The influence of maternal diet on offspring development and liver metabolism

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Abstract

Altering maternal nutrition affects fetal development and can have long-lasting effects on the offspring, potentially predisposing them to later metabolic disease. These effects can occur without affecting birth weight, although small for date offspring appear to be at increased risk. One mechanism linking changes in the maternal environment to an increased risk of later disease is enhanced exposure to glucocorticoids (GC). Tissue sensitivity to cortisol is regulated, in part, by the GC receptor (GR) and 11-beta-hydroxysteroid dehydrogenase (11βHSD) types 1 and 2. Several studies have shown the effects of maternal nutrient restriction on the programming of GC action in the offspring, however, dietary excess is far more characteristic of the diets consumed by contemporary pregnant women. The aim of this thesis was to provide a novel insight into the effects of moderate changes in the macronutrient ratio, within the maternal diet fed to pigs (whilst maintaining energy content), on offspring growth, development and liver metabolism until adolescence.

Fat supplementation (Fat supplemented (FS): 9 %; Control (C): 2.5 %) from day 0 until 110 of gestation, reduced maternal glucose tolerance at term and decreased the survival rate of piglets after birth, possibly due to hypoglycaemia. In addition, supplementing the maternal diet with protein (Protein supplemented (PS): 16.3 %; C: 12.3 %) also increased the incidence of postnatal mortality, with surviving offspring demonstrating an up-regulation of mRNA transcripts involved in GC sensitivity i.e. GC receptor and 11βHSD-1, in the liver. Furthermore, this thesis demonstrated no negative effects of accelerated postnatal growth on low-birth weight piglets as others have suggested.

In conclusion, this thesis has demonstrated a detrimental effect of fat and protein supplementation until day 110 of gestation on postnatal mortality. These findings could have profound consequences for the pig industry where reducing piglet mortality is of economic importance. In addition, an increased level of protein in the diet during gestation increases GC sensitivity in the offspring which may be indicative of excess GC exposure *in utero*. These types of adaptations could have significant implications in determining the programming effects of maternal diet on adult disease risk.

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Publications

INVITED REVIEW PAPERS

1. **ALMOND, K.L.**, BIKKER, B., LOMAX, M., SYMONDS, M.E. and MOSTYN, A. (2010) The influence of maternal diet on development and liver metabolism in the offspring. *Proceedings of the Nutrition Society* (In Press).

SHORT COMMUNICATIONS

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Abbreviations

GC	Glucocorticoid
HPA	Hypothalamic pituitary adrenal
CRH	Corticotrophin releasing hormone
ACTH	Adrenal corticotrophic hormone
CBG	Corticosteroid binding globulin
GR	Glucocorticoid receptor
11β-HSD	11β-Hydroxysteroid dehydrogenase
ER	Endoplasmic reticulum
C	Control
LP	Low-protein
NR	Nutrient restricted
BW	Body weight
TAG	Triglyceride
HF	High-fat
AUC	Area under the curve
HDL	High-density lipoprotein
IGF	Insulin-like growth factor
PPAR	Peroxisome proliferator-activated receptor
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
ACC	Acetyl-CoA carboxylase
FAS	Fatty acid synthase
FABP	Fatty acid-binding protein
VFA	Volatile fatty acid
TCA	Tricarboxylic acid
NEFA	Non-esterified fatty acid
EFA	Essential fatty acid
NRC	National Research Council
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GLUT	Glucose transporter
FATP	Fatty acid transport protein
FAT	Fatty acid transporter
ACBP	Fatty acyl-CoA binding protein
ACS	Fatty acid CoA synthetase
VLDL	Very low-density lipoproteins

COSHH	Control of Substances Hazardous to Health
SLS	Scientific Laboratory Supplies
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked-immunosorbent assay
GOD	Glucose oxidase
CV	Co-efficient of variance
CoA	Coenzyme A or Acyl-CoA
MEHA	3-methylN-(hydroxyethylanaline)
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
TMB	Tetramethylbenzidine
PCA	Perchloric acid
RT-PCR	Reverse transcriptase polymerase chain reaction
PCR	Polymerase chain reaction
cDNA	Complementary DNA
mRNA	Messenger RNA
RT	Reverse transcriptase
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
UV	Ultraviolet
T_m	Melting temperature
18S	18S ribosomal RNA
RPO	Ribosomal phosphoprotein
Cyclo	Cyclophylin
TAE	Tris-acetate EDTA
QPCR	Real-time PCR
Cp	Cycle point
E	Amplification efficiency
HK	Housekeeping
M	Gene expression stability measure
V	Average pair wise variation
SEM	Standard error of the mean
FS	Fat supplemented
PS	Protein supplemented
MCT	Medium-chain triglycerides
LCT	Long-chain triglycerides
NO	Nitric oxide
NOS	Nitric oxide synthase

IUGR	Intrauterine growth retardation
GTT	Glucose tolerance test
FTIR	Fourier Transform InfaRed
BMI	Body mass index
IR	Insulin receptor
FGR	Fractional growth rate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulphate-polyacryalmide gel electrophoresis
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulphate
HPR	Horseradish peroxidise
TEMED	Tetramethylethylenediamine
PBS	Phosphate-buffered saline
RIA	Radioimmunoassay
CPM	Counts per minute

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Chapter 1 – Literature review

This thesis reports a study of supplementing the gestational diet of pigs with either fat or protein on offspring growth and development, particularly focussing on liver metabolism. The introductory chapter will highlight the role of maternal nutrition on fetal programming, specifically the effects of fat and protein. It will be followed by an outline of the use of animals as models for humans in nutritional programming studies, particularly focussing on the pig. Finally, the liver structure and function will be described in detail.

1.1 Maternal nutrition and programming of adult disease

It is now well established that a sub-optimal environment *in utero* can have pronounced effects on the development of the fetus and thus confer greater risk of disease in later life. The process whereby a stimulus or insult at a sensitive or critical period of development has long term effects is termed programming. This was first investigated by the retrospective cohort studies of David Barker and colleagues during the late 1980's who established that individuals with low-birth weight, who were short or thin at birth, or who were small in relation to placental size, were at increased risk of metabolic disease such as hypertension and impaired glucose tolerance in adulthood (Figure 1.1) (Barker et al., 1990, Hales et al., 1991).

It is presumed that low-birth weight or disproportionate body size, are indicative of a lack of nutrients or oxygen during gestation, reflecting the adaptations that the fetus has made to sustain its normal development (Barker, 1995). The highest prevalence of impaired glucose tolerance and Type 2 Diabetes were seen in individuals who were small at birth and became obese as adults (Hales et al., 1991) suggesting that it is actually the mismatch between the pre and postnatal environment that causes these effects. This is further supported by later studies using the sheep as a model for maternal under-nutrition which have shown similar detrimental outcomes on the metabolism of the offspring who are adequately nourished, without any change in birth weight (Goplakrishnan et al., 2004, Gardner et al., 2005).

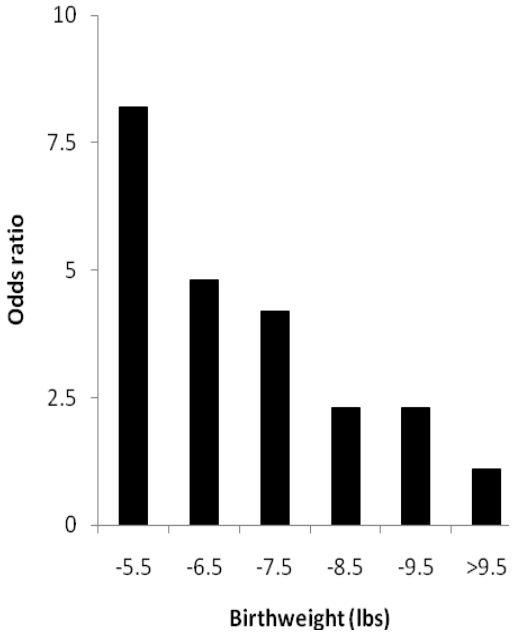

Figure 1.1 Odds ratio for developing impaired glucose tolerance or type 2 diabetes according to birth weight, among 370 men aged 64 years born in Hertfordshire (adjusted for body mass index) (Hales and Barker, 2001)

1.2 Maternal low-protein diets

Following on from these findings by Barker and colleagues, work began to investigate the cellular and molecular mechanisms behind this programming effect. Due to the importance of protein in growth and development, many studies, particularly in rats, focussed on the effects of maternal protein restriction on the offspring.

Protein restriction during pregnancy in rats has been shown to produce low-birth weight offspring with higher blood pressure in early adulthood (Table 1.1) (Langley-Evans et al., 1996, Langley-Evans et al., 1998, Bertram et al., 2001). In addition, another study which follows the pups to 18 months of age has demonstrated a reduction in offspring insulin sensitivity and perturbed triglyceride metabolism, shown by the increase in plasma cholesterol, and plasma and hepatic triglycerides, when rats where fed a low-protein diet (Table 1.1) (Ehruma et al., 2007). However, in contrast to the previous studies, birth weight was unaffected by maternal diet (Ehruma et al., 2007). Surprisingly, these changes have been demonstrated to take place despite no changes in overall energy intake of the mothers (Langley-Evans et al., 1998, Ehruma et al., 2007) or

differences in body weight or fat mass of the offspring (Ehruma et al., 2007).

1.2.1 The role of glucocorticoids (GC)

The precise mechanisms linking maternal under-nutrition, particularly low-protein diets, to adverse outcomes on the offspring are still unknown. However, studies in both sheep and rats have strongly suggested that glucocorticoids (GC) play a key role (Langley-Evans et al., 1996, Bertram et al., 2001, Whorwood et al., 2001). It is thought that fetal overexposure to maternal GC may trigger programming events *in utero* that establish persistent increases in GC hormone action in the offspring throughout life (Bertram et al., 2001, Whorwood et al., 2001).

GC are catabolic hormones, they promote gluconeogenesis and antagonise the actions of insulin to increase the concentration of glucose in the blood (Dimitriadis et al., 1997b). In addition, they are potent regulators of fetal growth and development, and are critical for promoting the maturation of fetal tissues, particularly the lung, to allow for adaptations to postnatal life (Ballard and Ballrad, 1972, Rheinisch et al., 1978). They also promote increased blood pressure by enhancing the activity of vasoactive hormones such as angiotensin II (Tangalakis et al., 1992).

GC excess has been linked to the clinical observations associated with the metabolic syndrome (Covar et al., 2000, Arnaldi et al., 2003). For example, patients with Cushing's disease who have increased secretion of cortisol, normally due to a pituitary tumour, can develop abdominal obesity, hypertension, hyperlipidemia, and insulin resistance (Arnaldi et al., 2003). Also, clinical administration of GC to treat acute and chronic inflammatory diseases has been associated with similar adverse metabolic effects (Covar et al., 2000). It therefore seems likely that programmed alterations in GC sensitivity may play a role linking maternal nutrient availability, fetal growth and metabolic disease risk.

Cortisol, the principal GC in humans, sheep, and pigs, but not rodents, is regulated by the activity of the hypothalamic pituitary adrenal (HPA) axis, a neuro-endocrine feedback loop, and is secreted in response to nutritional, physical and/or emotional stress, or low levels of circulating cortisol (Figure 1.2) (Bamberger et al., 1996). The first step of the HPA

axis is hypothalamic secretion of corticotrophin releasing hormone (CRH), which in turn triggers pituitary secretion of adrenal corticotrophic hormone (ACTH). ACTH is carried in the circulation to the adrenal cortex were it stimulates the secretion of cortisol. Most serum cortisol is bound to corticosteroid binding globulin (CBG) and only 'free' cortisol is active.

GC are also highly regulated at a tissue specific level by intracellular expression of GC receptor (GR), and the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes at the level of gene transcription (Figure 1.2) (Bamberger et al., 1996). There are two isoforms of 11ß-HSD, both are found in the endoplasmic reticulum (ER) and are responsible for the tissue-specific inter-conversion of cortisone to cortisol. 11β-HSD1 behaves as an 11-oxo-reductase, catalysing the conversion of cortisone to bioactive cortisol (Stewart and Krozowski, 1997). Conversely 11ß-HSD2 behaves as an 11-oxo-dehydrogenase and catalyses the opposite reaction (Stewart and Krozowski, 1997). The two isozymes are products of two different genes and have distinct tissue distributions, with 11β-HSD1 expressed primarily in the liver, adipose tissue, kidney and brain, and 11β-HSD2 mainly in the kidney and salivary glands (Walker and Stewart, 2003, Wang, 2005).

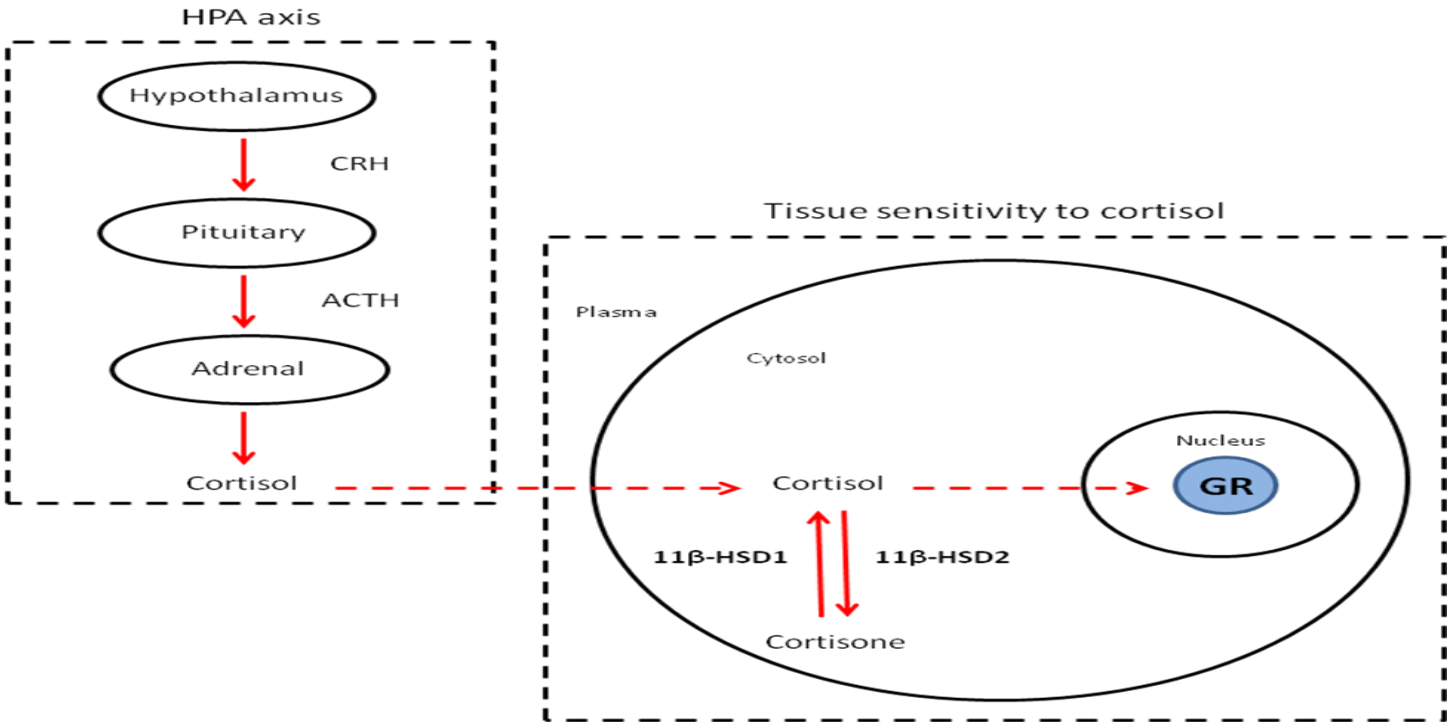

Figure 1.2 Diagrammatic representation of cortisol secretion via the hypothalamic pituitary adrenal (HPA) axis and its regulation at a tissue specific level. CRH = corticotrophin releasing hormone; ACTH = adrenal corticotrophic hormone; 11β-HSD = 11β-hydroxysteroid dehydrogenase; GR = glucocorticoid receptor

11β-HSD2 is expressed at high levels in feto-placental tissues and is thought to play a key role in protecting the fetus from overexposure to maternal cortisol (Stewart et al., 1995, Edwards et al., 1996, Langley-Evans et al., 1996, Condon et al., 1998, Whorwood et al., 2001). In the rat, the effects of the maternal low-protein diet on reducing offspring birth weight and programming of hypertension and dysregulation of glucose metabolism are thought to be mediated by the inhibition of placental 11β-HSD2 (Langley-Evans et al., 1996, Langley-Evans, 1998, Bertram et al., 2001, Ehruma et al., 2007). This is also seen in human studies were a positive relationship between placental 11β-HSD2 activity and fetal weight has been identified (Stewart et al., 1995).

Studies in both rats and sheep have shown that maternal diet programs increased GC sensitivity, at a tissue-specific level in both the fetal, neonatal and adult offspring (Bertram et al., 2001, Whorwood et al., 2001). This is determined by an increase in GR mRNA and protein expression and a reduction in 11β-HSD2 and/or 11β-HSD1 gene expression and/or activity. It is thought that this increase in GC sensitivity is due to a reduction in placental mRNA expression and enzyme activity of 11β-HSD2 (Bertram et al., 2001, Whorwood et al., 2001). In sheep, GR mRNA and protein expression is increased in the adrenals, liver, lungs, perirenal adipose tissue, and kidney of neonatal offspring born to ewes who were nutrient restricted during early-mid gestation (Table 1.1) (Whorwood et al., 2001). In addition, there was a 50 percent reduction in 11β-HSD2 mRNA expression in all tissues in which this key enzyme was found to be abundant, such as in the kidneys and adrenals (Whorwood et al., 2001). 11β-HSD1 expression was unaffected by maternal diet, except in perirenal adipose tissue, where there was a two-fold increase in mRNA abundance (Whorwood et al., 2001). Importantly, these effects were observed without any significant alterations in the maternal or fetal metabolic or endocrine environment (Clarke et al., 1998, Brameld et al., 2000). Similar findings were shown in rat studies when dams were protein restricted throughout gestation (Table 1.1) (Bertram et al., 2001). These offspring were shown to have decreased mRNA expression of 11β-HSD2 in the kidney from birth until adulthood (5 months of age), with no effect on 11β-HSD1 (Bertram et al., 2001). In addition, GR mRNA and protein expression were increased in peripheral tissues, such as the kidneys and lungs, from both late fetal (day 20) and neonatal offspring up to 12 weeks

of age (Bertram et al., 2001). Therefore, an increase in GC sensitivity in the offspring due to sub-optimal maternal nutrition is associated with an increased risk of metabolic disease (Figure 1.3) (Bertram et al., 2001, Whorwood et al., 2001).

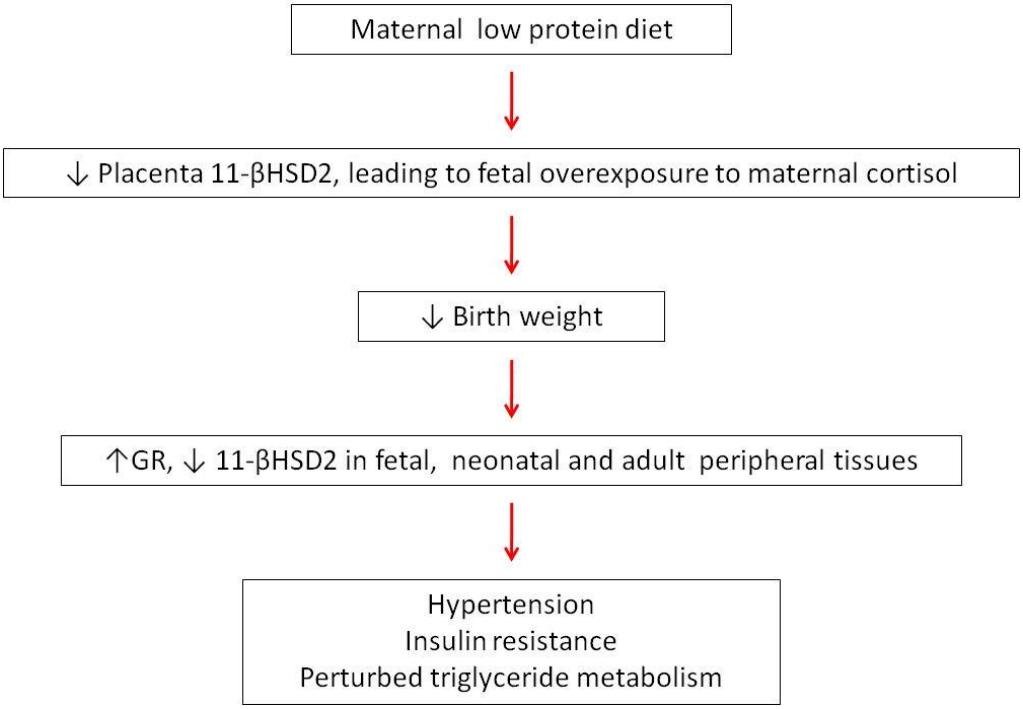

Figure 1.3 Role of maternal low-protein diet and glucocorticoids in the programming of offspring metabolic disease. 11β-HSD = 11β-Hydroxysteroid dehydrogenase; GR = Glucocorticoid receptor; Adapted from: (Bertram et al., 2001)

Table 1.1 Summary of effects of a maternal low-protein diet or nutrient- restriction during pregnancy on offspring development

		Placenta		Offspring			
Source	**Species and diet composition**	**Size**	**11β-HSD2**	**Birth weight**	**Age analysed**	**GC sensitivity**	**Phenotype**
Bertram et al. (2001)	Rats throughout pregnancy C: 18 % protein LP: 9 % protein	n/a	↓ mRNA	↓	Birth to 5 months	↑GR mRNA + protein (peripheral tissues) ↓ 11β-HSD2 mRNA (kidney)	↑ Blood pressure
Erhuma et al. (2007)	Rats throughout pregnancy C: 18 % protein LP: 9 % protein	n/a	n/a	n/a	1, 19 and 18 months	n/a	No difference in BW ↑ Insulin resistance ↑ Liver TAG
Langley-Evans et al. (1996 & 1999)	Rats throughout pregnancy C: 18 % protein LP: 9 % protein	↑	↓	↓	7 weeks	n/a	No difference in BW ↑ Blood pressure
Whorwood et al. (2001)	Sheep, days 28-77 of gestation C: 110 % Requirements NR: 50 % Requirements	↑	↓mRNA	No difference	Fetal (d77) and birth	↑GR and ↓ 11β-HSD2 mRNA (peripheral tissues)	↑ Kidney weight

C = Control; LP = Low-protein; NR = Nutrient restricted; BW = Body weight; ↑ = Increased in low-protein or nutrient restricted gestational groups; ↓ = Increased in low-protein or nutrient restricted gestational groups; GC = Glucocorticoid; 11β-HSD = 11β-Hydroxysteroid dehydrogenase; GR = Glucocorticoid receptor; TAG = Triglyceride

1.3 Maternal high-fat diets

This literature review has already discussed the link between maternal nutrition and programming of metabolic disease in the offspring. Human epidemiological studies first indicated that if you lived in a 'poor' industrial area your nutritional status was likely to be decreased (Barker and Osmond, 1986). This initiated several investigations, focussed on the associations of maternal protein-restriction or global under-nutrition, with birth weight and later disease. However, fetal nutrition and development may also be compromised by the imbalance of macronutrients in the maternal diet, with less obvious or measurable changes in neonatal phenotype.

1.3.1 High-fat intake and insulin resistance

The most common dietary imbalance in Western populations is excessive intake of dietary fat. There is widespread evidence in both human and animal studies to suggest that consumption of a high-fat diet increases the risk of developing insulin resistance, sometimes without an increase in fat mass (Maegawa et al., 1986, Storlien et al., 1986, Kraegen et al., 1991, Mayer et al., 1993, Mayer-Davis et al., 1997, Lovejoy, 2002, Storlien et al., 2006). Often this occurs without any change in energy intake (Maegawa et al., 1986, Kraegen et al., 1991), particularly in rodent studies, where, despite high-fat diets having an increased energy content (Maegawa et al., 1986), calorie intake between groups is often the same, or even reduced, perhaps due the reduction in palatability of the diets or the ability of the animals to effectively regulate their own energy intake.

Under normal conditions, pregnancy is an insulin resistant state due to constant utilisation of glucose by the fetus, resulting in maternal hypoglycaemia and progressive alterations in glucose metabolism (Frienkel, 1980, Leturque et al., 1980, Pere and Dourmad, 2000, Pere and Etienne, 2007). In addition, extra fat is deposited in the maternal body in preparation for increased energy demand during lactation, thus contributing to the insulin resistance (Jen et al., 1987). Consumption of a high-fat diet during pregnancy is thought to exaggerate this effect, shown by an increase in fasting plasma insulin concentrations in rats on day 20 of gestation (Table 1.2) (Guo and Jen, 1995, Taylor et al., 2003), resulting in an increase in glucose: insulin ratio (Guo and Jen, 1995). This occurred despite no difference in body weight of the dams (Guo and Jen, 1995,

Taylor et al., 2003). However, there was a larger fat mass in the dams fed a high-fat diet during pregnancy (Guo and Jen, 1995). In addition, fasting plasma corticosterone (rodent equivalent of cortisol) was significantly increased when dams were fed a high-fat diet during pregnancy (Taylor et al., 2003).

Maternal fat supplementation during pregnancy has also been shown to effect litter size, but with conflicting results between studies. Previous investigations have shown that feeding a high-fat diet to rats throughout gestation results in a reduction in litter size, although the reasons for this are not clear (Table 1.2) (French et al., 1952, Buckley et al., 2005). However, the amount of protein was slightly reduced in these studies, so this may be a contributing factor. Although, the improved quality of the protein in the high-fat diet should minimise this difference (French et al., 1952). Similarly, another study showed that feeding a high-fat diet throughout pregnancy and lactation, resulted in a reduction in the number of successful pregnancies by 12 percent (Table 1.2) (Khan et al., 2003). However, other studies have reported no effects of high-fat diet on litter size (Table 1.2) (Guo and Jen, 1995, Taylor et al., 2003, Taylor et al., 2005).

The effect of maternal fat supplementation on offspring birth weight is also subject to some controversy, with some studies reporting a reduction in birth weight (Table 1.2) (French et al., 1952, Taylor et al., 2003), whilst others showed no difference (Table 1.2) (Guo and Jen, 1995, Taylor et al., 2005, Buckley et al., 2005). Such a response is surprising, since in humans, gestational diabetes (characterised by impaired glucose tolerance during pregnancy in women without previously diagnosed diabetes), which is associated with maternal obesity (Chu et al., 2007), has been shown to result in fetal macrosomia (Figure 1.4) (Jovanovic and Pettitt, 2001, Schmidt et al., 2001, Franks et al., 2006). This is due to maternal hyperglycaemia which results in nutrient excess, as well as stimulating hyperinsulinemia (Calvo et al., 1998). Insulin-like growth factor-1 (IGF-1) production ultimately results in excess fetal growth (Menon and Sperling, 1996).

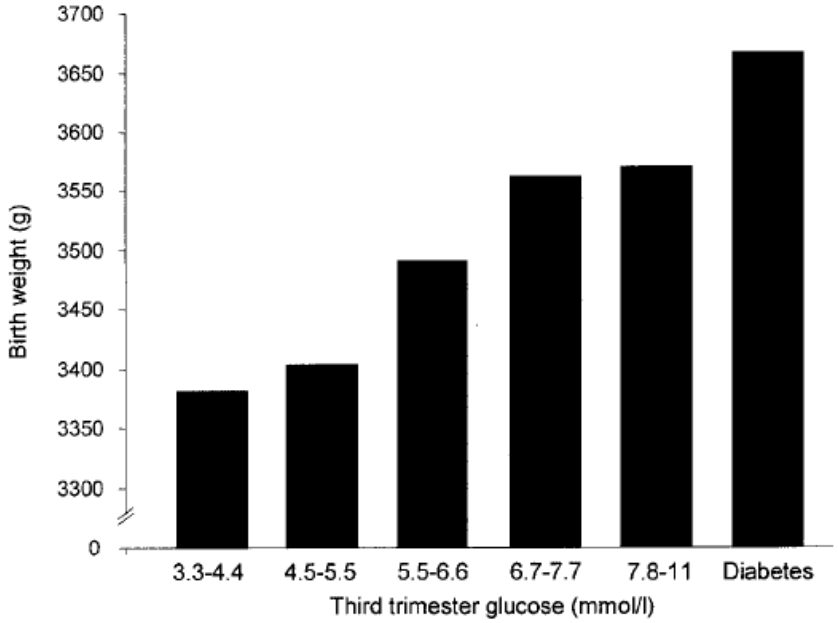

Figure 1.4 Association between maternal third-trimester plasma glucose following an oral glucose challenge (after 2 hours), and offspring birth weight in Pima Indians (Franks et al., 2006)

When evaluating the effects of fat supplementation during pregnancy, it is important to closely examine the composition of the diets fed to the rats. Most studies try to mimic the fat composition of the human diet (around 30 percent) which results in an unnaturally high fat content that would not usually be seen in the wild and would normally contain only 4-5 percent fat (French et al., 1952, Guo and Jen, 1995, Khan et al., 2003, Taylor et al., 2003, Taylor et al., 2005). The source and type of fat may also have important effects on the pregnancy outcomes, with many studies using animal lard as the fat supplement, in comparison to the control which uses vegetable oil (Khan et al., 2003, Taylor et al., 2003, Taylor et al., 2005). Finally, it is often not only the fat content of the diet which changes between the control and experimental groups, but also other components of the diet such as protein and carbohydrate quantities which would also be expected to affect the birth outcomes. For example, the high-fat diet in one study also had sucrose and corn starch added, and there was a reduction in fibre content in comparison the control diet (Guo and Jen, 1995). In addition, even if the carbohydrate and protein contents appear to be similar between experimental groups, the reduction in feed intake often caused by fat supplementation will significantly reduce the amount of carbohydrate or protein in relation to the control group (Khan et al., 2003, Taylor et al., 2003, Taylor et al., 2005).

Table 1.2 Summary of effects of maternal fat supplementation in rats throughout gestation

Source	Diet composition	Calorie intake	Maternal body weight	Maternal insulin	Litter size	Birth weight
Buckley et al. (2005)	C: 12 % fat HF: 59 % fat (safflower oil)	n/a	n/a	n/a	↓	No difference
French et al. (1952)	C: 100 % control diet (4 % fat) HF: 80 % control diet plus 20 % corn oil	No difference	n/a	n/a	↓	↓
Guo & Jen (1995)	C: 45g/Kg fat (unknown source) HF: 400 g/Kg fat (vegetable oil)	↓	No difference ↑ Fat mass	↑	No difference	No difference
Khan et al. (2003)	C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	No difference	n/a	n/a	↓	n/a
Taylor et al. (2003)	C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	No difference	No difference	↑	No difference	↓
Taylor et al. (2005)	C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	No difference	n/a	n/a	No difference	No difference

C = Control; HF = High-fat; ↑ = Increased in high-fat group; ↓ = Decreased in high-fat group

1.3.2 High-fat maternal diet and offspring development

There is now growing evidence to suggest that consumption of a high-fat diet during pregnancy may also contribute to metabolic abnormalities in the offspring. In humans, it is well documented that mothers with gestational diabetes, perhaps due to consumption of a high-fat diet or maternal obesity, have an increased risk of their offspring being of very high-birth weight and developing of obesity and impaired glucose tolerance at an early age (Figure 1.5) (Boney et al., 2005, Franks et al., 2006).

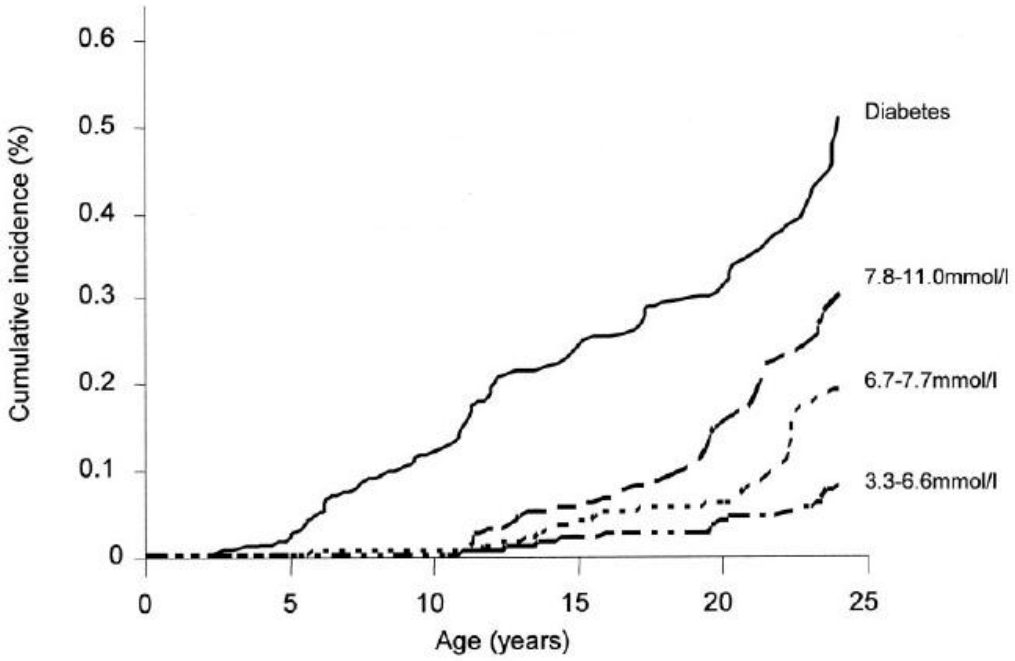

Figure 1.5 Cumulative incidence of type 2 diabetes stratified by category of maternal third trimester plasma glucose concentrations following a glucose tolerance test (2 hours after 75g oral glucose challange) (Franks et al., 2006)

Similar effects have been demonstrated by high-fat feeding in rats, where pups showed characteristics of the metabolic syndrome seen in humans, including; hypertension, hyperlipidemia and impaired glucose tolerance, despite no changes in birth weight (Table 1.3) (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). At birth, rat pups born to mothers fed a high-fat diet during pregnancy and lactation have significantly lower fasting glucose concentrations, with an increase in liver triglycerides (Table 1.3) (Guo and Jen, 1995). Similarly in another study, feeding a high ω-6 poly-unsaturated fat and low carbohydrate diet during pregnancy resulted in an increase in insulin secretion after an oral glucose challenge, increased

abdominal fat mass and a higher liver triglyceride content in males at 3 months of age (Table 1.3) (Buckley et al., 2005).

High-fat feeding throughout pregnancy and lactation, has been shown to increase the risk of hypertension (Table 1.3) (Khan et al., 2003, Khan et al., 2005) and reduce glucose tolerance (Taylor et al., 2005) in female offspring at 6 months and 1 year of age. This was accompanied by an increase in; body weight, adiposity and fasting plasma glucose and triglycerides (Khan et al., 2003, Taylor et al., 2005). These findings are similar to the outcomes shown in rat offspring born to mothers who were fed a low-protein maternal diet (Section 1.2) (Langley-Evans et al., 1996, Langley-Evans et al., 1998, Bertram et al., 2001).

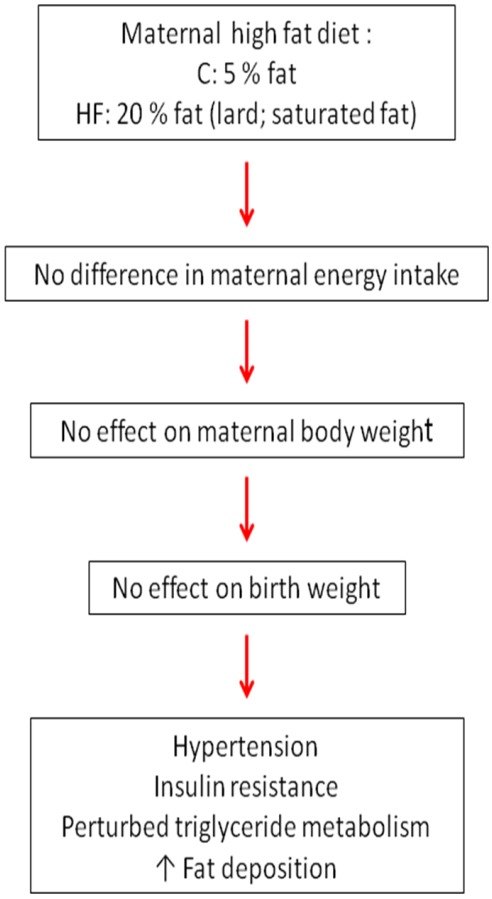

Figure 1.6 Role of maternal high-fat diet in the programming of offspring metabolic disease; C: Control; HF: High-fat; Adapted from (Khan et al., 2003, Taylor et al., 2005).

Table 1.3 Summary of effects of maternal fat supplementation in rats throughout gestation on offspring outcome

Source	Maternal diet composition	Age and sex analysed	Phenotype	Plasma profile	Liver TAG
Buckley et al. (2005)	Throughout pregnancy C: 12 % fat HF: 59 % fat (safflower oil)	Males 3 months	↑ Risk of insulin resistance ↑ Fat mass	No difference in fasting glucose and insulin ↑ Insulin AUC, no difference in glucose AUC	↑
Guo & Jen (1995)	Throughout pregnancy and lactation C: 45g/Kg fat (unknown source) HF: 400 g/Kg fat (vegetable oil)	Males and females Birth and weaning	↑ Fat mass	No difference in insulin or TAG at birth ↓ Glucose at birth ↑ Glucose and TAG at weaning	↑
Khan et al. (2003)	Throughout pregnancy and lactation C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	Males and females 6 months and 1 year	↑ Blood pressure in females	No difference	n/a
Khan et al. (2005)	Throughout pregnancy and lactation C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	Males and females 6 months	↑ Blood pressure in females ↑ Fat mass	No difference in lipid or glucose ↑ Insulin	n/a
Taylor et al. (2005)	C: 5 % fat (vegetable oil) HF: 25 % fat (animal fat)	Females 6 months and 1 year	↑ Insulin resistance (1 year) ↑ Fat mass	↑ Insulin and glucose ↑ TAG and ↓ HDL (1 year)	n/a

C = Control; HF = High-fat; ↑ = Increased in high-fat group; ↓ = Decreased in high-fat group; AUC, Area under the curve; TAG = Triglycerides; HDL = High-density lipoprotein

1.4 High-protein diets

1.4.1 Maternal protein supplementation

Following on from the investigations into the effects of maternal low-protein diets in rats on the programming of offspring, studies began into the effects of a high intake of protein during pregnancy. It was conceivable that if maternal consumption of a low-protein diet resulted in reduced birth weight and increased risk of hypertension, than a high-protein diet may produce the opposite and perhaps beneficial effects on the offspring. Surprisingly, in rats this appears not to be the case and an isocaloric maternal diet with increased protein ratio (high-protein: 40 % protein; control: 20 % protein) was associated with a reduction in birth weight of male offspring, followed by accelerated postnatal growth (Daenzer et al., 2001). In contrast to this, another study which fed a similar isocaloric, high-protein diet (high-protein: 40 % protein; control: 20 % protein) throughout gestation and lactation, demonstrated no effects on offspring birth weight (Thone-Reineke et al., 2006). However, the male offspring developed significantly higher blood pressure between 4 and 22 weeks of age (Thone-Reineke et al., 2006). In addition, glucose tolerance was measured in these animals at 22 weeks of age, with no significant difference between groups (Thone-Reineke et al., 2006). Another study in rats demonstrated no significant effects of a high protein, increased energy maternal diet (high protein: 54 % protein; control: 20 % protein) on offspring birth weight and blood pressure from 5 to 30 weeks of age (Zimanyi et al., 2002). This lack of significant findings could, perhaps, be due to the significant reduction in food intake seen in the high-protein fed dams, so that energy and protein intake was similar between groups (Zimanyi et al., 2002).

There is insufficient evidence in humans that increased protein intake or dietary protein supplementation during pregnancy affects offspring birth weight or postnatal growth due to the conflicting outcomes of epidemiological studies (Mathews et al., 1999, Kramer and Kakuma, 2003).

To date, no studies have examined the effect of a high-protein maternal diet on GC sensitivity in the offspring to see if it mimics the effects of a low-protein diet. This is probably due to the lack of a differential phenotype caused by maternal high-protein feeding.

1.4.2 High-protein intake in early life

There have been several investigations into the dietary manipulations during early life and the effects on growth and body composition of children as they age (Rolland-Cachera et al., 1995, Hoppe et al., 2004). Focus has been on the influence of protein in the diet, which, increases dramatically in infants during the period of complimentary feeding and the transition to the family diet (Alexy et al., 1999). In addition, formula-fed infants grow at a faster rate than breast-fed infants, perhaps due to an increase in protein intake in the formula-fed infants (Heinig et al., 1993).

It has been suggested that a higher than average protein intake (% of energy intake) at 2 years of age could increase the risk of adiposity by the age of 10 (Rolland-Cachera et al., 1995). The proposed mechanism behind this is that high-protein intake triggers secretion of IGF-1 and thereby accelerates growth and increases adipose tissue and muscle mass (Wabitsch et al., 1995, Dardevet et al., 1991). In contrast, a Dutch cohort study found no significant association between protein intake at 9 months of age and adiposity by 10 years, despite a significant association with body weight and IGF-1 concentrations (Hoppe et al., 2004). This difference in study outcome may be due to the differences in time that protein intake was measured during early life i.e. at 9 months compared with 2 years. This is further supported by Rolland-Cachera et al. (1995) who demonstrated no association between protein intake and later adiposity at 8 months of age. One potential limiting factor of these studies is the reliance on food diaries to record protein intake, and so the true protein intake may not have been accurately recorded. In addition, the role of breast-feeding versus formula feeding in early life may play a crucial role in determining adiposity and body weight in later childhood.

1.4.3 Benefits of high-protein diets in obese subjects

Obesity is often associated with insulin resistance, hyperlipidemia, hypertension and fatty liver disease (Grundy et al., 2004). This cluster of metabolic disorders, collectively known as metabolic syndrome, is known to increase the incidence of type 2 diabetes and cardiovascular disease (Grundy et al., 2004). Due to the growing rise in the incidence of obesity in the developed world, studies have begun to focus on the manipulation of

the macronutrient content of the diet to try and improve weight loss in obese individuals (Piatti et al., 1994).

Substituting dietary carbohydrate with protein has been shown to produce beneficial effects when combined with calorie restriction (Piatti et al., 1994, Parker et al., 2002). For example, insulin sensitivity and fat free-mass has been shown to increase in obese subjects who consumed a calorie restricted (3347 KJ/day) high-protein diet (high-protein: 45 % protein: 35 % carbohydrate) when compared to an isocaloric high-carbohydrate diet (control: 20 % protein: 60 % carbohydrate) for 21 days (Piatti et al., 1994). Similar effects were seen in obese women with type 2 diabetes who consumed a low-energy (6994 KJ/day), high-protein diet (high-protein: 30 % protein: 40 % carbohydrate; control: 16 % protein: 35 % carbohydrate) for 8 weeks followed by a 4 week energy-balanced period, however, this finding was not consistent in men (Parker et al., 2002). In contrast, energy restriction (6276 KJ/day) and an increased protein to carbohydrate ratio (high-protein: 30 % protein: 45 % carbohydrate; control: 16 % protein: 57 % carbohydrate) had no effects on insulin sensitivity or fat-free mass in obese patients with hyperinsulinemia, although, these patients were all normoglycemic (Farnsworth et al., 2003).

Similar findings were shown in obese rats where feeding a high-protein (high-protein: 59 % protein: 14 % starch; control: 21 % protein: 53 % starch, calorie restricted (30 %) diet reduced abdominal obesity, and plasma and hepatic triglycerides, to normal levels (Uebanso et al., 2009). Interestingly, feeding a similar high-protein and low-carbohydrate diet (high protein: 45 % protein: 19 % starch; control: 15 % protein: 41 % starch) to non-obese rats from 8-17 weeks results in no significant differences in glucose tolerance or body weight (Okitolonda et al., 1988).

To elucidate the cellular mechanisms underlying the effects of diet on obese subjects, the expression of genes involved in lipid homeostasis in the rat liver was examined (Uebanso et al., 2009). Genes involved in the regulation of fatty acid oxidation such as peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) and PPARα, were up-regulated in the liver of high-protein fed rats (Uebanso et al., 2009). In contrast, expression of genes involved in lipogenesis such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were reduced (Uebanso et al., 2009).

Furthermore, genes involved in fatty acid transport such as fatty acid-binding protein 1 (FABP1) were also expressed at levels that were significantly higher in the protein fed rats (Uebanso et al., 2009).

1.5 Animal models for nutritional programming

Animal models are required to examine the underlying physiological, biochemical, and molecular biological mechanisms behind the nutritional programming of offspring disease in a manner that would be unethical in humans (Litten-Brown et al., 2010). An advantage of using animal models is that the effect of nutrition can be assessed independently from confounding factors such as genetics and social status under controlled conditions. In general, animal responses to experimental procedures are qualitatively similar to humans, thus allowing more extreme dietary manipulation in animals to be used to predict what may happen in humans (Litten-Brown et al., 2010).

Various species have been used as models to study the effects of nutrition on fetal programming, the most common being the rat and the sheep (McMullen and Mostyn, 2009). Primates are the ideal animal model due to their similarities to humans, but long lifespan, expensive housing, and ethical considerations, limit their use (McMullen and Mostyn, 2009). Although rodents are small, inexpensive, and are ideal in multivariate experiments, there are numerous differences between rodents and humans (McMullen and Mostyn, 2009). One of the major limitations is that rodents are altricial animals, born with an undeveloped brain and endocrine system, with significant maturation of organs during the weaning period (McMullen and Mostyn, 2009). In contrast to the rat, sheep have a similar rate of pre- and postnatal growth to humans, and only produce one or two offspring, weighing between 3 and 6 kilograms, not unlike humans (McMullen and Mostyn, 2009).

