UNIVERSITY OF SOUTHAMPTON

Maternal factors influencing Metabolic Programming of the Blastocyst within the Uterine Environment

By

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ABSTRACT

Faculty of Health Medicine and Life Sciences

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It has been shown that maternal low protein diet in rodents confined to the pre-implantation period leads to reduced blastocyst lineage sizes and abnormal postnatal growth and physiology. Here, we investigated the effect of maternal diet on the environment experienced by blastocysts. Vaginal plug positive MF1 mice were fed either an 18% protein control diet or an isocaloric 9% low protein diet (LPD) until day 2.5 3.5 or 4.5 of pregnancy whereupon uteri were dissected out and uterine luminal fluid collected by direct sampling. Concentrations of the branch chain amino acids valine, leucine and isoleucine were significantly depleted on day 3.5 and 4.5 of pregnancy while methionine was elevated on day 4.5 in the uterine fluid of LPD mothers. Luminal fluid glucose concentrations were not significantly altered in response to LPD. These results suggest that programming may occur due to depletion of branched-chain amino acids in the uterine luminal fluids causing reduced signaling through the mTOR pathway thus causing embryos to undergo more catabolic rather than anabolic processes.

This study also measured amino acid levels in maternal serum on day 3.5 of pregnancy and observed the overall concentration of uterine fluid amino acids to be almost six times more than that of maternal serum. This suggests that active transport of amino acids is occurring across the uterine epithelium from serum to uterine fluid. Furthermore, The branch chain amino acids leucine, isoleucine and valine were also significantly depleted in maternal serum and of a similar concentration to that found in uterine luminal fluid. Unlike uterine luminal fluid glucose levels were found to be significantly elevated at day 3.5 of pregnancy and, the concentration of glucose in uterine luminal fluid was about half that of serum. Serum insulin levels were significantly depleted at day 3.5 of pregnancy.

Uterine FGF-II levels were increased in response to LPD treatment. This rise in uterine FGF-II levels could possibly aid implantation of the embryo. The observed rise in FGF-II levels correlates with increased activity of angiotensin converting enzyme (ACE) in uterine tissue. This increase in ACE activity is confined to the uterine RAS as circulating ACE activity is not increased. It was possible that any rise in ACE activity was due increased circulating corticosterone. This study failed to find any significant increase in corticosterone levels. Serum estrogen levels were significantly elevated on day 4.5 in

response to LPD treatment. Estrogen may be important for the development of uterine blood vessels and may work in synergy with FGF-II by promoting expression of FGFR1IIIc.

This study has identified significant alterations to direct environment of the peri-implantational embryo and within uterine tissue in response to LPD treatment. These alterations observed in response to LPD treatment may represent the early mediators of metabolic programming. However these early signals may cause Syndrome X phenotypes to become apparent post-reproductive peak if the environment predicted via metabolic programming is incorrect.

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Abbreviations

Full Name	Abbreviation	Full Name	Abbreviation
11β hydroxysteriod dedydrogenase-1	11βHSD-1	IGF receptor	IGFR
Adrenocorticotrophic hormone	ACTH	IGF binding protein	IGFBP
Angiotensin	ANG	Isotope coded affinity tag	ICAT
Angiotensin receptor	AT	Intergrated density value	IDV
Angiotensin converting enzyme	ACE	*In vitro* ferlilization	IVF
Birth weight	BW	Low birth weight	LBW
Bovine serum albumin	BSA	Low density lipoprotein	LDL
CCAAT/enhancer binding protein α	C/EBP	Low dilution	LD
Creb binding protein	CBP/p300	Low protein diet	LPD
Colony stimulating factor 1 receptor	CSF-1R	ligand-binding domain	LBD
Coronary heart disease	CHD	Luteinizing hormone	LH
Corticotrophin-releasing hormone	CRH	Luteotrophic hormone	LTH
DNA-binding domain	DBD	Macrophages	MF
Diastolic blood pressure	DBP	Mean arterial pressure	MAP
Developmental origins of heath and adult disease	DOHAD	Mineralocorticoid	MC
		Neuropeptide-Y	NPY
Fibroblast growth factor	FGF	Non-insulin-dependant diabetes mellitus	NIDDM
Glucagon like peptide	GLP	Normal protein diet	NPD
Glucocorticoid	GC	Predictive adaptive response	PAR
Glucocorticoid response element	GRE	Phosphate buffered saline	PBS
Heart rate	HR	phospholipase β1	PLCβ1
High dilution	HD	Polyacryliamide gel electrophoresis	PAGE
High performance liquid chromatography	HPLC	Protein kinase Cα	PKCα
Hepatocyte nuclear factor 1	HNF-1	Tissue plasminogen activator	PLAT
Horse radish peroxidase	HRP	Proopiomelanocortin	POMC
Hypothalamus-pituitary-adrenal	HPA	Renin angiotensin system	RAS
Insulin like growth factor	IGF	Renin binding protein	RnBP
Insulin response element	IRE	Systolic blood pressure	SBP
Intra uterine growth restriction	IUGR	Vascular endothelial growth factor	VEGF

Significant contributors to this investigation

Individual	Institution	Contribution
Dr Peter Humpherson	Department of Biology, University of York	Provided advice on preparation of samples for HPLC. Conducted HPLC and organised results for final analysis by myself at Southampton University
Dr Clive Osmond	MRC Environmental Epidemiology Unit, Southampton General Hospital	Provided advice on statistics. Specifically conducted data analysis of those results requiring normalisation (Fluid glucose), correlation analysis (serum data) or random effects regression analysis (amino acids in directly collected uterine fluid and all Western blots.
Dr Andrew Sharkey	Department of Pathology, University of Cambridge	Provided advice and training on how to conduct microarray investigations and analyse the results. However the actual investigations were conducted me.
Dr Fred Anthony	Maternal Fetal and Neonatal Physiology Deparment, University of Southampton	Conducted actual experiments for detection of serum estrogens, however. Data analysis was conducted by myself and Dr Clive Osmond. Also provided assistance for analysis of serum progesterone.
Miss Rose Panton	School of Biological Sciences, University of Southampton	Conducted all experiments for detection and semi-quantitative analysis of IGFBP-I and –II in uterine tissue under my supervision.

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Data fata sectus

Ubique quo fas et gloria ducunt

Chapter 1

Introduction

1.1 The developmental origins of health and disease

The linked disorders of diabetes type II, glucose intolerance, insulin resistance, obesity, hypertension, hyperinsulinemia and other cardiovascular disorders are all risk factors of "Syndrome X" (Reaven, 1988). Syndrome X is defined as "a cluster of changes associated with resistance to insulin-mediated glucose uptake [that] comprise a syndrome, which plays an important role in the etiology and clinical course of patients with non-insulin-dependent diabetes, high blood pressure, and coronary heart disease" (Reaven, 1993).

It is during development that mammals acquire the homeostatic mechanisms necessary for survival post parturition. Whilst *in utero*, these mechanisms are suggested to be susceptible to subtle but enduring alterations that can be associated with poor health during adulthood. These alterations are thought to be influenced by adjustments in maternal and fetal metabolism linked to nutritional deficiencies. The developmental origins of health and disease (DOHAD) hypothesis proposes that adult development of syndrome X type risk factors are strongly associated with distinct patterns of disproportionate fetal growth resulting from under nutrition during gestation (Barker 1995). In short, nutritional insult *in utero* may "programme" future disease in a mammal. Recent evidence also suggests that pharmacological insults may also have the capacity to "programme" adult disease.

1.1.1 Epidemiological evidence

Epidemiological investigations form the foundation of DOHAD; these studies were originally conducted in districts where midwifes were required to keep detailed records of postnatal characteristics. In Hertfordshire England, 5,645 men born between 1911-30 were traced and it was shown that normal term males who weighed less than 8.1 kg at one year were three times more likely to die of coronary heart disease (CHD) than those of heavier weight (Barker *et al*, 1989a). In another study using data from the British birth survey 1970 which included 9,921 10 year old males and 3,259 adult males, systolic blood pressure (SBP) was inversely correlated with birth weight (BW) (Barker *et al*, 1989b). This correlation between SBP and BW has been repeated in several

investigations (Leon *et al*, 1996; Rich-Edwards *et al*, 1997; Mogren *et al*, 2001). Other studies have also linked low BW to other Syndrome X risk factors such as, impaired glucose tolerance (Hales *et al*, 1991), non-insulin-dependant diabetes mellitus (NIDDM) (Robinson *et al*, 1992; Lithell *et al*, 1996), increased low density lipoprotein (LDL) cholesterol (Barker *et al*, 1996; Mogren *et al*, 2001; Suzuki *et al*, 2000) and even obesity (Ravelli *et al*, 1999; Leon, *et al*, 1996). All these disorders are associated with CHD. This predisposal to adult disease depends on low BW for gestational age, not on prematurity or on any complication *in utero*.

Whilst the studies discussed above provide evidence of an inverse correlation between BW and CHD/allied disorders, they do not directly prove a causal relationship between maternal nutrition and adult disease. BW is only a proxy for gestational nutrition or indeed any other factor that may programme a fetus for disease later in life. In fact, on the basis of these epidemiological studies it is not clear what the cause of low BW is and its relationship to adult disease. At this point it could be argued that the BW association to CHD might simply be a consequence of genotypes giving rise to connected phenotypes. While maternal nutrition and CHD are difficult to connect in a causal relationship through an apparent correlation between BW and CHD, it is well established that dietary behavior is influenced by a number of interrelated socioeconomic demographics and that identifiable population groups have different dietary behaviors (Barker *et al*, 1990b). Often poorer groups have worse diets than more affluent ones, a review by Jensen and Richter (2001) discusses the fact that calorie intake in the poorest quartile of women in the USA during pregnancy is up to 20 g less than more affluent socioeconomic groups. Perhaps not unsurprisingly, researchers have not rigorously attempted to link parental social class and other maternal lifestyle factors to CHD and BW. This is certainly because epidemiological investigators compiling data from middle-aged people simply do not have this kind of detailed information. However, one study by Koupilová *et al*, (1997) does attempt to resolve this problem by comparison of the BW to SBP relationship and sociodemographic factors at birth. Here it was found that even when results were simultaneously adjusted with sociodemographics at birth, sociodemographics at 50 and behaviour characteristics at age 50, only a slight reduction was seen in the relationship between BW and SBP. In a few cases researchers have

attempted to link CHD and social class as a possible indicator of environmental factors during postnatal life. Barker *et al*, (1989a) found no correlation between BW and social class at death and used this finding to argue that a persistent adverse environment from birth to death did not exist and therefore could not affect incidence of death from CHD. Leon *et al* (1996) found no consistent effects on the associations between BW and CHD when results were adjusted for education level and alcohol consumption. The results in these studies suggest that social class throughout life has no bearing on one's propensity to develop CHD in relation to BW. This conclusion is at odds with a study conducted by Wannamethee *et al*, (1996) where offspring's social class was highly correlated with paternal social class and that a greater risk of developing CHD was linked to manual paternal socioeconomic status.

Placental weight (PW):BW ratio is one of the strongest correlations uncovered during the investigation of DOHAD, a high PW:BW is closely linked to the risk factors for CHD (Barker *et al*, 1990b). The problem here is that linking the PW:BW ratio to maternal nutrition is vastly complicated by other maternal factors such as diabetes, smoking and obesity (Paneth & Susser, 1995), these factors are notoriously difficult to isolate in epidemiological investigations. Maternal smoking, for instance, has been shown to decrease BW and increase offspring's SBP by, on average, 1.54 mm Hg at age 18 compared with offspring from mothers who did not smoke (Williams & Poulton, 1999). Matters are further complicated by counter arguments found in the literature, and again maternal smoking is a case in point. Williams and Poulton (1999) themselves propose that smoking could indirectly affect BW though alteration of nutritional pathways, also smoking itself is linked to lower socioeconomic status (Logan & Spencer, 1996); thus, smoking may simply be an indicator of poorer nutritional status.

To negate many of these confounding factors such as smoking, diabetes, and socioeconomic class, the relationship between twins, CHD and BW has been investigated by several groups. Williams & Poulton (1999) studied twins BW relative to SBP and found that twins appeared to have lower blood pressure at ages 9 and 18 years than singletons ($P<0.05$). It can be logically argued that nutrition *in utero* is impaired where mothers carry more than one fetus, and here the investigators suggest that their

findings challenge the DOHAD hypothesis as twins have a lower BW to singletons. It should be remembered that this study concentrates on a relatively young sample group compared to other epidemiological investigations where participants have been at least 50+ years. Also, this study did not compare intra-sibling SBP's and BW, it only compared average BW and SBP in all twins. To complicate matters further, the lower BW of twins is not simply a matter of global competition for nutrients, type of twins and proximity of implantation have their own effects. Research by Loos *et al*, (2001) suggests that all types of twins (mono- or dizygotic) which have a peripheral insertion of umbilical cord weigh about 150 g less compared to those with a central insertion, also dizygotic twins have a higher rate of central cord insertion. Monozygotic dichorionic twins with fused placentas and peripheral cord insertion weigh about 300 g less than infants with separate placentas and central cord insertion, although in dizygotic twins placental fusion did not affect BW. Similarly to Williams and Poulton, (1999), a study conducted by Zhang *et al* (2001) failed to find any significant correlation between BW and blood pressure at age 7 years even between sibling twins, although the sample age again is very young. In another study, Poulter *et al*, (1999) looked at differences between twins blood pressure related to BW and found that there was a negative correlation between difference in BW and SBP or diastolic blood pressure (DBP) in monozygotic twins (this was not significant, possibly because the sample size was low). To some extent, this finding does lend itself to a separation of genetics and phenotype, suggesting a causal relationship between BW and blood pressure. In a different strata of this same study, a significant negative correlation was found between BW and blood pressure in all twins (monozygotic and dizygotic) who were not receiving hypertensive treatment ($P<0.05$). The major difference with this study is that it concentrated on adults (mean age 54) and not on children and adolescents and, as mentioned previously, hypertension and related disorders often only present themselves after the reproductive peak has passed. To a large extent, the intra-sibling comparisons by Zhang *et al*, (2001) and Poulter *et al*, (1999) negate the shortfalls of the Williams and Poulton (1999) study by quite logically using BW to proxy for fetal nutrition of individual twins, as suggested by Loos *et al*, (2001) work.

Some of the most compelling epidemiological evidence that maternal nutrition is the determinant connecting low BW and adult disease comes from the studies of areas affected by famine. Data from famine stricken areas allow researchers to produce results as close to experimental ones as possible. The most studied single event in relation to metabolic programming of Syndrome X is the Dutch Hunger Winter 1944-1945. Because precise records were kept concerning the amount of rations available and the precise timing of the nutritional insult, this event provides an almost unique model for the investigation of fetal programming of Syndrome X. Using this as a model, it was shown that people exposed to famine *in utero* had reduced glucose tolerance and that this was related to low BW (Ravelli *et al*, 1998). Additionally, those exposed early in gestation appeared to have a greater chance of developing CHD (Ravelli *et al*, 1999; Roseboom 2000).

1.1.2 Animal models

Animal studies have also been an important tool in establishing the link between maternal nutrition and adult disease as they negate the disadvantages of the lack of control of confounding variables inherent in epidemiological investigations. Rats exposed to low-protein diets (LPD) during perinatal development have, to a large extent, validated the theoretical link between maternal diet and adult disease. Langley and Jackson (1994) provided dams with diets containing a range of protein levels (18% [control], 12%, 9% & 6% by weight) for a 14 day period prior to mating and then throughout pregnancy; during lactation the mothers were transferred to a 20% protein diet. BW of the 6% diet was significantly lower than the other diets and at 9 weeks all LPD offspring were found to have an increased SBP over the controls (15-22 mm Hg). Similar results have been obtained from other studies into this phenomenon. Langley-Evans *et al*, (1996) showed that SBP was significantly higher (29 mm Hg) in the progeny of dams fed 9% than 18% protein diets for 14 days prior to mating until the start of lactation. Vehaskari *et al*, (2001) used dams fed on a 20% or 6% protein diet from gestational day 12 until term; at 8 weeks of age, the LPD offspring's SBP was elevated above the controls (20-25 mm Hg). Sayer *et al* (2001) found an increased SBP (15 mm Hg) in rats fed a LPD of 9% compared to 18% controls, also the female rats life span was reduced by 11%. Kwong *et al*, (2000) subjected rats to LPD but only for the pre-

implantation development period (i.e. fertilisation to blastocyst) yet this still replicated the previous observations for SBP and found that the female pups subjected to LPD *in utero* weighed significantly less than the control females up to 7 weeks of age.

Tonkiss *et al*, (1998) implanted radiotelemetric pressure transducers into the descending aorta of rats subjected to LPD *in utero*. This is superior to the tail cuff method previously used in most experiments to measure SBP because tail cuff requires that the animal be restrained at an elevated temperature for blood to flow to the tail; both are potential forms of stress. On the other hand, radiotelemeters were able to measure SBP, DBP, mean arterial pressure (MAP) and heart rate (HR). Dams were given either a 25% or 6% LPD for 5 weeks prior to mating through to parturition, pups were then fostered to well fed dams to limit the nutritional insult only to prenatal development; measurements were taken at 96 days of age. During the waking but not sleeping periods, the LPD offspring exhibited a small but significant elevation in DBP and HR compared to controls (4 mm Hg at most). Here, the small but significant differences in DBP contrast with previous findings of larger differences (>20 mm Hg), which may be due to the influence of stress. Nevertheless, prenatal nutritional insult did cause enduring differences in the regulation of blood pressure. In a second experiment, the rats were exposed to an olfactory stressor (ammonia). LPD rats showed a greater increase in SBP, DBP and MAP compared to controls during the first exposure to the stressor. At the second presentation of the stressor, readings for SBP, DPB and MAP converged, then, in the final presentation of the stressor, the SBP results were reversed. In short, the control rats exhibited a positive correlation between number of exposures and SBP, DPB and MAP while the LPD rats showed an inverse relationship. This relationship may be indicative of fundamental differences in the blood pressure regulatory systems of the two nutritional groups.

Other animal models have also been used to investigate the mechanisms of DOHAD. Persson and Jansson (1992) used a guinea pig model to investigate the effect of unilateral uterine artery ligation to create intrauterine growth retardation in 50% of the offspring. BW of artery-ligated littermates was reduced by over 20% compared to their normal littermates. At 4 months of age, HR was increased and intra-pair BW

differentials and intra-pair adult blood pressure differentials displayed a significant inverse correlation. In the sheep model, growth was shown to be slowed in late gestation fetuses from ewes undernourished for 10 days. This growth retardation began within three days of initiation nutritional insult; fetal growth resumed upon maternal re-feeding (Harding & Johnston, 1995). This shows that fetal growth patterns can be disrupted even with only a relatively small period of nutritional insult. Also, surgical limitation of placentome attachment sites in ewes have shown that fetal growth is differentially restricted with less restriction to the brain and more to the spleen and thymus; however, there are histological changes that occur in brain that are consistent with altered function (Rees *et al*, 1988).

Whilst animal models are useful for experimental control, they are limited by inter-species physiological differences. For instance, the sheep, a ruminant, derives its circulating blood glucose endogenously. Gluconeogenic substances such as short chain-fatty acids and amino acids are produced in the maternal liver as opposed to directly from the products of digestion. Reduced dietary intake leads to a large decrease in production of their substrates in the rumen; this can result in an accelerated drop of up to 40% in a ewe's blood glucose level overnight. While in fasting, pregnant women have been shown to lead to expedited nutrient usage; overnight fasting only causes approximately a 10% drop in blood glucose and complete starvation for up to 4 days is needed to elicit a 30% drop (Harding, 2001). Understanding of the differences between experimental models is therefore essential for contrasting human disease and induced animal disorders.

1.2 Metabolic programming

As already discussed, factors other than nutrition i.e. pharmacological factors such as smoking, can programme adult physiological alterations *in utero*. Thus, in this thesis the term "metabolic programming" is used to describe the fundamental biological processes that are believed to associate adult health to the nutritional experiences *in utero*. Metabolic programming in this context relates to an adaptive response to

particular maternal nutritional circumstances (Waterland & Garza 1998). Such adaptive responses are distinguished by three factors: 1) a critical ontogenic window where the incipient organism is vulnerable to a particular maternal nutritional insult (see **1.4 Critical periods**). 2) An enduring response to the nutritional environment *in utero*, continuing through to or manifesting in adulthood. 3) The existence of precise and measurable variables in response to the nutritional insult.

1.3 Evolution of metabolic programming

It is important to understand that while nutritional insult during embryonic growth is linked to adult diseases, these afflictions often only present themselves in chronic form after an organism's sexual peak has passed. As a result, reproductive fitness is not necessarily adversely affected. In terms of human evolution, when *Homo sapiens* appeared two hundred thousand years ago, life expectancy was not more than 40 years at best and reproductive peak was reached well before this (Lewin, 1998). Thus, the problems of Syndrome X which appear in our modern "extended life" would not have presented huge issues to our ancestors. In evolutionary terms, adaptations to insufficient nutrient supply *in utero* are a huge advantage for the fetus and mother.

Perhaps the simplest evolutionary explanation is that proposed be Metcalfe and Monaghan (2001) who summed up their concept of fetal programming as, "compensation for a bad start: grow now, pay later". In this concept of DOHAD, metabolic programming provides a mechanism whereby pregnancy can be maintained throughout periods of poor nutrition with the health consequences only apparent later in life. In evolutionary terms, these adaptations to sub-optimal nutrient supply provide a fitness advantage for both mother and fetus; for the mother as she has already invested a huge amount of resources in selecting a mate and conceiving/carrying the fetus, and for the fetus as it can survive to reproduce itself.

However, there is increasing speculation that there is more to the evolutionary and adaptive mechanisms of metabolic programming than Metcalfe and Monaghan's

(2001) definition allows. Thus far, I have described the programming of Syndrome X *in utero* as being a result of maternal and fetal adaptations to compromised nutrient supply necessary for survival. In fact the DOHAD hypothesis is potentially a far more complex evolutionary system than already eluded to. First proposed over 10 years ago (Hales & Barker, 1992), the Thrifty Phenotype Hypothesis proposes that the adaptations to maternal under nutrition by the fetus give rise to an energy-sparing phenotype; this effect is known as a "predictive adaptive response" (PAR) (Gluckman and Hanson, 2004). While this "thrifty" phenotype may be ideally suited to a sparse postnatal nutrient environment, if the offspring of a malnourished pregnancy develop postnatally in a nutrient-rich environment and do not undertake regular exercise, they could be at a higher risk of developing Syndrome X and its co-morbidities (Fig.1.3). It is proposed by Hales & Barker (2001) that suboptimal nutrient supply during pregnancy leads to a PAR in the offspring's glucose-insulin metabolism. An individual who had poor functional capacity for insulin secretion as a result of a PAR to prenatal under nutrition would not suffer the symptoms of Syndrome X if they continued to be poorly nourished and remained lean. Conversely, if this individual had an increased caloric intake and energy expenditure that did not match their prenatal PAR would lead this individual to become obese resulting in glucose intolerance and a predisposition to the symptoms of Syndrome X.

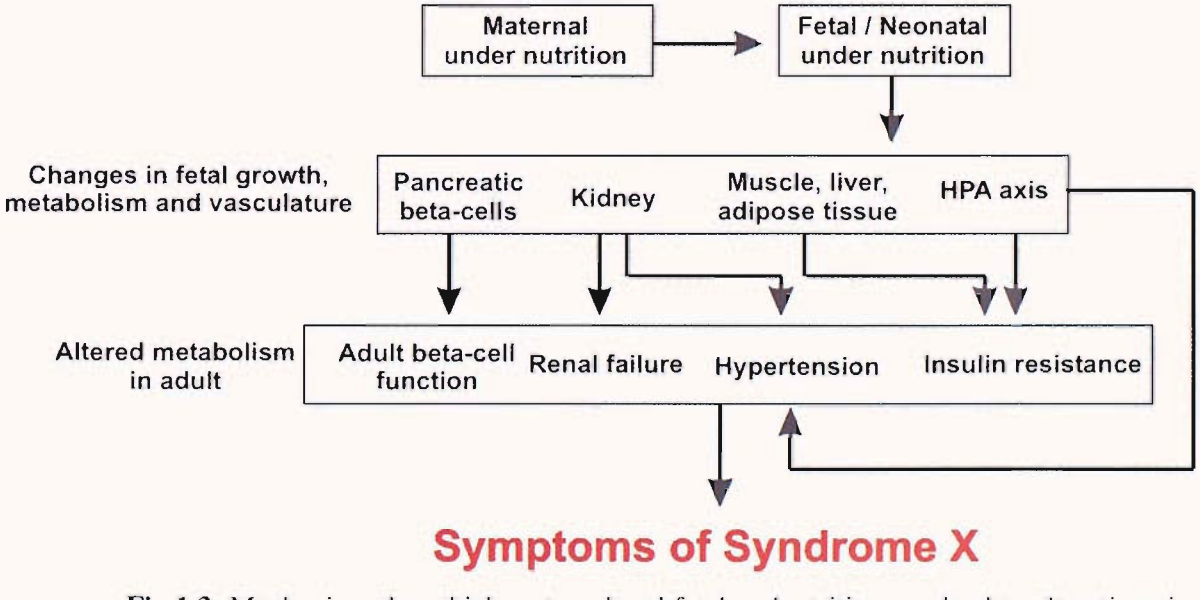

Fig.1.3: Mechanisms by which maternal and fetal malnutrition can lead to alterations in development *in utero*, energy metabolism post-parturition and symptoms of Syndrome X. (Adapted from Hales and Barker (2001)

The Thrifty Phenotype Hypothesis is distinct from the Thrifty Genotype Hypothesis, proposed by Neel (1962) to explain the high prevalence of diabetes type II in Native American populations. Whereas the thrifty phenotype is suggested to derive from nutritional state *in utero*, the thrifty genotype is proposed to originate from a "syndrome of impaired genetic homeostasis" (Neel *et al*, 1998). In DOHAD, the Thrifty Phenotype Hypothesis proposes that Syndrome X is brought about by environmental factors influencing developmental plasticity (Hales & Barker, 2001) while the Thrifty Genotype Hypothesis proposes that Syndrome X is brought about entirely by genetic factors (Neel, 1982). In short, the Thrifty Genotype Hypothesis proposes that the entire human population was evolved to subsist on a poor diet and deviation from this to a more "Westernised" diet results in the development of syndrome X.

In most economically developing countries, unprecedented social and economic change has swiftly affected dietary patterns and physical activity, which in turn has contributed to the current rise in Syndrome X. The prevalence of obesity in adults is 10-25% in most countries of Western Europe, 20%-25% in some countries in the Americas, and even higher elsewhere, over 50% in some island nations of the Western Pacific and native Americans tribes (WHO, 2002). This higher incidence of Syndrome X related to obesity has been linked to the rapid westernisation of diets and a decrease in physical activity due to mechanisation (Cockram 2000; Gill, 2001; Mohan *et al*, 2003). What is more, the increase in the occurrence of Syndrome X is not only distinct between different ethnic populations globally but also between those cohabiting in the same geographic region. Indeed, New Zealanders of Polynesian origin have a higher prevalence to obesity and type 2 diabetes mellitus than those of European origin (Rush *et al*, 2002). As already mentioned, there is a lower incidence of Syndrome X in European and even North American populations than in Pacific Islanders. Thus, it seems that "Westernisation" of diets alone is not enough to predispose people to a greater risk of developing Syndrome X.

The Thrifty Genotype Hypothesis is intriguing, and at first glance appears to provide an alternate explanation to the thrifty phenotype for the increase in Syndrome X

in ethnically diverse populations. However, as a stand-alone explanation for the prevalence of Syndrome X in worldwide populations, the Thrifty Genotype Hypothesis does have some serious shortfalls compared to its contemporary, the Thrifty Phenotype Hypothesis. Firstly, there are only weak arguments to support a link between NIDDM and genetics (Hales *et al*, 1997). There is no precedent where a genetic disease affects almost half of a number of geographically distinct populations, for example the Native Americans and Pacific Islanders (Ozanne & Hales 1998). Moreover, the thrifty genotype hypotheses should predict that one of the most Westernised populations of all, Europeans, should have a high rate of syndrome X. But in fact, as already pointed out, Europeans have comparatively low rate of Syndrome X; the Thrifty Phenotype Hypothesis actually predicts this because this population has had a comparatively stable diet for the last 100 years or so. Having said this, there is limited evidence to suggest that genetic thriftiness does exist for some historically limited dietary substances, for example cholesterol and salt (Broadhurst, 1997). Recently the population of Japan has had a tendency to consume more fat than in the past. In Japanese men, these changes have been associated with an elevated body mass index and increasing instance of Syndrome X (Shiwaku *et al*). There is circumstantial evidence that this rise in Syndrome X may be due to the alterations in diet interacting with a higher prevalence of polymorphisms for at least three genes that code for proteins thought to have roles in lipid and glucose metabolism: the beta 3-adrenergic receptor, the peroxisome proliferator-activated receptor gamma, and calpain-10 (Kawamori, 2002). Ultimately the Thrifty Genotype Hypothesis provides an interesting concept that may work in synergy with the Thrifty Phenotype Hypothesis to explain why Syndrome X is especially acute in certain ethnic populations, but in itself does not provide sufficient evidence to explain this phenomenon in such geographically diverse populations.

When contrasted with the Dutch Famine, another Second World War famine, the siege of Leningrad provides supportive evidence for both metabolic programming and the Thrifty Phenotype Hypothesis. During the siege of Leningrad the German army blockaded the city, now known as St Petersburg, between September 1941 and January 1944 preventing supplies from reaching the city for over 870 days. When the siege was in full force the bread ration was 250 g for workers and 125 g for others (Pavlov, 1965).

The average daily ration for most of the citizens of Leningrad during this time therefore provided around 300 calories and contained virtually no protein. Average male and female BW fell by 18% and 16% respectively (Antanov, 1947). Distinct to the situation in the Dutch hunger where epidemiological investigations have found strong associations between low birth weight (LBW), reduced glucose tolerance (Ravelli *et al*, 1998), chance of developing CHD (Ravelli *et al*, 1999; Roseboom 2000) and obstructive airways disease (Lopuhaä *et al*, 2000), the studies on the Leningrad cohort have failed to find any correlation between LBW and the symptoms of Syndrome X (Stanner *et al*, 1997; Stanner & Yudkin, 2001). At most, subjects exposed to famine during the siege showed evidence of mild endothelial dysfunction and a stronger influence of obesity on blood pressure (Stanner *et al*, 1997). Unlike the situation in the Dutch Hunger Winter where the famine was short, six months, and supply lines were re-established quickly after the siege, the situation in Leningrad was very different. At Leningrad, the siege lasted nearly two and a half years and, in contrast to the Dutch hunger winter, was inflicted on an already badly nourished population who remained malnourished for some time after the siege was lifted. As a result the Leningrad population was not subjected to the same rapid switches in diet that the Dutch population were. It can be argued that the Leningrad population was either of a thrifty phenotype PAR already or adapted one during the siege and that the environment post siege was more favorable to this. Consequently, they would not have been subjected to the same stress of being malnourished *in utero* and well nourished post parturition and, as a result, would not be predisposed to symptoms of Syndrome X. In short, their thrifty phenotype PAR would have been correctly forecast for their dietary environment.

1.4 Critical Periods

So far, we have established that nutritional insult during development can lead to an increased risk of developing Syndrome X in later life. We have also established a model whereby this can happen, the Thrifty Phenotype Hypothesis. This evolutionary mechanism, to some extent, attempts to predict postnatal environment and when it fails can exacerbate the symptoms of Syndrome X in adult life. To this point we have

described how developmental nutritional status can be linked to adult disease. However, it is also becoming clear that the timing and length of the nutritional insult during development can also have profound effects on the resulting Syndrome X risk factors exhibited.

1.4.1 Critical periods defined

During mammalian development there are four broad phases that are associated with the growth of specific cell types, organs, or even the entire organism. During the pre-implantation period, phase 1 of development and days 1-4 in mice, the single cell zygote divides to form a blastocyst containing distinct cell populations necessary for implantation, the trophectoderm and inner cell mass. After implantation during phase two, cells further differentiate as the embryo undergoes gastrulation, segmentation, somitogenesis and neuralation. The third phase, days 10-14 in mice, is associated with the development of internal organs and the growth of limbs. The final phase, days 14-19 in mice is characterised by bone formation, accelerated growth and maturation before birth. These events are just some of those essential to the development of a healthy mammal.

Due to the extreme consequences that disturbances in cellular differentiation can have on growth, the times where specific developmental events occur are known as "critical periods". A critical period can be as broad as the entirety of prenatal development (Cameron & Demerath, 2002) or as specific as the duration of embryo cavitation during blastocyst development depending on the context (Fig.1.4.1). For the purposes of this thesis a critical period must fulfill three criteria: 1) be a phase of developmental transition where the organism is vulnerable to an environmental insult. 2) affect developmental timing or structure in some way. 3) lead to permanent physiological change. It should be noted that this definition can, in addition to development *in utero*, include such stages as spermatogenesis/folliculargenesis and even post parturition events such as puberty.

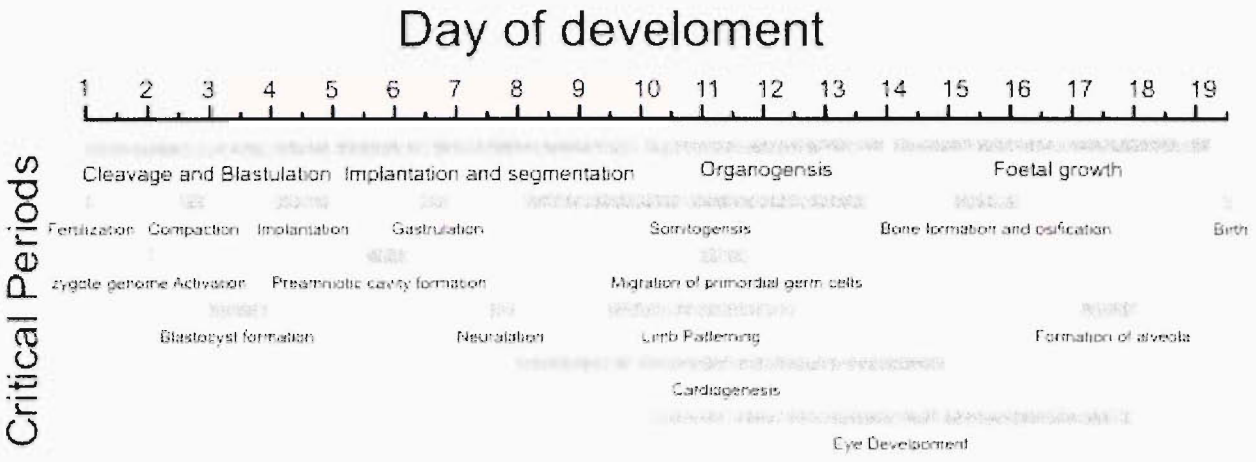

Fig.1.4.1: Diagram showing the chronology of a typical mouse pregnancy and examples of where various critical periods lie within it. Note that the critical periods do not necessarily lead on from one to another, rather they overlap and augment each other.

1.4.2 Critical periods and Syndrome X

Several authors have suggested that various patterns of disproportionate fetal growth are markers for distinct patterns of risk factors for CHD and Syndrome X. For example, neonates with a high asymmetry index (head size relative to trunk) had disorders in cholesterol metabolism (Barker *et al*, 1993), while neonates with a low ponderal index (weight relative to height) are more likely to develop insulin resistance post reproductive peak (Phillips *et al*, 1994; Litlell *et al*, 1996). In both of these cases the adult disease phenotypes were suggested to be related to reduced hepatic metabolism and reduced liver size. Stein *et al* (1975), suggested that infants with a low ponderal index were subjected to famine late in gestation and that this phenotype was exhibited because accumulation of adipose tissue and growth is greatest in the third trimester. When this finding is contrasted with the work of Phillips *et al* (1994) and Litlell *et al* (1996) it appears that a correlation could exist between timing of dietary restriction pre-parturition and adult disease phenotype. It is this concept that gives rise to the notion that dietary restriction during differing pre-parturition critical periods can programme specific phenotypes of Syndrome X.

Models based upon the Dutch Hunger Winter have also identified distinct risk factor phenotypes for Syndrome X that depend on the critical periods during which nutritional insult took place. Those exposed to the Dutch Hunger winter (1944-45) in early gestation demonstrated metabolic programming of higher fibrinogen

concentrations, lower plasma factor VII concentration (Roseboom *et al*, 2001a) and a greater atherogenic lipid profile (Roseboom *et al*, 2001b) than those exposed in later gestation. The occurrence of obstructive airways disease was shown to increase in people exposed to the famine *in utero* during early or mid gestation (Lopuhaä *et al*, 2000). Those exposed early in gestation also had a greater chance of developing CHD than those exposed later (Ravelli *et al*, 1999; Roseboom 2000). On the face of it our earlier supposition, that ponderal index is a marker of third trimester malnutrition and a risk marker for CHD in adulthood is at odds with the findings of Ravelli *et al* (1999). However, it is possible that those dietary disruptions of early critical periods or late critical periods can both have an influence on adult risk factors for CHD and that the ponderal index phenotype is only apparent when late critical periods are subject to dietary restriction. While studies conducted on offspring of the Leningrad siege showed no apparent correlation between Syndrome X and malnutrition *in utero*, associations were found to exist between malnutrition during puberty and CHD. Sparén *et al* (2003) found that men who experienced the Leningrad siege between ages 9-15 years of age had higher SBP and increased mortality rates from ischaemic heart disease and stroke. This research is supportive of our supposition that critical periods are not restricted to pre-parturition development and that metabolic programming can also occur in adolescent life.

1.4.3 The Thrifty Phenotype and Critical periods

Critical periods represent ontogenic windows where an organism can undergo permanent physiological alterations. Critical periods are numerous and span the entirety of pre-parturition and at least some of post-parturition development, thus there is huge scope for these ontogenic "choices" to be made in relation to environmental stimuli. Against the backdrop of the Thrifty Phenotype Hypothesis, critical periods provide the developmental plasticity whereby an organism can prepare for its postnatal or adult environment via metabolic programming (Fig.1.4.3). As already described, developmental decisions made during critical periods endure for the lifetime of the organism. It is when these judgements ultimately turn out to forecast incorrectly postnatal or adult nutritional environment that the organism has little capacity to adapt its physiology to its actual surroundings.

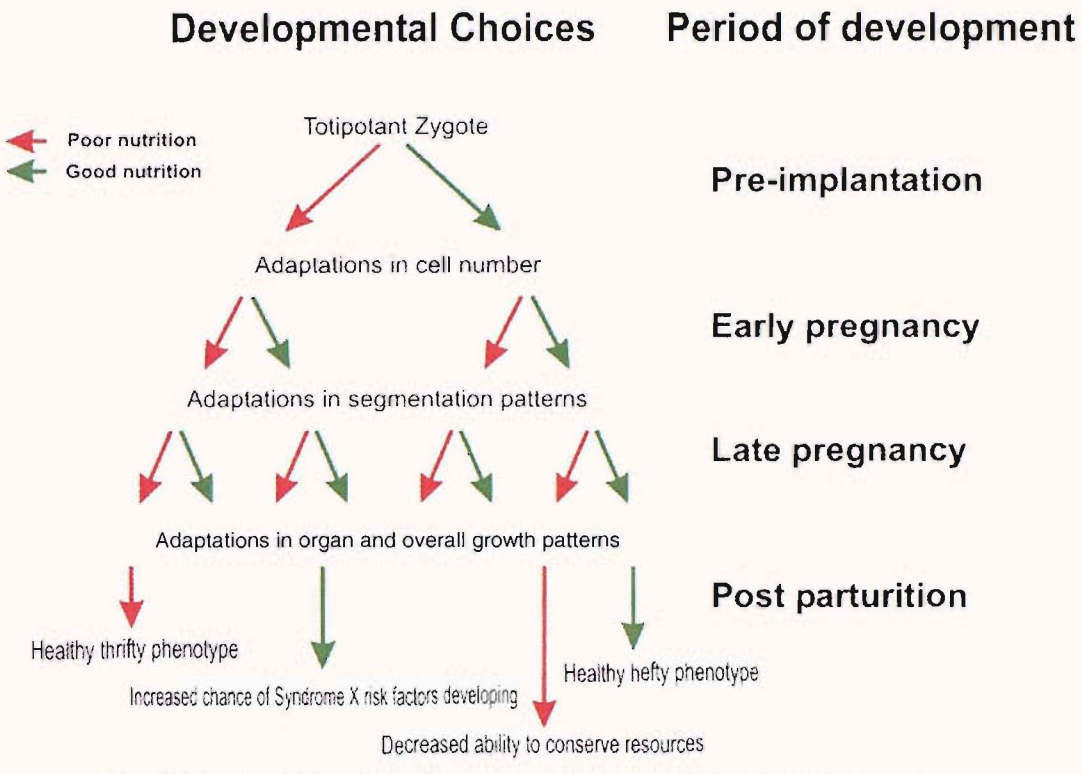

Fig.1.4.3: A schematic of some of the ontogenic metabolic programming windows available to an organism and how they may relate to post reproductive health. During each period of growth the organism is faced with a developmental "choice" that is dependant on current nutritional status; once made this "choice" is permanent. When the nutritional environment is consistently poor or consistently good then the phenotype derived from these developmental "choices" is ideally suited to its environment results in a healthy life. However, if the developmental nutritional environment is "confused" or is not predictive of postnatal environment then the organism will have had to make inappropriate "decisions" that are now fixed. It is when developmental plasticity is driven in these inappropriate directions that environmental maladaptation occurs and post-reproductive heath problems related to Syndrome X become a problem. For simplicity the phases of developmental plasticity, i.e. the critical periods, cover broad areas of growth in this example.

1.5 Reproductive physiology

Before proceeding to discuss specific mechanisms potentially involved in metabolic programming it is necessary to consider key aspects of mammalian reproductive physiology. In this section I will concentrate on the mouse, as it is our chosen animal model, though we will also consider data from other pertinent systems.

1.5.1 Structure of the oviduct and uterus

The oviduct (or Fallopian tube), is a narrow tube approximately 1.8 cm in length, it links the peri-ovarian space to the uterine horn and is suspended from the dorsal body wall by the mesotubarium (Rugh, 1991). It can be divided into three sections: the ampulla close to the ovarian bursa; the tightly coiled isthmus; and the intramural portion within the uterine wall. The ampulla opens next to the ovary into the periovarian space through a ciliated and fimbriated infundibulum. The ovary itself is suspended by ligament from the dorsal body wall. The coiled oviduct enters and projects into the uterus eccentrically at the dorsolateral wall slightly posterior to its rounded posterior end. This intramural section terminates in a protrusion known as the colliculus tubarius. The entire oviduct is covered in smooth muscle fibres, which cause peristaltic contractions that encourage movement of the ova towards the uterus. The ampulla itself is lined with simple, low columnar, non-ciliated cells, while the isthmus is lined with pseudostratified and low columnar epithelium and occasional ciliated cells, the intramural section is lined with simple, columnar epithelium.

In the mouse, the uterus (Fig.1.5.1) is a hollow Y shaped structure consisting of two lateral uterine horns known as cornua, which converge into a single median body, the corpus. The corpus lacks the tissue elements for implantation and is lined with cuboidal cells. The cornua progress anteromedially from the corpus dorsal to the urinary bladder and onto the oviducts. At the base of the corpus is the cervix, which is lined with stratified, squamous epithelium. This projects and opens into the vagina along the doso-ventral axis but not laterally where the lumen of the vagina extends into fornices. The endometrium, or mucosal layer in non-pregnant animals, is comprised of the lining epithelium, the lamina propria, and the uterine glands. The lining of the uterus is folded and lined with simple, columnar epithelium and many spiral, tubular, uterine glands. The myometrium is peripheral to the endometrium and consists of compact, circular muscles, an enclosing layer of loose connective tissue and finally the longitudinal muscles. The cornua are supported by the mesometria; these consist of broad ligaments through which blood vessels, lymph vessels and nerves pass at regular intervals.

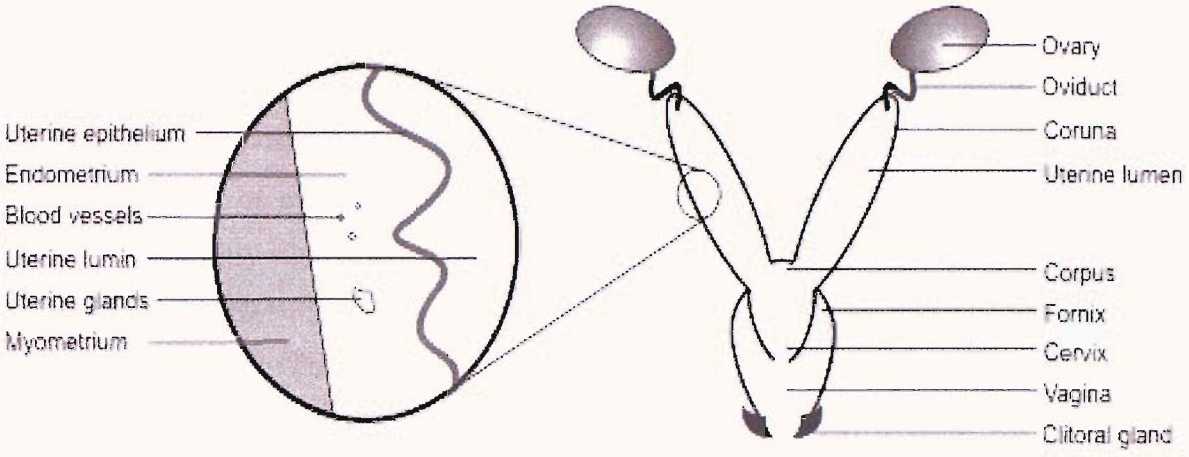

Fig.1.5.1: A basic schematic of the female mouse's genital system including an overall view of the structure and an exploded view of the structure of a uterine horn.

1.5.2 Oestrus cycle ovulation and mating

The vagina of the female mouse opens at about day 35 (5 weeks) and the first oestrus follows just after. Such weanlings are reluctant to accept a male for mating, and even if mating does occur it is unlikely to result in a viable pregnancy. Female mice usually reach reproductive maturity and can sustain a successful pregnancy from about 6 weeks of age.

The oestrus cycle of the rat and mouse are basically very similar to that of farm animals, except they show a remarkable feature in that the length of the oestrus cycle varies depending on whether the female mates or not. On the morning of the pro-oestrus part of the oestrus cycle there is a peak in oestradiol levels secreted from the Graafian follicle. This in turn causes a surge in luteinising hormone (LH) release from the anterior pituitary during the afternoon and leads to ovulation in the early hours the next day. If mating does not occur, the corpus luteum degenerates very quickly; as a result, progesterone secretion is kept to a minimum and the luteal phase of the ovarian cycle will only be 2-3 days long. If a female mouse is mated with a vasectomised male then her luteal phase will extend to 11-12 days. The explanation for this feature of mouse reproduction lies within the mechanical stimulus to the cervix provided by the penis; the presence of this stimulation is relayed via the central nervous system and causes the release of LH from the pituitary. LH extends the life of the corpus luteum, and progesterone is secreted for longer delaying the onset of menstrus. If mating was

successful, any fertilised oocytes now have the chance to develop and implant. This effect allows rats and mice to increase reproductive efficiency significantly because, without this abbreviation of the oestrus cycle, they would only be fertile once every 13-14 days instead of every 4-5 days. As their pregnancy is only about 20 days, this represents a highly significant economy. Additionally it should be noted that there is a secondary estrogen surge which occurs just before implantation on day 4 of pregnancy in rodents (Fig. 1.5.2). This estrogen surge was first accurately measured in rats by Shaikh and Abraham (1969) and has since been demonstrated to be critical for blastocyst implantation (Psychoyos *et al*, 1973a; Paria *et al*, 1993; Ma *et al* 2003). The oestrous cycle is summarized in Table.1.5.2 categorised by stage, characteristics and physiological purpose; hormonal changes are graphically shown in Fig.1.5.2.

Table 1.5.2: Stages of the oestrous cycle of the mouse. For each stage duration, physiological changes and overall purpose are described. Adapted from Rugh (1991).

Stage	Duration	Characteristics
Pro-oestrus	1-1.5 days	Anabolic, active growth in genital tract. Uterus is swollen and vaginal orifice is open. Largely nucleated and some cornified epithelium in vaginal smears.
Oestrus	1-3 days	Also known as on heat. Anabolic, active growth in genital tract. Swollen congested vulva and open vaginal orifice. No leukocytes but nucleated and cornified epithelium in vaginal smear. Ovulation occurs early in this stage.
Metestrus 1	1-5 days	Catabolic, degenerative changes in genital tract. Exclusively clumped cornified epithelium in vaginal smear.
Metestrus 2		Catabolic, degenerative changes in genital tract. Nucleated and cornified epithelium and leukocytes in vaginal smear.
Diestrus	2-4 days	Quiescent period of slow growth. Nucleated epithelium, leukocytes and some mucus in vaginal smear.

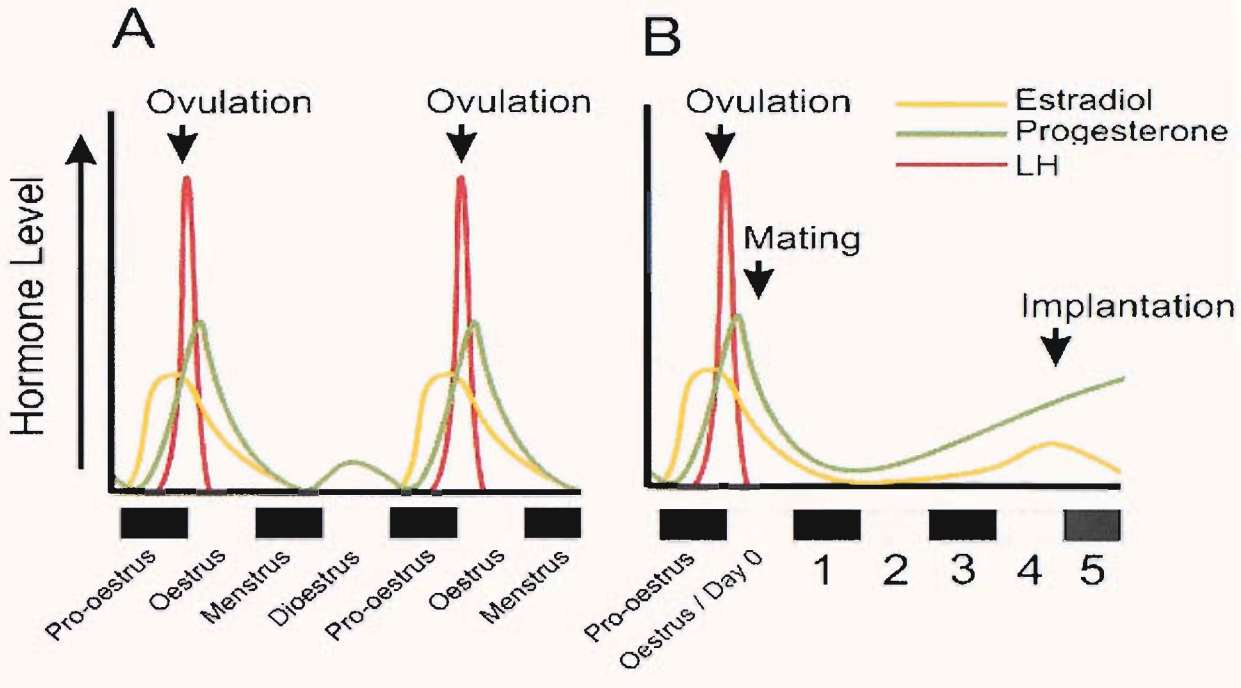

Fig.1.5.2: A) Hormone changes during the oestrous cycle of the mouse. When mating does not occur progesterone and oestrous levels diminish and the oestrous part of the cycle is cut to about one day. B) When mating occurs progesterone levels are maintained because of LH secretion from the anterior pituitary, additionally, there is a secondary surge of estadiol just prior to implantation.

Section 1.5.3 Embryonic Development

In mammals, ovulation occurs about 2 hours prior to fertilisation of the oocyte by a single sperm; this occurs in the ampulla region of the oviduct and denotes the start of embryonic development (Fig. 1.5.3). In mice, fertilisation occurs at approximately midnight and, in this study, is considered to be time point 0). When a sperm penetrates the zona pellucida of an oocyte, the posterior of the sperm's head fuses with the oocyte membrane. This elicits several processes, the most striking being the generation of Ca^{2+} wave oscillations which originate at the cortex and are propagated through the cytoplasm (Deguchi *et al*, 2000; reviewed by Dumollard *et al*, 2002).

There is a general consensus that, in the mammal, the Ca^{2+} wave oscillations are induced by the sperm depositing a factor into the oocyte during fertilisation (reviewed by Carroll, 2001). It would appear that this activating stimulus is either something that induces the egg to produce phospholipase C (PLC) or indeed introduction of PLC to the oocyte by the sperm itself (Jones, 1998). It has been recently shown that the latter

explanation is most probably correct (Larman *et al*, 2004) indeed PLC is present in mammalian sperm extracts and has been shown to activate Ca^{2+} release in sea urchin homogenates (Jones *et al*, 1998). In short, Carroll (2001) suggests that the Ca^{2+} oscillations are initiated by the sperm depositing a finite bolus of PLC into the egg. Ca^{2+} release in this system is driven by positive feedback by Ca^{2+} on Ca^{2+} through 1,4,5,-triphosphate receptors (IP3) and on the sperm derived PLC. In mammals the Ca^{2+} waves oscillations occur approximately once every 10-20 min for about 2-4 hrs post fertilisation (reviewed by Stricker, 1999). These Ca^{2+} waves trigger the release of cortical granules located beneath the oocytes plasma membrane initiating the "zona reaction". This involves cross-linking of zona glycoproteins including ZP3 such that it no longer binds sperm, thus preventing polyspermy. In mammals the primary fertilisation wave is followed by other secondary Ca^{2+} waves at intervals of 20-30 min lasting for up to 4 hrs, well after the completion of meiosis at the time of pronuclei formation (reviewed by Dumollard *et al*, 2002). Fertilisation also activates the second meiotic division and expulsion of the second polar body.

At about 24 hrs the first cleavage occurs; in the mouse this event is associated with activation of the embryonic genome (Schultz, 1993) and represents the conversion of the highly differentiated oocyte into totipotent blastomeres (Kaňka, 2003). In cows, sheep and humans, genome activation occurs between the 4 and 8 cell stages (Telford *et al*, 1990). Up until zygotic genome activation, the embryo almost exclusively relies on RNA and protein synthesised during oogenesis. During genome activation, three distinct processes, required for continued development, are accomplished (reviewed by Schultz, 2003). 1) Destruction of oocyte-specific transcripts. 2) Replacement of transcripts that are common to the early embryo. 3) Generation of transcripts novel to the developing embryo.

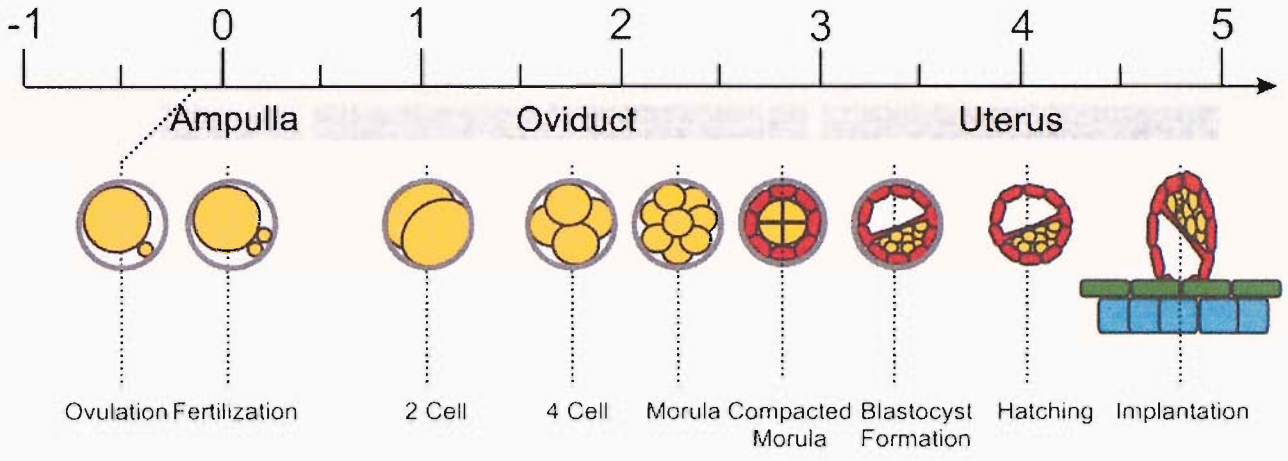

Fig. 1.5.3: Summary of preimplantation development of the mouse with time line, embryo morphology and embryo location at different stages.

Over the next 36 hrs the embryo further divides from 2 cells to form a 4-cell then 8-cell embryo (Fig. 1.5.3). Indeed, up to the 4-cell stage, single blastomeres have been shown to be able to develop to full grown mice, demonstrating that up until this stage the blastomeres are equipotent. However, by the early 8-cell stage the blastomeres become unable to develop individually (Kelly *et al*, 1977), demonstrating a loss of totipotency. Nevertheless, when blastomeres from 8-cell embryos are recombined with marked morulae they can still produce a wide range of tissues in the chimeric offspring (Kelly, 1977).

It is at the late 8-cell stage that the embryo begins the next major phase in its development, compaction. Compaction is the process whereby the blastomeres undergo major changes in adhesiveness such that they flatten and increase contact with each other. During this process, the cells of the developing embryo become polarised; this is signified by the development of distinctive apical and basal membranes. For a review of cell adhesion in the embryo, see Fleming *et al*, (2001). Scanning electron microscopy of compacted 8-cell blastomeres disassociated by culture in the absence of calcium reveal that the apical poles of the cells have microvilli while the inner lateral surfaces are smooth (Reeve and Ziomek, 1981). Additionally, at this stage of development, the apical poles also accumulate actin and endosomes while the nucleus moves towards the basal surface (Reve and Kelly, 1983; Johnson and Maro, 1985). Compaction is also characterised by the redistribution of E-cadherin from a pan-membrane distribution to only sites where the cellular membranes are in contact (Vestweber *et al*, 1987).

Around 60-65 hrs post fertilisation, the next round of cellular cleavage occurs. Some of the blastomeres of the compacted 8-cell embryo cleave orthogonal to the polarised axis to generate apical (exterior) and basal (interior) cells (Johnson and Ziomek, 1981). Due to the redistribution of cellular organelles discussed above the basal and apical cell types each inherit different membrane and associated components from their parent cell. Essentially the apical cells that inherit the apical membrane and cytocortex beneath maintain their polarised phenotype and become trophectoderm cells, the very first epithelium, while the basal cells are allocated to the inner cell mass (ICM) (Johnson and Ziomek, 1981; Johnson and Maro, 1985). Hence, it is at the 16-cell stage that the fate of the embryo's cells is first determined; consequently, this type of cell division known as differentiative division. It is also important to remember that conservative cell division also occurs at the the 8-cell stage; essentially conservative division gives rise to more cells of the same type. For the 32 cell embryo to form, another round of differentiative and conservative division occurs. Interestingly, the embryo can regulate the number of cells in the ICM and trophectoderm layers between the 16 and 32-cell stage. That is, if more cells exist in the trophectoderm than the ICM at the 16 cell stage more differentiative division will occur. Alternatively, if more cells exist in the ICM lineage at the 16 cell stage, more conservative divisions of trophectoderm cells will occur. This has the effect of balancing the number of cells in each embryonic lineage. The capacity of the embryo to regulate the numbers of cells in each lineage appears to be regulated by cellular contact and number (reviewed by Johnson and McConnell, 2004)

As the developing embryos pass from the oviduct to the uterus, around 72 hrs post fertilisation in the mouse, the 32-cell embryo is undergoing major developmental events. It is in the uterus that the embryo progresses from the compaction stage and becomes a blastocyst. For the blastocyst to form, tight junctions must assemble at the apicolateral contact regions between the newly differentiated trophectoderm cells (reviewed by Fleming *et al*, 2001). These tight junctions are an area of very close cellular contact and even partial membrane fusion that effectively forms a seal between the trophectoderm cells. By forming this barrier, paracellular diffusion is prevented and

the ICM is isolated from direct contact with the exterior environment. Once the tight junctions are in place the embryo begins the process of cavitation; essentially this is the formation of a fluid-filled cavity within the embryo beneath the trophectoderm known as the blastocoel. Formation of the blastocoelic cavity is widely thought to be due to the sodium pump, Na/K-ATPase, localised on the trophectodermal basolateral membrame setting up an osmotic gradient, causing water to enter into the embryo and occupy the extracellular spaces (reviewed by Watson and Barcroft, 2001). A blastocyst is composed of a hollow slightly oval ball of trophectoderm cells, within which is the ICM. The trophectoderm will go on to contribute to extraembrionic membrane systems such as the placenta, while the ICM is destined largely to become the fetus.

