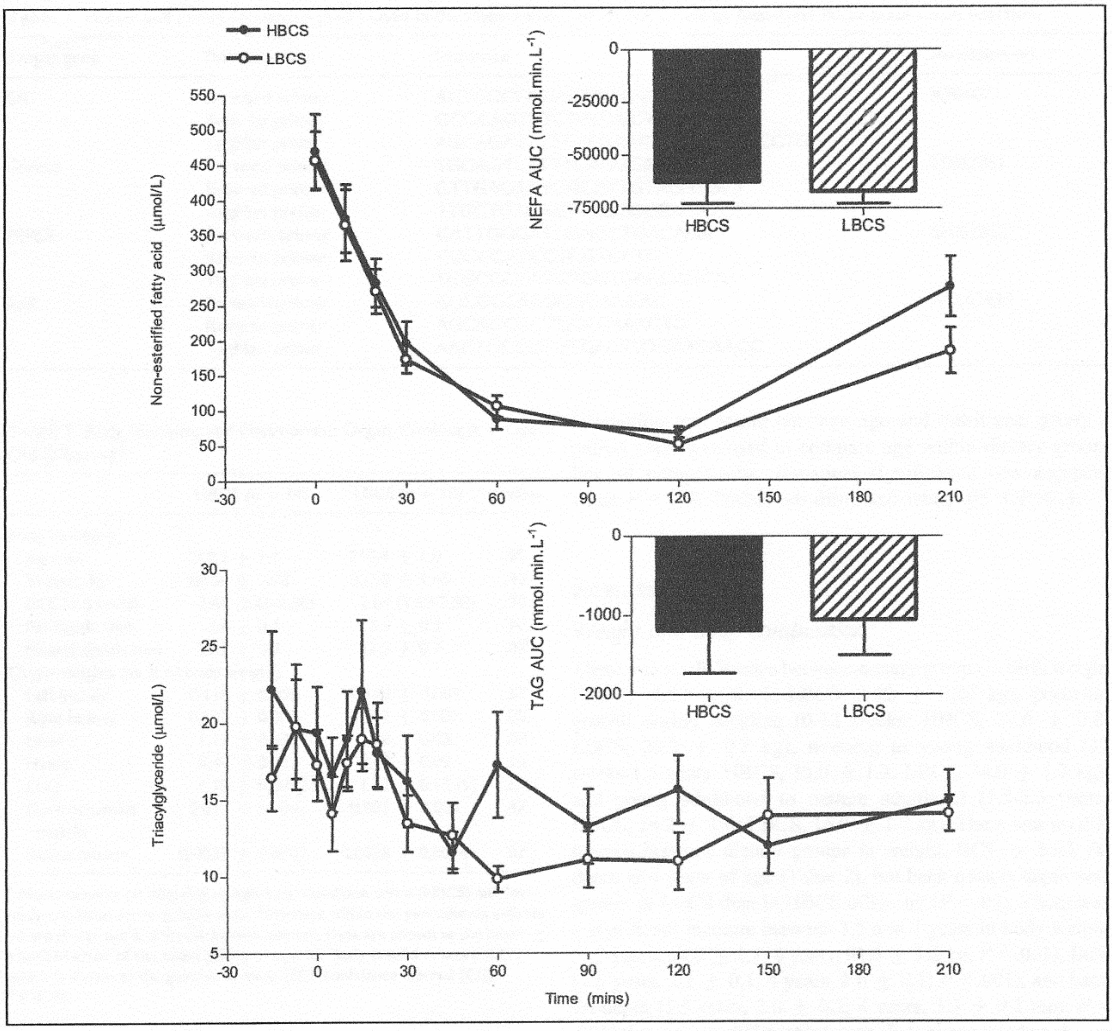

Figure 1. Plasma nonesterified fatty acid and triacylglyceride during the intravenous glucose tolerance test of offspring of higher body condition score (HBCS, filled bars/symbols) and lower body condition score (LBCS, hatched bars/open symbols) ewes. Data are shown as the mean ± standard error of the mean (SEM). Area under the curve (AUC) was calculated from baseline plasma levels prior to the glucose bolus.

separated by solid phase extraction. The TAG was converted into fatty acid methyl esters and were separated and analyzed by gas chromatography (HP6890 Hewlett Packard GC system with a BPX-70 column, Agilent Technologies, Cheshire, UK).

Insulin concentrations in plasma (25 µL, collected on EDTA tubes) and extracted pancreatic tissue were measured in duplicate by enzyme-linked immunosorbent assay (ELISA; DRG Sheep Insulin ELISA; ImmunoDiagnostic Systems, Tyne and Wear, UK). The range of the assay was 0.1 to 2.5 µg/L. The interassay and intraassay coefficients of variance were 7.0% and 4.4%, respectively.

Plasma cortisol (µg/dL) was measured in duplicate using an Immulite analyzer (DPC, UK) in 10 µL of plasma (collected by EDTA tubes) by a solid-phase, competitive chemiluminescent enzyme immunoassay, with an incubation cycle of 30 minutes. The range of the assay was 0.42 to 1.70 ng/mL and the intraassay coefficient of variance was 5.7%.

Data Analysis

Nonparametric data were log transformed prior to testing and are expressed as the geometric mean (95% confidence

Table 1. Primer and Probe Sequences (5′-3′) Used in the Measurement of mRNA Levels by Real-Time Polymerase Chain Reaction.

Target gene	Primer/probe	Sequence	Accession no.
GR	Forward primer	ACTGCCCCAAGTGAAAACAGA	X70407
	Reverse primer	GCCCAGTTTCTCCTGCTTAATTAC	
	TaqMan probe	AGAAGATTTTATCGAACTCTGCACCCCTGG	
G6Pase	Forward primer	TGGAGTCTTTTCAGGCATTGC	EF062861
	Reverse primer	CTTGAGACTGGCATTGTAGATGCT	
	TaqMan probe	TTGCTGAGACTTTCCGCCACATCCA	
PEPCK	Forward primer	GATTGGCATCGAGCTGACAGA	EF062862
	Reverse primer	CGCCCATCCTCGTCATG	
	TaqMan probe	TCGCCCTACGTGGTGACCAGCA	
InsR	Forward primer	ACCGCCAAGGGCAAGAC	AY162434
	Reverse primer	AGCACCGCTCCACAAACTG	
	TaqMan probe	AACTGCCCTGCCACTGTCATCAACG	

Table 2. Body Biometry and Postmortem Organ Weights in 4-Year-Old Offspring.[a]

	HBCS (n = 14)	LBCS (n = 10)	*P* value
Body biometry			
Age, wk	210.2 ± 1.1	210.4 ± 1.0	.90
Weight, kg	80.54 ± 1.78	82.50 ± 1.45	.43
BCS (1-5 units)	2.61 (2.41-2.88)	2.64 (2.50-2.80)	.96
Fat depth, mm	3.4 ± 0.4	3.3 ± 0.3	.90
Muscle depth, mm	29.2 ± 0.9	32.3 ± 0.7	.02[b]
Organ weights (as % of body weight)			
Left kidney	0.116 ± 0.003	0.110 ± 0.003	.57
Right kidney	0.113 ± 0.003	0.112 ± 0.002	.85
Liver	1.11 ± 0.03	1.08 ± 0.03	.89
Heart	0.44 ± 0.02	0.49 ± 0.03	.15
Lung	1.10 ± 0.09 (11)	1.17 ± 0.12 (9)	.62
Gastrocnemius muscle	0.077 ± 0.004	0.081 ± 0.003	.47
Soleus muscle	0.0037 ± 0.0002	0.0038 ± 0.0004	.87

[a] Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes. Numbers within the parentheses indicate where group size is different from maximum. Data are shown as the mean ± standard error of the mean (SEM), except for body condition score (BCS) which is shown as the geometric mean (95% confidence interval [CI]).

[b] $P < .05$.

intervals). Parametric data are expressed as the mean ± standard error of the mean. Area-under-the-curve (AUC) measurements for glucose, insulin, TAGs, and NEFA were calculated (GraphPad Prism, version 3, GraphPad Software Inc., San Diego) between 5 and 210 minutes (ie, following glucose administration) from baseline (preglucose bolus; ie, "incremental" AUC, Figure 1 and Table 3). For the purpose of comparison between these data and our previous study of these animals at 1.5 years,[8] we also report the AUC measurements from 0 mmol/L glucose to represent the total metabolic state. Dietary groups were compared with an unpaired Student *t* test (SPSS version 12, SPSS Inc, Chicago). To test the effect of age (ie, data at year 1.5 vs year 4)[8] and its interaction with dietary group, a repeated measures analysis of variance was used. Where a significant interaction was found between age and nutritional group a paired *t* test was used to compare age within dietary group. For all comparisons, statistical significance was accepted when $P < .05$. Trends are discussed when $.05 < P < .1$.

Results

Weight and Body Composition

There was no difference between dietary groups in birth weight (HBCS, 4.02 ± 0.14; LBCS, 3.92 ± 0.23 kg), postnatal growth during suckling (0-12 weeks: HBCS, 29.6 ± 0.8; LBCS, 29.3 ± 0.7 kg), weaning to young adulthood (12 weeks-1.5 years: HBCS, 35.0 ± 1.3; LBCS, 34.0 ± 1.7 kg), and young adulthood to mature adulthood (1.5-2.5 years: HBCS, 14.2 ± 1.6; LBCS, 16.9 ± 1.7 kg). There was no difference between dietary groups in weight, BCS, or back fat depth at 4 years of age (Table 2), but back muscle depth was greater in LBCS than in HBCS offspring ($P < .05$). There was a significant increase between 1.5 and 4 years in body weight (1.5 years, 68.0 ± 1.1; 4 years, 81.4 ± 1.2 kg; $P < .001$), BCS (1.5 years, 2.1 ± 0.1; 4 years, 2.6 ± 0.1; $P < .001$), and back fat depth (1.5 years, 2.0 ± 0.2; 4 years, 3.3 ± 0.2 mm; $P < .001$) that was not different between dietary groups (data shown are the combined dietary groups). For muscle depth, there was a significant interaction between age and dietary group whereby back muscle depth increased between 1.5 and 4 years in LBCS offspring only (1.5 years, 29.4 ± 0.9; 4 years, 32.3 ± 0.7 mm; $P < .05$).

There were no differences between the dietary groups in the weight of soleus and gastrocnemius muscles (Table 2), in gastrocnemius–femur length ratio (HBCS, 1.88 ± 0.08; LBCS, 2.04 ± 0.07) nor in gastrocnemius (HBCS, 13.13 ± 0.20; LBCS, 12.68 ± 0.24 cm) and soleus (HBCS, 2.73 ± 0.13; LBCS, 2.58 ± 0.18 cm) muscle circumference. There were no differences between the dietary groups in the major organ weights recorded (Table 2) nor in offspring body proportions (including. crown-rump length and abdominal circumference; data are not shown).

Table 3. Plasma Hormones and Nutrients Under Baseline (Fasted) Conditions and During the Glucose Tolerance Test.[a]

	HBCS (n = 14)	LBCS (n = 10)	*P* value
Basal plasma levels			
Glucose, mmol/L	4.06 ± 0.12	4.14 ± 0.14	.66
Insulin, pmol/L	102 (40-144)	91 (48-130)	.53
Insulin: glucose	28.02 ± 3.73	23.72 ± 3.05	.41
Cortisol, ng/mL	0.95 ± 0.11	0.93 ± 0.10	.13
TAG, µmol/L	20.49 ± 2.97 (13)	17.83 ± 2.23	.51
NEFAs, µmol/L	470.17 ± 53.07	457.08 ± 41.22	.86
Total protein, g/L	71.50 ± 0.99	73.60 ± 0.86	.14
Cholesterol, mmol/L	1.39 ± 0.09	1.46 ± 0.07	.53
HDL, mmol/L	0.84 ± 0.05	0.88 ± 0.04	.61
LDL, mmol/L	2.01 ± 0.08 (13)	2.11 ± 0.13	.51
Urea, mmol/L	5.61 ± 0.37	5.64 ± 0.35	.69
Glucose tolerance test			
Peak glucose, mmol/L	23.41 ± 0.43	24.04 ± 0.39	.31
Glucose AUC, mmol.min.L^{-1}	1193.00 ± 50.70	1259.00 ± 64.53	.42
Peak insulin, pmol/L	487.94 ± 49.81	598.72 ± 59.36	.16
Initial insulin AUC 0-5 min, nmol.min.L^{-1}	0.24 ± 0.07	0.24 ± 0.23	.96
Insulin AUC, nmol.min.L^{-1}	33.87 ± 4.50	43.98 ± 3.87	.12
Insulin AUC: glucose AUC	28.38 ± 3.56	35.98 ± 3.79	.16

[a] Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes. Area under the curve (AUC) calculations are from baseline. Data are shown as the mean ± standard error of the mean (SEM) except for basal insulin which is shown as the geometric mean (95% confidence interval [CI]). The number within indicates where n differs from column heading.