Due to the differences in the digestive system of sheep and humans, with sheep being ruminants, they have been used mainly to look at the effects of global under-nutrition during pregnancy rather than specific macronutrient manipulations such as low-protein or high-fat, seen in rat studies. The main difference between ruminants and non-ruminants is that they have a four-compartment stomach, ideally suited to digestion of fibre, particularly cellulose (McDonald et al., 1981). The first two chambers, the

rumen and reticulum, contain dense populations of microbes which secrete celulases that hydrolyse the beta-glycosidic bonds in fibre (McDonald et al., 1981). Break down of the fibre by these microbes produces volatile fatty acids (VFA) which are absorbed through the rumen wall and are the main energy source for ruminants (McDonald et al., 1981). The three main VFA are acetate, butyrate and propionate. Acetate and propionate can be transformed to ketones, which, if required, can then be converted to acetyl-coA and enter the citric acid cycle. In addition, VFA can be used for fatty acid syntheses and gluconeogenesis. Dietary protein is also fermented by the microbes predominantly producing ammonia, which the bacteria require for synthesising microbial protein, making it an efficient symbiotic relationship (McDonald et al., 1981).

The digested material from the rumen-reticulum then moves into the omasum where water is added and many inorganic materials are absorbed into the blood stream. Carbohydrates, such as sugars and starch, and proteins (including microbes) not digested in the rumen are transferred to the abosamum (McDonald et al., 1981). This is the direct equivalent to the monogastric stomach (for example that of the pig and human) and digestion occurs here in much the same way. The digestion of fat is limited in the rumen and most occurs in the small intestine (McDonald et al., 1981).

1.5.1 The pig as a model for nutritional programming

In recent years, the pig has become increasingly popular as an animal model for human disease (Litten-Brown et al., 2010). In this thesis the pig was used due to its similarities with humans in terms of the physiology and anatomy of the digestive system (Miller and Ullrey, 1987, Pond, 1991). The general architecture of the gastrointestinal tract of the pig and the human are similar. Notable differences include a higher proportion of cardiac mucosa lining the stomach of the pig, and the presence of a cecum in the pig compared with only the appendix in the human (Pond, 1991). The digestive functions of each segment of the gastrointestinal tract are similar with comparable enzyme activities and organ secretions (Pond, 1991). In addition, the endocrine and paracrine control of gastrointestinal tract growth, motility and overall function, appear to be similar (Pond, 1991). Finally, both pigs and humans are able to utilise some fibre as a source of

energy due to the fermentation that occurs in the large intestine (Pond, 1991).

The nutrient requirements during infancy, growth, reproduction, and lactation, are similar between the human and pig (Miller and Ullrey, 1987, Pond, 1991, Pond and Mersmann, 2001). In addition the neonatal pig is similar to the human infant with respect to the stage of development and function of several organs systems. The large litter size, which allows for multiple comparisons and birth weight studies, and the high growth rate after birth, also makes the pig an attractive candidate for nutritional intervention studies.

Table 1.4 A comparison of reproductive and general anatomical/physiological factors between humans and pigs; Adapted from (Litten-Brown et al., 2010)

	Human	**Pig**
Type of placenta	Disocid haemochorial	Diffuse epithelial chorial
Placental transfer		
Glucose	Occurs	Occurs
Amino acids	Occurs	Occurs
NEFA	Occurs	Limited occurrence
Immunoglobulins	Occurs	Does not occur
Length of gestation	38 weeks	115 days (16.5 weeks)
Number of offspring	1 to 2	8-12
Maturity at birth	Precocious	Precocious
Weight of offspring (Kg)	3.2	1.5
Possesses brown adipose tissue at birth	Yes	No
Adipose tissue (% body weight)	14	<2
Postnatal energy metabolism		
Total available lipid (g/Kg body weight)	150	11
Total available hepatic glycogen (g/Kg body weight)	3.78	2.50
Newborn period	28 days	14 days
Infant period	0.08 to 2 years	15 to 75 days
Juvenile period	3 to 13 years	76 to 115 days
Age at puberty (males)	13 to 16 years	5 to 18 months
Age at puberty (female)	11 to 14 years	6 months

NEFA = Non-esterified fatty acid

1.5.1.1 Placental structure

A primary determinant of fetal growth and development is the nutrient supply across the placenta, which in turn depends on the size, morphology, blood supply and transporter abundance of the placenta, and on the synthesis and metabolism of nutrient and hormones by the uteroplacental

tissues (Fowden et al., 2006). There are two characteristics used for the classification of placental structure, which differs amongst species (Bowen, 2000). These are; firstly, the gross shape of the placenta and the distribution of contact sites between fetal membranes and endometrium, and secondly, the number of layers of tissue between maternal and fetal vascular systems.

Just prior to the formation of the placenta, there are a total of six layers of tissue separating the maternal and fetal blood (Figure 1.7) (Bowen, 2000). There are three layers of fetal extraembryonic membranes in the chorioallantoic placenta of all animals, all of which are components of the mature placenta. These include the endothelium lining allantoic capillaries, connective tissue in the form of chorioallantoic mesoderm, and the chorionic epithelium, the outermost layer of fetal membranes. There are also three layers on the maternal side, but the number of these layers that are retained, and not destroyed by the process of placentation, varies greatly among species (Bowen, 2000).

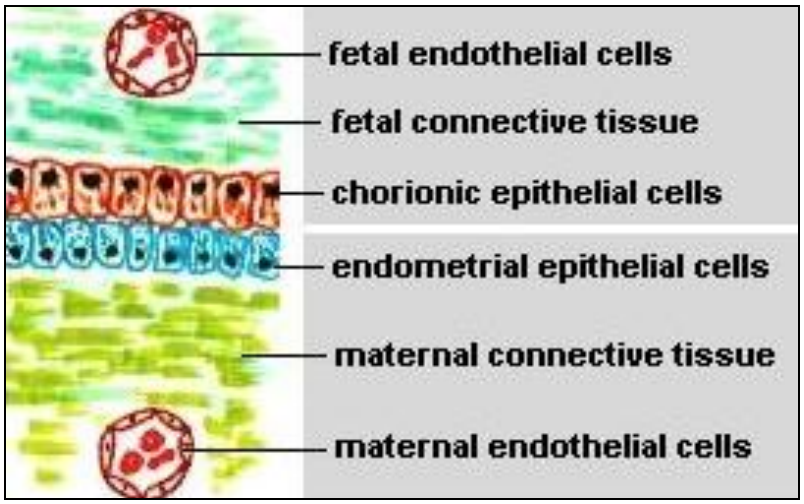

Figure 1.7 Diagrammatic representation of the structure of the placenta (Bowen, 2000)

In the human, the surface area of the placenta is limited to a discoid region, and belongs to the hemochorial category ((Litten-Brown et al., 2010, Bowen, 2000). This means the fetal chorionic epithelium is bathed in maternal blood because chorionic villi have eroded through maternal endothelium (Bowen, 2000). In contrast, pigs have a diffuse placenta where almost the entire surface of the allantochorion is involved in the formation of the placenta (Hughes and Varley, 1980, Bowen, 2000, Litten-

Brown et al., 2010). In addition, the chorionic epithelium of the pig fetus remains separated from the maternal blood by three layers of tissue (Bowen, 2000, Litten-Brown et al., 2010). There are also differences in the transport capabilities of both the human and pig placentas, and it is believed that there is limited transfer of non-esterified fatty acids (NEFA) in the pig (Mersmann and Pond, 2001). Another important difference is that the piglet is unable to acquire passive immunity from its mother *in utero* due to the inability to transport immunoglobulins across the placenta (Blecha, 2001).

1.5.1.2 Fetal growth and development

There is a well-defined, programmed, sequence of developmental changes that occur at specific times in the growth and development of the embryo and the fetus. The underlying principles of fetal maturation appear to be comparable between mammals, although there are quantitative differences between species (Litten-Brown et al., 2010).

In most species, the majority of growth occurs during the second half of pregnancy. In the human, fetal growth rate is relatively slow up to the 20th week of pregnancy but accelerates to reach a maximum around 30-36 weeks, declining thereafter until birth (Figure 1.8) (Book and Bustad, 1974, Litten-Brown et al., 2010). At birth the pig has not reached its peak growth rate and therefore there is no pre-parturition decline (Figure 1.8) (Book and Bustad, 1974, Litten-Brown et al., 2010). In litter-bearing species, the number of developing fetuses is inversely proportional to the ultimate size of each individual at full time. The pattern of fetal growth in pigs is determined by several factors including; the genome of the developing fetus, maternal nutrition and health, uterine capacity, and fetal position within the uterine horn (Litten-Brown et al., 2010).

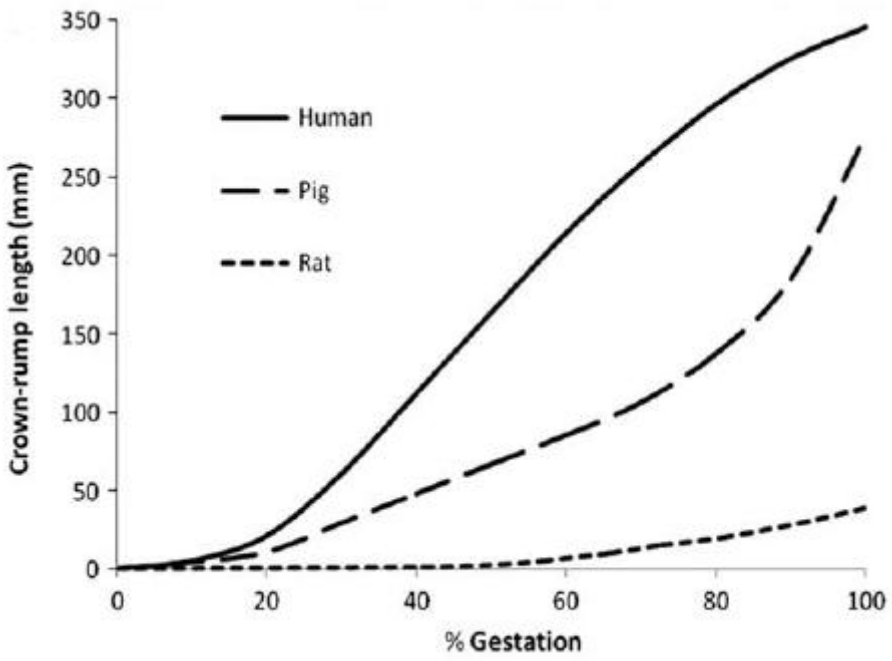

Figure 1.8 Comparison of the length of the human, pig and rat fetus as a function of gestational age; Adapted from (Book and Bustad, 1974)

Protein accumulation tends to occur early in human fetal development to reach its maximum around week 35, and precedes fat accumulation, most of which is subcutaneous, and only exceeds the weight of protein deposition by week 38 (Book and Bustad, 1974, Shulman, 1993). By term, the human infant has substantially more fat than the piglet (Shulman, 1993). In contrast to humans, body fat in the newborn pig is minimal, usually no more than 2 percent of body weight (Shulman, 1993), and most of this fat is structural and therefore unavailable for use as energy. However, within the first week of life, during the time of rapid growth, there can be a 10 to 20-fold increase in body fat (Pond and Mersmann, 2001). Similarly, rates of protein deposition and lean tissue growth are also very high during this time (Pond and Mersmann, 2001). Well fed piglets can double their birth weight in 7-10 days, whereas the infant doubles its birth weight by about 5 months of age (Shulman, 1993), thus piglets can provide an excellent model of accelerated growth and development.

1.6 Nutritional requirements of pigs

The ultimate goal of commercial pig production is the economical production of muscle tissue that is both a nutritious, healthy and palatable source of dietary protein for human consumption (Mitchell et al., 2001).

Consequently, adipose tissue is an inefficient by-product. The most expensive aspect of pig production is feed, and feed that is diverted to fat deposition is an input that is not rewarded by a cost-effective product. However, some adipose tissue is necessary for the welfare of the pig as it is an important source of insulation and energy storage.

Pigs are omnivorous, will consume most types of feed, have similar body composition to human adults, and have a propensity to obesity, making them excellent models for human nutrition (Pond, 1991, Yen, 2001). One major determinant of obesity in mammalian species, including humans and pigs, is energy intake. When energy intake exceeds energy utilisation, fat is deposited. Commercial pigs in developed countries are usually fed a low-fat, high-carbohydrate diet (Mitchell et al., 2001). Excess energy intake from these types of diets can lead to obesity, due to the pig's capacity to synthesise long-chain fatty acids from carbohydrates, primarily glucose. Feeding a low-protein diet leads to more fat deposition than those fed adequate protein (Ewan, 1991, Mitchell et al., 2001). An excessively high protein diet will also increase fat mass because the amino acids not used for protein synthesis will be de-aminated for fatty acid synthesis (Ewan, 1991, Mitchell et al., 2001).

1.6.1 Maternal nutrition

Optimal nutrient intake is not only the primary determinant of the normal growth and development, but is also important in reproduction and lactation. Until sexual maturity the nutrient requirements of pigs are largely designed to maximise growth and lean tissue deposition. The weight gain, and thus nutrient requirements, of the gestating sow should allow for the weight of the litter and the afterbirth, as well as maintenance of maternal body weight (Cunha, 1977, Ewan, 1991). The aim is that after parturition and lactation, sows should weigh approximately the same as they did prior to mating (Cunha, 1977). Gilts (1^{st} parity sows) have greater requirements than mature sows as their diet has to provide enough energy for their own growth as well as the growth of their developing fetuses (Cunha, 1977). The optimum weight gains for gilts and sows will vary depending on the original condition of the animal, the type of feed fed, the breed and size of the animal, and the number of offspring expected to be produced (Cunha, 1977). Nutrient demands in the sow are greatest during the last third of gestation when fetal growth is at a maximum (Cunha,

1977, Ewan, 1991, Pluske et al., 1995). It is normal commercial practice to restrict the feed intake of the sow throughout gestation in order to prevent excessive growth and fattening (Ewan, 1991).

The energy requirements to support lactation are three to four times greater than those of gestation (Cunha, 1977, Burrin, 2001). The nutrient requirements of the lactating period are a function of the sows need for growth and maintenance, plus the needs for milk production, which can represent 70 to 85 percent of the total energy required (Burrin, 2001). If insufficient energy is available, energy will be mobilised from the maternal tissues to sustain milk production, and weight loss will occur (Ewan, 1991). Generally, lactating sows are allowed *ad libitum* access to feed, and feed intake will determine if weight is gained or lost during lactation (Ewan, 1991).

1.6.2 Dietary energy sources

1.6.2.1 Carbohydrate

As mentioned previously, the major source of energy in a typical pig diet is carbohydrate. Carbohydrates are usually classified as simple (monosaccharides and disaccharides) or complex (oligosaccharides and polysaccharides). Simple carbohydrates are sugars and complex carbohydrates include starch, glycogen and cellulose (fibre).

In most developed countries, carbohydrates in pig diets are supplied in the form of starch which is present in cereal grains and their by-products, such as corn (maize), wheat, barley, rice, rye and oats (Burrin, 2001). Fibre is also used as alternative source of carbohydrates and is used mainly to reduce feed costs. Sources in pig diets include alfalfa, soybean hulls, oats, and wheat bran (Burrin, 2001). Fibre is not typically included in the diets of neonatal or growing pigs because it reduces growth by diluting the nutrient density (Ewan, 1991, Burrin, 2001). The most common use of fibre is in gestating sows where it minimizes excessive weight gain and serves as a laxative (Burrin, 2001).

1.6.2.2 Protein

The second most abundant nutrient required in the pig diet is protein (Burrin, 2001) which is an organic compound, composed of amino acids arranged in a linear chain and folded in a globular form. Different proteins

vary considerably in their amino acid composition (Burrin, 2001). Pigs are able to synthesise some amino acids in the body and these do not need to be provided in the diet; these are referred to as non-essential amino acids (Wu, 2009). Other amino acids cannot be synthesised by pigs, or at least not at the rate required, and these are referred to as essential (Wu, 2009). Some amino acids are only essential dietary components in certain situations such as during pregnancy or in very young pigs; these are known as conditionally essential amino acids (Wu, 2009). These include arginine, cysteine, glutamine and proline (Wu, 2009). There are 20 different amino acids that commonly occur in proteins, although only 10 of these are considered to be essential dietary components (Lewis, 1991, Burrin, 2001). The ideal amino acid composition is dependent on the physiological and reproductive function for which the amino acids are used, namely maintenance, growth, and milk synthesis (Table 1.5) (National Research Council (NRC), 1988).

Table 1.5 Amino acid requirements (% of diet) of sows during gestation and lactation; Adapted from (NRC, 1988)

Amino acid	Gestation	Lactation
Arginine	0	0.40
Histidine	0.15	0.25
Isoleucine	0.30	0.39
Leucine	0.30	0.48
Lysine	0.43	0.60
Methionine + Cystine	0.23	0.36
Phenylalanine + Tyrosine	0.45	0.70
Threonine	0.30	0.43
Trytophan	0.09	0.12
Valine	0.32	0.60

Dietary protein requirements are similar to that of a human diet and range from around 26 percent in neonates to 13 percent in adult diets (Burrin, 2001). Although pigs require amino acids for maintenance, growth, and reproduction, they are predominantly fed intact proteins to meet their requirements. As mentioned previously, cereal grains form the basis of most swine diets and these, as well as being a good source of starch, generally supply between 40 and 50 percent of the protein in the diets of

growing-finishing pigs (Lewis, 1991). The remaining protein usually comes from the addition of oilseed meals which are by-products of the oilseed industry (Seerley, 1991). In normal commercial practice, diets are often supplemented with crystalline amino acids such as lysine, tryptophan, methionine and valine, to obtain the ideal amino acid formulation (Lewis, 1991, Burrin, 2001).

1.6.2.2.1 Protein deficiency during gestation

Adequate protein during pregnancy is important to reduce neonatal mortality and ensure optimal piglet birth weight and growth. However, during pregnancy, the sow, unlike other species such as the rat, can effectively buffer the developing fetus against protein restriction, so that the development of the conceptus takes priority and will develop at the expense of maternal tissue (Cunha, 1977, Miller and Ullrey, 1987, Ewan, 1991). This is demonstrated by studies which demonstrate the effects of protein-restriction during pregnancy on the offspring. For example, a reduction in dietary protein content to 0.5 % in sows during pregnancy, whilst maintaining energy, content has no effects on litter size or piglet birth weight (Schoknecht et al., 1993, Schoknecht et al., 1994). In contrast, in gilts, the same protein restriction (0.5 % protein) has been shown to result in a reduction in piglet birth weight (Schoknecht et al., 1993). This difference is probably because gilts are still growing and so both the fetus and mother are competing for limited nitrogenous substrates to meet their needs. However, if this protein restriction of gilts occurs either in just early (days 1-44) or late (day 81 to term) pregnancy by 25 weeks of age the offspring have caught up to control progeny and there are no differences in body weight (Schoknecht et al., 1993).

Protein restriction during pregnancy, particularly in late gestation, appears to have a larger effect on subsequent milk production than birth weight, even if this restriction does not continue into lactation. This has been suggested because of the reduction in weaning weight when piglets were cross-fostered piglets from their control fed mothers onto those who were protein restricted (0.5 %) throughout gestation, despite no differences in birth weight (Schoknecht et al., 1993).

1.6.2.3 Fat

Most of the lipid in pig diets is in the form of triacylglycerols (triglycerides), the true fats (Pettigrew and Moser, 1991). Triglycerides are tri-esters of glycerol and fatty acids. Fatty acids are chains of carbon and hydrogen atoms with a carboxylic acid group at one end and a methyl group at the other. The properties of specific fat molecules depend on the particular fatty acids that constitute it (McDonald et al., 1981). Different fatty acids compose a different number of carbons in their chain, with the highest numbered Carbon, at the methyl end, known as ω-C. In addition, fats can also be saturated or unsaturated (Figure 1.9). When a fat is saturated the carbon atoms are bonded to as many hydrogen atoms as possible meaning that the bonds between the carbon atoms are all single bonds. The unsaturated fatty acids contain either one (monoenoic), two (dienoic), three (trienoic), or many (polyenoic) double carbon to carbon bonds in their chain (McDonald et al., 1981). Polyunsaturated fatty acids are fatty acids that contain two or more carbon to carbon double bonds. Omega-3-fatty acids have a double bond, three carbons away from the methyl carbon, whereas omega-6-fatty acids have it six carbons away. The main sources of polyunsaturated fatty acids are nuts, seeds, fish and leafy greens. Unsaturated fatty acids possess different physical properties from the saturated fatty acids; they have lower melting points and are chemically more reactive (McDonald et al., 1981).

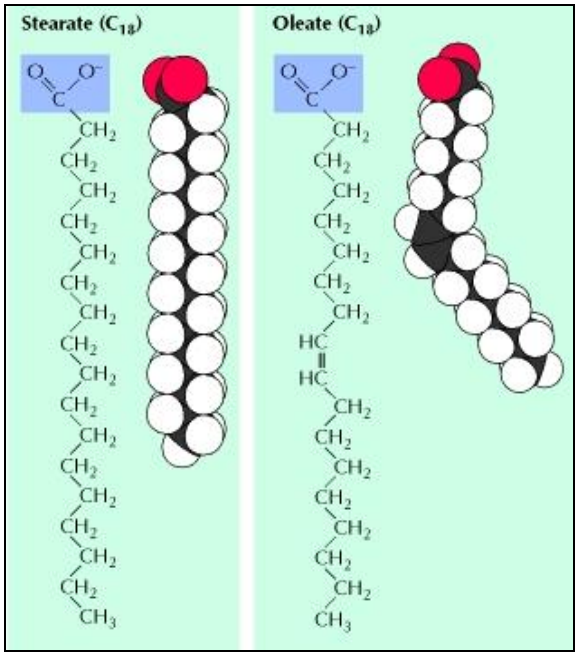

Figure 1.9 Examples of saturated (Stearate) and unsaturated fatty acids (Oleate) (Cooper, 2000)

The presence of a double bond in a fatty acid molecule means that the acid can exist in two forms depending on the arrangement of the hydrogen atoms attached to the carbon atoms of the double bond (McDonald et al., 1981). When both of the hydrogen atoms lie on the same side as the double bond the acid is said to be in the *cis* form (Figure 1.9), whilst if they lie on opposite sides it is in the *trans* form. Most of the naturally occurring fatty acids are in the *cis* form (McDonald et al., 1981).

There are three polyunsaturated fatty acids that are considered to be essential fatty acids (EFA); these are α-linoleic acid (n-6), linolenic acid (n-3), and arachadonic acid (n-6), which is synthesised from α-linoleic acid (Aflin-Slater and Aftergood, 1968, McDonald et al., 1981). EFA, like essential amino acids, are those fatty acids that cannot be biologically synthesised, or are synthesised in inadequate amounts to meet the animal's nutrient needs for growth, maintenance, and proper functioning of many physiological processes (Aflin-Slater and Aftergood, 1968). Alpha-Linoleic acid is the key essential fatty acid in the pig (Cunha, 1977). Arachadonic acid can be effective in preventing or curing a α-linoleic acid deficiency (Cunha, 1977). Alpha-Linoleic deficiency in pigs results in loss of hair, scaly dandruff-like dermatitis and slower growth rate in weaned pigs, probably due to less efficient feed utilisation (Hill et al., 1961, Leat, 1962, Sewell and McDowell, 1966). These investigations have led to the recommendation of α-linoleic acid requirements in swine by the National Research Council (NRC), which are around 0.1 percent of the total diet (NRC, 1988). These requirements can usually be met with a level of 1-1.5 percent fat in the diet (Cunha, 1977). Most diets consisting of grain and protein therefore, should supply enough α-linoleic acid for the pig (Cunha, 1977).

As well as the essential fatty acid requirements already discussed, fats are included in pig diets to increase the energy density, increase growth and food conversion efficiency, improve palatability, improve neonatal pig survival, and reduce airborne dust (Pettigrew and Moser, 1991, Burrin, 2001). However, as with other species, increased fat content will lead to increased fat deposition, even when there is equal energy intake (Mitchell et al., 2001). As mentioned previously, addition of fat to diet reduces feed intake without affecting the overall energy intake (Pettigrew and Moser,

1991). This reduction in feed intake may limit the intake of other essential nutrients.

1.6.2.3.1 Manipulation of dietary fatty acids in sow diets

Fats are found in various concentrations in major pig feedstuffs that are predominantly sources of carbohydrates and amino acids. Fats can also be added to the diet in the form of commercially available products such as animal fat and soy bean oil (Pettigrew and Moser, 1991). Most fats used in pig feeding contain longer-chain fatty acids of fourteen to twenty carbon molecules (Table 1.6) (Seerley, 1991). Fats and oils used in swine diets are derived from both animal and plant sources. Different fat sources differ in their fatty acid ratio (Table 1.6). Animal sources tend to be higher in saturated fatty acids, whilst those from plant sources are higher in polyunsaturated fatty acids (Table 1.6) (Burrin, 2001). The saturated to unsaturated ratio affects the melting point and energy value of fat as well as the fatty acid composition of the liver, fat, muscle, and milk (Seerley, 1991).

Table 1.6 Estimated percentage of major fatty acids and unsaturated to saturated fatty acid ratio in some fat sources used in the swine industry (Seerley, 1991)

	Carbons: Double bonds	Coconut oil	Palm oil	Soy bean oil	Corn oil	Safflower oil	Lard
Unsaturated: Saturated		0:1	1:1	6:1	7:0	8:1	1:5
Saturated fatty acids:							
Caprylic	8:0	7					
Capric	10:0	7					
Lauric	12:0	48					
Myristic	14:0	15	2				2
Palmitic	16:0	9	42	12	10	8	25
Stearic	18:0	2	4	2	2	3	10
Unsaturated fatty acids:							
Palmitoleic	16:1	6					2
Oliec	18:1		43	28	26	14	45
Polyunsaturated fatty acids:							
α-Linoleic (n-6)	18:2	1	9	50	56	75	11
Linolenic (n-3)	18:3			7	2		

Nutrient requirements for pigs give no recommendations for n-3 essential fatty acid requirements (Rooke et al., 1998). In human nutrition n-3 fatty acids, particularly docosahexaenoic acid (22:6 n-3), are important in late gestation as this is the time for rapid tissue deposition in organs that are highly reliant on n-3 such as the brain and retina (Makrides et al., 1994).

Commercial pig diets are based on cereals which are deficient in long-chain n-3 fatty acids. As the piglet also undergoes rapid brain tissue growth in late gestation n-3 fatty acids may also be important in sow nutrition (Passingham, 1985). One study has demonstrated the effects of the addition of tuna oil (30 g/kg), which is rich in n-3 fatty acids, instead of soy bean oil (30 g/kg) to an isocoloric sow diet from around 90 days of gestation until the end of the first week of lactation (Rooke et al., 1998). This study showed that tuna oil increased the proportion of n-3 fatty acids in both the maternal plasma and the milk. At birth, the offspring of these tuna oil fed sows had greater proportions of n-3 fatty acids in both their plasma and tissues such as the liver and the brain. This effect was thought to be independent of the colostrum intake as some piglets had not yet suckled at birth order was taken into account in the statistical model used. One explanation for this effect is that it is due to transfer of selected fatty acids across the placenta in late pregnancy, a process that was thought to be limited in pigs (Mersmann and Pond, 2001). Another study has demonstrated similar responses in plasma and tissue fatty acid proportions when diets containing an increased ratio of n-3 fatty acids in the form of salmon oil (0, 5, 10 and 20 g/ kg) to palm oil were fed from day 60 of gestation (Rooke et al., 2001a).

It has been hypothesised that a contributing factor to low piglet viability at birth may be an imbalance or deficiency of n-3 fatty acid supply to the pregnant sow (Rooke et al., 1998). However, in one study, the viability of piglets born to sows fed a tuna oil supplemented diet (30 g/kg) was significantly decreased when compared to a soy bean oil control (Rooke et al., 1998). This is an opposite result to what was hypothesised and it is believed that it could be linked to gestation length. It is thought that gestation length may be increased by an increased intake of n-3 fatty acids in the diet and so by inducing farrowing on days 113 and 114 gestation, and so those fed the soy-bean oil may have been closer to their natural farrowing date (Rooke et al., 1998). Further studies agree with this and

have demonstrated an increase in gestation length when salmon oil (500 g/kg) was added to the diet throughout gestation and lactation, with no effects on litter size (Rooke et al., 2001a). In addition, piglet birth weight was reduced when salmon oil was fed to the mothers, although surprisingly pre-weaning mortality was lower in these litters (Rooke et al., 2001a).

1.7 Liver metabolism

The liver was chosen in this thesis as the focus for further investigation into the effects of maternal diet on the offspring. This was due to its important role in whole body energy balance and metabolism. In addition, many studies have already shown an effect of nutritional programming on liver metabolism and gene expression in a range of species and models (Guo and Jen, 1995, Langley-Evans, 1996, Brameld et al., 2000, Bertram et al., 2001, Whorwood et al., 2001, Buckley et al., 2005, Ehruma et al., 2007)The present section will give a brief outline of the structure of the liver and will then go on to discuss the liver functions in terms of carbohydrate, lipid and protein metabolism.

1.7.1 Liver structure

The pig is a good experimental model for investigations on the liver because of its similarities to human liver in terms of size and morphology (Court et al., 2003). The liver is the largest and most metabolically complex organ in the body. It consists of many microscopic functional units, known as lobules, the number and size of which varies among species. Hepatocytes are responsible for the livers central role in metabolism and make up the bulk of the cells in the lobules.

Blood supply to the liver is from both the portal vein and hepatic artery (Figure 1.10). The blood entering the liver from the portal vein comprises all the blood returning from the small intestine, stomach, pancreas and spleen (Bowen, 2004), and makes up around 70 percent of the livers blood supply (Martin and Neuberger, 1957). This means that everything that is absorbed through the digestion process in the small intestine must pass through the liver, thus further highlighting its importance in metabolism. The remaining portion of the blood supply to the liver is from arterial blood via the hepatic artery, and so is rich in dissolved oxygen (Beath, 2003). After a meal, the portal vein delivers approximately twice the volume of

blood as the hepatic artery, equivalent to around 600 ml/min in an adult (Beath, 2003).

The terminal branches of the hepatic portal vein and hepatic artery empty together and mix as they enter the sinusoids in the liver. The sinusoids are distensible vascular channels lined with highly permeable endothelial cells and are bounded circumferentially by hepatocytes. As blood flows through the sinusoids, a considerable amount of plasma is filtered out into the spaces between the endothelial cells and the hepatocytes, known as the 'space of Disse' (Bowen, 2004). Blood from the sinusoids empties into the central vein of each lobule, these central veins merge into hepatic veins, which leave the liver and empty into the hepatic vena cava. Within the sinusoids are Kupffer cells, these play a very important role in the phagocytic system, which prevents invasion of the systemic circulation by bacteria (Bowen, 2002).

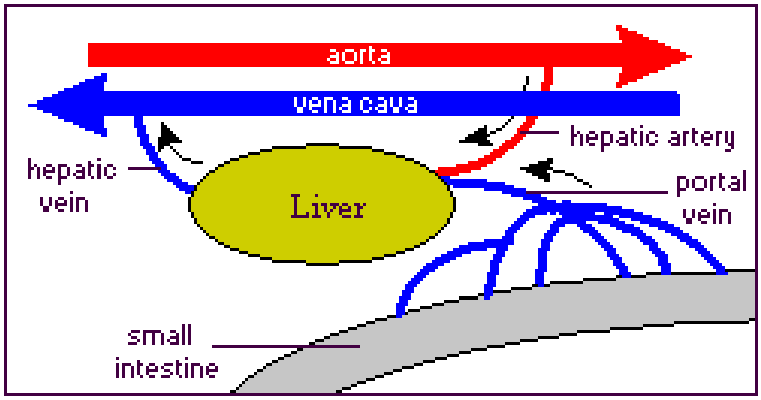

Figure 1.10 Diagrammatic representation of the hepatic vascular system; red blood vessels indicate oxygenated blood and blue blood vessels indicate de-oxygenated blood (Bowen, 2004)

The biliary system is a series of channels and ducts that conveys bile, a secretory and excretory product of hepatocytes, from the liver and into the lumen of the small intestine. Hepatocytes are arranged in 'plates' with their apical surfaces facing and surrounding the sinusoids (Figure 1.11) (Yen, 2001, Beath, 2003). The basal faces of adjoining hepatocytes are welded together by junctional complexes to form bile canaliculi (Figure 1.11) (Beath, 2003). The hepatocytes actively transport bile into the canaliculi, via specialised transport proteins, and those secretions flow parallel to the sinusoids but in the opposite direction to the blood (Beath, 2003). Bile collects in the bile ducts at the end of the canaliculi. Bile ducts thus begin

in close proximity to the terminal branches of the portal vein and hepatic artery, and this grouping is known as the portal triad (Yen, 2001).

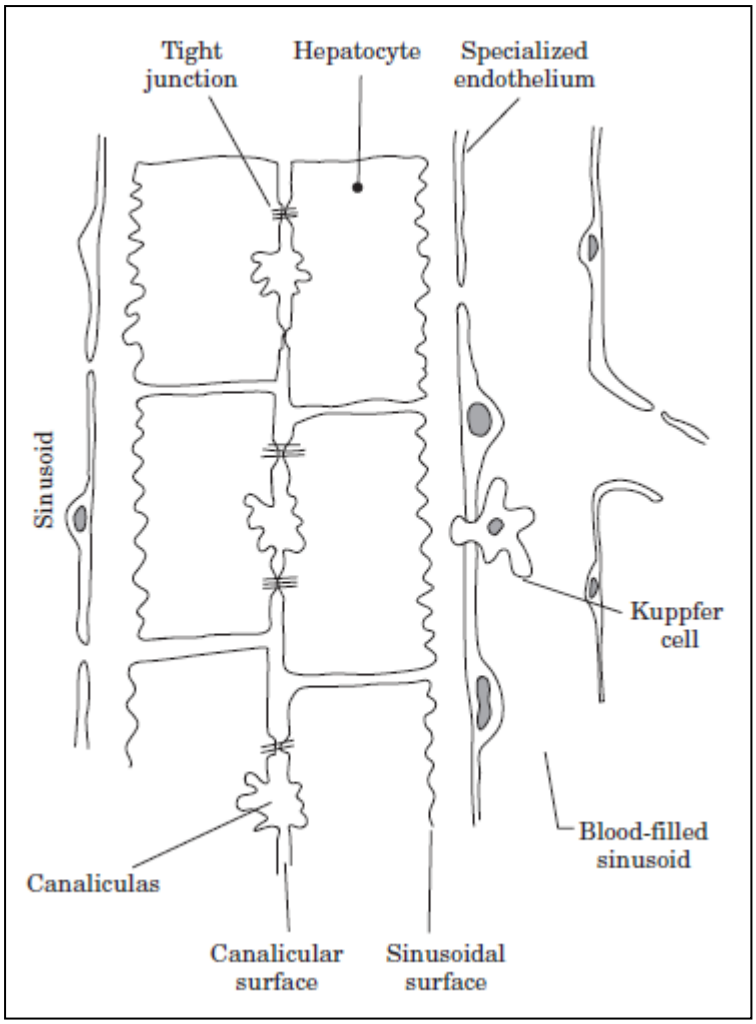

Figure 1.11 Structural arrangement of cells in the liver (Beath, 2003)

Attached to the liver is the gall bladder which stores and concentrates the bile made in the liver. Bile is involved in the emulsification of fats in the intestine. In addition, it aids in the secretion of many unwanted endogenous and exogenous compounds or toxins from the liver into the intestine, where they will be ultimately eliminated by the kidney (Bowen, 2001). Bile acids are derivatives of cholesterol, which is either ingested in the diet or synthesised in the liver (Bowen, 2001). Cholesterol is converted into the bile acids, cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (Bowen, 2001). This is then actively secreted into the canaliculi.

The liver has a pivotal role in lymph production in the body (Bowen, 2002). Lymph is a fluid which occupies the interstitial spaces between cells and

picks up unwanted materials such as fats and bacteria, and filters these substances out throughout the lymphatic system. Due to the large pores in the sinusoidal endothelial cells, fluid and proteins in the blood can flow freely through and into the space of Disse, forming lymph. Lymph flows through the space of Disse to collect in small lymphatic capillaries, which are associated with the portal triad, and from there into the systemic lymphatic system.

1.7.2 Liver function and metabolism

By birth, the architecture of the liver has been established for some time (Patten, 1948, Beath, 2003). *In utero*, it acts as the main source of red blood cell production, and even at birth, there are still active areas of haemotopoiesis (Beath, 2003). In human neonates, these areas usually disappear within six weeks after birth, being superseded by the bone marrow (Beath, 2003).

After the initial adaptations to circulatory changes have taken place, the liver starts to fulfil its role in maintaining homeostasis (Beath, 2003). Within the hepatocytes virtually all the reactions of intermediary metabolism can take place (Pesch and Topper, 1963). They play critical roles in synthesising molecules that are utilised elsewhere, in converting molecules from one type to another, and in regulating energy balance. As a result of all these reactions, the liver acts as a vital processing organ where nutrients absorbed from the gastrointestinal tract are transformed into materials required by other tissues in the body.

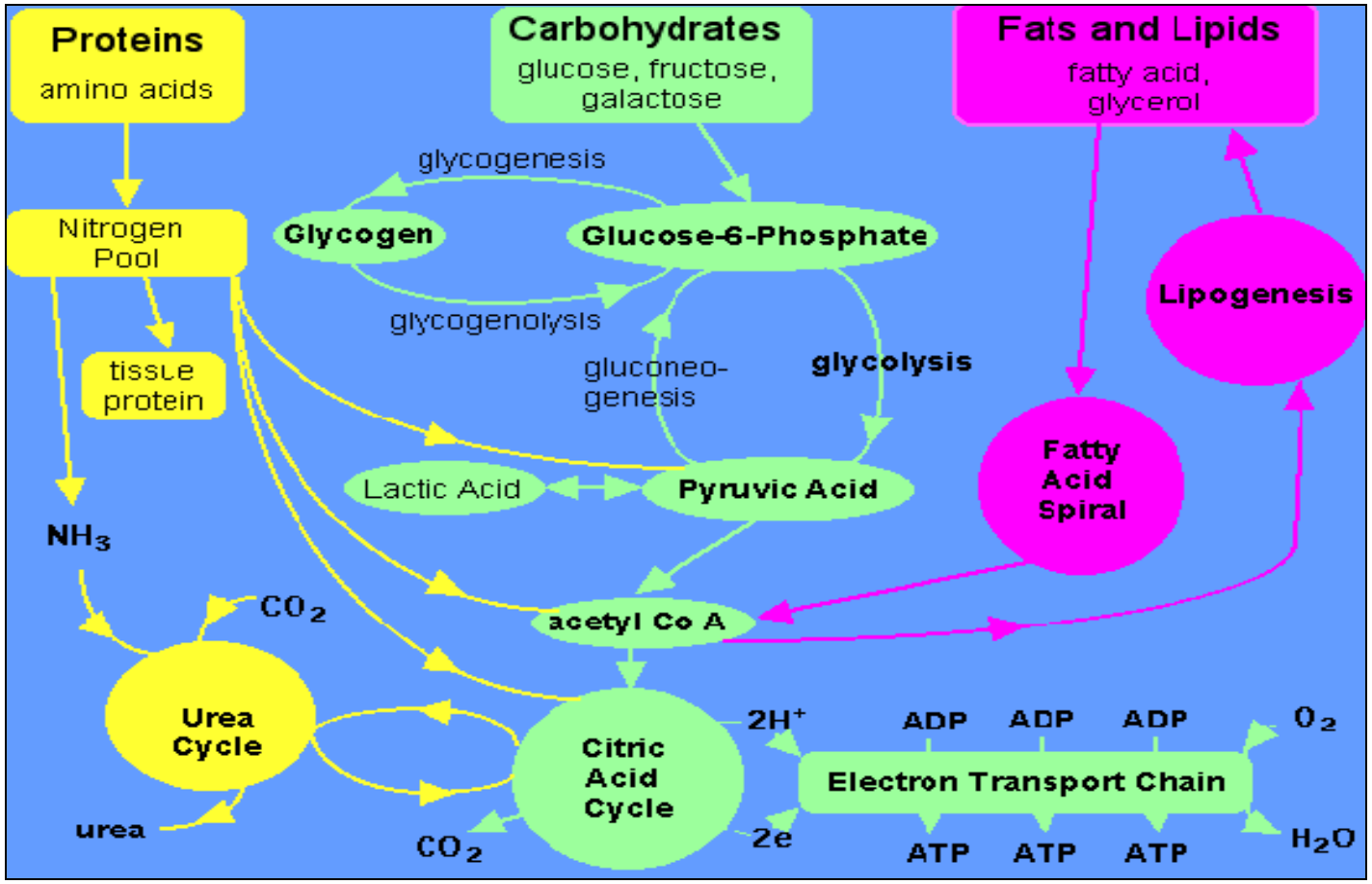

Figure 1.12 Overview of metabolism in the liver; ADP = Adenosine diphosphate ; ATP = Adenosine triphosphate; (Ophardt, 2003a)

1.7.2.1 Carbohydrate metabolism

The liver is the co-ordinating centre of carbohydrate metabolism. One of its major functions is to maintain plasma glucose concentrations within a normal range over both short (hours) and long (days and weeks) periods of time (Pesch and Topper, 1963, Bowen, 1995, Raddatz and Ramadori, 2007). This is achieved by a tightly regulated system of enzymes controlling either the breakdown or synthesis of glucose in the hepatoytes. This process is under the control of glucoregulatory mediators among which insulin plays a key role (Raddatz and Ramadori, 2007).

Excess glucose entering the blood, after the consumption of a large meal, is taken up by the liver and transformed to glycogen to be stored. This process is known as glycogenesis. Although glycogen is found in most tissues of the body, the liver has the highest glycogen content (Pesch and Topper, 1963) and thus provides a dynamic storehouse of energy. Glycogen suits this function as it can be rapidly synthesised from all available nutrients and is a source of glucose that can be utilised by all body tissues for energy. Also, because glycogen metabolism is under hormonal control, it is readily adaptable to the changing body needs (Pesch and Topper, 1963). If blood glucose levels begin to decline, the liver can utilise these glycogen stores through glycogenolysis, and export glucose back into the blood for transport to other tissues. Liver glycogen stores will deplete rapidly after birth in the newborn in order to maintain normoglycemia before the commencement of suckling (Figure 1.13) (Pesch and Topper, 1963, Okai et al., 1978, Mitchell et al., 2001). When hepatic glycogen reserves become exhausted, glucose can be synthesised from non-glucose polymers such as the gluconeogenic amino acids via gluconeogenesis. All amino acids except Leucine and Lysine are gluconeogenic.

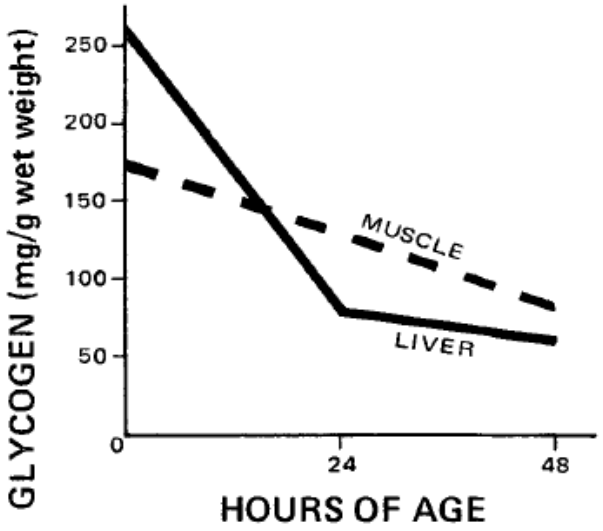

Figure 1.13 Tissue glycogen concentrations in piglets from birth to 2 days of age; adapted from (Okai et al., 1978)

In type 2 diabetes, as well as in liver disease, alterations in hepatic glucose metabolism occur due to insulin resistance, and include increased post-absorptive glucose production, together with diminished glucose uptake following carbohydrate ingestion (Raddatz and Ramadori, 2007).

1.7.2.1.1 Glucose transport in the liver

The transport of glucose across the plasma membrane is one of the most important cellular nutrient transport events, since glucose plays a vital role in cell homeostasis and metabolism. Virtually all mammalian cell membranes possess a facilitative transport system for the movement of glucose across the cell membrane down its chemical gradient either into or out of cells (Gould and Holmam, 1993). These transporters are specific for glucose and are not coupled to energy requiring components. Glucose transport into certain tissues is under acute and chronic control by circulating hormones. Defects in this transport system may underlie disease such as type 2 diabetes (Gould and Holmam, 1993). Glucose transport is mediated by a group of glucose transporters (GLUT) which are families of distinct genes and are expressed in a highly controlled tissue specific fashion (Table 1.7).