1.5.4 Early attachment and implantation

The receptivity for blastocyst implantation in mice is controlled by the synergistic action of progesterone and estrogen. The duration of the receptive phase is short in mice, only about 24 hrs. As a result, the uterus becomes receptive on day 4 of pregnancy or pseudopregnancy and proceeds to the refractory state on day five (Paria *et al*, 2002). As already discussed in section 1.5.2 the receptive period is preceded by a secondary surge of estrogen on day 4 of pregnancy. Yoshinaga (1988) defined the receptive uterus as being characterised by the following parameters: (1) Formation of bulbous protrusions on the apical surface of the luminal epithelium; (2) Secretion of the stage-specific glycoproteins by the luminal epithelium; (3) Readiness of stromal cells to decidualise when appropriate stimulation is applied; and (4) Reorganisation and changes of stromal extracellular matrix components so that stromal cells are conditioned for decidualisation and the appearance of basement membrane components in the matrix after decidualisation. This section is a very general review of embryo attachment and implantation, placentation is not dealt with here as it lies outside this investigations direct timeframe of interest. However for a general review of implantation including placentation see Johnson and Everitt, 1995.

As mentioned above endometrial receptivity and the process of implantation are strictly controlled by hormones, principally progesterone and estrogen (see Johnson and Everitt, 1995). Indeed it has been hypothesised by Mohamed *et al* (2004) that estrogen

regulates gene expression in the blastocyst through uterine factors, and identified Wnts as potential mediator of embryo-uterine communication during implantation. Overall however, Progestogenic domination is required if the blastocyst is to effectively bind to the uterine epithelium, because the changes in epithelial surface properties are in part mediated by this hormone. However, while progesterone is critical, additional input is also required from estrogen. It is well know that ovariectomised mice require injection of both progesterone and estrogen for the embryo to commence implantation. If only progesterone is administered then the blastocyst can remain in a quiescent state until estrogen is injected causing attachment and implantation to commence.

At around day 4.5-5.0, the mouse embryo hatches from the zona pellucida and begins implantation (Fig. 1.5.3). The first phase of embryo implantation in rodents and primates, so called invasive species, is attachment. Initial attachment of the embryo probably involves temporary adhesion between surface receptors and ligands on the embryonic/endometrial epithelium. The best characterised cell adhesion molecule on the luminal surface of the endometrium is $\alpha v\beta 3$ integrin which recognises extracellular matrix ligands containing the amino acid sequence arg-gly-asp. Its ligand osteopontin is co-localised with $\alpha v\beta 3$ and is thought to play an important role in endometrial/embryo signaling to facilitate embryo attachment to the uterine epithelium preceding invasion (reviewed by Lessey, 2002). Specifically the arg-gly-asp sequence has been associated with trophoblast attachment and subsequent outgrowth (Armant *et al*, 1986; Yelian *et al*, 1995). Illera *et al*, (2000) demonstrated that ablation of either $\alpha v\beta 3$ or its ligands binding sequence impairs attachment and hence implantation in the mouse. The leukemia inhibitary factor (LIF) receptor has also been shown to mediate the attachement process (Cullinan *et al*, 1996); indeed the presence of maternally expressed LIF is required for the decidualisation process to begin (Stewart *et al*, 1992). Other factors have also been implicated in the adhesion process, for example, Heparin binding-endothelial growth factor (Raab *et al*, 1996), insulin like growth factors (IGF's) and their growth factors (Crossey *et al*, 2002), Hoxa-10 (Benson *et al*, 1996) and forkhead transcription factor (Christian *et al*, 2002) (for an exhaustive review see Red-Horse *et al*, 2004). Additionally, embryo attachment and implantation is associated with a local rise in endometrial FGF-II levels (Paria *et al*, 2001). As for FGF receptors, FGFR1 has been

localised in rat uterine tissues including the stroma (Yazaki *et al*, 1993; Rider *et al*, 1995). Peri-implantation mouse blastocysts ubiquitously express transcripts of FGFR2, 3 and 4 (Rappolee *et al*, 1998). FGFR2 has been detected within the early postimplantation mouse embryo (Orr-Urtreger *et al*, 1993).

A few hours after the initial phase of attachment the process of implantation proper commences. By this time the luminal epithelium that is in contact with the conceptus has become eroded by trophectodermal processes that invade, isolate, solublise and then digests it. It is only during pregnancy that an interaction such as this occurs between two distinct organisms under normal physiological conditions. In rats, mice and humans, the endometrium exhibits hormone-mediated growth, differentiation and the transformation of endometrial stromal cells to decidual cells at embryo implantation sites (Abrahamsohn and Zorn, 1993). This process, known as decidualisation, involves extracellular matrix remodeling from interstitial-type to basal laminar-type components around the endometrial cells and a dynamic alterations in cell-cell adhesion (discussed by Matsumoto-Miyai *et al*, 2002). Indeed, decidualisation can be induced artificially by a combination of progesterone-treatment and a mechanical traumatisation of the uterine horn in the absence of estrogen (Shelesnyak, 1933). However, estrogen is obligatory for natural decidualisation to occur during normal pregnancy (Finn, 1966). In fact, The continued presence of estrogen reduces the dosage of estrogen required to maintain pregnancy post-implantation, (Milligan and Finn, 1997). This suggests that estrogen action via its receptor does not actually generate signals that are essential for implantation and decidualisation but, instead, modifies the sensitivity of uterine cells to the effects of progesterone. Kurita *et al* (2001) suggested that up-regulations of progesterone receptor in endometrial stroma are mediated through at least three mechanisms: 1) classical estrogen signaling through estrogen receptor α, 2) estrogen signaling through estrogen receptor ß, and 3) as a result of mechanical stimulation plus progesterone, which induces stromal cells to differentiate into decidual cells. Additionally, it should be noted that decidual cells are advantageously positioned to counteract invasion of the fetal trophoblast cells. Thus decidualisation along with this remodeling of the endometrial cell-matrix functions to protect against disproportionate trophoblast invasion (Kirby and Cowell, 1968).

Other molecules including the potent angiogenic factor VEGF have also been implicated in implantation. VEGF mRNA expression has been demonstrated to correlate temporarily and spatially with alterations in angiogenesis and vascular reactivity at implantation sites, and decidua (Jakeman *et al.*, 1993; Chakraborty *et al.*, 1995). Indeed, gonadotrophin mediated decreases in expression of VEGF mRNA have been linked to delayed implantation, reductions in size embryo implantation site size on days 5 and 6 of pregnancy and a prolonged gestational period (Sibug *et al*, 2002). The invasion of the embryo and formation of the decidum mark the completion of implantation. This process gives the embryo a physical hold within the uterus and an initial score of nutrition such that placentation can now occur ultimately leading to adjacent circulations and exchange of nutrients.

1.5.5 Factors known to affect embryo development

Most of the work that has elucidated the relationship between embryo development and amino acids has been accomplished though *in vitro* culture of embryos. It is clear from this research that human embryos require amino acids within their direct environment for successful growth and development to the blastocyst stage; even a brief exposure of five minutes to media devoid of amino acids can have a detrimental effect (Gardner and Lane, 1996). It appears that the non essential amino acids glycine, alanine, glutamate, taurine and other substrates of Na^+ dependant amino acid transport can not only protect mouse embryo from the mildly hypertonic environment that the embryo experiences *in utero*, but also can possibly act to actively communicate environmental conditions to the embryo (Van Winkle *et al*, 1990). Accumulation of amino acids results in a larger cellular volume and has been suggested to help the mouse embryo maintain a balance between anabolism and catabolism (Van Winkle *et al*, 1994). It has been demonstrated that cellular swelling of mouse embryos can promote anabolic processes such as protein synthesis and inhibit catabolic processes; on the other hand cellular shrinkage has the opposite effect, favouring catabolism over anabolism (Häussinger *et al*, 1994). During cavitation and blastocyst formation there is an increase in the essential amino acid transporters (reviewed by Van Winkle, 2001). This is probably partly due to the fact that embryos increase protein content in the 10 hours preceding implantation (Weitlauf, 1973). Van Winkle (2001) proposed that essential amino acids could act as

novel signaling elements in embryos and stimulate protein synthesis and accumulation through the mammalian target of rapamycin (mTOR) and its downstream targets p70 S6 and 4E-BP1 (discussed in Section 4.4). This model provides a mechanism whereby amino acid concentrations could signal maternal nutritional status and alter developmental plasticity during the pre-implantation critical period.

Glucose is an important factor in embryo development and provides an easily metabolised energy substrate. However, studies of embryonic nutrient uptake show that it is only at the blastocyst stage that glucose is the preferred nutrient; indeed prior to this, until the 8-cell stage, the early mouse embryo takes up pyruvate preferentially (Leese and Barton, 1984; Gardner and Leese 1986). Moreover, studies on the culture of preimplantation mouse embryos have established that the development of the zygote to the 2-cell stage has a requirement for the presence of pyruvate (Biggers *et al*, 1967). Lactate has been shown to be able to support embryonic development from the 2-cell stage (Whitten, 1957; Brinster, 1965), while glucose alone can support embryo development from the late 4-cell stage (Brinster and Thomson, 1966). While lactate alone is unable to support the first cleavage division, it is readily oxidized by both the 1- and 2-cell mouse embryo (Wales and Whittingham, 1967; Wales and Whittingham, 1973). Because of these data, there has been some debate about the need for culture media lacking glucose for IVF. In fact studies have shown that removal of glucose from early embryo media prior to compaction can actually improve embryo development (Coats *et al*, 1999; Porter *et al*, 2000). Porter *et al* (2000) found that embryos cultured in no glucose media from fertilisation to day 3 had better morphology compared to siblings cultured in low glucose media.

In addition to amino acids and glucose, growth factors have been shown to have a significant effect on embryo development. It has been demonstrated that addition of exogenous insulin (Harvey and Kaye, 1990) to embryo culture medum increases *in vitro* embryo cleavage rates, blastocyst formation and blastocyst outgrowth rate. Co-culture of embryos with epithelial cells has also been demonstrated to improve embryo quality *in vitro* (Joo *et al*, 2001). Liu *et al* (1999) have demonstrated that co-culture of human embryos with endometrial stromal cells from day 3 to day 5 of development significantly

increased blastocyst formation rate and significantly enhanced expression of IGF-I, IGF-II, IGF receptor 1 (IGF1R), and insulin receptor mRNA transcripts within the embryos. Insulin itself has been shown to enhance embryo development and increase blastocyst formation rates *in vitro* (Gardner and Sakkas,1993). In addition, insulin treated blastocysts have been shown to exhibit increased synthesis of specific proteins (Shi *et al*, 1994). Other growth factors have also been shown to stimulate embryo development; granulocyte-macrophage colony-stimulating factor is synthesised in the female genital tract and a study by Sjoblom *et al* (1999) showed that addition of 2 ng ml^{-1} of this growth factor to embryo culture medum increased blastocyst formation rates from 30% to over 70%.

1.5.6 Composition of the female genital tract fluid

The critical developmental processes of fertilisation, cleavage and blastocyst formation occur within oviductal and uterine fluid. It would be thought that such secretions would have been examined in depth. However, this is not the case, especially in mice. In mice, the fact that female genital tract secretions have not been studied in detail may be due to the fact that such fluids are only available in minute volumes; Hoversland and Weitlauf (1981) estimated that the volume of fluid in the pseudopregnant mouse uterus at around the normal time implantation is as little as 300 nl. In another study by Wales and Edirisinghe (1989) the volume of uterine fluid was shown to be nearly 9 µl on day one of pseudopregnancy but only about 2 µl from day 2-5 of pregnancy. Collectively, these data indicate that there is a large reduction in fluid volume between fertilisation and implantation. Indeed, Hoversland and Weitlauf (1981) also showed that the volume of fluid quickly rises to nearly 4 µl by between day 3 and 5 of pseudopregnancy if mice were not given progesterone injections. This reduction in reproductive fluid production during early pseudopregnancy has also been demonstrated in the uteri of rabbits (Oliphant *et al*, 1981; Gott *et al*, 1986). The minute volumes of reproductive fluids in the mouse not only make its collection of the genital fluids difficult but also complicate analysis of its components. As a result this section will draw much of its information from other species including rabbits and humans.

Osmolarity is similar to serum in diverse species, approximately 290 mOsm kg^{-1}; this value does not seem to be altered by hormonal variations (Menezo and Guerin, 1997). Mass *et al* (1979) demonstrated that the pH of oviduct fluid of rhesus monkeys is approximately 7.2 during the follicular phase sharply rises to approximately 7.8 during the leuteal phase. Electrolytes such as $K^+$and bicarbonate are approximately twice that of serum while Ca^{2+}, Mg^{2+} and Cl^- concentrations appear to be similar to serum (Menezo and Guerin, 1997; Leese, 1988). Overall protein content of oviductal fluid has been shown to be about 11 mg ml^{-1} this aproximately 5-10% of that's of serum Gardner and Leese (1990).

One of the major components of genital tract fluid are amino acids and these, as discussed in the previous section, have been shown to have profound effects on embryo development *in vitro*. One of the most striking observations of amino acids in uterine and oviductal fluid is the large amount of taurine present; this has been shown to be the case in mice (Domoulin *et al*, 1992; Guerin and Menezo, 1995), humans (Casslén and Nilsson, 1984) and in rabbits (Miller and Schultz, 1987). Other amino acids reported to be in abundance in the mouse reproductive tract include glycine and glutamine (Domoulin *et al*, 1992; Guerin and Menezo, 1995). Domoulin *et al* (1992) have additionally shown that the branch chain amino acids, so important for signaling in the mTOR system, are also present in mouse reproductive fluids. However, as already mentioned the fact that mouse reproductive fluid volumes are so small has forced previous researchers (Domoulin *et al*, 1992; Guerin and Menezo, 1995) to use flushing type approaches that make original dilutions impossible to know and thus only allow for estimation of amino acid balance in terms of percentage abundance. Where amino acid levels have been established conclusively in reproductive fluids, for example in the rabbit (Miller and Schultz, 1987), the concentration of amino acids appears to be a lot higher than in the maternal serum.

Amino acids have been hypothesised to have many roles in reproductive fluids as already discussed; branched chain amino acids are thought to act as novel signaling elements (Van Winkle, 2001). Glycine and alanine have been proposed to regulate intracellular pH and protect the embryo from osmotic stress (Bavister and McKiernan,

1993). Taurine and hypotaurine have been shown to enhance sperm capacitation (Mrsny *et al*, 1979) and increase embryo fertilisation rates in the Golden Hamster (Liebfried and Bavister, 1981). Additionally, hypotaurine is a free radical scavenger with the byproduct of this reaction being taurine (Fellman and Roth, 1985).

Unlike amino acids, glucose has been accurately measured in the reproductive tracts of mice; however, estimates of its concentration are variable. Gardner and Leese (1990) have estimated the concentration of glucose in the oviduct to be about 5.2 mM on day 2 of pregnancy where cumulus was not present and about 3.4 mM when cumulus was present. Gardner and Leese (1990) also estimated the concentration of pyruvate to be approximately 0.37 mM and the concentration of lactate to be approximately 4.79 mM in oviduct at day 2 of pregnancy. In contrast, Wales and Edirisinghe (1989) found uterine glucose concentration to be below assay range on day 1 of pregnancy rising to a concentration estimated to be about 1 mM on day 2 of pregnancy in the mouse. These very different results for glucose concentration within the oviduct and uterus at the same time point could mean that uterine and oviductal glucose concentrations are subject to differential regulation. In the human it appears that glucose concentrations rise between the oviduct and uterus during pre-implantation development (Gardener *et al*, 1996). Interestingly, and at odds with the findings for amino acids, glucose concentrations in the oviduct appear to be much lower in comparison to maternal serum, perhaps up to half the concentration (Menezo and Guerin, 1997). Korgun *et al* (2001) studied expression of glucose transporters within the rat uterus during the peri-implantation period of development and found that small amounts of the glucose transporter GLUT1 appeared on day 3 of pregnancy localised to the luminal epithelium and endometrial stroma. The expression of GLUT1 increased with decidsualisation. GLUT3 and 4 were both present throughout the pregnant rat uterus between conception and implantation, except GLUT3 was notably absent from smooth muscle and endometrial glands.

1.6 Effects of dietary protein restriction

In this section I will discuss the major physiological responses to dietary protein restriction. Low protein diet (LPD) is defined as, any situation where, only an animal's dietary protein is restricted to a level less than that required for normal healthy survival.

In general protein restriction elicits many of the same metabolic and hormonal reactions as the total dietary restriction does. Insulin levels will drop when rodents are treated with a LPD, although interestingly glucagon levels tend to stay about the same, though this does cause a relative decrease in the insulin:glucagon ratio (Kabadi, *et al*, 1976). After refeeding the plasma insulin levels return to normal showing that this effect is transient. Somatostatin levels will also rise in rats fed a LPD (Karabatas *et al*, 1992).

Rats fed an isocaloric LPDs have been shown to increase levels of total corticosteroids (Adlard and Smart, 1972; Tigner and Barnes, 1975) and levels of corticosterone (Herbert and Carrilo, 1982), and mice (Watson *et al*, 1983). These experiments were conducted on relatively young rodents i.e. less than 6 months and while corticosterone did increase in these models, evidence exists to suggest that those adrenocortical responses to this type of dietary insult are modulated with increase in age. Bandyopadhyay and Poddar (1998) found that while three and six month old rats fed a 5% LPD for 7 days exhibited an increase in plasma corticosterone, nine, twelve and eighteen month old rats exhibited no significant plasma corticosterone change. In fact when the LPD was administered for 30 days there was a significant drop in plasma corticosterone in the eighteen-month-old rats. Baldijão *et al* (1976), conducted experiments on pigs fed a LPD and found that while there was not a significant change in total corticosteroids there was a significant elevation of cortisol. NPY expression also increases in the basomedial hypothalamus when rats are fed an isocaloric diet (White *et al*, 1994).

Serum concentrations of amino acids have been shown to be altered in pregnant rats when treated with a LPD during both early (Kwong *et al*, 2000) and late pregnancy (Rees *et al*, 1999). Similarly Petrie *et al* (2002) found that circulating amino acid levels

are altered in serum of mice and rats administered a LPD during pre-implantation development. These findings are discussed in greater depth in Chapter 6. Additionally, it has been demonstrated that food intake will generally increase with treatment with LPD. This is thought to be due to protein and amino acids being the principle regulators of food intake (reviewed by Tome, 2004). Indeed, this phenomenon is largely accepted to be one of the major reasons the so-called "Atkins diet" works (Astrup *et al*, 2004). Basically eating a diet with a large proportion of protein actually quenches appetite; in fact you lose weight because you consume fewer calories. This is further discussed in terms of experimental intervention and metabolic programming studies in Chapter 3.

1.7 Summary of metabolic programming and Syndrome X

The major symptoms of metabolic programming of Syndrome X are hypertension, impaired glucose tolerance/type II diabetes and an increased body mass index. Several fundamental papers link LBW to Syndrome X disorders in adult life in both humans (Barker *et al*, 1989b; Hales *et al*, 1991; Ravelli *et al*, 1999) and in animal models (Langley and Jackson, 1994; Kwong *et al*, 2000; Ozanne and Hales, 2002). It is clear that there are of course inherent problems with the epidemiological investigations, such as birth weight being a less than adequate proxy for fetal environment (Huxley *et al*, 2002). Aside from the obvious difficulty of relating animal models to a human, laboratory studies on other mammals often suffer from unrealistic experimental interventions. The isocaloric low protein diet for instance has little resemblance to any deficient diet a human may encounter. In addition surgical interventions such as uterine artery ligation (Persson and Jansson, 1992) or limitation of placentome attachment sites (Rees *et al*, 1988) are even harder to directly relate to any situation a human may encounter. The various models of fetal programming can even give rise to data that would suggest that intrauterine growth retardation can have no effect on blood pressure (Edwards *et al*, 1999) or even lower blood pressure (Louey *et al*, 2000). At first glance it would appear that these results deride the link between uterine environment and adult disease and the benefit of such research to future public health planning. However, such simplistic critique of fetal programming studies loses sight of the fact that any difference

between control and experimental groups is indicative of a programming effect and through comparison of such results and contrasting of experimental conditions one can tease out the underlying mechanisms behind this phenomenon; surgical interventions on placentas for example, bypass many of the hormonal changes associated with dietary restriction.

What is clear from the studies to date is that there is no single phenotype of metabolic imprinting. The specific Syndrome X type symptoms exhibited depend largely on what critical periods were compromised and the PAR's made. Thus far most experiments have been limited to measuring outcomes in fetuses, neonates and adult offspring in an attempt to elucidate the molecular mechanisms underlying PAR's (Benedictsson *et al*, 1993; Bertrum *et al*, 2001; Langley-Evans, 1996a,b,c; Langley-Evans, 1997). There are far fewer papers which take account of maternal factors such as metabolism (Rees and Hay, 2002) or endocrine status (Kwong *et al* 2000), this is surprising because the maternal nutrient metabolism/mobilisation and endocrine status have been shown time after time to influence fetal growth (Reinisch, *et al*, 1978; Tangalakis *et al*, 1992). Some of the most interesting observations by Kwong *et al* (2000) are that a maternal LPD administered only for the pre-implantation period can affect embryo growth and cause metabolic programming effects in adult offspring. Indeed at this critical period of development maternal metabolic status is of great interest. It is possible that the uterine environment is determining the embryos growth and differentiation strategies that may have profound effects throughout fetal growth and beyond into adulthood.

1.8 Project Rationale

It is the aim of this investigation to examine metabolic programming of the early embryo following up on the work of Kwong *et al* (2000). Specifically this study aims to examine the peri-implantation critical period of embryo development to understand how this environment may alter in relation to maternal diet and what effect this may have on embryonic PAR's.

For this study the LPD mouse model will be used, that is the only experimental intervention will be dietary protein content; control group mice will be fed an 18% protein diet while experimental group mice will be fed an isocaloric 9% protein diet. In the first place this investigation seeks to characterise the most general effects of treating mice with a synthetic 9% LPD. Such parameters will include weight gain and food intake over the experimental period (Chapter 3). Perhaps the most challenging part of this project will be investigation into the peri-implatation embryos immediate environment, uterine fluid, including analysis of amino acids and glucose levels (Chapter 4). The volume of murine uterine luminal fluid is estimated to be only about 0.3 μl per uterine horn. However, this part of the investigation, in particular, could help to elucidate the mechanisms of signaling from the mother that possibly lead the peri-implatation embryo to make PAR's at this early stage of development. As a result, to complete this part of the investigation it will be necessary to develop novel methods to sample murine uterine luminal fluid and then select appropriately sensitive techniques to measure its constituents and validate results. This information is to be combined with analysis, via more traditional methodologies, of growth factors in uterine tissue to paint a vivid picture of how maternal nutritional status may affect the uterine endometrium and embryo implantation (Chapter 5). Constituents of maternal serum (Chapter 6) will also be measured not only to gain an appreciation for the general effects treatment with a LPD may have on maternal metabolism, but also to allow comparison with uterine fluid as a further method of verification that I am indeed sampling distinct fluids. Additionally, hormones and sex steroids will be measured in maternal serum. Finally this investigation utilises microarray technology as an exploitative means to investigate possible changes in gene expression in relation to treatment with a LPD (Chapter 7).

Chapter 2

Materials and methods

2.1 Animal treatments

Virgin female MF1 mice bred in house were maintained under controlled 12 hour lighting cycle (lights on 0700h and off at 1900h) and at a temperature of 24°C. Animals were allowed *ad libitum* access to standard rodent chow and tap water and were housed in groups of up to 12 from weaning to 7 weeks of age when experiments began. The virgin female MF1 mice were mated individually with male MF1 mice (Harlen UK Ltd, Bicester, UK) and conception was defined as the presence of a vaginal plug. All plug positive mice were assigned at random to a synthetic isocaloric experimental diet of either 9% protein (LPD) or control diet 18% (NPD) (Table 2.1) fed *ad libitum* and housed individually until either 3.5 or 4.5 days of pregnancy. Mice were weighed at time of plug and at 3.5 or 4.5 days of pregnancy. All animal experiments were performed in accordance with the provisions of Home Office licenses PPL30/1395 and PPL30/1967 granted under the 1986 Animal Procedures Act.

	9% protein diet (g)	18% protein diet (g)
Casein	90	180
Corn Starch	485	425
Sucrose	243	213
Fibre	50	50
Choline Chloride	20	20
DL-Methionine	50	50
AIN-76 Mineral Mix	200	200
AIN-76 Vitamin Mix	50	50
Corn Oil	100	100

Table 2.1: Ingredients of diets used for making 1 kg of synthetic isocaloric 9% experimental and 18% control diets (Langley and Jackson, 1994). All ingredients are supplied by Specialist Diet Services, Cambridge, UK

2.2 Serum collection and storage

Animals were culled on day 3.5 or day 4.5 post vaginal plug and blood was retrieved via vacupuncture. Blood was allowed to clot at 4°C for 30 mins, the blood was then centrifuged at 4°C for 10 mins at 10,000 g. Supernatant was removed and stored at -80°C.

2.3 Collection of uterine luminal fluid: perfusion method

At 4.5 days of pregnancy, mice were culled by dislocation of the neck, in accordance with schedule 1 of the 1986 Animal Procedures Act, and the time of cull recorded. The uterus was then promptly dissected out, placed on ice, and any mesometria tissue removed. Each uterine horn was then dissected away at the posterior from the corpus uteri and at the anterior approximately 1mm posterior from where the oviduct meets the coruna. Each uterine horn was then positioned on the outside of a closed petri dish filled with ice under a dissecting microscope. A 0.165 mm diameter Microfil needle (World Precision Instruments, Stevenage, UK) was placed 2 mm into the posterior end of the uterus. An ART Gel 20P Gilson tip (Promega, UK) was also placed 2 mm into the anterior end and both ends were secured using Sylko cotton thread (Coats Crafts, Darlington, UK) (Fig. 2.3). The Microfil needle was attached to a 101/UR peristaltic pump (Watson Marlow Bredel Products, UK) and phosphate buffered saline (PBS) (Dulbecco A; Oxoid, UK) was perfused through at 1.8 µl min^{-1}. 13 µl of luminal fluid mixed with perfused PBS was collected in the Gilson tip which was pre-marked at 13 µl. The collected fluid was then deposited in a 0.5 ml microfuge tube and centrifuged at 10000 g for 10 min at 4°C to remove any cellular contents. 10 µl of supernatant was removed and placed in a fresh 0.5 ml microfuge tube, the remaining 3 µl containing any cellular contents was retained for separate analysis; both microfuge tubes were stored at -20°C.

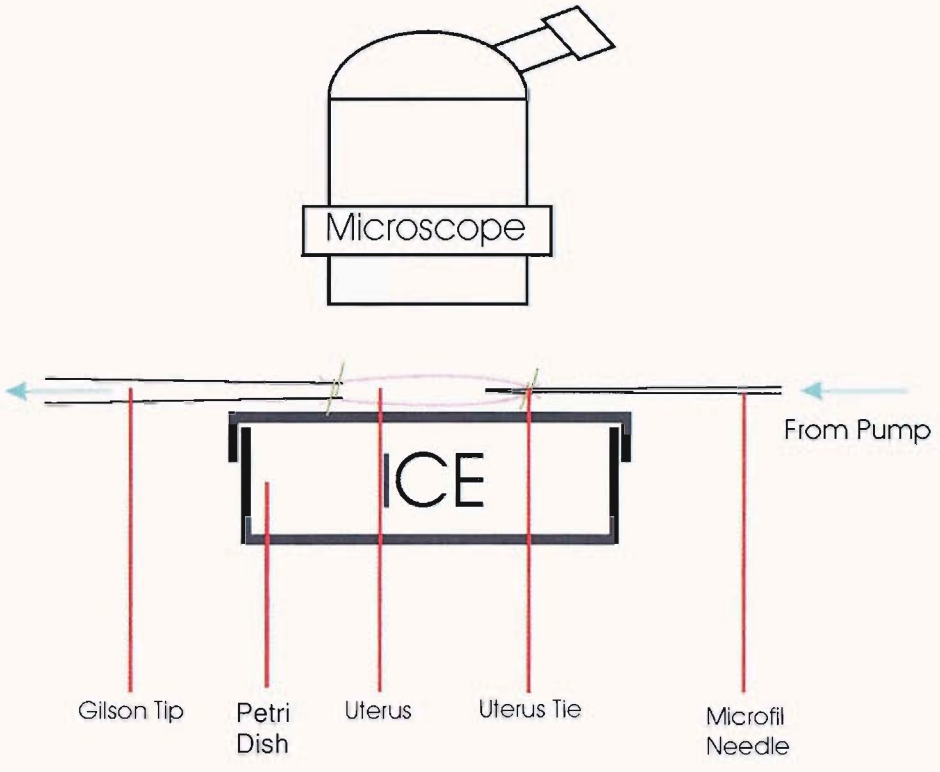

Fig.2.3: A schematic showing the perfusion method used to collect uterine luminal fluid. PBS is pumped through the MicroFil needle into the uterus. This flushes the uterine luminal fluid and cellular content into the Gilson tip. Once 13 µl of luminal fluid is collected, the Glison tip is removed and the fluid deposited into a 0.5 ml microfuge tube.

2.4 Collection of uterine luminal fluid: direct sampling method

2.4.1 Production and calibration of glass capillary collection tubes

Glass Pasteur pipettes were heated over a Bunsen burner and pulled to an approximate outside diameter of 0.5 mm ± 0.15 mm then scored and snapped to provide a straight tip. The tip end was then flame polished using a paraffin burner to remove any sharp edges.

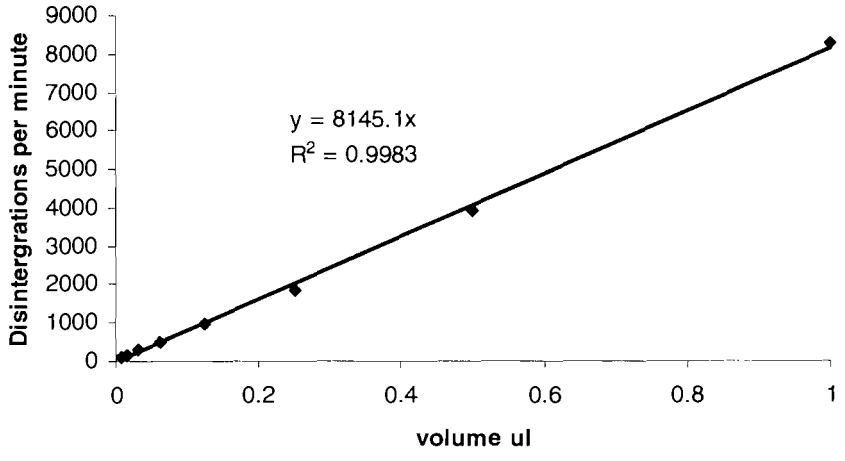

Fig. 2.4.1a: Disintegrations per minute plotted against volume of C^{14}-labeled sucrose. This standard curve was needed to link volumes to disintegrations min^{-1} such that the internal volume of the glass capillary tubes could be calibrated.

C^{14} sucrose (Sigma, UK) was used to calibrate the collection pipettes. This was diluted 1:20 from neat solution and stored in 100 µl aliquots at -20°C until needed. A serial dilution was prepared from 100 µl of the solution to create a calibration curve to relate volume (µl) to disintegrations min^{-1}. 1 µl from each dilution was placed on a piece of glass fibre filter paper (Whatman, UK) then placed into a scintillation vial with 5 ml of scintillation fluid. Disintegrations min^{-1} were measured using a LS6500 Gamma counter (Beckman, UK). Three serial dilutions were conducted and the standard curve constructed from the averages (Fig. 2.4.1a).

Individual pipettes were then calibrated by suspending them under a dissecting microscope and filling them to a distance of 1000 µm (measured with an eyepiece graticule) from the tip with C^{14} Sucrose. This was then completely drawn out of the tip with glass fibre filter paper and placed in a scintillation vial with 5 ml of scintillation fluid. Each pipette was then re-loaded with C^{14} Sucrose to 1000 µm from the tip. This time small amounts of fluid were drawn off using the filter paper such that the volumes removed were noted against the specific eyepiece graticule measurements. This allowed the consistency of the pipette bore to be established. Once again the filter paper was placed in scintillation vials with 5 ml of scintillation fluid. The disintegrations min^{-1} were then measured in each vial. Disintegrations were then converted to volume using

the calibration curve and individual standard curves were then constructed for each pipette; an example of which is shown in Table 2.4.1/Fig.2.4.1b.

Distance from tip (μm)	10000	8600	2000	1300
Count	2068	1754	452	300
Voulume (μl)	0.24	0.21	0.05	0.04

Table 2.4.1: An example of calibration results gained from one glass capillary tube. The count (disintegrations min^{-1}) from each tip is displayed cumulatively. i.e. 300 disintegrations min^{-1} [volume 1300μm to 0μm] + 152 disintegrations min^{-1} [volume 2000 μm to 1300 μm] = 452 disintegrations min^{-1} [the count for a 2000 μm volume of C^{14} labeled sucrose measured from the tip of the capillary]. The volume is calculated by using the calibration curve in Fig. 2.4.1b.

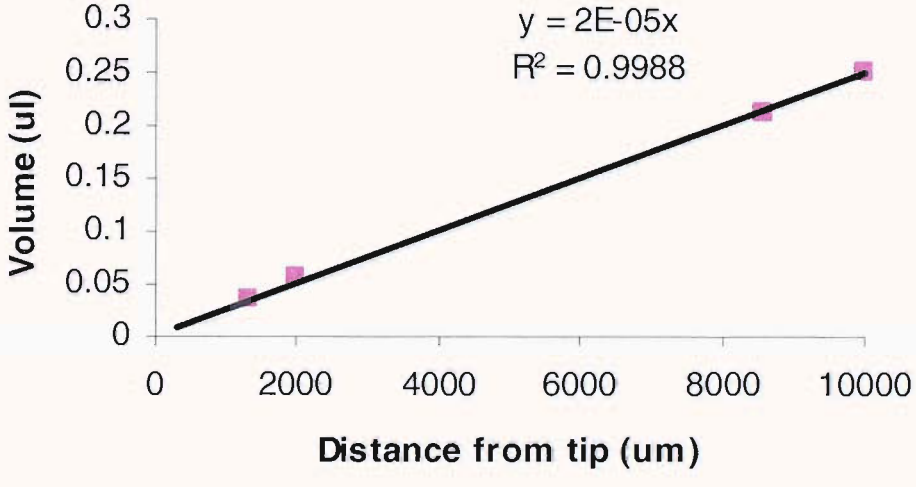

Fig. 2.4.1b: A sample calibration curve produced from Table 2.4.1 for a specific capillary tube. When uterine fluid is collected from a mouse the distance from the tip can be measured using a graticule and this calibration curve can be used to calculate the volume of uterine fluid.

2.4.2 Direct collection of and storage of uterine fluid

Mice fed on the NPD control diet or the LPD isocaloric experimental diet for 3.5 or 4.5 days were culled via cervical dislocation. The peritoneal cavity was opened and each uterine horn ligated with cotton thread posterior to the coruna. The uterus was then cut away from the ovary anterior to the ligation but posterior from the oviduct. This free end was then rinsed in ice cold PBS and blotted dry. The cotton thread was then removed and a collection pipette was then gently placed into the anterior of the uterine

horn and fed down the uterus to the corpus uteri. The pipette was then carefully removed and the distance the fluid occupied from the tip was measured under a dissecting microscope using an eyepiece graticule and recorded (for later conversion to volume using the pipettes standard curve). The uterine fluid was then ejected into an appropriate volume of PBS and centrifuged at 5,000g to separate any cellular contents, and supernatant was removed and placed in a separate microfuge tube. Both supernatant and cellular contents were snap frozen and stored at -80°C for future analysis.

A collection of fluid samples from anaesthesised animals was also developed by adapting a protocol similar to that used in rodent embryo transfer. In short, mice at 4.5 days post plug were administered with 2 µg kg-1 of Clenbuterol hydrochloride (also known as ventipulmin; Boehringer Ingelheim Ltd., UK); this is a smooth muscle relaxant given to relax the uterine musculature and aid insertion of the capillary tube. 10 minutes after the injection of 0.015 µg Clenbuterol hydrochloride per gram body weight, mice were injected with 7 µl Keteset and Acepromazine solution per gram body weight [375 µl 0.9% Saline, 100 µl, Ketaset (Fort Dodge, UK), 25 µl, Acepromazine(C-vet)]. Once the mice showed no response to pressure applied to the paw, approximately 2 cm^2 of hair was shaved from their dorsal midline, posterior to the kidneys. The mice were then placed on a flat 500 ml culture bottle containing water at 37.5°C (Falcon, UK). An incision of approximately 5 mm was made laterally over the spine. The skin was moved such that the incision was posterior to the kidney and above the fat pad distal to the ovary. Another incision was made in the body wall to facilitate exteriorisation of the ovary which was then clamped to hold it in place. A hole was made in the uterine horn posterior to the oviduct using a heated needle and a collection pipette was then gently fed into the uterine horn and carefully moved down the uterus to the corpus uteri. As above, the pipette was then carefully removed and the distance the fluid occupied from the tip was measured under a dissecting microscope. Unfortunately, this method was deemed to be unreliable as fluid collected tended to have a high degree of blood contamination. As a result, uterine fluid was only collected from mice culled by cervical dislocation, where little or no contamination from blood was observed.

2.5 Analysis of cellular contents of collected uterine luminal fluid

Analysis of cellular contents of uterine fluid was conducted in 'chambers' produced by Mr Adrian Wilkins, University of Southampton. Chambers were first used by Maro *et al* (1984) and are described in detail by Fleming *et al* (2002). Basically chambers consist of a steel washer sealed to a coverslip. The small quantities of cells collected during uterine fluid collection are stuck to the coverslip within the 'well' of the chamber and are thus protected during processing.

2.5.1 Formaldehyde fixation

Chambers were first rinsed with PBS then filled with 1.5 mg ml^{-1} poly-L-lysine hydrobromide (Sigma, UK) in PBS for 15 min, to make the coverslip base adhesive for fixed cells, before removal using a syringe and the chamber rinsed three times with PBS. The cellular samples were thawed at room temperature and 25 µl 4% formaldehyde (Sigma, UK) was added to the microfuge tube and left at room temperature for 15 min to fix the cells. The contents of the microfuge tube were then transferred into the chamber and left for 5 min to settle before a cover slip was placed on top and the chambers centrifuged for 30 min at 1,500 rpm. After centrifugation, the top coverslip was removed and the chamber washed three times with PBS. After washing, the chambers were flooded with 0.25% Triton X-100 in PBS for 15 min to permeabilise the cells, before a further wash in PBS. Chambers were then treated with 2.6 mg ml^{-1} NH_4Cl for 10 min to neutralise the fixative, the chambers were then washed three times with PBS. 30 µl primary antibody solution was added to the chamber and left for 1 hr at room temperature before washing three times with 0.1% Tween 20 in PBS over 30 min. 25 µl of secondary antibody plus 5 µl of Hoescht 33258 (for visualization of nuclei) was then added for 1 hr at room temp before washing three times with 0.1% Tween 20 in PBS over 30 min. Finally, 40 µl Citiflour (Citiflour Ltd) was added to the chamber; the chamber was then covered with a coverslip and sealed with clear nail varnish. Chambers were examined and photographed using a Leitz Diaplan fluorescence microscope.

2.5.2 Acetone fixation

Chambers were rinsed with PBS and filled with 0.1 mg ml-1 Concanavalin A (Sigma, UK) in PBS for 15 minutes, to make the base adhesive for living cells, before removal using a syringe and the chamber rinsed three times with PBS. The cellular samples isolated during the collection of uterine fluid (Sections 2.3 and 2.4) were thawed at room temp and suspended in 25 µl PBS. This suspension was then transferred to a chamber and left to settle for 5 min before a coverslip was placed on top and the chambers centrifuged at 1,500 rpm for 30 min. After centrifugation, the top cover slip was removed and the PBS removed. Chambers were then lowered into a bath of 100% acetone at -20°C and left in the freezer for 10 min. Chambers were then removed and the acetone replaced by washing three times with PBS over 15 min. Chambers were then immunostained and examined as in the formaldehyde fixation protocol.

2.6 Glucose measurement

Glucose concentrations in serum and directly collected uterine fluid (Sections 2.2 and 2.4) were measured by Dr Peter Humpherson at the Department of Biology, University of York, using a colorimetric enzymatic procedure Cobas-Mira S analyzer (Roche, Basel, Switzerland). The sample was mixed 1:1 with god-perid, a pH 7 buffer containing glucose oxidase, hydrogen peroxide and chromogen (Sigma, UK). First, glucose is oxidised using glucose oxidise and hydrogen peroxide is formed, which can be detected through a peroxidase catalysed reaction with the chromogen, p-aminoantipyrene and a phenol. This results in the formation of a red–violet reaction product that is measured at 546 nm. This assay is linear over a wide range. Stenninger *et al*, (2001) demonstrated that the within-run variation in glucose concentration is 1.8%, and the corresponding figure for between-run variation is 3.4%. The detection limit was set to 0.1 mmol l^{-1}.

2.7 Amino acids measurement

Amino acid concentrations within serum and uterine fluid (Sections 2.2, 2.3 and 2.4) were measured by Dr Peter Humpherson at the Department of Biology, University

of York. This was achieved using a Kontron 500 reverse phase high performance liquid chromatography (HPLC) system equipped with a F920 fluorescence detector and a 4.5x250 mm hypersil ODS-16 column (Jones chromatography, Hengoed, Mid Glamorgan, UK) (Fig. 2.7a). Derivatisation was accomplished through an automated reaction of the 25 µl sample and 25 µl of OPA reagent (10 µl 2-mercaptoethanol and 5ml of *o*-phthaldialdehyde). The elution gradient operated at 1.3 ml min^{-1} and consisted of two solvents; solvent A was composed of 1.8% tetrahydrofuran (Fischer Scientific, Loughborough, UK), 19.6% methanol and 78.6% sodium acetate (83 m mol l^{-1}, pH 5.9); solvent B consisted of 80% methanol and 20% sodium acetate. The two solvents were added at concentrations that varied according to time to allow for separation of amino acids, as shown in Table 2.7. Amino acids were excited at 330 nm and read at 450 nm. Unfortunately, this method did not allow for measurement of proline or cysteine. A typical chromatogram produced from HPLC of uterine luminal fluid is shown in Fig. 2.7b.

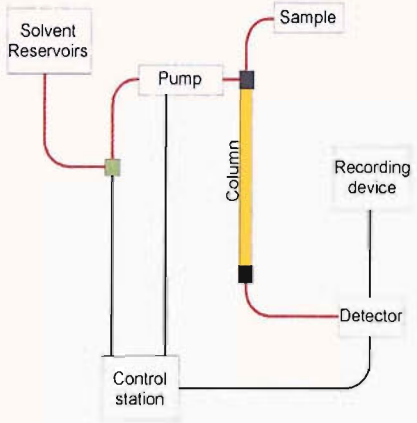

Fig. 2.7a: A schematic of a Kontron 500 reverse phase HPLC system. Black lines represent electrical links and red lines represent fluid tubes.

Time (mins)	% Reagent A	% Reagent B
0-9	100	0
9-12	100-86	0-14
12-19	18	14
19-24	18-50	14-50
24-28	50	50
28-40	50-0	50
40-44	0	100

Table. 2.7: The relative amounts of solvents A and B added according to time. The concentration of the 2 reagents are varied to allow for separation of amino acids which are differentially adsorbed by the column.

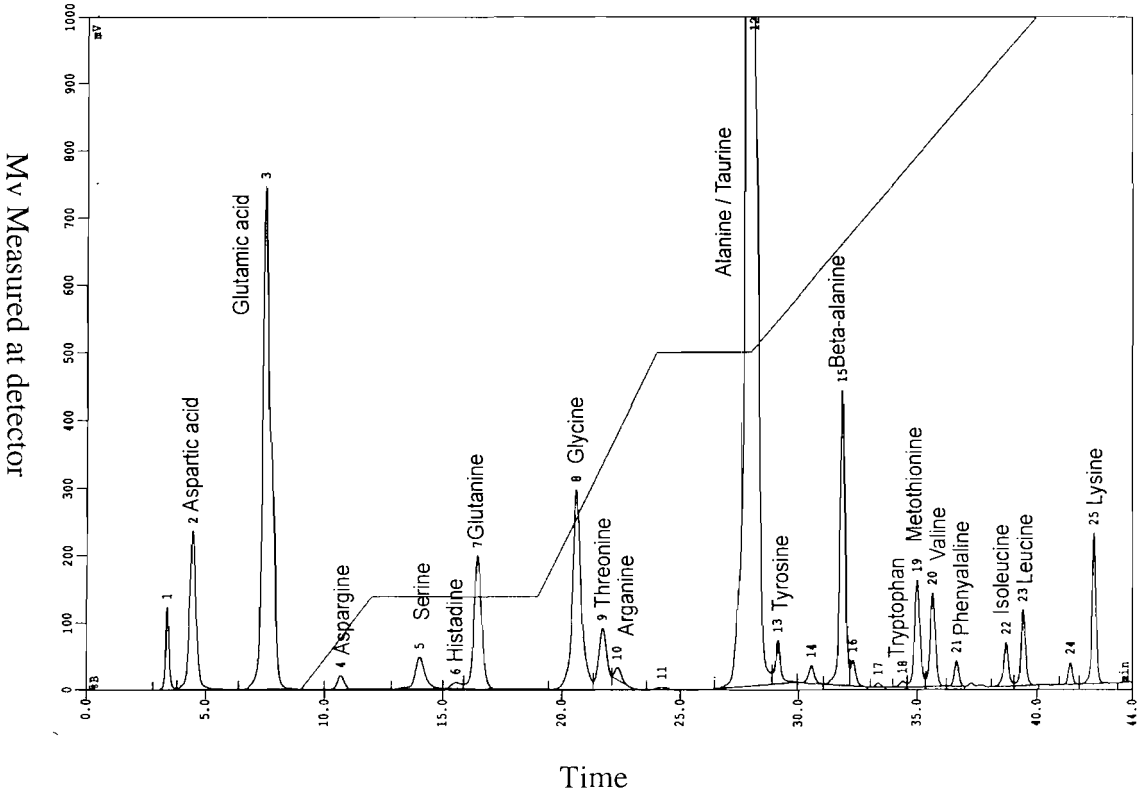

Fig. 2.7b : A typical trace generated from HPLC of uterine luminal fluid. Peaks are marked with their corresponding amino acid; unmarked peaks are unknown substances. mV read at detector are proportional to concentration. The stepped line is a visual representation of the amounts of reagent A and B (detailed in Table.2.7)

2.8 Protein detection

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of solubilised homogenised protein extracts of 3.5 or 4.5 day pregnant uterus was undertaken to investigate expression of proteins in various tissues semi-quantitatively. Uteri were dissected from mice on day 3.5 or 4.5 of pregnancy as described in Section 2.3, snap frozen and stored at -80°C until needed. For specific experiments other organs including heart, liver, kidney, and ovary were also dissected out, snap frozen and stored at -80°C.

2.8.1 Total protein extraction

Tissues were homogenised in 0.5 ml 1% w/v SDS using the Plus One Sample Grinding kit (Amersham) or a PowerGen 125 homogeniser (Fisher Scientific, UK) and then heated for 10 min at 105°C. Post heating, samples were microfuged at 10,000 g and

supernatant removed. Total protein of the supernatant was measured using the *DC* Protein Assay (Biorad). This is a colorimetric assay based on the Lowry assay (Lowry *et al*, 1951). Serial dilutions of 10 mg ml^{-1} BSA in PBS were used to generate the standard curve. Absorbence was read at 750 nm on a Dynatec MR 5000.

2.8.2 SDS-PAGE

A known quantity of total protein from each tissue lysate was loaded in duplicate onto either in-house made polyacrylamide gels with a 4% stack and a 10% or 15% resolve (Table 2.8.1). Gels were run for 1.5 h at 150 V in running buffer then electroblotted onto Highbond-ECL nitrocellulose sheets (Amersham, UK) for 17 h at 300 mA in transfer buffer (25mM Tris, 192mM Glycine, 0.1% (W/V) SDS, pH 8.3)

Table 2.8.1: Composition of gels for SDS-PAGE

Reagent	4%	10%	15%
Distilled H_2O	8.95 ml	6.02 ml	3.52 ml
Tris (ICN, UK)	Tris HCL pH6.8 3.75 ml	Tris HCL pH8.8 3.75 ml	Tris HCL pH8.8 3.75ml
30% Polyacrylamide (Proteome systems, UK)	2.0 ml	5.0 ml	7.5ml
10% SDS (ICN, UK)	0.15 ml	0.15 ml	0.15ml
10% APS (ICN, UK)	75 µl	75 µl	75 µl
TE MED (ICN, UK)	75 µl	7.5 µl	7.5 µl

2.8.3 Protein visualisation (Western Blotting)

After blocking for 1 h with 10% powdered milk in TBS/T (10mM Tris-HCL, 15mM NaCl, pH 8.3, 0.1% Tween), the nitrocellulose sheets were washed in TBS/T, placed in sealed plastic bags and then reacted with the appropriate primary antibodies overnight at 4°C on a rotating surface (see Section 5.2 for details). The nitrocellulose sheets were given 3 x 5 min washes in TBS/T and incubated for 1 h with either an appropriate HRP conjugated secondary antibody for chemiluminescence detection, or the appropriate fluorescent conjugated antibody for detection using the Licor Odyssey infrared laser detection and imaging system (see Section 5.2 for details).

Table 2.8.4: List of antibodies used throughout this investigation

Antibody	Supplier	Catalogue #	Dilution	Methods Section
Mouse α pan-cytokeratin	Sigma	P2871	1:400	4.2.1
Goat α mouse Alexa 488	Cambridge Bioscience	A-21121	1:500	
Rat α mouse FA/11	N/A	N/A	1:10	4.2.1
Goat α rat Alexa 488	Cambridge Bioscience	A-11006	1:500	
Rabbit α FGF-II	Santa Cruz	SC-1360	1:200	5.2.1
Goat α rabbit HRP	Sigma	A6154	1:400	
Rabbit α FGF-II	Santa Cruz	SC-1360	1:200	5.2.2
Goat α rabbit IR800	Rockland	611-132-003	1:10,000	
Mouse α Actin	Cal Biochem	CP47	1:5000	
Goat α mouse IR680	Molecular Probes	A-21059	1:10,000	
Rabbit α GR	Santa Cruz	SC-1004	1:500	5.2.3
Goat α rabbit IR680	Molecular Probes	A-21077	1:10000	
Mouse α Actin	Cal Biochem	CP47	1:5,000	
Goat α mouse IR800	Rockland	610-131-007	1:10,000	
Goat α IGFBP-I	Santa Cruz	SC-6000	1:300	5.2.4
Goat α mouse IR680	Molecular Probes	A-21059	1:10,000	
Goat α IGFBP-II	Santa Cruz	SC-6002	1:500	5.2.4
Rabbit α goat IR680	Molecular Probes	A-21088	1:10,000	
Mouse α Actin	Cal Biochem	CP47	1:5,000	
Goat α mouse IR800	Molecular Probes	610-131-007	1:10,000	

2.9 Angiotensin II converting enzyme (ACE) activity assay

To measure tissue angiotensin II converting enzyme activity, an assay adapted from Hurst and Lovel-Smith (1981) was employed. ACE is capable of hydrolysing the synthetic substrate Hip-His-Leu into histidyl-leucine and hippurate (Fig. 2.9). When cyanuric chloride (2-4-6 trichloro, 3-5 triazine) is added to a solution of hippurate a colorimetric reaction occurs without interference from any Hip-His-Leu which may be present. The intensity of this colorimetric reaction is proportional to the amount of hippurate present and can be accurately measured using a spectrophotometer.

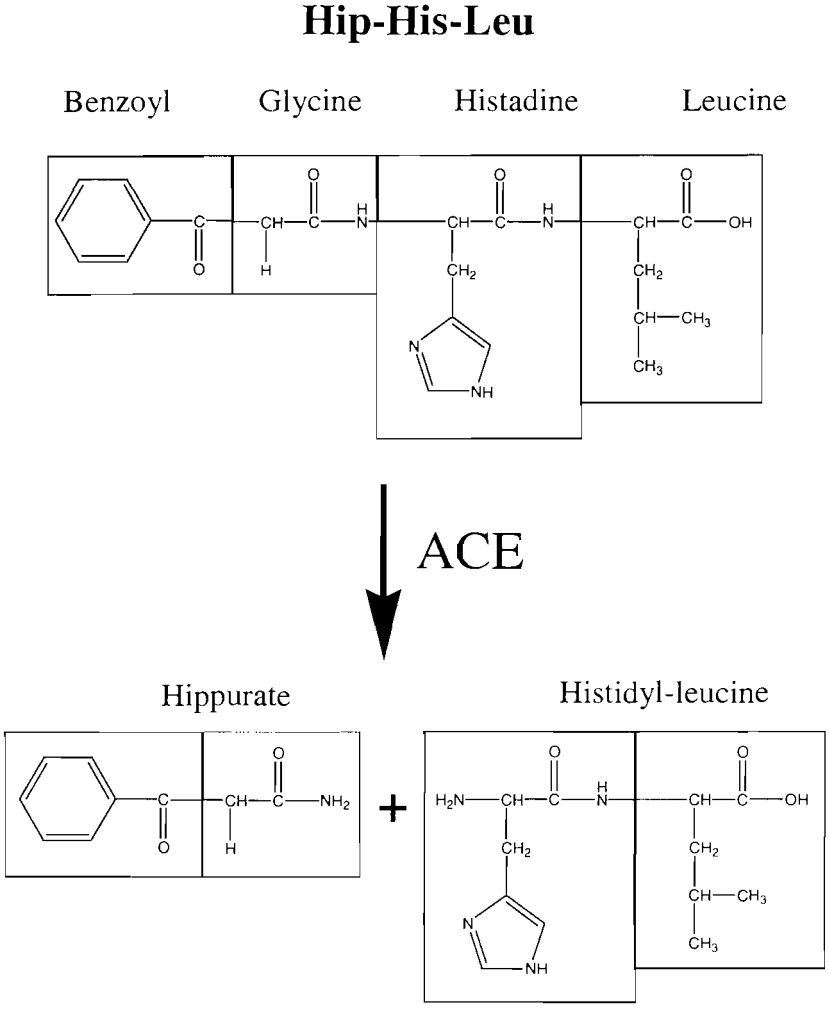

Fig. 2.9: Diagram showing the action of ACE on the synthetic substrate His-Hip-Leu. His-Hip-Leu is composed of benzyol, glycine, histadine and leucine. ACE hydrolyses the His-Hip-Leu such that it is split into hippurate and histadyl-leucine. The hippurate then reacts with cyanuric chloride to form a yellow colour. The formation of this chromogen is limited by the amount of hippurate as the cyanuric chloride is added in excess; as a result the absorbance of the final solution is proportional to the amount of hippurate present.

2.9.1 Tissue ACE assay

Uterine tissue previously stored at -80°C was placed in 300 µl of ice cold PBS and homogenised in either EDTA or EDTA-free complete protease inhibitor cocktail (Roche, UK) on ice using a PowerGen125 homogeniser (Fisher Scientific, UK). The tissue homogenate was then centrifuged at 4°C at 1 000 g for 1 min to remove any large pieces of unhomogenised uterine tissue. Supernatant was removed and stored on ice while total protein was measured using the DC protein assay as described in section 2.7.1.

Known concentrations of total protein were incubated at 4°C in a 2 ml microfuge tube with an appropriate volume of 300 mM KH_2PO_4, 150 mM NaCl, pH 8.3 such that the final volume was 250 µl. To this, 100 µl of 1 mM His-Hip-Leu in 300 mM KH_2PO_4, 150 mM NaCl, pH 8.3 was added to each microfuge tube. Immediately after mixing, the microfuge tubes were transferred to a shaking water bath at 37°C for a known time. The presence of chloride ions is an absolute necessity for ACE to hydrolyse His-Hip-Leu. As a result, at the end on the incubation time, 850 µl of ice cold NaCl-free 100 mM KH_2PO_4 was added to each tube to terminate the reaction and the samples were left on ice for 10 min. 250 µl of a solution of 3 g cyanuric chloride (2-4-6 trichloro 135 triazine) (Sigma) in 100 ml of dioxin (Sigma) was then added to each microfuge tube and left for 10 min at room temperature for the chromogen to develop. Samples were then centrifuged for 2 min at 5 000 g to remove precipitate. 4×200 µl of sample was then pipetted onto a microplate and the absorbance read at 420 nm using a Dinatech MR5000 plate reader.

Sample and standard absorbances were read at 420 nm against a blank of NaCl-free phosphate buffer and cyanuric chloride mixed 2:1. A serial dilution of 1 mM hippuric acid (sigma) in NaCl-free 100 mM KH_2PO_4 was used to create a standard curve (Fig. 2.9.1) which could be used to determine the amount of hippurate produced in each sample. The standard curve was linear over a wide range, Fig.2.9.1 shows a typical standard curve obtained.

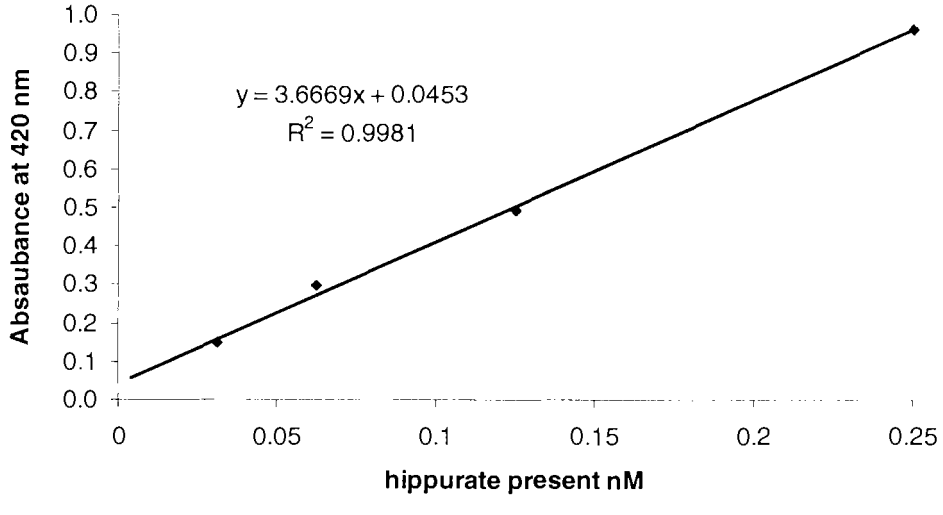

Fig. 2.9.1: Graph showing a standard curve for hippurate present. A serial dilution of 1mM hippuric acid was used to create a standard curve that could be used to determine the amount of hippurate produced in each sample.

2.9.2 Serum ACE assay

Appropriate volumes of serum were incubated at 4°C in a 1.5 ml microfuge tube with 300 mM KH_2PO_4, 150 mM NaCl, pH 8.3 such that the final volume was 37.5 µl. To this, 25 µl of 1 mM His-Hip-Leu in 300 mM KH_2PO_4, 150 mM NaCl, pH 8.3 was added to each microfuge tube. Immediately after mixing, the microfuge tubes were transferred to a shaking water bath at 37°C for a known time. At the end on the incubation time, 437 µl of ice cold NaCl-free 100 mM KH_2PO_4 was added to each tube to terminate the reaction and the samples were left on ice. After 10 min, 250 µl of a solution of 3 g cyanuric chloride (2-4-6 trichloro 135 triazine) (Sigma) in 100 ml of dioxin (Sigma, UK) was added to each microfuge tube and left for 10 min at room temperature for the chromogen to develop. Samples were then centrifuged for 2 min at 5,000 g to remove precipitate. 3×200 µl aliquots of each were loaded onto a microplate and the absorbance read at 420 nm using a Dinatech MR5000 plate reader. Blanks and standard curves used were identical to those used in the tissue ACE activity assay (Section 2.9.1)

2.10 Microarray analysis of uterine tissue

Collection and purification of RNA from uterine tissue was conducted with the assistance of Mr Daniel Miller at the University of Southampton, School of Biological Sciences. Assessing purity of RNA production, amplification of cDNA, Klenow labelling, hybridisation and scanning the microarrays was achieved with the help of Dr Andrew Sharkey at the University of Cambridge, Department of Pathology. Analysis and quantitation of microarray data was conducted with the assistance of Dr Gail Taylor Mr James Tucker and Mr Nethanial Street at the University of Southampton, School of Biological Sciences

2.10.1 Collection and Purification of RNA

To investigate and compare expression of a broad spectrum of genes in the uterus of mice fed the experimental 9% protein diet and the 18% protein control diet uterine horns were dissected out of mice at either day 3.5 or day 4.5 and stored in RNAlater (Qiagen LTD, UK).