Table 4. Minimum Plasma Nonesterified Fatty Acid (NEFA) and Triacylglyceride (TAG) Reached During the Intravenous Glucose Tolerance Test.[a]

	HBCS (n = 13)	LBCS (n = 10)	*P* value
Minimum NEFA, µmol/L	63.36 ± 8.38	51.74 ± 9.21	.37
Delta minimum NEFA, µmol/L (from baseline)	−406.82 ± 52.28	−405.34 ± 36.55	.98
Time to minimum NEFA, min	98.57 ± 7.97	120.00 ± 0.00	.03[b]
Minimum TAG, µmol/L	7.79 ± 0.85	6.59 ± 0.93	.36
Delta minimum TAG, µmol/L (from baseline)	−12.70 ± 2.87	−11.24 ± 2.06	.84
Time to minimum TAG, min	63.85 ± 9.56	80.50 ± 19.09	.41

[a] Measurements on offspring of higher body condition score (HBCS) and lower body condition score (LBCS) ewes. Data are shown as the mean ± standard error of the mean (SEM).

[b] $P < .05$, significant difference between dietary groups.

Basal Hormone and Nutrient Levels

There was no difference between the dietary groups in basal plasma hormone and nutrient levels (Table 3) nor in pancreatic insulin levels at postmortem (HBCS, 11.2 [5.4-12.9]; LBCS, 6.1 [3.3-8.2] nmol/g $P = .15$).

There was a small negative correlation between weight gain during suckling and basal glucose at 4 years (R^2 −.197, $P < .05$). Basal glucose (1.5 years, 3.7 ± 0.1; 4 years, 4.0 ± 0.1 mmol/L; $P < .01$), insulin (1.5 years, 39.3 ± 2.5; 4 years, 106.8 ± 10.2 pmol/L; $P < .001$), and basal insulin–glucose ratio (1.5 years, 10.7 ± 0.7; 4 years, 26.2 ± 2.5. $P < .001$) increased with age, regardless of dietary group (data shown are the combined dietary groups).

Response to a Glucose Load

Intravenous GTT. There was no difference between the dietary groups in peak glucose, peak insulin, or AUC analysis (from baseline; Table 3). For AUC calculations from 0 units (as index of total metabolic state), the lack of difference between the dietary groups persisted in glucose AUC (HBCS, 2030.07 ± 67.22; LBCS, 2123.19 ± 62.41 mmol.min.L^{-1}), initial insulin AUC 0 to 5 minutes (HBCS, 0.51 ± 0.06; LBCS, 0.64 ± 0.19 nmol.min.L^{-1}), insulin AUC (HBCS, 52.68 ± 5.77; LBCS, 66.07 ± 6.42 nmol.min.L^{-1}), and insulin:glucose AUC (HBCS, 25.80 ± 2.63; LBCS, 31.09 ± 2.96).

Weight gain between weaning and young adulthood was positively correlated with peak glucose (R^2 .175, $P < .05$) and insulin (trend only; R^2 .119, $P < .1$). There was a decrease with age in glucose AUC (from baseline; 1.5 years, 1425 ± 48; 4 years, 1226 ± 58 mmol.min.L^{-1}; $P < .01$) and initial insulin AUC 0 to 5 minutes (1.5 years, 0.51 ± 0.24; 4 years, 0.24 ± 0.08 nmol.min.L^{-1}.0-5 min, $P < .01$). There was a trend for an increase with age in insulin: glucose AUC (from baseline; 1.5 years 25.6 ± 1.6, 4 years, 31.5 ± 2.7 insulin:glucose; $P < .1$). These age effects were not affected by dietary group.

There was no difference between dietary groups in the overall TAG or NEFA response to a glucose load (Figure 1, AUC analysis). For NEFA, but not TAG, the minimum level was reached more quickly in HBCS than in LBCS group ($P < .05$; see Table 4).

Isolated muscle strips. As expected, basal ($P < .001$) and insulin-stimulated ($P < .01$) glucose uptake was greater in soleus than in vastus or gastrocnemius muscles. In all muscles, there was no difference in basal glucose uptake between the dietary groups (Table 5). In the soleus muscle, insulin-stimulated glucose uptake tended to be lower in the LBCS as compared to HBCS offspring ($P < .1$, Table 5), but there was no difference in uptake between the dietary groups in the vastus and gastrocnemius muscles.

Table 5. Glucose Uptake into Isolated Muscle Strips.[a]

	HBCS (n = 10)	LBCS (n =7)	P value
Basal glucose uptake, pmol/min/mg			
Soleus muscle	0.55 ± 0.07	0.43 ± 0.04	.20
Vastus muscle	0.24 ± 0.03	0.22 ± 0.03	.69
Gastrocnemius muscle	0.29 ± 0.07	0.25 ± 0.02	.57
Insulin stimulated glucose uptake, pmol/min/mg			
Soleus muscle	1.01 ± 0.06	0.84 ± 0.07	~.08
Vastus muscle	0.61 ± 0.07	0.54 ± 0.08	.46
Gastrocnemius muscle	0.56 ± 0.05	0.44 ± 0.04	.11

[a] Measurements on offspring of high body condition score (HBCS) and low body condition score (LBCS) ewes. Data are shown as the mean ± standard error of the mean (SEM), ~ $P < .1$.

Reevaluation of 1.5-Year Data

Five rams were removed from the cohort in the period of time following the study at 1.5 years.[8] Glucose handling data at 1.5 years were reanalyzed using only the animals that were studied at 4 years and showed that there were no differences between the groups in basal glucose (HBCS, 3.66 ± 0.06; LBCS, 3.80 ± 0.08 mmol/L $P = .18$) or initial insulin AUC 0 to 5 minutes (HBCS, 0.69 ± 0.08; LBCS, 0.60 ± 0.06 nmol.min.L^{-1}; $P = .41$) but that glucose AUC remained significantly increased in the LBCS group (HBCS, 2183 ± 31.0; LBCS, 2283 ± 25.2 mmol.min.L^{-1}; $P < .05$).

Muscle Morphology

In the vastus muscle, there was a reduction in total myofiber density, fast myofiber density ($P < .05$), and a trend for a reduction in slow myofiber density ($P < .1$) in the LBCS compared to HBCS offspring (Figure 2). Fast (HBCS, 2671 ± 110; LBCS, 2617 ± 360 μm^2) and slow (HBCS, 2969 ± 202; LBCS, 3319 ± 164 μm^2) myofiber CSA was similar between dietary groups. In the soleus muscle, slow myofiber density (Figure 2) and CSA (HBCS, 3793 ± 242; LBCS, 3665 ± 280 μm^2) were similar between dietary groups.

In the vastus muscle, capillary density tended to be reduced in the LBCS compared to HBCS offspring (Figure 2, $P < .1$). The capillary to myofiber ratio was significantly reduced in LBCS compared to HBCS offspring (Figure 2, $P < .05$). In the soleus muscle, capillary density and capillary to myofiber ratio were similar between dietary groups (Figure 2).

Gene Expression

Muscle and fat. Protein levels of Akt1 were lower ($P < .05$) and IGF-IR levels tended to be lower ($P < .1$) in the vastus muscle of LBCS offspring (Figure 3). The GLUT-4 protein levels were increased in the vastus muscle of LBCS offspring ($P < .001$). There were no differences in InsR, PI3-kinase p85 subunit, and PKCξ protein in the vastus muscle between dietary groups. Protein levels of Akt1 tended to be reduced in the abdominal fat of LBCS offspring ($P < .1$, Figure 3). There were no differences in GLUT-4, InsR, IGF-IR, PI3-kinase p85 subunit, and PKCξ protein in the abdominal fat between dietary groups.

Liver. The InsR mRNA levels were increased in liver of LBCS offspring (HBCS, 0.51 (0.37-0.65); LBCS, 0.72 (0.55-0.89) arbitrary units normalized to housekeeping gene, $P < .05$). There was no significant difference between dietary groups in liver GR, PEPCK, or G6Pase mRNA levels (data not shown).

Discussion

Epidemiological and animal studies implicate the fetal nutrient environment as a determinant of metabolic disease in later life. Maternal body composition is linked to maternal diet and food intake and is important since the mother provides nutrition for the fetus from both her dietary intake and her own body reserves. The offspring of mothers low body mass index have been shown to be at increased risk of insulin resistance.[2-4] Our previous observations showed that lowering the BCS of the ewe during pregnancy reduced glucose tolerance of young adult offspring.[8] In this study, we had the rare opportunity to study the same animals again in mature adulthood and found that rather than worsening, the LBCS animals displayed no signs of altered glucose metabolism, despite reduced myofiber, fast-fiber density, and capillary to myofiber ratio in the vastus lateralis muscle. Our findings suggest that this may be due in part to adaptations in the muscle insulin-signaling pathway.

An important and novel finding of this study is that the altered glucose handling at 1.5 years in offspring of LBCS mothers[8] did not persist into mature adult life, and indeed no other sheep study of glucose metabolism has followed up animals postnatally for so long following in utero undernutrition. The difference between dietary groups at 1.5 years in the response to a glucose load was still apparent when the data set was restricted only to the animals that were also studied at 4 years. Fatty acid metabolism (indicated by plasma NEFA and TAG levels) was similar between offspring groups, although the slightly longer time to minimum levels of NEFA during the GTT in LBCS animals may be a weak marker of altered insulin sensitivity in other organs such as adipose and liver. There was no difference between dietary group in insulin signaling pathway in adipose tissue, but we observed that hepatic InsR expression at the mRNA level was increased in LBCS animals. This was not associated with any change in fasting blood glucose and there was no effect on mRNA levels of genes involved with gluconeogenesis in the liver (ie, GR, PEPCK, and G6Pase). Increased liver insulin sensitivity could impact on the LCBS offspring response to dietary excess or deficiency through fatty acid synthesis, gluconeogenic, or glycogen storage pathways; however, this speculation was not tested in the current study design. In sheep offspring, exposure to a late gestation 50% undernutrition challenge increased insulin and glucose AUC at 1 year of age[17] and exposure to a 50%

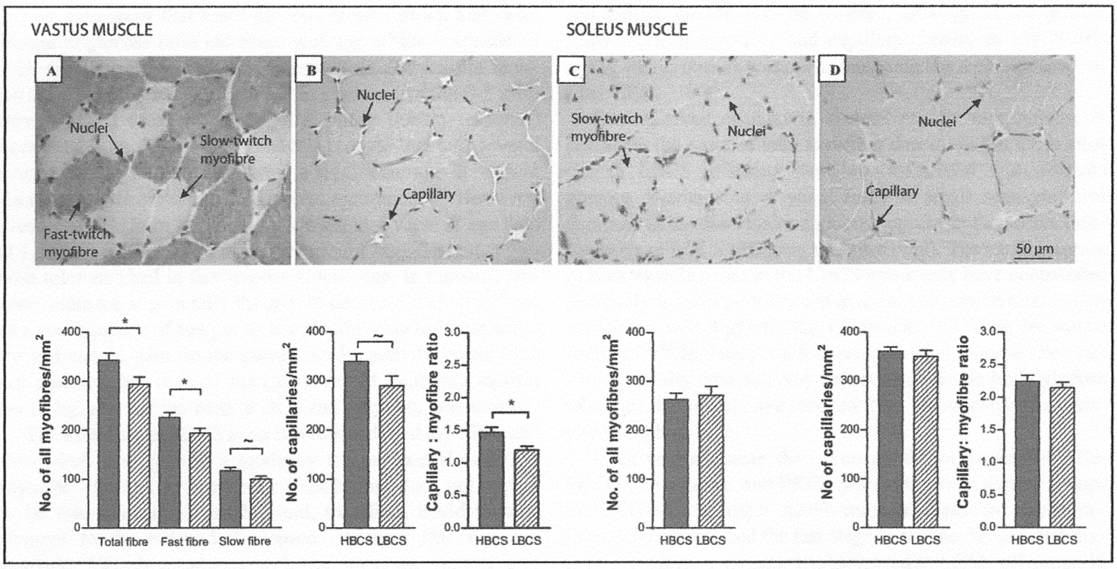

Figure 2. Myofiber and capillary density in the vastus (A and B) and soleus (C and D) muscles of offspring of high body condition score (HBCS, filled bars) and low body condition score (LBCS, hatched bars) ewes. Data are shown as the mean ± standard error of the mean (SEM), $*P < .05$ and $\sim P < .1$.