Table 1.7 Tissue specific expression of the glucose transporters (GLUT) in mammalian cells (Gould and Holmam, 1993)

Isoform	Tissue
GLUT 1	Placenta, brain, blood-tissue barrier; low levels in adipose tissue, muscle an liver
GLUT 2	Liver, pancreas, kidney and small intestine
GLUT 3	Brain; low-levels in placenta, liver, heart and kidney
GLUT 4	Muscle, heart and adipose tissue
GLUT 5	Small intestine; low-levels in brain, muscle and adipose tissue
GLUT 7	Hepatic microsomal glucose transporter

The primary transporter in the liver is GLUT 2. This is a low-affinity, high capacity transporter involved in the transport of glucose into and out of hepatocytes (Gould and Holmam, 1993). This means that it requires a high concentration of glucose to become activated, but, once activated; it can quickly remove large amounts of glucose from the blood. Together with the enzyme glucokinase, GLUT 1 is thought to act as a glucose-sensing mechanism that plays a role in blood glucose homeostasis, by responding to changes in blood glucose concentration and altering the rate of uptake into liver cells. In addition low levels of GLUT1 and GLUT3 are also expressed in the liver. These function effectively as unidirectional transporters, and are thought to work in tandem with each other in conditions where demand for glucose transport is high (Gould and Holmam, 1993). GLUT 7 is involved in the intracellular transport of glucose in the liver. When glucose is produced through both gluconeogenesis and glycogenolysis it is confined to the ER. Thus GLUT 7 is required to facilitate the transport of glucose across the ER membrane so it can be taken out of the hepatocyte (Gould and Holmam, 1993).

1.7.2.2 Lipid metabolism

The liver plays a key role in lipid metabolism which involves several pathways. It is the hub of fatty acid synthesis and lipid circulation through lipoprotein synthesis (Favarger, 1963, Nguyen et al., 2008). In addition, the liver is the principal site for fatty acid breakdown, which makes it a target site for many circulating lipids (Favarger, 1963). Furthermore, the liver has a key role in the production of phospholipids and in cholesterol metabolism (Favarger, 1963).

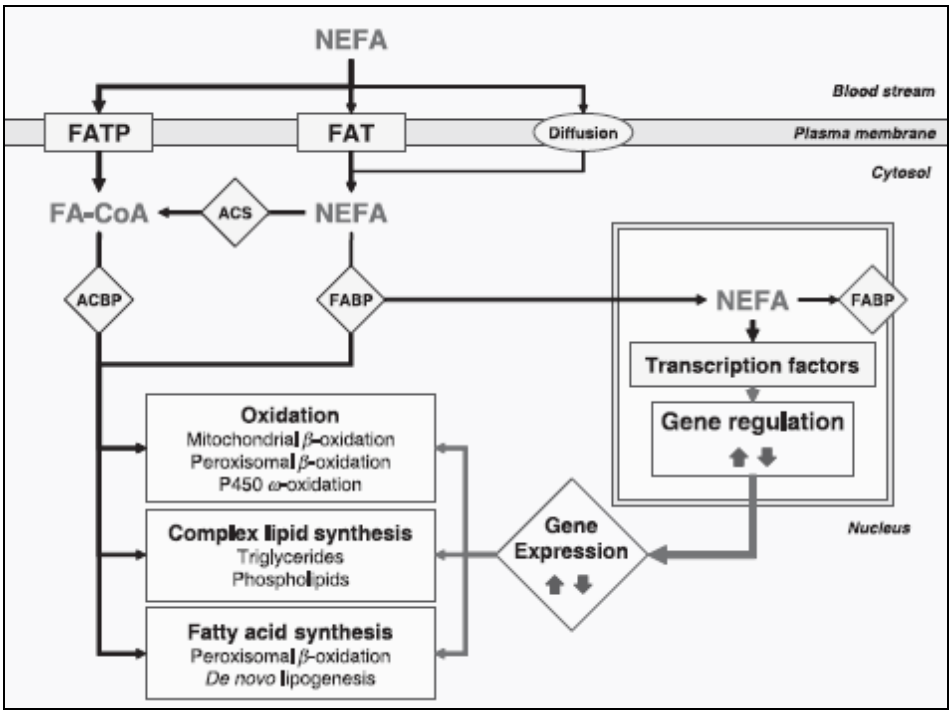

Figure 1.14 Fatty acid transport into the liver, intracellular activation, and main pathways of activated fatty acids; NEFA = Non-esterified fatty acid; FABP = Fatty acid-binding protein; FATP = Fatty acid transport protein; FAT: Fatty acid translocase; ACBP; Fatty acyl-CoA binding protein; ACS = Fatty acid CoA synthetase; (Nguyen et al., 2008)

The liver takes up NEFA from the blood directly in proportion to their concentration, usually through specialised transporters. Fatty acid transport proteins (FATP) are involved in uptake of long-chain fatty acids. Fatty acids taken up by FATP are preferentially channelled into triglyceride synthesis, suggestion a functional link between FATP fatty acid uptake and lipid storage (Kim et al., 2004). In addition, fatty acid translocase CD36 (FAT/CD36) is also involved in fatty acid uptake into the cell when fatty acid and/or insulin plasma concentrations are raised (Bonen et al., 2004). FAT/CD 36 translocation to the hepatocyte membrane is significantly associated with insulin resistance and hyperinsulinaemia in patients with non-alcoholic fatty liver disease (Miquilena-Colina et al., 2011).

Within the hepatocytes, these fatty acids are bound to FABP1 and acyl CoA binding protein, which transport them into intracellular compartments for metabolism (Nguyen et al., 2008). In addition, they may also be taken into the nucleus where they regulate gene expression by controlling the activity or abundance of key transcription factors (Nguyen et al., 2008). Many transcription factors have been identified as prospective targets for

fatty acid regulation including PPARs (Schoonjans et al., 1996). PPARα is highly expressed in the nucleus of hepatocytes, where it regulates a set of enzymes that are crucial for fatty acid transport and oxidation (Nguyen et al., 2008).

Lipogenesis is tightly controlled by hormonal and nutritional conditions. Briefly, high carbohydrate diets induce, whereas fasting or fat feeding inhibit lipogenesis (Nguyen et al., 2008). It is especially dependent on concentration and tissue sensitivity to insulin (Nguyen et al., 2008). Fatty acids synthesised in the liver are exported through lipoprotein production, and thus provide an energy source and structural components for membrane building (Nguyen et al., 2008). There is a close relationship between the rate of fatty acid synthesis and the activity of the enzyme FAS. FAS is expressed in both liver and adipose tissue. In humans, the major site of fatty acid synthesis is in the liver (Patel et al., 1975), whereas in pigs it is in the adipose tissue (O'Hea and Leveille, 1969). It is regulated by hormones, nutritional status and the intracellular fatty acid concentration. For example, insulin activates FAS and glucagon inhibits it (Nguyen et al., 2008).

Fatty acids may be oxidised in the liver, either in the mitochondria or the peroxisomes. Two main factors regulating the degree to which fatty acids are oxidised in the liver are their supply to the liver, via lipolysis, and the partitioning between oxidation and microsomal esterification (Nguyen et al., 2008). Fatty acid oxidation occurs through the β-oxidation pathways, resulting in the formation of acetyl-CoA. The acetyl-CoA can then be oxidised completely in the citric acid cycle, in order to produce energy. Peroxisomal β-oxidation is responsible for the metabolism of very long chain fatty acids, whereas mitochondrial β-oxidation oxidises short, medium and long chain fatty acids (Nguyen et al., 2008). When carbohydrates are scarce the liver has the ability to produce ketone bodies from the acetyl-CoA formed during β-oxidation through ketogenesis (Nguyen et al., 2008). These are then transported to other tissues in the body so that they can be reconverted to acetyl-CoA to produce energy via the citric acid cycle (Nguyen et al., 2008). An accumulation of ketone bodies in the blood is known as ketosis. When these reach very high levels they will lower the pH of the blood to dangerously acidic levels, this is called ketoacidosis.

The liver is able to secrete lipids by transforming them to very low-density lipoproteins (VLDL). Four sources of fatty acids are used for lipoprotein synthesis, these are fatty acids synthesised in the liver through lipogenesis, cytoplasmic triglyceride stores, fatty acids derived from lipoproteins taken up by the liver, and free fatty acids in the plasma (Julius, 2003). Lipid components that are synthesised in the smooth endoplasmic reticulum are added, by the microsomal triacylglycerol transfer protein, to apoprotein B (White et al., 1998). After being carried to the Golgi apparatus in transport vesicles, the apoproteins are glycosylated. Secretory vesicles bud off the Golgi membrane, migrate to the sinusoidal membrane of the hepatocyte, then fuse with the membrane and release VLDL into the blood. It has been suggested that the rate of secretion of VLDL is proportional to the rate of fatty acid synthesis (Nguyen et al., 2008). In addition, VLDL formation is thought to be stimulated by an increase in plasma NEFA concentration (Julius, 2003). This situation would be expected to be exacerbated in an insulin-resistant state, which also promotes increased stability of nascent apoprotein B and enhances the gene expression of microsomal triacylglycerol transfer protein (Taghibiglou et al., 2000).

The accumulation of lipid droplets in hepatocytes results in hepatic steatosis. This may develop as a consequence of multiple dysfunctions such as alterations in PPARα sensing and β-oxidation, VLDL secretion, and pathways involved in the synthesis of fatty acids (Nguyen et al., 2008). In addition, an increase in the circulating pool of non-esterified fatty acids may also be a major determinant in the pathogenesis of fatty liver disease (Nguyen et al., 2008).

1.7.2.3 Protein metabolism

Protein metabolism in the liver is just as important as glucose and lipid metabolism. Proteins make up the structural tissue for muscles and tendons, transport oxygen or haemoglobin in the blood, catalyse all biochemical reactions as enzymes, and regulate reactions as hormones (Ophardt, 2003a). The body must be able to synthesise proteins, amino acids, and other non-protein nitrogen containing molecules for growth, replacement and repair. The liver plays an important role in protein synthesis (Tarver, 1963), with the exception of immunoglobulins, nearly all

of the plasma proteins are made in the liver (Martin and Neuberger, 1957, Beath, 2003). Albumin, the major plasma protein, is synthesised almost exclusively by the liver (Tarver, 1963, Bowen, 1995). In addition, excess protein can be used to supply energy or build reserves of glucose, glycogen, or lipids.

After digestion, proteins and amino acids are transported to the liver, via the portal vein. They are rapidly taken up by the sinusoidal hepatocytes where they can be catabolised by deanimation or transamination (Beath, 2003). Deamination is the process whereby amino acids are converted to their corresponding keto acids by the removal of the amine functional group and replacement with the ketone group. The ammonia produced during this process is toxic and must be removed from the body by conversion to urea in the liver (Tarver, 1963); this is excreted via the urine. Transamination refers to the transfer of an amine group from an amino acid to a ketone group of another acid, using enzymes known as transaminases. These reactions are used for the production of non-essential amino acids in the body (Ophardt, 2003b). Once these keto acids have been formed by either deamination or transamination they can be used for several purposes. The most obvious purpose is that they complete metabolism to form carbon dioxide and water in the citric acid cycle (Tarver, 1963). However, if there are excess proteins in the diet then these keto acids can be used to synthesise glucose or lipids through gluconeogenesis or lipogenesis, depending on the type of amino acid (Tarver, 1963, Bowen, 1995).

1.8 Conclusions and future perspectives

Overall, the current research in this area emphasises the importance of maternal nutrition on the development of the embryo, placenta and fetus. Importantly, the consequences of sub-optimal nutrition through alterations in the macronutrient content of the maternal diet will not simply be reflected in altered neonatal body composition and increased mortality, but are likely to continue into adulthood and confer greater risk of metabolic disease. The mechanisms behind this nutritional programming are beginning to be elucidated, with particular focus on GC action and insulin sensitivity. The liver is of key importance in these studies due to its primary role in metabolism and maintaining whole body energy balance. Despite the similarities between pigs and humans in terms of digestive

physiology, rats and sheep are still the animals of choice for these studies. In addition, the manipulation of the macronutrient content in these studies, be it through protein restriction or fat supplementation, is often very severe, with experimental diets which are very different to what the animal would normally receive.

1.9 Aims and hypothesis

The aim of this study was to provide a novel insight into the effects of moderate changes in the macronutrient ratio of the maternal diet fed to pigs, whilst still maintaining energy content, on offspring development and liver metabolism until adolescence. In addition, the effect of offspring birth weight on liver metabolism will be examined. As well as providing insights into potential mechanisms for nutritional programming in humans, this study will also have implications for the pig industry where optimum maternal nutrition to improve offspring viability and growth are of key importance.

This study had two main hypotheses; the first was that increasing the fat content of the maternal diet would reduce litter size and increase the risk of perturbations in liver metabolism in the offspring, possibly due to alterations in insulin sensitivity. It was hypothesised that low-birth weight offspring will be at increased risk. The second hypothesis was that protein supplementation of the diet would have beneficial effects on offspring survival and development.

Chapter 2 - Materials and methods

This chapter describes the materials and methods used throughout the study, with a brief description of underlying theory for more novel methods. All methods have been validated and supporting data (where appropriate) are also included.

All animal procedures, data and sample collection, and milk composition analysis were carried out by staff at Schothorst Feed Research in the Netherlands. Insulin enzyme-linked-immunosorbent assays (ELISA) on the piglet plasma samples were carried out by Dr Hernan P. Fainberg and Miss Mehreen Azhar carried out the glycogen extraction and analysis in the liver.

2.1 Study protocols

All animal procedures, described in this thesis, were approved by the Ethics Committee for Animal Experiments of the Animal Sciences Group of Wageningen Research Centre, and conducted at Schothorst Feed Research in the Netherlands. All laboratory procedures were carried out at The University of Nottingham under the United Kingdom Control of Substances Hazardous to Health (COSHH: SI NO 1657, 1988) code of laboratory practice.

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Company (Gillingham, UK) and equipment was obtained from Scientific Laboratory Supplies (SLS). Details of all suppliers can be found in the Appendix. Unless otherwise stated all aqueous solutions were made up in distilled water (dH_2O)

2.2 Animal study

Yorkshire x Landrace sows were used in this study. Sows of parity 2 to 6 were selected and equally distributed amongst the 3 treatments, with a mean parity of 3.8 (SEM 0.24). Each week one sow from each treatment was artificially inseminated with pooled semen from multiple sires, as is normal practice in the pig industry. Pregnancy was confirmed when sows did not return to oestrus within 24 days after insemination. If sows failed to become pregnant on the first attempt they were removed from the trial and another sow was mated for that treatment. This process continued

weekly until there were 8 sows per treatment. Throughout gestation all sows were maintained in gestation crates. Sows were observed daily during lactation and gestation for abnormalities and any clinical signs of sickness. Medical treatments were applied after consultation with a veterinarian, and registered in an animal log book.

The experimental treatments started at the day of breeding when sows (n=24) were randomly allocated to one of three isocaloric gestational diets differing in starch, fat and protein content (Table 2.1; see appendix C for full dietary composition). Sows were fed according to an increasing feeding scheme in gestation, 25.1 MJ/day from mating (day 0) until day 70 and 32.6 MJ/day from day 70 to 110. Due to the higher energy content of the increased fat diet, the daily feed allowance was reduced in this group throughout gestation to insure equal energy intake (Table 2.1). Sows in parity 2 received 95 % of the feeding scheme. Diets were prepared in a factory for research diets according to the National Research Council (NRC) energy and nutrient recommendations for gestating and lactating sows. The control diet had a high starch content, as is normal practice in the commercial pig industry. Starch was included from cereals or tapioca. Palm oil was fed to increase the fat content; this has an equal ratio of saturated to unsaturated fatty acids (Chapter 1; Section 1.6.1.3). In addition, the fat supplemented diet had a reduced starch content, to maintain equal energy intake. Increased protein content was derived from the addition of lupins, a type of legume, and sunflower seed meal to the diet. These were selected due to their high arginine and glutamine amino acid content. All diets were analysed for proximate components prior to feeding and met adequate essential amino acid, fatty acid, vitamin and mineral requirements.

Table 2.1 Composition and quantity of the experimental diets with alterations in macronutrient ratio

		Feed quantity during gestation (Kg/day)			Diet composition (MJ/Kg)		
Gestational diet	**Code**	**Day 0-40**	**Day 40-70**	**Day 70-110**	**Starch**	**Fat**	**Protein**
Control (n=8)	C	2.89	2.89	3.75	5.37	0.93	2.09
Fat supplemented (n=8)	FS	2.51	2.51	3.26	2.74	3.29	2.07
Protein supplemented (n=8)	PS	2.89	2.89	3.75	5.39	1.37	2.77

All treatment diets were fed from the day of mating (day 0) until day 110 of gestation. All sows received the same diet for lactating sows (Table 2.3 see appendix C for full dietary composition) from day 110 of gestation and throughout lactation. The feeding level was reduced before farrowing and gradually increased post farrowing as is normal practice in the pig industry (Table 2.3). Feed intake of each sow was recorded throughout pregnancy and lactation.

Table 2.2 Feed allowance of sows from day 110 of gestation until the end of lactation

Day	Feed allowance (Kg/day)
111-113	3.2
114-115	2.5
Farrowing	2.0
1	2.5
2	3.0
3	3.5
4	4.0
5	4.5
6	5.0
7	5.5
8	5.5
9-10	6.0
11-28	*Ad libitum*

One week before the expected farrowing date, sows were moved to farrowing crates. All sows farrowed naturally and piglets were allowed to suckle. If litter size exceeded 13 live born piglets, the additional piglets were removed before day 3 post-farrowing because of the maximum number of functional teats. Body weight of piglets at weaning (28 days), 63 days and 6 months was recorded.

Standard creep feed was supplied to the piglets from two weeks of age (Table 2.3; see appendix D for nutrient composition). At weaning (mean lactation length = 28 days), piglets were group housed in pens with pigs of similar body weights, and received a commercial post-weaning diet, from 3 weeks post-weaning (Table 2.3; see appendix D for nutrient composition). At 5-6 weeks post-weaning (approximately 25 Kg) piglets were weighed and placed in one pen with piglets of mixed sex and equal bodyweights, in a barn for growing-finishing pigs. These pigs received a grower diet (Table

2.3; see appendix D for nutrient composition) and subsequently a commercial diet for finishing pigs until they reached 6 months of age (around 110 Kg is commercial slaughter weight; Table 2.3; see appendix D for nutrient composition).

At 6 months of age, in pigs that were not selected for tissue sampling (Section 2.3), back fat thickness was measured ultrasonically using the P2 method (back fat measured at 5cm from the midline over the last rib), with a Lean-Meater ® (Renco Corp. Minnesota, U.S.A.).

Table 2.3 Basic composition of sow and piglet diets (MJ/Kg)

	Diet composition (MJ/Kg)				
	Sow	Piglet			
	Lactation	Creep feed	Post-weaning	Grower diet	Finisher diet
Starch	5.42	5.65	6.12	6.44	6.39
Fat	2.33	2.78	2.20	1.82	1.69
Protein	2.74	2.99	2.98	2.89	2.72

2.3 Collection of plasma and tissue samples

2.3.1 Collection of maternal plasma

Blood samples were taken from sows in the morning before feeding, 4 days prior to mating, and on days 40, 70 and 108 of gestation. Samples were taken via a catheter, placed in the ear one day prior to sampling; this avoids excess stress to the sow at the time of sampling. Before a sample was taken, the catheter was flushed with saline to remove any blockages and an 8 ml sample was collected. The EDTA (ethylenediaminetetraacetic acid) tubes containing the blood samples were centrifuged at 1800 g in a bench top centrifuge for 15 minutes at 4 °C. Following centrifugation the plasma was removed, frozen in eppendorf tubes and stored at -20 °C for subsequent analysis.

2.3.2 Collection of piglet samples

A low and median-birth weight piglet in each litter were selected for tissue and plasma sampling either at 1 week ± 2 days or 6 months of age. The piglet with the lowest birth weight of each litter was always selected for sampling at 1 week of age, and this was independent of the animal's sex. The next step was to select a median-birth weight piglet from the litter, of which there were usually several animals around the same weight. At this stage, if possible, piglets were matched for sex with the low-birth weight

piglet which had already been selected. At this time, piglets were pre-selected for sampling at 6 months of age. For this process, the piglet with the next lowest birth weight was selected, and again a median-birth weight piglet was chosen as before. Males selected for sampling at 6 months of age were not castrated. In the EU (not UK) castration is usual practice in the pigs to help reduce fighting and to eliminate 'boar' odour which might taint the taste of the meat. Offspring selected for sampling at 1 week of age were not fasted as they were still suckling from their mothers. However, offspring at 6 months of age were fasted for 12 hours prior to sampling.

Piglets selected for sampling were sedated with a ketamin-xylazine combination (Ketamin 10%, Alfasan International BV, Woerden, The Netherlands) injected intramuscularly. They were then weighed and a blood sample was taken intracardially prior to euthanasia with T-61 dose (Intervet, Boxmeer, The Netherlands). The Ethylenediaminetetraacetic acid (EDTA) tubes containing the blood samples were centrifuged at 1800 g in a bench top centrifuge for 15 minutes at 4 °C. Following centrifugation the plasma was removed, frozen in eppendorfs and stored at -20 °C for subsequent analysis. The liver, kidneys, pancreas, heart, spleen, brain, stomach, lungs, adrenals, gonads, thyroid, skeletal muscle (biceps fermoris and longissimus dormis) and a sample of subcutaneous adipose tissue were then removed from these animals and weighed. Organs were snap-frozen in liquid nitrogen prior to storage at -80°C for further analysis.

2.4 Analysis of plasma samples

All plasma samples were deforested slowly on ice and auto-analysed (Randox RX Imola, Randox Laboratories Ltd. Co Antrim, UK) for glucose, triglycerides (TAG), non-esterified fatty acids (NEFA) and cholesterol using quantitative enzymatic colorimetric assays supplied in kits (Randox Laboratories Ltd. Co Antrim, UK). To limit freeze-thaw cycles of the plasma, all assays for each sample were carried out at the same time. In addition, samples were analysed for Insulin using a porcine specific ELISA (Mercodia Porcine Insulin ELISA, Mercodia AB, Uppsala, Sweden).

Repeats were carried out for each assay in order to determine co-efficient of variance. To measure inter-assay variation, one sample was chosen and repeated each time the assay was carried out. In addition, intra-assay

variation was measured by running the same sample several times in the same assay.

2.4.1 Glucose

Glucose concentrations were determined using the enzyme glucose oxidase (GOD) which catalyses the transformation of glucose and water in the presence of oxygen, to gluconic acid and hydrogen peroxide. The hydrogen peroxide formed reacts with 4-aminoantipyrine and p-hydroxybenzene sulphonate, which is catalysed by peroxidise, to form a red-violet quinoeimine dye as an indicator with maximal absorbance at 500 nm. The intensity of the dye is directly proportional to the glucose concentration in the sample. The inter-assay co-efficient of variance (CV) for glucose was 1.6 %, and the intra-assay CV was 1.0 %.

2.4.2 Non-esterified fatty acids (NEFA)

Non-esterified fatty acid (NEFA) concentrations were determined using the enzyme acyl-CoA synthetase, which when added to plasma allows NEFA to form thiol esters of coenzyme A (CoA; known as acyl-CoA) in the presence of adenosine triphosphate (ATP), magnesium cations and CoA. Acyl-CoA is oxidised by acyl–CoA oxidase to produce hydrogen peroxide. Peroxidase enzymes then catalyse the oxidative condensation of 3-methylN-(hydroxyethylanaline) (MEHA) with 4-aminoantipyrine in the presence of hydrogen peroxide to from a purple coloured adduct with a maximal absorbance at 550 nm. The inter-assay CV for NEFA was 4.9 %, and the intra-assay CV was 0.4 %.

2.4.3 Triglycerides (TAG)

Triglyceride (TAG) concentrations were determined after enzymatic hydroylisis with lipases which break down the triglycerides to glycerol and fatty acids. Glycerol kinase (GK) then catalyses the transfer of a phosphate from ATP to glycerol. The resulting glycerol-3-phosphate is oxidised by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate and hydrogen peroxide. Peroxidise then catalyses the reaction of hydrogen peroxide with 4-aminophenazone and chlorophenol producing quinoneimine dye with a maximal absorbance at 540 nm. The inter-assay CV for TAG was 2.8 %, and the intra-assay CV was 1.4 %.

2.4.4 Cholesterol

Cholesterol concentrations were determined after enzymatic hydrolysis to produce cholestene-3-one and hydrogen peroxide. The indicator quinoneimine is formed from hydrogen peroxide 4-aminoantipyrine in the presence of phenol and peroxidise. The resulting quinoneimine dye produced is directly proportional to the cholesterol concentration when measured at 600 nm. The inter-assay CV for cholesterol was 2.4 %, and the intra-assay CV was 1.0 %.

2.4.5 Insulin

Plasma insulin concentrations were determined in the sows at 108 days of age and piglets at 6 months of age, using a commercially available ELISA kit (Mercodia Porcine Insulin ELISA, Mercodia AB, Uppsala, Sweden). Analysis of the 6 month samples was kindly carried out by Dr Hernan P. Fainberg due to time constraints.

This technique works by immobilising two monocolonal antibodies, directed against separate antigenic determinants of the insulin molecule, on a 96-well plate. During incubation, the insulin in the plasma sample binds with the peroxidise-conjugated anti-insulin antibodies. After washing with a mild detergent solution to remove any unbound enzyme labelled antibody, the bound peroxidise-conjugate is detected by a reaction with 3,3'-5,5'-tetramethylbenzidine (TMB). The reaction is stopped by the addition of acid, giving a colorimetric endpoint that can be read spectrophotometrically. Using known standards, a binding curve can be generated, which allows the unknown concentrations of insulin in the plasma to be determined.

2.4.5.1 Insulin enzyme-linked-immunosorbent assay (ELISA) procedure

In all plates, a standard curve (0-1.5 µg/L) was used to determine the insulin concentrations of the samples. 25 µl of the correct standard or sample was carefully pipetted into the appropriate wells of an antibody bound 96-well plate (Mercodia Porcine Insulin ELISA, Mercodia AB) and 100 µl of enzyme conjugate (Mercodia Porcine Insulin ELISA, Mercodia AB) solution added. All samples were run in duplicate in order to establish intra-assay variability. A CV of less than 5 % was considered acceptable and samples were repeated if the CV was higher than this (see section

2.6). Plates were incubated at room temperature whilst being agitated for 2 hours, and then washed 6 times with 700 µl of wash buffer (Mercodia Porcine Insulin ELISA, Mercodia AB) per well. After the final wash plates were inverted and tapped firmly against absorbent paper to ensure all buffer was removed. 200 µl of substrate TMB (Mercodia Porcine Insulin ELISA, Mercodia AB) was then added to each well and plates incubated for 15 minutes at room temperature. Following this, 50 µl of Stop Solution (Mercodia Porcine Insulin ELISA, Mercodia AB) was added to each well and the plate was agitated for approximately 5 seconds to ensure mixing. Absorbance was then measured at 450 nm using the automated plate reader (µQuant, BIO-TEK Instruments Inc. Vermont, USA). Insulin concentrations were determined following linear regression analysis of the standard curve.

2.5 Analysis of piglet tissue samples

The preparation and sorting of tissue samples prior to analysis was carried out on dry ice so no significant thawing of the samples occurred. For each analysis, the required amount of frozen tissue was cut from the sample and the remainder returned to the freezer.

2.5.1 Lipid extraction and triglyceride (TAG) assay

Liver lipid content was assessed using a modified version of the Folch assay (Folch et al., 1957) followed by colorimetric analysis for TAG content using a commercially available kit (Randox Laboratories Ltd.). The extraction procedure exploits the interaction between chloroform-methanol-water and cellular lipids for the separation of the homogenised tissue into two phases in which the lower phase is the total pure lipid extract (Folch et al., 1957). After homogenisation and gravity filtration, due to the polarity and density of the suspension solution and the lipids, the cellular debris can be separated by adding water (Folch et al., 1957). The chloroform-methanol solution can be removed by applying nitrogen steam. TAG concentrations of the extracted lipid samples were determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidise (Section 2.4.3).

2.5.1.1 Lipid extraction procedure

Samples (0.5 g) were sectioned from liver and muscle tissue and thawed in 2 ml of cold chloroform:methanol (2:1). The tissues were then completely disrupted using a Dispomix® (Thistle Scientific Ltd. Glasgow, UK) closed system homogeniser. The homogenate was agitated for 15-20 minutes at room temperature then filtered to recover the liquid phase. The filter paper was washed further with 8ml solvent, to a final volume 20 times the tissue sample. The solvent was then washed with 0.2 volumes of H_2O and vortexed for a few seconds. The resulting mixture was centrifuged at 1160 g for 10 minutes at room temperature to separate the homogenate into two phases. The upper phase was discarded and the lower phase which is total pure lipid extract was centrifuged for a further 10 minutes and any remaining impurities removed. Samples were dried under nitrogen steam and then re-dissolved in a mixture of tert-butanol: triton X-100 (3:2).

2.5.1.2 Triglyceride (TAG) assay procedure

This assay was carried out in sterile 96-well plates (ABgene Ltd. UK, Epsom, UK). In all plates, a standard curve (0-390 mg/dL) was used to determine the TAG concentrations of the samples. 2 µl of the correct concentration of standard or lipid of the unknown samples was carefully pipetted into each well of the plate and 200 µl of TAG reagent (Randox Laboratories Ltd.) added, ensuring no bubbles were formed. All samples were run in triplicate in order to establish intra-assay variability. A CV of less than 5 % was considered acceptable and samples were repeated if the CV was higher than this (Section 2.6). Due to the large sample number, inter-assay variability was established by running one lipid sample on all plates (CV = 8.8 %). Plates were incubated at 37 ºC whilst being agitated for 5 minutes. Absorbance was then measured at 500 nm using the automated plate reader. TAG concentrations were determined following linear regression analysis of the standard curve. TAG concentrations were expressed as a ratio of TAG found in micrograms and weight in grams of the sample previously dissected (mg/g).

2.5.2 Glycogen extraction and assay

Liver glycogen was extracted using a modified version of the method first used to extract glycogen from muscle cells (Dalrymple and Hamm, 1972). This work was carried out by Miss Mehreen Azhar as part of her BmedSci (hons) project.

Neutralized Perchloric acid (PCA, Thermo Fisher Scientific Ltd. Leicestershire, UK) homogenates of liver were mixed with the enzyme amyloglucosidase which extracts the glycogen and reduces it to glucose. A glucose enzymatic colorimetric assay was then carried out to determine glucose content and by using reduced glycogen standards this can easily be converted to glycogen concentration. In order to eliminate any free glucose in the tissues, each sample is run with a control which is not treated with the enzyme; this can then be taken into account when calculating the glycogen content.

2.5.2.1 Glycogen extraction procedure

Samples (0.5 g) were sectioned from liver and thawed in 2 ml of PCA (8 % (v/v)). The tissues were then completely disrupted using a Dispomix® closed system homogeniser. After addition of a further 3 ml of PCA (8 % (v/v)), the tubes were vortexed briefly and spun at 3220 g for 1 minute at 4 °C. 1 ml of the supernatant was removed to a fresh 1.5 ml eppendorf and neutralised (pH 7) with 100 µl of saturated sodium bicarbonate solution. Sufficient quantities of sodium acetate (0.2 M) were then added to the neutral supernatant to reduce the pH to 4.8, the optimal pH for amyloglucosidase, and the volume made up to 400 µl with H_2O. 200 µl of the supernatant was transferred to a fresh 1.5 ml eppendorf so each sample had two tubes. To one of these sets of tubes 5 µl of amyloglucosidase (80 U/ml) was added and gently tapped to mix. The other set of tubes acted as the free glucose controls and so 5 µl of H_2O was added. All samples were incubated at 37 °C for 30 minutes whilst agitating at low speed, thus allowing for optimal reduction of glycogen to free glucose, and then boiled at 100 ºC for 5 minutes to stop the reaction. Samples were then centrifuged at 13250 g for 10 minutes at 4 °C and the supernatant collected.

2.5.2.2 Glucose assay procedure

This assay was carried out in sterile 96-well plates. In all plates, a standard curve was used to determine the glucose concentrations of the samples. Glycogen standards (0–200 µg/µl) were prepared using bovine liver glycogen in neutralised PCA solution (8 % PCA (v/v) neutralised with saturated sodium bicarbonate). All standards were treated with aminoglucosidase to reduce them to glucose as shown above.

2 µl of the correct concentration of standard or sample was carefully pipetted into each well of the plate and 200 µl of glucose reagent (Randox Laboratories Ltd.) added, ensuring no bubbles were formed. All samples were run in duplicate in order to establish intra-assay variability. A CV of less than 5 % was considered acceptable and samples were repeated if the CV was higher than this (Section 2.6). Due to the large sample number inter-assay variability was established by running one sample on all plates (CV<10 %). Plates were incubated at 37 ºC whilst being agitated for 10 minutes. Absorbance was then measured at 500 nm using the automated plate reader. Glucose concentrations were determined following linear regression analysis of the standard curve. The free glucose concentration was then subtracted from the total glucose reading and glycogen concentrations were attained using the standards. Results were expressed as a ratio of glycogen found in micrograms and weight in grams of the sample previously dissected (mg/g).

2.5.3 RNA extraction

Total RNA was extracted from liver tissue using Tri Reagent (Sigma-Aldrich), a modified version of the single step acidified phenol-chloroform homogenisation/precipitation method (Chomczynski, 1987). Tri Reagent contains guanidine thiocyanate and phenol, which dissolves RNA during tissue homogenisation. The addition of chloroform separates the homogenate into three phases. RNA can then be precipitated from the aqueous phase containing the RNA, which can be further precipitated.

In order to ensure any DNA that may be contaminating the RNA is removed, the RNA extract was treated with DNase before determination of the RNA integrity using spectrophotometric absorbance (Nanodrop® ND-1000 spectrophotometer, Labtech International Ltd. East Sussex, UK) to fully confirm quantity and quality. The absorbance at 260 nm relative to RNA free water alone was measured. In addition, the absorbance of each sample at 280 nm was calculated. This allowed the calculation of the ratio of optical density of the samples at 260:280 nm. Spectrophotometric absorbencies at 260 nm reflect nucleic acids, whereas that at 280 nm reflects protein contaminants. A ratio ≥1.8 was regarded as satisfactory (Wilkinson, 1995). RNA concentrations were automatically calculated using the absorbance at 260 nm using the software (Nano D2000, Labtech

International Ltd.) with the spectrophotometer, where one unit of optical density reflects 40 µg RNA/ml.

$$\text{RNA concentration of the stock extraction (µg/µl)} = \frac{(\text{Optical density at 260nm} \times 40\text{µg/ml}) \times (\text{ml of sample})}{\text{Original stock volume added (µl)}}$$

Due to the high glycogen content of the liver, which makes RNA extracts yellow in colour, glycogen had to be removed from the samples prior to spectrophometric analysis. This procedure was carried out using addition of sodium acetate.

In order to minimise the risks of contamination and degradation of RNA, the environment and reagents had to be ribonuclease-free. Several measures were taken to achieve and maintain this, including; the use of RNA free water, the treatment of tips and eppendorff tubes whilst wearing gloves to protect samples from RNases in human skin, the use of the laminar flow cabinet to set up reactions, and autoclaving all solutions and equipment before use. Moreover, all samples are maintained on ice throughout and aliquots of RNA containing solutions are prepared at the time of extraction to minimise the need for repeated freezing and thawing, which is known to lead to RNA degradation (Wilson and Walker, 2001).

2.5.3.1 RNA extraction procedure

RNA preparation

Samples (between 0.05 and 0.1 g) were sectioned from liver tissue, and thawed in 1 ml Tri-reagent (Sigma-Aldrich). The tissues were then completely disrupted using a Dispomix® closed system homogeniser.

Once homogenised, 200 µl of chloroform was added, vortexed and then incubated at room temperature for 15 minutes. After incubation, the samples were centrifuged in a microcentrifuge at 11300 g for 15 minutes at 4 °C. Once centrifuged, the top aqueous phase was then carefully transferred to fresh 1.5 ml sterile eppendorf tubes, taking care not to disturb the interface. 500 µl isoproponal was added and the tubes were allowed to stand for 10 minutes at room temperature to precipitate the RNA.

Following this, the samples were centrifuged at 11300 g for 10 minutes at 4 °C. The supernatant was decanted and the RNA precipitate was washed in 1 ml of ice cold 70 % (v/v) ethanol and centrifuged at 11300 g for 5 minutes at 4 °C. The ethanol supernatant was decanted and the RNA pellets allowed to air dry before reconstitution with 250 µl sterile RNA free water and freezing at -80 °C to facilitate re-suspension.

Glycogen removal

To each 250 µl RNA sample, 750 µl of 4 M sodium acetate was added, vortexed, and left at 20 °C overnight. The next day the samples were centrifuged at 7840 g for 20 minutes at 4 °C, this allows reformation of the RNA precipitate. The supernatant was decanted and the precipitate washed with 1 ml of 75 % (v/v) ethanol. Following this, the samples were centrifuged at 7840 g for 20 minutes at 4 °C. The ethanol supernatant was decanted and the RNA pellets allowed to air dry before reconstitution with 20 µl RNA free water and freezing at -80 °C to facilitate re-suspension.

DNase treatment of RNA

Working on ice, 1 µl of DNase buffer and 1 µl DNase (1 U/µl) were aliquoted into each tube followed by 8 µl of the RNA sample. The samples were then vortexed and allowed to incubate for 15 minutes at room temperature to allow the DNase to remove any DNA that may be contaminating the RNA. After incubation, 1 µl of DNase Stop solution was added to each tube, vortexed and transferred to the PCR machine (Techne Thermal Cycler, Bibby Scientific Ltd. Staffordshire, UK) and heated at 70 °C for 10 minutes to denature both the DNase 1 and the RNA. The samples were then placed on ice.

Spectrophotometric analysis of RNA

1 µl aliquots of each sample were used and spectrophotometric absorbance measured using a Nanodrop® spectrophotometer.

2.5.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a molecular biology technique that allows generation of complimentary DNAs (cDNAs) from messenger RNA (mRNA) for cloning using the reverse transcription enzyme Superscript II (Invitrogen Ltd. Paisley, UK); the cDNA can then be amplified by PCR (Polymerase Chain Reaction).

2.5.4.1 Reverse transcriptase polymerase chain reaction (RT-PCR) procedure

0.2 ml sterile eppendorf tubes were labelled with the individual sample ID. Working on ice, RNA samples were prepared to obtain a 3 µg of RNA in a 3 µl volume of RNA free water using the RNA concentrations obtained from the spectrophotometric absorbance at 260 nm (Section 2.5.3). Two negative controls were also included at this point; the 'no RNA' and 'no superscript' control. 9.5 µl of RT (reverse transcriptase) master mix (Invitrogen Ltd.) was aliquoted into each tube, vortexed and centrifuged briefly at 1250 g. This RT master mix consisted of 1 µl random hexamers primers (Roche Co. Basel, Switzerland), 1 µl deoxyribonucleotide triphosphate (dNTP; final concentration 10 nmpol/l) and 7.5 µl RNase free water. The tubes were then transferred to the PCR machine and heated to 65 °C for 10 minutes. 4 µl 5 x first strand buffer (Invitrogen Ltd.) and 2 µl dithiothreitol (DTT; 0.1M) were then added on ice. Samples were incubated for 32 minutes at room temperature before addition of 0.75 µl of Superscript II (200 U/µl) and 0.75 µl RNA free water. Reverse transcription was allowed to proceed for 45 minutes at 42 °C, then for 15 minutes at 70 °C. The reaction was then stopped and cooled to 8 °C.

2.5.5 Polymerase chain reaction (PCR)

PCR is a basic technique that results in the selective amplification of a chosen region of a DNA molecule (Bustin, 2004). Any region of the DNA molecule can be chosen, provided the sequences at the borders of the selected regions are known. The vast diversity of PCR methodology makes it a powerful tool to amplify small amounts of DNA or mRNA for a variety of analysis (Bustin, 2004). Since many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression, the ability to quantitate transcription levels of specific genes is essential to any research into gene function (Bustin, 2004).

PCR involves two primers (single-stranded DNAs) which are complimentary to opposite strands of the double stranded DNA sequence to be amplified (Bustin, 2004). It therefore presupposes knowledge of the genome sequence. The use of thermostable *Taq* Polymerase enzyme allows the double stranded DNA to be separated and subsequently permits the primers to anneal to their respective sequences with minimal loss of

enzymatic activity (Saiki et al., 1988). During this reaction, the polymerase enzyme synthesises the new DNA strand by adding nucleotides after binding the primers. The basic process of PCR is carried out over several cycles of denaturation, hybridisation and synthesis resulting in the eventual synthesis of several hundred million copies of the desired DNA fragment (Wilkinson, 1995, Wilson and Walker, 2001).

The products of each PCR reaction were tested using agarose gel electrophoresis in order to assess the size and abundance of the PCR product, and to check for DNA contamination. One clear band should be visible for each PCR reaction, more than one band or smearing on the gel indicates DNA contamination. DNA molecules carry a negative charge, which enables their separation in an electric field, with the smaller molecules moving faster and migrating farther than the larger ones in the direction of the cathode. The gel contains ethidium bromide which fluoresces under ultra violet (UV) light when intercalated in DNA. The DNA can then be visualised using a UV transilluminator CCD camera (Fujifilm luminescent image analyser LAS-1000 V1.01, Fuji Photo Film Co. Ltd. Tokyo, Japan).

The PCR product was then extracted from the gel and purified using the QIAquick® gel extraction kit (Qiagen, West Sussex, UK). This combines spin-column technology with the selective binding properties of a silica membrane which absorbs the DNA in the presence of high concentrations of salt, while allowing contaminants to pass through; after further purification steps, the purified DNA can then be eluted.

Primer design

Oligonucleotide primers are sequences of nucleic acids, essential for the first step of DNA replication by polymerase reaction. They are required because their binding to the genome marks the point where the DNA polymerase starts or finishes the imprinting of the new DNA sequence. The DNA polymerase starts replication at the 3' end of the primer, and then copies to the opposite strand.

One of the parameters for optimal polymerase amplification is similar melting temperature (T_m) of both primers, to ensure annealing to the DNA occurs at the same time. A significant difference between temperatures

may result in an incorrect sequence of DNA or failure to extend at all. Correct T_m also prevents formation of primer dimmers, where the primers anneal to each other during the reaction. Optimal primer length is between 15-30 bases with a cytosine (C) and guanine (G) content (the number of Cs and Gs in the primer as a percentage of the total bases) around 30-80 % to avoid the formation of loops and mishybridisation of the primers. Primer sequences should also be unique to select for a specific region of DNA. A common method to check for this is to use an NCBI tool, Primer-BLAST (http://blast.ncbi.nlm.nih.gov/), which will indicate all possible regions that the primer might bind to, and indicate any intron/exon boundaries, avoiding any amplification of genomic DNA. Alternatively, software such as Beacon Designer can be used to design specific primers.

All primer sequences used were taken from previously published literature and then optimised for this study. The oligionucleotides were purchased from Sigma-Genosys (Sigma Genosys Ltd. Suffolk, UK) and were re-suspended with the appropriate volume of RNA free water to a stock concentration of 100 µM. Each PCR product was sent for DNA sequencing for the validation of the primers using the QMC sequencing service. The resulting chromatogram was then analysed using Chromas Ligte computer software (http://www.technelyium.com.au) and checked for the correct genes using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Table 2.4 describes the sequence and annealing temperature of each primer used during this project.

Table 2.4 Primer sequences used in this thesis

Gene	Abbreviation	Primer sequences (5'-3')	Annealing temp. (°C)	Ascension number
18S ribosomal RNA	18S	F: GATGCGGCGGCGTTATTC	60	AK2372246
		R: CTCCTGGTGGTGCCCTCC		
Ribosomal phosphoprotein	RPO	F: CAGCAAGTGGGAAGGTGTAATC	60	NM_001098598
		R: CCCATTCTATCATCAACGGGTA		
Cyclophylin	Cyclo	F: GCACTGGTGGCAAGTCCAT	60	AY008846
		R: AGGACCCGTATGCTTCAGGA		
Glucocorticoid receptor	GR	F: GCTGCTGGTCCTGCTGCTC	60	AF141371
		R: CCTTCACATTCGGCTGCTCTGG		
11beta-Hydroxysetroid Dehydrogenase type 1	11-BHSD1	F: CGCAGAAGCATGGAGGTCAAC	60	NM_214248
		R: AATAGGGAGCAACAAGTCGAT		
11beta-Hydroxysetroid Dehydrogenase type 2	11-BHSD2	F: ACCGTGTTGGAGTTGGATAGC	60	NM_213913
		R: CTGGCAGTGTGGACCTTGG		
Insulin receptor	IR	F: AGCCTGCGAGAGCGGATC	60	AF102858
		R: AGCCATCAATTCCATCACCAC		
Peroxisome proliferator-activated receptor alpha	PPARα	F: CGGAGAACCATTCGGCTAAAG	60	NM_001044526
		R: CGAGAGGCACTTGTGGAAACG		
Fatty acid binding protein 1	FABP1	F: CCAAGGTCGTCCAGAATGAG	60	AY960623
		R: CTGAACCACTGTCTTGACCTT		

2.5.5.1 Polymerase chain reaction (PCR) procedure

As previously described, the PCR reaction was performed working on ice, wearing gloves and using filter tips. The reaction was performed in a final volume of 20 µl using 1 µl of cDNA, 10 µl of PCR master mix (ABgene Ltd.), 1 µl of forward and 1 µl reverse primer, at a final concentration of 100 µmol/l in 7 µl of RNA free water. The tubes were then transferred to the PCR machine (Touchgene Gradient, Techne). PCR conditions are described in table 2.5.

Table 2.5 Polymerase chain reaction (PCR) standard program

Step	Temperature (°C)	Time
Initiation	105	4 minutes
Enzyme activation	95	15 minutes
	45 cycles	
Denaturing	94	30 seconds
Annealing	60	30 seconds
Primer extension	72	60 seconds
Final extension	72	7 minutes
Hold	8	Indefinitely

2.5.5.2 Gel electrophoresis of DNA

A gel was made by dissolving agarose (3 % (w/v) agarose pH7) in 1x TAE (tris-acetate EDTA) buffer (2 M Tris([hydroxymethyl] amino-methane, 0.2 % (w/v) SDS, 1 M glacial acetic acid, 0.5 M Na_2 EDTA pH8, made in deionsate water to 100ml) and heating. The solution was cooled before adding ethidium bromide to a final concentration of 5 µg/ml. The agarose was then immediately poured into a horizontal gel tank with the appropriate size comb inserted, allowing wells to form. Once the gel had set, the comb was removed, and the gel covered with 1x TAE.