RNA was extracted using the RNeasy kit (Qiagen LTD, UK); unfortunately no details could be obtained as to the composition of individual buffers in this kit. The method in short is as follows: Uterine tissue was removed from RNAlater and weighed. Up to 40 ng was then transferred to a 2 ml microfuge tube containing 600 µl GITC-containing buffer with β-Mercaptoethanol (Quigen, UK) then disrupted and lysed using a PowerGen125 homogeniser (Fisher Scientific, UK); this also has the effect of shearing genomic DNA and reducing viscosity. Each sample tissue lysate was then centrifuged for 3 min at 10,000 g and the supernatant transferred to a new 0.5 ml microfuge tube. 600 µl of ethanol (Sigma, UK) was then added to each supernatant and mixed via pipetting. 600 µl of each sample was then added to separate RNeasy columns placed in 2 ml collection tubes and centrifuged for 1 min at 10,000 g; the flow-through was discarded and the procedure repeated with the remaining 600 µl of sample. 700 µl of RW1 buffer was added and each column centrifuged for 1 min at 10,000 g to wash. Each column was then transferred to a new 2 ml collection tube and 500 µl of RPE buffer containing ethanol was added to the column before centrifuging for 1 min at 10,000 g. Another 500 µl of RPE buffer containing ethanol was added and the column centrifuged for 2 min. Columns were then transferred to new 1.5 ml microfuge tubes and 50 µl of RNase free water (Sigma, UK) was added to the columns. RNA was eluted by centrifuging for 1 min at 10,000 g. The quantity and purity of RNA was assessed using a BioPhotometer (Eppendorf, UK).

2.10.2 Production of cDNA (1st strand synthesis) and cDNA amplification

Microarrays require that an experimental sample be compared against a reference sample. Each of the experimental samples consited of 500 ng of total RNA from a single uterine sample placed in a 0.5 ml microfuge tube. Reference samples were produced by pooling together 500 ng of RNA from five 18% control samples; from this master mix,

three 500 ng aliquots were placed in 0.5 ml microfuge tubes for cDNA production. This pooling of the control samples should reduce variation in the final analysis because each experimental sample will be compared to the same reference rather than another individual. Note experimental samples were not pooled but were assayed against the pooled controls.

To each tube, 1 µl CDS Primer [5'-AAG CAG TGG TAT CAA GCG AGA GTA $C\underline{T}_{(30)}N_{(-1)}N$ (note N=A,C,G or T; $N_{(-1)}$ = A,G or C] (Clontech, UK), 1 µl TS primer [5'-d(AAG CAG TGG TAT CAA CGC AGA GTA CGC)r(GGG) note last 3 underlined bases are RNA] (Clontech, UK), 2 µl RNase-free water (Sigma, UK) was added on ice. The microfuge tubes were then incubated for 2 min at 72°C before placing back on ice. 2 µl 5×First Strand Buffer (Clontech, UK), 1 µl 20 nM DTT (Clontech, UK) in RNase-free water, 1 µl 10 mM dNTPs (Invitrogen, UK), 1 µl Powerscript reverse transcriptase (Clontech, UK) was added to each tube before tubes were incubated for 60 min at 42°C in a Perkin-Elmer DNA Thermal Cycler 9600 (Norwalk, CT), samples were then placed back on ice.

To each 2 µl experimental and reference sample from first strand synthesis was added 75 µl RNase-free water, 10 µl 10×PCR Buffer II (Applied Biosystems, UK), 2 µl 10 mM dNTPs, 4 µl 11A Primer [AAG CAG TGG TAT CAA CGC AGA GT] (Clontech, UK), 5 µl 25 mM $MgCl_2$ (Applied Biosystems, UK), 2 µl AmpliTaq (5 U/µl; Applied Biosystems, UK). A Perkin-Elmer DNA Thermal Cycler 9600 was used to amplify cDNA using programme: 95°C 1 min (activate enzyme), 95°C 10 sec, 65°C 10 sec [15 cycles]; final 68°C 6 min, hold 4°C. The reference samples were then pooled, mixed and aliquoted for analysis while experimental samples were stored individually. Samples and references were stored at -20°C until labelling.

2.10.3 Klenow Labelling of cDNA

In a 0.5 ml microfuge tube, 20 µl of Random Primer Buffer (Invitrogen, UK) was added to 21 µl of amplified cDNA from each experimental trial to be arrayed, and an equal number of reference trials. These were incubated for 5 min at 95°C and placed

back on ice. 2 µl (2 nmol) Cy5 labelled dCTP was added to the experimental trials and 2 µl (2 nmol) Cy3 labelled dCTP was added to the reference trials (Amersham Biosciences, UK). 5 µl of 10×Low C-dNTPs (Invitrogen, UK) and 1 µl Klenow (40 U/µl) (Invitrogen, UK) were added to both experimental and reference trials. References and experimental trials were then incubated for 2 hrs at 37°C. After incubation, 5 µl of stop buffer was added (Invitrogen, UK).

G50 columns (Amersham Biosciences, UK) were placed in 2 ml microfuge tubes and the total volume of samples and references were pipetted onto the G50 columns (Amersham Biosciences, UK); these were centrifuged at 2,000 g (this caused any unbound C-dNTPs to be held in column and only labelled cDNAs to be collected). The total volume of each experimental sample was then combined with a reference sample and 5 µl Mouse Cot-1 DNA (Invitrogen, UK), 10 µl 3M Na acetate pH 5.2 and 250 µl 100% ethanol were added. Samples were then left at -20°C for 2 hrs for cDNA to precipitate. Samples were centrifuged at 20,000 g at 4°C for 15 min. Supernatant was removed and the pellet was washed twice with 750 µl 75% ethanol and allowed to air dry in the dark.

2.10.4 Preparation of cover slips/slides and hybridisation

M Series Cover Slips (Erie Scientific Co, NH, USA) were washed in 70% ethanol then rinsed twice in Millipore water and once in isopropanol and allowed to air dry. Cover slips were applied to the GAPS II slides printed with Mouse cDNAs (BBSRC Array Group, Cambridge University, Department of Pathology, UK) such that they covered the printed cDNAs (slides described in detail in Chapter 7, Micro Arrays). Hybridisation buffer (40% deionised formamide, 5×SCC (1×SCC is 75 mM NaCl, 7.5 mM sodium citrate, pH 7.2), 5×Denhardt's (1×Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1 mM Pyrophosphate, 50 mM Tris, pH 7.4, 0.1% SDS) was warmed to 50°C and 50 µl was added to each array such that it was drawn between the cover slip and slide by capillary action. Arrays were then incubated at 50°C for 2 hrs in a humidified box. After incubation, the cover slips were removed from the arrays in 2×SCC and washed/rinsed/air dried as before. The array slides were washed in 2×SCC for 5 min then rinsed the same way as the cover slips before air drying in dark.

The air dried cDNA pellet was re-suspended in 46 µl hybridisation buffer and 2 µl Cot-1 DNA, 1 µl PolyA (Invitrogen, UK), 1 µl yeast tRNA (Invitrogen, UK), at 50°C. Re-suspended cDNA was then incubated for 5 min at 95°C and for 5 min at 50°C. Cover slips were re-applied to the array slide and the 50 µl of probe added to each array such that it was drawn between the cover slip and slide by capillary action. Arrays were then hybridised for 16 hrs at 50 °C in a humidified box. After hybridisation was completed, cover slips were removed from array slides by immersion in 2×SCC. Array slides were then washed twice in 2×SCC in the dark for 5 min, twice in 0.1×SCC/0.1% SDS in the dark for 5 min, and twice in 0.1×SCC in the dark for 5 min. Array slides were then rinsed briefly twice in Millipore water and once in isopropanol. Arrays were then dried by centrifugation for 2 min at 10 g and stored in the dark until scanning.

2.10.5 Reading and analysis of array

Gene features on the array were scanned and digitized using a Genepix 4100 A Scanner (Axon Instruments, UK) using GenePix Pro 4.1 software (Axon Instruments, UK). Spots of an integrated density of less than 500 in either channel were ignored as were spots less than 100 µm in diameter to eliminate false positives and unreliable readings close to background that would interfere with the analysis. Data files were then converted from .gpr format to .mev format using Express Converter software (TIGR, USA) and imported into TIGR MIDAS software (TIGR, USA) for background subtraction and LOWESS normalisation (Quackenbush, 2002). Statistical differences in gene expression levels (intergrated intensity values) were analysed using the Students t-test function in Microsoft Excel and were considerd to be significantly different if they had a $p<0.05$.

2.11 Statistics

2.11 Data normalisation

Data was analysed for normality using the Kolmogorov-Smirnov test (SPSS V12.0) and can be assumed to be normally distributed unless stated. Data found not to

be normally distributed (specifically data in Section 4.3.5) normalised by replacing the values with normal scores. The first step in this technique is to generate a set of random numbers with an equal 'n' to the original set. This set of random numbers has a mean of zero and a standard deviation of 1. The next step is to replace the highest number in the original data set with the highest number in the normal data set, and then the next highest number in the original data set is replaced with the next highest number in the normal data set, and so on until all the values in the original data set have been replaced. Once this is done the data can be analysed by normal statistical means.

2.11.1 Statisical analysis

All data, except those in Chapters 3 and 7, were analysed by Dr Clive Osmond, Senior statitisian, MRC Environmental Epidemiology Unit, University of Southampton. Statistical analysis used either a students t-test (SPSS V12.0) where repeats were simply aliquots of the same sample (i.e. maternal serum, Chapter 6) or random effects regression analysis (STATA) where repeats were collected from different parts of the same organ (i.e left and right uterine horns, Chapter 4) or repeats were measured in separate assays (i.e westen blotting, Chapter 5). The random effects regression analysis takes account of variability derived from different hierarchical data levels (Kwong *et al*, 2004). Bivariate correlations were analysed using SPSS V12.0 (section 6.3.10). All data was considered to be significantly different if $p<0.05$. Data in Chapter 3 was analysed using Students t-test in Microsoft Excel. Microarray data in Chapter 7 was analysed using the Students t-test in Microsoft Excel (See Sections 2.10.5 and 7.2 for detailed explanation of microarray analysis).

Chapter 3

General observations of experimental mice

3.1 Introduction

Previous studies have suggested that administration of a low protein diet increases food intake in non pregnant animals (Colombo *et al*, 1992; Hillgartner and Romsos, 1987; Meliza *et al*, 1974; Swick and Gribskov, 1983; White *et al*, 1994; White *et al*, 1998; White *et al*, 2003). However, reports on food intake in metabolic programming investigations have been somewhat contradictory, some investigations have found no significant differences between LPD and NPD diets (Kwong *et al*, 2000; Langley-Evans, 2000; Rees *et al*, 1999; Sayer *et al*, 2001) and some have found a decrease in food intake where animals have been fed a LPD (Langley and Jackson, 1994; Stephen and Nagy, 1996). However, the decreased food intake in relation to dietary protein restriction observed by Langley and Jackson (1994) and Stephen and Nagy (1996), may be explained by the results of Fangyan *et al* (2000). Langley and Jackson (1994) observed significant decreases in food intake where rats were administered a 6% LPD. Similarly Stephen and Nagy (1996) observed a decrease in food intake where rats were administered a 0.5% LPD. Du *et al* (2000) fed rats 2%, 5%, 8%, 10%, 15% vs. 20% casein diets and demonstrated that food intake in rodents over the range of dietary protein appears to follow a quasi bell-shaped response curve with maximum food intake occurring in rats fed 8–10% casein diet and food intake being severely reduced in rats fed the 2% casein diet. As a consequence the reduction in food intake observed by Langley and Jackson (1994) and Stephen and Nagy (1996) may be a result of severe protein malnourishment.

Results are similarly confused where maternal weight gain is concerned. Some investigations imply that there is no significant difference in maternal weight gain throughout pregnancy where a LPD is administered (Kwong *et al*, 2000; Rees *et al*, 1999). Other investigators report that there is a significant decrease in maternal weight gain where a LPD is administered during gestation (Langley-Evans, 2000; Sayer *et al*, 2001; Langley and Jackson, 1994)

To allow us to understand better our model of dietary protein restriction and aid analysis in later chapters, it is important to characterise the most general aspects of our

experimental model. In this section the impact of maternal LPD on maternal food intake and weight are therefore measured.

3.2 Materials and methods

3.2.1 Weight gain in experimental mice

Mice were weighed on day 0.5 of pregnancy and every day up to day of cull. Results are expressed as weight change in g. Statistical differences were assessed as described in Section 2.11 using the students t-test to measure intra-group and one way ANOVA to measure inter-group differences.

3.2.2 Food intake of experimental mice

Food intake of mice was monitored by weighing food placed into the cage with the mouse at day 0.5 and then measuring the food remaining in the cage on the day of cull. Statistical differences were assessed as described in Section 2.11 using the students t-test to measure intra-group and one way ANOVA to measure inter-group differences.

3.3 Results

3.3.1 Weight gain in experimental mice

The weight of mice administered the LPD and NPD were taken on day of plug and day of cull to assess the effect of protein restriction on weight gain. No significant differences were observed in weight gain between mice fed the LPD and the NPD at any individual time point. Both groups of mice gained a significant and equivalent amount of weight over the experimental period. In the case of those mice fed the 9% LPD weight was significantly increased between day 2.5 and day 4.5 ($p<0.05$). In contrast, mice fed the 18% NPD gained a significant amount of weight between day 2.5 and day 3.5.

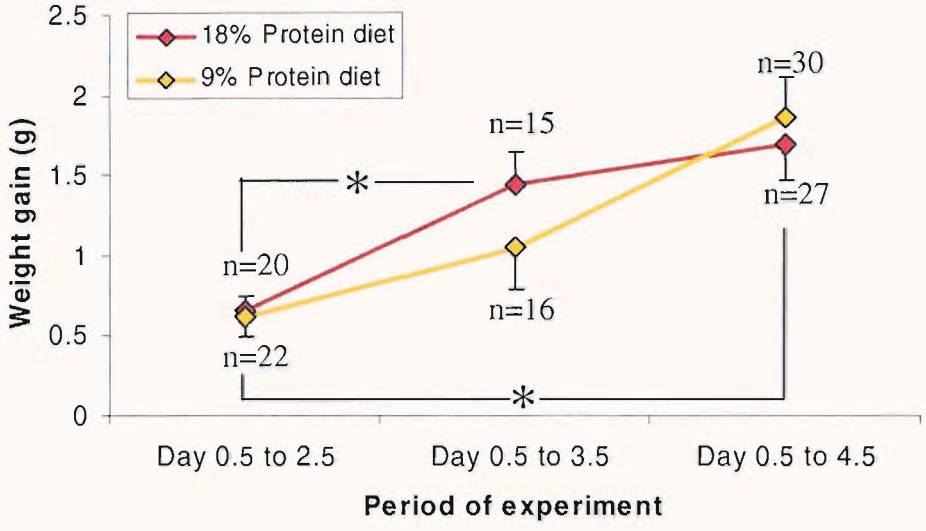

Fig. 3.3.1: Weight gain in grams for mice fed either the 18% NPD or the 9% LPD between day 0.5-2.5, 0.5-3.5, 0.5-4.5. No significant differences were observed in weight gain between the 9% LPD and 18% NPD over any time period of diet administration. However, those treated with LPD exhibited a significant gain in weight between day 0.5 and 4.5 while those treated with the NPD exhibited a significant weight gain between day 0.5 and 3.5. *=p<0.05. Error bars are SEM

3.3.2 Food intake of experimental mice

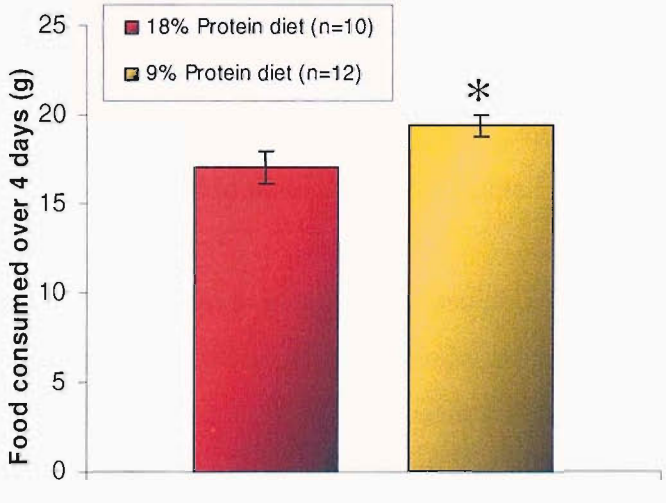

Fig. 3.3.2: Food intake of mice fed the 18% NPD and 9% LPD between day 0.5 and 4.5 post plug. Mice fed the 9% LPD consume significantly more food over the 4 days of diet treatment than those fed the 18% NPD (*=p<0.05). Error Bars are SEM

It was possible that administration of a LPD may affect the appetite of animals and therefore the amount and balance of nutrients that they would ultimately have

available. As a result, monitoring of food intake was undertaken of mice fed the 9% LPD compared to those fed the 18% NPD. Over 4 days diet treatment (day 0.5 to day 4.5 post plug) it was found that mice fed the 9% LPD consume significantly more food than those fed the 18% NPD (18% NPD 17.07±0.88g vs. 9% LPD 19.40±0.64g; $p<0.05$) (Fig. 3.3.2). This represents a ≈14% greater food intake in mice fed the 9% LPD compared to those fed the control 18% NPD.

3.4 Discussion

As previously discussed in section 3.1 there have been conflicting reports regarding weight gain in rodents fed 18% protein diets compared to those fed 9% isocaloric protein diets. As a result I measured weight gain in mice administered the synthetic 18% NPD and 9% LPD. No significant differences were observed in weight gain between the two diets over either 2, 3 or 4 days of administration. Previous studies that have found a significant difference in maternal weight gain have only found it where synthetic diets have been administered over at least 18 days of pregnancy (Langley-Evans, 2000; Sayer *et al*, 2001; Langley and Jackson, 1994). Where metabolic programming studies have measured maternal weight gain over the pre-implantation period (Kwong *et al*, 2000) and the first seven days on pregnancy (Sayer *et al*, 2001), no significant differences were observed in maternal weight gain.

Because previous studies have given inconsistent reports of food intake in response to dietary protein and because mice in our model are allowed to feed *ad libitum* food intake was measured in a limited group of mice over 4 days from day 0.5 to day 4.5 post plug. The results from this study show a small yet significant increase in food intake in mice administered the 9% LPD diet compared to those fed the 18% NPD. The present study varies somewhat from most of the previously published data on dietary intake during metabolic programming studies (Langley-Evans, 2000; Langley and Jackson, 1994; Rees *et al*, 1999; Sayer *et al*, 2001; Stephen and Nagy, 1996) as administration of synthetic diets was for just the first four days of pregnancy. Previous reports have suggested that dams fed a LPD do consume significantly more food than

those fed a NPD during the first week of pregnancy and diet administration (Desai *et al*, 1996). The only other published study, by Kwong *et al* 2000, where food intake was measured in dams over the pre-implantation period comparing a LPD and NPD found no significant differences in food intake.

It has previously been shown that increased food intake and body fat in relation to low dietary protein are associated with an elevation of neuro peptide Y (NPY) gene expression in the basomedial hypothalamus (White *et al*, 1994; White *et al*, 1998). Exogenous NPY is a potent stimulus for food intake and body fat accumulation (Kalara *et al*, 1991) and it is possible that NPY has a causative role in the increase in food intake and body fat that is associated with moderately low-protein diets. NPY levels are influenced by levels of GCs (Strack *et al*, 1995) and it may be inferred that the increase in food intake observed could be due to increased expression of NPY stimulated by elevated GCs. However, serum GCs were not found to be elevated in this dietary protein restriction model (section 6.3.4). Insulin has been shown to inhibit the action of NPY (Schwartz, 1992) and has been shown to be significantly reduced in this study in maternal serum at 3.5 days post plug where mice have been administered a 9% LPD compared to 18% NPD controls (section 6.3.3). Hence, the observed decrease in insulin in this study could be linked to an increase in appetite and food intake, possibly mediated by NPY.

This chapter has examined the most general outcome measures of weight gain and food intake to build a better picture of the responses of MF1 mice to the administration of a 9% LPD and an 18% NPD. Maternal weight gain over 2, 3 and 4 days of synthetic diet administration did not significantly differ between mice fed the 9% LPD and 18% NPD. However, maternal food intake was seen to be significantly increased where mice were fed the 9% LPD compared to 18% NPD controls. I speculate that this increase in food intake may be due to an increase in circulating NPY as a consequence of the decrease in serum insulin observed at day 3.5.

Chapter 4

Analysis of uterine fluid

4.1 Introduction

The contribution of amino acids, glucose and growth factors to embryonic development have been previously discussed in Section 1.5.6. The first phase of my investigation into fetal metabolic programming focuses on collection and analysis of such metabolites in uterine luminal fluid. Components of oviduct and uterine fluid have been studied in great detail in large mammals including heifers (Elhassan *et al*, 2001), ewes (Hill *et al*, 1997) and humans (Casslen & Nilsson, 1984; Gardner *et al*, 1996). Previous investigations have characterised various components of both mouse reproductive fluids including glucose in the uterus (Wales & Edirisinghe, 1989) and oviduct (Gardner and Leese, 1990), pyruvate in the uterus (Wales & Edirisinghe, 1989) and oviduct (Gardner and Leese, 1990) and cations in the uterus (Wales & Edirisinghe, 1989). However, investigations to examine the amino acid composition of mouse oviduct luminal fluid (Dumoulin *et al*, 1992; Guerín *et al*, 1995) have always been confounded because samples either had to be pooled for analysis or the exact dilution factors were unknown. As a result, the information on oviduct fluid amino acid concentration has in the past been expressed as a percentage of total amino acids (Fig. 4.3.2c)

Other studies conducted on embryos *in vitro* have shown that culture media composition can have a direct effect on cell allocation and embryo development. For instance, amino acids (Devreker *et al*, 2001; Lane *et al*, 2001; Lane and Gardener 1997) and glucose (Porter *et al*, 2000; Coats *et al*, 1999) have been shown to affect embryo development in culture. Amino acids have also been shown to affect viability of mouse and rat embryos after transfer (Mehta and Kiessling, 1990; Zhang and Armstrong, 1990; Gardner and Lane, 1993; Lane and Gardner, 1994; Lane and Gardener 1997), although viability studies on ruminant embryos have not been reported.

Kwong *et al* (2000) demonstrated that administration of a low protein diet during pre-implantation development could have a direct effect on blastocyst cell number and cell allocation. It is reasonable to expect that there could be alterations in the composition of luminal fluid in response to altered dietary treatment. Ultimately, any

alterations in the composition of the uterine luminal fluid could have a profound effect on embryo growth and development including cell allocation and even DNA methylation. As such, luminal fluid is a logical place to begin my investigations as its composition could directly affect pre-implantation embryo development and lead to responses which ultimately result in metabolic programming of a thrifty phenotype.

4.2 Methods

4.2.1 Analysis of cellular contents: Perfusion method

Uterine fluid was collected by the perfusion method as described in section 2.5. Due to the small amount of fluid and cells retrieved from within each uterine horn the analysis of the cell types within the luminal fluid was conducted in 'chambers'.

For immunodetection and classification of epithelial cells, the formaldehyde fixation protocol was used (section 2.5.1). A mouse monoclonal anti-pan-cytokeratin (Sigma, UK) antibody was used at a dilution of 1:400 for detection of epithelial cells and was added for 1 hr at room temp; secondary antibody was goat anti mouse immunoglobulin coupled to Alexa 488 (Cambridge Bioscience, UK) used at a dilution of 1:500 and was added for 1 hr at room temp.

For immunodetection and classification of macrophages, the acetone fixation method was used (section 2.5.2). A rat anti mouse FA/11 monoclonal antibody was used at a dilution of 1:10 for detection of activated macrophages (Smith and Koch, 1987; Rabinowitz and Gordon, 1991) for 1 hr at room temp; secondary antibody was goat anti rat Alexa 488 (Cambridge Bioscience, UK) used at a dilution of 1:500 for 1 hr at room temp.

To evaluate antibody specificity and cellular phenotype (epithelial or macrophage origin), cells from fresh perfusions were added to 200 µl T6+BSA media (5.5 mM Glucose, 80 mM Na Cl, 0.5 mM Mg Cl_2, 18.2 mM Lactate, 0.27 mM Pyruvate, 4 mg l^{-1} Bovine Serum Albumin (All ingredients from Sigma, UK)) and incubated on a coverslip

for 24 hours at 37°C and 5% CO_2 so that the cells would attach to the surface before fixation and staining. The cover slips were removed from the incubator and washed three times in PBS. After washing, the cells adhered to the slides were fixed in formaldehyde, there after the cells were immunolabelled as described above. Cells were abundant on all slides as denoted by nuclear Hoechst staining.

4.2.2 Analysis of cellular contents: Direct collection method

Cellular debris was analysed as previously described above except that luminal fluid was collected using the direct collection method (section 2.4) and propidium iodide (Sigma, UK) was used to visualise nuclei instead of Hoescht stain as in section 2.5.

4.2.3 Amino acid concentrations in luminal fluid: Perfusion method

5 µl of perfused uterine luminal fluid was used from the original 10 µl aliquot stored at the same time as the preparation of the glucose samples for analysis to minimise freezing and thawing. Pilot studies showed that taurine was present at a high concentration compared to other amino acids such that it could not be measured with other amino acids; also, this high concentration could often obscure the analysis of alanine concentration due to the large taurine peak amalgamating with the alanine peak. As a result, the 5 µl of fluid used was split into 4 aliquots, 2 x 0.25 µl high dilution (HD) for specific analysis of taurine and alanine and 2 x 2.25 µl low dilution (LD) to measure all other amino acids. To check that our dilutions were correct, 5 µl of 125 µM D-homoserine in PBS was added to the 5 µl of fluid prior to splitting into separate aliquots. Each separate aliquot was diluted up to 20 µl in PBS then 5 µl of 25 µM α-amino butyric acid in PBS was added so that the efficiency of HPLC could be assessed in each run (i.e. the know concentration of α-amino butyric acid allowed the size of its peak to be assessed across individual chomatograms) . After dilution, the HD aliquots had a D-homoserine concentration of 1.1 µM and the LD aliquots had a concentration of 10 µM; both the HD and LD had an α-amino butyric acid concentration of 5 µM. D-Homoserine and α-amino butyric acid were selected because neither is produced naturally and neither would interfere with the detection of other amino acids. Amino acid concentrations were measured as described in Section 2.3.

4.2.4 Amino acid concentration in luminal fluid: Direct collection method

For amino acid analysis, fluid was collected and measured as discussed in section 2.4. Uterine fluid collected from a single uterine horn was then ejected into 25 µl of PBS in a 0.5 ml microfuge tube using a mouth pipette and placed on ice. The procedure was then repeated for the other uterine horn. Once both samples had been collected the microfuge tubes were centrifuged as before (section 4.2.5). After centrifugation, the supernatant was removed and placed in a HPLC tube and stored at -80°C until analysis. Amino acid concentrations were measured as described in Section 2.7.

4.2.5 Glucose concentration in luminal fluid: Direct collection method

For glucose analysis, fluid from a single uterine horn was collected as described in section 2.4, including an assessment of fluid volume collected, then ejected into 60 µl of PBS in a 0.5 ml microfuge tube using a mouth piece and placed on ice. The procedure was then repeated for the other uterine horn. Once both samples had been collected, the microfuge tubes were centrifuged at 4°C for 10 min at 5,000 g to remove cellular contents. After centrifugation, the supernatant was removed and stored at -80°C until analysis. Analysis of glucose concentrations was conducted as described in section 2.6

4.2.6 Insulin concentration in luminal fluid: Direct collection method

For insulin analysis, fluid was collected as described in section 2.4 including an assessment of fluid volume collected. In contrast to Sections 4.2.4 and 4.2.5 fluid from both horns was combined in 5 µl of PBS in a 0.5 ml microfuge tube using a mouth piece and placed on ice. The microfuge tubes were centrifuged at 4°C for 10 min at 5,000 g to remove cellular contents. After centrifugation, the supernatant was removed and stored at -80°C until analysis. Uterine fluid insulin was measured using a Rat insulin sandwich ELISA kit (Crystal Chem International, IL, USA). This kit is also 100% reactive with mouse insulin. Insulin is detectable from 156-10,000 pg ml^{-1}. Absorbance was measured on a Dinatech MR5000 plate reader, measuring wavelength at 480 nm and subtracting wavelength at 630 nm.

4.3 Results

4.3.1 Analysis of cellular contents of luminal fluid collections: Perfusion method

Non-apoptotic cellular lysis is know to be accompanied by, amongst other factors, the release of large amounts of amino acids especially asparagine, glutamine and glutamate. It is possible that the method employed to retrieve uterine luminal fluid may cause mechanical damage to the cells within the uterine wall and hence release amino acids, glucose and other factors which would interfere with the analyses. I therefore, conducted pilot studies to ascertain the extent and types of cellular contents collected in uterine fluid. These data are not related to dietary treatment. Cellular contents of samples isolated from perfusion collection of uterine fluid were examined using immunofluorescence microscopy.

Immunodetection in chambers using anti-cytokeratin antibody on frozen cells from perfused uterine fluid revealed the presence of large aggregations of epithelial cells; these were visible in four of the six chambers examined, Fig. 4.3.1a-c shows typical results. Immunodetection of macrophages originally used the same protocol as for detection of epithelial cells, however, while nuclear Hoechst staining did show that cells were present, macrophages were not identifiable. Where uterine cells were isolated and grown on cover slips for 24 hrs, large numbers of epithelial cells were observed as denoted by clearly visible cytoskeletal elements (Fig.4.3.1 D-F). Cover slips stained with FA/11, however, were negative, indicating the absence of macrophages despite the clear presence of cells indicated by nuclear Hoechst staining (data not shown). As a result, the acetone fixation protocol was adopted (section 2.5.2). Using the acetone fixation protocol only one macrophage was located in one of the six chambers examined (Fig.4.3.1 G-H).

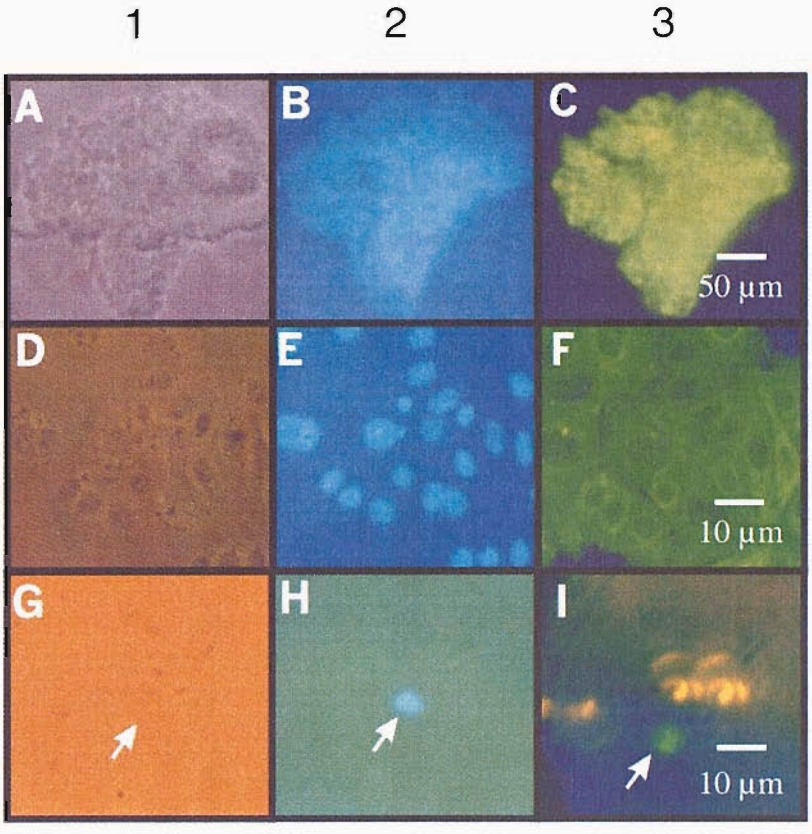

Fig. 4.3.1: Examination of cell types collected from uterine luminal fluid. Column 1 is brightfield photos, column 2 is UV visualisation of nuclei from Hoechst staining and column 3 is immunodetetion of cells. A-C is an epithelial aggregate from frozen cells collected from a perfused uterus. D-F is are uterine epithelial cells isolated from perfused uterine fluid and grown on a slide for 24 hours in T6+BSA. G-H is an activated macrophage from frozen cells isolated from perfused uterine fluid (arrow indicates macrophage). Primary antibodies: Mouse α pan-cytokeratin/Rat α mouse FA/11. Secondary antibodies: Goat α mouse Alexa 488/Goat α rat Alexa 488

Table 4.3.1: Number of epithelial cell aggregates and macrophages isolated from perfusion collection method uterine fluid.

	Number counted/chambers used	Average per chamber
Epithelial cells	15/6	2.5
Macrophages	1/6	0.17

4.3.2 Amino acid composition of uterine fluid: Perfusion collection method

The effect of maternal LPD on the composition of amino acids in the peri-implantation uterine luminal fluid was assessed. Amino acid assays were conducted at the University of York using a Kontron 500 reverse phase HPLC. Perfusions were analysed from 11 mice fed the control 18% protein diet and from 10 mice fed the experimental isocaloric 9% protein diet. Amino acid concentrations obtained from the

two uterine horns for each mouse were averaged (Fig. 4.3.2a). The effects of the maternal LPD on average concentration of individual amino acids per mouse were then analysed using the Students t-test. No significant differences in amino acid concentrations could be detected between treatments when absolute concentrations of amino acids were analysed. There was also no significant difference between the total concentration of essential and non-essential amino acids between treatments (Fig. 4.3.2b)

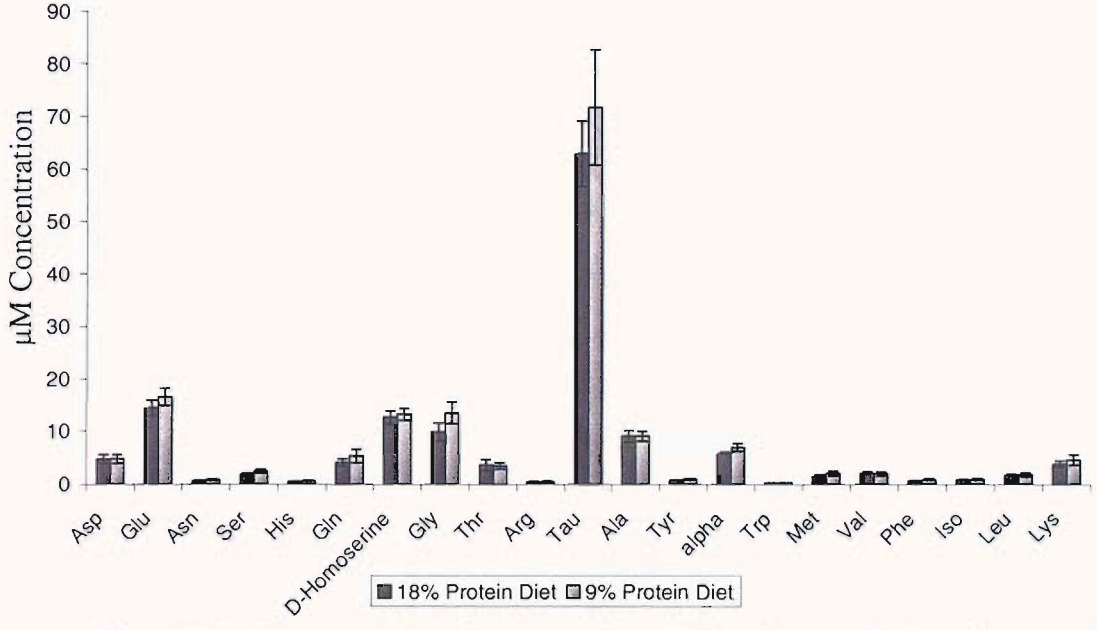

Fig. 4.3.2a: Concentrations of amino acids within uterine luminal perfusions collected from mice fed an 18% NPD and a 9% LPD at 4.5 days post vaginal plug are plotted. Error bars represent SEM. No significant differences are apparent between 9% LPD and the 18% NPD control diet

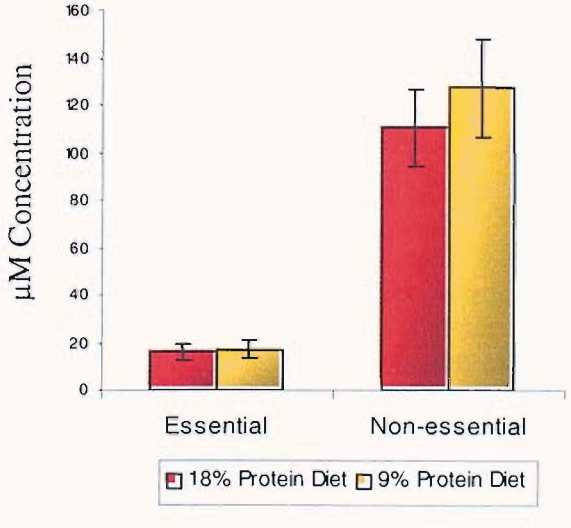

Fig. 4.3.2b: Concentrations of essential and non-essential amino acids in uterine fluid perfused from mice fed an 18% NPD and a 9% LPD at 4.5 days post vaginal plug. No

significant differences in amino acid concentrations between the 9% LPD and 18% NPD control diet. Error bars represent SEM.

It was observed that the concentrations of amino acids measured between perfusions from the two uterine horns of the same animal varied considerably. However, when these concentrations were converted to percentages of total amino acids measured the variation between uterine horns was reduced (data not shown). This variation was concluded to be due to differential dilution of the luminal fluid during perfusion of uterine horns. While 13 µl of fluid was always collected from each perfusion, the amount of fluid left in each horn and the effect of any possible small leaks from the collecting system could not be accounted for. As a result, dilution effects could not be controlled adequately using this method. Consequently, when using the perfusion method for collection of uterine luminal fluid, the proportion of each amino acid in the overall milieu may be a better measure of compositional changes in uterine luminal fluid due to LPD diet. Indeed, if the overall results obtained are converted to percentages and compared to published results of oviduct flushings (Dumoulin *et al*, 1992; Guerin *et al*, 1995) consistency between the two data sets is apparent (Fig. 4.3.2c).

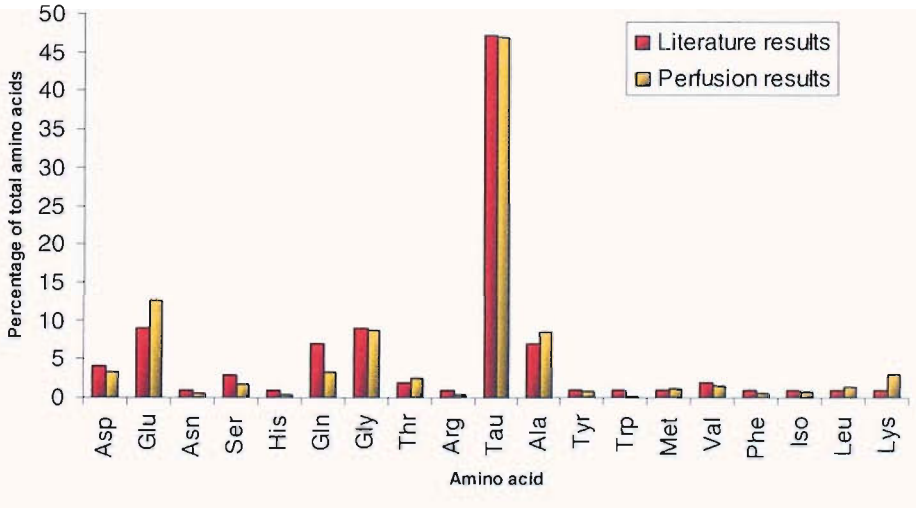

Fig. 4.3.2c: Amino acids, expressed as a percentage of total, collected by perfusion method from mouse uteri 4.5 days post vaginal plug compared with previously published data. Amino acids in the oviduct and their relative amounts are expressed as percentages averaged from two studies (Dumoulin *et al*, 1992; Guerin *et al*, 1995) and compared to the relative amounts of amino acids obtained from luminal flushings 4.5 days post-vaginal plug and analysed using HPLC. Results are derived from luminal fluid of mice fed the 18% control diet.

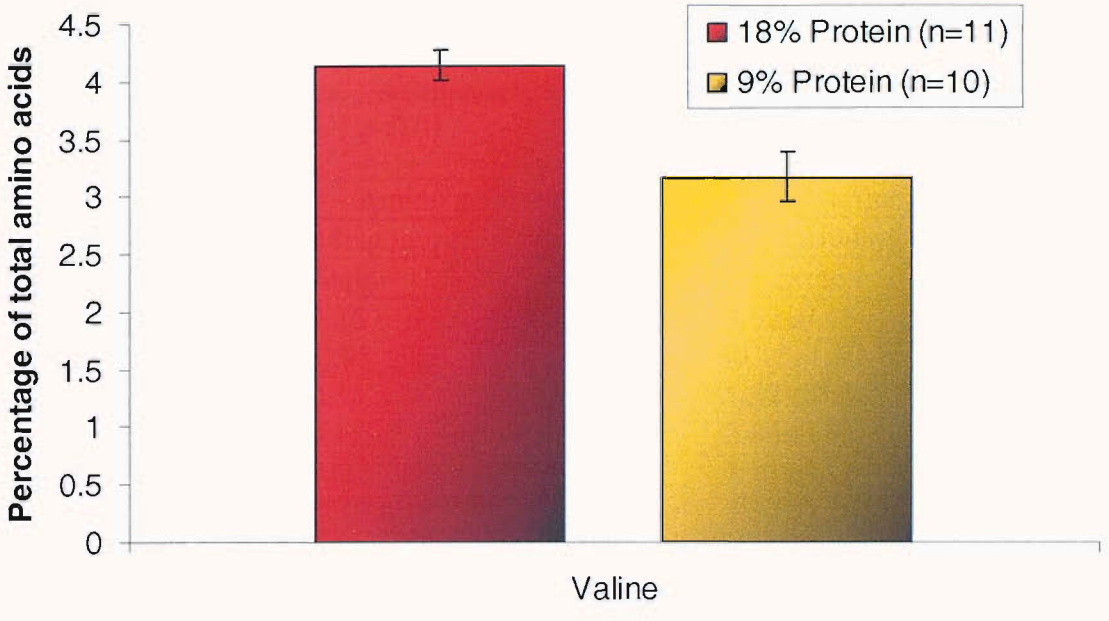

Fig. 4.3.2d: Valine, expressed as a percentage of total amino acids, is decreased in uterine luminal fluid perfused from mice fed the 9% LPD compared to those fed the 18% NPD where taurine and alanine are included in the analysis. Fluid was perfused from uteri 4.5 days post-vaginal plug. Error bars represent SEM. * $P<0.05$

Amino acids converted to percentages and averaged between left and right horns for each animal were subjected to an arc-sin transformation and statistical differences between the proportions of each amino acid were measured using the Students t-test (Table. 4.3.2a). Using this method of analysis, the proportion of valine was found to be significantly depleted (LPD 1.34% vs. control 1.86%; $p<0.05$) in fluid perfused from the peri-implantation uteri of the 9% LPD group compared to the 18% NPD control group (Fig. 4.3.2d). No significant differences were observed in the percentage of essential and non-essential amino acids between the two treatment groups.

Table 4.3.2a: Amino acids expressed as a proportion of total amino acid concentration in uterine fluid collected by the perfusion method. Red text indicates an essential amino acid. Data are presented either with or without the inclusion of taurine/alanine which can confound statistical analysis due to the proportionately higher degree of variation they can exert on the other amino acids. * $p<0.05$; † $p<0.01$

	Amino acid as a percentage of total amino acids			
	Including taurine/alanine		Not including taurine/alanine	
Amino Acid	18% protein	9% protein	18% protein	9% protein
Aspartic acid	3.68±0.26	3.09±0.29	8.09±0.46	7.30±0.5
Glutamic acid	13.55±0.98	11.63±0.71	30.43±1.50	28.49±1.69
Asparagine	0.59±0.38	0.55±0.06	1.32±0.05	1.30±0.08
Serine	1.68±0.16	1.66±.013	3.86±0.32	4.03±0.26
Histidine	0.51±0.09	0.45±0.05	1.13±0.12	1.08±0.11
Glutamine	3.17±0.29	3.33±0.53	7.37±0.76	7.98±1.22
Glycine	8.61±0.84	9.02±0.50	19.29±1.00	22.02±1.59
Threonine	2.81±0.30	2.35±0.24	6.37±0.55	5.49±0.30
Arginine	0.28±0.05	0.31±0.082	0.63±10	0.68±0.15
Taurine	45.5±2.48	48.13±2.59	NA	NA
Alanine	9.06±0.97	8.02±0.13	NA	NA
Tyrosine	0.82±0.08	0.81±1.30	1.88±0.21	1.86±0.22
Tryptophan	0.23±0.02	0.19±0.024	0.52±0.03	0.45±0.04
Methionine	0.19±0.11	1.29±0.277	2.69±0.25	2.89±0.12
Valine	1.86±0.13*	1.34±0.14*	4.14±0.13†	3.17±0.21†
Phenylalanine	0.65±0.05	0.67±0.09	1.46±0.05	1.58±0.11
Isoleucine	0.80±0.06	0.67±0.07	1.81±0.06*	1.58±0.19*
Leucine	1.56±0.11	1.28±0.14	3.52±0.11*	3.02±0.25*
Lysine	3.31±0.32	3.03±0.12	7.55±0.44	7.07±0.71
Essential	12.55±0.48	12.42±1.39	69.76±0.87	72.27±1.92
Non-essential	88.22±0.43	87.74±1.41	30.23±0.86	27.75±1.91

The major problem with analysing each amino acid as a percentage of total amino acids is that the proportion and variability of all amino acids directly influences results of an individual amino acid. Indeed, a decrease in one amino acid will cause an apparent increase in the other amino acids. This effect is especially problematic if one amino acid is subject to high variability, as this variability will be transferred to all the other amino acids within the assay, obscuring possible pertinent changes in relative amino acid proportions. In this experiment it was found that taurine not only contributed about 50% of the total amino acids present in perfused luminal fluid its SEM was also up to 5 times greater than most other amino acids within the analysis. While this larger SEM does reflect its higher concentration, it confounds analysis of other amino acids in terms of a proportionate analysis. Alanine also presented a confounding influence due to its

"shouldering" of taurine (i.e. the two peaks occur alongside each other in HPLC data sheets). As a result, the proportions of amino acids present in perfused luminal fluid from the 9% LPD and the 18% NPD groups were analysed with taurine and alanine excluded in an attempt to reduce the variation of the other amino acids (Table 4.3.2a). When amino acids were analysed in this way, in addition to valine (LPD 3.17% vs. control 4.14%; $p<0.01$), leucine (LPD 3.02% vs. control 3.52%; $p<0.05$) and isoleucine (LPD 1.58% vs. control 1.81%; $p<0.05$) also exhibited a significant reduction as a proportion of total amino acids in the LPD samples (Fig. 4.3.2e).

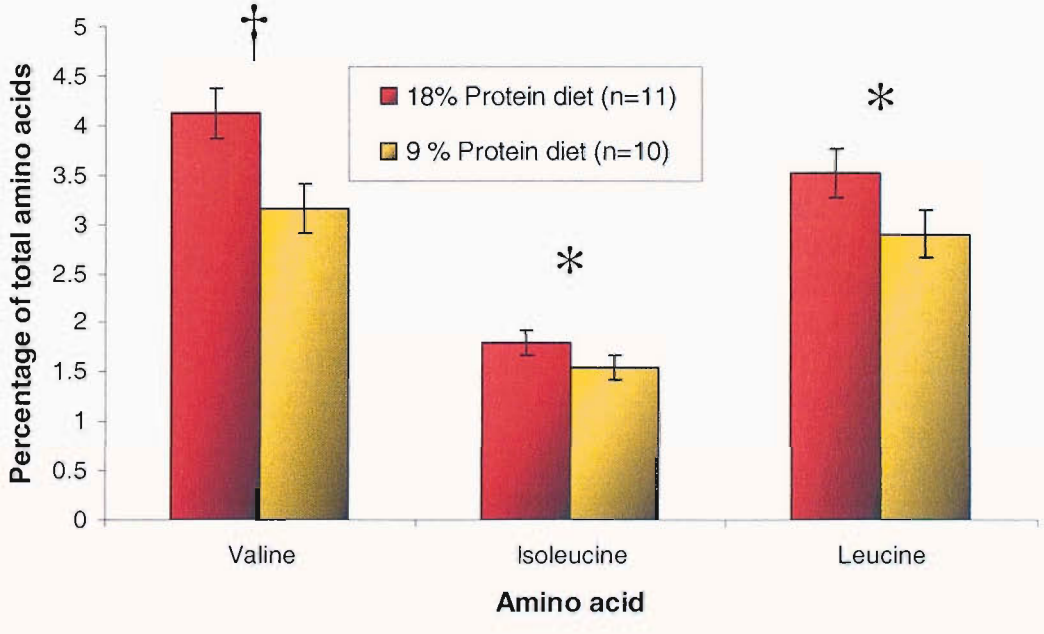

Fig. 4.3.2e: The proportions of alanine, valine, isoleucine and leucine are significantly reduced in uterine luminal fluid when taurine and alanine are removed from the analysis. The relative proportions of amino acids perfused from uteri 4.5 days post plug with taurine and alanine excluded are displayed. Error bars represent SEM. * $p<0.05$; † $p<0.01$

A further concern with the perfusion method was the length of time and variation in time that a single collection might take to complete, between 15 and 45 min. It was possible that over the course of the perfusion, the uterus may be leaching amino aids into the lumen and confounding readings. As a result, amino acid levels (expressed as a percentage of total amino acids) were compared between either left or right uterine horns perfused immediately after cull and the counterpart uterine horns perfused 30 min after cull (n=5) during which time the uterine horns were kept on ice. Fluid collected in this way was analysed by HPLC statisticaly evaluated by paired t-test (Table 4.3.2b).

Table 4.3.2b: Amino acids, expressed as a percentage of total amino acid concentration, in uterine luminal fluid either perfused from the uterus immediately after cull or after 30 min with uterine horns kept on ice. Red text indicates an essential amino acid.

	Amino acid as a percentage of total amino acids	
Amino Acid	T + 0 min	T + 30 min
Aspartic acid	4.39±0.96	4.40±0.68
Glutamic acid	12.20±0.81	11.40±0.38
Asparagine	0.49±0.03	0.46±0.05
Serine	1.65±0.38	1.88±0.34
Histidine	0.51±0.053	0.50±0.04
Glutamine	2.94±0.40*	3.89±0.54*
Glycine	9.12±0.77	9.96±0.28
Threonine	2.92±0.33	2.90±0.34
Arginine	0.28±0.08	0.35±0.05
Taurine	50.46±3.46	49.49±1.87
Alanine	6.35±0.51	5.08±1.08
Tyrosine	0.82±0.08	0.86±0.017
Tryptophan	0.19±0.02	0.21±0.02
Methionine	0.97±0.12	0.95±0.16
Valine	1.53±0.19	1.45±0.07
Phenylalanine	0.49±0.052	0.51±0.02
Isoleucine	0.75±0.08	0.69±0.03
Leucine	1.47±0.23	2.46±1.16
Lysine	2.72±0.23	2.50±0.148
Essential	12.21±1.19	12.84±1.39
Non-essential	88.81±1.11	88.17±1.39

Overall, using this method of analysis, the proportion of glutamine was found to be significantly elevated (0 min 2.94±0.04% vs. +30 min 3.89±0.54%; $p<0.05$) in fluid perfused from peri-implantation uteri 30 min post cull compared with those perfused immediately (fig. 4.3.2f). No differences were found for any other amino acid or for essential and non-essential amino acids.

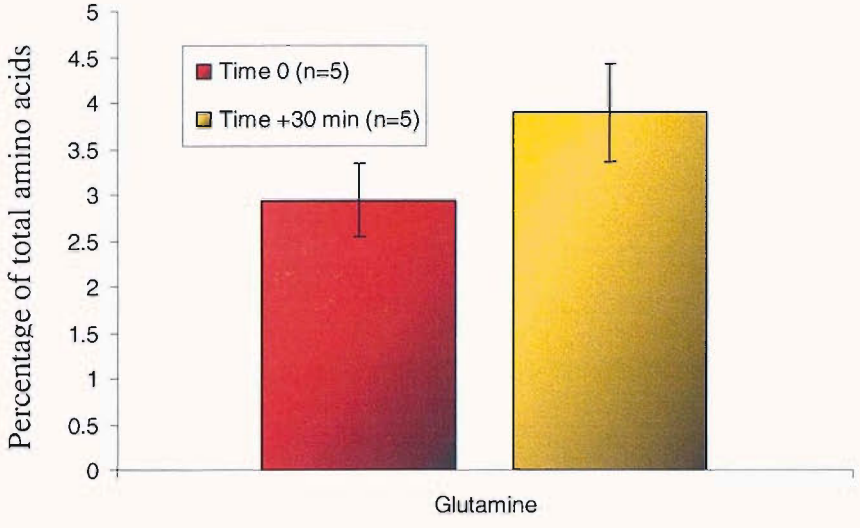

Fig 4.3.2f: The proportion of glutamine is significantly increased ($p<0.05$) in uterine luminal fluid perfused from mice 4.5 days post plug fed an 18% NPD immediately after cull compared to those perfused 30 min post cull. Error bars represent SEM.

4.3.3 Analysis of cellular contents of luminal fluid: Direct collection method

Immunodetection in chambers using anti-cytokeratin antibody on freshly isolated cells from directly collected uterine fluid once again revealed the presence of large aggregations of epithelial cells. These were visible in all four of the chambers examined (Table 4.3.3), Fig. 4.3.3a & b show typical results. Of the four chambers examined for the presence of macrophages, all were positive (Table 4.3.3)

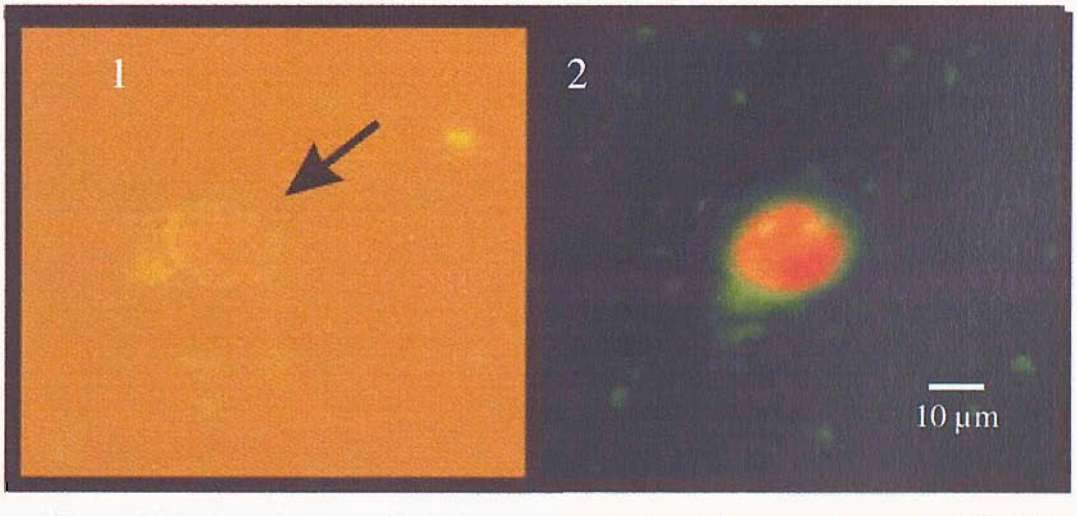

Fig. 4.3.3a: Examination of cellular contents isolated from uterine luminal fluid by the direct collection method and stained for macrophages. 1) Bright field view. 2) Combined channel view, red staining is nucleus (propidium iodide), green staining is FA11 expressed on the cellular membrane of the activated macrophage. Primary antibody: Rat α mouse FA/11. Secondary antibody: Goat α rat Alexa 488

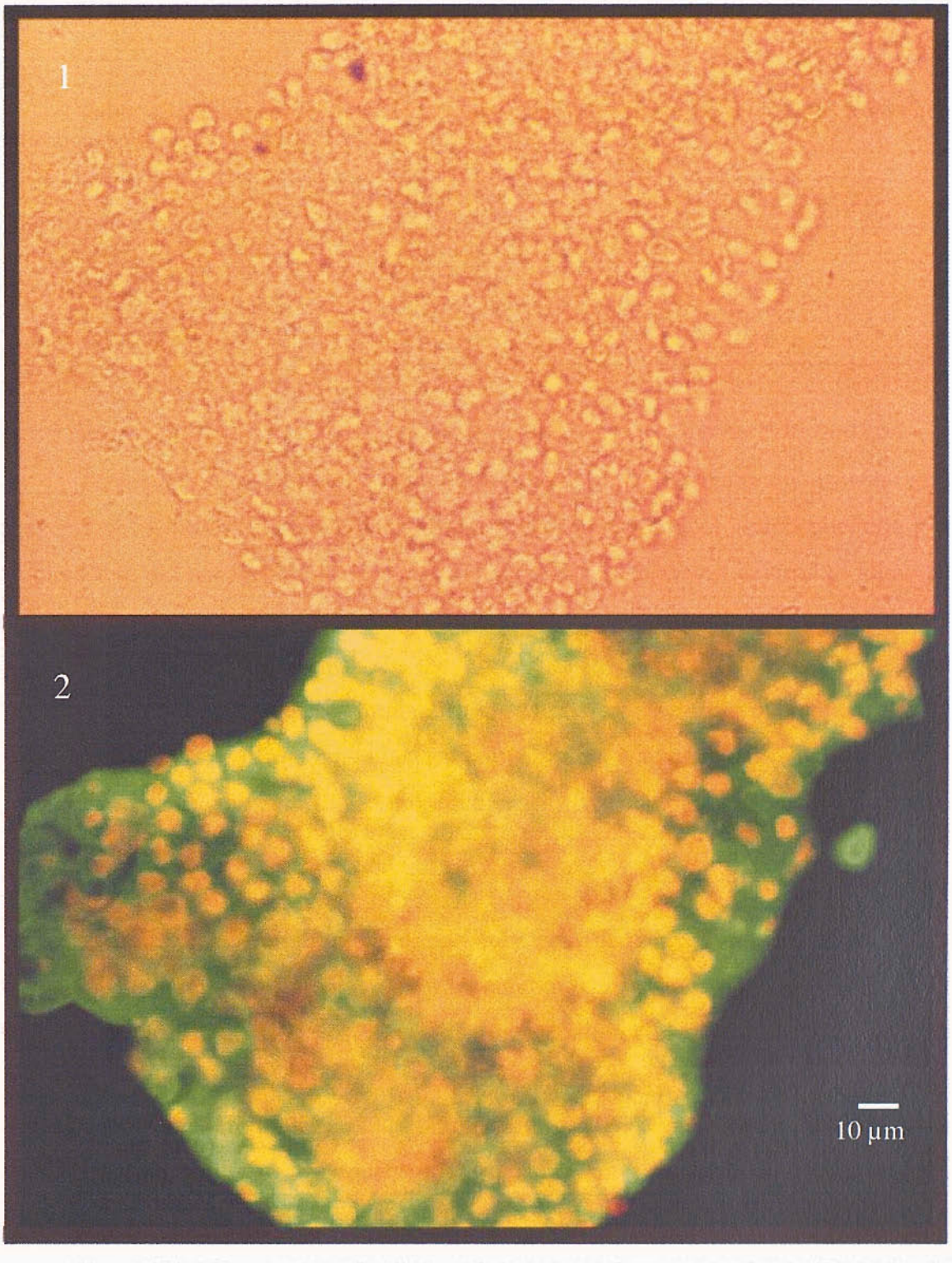

Fig. 4.3.3b: Examination of cellular debris isolated from uterine luminal fluid by the direct collection method and stained for epithelial cytokeratin filaments. 1) Bright field view of a "raft" of epithelial cells. 2) Combined channel view, red staining represents nuclei (propidium iodide), green staining is cytokeratin a cytoskeletal component of epithelial cells. Primary antibody: Mouse α pan-cytokeratin Secondary antibody: Goat α mouse Alexa 488

Table 4.3.3: Number of epithelial cell aggregates and macrophages isolated from direct collection method uterine fluid.