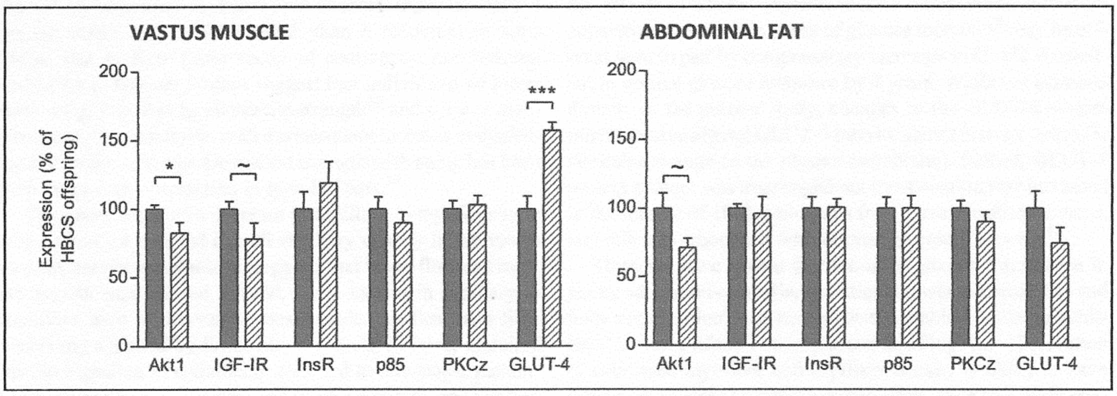

Figure 3. Insulin signaling pathway protein expression in the vastus muscle and abdominal fat of offspring of high body condition score (HBCS, filled bars) and low body condition score (LBCS, hatched bars) ewes. Data are shown as the mean ± standard error of the mean (SEM). $\sim P < .1$, $*P < .05$, and $***P < .001$.

reduction from 28 to 78 dGA increased glucose AUC in 63- and 250-day-old offspring.[24] Glucose tolerance was reduced among men and women exposed to famine during late gestation, and this was more pronounced in those exposed to famine, who then became obese as adults.[25] In rats, glucose tolerance of offspring of mothers fed a low-protein diet was improved at 6 to 9 weeks[26,27] unaltered at 44 weeks[27] and significantly worse by 17 months and exacerbated by an overabundant postnatal diet.[28,29] Sheep are ruminants but have advantages as a model in that singleton offspring can be studied and also they develop key organ systems fully prenatally. In the present study, back fat and muscle and BCS increased modestly with age but at the 4 year study the animals still were of average BCS. It remains possible that if they had become obese differences between the lower and higher condition score groups in glucose handling may have persisted or worsened with age. Thus, overall these findings provide additional insight into our understanding of the influence of nutrition in early life on the risk of disorders of metabolism in adult life such as obesity and type 2 diabetes.[19]

Our data show that basal glucose, basal insulin, and basal insulin to glucose ratio increased with age which is consistent with signs of altered glucose homeostasis and insulin resistance. The initial insulin response to glucose (insulin 0-5 minutes AUC, a measure of the first phase insulin response) decreased from 1.5 to 4 years and this is consistent with several human studies that have reported a significant age-dependent decrease in glucose-stimulated insulin secretion.[30-32] However, glucose AUC (from baseline) was lower at 4 years of age than at 1.5 years (independent of dietary group) suggesting that glucose tolerance had in fact improved with age. In humans, glucose tolerance is generally thought to decrease with age,[33] not as a consequence of age per se but possibly due to lower activity and weight gain. In the current study, activity levels were not assessed but it is of note that weight gain was modest (~10 kg) and the increase in BCS and adiposity was small.

The offspring of LBCS ewes had reduced total myofiber and fast-twitch density, and a tendency for reduced slow-twitch myofiber density, in the vastus muscle that does not appear to be due to changes in CSA and, therefore, could involve changes to the extracellular space, although this was not assessed. This is consistent with our previous findings that early and late gestation undernutrition periods reduced fiber density in the late gestation fetus.[11] A reduction in fast fiber number has been seen in the vastus lateralis muscle of 14-day-old lambs following a 50% nutrient reduction at 30 to 70 dGA.[34] A reduction in fast fibers is more likely to have a greater effect on muscle strength than a reduction in slow fibers, due to their faster speed of contraction and reduced resistance to fatigue. Studies suggest that individuals of lower birth weight have reduced muscle strength[35] and a lower myofiber score[36] in adult life, with a consequent increase in disability and frailty with age. Decreased strength with aging has been associated with a reduction in type II fibers.[37]

Our observation of a decrease in capillary to myofiber ratio and a trend for reduced overall capillary density in the vastus muscle, but not in the soleus, suggests that blood flow and muscle growth were related. Indeed, the reduction in capillary to myofiber ratio in the vastus muscle indicates that each fiber was being supplied by fewer blood vessels. In mice, impaired insulin signaling in endothelial cells led to attenuated insulin-induced capillary recruitment and insulin delivery, and reduced glucose uptake in skeletal muscle.[38] Our observation of decreased Akt1 (significant) and IGF-IR (trend only) levels in LBCS offspring vastus muscle are consistent with decreased muscle growth. There was a trend for only Akt1 to be lower in LBCS offspring abdominal fat. Myofiber hypertrophy and hypertrophy-associated vascular endothelial growth factor (VEGF) expression is inhibited by a dominant-negative mutant of Akt1 and conversely, transduction of a constitutively active form of Akt1 increase myofiber size and VEGF production.[39] Hypertrophy of myotubes, in response to IGF-I or insulin (both ligands of IGF-IR), increases secretion of VEGF.[39] The VEGF is implicated in capillary remodeling/angiogenesis.[40] Interestingly, Akt1 has also been implicated in angiogenesis.[41] Thus, Akt1 is a mechanism through which blood vessel recruitment and muscle growth may be coupled, although in the present study reduced myofiber and capillary density in LBCS offspring vastus muscle was not accompanied by a change in myofiber CSA.

Our observed changes in skeletal muscle morphology, in particular the reduced total myofiber density in hindlimb muscle, in LBCS offspring were not associated with reduced glucose tolerance at 4 years (only a small nonsignificant decrease in insulin-sensitive glucose uptake in the soleus muscle strips of LBCS offspring was observed). The small increase in back muscle depth in the LBCS group may have contributed positively to glucose tolerance in these animals, similar to that associated with hypertrophy of muscle following resistance training.[42,43] In theory, such improvement in glucose tolerance could partially attenuate any reduction in whole body glucose tolerance that might have resulted from reduced myofiber density in limb muscle.

In the vastus muscle, there were no differences in InsR, PI3-kinase p85 subunit, and PKCζ protein between dietary groups but GLUT-4 (the major insulin-responsive glucose transporter in skeletal muscle and the last stage in the insulin pathway signaling to glucose uptake) was increased in LBCS offspring. In contrast, there was no effect on these key genes in abdominal fat. In contrast, previous studies observed reduced skeletal muscle GLUT-4 in much younger (8 months) lambs following early gestation maternal undernutrition.[13] So we speculate that the effects of altered skeletal muscle composition that were apparent at 1.5 years in terms of glucose tolerance[8] may be offset at least in part by compensatory increases in GLUT-4 resulting in normal glucose tolerance by 4 years. While not assessed directly in the present study, changes to the GLUT-4 system might involve altered GLUT-4 translocation (from intracellular vesicular storage to the plasma membrane). Indeed, GLUT-4 protein content was unchanged but translocation was increased in the muscle of 10-day-old pups from protein restricted dams, and this was associated with normal glucose tolerance.[44]

Thus, we have shown that the mild glucose intolerance in young adulthood caused by gestational lower maternal diet and body composition[8] was no longer detectable by mature adulthood in terms of total body glucose handling. Our observation of decreased myofiber and capillary density is likely to have originated in utero and at the young adult stage may have contributed to altered glucose handling—but by mature adulthood we speculate that the observed upregulation of GLUT-4 and increase in back muscle depth may form part of a compensatory mechanism to regulate glucose homeostasis. However, these adaptations may have limitations in an overabundant postnatal environment, where there would be increased propensity for weight gain and obesity. These findings have implications for dietary choices of mothers, even prepregnancy.

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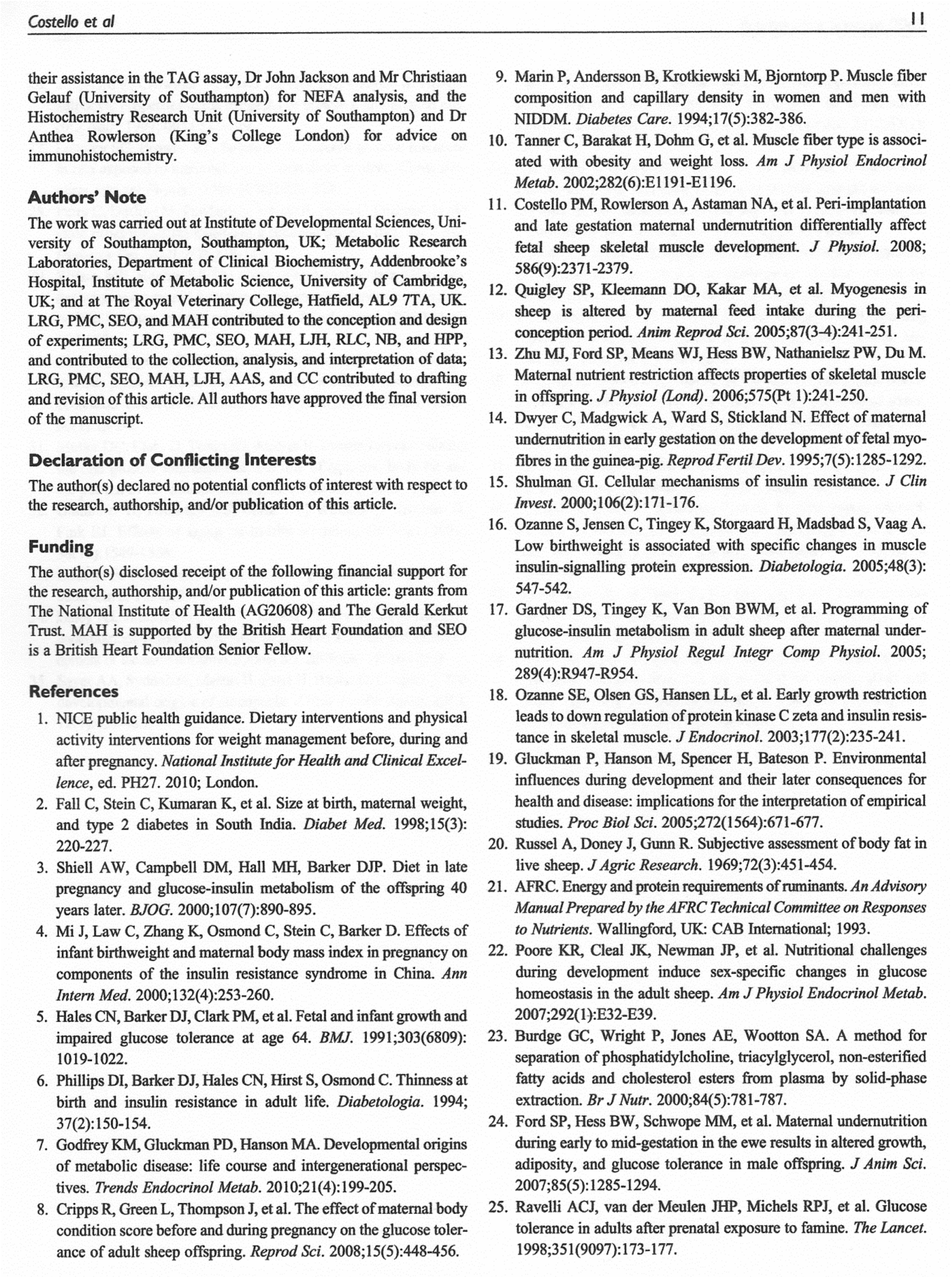

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Authors' Note

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Differential Pathways to Adult Metabolic Dysfunction following Poor Nutrition at Two Critical Developmental Periods in Sheep

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Abstract

Epidemiological and experimental studies suggest early nutrition has long-term effects on susceptibility to obesity, cardiovascular and metabolic diseases. Small and large animal models confirm the influence of different windows of sensitivity, from fetal to early postnatal life, on offspring phenotype. We showed previously that undernutrition in sheep either during the first month of gestation or immediately after weaning induces differential, sex-specific changes in adult metabolic and cardiovascular systems. The current study aims to determine metabolic and molecular changes that underlie differences in lipid and glucose metabolism induced by undernutrition during specific developmental periods in male and female sheep. Ewes received 100% (C) or 50% nutritional requirements (U) from 1–31 days gestation, and 100% thereafter. From weaning (12 weeks) to 25 weeks, offspring were then fed either *ad libitum* (CC, UC) or were undernourished (CU, UU) to reduce body weight to 85% of their individual target. From 25 weeks, all offspring were fed *ad libitum*. A cohort of late gestation fetuses were studied after receiving either 40% nutritional requirements (1–31 days gestation) or 50% nutritional requirements (104–127 days gestation). Post-weaning undernutrition increased *in vivo* insulin sensitivity, insulin receptor and glucose transporter 4 expression in muscle, and lowered hepatic methylation at the delta-like homolog 1/maternally expressed gene 3 imprinted cluster in adult females, but not males. Early gestational undernutrition induced lower hepatic expression of gluconeogenic factors in fetuses and reduced *in vivo* adipose tissue insulin sensitivity in adulthood. In males, undernutrition in early gestation increased adipose tissue lipid handling mechanisms (lipoprotein lipase, glucocorticoid receptor expression) and hepatic methylation within the imprinted control region of insulin-like growth factor 2 receptor in adulthood. Therefore, undernutrition during development induces changes in mechanisms of lipid and glucose metabolism which differ between tissues and sexes dependent on the period of nutritional restriction. Such changes may increase later life obesity and dyslipidaemia risk.