20 µl of PCR product was mixed with 5 µl of 5x loading buffer (0.0016 % (v/v) saturated bromophenol blue, 0.15 % (w/v) EDTA, 20 % (v/v) glycerol) and loaded into separate lanes along with a 1 Kb size marker (ABgene Ltd.). Electrophoresis was then performed at constant 120 V for 1 hour. For the visualisation of DNA, the gel was exposed to a UV transilluminator CCD camera. The size of PCR product was then estimated by comparison to the DNA marker bands.

2.5.5.3 DNA extraction procedure

After visualisation, the bands were cut out of the gel and transferred into sterile 1.5ml eppendorfs. The weight of each gel slice was noted and the DNA was extracted using a QIAquick® gel extraction kit, following the instructions. In summary, 3x volume of buffer QG (pre-made buffer contained within the Qiagen kit (QG not defined)) was added to each tube, vortexed and incubated at 50 °C for 10 minutes, vortexing at regular intervals. Once the agarose had completely dissolved, 1x volume of isopropanol was then added to precipitate the DNA. The samples were then transferred into sterile spin columns (contained within the kit), and centrifuged at 13,250 g for 1 minute. The resulting flow-through was discarded and a further 500 µl of buffer QG was added to the spin columns and centrifuged again at 13,250 g for 1 minute to ensure the removal of all traces of agarose.

Once centrifuged, the flow-through was again discarded and the samples washed in 750 µl of ethanol based buffer PE (pre-made buffer contained with the kit; PE not defined). The samples were then centrifuged at 13,250 g for 1 minute and the resulting-flow through discarded. Finally, the columns were transferred into sterile 1.5 ml eppendorfs and 30 µl of RNA free water was added directly onto the membranes of each spin column; these were left to stand for 1 minute, and then centrifuged at 13,250 g for 1 minute to elute the DNA.

Once extracted, the DNA concentrations were determined using the nanodrop® spectrophotometer (Section 2.5.3.1), and subsequently used for making standards to be used on the real-time PCR machine.

2.5.6 Real-time polymerase chain reaction (QPCR)

Real time PCR (QPCR) uses a signal, normally fluorescence, which increases with the increased amount of DNA formed by PCR (Bustin, 2004). When the signal rises above a set background, it is detected. The signal normally rises in a sigmoidal way, until it reaches its plateau (Figure 2.1). During the linear increase, the signal can be compared with the signal from other samples. A standard curve with known DNA concentrations (Figure 2.2) is usually run on the same 96-well plate, to compare the signal of the samples and to calculate the efficiency of the reaction (Bustin, 2004). The signal is normally measured at a threshold level called the cycle

point (Cp). The cycle number at which the signal crosses the Cp is correlated to the initial concentration of DNA in the template (Higuchi et al., 1993). The plotting of the Cp values, versus the log of the cDNA concentration of the standard curve creates a slope, which reflects the amplification efficiency (E). E can be calculated by the formula shown below (Equation 2.1) (Pfaffl, 2001) and should be between 95-100 % (1.95 ≥ E ≥ 2).

$$E = 10^{[-1/slope]}$$

Equation 2.1 Calculation of the real-time polymerase chain reaction (QPCR) efficiency (E); Slope = gradient of standard curve (Pfaffl, 2001)

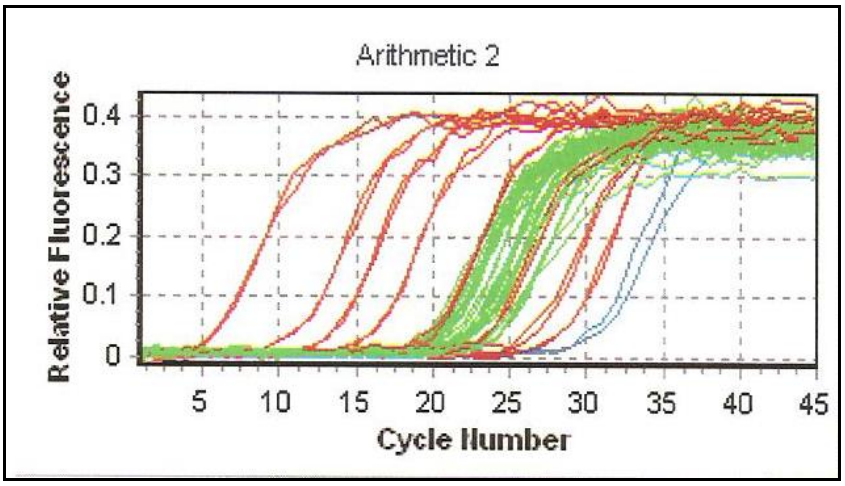

Figure 2.1 Typical real-time polymerase chain reaction (QPCR) result. Red: standard curve; green: unknown samples and blue: negative control

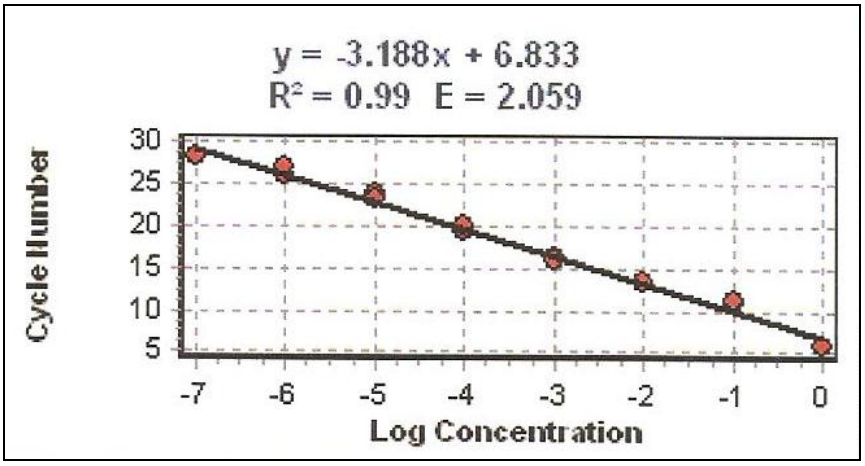

Figure 2.2 Typical standard curve from real-time polymerase chain reaction (QPCR**)**, indicating the appropriate R^2, efficiency (E), and slope values

DNA binding agents, like SYBR green, bind specifically to double stranded DNA; they emit a fluorescent signal once bound. During each cycle of PCR, the fluorescent signal increases with the amount of double stranded DNA. As with conventional PCR, the specificity of the reaction is determined entirely by its primers; however the PCR can be verified by a melting curve that allows comparison of the melting temperatures of the specific product and any suspected non-specific products (Ririe et al., 1997, Al-Robaiy et al., 2001). The reason for this is that different length products and products of different sequences will melt at different temperatures and will be observed as distinct peaks (Figure 2.3); therefore, a single dissociation peak should be present to confirm specificity (Ririe et al., 1997).

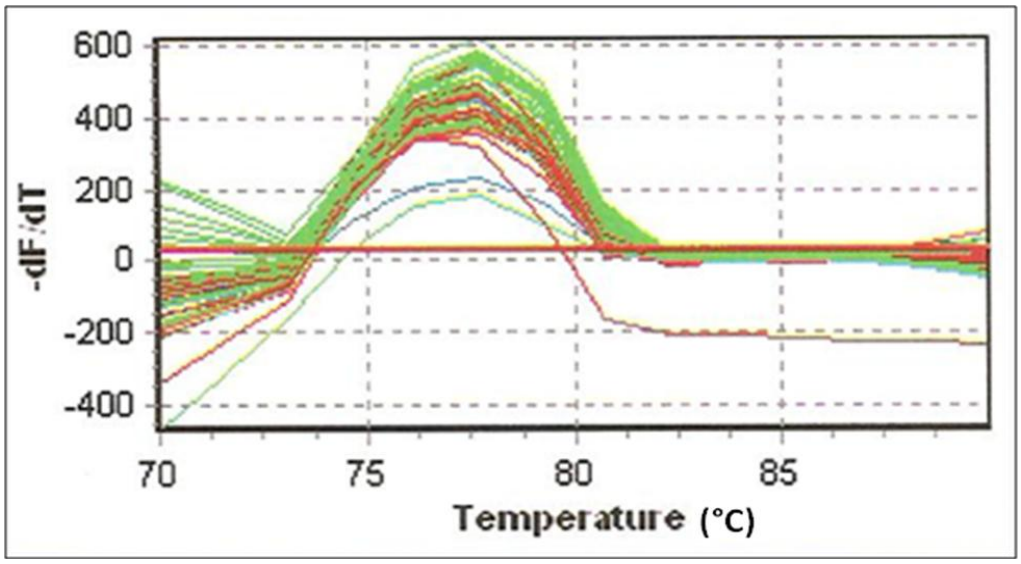

Figure 2.3 A typical dissociation peak from real-time polymerase chain reaction (QPCR); Negative first derivative of the melt curve which measures a cycle to cycle decrease in fluorescence as temperature is increased; -dF/dT = negative of the rate of change in fluorescence (dF) as a fraction of temperature (dT); Red: standard curve; green: unknown samples and blue: negative control

When comparing samples, at least one housekeeping (HK) gene should be used to rule out any pipetting errors and to normalise for any variation in RNA abundance between samples (Bustin, 2004). HK genes should be highly expressed and not altered by the treatment in question (Bustin, 2004). For all gene expression data analysed in this study, three HK genes 18S ribosomal RNA (18S), Ribosomal phosphoprotein (RPO) and cyclophylin (cyclo), were used. All Cp values (both target and HK genes) were first transformed to normalised relative expression levels using the delta-Cp method (Equation 2.2), where the Cp value of your gene is

related to the control Cp (sample with the highest expression (lowest Cp value)). The Microsoft excel program geNorm was then used to calculate the gene expression stability measure (M) for a HK gene as the average pair wise variation (V) for that gene with all other tested HK genes (Vandesompele et al., 2002). Exclusion of the gene with the highest M value allows ranking of the genes according to their expression stability. A gene expression normalisation factor was then calculated for each tissue sample, based on the geometric mean of the two most stable reference genes.

$$\text{Delta-Cp} = E^{\wedge(\text{control Cp} - \text{sample Cp})}$$

Equation 2.2 Calculation of Delta-Cp; E = Efficiency (calculated in Equation 2.1); control Cp = Crossing point of the sample with the highest expression (lowest CP value)

The relative gene expression of each sample was then calculated using a mathematical model for relative quantification developed by (Pfaffl, 2001), where the normalised relative expression values for each sample (delta-Cp) were divided by the normalisation factor of the reference genes for that specific sample (Equation 2.3).

$$\text{Relative gene expression} = \frac{\text{Delta-CP}}{\text{Normalisation factor of reference genes}}$$

Equation 2.3 Calculation of relative gene in a real-time polymerase chain reaction (PCR); Delta-CP calculated from Equation 2.2; Normalisation factor of reference genes = geometric mean of the two most stable reference genes

2.5.6.1 Real-time polymerase chain reaction (QPCR) procedure

In all runs, a standard curve was used to enable the efficiency of the reaction to be calculated. For each gene, DNA that made up the standard curve was obtained from DNA extracted from the agarose gels. A serial dilution, 1:10 of the DNA is completed, until a set of 9 standards is achieved; the lowest standard having a concentration of 1×10^{-9} ng/µl.

QPCR reactions were carried out in a final volume of 15 µl, which included 1 µl of both forward and reverse primers, 7.5 µl of SYBR green master mix (Qiagen), and 4.5 µl of RNA free water. Fourteen µl of this mix was carefully pipetted into each well of a sterile 96-well plate and 1µl of cDNA

of the unknown samples or 1 µl of the correct concentration of standards was used, ensuring no bubbles were formed. Furthermore, a cDNA negative control was also run on each plate to check for any contamination; RNA free water was used instead of cDNA for this. All samples were run in duplicate in order to establish intra-assay variability. A CV of less than 5 % was considered acceptable and samples were repeated if the CV was higher than this (Section 2.6). Due to the large sample number inter-assay variability was established by running one cDNA sample on all plates for that gene (CV<10 %).

Once the plates were made, they were heat-sealed and placed in the PCR machine (Quantica™, Techne). PCR conditions are described in Table 2.6.

Table 2.6 Real-time polymerase chain reaction (QPCR) standard program

Step	Temperature °C	Time
Denaturing	95	15 minutes
	45 cycles	
Denaturing	94	30 seconds
Annealing	60	30 seconds
Extension	72	60 seconds
Melt curve	Ramp from 75-95	15 minutes
Hold	8	Indefinitely

The data collected from the QPCR reaction was exported to an Excel datasheet and the relative gene expression for each sample calculated.

2.6 Statistical analysis

To assist in the study design and to limit the sample sizes that would be required in each group and not erroneously accept or reject the null hypothesis, a power calculation was performed. Sample size was calculated to assess the effect of measured variables obtained from a dietary intervention or group at 80 % power. The sensitivity of the power calculation is a function of the group sizes, statistical controls and reliability of the measures. Therefore, by increasing the sample size it is possible to increase the statistical power. In the research literature, it is generally accepted that a power of 80 % is adequate. After performing power calculations using Samplepower TM (SPSS Inc. IL, USA), it was decided that 8 animals in each group would be sufficient. This is based on data

from a prior study investigating metabolic gene expression in adipose tissue from small and normal piglets. A difference of at least 34 % was observed (25±4; 16.5±5 gene as a % of 18S), groups of 8 will have 96.4 % statistical power to yield a statistically significant result ($P<0.05$).

Previous to any statistical analysis, all the values collected in each assay were transferred to an excel datasheet. The CV of all the duplicate values were analysed and a CV < 5 % confirmed low variation between duplicates and the reproducibility of the assay. The data points that fulfil this requirement were used for the analysis.

All data were analysed using the SPSS for windows package, version 18.0. The distribution of the data collected in each assay was the main factor for the chosen statistical test to perform. Data were tested for normal distribution using the Kologorov-Smirnov test and a $P \geq 0.05$ confirmed normality. Depending on the significance of this analysis, parametric or non-parametric tests were utilised for the analysis of the data. For consistency, the data in these studies are presented as mean and standard error (SEM).

Throughout this study, in order to examine the effect of increased fat or protein during gestation, the means of the fat supplemented (FS) or protein supplemented (PS) groups were compared with the control (C), but not with each other. In addition, differences between small and median-birth weight offspring were investigated by comparing the small and median-birth weight animals selected for each dietary group. Thus, depending on their type of distribution based on the significance of the Kologorov-Smirnov test, either an independent parametric (T-test) or non-parametric test (Mann Whitney U) was assigned to compare each group with the control. When an independent T-test was applied, it was followed by a Leven's test to evaluate equal variances. If the test result was $P \geq 0.05$, equal variance between the groups could be assumed. More specific tests are detailed in the relevant results chapters and further explanation is given when relevant.

Chapter 3 - Impact of maternal nutrition on neonatal mortality

3.1 Introduction

The piglet has a higher rate of neonatal mortality than many other animals (Table 3.1). Most of these occur in the very early postnatal period, with 50 percent of all pre-weaning deaths taking place within the first 3 days after parturition, and 90 percent within the first week of life (Varley, 1995). Multiparous species such as the pig have evolved to allow for relatively high losses as a natural selection mechanism (Varley, 1995). Cattle, sheep and humans produce fewer offspring and therefore display increased maternal investment to individual offspring, thus resulting in lower mortality rates (Varley, 1995). In addition, there is also the availability of advanced, medical technology to promote high survival rates in many human populations. However, in animal production, this is much more difficult and not considered to be cost-effective.

Table 3.1 Neonatal mortality for different species (Varley, 1995)

	Neonatal mortality (%)	
Species	**Average**	**Range**
Pig	13	5-20
Human	2	1.5-7
Cattle	4	0-10
Sheep	5	3-8

3.1.1 Maternal nutrition and neonatal mortality

Optimal feeding during pregnancy is important because it determines the size and viability of piglets at birth (Pluske et al., 1995). It also has a key role in determining the subsequent milk production potential during lactation (Pluske et al., 1995), which will in turn determine the offspring's growth. Piglets are born with very low energy reserves (Chapter 1; Section 1.5.1.2) and in the absence of immediate and adequate nutrition, hypoglycaemia will occur (Varley, 1995). The availability of body reserves and the ability of the piglet to utilise these, determines the extent to which it avoids hypoglycaemia and hypothermia and hence survival. Therefore, emphasis has been placed on increasing the piglets limited neonatal energy stores to enhance birth weight and/or survival by manipulating sow nutrition during gestation.

In gilts, increasing the feed intake during gestation will increase average piglet birth weight, with a maximum birth weight achieved by feeding around 1.9 Kg/day (Figure 3.1) (Baker et al., 1969), although in this study, the level of feed intake had no effect on litter size or piglet mortality (Baker et al., 1969). Similar results have been observed in sows, although a maximum birth weight is not achieved until food intake is greater than observed with gilts (Pond, 1973).

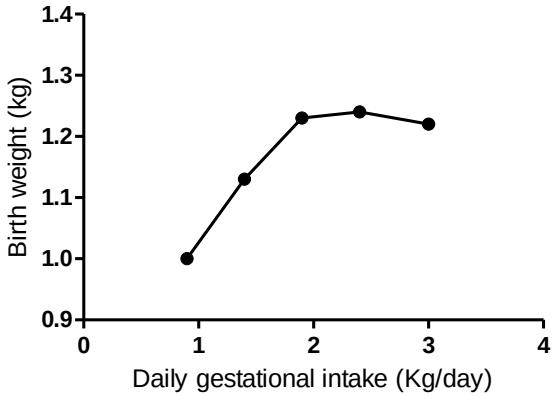

Figure 3.1 Relationship between daily food intake of gilts during pregnancy and piglet birth weight (Baker et al., 1969)

3.1.1.1 Fat supplementation

The effects of a high-fat intake during pregnancy on maternal glucose tolerance and offspring birth weight and mortality in rat nutritional programming studies have already been described in Chapter 1 of this thesis. Briefly, consumption of a high-fat diet during pregnancy increases the risk of insulin resistance in the mothers which may be linked to a reduction in litter size and an increase in offspring mortality (French et al., 1952, Guo and Jen, 1995, Khan et al., 2003, Taylor et al., 2003, Taylor et al., 2005). Despite gestational fat supplementation being detrimental in rat programming studies, it is widely used in the commercial pig industry to reduce offspring mortality, although the levels of fat used are much lower.

As previously mentioned (Chapter 1; Section 1.5.1.2), piglets are born with a very small amount of stored lipid that they can use for energy. They have large energy stores in the form of glycogen but much of this is depleted during the first 2 days after birth (Chapter 1; Section 1.7.2.1) (Okai et al., 1978). It has been previously suggested that maternal fat supplementation, may improve the pre-weaning survival of piglets by improving their energy status (Seerley et al., 1974). However, this has

been demonstrated not to be the case, and, supplementing the diet with fat during late gestation, whilst maintaining energy content, has no effect on maternal plasma glucose concentrations, or piglet birth weight and liver glycogen content (Seerley et al., 1974, Boyd et al., 1978, Seerley et al., 1981, Azain, 1993). There is a slight increase in carcass fat concentration slightly after birth (Seerley et al., 1974), but this increase is so small it is unlikely to be of practical importance (Pettigrew, 1981).

The most consistent effect of feeding fat during late gestation and/or lactation is to increase the milk yield and fat content of both the colostrum and the milk (Seerley et al., 1974, Pettigrew, 1981). Such increases may account for an improvement in the survival rate of piglets born to sows whose diets were supplemented with fat during late gestation and lactation (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993). This effect was largest within herds where the expected survival rate of piglets is low (less than 80 %) (Pettigrew, 1981). A greater effect on the survival rate of low-birth weight piglets (less than 1 Kg) than on their larger litter mates has been seen in most studies (Seerley et al., 1974, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993). However, these results need to be interpreted with caution as not all studies produced positive results on piglet survival, and negative responses to fat supplementation are often not published (Pettigrew, 1981).

Table 3.2 Summary of effects of maternal fat supplementation in pigs, during late gestation and lactation on offspring survival rate

Source	Seerley et al. (1974)	Seerley et al. (1981)	Boyd et al. (1978)	Azain et al. (1993)
Diet	Gestation day 109 – parturition C: 40 % corn starch FS: 40 % corn oil	Gestation day 109 - end of lactation C: 5.3 % fat FS (corn oil): 12.4 % fat FS (lard): 13.7 % fat	Gestation day 109 – day 14 lactation C: 15 % corn starch FS: 15 % corn oil	Gestation day 109 - day 7 lactation C: 2 % soy bean oil FS (MCT): 2 % soy bean oil + 10 % MCT FS (LCT): 12 % LCT (soy bean oil)
Calorie intake	No difference	↑	No difference	No difference
Maternal glucose	No difference	n/a	No difference	No difference
Birth weight	No difference	No difference	No difference	No difference
% Milk lipid	↑	No difference	n/a	No difference
Liver glycogen	No difference	No difference	No difference	n/a
% Total lipid	↑	No difference	No difference	n/a
Piglet glucose	↑	n/a	n/a	↑
Survival rate	↑	↑	↑	↑

C = Control; FS = Fat supplemented; ↑ = Increased in fat-supplemented groups; MCT = Medium-chain triglycerides; LCT = Long-chain triglycerides

3.1.1.2 Amino acid supplementation

At present, there is limited and conflicting evidence demonstrating the effects of protein supplementation during pregnancy on offspring outcome (Chapter 1; Section 1.4.1), with most work instead, focussing on the effects of high-protein diets during childhood or on obesity in adults (Chapter 1; Sections 1.4.2-1.4.3). Recent studies have begun to focus on the role of specific amino acids, during pregnancy, and thus investigate the consequences of supplementing in order to provide beneficial outcomes for the offspring.

Amino acids are not only the building blocks of proteins in cells, but are also precursors for the syntheses of nitrogenous substances, polyamines, creatine, dopamine and catecholamines, essential for whole-body energy homeostasis (Wu, 2009, Wu et al., 2010). One key amino acid thought to be crucial in placental and fetal development is arginine, probably due to its role as an essential precursor for nitric oxide (NO) and polyamine production (Figure 3.2) (Wu and Morris, 1998, Wu et al., 2004, Wu, 2009). Glutamine is a precursor for arginine synthesis, and is also important in its own right for polyamine production (Wu and Morris, 1998). NO and polyamines are essential for angiogenesis, embryogenesis, placental trophoblast growth, regulating placental-fetal blood flow, and thus, the transfer of nutrients and oxygen from the mother to the fetus (Roselli et al., 1998, Bird et al., 2003, Wu et al., 2010). Consequently, NO and polyamine synthesis have a key role in fetal growth and perinatal survival (Wu et al., 2006). This is demonstrated by knocking out the NO synthase (NAS) gene, which results in inhibition of NO synthesis in pregnant mice and causes intrauterine growth retardation (IUGR) (Hefler et al., 2001). A large abundance of arginine has been discovered in porcine allantoic fluid during early gestation (Wu et al., 1996), further supporting the important role of arginine in fetal growth and development.

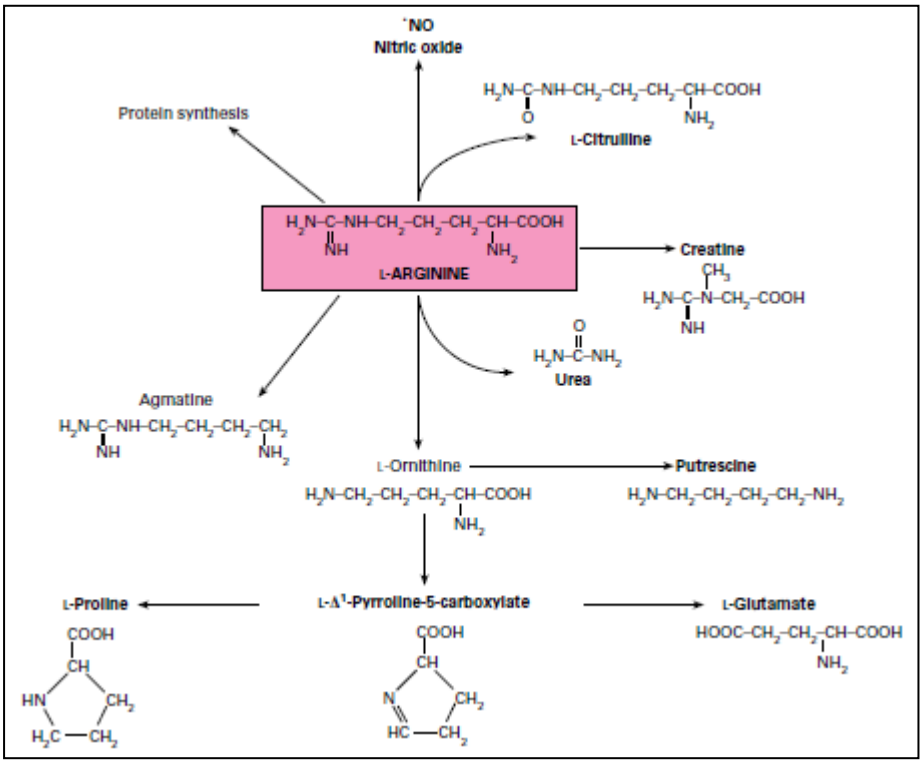

Figure 3.2 Metabolic fates of arginine in mammalian cells (Wu and Morris, 1998)

It is normal commercial practice in the swine industry, to restrict feed intake of sows during pregnancy, to prevent excessive weight gain (Ewan, 1991). Therefore, in some cases animals do not receive sufficient amino acids to support optimal embryonic and fetal survival throughout gestation (Kim et al., 2007). Because of extensive catabolism of arginine by arginase in the small intestine, only a percentage of dietary arginine will enter the portal circulation of the pregnant animal (Bergan and Wu, 2009). Therefore, it was hypothesised that dietary supplementation with arginine would improve reproductive performance. Several lines of experimental evidence support this hypothesis. Firstly, a recent study with pregnant sows demonstrated that dietary supplementation with 1 percent arginine from 30 days of gestation until term decreased the number of stillborn piglets (Mateo et al., 2007). Similarly in rats, supplementing the maternal diet with 1.3 percent arginine, either throughout the whole of pregnancy or during days 1 to 7 of gestation, increased the litter size and number of live-born pups, with no effect on birth weight (Zeng et al., 2008). Conversely, dietary supplementation with 0.8 percent arginine from the beginning of pregnancy has been shown to produce detrimental effects by reducing embryonic survival rate and thus litter size in pregnant sows on day 25 of gestation (Li et al., 2010).

3.1.2 Aims and hypothesis

In summary, fat supplementation, during late gestation and lactation, has been used in the commercial pig industry in an attempt to reduce the high rates of piglet mortality for many years. This is the first study to investigate the effects of fat supplementation in sows, throughout most of gestation (until day 110), on maternal weight gain, glucose tolerance at term and piglet mortality. It is hypothesised that there will be an increase in maternal body weight and back fat throughout gestation, although this will not be different with fat supplementation due to the isocaloric nature of the diets. Glucose tolerance will be reduced near to term in the fat supplemented animals, which may result in an increase in postnatal mortality, particularly in the first few days after birth.

In contrast to the studies investigating the effects of fat supplementation on offspring mortality, the effects of protein supplementation are relatively unknown. However, recent studies have demonstrated the beneficial effects of arginine supplementation in reducing mortality and increasing offspring birth weight. The second aim of this chapter is to examine the effects of supplementing the sow diet with a protein source that is high in arginine and glutamine, on offspring mortality and birth weight. It is hypothesised that this will result in a slight increase in offspring birth weight and thus a reduction in neonatal mortality.

3.2 Methods

A full description of the study design and plasma sampling and analysis methods are described in Chapter 2.

3.2.1 Measurement of maternal body weight and back fat

The body weight and back fat measurements of all sows were recorded 4 days prior to mating (-4) and on days 40, 70 and 108 of gestation. Back fat was recorded ultrasonically using the P2 method (back fat measured at 5cm from the midline over the last rib), with a Lean-Meater ® (Renco Corp. MA, U.S.A.).

3.2.2 Analysis of maternal plasma

Plasma was sampled on days -4, 40, 70 and 108 days of gestation and auto-analysed for glucose, non-esterified fatty acids (NEFA), triglycerides (TAG), cholesterol as described in Chapter 2, Sections 2.3-2.4. In addition, fasting plasma insulin concentrations were determined at 108 days of gestation as previously described (Chapter 2; Section 2.4.5).

3.2.2.1 Glucose tolerance test (GTT)

Sows fed the control and fat supplemented diets were tested for glucose tolerance on day 108, towards the end of gestation. Protein supplemented sows were not tested for glucose tolerance due to commercial constraints. As with the blood sampling, one day prior to sampling, a catheter was placed in the sow's ear. The first blood samples were taken every minute, for 4 minutes prior to glucose administration; the catheter was flushed with saline between sampling. Blood from each time point was tested for glucose using indicator stick (Precision Xtra Plus, Abbott Laboratories, IL, USA) and an automated glucose reader (Precision Xceed, Abbott Laboratories). Glucose solution (Dextrose monohydrate, 495.4 g/L; AVEBE, Veendam, The Netherlands) was then infused intravenously over a 5 minute period. The catheter was flushed with saline and 15 blood samples were then taken over a period of an hour. Glucose concentrations were measured as before, in order to determine the rate of glucose clearance of the sows.

3.2.3 Measurements at birth

The gestation length, total number of live and still born piglets, and birth weight were recorded. In addition, any piglet deaths during the lactation

period were recorded and the reason for death noted as one of 5 categories which were low-viability, trauma, scours (diarrhoea), deformity, or other (unknown diagnosis).

3.2.4 Milk sampling and composition analysis

Milk composition analysis was carried out by employees from Schothorst Feed Research. In all sows, milk was sampled, after milk let down via an intravenous oxytocine injection (10 I.U./mL; Eurovet Animal Health, Bladel, The Netherlands) into the ear on day 2 and 24 of lactation. The fat, protein and lactose concentrations were determined in the fresh milk by infared analysis, using a Fourier Transform InfaRed (FTIR) interferometer (MilkoScan^{TM}, Foss Electric, Hillerød, Denmark). This works by sending out light of the specific wavelength to be detected, which differs for fat, protein, and lactose, and measuring the absorbance at this wavelength.

3.2.5 Statistical analysis

Details of all statistical test performed are described in Chapter 2, Section 2.6.

3.3 Results

3.3.1 Maternal growth

All sows consumed most of the food offered to them throughout gestation (Table 3.3). This meant that as the diets were isocaloric, (except FS diet which was slightly higher in energy, counteracted by a reduced feed allowance) energy intake was similar between groups throughout gestation.

Table 3.3 Average daily feed intake of sows throughout gestation

Gestational day	C	FS	PS
	(Kg/day)	(Kg/day)	(Kg/day)
0-40	2.82±0.02	2.47±0.02	2.81±0.02
40-70	2.86±0.02	2.47±0.02	2.85±0.02
70-110	3.71±0.02	3.16±0.07	3.70±0.03

Values are means ± SEM for control (C; n=8), fat supplemented (FS; n=8), and protein supplemented (PS; n=8) sows

Prior to mating and the beginning of this study, there was no difference in body weight or back fat thickness between the sows (Table 3.4). As expected, both maternal body weight and back fat increased throughout gestation (Table 3.4), with a total increase in body weight of around 72-79 Kg and an increase in back fat of 3-4 mm (Table 3.4). Fat or protein supplementation did not affect mean body weight or back fat throughout gestation at any time point (Table 3.4). The increase in body weight was largest during the final part of gestation (day 70-80) when compared with early gestation (prior to mating-day 40), this is shown by a higher fractional growth rate during this time (Table 3.4). In contrast, the increase in back fat followed an opposite pattern, with the largest increases being seen in early gestation (Table 3.4). However, this is not the case in the protein supplemented group, where a similar increase in back fat was seen during both early and late gestation resulting in a significant increase in the growth rate of the back fat of sows during late gestation when compared to the control animals (Table 3.4).

Table 3.4 Body weights, back fat and growth rates throughout gestation

	C	FS	PS
Prior to mating:			
Body weight (Kg)	215.50±13.42	209.88±9.25	204.00±9.69
Back fat (mm)	13.88±0.86	13.81±0.59	12.94±1.05
Day 40 of gestation:			
Body weight (Kg)	236.63±11.76	232.63±9.61	224.63±9.32
Back fat (mm)	15.63±1.01	15.50±0.63	14.38±0.91
Day 70 of gestation:			
Body weight (Kg)	257.75±10.82	254.63±8.60	251.75±9.22
Back fat (mm)	16.44±1.26	16±0.56	15.81±1.04
Day 108 of gestation:			
Body weight (Kg)	287.75±10.47	281.88±8.70	282.88±9.03
Back fat (mm)	17.31±1.27	16.69±0.46	17.31±1.09
Fractional growth rate (prior to mating-108 days):			
Body weight (Kg/day/Kg)	$3.3x10^{-3}±5x10^{-4}$	$3.3x10^{-3}±4x10^{-4}$	$3.7x10^{-3}±4x10^{-4}$
Back fat (mm/day/mm)	$2.3x10^{-3}±2x10^{-4}$	$2.0x10^{-3}±3x10^{-4}$	$3.4x10^{-3}±7x10^{-4}$
Fractional growth rate (prior to mating-40 days):			
Body weight (Kg/day/Kg)	$2.7x10^{-3}±6x10^{-4}$	$2.8x10^{-3}±3x10^{-4}$	$2.6x10^{-3}±4x10^{-4}$
Back fat (mm/day/mm)	$3.1x10^{-3}±5x10^{-4}$	$3.1x10^{-3}±2x10^{-4}$	$3.1x10^{-3}±2x10^{-4}$
Fractional growth rate (70-108 days):			
Body weight (Kg/day/Kg)	$3.8x10^{-3}±4x10^{-4}$	$3.5x10^{-3}±4x10^{-4}$	$4.2x10^{-3}±6x10^{-4}$
Back fat (mm/day/mm)	$1.7x10^{-3}±3x10^{-4}$	$1.4x10^{-3}±7x10^{-4}$	$3.1x10^{-3}±5x10^{-4*}$

Values are means ± SEM taken 4 days prior to mating and on days 40, 70 and 108 of gestation for control (C; n=8), fat supplemented (FS; n=8), and protein supplemented (PS; n=8) sows; Statistical differences between PS and the control are denoted by * P<0.05 (Mann-Whitney)

3.3.2 Fasted plasma metabolite concentrations

In some groups, the number of sows does not equal 8 (Table 3.5). This was due to the catheter in the sow's ear not working properly. Either there were problems with initial insertion, or withdrawing blood due to a blockage.

Fasted plasma glucose and TAG concentrations remained constant throughout gestation (Table 3.5). A similar pattern was seen with plasma total cholesterol; however there was a decrease in concentration towards term at 108 days of gestation (Table 3.5). There was a sharp decrease in plasma NEFA during the first 40 days of gestation (Table 3.5). Concentrations then levelled out and remained constant throughout gestation (Table 3.5).

Supplementing the maternal diet with fat had no effect on the fasting plasma concentrations of glucose, TAG or NEFA throughout gestation (Table 3.5). Total cholesterol plasma concentrations were raised throughout gestation in the fat supplemented group (Table 3.5). However, levels were consistently higher before mating took place on day 0, so this is probably not a dietary effect.

Protein supplementation had no effect on glucose, TAG or cholesterol concentrations throughout gestation (Table 3.5). There was a reduction in NEFA concentration at 108 days of gestation in the protein supplemented group (Table 3.5).

Dietary supplementation with either fat or protein had no effect on maternal fasting plasma insulin concentrations (μg/ml) at 108 days of gestation (C: 0.46±0.01; FS: 0.48±0.01; PS: 0.48±0.18).

Table 3.5 Fasted plasma concentrations of glucose, triglycerides (TAG), non-esterified fatty acids (NEFA) and cholesterol during gestation

Gestational day	Gestational diet	Glucose (mM)	TAG (mM)	NEFA (mM)	Cholesterol (mM)
-4	C (n=5)	3.88±0.57	1.48±0.30	1.12±0.47	2.10±0.12
	FS (n=8)	3.58±0.51	1.57±0.20	1.46±0.36	2.46±0.07*
	PS (n=7)	3.80±0.32	1.63±0.33	1.24±0.21	2.49±0.19
40	C (n=8)	3.93±0.12	1.27±0.09	0.27±0.05	2.15±0.09
	FS (n=8)	4.11±0.08	1.52±0.32	0.27±0.02	2.61±0.14*
	PS (n=8)	3.90±0.21	1.30±0.08	0.21±0.02	2.10±0.11
70	C (n=8)	3.90±0.10	1.24±0.05	0.19±0.01	2.03±0.09
	FS (n=8)	3.98±0.10	1.38±0.13	0.28±0.02*	2.44±0.14*
	PS (n=8)	3.71±0.19	1.34±0.15	0.24±0.02	2.00±0.11
108	C (n=7)	3.93±0.10	1.38±0.13	0.32±0.06	1.57±0.06
	FS (n=8)	3.70±0.17	1.41±0.15	0.38±0.09	1.94±0.15*
	PS (n=8)	3.97±0.10	1.34±0.08	0.15±0.04*	1.83±0.11

Values are means ± SEM taken 4 days prior to mating and on days 40, 70 and 108 of gestation; Data was statistically tested using unpaired T-tests between control (C) and fat supplemented (FS) or protein supplemented (PS) groups and differences are: * $P<0.05$

3.3.3 Glucose tolerance tests

Two sows were unable to be tested for glucose tolerance due to a blockage of the catheter in their ear. As previously stated, protein supplemented sows were not tested for glucose tolerance.

Following intravenous glucose administration, at 108 days of gestation, the glucose area under the curve was greater in those sows receiving the supplemented fat diet (Figure 3.3 and 3.4), indicating a slower rate of glucose clearance in these animals. The basal glucose concentrations and the peak glucose concentrations achieved in both the control and fat supplemented groups were not different (Table 3.6).

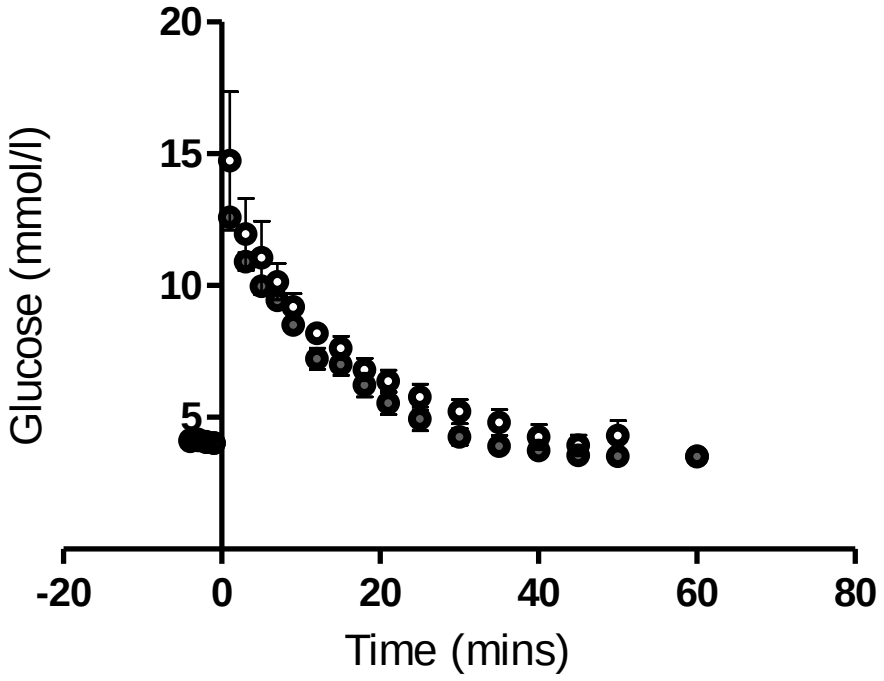

Figure 3.3 Plasma glucose after intravenous glucose tolerance test (See Chapter 3; Section 3.2.2.1) in control ● or fat supplemented ○ sows at 108 days of gestation; Values are means ± SEM

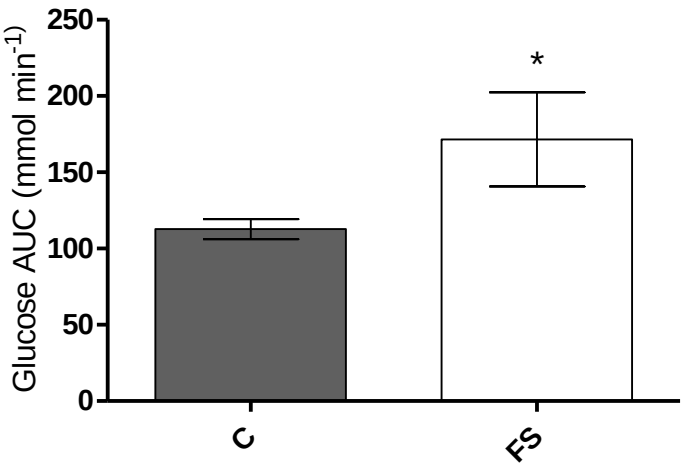

Figure 3.4 Area under the curve (AUC) following bolus administration of glucose (See Chapter 3; Section 3.2.2.1) to control (C; n=8) or fat supplemented (FS; n=6) sows at 108 days of gestation; Values are means ± SEM; Statistical differences are denoted by * P<0.05 (T-test)

Table 3.6 Derived values from intravenous glucose tolerance test (See Chapter 3; Section 3.2.2.1) at 108 days of gestation

	C	FS
Basal glucose, mM	4.14±0.17	5.05±0.99
Max. Peak, mM	12.48±0.25	14.73±2.62

Values are means ± SEM for control (C; n=8) and fat supplemented (FS; n=6) fed sows

3.3.4 Milk composition

The sow's milk on day 2 of lactation contained approximately 8 % fat, 5 % lactose and 6 % protein (Table 3.7). Fat or protein supplementation had no effect on this composition (Table 3.7).

Table 3.7 Milk composition on day 2 of lactation

Milk composition (%)	C	FS	PS
Fat	7.80±0.56	7.87±0.56	7.46±0.71
Lactose	5.35±0.17	4.85±0.33	5.05±0.15
Protein	5.55±0.24	6.32±0.51	5.95±0.14

Values are means ± SEM for control (C; n=8), fat supplemented (FS; n=8), or protein supplemented (PS; n=8) sows

3.3.5 Birth weight and postnatal mortality

Gestation length was unaffected by maternal diet (C: 117.4±0.32; FS: 116.1±0.67; PS: 115.9±0.77). The average litter size was 15-16 piglets, and this was unaffected by fat or protein supplementation during pregnancy (Table 3.8). In both the control and protein supplemented

groups, there was an equal distribution of males and females amongst litters (Table 3.8). However, maternal fat supplementation tended to increase the number of male offspring within a litter (Table 3.8).

Maternal fat or protein supplementation had no effect on offspring birth weight, with the average birth weight remaining around 1.2 kg (Table 3.8). Birth weight distribution is usually normally distributed, and this can be seen from the data and was unaffected by fat or protein supplementation (Figure 3.5). Female offspring tended to be lighter than the male offspring in the control and fat supplemented group, with a higher percentage of very low-birth weight (<800g) female offspring (Table 3.8; Figure 3.5). However, this is not seen in the protein supplemented group, where birth weight was similar between gender (Table 3.8; Figure 3.5).

Table 3.8 Litter size, offspring birth weight and sex ratio

	C	**FS**	**PS**
Litter size	14.88±0.78	16.38±0.75	16.00±1.07
Males	7.00±0.59	9.00±0.65**	8.50±0.92
Females	7.88±0.85	7.38±0.68	7.50±0.78
Mean birth weight (Kg)	1.22±0.04	1.25±0.07	1.21±0.06
Males	1.26±0.07	1.30±0.08	1.22±0.08
Females	1.16±0.03*	1.20±0.06	1.21±0.04

Values are means ± SEM for control (C; n=8), fat supplemented (FS; n=8), or protein supplemented (PS; n=8) gestational groups; Statistical differences between males and females are denoted by * P<0.05 (T-Test); Statistical differences between FS and the control are denoted by ** P<0.05 (T-Test)

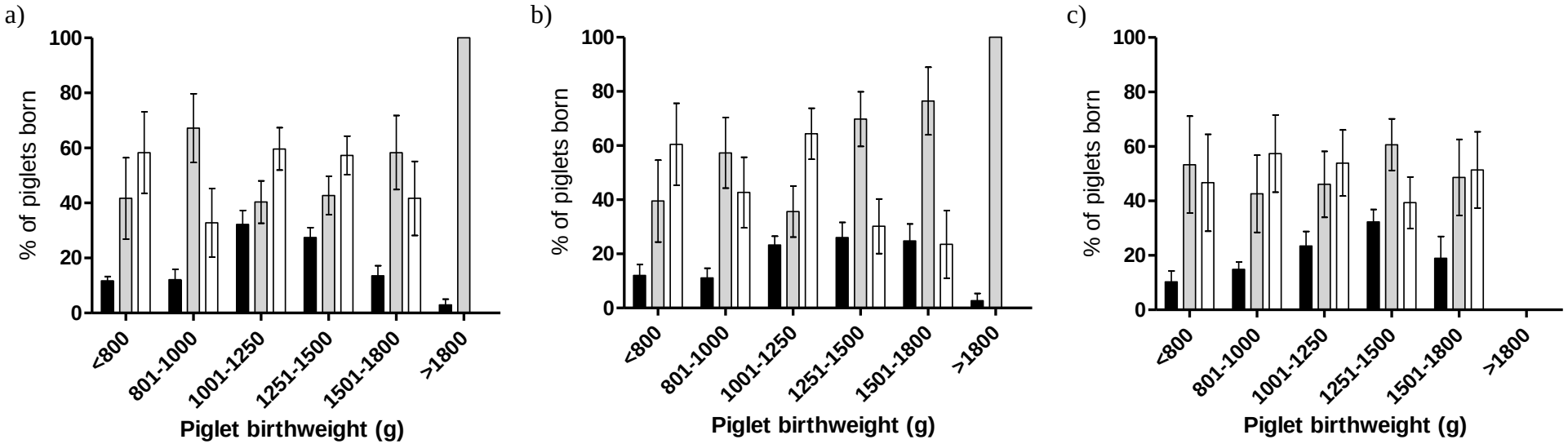

Figure 3.5 Relationships between the percentage of piglets born in each weight category (■) and percentage of male (▣) and female (□) piglets in that specific weight category for control (a), fat supplemented (b), or protein supplemented (c) gestational groups; Values are means ± SEM

The average number of stillborn piglets per litter was 8-10 %, with the average piglet mortality from birth until weaning (day 28) around 4 % for the control group (Table 3.9). Despite maternal diet having no effect on the number of stillborn piglets per litter (Table 3.9), the number of piglets who died after birth was significantly increased with both fat and protein supplementation (Table 3.9). 92 % of these deaths occurred within the first 24 hours after birth in the fat supplemented group and 80 % in the protein supplemented group. These piglets tended to be those with the lowest birth weight of the litter (Figure 3.3). Male piglets tended to account for a higher percentage of the postnatal mortality in all groups (Table 3.9). The primary cause of piglet mortality was trauma caused by entrapment by the sow, although the reasons for this entrapment are not known (Table 3.9). One litter in the fat supplemented group had a high percentage of piglet deaths from a muscle deformity known as spraddled legs (Table 3.8).