	Number counted/chambers used	Average per chamber
Epithelial cells aggregates	12/4	3
Macrophages	8/4	2

4.3.4 Amino acids composition of uterine fluid: Direct collection method

To acquire more precise measurements of glucose, amino acids and growth factor concentrations within uterine luminal fluid, a second method of fluid collection was required that did not include its dilution. This second method was devised to allow for precise measurement of the volume of fluid collected. This technique was originally piloted on anaesthetised animals in the hope that this would avoid tissue necrosis affecting the composition of the luminal fluid. However, it was discovered that uterine fluid samples collected from anaesthetised animals were contaminated with blood. This problem was not apparent in samples collected from animals culled via cervical dislocation.

The direct collection method not only provided measurements of the exact volume of the sample collected, it also was found to reduce dramatically the variation in the samples measured. This was observed within the fluid collected from different horns of the same mouse (Fig 4.3.4a). The balance of amino acids (amino acid as percentage of total amino acids from uterine fluid of mice fed the 18% control diet) collected by this method is comparable to previously published data on amino acid levels in oviduct (Dumoulin *et al*, 1992; Guerín *et al*, 1995) and to results previously obtained from the perfusion collection method (Fig 4.3.4b). Additionally it was found that there was little correlation between the balance of amino acids in uterine luminal fluid and maternal serum, both in terms of percentage and absolute concentration (Fig. 4.3.4c and d)

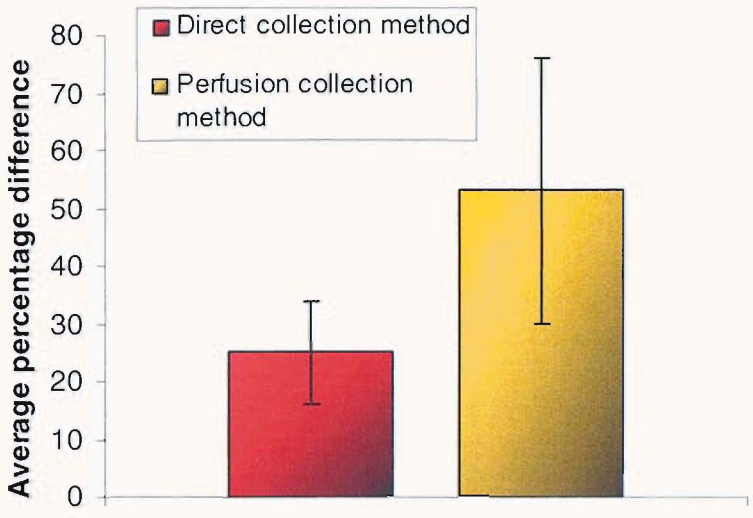

Fig 4.3.4a: Average percentage difference in concentration between amino acids collected from each uterine horn of individual mice using the perfusion method and the direct collection method. Note that the direct collection method resulted in decreased average percentage difference between the concentrations of amino acids collected from each uterine horn from an individual mouse. Results are expressed as a percentage as the *direct collection* yielded concentrations approximately ×100 that of the *perfusion method*.

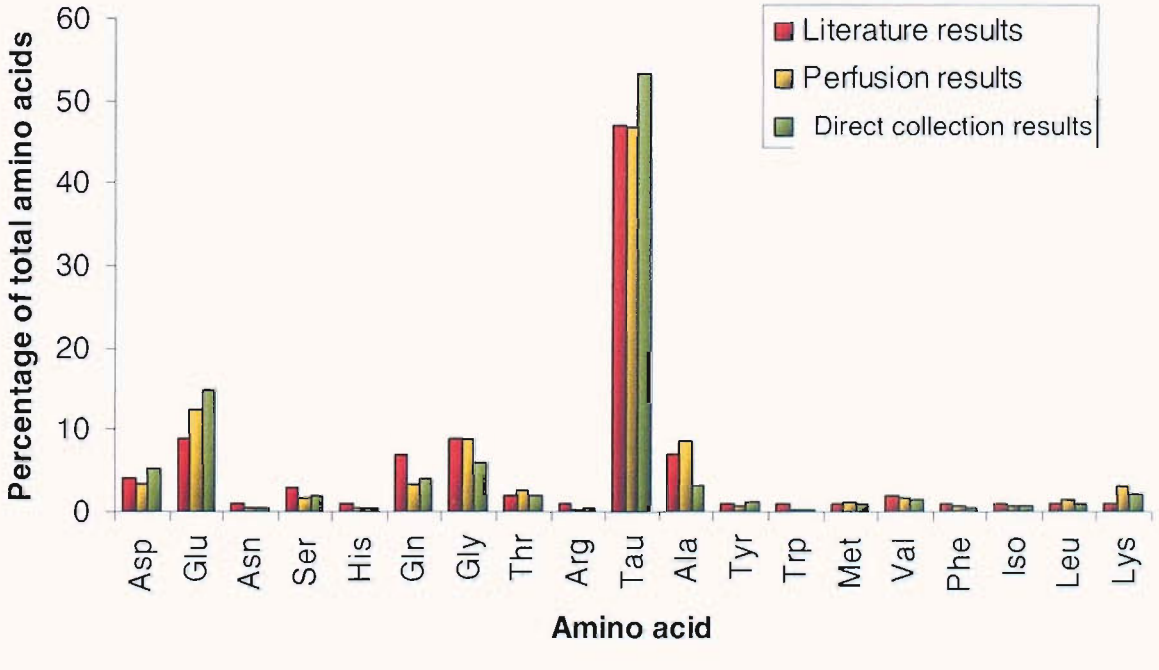

Fig. 4.3.4b: The balance of amino acids in uterine luminal fluid. Note that similar results have been obtained using the perfusion method and the direct collection method and that these results correlate with published literature results for oviduct (Dumoulin *et al*, 1992; Guerin *et al*, 1995). Perfusion method and direct collection method results are derived from luminal fluid of mice fed the 18% control diet.

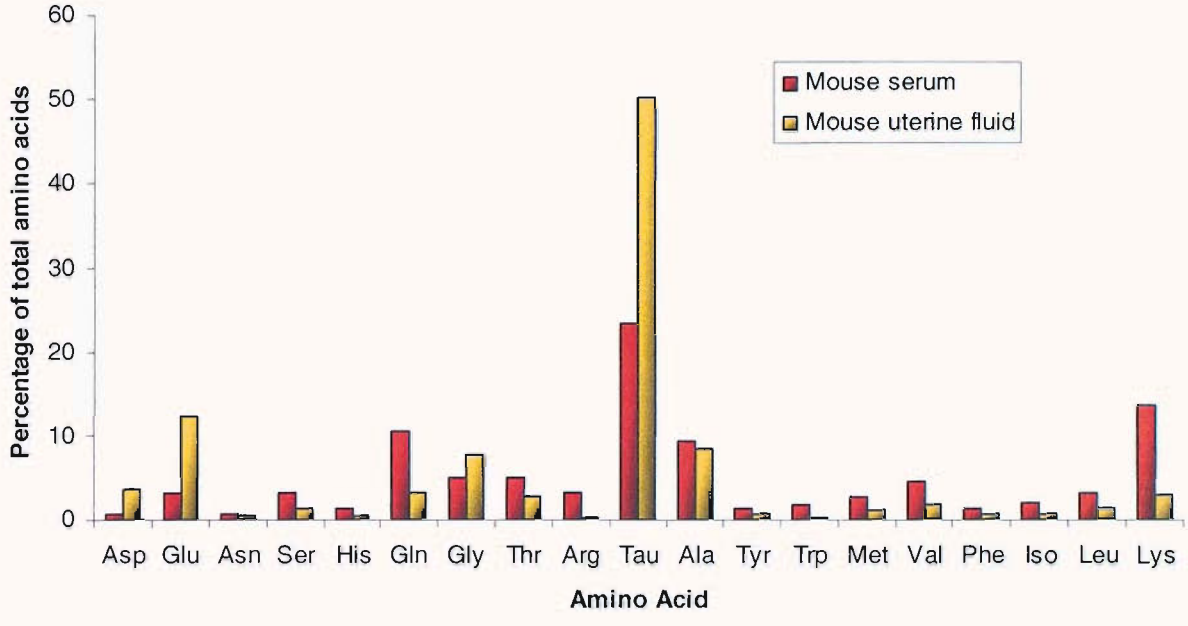

Fig. 4.3.4c: Relative balance of amino acids in uterine luminal fluid and serum. These data suggests that there is little or no contamination of uterine fluid by blood. Serum amino acid results converted to percentages from data presented in section 6.2.7.

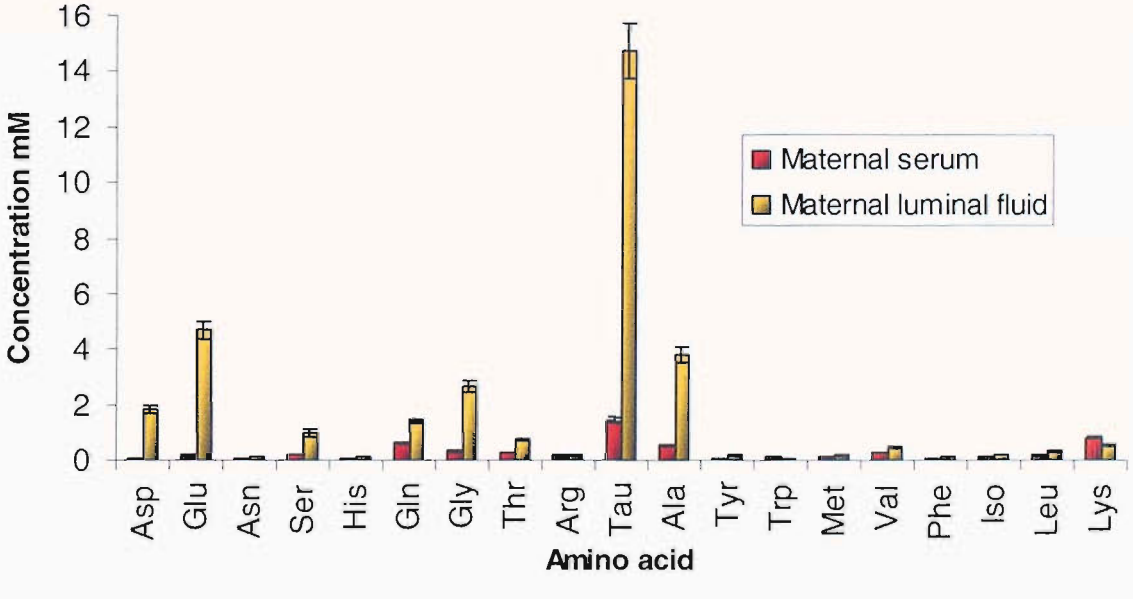

Fig. 4.3.4d: Amino acid concentrations in serum and uterine fluids of mice fed an 18% NPD collected on day 3.5 post plug.

Table 4.3.4: Amino acid concentrations within uterine luminal fluid of mice at either 2.5, 3.5 or 4.5 days of pregnancy fed either the 18% NPD or the 9% LPD diet collected using the direct collection method. Red text indicates an essential amino acid. Differences between diet treatment at individual time points represented by *=$p<0.05$; †=$p<0.005$. Differences between time points within the same diet treatment represented by a=$p<0.05$; b=$p<0.01$; c=$p<0.001$

	Amino acid Concentration (mM)					
	2.5 days		3.5 days		4.5 days	
Amino Acid	18% protein	9% protein	18% protein	9% protein	18% protein	9% protein
n	10	10	12	13	15	15
Aspartic acid	1.46±0.27	1.57±0.14	1.83±0.17c	1.54±0.12c	1.25±0.05c	1.17±0.08c
Glutamic acid	3.72±0.56	3.83±0.33	4.72±0.31c	4.04±0.38	3.47±0.16c	3.44±0.15
Asparagine	0.11±0.02	0.12±0.01	0.14±0.011a	0.13±0.02	0.10±0.01a	0.12±0.01
Serine	0.61±0.23	0.73±0.19	0.97±0.15b	0.76±0.09b	0.41±0.04b	0.42±0.04b
Histidine	0.28±0.06b	0.26±0.05c	0.14±0.02ba	0.11±0.01c	0.09±0.01a	0.09±0.01
Glutamine	0.91±0.16	1.07±0.11	1.41±0.09c	1.22±0.09	0.9±0.05c	0.97±0.05
Glycine	2.91±0.42b	3.45±0.53b	2.68±0.22bc	2.25±0.24b	1.3±0.09c	1.47±0.12b
Threonine	0.28±0.05c	0.32±0.05c	0.72±0.05ca	0.57±0.05cb	0.47±0.05a	0.42±0.05b
Arginine	0.18±0.03	0.18±0.02	0.16±0.02c	0.14±0.02c	0.13±0.01	0.12±0.1c
Taurine	11.19±1.17	13.51±1.76	14.74±1.00	12.09±0.90	12.07±1.00	11.15±0.59
Alanine	1.20±0.23c	1.33±0.21c	3.80±0.27c	3.32±0.34c	0.73±0.08c	0.74±0.05c
Tyrosine	0.43±0.11b	0.34±0.06b	0.18±0.01b	0.21±0.03b	0.33±0.10	0.20±0.02
Tryptophan	0.05±0.01	0.05±0.01	0.06±0.01	0.07±0.01	0.07±0.02	0.06±0.1
Methionine	0.14±0.02	0.20±0.04	0.18±0.01	0.21±0.02	0.20±0.01†	0.27±0.02†
Valine	0.61±0.08b	0.51±0.07a	0.45±0.03†b	0.31±0.02†a	0.36±0.02†b	0.27±0.02†a
Phenylalanine	0.17±0.03	0.17±0.02	0.14±0.01b	0.12±0.01a	0.10±0.01b	0.10±0.01a
Isoleucine	0.20±0.04	0.19±0.03	0.21±0.02*b	0.16±0.01*a	0.16±0.01*b	0.13±0.01*a
Leucine	0.42±0.05	0.42±0.06b	0.32±0.02*	0.24±0.01*b	0.26±0.01*	0.23±0.02*
Lysine	0.39±0.0b	0.39±0.04	0.50±0.02b	0.51±0.08	0.46±0.03	0.50±0.02
Essential	2.68±0.38	2.64±0.30	2.88±0.17a	2.15±0.27	2.33±0.13a	2.18±0.10
Non-essential	22.31±2.86	25.76±2.82	26.27±1.21a	21.49±1.21b	20.08±1.69a	18.00±0.93b
Total	24.77±3.08	28.40±3.87	29.15±3.27a	23.86±1.41a	22.42±1.82a	20.19±1.04a

Uterine luminal fluid of mice treated with either the 18% NPD or the 9% LPD and collected using the direct collection method was analysed for amino acid concentrations as previously described (Table 4.3.4). No significant differences were observed between amino acid concentrations in uterine luminal fluid of mice fed the LPD and NPD on day 2.5 of pregnancy. It was found that on day 3.5 the essential amino acids valine (LPD 0.31±0.02 mM vs. 0.45±0.03 mM control; $p<0.005$), isoleucine (LPD 0.16±0.01 mM vs. 0.21±0.02 mM control; $p<0.05$) and leucine (LPD mM 0.24±0.01 vs. 0.32±0.02 mM control; $p<0.05$) were significantly depleted in mice fed the 9% LPD compared to controls (Fig. 4.3.4e).

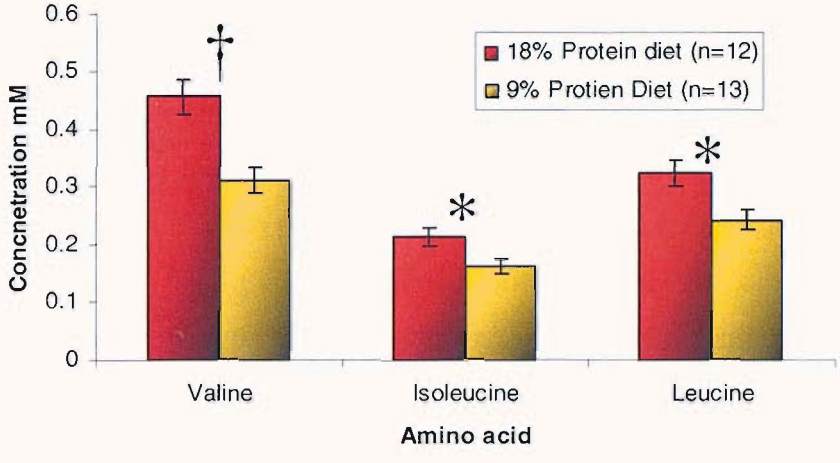

Fig 4.3.4e: Amino acids which display a significant decrease in concentration within uterine luminal fluid on day 3.5 of pregnancy in response to maternal LPD. Fluid was collected using the *direct collection method*. *=$p<0.05$; †=$p<0.005$

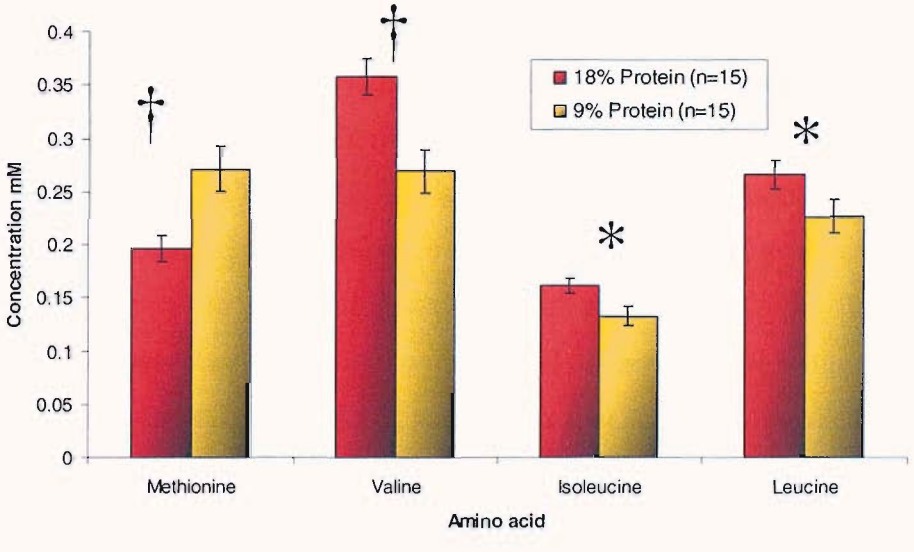

Fig. 4.3.4f: Amino acids which display a significant difference in concentration within uterine luminal fluid on day 4.5 of pregnancy in response to maternal LPD treatment. Fluid was collected using the *direct collection method*. *=$p<0.05$; †=$p<0.005$

The analysis conducted at day 4.5 of development yielded similar results, valine (LPD 0.31±0.02 mM vs. 0.45±0.03 mM control; $p<0.005$), isoleucine (LPD 0.13±0.01 mM vs. 0.16±0.01 mM control; $p<0.05$) and leucine (LPD 0.31±0.02 mM vs. 0.45±0.03 mM control; $p<0.05$) all exhibited reductions in concentrations in the 9% LPD samples compared to the 18% NPD samples. Additionally, it was found that methionine (LPD

0.31±0.02 mM vs. 0.27±0.02 mM control; $p<0.005$) was significantly increased in uterine luminal fluid from mice administered the 9% LPD (Fig. 4.3.4f).

Between day 2.5 and 3.5 the concentration of essential amino acids and total amino acids significantly increased ($p<0.05$) in uterine luminal fluid of mice treated with the 18% NPD; however, no such pattern was observed in the uterine fluid of mice treated with the 9% LPD (Figs. 4.3.4g and i). Within uterine luminal fluid of mice treated with the 18% NPD the individual amino acids glutamine, threonine, alanine and lysine were significantly elevated in concentration while histadine, tyrosine, and valine were significantly decreased in concentration (Fig. 4.3.4j). Similarly in the uterine luminal fluid of mice treated with the 9% LPD the concentration of threonine and alanine was significantly increased and the concentration of histidine, tyrosine, glycine, leucine and valine were significantly decreased.

In contrast, the concentration of non-essential amino acids and total amino acids was significantly reduced ($p<0.05$) between day 3.5 and 4.5 in the uterine luminal fluid of mice treated with the NPD and LPD (Figs. 4.3.4g and I). Additionally, between day 3.5 and 4.5 a significant depletion ($p<0.05$) in the concentration of total essential amino acids was observed in the uterine luminal fluid of mice treated with the NPD; this difference was not observed in uterine luminal fluid of mice treated with the LPD (Fig. 4.3.4h). Depletions in individual amino acids were also observed between day 3.5 and day 4.5 of pregnancy (Table 4.3.4). In mice fed the 18% NPD 11 amino acids namely, aspartic acid, glutamic acid, asparagine, serine, histidine, glutamine, glycine, threonine, valine, phenylalanine and isoleucine were significantly depleted on day 4.5 compared to day 3.5 (Fig.4.3.4h). In mice fed the 9% LPD, 7 amino acids, aspartic acid, serine, glycine, threonine, valine, phenylalanine and isoleucine were significantly depleted on day 4.5 compared to day 3.5; glutamic acid, glutamine, histidine and serine did not exhibit a significant depletion in concentration (Fig. 4.3.4k).

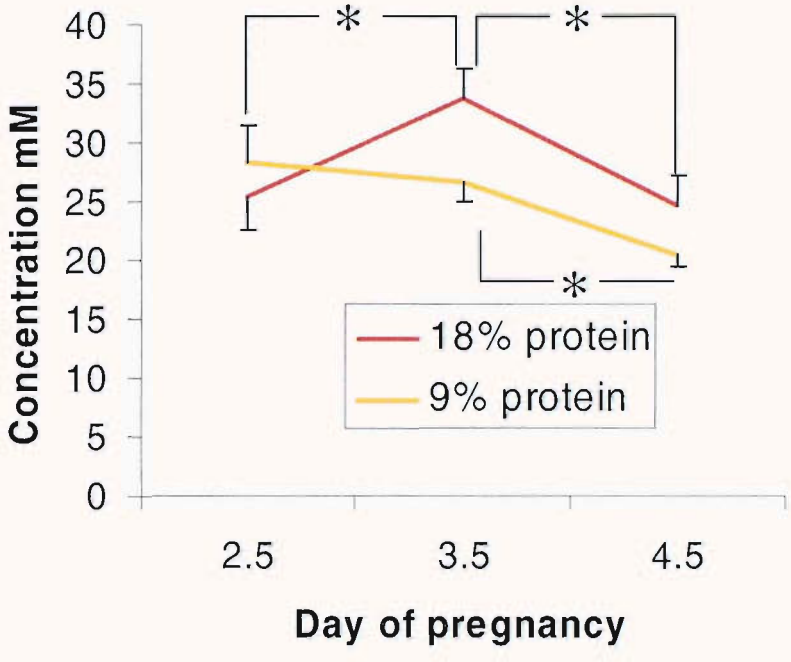

Fig. 4.3.4g: Change in concentration of total amino acids in uterine luminal fluid between day 2.5 and day 4.5. Total amino acids rise between day 2.5 and 3.5 of pregnancy then fall significantly between day 3.5 and 4.5 in uterine fluid of mice fed the 18% NPD. In contrast mice fed the LPD only exibit a significant drop in amino acid contentration between day 3.5 and 4.5. Fluid collected using the direct collection method. Error bars are SEM. *=p<0.05.

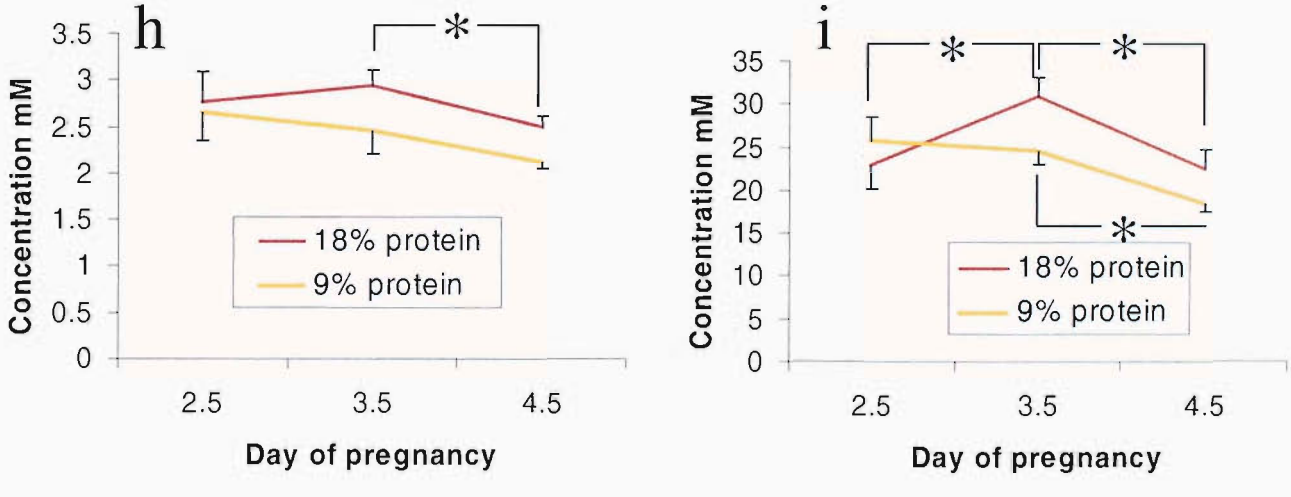

Fig. 4.3.4h & I: Change in concentration of essential (h) and non-essential amino acids (i) in uterine luminal fluid between day 3.5 and day 4.5. Fluid collected using the *direct collection method*. *=p<0.05.

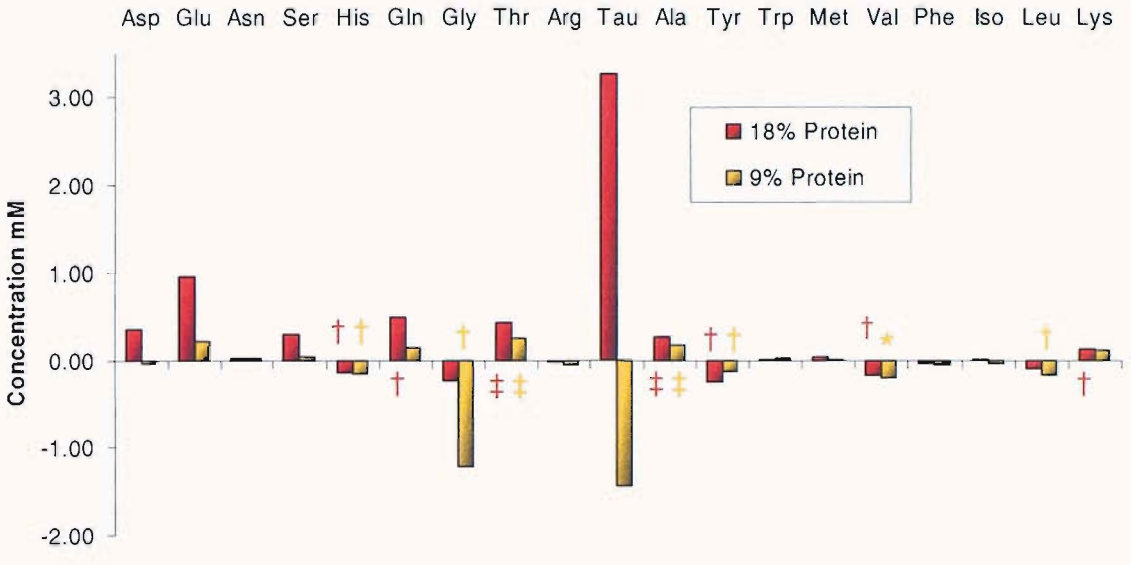

Fig 4.3.4j: Change in concentration of individual amino acids in uterine luminal fluid between day 2.5 and day 3.5. Fluid collected using the direct collection method. *=$p<0.05$; †=$p<0.01$ ‡=$p<0.001$. Note, no error bars are shown as only the overall difference is displayed because data is not paired.

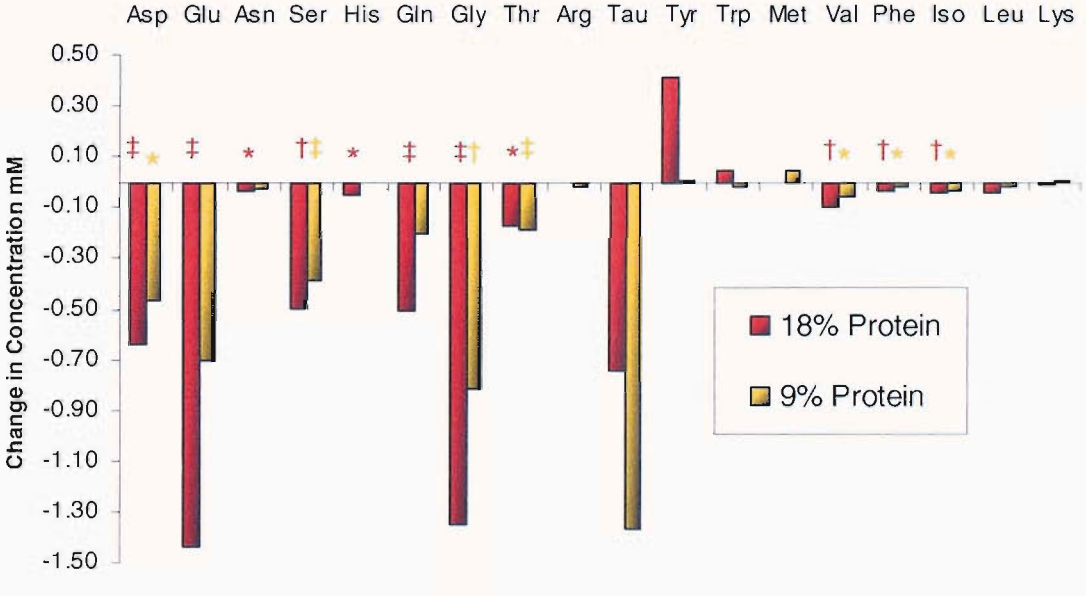

Fig 4.3.4k: Change in concentration of individual amino acids in uterine luminal fluid between day 3.5 and day 4.5. Fluid collected using the direct collection method. *=$p<0.05$; †=$p<0.01$ ‡=$p<0.001$. Note, no error bars are shown as only the overall difference is displayed because data is not paired.

4.3.5 Uterine luminal Glucose: Direct Collection method

Uterine luminal glucose levels were measured by collecting fluid via the direct collection method (Section 2.4) and analysis of the samples using the Cobas Mira (Section 2.6). Glucose concentrations were measured in the uterine fluid of mice fed either the 18% NPD or the 9% LPD on either day 2.5, 3.5 or 4.5 of pregnancy. Pilot studies conducted on fluid collected from uteri on day 4.5 of pregnancy suggested that samples of fluid from single uteri could be accurately and consistently analysed for glucose content by dilution into 60 µl of PBS (Table 4.3.5a). However, when glucose concentrations were measured in samples collected from day 3.5, significantly more samples were found to be below the detection threshold (Table 4.3.5b). The same phenomenon was also observed on day 2.5 to an even greater extent (Table 4.3.5c). Samples that do contain some glucose but are below detection limits are labelled "LOW" in Tables 4.3.5a-c. Overall, ≈70% of samples were below detection limits on day 2.5 while only ≈12% of samples were below detection limits on day 4.5. To overcome this problem the data was normalised by replacing all 110 values with normal scores (see Section 2.11). After normalisation, no significant differences were found to exist in glucose concentrations between the two diets at any time point. However, glucose concentrations were found to significantly rise between all time points in both dietary treatments; this is shown in relative terms using the normalised data in Fig.4.3.5.

Table 4.3.5a: Non-normalised data obtained from analysis of glucose levels in day 4.5 uterine fluid. The value 'Low' denotes glucose is present but below detection limits. 'No fluid' denotes that no uterine fluid was collected from that uterine horn.

18% Protein			9% Protein		
Sample code	Left	Right	Sample code	Left	Right
A	4.1	5.6	E	6.4	6.1
B	3.5	4.2	F	2.3	2.9
C	6.1	7.6	G	9.5	7.3
D	9.6	7.2	I	7.2	5.2
H	0.3	LOW	J	4.8	4.1
AN	8.5	0.95	AL	8.6	0.1
AO	0.6	LOW	AP	2.4	LOW
			AQ	LOW	No fluid
			AR	2.1	2.0

Table 4.3.5b: Non-normalised data obtained from analysis of glucose levels in day 3.5 uterine fluid. The value 'Low' denotes glucose is present but below detection limits. 'No fluid' denotes that no uterine fluid was collected from that uterine horn.

18% Protein			9% Protein		
Sample code	Left	Right	Sample code	Left	Right
B	21.3	2.9	A	0.83	LOW
D	0.8	0.4	C	0.225	0.277
F	2.2	LOW	E	6.2	9.17
H	3.5	LOW	G	8.0	4.0
J	2.0	4.2	I	1.25	0.56
L	3.2	3.1	K	LOW	LOW
M	1.85	No fluid	N	0.78	0.55
Q	1.48	2.0	O	3.1	LOW
R	0.9	LOW	P	LOW	1.4
S	1.95	0.97	V	No fluid	LOW
T	LOW	2.7	W	0.65	LOW
U	LOW	LOW	X	0.29	No fluid

Table 4.3.5c: Non-normalised data obtained from analysis of glucose levels in day 2.5 uterine fluid. The value 'Low' denotes glucose is present but below detection limits. 'No fluid' denotes that no uterine fluid was collected from that uterine horn.

18% Protein			9% Protein		
Sample code	Left	Right	Sample code	Left	Right
AD	1.23	0.92	AC	Low	Low
AF	Low	Low	AE	0.067	Low
AG	0.43	2.52	AH	Low	0.45
AK	Low	Low	AI	Low	Low
AL	0.74	Low	AJ	Low	1.8
AM	Low	Low	AN	0.85	Low
AO	1.48	Low	AR	0.62	Low
AP	Low	Low	AS	Low	Low
AQ	Low	Low	AT	Low	Low

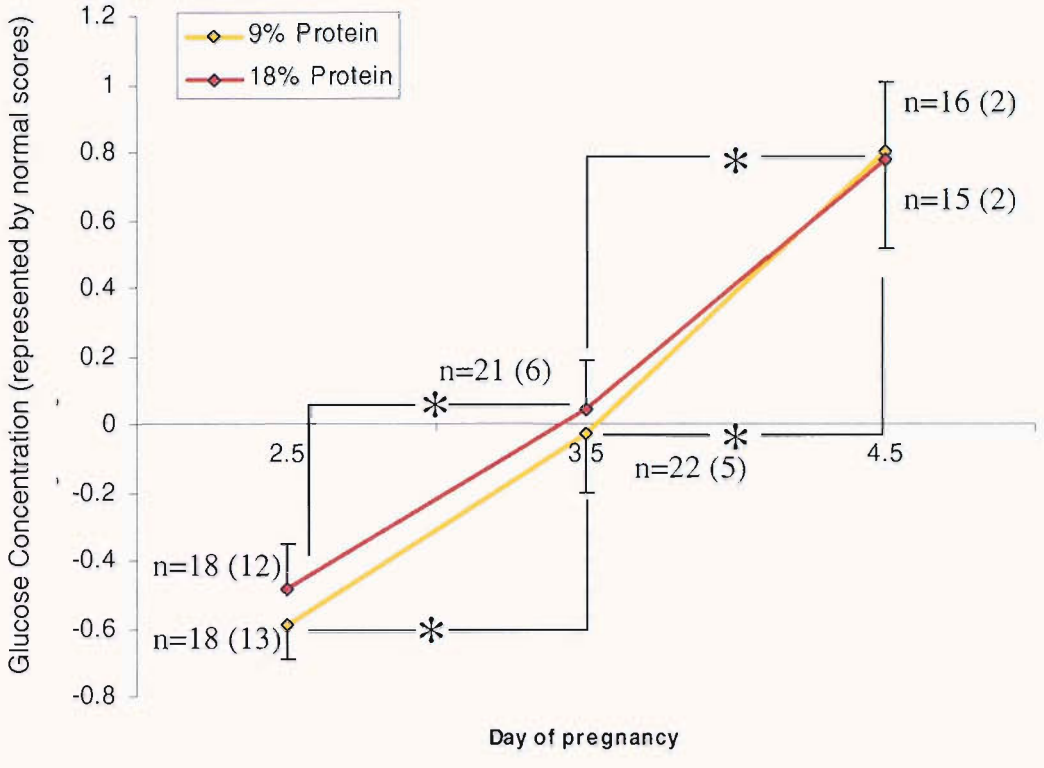

Fig.4.3.5: Relative glucose concentrations in the uterine luminal fluid of mice fed either an 18% NPD or a 9% LPD for either 2.5 3.5 or 4.5 days of pregnancy using normalised data. Numbers in brackets show the total number of readings that were below detection limits. After normalisation of data (described in text) No significant differences were observed in uterine luminal glucose concentrations between mice fed the experimental and control diets. However, glucose concentrations were found to significantly elevate between day 2.5 to 3.5 and day 3.5 to day 4.5. *=$p<0.001$.

4.3.6 Uterine Insulin: Direct Collection method

As already discussed in Section 1.5.5 insulin is an important modulator of embryo growth and development. Hence, I attempted to measure its concentration in uterine luminal fluid in relation to dietary treatment. Insulin concentrations were measured in the uterine fluid of mice fed either the 18% NPD or the 9% LPD on day 3.5 of pregnancy. Insulin concentrations were estimated to be approximately 2.8±0.3 ng ml^{-1} in mice treated with the 9% LPD and 3.1±0.3 ng ml^{-1} in mice treated with the 18% NPD with no significant difference being observed between the two groups. However, it was also noted that when insulin concentration was assessed the optical densities (OD) of the samples (prior to conversion to actual concentrations) were on average below that of the lowest sample and only just above background readings (Fig. 4.3.6). For this reason a second experiment was undertaken to assess the insulin concentration in uterine fluid

collected and pooled from 5 separate mice on day 3.5 of pregnancy. Logically if readings of insulin concentration were accurate in the first experiment then OD readings should increase to give insulin concentrations equivalent to approximately 5 times that of the first experiment. Unfortunately, the second experiment failed to verify the original results and yielded almost identical OD readings to the first experiment (Fig. 4.3.6), suggesting that insulin levels in mouse uterine luminal fluid are below detection limits using this methodology.

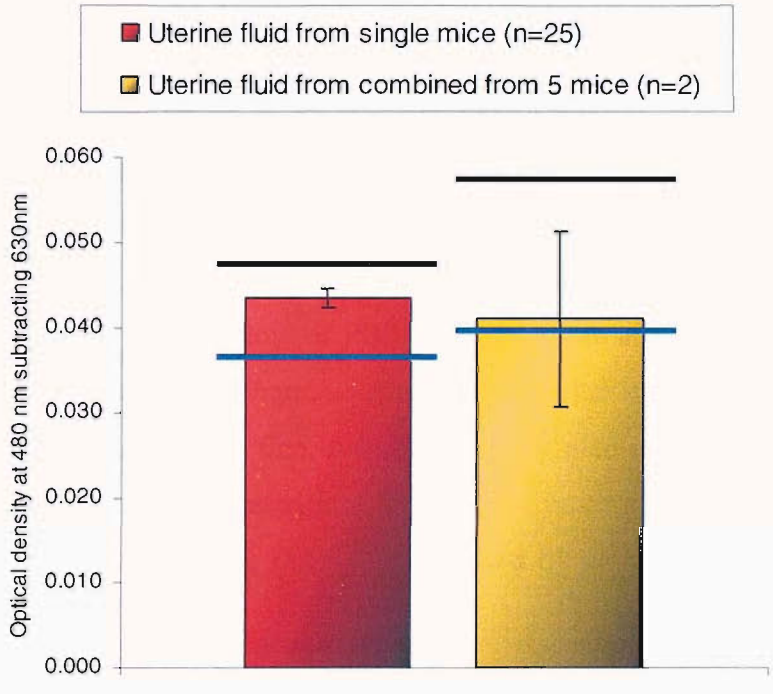

Fig. 4.3.6: The optical densities (OD) of uterine fluid samples assayed for insulin concentration showing the OD of the lowest standard (0.1 ng, black line) and the OD of the background (blue line). Data here represents 2 separate experiments, firstly where insulin was measured in uterine fluid from single mice and secondly where uterine fluid was pooled from 5 mice for analysis. There is no significant difference between the OD of these experiments. Data here is pooled from fluid collected from mice treated with both the LPD and NPD on day 3.5 of pregnancy.

4.4 Discussion

The pre-implantation embryo enters the uterus approximately 72 hrs post fertilisation and, until implantation, relies almost solely on the content of luminal fluid to survive and develop. As a result, this environment is critical for investigation of the

mechanisms of pre-implantation metabolic programming. Two novel methods of uterine fluid collection were developed that limit damage to the cellular structure of the uterus. Using these methods it has been possible to utilise a precise dietary regime designed for metabolic programming studies (Langley and Jackson, 1994; Rees *et al*, 1999) to illuminate any links that may exist between metabolic programming of the early embryo (Kwong *et al*, 2000) and the peri-implantation uterine environment.

Using two methods for collection of uterine luminal fluid, perfusion and direct, it was possible to examine in some detail the composition of the fluid and compare the effects of two dietary treatments on its composition. However, it is important that the perfusion method be as gentle as possible to minimise damage to the cellular structure of the uterus and thereby avoid artifacts and any sources of error that may occur in fluid composition as a result. The perfusion collection procedure for uterine luminal fluid relies upon an extremely gentle flow of PBS to flush, dilute and collect the contents of the uterine milieu and minimise harm to the interior of the uterus (section 2.3). The direct collection procedure uses a fine, flame polished, pulled glass tube (approximately 0.5 mm in diameter) that is threaded into the uterus from a point just posterior to the oviduct down to the corpus uteri; fluid was collected by capillary action (section 2.4-2.5).

To ensure that the collection procedures were causing the least possible damage to the uterus itself and avoid any possible associated alterations in the collected contents of the uterus, the cellular debris from several pilot perfusions were examined by mounting the cells in chambers and using immunofluorescence techniques (section 2.6). Using the perfusion collection method, sheets of epithelial cells were clearly visible in all of the chambers examined (average 2.5 per chamber) suggesting that the collection procedure may have a detrimental effect due to the dislocation of some 'rafts' of epithelial cells. However, it is unclear if these cells were dislodged by the collection procedure or perhaps, by natural renewal and hence were already free floating in the uterine milieu. The fact that activated macrophages were difficult to isolate and identify using the perfusion method (average 0.17 per chamber) suggests that there is only very limited damage being inflicted on the interior of the uterus by this collection procedure.

While using the direct collection method, sheets of epithelial cells were also clearly visible in the chambers but at approximately the same frequency as in the perfusion method (average 3.0 per chamber). Alternatively, the number of macrophages identified was much higher using the direct collection method (average 2 per chamber).

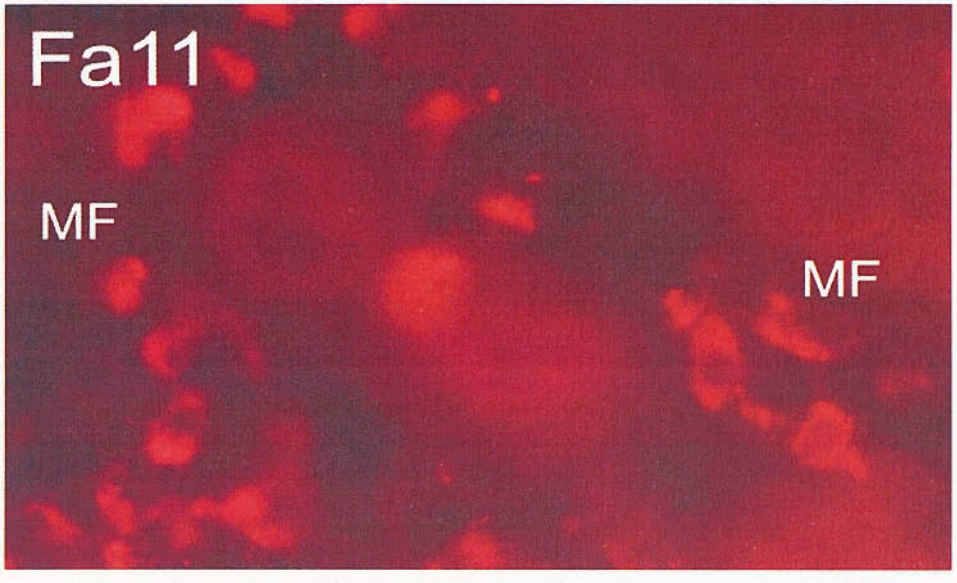

Fig. 4.4a: Section of paraffin fixed uterine tissue immunostained with Fa11 antibody showing the presence of activated macrophages (MF) in the endometrium. Picture courtesy of Wild A.E. and Cleal J. (per. com.)

Macrophages are largely confined to the uterine myometrium but are present in the endometrium increasing in number in association with decidualisation (Hunt *et al*, 1985). FA/11 only detects activated macrophages (Smith and Koch, 1987; Rabinowitz and Gordon, 1991) and it could be argued that the immunofluorescence assay simply did not allow for adequate identification of dislodged macrophages. In spite of this argument, unpublished data by Wild and Cleal (personal communication), that used FA/11 for immunodetection of macrophages in sections of mouse uteri suggest that activated macrophages are localised both within the uterine myometrium and endometrium (Fig. 4.4a).

Since activated macrophages are located within endometrium, these data suggest that both methods of uterine fluid collection are causing some tissue damage. Only one activated macrophage could be identified using the direct collection method compared to eight when using the perfusion method, these data suggest that the direct collection

method is causing relatively more damage to uterine tissues than the perfusion collection method. Ultimately, even if the direct collection method does cause more damage to uterine tissues than the perfusion collection method, there were no significant differences in percentage amino acid balance between the two techniques (Fig. 4.3.4c). Additionally, the percentage amino acid balance data shows close correlation to previously published data on amino acid content of oviduct fluid (Dumoulin *et al*, 1992; Guerin *et al*, 1995). Thus, any damage caused to uterine tissues in the process of luminal fluid collection is probably negligible in either of the collection methods used.

A possible concern with the perfusion method was the variable amounts of time that collection of uterine fluid took place (15-45 min), as a result additional controls were undertaken. Uteri were dissected from mice and luminal fluid collection initiated immediately from one uterine horn while the other uterine horn was kept on ice until for 30 min before collection of luminal fluid. It was found that glutamine was significantly elevated as a percentage of total amino acids in the uterine horns that were perfused after a 30 min delay; no significant alterations in percentage balance of other amino acids were detected. It is possible that this represents a leaching of glutamine from uterine tissues into the luminal fluid after the animal was culled. When percentage amino acid balance is compared between the perfusion method and the direct collection method glutamine concentration shows no significant difference.

The significance of amino acids on embryo development in vitro has been briefly discussed in Section 1.5.6. Here, I have measured amino acid concentrations in uterine fluid in relation to diet. In both the perfusion and direct collection methods, taurine was the most abundant amino acid accounting for up to half of total amino acids while glutamate, glutamic acid, glycine and alanine also contributed significant quantities to uterine amino acid milieu. The essential amino acids only comprised approximately 17% of total measurable amino acids. The perfusion method of collecting uterine luminal fluid did not allow for analysis of actual amino acid concentrations due to the fact that diluton factors were unknown. While no significant differences were observed in percentage amino acid balance between the two methods of uterine luminal fluid collection, the direct collection method has the significant advantage of allowing

measurement of amino acid concentrations rather then just percentage balance. When amino acid *percentage balance* obtained from uterine luminal fluid was compared to percentage amino acid balance obtained from maternal serum, it is clear that the two patterns are distinct (Fig. 4.3.4d), suggesting that there was little or no contamination of luminal fluid samples with blood. When amino acid *concentrations* are compared between the direct collection method (Table 4.3.4a) and maternal serum (Table 6.3.9), again it becomes clear that amino acid composition of the two fluids is completely different (Fig. 4.3.4e); most strikingly, much higher concentrations of asparagine, glutamine, serine, glutamic acid, glycine, taurine and alanine are present in uterine fluid. This data suggests that these non-essential amino acids are actively transported from the maternal blood to the uterine luminal fluid. It appears that the essential amino acids and the other non-essential amino acids are at similar concentrations in both the luminal fluid and maternal serum.

Using the perfusion collection method, it was found that the percentage of valine was significantly reduced in the luminal fluid of mice fed a 9% LPD compared to controls at day 4.5 post plug. Where taurine and alanine were omitted from analysis of the perfused luminal fluid, isoleucine and leucine displayed significant reductions in terms of percentage total amino acids in addition to valine in mice fed a 9% LPD compared to controls. The direct method of collecting uterine luminal fluid gave similar results, demonstrating a significant depletion of valine, leucine and isoleucine at 3.5 and 4.5 day post plug. Furthermore, the direct collection method highlighted a significant elevation of methionine in uterine luminal fluid at 4.5 days post plug. Valine, leucine and isoleucine have also been demonstrated in this investigation to be depleted in the serum of mice fed a 9% LPD compared to controls at 3.5 days post plug (section 6.3.7). Previous metabolic programming investigations conducted on rats also show that these amino acids are depleted around the peri-implantation period in maternal serum in response to maternal LPD (Kwong *et al*, 2000). The fact that valine, isoleucine and leucine are depleated in both the uterine fluid and maternal serum is probably no conincidence. Data from other studies have shown that transplacental transfer rates of selected amino acids, in particular the branched-chain amino acids, are directly dependent on their concentrations in the maternal plasma throughout the physiological

range of maternal and fetal amino acid concentrations in pregnant sheep (Jozwick *et al*, 1999; Patti *et al*, 1998). In contrast, this study showed that methionine concentration increased in uterine fluid but decresed in maternal serum on day 4.5 of pregnancy.

Overall, reduction in availability of leucine, isoleucine and valine could reduce protein synthesis in the peri-implantational embryo due to reduction in signaling through the serine-threonine protein kinase, mTOR (also known as FRAP or RAFT1). Included in the targets of mTOR are p70 S6 and 4E-BP1, downstream these regulate transcriptional machinery and translational efficiency (Gingras *et al*, 1999; Proud, 2002; Fig 4.4b). Activation of mTOR causes phosphorylation of the S6 kinases S6K1 and S6K2 leading to their disassociation from translation initiation factor p70 S6 kinase (Avruch *et al*, 2001). p70 S6 kinase is associated with upregulation of translation of mRNAs that have an uninterrupted stretch of pyrimidine residues next to the 5' 50-cap structure; these mRNAs are known as terminal oligopyrimidine mRNAs (Meyuhas, 2000). Proteins included in the family of terminal oligopyrimidine mRNAs include ribosomal proteins, elongation factors, and poly(A) binding protein, i.e. these proteins comprising the machinery of mRNA translation (Kimball and Jefferson, 2004). Similarly, mTOR phosphorylates 4E-BP1 (also known as PHAS-1), causing this protein to disassociate from eIF-4E. Once inhibition by 4E-BP1 is removed due to its phosphorylation, eIF-4E is free to associate with other proteins such as the translation factor eIF-4G to form productive translation initiation factor complexes that are necessary for normal cap-dependant protein synthesis (Gingras *et al*, 1999). In short, eIF-4E acts to increase the rate of translation.

While not yet completely defined, mTOR, appears to be a point of convergence for both insulin (Scott *et al*, 1998; Nave *et al*, 1999) and amino acid signaling (Hara *et al*, 1998; Peyrollier *et al*, 2000; Xu *et al*, 2001). The branched chain amino acids leucine, isoleucine and valine have been shown to up regulate the activity of mTOR stimulated protein synthesis (Fig. 4.4b) and this response can be ablated by application of rapamycin, an inhibitor of mTOR (Tremblay and Marette, 2001). Leucine is the most potent regulator of mTOR, mediated activities with isoleucine and valine having a lesser effect (Lynch *et al*, 2000). While it is clear that the mTOR pathway is regulated by

branched chain amino acids, the evidence for direct control is fairly circumstantial. Yoshizawa (2004) suggested that amino acids may modulate the activity of the newly discovered protein raptor, which interacts with mTOR and plays a central role in signaling from mTOR to S6K1 and 4E-BP1. Results suggest that insulin plays a permissive function in leucine-induced protein synthesis. Insulin appears to facilitate, however is not required for, branch chain amino acid stimulation of 4E-BP1 and S6 phosphorylation (Yoshizawa *et al*, 2002). Additionally, it has been shown that leucine administration stimulates the phosphorylation of 4E-BP1 and S6 in the liver of non-diabetic, but not diabetic, rats (Yoshizawa *et al*, 2002). As a result, Yoshizawa (2004) suggested that in the liver, insulin is necessary for mediating the amino acid induced 4E-BP1 and S6K1 phosphorylation.

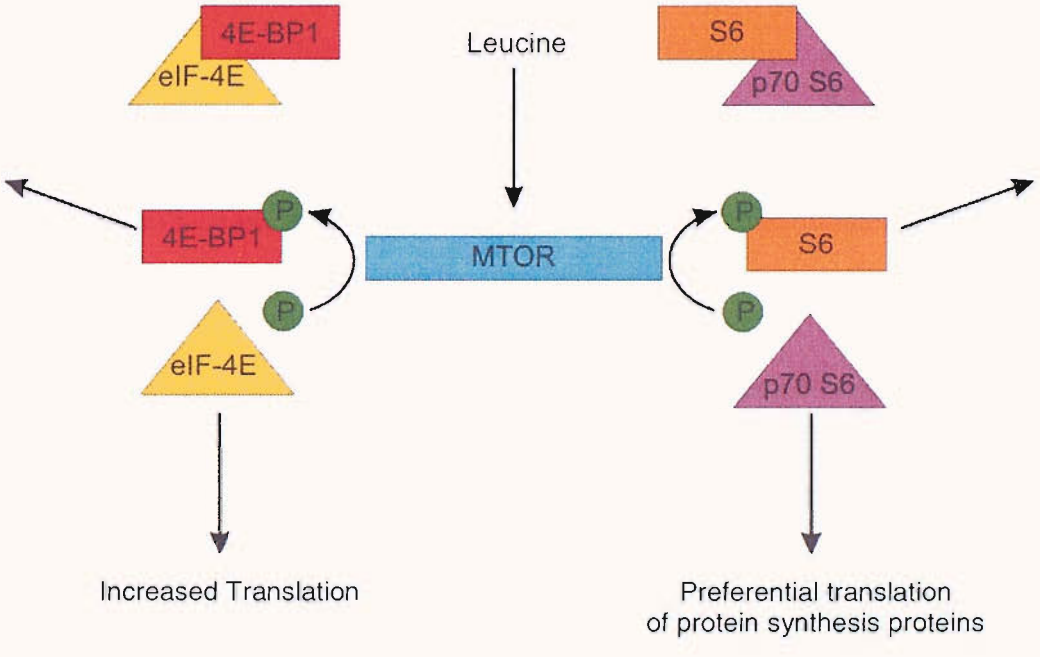

Fig. 4.4b: The serine-threonine protein kinase mTOR phosphorilates 4E-BP1 and p70 S6 causing an increase in both the machinery for protein synthesis and protein synthesis as a whole.

Whatever the specific mechanism of amino acid regulation of the mTOR pathway it is clear that branched-chain amino acids can have a profound development effect on embryo development and implantation. Supplementation of embryo culture media with branch chain amino acids has been shown to increase cell numbers (Biggers *et al*, 2000; Ho *et al*, 1996; Summers *et al*, 2000) and aid hatching (Biggers *et al*, 2000; Ho *et al*,

1996; Summers *et al*, 2000). Arginine and leucine have been shown to be important for stimulation of trophoblast outgrowth (Gwatkin 1966; Naeslund, 1979; Martin and Sutherland, 2001) and leucine specifically has been shown to regulate insulin receptor signaling (Patti *et al*, 1998), protein synthesis and cell cycle progression (reviewed by Martin *et al*, 2003). In the developing embryo, it is most probably that these effects are mediated through the mTOR pathway. Martin and Sutherland (2001) demonstrated that leucine-dependant trophoblast outgrowth could be ablated by administration of the mTOR inhibitor rapamycin. The observed reduction in branched chain amino acids in the uterine luminal fluids of mice fed the 9% LPD could well be causing the developing embryos to exhibit many of these effects and undergo more catabolic rather than anabolic processes. Indeed, it is possible that the reduction in blastocyst cell number observed by Kwong *et al* 2000, in response to LPD administration, was due to reductions in branch chain amino acids in uterine luminal fluids. This study has also shown that maternal serum insulin is significantly reduced in response to administration of LPD up to day 3.5 post plug (section 6.3.3). Indeed, any decline of embryo anabolism could be compounded if this reduction in serum insulin is mirrored in uterine luminal fluid due to the removal of insulin's stimulatory effect on mTOR signaling. Unfortunately this study was unable to measure insulin leves in uterine luminal fluids.

In addition, a significant elevation of methionine was observed in the uterine luminal fluid at day 4.5 post plug of mice administered the 9% LPD. This elevation in methionine concentrations could be explained by three hypotheses. 1) The fact that mice fed the 9% LPD consume more food than their counterparts fed the 18% NPD (Section 3.3.2), coupled with the fact that both synthetic diets are supplemented with extra methionine (Table 2.1) could lead to excess methionine in maternal serum and hence the uterine luminal fluid. 2) Alternatively, it is possible that the excess of methionine is a by-product of increased cysteine production in response to the low amounts of cysteine present in the synthetic 9% LPD (Rees, 2002; Fig. 4.4c; discussed in section 6.4). In short, Rees (2002) predicts an increase in methionine levels because this is the most effective way to remove the toxic amino acid homocysteine from tissues. Maternal levels of circulating homocystine have been shown to approximately double in rodents fed a LPD (Petrie *et al*, 2002). Since this study has failed to demonstrate an increase in

circulating maternal methionine concentrations, in fact methionine concentrations were shown to decreases in maternal serum (section 6.3.7), it seems unlikely that this explanation is correct. However, it is possible that the increased concentration of methionine on day 4.5 post plug is representative of a local system whereby toxic homocysteine concentrations are being actively reduced in the uterine environment. 3) Previously, Jozwick *et al* (2001) have demonstrated in sheep that branch chain amino acids can in fact inhibit umbilical uptake of methionine. It is possible that a similar system is operating within the peri-implantation uterus and the significant depletions in valine, isoleucine and leucine concentration infact allow for a greater rate of methinine transportation into the uterine luminal fluid.

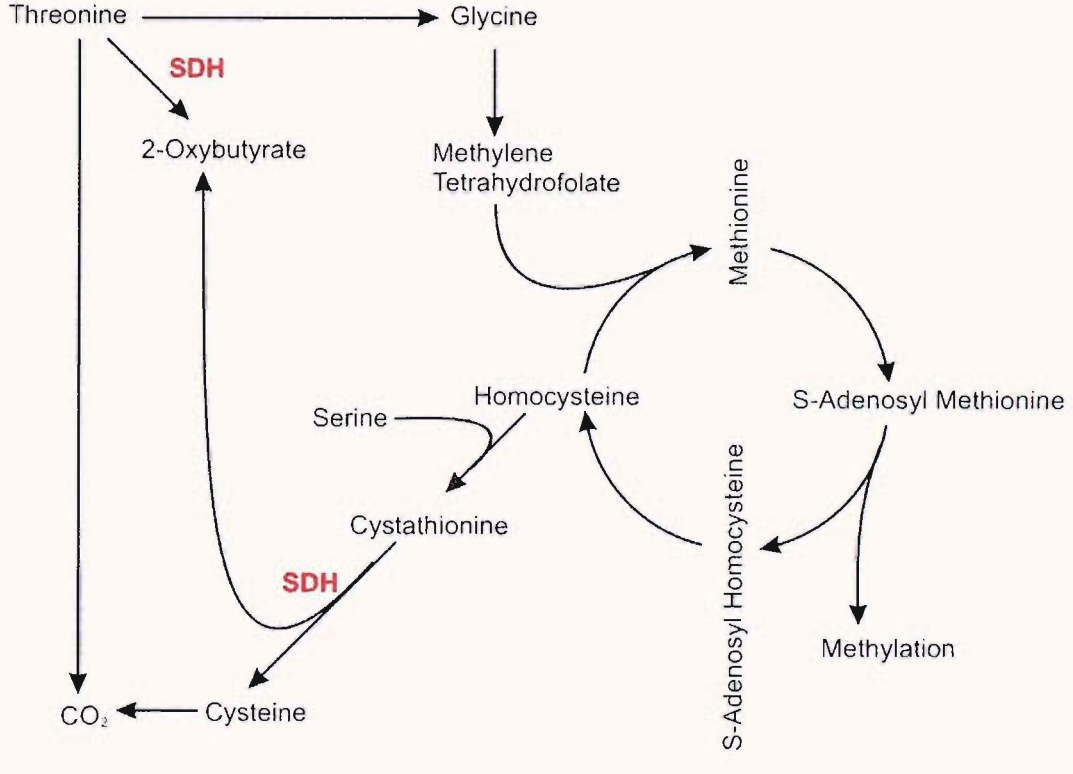

Fig. 4.4c: Pathways for the production of cysteine from threonine and removal of homocysteine. The enzyme serine-threonine dehyrogenase (SDH) is and its actions are highlighted. Adapted from Rees (2002)

Embryo culture media supplemented with amino acids has been shown to have a profound effect on imprinted gene methylation (Doherty *et al*, 2000). Rees *et al* (2000) demonstrated that, in certain tissues, offspring from dams administered a LPD show an

altered pattern of DNA methylation. It is possible that the increased methionine observed here in the uterine luminal fluid of mice fed the 9% LPD correlated with increased DNA methylation. Indeed the consequences of altered DNA methylation could be wide ranging for gene imprinting. Indeed Rees *et al* (2000) noted that the fetuses from LPD mothers had hypermethylated DNA in liver extracts and that there was an increase in threonine oxidation suggestive of excessive dietary methionine. Two of the most described imprinted genes are that of IGF-II and its receptor IGF-IIR/mannose 6-phosphate receptor. Deletion of the methylation-sensitive region activates the silenced maternal copy of the IGF-II gene, increasing IGF-II production and leading to fetal overgrowth (reviewed by O'Dell and Day, 1998). It is possible methylation of IGF-II is altered in the early embryo as a result of maternal exposure to a LPD during pre-implantation development. This could offer an explanation for the reduced birth weight of offspring from dams fed a LPD during the pre-implantation development period (Kwong *et al*, 2000)

Using the direct collection method, a significant depletion of amino acid concentration was observed between day 3.5 and 4.5 in essential, non essential (Fig. 4.3.4h) and total amino acids (Fig. 4.3.4h) in the uterine luminal fluid of mice fed the 18% control NPD. In contrast, mice fed the 9% LPD only exhibited a depletion of essential amino acids (Fig. 4.3.4h) between day 3.5 and day 4.5 in uterine luminal fluid. The reason for this distinction appears to be two fold, 1) Fewer amino acids are significantly depleted in uterine luminal fluid between day 3.5 and 4.5 where mice are fed a 9% LPD (Fig. 4.3.4j). 2) Those amino acids that were significantly depleted in the uterine luminal fluid of mice fed the 9% LPD show a smaller depletion than those that were significantly depleted in the uterine luminal fluid of mice fed the 18% NPD (Fig. 4.2.4j). These results suggest that there is a 'basal' concentration of amino acids around the time of implantation and that mice fed the 9% LPD reach this 'basal' state sooner than mice fed an 18 % NPD. Taken as a whole, these data suggest not only that there is a temporal concentration pattern for amino acids in uterine luminal fluid during normal pre-implantation development but also that this pattern can be disrupted by administration of a LPD.