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Introduction

The worldwide incidence of obesity nearly doubled between 1980 and 2008, affecting both developed countries and developing societies that are undergoing socio-economic transition [1]. Strategies to reduce obesity by promoting healthy eating and an active lifestyle are producing disappointing results [2] and lifestyle factors do not appear to have the same impact on obesity in all individuals. Epidemiological and animal studies demonstrate that early life nutrition can exert a major influence on susceptibility to obesity, cardiovascular and metabolic diseases in adulthood [3,4]. Thus, knowledge of an individual's nutritional pathway during development may be beneficial in devising effective strategies to reduce the risk of obesity and its consequences.

The timing of nutritional challenges during development dictates the type, direction and magnitude of changes in the offspring's metabolic and cardiovascular phenotype. Mid- and late-gestation exposure to the Dutch Famine reduced birth weight and glucose tolerance in adulthood, which worsened with obesity [5] while early gestation famine exposure did not affect birth weight, but increased coronary heart disease prevalence and atherogenic risk factors [6,7]. Postnatally, nutrition and growth patterns also influence later metabolic health, with reduced birth size, persistent thinness in infancy but accelerated childhood growth and later obesity conferring increased risk of insulin resistance and dyslipidaemia [8–11]. Malnutrition in the first year also reduces glucose tolerance and insulin sensitivity in young men, independent of birth weight [12]. As well as investigating low birth weight and thinness at birth [13,14] it is now clear that a range of possible developmental pathways to poor health in adulthood should be considered. These include the periconceptional and fetal periods and/or early postnatal infant life and beyond.

Animal models have confirmed the influence of different windows of sensitivity on offspring phenotype and work towards elucidating mechanisms in different tissues [15–20]. In sheep, undernutrition either during the first month of gestation or immediately after weaning (before puberty/adolescence) induces differential, sex-specific changes in metabolic and cardiovascular systems in adulthood. In males, cardiovascular abnormalities such as left ventricular hypertrophy were observed following exposure to undernutrition in early gestation (with no effect on birth weight) which was ameliorated if postnatal nutrition was relatively 'matched' to that prenatally [21]. In contrast, post-weaning undernutrition improved glucose tolerance and insulin sensitivity in both young and mature females, regardless of early gestation undernutrition [22]. Small for gestational age human infants also show increased sensitivity to insulin [23]. In females particularly, this may help return body weight towards a threshold necessary for successful reproductive function [23]. No such adaptation was observed in male sheep undernourished post-weaning [22], suggesting they must employ different strategies to regain body weight. Although an increase in fat was observed in young but not mature adult females exposed to both early gestation and post-weaning undernutrition [22], metabolic abnormalities may become apparent before overt obesity [24]. We suggest that enhanced insulin sensitivity in postnatally undernourished females may facilitate body weight recovery but increase their susceptibility to later obesity so long as nutrient supply is abundant. Indeed alterations in glucose metabolism and liver function in aged sheep exposed to maternal undernutrition have been observed only following a later period of unlimited feeding [25].

Epigenetic regulation by DNA methylation provides a mechanism linking suboptimal nutrition with long-lasting changes in specific gene expression and later phenotype [26,27,28]; for example epigenetic markers in neonatal blood and cord tissue have been linked with later adiposity and cardiometabolic risk factors [29,30]. Furthermore, hepatic epigenetic modifications are detectable in early postnatal life following maternal undernutrition, before the phenotypic changes appear within the liver or other metabolic tissues, suggesting an interaction between the later environment and these modifications [24,31]. The influence of early postnatal diet on epigenetic mechanisms remains little studied [32].

To develop biomarkers of risk and suggest preventative measures for obesity requires a detailed molecular phenotype in metabolically active tissues and understanding of lipid metabolism that may predispose to inappropriate adipose deposition. Large animal models allow links between specific molecular and epigenetic alterations in a range of tissues and whole animal phenotype. The aim of this study was therefore to determine underlying differences in lipid metabolism in male and female sheep following early gestation and/or post-weaning undernutrition, both *in vivo* and at the molecular level. We have measured circulating non-esterified fatty acid (NEFA) and triacylglycerol (TAG) during a glucose tolerance test (GTT) and mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ; regulation of adipocyte differentiation), lipoprotein lipase (LPL; hydrolyses triglycerides and regulates their movement into adipocytes) and glucocorticoid receptor (GR; mediates glucocorticoid effects on adipose lipid accumulation and mobilisation) in metabolically active tissues. The mechanistic basis of the persistent, sex-specific effect of post-weaning undernutrition on metabolic homeostasis was also examined in tissues that regulate glucose and lipid uptake by measuring insulin receptor (IR), glucose transporter 4 (GLUT4), GR, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). We have also investigated epigenetic modifications of hepatic genes regulating metabolic homeostasis and body composition by measuring methylation of imprinting control regions (ICRs) of the insulin-like growth factor (IGF) 2/H19 and insulin-like growth factor-2 receptor (IGF2R) imprinted clusters and of the delta-like homolog 1 (DLK1)/maternally expressed gene 3 (MEG3) imprinted cluster, which are involved in fetal and early postnatal growth and in adipocyte development, respectively, and have been linked with metabolic abnormalities and changes in adiposity [33–35]. We proposed that for females undernutrition prior to puberty will have a greater impact on mechanisms that control lipid and glucose metabolism, given the importance of maintaining adequate body condition for future reproductive success. In males, we proposed early gestation undernutrition will have a greater impact on mechanisms regulating adiposity, due to the relatively greater need for nutrients to drive their faster growth rate during gestation. We found that exposure to both gestational and post-weaning undernutrition induces sex- and tissue-specific effects on lipid and glucose handling mechanisms that may increase obesity and dyslipidaemia risk in later life.

Materials and Methods

Study of Adult Offspring Exposed to Early Life Undernutrition

Animals. All procedures were carried out in accordance with the regulations of the UK Home Office Animals (Scientific Procedures) Act, 1986 (PPL 30/1858). The data in this study comes from the same cohort of sheep previously studied by us and for which other data have been published elsewhere [21,22,36]. Fifty-nine Welsh Mountain ewes in their second or third parity (2–3 years old), carrying singleton and twin pregnancies, and of uniform good body condition score (BCS; ~3 on a scale of 1–5 [37]) were used in this study. Full details about ewe mating, ewe and lamb housing and diets are presented elsewhere [22]. All manipulations of ewes and of lambs until 1.5 years of age took place at the Royal Veterinary College, North Mymms. A flow diagram showing the allocation of ewes and their offspring to the different dietary regimes is presented in Figure S1.

Before conception, ewes were randomly assigned to a control or a dietary restricted group. Control ewes (early gestation control, C; $n = 29$; 12 singleton (s), 17 twin (t)) received 100% nutritional requirements before and throughout gestation. From 1–31 days gestation (dGA; term = 147 days), ewes in the dietary restricted group (early gestation undernutrition, U; $n = 31$; 15 s, 15 t) received 50% nutritional requirements and then 100% requirements for the remainder of gestation. From −7 dGA, ewes were weighed weekly. The starting pelleted diet ration for each ewe was calculated using RUMNUT software (Ruminant Nutrition) version 5 for sheep (AT Chamberlain, Hampshire) based on initial body weight. This ration was adjusted weekly according to their weight measurement. The RUMNUT software was based on AFRC Guidelines for pregnant sheep [38] and incorporated adjustments for weight gain according to gestational age. Water was provided *ad libitum*. Ewes were individually penned from −7 to 37 dGA and group housed thereafter with animals at a similar gestational age. Ewes delivered and suckled lambs naturally while receiving 100% nutritional requirements, with further feed ration adjustments according to lactational needs [38].

A total of 77 male and female, singleton and twin, lambs were included in the study (Figure S1). Lambs were weaned at 12 weeks, after gradual introduction of the post-weaning diet [22]. Lambs from C and U ewes were grouped with approximately 10 others of similar body weight and post-weaning treatment group in open barns. For each lamb, an individual linear weight trajectory was calculated for the period 12–25 weeks that was based on weights taken at birth, 4, 8 and 12 weeks. Lambs were assigned to either a post-weaning control diet (yielding early gestation control/post-weaning control, CC; early gestation undernutrition/post-weaning control, UC) or post-weaning undernutrition diet (early gestation control/post-weaning undernutrition, CU; early gestation undernutrition/post-weaning undernutrition, UU). Post-weaning control lambs were fed 100% nutritional requirements to follow their individual trajectory from 12–25 weeks and post-weaning undernutrition lambs were fed a restricted pelleted diet such that body weight was reduced to 85% of their individual target weight from 12–25 weeks. Lambs had free access to hay throughout. To keep body weight on the desired trajectory, lambs were monitored individually by weekly weighing and feed ration adjustment. If necessary, lambs were temporarily removed to individual pens to maintain body weight at the desired level. For twin lambs, one twin was assigned to the post-weaning control diet and the other to the post-weaning undernutrition diet, except for twin pairs from 14 ewes in which only one offspring of the pair was used (Figure S1). The number, sex and proportion of singleton to twin lambs in each group are as follows; males: CC, *n* = 13 (5 s, 8 t), CU, *n* = 8 (3 s, 5 t), UC, *n* = 8 (4 s, 4 t), UU, *n* = 11 (5 s, 6 t); females: CC, *n* = 9 (2 s, 7 t), CU, *n* = 7 (2 s, 5 t), UC, *n* = 10 (2 s, 8 t), UU, *n* = 11 (4 s, 7 t).

From 25 week onwards, all lambs were returned to larger group housing and received 100% nutritional requirements. All lambs were weighed at 35 weeks and just prior to experiments (see below) at 16.5±0.1 months (1.5 years) and 29.6±0.2 months (2.5 years) of age. At each experimental age, body condition score was assessed manually and fat and muscle depths were measured by ultrasound in the third lumbar region by a small number of experienced animal technicians, as described previously [22].

For technical reasons e.g catheter loss prior to *in vivo* experiments or difficulty with laboratory procedures, data from the full number of animals in each group were not always achieved. The number of observations for each data set is indicated in the legend of each table or figure or within the text of the results section.

Surgical techniques. As described previously [22], surgery at 9.9 months (to create carotid artery loops for repeated arterial access and to vasectomise male lambs) and experiments at 1.5 years of age were performed at the Royal Veterinary College and experiments at 2.5 years were performed at the University of Southampton. Prior to each set of experiments, sheep were moved into individual cages and acclimatised for 4–6 days, with no change in their feeding regime. Indwelling carotid artery and jugular vein catheters were inserted under general anaesthesia (at 1.5 years: 2–4% halothane in O_2 by face mask; at 2.5 years: induced by thiopentone sodium (10 $mg.kg^{-1}$, *i.v.*) and maintained by 2–4% halothane in O_2 by endotracheal tube). Antibiotic treatment (15 $mg.kg^{-1}$, *i.m.*; Betamox) was administered to all sheep. At 1.5 years, catheters were maintained for 3 days then removed prior to sheep returning to group housing. At 2.5 years, catheters were maintained for up to 17 days during a series of experiments and subsequent *post mortem* that are described elsewhere [21,22,39].

Experimental protocol. At 1.5 and 2.5 years of age, a glucose tolerance test (GTT) was performed following an overnight fast (16.00 h–09.00 h). Glucose (0.5 g.kg body $weight^{-1}$) was administered as an intravenous bolus over 3 minutes and arterial blood samples (3 ml into chilled EDTA tubes) were collected immediately before (0 min) and at 10, 20, 30, 60, and 120 min after the start of the glucose administration (time 0). All blood samples were centrifuged immediately (10 min at 4°C) and plasma was stored at –20°C for later laboratory analysis.

Study of Fetuses Exposed to Gestational Undernutrition

Animals. A separate cohort of 23 Welsh Mountain primiparous ewes, carrying singleton fetuses, of uniform body condition score were used for collection of late gestation fetal tissues. As described previously [40], ewes were randomly assigned to a control or one of two dietary restricted groups, housed individually on straw and from −16 dGA (adjusted to gestational age) were fed a complete pelleted diet (89.2% dry matter as fed, providing 10.7 MJ.(kg dry $matter)^{-1}$ (metabolisable energy) and 14.8% protein) with water available *ad libitum*. Control ewes (C, *n* = 8; 4 male and 4 female fetuses) were fed 100% nutritional requirements throughout gestation. Early gestation nutrient-restricted ewes (E, *n* = 9; 5 male and 4 female fetuses) were fed 40% of nutrient requirements from 1−31 dGA and late gestation nutrient-restricted ewes (L, *n* = 6; 4 male and 2 female fetuses) were fed 50% of nutrient requirements from 104−127 dGA (term ~147 dGA). At all other times, ewes received 100% nutrient requirements. Full details about ewe mating, surgical and experimental procedures in these fetuses that are not presented in the current study are described elsewhere [40,41].