Table 3.9 Stillbirths and postnatal mortality until 28 days of age

	C	**FS**	**PS**
Stillbirths (% of litter)	9.45±2.28	7.91±2.78	10.05±2.83
Males	5.32±2.30	3.49±1.94	7.48±2.92
Females	4.13±1.23	4.42±1.74	3.30±1.28
Postnatal mortality (0-28 days) (% of litter)	3.95±1.66	17.51±6.00*	11.68±2.93*
Males	3.05±1.66	10.31±4.30	6.12±2.82
Females	0.89±0.89	7.19±3.43	5.55±1.36
Reasons for mortality (% of litter):			
Trauma	3.11±1.67	8.84±3.0	5.78±3.50
Low viability	0.83±0.83	4.85±2.55	3.40±1.68
Deformity – Spraddled legs	n/a	3.13±3.13	1.88±1.24
Scours	n/a	n/a	0.63±0.63
Unknown	n/a	0.69±0.69	n/a

Values are means ± SEM for control (C; n=8), fat supplemented (FS; n=8) or protein supplemented (PS; n=8) gestational groups; Statistical differences between FS or PS and the control are denoted by * $P<0.05$ (Mann-Whitney)

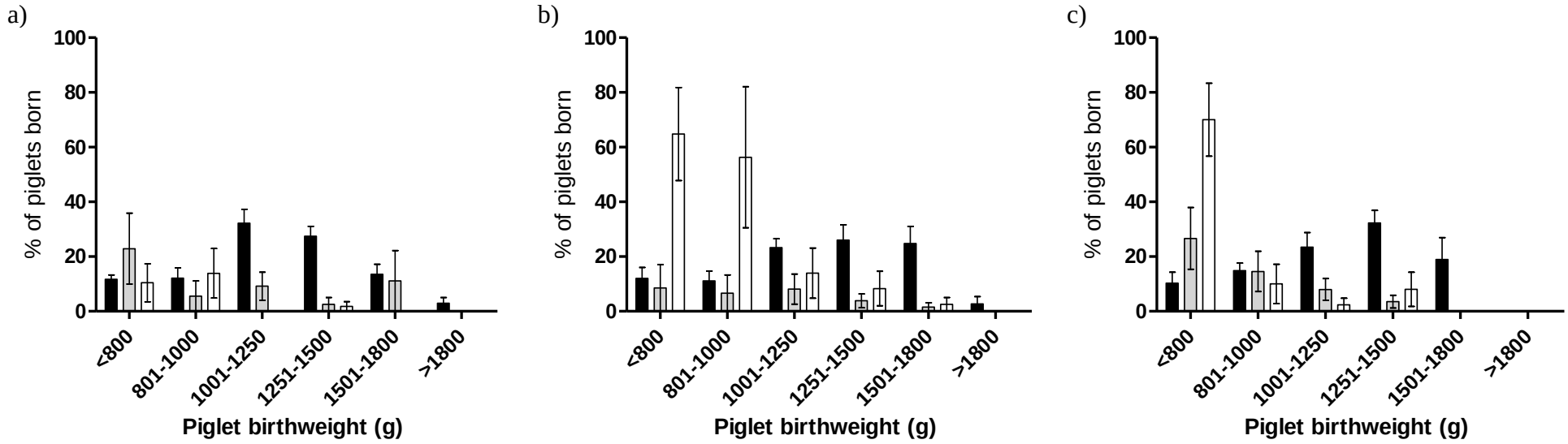

Figure 3.6 Relationships between the percentage of piglets born in each weight category (■) and percentage stillbirths (▩) and pre-weaning mortality (□) for that specific weight category for control (a), fat supplemented (b), or protein supplemented (c) gestational groups; Values are means ± SEM

3.4 Discussion

3.4.1 Fat supplementation

Maternal fat supplementation has contrasting effects in both rat and pig studies, with differing results even within species. In the rat model, fat supplementation throughout pregnancy has been shown, in some cases, to reduce the number of successful pregnancies and/or offspring birth weight (French et al., 1952, Khan et al., 2003, Taylor et al., 2003), whilst other studies have reported no effects until the offspring have been followed up in later life (Guo and Jen, 1995, Taylor et al., 2005). In contrast to this, fat supplementation to pigs during the last few days of gestation and lactation is thought to improve neonatal survival, possibly by an increase in lipid or glycogen stores at birth, or through an increase in milk yield or fat content of the milk (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993). There are many reasons why such large differences between rat and pig studies may have been observed; these include the timing of supplementation, the source and quantity of the fat, and the energy intake of the animals.

The sole purpose of the previous investigations in pigs was to improve neonatal survival and so, in contrast to this study and past rodent studies, fat was only supplemented in the final week of gestation, as opposed to most of the gestational period, and a normal diet was fed to all animals the rest of the time. In this investigation, the control diet fed to the pigs contained 2.5 percent fat (w/w), with the fat supplemented diet containing 9 percent fat (w/w). These are similar to the levels seen in the previous pig supplementation investigations (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, , Azain, 1993). In contrast, a typical rodent diet for fat supplementation investigations would contain around 20-25 percent fat (w/w), usually in the form of animal fat (Taylor et al., 2003). Outcomes from this would be measured against animals fed a control diet containing just 5 percent fat (w/w), usually from a vegetable source. This is probably because the rat is being used as a model for nutritional programming, therefore, the level of fat that is fed is often much higher than in the pig in order to mimic human fat consumption.

In addition, in this study, and most previous investigations into fat supplementation in pigs (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993,), all diets were isocaloric, so

energy intake was identical between groups throughout gestation. In order to do this, starch content had to be reduced by almost half, from 31.6 (w/w) percent in the control diet, to 16.1 percent (w/w) in the fat supplemented group. However, protein content remained constant at 12 percent (w/w), this is within recommended protein quantity guidelines for pigs (Burrin, 2001). In addition, the source of protein was the same between diets, ensuring that the amino acid composition remained constant. However, in rodent studies (Khan et al., 2003, Taylor et al., 2003), despite the diet compositions being similar between groups, with just an increase in fat content, there is often a reduction in food intake by the rats, due to the higher energy content and often reduced palatability of these diets. This in turn will reduce the carbohydrate, and perhaps more importantly, the protein intake of these animals, perhaps explaining the reduction in successful pregnancies, litter size and birth weight.

3.4.1.1 Maternal glucose tolerance

To meet the increasing demand for glucose by the fetuses, due to higher energy needs, insulin sensitivity and glucose tolerance decreases towards the end of gestation (Frienkel, 1980, Leturque et al., 1980, Pere and Dourmad, 2000, Pere and Etienne, 2007). This effect has been shown to be exaggerated in animal experiments using rats and pigs investigating the outcomes of feeding additional fat during gestation (Guo and Jen, 1995, Taylor et al., 2003, van der Peet-Schwering et al., 2004). For example in one pig study, which fed additional fat (fat-supplemented: 9.6 % fat; control: 5.2 % fat), to pigs from day 85 of gestation, there is a reduction in glucose tolerance at 108 days of gestation, shown by an increase in the area under the curve, with no effect on basal glucose or maximum peak glucose concentrations (van der Peet-Schwering et al., 2004). Due to the higher energy intake of sows fed the fat supplemented diet, these animals gained more weight and had a larger increase in back fat thickness, during the period of nutritional intervention than the control animals (van der Peet-Schwering et al., 2004). Similarly, rat studies have shown that consumption of a high-fat diet (high-fat: 25 % fat; control: 5 % fat) throughout pregnancy, causes an increase in maternal basal insulin concentrations on day 20 of gestation, suggesting hyperinsulinemia in these mothers (Taylor et al., 2003). This occurred despite no differences in weight gain between these animals (Taylor et al., 2003).

The rate at which glucose is used by maternal tissues decreases during gestation and this allows for improved placental transfer of glucose (Pere and Dourmad, 2000) that could result in fetal hyperinsulinemia (van der Peet-Schwering et al., 2004). This in turn can cause extreme hypoglycaemia immediately after birth, thus resulting in postnatal death (van der Peet-Schwering et al., 2004) as demonstrated in both human and pig studies where increased birth weight and postnatal mortality was seen in mothers who were less glucose tolerant (Kemp et al., 1996, Schmidt et al., 2001). It would be logical to therefore conclude that supplementing the maternal diet with fat during gestation may reduce maternal glucose tolerance and increase postnatal mortality.

In agreement with previous findings (van der Peet-Schwering et al., 2004), this study has demonstrated a reduction in glucose tolerance at term, when sows were fat supplemented from mating until day 110 of gestation. However, the area under the curve and maximum peak after glucose administration is very different between studies. This is probably because previous investigations by van der Peet-Schwering et al. (2004) used pelleted glucose (3 g/Kg $BW^{.75}$) and fed this to animals before glucose testing thus producing much lower average maximum glucose peak and area under the curve concentrations than observed in this study where glucose solution (123.85 g/L BW) was injected intravenously. In addition, basal glucose concentrations were much lower (average 2.5 mmol/L; (van der Peet-Schwering et al., 2004) in comparison to the normal concentrations (4-5 mmol/L) seen in this and other investigations in pigs (Pere and Dourmad, 2000, Pere and Etienne, 2007), and this could also account for differences seen in area under the curve. The reasons for such pronounced differences are unclear as pig breed, body weight, back fat, and timing of plasma sampling are all comparable between studies. The only notable difference is in sampling technique, were that in our study blood was sampled from a vein in the ear, and in the study by van der Peet-Schwering et al. (2004) it was taken from an incision in the tail. The changes in glucose tolerance observed in this study occurred despite fat supplementation having no effect on maternal body weight, back fat, or plasma glucose, NEFA, TAG and cholesterol concentrations, throughout gestation, probably due to identical energy intake between the gestational groups. In addition, in contrast to previous investigations in rats (Taylor et al., 2003), basal insulin concentrations were not affected by maternal fat

supplementation. Unfortunately, it was not possible to determine insulin sensitivity in these mothers, by examining the insulin area under the curve following glucose administration, because blood samples taken and tested for glucose were not stored for further analysis.

3.4.1.2 Piglet mortality

Fat supplementation increased piglet mortality by around 14 percent, despite having no effects on gestation length, litter size or birth weight. Most of these deaths occurred within the first day of life, thus further supporting the hypothesis of hypoglycaemia at birth due to reduced maternal glucose tolerance. This is in contrast to previous studies which have indicated a protective effect of fat supplementation during pregnancy on postnatal survival, although these studies also see no differences in piglet birth weight (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993,). In addition, van der peet-Schwering at al. (2004) demonstrated no effects of fat supplementation on postnatal mortality, despite these mothers showing reduced glucose tolerance and producing a higher number of stillborn offspring. The contrasting results in postnatal mortality between studies may perhaps be due to the much longer length of fat supplementation in this study, thus causing greater detrimental results.

The two major reasons for piglet mortality between 0 and 3 days of age are low viability and trauma (Vaillancourt et al., 1990). Low viability is classified according to PigCHAMP^{TM} standardised pre-weaning mortality form, as weakness from very low-birth weight and/or starvation. Trauma is classified as being laid on, savaged or injured by the mother. Scours and infection are the major cause of death in piglets older than 7 days of age (Vaillancourt et al., 1990). The outcomes of this study agree with this; with 50 percent of deaths in offspring of fat supplemented mothers, caused by trauma, and 28 percent caused by low viability and the failure to suckle, perhaps due to hypoglycaemia at birth.

Studies investigating the cause of death in neonatal piglets need to be interpreted with caution. One reason for this is that subjective appraisal is involved in determining the cause of death. For example in one study, pig producers from 11 herds submitted 155 piglets thought to be stillborn (Vaillancourt et al., 1990). With further examination, 13 of these piglets

were found to have been born alive, and one piglet was mummified. In summary, 9 percent of all piglets submitted as stillborn were classified incorrectly. In addition, the recorded cause of death is often only the terminal factor, and there may have been one or more other predisposing influences leading up to death (Vaillancourt et al., 1990, Varley, 1995). For example, it is possible that those deaths associated with trauma, are a result of low viability and a failure to suckle resulting in a reduction in glucose intake thus reducing piglet cognition and ability to move away quickly and, ultimately, being trapped by the sow as she lies down. This is further supported by English and Smith (1975), who used a 'case history' approach to monitor the factors leading to the death of 236 piglets. They discovered that whilst crushing and trampling by the sow were involved in 35 percent of the deaths, nearly half of these animals had already been weakened, usually by malnutrition, before being crushed (English and Smith, 1975).

Within a litter, piglets that are light at birth, have a higher risk of mortality (Milligan et al., 2002). In agreement with this, in this study, low-birth weight piglets (<1 Kg) accounted for the majority of deaths. Smaller piglets will have less energy reserves, and probably less ability to suckle than the larger piglets (Milligan et al., 2002), and thus it is possible that they may be more susceptible to hypoglycaemia than their larger litter mates. This is further supported by human cohort studies, which suggest that the risk of developing hypoglycaemia after birth is highest in low-birth weight babies (Lubchenco and Bard, 1971). In addition, piglet hierarchy within the litter is established soon after birth, with the smallest piglets tending to be lowest in the hierarchy order and therefore receiving less milk than their larger littermates due to competition for the most productive teats (Mitchell et al., 2001).

As demonstrated in previous studies (Bereskin et al., 1973), male piglets tend to be heavier at birth than female piglets. It would therefore be logical to conclude that males would have a survival advantage over females. However, this is not the case with this study agreeing with the findings of Bereskin et al. (1973) where a higher proportion of piglet mortality occurred in males. This could perhaps be because the low-birth weight male piglets tended to be the runts of the litter who are not as well

adapted to postnatal life, with less energy reserves, than their female litter mates who were genetically programmed to be of lower birth weight.

3.4.1.3 Milk composition

It has been suggested that maternal fat supplementation may affect milk composition by increasing the fat content (Pettigrew, 1981). However, milk composition of the sows was unaffected by fat supplementation in this study. This is probably due to the dietary interventions ceasing at 110 days of gestation and all sows being fed the same diet from this period until the end of lactation.

3.4.2 Protein supplementation

Investigations into the effects of protein supplementation in pregnancy are limited, and most are linked to the role of key amino acids during pregnancy. It has been previously suggested in pigs and rats, that supplementing the maternal diet with arginine, provides beneficial effects on reproductive performance by increasing litter size and reducing the number of stillborn animals (Mateo et al., 2007, Zeng et al., 2008). This is probably due to the role of arginine in nitric oxide and polyamine production and therefore its essential role in fetal development (Roselli et al., 1998, Wu and Morris, 1998, Bird et al., 2003, Wu et al., 2004).

Whole protein sources of legumes and sunflowerseed meal were added to the gestational diets for protein supplementation in this study. These were selected as they are rich in arginine and glutamine. The final diet composition contained 1.2 percent arginine (control: 0.7 % arginine) and 3.5 % glutamine (control: 2.2 % glutamine). In addition, concentrations of all other amino acids were increased slightly because a whole protein source was fed, although it must be noted that minimum essential amino acid requirements were met for all gestational diets.

3.4.2.1 Maternal body weight and back fat

As expected, and demonstrated in other studies (Mateo et al., 2007), maternal body weight and back fat thickness increased gradually throughout gestation. This increase in maternal body weight is highest during the final portion of gestation (day 70-108) and coincides with the increase in maternal feed intake and the maximal period of fetal growth (Book and Bustad, 1974, Litten-Brown et al., 2010). Protein

supplementation had no effect on maternal body weight throughout gestation. This is similar to other findings which show no effects of arginine supplementation on maternal body weight (Mateo et al., 2007, Zeng et al., 2008). In contrast to the pattern of a higher rate of maternal body weight gain in late gestation, the rate of maternal back fat thickness gain is highest during early gestation (mating-day 40). However, this study has demonstrated a much higher rate of increase in the back fat thickness of protein supplemented mothers during late gestation; this increase is comparable to the growth seen in early gestation. Despite this, back fat is identical between sows at 108 days of gestation. This increase in back fat coincides with a decrease in plasma NEFA concentration in these animals at 108 days of gestation, perhaps due to utilisation of the NEFA to convert to triglycerides for storage.

3.4.2.2 Reproductive performance

In contrast to previous findings, this study has shown no effects of protein supplementation, with a protein source rich in arginine, on litter size, number of live-born piglets produced, or piglet birth-weight. This is probably due to the reduced amount of arginine in the diets in comparison to other studies which fed around 1 percent arginine as a concentrated, crystalline amino acid (Mateo et al., 2007, Zeng et al., 2008). Another point to note is that previous investigations did not add any arginine to their control diets (Mateo et al., 2007, Zeng et al., 2008), this is in accordance with the minimum amino acid requirements during pregnancy in swine (NRC, 1988). Therefore, it is possible that the results produced are actually detrimental effects from the lack of arginine in the control group rather than positive effects on the supplemented group.

The key finding from this study is that protein supplementation until day 110 of gestation resulted in a rise in neonatal mortality rates, although this increase was not as high as with fat supplementation. The reasons for this mortality are as yet unclear, although it is possible that it may be linked to the increase in maternal back fat and reduction in NEFA plasma concentrations, seen at 108 days of gestation.

These results contradict previous investigations where no detrimental effects of arginine supplementation on mortality have been recorded (Mateo et al., 2007, Zeng et al., 2008). However, it must be noted that

previous studies have solely been looking at the influence of arginine supplementation and so the control diet has been balanced for nitrogen content by the addition of alanine. This study was designed to look at the effects of global protein supplementation and so sows were fed 16.3 percent protein as opposed to the control diet which contained 12.3 percent protein. Therefore, nitrogen content is clearly increased in the supplemented diet. In addition, as with fat supplementation, not all macronutrient components could stay the same to maintain energy content and health of the sow. As a result, protein supplemented sows received 1.2 percent more fat and 1.4 percent less fibre than those fed the control diet and this may influence postnatal mortality.

It is also important to consider the differences in the time course for protein supplementation between these experiments. This is the only study to investigate the effects of supplementation throughout the majority of the gestational period. In addition, the only other study in pigs, which supplemented arginine from the first day of gestation, produced negative effects, shown by a reduction in litter size at 25 days when pregnancy was terminated (Li et al., 2010). Therefore, it may be, the long-time period of protein supplementation, particularly early in gestation, the increased protein or fat content, or the reduction in fibre content which causes the high piglet mortality rates seen in this study.

3.4.3 Conclusions

In conclusion, this study has demonstrated that fat supplementation throughout pregnancy, reduces maternal glucose tolerance towards term and decreases the survival rate of piglets after birth, possibly due to the development of hypoglycaemia in these offspring. Furthermore, supplementing the maternal diet with protein also increased the incidence of postnatal mortality. These findings may have consequences for the pig industry where reducing piglet mortality is of key economical importance and where fat and protein supplementation were previously considered to be beneficial practice (Pettigrew, 1981, Wu et al., 2010). In addition, glucose intolerance in the mother is thought to be a risk factor for development of metabolic disease in the offspring (Chapter 1.3.1) (Boney et al., 2005, Franks et al., 2006), and so the aim of next chapter is to follow up the effects of maternal fat supplementation by observing the outcomes of the offspring.

Chapter 4 - The effect of maternal fat supplementation on offspring development

4.1 Introduction

The consequences of supplementing the pig diet with fat during pregnancy on the programming of piglet development have thus far not been investigated. Studies have instead focussed on changing the fatty acid profile of the offspring by altering the polyunsaturated fatty acid content of the maternal diet (Chapter 1; Section 1.6.3.2.1) (Rooke et al., 1998, Rooke et al., 2001a, Rooke et al., 2001b). Or, on the supposed beneficial effects of fat supplementation on increasing offspring survival rate, which is of key economical importance in the pig industry (Chapter 3) (Seerley et al., 1974, Pettigrew, 1981, Boyd et al., 1978, Seerley et al., 1981, Azain, 1993). Despite this, the present study has previously reported detrimental effects of fat supplementation during pregnancy on maternal glucose tolerance and neonatal mortality (Chapter 3). Briefly, fat supplementation caused a reduction in maternal glucose tolerance at term, perhaps leading to hypoglycaemia in the piglets, providing the link to the increase in neonatal mortality that was observed within the first 24 hours after birth.

4.1.1 Maternal fat supplementation and programming of offspring metabolism

It has been well documented that offspring born to mothers who develop gestational diabetes, often due to intake of a high fat diet and/or maternal obesity, have increased risk of developing metabolic complications such as obesity and impaired glucose tolerance at an early age (Chapter 1; Section 1.3.2) (Boney et al., 2005, Franks et al., 2006). However, these offspring are usually of very high-birth weight due to fetal macrosomia caused by hyperglycaemia in the mother (Jovanovic and Pettitt, 2001, Schmidt et al., 2001, Franks et al., 2006).

In addition, investigations into high-fat feeding during pregnancy in rats have demonstrated similar detrimental effects on the metabolism of the offspring, despite there being no effect on birth weight (Chapter 1; Section 1.3.2) (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). These findings are similar to those seen in the offspring of maternal protein restricted or nutrient restricted rats and sheep (Chapter 1; Section

1.2)(Langley-Evans et al., 1996, Langley-Evans et al., 1998, Bertram et al., 2001, Whorwood et al., 2001).

Due to the similarities seen between the high-fat and low-protein models of developmental programming, which leads to a phenotype with features of the metabolic syndrome, it has been proposed that a there is a common underlying mechanism such as perturbed glucocorticoid (GC) activity and insulin resistance (Khan et al., 2003, Taylor et al., 2003). This is supported by the elevation in plasma corticosterone seen at 20 days of gestation in fat supplemented dams (Taylor et al., 2003). Furthermore, feeding a high-fat diet to non-pregnant rats leads to both basal and stress-induced activation of the hypothalamic pituitary adrenal (HPA) axis, thus elevating adrenal GC production (Pascoe et al., 1990, Tannenbaum et al., 1997). However, male offspring of coconut oil-fed dams, which is high in saturated fatty acids (Chapter 1; Section 1.6.1.3), show increased blood pressure but do not show evidence of altered HPA axis activation in the hypothalamus or liver (Langley-Evans, 1996), suggesting perhaps that another mechanism may be involved.

4.1.2 Birth weight and offspring development

Pigs exhibit the most severe naturally occurring intrauterine growth retardation (IUGR) of all livestock species (Wu et al., 2006). IUGR or 'runt' piglets are defined as weighing 1 kg or less, and less than 65 percent of the birth weight of the larger litter-mate controls (Hegarty and Allen, 1978). Studies have shown that the within-litter weight distribution seen at birth is already established by the end of the embryonic stage of pregnancy (day 35) (Van der Lende et al., 1990, Finch et al., 2002).

As previously reported in this thesis, low birth weight piglets have a higher incidence of mortality (Chapter 3). Those small piglets that do survive will grow more slowly than their heavier litter mates, often due to their inability to compete with larger piglets (McGlone et al., 2001, Gondret et al., 2006). Thus it is normal husbandry practice, as demonstrated in this study, to separate litters after weaning and group them according to birth weight (McGlone et al., 2001). It is then possible to feed these animals appropriately so that they achieve their target weight and body composition in the shortest time possible (McGlone et al., 2001). However, keeping pigs in groups based on body weight decreases their ability to

establish a hierarchy and leads to increased fighting and stress often resulting in reduced feed intake and growth, wounds, and occasionally death (McGlone et al., 2001). And often, despite this grouping, growth rate is still reduced in very low-birth weight piglets when compared to their litter mates (Hegarty and Allen, 1978, Powell and Aberle, 1980).

In addition, it has been documented that piglets weighing less than 1 Kg at birth, not only grow more slowly, but also less efficiently, meaning that they are more likely to produce a carcass with a higher proportion of fat and less muscle than their larger birth weight piglets who were slaughtered at the same weight (96 Kg) (Powell and Aberle, 1980). This was demonstrated by a higher marbling score and percentage lipid in the longissimus muscle of the runt piglets (Powell and Aberle, 1980), despite no differences in back fat depth or *longissimus* muscle area between birth weight groups (Powell and Aberle, 1980).

4.1.2.1 Accelerated postnatal growth

It is well documented in humans that low-birth weight infants, who experienced growth restriction *in utero*, have a greater risk of obesity and developing metabolic disorders such as impaired glucose tolerance and hypertension in later life (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001). This relationship between low-birth weight and/or thinness at birth and the increased risk of metabolic disease in later life has been associated with accelerated growth during childhood (Hales and Barker, 2001). It can be best explained by examining birth weight and growth data from a cohort study of 7086 men and women born between 1924 and 1933 in Helsinki, Finland (Forsen et al., 2000). Figure 1.2 from this article shows the childhood growth of 471 men and women who developed type 2 diabetes. Height, weight and body mass index (BMI; weight/height^2) are expressed as Z-scores, which represent the differences, expressed as standard deviations, from the mean value of the entire cohort, which is set at zero. Small or large children who grow at a steady rate, relative to the mean, would follow a horizontal path on the figure. Children, who later developed type 2 diabetes, having been small or thin at birth, continued to have low rates of growth in infancy but from 7 years onwards showed accelerated growth in both weight and height (Forsen et al., 2000). By the age of 15 years, both boys and girls who later developed type 2 diabetes, were above average for the cohort (Forsen et

al., 2000). One explanation for this effect is that fetal growth restriction lead to a reduction in pancreatic cell number and that subsequent accelerated growth and weight gain during childhood, leads to excessive metabolic demand on this limited cell mass (Hales and Barker, 2001).

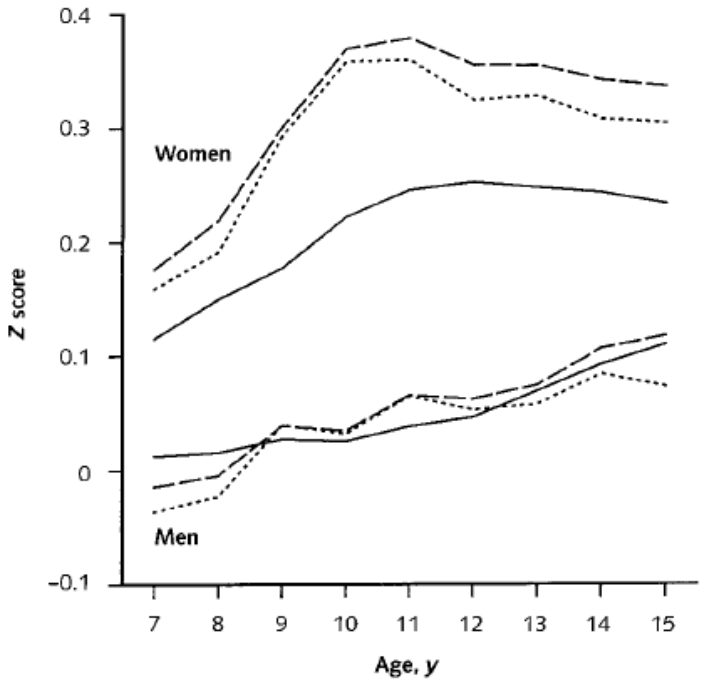

Figure 4.1 Height (solid line), weight (dashed line), and body mass index (dotted line) during childhood of 286 men and 185 women who later developed type 2 diabetes (Forsen et al., 2000)

4.1.3 Aims and hypothesis

In summary, maternal fat supplementation of rats throughout pregnancy, by the addition of fats containing a high proportion of saturated fatty acids to the diet, has been shown to produce offspring with metabolic abnormalities at 6 months and 1 year of age (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). This is similar to the effects seen in protein restricted or nutrient restricted rats and sheep (Langley-Evans et al., 1996, Langley-Evans et al., 1998, Bertram et al., 2001, Whorwood et al., 2001), thus indicating a similar mechanism such as altered GC sensitivity or insulin resistance in the offspring. At present there are currently no follow-up investigations into the effects of maternal fat supplementation on piglet outcomes in later life, either for the development of the pig industry or when using the piglet as a model for human infants.

The present study has already described how fat supplementation of pigs throughout pregnancy, by the addition of palm oil in the diet, increased maternal glucose tolerance and increased the rate of neonatal mortality (Chapter 3). This chapter will therefore focus on the effects of maternal fat supplementation on piglet development until 6 months of age that is hypothesised that fat supplementation to no effects on the offspring at one week of age, but, by 6 months of age, these offspring could show signs of developing metabolic disease. Primary markers would be an increased body weight due to a higher fat mass, with perturbed glucose/lipid homeostasis shown by raised circulating plasma levels of glucose, insulin and triglycerides (TAG).

To elucidate the mechanism behind this, this chapter will focus on the effects of maternal fat supplementation on the piglet liver due to its importance in whole body energy balance and metabolism. It was hypothesised that by 6 months of age, expression of genes involved in GC sensitivity such as GC receptor (GR) and 11beta-Hydroxysteroid Dehydrogenase (11β-HSD) type 1 will be increased in offspring born to fat supplemented sows. In addition there will be an increase in liver triglycerides with a reduction in genes involved in fatty acid oxidation and uptake such as peroxisome proliferator-activated receptor alpha (PPARα) and fatty acid-binding protein 1 (FABP1). Finally, insulin sensitivity may be reduced and so gene expression of insulin receptor (IR) might be lower.

Compensatory growth is encouraged in the pig industry and so it is hypothesised that by 6 months of age, the low-birth piglets will be of similar body weight to their median-birth weight litter mates. Low-birth weight offspring have been shown to be at a higher risk of obesity and metabolic complications, due to accelerated postnatal growth (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001). This chapter will also investigate the effects of maternal fat supplementation in piglets of low-birth weight. It is hypothesised that the low-birth weight piglets will be most affected by maternal diet, shown by an exaggerated response to fat supplementation, when compared to their average birth weight litter mates.

4.2 Methods

4.2.1 Method overview

A full description of the study design and laboratory protocols is described in Chapter 2. Briefly, one low and one median-birth weight piglet in each litter born to sows fed either the control or fat supplemented gestational diets were selected for tissue sampling at 1 week ± 2 days and 6 months of age. Plasma and liver tissue samples were taken and processed as described in Chapter 2, Section 2.3.1. Plasma was auto-analysed for glucose, nonesterified fatty acids (NEFA), TAG and cholesterol as described in Chapter 2, Section 2.4. In addition, fasting plasma insulin concentrations were determined at 6 months of age by Dr Hernan P. Fainberg (Chapter 2; Section 2.4.5). Liver samples were analysed for lipid and glycogen content as described in Chapter 2, Section 2.5. Glycogen analysis was carried out by Miss Mehreen Azhar In addition, liver gene expression of GR, 11β-HSD1, PPARα, FABP1, and IR was measured (Chapter 2; Sections 2.5.3-2.5.6). In pigs that were not selected for tissue sampling, back fat thickness was measured at 6 months of age (Chapter 2; Section 2.2).

4.2.2 Statistical analysis

Full details of all statistical tests performed are described in Chapter 2.6.

For this chapter, in order to examine the effect of maternal diet on the offspring selected for tissue sampling at 1 week or 6 months of age, either the low or median-birth weight piglets from the control group were compared with the appropriate low or median piglets from the fat supplemented group (Table 4.1). This was carried out using either an unpaired T-test or a Mann-Whitney test, depending on whether the data was parametric or non-parametric. In addition, to compare differences between piglet birth weights, data from both low and median-birth weight piglets were compared for either the control or fat supplemented groups (Table 4.1), again using T-tests of Mann-Whitney Tests. It was not appropriate to statistically compare data obtained at 1 week and 6 months of age as, although all techniques were kept the same, these tissues were analysed separately in the lab due to a delay between the tissues arriving.

Pearson's correlation coefficient was used to demonstrate relationships between birth weight, growth rate, and back fat thickness. For this calculation all piglets born to sows from either the control or fat

supplemented groups, which were not selected for tissue sampling at 6 months of age, were used.

Table 4.1 Outline of groups that were compared in this chapter

	C (L) 1 week	**C (M) 1 week**	**C (L) 6 months**	**C (M) 6 months**	**FS (L) 1 week**	**FS (M) 1 week**	**FS (L) 6 months**	**FS (M) 6 months**
C (L) 1 week	n/a	**Yes (weight)**	No	No	**Yes (diet)**	No	No	No
C (M) 1 week	**Yes (weight)**	n/a	No	No	No	**Yes (diet)**	No	No
C (L) 6 months	No	No	n/a	**Yes (weight)**	No	No	**Yes (diet)**	No
C (M) 6 months	No	No	**Yes (weight)**	n/a	No	No	No	**Yes (diet)**
FS (L) 1 week	**Yes (diet)**	No	No	No	n/a	**Yes (weight)**	No	No
FS (M) 1 week	No	**Yes (diet)**	No	No	**Yes (weight)**	n/a	No	No
FS (L) 6 months	No	No	**Yes (diet)**	No	No	No	n/a	**Yes (weight)**
FS (M) 6 months	No	No	No	**Yes (diet)**	No	No	**Yes (weight)**	n/a

Yes (weight) = Groups that have the same maternal diet but were in different birth weight categories (L = Low-birth weight; M = Median-birth weight) can be compared to examine the effect of birth weight; Yes (diet) = Groups that have different maternal diets (C = control; FS = Fat supplemented) but were in the same birth weight category were compared to determine the effect of maternal diet.

4.3 Results

4.3.1 Sex ratio

Unfortunately, sex was not equally distributed amongst dietary groups (Table 4.2). This was due to the method of the piglet selection process (See Chapter 2; Section 2.31) which resulted in uneven sex distribution in the control group, with more female piglets selected for sampling at 1 week of age then males (Table 4.2). This occurred because, in this group despite sex distribution being equal amongst the litter (Chapter 3; Table 3.8), females tended to have a lower birth weight (Chapter 3; Table 3.8), with a higher proportion of female piglets weighing less than 800 g (Chapter 3; Figure 3.5.a). In addition to this, in this group, male piglets that were of low-birth weight had a decreased survival rate when compared to their female, low-birth weight litter mates (Chapter 3; Table 3.9). These factors meant that, on most occasions, the low-birth weight piglet selected for sampling at 1 week of age was more likely to be female than male (Table 4.2). The median-birth weight piglets that were then selected were matched for sex with these animals (Table 4.2). In this control group, there were more male piglets between the birth weight categories of 801-1000 g (Chapter 3; Figure 3.5.a), meaning that when piglets were selected for sampling at 6 months of age the next lowest birth weight piglet was usually within this category and therefore, was more likely to be male. This meant that there were more male piglets selected in this group, with a lower number of females (Table 4.2).

In the fat supplemented group, there was a higher ratio of male piglets to female piglets within the litters (Chapter 3; Table 3.8). This meant that although, as with the control group, there were a higher proportion of low-birth weight females (Chapter 3; Figure 3.5.b) and the males had a slightly higher risk of postnatal mortality (Chapter 3; Table 3.9), sex ratio was fairly balanced between the groups of piglets that were selected for sampling at 1 week and 6 months of age (Table 4.2).

For this chapter, except where otherwise shown, males and females were considered together.

Table 4.2 Sex ratio of piglets selected for tissue and plasma sampling

		Number of males		Number of females	
		Low-birth weight	Median-birth weight	Low-birth weight	Median-birth weight
C	**1 week**	1	1	7	7
	6 month	6	6	2	2
FS	**1 week**	4	5	4	3
	6 month	3	5	5	3

C = Control; FS = Protein supplemented

4.3.2 Body weight and accelerated growth

Despite there being no significant difference in body weight at 6 months, between male and female pigs, or with fat supplementation (Table 4.3), male piglets that had been castrated tended to have increased back fat thickness at 6 months of age when compared to their female litter mates (Table 4.3). However, back fat thickness of the offspring, measured at 6 months of age, was unaffected by maternal fat supplementation throughout pregnancy (Table 4.3). In addition, back fat thickness was not correlated with offspring growth rate from birth to 6 months (C: r=0.17, P=0.30; FS: r=-0.46, P=0.75).

Table 4.3 Body weight and back fat thickness at 6 months of age in piglets not selected for tissue sampling

	C		FS	
	Females	**Males**	**Females**	**Males**
BW at 6 months (Kg)	116.10±0.73	113.97±0.88	114.69±0.73	116.14±0.71
Back fat (mm)	14.51±0.55	17.73±0.94*	13.67±0.45	15.85±0.49*

Values are means ± SEM for control (C; n=8) and fat supplemented (FS; n=8) gestational groups; Data was statistically tested for each gestational group using unpaired T-tests between the male and female piglets, differences are: *P<0.05; BW = Body weight

Birth weight of piglets selected for tissue sampling was not different between dietary groups at 1 week or 6 months of age (Table 4.4). However, due to the study design, birth weight of piglets selected at 1 week of age was often lower than that of piglets selected at 6 months of age (Table 4.4). In accordance with the study design, low-birth weight piglets weighed significantly less than the median-birth weight piglets at 1 week of age (Table 4.4). However, by 9 weeks of age, there was no significant difference in body weight between the low and median-birth weight piglets selected for sampling (Table 4.4), this continued to 6 months of age. This is due to accelerated growth in the low-birth weight

animals, demonstrated by strong negative correlation between birth weight and growth rate to 6 months of age, in both control and fat supplemented gestational groups (Figure 4.2).

Table 4.4 Birth weight, body weight at 9 weeks of age and body weight at euthanasia in piglets sampled at 1 week and 6 months of age

			Birth weight (Kg)	**BW at 9 weeks** (kg)	**BW at euthanasia** (Kg)
C	**1 week**	**Low** (n=8)	0.72±0.07	n/a	1.12±0.13
		Median (n=8)	1.25±0.06*	n/a	2.13±0.13*
	6 month	**Low** (n=7)	0.99±0.07	22.44±0.93	115.21±5.92
		Median (n=7)	1.37±0.05*	24.77±1.46	120.14±3.27
FS	**1 week**	**Low** (n=8)	0.90±0.07	n/a	1.65±0.20**
		Median (n=8)	1.27±0.12*	n/a	2.61±0.25*
	6 month	**Low** (n=7)	1.05±0.10	23.85±2.23	115.86±5.06
		Median (n=7)	1.38±0.05*	27.33±2.20	125.86±4.81

Values are means ± SEM for control (C) and fat supplemented (FS) gestational groups; Data was statistically tested at 1 week and 6 months using unpaired T-tests between the low and median-birth weight piglets and differences are: *P<0.05; In addition, differences between C and FS, low or median groups are denoted by: ** P<0.05 (T-test); BW = Body weight

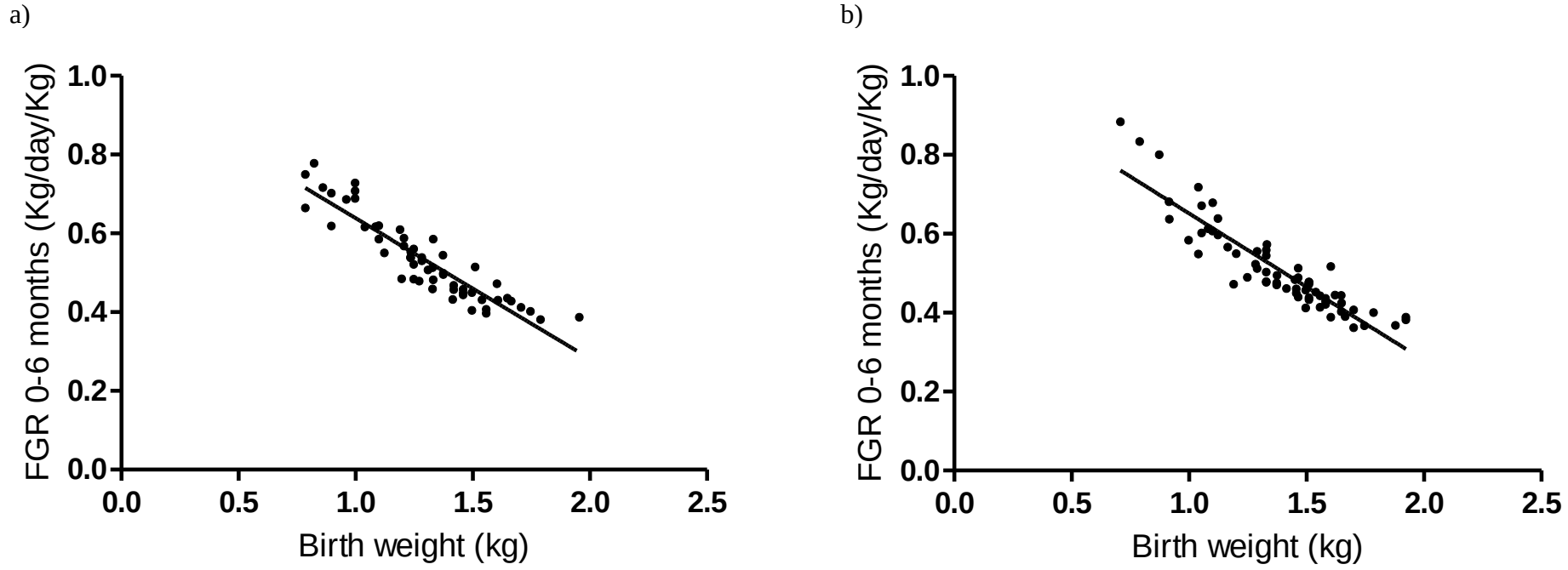

Figure 4.2 Relationship between piglet birth weight and fractional growth rate (FGR) to 6 months of age in piglets born to sows fed (a) the control (n=8) (r=-0.92; P<0.001) or (b) the fat supplemented (n=8) (r=-0.91; P<0.001) gestational diet (Pearson's correlation coefficient)

4.3.3 Plasma analysis

Plasma concentrations of glucose, TAG, NEFA, and cholesterol, were raised at 1 week, when compared to samples taken at 6 months of age (Table 4.5). Maternal fat supplementation did not affect plasma metabolite concentrations in the offspring, at either 1 week or 6 months of age (Table 4.5). Plasma metabolite concentrations were unaffected by birth weight, except cholesterol, which was raised at 1 week in the low-birth weight offspring born to control diet fed mothers (Table 4.5). Concentrations were then decreased in comparison to the median-birth weight piglets by 6 months of age (Table 4.5).

Maternal dietary supplementation with fat did not affect fasting plasma insulin concentrations (μg/ml) in offspring at 6 months of age (Low: C: 0.16±0.03; FS: 0.15±0.03; Median: C: 0.22±0.08; FS: 0.22±0.08). Unfortunately, low and median-birth weight piglets could not be compared as due to the number of samples, they were analysed on separate 96-well plates, with two different insulin kits. When inter-assay variation was determined with one sample on both plates the CV was large (16.3 %) and so therefore, it would not be appropriate to compare these groups.

Table 4.5 Fasted plasma concentrations of glucose, triglycerides (TAG), nonesterified fatty acids (NEFA) and cholesterol in low and median-birth weight piglets, sampled at 1 week and 6 months of age

			Glucose (mM)	TAG (mM)	NEFA (mM)	Cholesterol (mM)
C	**1 week**	**Low** (n=7)	7.52±0.40	3.64±0.71	1.18±0.15	4.77±0.34
		Median (n=8)	7.97±0.23	3.16±0.58	0.99±0.11	3.60±0.36*
	6 month	**Low** (n=8)	4.22±0.47	1.14±0.22	0.96±0.14	2.36±0.37
		Median (n=8)	4.71±0.41	1.26±0.09	0.94±0.15	3.16±0.08*
FS	**1 week**	**Low** (n=8)	7.37±0.46	4.12±1.27	1.14±0.24	4.39±0.34
		Median (n=8)	8.14±0.45	3.78±0.96	1.17±0.32	4.17±0.18
	6 month	**Low** (n=8)	4.23±0.31	1.51±0.12	0.79±0.17	2.94±0.11
		Median (n=8)	4.28±0.24	1.10±0.22	0.98±0.18	2.06±0.48

Values are means ± SEM for control (C) and fat supplemented (FS) gestational groups; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: *P<0.05

4.3.4 Liver analysis

4.3.4.1 Liver weight and composition

Maternal fat supplementation had no effect on the liver weight or liver fat and glycogen content of offspring at 1 week or 6 months of age (Table 4.6). It was not possible to measure liver glycogen content at 6 months of age as these animals were fasted over night and so glycogen content was below the level of detection and therefore, could not be measured on the standard curve. As would be expected, low-birth weight piglets had smaller livers than median-birth weight piglets (Table 4.6). However, when liver weight was assessed as a proportion of body weight, there were no birth weight differences (Table 4.6). By 6 months, liver weight was similar between low and median-birth weight piglets (Table 4.6); this corresponds to the similarity in body weight of these animals seen at this time (Table 4.4). There was no relationship between piglet birth weight and liver TAG or glycogen content at 1 week or 6 months of age in the control or fat supplemented groups (Table 4.6).