The impact of glucose on embryo development has been discussed in Section 1.5.6. This (section 6.3.8) and previous studies (Kwong et al, 2000) have shown that serum glucose levels are elevated in response to administration of a LPD. Women with poorly managed diabetes mellitus have a higher incidence of congenital abnormalities (reviewed by Greene, 1993) and spontaneous abortion (Mills *et al*, 1988). Indeed, it has also been demonstrated, in mice, that maternal hyperglycaemia can adversely affect embryo development. Such adverse effects can manifest themselves as reduced blastocyst formation rates (Diamond *et al*, 1989) and an increase in the probability of congenital malformations (Moley *et al* 1991). These effects can be mitigated by insulin treatment prior to superovulation (Moley *et al* 1991). In vitro culture of mouse embryos in elevated glucose concentrations have only been shown to partially recreate these effects (Diamond *et al*, 1991). However, intraembryonic levels of glucose and Krebs cycle metabolites are elevated and correlate with the degree of developmental delay observed in mouse embryos cultured in elevated glucose media (Moley *et al*, 1996). *In vitro* culture of mouse embryos in media containing metabolic intermediates such as ketones replicates the observations of retarded development of embryos obtained from diabetic mothers (Moley *et al* 1994). It has also been demonstrated that preimplantation mouse embryos retrieved from diabetic mothers, exhibit a decrease in glucose utilisation that is directly derived from a decrease in glucose transport at both the mRNA and protein levels (Moley *et al*, 1998). Syndrome X type effects predicted by the DOHAD hypothesis have also been shown to occur in offspring of diabetic mothers. For example, Iwase *et al* (1995a) demonstrated that offspring of diabetic dams exhibited lower BW, higher SBP at 6 months and increased heart rate compared to controls. Indeed Iwase *et al*, (1995b) also demonstrated that the offspring of diabetic dams had a shorter lifespan than controls.

Glucose concentrations were measured in uterine luminal fluid of mice treated with either the LPD or NPD for either 2.5, 3.5 or 4.5 days of pregnancy. This was done to examine the possibility that hyperglycaemia in uterine luminal fluid may lead to the metabolic programming effects observed by Kwong *et al* (2000) in offspring of mothers treated with an LPD. It was found that the number of glucose measurements below detection limits were inversely proportional to the day of pregnancy. Consequently, the

data for day 4.5 gives a fairly robust measurement of glucose concentrations, however, it is reasonable to assume that the glucose measurements for day 3.5 and 2.5 are at best estimates. As a result of there being so many samples that contained glucose readings below detection thresholds, these data had to undergo a transformation to normalise them for statistical analysis. The current study estimated uterine luminal glucose concentration in normalised data to be approximately 1 mmol/l on day 2.5 of pregnancy; this is very similar to an estimate by Wales and Edirisinghe (1989) for the same time point. Moreover, Wales and Edirisinghe (1989) also found that glucose levels rose between day 1 and day 2 of pregnancy in the mouse suggesting that the observed increase in glucose concentrations in this study is indeed occurring *in vivo*. Overall, it appears that the concentration of glucose in uterine luminal fluid (≈5.4 mmol/l) is about half the concentration of glucose in maternal serum (≈10.5 mmol/l) on day 4.5 of pregnancy. Whilst no significant differences were found to exist between dietary treatments it is nevertheless clear that glucose levels rise dramatically between day 2.5 and day 4.5 of pregnancy. The exact extent of the rise in uterine glucose levels between days 2.5 and 4.5 should be the subject of further investigation. Additionally, the possibility that the balance of metabolic intermediates such as ketone bodies should also be investigated to establish if they infact have any role to play in peri-implantation metabolic programming.

As discussed in Section 1.5.6, Korgan *et al* (2001) have demonstrated that the expression of the glucose transporter GLUT1 increases in the luminal epithelium and decidual tissue of the rodent between day 3 of pregnancy and implantation. It is reasonable to surmise that this increase in GLUT1 expression leads to a rise in glucose transport across the luminal epithelium and is responsible for the increase in glucose concentration observed in uterine fluid between day 2.5 and day 4.5. Other species have also demonstrated cycle-dependent changes in reproductive glucose concentrations. In the sow glucose levels have been shown to decrease dramatically in oviductal fluids post ovulation (Nichol *et al*, 1998). In the human, more interestingly, Gardner *et al* (1996) found that glucose levels decreased between the follicular and midcycle stages before increasing again up to the luteal phase. It should, however, be noted that Gardner *et al* (1996) found no cycle-dependent changes in *uterine* glucose levels in the human. These

findings could represent a divergence of function in glucose control between the rodent and the human uterus in the early stages of pregnancy.

Results obtained here seem to indicate that the environment of the peri-implantational embryo is indeed altered in response to application of a 9% LPD. The amino acid profile of the uterine luminal fluid of mice fed a 9% LPD appears to be depleted in the branched chain amino acids and an increase in methionine concentrations. It is possible that these alterations to the embryo environment could cause alterations in embryo metabolism and DNA methylation. Glucose levels were also measured in uterine luminal fluid at day 4.5 post plug. However, no significant differences in glucose concentrations were observed, although they did appear to rise significantly during the preimplantation period. The observed alterations to normal embryo environment could impact on developmental plasticity and have a role to play in metabolic programming of a Thrifty Phenotype. Thus, these alterations to uterine luminal fluid might be causal of the Syndrome X type symptoms observed when rats have been administered a LPD during the pre-implantation development period alone (Kwong *et al*, 2000).

Chapter 5

Uterine tissue

5.1 Introduction

The uterine tissues, in particular the endometrial epithelium and stroma, are of importance to this project as they must be synchronised to embryo development and receptive to blastocyst implantation. "Implantation is a process by which the embryo makes close physical contact with the maternal endometrium for the establishment of pregnancy" (Paria *et al*, 2000). Endometrial receptivity as defined by Psychoyos (1973b), is a self-limited period in which the endometrial epithelium permits blastocyst adhesion. There is no evidence to suggest that endometrial receptivity is ablated by administration of a LPD, indeed studies have shown that there is no significant difference in litter size (Kwong *et al*, 2000). However, it is possible that there may be subtle alterations in the physiology of the uterine endometrium and epithelia or even the timing of endometrial receptivity in response to administration of a LPD.

Few studies have investigated the relationship between uterine physiology and diet and those that have concern themselves with late pregnancy. Where rats were subject to global dietary restriction during pregnancy it was found that uterine blood flow was impaired due to reduced vascular remodelling just prior to term (Ahokas *et al*, 1984). This decreased uteroplacental blood flow associated with diet restriction is the result of increased alpha-adrenergic vasomotor tone (Ahokas *et al*, 1986). Itoh *et al* (2002) demonstrated that administration of a LPD to rats until day 18 or 19 of pregnancy reduced the vasodilatory response of uterine arteries to vascular endothelial growth factor (VEGF); this was hypothesised to be due to attenuation of the nitric oxide component of VEGF-induced vasorelaxation. Administration of a LPD has also been shown to decrease significantly the concentration of nuclear estrogen receptor (Singh *et al*, 1980) and nuclear progesterone receptor (Singh *et al*, 1981) on day 15 and 20 of dietary protein restriction.

This chapter deals with the impact of a LPD on peri-implantation uterine tissue growth factors, steroid receptor and elements of the IGF axis that may impinge on uterine physiology, implantation receptivity and contribute to metabolic programming.

5.2 Methods

5.2.1 SDS-PAGE and western blotting of FGF-II: Chemiluminescence

Uteri were removed and total protein extracted, gels run and transferred and blotting undertaken as described in Section 2.8. Four uterine protein extracts at day 3.5 (2 × 9% and 2 × 18%) were loaded in duplicate on each gel along with 1 lane of Precision Plus MW size markers (Biorad, UK) and one lane containing a "standard uterine sample" (produced from uterine horns of a mouse fed 18% control diet for 3.5 days post plug) that would be loaded on all gels (Table 5.2.1). After transfer and blocking, the membranes were incubated in MTBS/T containing 1:200 rabbit anti-fibroblast growth factor (FGF)-II IgG (Santa Cruz), or 1:200 rabbit anti-FGF-II IgG with 1:200 blocking peptide, or MPBS/T alone, overnight at 4°C on a rotating surface. After washing, the membranes were incubated with goat anti-rabbit HRP conjugated secondary antibody diluted 1:400 (Sigma). After further washes, 2 ml of SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added to each membrane for 10 min. Proteins were visualised by exposing Biomax Light 1 film (Sigma, Kodak) to each membrane for a known period of time. Proteins were quantified using an Alpha Imager and the integrated density of experimental bands was adjusted against the standard uterine sample. A Students t test was used to compare relative amounts of FGF-II isoforms between dietary interventions.

Table 5.2.1: A schematic showing order of samples loaded on to each gel for visualisation of FGF-II. Set 2 is a repeat of set 1.

	Set 1				Set 2				
MW markers	9% A	18% A	9% B	18% B	9% A	18% A	9% B	18% B	Standard sample

5.2.2 SDS-PAGE and western blotting of FGF-II: Infrared imaging

Uteri were removed and total protein extracted, gels run and transferred and blotting undertaken as described in Section 2.8. After blocking, membranes were incubated in MTBS/T containing 1:200 rabbit anti-FGF-II IgG 2 and 1:5000 mouse anti-actin IgG (Cal Biochem) as a loading control at 4°C overnight. Membranes were incubated at room temperature in the dark for 1 h in secondary antibody; IR800

conjugated goat anti-Rabbit IgG (1:10000) (Rockland INC) and IR680 conjugated goat anti mouse IgG (1:10000) (Molecular Probes) in MPBS/T, and washed as before. Proteins were visualised using the Odyssey infrared imaging system and quantified using its corresponding software (Licor, UK).

5.2.3 SDS -PAGE and western blotting of glucocorticoid receptor: Infrared imaging

For detection of glucocorticoid receptor, proteins were extracted and gels transferred as detailed in section 2.8, however gels used had a 4% stacking gel and 10% resolving gel. After blocking, membranes were incubated in 1:500 dilution of rabbit anti-GR IgG antibody (Santa Cruz Biotech, UK) and mouse anti-actin IgG 1:5000 (Cal Biochem) at 4°C overnight. Membranes were then incubated with 1:10,000 IR800 conjugated goat anti-Rabbit IgG (Rockland INC) and 1:10,000 IR680 conjugated goat anti mouse IgG (Molecular Probes) in the dark at room temperature for 1 h. Proteins were visualised using the Odyssey infrared imaging system and quantified using its corresponding software (Licor, UK).

5.2.4 SDS-PAGE and western blotting of IGFBP-I: Infrared imaging

Visualisation of IGFBP-I was conducted by Miss Rose Panton (Biology undergraduate) under my supervision. Gels were loaded, run and transferred as described in section 2.8. After blocking, membranes were incubated in MPBS/T containing 1:300 goat anti-IGFBP-I IgG (Santa Cruz Biotech. UK) at 4°C overnight. Membranes were then incubated at room temperature in the dark for 1 h in secondary antibody; IR680 conjugated rabbit anti-goat IgG (Molecular Probes) in MPBS/T, and washed as before.

5.2.5 SDS-PAGE and blotting of IGFBP-II: Infrared imaging

Detection and visualisation of IGFBP-II was conducted by Miss Rose Panton (Biology undergraduate) under my supervision. Gels were loaded, run and transferred as described in section 2.8. Membranes were incubated with 1:500 goat anti-IGFBP-II IgG (Santa Cruz Biotech. UK) and 1:5000 mouse anti-actin IgG (Cal Biochem) at 4°C overnight. Membranes were then incubated at room temperature in the dark for 1 h in

secondary antibody; IR680 conjugated rabbit anti-goat IgG (Molecular Probes) and IR800 conjugated goat anti mouse IgG (Rockland INC) in MPBS/T, and washed as before.

5.2.6 Tissue ACE Assay

Tissue ACE assay was conducted as described in Materials and Methods section 2.9.1. For this experiment, pilot studies were conducted to optimise both time and total protein loaded, see section 5.3.6.

5.3 Results

5.3.1 Uterine expression of FGF-II: chemiluminescent detection

To examine the expression of FGF-II within the uterine horn 3.5 days post plug, I conducted experiments using SDS-PAGE and immuno blotting of uterine protein extracts. Controls were conducted that confirmed primary and secondary antibody specificity; no bands were evident in controls where primary or secondary antibody were omitted or where FGF-II antibody was co-incubated with FGF-II peptide (Fig. 5.3.1a-d). Three splice variants of FGF-II were detected of mass 18, 22.5 and 22 kDa (Rifkin and Moscatelli, 1989; Samathanam *et al* 1998; Delrieu, 2000).

Two experiments were conducted to optimise conditions. 1) 20, 30, 40, 50 or 60 µg of total protein were run, transferred and blotted and membranes were exposed for 5 min to Biomax Light 1 film (Sigma, Kodak) (Fig. 5.3.1e). 2) Gels loaded with 45 µg of total protein were run, transferred and blotted and membranes were exposed to Biomax Light 1 film for either 1, 2, 5, 10 and 15 min (Fig 5.3.1f). Upon the basis of the optimisations, the experiment itself was conducted by loading 45 µg of total protein into each lane of the gel and exposing the membranes to Biomax Light 1 film for 5 min.

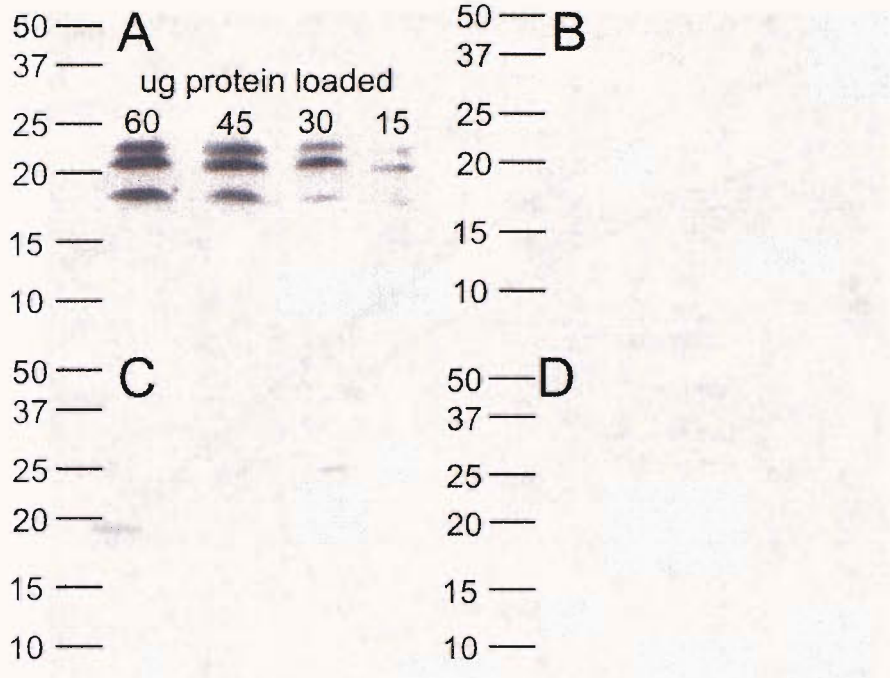

Fig. 5.3.1a-d: Western Blot of mouse uterine tissue incubated with (A) FGF-II primary antibody for 16 hrs at 4°C and secondary antibody for 1hr at room temperature. (B) Incubated with primary antibody only for 16 hrs. (C) Incubated with secondary antibody only for 1hr at room temperature. (D) Incubated with primary antibody and peptide for 16 hrs at 4°C and secondary antibody for 1hr at room temperature. Primary: Rabbit α FGF-II Secondary Goat α rabbit HRP

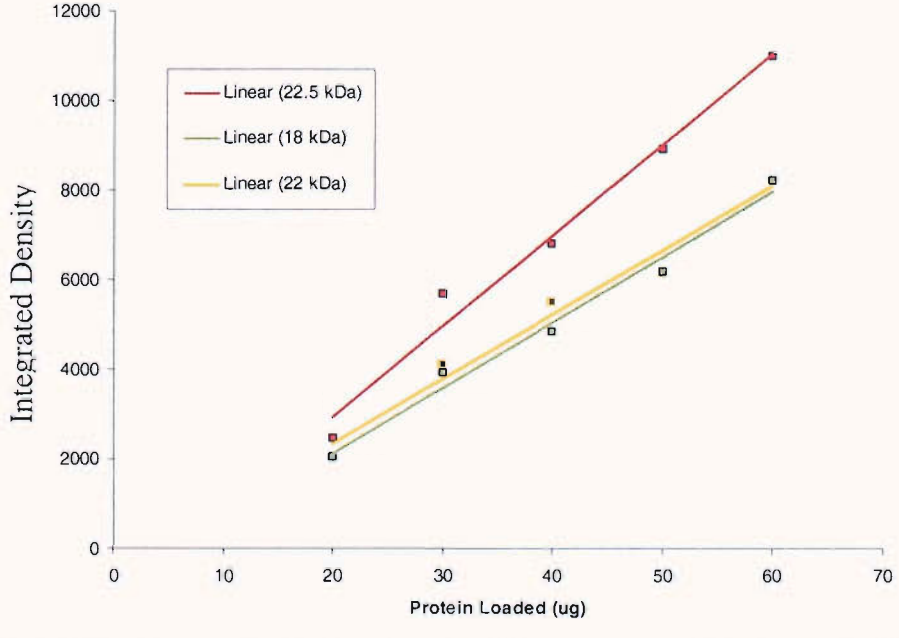

Fig. 5.3.1e: Integrated density values for Western blots of FGF-II using 20, 30, 40, 50 and 60 µg of total protein from uterine tissue blotted and probed with FGF-II primary antibody. Bands were visualised by exposing membranes to Biomax light 1 film for 5 min. Each splice variant of FGF-II exhibits a wide linear detection range. On the basis of these results it was decided that 45 µg of total protein should be loaded onto the gel.

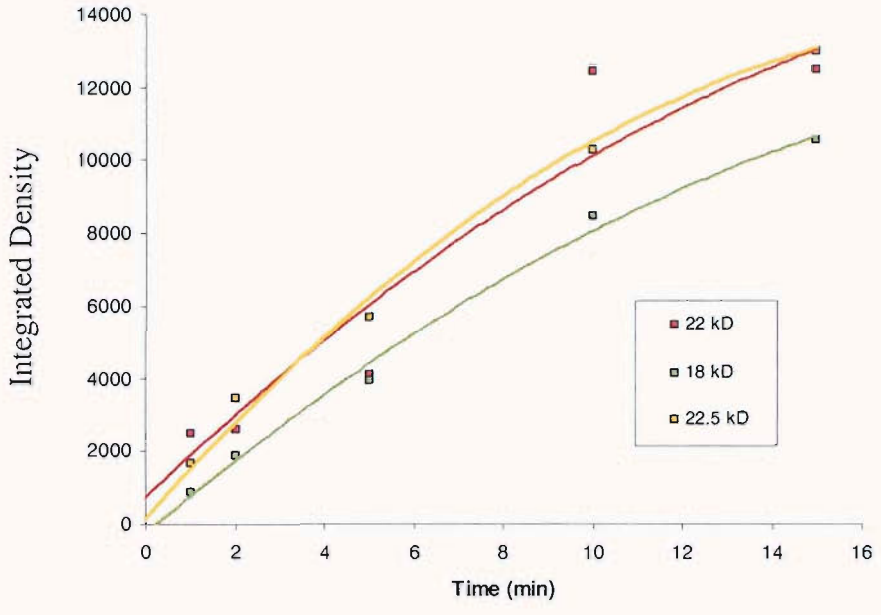

Fig. 5.3.1f: Integrated density values for Western blots of 45 µg of total protein from uterine tissue Western Blotted and probed with FGF-II primary antibody. Bands were visualised using 1, 2, 5, 10 and 15 min exposures of membranes to Biomax light 1 film. Each splice variant of FGF-II exhibits a wide linear detection range up to 10 min where the saturation of the signal begins. On the basis of these results it was decided that a 5 min exposure was best for detection of FGF-II splice variants

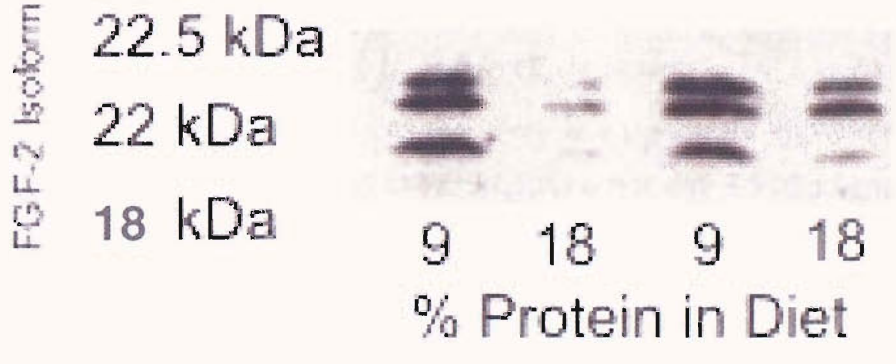

Fig. 5.3.1g: Typical pattern of uterine FGF-II expression visualised on Biomax Light-1 film following chemiluminescent amplification. Note that the 18, 22 and 22.5 kDa splice variants are visible in all samples and the signal appears to be stronger in the mice fed the 9% LPD. Primary: Rabbit α FGF-II Secondary Goat α rabbit HRP

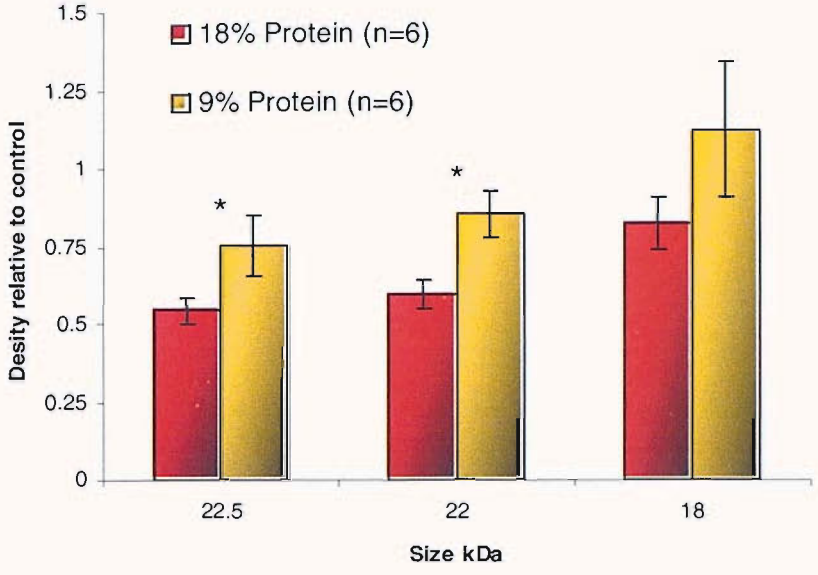

Fig. 5.3.1h: Relative uterine expression of FGF-II spice variants at day 3.5 of pregnancy using chemiluminescent detection. * = A significant elevation of the 22.5 and 22 kDa spice variants was detected ($p<0.05$) Error bars are SEM.

The 18, 22 and 22.5 kDa splice variants of FGF-II were clearly visible in animals fed either the 9% LPD or the 18% NPD (Fig. 5.3.1g). Additionally, it was found that the levels of the 22 and 22.5 kDa splice variants of FGF-II were significantly elevated in the uterine horns of mice fed the 9% LPD (Fig. 5.3.1h). The amount of the 18 kDa protein was not significantly altered in response to administration of the 9% LPD.

5.3.2 Uterine expression of FGF-II: infrared detection

To confirm previous observations that there is a significant elevation in the 22 and 22.5 kDa splice variants of FGF-II in the uterine horn at day 3.5 of pregnancy and to validate the use of the Odyssey infrared detection system (Licor, UK) for SDS-PAGE protein detection, further experiments were conducted into the levels of FGF-II protein in the uterus. In this experiment, actin was used as an internal loading control for FGF-II. As in the chemiluminescent detection system (Section 5.3.1), three splice variants of FGF-II were detected of mass 18, 22.5 and 22 kDa. No bands were evident in controls where primary antibody was omitted or where FGF-II antibody was co-incubated with FGF-II peptide (Fig. 5.3.2a-d).

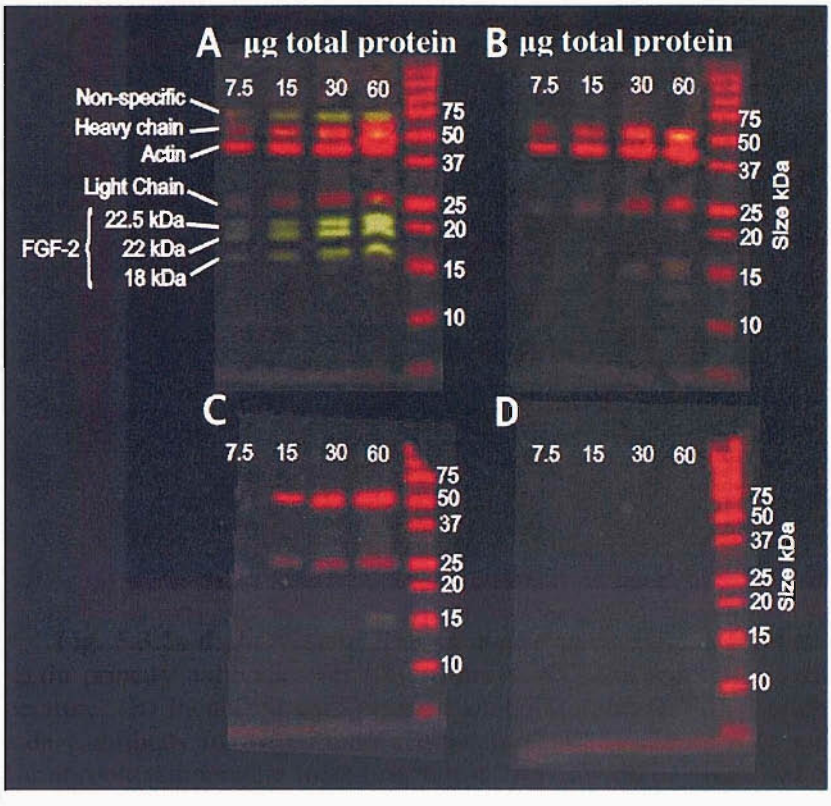

Fig. 5.3.2a-d: A Western Blot of mouse uterine tissue incubated with FGF-II primary and actin primary antibodies for (A) 16 hrs at 4°C and secondary antibodies for 1hr at room temperature. (B) Incubated with primary antibodies and FGF-II peptide for 16 hrs at 4°C and secondary antibody for 1hr at room temperature. (C) Incubated with secondary antibodies only for 1hr at room temperature [note that mouse IgG can still be visualised by actin secondary]. (D) Incubated with only primary antibody for 16 hrs at 4°C. Primary: Rabbit α FGF-II Secondary Goat α rabbit IR800

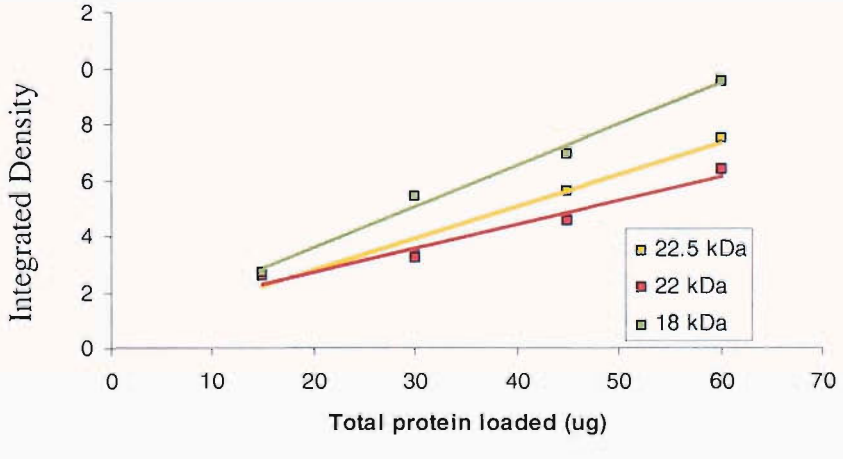

Fig. 5.3.2e: Integrated density values for Wesetern blot of 15, 30, 45 and 60 µg of total protein from uterine tissue blotted and probed with FGF-II primary antibody. Bands were visualised using the Licor Odyssey infrared detection system. The signal for all of the splice variants is linear over all the protein amounts loaded.

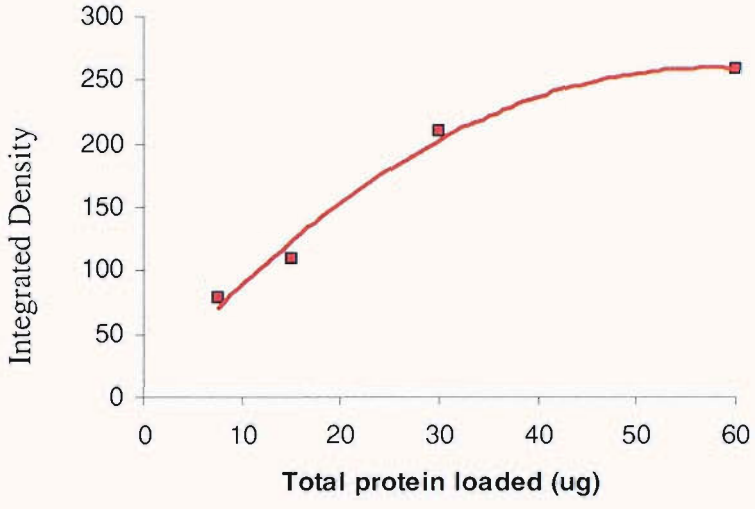

Fig. 5.3.2f: Integrated density values for Western blot of 7.5, 15, 30 and 60 µg of total protein from uterine tissue blotted and probed with actin primary antibody. Bands were visualised using the Licor Odyssey infrared detection system. The signal for actin is linear between 10 and 30 µg of protein loaded before saturation occurs between 30 and 60µg protein loaded. On the basis of these results and those from Fig 5.3.2e it was decided that 25 µg of total protein should be loaded onto the gel.

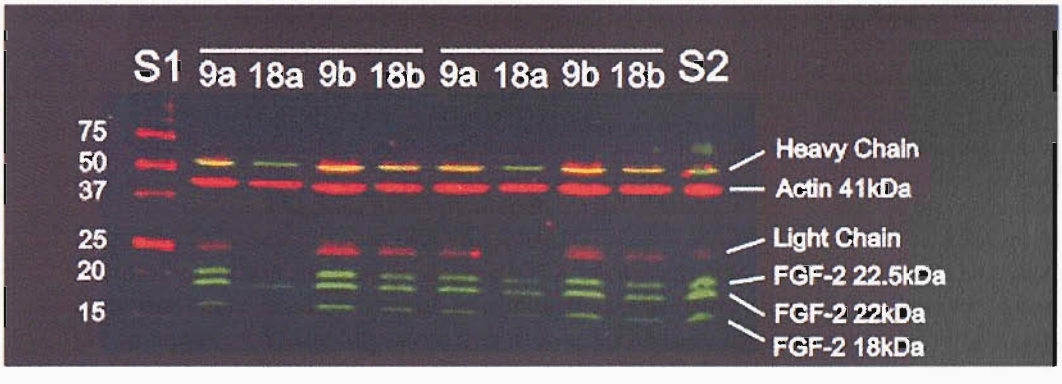

Fig.5.3.2g: Typical pattern of uterine FGF-II and actin expression visualised using the Odyssey infrared imaging system. FGF-II splice variants are of a lower intensity in the samples extracted from uteri of 18% NPD mice compared to those extracted from 9% LPD mice. Note that there is a high degree of similarity between the signals obtained from replicates within the same gel. Additionally, note that mouse IgG heavy/light chains are visible along with FGF-II splice variants. Primary antibodies: Rabbit α FGF-II/Mouse α Actin. Secondary antibodys: Goat α rabbit IR800/Goat α mouse IR680

FGF-II signal was optimised by loading 15, 30, 45 or 60 µg of total protein, while actin was optimised by loading 7.5, 15, 30, 60 µg of total protein. On the basis of the optimisations (Fig 5.3.2e & f), the experiment itself was conducted by loading 25 µg of 4× day 3.5 uterine protein extracts as described in Section 5.2.1 and shown in Table

5.2.1. Similar to the chemiluminescent detection protocol (Section 5.3.1), the 18, 22 and 22.5 kDa splice variants of FGF-II were clearly visible in samples collected from both mice fed the 9% LPD and the 18% NPD. Actin expression was not significantly altered in relation to diet (data not shown). In accord with the previous chemiluminescent detection experiment, levels of the 22 and 22.5 kDa splice variants were found to be significantly elevated in the uterine horns of mice fed the 9% protein LPD when adjusted for actin intensity; levels of the 18 kDa protein were once again found not to alter significantly.

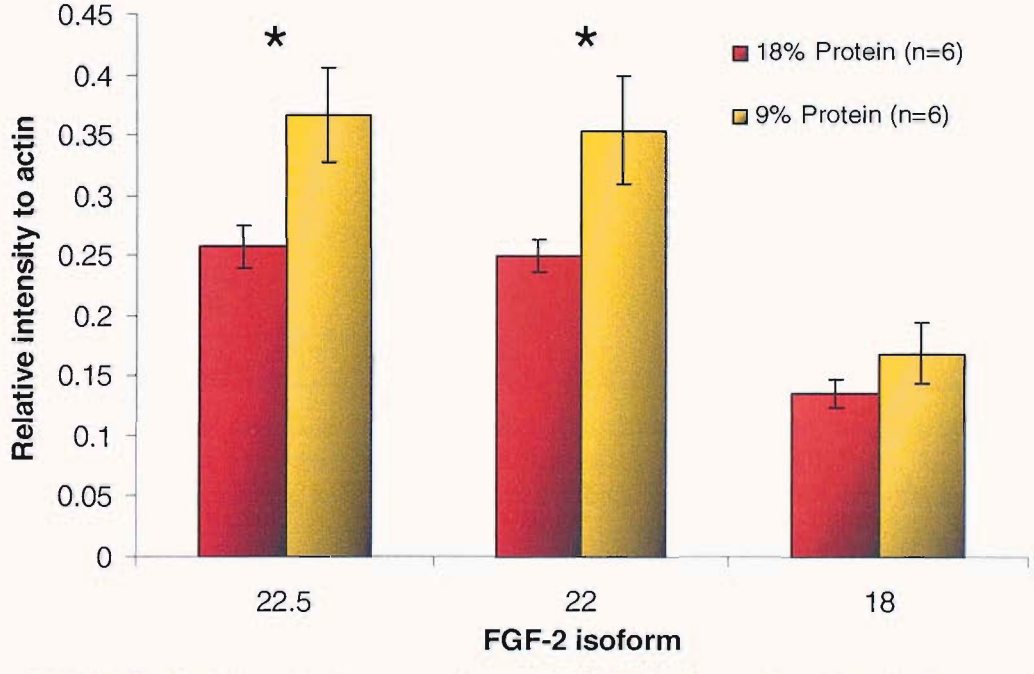

Fig 5.3.2h: Relative uterine expressions of FGF-II splice variants in relation to actin at day 3.5 of pregnancy using infrared detection. As with the chemiluminescent detection method, a significant elevation in FGF-II was found in the 22.5 and 22 kDa splice variants. *=$p<0.05$ Error bars are SEM.

5.3.3 Glucocorticoid receptor infrared imaging

Glucocorticoids and their receptors play important roles in stress responses and regulation of metabolism; thus, experiments to ascertain the relative amounts of GR were undertaken. GR was readily detected in uterine homogenates of both mice fed the 9% LPD and 18% NPD (Fig. 5.3.3a) during experiments to optimise conditions for quantification of GR levels in relation to diet. GR and actin signal were optimised by loading 3.75, 7.5, 15 and 30 µg of total protein (Fig 5.3.3b & c). Upon the basis of the optimisations, relative amounts of GR in the uteri of mice fed either an 18% NPD or 9%

LPD were assayed by loading 20 µg total protein from day 3.5 uterine protein extracts. No bands were visualised where membranes were incubated either with no primary or no secondary antibody (data not shown). No significant differences were observed in uterine GR levels between dietary treatments (Fig 5.3.3d).

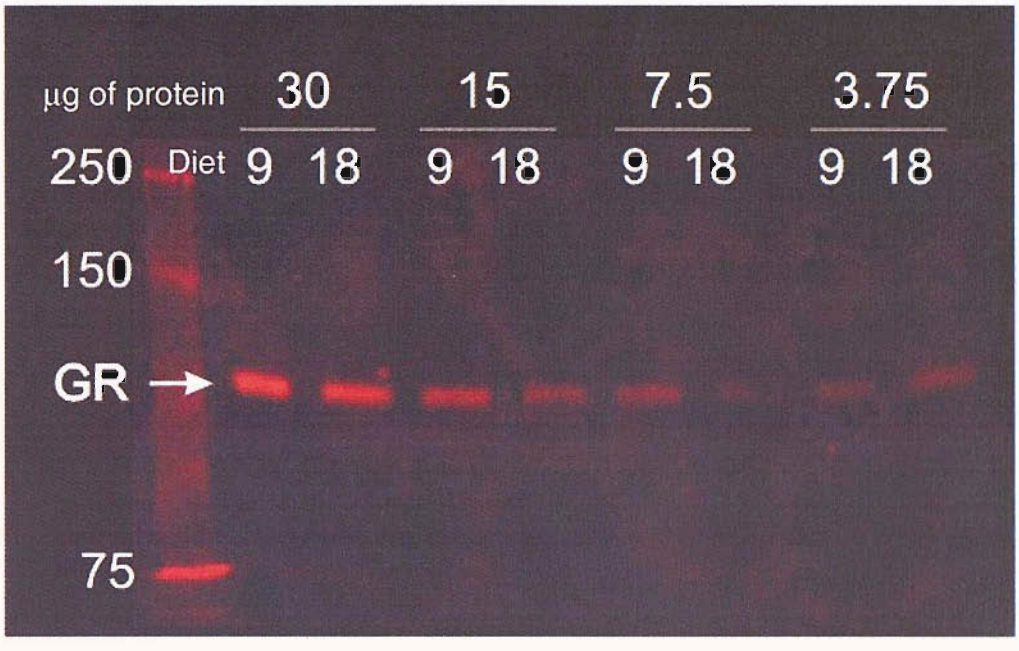

Fig. 5.3.3a: GR signal as visualised using the Odyssey infrared imaging system. GR bands are visible at the expected point, around the 100 kDa marker (GR = 92 kDa). Primary antibodys: Rabbit α GR/Mouse α Actin Secondary antibodies: Goat α rabbit IR680/Goat α mouse IR800

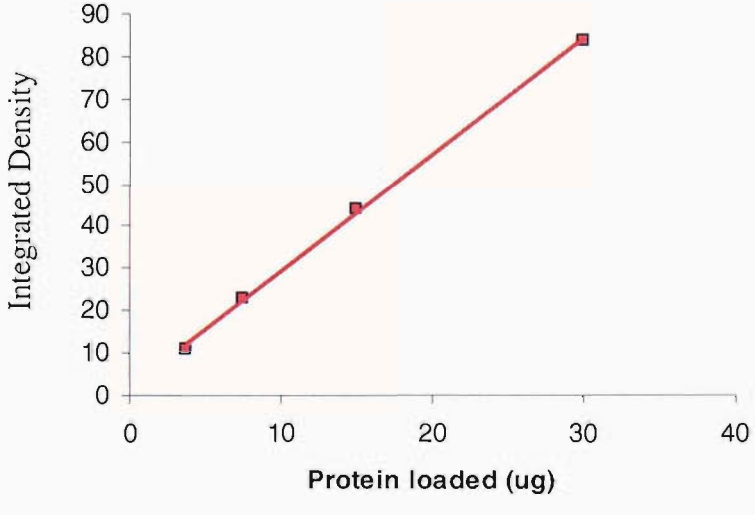

Fig.5.3.3b: Integrated density values obtained for uterine GR against total uterine protein loaded. The signal for GR is linear over all protein quantities loaded from 3.75 to 60µg.

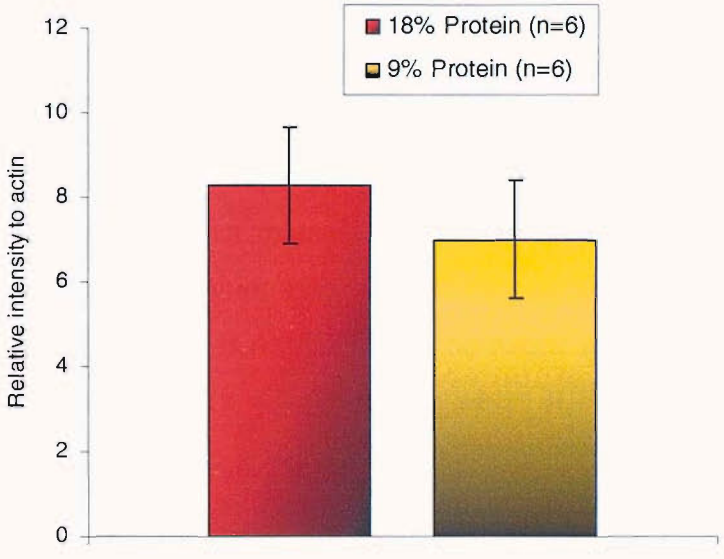

Fig.5.3.3c: Uterine expression of GR in relation to actin at day 3.5 of pregnancy using infrared detection. No significant differences were observed in GR expression between diet treatments

5.3.4 IGFBP-I infrared imaging

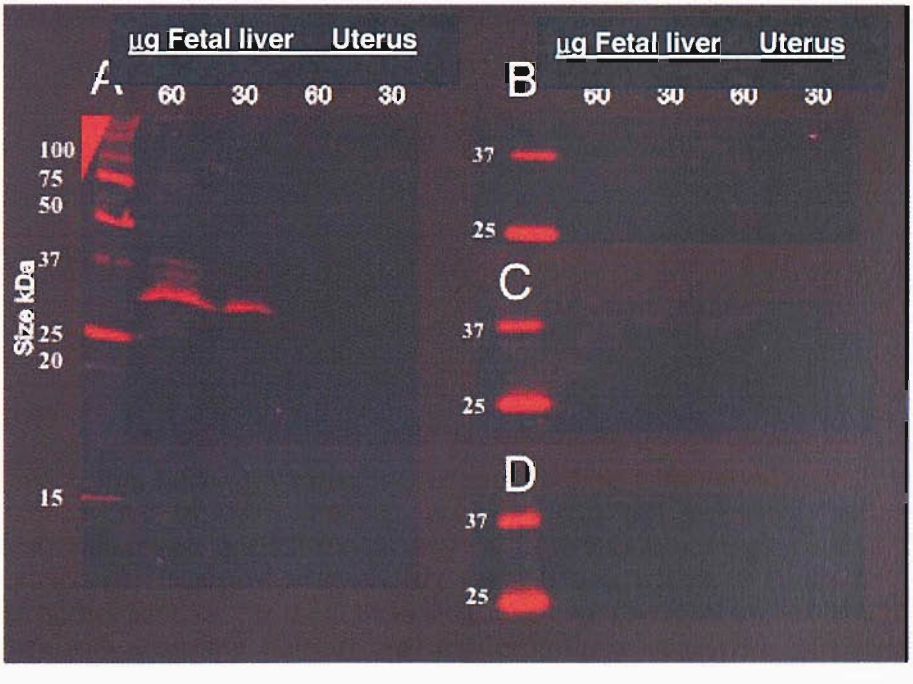

Fig. 5.3.4a-d: Western Blot of mouse uterine tissue incubated with (A) IGFBP-I primary antibody for 16 hrs at 4°C and rabbit anti-goat IgG secondary antibody for 1hr at room temperature; note that IGFBP-I is only visible in fetal liver samples not in day 3.5 uterine tissue extract. (B) Incubated with primary antibody and IGFBP-I peptide for 16 hrs at 4°C and secondary antibody for 1hr at room temperature. (C) Incubated in MPBS/T for 16 hrs at 4°C and then with secondary antibody only for 1hr at room temperature. (D) Incubated with primary antibody for 16 hrs at 4°C then in MPBS/T for 1 hr at room temp. Primary: Goat α IGFBP-I. Secondary: Goat α mouse IR680

Insulin like growth factor binding proteins (IGFBPs) play an important role in the regulation of IGFs in terms of attenuation of action and protein half life in both circulation and within local systems. SDS-PAGE and blotting for detection of IGFBP-I was undertaken to assess any impact maternal LPD may have on expression of this protein in the uterus of peri-implantation mice. In optimisation experiments using fetal liver sample, a band for IGFBP-I was detectable but no bands were evident in controls where primary or secondary antibody was omitted or where IGFBP-I antibody was co-incubated with IGFBP-I peptide (Fig. 5.3.4a-d).

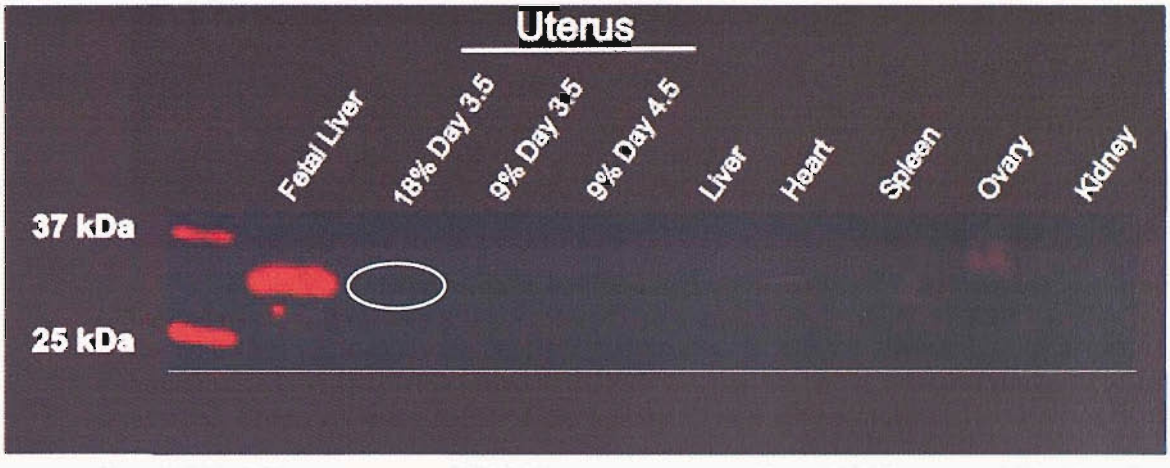

Fig. 5.3.4e: Expression of IGFBP-I in protein extracts from fetal liver, peri-implantation uterine tissue and various adult organs (45 µg total protein loaded). IGFBP-I is clearly visible in the fetal liver positive control and is faintly visible in day 3.5 uterine tissue extract from a mice administered the 18% NPD (ringed). IGFBP-I is not detectable in any of the other tissue extracts assayed. Primary: Goat α IGFBP-I. Secondary: Goat α mouse IR680

IGFBP-I was found to be practically undetectable in peri-implantation uterine tissue run along side fetal liver sample. Similary IGFBP-I was undetectable in a range of other tissues (Fig.5.2.4e). After various optimisations, IGFBP-I was only faintly visualised in one uterine sample and in no other tissues apart from fetal liver (Fig. 5.3.4a-e).

5.3.5 IGFBP-II: infrared imaging

As another regulatory protein of the IGF axis, IGFBP-II was assayed for alterations in protein expression in relation to administration of a LPD. No bands were evident in controls where primary antibody was omitted or where IGFBP-II antibody

was co-incubated with IGFBP-II peptide (Fig.5.3.5a-d). Control results for actin were as Fig 5.3.2a-d. IGFBP-II was clearly visible in protein extracts obtained from fetal liver and used as a positive control (Fig. 5.3.5e). While IGFBP-II was not detectable in any uterine extracts collected at 3.5 days of pregnancy it was detectable in uterine tissue collected at 4.5 days (Fig. 5.3.5e); IGFBP-II was also detectable in adult liver, ovary and kidney (Fig. 5.3.5e).

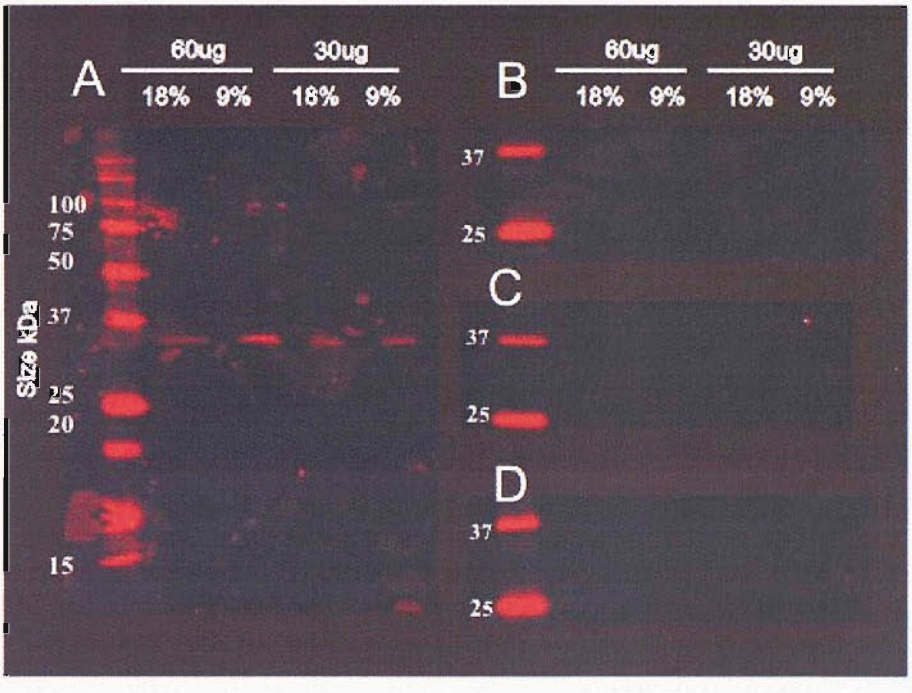

Fig. 5.3.5a-d: Controls for infrared visualisation of IGFBP-II. (A) Incubated with goat ant-IGFBP-I and antibody for 16 hrs at 4°C and rabbit anti-goat IgG secondary antibody for 1hr at room temperature. (B) Incubated with primary antibody and IGFBP-II peptide for 16 hrs at 4°C and secondary antibody for 1hr at room temperature. (C)Incubated in MPBS/T for 16 hrs at 4°C and then with secondary antibody only for 1hr at room temperature. (D) Incubated with primary antibody for 16 hrs at 4°C then in MPBS/T for 1 hr at room temp. Primary: Goat α IGFBP-II. Secondary: Goat α mouse IR680

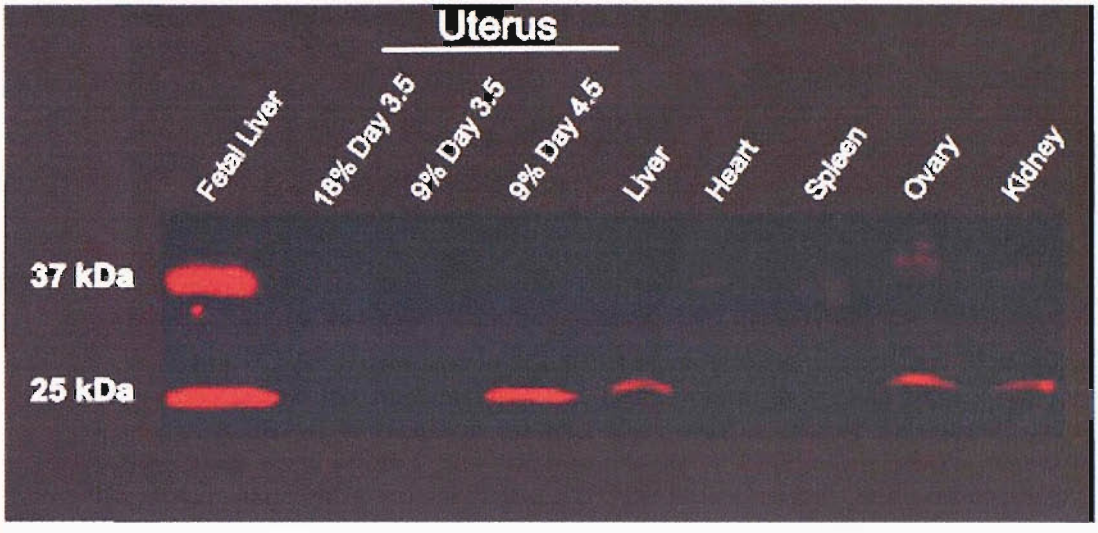

Fig. 5.3.5e: Expression of IGFBP-II in protein extracts of fetal liver, peri-implantation uterine tissue and the adult organs liver, heart, spleen, ovary and kidney (45 µg total protein loaded). IGFBP-II is visible in the fetal liver positive control and is also visible in day 4.5 uterine tissue extract from a mice administered the 18% NPD. IGFBP-II is also detectable in the liver, ovary and kidney. Primary: Goat α IGFBP-II. Secondary: Goat α mouse IR680

IGFBP-II and actin signals were optimised by loading 7.5, 15 and 30 µg of total protein (Fig 5.3.5f & g). Upon the basis of the optimisations, relative amounts of IGFBP-II in the uteri of mice fed either an 18% NPD or 9% LPD were assayed by loading 20 µg total protein of day 4.5 uterine protein extracts into each well of the gel in duplicate as shown in Table 5.2.1. . IGFBP-II was not detectable in extracts obtained on 3.5 days post plug)

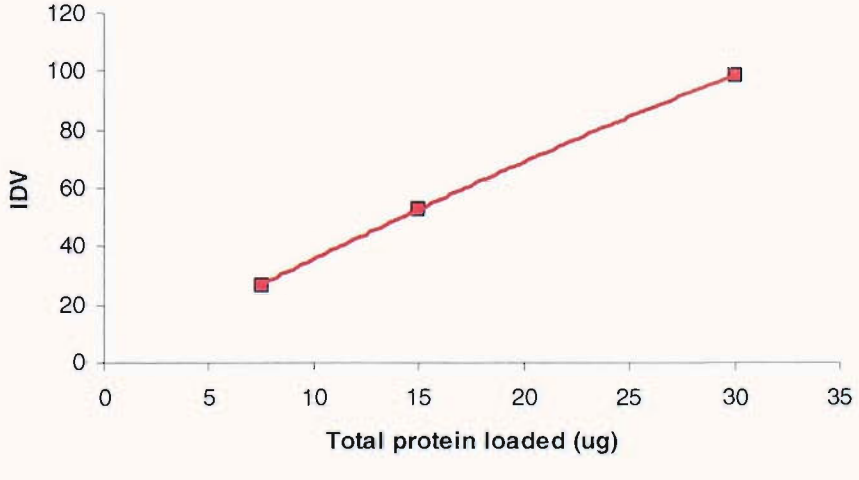

Fig. 5.3.5f: Integrated density value (IDV) of uterine IGFBP-II uterine signal against total uterine protein loaded. The signal is linear over all the protein amounts loaded.

Fig. 5.3.5g: Integrated density value (IDV) of uterine actin signal against total uterine protein loaded. The signal obtained is static between 7.5 and 15 µg loaded and is assumed to be early s-phase of the signal curve, however ,the signal doubles between 15 and 30 µg loaded and is taken to be part of the linear range.

Levels of IGFBP-II were measured by Western blotting of uterine tissue from mice fed either the 9% LPD or the 18% NPD at 4.5 days of pregnancy (Fig 5.3.5h). No significant differences were observed in uterine IGFBP-II levels between dietary treatments even though IGFBP-II levels in the uterine tissue of mice fed the 9% LPD were on average twice those of the mice fed the 18% NPD (Fig. 5.3.5i). On the whole, only relatively small variations were observed in IGFBP-II levels between replicates of the same animal (Table 5.3.5). However, large variations in IGFBP-II levels existed between individual mice in each of the treatment group (Table 5.3.5).

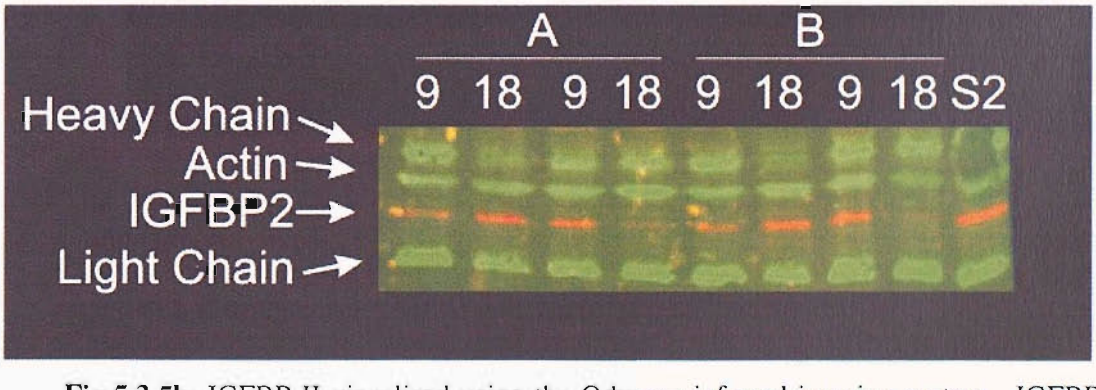

Fig.5.3.5h: IGFBP-II visualised using the Odyssey infrared imaging system. IGFBP-II is visualised in red while actin is visualised in green. Note that there is a high degree of similarity between the signals obtained from replicates in the same gel (set A compared to set B). Additionally, note that actin mouse heavy/light chains are visible along with IGFBP-II splice variants. Primary: Goat α IGFBP-I. Secondary: Goat α mouse IR680

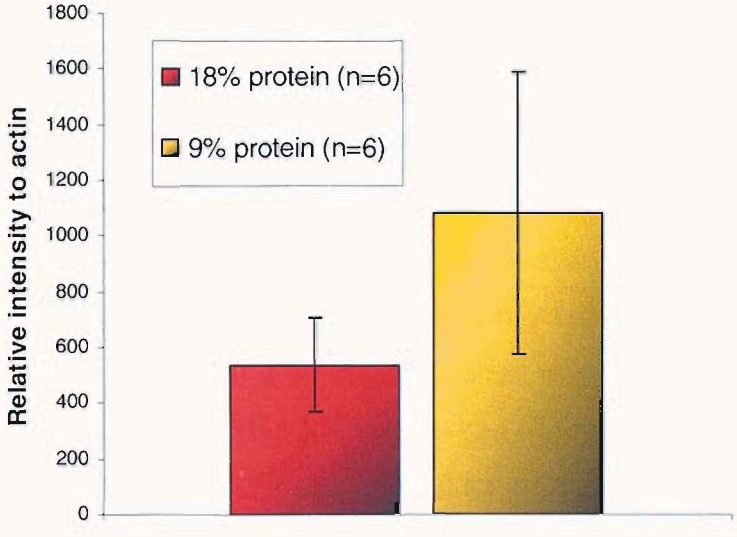

Fig. 5.3.5i: Relative uterine expressions of IGFBP-II splice variants in relation to actin at day 4.5 of pregnancy using infrared detection. There is no significant difference in the levels of uterine IGFBP-II relative to actin between mice fed an 18% NPD or a 9% LPD. Even though the average level of IGFBP-II relative to actin in uterine homogenates of mice fed a 9% LPD are nearly twice that of those fed the 18% LPD large variations are evident in IGFBP-II levels between individual mice (Table 5.3.5). Error bars are SEM.