Post Mortem Procedures

At the completion of all experimental protocols, *i.v.* pentobarbitone sodium overdose (200 $mg.ml^{-1}$ Pentoject, Animalcare Ltd, UK) was used to kill adult sheep (2.5 years) or pregnant ewes and their fetuses (at 127 dGA). Samples of left and right lobes of the liver were removed from both adults and fetuses and skeletal (flexor digitorum longus) muscle and perirenal adipose tissue was removed from adults. All tissues were snap frozen in liquid nitrogen and stored at −80°C.

Laboratory Analysis

mRNA expression analysis. Total RNA was extracted from adult muscle, adult adipose tissue and fetal and adult left and right liver lobe using the TRIzol method and reverse transcribed into cDNA. Real-time RT PCR (ABL Prism 7700 and 7500 Sequence Detection System, Applied Biosystems Step One Plus) was used to measure mRNA expression for IR in all adult and fetal tissues, GLUT4 in adult muscle and adipose tissue, GR in adult adipose tissue and adult and fetal liver, LPL and PPAR-γ in adult adipose tissue, PEPCK and G6Pase in adult and fetal liver, IGFII and IGF2R in the left lobe of adult liver. Primers and probes (Table S1) were designed using Primer Express Software (Applied Biosystems, USA). GR in adult adipose tissue, IGFII and IGF2R gene expression and expression of housekeeping genes were measured using SYBR Green (JumpStart Taq ReadyMix, Sigma Aldrich S4438). Gene expression in adult muscle and adipose tissues was normalised to the geometric mean of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression, as determined using a GeNorm normalising kit (Primer Design, Southampton, UK) validated for use in the sheep. In adult and fetal liver, gene expression was normalised to the geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein 19 large subunit (RPL-19) and β-actin mRNA expression, as determined using the GeNorm normalising kit. GR gene expression in adult adipose tissue was performed before the GeNorm kit became available so these data were normalised to 18S mRNA expression. Coefficients of variation ranged from 6% to 10%.

PCR-based methylation analysis. PCR was used to amplify regions within the imprinting control regions (ICRs) of the DLK1/MEG3, IGF2R and IGF2/H19 imprinted clusters (Table S1) then DNA methylation levels were analysed by pyrosequencing using a PyroMark Q96 (Biotage/Qiagen), as described previously [42]. In brief, genomic DNA from adult left liver lobe was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research), following manufacturer's guidelines. The expression of the DLK1/MEG3 imprinted cluster is regulated, like the IGF2/H19 cluster, by binding of CTCF within the ICR region, which is itself influenced by the presence or absence of DNA methylation at CTCF binding sites. We identified a potential CTCF site within the MEG3 ICR, based upon reported CTCF consensus sequences [43–45] that contained CpG dinucleotides 2, 3, and 4, and was directly adjacent to CpG1 within the pyrosequencing assay. Methylation at the H19 loci was examined across CTCF binding site IV, which has previously been shown to regulate H19 expression [44,46]. Methylation within the ICR of IGF2R, a 2.3 Kb CpG island regulates IGF2R expression in sheep [46,47].

Determination of DNA methylation within the GR promoter region was undertaken prior to the availability of the above methodology, therefore for this data set methylation-sensitive PCR was used. PCR was used to amplify DNA extracted from adult left and right liver lobes and treated with methylation-sensitive restriction enzymes (AciI and HpaII). GR primers were designed to amplify two regions within the CpG island within the GR promoter region, based on a partial ovine sequence determined by express sequence tag mapping to the bovine sequence (Table S1).

Plasma lipid analysis. Plasma NEFA and TAG concentrations during GTT at 1.5 and 2.5 years were measured to assess insulin-induced inhibition of lipolysis in adipose tissue. A randomly chosen subset of animals was included for these measurements. Plasma NEFA concentrations were measured by a spectrophotometric two-stage enzymatic assay kit (Wako NEFA HR2, ACS-ACOD method; Alpha Laboratories, Eastleigh, UK) on a Konelab 20 autoanalyser (Thermo Fisher Scientific, Hemel Hempstead, UK). This method was not sensitive enough to measure TAG concentrations. Therefore, gas chromatography (HP 6890 Series GC System equipped with a flame ionisation detector and 7683B Series Injector) was used, following separation of lipid classes by solid phase extraction, to measure plasma TAG concentrations (extracted from 0.4 g plasma, made up to 0.8 ml) or adipose total lipid content (extracted from 20 mg adipose tissue), as described previously [48].

Data Analysis

For NEFA and TAG analysis during GTT in adult sheep, summary measures (baseline, slope, maximum fall from baseline and area under curve relative to baseline (AUC)) were calculated. For all adult data except liver, the effects of four main factors were tested (early gestation diet, post-weaning diet, sex and offspring number (i.e. singleton or twin)) using multifactorial analyses of variance (4-way ANOVA). For adult liver, the same factors were examined using multifactorial analyses of variance for repeated measures (RM ANOVA), with liver lobe as the repeated measure. For fetal liver, the main factors of maternal dietary group and fetal sex were used in a RM ANOVA, with liver lobe as the repeated measure. Where significant effects of a main factor or interactions between the main factors were found, further analyses were performed within subsets of the data. Linear regression analysis was used to examine relationships between two factors: this was done within each sex, across all nutritional groups. Statistical analyses were performed using GraphPad Prism version 5 and SPSS version 11. Data are expressed as mean ± S.E.M. and significance was accepted at $p<0.05$, with a trend noted when $0.05<p<0.1$.

Results

Lipid Metabolism during Glucose Tolerance Test

Diminished insulin-induced inhibition of lipolysis in adult males and females undernourished in early gestation. Plasma NEFA and TAG concentrations during a glucose tolerance test (GTT), performed in both young (1.5 years) and mature (2.5 years) adulthood [22] were determined as a surrogate measure of insulin-induced adipose tissue lipid metabolism. The insulin-induced fall in NEFA output during GTT was reduced ($p<0.05$) at 2.5 years in sheep exposed to early gestation undernutrition, regardless of post-weaning nutrition (Fig. 1B). There was no effect of sex on this response. The slope from 10–30 min during the GTT tended to be shallower in UC and UU groups (-9.4 ± 0.9 *vs.* -7.2 ± 1.4 μmol.l.min^{-1}, $p<0.1$). Basal NEFA and TAG concentrations at 2.5 years were not influenced by early life nutrition (Table 1). There was no effect of early gestation undernutrition on plasma NEFA and TAG concentrations during the GTT at 1.5 years. Faster relative growth during the first 12 postnatal week was associated with reduced NEFA AUC at 2.5 years ($r^2=0.20$, $p<0.05$). Low birth weight was associated with increased NEFA AUC at 2.5 years ($r^2=0.16$, $p<0.005$).

Increased insulin sensitivity in mature adult females undernourished post-weaning. At 1.5 years, there were no overall effects of post-weaning nutrition or sex on plasma NEFA or TAG concentrations before (Table 1) or during GTT (data not shown). Basal NEFA and TAG concentrations in 1.5 year-old males and females combined were positively related to current fatness (back fat depth corrected for body weight; $r^2=0.16$ and 0.19, respectively; $p<0.01$). At 2.5 years, in females only, the maximum fall in plasma TAG concentrations during GTT was greater (indicating increased insulin sensitivity) in animals undernourished only in the post-weaning period (CU, 11.5 ± 5.5 μg.ml^{-1}) compared to controls (CC, 8.7 ± 1.4 μg.ml^{-1}). Adipose tissue lipid content was also reduced ($p<0.05$) in CU compared to CC females at 2.5 years (Table 2).

Tissue Expression of Factors Involved in Lipid Metabolism

Increased adipose tissue lipid handling factors in adult males undernourished in early gestation. In males, but not females, both LPL and GR mRNA levels in adipose tissue at 2.5 years were significantly higher in sheep exposed to early gestation undernutrition (Fig. 2). This was not associated with an effect on adipose tissue lipid content (Table 2). PPARγ (Table 2), LPL (Fig. 2) and GR mRNA (1.49 ± 0.05 *vs.* 0.97 ± 0.05 arbitrary units) levels in adipose tissue at 2.5 years were significantly ($p<0.001$) higher in males than females. Faster relative growth during the first 12 postnatal weeks was associated with higher GR mRNA levels in adipose tissue ($r^2=0.11$, $p<0.05$).

Adipose tissue factors are unaffected by post-weaning undernutrition. There were no significant effects of post-weaning nutrient restriction on mRNA levels of PPARγ, LPL or GR in adipose tissue of either male or female sheep at 2.5 years (Fig. 2, Table 2).

Tissue Expression of Factors Involved in Glucose Metabolism

Hepatic gluconeogenic gene expression during fetal but not adult life is reduced by early and late gestation undernutrition. In a cohort of late gestation (127 days) singleton fetuses exposed to a 40% nutrient restriction in early gestation (compared to 50% in the adult cohort), PEPCK mRNA levels were reduced ($p<0.05$) in both hepatic lobes (Fig. 3). Levels

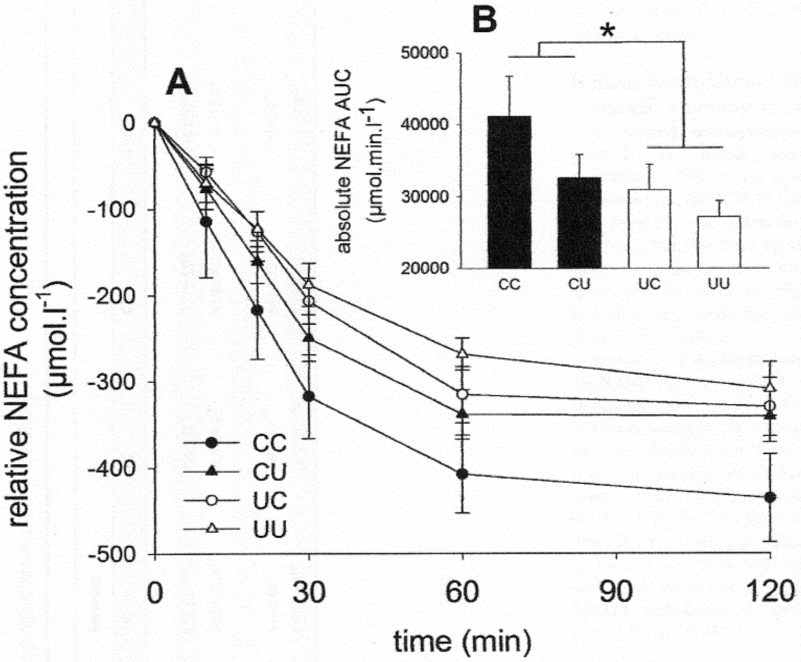

Figure 1. *In vivo* lipid handling in 2.5 year-old sheep. (A) Plasma non-esterified fatty acid (NEFA) concentrations relative to baseline in male and female sheep combined during a glucose tolerance test at 2.5 years. Glucose (0.5 g.kg^{-1}) was given at time 0. (B) Absolute area under the NEFA response curve (AUC); *p<0.05, early gestation undernutrition groups less than early gestation control groups. CC: *n* = 13; CU: *n* = 14; UC: *n* = 15; UU: *n* = 13). Values are mean ± S.E.M.
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of GR (p<0.01) and G6Pase (p<0.05) mRNA in fetal liver were also reduced by early gestation undernutrition, but in a lobe-specific manner. PEPCK, GR and G6Pase mRNA levels were similarly reduced in fetuses exposed during late gestation (104–127 days) (Fig. 3).

In adult tissues, early gestation undernutrition had no effect on IR mRNA levels in muscle (Fig. 4), adipose tissue or liver (Table 2), or on hepatic GR, PEPCK or G6Pase mRNA levels (Fig. 2, Table 2). While being a twin had no bearing on adult glucose tolerance in this model, GLUT4 mRNA levels in muscle were elevated in adult female twins undernourished in early gestation (UC and UU combined (*n* = 11), 1.28±0.08 *vs.* CC and CU combined (*n* = 12), 0.90±0.08 arbitrary units; p<0.005). Early gestation undernutrition increased PEPCK mRNA levels (p<0.05) in the adult right liver lobe compared to controls, but only in singleton males (0.31±0.04 *vs.* 0.18±0.04 arbitrary units).