Table 4.6 Liver weight, triglyceride (TAG) and glycogen content in piglets at 1 week and 6 months of age

			Liver weight (Kg)	Liver weight (% of BW)	Liver TAG (mg/g)	Liver glycogen (mg/g)
C	**1 week**	**Low** (n=8)	0.05±0.008	4.41±0.40	1.98±0.40	75.8±19.6
		Median (n=8)	0.10±0.008*	4.82±0.19	1.17±0.37	91.8±13.8
	6 month	**Low** (n=8)	2.48±0.12	2.11±0.10	0.84±0.17	-
		Median (n=8)	2.51±0.12	2.03±0.06	0.97±0.18	-
FS	**1 week**	**Low** (n=8)	0.07±0.010	4.42±0.25	1.25±0.40	92.7±17.9
		Median (n=8)	0.12±0.008*	4.53±0.23	0.80±0.11	50.2±16.9
	6 month	**Low** (n=8)	2.44±0.12	2.11±0.07	0.95±0.22	-
		Median (n=8)	2.56±0.10	2.07±0.05	0.60±0.14	-

Values are means ± SEM for control (C) and fat supplemented (FS) gestational groups; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: *P<0.01; BW = Body weight

4.3.4.2 Liver gene expression

Gene expression of GR, 11β-HSD1, PPARα, and IR, in the piglet liver at 1 week and 6 months of age was unaffected by maternal fat supplementation and offspring birth weight (Figure 4.3, 4.4, 4.5 and 4.7).

FABP1 liver mRNA expression was increased at 1 week of age in median-birth weight piglets born to sows fed a control diet when compared to those fed fat supplemented gestational diets (Figure 4.6). However, by 6 months of age, fat supplementation or weight at birth had no effect on gene expression (Figure 4.6).

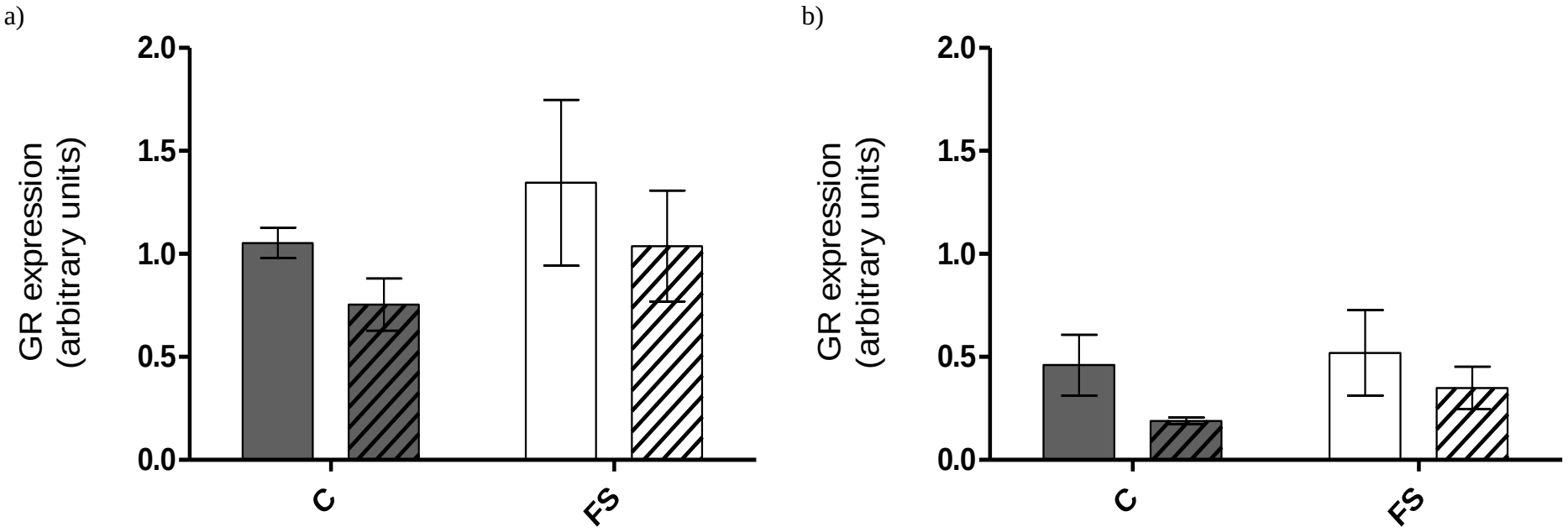

Figure 4.3 Liver Glucocorticoid receptor (GR) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or fat supplemented (FS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of GR mRNA using the genorm strategy; 18S, ribosomal phosphoprotein (RPO) and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM

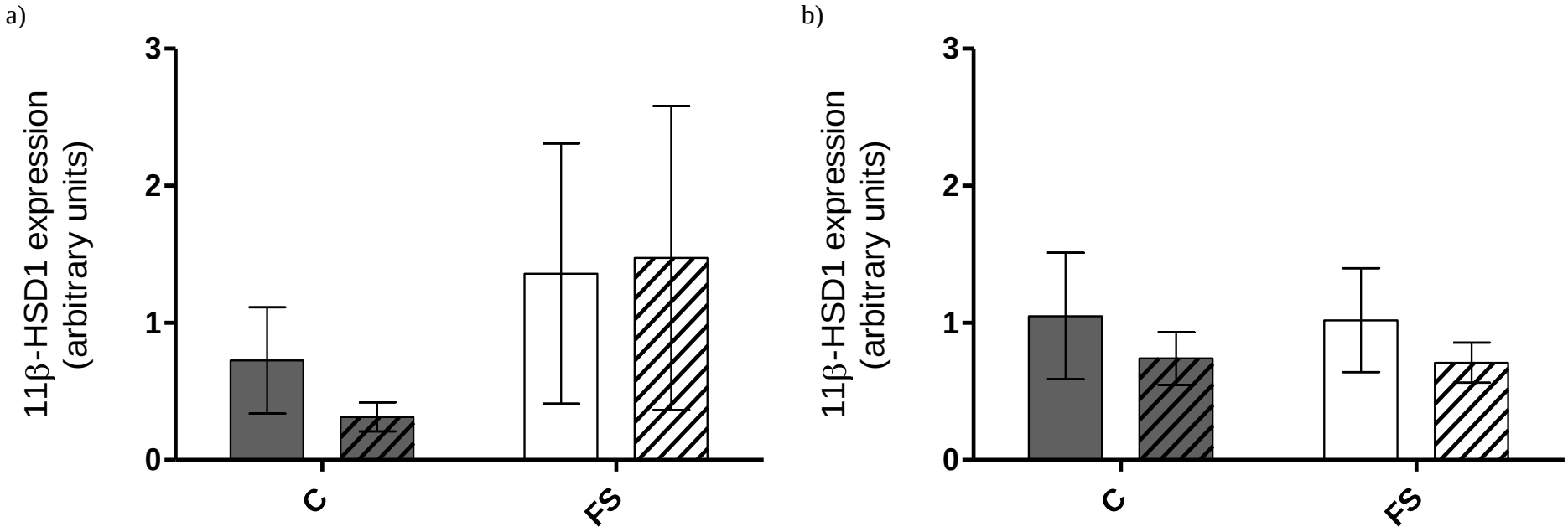

Figure 4.4 Liver 11beta-Hydroxysetroid Dehydrogenase type 1 (11β-HSD1) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or fat supplemented (FS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of 11β-HSD1 mRNA using the genorm strategy. 18S and cyclophylin were used as housekeeping genes, and results are given in arbitrary units. Values are means ± SEM

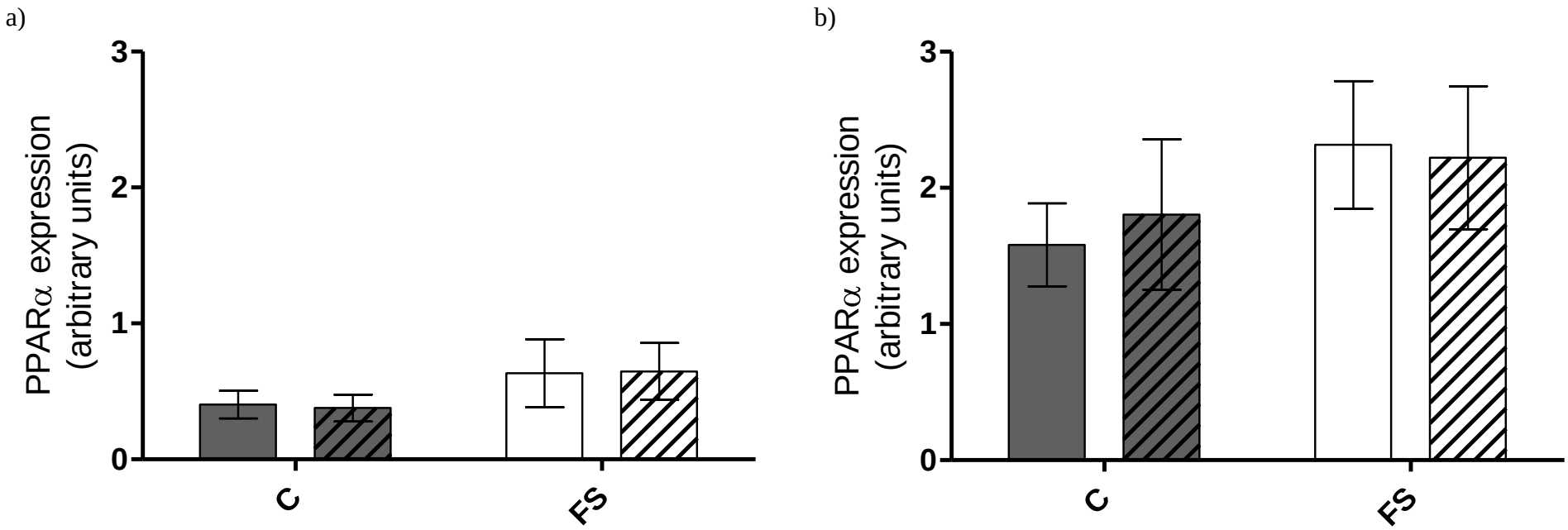

Figure 4.5 Liver Peroxisome proliferator-activated receptor alpha (PPARα) in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or fat supplemented (FS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of PPARα mRNA using the genorm strategy; 18S and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM

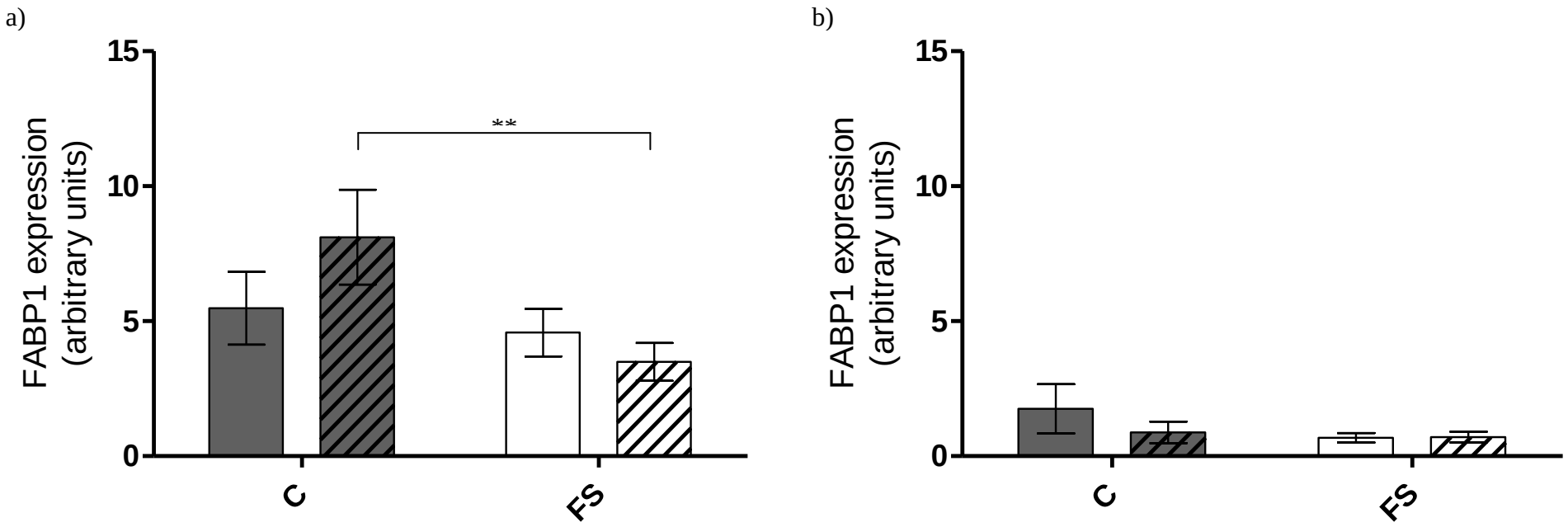

Figure 4.6 Liver Fatty acid-binding protein 1 (FABP1) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or fat supplemented (FS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of FABP1 mRNA using the genorm strategy; 18S, ribosomal phosphoprotein (RPO) and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM; Statistical differences between C and FS groups are denoted by ** P<0.05 (T-Test)

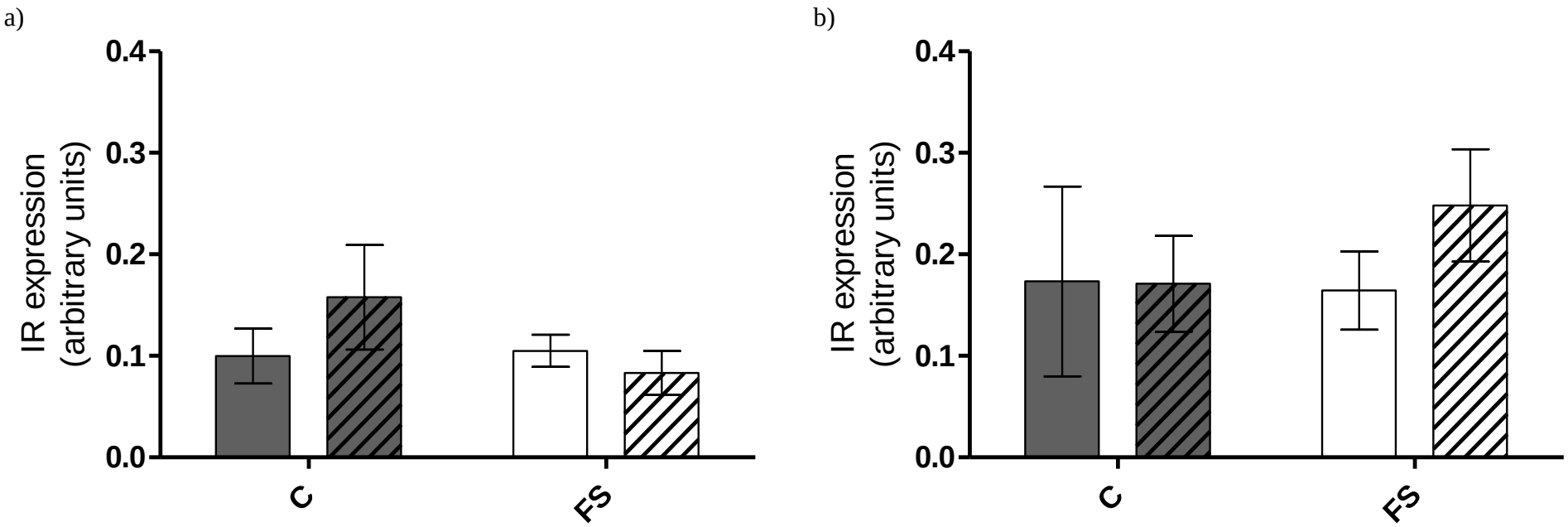

Figure 4.7 Liver Insulin receptor (IR) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or fat supplemented (FS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of IR mRNA using the genorm strategy; 18S, ribosomal phosphoprotein (RPO) and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM

4.4 Discussion

4.4.1 Maternal fat supplementation and offspring development

In rats, feeding a high-fat diet throughout pregnancy, has been demonstrated to produce offspring with a greater risk of developing metabolic complications such as obesity, hyperlipidemia, hypertension, impaired glucose tolerance and insulin resistance by 6 months to a year of age (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). These conditions are characterised by an increase in abdominal fat mass, raised plasma TAG, glucose and insulin concentrations, and an increase in liver triglyceride content in the offspring (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005), often by the time of weaning (Guo and Jen, 1995).

Previously, this study has demonstrated reduced maternal glucose tolerance at term and increased postnatal mortality with moderate, dietary fat supplementation (Chapter 3). In humans, reduced glucose tolerance during pregnancy has been linked with a higher risk of obesity and insulin resistance in the offspring (Boney et al., 2005, Franks et al., 2006).

This study was the first in pigs to investigate the effects of maternal fat supplementation throughout pregnancy on growth and development of the offspring, and in contrast, did not show any differences in body weight or fat mass at 1 week or 6 months of age. However, fat mass in these animals was only measured as back fat thickness and the deposition of fat in other fat depots was not measured. Often in the high-fat, rat studies, the area of fat that was increased was the abdominal fat (Buckley et al., 2005, Khan et al., 2005, Taylor et al., 2005), thus accounting for the overall increase in fat mass and potentially explaining why no differences were seen in this study. However, in addition, maternal fat supplementation had no affect on plasma glucose, TAG, NEFA, cholesterol or insulin levels in the offspring. Similarly, no differences were detected in liver TAG content; suggesting that in this study, maternal fat intake during pregnancy had no effect on offspring metabolism.

In order to elucidate the mechanisms behind the effects of maternal fat supplementation in rats, it was hypothesised that they may be due to perturbed GC metabolism, similar to the effects demonstrated by feeding a low-protein diet or nutrient restricting mothers during pregnancy (Bertram et al., 2001, Whorwood et al., 2001), although to date, no studies have

tested this hypothesis. These offspring demonstrated an increase in gene expression of GR in tissues such as the liver and lungs, and a reduction in 11β-HSD2 in tissues such as the kidney. This hypothesis was further supported by studies which demonstrated an increase in plasma GC when a high-fat diet is consumed (Pascoe et al., 1990, Tannenbaum et al., 1997, Taylor et al., 2003).

As would be expected, due to the lack of difference between fat mass, plasma profile, and TAG content of the liver, this study demonstrated no difference in GC sensitivity in offspring born to fat supplemented sows. This was concluded as there was no difference in gene expression of GR or 11β-HSD1 in the livers of these animals. It was not possible to measure expression of 11β-HSD2 mRNA in the livers of these offspring, as it is expressed at such low levels. In addition, expression of genes involved in lipid metabolism such as PPARα, and also genes involved in insulin sensitivity in the liver such as the insulin receptor, were unaffected by maternal diet.

FABPs are the most abundant proteins in the cytosol of cells and play an important role in fatty acid uptake, oxidation and storage (Chapter 1; Section 1.7.2.2) (Atshaves et al., 2010). At 1 week of age mRNA expression of FABP1 was increased in the median-birth weight offspring, born to sows that were fed the control diet, in comparison to those who were fat supplemented. The reason for this could be that it is a normal up-regulation of gene expression, caused by commencement of suckling in order to allow the offspring to take up and process the increase in circulating lipids through milk digestion. This is supported by Gordon et al. (1985) who demonstrated an increase in liver and intestinal FABP1 mRNA expression during the first 24 hours after birth in rats (Figure 4.8). It is possible that those offspring born to sows that were fat supplemented throughout gestation do not show the same up-regulation as they are already adapted to a high-fat diet. However, it is unclear as to why this expression pattern is only demonstrated in the median-birth weight piglets and not those of low-birth weight. In agreement with the expression profiles of other genes tested, by the age of 6 months, piglet FABP1 was unaffected by maternal fat supplementation.

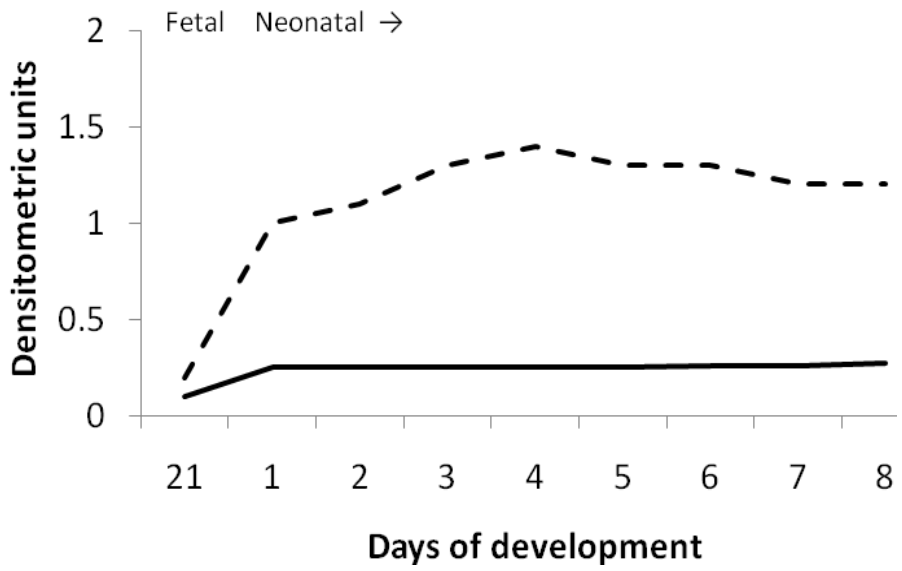

Figure 4.8 Developmental regulation of liver FABP (FABP1) mRNA accumulation in the liver (solid line) and small intestine (dashed line) of rats (Gordon et al., 1985)

Aside from the obvious species differences, there are a number of potential reasons for the contrasting outcomes demonstrated between this study and previous rat studies, which are outlined below.

4.4.1.1 Fatty acid composition of supplemented diets

The type of fat which is used to supplement the maternal diet is crucial in determining the outcome on the offspring and one reason for the contrasting results between study outcomes could be due to the different type of fat used. In most rat studies the high-fat diet is prepared through the addition of lard, which is high in saturated fatty acids (Chapter 1; Section 1.6.1.3) (Khan et al., 2003, Taylor et al., 2003, Taylor et al., 2005). This is probably because diets high in saturated fat have been demonstrated, in both humans and rats, to be detrimental to health by increasing the risk of obesity, reducing insulin sensitivity, and increasing the risk of cardiovascular disease (Maron et al., 1991, Parker et al., 1993, Guo and Jen, 1995, Hu et al., 1999, Taylor et al., 2003). In agreement with this, studies investigating the effects of fat supplementation on growing-finishing pigs (20 to 100 Kg), which used fat from animal sources that were high in saturated fat, demonstrated that fat supplementation, by as little as 5 to 6 percent of the pig diet, results in increased daily weight gain to feed ratio, although this also correlates with an unfavourable increase in back fat thickness of these animals (Stahly and Cromwell, 1979, De la Llata et al., 2001).

In contrast to rat studies, investigations into the effects of additional fat supplementation in the diet of pregnant sows tended to use fat sources that were rich in polyunsaturated fatty acids, such as corn oil and soy bean oil (Chapter 1; Section 1.6.1.3) (Seerley et al., 1974, Boyd et al., 1978, Seerley et al., 1981, Azain, 1993), and so potentially providing a link between the differential findings observed. Diets with a high ratio of polyunsaturated fatty acids, such as linoleic and linolenic acid, to saturated fatty acids, have been demonstrated to provide beneficial health effects, such as reducing cardiovascular disease risk in humans (Hu et al., 1999, Siscovick et al., 2000), perhaps due to their mechanism of lowering plasma cholesterol levels (Hegsted et al., 1965, Mattson and Grundy, 1985). This is further supported by another study which demonstrated that diets high in saturated fat produce the most severe insulin resistance in rats and, supplementation with fish oils, which are high in omega-3 fatty acids, has been shown to completely alleviate this effect (Storlien et al., 1987). In addition, dietary supplementation of growing pigs with linoleic acid has also been shown to be beneficial by reducing obesity, shown by a reduction in back fat, and also results in an increase in daily weight gain to feed ratio (Ostrowska et al., 1999), this has also been demonstrated in mice (West et al., 1998).

This study used palm oil, which has an equal ratio of saturated to unsaturated fatty acids (Chapter 1; Section 1.6.1.3), to supplement the diet of the sows. Therefore, this could potentially be a factor to explain why no detrimental effects were seen in the piglets in terms of increased back fat or glucose and lipid homeostasis.

4.4.1.2 Severity and timing of nutritional manipulation

Another factor which may explain the difference in outcomes between this investigation and the rat high-fat feeding studies is the severity of the nutritional manipulation, which in the rats is much more severe. This has previously been discussed in Chapter 3; Section 3.4.1. In addition, as previously mentioned (Chapter 3; Section 3.4.1), the high energy content of the rat diets, and the low starch ratio of the fat-supplemented diets in this study, may also play a role the outcome in the offspring. Therefore, it could be the reduced protein intake of the fat-fed dams during gestation, due to a reduction in food intake that may result in the programming

effects demonstrated on the offspring. This would further explain why the phenotype of the offspring is similar to that of those born to mothers who consumed a low-protein diet throughout pregnancy. In this study, protein intake remained the same between the control and fat supplemented mothers and so could explain why no detrimental effects were seen in these offspring.

The period of nutritional intervention in the majority of the rat studies persisted throughout gestation and lactation (Guo and Jen, 1995, Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). Pups were then weaned onto a standard chow diet, usually containing around 5 percent fat. This period was longer than demonstrated in this study, where, at 110 days of gestation, the intervention stopped and all sows received the same diet until piglets were weaned. As previously discussed, in this study, maternal-fat supplementation had no effect on the composition of the sow's milk during lactation (Chapter 3). It could be that fat supplementation of the maternal diet during the gestation and lactation period may have a more significant effect on the programming of the offspring than feeding a high-fat diet during the gestation period alone. This is demonstrated by Khan et al. (2005) who showed that endothelial function in rats was most severely compromised in those offspring born to dams who consumed a fat-rich diet during the lactation period alone. This may be due to an effect of fat supplementation on increasing milk yield and fat content (Seerley et al., 1974, Pettigrew, 1981).

4.4.1.3 Sex and age of offspring sampled

Most rat studies which have demonstrated that maternal fat-rich diets leads to the programming of hypertension and reduced glucose tolerance have only demonstrated these effects in female offspring (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). Results from this study contain data from both male and female offspring as there was no significant difference between outcomes in males and females. This lack of significance may reflect the reduced number of animals, especially males, in some of the groups. In addition, when you consider females by themselves, there are no differences between piglets from the control and fat supplemented maternal groups. However, this study was not powered to investigate sex differences and so it is possible that more animals per group would be required to see such differences.

This study investigated the offspring at 1 week and 6 months of age. Gilts generally reach puberty at around 6 to 7 months of age (Bazer et al., 2001). In boars pubertal development begins around 3 months of age, and sperm production is maximal around 2 years of age (Bazer et al., 2001). Therefore, sampling the pigs at 6 months of age is the equivalent to the beginning of adolescence in humans. This time point for tissue sampling was chosen because in the pig industry, 6 months coincides with the time that these animals are nearing/or have reached their optimal slaughter weight of around 110-115 Kg (McGlone et al., 2001). Therefore, piglet development and body composition up until this time is crucial.

Studies in rats which have shown significant programming effects of high-fat feeding during gestation have investigated the offspring at 6 months or 1 year of age (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). In contrast to pigs and humans, development in rats is much faster as they have a much shorter life-span, around 2.5 to 3 years in captivity (Anne, 2010). By the age of 6 weeks, rats are sexually mature (Anne, 2010). This means that this sampling period of 6 months and 1 year is equivalent to around 18 and 30 years of age in a human (Anne, 2010), which is much later than in this study, possibly providing another factor to explain the difference in outcomes between studies.

4.4.1.4 Link to piglet mortality

Chapter 3 of this thesis has already described how maternal fat supplementation throughout pregnancy decreased sow glucose tolerance at term and increased offspring postnatal mortality. This may provide an important explanation as to why no programming effects were observed between offspring born to control or fat supplemented mothers. It is possible that those piglets that were more severely affected by maternal fat supplementation died within a few hours after birth. This phenomenon is not seen in the rat studies, where increases in offspring mortality are not noted with high-fat feeding (Guo and Jen, 1995, Khan et al., 2003, Taylor et al., 2003, Buckley et al., 2005, Khan et al., 2005, Taylor et al., 2005).

4.4.2 The effects of accelerated postnatal growth in low-birth weight animals

In the pig industry, a high postnatal growth rate is encouraged and, after weaning, piglets are separated by weight and fed in order to achieve the optimal slaughter weight and body composition in the shortest time possible (McGlone et al., 2001). This commercial husbandry practice was used in this study. However, often the low-birth animals grow less efficiently, and are more likely to produce a carcass with a higher proportion of fat than their larger birth weight piglets (Powell and Aberle, 1980, Gondret et al., 2006).

This study has observed a relative increase in growth rate of low-birth weight infants so that, by the age of 9 weeks, there was no significant difference in body weight between low and median-birth weight animals. However, in contrast to what might have been expected, a higher growth rate was not associated with an increase in back fat thickness in these animals. However, measuring body fat by examining back fat thickness may not be the best indicator of fat mass and carcass composition in pigs. This is supported by Powell and Aberle (1980), who demonstrated that often back fat thickness is unchanged between piglets of different birth weights at slaughter, but these animals may show increased lipid in the muscle, demonstrated by a higher marbling score. Therefore, the piglets in this study may have adverse effects of rapid postnatal growth but it is possible that this is not reflected in terms of back fat thickness.

Low-birth weight and accelerated postnatal growth has also been linked to a higher risk of obesity and metabolic complications in later life in the human population (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001). Similarly, maternal nutrient restriction in rats produces low-birth weight offspring with similar detrimental metabolic outcomes including hypertension and reduced glucose tolerance (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Bertram et al., 2001,). Therefore, it was hypothesised that the low-birth weight piglets would be at increased risk of obesity and show increased GC sensitivity than their median-birth weight litter mates. However this was not demonstrated to be the case, with no differences between low and median-birth weight offspring in any of the variables tested.

4.4.3 Conclusions

This was the first study in pigs to investigate the programming effects of a fat supplemented maternal diet during gestation, and demonstrated no effects on offspring growth or liver metabolism up to 6 months of age. These results are in contrast to those studies which have fed high-fat diets to rats and demonstrated significant programming effects on the offspring, such as increased fat mass, reduced glucose tolerance and hypertension (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). This was surprising considering that it has previously been demonstrated that fat supplementation reduced maternal glucose tolerance and increased the rate of offspring postnatal mortality (Chapter 3).

In addition, this study has not demonstrated any negative effects of accelerated postnatal growth which was encouraged through husbandry practice. By 6 months of age the low-birth weight piglets had reached the same body weight as their litter mates, but with no increases in back fat thickness or GC sensitivity as might have been expected. Future work will determine whether there are more subtle differences in carcass quality through analysis of the muscle composition. This could have important implications on the normal commercial practice in the pig industry of encouraging rapid growth by grouping pigs based on their body weights at weaning.

Chapter 5 - The effect of maternal protein supplementation on offspring development

5.1 Introduction

5.1.1 Low-protein maternal diets

Maternal protein restriction has been demonstrated in the rat to reduce offspring birth weight and programme offspring to have a greater risk of developing metabolic complications such as obesity, hypertension and insulin resistance in later life (Chapter 1; Section 1.2) (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Bertram et al., 2001, Ehruma et al., 2007). The mechanism behind this is thought to be due to a reduction in placental 11β-hydroxysteroid dehydrogenase (11β-HSD) 2, which normally serves to protect the fetus from overexposure to maternal glucocorticoids (GC) (Chapter 1; Section 1.2.1) (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Bertram et al., 2001). Consequently, the fetus is exposed to excess maternal GC which in turn, increases the offspring's sensitivity to GC at a tissue specific level by increasing the gene expression of GC receptor (GR) and 11β-HSD1, and decreasing expression of 11β-HSD2 (Chapter 1; Section 1.2.1) (Bertram et al., 2001). Similar outcomes have also been demonstrated through global maternal under-nutrition in the sheep model, although differences in offspring birth weight were not observed (Whorwood et al., 2001).

5.1.2 Maternal protein supplementation

In the Western World, dietary protein intake exceeds the recommended levels, especially amongst young people (Rolland-Cachera et al., 2000). It is therefore, crucial to investigate the impacts of high-protein maternal diets on fetal programming. Following on from the low-protein studies, it is conceivable that increasing the protein ratio in the maternal diet may have beneficial effects on the offspring. However, this appears to not be the case although investigations into the influence of protein supplementation during pregnancy are limited, with conflicting outcomes between studies (Chapter 1; Section 1.4.1).

This study has previously demonstrated that maternal protein supplementation throughout pregnancy in the sow, increases offspring postnatal mortality with no effects on piglet birth weight (Chapter 3). Conversely, in rats, a reduction in offspring birth weight, similar to that

observed with protein restriction, has been demonstrated when dams were fed an isocaloric, high-protein diet (40 %) throughout gestation (Daenzer et al., 2001). These offspring showed accelerated postnatal growth and by the age of 9 weeks had increased fat mass in comparison to those born to mothers fed an adequate amount of protein (20 %) during gestation (Daenzer et al., 2001). In contrast, other rat studies have not seen any effects of maternal protein supplementation on offspring birth weight (Zimanyi et al., 2002, Thone-Reineke et al., 2000), but have shown an increase in blood pressure of the male pups by 4 weeks of age (Thone-Reineke et al., 2006).

As yet, no studies have demonstrated the effects of increased dietary protein content on GC sensitivity in the offspring to examine whether it mimics the effects of maternal protein restriction. This is probably due to the contrasting results of protein supplementation in both humans and rats (Mathews et al., 1999, Daenzer et al., 2001, Zimanyi et al., 2002, Kramer and Kakuma, 2003, Thone-Reineke et al., 2006). In addition, to date, there are currently no investigations into the effects of maternal protein supplementation on piglet development.

5.1.3 Aims and hypothesis

In summary, consuming a low-protein diet throughout gestation is associated with a reduction in birth weight and an increased risk of metabolic disease in the offspring (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Bertram et al., 2001, Ehruma et al., 2007). At present, there are conflicting results to demonstrate the effects of high-protein diets during pregnancy (Mathews et al., 1999, Daenzer et al., 2001, Zimanyi et al., 2002, Kramer and Kakuma, 2003, Thone-Reineke et al., 2006). To date, no studies have investigated the effects of an increased protein intake on GC sensitivity or metabolism in the offspring.

This study has previously described how maternal protein supplementation of sows, by the addition of protein sources which were rich in arginine and glutamine, increased neonatal mortality (Chapter 3). The present chapter will focus on the effects of protein supplementation throughout pregnancy on the programming of piglet development until 6 months of age, particularly focussing on GC sensitivity to determine whether protein supplementation has similar or contrasting effects to the protein restricted

rat model. For this the liver was investigated, due to its importance in whole body energy balance and metabolism, and also because previous studies have demonstrated differences in GR and 11β-HSD1 gene and protein expression in this tissue with protein or nutrient restriction (Bertram et al., 2001, Whorwood et al., 2001).

As protein supplementation had no effects on average birth weight (Chapter 3) it was hypothesised that there would be no subsequent differences in body weight or growth rate of these piglets. It has previously been demonstrated that low-birth weight piglets grow at a faster rate than their median-birth weight piglets and so by 6 months of age are of a similar body weight (Chapter 4). This practice is encouraged in the pig industry by grouping of piglets according to body weight and encouraging growth accordingly (Chapter 4) (McGlone et al., 2001). It is hypothesised that this accelerated postnatal growth will also be demonstrated in those low-birth weight piglets born to protein supplemented sows.

As previously described, low-birth weight offspring often have increased risk of obesity and metabolic complications, due to accelerated postnatal growth (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001). In addition, metabolic complications in offspring born to protein restricted mothers, is accompanied by a reduction in birth weight followed by accelerated growth (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Bertram et al., 2001). Therefore it is hypothesised that the low-birth piglets born to protein supplemented sows will be at increased risk of developing metabolic complications in later life, similar to those effects demonstrated with protein restriction. These offspring may have increased fat mass, demonstrated by an increase in back fat thickness. Insulin sensitivity may be reduced and so plasma insulin, glucose and triglycerides (TAG) will be increased, possibly with a reduction in gene expression of insulin receptor (IR). In addition, these piglets will show increased GC sensitivity in their liver, demonstrated by a higher mRNA expression of GR and 11β-HSD1 in the low-birth weight, protein supplemented group. However, an increase in circulating plasma cortisol is not expected in these animals. In addition there will be an increase in liver triglycerides with a reduction in genes involved in fatty acid oxidation and uptake such as Peroxisome proliferator-activated receptor alpha (PPARα) and Fatty acid-

binding protein 1 (FABP1). It is further hypothesised that median-birth weight offspring will be unaffected by maternal protein supplementation.

5.2 Methods

5.2.1 Method overview

A full description of the study design and laboratory protocols is described in Chapter 2. Briefly, one low and one median-birth weight piglet in each litter born to sows fed either the control or protein supplemented gestational diets were selected for tissue sampling at 1 week ± 2 days and 6 months of age. Plasma and liver tissue samples were taken and processed as described in Chapter 2, Section 2.3.1. Plasma was auto-analysed for glucose, nonesterified fatty acids (NEFA), TAG and cholesterol as described in Chapter 2, Section 2.4. In addition, fasting plasma insulin concentrations were determined at 6 months of age by Dr Hernan P. Fainberg (Chapter 2; Sections 2.4.5). Liver samples were analysed for lipid and glycogen content as described in Chapter 2, Section 2.5. Glycogen analysis was carried out by Miss Mehreen Azhar. In addition, liver gene expression of GR, 11β-HSD1, PPARα, FABP1, and IR, was measured (Chapter 2; Sections 2.5.3-2.5.6). In pigs that were not selected for tissue sampling, back fat thickness was measured at 6 months of age (Chapter 2; Section 2.2).

5.2.2 Protein extraction from frozen tissue

Due to the significant result seen with GR gene expression between the median-birth weight piglets at 6 months of age (Section 5.3), total protein was extracted from the liver of these piglets and analysed for GR protein expression. In addition, in order to act as a positive control for each sample, β-actin protein expression was also determined for each sample. Actin is expressed in all eukaryotic cells and is the major component of the cytoskeleton. β-actin is known as the cytoplasmic actin, and is expressed predominantly in non-muscle cells such as the liver.

The extraction of cell proteins requires efficient cell lysis and protein solubilisation, whilst avoiding protein degradation and interference with protein immuno-reactivity and biological activity. For this study, we have used CelLytic^{TM}MT mammalian tissue lysis/extraction reagent to enable the extraction of whole cell tissue proteins. The lysis buffer consists of a dialyzable mild detergent at a low concentration, for minimal interference with protein interactions and biological activity. The reagent also contains bicine, a buffer which is preferable for biological activity. Crude cell extracts contain a number of endogenous enzymes, such as proteases,

which are capable of degrading the proteins of interest. A protease inhibitor cocktail is also added to the buffer which contains inhibitors of these enzymes to increase the protein yield.

5.2.2.1 Whole-cell lysate procedure

Between 100–200 g of frozen liver tissue was used for this assay. To each 100 mg of tissue 1 ml of CelLytic^{TM}MT mammalian tissue lysis/extraction reagent and 5 µl of Protease Inhibitor Cocktail were added. The tissues were then completely disrupted using a Dispomix® closed system homogeniser. The homogenate was transferred to 1.5 ml sterile eppendorf tubes and centrifuged at 7840 g for 10 minutes at 4 °C. The supernatant was then transferred to a fresh 1.5 ml eppendorf tube and stored at -20 °C until subsequent analysis.

5.2.3 Protein concentration determination

The protein concentrations were determined using the bicinchoninic acid (BCA) assay (Smith et al., 1985, Wiechelman et al., 1988). The assay is based on changes in absorbance produced by reactions between the BCA reagent and the protein, resulting in a colour change in the sample from green to purple, proportional to the protein concentration. The benefit of using this assay is that it is less susceptible to interference from detergents and buffers used in the homogenisation of the protein sample. The BCA assay measures the reduction of Cu^{2+} to Cu^{+} by the amino acids cysteine, tryptophan and tyrosine in alkaline solutions of protein. The BCA reagent forms a purple complex with Cu^{1+}, which has a strong absorbance at 562 nm.

5.2.3.1 Bicinchoninic (BCA) assay procedure

Protein samples were diluted to 1 in 20 using 0.9 % saline to ensure concentrations fell within the linear range. A standard curve (0–1 mg/ml) was prepared using bovine serum albumin (BSA) in 0.9 % saline. Solutions A (1 % BCA, 2 % sodium carbonate, 0.16 % sodium tartrate and 0.4 % NaOH, pH 11.25 with 10 % sodium bicarbonate) and B (4 % copper sulphate) were prepared and a ratio of 100A:2B was mixed on the day of the assay. 2 µl of the correct concentration of standard or protein of the unknown samples was carefully pipetted into each well of a sterile 96-well plate and 200 µl of A: B mix added, ensuring no bubbles were formed. All samples were run in duplicate in order to establish intra-assay variability.

Plates were incubated at 37 ºC whilst being gently agitated for 30 minutes. Absorbance was then measured at 570 nm using the automated plate reader. Protein concentrations were determined following linear regression analysis of the standard curve and corrected for the initial dilution.

5.2.4 Western blotting

The abundance of selected proteins can be measured using sodium dodecyl sulphate-polyacryalmide gel electrophoresis (SDS-PAGE) (Burnette, 1981). During the first step the proteins are separated by their molecular weight using electrophoresis. After the separation, the proteins are transferred to a membrane (usually nitrocellulose or polyvinylidene fluoride (PVDF)) and detected by specific antibodies. The second antibody is conjugated with an enzyme and visualised using an enhanced chemiluminescence detection system. In order to prove the reproducibility of the assay, each Western blot was performed in duplicate for these studies.

Sodium dodecylsulphate (SDS) is an ionic detergent which gives a negative charge and denatures proteins, thus allowing the complete separation of proteins by their molecular weight (Burnette, 1981). In addition, SDS binds specifically in a mass ratio of 1.4 g SDS:1 g protein. In so doing, SDS confers a negative charge to the polypeptide in proportion to its molecular weight (Burnette, 1981). It is usually necessary to reduce disulphate bridges before they adopt the random-coil configuration necessary for separation by size (Burnette, 1981), this is done by adding β-mercaptoethanol. To ensure complete denaturation, it is also necessary to boil this mixture at 100 ºC for 10 minutes.

Once the proteins have been denatured and are negatively charged, they are loaded into the wells of a polyacrylamide gel. Together with the samples, a reference sample and protein marker or ladder (Bio-Rad Laboratories Ltd. Hertfordshire, UK) are also loaded. The reference sample used is a single protein sample used for all gels and enables the calculation of intra-assay coefficient of variance. The marker is a mixture of proteins, with known molecular weights and is used to confirm the target protein size. The SDS-protein complexes are then electrophoresed, with proteins moving towards the anode at a rate inversely related to their molecular weight. The resolution of the gel depends on the concentration of the

acrylamide. This determines the pore size, which can increase the separation between lower molecular weight proteins.

Proteins separated by SDS-PAGE can be transferred to a nitrocellulose or PVDF membrane for immunoblotting analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separated on the SDS-PAGE. The efficiency of the transfer can be tested by staining the membrane with Ponceau S dye and the gel with Coomassie blue

To detect a specific antigen blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane. To reduce non-specific binding, the membrane is rinsed at the end of the incubation in order to remove unbound or unspecific antibodies. The membrane is then exposed to a secondary antibody in order to detect the complex antibody target protein. Secondary antibodies are produced from a different animal species and are able to bind to the heavy chains of the primary antibody. In addition, the secondary antibody is commonly attached to a reporter enzyme such as horseradish peroxidise (HPR). Finally, after excess secondary antibody is washed free of the membrane, a chemiluminescent substrate is added which emits light upon reaction with the enzyme/antibody conjugate, resulting in a visible band where the primary antibody bound to the protein.

5.2.4.1 Western blot procedure

Gel preparation

Polyacrylamide gels were cast using a dual gel caster. This casting system consists of two glass plates: a small one sits on top of the larger one with a gap between. Both plates were vertically assembled within the casting apparatus according to the manufacturer's instructions (mini-PROTEAN® 3 cell, BioRad laboratories Ltd). The 12 % acrylamide resolving gel (10 ml of resolving gel: 3.3 ml dH_2O, 4.0 ml of 30 % acrylamide-bisacrylamide solution, 2.5 ml 1.5M Tris (pH 8.8), 100 µl 10 % (w/v) SDS, 100 µl 10 % (w/v) ammonium persulphate and 4 µl N,N,N',N'-tetramethylethylenediamine (TEMED)) was poured between the plates to a level of 3 cm below the top. To avoid gel-oxidation and to ensure a uniform surface, 2 ml of water-saturated butanol was placed on top of the gel. The

polymerisation of the resolving gel was conducted at room temperature for 45 minutes. After this time, the butanol was decanted and the gel surface rinsed with dH_20 and blotted dry. Stacking gel (10 ml of stacking gel: 6.8 ml dH_2O, 1.7 ml of 30 % acrylamide-bisacrylamide solution, 1.25 ml 1.0M Tris (pH 6.8), 100 µl 10 % (w/v) SDS, 100 µl 10 % (w/v) ammonium persulphate and 4 µl TEMED) was poured on top of the resolving gel and a 15 well comb inserted in order to produce the loading wells. This was then left to polymerise at room temperature for 15 minutes.