Table 5.3.5: IGFBP-II levels relative to actin in individual mice. The level of IGFBP-II relative to actin is not subject to much variation between replicates from individual mice. However, measurements between mice in both treatment groups are subject to a large degree of variation.

9% protein diet		18% protein diet	
Mouse #	IGFBP-II relative to actin	Mouse #	IGFBP-II relative to actin
1	441.3±72.1	7	800.9±84.1
2	691.9±79.5	8	140.6±31.37
3	1110.7±246.9	9	957.7±394.32
4	2455.5±1222.7	10	1722.0±281.1
5	5187.7±2009.8	11	417.1±236.7
6	264.4±61.8	12	161.1±25.8
13	305.2±44.5	17	524.0±56.5
14	70.6±5.59	18	53.3±2.67
15	196.1±26.7	19	579.1±90.36
16	106.1±9.1	20	16.1±6.646.64
Average	1691.1±769.2	Average	699.97±245.29

5.3.6 Angiotensin II converting enzyme assay

To characterise any possible changes in the regulation of the local uterine renin angiotensin system (RAS), experiments were undertaken to assay the relative activity of ACE in maternal uterine tissue in relation to diet. Protein from the left uterine horns from mice fed the 18% NPD or 9% LPD were extracted in buffer containing EDTA, while protein from the right uterine horns from these animals was extracted in buffer not containing EDTA. To optimise the technique for uterine tissue, either 300, 150 or 75 µg of uterine protein extracted from uterine horns in buffer either with or without EDTA was assayed for ACE activity over a 60 min incubation as described in the tissue ACE section of Materials and Methods (Fig 5.3.6a). 300 µg of total protein of uterine extract in buffer either with or without EDTA was assayed for ACE activity over either a 15, 30, 60 or 120 min incubation (Fig 5.3.6b).

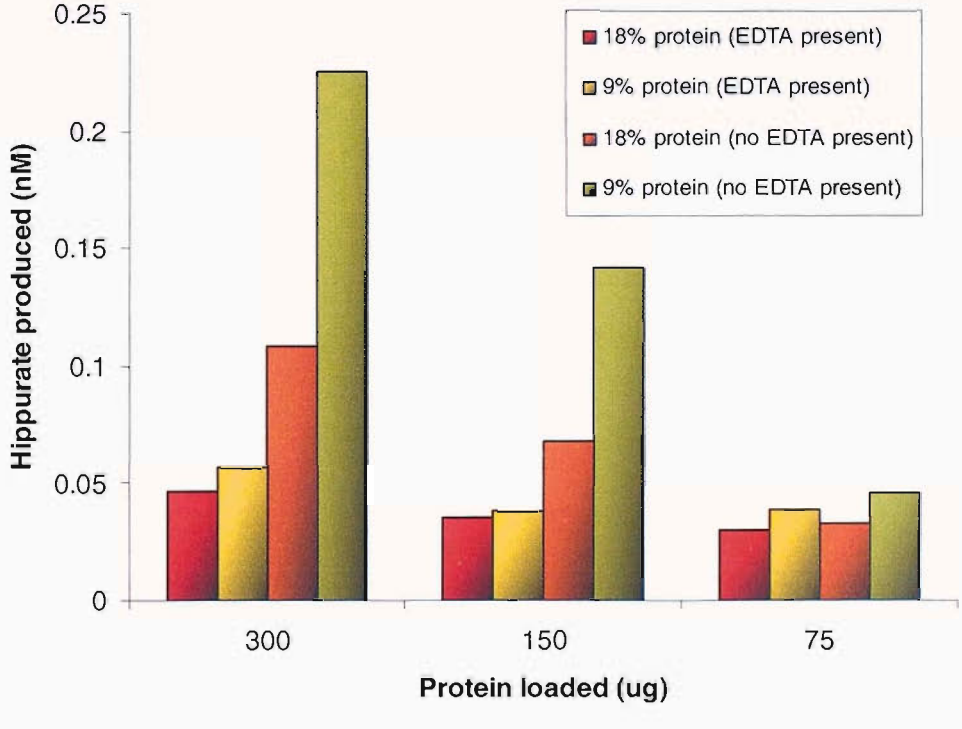

Fig. 5.3.6a: ACE activity in uterine tissue over the course of a 60 min incubation in relation to total protein added. The samples where EDTA was added show a reduced capacity of ACE to convert Hip-His-Leu to hippuric acid.

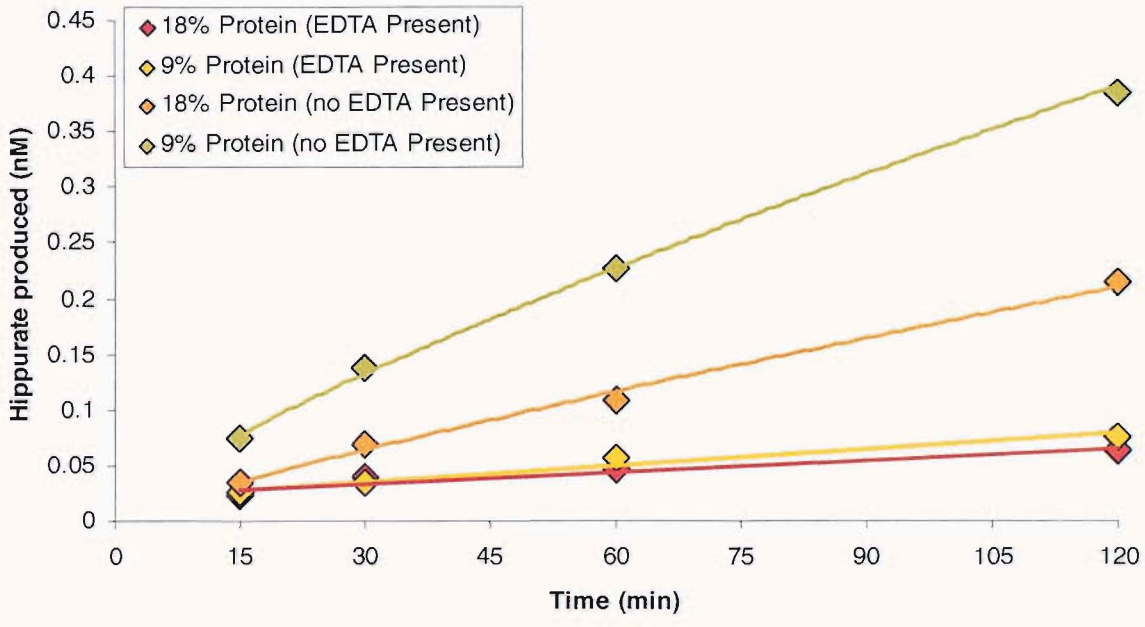

Fig. 5.3.6b: ACE activity over a time course incubation of 15-120. Samples where no EDTA was added to the extraction buffer are linear between 15 and 60 min. The samples where EDTA was added show a reduced capacity of ACE to convert Hip-His-Leu to hipputic acid.

From the pilot studies, the amount of total protein to be used and the incubation time was selected. As a result of these observations, the experiments to assess local uterine changes in ACE activity were conducted using 300 µg of protein and a 60 min incubation time in buffer containing no EDTA. Overall, mice fed the experimental 9% LPD until 3.5 days of pregnancy were found to have significantly increased rate of ACE activity in uterine tissue (Fig. 5.3.6)

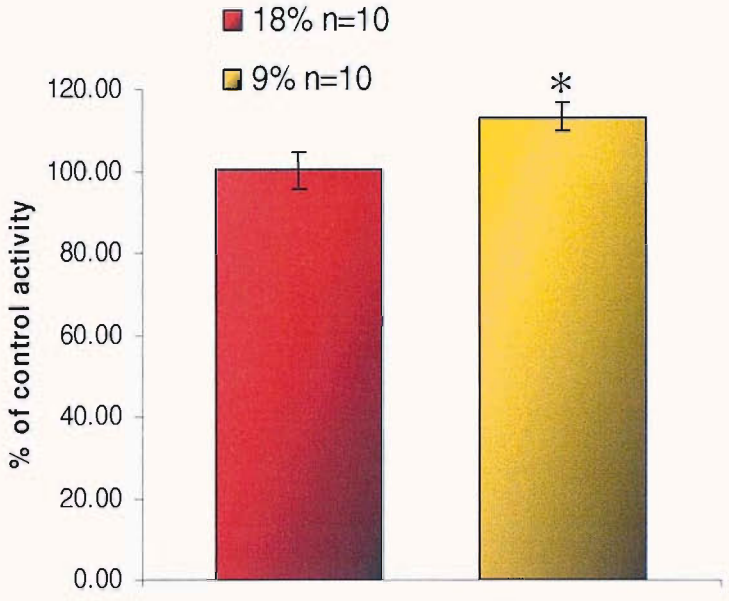

Fig 5.3.6c: Percentage activity of ACE in uterine tissue at 3.5 days of pregnancy following maternal treatment with either a 9% LPD or an 18% NPD. ACE activity is significantly elevated in uterine tissue 4.5 days post plug from mice fed a 9% LPD compared to controls fed a 18% NPD. *=$p<0.05$

5.3.7 Uterine weights and protein extracted

The wet weight of each uterine horn extracted on day 3.5 of pregnancy for western blot analysis was measured before snap freezing to see if dietary treatment had any impact on reproductive organ weight. No significant differences were observed in total wet weight of uterine horns extracted from animals fed either dietary treatment (Fig. 5.3.7a). However, it was found that significantly less total protein was extracted from uteri taken from mice fed the 9% LPD up to day 3.5 of pregnancy compared to the 18% NPD controls (Fig. 5.3.7b). In terms of total protein per gram uterine wet weight no significant differences were observed between dietary treatments (Fig. 5.3.7c).

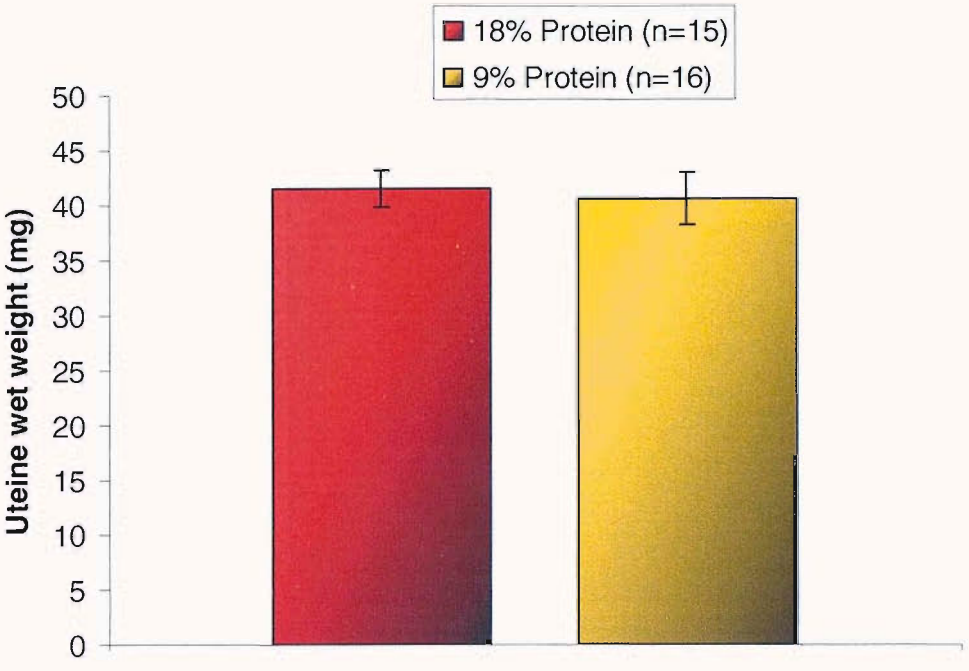

Fig 5.3.7a: The average weight of both uterine horns extracted from mice fed either the 9% LPD or the 18% NPD for the first 3.5 days of pregnancy. No significant differences were observed in uterine weights between dietary treatments.

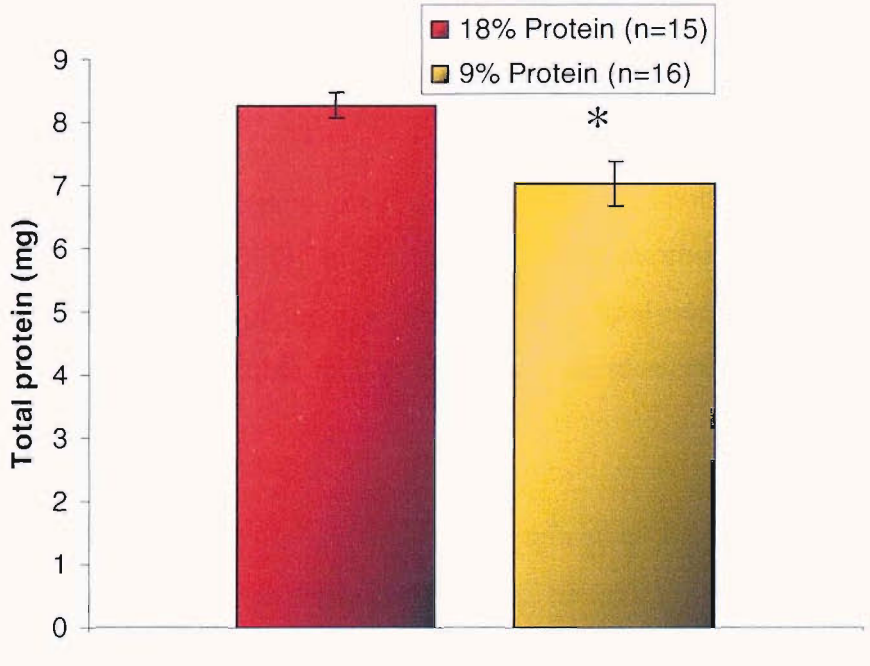

Fig 5.3.7b: The average amount of total protein extracted from uterine horns obtained from mice fed either the 9% LPD or the 18% NPD for the first 3.5 days of pregnancy. Significantly less total protein was extracted from uterine horns taken from mice treated with the 9% LPD compared to 18% NPD controls. *=$p<0.05$

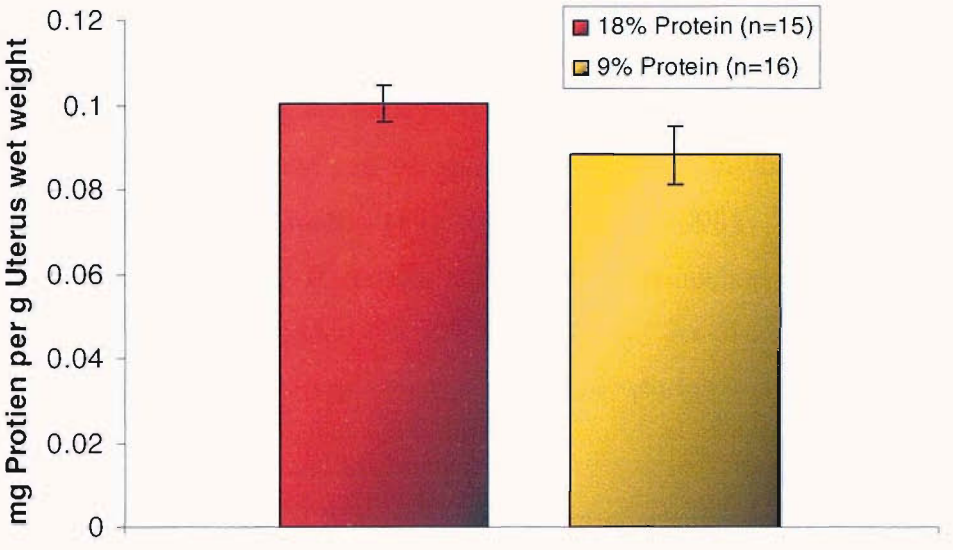

Fig 5.3.7c: The average amount of total protein extrated per gramme uterus wet weight from uterine horns obtained from mice fed either the 9% LPD or the 18% NPD for the first 3.5 days of pregnancy. No significant differences were observed in the total protein:uterine wet weight ratio between dietary treatments.

5.4 Discussion

The tissues of the uterus play a crucial role in modulation of the embryo environment and interact with the implanting blastocyst to produce a viable pregnancy. As a result, various proteins of the murine uterus were analysed in relation to treatment with a LPD to elucidate possible factors that may lead to metabolic programming of a Syndrome X phenotype.

FGF-II is a multifunction cytokine implicated in the differentiation and proliferation of a broad range of mesodermal and neuronal cell types (Delrieu, 2000). The FGF-II is encoded by a single gene and produces several splice variants. The high molecular weight isoforms of size 22, 22.5, 24 (Florkienwicz and Sommer, 1989; Prats *et al*, 1989) and 34 kDa (Arnaud *et al*, 1999) are produced via multiple CUG translation start codons (Fig. 5.4a). Alternatively, the 18 kDa splice variant is produced from an AUG start codon 3' to the high molecular weight splice variants CUG codons

(Florkienwicz and Sommer, 1989; Prats *et al*, 1989) (Fig. 5.4a). Thus, high molecular weight splice variants are co-linear amino-terminal extensions of the 18 kDa isoform (Delrieu, 2000). Notwithstanding the lack of a traditional secretory signal peptide, the 18 kDa splice variant undergoes exocytosis through the endoplasmic reticulum via a Golgi-independent pathway and can be localised in various biological fluids including plasma and the extracellular matrix (Florkienwicz *et al*, 1998). The higher molecular weight splice variants of FGF-II contain a 37 amino acid nuclear localisation sequence located 5' to the AUG start codon (Bugler *et al*, 1991). As discussed in Section 1.5.4 FGF-2 has been demonstrated to be of some importance during reproductive events.

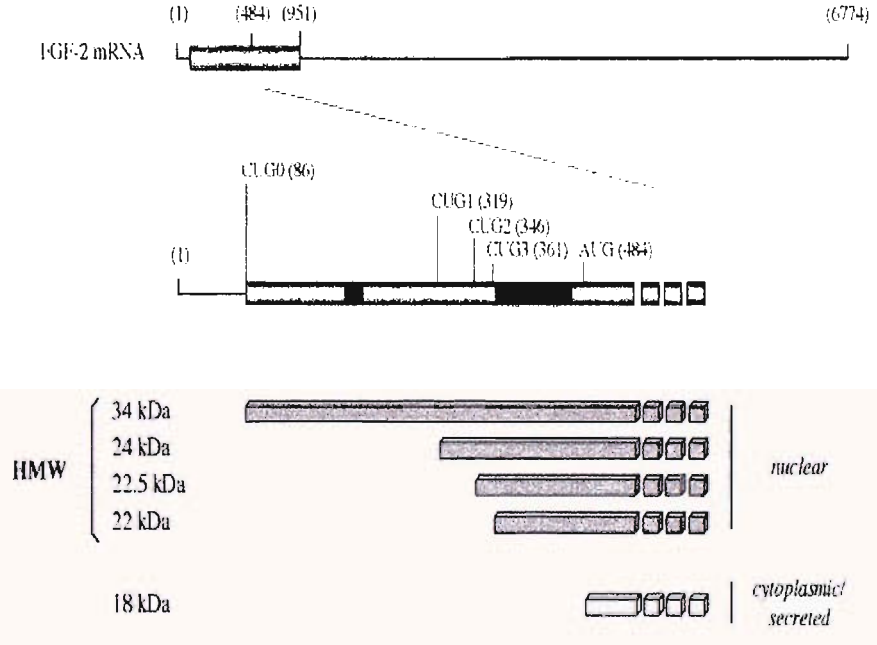

Fig 5.4a: The human FGF-II gene. Top: 6774 nucleotide mRNA FGF-II showing the translated region (grey box) and the 5' and 3' untranslated regions (black lines). Middle: 5' section of the FGF-II mRNA showing the 5' untranslated region, translated regions (boxes) nuclear localisation sequences (black boxes) and AUG/CUG translational start codons that account for different splice variants. Bottom: the high molecular weight (grey boxes) 18 kDa (open boxes) splice variants of the FGF-II proteins corresponding to mRNA domains. Reproduced from Delrieu, 2000. Note Human Rat and Mouse FGF-II share 97% sequence Homology (Wright, 2001)

FGF-II has been shown to control various biological activities in various cell types including proliferation, differentiation, migration, vasculogenesis, angiogenesis, blood vessel remodelling and wound healing (Szebenyi and Fallon, 1999). FGF-II is also well known to be a peptide of some importance during reproduction. It has been shown to be present in the uterus of many mammals including primates (Samathanam *et*

al, 1998), pigs (Katsahambas and Hearn, 1996) and rabbits (Grundker and Kirchner, 1996). FGF-II has been localised to the endometrial stroma underlying the luminal epithelium of the mouse uterus (Wordinger *et al*, 1992; Paria *et al*, 2001; A.E. Wild and J. Cleal, pers. com) and blood vessels within the endometrium (A.E. Wild and J.Cleal, pers. com.) and has been shown to co-localise with macrophages in the murine endometrium (Wild and Cleal, personal communication) (Fig. 5.4b).

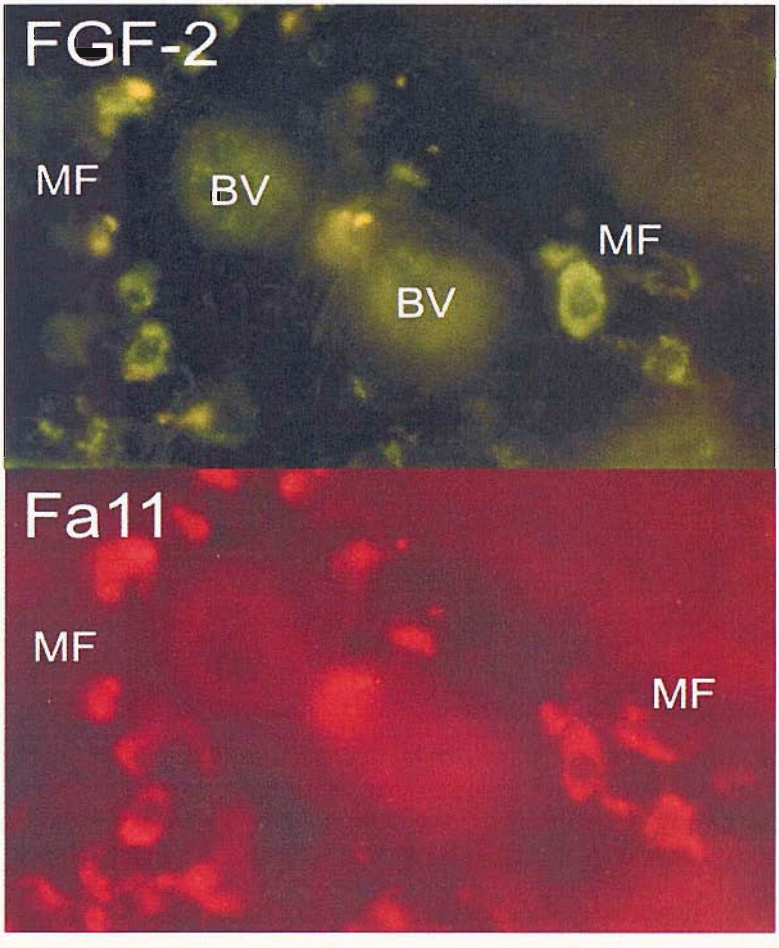

Fig. 5.4b: Section of paraffin fixed uterine endometrial tissue immunostained for both FGF-II and Fa11 (detects activated macrophages; section 4.3.1 and 4.3.2). Note that FGF-II is present within the uterine endometrium and co-localises with both activated macrophages and blood vessels. Picture courtesy of A.E. Wild and J. Cleal (personal communication)

SDS-PAGE and subsequent blotting allowed detection of three splice variants of FGF-II in uterine tissue on day 3.5 of pregnancy; these were the 18, 22 and 22.5 kDa isoforms of the protein. Semi-quantitative measurement by chemiluminescent protein detection demonstrated that levels of the 22 and 22.5 kDa splice variants of FGF-II were significantly increased in the uterine tissue of mice treated with a 9% LPD relative to

controls fed an 18% NPD. Levels of the 18 kDa splice variant showed no significant difference between the two dietary treatments. The elevation observed in the 22 and 22.5 kDa splice variants of FGF-II was also seen where FGF-II protein levels were measured using an infrared detection system. The infrared detection system offered significant advantages over the traditional chemiluminescent system of protein detection including lower background, higher sensitivity and direct detection of proteins from membranes. Moreover, the infrared detection system allowed simultaneous visualisation of two proteins. In this case, actin in addition to FGF-II; as a result, FGF-II measurements could be adjusted for loading error as the house-keeping protein actin was shown not to alter in response to diet. This elevation of high molecular weight isoform of FGF-II in mice fed a 9% LPD could represent a form of compensation by the uterus to dietary challenge. As mentioned above, FGF-II is a potent inducer of vascular remodelling. The rise in FGF-II in response to 9% LPD treatment may facilitate implantation and/or nutrient supply to the embryo under dietary challenge. The fact that the secreted 18 kDa low molecular weight isoform was shown not to alter significantly in relation to dietary treatment suggests that the embryo does not experience altered FGF-II signalling, at least during the pre-implantation period. Indeed, I have also shown that VEGF protein is significantly elevated in maternal serum (Section 6.3.7) on day 4.5 of pregnancy.

Two possible pathways may explain the observed elevation of FGF-II levels in relation to diet; both would depend upon an increase in availability of circulating corticosterone signalling in response to treatment with a 9% LPD. In the first pathway (Fig. 5.4c; red lettering), a rise in circulating GCs causes increased levels of the colony stimulating factor 1 receptor (CSF-1R) (Sapi *et al*, 1995) possibly by binding to a composite Glucocorticoid receptor element (GRE) on its proto-oncogene *C-fms* (Flick *et al*, 2002). Additionally, studies on cultured human endometrial cells also suggest that levels of C-fms and its ligand, CSF-1, are increased in response to GCs (Gill *et al*, 2001b). Increases in these proteins lead to an increase in macrophage proliferation (Wood *et al*, 1992) within the uterus. Activated macrophages have been shown to co-localise with FGF-II in the uterus (A.E. Wild and J. Cleal, per. com.) (Fig. 5.4b) and this is suggestive of macrophage production of FGF-II. Consequently, increased macrophage

numbers would lead to increased production of FGF-II. However, A.E. Wild and J. Cleal (per. com.) failed to demonstrate a significant rise in macrophages within the uterine endometrium by examination of paraffin sections. As a result, the second pathway may be considered (Fig. 5.4c; black lettering) where elevated GC's increase ACE levels (Fishel *et al*, 1995; Barreto-Chaves *et al*, 2000, Barreto-Chaves *et al*, 2001). This increase in ACE levels then causes an increase in the conversion of angiotensin I (ANGI) to ANGII. This in turn may lead to increased binding of ANGII to the AT 1 receptor and through intracellular signalling FGF-II production is increased (Peifley and Winkles 1998; Peng *et al*, 2001).

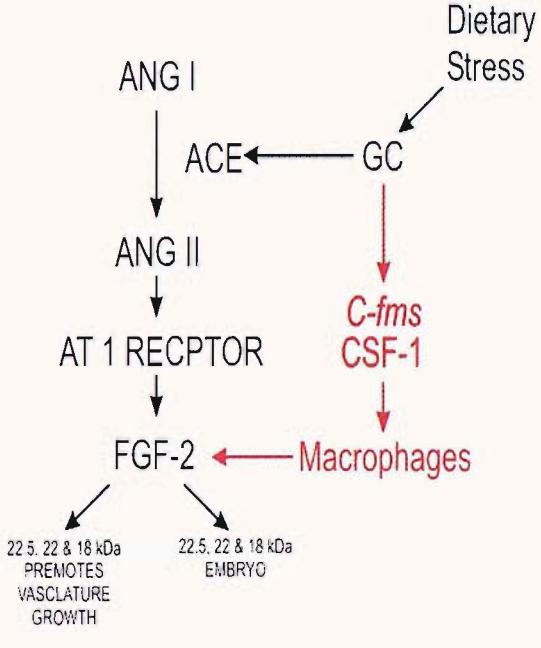

Fig 5.4c: Two theoretical pathways under investigation whereby FGF-II levels increase in response to 9% LPD treatment. Pathway I (red lettering): Increased GCs cause an increase in the levels of C-fms and CSF-1, these in turn lead to increased numbers of macrophages and expression of FGF-II. Pathway II (black lettering): Elevated GCs cause an increase in the amount of ACE protein. ACE converts ANG I to ANG II which in turn binds to the AT1 receptor causing intracellular signaling and a rise in FGF-II levels.

Indeed, further investigation demonstrated that at day 3.5 of development the activity of ACE was significantly elevated in the uterine tissues of mice fed the 9% LPD. While the main role of ACE in mammals is still thought to be as a component of the RAS which controls blood pressure, Corvol *et al* (1995) suggested the relevance of ACE to the animal must be much more complex than this; two reasons were cited. One reason

is the ACE gene in itself is very complex, and second that the gene and product are present in species which lack an identifiable renin angiotensin system (RAS). ACE is anchored by its C-terminal transmembrane domain with its active sites exposed to the extracellular spaces. However, ACE also exists in a soluble circulating form and is produced by specific proteolytic cleavage extracellular to its C-terminal transmembrane anchor probably by a zinc metalloprotease 'secretase' (reviewed by Turner & Hooper, 2002). Furthermore, it is worth noting at this stage, that the increase in ACE activity observed in uterine tissue in this study was not mirrored in maternal serum (section 6.3.2). This suggests that any increase in conversion of ANG I is confined to the local uterine RAS rather than being a systemic response to LPD. However, when corticosterone was assayed in maternal serum from mice fed either the 9% LPD or 18% NPD, no significant differences were observed (section 6.3.4), suggesting that circulating corticosteroids *per se* are not the primary cause of an elevation in FGF-II.

It has been suggested that within the uterus Ang II may be involved with implantation and, moreover, control of uterine blood flow during pregnancy (Hagemann *et al*, 1994). Indeed, experiments by Squires and Kennedy (1992) suggest that there is a requirement for ANG II during decidualisation in rats. It has been shown that there is a small yet detectable quantity of ACE within uterine tissues (Cushman and Cheung, 1971) which localised mainly to uterine blood vessels (Moeller *et al*, 1992). ANG II receptors are present primarily within the myometrium, uterine glands and at a low level in the endometrium of the rat uterus. ANG II receptors are also abundant in the stroma of the mouse uterus, [both the AT-1 and AT-2 subtypes] (Speth *et al*, 1999).

In lieu of the results obtained during the analysis of corticosterone levels in maternal serum, it was decided that analysis of glucocorticoid receptor levels in relation to dietary treatment should be undertaken. The glucocorticoid receptor (GR), of mass 95kDa (Arriza, *et al*, 1987), is a member of the steroid hormone receptor superfamily, which also includes progesterone receptor and androgen receptor (Evans, 1988). It has a modular structure consisting of a DNA-binding domain (DBD), a ligand-binding domain (LBD), a nuclear localisation domain, and several transcriptional activation functions (Beato *et al*, 1995). GR is expressed in most tissues and has an affinity constant for GCs

well within the normal physiological band, resulting in the strength of signal from the GR being proportionate to the amount of circulating GC (Beato *et al*, 1995). When unbound to GCs, the GR resides in the cytoplasm where it complexes with several other ancillary proteins, including heat-shock protein 90 (Gustafsson *et al*, 1989). When the receptor binds to GC, any ancillary proteins that are bound to it are released and the ligand-receptor complex proceeds into the nucleus; this is by virtue of a nuclear localisation sequence located in the DBD. Once in the nucleus, the GR will associate with a second ligand-receptor complex forming a homodimer. The dimerised receptor can then interact with DNA and influence transcription directly through several types of (GREs). The induction kinetics of this system are relatively slow, usually between 24-48 hours, suggesting that this mode of gene activation is more likely to be involved in longer-term developmental and anti-inflammatory processes. Because of this relatively slow mode of action, it is not extensively believed that this method of transcriptional activation plays a role in the more rapid anti-inflammatory activity of GCs (Newton, 2000). Additionally, another splice variant of GR exists known as GRβ; this differs from GRα only at the carboxyl terminal. However, it appears that this splice variant is not expressed in mice, apparently because the GRβ exon belongs to the 3'-untranslated region of the murine GR gene (Otto *et al*, 1997). GR mRNA has been identified in the uterus (Gunin *et al*, 2003) and GR protein has been specifically localised in the luminal epithelium, endometrial stroma and myometrial smooth muscle, but not the endometrial glands (Korgan *et al*, 2003). This investigation has shown also that GR is present in uterine tissues of the mouse however its expression was not found to significantly alter in relation to dietary treatment.

It should also be pointed out that estrogen has also been shown to positively regulate FGF-II expression in human fibroblasts (Fujimoto *et al*, 1997a; Fujimoto *et al*, 1997b) and in ovariectomised ewes (Reynolds *et al*, 1998). While an elevation in circulating estrogen has been found to exist in this study (see Section 6.2.5) it was found on day 4.5 of pregnancy not on day 3.5 of pregnancy where the elevation in FGF-II is observed. As a result, the elevation in FGF-II and serum estrogen may not be physiologically related. However, estrogen can regulate splice variants of the FGF1R gene (discussed in Section 6.4).

The IGF axis plays an important role in reproductive physiology. The behavior of the IGF axis is regulated by six well characterised IGF-binding proteins (IGFBP), these are designated IGFBP-I through -VI. IGFBPs modulate IGF availability and activity (Jones and Clemmons, 1995) Modulation of IGF availability to the IGF receptors by IGFBPs is due to the fact that the IGFBPs bind the IGFs with much higher affinity than their receptors (Clemmons, 1997). IGFBPs can also serve to increase the half-life of IGFs. For example, plasma IGF-I half life can be increased to 15 hours when bound by IGFBP-II compared with 30 minutes for unbound IGF-I (Guler *et al*, 1989); this mechanism also serves to provide a pool of IGFs. Several of the IGFBPs have also been shown to bind a variety of extracellular matrix components, cell association molecules and putative receptors; these interactions are collectively known as IGFR-independent actions. IGFBPs -I and -II both have an Arg-Gly-Asp intergrin-binding motif at their C-terminus. In IGFBP-I, this has been shown to stimulate cell migration of human trophoblasts through the fibronectin receptor (Irving and Lala, 1995).

To a large extent the IGF axis is under nutritional control. Studies have shown that when either a LPD, a single essential amino acid deficient diet or a plant protein diet are fed to rodents, IGF-I synthesis is reduced (Thissen, *et al*, 1994; Underwood *et al*, 1994) and IGFBP-I synthesis is increased (Kato, *et al*, 1991). Isolated rat hepatocytes have been shown to decrease IGF-I secretion increase secretion of IGFBP-I (Jousse *et al*, 1998) when one or more essential amino acids are in short supply. Evidence from several studies seems to support the idea that the IGF axis is regulated by factors linked to the availability of essential amino acids (Takenaka *et al*, 2000). For example, the IGFBP-I gene itself has at least 4 promoters, an insulin response element (IRE), a GRE, a cAMP response element and a hepatocyte nuclear factor-1 binding site (Lee *et al*, 1993). Takenaka *et al* (2000) cultured hepatic cells in medum with minimal amino acids and determined that the cis-acting sequence involved in the amino acid signal was in the same region as the IRE and GRE, located in the 5' flanking region of IGFBP-I. This model system excludes the influence of external endocrine factors in the response to amino acid deprivation in vitro and suggests the existence of an amino acid response element in this gene. GCs stimulate IGFBP-I while insulin represses its expression

(Orlowski *et al*, 1991). Insulin suppresses IGFBP-I transcription in a dominant fashion so that it will not only suppress basal IGFBP-I production but also GC stimulated production (Unterman *et al*, 1992). It is quite possible that regulation of IGFBP-I in vivo is achieved through a cross-talk mechanism involving GCs, insulin and amino acids. Similarly to IGFBP-I, IGFBP-II has been shown to be under nutritional control. IGFBP-II transcription has been shown to increase up to 40% in response to severe dietary protein deprivation (Ketelslegers *et al*, 1996; Clemmons, 1997) and increase up to 75% in the serum of severely food restricted guinea pigs. (Sohlstrom *et al* 1998)

Given the potential for IGFBP-I and II to be under complex nutritional control, they presented themselves as ideal candidates for research with respect to metabolic programming. IGFBP-I was found to be expressed at very low levels in uterine tissue at day 3.5 and was undetectable at day 4.5 pregnancy. However, these low levels of detection for IGFBP-I were not reflected in samples of fetal liver assayed as a positive control. This suggested that the antibody was capable of detecting IGFBP-I but those levels of expression in the uterus are very low around the peri-implantation period. These low levels of IGFBP-I expression in the uterus prior to implantation are perhaps not surprising as studies in primates have demonstrated that IGFBP-I expression is a conceptus mediated event (Kim, 2004; Tarantino *et al*, 1992). Unfortunately, the low levels of detection obtained for IGFBP-I in the uterus prevented further quantitative analysis its expression in relation to treatment with a LPD. It is possible that these low levels of detection in this investigation represent the fact that the uterus produces non-phosphorylated and 3 lesser-phosphorylated variants of IGFBP-1 (reviewed by Carter *et al*, 2004). Indeed, the antibody used in this investigation may only effectively recognise the fully phosphorylated version of IGFBP-1 produced by the liver.

In contrast to IGFBP-I, IGFBP-II was not detectable in uterine tissue at 3.5 days pregnancy, yet it was clearly visible in uterine tissue on day 4.5 pregnancy. Even though the average amount of IGFBP-II in mice fed the 9% LPD was nearly twice that of those fed the 18% NPD this difference was not significant. The large variations in IGFBP-II expression were observed between animals, not within the replicates of individual animals, suggesting that the experimental methodology was sound and that the large

amount of variation was due to some natural phenomenon. In previous studies IGFBP-II mRNA has been detected at low levels by *in situ* hybridisation in mouse uteri prior to implantation (Damario *et al*, 1998) and at much higher levels in decidua post-implantation in the mouse (Damario *et al*, 1998) and the rat (Cerro and Pinter, 1997). The fact that IGFBP-II only becomes detectable in this study at the peri-implantational period on day 4.5 post plug is suggestive that expression of this protein is linked to implantation events. It is possible due to the specific and dramatic up regulation of IGFBP-II at implantation, the timing of ovulation becomes much more of an issue with respect to IGFBP-II measurement in the uterus. In short, it is possible that even a short difference between the ovulation times of the mice used in this experiment may introduce the large variations in IGFBP-II expression observed in this experiment. It is possible that IGFBP-II levels are indeed elevated with respect to 9% LPD treatment in this experimental model, but that variation induced by slight differentials in developmental timing are interfering with its detection. Was shown to be significantly elevated in response to LPD treatment this could suggest that implantation is occurring sooner in the LPD treated animals.

As discussed above, several studies have shown that IGFBP-II levels can increase in response to dietary insult (Ketelslegers *et al*, 1996; Clemmons, 1997; Sohlstrom *et al* 1998). While these earlier studies used far harsher dietary treatments than my model of metabolic programming they serve to show that diet can have an effect on IGFBP-II levels. Additionally IGFBP-II levels have been shown to be positively regulated by estrogen (Adesanya *et al*, 1996). This study has observed a significant elevation of serum estrogen concentrations on day 4.5 post plug (section 6.2.5) which could be interpreted as circumstantial evidence for increased IGFBP-II levels. Ultimately, IGFBP-II was not found in this experiment to be significantly different in the uterus; however, this may be a function of its rapid up-regulation during implantation and the inability of the experimental protocol to control for small differences in the timing of ovulation.

As already discussed above significant elevations were observed in uterine FGF-II and ACE activity in uteri of mice treated with the 9% LPD compared to those fed the

18% NPD up to day 3.5 of pregnancy. However one could speculate that these observed elevations may be an artefact of altered uterine structure specifically relating to uterine weight and total protein content; as a result data relating to uterine wet weights and total protein content was analysed for uteri extracted on day 3.5 of pregnancy. This analysis found no significant differences in the total wet weight of uteri extracted on day 3.5, however, significantly less total protein was extracted from the uteri of mice treated with the 9% LPD. This finding could call into question the biological significance of the elevations observed. Indeed, the possibility exists that the observed elevations in FGF-II and ACE activity may simply be artefacts of adding proportionately more of the uterine total protein to the experiments conducted. As a result the relationship between uterine weight and total protein extracted was analysed. Here, it was found that no significant differences existed between dietary treatments of the ratio of total protein:uterine wet weight. This finding suggests that while the uteri from 9% LPD treated mice do contain less protein, the total amount of protein per unit uterine weight is comparable between dietary treatments. The consequence of this is that it seems likely that the observed significant elevations in FGF-II and ACE activity are indeed biologically relevant and not simply artefacts of the experimental design.

Nevertheless the fact that total protein appears to be significantly reduced in the uteri of mice treated with the 9% LPD while total uterine weight remains comparable does raise some interesting questions. The most obvious explanation for this is that uteri from 9% LPD treated mice may have less total protein but greater water retention thus leading to the observed equivalence in uterine weight between dietary treatments. It is fascinating that increased uterine water retention is a consequence of elevated estrogen (reviewed by Couse and Korach, 1999). Conversely, it has also been shown that administration of anti-estrogen chemicals can reduce uterine weight due to reduced water retention (Janessens *et al*, 1984). This study has also found circulating estrogen to be elevated on day 4.5 of pregnancy (section 6.). While the observations for uterine weight and total protein were taken on day 3.5 not day 4.5 it is possible that elevations in estrogen levels may go some way to explaining these observed phenomenon. In addition, Hastings *et al* (2003) have shown that estrogen induced uterine water retention is not

mediated via the action of VEGF, which this study has also been shown to be elevated within maternal serum.

In this Chapter I have described elevations in uterine FGF-II and ACE activity in response to administration of maternal LPD. These data taken together with evidence in Chapter 6 that VEGF and estrogen are elevated in maternal serum appear to suggest that the mice treated with the LPD are exhibiting increases in angiogenesis and vascular remodeling. These modulations in expression of these molecules that attenuate vascular genesis in the uterus could form the basis of alterations designed to aid embryo implantation. Indeed the results of Chapter 4 suggest that the amino acid composition of the uterine fluid is altered in response to LPD such that an embryo developing within this environment may have lower protein synthesis and consequently be less prepared for implantation. In this situation it would be advantageous for the uterine tissue not only to be receptive to implantation but also ready to supply any extra nutrients the embryo may require.

Chapter 6

Maternal Serum

6.1 Introduction

Thus far I have investigated specific systems local to the uterus that may influence metabolic programming. I have not, however, defined the metabolic and endocrine status of the mother during administration of the LPD. Is has been shown that dietary protein restriction can increase serum corticosterone (Herbert & Carrillo, 1982), lower serum albumin (Ramos *et al*, 2000), lower serum insulin (Reis *et al*, 1997), lower serum IGF-I (Bourrin *et al*, 2000; Filho *et al*, 1999; Fliesen *et al*, 1989) and increase serum IGFBP-I (Filho *et al*, 1999). Administration of LPD has also been shown to decrease levels of very low density lipoprotein-triacylglycerols suggesting that limiting of lipid storage occurs in adipose tissues (Boualga *et al*, 2000).

Where metabolic programming has been studied throughout pregnancy, it has been shown that serum concentrations of branch-chain amino acids can be depleted and that threonine in particular can be depleted by up to 50% close to term in rats fed a 9% LPD (Rees *et al*, 1999). Serum of rats fed a LPD for the pre-implantation period were found to have increased serum glucose, decreased serum insulin and a depletion of essential amino acids (Kwong *et al*, 2000). Similarly, Petrie *et al* (2002) found perturbations in circulating amino acid levels and additionally demonstrated that homocysteine levels are significantly elevated in serum of mice and rats administered a LPD during pre-implantation development.

The adaptations that the mother makes to accommodate administration of a LPD regime are key to establishing the basis of our observations within the uterine fluid and uterine tissue. As a result, this chapter attempts to understand composition of maternal serum with respect to dietary treatment so I can further refine our results to date and investigate the mechanisms of metabolic programming.

6.2 Methods

6.2.1 Total protein

Serum was collected from mice fed either the LPD or NPD diet and culled 2.5, 3.5 or 4.5 days of development (Section 2.2). Serum was diluted 1:20 in PBS to ensure that readable protein levels were within the linear range. The DC protein assay kit (Biorad, UK) microplate assay protocol was used to determine total protein levels. Serum samples were loaded onto the microplate in triplicate. Serial dilutions of 10 mg ml^{-1} BSA in PBS were used to generate standard curves.

6.2.2 Serum angiotensin converting enzyme assay

Serum was collected and prepared (section 2.2) and the serum ACE assay was conducted as described in the serum ACE section of the Materials and Methods (section 2.9).

6.2.3 Serum Insulin

Serum insulin was measured using a Rat insulin sandwich ELISA kit (Crystal Chem International, IL, USA), the same kit used for the measurement of uterine fluid insulin (Sections 4.2.6 and 4.3.6). This kit is also 100% reactive with mouse insulin. Insulin is detectable from 156-10,000 pg ml^{-1}. Absorbance was measured on a Dinatech MR5000 plate reader, measuring wavelength at 480 nm and subtracting wavelength at 630 nm.

6.2.4 Serum Corticosterone

Serum corticosterone was measured using a ^{125}I RIA kit (ICN Biomedicals, UK). This kit works by way of competitive binding between serum corticosterone and ^{125}I labeled corticosterone and is accurate betweeen 25-1000 ng ml^{-1}. Samples were measured in duplicate using a 1274 RiaGamma (LKB-Wallac, Finland).

6.2.5 SerumEstrogen

Serum estrogen levels were measured in mouse serum 2.5, 3.5 and 4.5 days of pregnancy by Dr Fred Anthony at the Maternal Fetal and Neonatal Physiology Deparment, University of Southampton, using 3rd Generation Estradiol RIA (DSL, UK). This is a competitive binding assay between serum corticosterone and ^{125}I labeled estradiol and is accurate from 1.5-150 pg ml^{-1}. The assay was modified to measure estrogen in smaller volumes of serum. In short, 20 µl of standards, controls or unknowns were pipetted into appropriately labeled 12 × 75 mm glass test tubes. 20 µl estradiol antiserum was added to all tubes except those tubes used for non-specific binding and total count controls. Tubes were incubated for 4 hrs at 4°C. 20 µl of ^{125}I labeled estradiol was added to all tubes. Tubes were incubated for 20 hrs at 4°C. 500 µl of precipitating reagent was added to all tubes except total count tubes. Tubes were incubated at room temperature for 20 min then centrifuged at 1,500 g. After centrifugation, supernatant was aspirated and ^{125}I-labeled estradiol measured using a 1274 RiaGamma gamma counter (LKB Wallac, UK).

6.2.6 Serum Progesterone

Serum progesterone was measured using a 17α-OH progesterone ^{125}I RIA kit (DSL, UK). This kit works by way of competitive binding between serum progesterone and ^{125}I labeled corticosterone and is accurate between 0.1-20 ng ml^{-1}. Samples were measured in duplicate using a 1274 RiaGamma (LKB-Wallac, Finland).

6.2.7 Serum VEGF

Serum VEGF levels were measured using a pan VEGF ELISA kit (R&D systems, UK). VEGF is detectable from 156-10,000 pg ml^{-1}. Absorbance was measured on a Dinatech MR5000 plate reader, measuring wavelength at 480 nm and subtracting wavelength at 630 nm.

6.2.8 Serum Glucose

Serum samples were diluted 1:100 in PBS and sent to Dr Peter Humpherson on dry ice at the Department of Biology, University of York for analysis using Cobas-Mira S analyzer (see section 2.6).

6.2.9 Amino acids

Serum Samples were diluted 1:25 in PBS and sent to Peter Humpherson on dry ice at the Department of Biology, University of York for HPLC analysis (see section 2.7).

6.3 Results

6.3.1 Total Protein

Since our dietary model relies on protein restriction in the experimental animals it was logical to measure total protein in maternal serum. Total protein levels within maternal serum were found to be significantly elevated ($p<0.05$) in animals fed the experimental LPD (n=13) compared to those fed the control NPD (n=13) at day 2.5 but not at days 3.5 or 4.5 post plug (Fig 6.3.1). Additionally, animals fed the LPD displayed a significant depletion in total protein levels between day 2.5 (n=13) and 4.5 (n=18) of pregnancy ($p<0.05$).

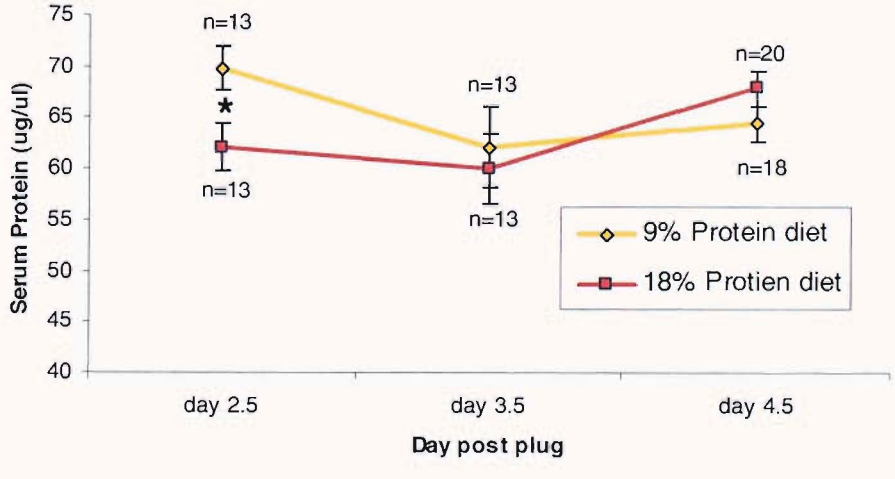

Fig 6.3.1: Time course showing changes in serum total protein levels between day 2.5 and day 4.5 of pregnancy. A significant elevation of total protein was observed between the 9% protein LPD and 18% protein NPD groups on day 2.5 post plug. Additionally, a significant depletion of total protein between days 2.5 and 4.5 post plug was observed in the 9% protein LPD group. Error Bars are SEM.

6.3.2 Serum ACE activity assay

Because a significant elevation in the activity of ACE was observed in uterine tissue of mice fed the experimental 9% LPD (section 5.3.6), it was decided that serum

activity of this enzyme should also be measured to ascertain if this effect was due to local or global changes in ACE activity. For this experiment, pilot studies were undertaken to optimise the experimental conditions. Serum at 2.5, 1.25, 0.625 or 0.313 µl volumes from a mouse fed the 18% NPD was assayed for ACE activity using an incubation of 120 min at 37°C as described in the serum ACE section of the Materials and Methods (Fig. 6.3.2a). Additionally, 2.5 µl of serum from a mouse fed the 18% NPD was assayed for ACE activity using incubations of either 15, 30, 60 or 120 min at 37°C (Fig. 6.3.2b). Based on the observations gained during these optimisations, it was decided to measure the activity of ACE using 2.5 µl of serum incubated in a shaking water bath at 37°C for 40 min. No significant difference was observed in ACE activity in the serum of mice fed the LPD (n=12) compared to mice fed the NPD (n=13) for 3.5 days post plug (Fig. 6.3.2c).

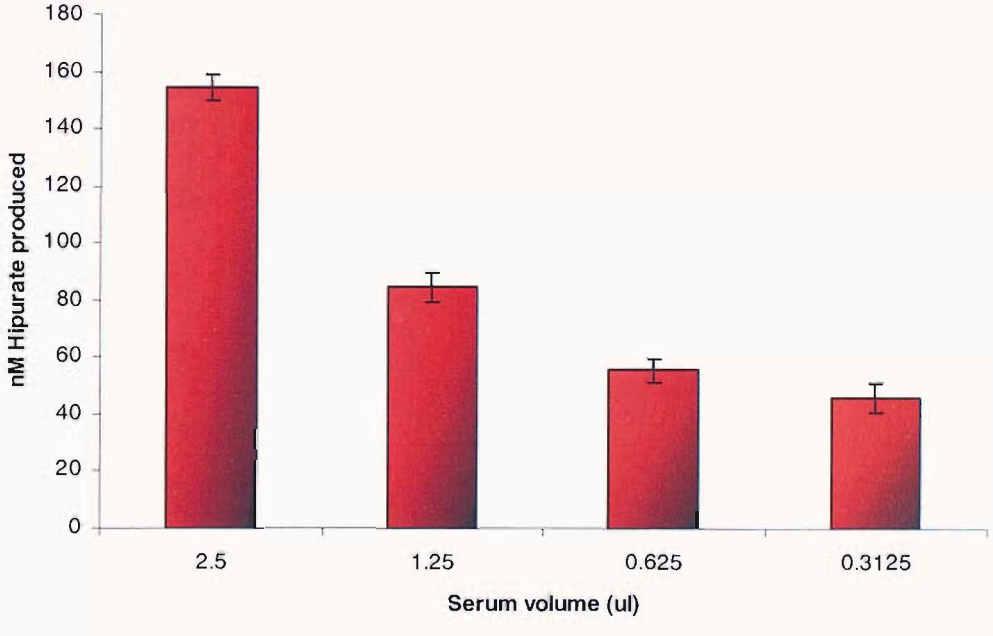

Fig. 6.3.2a: ACE activity within varying volumes of serum obtained from a mouse fed the 18% LPD over an incubation of 120 min. The concentration of hippurate produced is proportional to the volume of serum used. Error bars are SEM.

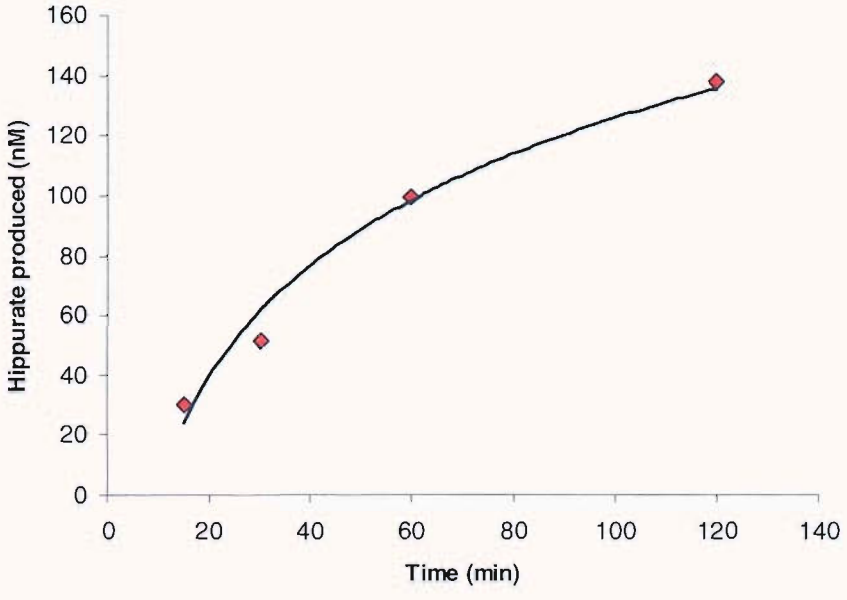

Fig.6.3.2b: ACE activity over time within 2.5 µl of maternal serum over incubations of 15, 30, 60 and 120 min. The liniear range for this assay extends from 15-60 min.

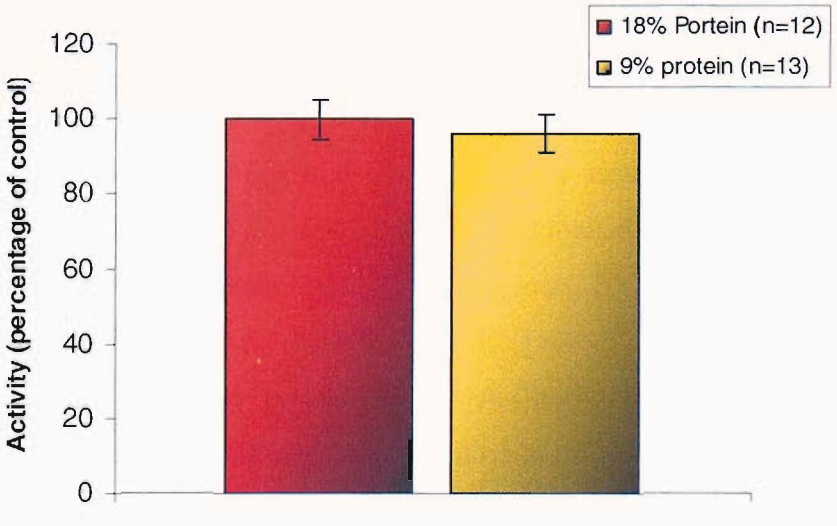

Fig 6.3.2c: Serum ACE activity of mice fed either the experimental 9% protein LPD or the control 18% protein NPD until day 3.5 of pregnancy. No significant differences were observed. Error bars are SEM.

6.3.3 Serum Insulin

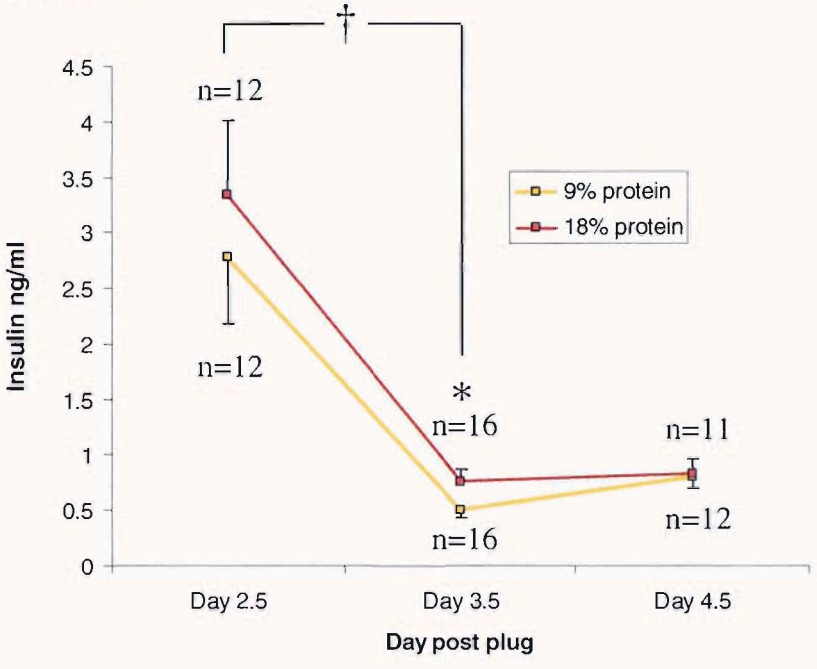

Fig.6.3.3a: Serum insulin levels at 2.5, 3.5 and 4.5 days of pregnancy in maternal serum of mice fed either the 9% protein LPD or the 18% protein NPD. Serum insulin is significantly reduced in mice fed a LPD compared to NPD control mice at day 3.5 of pregnancy. Error Bars are SEM. Additionally, there is a striking reduction in serum insulin levels between days 2.5 and 3.5 of pregnancy. * = $p<0.05$; †=$p<0.001$

A previous study has suggested that there is a significant depression in the maternal serum insulin levels of rats fed a 9% LPD compared to those fed an 18% NPD during pre-implantation development period (Kwong *et al*, 2000). I measured the serum insulin levels of mice fed either the experimental 9% protein LPD (n=16) or the control 18% protein NPD (n=16) for 3.5 days post plug. A significant depletion of insulin was observed in the serum of mice fed the LPD compared to those fed the NPD (Fig. 6.3.3a). What is more, a significant reduction in serum insulin levels was observed between day 2.5 and day 3.5 of pregnancy; this reduction in serum insulin was observed in the serum of mice treated with both diets (Fig. 6.3.3a). Standard curves in all three experiments were linear and reproducible (Fig. 6.3.3b), suggesting that conversion of absorbance values to insulin concentration was not a factor in this result.