Gluconeogenic genes in liver differs between the lobes. There was differential expression of gluconeogenic genes between the fetal liver lobes, with G6Pase (p<0.01) and GR (p = 0.065) expression overall greater in the left (29.09±5.93 and 8.85±0.66, respectively) compared to the right (20.34±4.15 and 7.18±0.73 arbitrary units, respectively) liver lobe. Regardless of early gestation or post-weaning undernutrition, in adult liver GR mRNA levels overall were greatest (p<0.001) in the right lobe compared to left lobe in males and females (males: 7.14±0.53 *vs.* 3.24±0.23; females: 3.56±0.25 *vs.* 2.30±0.16 arbitrary units) and PEPCK mRNA levels were greatest (p<0.05) in the left lobe compared to right lobe in males (0.27±0.02 *vs.* 0.22±0.02 arbitrary units). Both GR and PEPCK mRNA levels overall were higher (p<0.001) in adult male than female liver, regardless of lobe (5.19±0.22 *vs.* 2.93±0.26 and 0.24±0.02 *vs.* 0.14±0.02 arbitrary units, respectively) and in female twins compared to singletons (3.89±0.26 *vs.* 3.24±0.43 arbitrary units (right liver lobe) and 0.21±0.02 *vs.* 0.10±0.03 (left liver lobe), respectively, see also Table 2). DNA methylation within the GR promoter region in adult liver was not affected by lobe, nutrition, sex or offspring number (Table 2).

Increased insulin signalling factors in muscle in adult females undernourished post-weaning. Post-weaning undernutrition in adult female, but not male sheep, regardless of early gestation undernutrition, significantly (p<0.05) increased mRNA levels of IR and GLUT4 in skeletal muscle but not in peri-renal adipose tissue (Fig. 4, Table 2). Reduced growth rate during the period of post-weaning undernutrition from 12–25 weeks was also directly associated with increased IR and GLUT4 mRNA levels in muscle from females ($r^2 = 0.17$, p<0.05 and $r^2 = 0.22$, p<0.01, respectively). The effect of post-weaning undernutrition on GLUT4 mRNA levels was strongest in singleton sheep, as revealed by investigation of a significant (p<0.05) interaction between post-weaning undernutrition and offspring number: CU and UU singletons combined, 1.25±0.10 (*n* = 4) *vs.* CC and UC singletons combined, 0.74±0.12 (*n* = 4) arbitrary units. Post-weaning nutrition restriction had no effect in the liver on IR, GR, PEPCK or G6Pase mRNA levels (Table 2), except in the right liver lobe where PEPCK mRNA levels in singleton male sheep were reduced by post-weaning undernutrition (CU and UU combined,

Table 1. Basal plasma NEFA and TAG concentrations in young (1.5 years) and mature (2.5 years) adult male and female sheep.

	Males				Females			
	CC	CU	UC	UU	CC	UC	CU	UU
1.5 Years:								
NEFA (µmol.l^{-1})	$792\pm70^{(5)}$	$647\pm53^{(5)}$	$747\pm104^{(5)}$	$748\pm132^{(5)}$	$828\pm93^{(6)}$	$914\pm72^{(5)}$	$907\pm52^{(5)}$	$815\pm90^{(5)}$
TAG (µmol.l^{-1})	$36.4\pm4.0^{(6)}$	$27.1\pm6.5^{(4)}$	$5.3\pm5.3^{(6)}$	$34.9\pm11.6^{(6)}$	$38.2\pm11.4^{(5)}$	$38.6\pm4.9^{(5)}$	$33.9\pm4.0^{(5)}$	$41.5\pm12.0^{(5)}$
2.5 Years:								
NEFA (µmol.l^{-1})	$482\pm65^{(7)}$	$410\pm35^{(8)}$	$375\pm52^{(8)}$	$384\pm46^{(8)}$	$544\pm83^{(6)}$	$448\pm49^{(6)}$	$443\pm52^{(7)}$	$399\pm50^{(6)}$
TAG (µmol.l^{-1})	$22.8\pm3.2^{(9)}$	$30.8\pm6.7^{(6)}$	$19.0\pm1.8^{(6)}$	$27.3\pm5.0^{(7)}$	$25.0\pm2.3^{(6)}$	$29.9\pm7.5^{(5)}$	$17.4\pm3.2^{(7)}$	$21.3\pm1.6^{(6)}$

Data are mean ± S.E.M. Values in brackets represent number of observations.

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0.16±0.02 ($n = 8$) *vs.* CC and UC combined, 0.31±0.06 ($n = 10$) arbitrary units).

Hepatic Methylation Pattern of Factors Regulating Metabolic Homeostasis and Body Composition

Increased methylation of imprinted genes regulating growth in adult males undernourished in early gestation. There was a significant ($p<0.05$) increase in methylation within the ICR of IGF2R at CpG 11, 13 and 15 in left liver lobe from 2.5 year-old males undernourished in early gestation but not from females (Fig. 5). Early gestation undernutrition had no effect on methylation at the IGF2/H19 or DLK/MEG3 loci in adult liver in either sex (Fig. 5). There were no effects of early gestation undernutrition on IGFII or IGF2R mRNA levels in adult liver (Table 2).

Decreased methylation of adipocyte development factor DLK/MEG3 in adult females undernourished post-weaning. In female but not male sheep, post-weaning undernutrition significantly reduced hepatic DLK/MEG3 methylation at CpG dinucleotides 1, 2, 3, and 4 (Fig. 5), an effect that could reduce expression of DLK1. There was no significant effect of post-weaning undernutrition on IGF2R or IGF2/H19 imprinted clusters (Fig. 5). Poor growth in females between 12 and 25 weeks was associated with decreased DLK/MEG3 methylation at CpG 2, 3 and 4 ($r^2 = 0.24$, 0.29 and 0.18, respectively, $p<0.005$). There was a positive relationship in 2.5 year-old males between DLK/MEG3 methylation at CpG 4 in left liver lobe and muscle depth ($r^2 = 0.13$, $p<0.05$).

Discussion

This large animal study is unique in its comparison of the effects of a postnatal nutrient restriction challenge in the context of a previous prenatal nutritional challenge. We have shown that exposure to both early gestation and post-weaning undernutrition have sex- and tissue-specific effects on mechanisms of lipid and glucose metabolism, effects that may increase the risk of obesity and dyslipidaemia in later life. The presence of sex differences in the current study confirms differential effects on metabolic and cardiovascular parameters previously reported in sheep [21,22], and goes further to suggest that males and females adopt different strategies in the face of reduced nutrition at critical windows of development.

We showed previously that female sheep undernourished in the post-weaning period make metabolic adaptations, improving their glucose handling, that persist from young through to mature adulthood [22]. In the current study, we show that this effect is likely brought about by enhanced insulin-induced glucose uptake specifically into muscle, via increased insulin receptor and glucose transporter 4 mRNA levels. Factors which are known to be involved in hepatic insulin sensitivity, in the regulation of gluconeogenesis and in adipose tissue differentiation and lipid hydrolysis, were unaffected by post-weaning undernutrition in this study. The effect in muscle is consistent with studies of improved insulin signalling capacity in intra-uterine growth restricted fetal sheep [49] and in rat offspring exposed to maternal low protein diet [19], and provides a mechanism whereby post-weaning undernutrition in females induces adaptations in muscle that enhance nutrient uptake and storage. If this effect was initiated shortly after the post-weaning challenge, prior to puberty, it may provide a strategy to facilitate the return to control body weight observed by 1.5 years (young adulthood) [22], which could be critical to ensure reproductive success. However with ageing and/or an over-abundant nutrient environment, these mechanisms

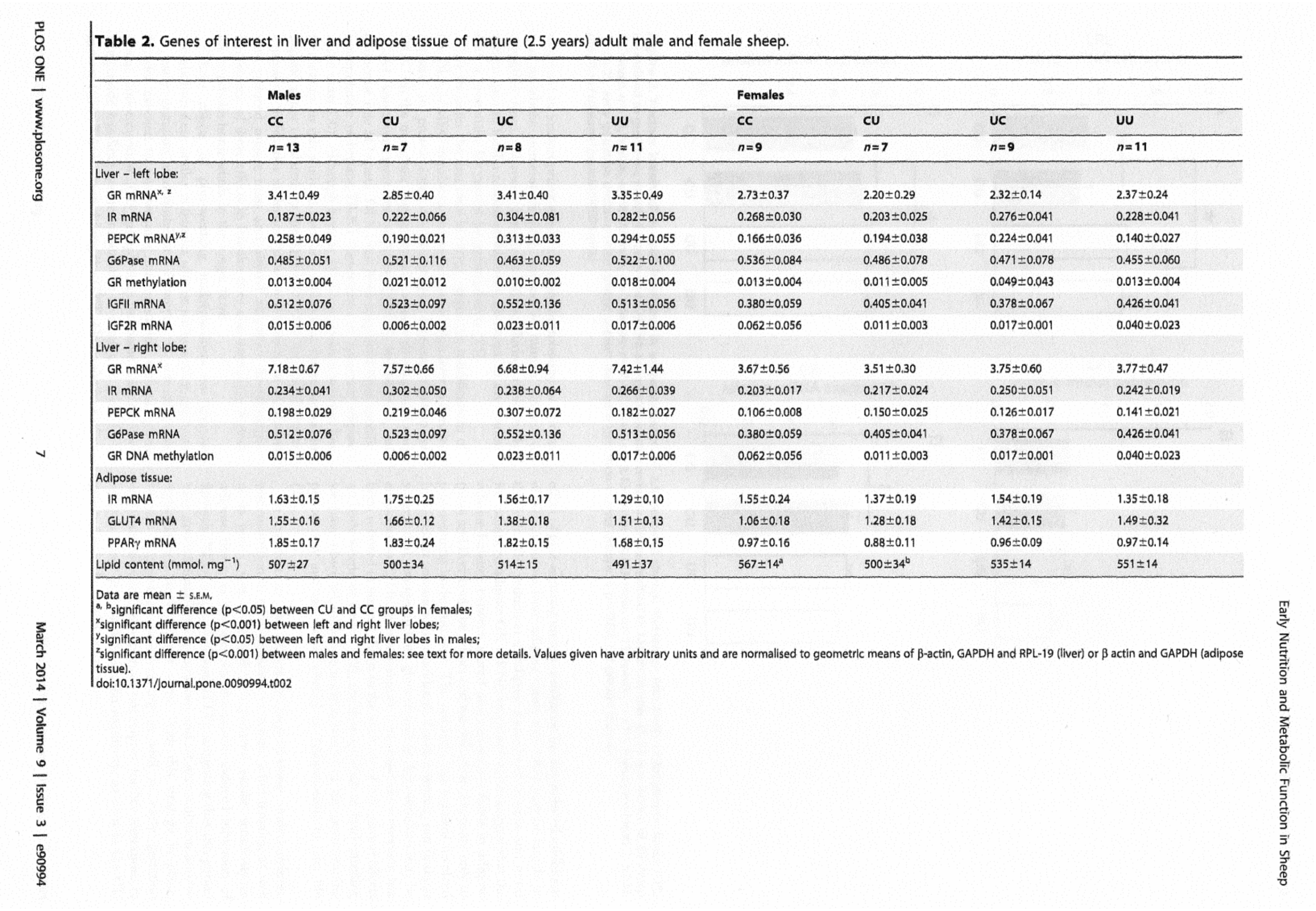

Table 2. Genes of interest in liver and adipose tissue of mature (2.5 years) adult male and female sheep.

	Males				Females			
	CC	CU	UC	UU	CC	CU	UC	UU
	n = 13	*n* = 7	*n* = 8	*n* = 11	*n* = 9	*n* = 7	*n* = 9	*n* = 11
Liver – left lobe:								
GR mRNA$^{x, z}$	3.41±0.49	2.85±0.40	3.41±0.40	3.35±0.49	2.73±0.37	2.20±0.29	2.32±0.14	2.37±0.24
IR mRNA	0.187±0.023	0.222±0.066	0.304±0.081	0.282±0.056	0.268±0.030	0.203±0.025	0.276±0.041	0.228±0.041
PEPCK mRNA^{y,z}	0.258±0.049	0.190±0.021	0.313±0.033	0.294±0.055	0.166±0.036	0.194±0.038	0.224±0.041	0.140±0.027
G6Pase mRNA	0.485±0.051	0.521±0.116	0.463±0.059	0.522±0.100	0.536±0.084	0.486±0.078	0.471±0.078	0.455±0.060
GR methylation	0.013±0.004	0.021±0.012	0.010±0.002	0.018±0.004	0.013±0.004	0.011±0.005	0.049±0.043	0.013±0.004
IGFII mRNA	0.512±0.076	0.523±0.097	0.552±0.136	0.513±0.056	0.380±0.059	0.405±0.041	0.378±0.067	0.426±0.041
IGF2R mRNA	0.015±0.006	0.006±0.002	0.023±0.011	0.017±0.006	0.062±0.056	0.011±0.003	0.017±0.001	0.040±0.023
Liver – right lobe:								
GR mRNA^{x}	7.18±0.67	7.57±0.66	6.68±0.94	7.42±1.44	3.67±0.56	3.51±0.30	3.75±0.60	3.77±0.47
IR mRNA	0.234±0.041	0.302±0.050	0.238±0.064	0.266±0.039	0.203±0.017	0.217±0.024	0.250±0.051	0.242±0.019
PEPCK mRNA	0.198±0.029	0.219±0.046	0.307±0.072	0.182±0.027	0.106±0.008	0.150±0.025	0.126±0.017	0.141±0.021
G6Pase mRNA	0.512±0.076	0.523±0.097	0.552±0.136	0.513±0.056	0.380±0.059	0.405±0.041	0.378±0.067	0.426±0.041
GR DNA methylation	0.015±0.006	0.006±0.002	0.023±0.011	0.017±0.006	0.062±0.056	0.011±0.003	0.017±0.001	0.040±0.023
Adipose tissue:								
IR mRNA	1.63±0.15	1.75±0.25	1.56±0.17	1.29±0.10	1.55±0.24	1.37±0.19	1.54±0.19	1.35±0.18
GLUT4 mRNA	1.55±0.16	1.66±0.12	1.38±0.18	1.51±0.13	1.06±0.18	1.28±0.18	1.42±0.15	1.49±0.32
PPARγ mRNA	1.85±0.17	1.83±0.24	1.82±0.15	1.68±0.15	0.97±0.16	0.88±0.11	0.96±0.09	0.97±0.14
Lipid content (mmol. mg^{-1})	507±27	500±34	514±15	491±37	567±14^{a}	500±34^{b}	535±14	551±14

Data are mean ± S.E.M.