The polymerised gels were transferred to a vertical electrophoresis unit (BioRad laboratories Ltd.). The reservoir and sample wells were filled with 1 x running buffer (25 M Tris, 250nM Glycine and 0.1 % (w/v) SDS).

Sample preparation and electrophoresis

The protein concentrations of whole cell samples were determined using the BCA assay. Aliquots of each sample were diluted in homogenisation buffer (celLytic™ MT), to a concentration of 6.3 mg protein/ml in a final volume of 20 µl, this concentration equates to 15 µg protein in each well and ensures an equal protein load for each sample. 50 µl of protein dissociation buffer (10 % (v/v) glycerol, 2 % (w/v) SDS and 5 % (v/v) β-mercaptoethanol made up to volume in 50 mM Tris (pH 6.8)) and 14 µl glycerol-bromophenol blue (16 % (v/v) glycerol and 0.001 % (w/v) bromophenol blue in dH_2O) was added to each sample which was then incubated at 100 ºC for 10 minutes.

Before loading the protein samples, the wells were cleansed of un-polymerised acrylamide using a Pasteur pipette and the running buffer. 10 µl of sample, reference or molecular weight marker was loaded into each lane; empty lanes were loaded with 10 µl of glycerol-brompohenol blue to ensure even running. The gels were then electrophoresed at 200 V for 45 minutes, or until the dye front was sufficiently near the bottom of the gel.

Electrophoresis transfer onto PVDF membrane

Proteins from the polyacrylamide gel were transferred to a PVDF membrane (Immobilon®-P Transfer Membrane, Millipore, MA, USA) using electroblotting. Before blotting, the PVDF membrane was immersed in 100 % methanol for 15 seconds and transferred to dH_2O for 2 minutes. After this period, the membrane was transferred, along with blotting paper, to

transfer buffer to equilibrate (25 mM Tris, 192 mM glycine, 10 % methanol made up to volume with dH_2O). The gel and the membrane were sandwiched between a layer of blotting paper soaked in transfer buffer and supported by two scotch- Brite pads (3M, Bracknell, UK). This 'sandwich' was placed in a cassette and inserted into the blotting tank (BioRad laboratories Ltd) in the order cathode – gel – membrane – anode (Figure 5.1), to allow the negatively charged polypeptides onto the membrane. The tank was filled with transfer buffer and electrophoresed at 80 V for 1 hour. A cool water stream was passed through the high voltage transfer tank so that the proteins were not denatured by the blotting process.

(+) Positive electrode (Anode)
********** Plastic cassette
IIIIIIIIIIIIIIIIIIIIIIIII Scotch-Brite pads
1 Sheet blotting paper
PVDF membrane
Polyacrylamide gel
1 Sheet blotting paper
IIIIIIIIIIIIIIIIIIIIIIIII Scotch-Brite pads
********** Plastic cassette
(-) Negative electrode (Cathode)

Figure 5.1 Schematic diagram of a western blotting transfer unit

Protein staining

Following electrophoresis and blotting, the proteins on the PVDF membrane were reversibly stained and visualised. The membrane was incubated for 1 minute at room temperature with 10 % Ponceau-S red stain (2 % (w/v) Ponceau- S, 30 % (w/v) trichlroacetic acid, 30 % (w/v) sulphosalicylic acid in dH_2O, further diluted prior to use to 1:10 with dH_2O). Ponceau- S stain binds to protein and reveals a series of multiple bands arising vertically in lanes from each well, thus allowing verification of even blotting. Following the visualisation, it is possible to de-stain the Ponceau-S by rinsing the membrane with 0.1 M NaOH. The final confirmation of the efficiency of the transfer was assessed by staining the gel with Coomassie brilliant blue (0.1 % Simple Blue™, Invitrogen, CA, USA, in 50 % methanol and 7 % acetic acid in dH_2O) for 2 minutes. This procedure stains any proteins remaining on the gel after electroblotting and so confirms the complete transfer of polypeptides.

Immunodetection

In order to perform rapid immunodetection without blocking to prevent potential non-specific binding, the PVDF membrane must be dried thoroughly. To do this the membrane was soaked in 100 % methanol for 10 seconds to drive out the water, and then placed on a piece of filter paper to allow the methanol to evaporate for 15 minutes. Once dried the membrane was incubated overnight at 4 °C with 10 ml of primary antibody diluted in blocking buffer (1 % BSA in PBS (phosphate-buffered saline; 10 mM NA-phosphate, pH 7.2, 0.9 % NaCl) containing 0.05% (v/v) Tween-20).

Each antibody was validated by measuring the band that it generated against the protein ladder. If the identified band was a similar molecular weight to the protein interest as published in the literature then the antibody was used. In addition, the result of this assay was compared with the non-specific bands produced by immunoblots incubated with non-immune rabbit serum. To optimise the incubation conditions of the antibodies, identical gels were exposed to different concentrations of primary antibodies, incubation times and temperatures (Table 5.1).

Table 5.1 Technical details of the antibodies used

Antibody	Supplier	Catalogue number	Protein type	Protein size (kDa)	Primary antibody dilution factor
β-Actin	Source Bioscience AUTOGEN, UK	ABE933	Cytosol	43	1:2000
GR	Thermo Fisher Scientific Ltd, UK	PA1-511A	Nuclear	97	Variety tested

At the end of the incubation with the primary antibody, the membrane was washed twice in PBS, before the addition of the secondary antibody (1:5000 dilution of Protein G (BioRad laboratories Ltd.) in 1 % BSA in PBS containing 0.05% (v/v) Tween-20) and incubated for 30 minutes at room temperature. The secondary antibody was discarded and the membrane was washed twice in PBS.

Enhanced Chemiluminescence

The membrane was incubated in chemiluminescent substrate reagents (1:1 of reagents 1 and 2, Millipore) for 5 minutes. The excess reagent was drained and the membrane placed in the CDD camera to generate a digital

image. The optimum time of exposure for all immunoblots was 30 seconds. The image created by this process was analysed by densitometry (Aida version n2.0, Raytek Scientific Ltd., Sheffield, UK), which evaluates the density of the bands. The intensity value of each band reflects the abundance of the polypeptide within the initial sample.

5.2.5 Plasma cortisol

Due to the significant differences in GR and 11β-HSD1 mRNA expression in the liver, plasma cortisol expression was analysed at 6 months of age using a GammaCoat™ Cortisol radioimmunoassay (RIA) kit (GammaCoat™ [^{125}I] Cortisol Radioimmunoassay Kit, Diasorin, Minnesota, USA). RIA is a very sensitive technique, first developed by Yallow and Berson (1960) to measure concentrations of antigens, such as hormones, in the blood. This technique works by mixing a known amount of radioisotope-tagged cortisol or tracer with a specific antibody, where the antibody is immobilised onto the lower inner wall of the GammaCoat tube. The tracer and antibody chemically bind together. Unknown plasma samples and calibrators of known concentrations are then added to the tubes. The cortisol in the samples competes with the radiolabelled antigen for binding sites and displaces some of the I^{125}-tagged cortisol. The radioactivity of the free-tagged cortisol can then be measured using a gamma counter. Using known standards, a binding curve can then be generated, which allows the unknown concentrations of cortisol in the plasma to be determined.

5.2.5.1 Cortisol radioimmunoassay (RIA) procedure

All reagents were allowed to reach room temperature and were mixed thoroughly prior to use. With all assays, a standard curve (1-60 µg/dL) was used to determine the cortisol concentrations of the samples. All samples were run in duplicate in order to establish intra-assay variability. A co-efficient of variance (CV) of less than 5 % was considered acceptable and samples were repeated if the CV was higher than this (see section 2.6). For all assays the first 2 tubes were left blank as these were the Total Count tubes. 10 µl of Cortisol Serum Blank (GammaCoat™ [^{125}I] Cortisol Radioimmunoassay Kit, Diasorin), Cortisol Serum Calibrator (GammaCoat™ [^{125}I] Cortisol Radioimmunoassay Kit, Diasorin), or unknown plasma sample were carefully pipetted into the correct tubes, and 1 ml of tracer-buffer reagent (GammaCoat™ [^{125}I] Cortisol Radioimmunoassay Kit, Diasorin) added. Tubes were then vortexed for a few seconds at a low

speed, and incubated for 45 minutes in a water bath maintained at 37 °C. All tubes, expect the Total Counts, were aspirated, ensuring all liquid traces were removed. All tubes were then counted in a gamma counter (Wizard^{TM} 1470 Automatic Gamma Counter, PerkinElmer, Massachusetts, USA) for 1 minute in a window suitable adjusted for iodine-125. Cortisol concentrations were determined following analysis of the standard curve.

5.2.6 Statistical analysis

Full details of all statistical tests performed are described in Chapter 2.6.

For this chapter, in order to examine the effect of maternal diet on the offspring selected for tissue sampling at either one week or 6 months of age, either the low or median-birth weight piglets from the control group were compared with the appropriate low or median piglets from the protein supplemented group (Table 5.2). This was carried out using either an unpaired T-test or a Mann-Whitney test, depending on whether the data was parametric or non-parametric. In addition, to compare differences between piglet birth weights, data from both low and median-birth weight piglets were compared for either the control or protein supplemented groups (Table 5.2), again using T-tests of Mann-Whitney Tests. As previously mentioned in Chapter 4, it was not appropriate to statistically compare data obtained at 1 week and 6 months of age as, although all analysis techniques were kept the same, these tissues were analysed separately in the lab.

Pearson's correlation coefficient was used to demonstrate relationships factors such as birth weight, growth rate to 6 months of age, and back fat thickness. For this calculation all piglets born to sows from either the control or protein supplemented groups, which were not selected for tissue sampling at 6 months of age, were used.

Table 5.2 Outline of groups that were compared in this chapter

	C (L) 1 week	**C (M) 1 week**	**C (L) 6 months**	**C (M) 6 months**	**PS (L) 1 week**	**PS (M) 1 week**	**PS (L) 6 months**	**PS (M) 6 months**
C (L) 1 week	n/a	**Yes (weight)**	No	No	**Yes (diet)**	No	No	No
C (M) 1 week	**Yes (weight)**	n/a	No	No	No	**Yes (diet)**	No	No
C (L) 6 months	No	No	n/a	**Yes (weight)**	No	No	**Yes (diet)**	No
C (M) 6 months	No	No	**Yes (weight)**	-	No	No	No	**Yes (diet)**
PS (L) 1 week	**Yes (diet)**	No	No	No	n/a	**Yes (weight)**	No	No
PS (M) 1 week	No	**Yes (diet)**	No	No	**Yes (weight)**	n/a	No	No
PS (L) 6 months	No	No	**Yes (diet)**	No	No	No	n/a	**Yes (weight)**
PS (M) 6 months	No	No	No	**Yes (diet)**	No	No	**Yes (weight)**	n/a

Yes (weight) = Groups that have the same maternal diet but were in different birth weight categories (L = Low-birth weight; M = Median-birth weight) can be compared to examine the effect of birth weight; Yes (diet) = Groups that have different maternal diets (C = control; PS = Protein supplemented) but were in the same birth weight category were compared to determine the effect of maternal diet.

5.3 Results

5.3.1 Sex ratio

As described in Chapter 4, Section 4.3.1, offspring sex was not equally distributed amongst dietary groups, due to the method of selection process of low and median-birth weight piglets for tissue and plasma sampling. The reasons for unequal sex ratio, in the control group have previously been discussed (Chapter 4, Section 4.3.1). In contrast to both the control, and fat supplemented groups, there was no difference between male and female average birth weight for the litter in the protein supplemented group (Chapter 3; Table 3.8). In addition, there was a slightly higher ratio of very low-birth weight (<800 g) males to females in the litters from protein supplemented mothers (Chapter 3; Figure 3.5.c). This meant that when piglets were selected for sampling at one week of age, the lowest birth weight piglet of the litter was usually male (Table 5.3). In contrast to this, of the piglets that were selected for sampling at 6 months of age, a greater proportion was female (Table 5.3). This resulted in a reversal of sex ratio when comparing the control group with the protein supplemented group at both 1 week and 6 months of age (Table 5.3).

For this chapter, except where shown, males and females were considered together.

Table 5.3 Sex ratio of piglets selected for tissue and plasma sampling

		Number of males		Number of females	
		Low-birth weight	Median-birth weight	Low-birth weight	Median-birth weight
C	**1 week**	1	1	7	7
	6 month	6	6	2	2
PS	**1 week**	7	6	1	2
	6 month	2	3	5	4

C = Control; PS = Protein supplemented

5.3.2 Body weight and accelerated growth

Despite there being no significant difference in body weight at 6 months between male and female pigs or with protein supplementation (Table 5.4), male piglets that had been castrated tended to have increased back fat thickness at 6 months of age when compared to their female litter mates (Table 5.4). However, back fat thickness of the offspring, measured at 6 months of age, was unaffected by maternal protein supplementation

throughout pregnancy (Table 5.4). In addition, there was no correlation between back fat thickness and offspring growth rate from birth to 6 months (C: r=0.17, P=0.30; PS: r=0.23, P=0.12).

Table 5.4 Body weight and back fat thickness at 6 months of age in piglets not selected for tissue sampling

	C		PS	
	Females	**Males**	**Females**	**Males**
BW at 6 months (Kg)	116.10±0.73	113.97±0.88	115.76±0.76	115.97±1.14
Back fat (mm)	14.51±0.55	17.73±0.94*	14.28±0.46	17.73±0.78*

Values are means ± SEM for control (C; n=8) and fat supplemented (PS; n=8) gestational groups; Data was statistically tested for each gestational group using unpaired T-tests (C group) or Mann-Whitney tests (PS group) between the male and female piglets, differences are: *P<0.05; BW = Body weight

Birth weight of piglets selected for tissue sampling at 6 months of age was not different between dietary groups (Table 5.5). In those piglets selected for sampling at 1 week of age, birth weight was slightly higher in the low-birth weight piglets born to protein supplemented sows when compared to the control group, although this is not significant (Table 5.5). However, by 1 week of age this difference in body weight had become significant (Table 5.5). In accordance with the study design, low-birth weight piglets weighed significantly less than the median-birth weight piglets at 1 week of age (Table 5.5). However, by 9 weeks of age, there was no significant difference in body weight between the low and median-birth weight piglets selected for sampling (Table 5.5), this continued to 6 months of age. As described in chapter 4, it is thought that this is due to accelerated growth in the low-birth weight animals, demonstrated by strong negative correlation between birth weight and growth rate to 6 months of age, in both control (Chapter 4; Figure 4.2a) and protein supplemented gestational groups (Figure 5.2).

Table 5.5 Birth weight and body weight at euthanasia in piglets at 1 week and 6 months of age

			Birth weight (Kg)	BW at 9 weeks (kg)	BW at euthanasia (Kg)
C	**1 week**	**Low** (n=8)	0.72±0.07	n/a	1.12±0.13
		Median (n=8)	1.25±0.06*	n/a	2.13±0.13*
	6 month	**Low** (n=7)	0.99±0.07	22.44±0.93	115.21±5.92
		Median (n=7)	1.37±0.05*	24.77±1.46	120.14±3.27
PS	**1 week**	**Low** (n=8)	0.90±0.04	n/a	1.66±0.17**
		Median (n=8)	1.30±0.06*	n/a	2.53±0.20*
	6 month	**Low** (n=7)	1.11±0.08	25.33±2.17	119.92±2.45
		Median (n=7)	1.34±0.07*	28.97±2.32	128.92±4.11

Values are means ± SEM for control (C) and fat supplemented (PS) gestational groups; Data was statistically tested at 1 week and 6 months using unpaired T-tests or Mann-Whitney tests between the low and median-birth weight piglets and differences are: *P<0.05; In addition, differences between C and PS, low or median groups are denoted by: ** P<0.05 (T-test); BW= Body weight

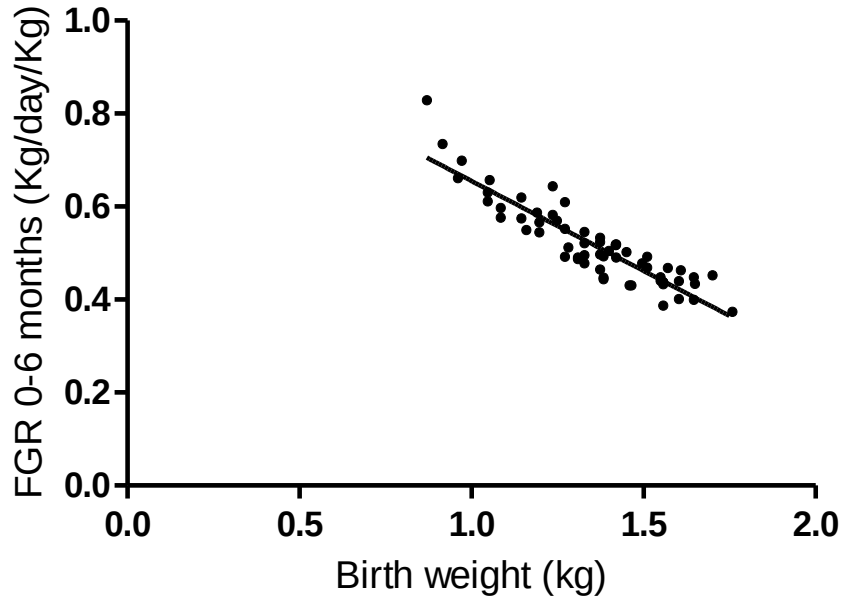

Figure 5.2 Relationship between piglet birth weight and fractional growth rate (FGR) to 6 months of age in piglets born to sows fed the protein supplemented (n=8) (r= -0.91; P<0.001) gestational diet (Pearson's correlation coefficient)

5.3.3 Plasma analysis

Plasma concentrations of glucose, TAG, NEFA, and cholesterol, were raised at 1 week, when compared to samples taken at 6 months of age, in both the control and protein supplemented groups (Table 5.6). Maternal protein supplementation or offspring birth weight did not affect plasma metabolite concentrations in the offspring, at either 1 week or 6 months of age (Table 5.6). In addition, maternal protein supplementation did not affect fasting plasma insulin concentrations (μg/ml) in offspring at 6 months of age (Low: C: 0.16±0.03; PS: 0.15±0.02; Median: C: 0.22±0.08; PS: 0.26±0.07). As previously explained (Chapter 4; Section 4.3.3), it was not possible to compare low and median-birth weight plasma samples as these were carried out on separate assay plates with different kits.

It was not possible to determine cortisol concentrations for these piglets at 6 months of age. This was due to a problem with the standard curve that was generated, which meant that the counts per minute (CPM) reading for each of the calibrators was much lower (around half) than the expected values given in the literature. The reasons for this are unclear as the protocol was followed, and the calibrators all came as part of the kit readymade up to the correct concentrations, so there was no dilution involved where it may have been possible to make a mistake. This meant that the CPM which were read for the unknown plasma samples were all too high to be read on the curve generated, which went up to around 8,500 CPM for the 1 μg/dL cortisol calibrator. Analysis of the raw data generated for each sample demonstrates similar CPM values to the examples provided in the instruction manual for this kit. Unfortunately, due to time constraints it was not possible to repeat this assay. In addition, as there was no difference between CPM values generated (C: low: 7432.81±598.71, median: 7680.69±327.49; PS: low: 8976.86±620.21, median: 7436.57±649.28), this suggested that maternal protein supplementation had no effect on cortisol concentrations at 6 months of age.

Table 5.6 Fasted plasma concentrations of glucose, triglycerides (TAG), nonesterified fatty acids (NEFA) and cholesterol in low and median-birth weight piglets, sampled at 1 week and 6 months of age

			Glucose (mM)	TAG (mM)	NEFA (mM)	Cholesterol (mM)
C	**1 week**	**Low** (n=7)	7.52±0.40	3.64±0.71	1.18±0.15	4.77±0.34
		Median (n=8)	7.97±0.23	3.16±0.58	0.99±0.11	3.60±0.36*
	6 month	**Low** (n=8)	4.22±0.47	1.14±0.22	0.96±0.14	2.36±0.37
		Median (n=8)	4.71±0.41	1.26±0.09	0.94±0.15	3.16±0.08*
PS	**1 week**	**Low** (n=8)	8.18±0.60	3.73±0.43	1.04±0.17	4.16±0.33
		Median (n=8)	8.36±0.59	3.08±0.42	0.93±0.12	3.44±0.30
	6 month	**Low** (n=7)	4.42±0.35	1.58±0.14	0.83±0.19	2.53±0.37
		Median (n=7)	4.40±0.61	1.41±0.13	1.04±0.19	2.64±0.38

Values are means ± SEM for control (C) and protein supplemented (PS) gestational groups; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: *P<0.01

5.3.4 Liver analysis

5.3.4.1 Liver weight and composition

At 1 week of age low-birth weight piglets, born to sows fed protein supplemented diets, had heavier livers than those born to control mothers (Table 5.7). This is probably accounted for by the higher body weight of these animals (Table 5.5) as this difference disappears when liver weight is expressed as a percentage of body weight (Table 5.7). Despite no significant differences in body weight at 6 months of age (Table 5.5), liver weight, expressed as a percentage of body weight is increased in median-birth weight piglets born to sows that were protein supplemented (Table 5.7). As previously shown, low-birth piglets had smaller livers than median-birth weight piglets, with no difference in liver weight being noted at 6 months of age (Table 5.7). Liver triglyceride and glycogen content were unaffected by maternal protein supplementation or piglet birth weight (Table 5.7). As previously explained in Chapter 4, it was not possible to measure liver glycogen content at 6 months of age in these animals as it was below the level of detection.

Table 5.7 Liver weight, triglyceride (TAG) and glycogen content in piglets at 1 week and 6 months of age

			Liver weight (Kg)	Liver weight (% of BW)	Liver TAG (mg/g)	Liver glycogen (mg/g)
C	**1 week**	**Low** (n=8)	0.05±0.008	4.41±0.40	1.98±0.40	75.8±19.6
		Median (n=8)	0.10±0.008*	4.82±0.19	1.17±0.37	91.8±13.8
	6 month	**Low** (n=8)	2.48±0.12	2.11±0.10	0.84±0.17	n/a
		Median (n=8)	2.51±0.12	2.03±0.06	0.97±0.18	n/a
PS	**1 week**	**Low** (n=8)	0.08±0.001**	4.76±0.30	1.17±0.23	58.9±14.0
		Median (n=8)	0.11±0.010*	4.49±0.21	0.82±0.20	85.4±15.3
	6 month	**Low** (n=8)	2.47±0.09	2.08±0.06	0.62±0.13	n/a
		Median (n=8)	2.81±0.16	2.24±0.08**	0.61±0.14	n/a

Values are means ± SEM for control (C) and fat supplemented (PS) gestational groups; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: *P<0.01; In addition, differences between C and PS, low or median groups are denoted by: ** P<0.05 (T-test); BW = Body weight

5.3.4.2 Liver gene expression

At 6 months of age there was an inverse relationship between birth weight and the effects of protein supplementation on GR mRNA expression. In the low-birth weight piglets, maternal protein supplementation reduced gene expression of GR (Figure 5.3.b). Conversely, in the median-birth weight piglets, maternal protein supplementation increased GR expression when compared to the control piglets (Figure 5.3.b). This resulted in significantly higher mRNA GR expression in the median-birth weight piglets born to protein supplemented sows, when compared to their low-birth weight litter mates (Figure 5.3.b). Similar, GR expression patterns are seen in piglets at 1 week of age; however, there was no significant difference (Figure 5.3.a).

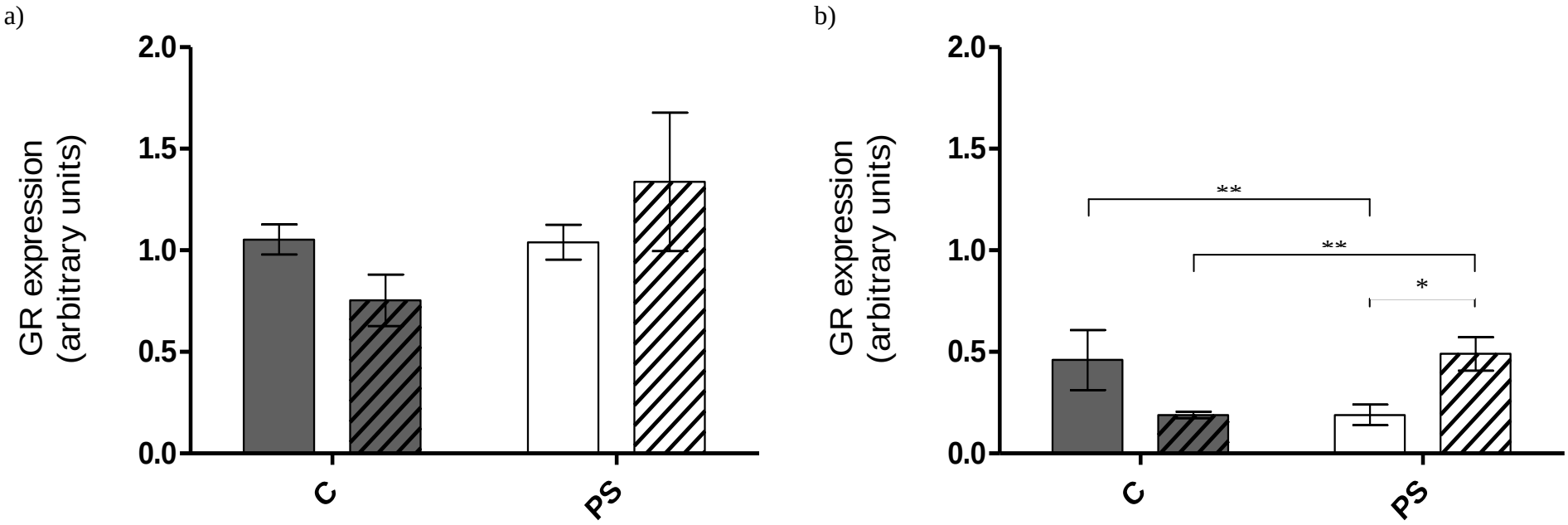

Figure 5.3 Liver Glucocorticoid receptor (GR) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or protein supplemented (PS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of GR mRNA using the genorm strategy; 18S, ribosomal phosphoprotein (RPO) and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: $^{*}P<0.05$; In addition, statistical differences between C and PS groups are denoted by ** $P<0.05$ (Mann-Whitney (low-birth weight); T-Test (median-birth weight))

11β-HSD1 showed similar gene expression patterns in piglets at 1 week and 6 months of age (Figure 5.4.a and 5.4.b). Similar to its effects on GR expression, maternal protein supplementation increased mRNA expression of 11β-HSD1 in median-birth weight piglets, although, small birth weight piglets were unaffected (Figure 5.4.a and 5.4.b). This resulted in higher expression of 11β-HSD1 in median-birth weight piglets, born to mothers fed a protein supplemented diet, when compared to their low-birth weight litter mates (Figure 5.4.a and 5.4.b).

Maternal protein supplementation had no effect on PPARα mRNA expression in the liver at 1 week or 6 months of age (Figure 5.5.a and 5.5.b). In low-birth weight piglets at 1 week of age, gene expression of FABP1 was decreased when sows were fed a protein supplemented diet throughout gestation (Figure 5.6.a). In addition, FABP1 expression is higher in median-birth weight piglets at 1 week of age, when compared to low-birth weight piglets (Figure 5.6.a). Birth weight or maternal diet had no effect on offspring FABP1 mRNA expression at 6 months of age (Figure 5.6.b).

Maternal diet had no effect on piglet mRNA expression of IR at 1 week or 6 months of age (Figure 5.7.a and 5.7.b). Similar to GR and 11β-HSD1 expression, expression of the IR gene was increased at 6 months of age in median-birth weight piglets, born to sows fed a protein supplemented diet, than their low-birth weight litter mates (Figure 5.7.b). 11β-HSD2 gene expression was measured in liver tissue at both 1 week and 6 months but was too low to be detected. This was expected as 11β-HSD2 is not highly expressed in the liver.

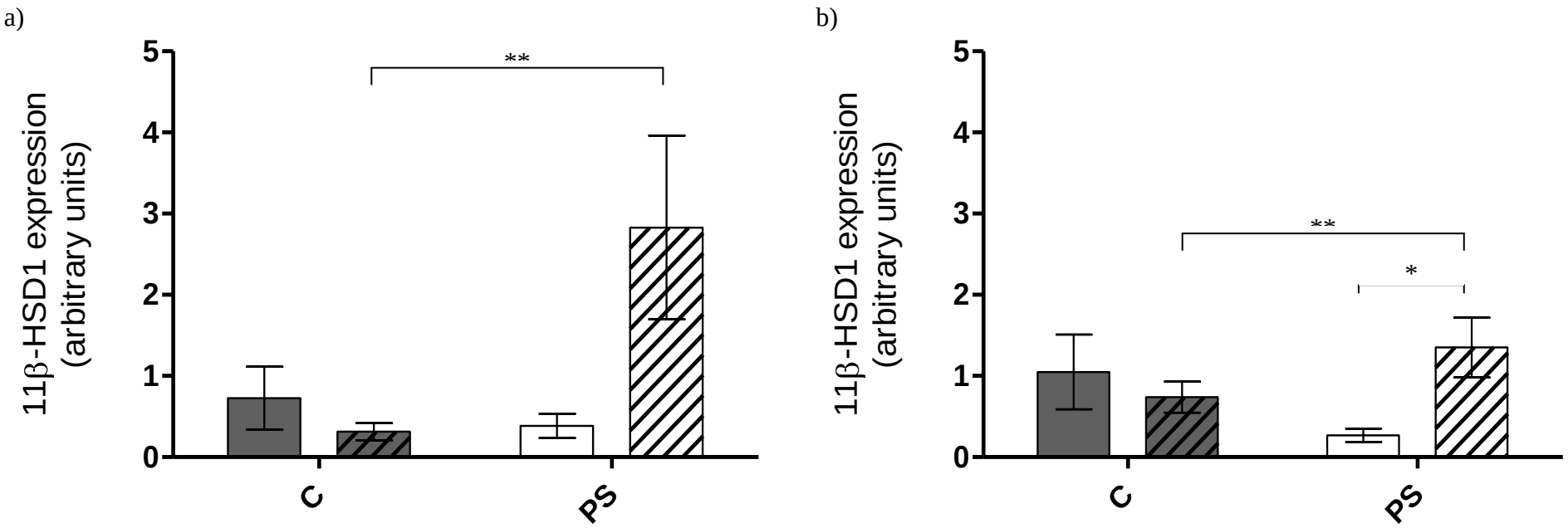

Figure 5.4 Liver 11beta-Hydroxysetroid Dehydrogenase type 1 (11β-HSD1) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or protein supplemented (PS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of 11β-HSD1 mRNA using the genorm strategy. 18S and cyclophylin were used as housekeeping genes, and results are given in arbitrary units. Values are means ± SEM; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: $^{*}P<0.05$; In addition, statistical differences between C and PS groups are denoted by ** $P<0.05$ (Mann-Whitney)

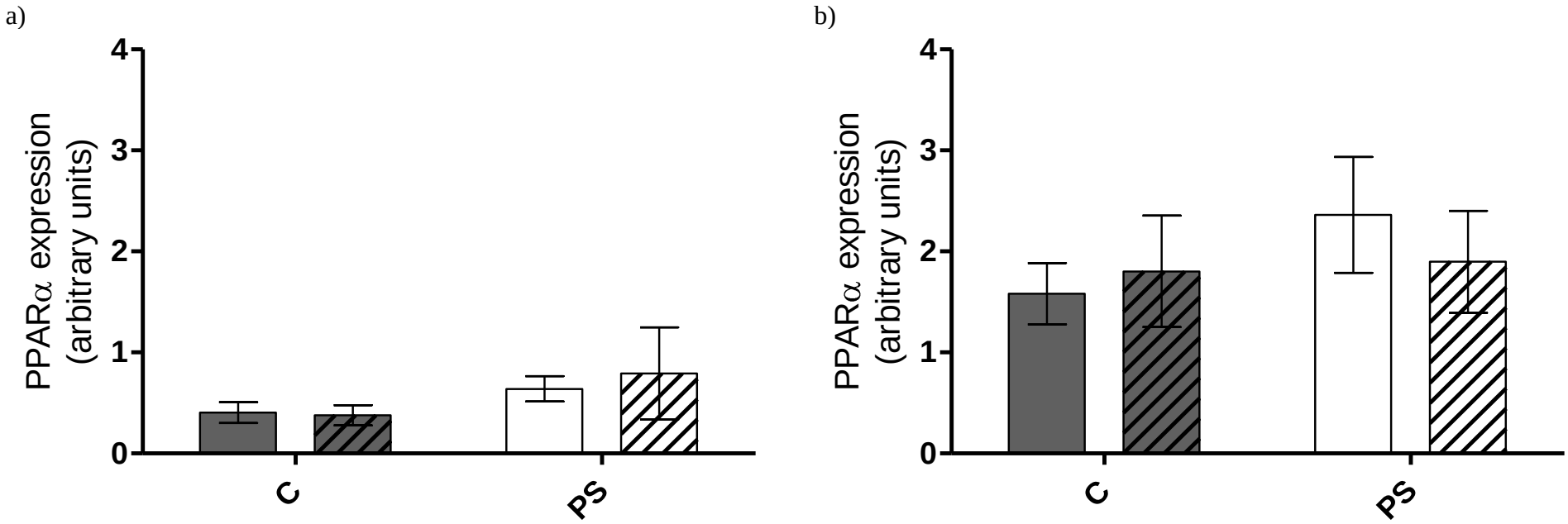

Figure 5.5 Liver Peroxisome proliferator-activated receptor alpha (PPARα) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or protein supplemented (PS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of PPARα mRNA using the genorm strategy; 18S and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM

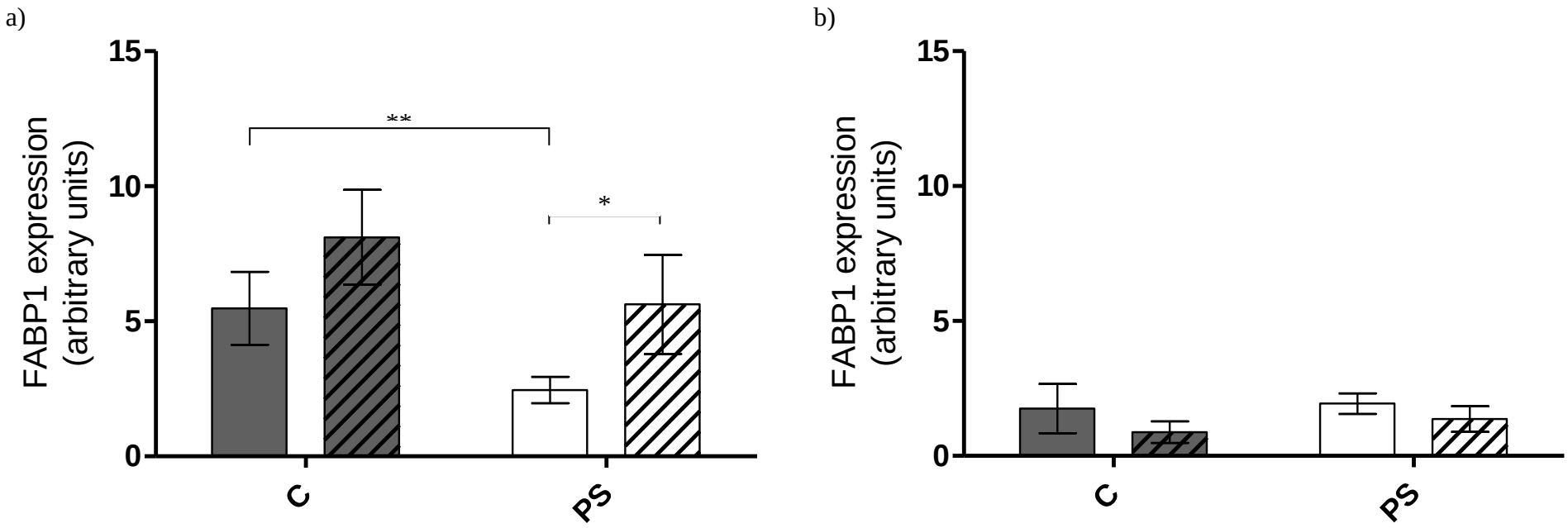

Figure 5.6 Liver Fatty acid-binding protein 1 (FABP1) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or protein supplemented (PS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of FABP1 mRNA using the genorm strategy. 18S and cyclophylin were used as housekeeping genes, and results are given in arbitrary units. Values are means ± SEM; Data was statistically tested at 1 week and 6 months using Mann-Whitney tests between the low and median-birth weight piglets and differences are: $^{*}P<0.05$; In addition, statistical differences between C and PS groups are denoted by ** $P<0.05$ (T-test)

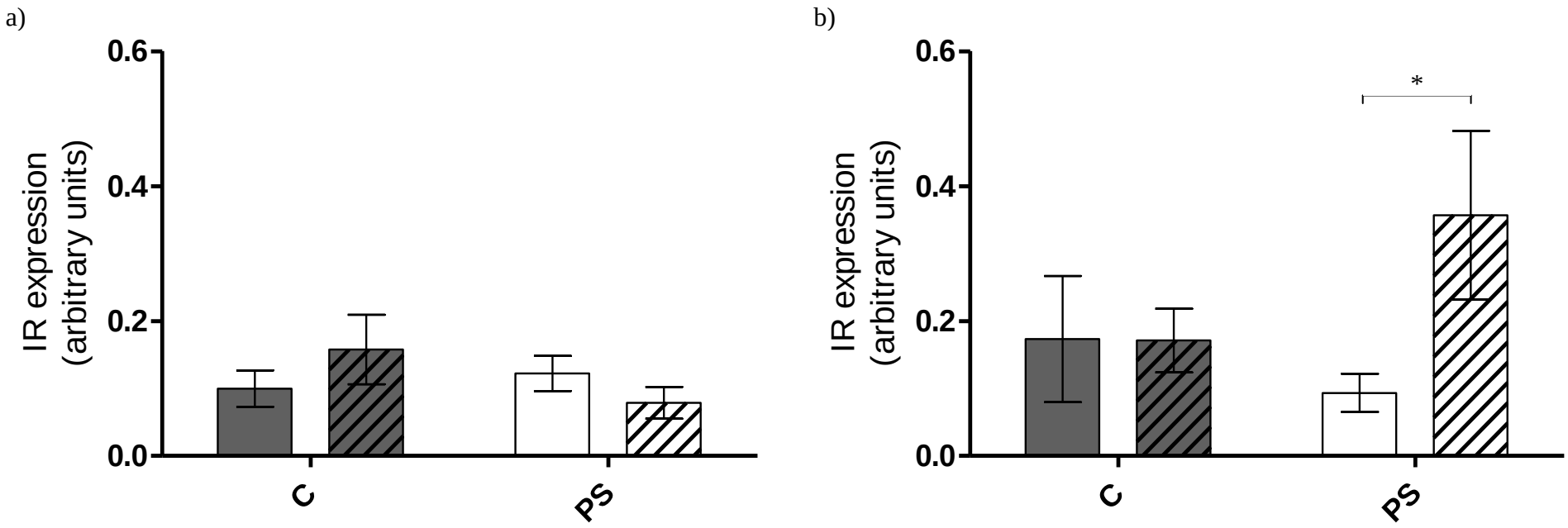

Figure 5.7 Liver Insulin receptor (IR) mRNA expression in low ☐ and median ▨ birth weight piglets, born to sows fed control (C; n=8) or protein supplemented (PS; n=8) gestational diets, sampled at (a) 1 week and (b) 6 month of age; Relative quantification of IR mRNA using the genorm strategy; 18S, ribosomal phosphoprotein (RPO) and cyclophylin were used as housekeeping genes, and results are given in arbitrary units; Values are means ± SEM; Data was statistically tested at 1 week and 6 months using T-tests between the low and median-birth weight piglets and differences are: $^{*}P<0.05$

5.3.4.3 Protein expression of glucocorticoid receptor (GR)

The GR antibody used in this study has been demonstrated to detect a protein which is around 97 kDa (Fan et al., 2010). Unfortunately, despite previously published data suggesting that the primary GR antibody used worked in pigs (Weaver et al., 2000) and also rats (Fan et al., 2010) and that the amino acid sequence was 100 percent homologous to pigs, demonstrated by a sequence search using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it was not possible to determine GR protein expression in the liver of these piglets at 6 months of age. This was due to the antibody creating a series of multiple protein bands which were visible on the membrane even when the antibody was used at a variety of dilutions (Figure 5.8). In addition, the strongest of these bands was not at 97 kDa but was instead around 50 kDa, suggesting that further dilution of the primary antibody would not have been appropriate (Figure 5.8).

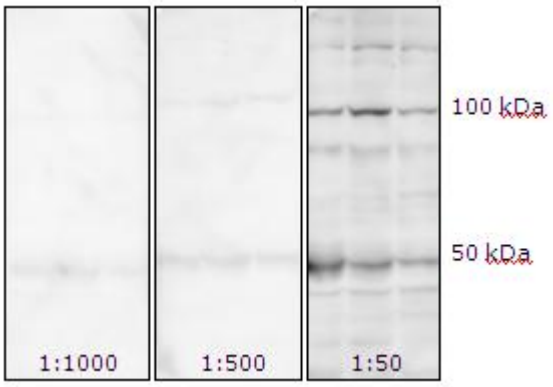

Figure 5.8 Representative image of a Western blot for glucocorticoid receptor (GR); 3 dilutions of primary antibody tested: 1:1000, 1:500, 1:50; 1:5000 dilution of secondary antibody (Protein G); 15 µg protein in each lane

In order to test the quality of the extracted liver proteins, a β-actin antibody was used. This worked for all the samples, demonstrated by a strong band around 44 kDa (Figure 5.9), further suggesting that there was a problem with the GR antibody used in this study, rather than the protein extractions.

Due to time constraints it was not possible to order another GR antibody and test this with the 6 month liver samples.

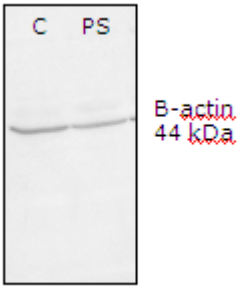

Figure 5.9 Representative image of a Western blot for β-actin; 1:2000 dilution of primary antibody; 1:5000 dilution of secondary antibody (Protein G); 15 µg protein in each lane; C = control; PS = Protein supplemented

5.4 Discussion

Despite the increase in dietary protein in the Western population (Rolland-Cachera et al., 2000), investigations into protein supplementation during pregnancy are limited, with conflicting outcomes between studies (Daenzer et al., 2001, Zimanyi et al., 2002, Thone-Reineke et al., 2006). This is the first study in pigs to investigate the effects of maternal protein supplementation during gestation on offspring development. Previous studies have instead focussed on the function of dietary protein during pregnancy on offspring survival and litter size as this is of key economical importance in the pig industry. This is demonstrated by studies in both rats and pigs which have suggested a role for specific amino acid supplementation, particularly arginine, during pregnancy, in improving offspring survival rate (Mateo et al., 2007, Zeng et al., 2008). In contrast to this, this study has previously demonstrated an increase in neonatal mortality, particularly of low-birth weight offspring, when sows were protein supplemented with an arginine rich source (Chapter 3).

5.4.1 Growth and development of the offspring

This study demonstrated no effects of maternal protein supplementation on the body weight of the piglets at 6 months of age. This was expected as no differences in birth weight or litter size were previously observed (Chapter 3). A link between a maternal protein rich diet and offspring adiposity has previously been demonstrated, but this was probably linked with accelerated postnatal growth due to a reduction in birth weight of the offspring (Daenzer et al., 2001).

As hypothesised, and as previously observed (Chapter 4), low-birth weight offspring demonstrated accelerated postnatal growth so that by 9 weeks of age, there were no significant differences in body weight between piglets. This increase in growth rate, however, was not associated with an increase in fat mass, measured by back fat thickness, in the low-birth weight offspring as might have been expected from evidence in both the pig industry (Powell and Aberle, 1980) and human cohort studies (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001). However, it is possible that back fat thickness is not a good indicator for an increase in fat composition of the pig and that another method for the measurement of adiposity may have been more appropriate (Chapter 4; Section 4.4.2).

5.4.2 Glucocorticoid (GC) sensitivity

Offspring born to sows fed a protein rich diet throughout pregnancy demonstrated an increase in gene expression of both GR and 11β-HSD1 at 6 months of age, suggesting that these animals were more sensitive to GC. Similar affects have been observed in offspring born to mothers who were protein restricted or that were under-nourished during pregnancy (Bertram et al., 2001, Whorwood et al., 2001). It is thought that this is due to enhanced transfer of maternal GC to the fetus, resulting in an increase in GC sensitivity in the peripheral tissues of the offspring (Bertram et al., 2001, Whorwood et al., 2001). Unfortunately, it was not possible to measure fasting plasma cortisol in these animals to see if this was raised. However, previous studies that have measured offspring plasma cortisol have demonstrated that it is usually unaffected by maternal nutrient restriction (Gilbert et al., 2005, Gardner et al., 2006), suggesting perhaps that similar results may have been noted in this study. Analysis of the CPM data obtained from the cortisol assay, suggests that this may be the case. However, this is not conclusive as there was obviously a problem with the assay and although it seemed only to be concerned with the standard curve, it is possible that it was a problem with the assay as a whole and so this data should be interpreted with caution.