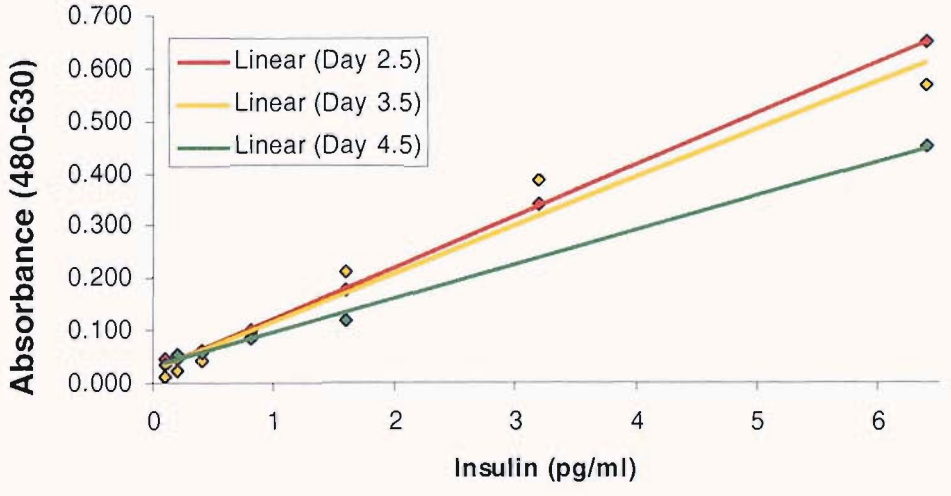

Fig.6.3.3b: The standard curves used to calculate insulin values in each of the three ELISAs conducted. The standard curves are repeatable and reproducible for each ELISA conducted for each time point.

This experiment was conducted by assaying insulin levels at each time point on a separate ELISA plate. To ensure that the large differences observed in serum insulin levels between day 2.5 and 3.5 were not due to inter-assay varation, a fourth ELISA was carried out containg samples from all three timepoints. It is worth noting at this point that this ELISA was carried out late in the course of this investigation and as a result insufficient serum remained to repeat the experiment. Consequently, only a subset of serum samples collected from mice treated with the 18% LPD were used in the verification. Insulin values were still significantly higher in maternal serum on day 2.5 compared to the other time points (Fig 6.3.3c), this suggests that the observed drop in serum insulin between days 2.5 and 3.5 is occurring and is not just a result of inter assay variation.

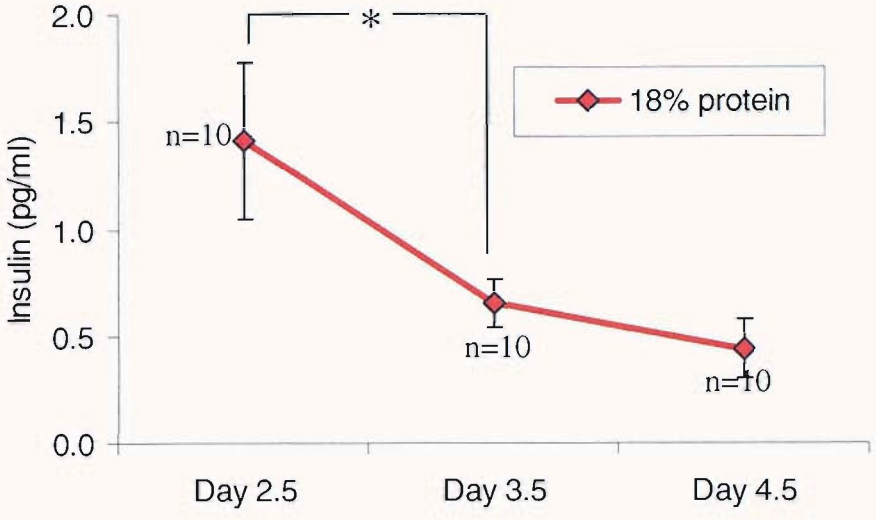

Fig.6.3.3c: Serum insulin from mice fed the 18% NPD until either 2.5, 3.5 or 4.5 days of pregnancy assayed on a single ELISA. A significant drop in serum insulin levels was observed between day 2.5 and day 3.5 of pregnancy and verifies the results shown in Fig.6.3.3a. Error bars are SEM. *=$p<0.05$

6.3.4 Serum Corticosterone

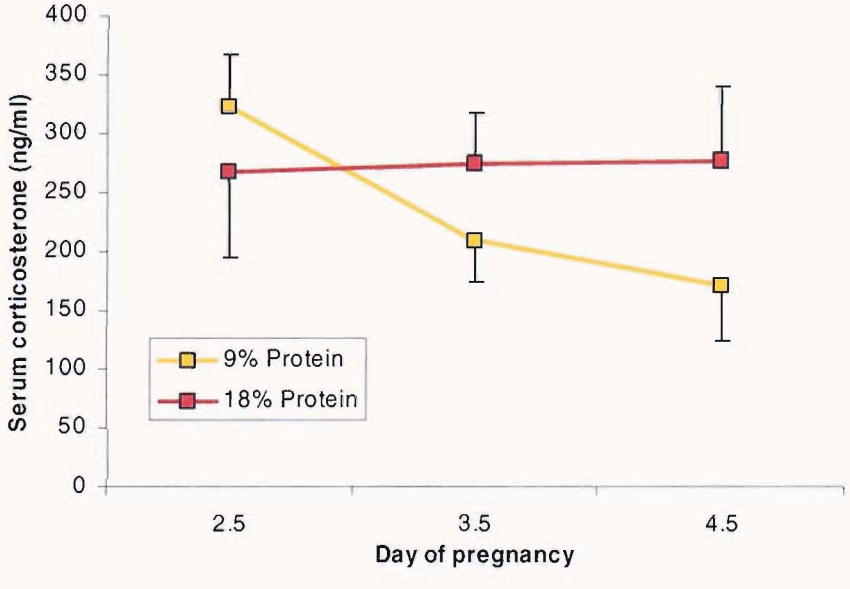

Fig 6.3.4: Corticosterone concentrations in mice fed either the 9% protein LPD or the 18% protein NPD for either 2.5, 3.5 or 4.5 days of pregnancy. No significant differences were observed in the concentrations of serum corticosterone between the two dietary regimes or within the dietary treatments at different time points. Error bars are SEM.

Corticosterone, one of the major components of the HPA axis, has been implicated in responses to LPD (Herbert & Carrillo, 1982) and is a potential mediator of

FGF-II levels through attenuation of ACE levels (Fig. 5.4b). Serum corticosterone levels were assessed in mice fed the LPD and the NPD, at 2.5, 3.5, and 4.5 days of pregnancy. No significant differences in serum corticosterone levels were observed between the different dietary regimes or indeed between time points in individual mice.

6.3.5 Serum estrogen

As discussed in Sections 1.5.2 and 1.5.4, estrogen is a potent mediator of many reproductive events. As a result, serum estrogen was measured on day 2.5, 3.5 and 4.5 of pregnancy. Serum estrogen concentration was found to be significantly higher in mice fed the 9% LPD at 4.5 days post plug ($p<0.05$) but not at 2.5 or 3.5 days of pregnancy. Moreover, there is a significant drop in serum estrogen levels of mice fed the 18% NPD between day 2.5 and day 4.5 of pregnancy. This drop in serum estrogen is physiologically normal (Section. 1.5.2) however, there is also an estrogen surge around implantation (Fig. 1.5.2) it is possible that this is being measured in the mice treated with the 9% LPD but not the 18% NPD.

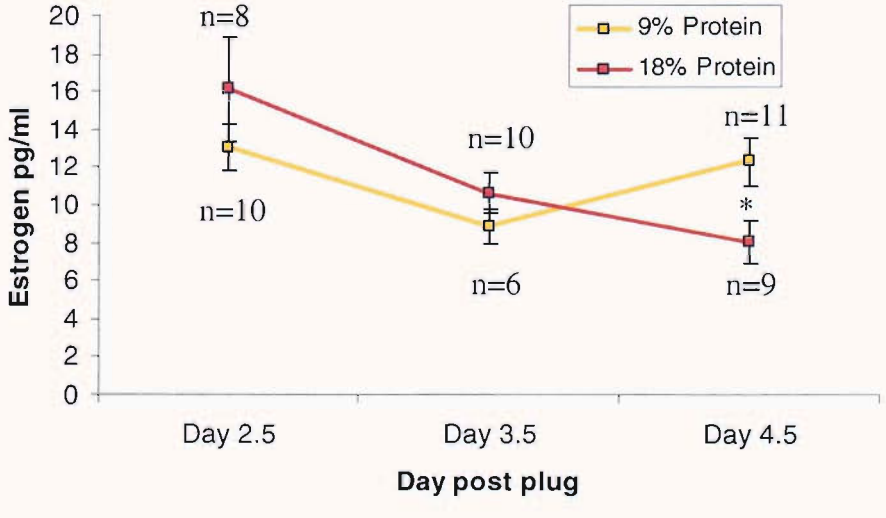

Fig. 6.3.5: Serum estrogen levels in mice fed either a 9% LPD or an 18% NPD from plug days 2.5, 3.5 and 4.5 of pregnancy. Estrogen is significantly elevated in mice fed the 9% LPD on day 4.5 of pregnancy. Additionally, there is a significant drop in estrogen concentration between day 2.5 and 4.5 post plug in serum of mice fed the 18% NPD, this reduction is not apparent in mice fed the 9% LPD. *=$p<0.05$ error bars are SEM.

6.3.6 Serum Progesterone

Progesterone is also a major regulator of reproductive events (discussed in Section 1.5.2 and 1.5.4), as a result, its levels in maternal serum were measured on day

2.5, 3.5 and 4.5 of pregnancy. Serum progesterone levels were found to be unaltered with respect to diet or time point within the same dietary treatment (Fig. 6.3.6).

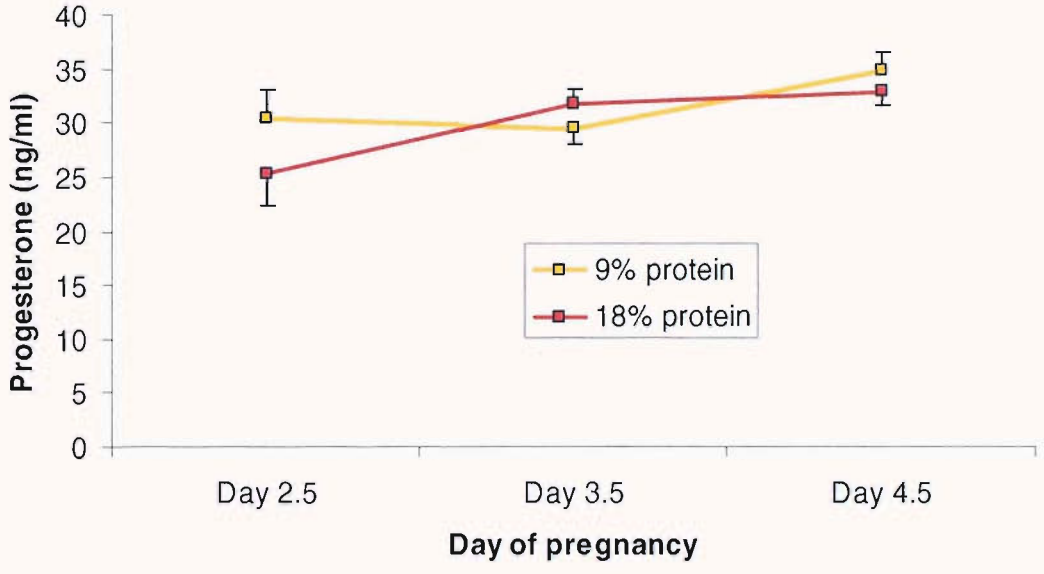

Fig 6.3.6: Serum progesterone levels in mice fed either a 9% LPD or an 18% NPD from plug days 2.5, 3.5 and 4.5 of pregnancy. There are no significant differences in serum progesterone levels between dietary treatments or between time points. Error bars are SEM.

6.3.7 Serum VEGF

VEGF is a powerful endothelial cell-specific mitogen and mediator of angiogenesis (Hyder *et al*, 2000). VEGF also appears to work in synergy with FGF-II, hence VEGF was measured in serum of mice treated with either the 9% LPD or 18% NPD. While two microplates were utilised for this ELISA, serum samples from both days and both dietary treatmens were assigned to each plate. As an additional control an extra serum sample was plated in duplicate on each microplate to assess inter-assay variation. Inter assay differences were minimal <5% was observed. Serum VEGF was found to be significantly elevated on day 3.5 of pregnancy in mice treated with the 9% LPD (Fig. 6.3.7). No significant differences between VEGF levels were observed between individual time points within the same dietary treatment group.

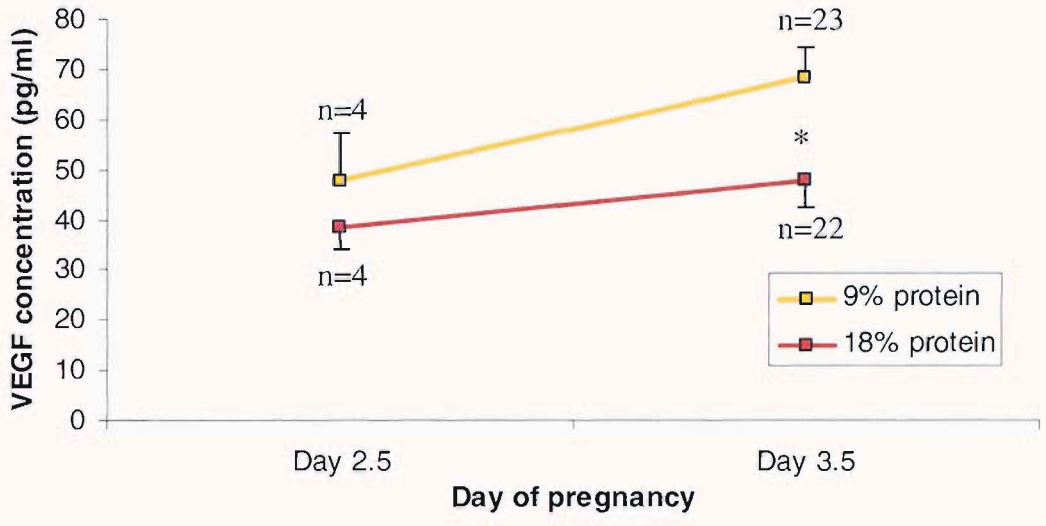

Fig.6.3.7: Serum VEGF concentration on day 2.5 and 3.5 of pregnancy in mice fed either the 9% protein LPD or the 18% protein NPD. Serum VEGF concentration is significantly higher in mice treated with the LPD on day 3.5 of pregnancy. Error bars are SEM . *=p<0.02

6.3.8 Serum Glucose

A previous study has indicated that there is a significant elevation in the maternal serum glucose levels of rats fed a 9% LPD compared to those fed an 18% NPD during pre-implantation development period (Kwong *et al*, 2000). Serum glucose levels were measured in mice fed the 9% LPD (n=19) and mice fed the 18% NPD (n=20), at 2.5, 3.5 or 4.5 days post plug. Serum glucose levels were found to be significantly elevated in mice administered the LPD compared to those treated with the NPD at day 3.5 post plug ($p<0.05$) (Fig. 6.3.8).

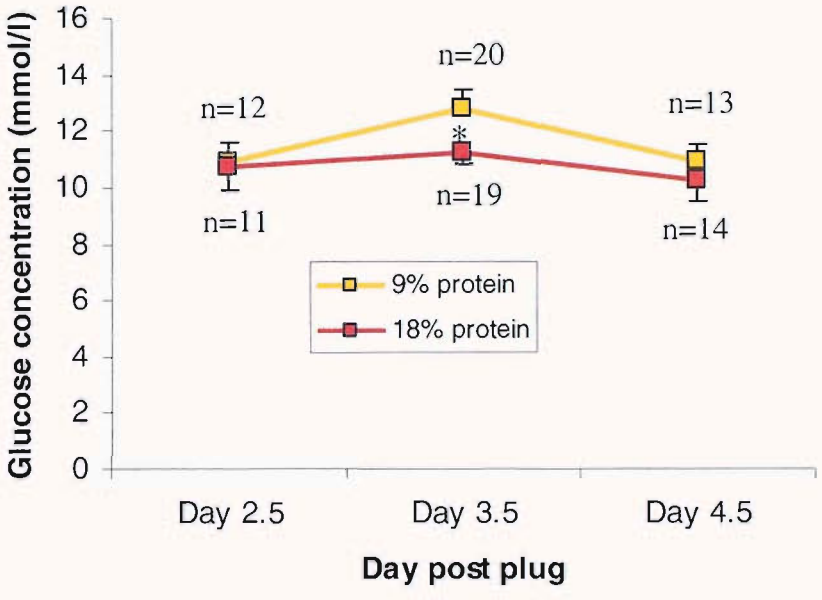

Fig. 6.3.8: Serum glucose levels assayed at either 2.5, 3.5 or 4.5 days of pregnancy in mice fed either a 9% LPD or a 18% NPD. Serum glucose is elevated in mice fed the LPD compared to those fed the NPD fed control mice at 3.5 days of pregnancy. Error bars are SEM. *=$p<0.05$.

6.3.9 Serum amino acids

Serum amino acids have previously been shown to alter in pregnant rats in relation to a LPD administration during pre-implantation development (Kwong *et al*, 2000). Additionally, serum amino acid levels were found to be useful in assessment of potential contamination of maternal uterine luminal fluid by blood (Fig. 4.3.4d).

No significant differences were observed in individual serum amino acid concentration on day 2.5 of pregnancy (Table 6.3.9). Serum concentrations of the amino acids histidine, arginine, alanine, tyrosine, tryptophan, valine, phenylalanine, isoleucine, leucine and lysine were observed to be significantly depleted in mice fed the 9% protein LPD compared to those fed the 18% protein NPD on day 3.5 of pregnancy (Fig. 6.3.9a). No amino acids were observed to be significantly elevated in mice fed the LPD compared to those fed the NPD on day 3.5 of pregnancy (Table 6.3.9). On day 4.5 of pregnancy it was oberved that the amino acids glutamine, tyrosine, valine, isoleucine and leucine were reduced in maternal serum of mice fed the LPD compared to those fed the

NPD (Fig. 6.3.9b). No amino acids were observed to be significantly elevated in mice fed the LPD compared to those fed the NPD on day 4.5 of pregnancy (Table 6.3.9)

It was observed, on day 3.5 of pregnancy, that there was a significant reduction in the overall concentration of essential amino acids in the serum of mice fed the 9% protein LPD compared to those administered the 18% protein NPD; no corresponding significant reduction was observed in the concentration of non-essential amino acids or in total concentration of amino acids in maternal serum (Table 6.3.9; Fig.6.3.9b). In contrast, on day 4.5 of pregnancy, essential, non essential and total amino acids were all reduced in the serum of mice fed the 9% LPD compared to those fed the 18% NPD.

Table 6.3.9: Serum concentrations of free amino acids measurable by HPLC from mice fed either the 18% protein NPD or the 9% protein LPD at day 2.5, 3.5 or 4.5 of pregnancy. Values shown with SEM. Red text indicates an essential amino acid. Differences between diet treatment at individual time points represented by *=p<0.05;† =p<0.01; ‡=p<0.001. Differences between time points within the same diet treatment represented by a=p<0.05; b=p<0.01; c=p<0.001

	Amino acid Concentration (mM)					
	2.5 days		3.5 days		4.5 days	
Amino Acid	18% protein	9% protein	18% protein	9% protein	18% protein	9% protein
n	11	9	15	11	10	11
Aspartic acid	0.053±0.007	0.049±0.009	0.045±0.005	0.051±0.006	0.046±0.008	0.035±0.003
Glutamic acid	0.133±0.029	0.114±0.026a	0.225±0.002	0.243±0.002ac	0.154±0.027	0.096±0.008c
Asparagine	0.044±0.009	0.037±0.004	0.050±0.004	0.046±0.003a	0.040±0.005	0.032±0.003a
Serine	0.104±0.020b	0.099±0.013b	0.246±0.019b	0.216±0.015b	0.118±0.015b	0.099±0.012b
Histidine	0.052±0.009a	0.050±0.007	0.095±0.008†a	0.070±0.004†	0.054±0.005a	0.044±0.005
Glutamine	0.416±0.075a	0.415±0.093	0.683±0.046a	0.684±0.051b	0.473±0.037*a	0.407±0.029*b
Glycine	0.112±0.020c	0.108±0.013c	0.365±0.035c	0.335±0.027c	0.123±0.11c	0.116±0.020c
Threonine	0.313±0.049	0.282±0.037	0.348±0.022a	0.272±0.039	0.388±0.294a	0.287±0.029
Arginine	0.145±0.024	0.125±0.014	0.205±0.016*	0.165±0.019*	0.169±0.024	0.140±0.17
Taurine	0.763±0.064c	0.851±0.060c	1.610±0.149c	1.752±0.123c	0.538±0.38c	0.493±.020c
Alanine	0.392±0.059a	0.442±0.058	0.618±0.052*a	0.509±0.033*a	0.386±0.031a	0.338±0.019a
Tyrosine	0.077±0.013	0.070±0.010	0.090±0.082*	0.071±0.006*	0.077±0.008*	0.059±0.003*
Tryptophan	0.086±0.013a	0.077±0.010	0.133±0.007†b	0.105±0.008†	0.078±0.006b	0.067±0.008
Methionine	0.136±0.019	0.142±0.018c	0.169±0.022*	0.112±0.017*c	0.119±0.013	0.096±0.011
Valine	0.255±0.039	0.177±0.024	0.297±0.014‡	0.185±0.016‡	0.238±0.024†	0.148±0.015†
Phenylalanine	0.067±0.009	0.057±0.007	0.087±0.005‡	0.067±0.005‡	0.061±0.006	0.047±0.005
Isoleucine	0.116±0.018	0.085±0.011	0.133±0.006‡	0.089±0.008‡	0.108±0.011*	0.076±0.010*
Leucine	0.162±0.024	0.117±0.015	0.212±0.011‡a	0.143±15.91‡	0.147±0.015*a	0.102±0.013*
Lysine	0.385±0.083c	0.285±0.057b	0.943±0.80*c	0.711±0.069*bc	0.286±0.068c	0.255±0.063c
Essential	1.686±0.257a	1.392±0.185	2.667±0.177‡ab	1.834±0.141‡	1.649±0.143*b	1.201±0.187*
Non-essential	2.098±0.280b	2.190±0.211b	3.954±0.285bc	3.7652±0.220bc	1.955±0.148*c	1.556±0.149*c
Total	3.784±0.529b	3.583±0.396a	6.190±0.590bc	5.600±0.318ac	3.604±0.245*c	2.757±0.325*c

Between day 2.5 and 3.5 of pregnancy a significant elevation in non-essential and total amino concentration was observed in the serum of mice treated with both the LPD and NPD (Figs. 6.3.9d and e). Mice treated with the 18% NPD also exhibited a significant increase in essential amino acid concentration between day 2.5 and 3.5 of pregnancy; this was not observed in those mice treated with the 9% LPD. In the serum of mice treated with the 18% NPD, concentrations of the individual amino acids, serine, histidine, glutamine, glycine, taurine, alanine, tryptophan, and lysine were all significantly elevated between day 2.5 and 3.5 of pregnancy (Fig. 6.3.9f). In the serum of mice treated with the 9% LPD between day 2.5 and 3.5 of pregnancy the concentrations of the individual amino acids glutamic acid, serine, glycine, taurine, lysine were significantly increased while methionine concentrations were observed to significantly decrease.

In contrast, between day 3.5 and 4.5 of pregnancy a significant decrease in the concentration of non-essential and total amino acids was observed in the serum of mice treated with both the 18% NPD and the 9% LPD (Figs. 6.3.9d and e). Additionally the concentration of essential amino acids was significantly decreased between day 3.5 and 4.5 of pregnancy in the serum of mice treated with the 18% NPD; this trend was not observed in the serum of mice treated with the 9% LPD. In the serum of mice treated with the 18% NPD, concentrations of the individual amino acids, serine, histidine, glutamine, glycine, taurine, alanine, tryptophan, leucine and lysine were all significantly decreased while concentrations of the amino acid threionine were significantly elevated between day 3.5 and 4.5 of pregnancy (Fig. 6.3.9g). In the serum of mice treated with the 9% LPD between day 2.5 and 3.5 of pregnancy, the concentrations of the individual amino acids glutamic acid, asparginine, serine, glutamine, glycine, taurine, alanine and lysine were all significantly decreased

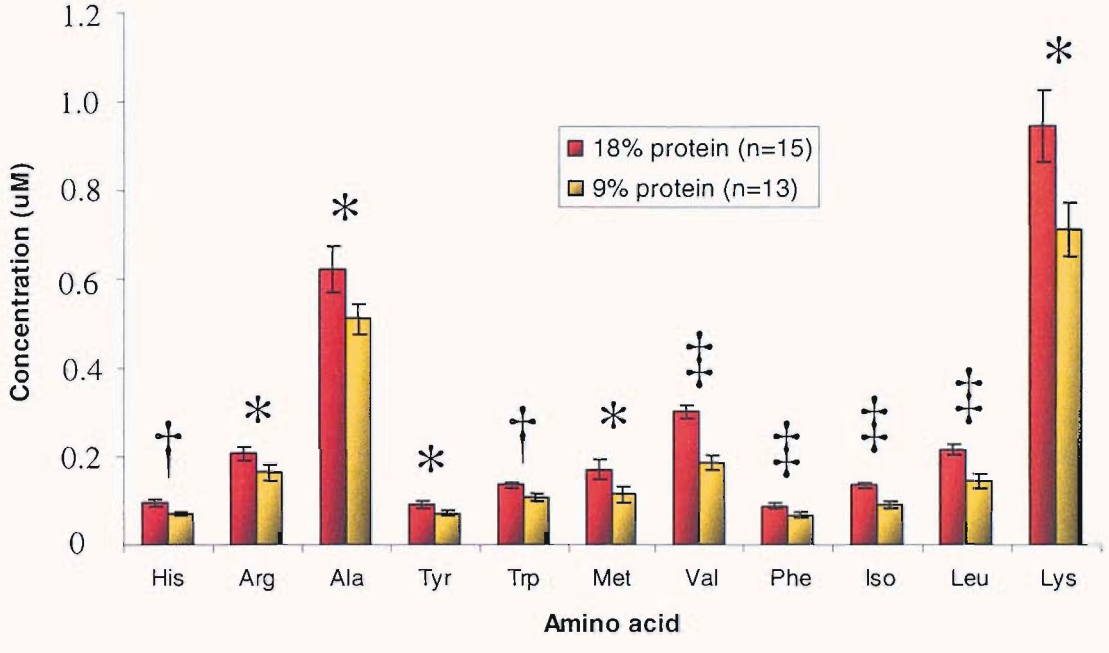

Fig 6.3.9a: Concentrations of serum amino acids that significantly change in mice fed either the 9% protein compared with the 18% protein NPD at day 3.5 of pregnancy. Error bars are SEM. *=p<0.05; † =p<0.01; ‡=p<0.001

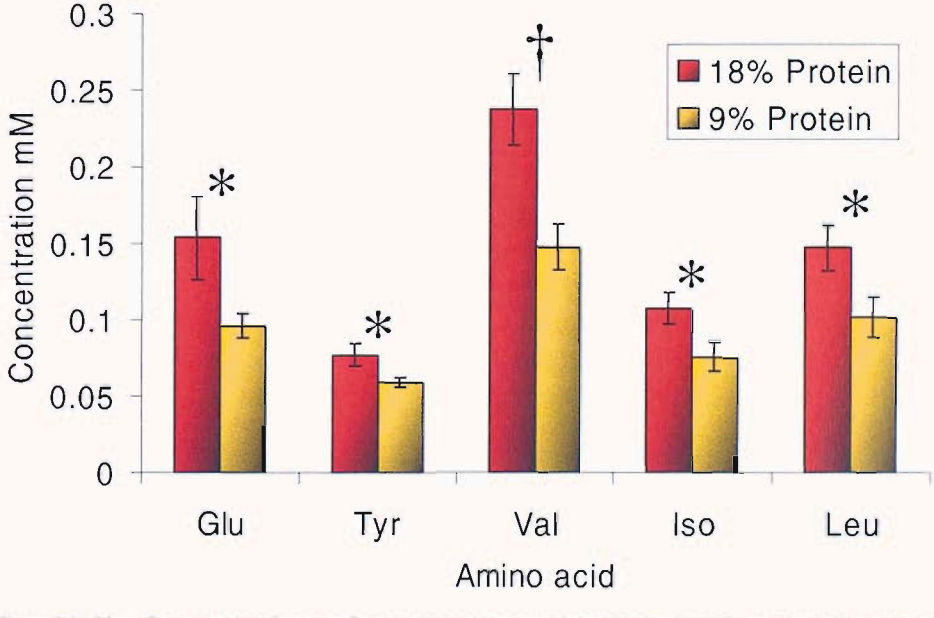

Fig. 6.3.9b: Concentrations of serum amino acids that significantly change in mice fed either the 9% protein compared with the 18% protein NPD at day 4.5 of pregnancy. Error bars are SEM. *=p<0.05; † =p<0.01; ‡=p<0.001

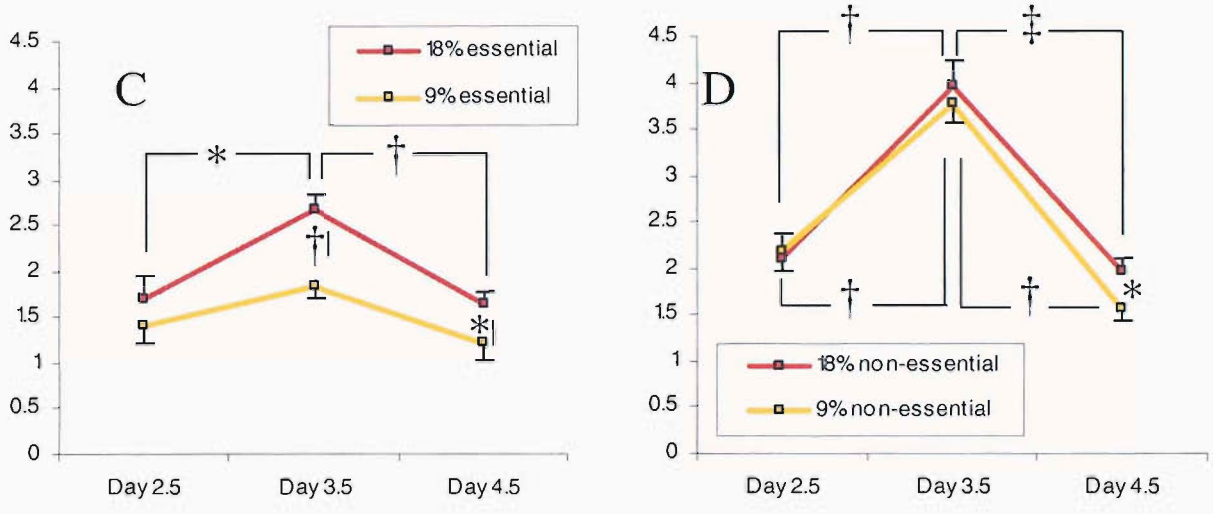

Fig 6.3.9c & d: c) Total concentration of essential amino acids and d) non-essential amino acids in the serum of mice fed either the LPD or the NPD until 2.5, 3.5 or 4.5 days of pregnancy. Error bars are SEM. *= $p<0.05$ †=$p<0.001$

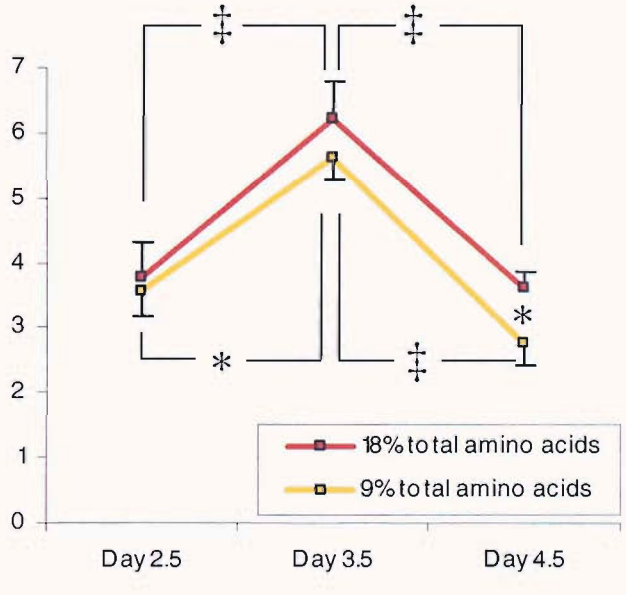

Fig 6.3.9e: Total concentration of amino acids in the serum of mice fed either the LPD or mice fed the NPD at 3.5 days post plug Error bars are SEM. *=$p<0.05$

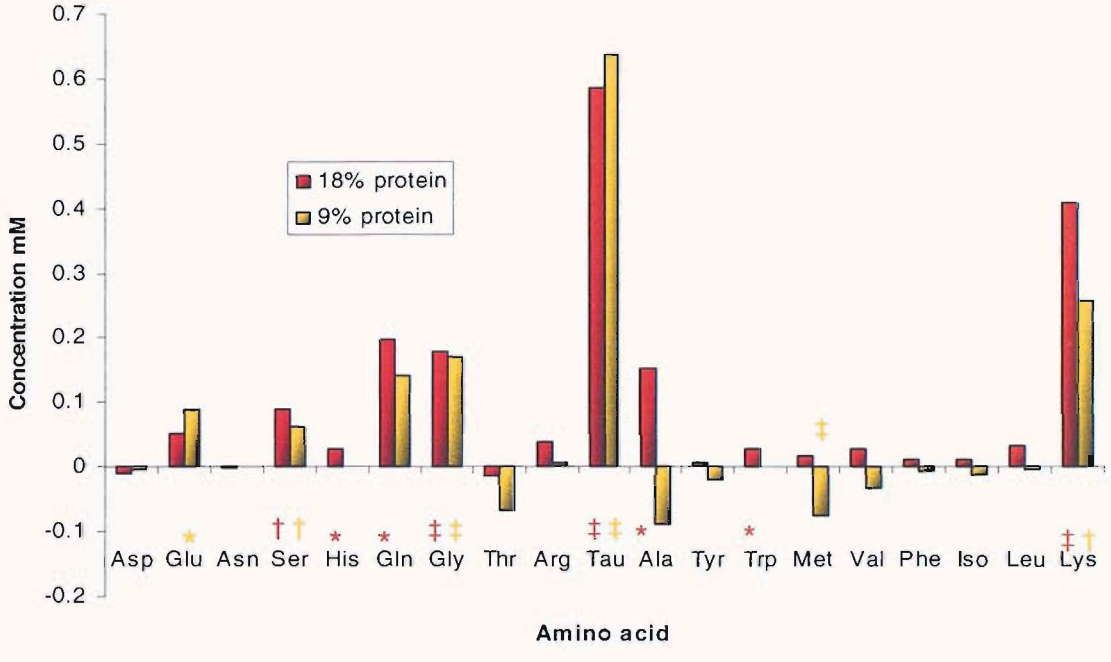

Fig 6.3.9f: Change in concentration of individual amino acids in maternal serum between day 2.5 and day 3.5. *=$p<0.05$; †=$p<0.01$ ‡=$p<0.001$. Note, no error bars are shown as only the overall difference is displayed because data is not paired.

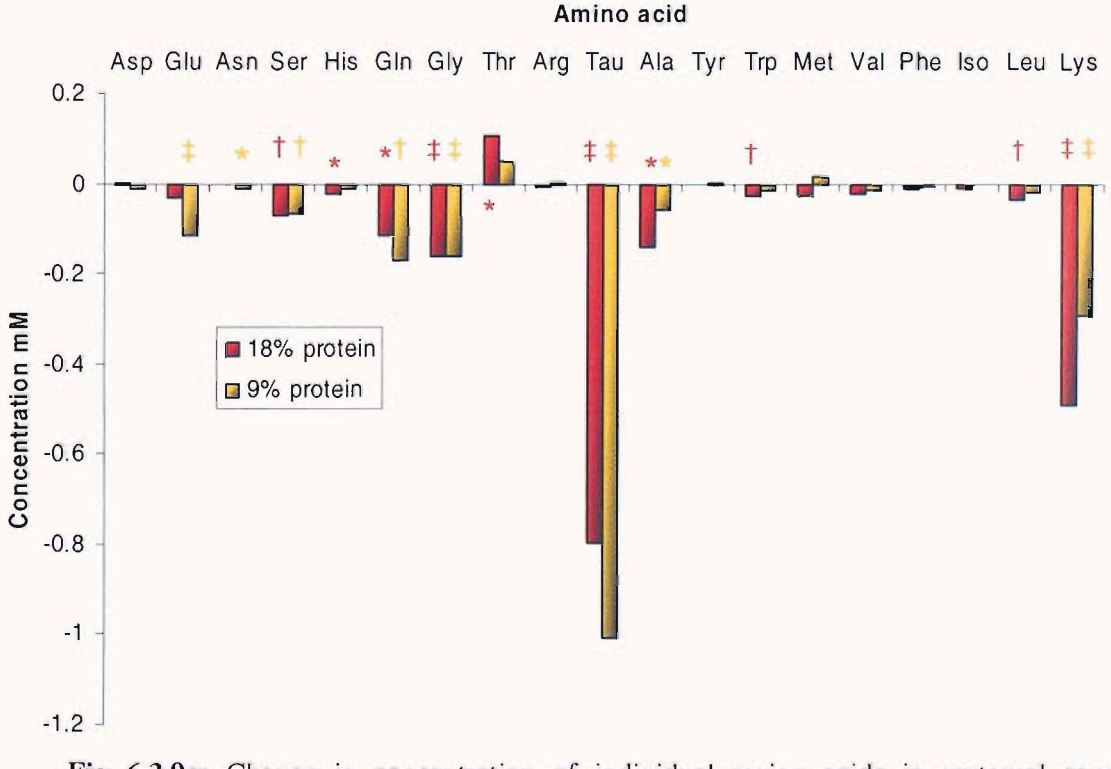

Fig 6.3.9g: Change in concentration of individual amino acids in maternal serum between day 3.5 and day 4.5. Fluid collected using the direct collection method. *=$p<0.05$; †=$p<0.01$ ‡=$p<0.001$. Note, no error bars are shown as only the overall difference is displayed because data is not paired.

6.3.10 Correlations between serum components

Individual components of the maternal serum that were measured in serum during the course of this investigation were also analysed against each other to highlight any potentially interesting correlations. Serum protein was found to have a significant positive correlation with serum corticosterone ($p<0.05$) when data was analysed across all three time points; individual dietary treatments also exhibited positive correlations (Fig. 6.3.10a). Similarly, serum insulin was found to have a significant positive correlation with serum estrogen ($p<0.05$) when data was analysed across all three time points; individual dietary treatments also exhibited positive correlations (Fig. 6.3.10b). In contrast, a significant negative correlation was found to exist between serum insulin concentration and serum ACE activity on day 3.5 of pregnancy ($p<0.05$); the individual dietary treatments also displayed similar correlations (Fig. 6.3.10c). Serum amino acids were also analysed in this way, however, it was found that each amino acid was highly significantly correlated against every other amino acid; as a result the data is not shown here.

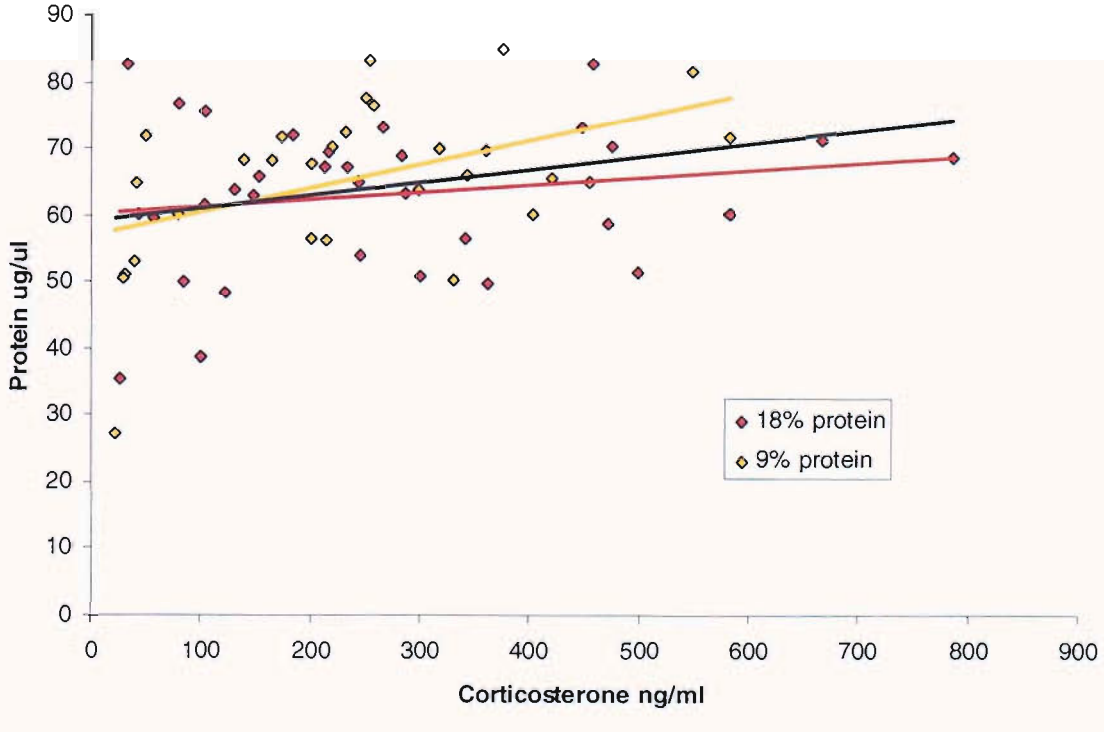

Fig. 6.3.10a: Concentrations of total protein and corticosterone are positively correlated in maternal serum between day 2.5 and 4.5 of pregnancy (black line; $p<0.05$). Data pooled from both the LPD and NPD diets and also across days for analysis, however, correlations for individual dietary treatments are also shown (coloured lines).

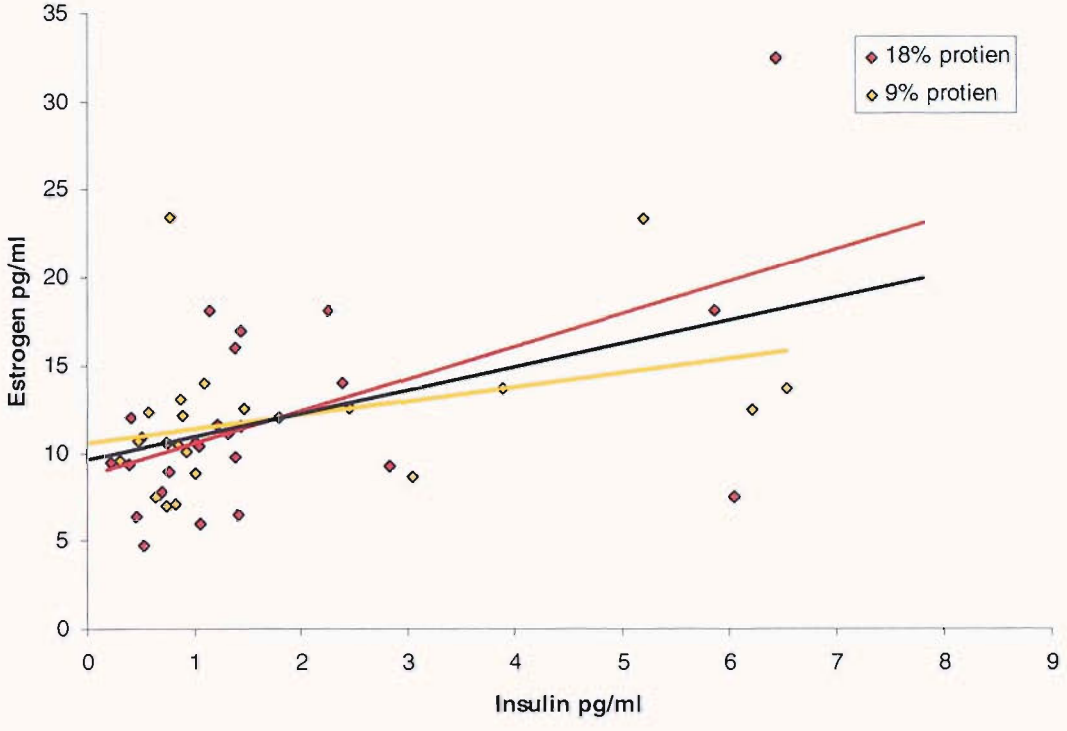

Fig. 6.3.10b: Concentrations of estrogen and insulin are positively correlated in maternal serum between day 2.5 and 4.5 of pregnancy (black line; $p<0.05$). Data pooled from both the LPD and NPD diets and also across days for analysis, however, correlations for individual dietary treatments are also shown (coloured lines).

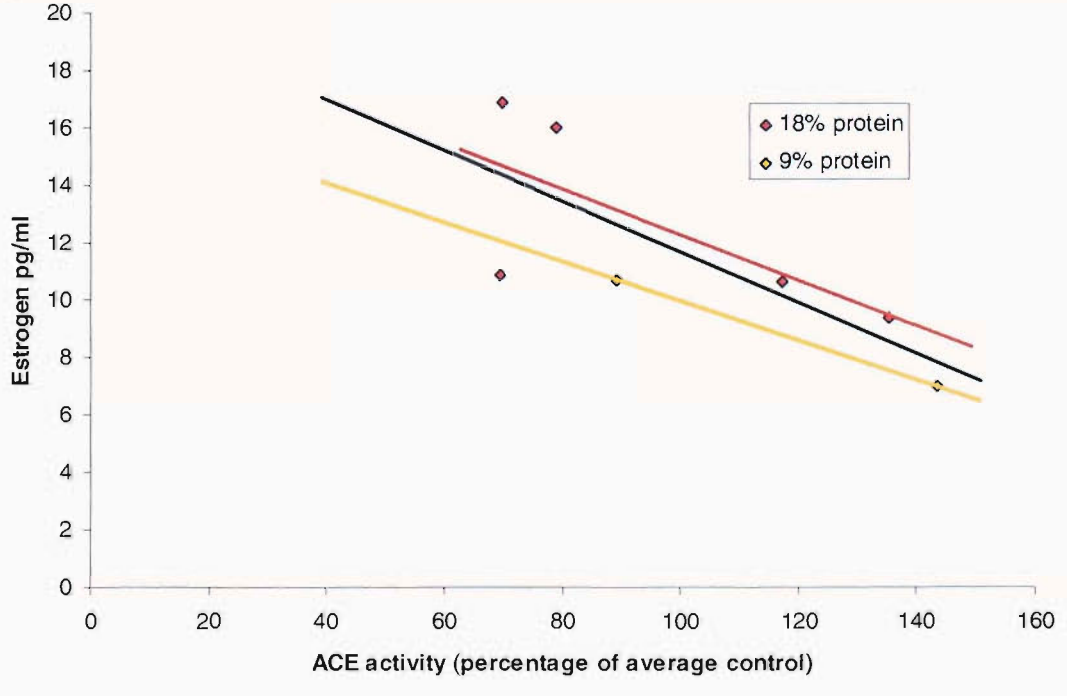

Fig. 6.3.10b: Estrogen concentrations and ACE activity are negatively correlated in serum maternal serum on 3.5 of pregnancy ($p<0.05$). Data pooled from both the LPD and NPD diets, however, correlations for individual dietary treatments are also shown (coloured lines).

6.4 Discussion

Thus far, this study has concentrated on factors within the uterine environment that may be altered in response to administration of a LPD, because of the direct impact uterine factors may have on embryo growth and developmental plasticity. However, the effects of administration of a LPD are not just confined to the maternal reproductive organs, they can cause much more general alterations in metabolism. Indeed, it is these alterations to maternal metabolism that are probably causal of the effects observed within the uterine environment. As a result, this chapter deals with measurements of metabolic markers and possible signaling molecules in maternal serum that may be altered in response to treatment with a LPD.

This experimental dietary model, at its most basic level, deprives the mice fed the LPD of protein compared to those mice fed the NPD. Total protein was found to be significantly elevated in mice fed the LPD at 2.5 days post plug. However, no significant differences in serum total protein levels were observed on day 3.5 or 4.5 post plug. Previous studies have demonstrated a drop in serum protein concentration when rodents have been administered a LPD for six weeks or more (Rossi *et al*, 1980; Sarisnerand *et al*, 1990). These observations may possibly be due to a decrease in serum albumin which has also been observed in many studies where a LPD has been administered (Rossi *et al*, 1980; Isozaki *et al*, 1993; Ramos *et al*, 2000). However, I am not aware of any studies that have measured serum protein levels in relation to dietary protein over the comparatively short time of 4 days. It is possible that the observed early elevation in serum total protein represents an increase in serum albumin content; albumin being the most abundant protein in serum. The promoter for albumin contains several regulatory domains including one for hepatocyte nuclear factor 1 (HNF-1) and one for CCAAT/enhancer binding protein α (C/EBP) (Tronche *et al*, 1990). Ogawa *et al* (1999) starved rats for 1 or 3 days and demonstrated that when rats are starved for 1 day HNF-1 and C/EBP mRNA levels are increased. Moreover, those rats starved for 3 days showed a significant decrease in C/EBP mRNA. Indeed, using microarray analysis, this study has also found C/EBP mRNA in uterine tissue to be significantly depleted on days 3.5 and 4.5 of pregnancy (Section 7.3, Table 7.3c). However, it should be noted that the

observations on day 1 of starvation were not concomitant with a significant increase in circulating albumin levels. It is possible that even the very mild LPD insult applied in this study is enough to provide sufficient dietary stress to increase levels of HNF-1 and/or C/EBP transiently on day 2.5 post plug. This could lead to increased serum albumin and thus explain the transient increase in serum total protein.

ACE activity was analysed in maternal serum because of its potential to mediate the expression of FGF-II in uterine tissues (section 5.4; Fig. 5.4b). As already discussed, in addition to ACE being present as a transmembrane protein, it also exists in a soluble circulating form produced by proteolytic cleavage extracellular to its C-terminal transmembrane anchor, probably by a zinc metalloprotease 'secretase' (reviewed by Turner & Hooper, 2002). No significant differences in serum ACE activity were observed between dietary treatments on day 3.5 of pregnancy, suggesting that the elevation in activity observed in uterine homogenates represents local alterations to the uterine renin angiotensin system. This opposes the idea that the elevation observed in uterine ACE activity simply reflects an increase in circulating ACE due to either a global ACE up-regulation or increased activity of the 'secretase' responsible for ACE cleavage. ACE ativity was demonstrated to be negatively correlated with serum estrogen concentration. Previous investigations have shown estrogen has a biphasic effect on the RAS of rodents because it both increases levels of angiotensinogen and renin while decreasing the activity of ACE (reviewed by Brosnihan *et al*, 1999). The net result of these effects is probably to mitigate the hypertensive effects of estrogen during reproductive events.

Significant decreases have been reported in serum insulin of rats (Rothwell *et al*, 1983; Tse *et al*, 1995; Reis *et al*, 1997) and pigs (Atinmo *et al* 1976) administered LPD for prolonged periods of time. There have been reports of significant elevations in serum glucose in response to long term administration of a LPD (Tse *et al*, 1995), however, most studies have found no significant changes in serum glucose levels (Ferreira *et al*, 2003; Delghingaro-Augusto *et al* 2004). Kwong *et al* (2000) demonstrated that administration of a LPD for the first 4 days of pregnancy could decrease serum insulin and increase serum glucose levels at day 3.5 of pregnancy. Claeyssens *et al* (1992),

demonstrated that weanling rats fed a LPD for 3 weeks had a significantly lowered serum insulin:glucagon ratio compared to controls. It has recently been suggested that a normal insulin level for a mouse can be considered to be anything less than 5 ng ml^{-1} (Cheverud *et al*, 2004). Indeed in the investigation conducted by Cheverud *et al*, (2004) both serum insulin and glucose were both subject to a large degree of variation even within control mice. This study found serum insulin levels to be between 1 ng ml^{-1} and 4.5 ng ml^{-1}. In this study it was also found that serum insulin was decreased in mice treated with the LPD for the first 3.5 days of pregnancy. On the other hand, a large decrease in serum insulin was observed in animals treated with both the LPD and NPD between day 2.5 and 3.5. This large drop in serum insulin levels was confirmed by analysis of insulin in a small subset of serum samples collected across day 2.5, 3.5 and 4.5. The large reduction in serum insulin between day 2.5 and 3.5 perhaps calls into question the biological significance of the relatively small difference observed between the two dietary regimes on day 3.5. It is of worth to note that no overall decrease in serum insulin levels was observed between day 2.5 and 3.5 when rats were subjected to an identical experimental treatment (Kwong *et al*, 2000). In itself, the decrease in serum insulin between day 2.5 and 3.5 may be a reaction to the switch from the natural to the synthetically derived diet on day 0.5 of pregnancy. However, it seems unlikely that this drop in serum insulin levels is not related to serum glucose levels as these are fairly constant between day 2.5 and 4.5. This phenomenon perhaps deserves greater investigation through analysis of serum insulin and glucose levels at day 1 and 1.5 of pregnancy.

Nevertheless, the possibility exists that a lowered insulin:glucagon ratio exists on day 3.5 in the mice administered the LPD in this study; if this is the case it would be logical to expect serum glucose levels to be increased in response to LPD treatment due to increased gluconeogenesis. Additionally, it should also be remembered that the LPD is balanced for energy compared to the NPD; in short, this means that the LPD contains more sugar per unit weight (Section 2.1). Indeed this study has also found glucose levels to be elevated on day 3.5 of pregnancy in the serum of animals treated with the LPD. Cheverud *et al*, (2004) have suggested that a serum glucose level of 8.0 mmol/l is at the high end of the normal range, this investigation has found serum glucose levels to be

slightly elevated at approximately 11 mmol/l. While it is easy to understand why serum glucose may be elevated within maternal serum in terms of serum insulin, if biologically significant, it is less simple to explain the actual drop in serum insulin on day 3.5 itself. Then again, leucine has been shown to be a powerful stimulator of insulin release. It is hypothesised that leucine metabolism via the Krebs' cycle or mitochondrial oxidation of leucine in β-cells can elevate the ATP/ADP ratio which in turn stimulates insulin release (Gao *et al*, 2003; Li *et al*, 2003). Moreover, it has been previously demonstrated that LPD treatment can impair leucine-mediated insulin secretion (Reis *et al*, 1997). Leucine has been shown to be significantly reduced in the serum of animals treated with the LPD in this study (Section 6.3.7) and in other studies of LPD treatment during early pregnancy (Kwong *et al*, 2000; Petrie *et al*, 2002).

Rats administered a LPD have demonstrated poor tolerance to orally administered glucose (Okitolonda *et al*, 1987) related to significantly reduced insulin secretion from β-cells (Swenne *et al*, 1987; Carneiro *et al*, 1995; Ferreira *et al*, 2003, Delghingaro-Augusto *et al* 2004). This blunted insulin response to glucose has in turn been related to a reduction in pancreatic β-cell mass (Swenne *et al*, 1992) and a lower responsiveness to glucose by the remaining β-cells (Picarel-Blanchot *et al*, 1995). While it is unlikely, in this model, that 3 days of LPD treatment could significantly alter β-cell mass it is possible that the observed reduction in insulin in response to LPD is due to poor insulin secretion. Studies have shown altered expression of genes involved in insulin secretion. For example, Ferreira *et al* (2003) showed that protein kinase Cα (PKCα) and phospholipase β1 (PLCβ1) mRNA levels were significantly reduced and these reductions were correlated with reduced insulin secretion in the islets of rats treated with a LPD for 8 weeks. Both PKCα and PLCβ1 are potentially involved in insulin secretion (reviewed by Zawalich and Zawalich, 1996). Using microarray technology Delghingaro-Augusto *et al* (2004) demonstrated reduced expression of voltage-gated K^+ channel proteins. Voltage-gated K^+ channels are thought to play an important role in insulin secretion stimulated by glucose. An increase in the ATP/ADP ratio through glucose metabolism closes K^+ ATP channels and depolarises β-cells; this depolarisation leads to the opening of voltage-dependent Ca^{2+} channels and an influx of calcium that triggers insulin granule exocytosis (reviewed by Miki *et al*, 1999). Hence, it is possible

that altered leucine signaling and gene expression may act in synergy to lower insulin levels in mice treated with a LPD during the early stages of pregnancy.

Food restriction studies in mammals have generally shown that a poor diet causes an increase in circulating GC levels and activity of the HPA axis (Fichter and Pirke, 1986; Gold *et al*, 1986; Berga *et al*, 2001). Treatment specifically with a LPD has also been shown to increase levels of corticosterone (Herbert & Carrillo, 1982; Bandyopadhyay and Poddar, 1998). During starvation, serum glucagon and glucagon like peptide-1 levels increase; both in turn cause an increase in circulating levels of GCs and mineralocorticoids by stimulating release of corticotrophin releasing hormone, adrenocorticotrophic hormone and arginine-vasopressin (Rao, 1995; Larsen *et al*, 1997). These increased levels of GCs act on the β-cell and inhibit insulin release (Lambillotte *et al*, 1997; Pierlussie *et al*, 1986; Billaudel *et al*, 1984). Long-term exposure to dexamethasone inhibits insulin biosynthesis and decreases the levels of preproinsulin mRNA (Philippe and Missotten, 1990; Philippe *et al*, 1991; Delaunay *et al*, 1997) and this happens in a dose dependant manner (Jeong *et al*, 2001). This inhibition of insulin production is certainly in part due to transcriptional down-regulation (Goodman *et al*, 1996) but it is also suggested that GCs can inhibit insulin biosynthesis by destabilising preproinsulin mRNA (Philippe and Missotten, 1990). Additionally, it has been shown that GCs can increase ACE levels (Fishel *et al*, 1995; Barreto-Chaves *et al*, 2000, Barreto-Chaves *et al*, 2001) causing increased conversion of ANGI to ANGII. Corticosterone levels in control mice have previously been reported to be approximately 200 ng ml^{-1} (McNamara and Lenox 2004, strain C57BL/6); this study broadly concurred with these previous findings by showing serum corticosterone to be $\approx$260 ng ml^{-1} in control animals. Given the previous studies on food restriction and the fact that this study has identified responses of insulin and ACE that could be strongly associated with elevated GC levels, it was of some surprise that no significant difference was observed in serum corticosterone concentrations at day 3.5 of pregnancy in relation to dietary treatment. Having said this, other metabolic programming studies have also failed to find a significant elevation in maternal corticosterone at 14 days post plug in the rat (Fernandes-Twinn *et al*, 2003). Yet when a LPD was administered to rats from day 14, maternal corticosterone levels have been found to be significantly elevated at term

(Lesage *et al*, 2003). This particular result could be due to the added stress the LPD would present during the third trimester where fetal growth rate is at its highest. While this study found no significant alterations in corticosterone levels in relation to diet, it is worth noting that corticosterone levels were found to be significantly positively correlated with serum total protein. This may be partly explained by the observation of Martinez *et al* (1995) that rats fed a diet with a poor protein source exhibited reduced corticosterone levels and an overall lower rate of whole body protein synthesis.

Estrogen is a major reproductive hormone produced mainly by the ovaries and also by the placenta during the later stages of pregnancy (discussed in Sections 1.5.2 and 1.5.4). Studies where LPD have been administered for extended periods of time have shown large drops in estrogen concentration (Ammann *et al* 2000; Ammann *et al* 2002). In contrast, estrogen has previously been demonstrated to be significantly elevated during late pregnancy in dams fed a LPD identical to the one used in this study (Fernandez-Twinn *et al*, 2003). Mice fed the 18% NPD diet displayed a significant drop in serum estrogen levels between day 2.5 and day 4.5 of pregnancy. This overall pattern of decreasing serum estrogen is physiologically normal however an estrogen surge is also expected just prior to implantation on day 4.5 (Section 1.5.2). In fact, serum estrogen concentrations were significantly higher in mice fed the 9% LPD at 4.5 days of pregnancy compared to controls fed the 18% NPD. It is possible that at this time point we are simply not observing the estrogen surge in mice treated with the 18% NPD and that the elevation in serum estrogen observed in mice treated with the 9% LPD is further evidence that implantation is occurring earlier in these animals (see Section 5.4). Also, estrogen in conjunction with progesterone has been shown to be a suppressor of insulin secretion by β-cells (Sorenson *et al*, 1993) and may also help to explain the apparent drop in circulating insulin observed on day 3.5 of pregnancy. Because of these previous findings it is of some surprise that serum estrogen was found to positively correlate with serum insulin levels in this study. Serum progesterone levels were also measured in this study but were found to not be significantly altered by treatment with a 9% LPD.