[a, b] significant difference ($p<0.05$) between CU and CC groups in females;

[x] significant difference ($p<0.001$) between left and right liver lobes;

[y] significant difference ($p<0.05$) between left and right liver lobes in males;

[z] significant difference ($p<0.001$) between males and females: see text for more details. Values given have arbitrary units and are normalised to geometric means of β-actin, GAPDH and RPL-19 (liver) or β actin and GAPDH (adipose tissue).

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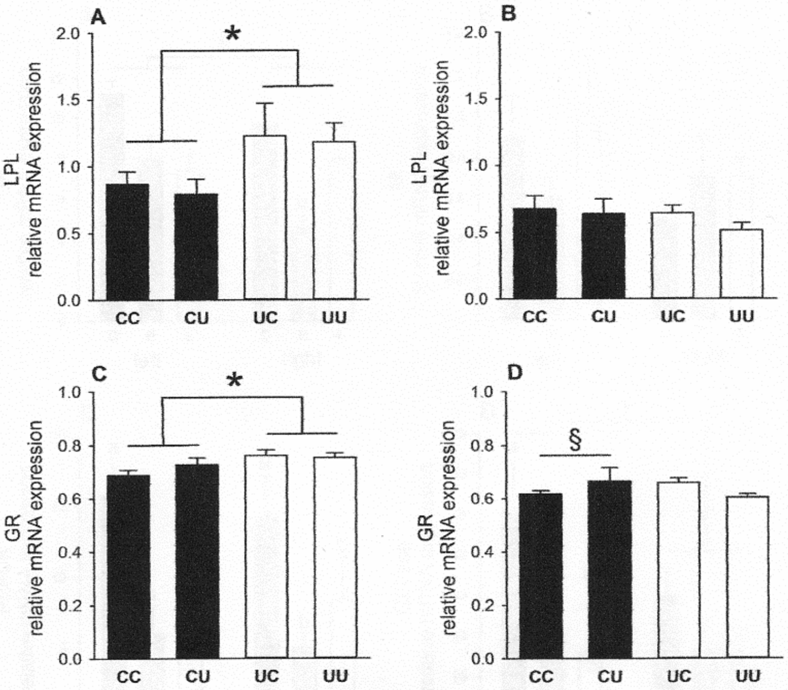

Figure 2. Factors involved in lipid handling in adipose tissue of 2.5 year-old sheep. Messenger RNA levels of lipoprotein lipase (LPL, normalised to geometric mean of β actin and GAPDH) and glucocorticoid receptor (GR, normalised to 18S) from male (A, C) and female (B, D) sheep (CC: male, $n = 13$, female, $n = 9$; CU: male, $n = 7$, female $n = 7$; UC: male, $n = 8$, female, $n = 9$; UU: male, $n = 11$, female, $n = 11$). Values are mean ± S.E.M. *$p < 0.05$, early gestation undernutrition groups greater than early gestation control groups. § $p = 0.08$, CU greater than CC.
doi:10.1371/journal.pone.0090994.g002

may lead to inappropriate effects on body composition or fatness and metabolic homeostasis [25]. Indeed in human infants, a transition from enhanced insulin sensitivity during rapid early weight gain to insulin resistance has been observed, in association with a propensity for central fat accumulation [23].

We show for the first time in sheep, epigenetic modifications of the DLK1/MEG imprinted cluster in mature adult liver of females exposed to post-weaning undernutrition. Genetic ablation of DLK1 in the myogenic lineage impairs muscle development and DLK1 knock-out increases adiposity [34,35]. In addition, an endocrine role of hepatic DLK1 has been suggested since specific manipulation of hepatic DLK1 expression can influence adipose tissue mass [50]. Although hepatic DLK1 expression was too low to measure in this study, it may be possible that the reduction in hepatic genes regulated by the DLK1/MEG3 imprinted cluster expression in females undernourished post-weaning is associated with reduced muscle mass and altered adipocyte development. Persistence of these effects in females may also negatively impact on metabolic homeostasis in the longer term.

These effects of post-weaning undernutrition on glucose metabolism were similar in females with or without prior exposure to early gestation undernutrition. However, in mature adult females exposed to post-weaning undernutrition without gestational undernutrition (CU group), adipose tissue and/or liver triglyceride handling was more sensitive to insulin, as given by a greater fall in TAG output during the GTT. Given the lack of an effect of post-weaning undernutrition on insulin-induced inhibition of NEFA output from adipose tissue, this effect may be largely in the liver. The reduction in adipose tissue lipid content and trend for enhanced adipose GR mRNA levels in females suggest that the relative balance between stored and circulating lipids in adipose tissue and liver could be influenced specifically by exposure only to post-weaning undernutrition. The potential mechanisms involved in this effect remain to be elucidated. Taken together, our findings suggest that changes in both glucose and lipid metabolism may be employed in CU females to enhance body mass once nutrition has been restored to normal. Even in the absence of any current effects on body weight or fatness [22], the dysregulation of lipid handling we have observed following undernutrition in the post-weaning period may increase risk of obesity or dyslipidaemia in later life [24].

In contrast to the effects of post-weaning undernutrition, undernutrition in early gestation in mature adult females, as well as in males, was associated with reduced insulin sensitivity in adipose tissue, as revealed by the insulin-induced inhibition of lipolysis (NEFA response during GTT), suggesting that this period may also influence lipid metabolism and fat accumulation and/or dyslipidaemia in the longer term. With this strategy in place, females exposed to post-weaning as well as early gestation undernutrition (UU group) may not require further alterations in adipose tissue and/or liver insulin sensitivity, as seen in the CU group, for body weight recovery.

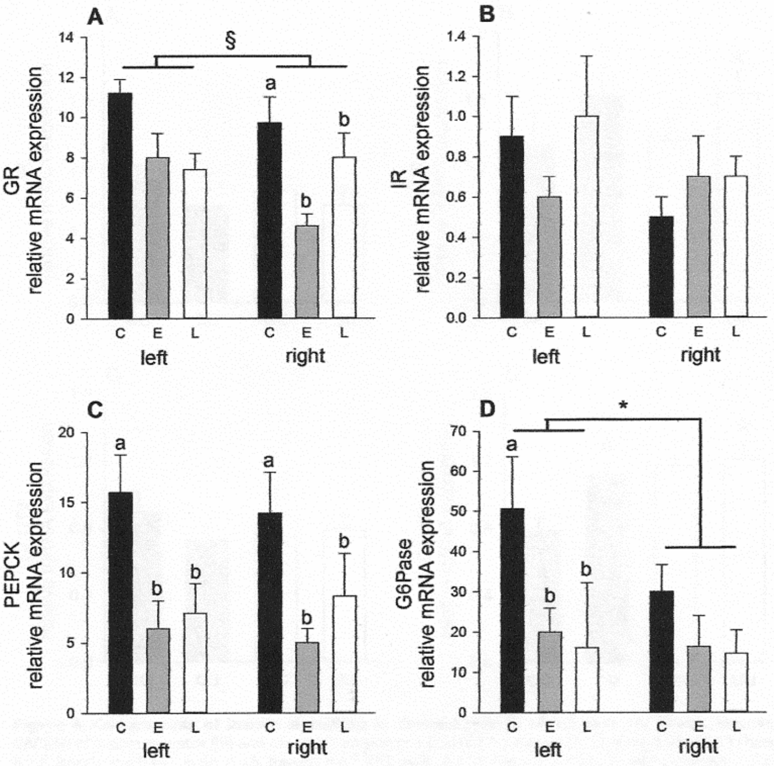

Figure 3. Factors involved in glucose homeostasis in liver of 127 dGA fetal sheep. Messenger RNA levels (normalised to geometric mean of β actin, GAPDH and RPL-19) of glucocorticoid receptor (GR; A), insulin receptor (IR; B), phosphoenolpyruvate carboxykinase (PEPCK; C) and glucose-6-phosphatase (G6Pase; D) in left and right liver lobes of control (C, $n=8$), early gestation undernutrition (E, $n=9$) and late gestation undernutrition (L, $n=6$) fetuses (males and females combined). Values are mean ± S.E.M. Different letters denote significantly different values based on nutritional group ($p<0.05$). *$p<0.01$, § $p=0.065$; difference between left and right liver lobes.
doi:10.1371/journal.pone.0090994.g003

In male sheep, we proposed that early gestation rather than post-weaning undernutrition would have a greater impact on mechanisms regulating adiposity. Males may have a heightened sensitivity to nutrient restriction in early gestation, when their growth rate is faster than females [51], potentially causing them to make adaptations to enhance their ability to optimise growth and body composition in later nutritionally adequate times. We demonstrated previously that males exposed to early gestation undernutrition have a relatively faster growth rate in the first 12 postnatal weeks without any change in birth weight [21]. In the current study we found that insulin sensitivity in adipose tissue, as revealed by the insulin-induced inhibition of lipolysis (NEFA response during GTT), was reduced in mature adult males undernourished in early gestation alongside increases in LPL and GR mRNA levels. Although these molecular effects were small and may not be reflected in subsequent protein expression, they were also associated with faster early postnatal growth. In this cohort, we did not observe long-term effects of early nutrition on fat depth [22], although this measurement was limited to one subcutaneous region, nor was there an effect on actual lipid content in adipose tissue at 2.5 years. However, lipid content may be maintained by a balance between the actions of glucocorticoids and LPL. Changes in glucocorticoid sensitivity and the ability to hydrolyse and transport triglycerides in early gestation-undernourished males could lead to changes in fat distribution or fat deposition in other organs such as the liver, effects that may be exacerbated by a subsequent abundant diet or with ageing. Indeed, the effect of early gestation undernutrition on adipose insulin sensitivity in males only became apparent at 2.5 years of age. The possibility of an altered plasma lipid profile in these animals, alongside our previous observations that exposure to early gestation undernutrition influences cardiac morphology and coronary function in adult male sheep [21], may contribute to a risk of cardiovascular disease. For female sheep exposed to early gestation undernutrition, the insulin-induced fall in NEFA during GTT was similar to that in males however there were no changes in LPL or GR mRNA levels in adipose tissue. While this may suggest these females do not have the same risk of dyslipidaemia, the implication of these differences requires further investigation. Our molecular studies of adipose tissue utilised the perirenal depot; given the differences in metabolic profiles between fat depots [52], the possibility that early gestation or post-weaning undernutrition influenced adipose tissue metabolism and/or

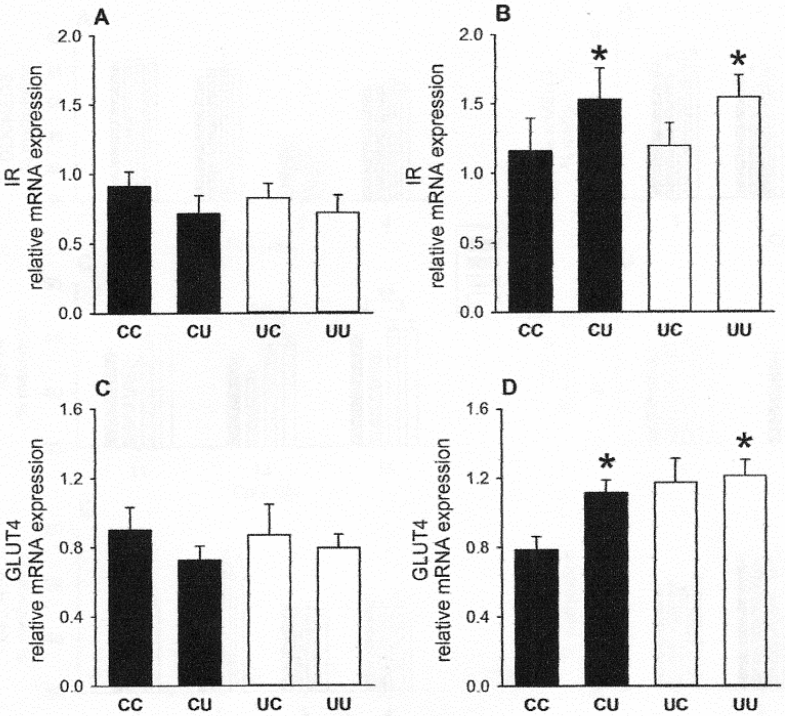

Figure 4. Components of insulin signalling in skeletal muscle of 2.5 year-old sheep. Messenger RNA levels (normalised to β actin and GAPDH) of insulin receptor (IR) and glucose transporter 4 (GLUT4) from male (A, C) and female (B, D) sheep (CC: male, $n = 13$, female, $n = 8$; CU: male, $n = 7$, female $n = 7$; UC: male, $n = 6$, female, $n = 7$; UU: male, $n = 11$, female, $n = 10$). Values are mean ± S.E.M. $^*p < 0.05$, post-weaning undernutrition groups greater than post-weaning control groups.
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deposition elsewhere in the body cannot be excluded from the current study.