GC have important functions within the body, such as increasing blood pressure and increasing plasma glucose concentration (Chapter 1; Section 1.2.1) (Tangalakis et al., 1992, Dimitriadis et al., 1997a), and are crucial to controlling whole body metabolism. As a result, an increase in GCs and/or GC sensitivity has been linked to an increased risk of metabolic disease such as obesity, hypertension and insulin resistance (Covar et al., 2000, Bertram et al., 2001, Whorwood et al., 2001, Arnaldi et al., 2003). Therefore, it would have been expected that those animals demonstrating an increase in GC sensitivity, would have an increase in fat mass and perhaps, perturbed lipid and glucose homeostasis, demonstrated by an increase in circulating insulin, glucose and triglycerides, and perhaps a reduction in genes involved in the lipid uptake and oxidation pathways. However, the consequences of this increase in GC sensitivity where not apparent in the variables that were measured in these animals, at 6 months of age, with no difference observed back fat thickness, plasma insulin or metabolites, and expression of FABP1, PPARα and IR mRNA. Despite this, liver weight was increased in the median-birth piglets born to

sows that were protein supplemented, although the reason for this increase is not known. It was previously thought to perhaps be due to hepatic accumulation of lipids, as hepatic steatosis is common in patients with metabolic syndrome (Marceau et al., 1999), however this was proven not to be the case as the triglyceride content of the liver was not statistically different between dietary groups.

There are several possible reasons as to why no consequences of the increased GC sensitivity were noted in these animals. The first explanation is that these differences in gene expression may not translate into differences in protein expression. It was not possible to test this hypothesis as the antibody for GR did not produce specific bands with these tissue samples. Unfortunately, due to time constraints, it was not appropriate to test any other antibodies, especially as the antibody used in this study was 100 percent homologous to the pig amino acid sequence. In addition, the GR antibody tested had previously been demonstrated to work in pigs, although this study was investigating brain tissue expression (Weaver et al., 2000). However, in this paper, multiple bands were also detected, at 95 and 87 kDa, and it is unclear whether these coded for the functional GR protein (Weaver et al., 2000). Another investigation which claimed to have detected GR in rats may also be called into question as, in this study, the membrane was divided into sections before immunodetection and only the area of interest, around 97 kDa, was exposed to the GR antibody (Fan et al., 2010). Therefore, multiple bands may not have been detected (Fan et al., 2010).

Increases in gene expression of GR and 11B-HSD1 were observed at both 1 week and 6 months of age, although GR expression at 1 week of age was not significant, suggesting that this is a programming effect of the protein rich diet fed to the mothers of these piglets. The second explanation as to why no consequences of GC sensitivity were noted in this study could be that these piglets were analysed at 6 months of age and that they were still relatively young. Often, detrimental programming effects such as increased blood pressure, cardiovascular disease, and insulin resistance, in both humans and rats, are not observed until adulthood (Erikson et al., 2000, Forsen et al., 2000, Hales and Barker, 2001, Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005, Ehruma et al., 2007). Therefore, as these animals were analysed during puberty (6 months of age), as this is when

they have reached commercial sale weight, it is possible that the potential negative effects of increased GC sensitivity such as an increase in circulating triglycerides and an increase in fat mass, may not have been noted until they were much older.

In addition, programming effects are often only demonstrated when there is a mismatch between the fetal and the postnatal environment, perhaps caused by under-nutrition or growth restriction in utero (Hales et al., 1991, Langley-Evans et al., 1996, Langley-Evans et al., 1999, Erikson et al., 2000, Forsen et al., 2000, Bertram et al., 2001, Ehruma et al., 2007). In this study, the diets fed to these animals post-weaning contained around 16 to 17 percent crude protein, to provide optimal growth of the piglets during this period. And so, those animals born to sows subjected to protein supplementation, actually received similar dietary protein levels in postnatal life. It is therefore possible that although these animals were programmed *in utero* to be more sensitive to GC by the feeding of a protein rich diet, actually these animals were not subjected to any detrimental effects of this because they went on to receive similar protein levels as neonates and post-weaning.

The low-birth weight offspring who demonstrated accelerated postnatal growth had lower mRNA expression of GR, 11β-HSD1, and IR than their median-birth weight litter mates of the same body weight. Therefore, it seems that the median-birth weight animals are more affected by maternal protein supplementation than the low-birth weight animals. This was surprising considering the evidence linking low-birth weight and accelerated growth to an increase in metabolic abnormalities in adulthood (Langley-Evans et al., 1996, Langley-Evans et al., 1999, Erikson et al., 2000, Forsen et al., 2000, Bertram et al., 2001, Hales and Barker, 2001) and studies which show an increase in GC sensitivity in the offspring, demonstrated by an increase in GR and 11β-HSD1 in certain tissues such as the liver (Bertram et al., 2001). However, it is probable that these effects are mediated through the low-protein maternal diet or under-nutrition during pregnancy, rather than just the effects of low-birth weight alone. This is further supported by Whorwood et al. (2001) who demonstrated an increase in GC sensitivity at birth in the offspring of under-nourished sheep, despite there being no differences in offspring weight.

5.4.3 Effect of piglet sex

Piglet sex is an important determinant of adult body composition. Generally, the amount of fat is less in intact males or boars, than in intact females or gilts, with castrated males or barrows having the most amount of fat (Siers, 1975, Mitchell et al., 2001). In addition, gilts grow at a slower rate than boars and barrows, and so the time taken for gilts to reach a slaughter weight of around 99 Kg from a starting weight of 27 Kg is significantly longer (Siers, 1975).

In this study there was a mixture of males and females. The males that were used for tissue and plasma analysis at 6 months of age were not castrated; this was in order to try to balance out some of the effects of sex that might be demonstrated, as all females remained intact. Male piglets that were not used for tissue analysis were castrated as is normal practice in the pig industry (Chapter 2; Section 2.3.1). As expected, this study demonstrated an increase in back fat thickness in castrated males at 6 months of age in comparison to the females. This effect was noted regardless of the diet that the mothers received (see also Chapter 4). Unfortunately, back fat thickness was not measured in those animals selected for sampling so it is unclear as to whether the males would have had reduced thickness in comparison to the females as would be expected (Siers, 1975).

Knowing that sex has significant effect on piglet body composition, it is also possible that it could also play a fundamental role in determining the effects of nutritional programming. This is further supported by studies in rats which have demonstrated a protective role for female sex hormones against the development of diet-induced insulin resistance and associated disorders such as hypertension (Galipeau et al., 2002, Hevener et al., 2002).

Unfortunately, male and female ratio of piglets selected for further tissue and plasma analysis was not equal between the control and protein supplemented groups at 1 week or 6 months of age. There was a high ratio of males at 1 week of age and a higher ratio of females at 6 months of age in the median-birth weight piglets born to protein supplemented mothers, relative to the control group. Therefore, it is possible that sex could have had an effect on the results so that the significant differences observed

between the gene expression of control and protein supplemented groups, could be a factor of sex rather than dietary intervention. In order to try to disprove this, sex differences between dietary groups for all variables were measured and there was no difference between males and females. In addition, if males and females were examined separately there were no effect of maternal diet on gene expression of GR and 11β-HSD1 as previously demonstrated. However, the reason that no differences were noted was probably because this study was not powered to look at sex differences and some groups had relatively low numbers of males or females. If both the effects of sex and birth weight had been investigated the number of animals needed in this study would have been significantly higher, also resulting in a lot of animal wastage. Despite this, as this study demonstrated similar results at both 1 week and 6 months of age in terms of gene expression, and within these groups at this time the ratio of males to female was reversed, it should be concluded that this was more likely to be an effect of maternal protein supplementation rather than offspring sex.

5.4.4 Conclusions

This was the first study to investigate the programming effects of feeding a protein rich diet to pigs throughout pregnancy. It has demonstrated an up-regulation of genes involved in GC sensitivity, such as GR and 11β-HSD1, in the liver, but has as yet, not detected any other significant changes in these piglets, with no differences observed in body weight, back fat thickness or liver metabolism. Surprisingly, these effects were only observed in the median-birth weight piglets. This increase in GC sensitivity was similar to the programming effects observed by maternal protein restriction or global under-nutrition during pregnancy (Bertram et al., 2001, Whorwood et al., 2001). Possibly suggesting that the type of nutritional insult pregnancy is not important and that maternal under-nutrition may cause similar programming effects as over-nutrition.

In both humans and animal models, alterations in GC sensitivity have been associated with a higher risk of developing metabolic abnormalities (Walker et al., 1998, Bertram et al., 2001, Whorwood et al., 2001, Rask et al., 2002). Therefore, these findings could have important implications in determining the programming effects of maternal diet on human disease risk.

The importance of these findings for the pig industry are not clear, because no phenotypic consequences were observed in the parameters measured between offspring of protein supplemented, compared to control fed mothers. Therefore, it is likely that meat quality would not be affected by such minor alterations in maternal dietary composition, although further work is currently being carried out to investigate the effects on muscle quality to validate this. However, this study may have important implications if these offspring were to become breeding stock and were allowed to grow and develop past 6 months of age.

Chapter 6 - Conclusions

6.1 General conclusions

The aim of this study was to demonstrate the effects of moderate changes in the macronutrient ratio of the maternal diet, whilst still maintaining energy content, on offspring development and metabolism until adolescence. This was achieved by assessing body composition, the expression of key genes involved in glucocorticoid (GC) sensitivity and metabolism in the liver, as well as analysing the metabolic profile of the plasma. In addition, the effect of piglet birth weight and subsequent postnatal growth on liver metabolism was investigated. This chapter outlines the key findings from this study and aims to determine why some of these outcomes may have been different from what was previously expected.

6.1.1 Fat supplementation

One of the main findings of this study was the effect of maternal fat supplementation on glucose tolerance of the sow at term and the implications for offspring mortality (Chapter 3). Recent investigations in pigs have demonstrated the negative effects of fat supplementation during pregnancy on maternal glucose tolerance (van der Peet-Schwering et al., 2004). This is similar to the effects of obesity and/or fat consumption during pregnancy in both humans and rats on the increased risk of developing abnormal glucose homeostasis (Guo and Jen, 1995, Taylor et al., 2003) and/or gestational diabetes (Chu et al., 2007). In agreement, with these findings, this study has demonstrated that an increased fat ratio of the diet from mating until day 110 of gestation results in a reduction in maternal glucose tolerance.

Previously, fat supplementation was considered beneficial during pregnancy to reduce piglet mortality, although the intervention period of these studies were only during the last few days of gestation and throughout lactation (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993). However, in contrast to these previous observations, piglet mortality was increased in the litters of fat supplemented mothers. The reason for this increase in mortality was hypothesised to be due to enhanced supply of maternal glucose to the fetus, causing hyperinsulinemia which leads to hypoglycaemia in these

offspring at birth (Pere and Dourmad, 2000, van der Peet-Schwering et al., 2004). This was further supported by the finding that the majority of the piglet deaths occurred within the first 24 hours after birth, and that those animals that died were of low-birth weight (Lubchenco and Bard, 1971). However, we were unable to prove or disprove this, as plasma samples were not taken from these animals at birth due to the financial and time constraints with such a large sample size in a commercial pig environment.

In order to further develop this study, the effects of maternal fat supplementation on postnatal piglet development were examined (Chapter 4). Investigations in rats have demonstrated that feeding a high-fat diet during pregnancy increases the risk of metabolic abnormalities such as obesity, hypertension and impaired glucose tolerance in the pups (Khan et al., 2003, Khan et al., 2005, Taylor et al., 2005). Similar effects have been noted in offspring born to mothers with impaired glucose tolerance (Boney et al., 2005, Franks et al., 2006). Following on from the previous observations noted in this study, demonstrating reduced glucose tolerance and increased neonatal mortality, it was hypothesised that maternal fat supplementation would result in piglets who had increased fat mass and perturbed glucose and lipid homeostasis. However, this study demonstrated no differences between piglets born to sows fed a control or fat supplemented diet in the parameters measured at 6 months of age.

6.1.2 Protein supplementation

Despite the tendency for dietary protein intake to exceed recommended values in the Western world, particularly in younger women (Rolland-Cachera et al., 2000), investigations into the outcomes of a high-protein diet during pregnancy are limited, with conflicting results between studies (Mathews et al., 1999, Daenzer et al., 2001, Zimanyi et al., 2002, Kramer and Kakuma, 2003, Thone-Reineke et al., 2006). It is often not the amount of dietary protein which is important in determining the outcomes, but the amino acid composition of the diet (Burrin, 2001). Therefore, previous studies have focussed on the effects of supplementing the maternal diet with specific amino acids, particularly arginine (Mateo et al., 2007, Zeng et al., 2008), on increasing litter size and reducing the number of stillborn offspring.

Surprisingly, this study demonstrated an increase in postnatal mortality when sows were fed a diet with an increased ratio of a protein source that was rich in arginine, with no effect on litter size or number of stillborn piglets (Chapter 3). The reasons for this increase in neonatal mortality are unclear, although it may be linked to an increase in maternal back fat and decrease in plasma non-esterified fatty acids (NEFA) during late gestation.

The final important finding of this study was an increase in GC receptor (GR) and 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) gene expression in the livers of the offspring born to sows that were fed a protein rich diet, thus suggesting increased GC sensitivity in these animals (Chapter 5). These results were similar to the programming effects demonstrated by under-nutrition and protein restriction in both the rat and sheep models (Bertram et al., 2001, Whorwood et al., 2001). Despite the apparent increase in GC sensitivity in this study, no consequential effects on offspring metabolism seemed to accompany this as might have been expected (Covar et al., 2000, Bertram et al., 2001, Whorwood et al., 2001, Arnaldi et al., 2003), perhaps because the animals were too young at 6 months of age for any detrimental effects to have become apparent (Chapter 5).

6.2 Study limitations and future perspectives

6.2.1 Maternal dietary manipulations

This study was unique in that the manipulations in the maternal diet were subtle and energy content was maintained. The primary reason for this was because this study, as with a high proportion of large animal investigations, relied on collaboration with industry. Therefore, extreme manipulations of the maternal diet which have the potential to be detrimental to the sow's health and welfare, and the meat quality of the offspring, would not be economically viable. This could provide a plausible reason as to why there were differences between the more severe programming outcomes demonstrated in rat studies.

In order to maintain energy content between diets it was necessary to adjust the ratio of other dietary components such as starch content (Appendix C). For example, in the fat supplemented diet, starch content was reduced by half in comparison to the control diet (FS: 161 g/Kg; C: 316 g/Kg), due to a reduction in the tapioca content. This was further

complicated by the use of whole food sources in the diet. The bulk of pig feed is usually supplied in the form of cereal grains which contain mainly starch, but some protein. In order to increase the protein content of the diet, by-products of the oilseed industry are often used, such as soybean and rapeseed meal. These different oilseed meals contain different quantities of fibre, starch and oils. This meant that when the diet was protein supplemented with lupins and sunflower seed meal, the quantities of other ingredients were reduced such as rapeseed oil, soy bean hulls and sugar beet pulp. This resulted in slight alterations in the fat and fibre ratio between the control and protein supplemented diet. Therefore, it is possible that it is perhaps not the increased fat or protein in the diet which has caused these effects, but it is actually the differing ratio of other key nutrients in the diets. This is further supported by population studies in humans which demonstrate an increased risk of developing non-insulin-dependent type 2 diabetes mellitus when a high-fat, low-carbohydrate diet is consumed (Marshall et al., 1991).

6.2.2 Dietary intervention period

The dietary intervention period for this study was from 0 to 110 days of gestation. From day 110 of gestation all sows then received the same diet until the end of lactation. This meant that there was no difference in milk composition between dietary treatments (Chapter 3). Maternal diet is known to affect milk composition (Burrin, 2001) and it has been suggested by several studies that fat supplementation of sows during late gestation and lactation improve milk yield and fat content of the milk, which may account for a reduction in offspring mortality rate (Chapter 3) (Seerley et al., 1974, Boyd et al., 1978, Pettigrew, 1981, Seerley et al., 1981, Azain, 1993). Therefore, it is possible that the large increase in piglet mortality may have been reduced by extending the period of fat supplementation throughout lactation and this could be the focus of future studies.

6.2.3 Sex ratio

As previously discussed, sex ratio amongst offspring selected for tissue analysis at 1 week and 6 months of age was not equal (Chapter 5). This was due to the selection method which, due to constraints on the number of animals that could be included, was based entirely on birth weight, where the lowest birth weight piglet was always selected regardless of sex. It is unlikely that piglet sex was a factor contributing to the significant

outcomes observed in the offspring born to sows who received the protein supplemented diet, although this cannot be ruled out. Therefore, a further study would be required to elucidate whether sex had a role to play in these programming mechanisms. This could be done by only selecting male or female offspring to analyse, or to include more animals in the study so that there would be an increased likelihood of having a more even male to female offspring ratio. However, there would be potential ethical concerns with conducting this kind of trial as a large number of animals would be required and the amount of wastage would be high if both birth weight and animal sex were piglet selection criteria.

6.2.4 Different tissue responses

Patients who have the metabolic syndrome have been demonstrated to have greater risk of non-alcoholic fatty liver disease (Hamaguchi et al., 2005). It has been proposed that this reflects a failure of the normal partitioning of excess fat exclusively to the adipose tissue, resulting in ectopic fat storage in the liver, muscle and pancreas (Hamaguchi et al., 2005). It has previously been discussed how an increase in GCs and/or GC sensitivity is linked to an increased risk of metabolic disease (Covar et al., 2000, Bertram et al., 2001, Whorwood et al., 2001, Arnaldi et al., 2003). For this reason, this study focussed on the effects of dietary supplementation during gestation on the livers of the offspring, particularly focussing on GC sensitivity. Despite demonstrating changes in gene expression of GR and 11β-HSD1 with protein supplementation no other effects were noted in the liver. It is possible that these effects may not have been noted until the offspring were older or had begun to consume a diet which was lower in protein content once optimal growth was no longer the key objective (Chapter 5) (Burrin, 2001). However, differences of maternal diet manipulation in other offspring tissues may be noted at an earlier age; one such tissue is the muscle. For example, one study in rats has demonstrated that feeding a cafeteria diet, which consists of *ad libitum* intake of highly palatable high-fat or high-sugar foods, to rats throughout gestation and lactation resulted in pups who had a 25 percent reduction in muscle cross-sectional area with an increase in intramuscular lipid content at weaning, despite no effects on birth weight or growth rate (Bayol et al., 2005). However, food intake of the dams was not measured and so overall maternal energy and fat intake, which would normally increase, could not be taken into account (Bayol et al., 2005).

The ultimate goal of meat animal production is the economical production of muscle tissue that is both a nutritious and palatable source of dietary protein for human consumption (Mitchell et al., 2001). By the time of birth, the number of muscle fibre cells in the pig is fixed (Mitchell et al., 2001). However, during postnatal growth, the muscle fibres increase in both length and width, this is also accompanied by an increase in intramuscular lipid content, which should be limited (Mitchell et al., 2001). Previous studies in pigs have focussed on trying to improve muscle quality by increasing maternal food intake during mid-gestation, but have found no effects of on muscle characteristics or meat quality (Nissen et al., 2003). In addition, low-birth weight piglets have been shown to have reduced muscle weights and increased muscle lipid content when compared to their higher birth weight littermates at slaughter (Gondret et al., 2006). Therefore, future work is currently underway to determine the effects of maternal fat and protein intake, and offspring birth weight, on the gene expression and composition of the muscle.

6.3 Concluding remarks

This study has demonstrated the role of fat and protein supplementation during pregnancy. The findings from this study could have consequences for the pig industry, particularly when trying to reduce piglet mortality. In addition, these findings could have important implications in determining the programming effects of maternal diet on human disease risk.

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Appendices

A. Abstracts and conferences attended

The effect of increased maternal dietary intake during pregnancy on offspring birth weight and neonatal survival. By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Poster presentation – Nutrition Society Annual Summer meeting, Nottingham, UK, July 2008

Altering maternal nutrition affects fetal development and may have long-lasting effects on the offspring, potentially predisposing them to later disease such as diabetes and obesity. Studies have shown that these effects may take place without alterations in birth weight (1), although smaller-birth-weight offspring appear to be at increased risk (2). There is limited literature on the effects of increasing the fat and protein content of the gestational diet on birth weight and postnatal survival.

Pregnant sows were fed one of six isoenergetic diets (Table) that differed only in composition and quantity fed during the first and final trimester of gestation. Sows fed on a low–high feeding scheme were fed 2.7 kg/d between day 0 and day 70 of gestation and 3.5 kg/d between day 70 and day 110 of gestation. Sows fed on a high–low feeding scheme were fed 3.5 kg/d between day 0 and day 40 of gestation and 2.7 kg/d between day 40 and day 110 of gestation. All sows received a standard diet from day 110 and throughout lactation. All sows delivered naturally and all piglets were weighed. At 7 ±3 d one small and one median piglet per sow were selected and weighed. All preliminary data was analysed using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

Treatment	Diet description	Feeding scheme	No. of sows
1 (Control)	High starch	Low–high	8
2	High fat	Low–high	8
3	High protein	Low–high	8
4	High starch	High–low	8
5	High fat	High–low	8
6	High protein	High–low	8

There was no effect of diet on piglet birth weight or litter size. Maternal diet had no effect on the growth rate of the small piglets. Median-birth-weight piglets born to sows fed diet 5 (151.4g (SEM 10.4)) had a significantly reduced (P<0.05) growth rate up to 7 d when compared to sows fed diet 1 (control diet, 188.5g (SEM 12.7)) and diet 2 (226.2g (SEM 15.2)). Median-birth-weight piglets had a significantly increased (P<0.05) growth rate up to 7 d when compared with the smaller piglets born to sows fed diet 1 (control diet; small, 91.0g (SEM 19.8); median, 188.5 (SEM 12.9)), diet 2 (small, 126.5g (SEM 18.3); median, 226.2g (SEM 15.2)), diet 4 (small, 108.8g (SEM 17.1); median, 189.4g (SEM 14.8)) and diet 5 (small, 98.3g (SEM 15.6); median, 151.4g (SEM 10.4)). Maternal diet had no effect on the percentage of stillborn piglets per litter. There was a significant increase (P<0.05) in the percentage of postnatal deaths per litter in sows fed diet 2 (16.81 (SEM 5.87)), diet 3 (1.88 (SD 0.52)) and diet 6 (8.83 (SEM 1.72)) when compared with diet 1 (control, 3.95 (SEM 1.66)).

Maternal nutrition during gestation appears to have no major effect on offspring birth weight or growth rate up to 1 week of age. This outcome may be a result of the sow compensating for any dietary change by compromising her own metabolism in order to avoid any detrimental effects on offspring birth weight. It may be interesting to compare sow body condition and glucose tolerance during pregnancy to confirm this notion. Smaller-birth-weight piglets tend to grow more slowly than median piglets during the first week of life [(3)]. High fat feeding of the sow during late gestation and high protein feeding in either early or late gestation increases the percentage of piglet mortalities in the litter. The prospective findings from the ongoing study will inform further on the influence of increasing dietary components during pregnancy.

1. Whorwood CB, Firth KM, Budge H & Symonds ME (2001) *Endocrinology* **142**, 2854–2864.
2. Phillips DIW, Barker DJP, Fall CHD, Seckl JR, Whorwood CB, Wood PJ & Walker BR (1998) *J Clin Endocrinol Metab* **83**, 757–760.
3. Mostyn A, Litten JC, Perkins KS, Euden PJ, Corson AM, Symonds ME & Clarke L (2005) *Am J Physiol Regul Integr Comp Physiol* **288**, R1536–R15

The influence of changes in macronutrient intake during gestation on gene expression of on glucocorticoid receptor (GR) and 11-beta-hydroxysteroid dehydrogenase 1 and 2 (11βhsd1 and 2) in the livers of neonatal pigs; effect of size at birth By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Poster presentation – The Society for Endocrinology Annual meeting, Harrogate, UK, March 2009

Background

Altering maternal nutrition affects fetal development, potentially predisposing them to later metabolic disease. Tissue sensitivity to cortisol is regulated, in part, by GR and 11βhsd1 and 2, and GR and 11βhsd1 expression is known to be raised both with obesity and type 2 diabetes.

Objectives

To examine whether gestational diet influences GR and11βhsd1 and 2 mRNA expression in the neonatal liver.

Methods

Pregnant sows were fed one of six isoenergetic diets (Table). Sows fed on a low–high feeding scheme were fed 2.7 kg/d between day 0 and day 40 of gestation and 3.5 kg/d between day 70 and day 110 of gestation. The dietary regimes were reversed for sows fed on a high–low feeding scheme. At 7 days one small and one median piglet per litter were selected, humanely euthanized and its liver sampled. GR and 11βhsd1 and 2 mRNA expression was quantified by real-time PCR. Values are presented as arbitrary units.

	Control	***2***	***3***	***4***	***5***	***6***
	(n=8)	(n=8)	(n=8)	(n=8)	(n=8)	(n=8)
Diet description	High starch	High fat	High protein	High starch	High fat	High protein
Feeding scheme	Low-high	Low-high	Low-high	High-low	High-low	High-low

Results

Median size piglets born to sows fed high protein had increased ($p<0.05$) expression of GR (control: 0.57±0.15; 3: 1.05±0.19; 6: 1.70±0.44) and 11βhsd1 (control: 2.47±0.76; 3: 6.53±1.44; 6: 12.12±2.20) compared to controls. In contrast, expression of 11βhsd2 in the liver of the small piglets born to sows fed high levels of the high fat diet during late gestation (diet 5) was increased ($p<0.05$) when compared to sows fed the same diet at high levels during early gestation (diet 2) (2: 0.10±0.04; 5: 0.30±0.06).

Conclusion

Changes in GR and 11βhsd1 expression indicate increased sensitivity to glucocorticoids in the liver at 1 week of age in piglets born to sows fed a high protein diet during gestation. Further studies are currently in progress to investigate whether these changes persist into adulthood.

The effect of increased maternal dietary fat intake during pregnancy on glucose tolerance near term and offspring birth weight By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Poster presentation – Nutrition Society Scottish meeting, Edinburgh, UK, April 2009

Diabetes during pregnancy in humans has been linked to adverse effects on the offspring such as fetal macrosomia, and impaired glucose tolerance and obesity in later life (Dabelea et al., 2000). Previous studies in pigs have shown that high fat feeding during late gestation can reduce glucose tolerance at term (van der Peet-Schwering et al., 2004), but the long term effects on the offspring are not known. This study examines whether increased fat intake during pregnancy influences glucose tolerance near to term and offspring birth weight.

Pregnant sows were fed one of four isoenergetic diets (Table) that differed only in composition and quantity fed during the first and final trimester of gestation. Sows fed on a low–high feeding scheme were fed 2.7 kg/d between day 0 and day 70 of gestation and 3.5 kg/d between day 70 and day 110 of gestation. Sows fed on a high–low feeding scheme were fed 3.5 kg/d between day 0 and day 40 of gestation and 2.7 kg/d between day 40 and day 110 of gestation. On day 108 of gestation glucose (0.5 g/kg; i.v.) was administered, via a catheter inserted into the sows ear, and regular blood samples were taken for 1 hour. Blood from each time point was tested using a glucometer, in order to determine the rate of glucose clearance.

Diet description	*High starch (low-high) (n=8)*	*High fat (low-high) (n=5)*	*High starch (high-low) (n=7)*	*High fat (high-low) (n=8)*
Basal glucose conc. (mmol/l)	4.14±0.17	4.6±0.09	4.13±0.12	4.06±0.13
Glucose AUC (mmol min^{-1})	112.75±6.53^{a}	140.80±3.65^{b}	134.57±10.83^{a}	165.88±11.47^{b}
Birth weight of offspring (kg)	1.20±0.06	1.28±0.06	1.23±0.09	1.21±0.06

All results are expressed as means±SEM. Different letters denote values that are significantly different from each other (Mann-Whitney U test)

Gestational diet had no effect on basal glucose concentrations, but resulted in glucose intolerance in mothers, irrespective of the timing of the additional fat. Birth weight was unaffected by gestational diet or sow glucose tolerance.

Feeding a high fat diet throughout gestation impairs glucose tolerance without any immediate effects on birth weight. The prospective findings from this ongoing study will inform further on the long-term effects that impaired maternal glucose tolerance may have on her offspring.

1. Dabelea D, Knowler WC, Pettit DJ: **Effects of diabetes in pregnancy on offspring: follow up research in Pima Indians** *Journal of maternal-fetal medicine* 2000, **9**(1):83-88.
2. van der Peet-Schwering CMC, Kemp B, Binnendijk GP, den Hartog LA, Vereijken PFG, Verstegen MWA: **Effects of additional starch or fat in late-gestating high nonstarch polysaccharide diets on litter performance and glucose intolerance in sows**. *Journal of Animal Science* 2004, **82**:2964-2971.

The effect of stage of gestation on feeding high-protein on hepatic glucocorticoid sensitivity in the neonate and later metabolic homeostasis in the young adult offspring By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Poster presentation –6th International Conference on Developmental Origins of Health and Disease, Santiago, Chile, Nov 2009

Objectives Altering maternal nutrition affects fetal development, potentially predisposing offspring to later metabolic disease. Glucocorticoid (GC) excess has been linked to the clinical observations associated with the metabolic syndrome. Tissue sensitivity to cortisol is regulated, in part, by GR and 11βhsd1 and 2. Several studies have shown the effects of maternal nutrition on the programming of GC action in the offspring (Gnanaligham et al., 2005, Whorwood et al., 2001). The hypothesis is that feeding a high protein diet during gestation alters development of GC sensitivity.

Methods Pregnant sows were fed one of four isoenergetic diets (Table) that differed in composition and quantity fed during the first and final trimester of gestation. Sows fed on a low–high feeding scheme were fed 2.7 kg/d between day 0 and day 40 of gestation and 3.5 kg/d between day 70 and day 110 of gestation. The dietary regimes were reversed for sows fed on a high–low feeding scheme. At 7 days and 6 months, one median piglet per litter were selected, humanely euthanized, a blood sample taken and its liver sampled. GR and 11βhsd1 and 2 mRNA expression was quantified by real-time PCR (Values are presented as arbitrary units). Plasma concentrations of glucose and non-esterified fatty acids (NEFA) were assessed using enzymatic-colorimetric assays. Offspring that were not selected for tissue sampling were raised commercially and body composition was recorded at the slaughterhouse.

	C (n=8)	***HP(L-H)*** (n=8)	***C(H-L)*** (n=8)	***HP(H-L)*** (n=8)
Diet description	High starch	High protein	High starch	High protein
Feeding scheme	Low-high	Low-high	High-low	High-low

Results Piglets born to sows fed high protein had increased ($p<0.05$) liver expression of GR and 11βhsd1 at 1 week. At 6 months of age these piglets born to sows with reduced feed intake in late gestation had decreased glucose and increased NEFA plasma concentrations and diameter of the longissimus dorsi muscle (table).

	C	***HP(L-H)***	***C(H-L)***	***HP(H-L)***
GR/18S (arbitrary units)	0.57 ± 0.11[a]	1.05 ± 0.19[b]	0.83 ± 0.18	1.70 ± 0.44[b]
11BHSD-1/18S (arbitrary units)	2.47 ± 0.76[a]	6.53 ± 1.44[b]	4.74 ± 1.05[a]	12.12 ± 2.20[b]
Glucose (mmol/l)	4.71 ± 0.41[a]	4.40 ± 0.61	4.55 ± 0.23	3.75 ± 0.16[b]
NEFA (mmol/l)	0.93 ± 0.15[a]	1.04 ± 0.19	1.17 ± 0.14	1.41 ± 0.07[b]
Muscle diameter (mm)	56.35 ± 0.84[a]	58.30 ± 0.89	58.99 ± 0.94[b]	61.79 ± 1.15[b]

All results are expressed as means ± SEM. Different letters denote significant differences ($P<0.05$) between groups (Mann-Whitney U test).

Conclusions Feeding a high-protein diet during pregnancy programs increased hepatic cortisol sensitivity in the newborn. Surprisingly this was not associated with any major long term adverse metabolic outcomes. Indeed, it appeared to improve glucose tolerance, an adaptation possibly mediated by increased muscle growth. Studies are currently in progress to further investigate the potential effects on insulin responsiveness together with liver and muscle metabolism.

1. M. G. Gnanaligham *et al.*, *Am J Phys.,* 289: 1407-1415, 2005.
2. C. B. Whorwood *et al*., *Endocrinology*., 142(7): 2854-2864, 2001.

Glucose tolerance near term and offspring birth weight; effect of high-fat feeding during pregnancy By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Poster presentation –6th International Conference on Developmental Origins of Health and Disease, Santiago, Chile, Nov 2009

Introduction Diabetes during pregnancy in humans has been linked to adverse effects on the offspring such as fetal macrosomia, and impaired glucose tolerance and obesity in later life (Dabelea et al., 2000). Previous studies in pigs have shown that high fat feeding during late gestation can reduce glucose tolerance at term (van der Peet-Schwering et al., 2004), but the long term effects on the offspring are not known. This study examines whether increased fat intake during pregnancy influences glucose tolerance near to term and offspring birth weight. The hypothesis is that increased fat intake will decrease glucose tolerance without effecting birth weight.

Methods Pregnant sows were fed one of four isoenergetic diets (Table). Sows fed on a low–high feeding scheme were fed 2.7 kg/d between day 0 and day 40 of gestation and 3.5 kg/d between day 70 and day 110 of gestation. The dietary regimes were reversed for sows fed on a high–low feeding scheme. On day 108 of gestation glucose (0.5 g/kg; i.v.) was administered, via a catheter inserted into the sows ear, and regular blood samples were taken for 1 hour. Blood from each time point was tested using a glucometer, in order to determine the rate of glucose clearance.

	C (n=8)	***HF(L-H)*** (n=8)	***C(H-L)*** (n=8)	***HF(H-L)*** (n=8)
Diet description	High starch	High fat	High starch	High fat
Feeding scheme	Low-high	Low-high	High-low	High-low

Results Gestational diet had no effect on basal glucose concentrations, but resulted in glucose intolerance in mothers, irrespective of the timing of the additional fat. Birth weight was unaffected by gestational diet or sow glucose tolerance.

	C	*HF(L-H)*	*C(H-L)*	*HF(H-L)*
Basal glucose conc. (mmol/l)	4.14 ± 0.17	4.6 ± 0.09	4.13 ± 0.12	4.06 ± 0.13
Glucose AUC (mmol min^{-1})	112.75 ± 6.53^{a}	140.80 ± 3.65^{b}	134.57 ± 10.83^{a}	165.88 ± 11.47^{b}
Birth weight of offspring (kg)	1.20 ± 0.06	1.28 ± 0.06	1.23 ± 0.09	1.21 ± 0.06

All results are expressed as means±SEM. Different letters denote values that are significantly different from each other (Mann-Whitney U test)

Conclusions Feeding a high fat diet throughout gestation impairs glucose tolerance without any immediate effects on birth weight. The prospective findings from this ongoing study will inform further on the long-term effects that impaired maternal glucose tolerance may have on her offspring.

1. D. Dabelea et al., *J Matern Fetal Med.,* 9(1): 83-88, 2000.
2. C. M. C. van der Peet-Schwering., *J Anim Sci.,* 82: 2964-2971, 2004.

The influence of changing the macronutrient content of the maternal diet on offspring development and liver metabolism By Kayleigh Almond[1], Paul Bikker[2], Michael Lomax[1], Michael Symonds[1] and Alison Mostyn[1], [1]*University of Nottingham, Nottingham, UK and* [2]*Schothorst Feed Research, Lelystad, The Netherlands*

Oral presentation –The Neonatal Society Autumn meeting, London, UK, Nov 2009

Objectives Altering maternal nutrition affects fetal development, potentially predisposing offspring to later metabolic disease. Glucocorticoid (GC) excess has been linked to the clinical observations associated with the metabolic syndrome. Tissue sensitivity to cortisol is regulated, in part, by glucocorticoid receptor (GR) and 11-beta- hydroxysteroid-dehydrogenase (11BHSD) types 1 and 2. Several studies have shown the effects of maternal nutrient restriction on the programming of GC action in the offspring (Gnanaligham et al., 2005, Whorwood et al., 2001), however, dietary excess is far more characteristic of the diets consumed by a substantial number of pregnant women. The hypothesis of this study is that consuming a high-fat or protein diet during gestation decreases maternal glucose tolerance and increases offspring liver GC sensitivity potentially altering lipid metabolism.

Methods Pregnant sows were fed one of three isoenergetic diets (Table 1), that altered only in starch, protein and fat content, between days 0 and 110 of gestation (term = 115 days). Glucose tolerance tests were carried out on day 108 of gestation in sows who received high fat and control diets. All sows delivered naturally and piglets were weighed at birth. At 7 days, one median piglet per litter were selected, humanely euthanized, a blood sample taken and its liver sampled. GR, 11BHSD-1 and 2, and peroxisome-proliferator-activated-receptor alpha (PPARα) gene expression were quantified by real-time PCR. Triglyceride (TAG) content was measured using spectrophotometric analysis following Folch extraction [3]. Plasma concentrations of glucose, non-esterified fatty acids (NEFA) and TAG were assessed using enzymatic-colorimetric assays. Offspring that were not selected for tissue sampling were raised commercially and body weight and composition was recorded at the slaughterhouse. All results were expressed as means ± SEM. Significant differences between groups

were determined using either a T-test (parametric) or Mann-Whitney U test (non-parametric).

Maternal diet	Starch (%)	Fat (%)	Protein (%)
Control	31.6	2.5	12.3
High fat	16.1	8.9	12.2
High protein	31.7	3.7	16.3

Table 1: Maternal diets

Results Consuming a high fat gestational diet reduced ($p<0.05$) maternal glucose tolerance at term. Offspring mortality was increased ($p<0.05$) when mothers consumed a high fat or protein diet. Birth weight, body weight at 6 months and growth rate was unaffected by maternal diet. Offspring born to mothers receiving a high protein diet had increased ($p<0.05$) liver expression of GR and 11BHSD-1 at 1 week. Although not significant there was a trend towards a decrease in TAG content and increase in PPARα gene expression in the livers of offspring at 1 week of age born to mothers who consumed either the high fat or protein diets.

Conclusions Feeding a high-fat diet reduces glucose tolerance in the mothers at term; it is unclear what effect high protein feeding has. Neonatal survival appears to be compromised when mothers consume a high-fat, or protein diet throughout gestation. Feeding a high-protein diet during pregnancy programs increased hepatic cortisol sensitivity in the newborn. Future work will further determine the effect of high-fat or protein maternal diets on offspring liver metabolism at 6 months of age.

B. Details of suppliers

Abbot Laboratories, 100 Abbot Park Road, Abbot Park, Illinois 60064-3500, USA; www.abbot.com

ABgene Limited UK, ABgene House, Blenhem Road, Epsom, KT19 9AP; www.abgene.com

Alfasan International BV, Kuipersweg 9, JA 3440, Woerden, The Netherlands, PO Box 78 3440 AB; www.alfasan.com

AVEBE, 9640 AA Veendam, The Netherlands; www.avebe.com

Bibby Scientific Ltd. Beacon Road, Stone, Staffordshire, UK, ST15 O5A; www.bibby-scientific.com

BioRad Laboratories Ltd. Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX; www.bio-rad.com

Bio Tek Instruments Inc. 100 Tigan Street, Winoski, Vermont 05404, USA; www.biotek.com

Diasorin Inc. 1951 Northwestern Avenue, P. O Box 185, Stillwater, Minnesota, USA, 55082-0285; http://www.diasorin.com/en

FOSS, Slangerupgade 69, Postbox 260, DK-3400, Hillerød, Denmark; www.foss.co.uk

Fuji Photo Film Co. Ltd. 7-3, Akasaka 9-chome, Minato-ku, Tokyo 107-0052, Japan; www.fujifilm.com

Intervet, Wim de Körverstraat 35, 5831 AN Boxmeer, The Netherlands; www.intervet.com

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Buisness Park, Paisley, United Kingdom, PA4 9RF; www.invitrogen.com

Labtech International Ltd. Acorn House, The Broyle, Ringmer, East Sussex, UK, BN8 5NN; www.labtech.co.uk

Mercodia A B, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden; http://www.mercodia.se

Millipore, 290 Concord Road, Billerica, Massachusetts 01821 USA; www.millipore.com

PerkinElmer, 940 Winter Street, Waltham, Massachusetts 02451, USA; http://www.perkinelmer.co.uk

Qiagen, Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ; www.qiagen.com

Randox Laboratories Ltd, 55 Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY; www.randox.xom

Raytek Scientific Ltd. 26 Norton Park View, Sheffield, UK, S8 8GS; www.raytek.co.uk

Renco Corp. 116 3rd Ave. N. Minneapolis, Minnesota, USA, 55401; www.rencocorp.com

Roche Co. Konzern-Hauptsitz, Grenzacherstrasse 124, CH-4070 Basel, Switzerland; www.roche.com

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT; www.sigm-aldrich.com

Sigma-Genosys Ltd, Sigma-Aldrich House, Homefield Business Park, Homefield Road, Haverhill, Suffolk, CB9 8QP; www.sigma-genosys.com

SPSS inc. 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606, USA; www.spss.com

Source Bioscience AUTOGEN, 1 Orchard Place, Nottingham Business Park, Nottingham, UK, NG8 6PX; www.autogenbioclear.com

Thermo Fisher Scientific Ltd/Across Ltd, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG; www.fisher.co.uk

Thistle Scientific Ltd. DFDS House, Goldie Road, Uddingston, Glasgow, G71 6NZ; www.thistlescientific.co.uk

3M United Kingdom PLC, 3M Centre, Cain Road, Bracknell, UK, RG12 8HT; www.solutions.3m.co.uk

C. Full composition of experimental diets

	C	FS	PS	Lactation
Feed stuff	**%/Kg of diet**	**%/Kg of diet**	**%/Kg of diet**	**%/Kg of diet**
Tapioca	28.1	3.1	28.1	-
Rapeseed meal	10.0	10.0	5.0	4.0
Lupins	-	-	12.0	-
Sunflower seed meal	4.0	4.0	16.4	2.0
Soybean hulls	13.0	17.0	3.0	2.0
Sugar beet pulp	10.0	10.0	-	2.0
Palm oil	-	6.6	-	3.1
Soybean oil	0.5	0.5	1.1	0.97
Maize	-	-	-	10.0
Soybean meal	-	-	-	11.7
Wheat	10.0	10.0	10.0	26.4
Barley	10.0	10.0	10.0	15.0
Wheat middling's	15.0	15.0	15.0	15.0
Molasses	4.0	4.0	4.0	4.0
Monocalciumphosphate	0.22	0.22	0.22	0.48
Salt	-	-	-	0.37
Limestone	0.50	0.60	0.73	1.56
Premix vit. + min.	0.5	0.5	0.5	0.5
Lysine (25%)	0.17	0.17	-	-
Lysine-HCl (L, 79%)	-	-	-	0.17
Threonine (L, 98%)	-	-	-	0.02
Phytase	0.5	0.5	0.5	0.5
Threonine 15% 2390*	0.01	0.01	-	-
Sodium bicarbonate	0.6	0.6	0.6	0.16
Diet composition	**g/Kg**	**g/Kg**	**g/Kg**	**g/Kg**
Nutrients				
Ash	64	52	63	61
Crude protein	123	122	163	161
Crude fat	25	89	37	63
Crude fibre	120	121	106	52
ID starch + sugar	368	212	361	367
Sugar	61	60	56	59
Starch	316	161	317	319
AID LYS	4.03	4.12	4.99	7.10
AID MET	1.57	1.58	2.11	2.14
AID M+C	3.20	3.24	4.29	4.47
AID THR	2.62	2.68	4.01	4.29
AID TRP	0.98	0.99	1.36	1.53
LYS	5.92	5.98	6.63	8.58

MET	2.07	2.04	2.60	2.55
CYS	2.46	2.46	2.98	3.05
M & C	4.53	4.50	5.59	5.60
THR	4.54	4.50	5.78	5.87
TRP	1.48	1.46	1.84	1.96
ILE	4.37	4.35	6.16	6.13
ARG	7.10	7.04	12.39	9.83
FEN	5.05	5.02	6.88	7.35
HIS	3.16	3.13	4.08	4.09
LEU	7.81	7.76	10.59	11.46
TYR	3.81	3.86	4.93	5.15
VAL	5.84	5.79	7.42	7.49
ALA	5.39	5.27	6.75	7.07
ASP	9.57	9.53	13.82	13.53
GLU	22.04	21.74	32.46	32.66
GLY	6.17	6.26	7.94	7.09
PRO	7.66	7.64	9.01	11.08
SER	5.44	5.46	7.25	7.49
Ca	6.78	6.70	6.78	9.31
Na	2.00	1.98	1.90	2.17
Ferm CHO	235	234	187	145
Ind. CHO	122	110	132	80
Dig. P	2.47	2.41	2.63	3.27
NSP	343	330	300	209
dig. NSP	224	223	173	132
dEB	301	280	302	220
C18:2	7.7	14.6	13.2	15.7
C18:3	1.04	1.22	1.49	1.46
C18:2+3	8.8	15.8	14.7	17.1
C16+18>0	12.59	44.20	20.76	33.83
C16+18.0	2.42	30.26	3.44	16.92

Control (C); fat supplemented (FS); protein supplemented (PS)

D. Nutrient composition (g/kg) of piglet diets

	Creep	Post-weaning	Grower	Finisher
Net energy (MJ)	10.42	10.04	9.70	9.50
Ash	57.25	55.54	55.56	48.79
Crude protein	175.61	175.15	169.98	160.03
Crude fat	75.08	59.45	49.12	45.74
Crude fibre	29.11	39.75	44.26	58.09
ID starch + sugar	402.20	409.71	416.18	409.96
Starch	332.54	359.84	378.95	376.09
AID LYS	11.33	10.26	8.80	7.16
AID MET	4.32	3.54	3.03	2.38
AID M+C	6.67	5.95	5.40	4.68
AID THR	6.57	5.86	5.23	4.28
AID TRP	2.20	1.94	1.64	1.34
Ca	7.96	7.01	7.01	4.84
Dig. P	3.87	3.57	2.85	1.88
NSP	152.33	172.10	179.20	213.70