In the current study, as well as examining the effects of both pre- and postnatal undernutrition on adult liver, we examined fetal liver in a separate cohort of sheep exposed to maternal undernutrition at 40% of control levels (compared to a 50% reduction in the study of adult offspring). Both left and right liver lobes were examined, except for the epigenetic analyses. There is unequal provision of umbilical venous blood between the liver lobes in fetal life, with predominantly more going to the left lobe, such that each lobe may be differentially sensitive to changes in maternal nutrient provision. Nutrient restriction in both early and late gestation reduced key gluconeogenic enzymes in late gestation liver and there was differential expression between the fetal liver lobes. These effects did not persist into adult life and indeed, as with the lobar differences in gene expression patterns, appear even to switch direction over the life course. We therefore show that the developing liver makes adaptations to reductions in maternal nutritional status and there is differential expression between the lobes. However, although these hepatic effects imply potential changes in gluconeogenic capacity, as well as growth potential [53], in response to maternal undernutrition, they may not translate to significant functional significance given the absence of changes in basal glucose in either exposed fetuses or adults [22,40]. However, we must be cautious making direct comparisons between the fetal and adult cohorts reported here given the difference in the level of maternal nutritional challenge imposed. In addition, primiparous, singleton-bearing ewes were used in the fetal study, whereas older ewes carrying both singletons and twins were used in the adult study. Given findings that birth order poses a non-modifiable risk for obesity [54], the relative maternal constraint experienced by first born offspring may have imposed an additional environmental challenge to the fetuses exposed to maternal undernutrition.

The role of the liver as a sensor of maternal nutrient status is suggested in studies linking fetal liver blood flow with subsequent offspring fat mass [55,56]. Experimental manipulation of fetal liver blood perfusion and reduction in maternal diet in sheep have both been shown in sheep to influence hepatic IGF expression [57,58], with potential long term effects on offspring growth and metabolism. In addition, reduced DNA methylation at the IGF2/H19 imprinted locus has been reported in humans following the Dutch Famine [59] and in fetal and postnatal sheep in response to altered maternal diets [46,60]. Unlike findings in lamb adrenal tissue [46], no effect of early gestation undernutrition was observed at the IGF2/H19 locus in adult sheep liver. However, methylation of IGF2R in liver of early gestation undernourished males was increased, which could increase IGF2R expression. Since this receptor acts to limit circulating IGF2 availability [61], this increase may influence growth potential or muscle growth. Although we were not able to demonstrate an effect of early gestation undernutrition on hepatic IGF2R gene

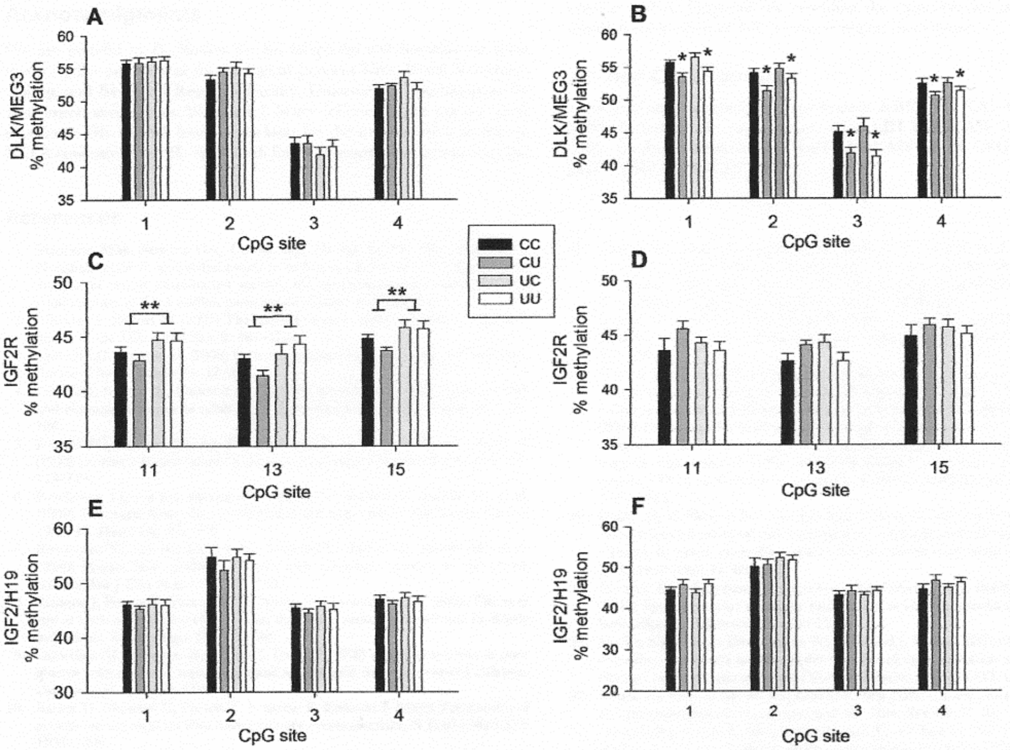

Figure 5. Epigenetic modification of genes regulating metabolic homeostasis and body composition in liver of 2.5 year-old sheep. Methylation of CpG dinucleotides within the ICRs of the DLK1/MEG3 (A, B), IGF2R (C, D) and IGF2/H19 (E, F) imprinted regions in male (A, C, E) and female (B, D, F) sheep (CC: male, $n = 11$, female, $n = 9$; CU: male, $n = 7$, female $n = 7$; UC: male, $n = 8$, female, $n = 9$; UU: male, $n = 10$, female, $n = 11$). Values are mean ± S.E.M. *$p < 0.05$, post-weaning undernutrition less than post-weaning control; **$p < 0.05$, early gestation undernutrition greater than early gestation control.

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expression, possibly because the affected CpG dinucleotides were not contiguous, others have also shown sex-specific effects on IGF2R expression and muscle area in mature cattle exposed to a maternal low protein diet in the first trimester [62]. Whether methylation patterns of these growth factors during early life were involved in control of lean *vs.* fat mass and growth patterns prior to tissue collection at 2.5 years remains unknown.

The current study did not identify any mechanisms that would enable male sheep exposed to post-weaning undernutrition to catch up once nutrition was restored to normal. The absence of any effects of undernutrition in this period on glucose tolerance and peripheral insulin sensitivity suggests that these males must employ other mechanisms to regain body weight following the nutritional challenge. All sheep were allowed access to the same food rations after the nutritional challenge but feed intake of individual animals was not determined when group-housed. It remains possible that the regulation of appetite was influenced by the post-weaning nutrient challenge [63].

Although we [22] and others [16] have shown no influence of the early gestation nutritional environment or being a twin on whole body glucose tolerance, we observed here some differences between singleton and twin offspring e.g. glucose transporter 4 mRNA levels in muscle, that were increased following early gestation undernutrition but only in twins. Twins face an additional nutritional constraint during gestation, with lower fetal glucose levels in late gestation and reduced birth weight [64] suggesting that the double insult of being a twin and facing gestational undernutrition may be required to induce such an effect [65]. Enhanced glucose uptake in muscle of these animals may contribute to the increased insulin sensitivity observed in twins in this cohort [22]. For singleton offspring, the strongest influence on glucose transporter 4 expression was nutrient restriction in the post-weaning period.

In conclusion, undernutrition during development induces changes in mechanisms of lipid and glucose metabolism which differ between tissues and sexes contingent on the period of nutritional restriction. Such changes may increase later life obesity and dyslipidaemia risk.

Supporting Information

Figure S1 Flow diagram showing the total number of ewes and their offspring used in the study of adult sheep exposed to early gestation and/or post-weaning undernutrition.
(DOCX)

Table S1 Target gene primer and probe cDNA sequences for real-time PCR, methylation-sensitive PCR and pyrosequencing.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KRP JKC KAL GCB MAH LRG. Performed the experiments: KRP LJH RJSM AW AB LF JKC LRG. Analyzed the data: KRP LJH RJSM AW AB LF LRG. Wrote the paper: KRP GCB MAH LRG.

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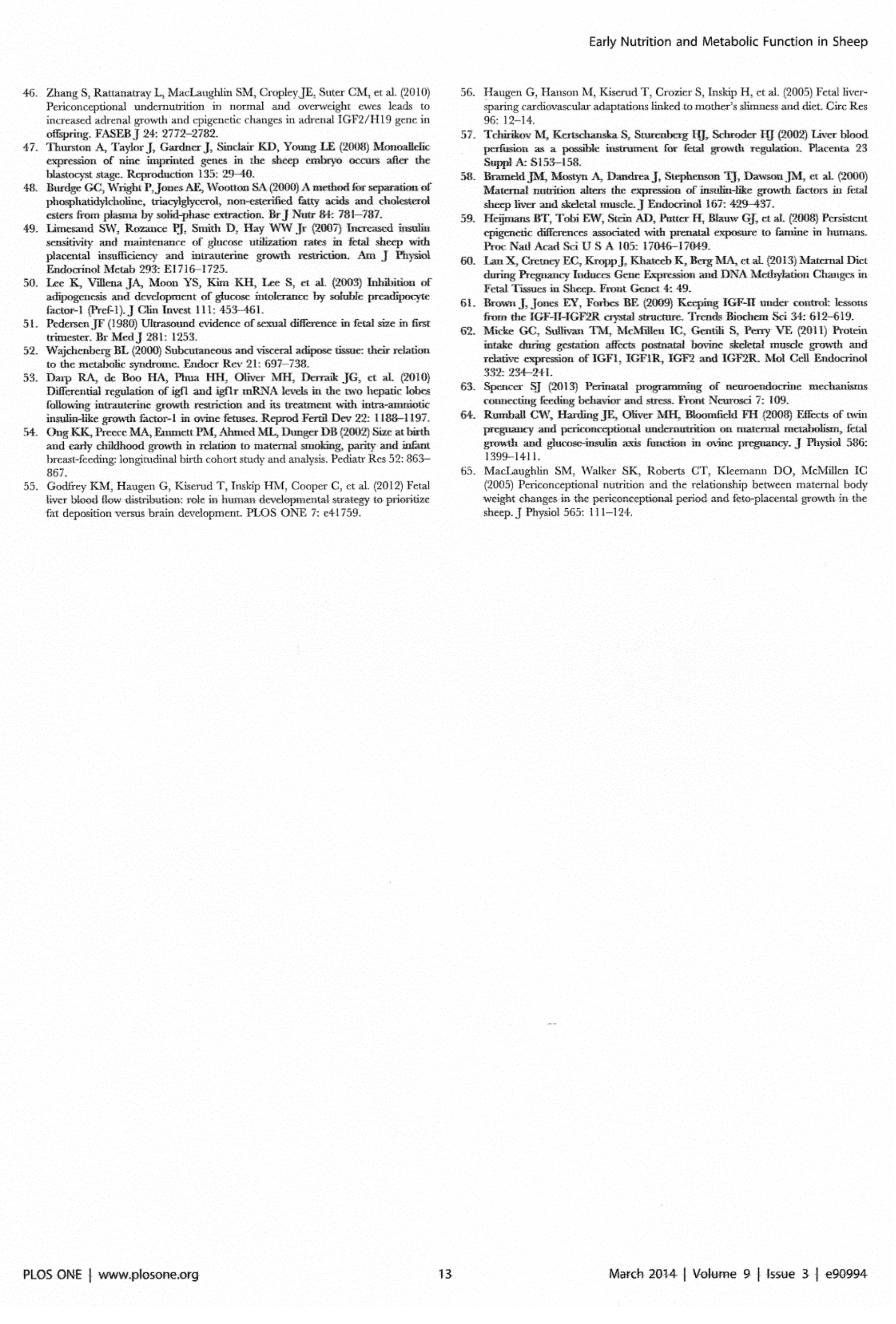

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