Perhaps more interesting is estrogen's affiliation with uterine cell proliferation. The mitogenic effects of estrogen on uterine epithelium are mediated indirectly through

its binding to ER$_\alpha$ in stroma (Cooke *et al*, 1997). This leads to epithelial proliferation through an as yet undefined mechanism (Cooke *et al*, 1998). Furthermore, it has been reported that estrogen increases epithelial proliferation in co-cultures of human uterine stromal and epithelial cells, but had no mitogenic effect on epithelium alone (Pierro *et al*, 2001). Conversely, it has also been shown that endogenous estrogen deficiency reduces proliferation and enhances apoptosis-related death in vascular smooth muscle cells (Ling *et al*, 2004). Estrogen signaling is critical to uterine tissue function; in knockout mice that lack estrogen receptor α, the uterus is present but is hypoplastic and unresponsive to estradiol (Lubahan *et al*, 1993). Additionally estrogen receptors have been localised to the vasculature in the uterus suggesting a role for estrogen in the development of uterine blood vessels (reviewed by Rogers and Abberton, 2003). Indeed, vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen, has an estrogen response element (Hyder *et al*, 2000). Fascinatingly, estrogen has been shown to promote the expression of FGFR1IIIc in the porcine endometrium (Welter *et al*, 2004). FGFR1 splice variants have also been located in the rodent uterine stroma (Rider *et al*, 1995). These data suggest that the elevated serum estrogen observed in this study may also have a role in any increased mitogenesis or vascular genesis within the uterine stoma or vasculature in response to treatment with a LPD.

As mentioned above, VEGF is an endothelial cell-specific mitogen and a potent mediator of vascular genesis (Senger *et al*, 1983; Leung *et al* 1989). In reproductive tissues, VEGF has been demonstrated to enhance follicular development by promoting an enhanced ovarian vascular network and diminishing apoptosis (Quintana et al, 2004). VEGF mRNA has also been localised within compartments of the rat (Karuri *et al*, 1998) and mouse (Chakraborty *et al*, 1995; Halder *et al*, 2000) uterus throughout the oestrus cycle and during early pregnancy. In rodents, VEGF has also been shown to be the main factor responsible for increased endometrial vascular permeability up to implantation (Rabbani and Rogers, 2001). Serum VEGF concentrations have previously been shown to be ≈40 pg ml^{-1} in control mice (Toyoda *et al*, 2001) this study's findings are almost identical with respect to controls with MF1 female mice having a serum VEGF concentration of ≈40 pg ml^{-1}. Moreover, this study has also shown serum VEGF to be significantly elevated on day 4.5 of pregancy in mice treated with the LPD suggesting

that there is indeed an elevation in angiogenesis in response to suboptimal diet during early pregnancy.

VEGF has classically been shown to be co-expressed with FGF-II during vascular genesis and wound healing (discussed by Toyoda et al, 2001) VEGF, FGF-II and their receptors have also been isolated in the uterus of various mammals including the rat, mouse and primate (reviewed by Ferrara and Davis-Smyth, 1997; discussed by Wei *et al*, 2004). Specifically Wei *et al* (2004) have demonstrated that VEGF and FGF-II are co-localised in epithelial cells, stromal cells, and blood vessels in the endometrium during the menstrual cycle and early pregnancy of the rhesus monkey. This study has shown splice variants of FGF-II are elevated in the uterus on day 3.5 of pregnancy (sections 5.3.1 and 5.3 2). The relationship between co-expression VEGF and FGF-II appears less to be the case of one causing the expression of the other and more the case that expression of these two mitogens is driven by some diverse and some common factors leading to synergistic expression and function during vascularisation. As already mentioned, VEGF can be directly stimulated by estrogen via an estrogen response element on its gene (Hyder *et al*, 2000), however, VEGF expression can also be modulated by a startling array of proteins, hormones and simple molecules like nitric oxide (for a full review see Jośko and Mazurek, 2004). In this discussion I will only deal with the factors most pertinent to this investigation. In addition to estrogen ANG-II has been shown to upregulate VEGF expression, this is probably mediated through the AT-I and not the AT-II receptor (Chua *et al*, 1998). As mentioned above, in this study ACE activity has been shown to be enhanced in LPD animals in a tissue specific manner on day 3.5 of pregnancy (sections 5.3.6 and 6.3.2). This suggests that a higher concentration of ANG-II may also exist on day 4.5 of pregnancy, at least in the uterus, of mice administered the LPD leading to elevated VEGF production. Moreover, it should not be forgotten that in addition to VEGF, ANG-II can also upregulate FGF-II expression (discussed in section 5.4; Fig. 5.4c). Thus ANG-II may be another possible source of the observed synergistic upregulation of VEGF and FGF-II. The transcription factor SP1 has been shown to play a significant role in VEGF induction (Pal *et al*, 1998), it should also be noted that elevated SP1 also precedes the increased synthesis of FGF-II and VEGF during the healing of duodenal ulcers (Szabo *et al*, 2000).

It is well known that a LPD can cause alterations in serum amino acid concentration when administered for prolonged periods of time (Delghingaro-Augusto *et al*, 2004). Investigations have also demonstrated that a LPD can alter serum amino acid concentration during pregnancy. Rees *et al* (1999) fed dams a LPD up to day 19 of pregnancy and demonstrated that glycine, isoleucine, leucine, and valine concentrations were slightly yet significantly reduced, threonine was markedly reduced by nearly 50%, while glutamine levels were significantly increased. However, within the IUGR fetus itself, only free concentrations of threonine were reduced significantly. Interestingly, non-pregnant dams fed the LPD for an equivalent amount of time did not display the same dramatic drop in serum threonine concentrations found in their pregnant counterparts. Overall, administration of a LPD during pregnancy did not cause a severe decrease in maternal serum concentrations of amino acids, in fact many essential amino acid concentrations were maintained, possibly through mobilisation of maternal stores. It is most intriguing that free lysine concentrations, which are known to approximately double during pregnancy, seemed to be unaffected by administration of a LPD diet. Kwong *et al* (2000) demonstrated very similar results to Rees *et al* (1999) in that dams fed a LPD for only the first four days of pregnancy exhibit alterations to specific amino acid concentrations in maternal serum; isoleucine, leucine, methionine, proline, threonine, and valine concentrations were all reduced while glutamine levels were increased. Furthermore these alterations observed by Kwong *et al* (2000) in serum amino acid concentrations were concurrent with classic symptoms of metabolic programming observed within the offspring of these pregnancies, such as increased SBP and altered growth trajectories. Previous studies have also reported a depletion of fetal serum taurine in response to maternal treatment of a LPD (Bertin *et al*, 2002).

Supplementation of a maternal LPD with extra threonine did not cause any improvement in growth rates of fetuses subjected to a LPD *in utero*, but did reduce the oxidation of threonine by the maternal liver to levels comparable to controls (Rees *et al*, 2000). The failure to rescue growth with a LPD supplemented with threonine coupled with hypermethylation of fetal DNA suggests that it is not a deficiency of threonine causing the metabolic imprinting effects observed. Indeed, Rees *et al* (2000) noted that

the fetuses from LPD mothers had hypermethylated DNA in liver extracts and that there was an increase in threonine oxidation suggestive of excessive dietary methionine. Furthermore, this study has shown methionine to be significantly elevated in the uterine luminal fluid of mice treated with a LPD for 4.5 days of pregnancy (section 4.4). Fetal pancreatic islet size and islet-cell proliferation have been shown to be reduced and apoptosis increased where dams have been treated with a LPD during gestation (Snoeck *et al*, 1990; Petrik *et al*, 1999). Based on the observations by Bertin *et al*, (2002) that serum taurine is reduced in fetal serum, Boujendar *et al*, (2002) undertook taurine supplementation in drinking water and found it to mitigate the effects of LPD treatment on fetal pancreatic islets.

As in uterine luminal fluid (Section 4.3.4), taurine was the most abundant amino acid in maternal serum with glutamine and alanine making up a significant contribution. However, unlike uterine luminal fluid, lysine also contributed significantly to amino acid concentration in maternal serum. Essential amino acids contributed about 40% of total amino acids in maternal serum, a significantly larger proportion ($p<0.001$) than the 17% contribution in uterine luminal fluid. As discussed in Section 4.4 the overall concentration of amino acids in maternal serum is much less than that found in the maternal luminal fluid suggesting active transport of amino acids. Overall, all amino acids were found to correlate positively with each other. In short this data tells us that when you increase the concentration of one amino acid the concentration of the all the rest is also increased. This pattern is probably indicative of experimental abnormalities rather than being of any biological importance. Indeed it is possible that even slight variations between haemolysis in samples could lead to such a pattern.

This study found that there were no significant differences in amino acid concentration between dietary treatments on day 2.5 of pregnancy. The serum amino acids histidine, arginine, alanine, tyrosine, tryptophan, valine, phenylalanine, isoleucine, leucine and lysine were all found to be significantly depleted on day 3.5 post plug in mice fed the 9% LPD compared to those fed the 18% NPD. Overall, a significant depletion in essential but not non-essential amino acids was observed on day 3.5 where mice were fed the 9% LPD relative to mice fed an 18% NPD. On day 4.5 of pregnancy

fewer amino acids were found to be significantly different in relation to dietary treatment. Glutamine, tyrosine, valine, isoleucine and leucine concentration were all found to be significantly reduced in the serum of mice treated with the LPD compared to controls. The fact that fewer serum amino acids are significantly reduced in concentration on day 4.5 of pregnancy suggests that there is more production of amino acids either by proteolysis or by *de novo* synthesis. It is also interesting to note that all the amino acids found to be significantly reduced in concentration on day 3.5 of pregnancy excepting alanine and tyrosine are essential amino acids. Furthermore, it also worth noting that those that are depleted both on day 3.5 and day 4.5 of pregnancy in maternal serum are solely essential amino acids. It is worth mentioning that circulating maternal levels of the toxic amino acid homocystine are elevated to approximately double in rodents fed a LPD (Petrie *et al*, 2002). This has been hypothesised to be due to the conversion of methionine to cysteine (Rees, 2002). However, I have as yet been unable to measure homocystine in uterine luminal fluid or maternal serum.

Serum amino acid concentration was also found to change over the preimplantation period. In both the serum of mice treated with the NPD and LPD, total amino acid concentrations significantly rose between day 2.5 and 3.5 of pregnancy then significantly fell again between day 3.5 and 4.5 of pregnancy. This rise then fall in amino acid concentrations appears to be mainly driven by significant changes in non-essential amino acid concentration across the experimental period (Figs 6.7.3c, d and e). In mice fed the NPD, serum essential amino acid concentrations followed the same trend as the non essential amino acids, significantly rising between day 2.5 and 3.5 of pregnancy before significantly falling between day 3.5 and 4.5; this pattern was not observed in mice treated with the LPD where serum essential amino acids did not alter significantly between any time points. It would seem that this pattern of rising then falling serum amino acid concentrations could be a natural phenomenon of preimplantation development. Indeed, this study has also found a very similar pattern of amino acid concentrations within uterine luminal fluid over the same time period (Fig. 4.3.4g-h). Furthermore, it would seem likely that these alterations in serum amino acid concentrations in fact form the basis of the very similar changes observed in uterine luminal fluid amino acid concentration (discussed in Section 4.4). However, the overall

purpose of these changes in amino acid concentrations observed in maternal serum and uterine luminal fluid still remains unclear.

Could these transient changes in serum amino acid concentrations on day 3.5 of pregnancy represent a mobilisation of nutrients solely to influence the composition of uterine luminal fluid? This would seem unlikely as gross changes in maternal amino acid balance would be a hugely inefficient method of altering the embryo environment when changes in amino acid transport could perhaps work much better. It would seem more likely that any large rise then fall in circulating and uterine fluid amino acids would be much more consistant with wholesale nutrient redistribution in preparation for pregnancy. Kwong *et al* (2000) also measured amino acid concentrations in rat maternal serum during the preimplantation period but failed to observe any such pattern. However, it should be noted that Kwong *et al* (2000) only measured serum amino acid concentrations on day 2.5 and 4.5 of pregnancy so could have missed any rise in amino acid concentrations that could have occurred on day 3.5 of pregnancy. If indeed the transient increase in serum amino acid concentration on day 3.5 is due to increased protein breakdown one would expect to observe increased ammonium levels in maternal serum too. This is because when proteins are broken down by cells throughout the body by deamination, ammonia is formed as a waste product and released into the blood. This study did not measure serum ammonium levels. However it did measure, perhaps the next best thing, glutamine levels. As ammonium is toxic to the body, it must be efficiently disposed of. In many tissues, ammonium combines with glutamate to make glutamine, which then circulates to the liver. Once in the liver, glutamine is broken down to glutamate and ammonium again and the ammonium is formed into urea for excretion. In this study it was observed that on day 4.5 there was a significantly less glutamine in the serum of mice treated with the LPD. This could represent the fact that less ammonium was produced during any major protein degradation event occurring on day 3.5 of pregnancy. Indeed, previous studies have demonstrated that during protein deprivation in rats, rates of protein turnover, incuding both synthesis and degradation, decrease. For example, in one study rats were given an essentially protein-free diet. After 1 day, protein synthesis had dropped by 25-40%. After 3 days, protein breakdown and oxidation had decreased by 30-45% (Mortimore and Poso, 1987). While this study is

extreme compared to the current investigation, it does suggest that protein turnover in our model of protein restriction could be reduced. Additionally, studies in humans have also shown that protein turnover can be reduced very quickly in response to dietary protein restriction (Taveroff *et al*, 1994).

This chapter details several metabolic and hormonal pathways which may contribute to the control of peri-implantation metabolic programming and several interesting points were raised. A transient elevation was observed in serum total protein in those mice treated with the 9% LPD which may be linked to an increase in albumin levels. As Kwong *et al* (2000) have previously shown in the rat serum levels of insulin were elevated and glucose levels ware decreased on day 3.5 of pregnancy in the serum of mice treated with a 9% LPD. Similarly, serum amino acid levels were also found to alter in relation to dietary treatment. Though perhaps of more interest than any changes in amino acid levels in the serum is the observation that the branch chain amino acids are significantly reduced in both maternal serum and uterine fluid to a similar extent. This suggests that alterations to amino acid concentrations in the uterine fluid are directly linked to those in the serum. However it was also found that serum ACE activity was not altered in those mice treated with the LPD, suggesting that the elevations in ACE activity noted in uterine tissue are indeed a local phenomenon. Overall, the experiments detailed in this chapter demonstrate that specific differences do exist in the serum components of mice treated with a 9% LPD compared to controls and better help us to understand the regulation of peri-implantation metabolic programming.

Chapter 7

Microarrays

7.1 Introduction

Thus far this study has exclusively investigated non-genetic measures of possible maternal adaptations to treatment with an LPD. As already discussed it has been shown that there are some interesting alterations in uterine amino acid concentrations, uterine protein expression and constituents of maternal serum. All of these outcomes may play a part in metabolic programming of the early embryo. While this investigation has repeatedly hypothesised about the possible pathways which may underlie these alterations it has as yet failed to illuminate the genetic components of such mechanisms.

Several techniques exist that could assist in the investigation of genetic regulation of maternal responses to dietary treatment. RT-PCR and northern blots are useful in the investigation of regulation of specific genes and can provide highly accurate determinations of relative expression. However, their underlying methodology limits their application to the investigation of one gene at a time. Alternatively, the relatively new technology of cDNA microarrays can provide a powerful tool with which to dissect the molecular mechanisms underlying biological pathways by comparing the expression of thousands of genes in a single experiment (Ferea and Brown, 1999)

One of the first applications of microarrays was in the investigation of cell-cycle regulated genes using the full genome of yeast (Spellman *et al*, 1998). Since that time microarray technology has been used to investigate a huge number of biological mechanisms in eukaryotes and prokaryotes. Such investigations are as diverse as experiments on identification of new genes regulated by light color in cyanobacterium (Stowe-Evans *et al*, 2004) to quantitative analysis of tumor mitochondrial RNA (Han *et al*, 2005). In this chapter microarray technology is utilised as an exploratory tool to investigate the possible components of metabolic programming in the preimplantation uterus at the genetic level.

7.2 Materials and methods

7.2.1 Microarrays

Chip 1 of the NIA 15K Mouse microarray based on the cDNA Clone Set generated by Tanaka *et al*, 2000 from embryos and placenta upto day 12.5. The Characteristics of this chip are: 15,264 (7860 on chip 1) "unique" cDNA clones were rearrayed among 52,374 ESTs from pre- and peri-implantation embryos, E12.5 female gonad/mesonephros, and newborn ovary of *Mus musculus*. Up to 50% of the genes on this array are derived from novel genes. Genes have an average insert size of ≈1.5 kb. Clones were sequenced from 5' and 3' termini to obtain longer reads and verify sequence. Clone names are from H3001A01 to H3159G07. For full details see Tanaka *et al*, 2000 and Kargul *et al*, 2001 for details. Interactive information on all the genes present can also be found at: **http://lgsun.grc.nia.nih.gov/cDNA/15k.html**.

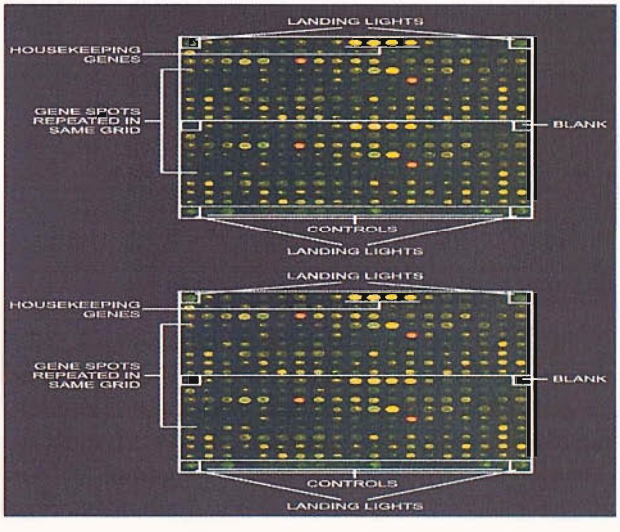

Fig 7.2.1: An anotated diagram of a typical scan from one of the 48 grids printed on the first chip of the NIA 15K Mouse microarray. House keeping genes (actin, myosin, ubiquitin, and beta-2 microglobulin) are spotted onto each grid of each arrays to act as an interna contorl and to endure homoginaoty of results. Blanks are spotted with double distilled water to act as a negative control. Landing lights are spots of Cy5 to assist grid alignment post scanning. Each set of genes is printes in duplicate in each grid.

7.2.2 Analysis

Microarray chips were hybridised with cDNA from uterine tissue 3.5 post plug (18% protein NPD n=4; 9% protein LPD n=5) as described in section 2.10. This microarray contains 7860 genes printed in duplicate; the design of this chip is described in Fig. 7.2.1. Scanning, normalisations and analysis were done as described in section 2.10.5

7.3 Results

The relative expression of 7945 potential genes was examined in day 3.5 and 4.5 uterine tissue to investigate what impact dietary treatment may have on the regulation of genes in uterine tissue. Data was normalised using the lowess method; in short this method of normalisation compensates for imbalances between cy3 and cy5 dyes. Such imbalances could be caused, for example, by overloading of one set of labelled cDNA's or variations in dye fluorescence between batches. Overall the raw data was well suited to lowess normalisation as there is little variation in EST's expression between dietary treatments; i.e. most of the EST's have almost a 1:1 expression ratio between dietary treatments. However, it is possible to see a distinct shift towards the cy3 dye in the raw data in both the data for day 3.5 and 4.5. This can be recognised by the fact that most of the data lays below the red line denoting 1:1 EST expression (Fig.7.3a). This shift is removed from the data post normalisation (Fig.7.3b).

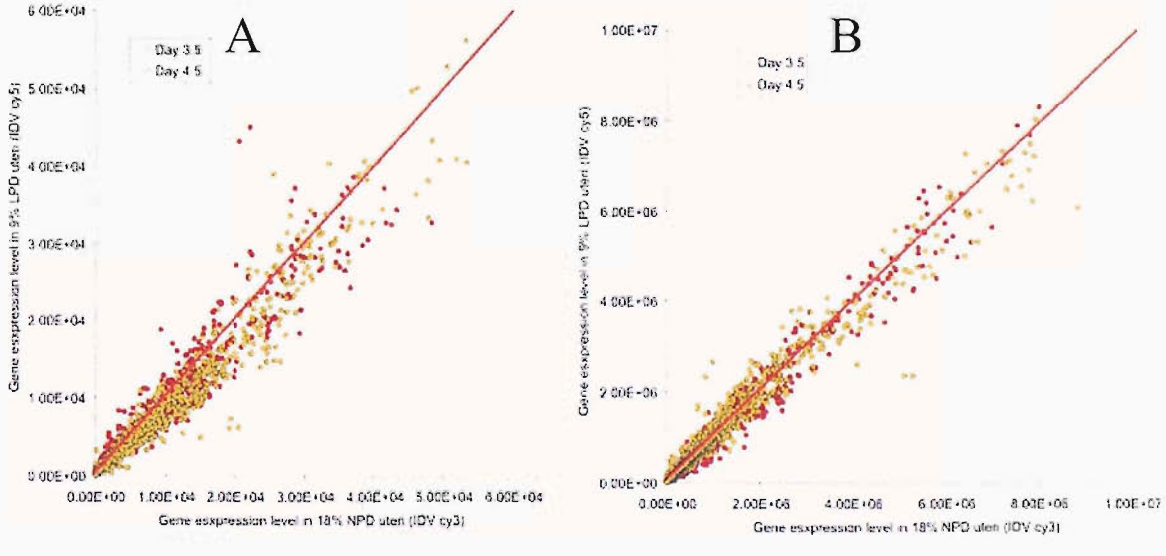

Fig.7.3a & b: Expression profiling of the 7860 EST's printed on the NIA 15K Mouse micro array in relation to dietary treatment and day of pregnancy. A) Prior to Lowess normalisation and B) after lowess normalisation. Red dots are day 3.5 (n=5) data while yellow dots are day 4.5 data (n=4). The red line illustrates where 1:1 gene expression. Note that while the arbitary intergrated density value (IDV) units change after normalisation it is the overall pattern of gene expression that is important.

After normalisation, of the 7945 expressed sequence tags (EST's) analysed 7775 were expressed on day 3.5 of pregnancy and 7861 were expressed on day 4.5 of

pregnancy. Overall, of those EST's that were expressed 690 genes were identified that significantly altered expression on day 3.5 or 4.5 in relation to dietary treatment. Of the 690 EST's found to alter significantly 139 had altered expression on day 3.5 of pregnancy, 413 had altered expression on day 4.5 of pregnancy and 138 had altered expression on day 3.5 and 4.5 of pregnancy. Of the EST's that showed significant alterations in expression on day 3.5 of pregnancy a total of 234 EST's significantly decreased in expression while 43 significantly increased in expression. Of the EST's that showed significant alterations in expression on day 4.5 of pregnancy total of 321 genes decreased in expression while 230 increased in expression.

Of those 690 EST's found to significanly alter in expression in relation to diet, 260 have been previously described in some way; of these 66 altered on day 3.5 alone (Table 7.3 a), 138 altered on day 4.5 alone (Table 7.3 b) and 56 altered both on day 3.5 and 4.5 (Table 7.3 c). The other 430 of the EST's which were been were identified to alter expression in relation to diet have not been previously described; 83 of these altered on day 3.5 alone (Table 7.3 d), 275 altered on day 4.5 alone (Table 7.3 e) and 72 altered both on day 3.5 and 4.5 (Table 7.3 f). Overall 170 more EST's significantly altered in expression in relation to diet on day 4.5 than on day 3.5; this represents an increase of 65% in the number of genes affected by dietary treatment. The known EST's found to significantly alter in expression have been sorted into functional classifications based on adaptations of the systems used by Kargul *et al* (2001) and Mu *et al* (2001) and upon the nucleotide and protein descriptions found at **http://lgsun.grc.nia.nih.gov/cDNA/15k.html** (Fig. 7.3c; Tables 7.3a-c; Key in table 7.3g).

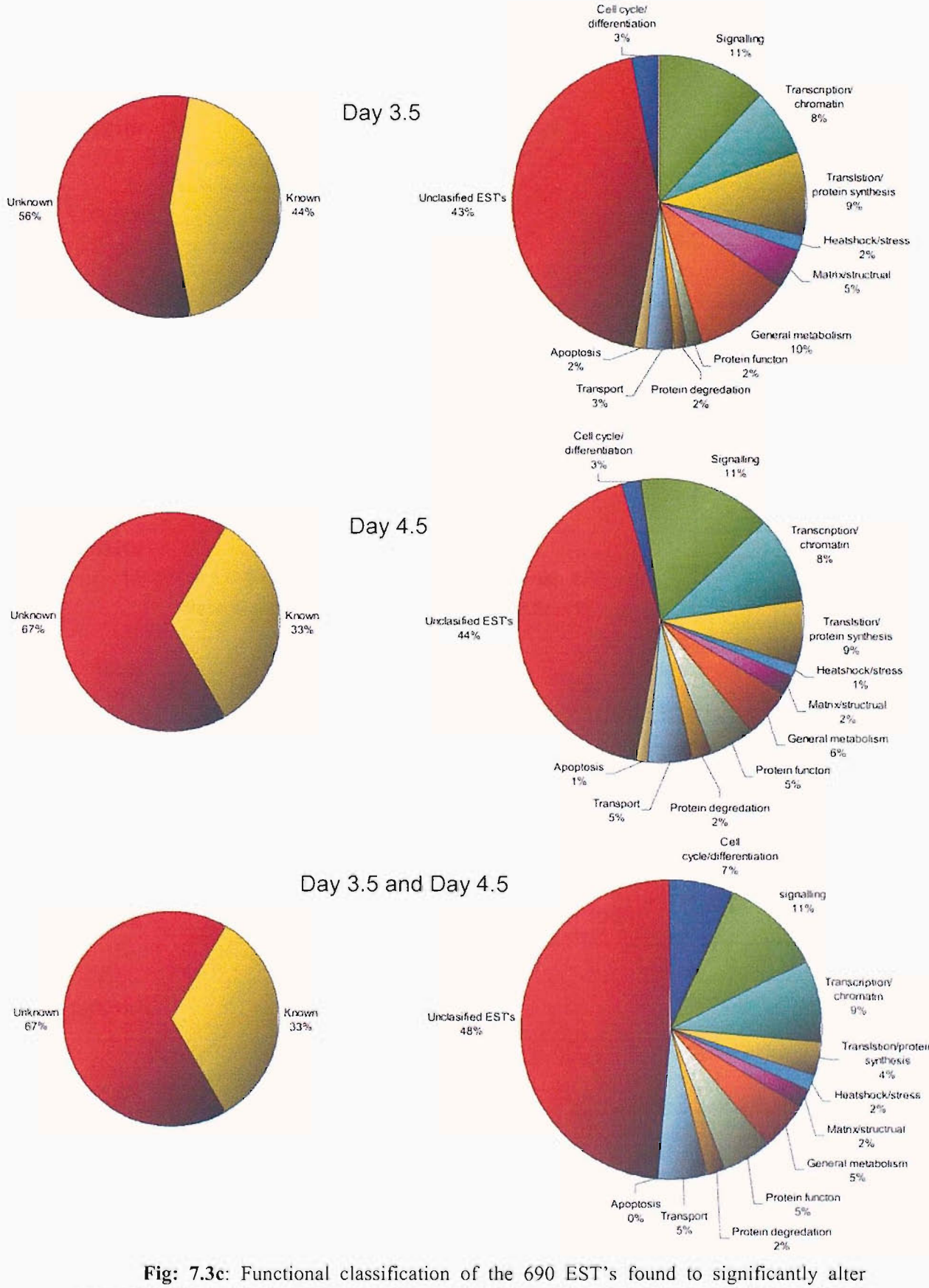

Fig: 7.3c: Functional classification of the 690 EST's found to significantly alter expression in relation to dietary treatment on day 3.5 and 4.5 of pregnancy. Left hand charts: proportions of known (previously described) and unknown EST's. Right hand charts: functional breakdown of those 260 known genes in Tables 7.3a-c. Unclassified EST's include previously described EST's with unknown function, hypothetical genes and genes with insufficient information to adequately classify

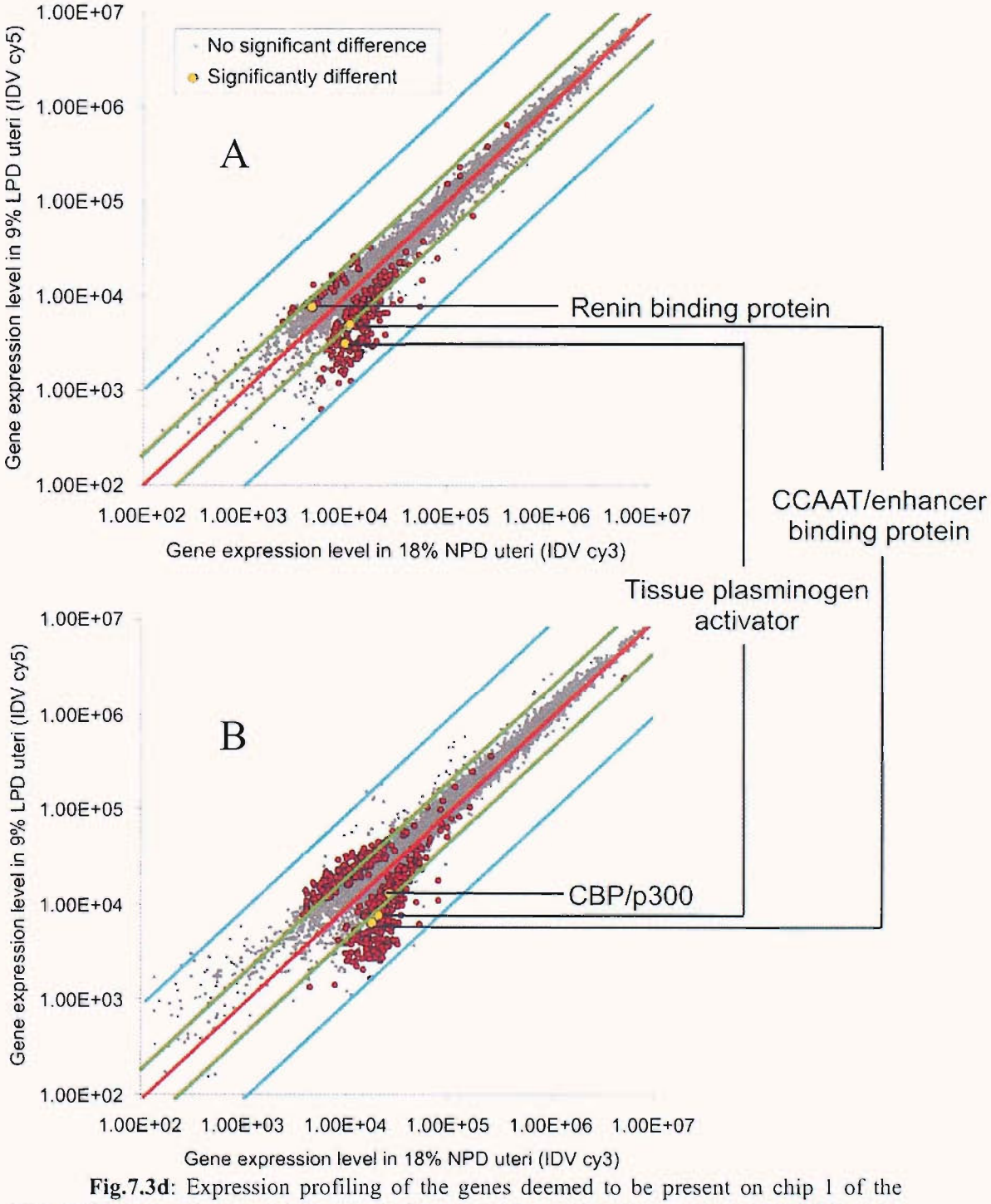

Fig.7.3d: Expression profiling of the genes deemed to be present on chip 1 of the NIA 15K Mouse microarray between uteri extracted from mice treated with a 18% NPD and a 9% LPD on day 3.5 (A) and day 4.5 (B). For each gene, average expression levels IDV (arbitrary units) were calculated from five independent hybridisations on day 3.5 and 4.5 for 18% NPD and 9% LPD uteri. Genes that show significantly different expression levels between 18% NPD and 9% LPD uteri ($p<0.05$) are displayed as red spots; other genes are displayed as gray spots. Genes that alter significantly are shown in Tables 7.3a-f. Labled genes (yellow dots) are discussed in Section 7.4. The Red Line indicates a 0-fold difference in gene expression; green lines indicate 2-fold expression differences; blue lines indicate 10-fold expression differences.

Table 7.3a: List of known genes exhibiting a significant difference in expression between dietary treatments on day 3.5 of pregnancy. Letter after clone name is EST functional clssification information, Key in Table 7.3g. EST's described in discussion are underlined.

Clone name	Description	Fold change 3.5	Clone name	Description	Fold change 3.5
H3058C03 (a)	*Mus musculus* Bcl2-like 10 (Bcl2l10), mRNA	0.58	H3056C01 (b)	*Mus musculus* formin 2 (Fmn2), mRNA	0.57
H3059G08 (b)	Monocyte to macrophage differentiation factor 2 (MMD2)	0.52	H3021H03 (c)	*Mus musculus* Ras suppressor protein 1 (Rsu1)	0.55
H3051F01 (c)	*Mus musculus* ankyrin repeat domain 6 (Ankrd6), mRNA	1.43	H3079C07 (c)	GTP RHO BINDING PROTEIN 1	0.61
H3058C09 (c)	*Mus musculus* Jun oncogene (Jun), mRNA	0.70	H3021G11 (c)	*Mus musculus* calreticulin (Calr), mRNA	0.24
H3079D07 (e)	*Mus musculus* RB1-inducible coiled-coil 1 (Rb1cc1), mRNA	0.58	H3051E08 (e)	Similar to: *Mus musculus* mRNA for PEBP2a1 protein, complete cds	1.75
H3077E05 (d)	*Mus musculus* phosphodiesterase 3A, cGMP inhibited (Pde3a), mRNA	0.37	H3058G02 (e)	*Mus musculus* T-cell lymphoma breakpoint 1 (Tcl1), mRNA	0.39
H3054A06 (d)	HN1 like {Homo sapiens}	2.13	H3076H12 (d)	*Mus musculus* Treacher Collins Franceschetti syndrome 1, homolog (Tcof1), mRNA	0.61
H3024F12 (d)	*Mus musculus* similar to Transcription factor BTF3 (RNA polymerase B transcription factor 3) (LOC218490), mRNA	0.40	H3028H03 (d)	SNRNA ACTIVATING PROTEIN COMPLEX 50 KDA SUBUNIT SNAPC 50 KDA SUBUNIT PROXIMAL SEQUENCE ELEMENT BINDING TRANSCRIPTION FACTOR BETA SUBUNIT PSE BINDING FACTOR BETA SUBUNIT PTF BETA	0.50
H3024B08 (e)	acidic ribosomal phosphoprotein P0	0.39	H3076C08 (e)	PROTEASOME SUBUNIT BETA TYPE 8 EC 3.4.25.1 PROTEASOME COMPONENT C13 MACROPAIN SUBUNIT C13 MULTICATALYTIC ENDOPEPTIDASE COMPLEX SUBUNIT	0.45
H3079H11 (e)	40S RIBOSOMAL PROTEIN S3A BA108L7.8 (NOVEL PROTEIN (TRANSLATION OF CDNA FLJ10512 (EM:AK001374))) (CDNA FLJ10512 FIS, CLONE NT2RP2000658) homolog [*Homo sapiens*]	1.49	H3030E08 (e)	*Mus musculus* ribosomal protein L26 (Rpl26)	1.56
H3073H01 (c)		0.74	H3077E02 (e)	*Mus musculus* peptidylprolyl isomerase (cyclophilin)-like 1 (Ppil1), mRNA	0.18
H3080H01 (f)	*Mus musculus* ubiquinol-cytochrome c reductase core protein 1 (Uqcrc1), mRNA	0.51	H3078A10 (g)	TRUNCATED BRE ALPHA A3+ ISOFORM homolog [Homo sapiens]	0.63
H3058E07 (h)	*Mus musculus* microtubule-associated protein 4 (Mtap4), mRNA	1.64	H3056E01 (h)	*Mus musculus* actin related protein 2/3 complex, subunit 5 (165 kDa) (Arpc5), mRNA	0.18
H3073G09 (h)	ECHINODERM MICROTUBULE ASSOCIATED PROTEIN LIKE EMAP	0.56	H3022C01 (i)	*Mus musculus* phosphate cytidylyltransferase 1, choline, alpha isoform (Pcyt1a), mRNA	0.46
H3032F02 (j)	*Mus musculus* renin binding protein (Renbp)	1.61	H3030C07 (i)	*Mus musculus* like-glycosyltransferase (Large)	1.67
H3059B01 (i)	*Mus musculus* hydroxysteroid dehydrogenase-1, delta<5>-3-beta (Hsd3b1), mRNA	0.52	H3066H02 (i)	*Mus musculus* BRG1/brm-associated factor 53A (Baf53a-pending), mRNA	0.55
H3055B10 (i)	1 AMINOCYCLOPROPANE 1 CARBOXYLATE	0.72	H3077G07 (i)	*Mus musculus* envoplakin (Evpl), mRNA	0.59
H3022E04 (j)	*Mus musculus* protein phosphatase, EF hand calcium-binding domain 2 (Ppef2), mRNA	0.50	H3038D07 (k)	*Mus musculus* major vault protein (Mvp), mRNA	2.27
H3023A07 (k)	*Mus musculus* solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 (Slc3a2), mRNA	0.43	H3022E09 (l)	ADAMTS EC 3.4.24.- A DISINTEGRIN AND METALLOPROTEINASE WITH THROMBOSPONDIN MOTIFS ADAM	0.39
H3024D04 (l)	BINDING	0.22	H3078F01 (l)	FINGER PROTEIN TRIPARTITE MOTIF PROTEIN	0.60
H3071G10 (l)	*Mus musculus* similar to OVOSTATIN PRECURSOR (OVOMACROGLOBULIN) (LOC232400), mRNA	0.55	H3055E11 (l)	similar to CDNA FLJ14745 FIS, CLONE NT2RP3002785, WEAKLY SIMILAR TO LETHAL(2)DENTICLELESS PROTEIN [Homo sapiens]	0.52
H3029D07 (l)	*Mus musculus* DNA segment, Chr 11, ERATO Doi 175, expressed (D11Ertd175e), mRNA	1.92	H3021H09 (l)	*Mus musculus* expressed sequence AW545363 (AW545363), mRNA	0.37
H3029G06 (l)	WUGSC:H05 1 PROTEIN	2.04	H3077B05 (l)	similar to P16H6 (FRAGMENT) [Xenopus laevis]	0.65
H3063D11 (l)	*Mus musculus* expressed sequence AU022428	0.25	H3021H12 (l)	*Mus musculus* expressed sequence AL022848	0.42
H3059H11 (i)	*Mus musculus* hypothetical protein MGC38585	0.58	H3055D02 (l)	*Mus musculus* hypothetical protein MGC36398	0.59
H3066E12 (l)	*Mus musculus* hypothetical protein MGC28084	0.47	H3053G06 (l)	MG53D08.R1 (FRAGMENT)	1.82
H3022G10 (l)	HYPOTHETICAL 12.3 KDA PROTEIN (FRAGMENT).	0.52	H3080F06 (l)	MSZF73-1 (FRAGMENT)	0.28
H3076H03 (l)	Similar to: *Mus musculus*, RIKEN cDNA 2010008E23 gene, clone MGC:36813 IMAGE:4209499, mRNA, complete cds	0.41	H3021D10 (l)	RIKEN cDNA 2610024N24 gene	0.31
H3078D02 (l)	*Mus musculus* cDNA sequence BC004056	0.63	H3002A01 (l)	*Mus musculus* RIKEN cDNA 1110002H15 gene	0.30
H3080G01 (l)	*Mus musculus* RIKEN cDNA 2810405O22	0.21	H3056H10 (l)	*Mus musculus* RIKEN cDNA 4933411J24	0.69
H3074G11 (l)	*Mus musculus* RIKEN cDNA 5730403J10	2.00	H3021D09 (l)	*Mus musculus* RIKEN cDNA 2310004K06 gene	0.47
3002A03 (l)	*Mus musculus* RIKEN cDNA 1110002B05	0.63	H3066G12 (l)	*Mus musculus* RIKEN cDNA 4432405K22	0.35
H3002H07 (l)	*Mus musculus* RIKEN cDNA 1700019D03 gene	0.67	H3051C08 (l)	RIKEN cDNA 2310067E08 gene / putative	1.79

Table 7.3b: List of known genes exhibiting a significant difference in expression between dietary treatments on day 4.5 of pregnancy. Letter after clone name is EST functional clssification information, Key in Table 7.3g. EST's described in discussion are underlined.

EST clone name	Description	Fold change 4.5	EST clone name	Description	Fold change 4.5
H3072H10 (a)	*Mus musculus* similar to Beta-arrestin 2 (Arrestin, beta 2) (LOC216869), mRNA	0.17	H3022C11 (a)	*Mus musculus* macrophage erythroblast attacher (Maea), mRNA	0.76
H3041C04 (b)	weakly similar to RB-BINDING PROTEIN [*Homo sapiens*]	0.48	H3056F01 (b)	*Mus musculus* growth differentiation factor 9 (Gdf9), mRNA	0.49
H3030H12 (b)	*Mus musculus* orthodenticle homolog 2 (Drosophila) (Otx2), mRNA	0.51	H3058A10 (c)	*Mus musculus* homeodomain interacting protein kinase 3 (Hipk3), mRNA	0.54
H3071G11 (c)	*Mus musculus* RAP2B, member of RAS oncogene family, mRNA	0.22	H3028F06 (c)	*Mus musculus* RAB17, member RAS oncogene family, mRNA	1.69
H3022A12 (c)	*Mus musculus* timeless homolog (*Drosophila*) (Timeless), mRNA	0.28	H3046D01 (c)	*Mus musculus* similar to TIM1 (LOC210535), mRNA	2.13
H3026F12 (c)	*Mus musculus* nuclear receptor subfamily 6, group A, member 1 (Nr6a1), mRNA	0.41	H3076H06 (c)	*Mus musculus* adenylate kinase 4 (Ak4), mRNA	0.20
H3038F12 (c)	hypothetical Protein kinase-like (PK-like) structure containing protein	2.56	H3041B11 (c)	*Mus musculus* mevalonate (diphospho) decarboxylase (Mvd), mRNA	1.45
H3038H09 (c)	*Mus musculus* activin receptor IIA (Acvr2), mRNA	2.04	H3077E08 (c)	Similar to: *Mus musculus* epidermal growth factor receptor, related sequence (Egfr-rs), mRNA	0.25
H3076F12 (c)	REGULATOR OF G PROTEIN SIGNALING	0.29	H3072G09 (c)	*Mus musculus* potassium voltage-gated channel, subfamily H (eag-related), member 1 (Kcnh1), mRNA	0.26
H3033D07 (c)	RECEPTOR PROTEIN TYROSINE KINASE ERBB EC 2.7.1.112 C	0.29	H3018C06 (c)	*Mus musculus* low density lipoprotein receptor-related protein 1 (Lrp1), mRNA	2.86
H3054C01 (c)	*Mus musculus* nuclear receptor subfamily 2, group E, member 3 (Nr2e3), mRNA	2.17	H3022D05 (c)	*Mus musculus* cytotoxic T lymphocyte-associated protein 2 alpha (Ctla2a), mRNA	0.67
H3051F02 (c)	*Mus musculus* SH2 domain containing 3C (Sh2d3c), mRNA	1.82	H3052D08 (c)	*Mus musculus* CUB and Sushi multiple domains 1 (Csmd1), mRNA	2.56
H3059G09 (c)	kinesin family member C5A	0.16	H3042B08 (c)	Similar to: *Mus musculus* checkpoint kinase 1 homolog (S. pombe) (Chek1), mRNA	0.56
H3076E12 (d)	*Mus musculus* regulatory factor X, 1 (influences HLA class II expression) (Rfx1), mRNA	0.54	H3040E06 (d)	*Mus musculus* high mobility group AT-hook 2 (Hmga2), mRNA	1.85
H3076H08 (d)	*Mus musculus* Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (Cited4), mRNA	0.48	H3030G12 (d)	*Mus musculus* histone cell cycle regulation defective homolog A (S. cerevisiae) (Hira), mRNA	0.17
H3072H12 (d)	*Mus musculus* E2F transcription factor 5 (E2f5), mRNA	0.57	H3066D01 (d)	*Mus musculus* nuclear DNA binding protein (C1d-pending), mRNA	0.22
H3018D07 (d)	DNA2 LIKE HOMOLOG DNA REPLICATION HELICASE LIKE HOMOLOG	1.59	H3021F06 (d)	DNA LIGASE IV EC 6.5.1.1 POLYDEOXYRIBONUCLEOTIDE SYNTHASE	0.40
H3044D06 (d)	*Mus musculus* POU domain, class 6, transcription factor 1 (Pou6f1), mRNA	1.85	H3076H01 (d)	PAI-1 MRNA-BINDING PROTEIN homolog [*Homo sapiens*]	0.28
H3054D02 (d)	*Mus musculus* H3 histone, family 3B (H3f3b), mRNA	1.89	H3036F04 (d)	Similar to: *Mus musculus* zinc finger protein 42 (Zfp42), mRNA	2.22
H3078A11 (d)	*Mus musculus* pericentrin 2 (Pcnt2), mRNA	0.44	H3076A12 (d)	*Mus musculus* T-box 20 (Tbx20), mRNA	0.78
H3079H10 (e)	Similar to: *Mus musculus* eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y), mRNA	0.53	H3051D07 (e)	DJ382I10.5 NOVEL PROTEIN SIMILAR TO ARGINYL TRNA SYNTHETASE ARGININE TRNA LIGASE, EC 6.1.1.19 ISOFORM SLA/LP	1.52
H3045E04 (e)	*Mus musculus* Y box protein 2 (Ybx2), mRNA	1.92	H3035A12 (e)		0.68
H3052H07 (e)	SERYL-AMINOACYL-TRNA SYNTHETASE 2.	2.17	H3053A07 (e)	*Mus musculus* spinocerebellar ataxia 1 homolog (human) (Sca1), mRNA	1.92
H3077E03 (e)	*Mus musculus* poly(rC) binding protein 1 (Pcbp1), mRNA	0.24	H3059B05 (e)	PERIPHERIAL BENZODIAZEPINE RECEPTOR ASSOCIATED PROTEIN.	0.64
H3051D06 (e)	CDNA FLJ10299 FIS, CLONE NT2RM2000013, MODERATELY SIMILAR TO DNA-DIRECTED RNA POLYMERASE III 128 KDA POLYPEPTIDE (EC 2.7.7.6) homolog [Homo sapiens]	2.13	H3024H01 (e)	*Mus musculus* immature colon carcinoma transcript 1 (Ict1), mRNA	0.44
H3053B06 (f)	*Mus musculus* ubiquitination factor E4B, UFD2 homolog (S. cerevisiae) (Ube4b), mRNA	2.22	H3055E09 (f)	TROPHININ ASSOCIATED PROTEIN TASTIN TROPHININ ASSISTING	0.52
H3073G07 (f)	*Mus musculus* granzyme M (lymphocyte met-ase 1) (Gzmm), mRNA	0.23	H3051F10 (g)	*Mus musculus* chemokine (C-X-C motif) ligand 1 (Cxcl1), mRNA	1.75
H3024H12 (g)	heat shock 70kD protein 8	0.25	H3033H12 (h)	weakly similar to OPIOID BINDING PROTEIN/CELL ADHESION MOLECULE PRECURSOR (OBCAM) (OPIOID-BINDING CELL ADHESION MOLECULE) (OPCML) [Rattus norvegicus]	3.33
H3066H01 (h)	*Mus musculus* phosphatidylinositol glycan, class F (Pigf), mRNA	0.20	H3055H03 (h)	BARDET BIEDL SYNDROME 4	0.15
H3024A06 (i)	*Mus musculus* alcohol dehydrogenase 5 (Adh5), mRNA	0.49	H3077B06 (i)	*Mus musculus* long chain fatty acyl elongase (Lce-pending), mRNA	0.65
H3026D05 (i)	*Mus musculus* N-myristoyltransferase 1 (Nmt1),	1.85	H3063D10 (i)	*Mus musculus* glucan (1,4-alpha-), branching enzyme 1 (Gbe1), mRNA	0.46
H3059G03 (i)	*Mus musculus* transferrin receptor (Trfr), mRNA	0.17	H3055A10 (i)	CYTOSOLIC PHOSPHOLIPASE A2	0.61

Table 7.3b: Continued

Clone name	Description	Fold change 4.5	Clone name	Description	Fold change 4.5
H3029B12 (i)	PROTEIN ARGININE N METHYLTRANSFERASE	2.00	H3019F07 (c)	*Mus musculus* serine/threonine kinase 10 (Stk10), mRNA	2.44
H3022G06 (j)	*Mus musculus* ubiquitin conjugating enzyme 7 interacting protein 5 (Ubce7ip5-pending), mRNA	0.71	H3055E02 (j)	TETRATRICOPEPTIDE REPEAT PROTEIN	0.19
H3055E12 (j)	*Mus musculus* f-box and leucine-rich repeat protein 12 (Fbxl12), mRNA	0.14	H3034G11 (j)	Similar to: *Mus musculus* gene for AMY-1, complete cds	1.89
H3021F01 (j)	*Mus musculus* aspartyl aminopeptidase (Dnpep), mRNA	0.63	H3014E06 (j)	*Mus musculus* UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4 (B3galt4), mRNA	2.08
H3052H01 (j)	*Mus musculus* calneuron 1 (Caln1), mRNA	0.54	H3051A02 (k)	*Mus musculus* secretogranin III (Scg3), mRNA	1.72
H3045B12 (k)	*Mus musculus* solute carrier family 12, member 7 (Slc12a7), mRNA	2.27	H3041A11 (k)	SODIUM/DICARBOXYLATE COTRANSPORTER NA + /DICARBOXYLATE	1.69
H3001A02 (k)	*Mus musculus* secretin (Sct), mRNA	0.51	H3024E12 (k)	*Mus musculus* monocarboxylate transporter 4 (MCT4), mRNA	0.56
H3078E05 (k)	DYNEIN HEAVY CHAIN, CYTOSOLIC DYHC CYTOPLASMIC DYNEIN HEAVY	0.43	H3051G01 (k)	IMPORTIN ALPHA SUBUNIT KARYOPHERIN ALPHA	1.92
H3045H07 (l)	Similar to: *Mus musculus* adult male testis cDNA, RIKEN full-length enriched library, clone:4930572N12 product:unknown EST, full insert sequence	1.79	H3045H07 (l)	Similar to: *Mus musculus* adult male testis cDNA, RIKEN full-length enriched library, clone:4930572N12 product:unknown EST, full insert sequence	1.79
H3024C06 (l)	*Mus musculus* teratocarcinoma expressed, serine rich (Tera-pending), mRNA	0.33	H3076G02 (l)	hypothetical SWAP / SURP containing protein	0.09
H3059E11 (l)	Similar to: *Mus musculus* adult male thymus cDNA, RIKEN full-length enriched library, clone:5830463N04 product:unknown EST, full insert sequence	0.46	H3072H07 (l)	Similar to: *Mus musculus* adult male olfactory brain cDNA, RIKEN full-length enriched library, clone:6430505D04 product:unknown EST, full insert sequence	0.32
H3035H12 (l)	hypothetical Proline-rich region/RING finger containing protein	0.64	H3039D12 (l)	weakly similar to L1 RETROPOSON, ORF2 MRNA (FRAGMENT) [*Rattus norvegicus*]	1.92
H3054F01 (l)	Similar to: *Mus musculus* adult female vagina cDNA, RIKEN full-length enriched library, clone:9930120K16 product:unclassifiable, full insert sequence	1.79	H3057B01 (l)	Similar to: *Mus musculus* 13 days embryo male testis cDNA, RIKEN full-length enriched library, clone:6030427F01 product:unknown EST, full insert sequence	0.68
H3033E05 (l)	Hypothetical DEAD/DEAH box helicase containing protein	0.18	H3057A03 (l)	DNA SEGMENT, CHR 16, WAYNE STATE UNIVERSITY 83, EXPRESSED.	0.45
H3062G09 (l)	Similar to CG1-62 protein [*Homo sapiens*]	0.38	H3002H01 (l)	NUCLEOLAR PROTEIN GU2.	0.32
H3038B11 (l)	*Mus musculus* hypothetical protein MGC38847 (MGC38847), mRNA	1.45	H3060H05 (l)	hypothetical Protein kinase-like (PK-like) structure containing protein	0.17
H3076G02 (l)	hypothetical SWAP / SURP containing protein	0.09	H3031F07 (l)	CGTHBA PROTEIN 14 GENE	2.08
H3079A12 (l)	Similar to: *Mus musculus* LOC216944	0.72	H3063D11 (l)	*Mus musculus* expressed sequence AU022428	0.21
H3047G07 (l)	SIMILAR TO HYPOTHETICAL PROTEIN FLJ20302 homolog [Homo sapiens]	1.61	H3059G04 (l)	KIAA0280 PROTEIN (FRAGMENT) homolog [Homo sapiens]	0.25
H3066G04 (l)	*Mus musculus* hypothetical protein MGC32471	0.54	H3036B02 (l)	*Mus musculus* RIKEN cDNA 4932431F02 gene	1.72
H3002A11 (l)	*Mus musculus* RIKEN cDNA 5830436K05 gene	0.49	H3066G12 (l)	*Mus musculus* RIKEN cDNA 4432405K22 gene	0.39
H3030G04 (l)	*Mus musculus* RIKEN cDNA 5730589L02 gene	1.72	H3071C01 (l)	*Mus musculus* RIKEN cDNA 1700003M02 gene	0.23
H3078H01 (l)	*Mus musculus* RIKEN cDNA 2410007P03 gene	0.56	H3077E12 (l)	*Mus musculus* RIKEN cDNA 2810406C15 gene	0.21
H3069H05 (l)	*Mus musculus* RIKEN cDNA 2310015A05 gene	0.39	H3037B01 (l)	*Mus musculus* RIKEN cDNA 4930402F02 gene	0.43
H3053D06 (l)	*Mus musculus* RIKEN cDNA 1810048P08 gene	3.13	H3015C07 (l)	*Mus musculus* RIKEN cDNA 1200014H14 gene	1.56
H3028E07 (l)	*Mus musculus* RIKEN cDNA 3110082I17 gene	1.22	H3077A12 (l)	*Mus musculus* RIKEN cDNA 4930415G15 gene	0.60
H3042D09 (l)	RIKEN cDNA 5730481H23 gene	1.64	H3021H02 (l)	*Mus musculus* RIKEN cDNA 1200008A18 gene	0.25
H3072F06 (l)	RIKEN cDNA 2610103K11 gene	0.59	H3011C04 (l)	*Mus musculus* RIKEN cDNA 0610038D11 gene	1.49
H3062E10 (l)	RIKEN cDNA 2210010C17 gene	0.56	H3053G09 (l)	SIMILAR TO RIKEN CDNA 0I2	0.57
H3013F06 (l)	Similar to: *Mus musculus*, RIKEN, clone:C130023C23 product:unknown EST, full insert sequence	1.64	H3054A08 (l)	Similar to: *Mus musculus* similar to CG1874-PA [*Drosophila melanogaster*] (LOC330832),	2.13
H3078G01 (l)	*Mus musculus* expressed sequence AI428139	0.66	H3060C12 (l)	*Mus musculus* LOC210415	0.50
H3078G01 (l)	*Mus musculus* expressed sequence AI428139	0.66	H3016D05 (l)	Similar to: *Mus musculus* hypothetical protein 6030430O11	1.56
H3079B06 (l)	*Mus musculus* LOC231423	0.61	H3028B05 (l)	Similar to: *Mus musculus* LOC224609 mRNA	1.19
H3072H06 (l)	*Mus musculus* LOC209550	0.26	H3080F06 (l)	MSZF73-1 (FRAGMENT).	0.26
H3072B12 (l)	*Mus musculus* LOC244840	0.47	H3063F03 (l)	*Mus musculus* LOC232186	2.33
H3058C10 (l)	hypothetical BRCT domain containing protein	0.58	H3047F05 (l)	AF 9	1.52
H3032B07 (l)	hypothetical Phosphotyrosine interaction (PID or PI) containing protein	1.67	H3010A08 (l)	*Mus musculus* testis expressed gene 19 (Tex19), mRNA	0.65
H3071C08 (l)	E Y 2	0.38			

Table 7.3c: List of known genes exhibiting a significant difference in expression between dietary treatments on day 3.5 and 4.5 of pregnancy. Letter after clone name is EST functional clssification information, Key in Table 7.3g. EST's described in discussion are underlined.

Clone Name	Description	Fold change 3.5	Fold change 4.5	Clone name	Description	Fold change 3.5	Fold change 4.5
H3078D07 (b)	*Mus musculus* immediate early response 5 (Ier5), mRNA	0.56	0.50	H3031B07 (b)	*Mus musculus* twisted gastrulation protein (Twg-pending), mRNA	2.17	2.50
H3060H04 (b)	*Mus musculus* mitogen activated protein kinase binding proten 1 (Mapkbp1), mRNA	0.21	0.15	H3058A09 (b)	M PHASE PHOSPHOPROTEIN 9	0.55	0.55
H3080E06 (c)	*Mus musculus* plasminogen activator, tissue (Plat), mRNA	0.32	0.36	H3072G01 (c)	*Mus musculus* kinesin superfamily protein 18A (Kif18A), mRNA	0.47	0.43
H3056H06 (c)	*Mus musculus* EGF-like module containing, mucin-like, hormone receptor-like sequence 4 (Emr4), mRNA	0.56	0.45	H3059G06 (c)	*Mus musculus* metal response element binding transcription factor 1 (Mtf1), mRNA	0.16	0.12
H3071H09 (c)	*Mus musculus* G protein-coupled receptor 1 (Gpr1), mRNA	0.48	N/A	H3071H08 (c)	*Mus musculus* G protein-coupled receptor 1 (Gpr1), mRNA	N/A	0.16
H3059G10 (d)	DNA2 LIKE HOMOLOG DNA REPLICATION HELICASE LIKE HOMOLOG	0.47	0.40	H3024F12 (d)	*Mus musculus* similar to Transcription factor BTF3 (RNA polymerase B transcription factor 3) (LOC218490), mRNA	0.40	0.31
H3059F06 (d)	*Mus musculus* H1 histone family, member O (oocyte-specific) (H1fo-pending), mRNA	0.48	0.35	H3056E07 (d)	CCAAT/enhancer binding protein (C/EBP)	0.44	0.34
H3066H11 (d)	*Mus musculus* mutS homolog 4 (E. coli) (Msh4), mRNA	0.36	0.26	H3076G01 (e)	C TERMINAL MODULATOR	0.36	0.39
H3033G03 (e)	*Mus musculus* FK506 binding protein 4 (59 kDa) (Fkbp4), mRNA	0.54	0.30	H3078C01 (f)	TEB4 PROTEIN homolog [*Homo sapiens*]	0.50	0.42
H3060H03 (g)	*Mus musculus* T-cell lymphoma breakpoint 1 (Tcl1), mRNA	0.16	0.15	H3080F01 (h)	*Mus musculus* zinc finger, DHHC domain containing 7 (Zdhhc7), mRNA	0.18	0.30
Ctl141002 01_O22 (i)	*Mus musculus* hydroxysteroid dehydrogenase-1, delta<5>-3-beta (Hsd3b1), mRNA	0.43	0.38	H3063C09 (i)	*Mus musculus* UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6 (B4galt6), mRNA	0.38	0.34
H3060H07 (i)	*Mus musculus* spermatid-specific thioredoxin 1 (Sptrx1), mRNA	0.20	0.27	H3076C01 (f)	26S protease regulatory subunit	0.41	0.42
H3059F01 (j)	*Mus musculus* ubiquitin carboxy-terminal hydrolase L1 (Uchl1), mRNA	0.48	0.52	H3031D07 (f)	26S proteasome non-ATPase regulatory subunit 1	1.96	1.75
H3077E04 (k)	*Mus musculus* karyopherin (importin) alpha 1 (Kpna1), mRNA	0.24	0.20	H3021F05 (k)	*Mus musculus* karyopherin (importin) alpha 2 (Kpna2), mRNA	0.36	0.25
H3021E11 (k)	*Mus musculus* ATPase, H+ transporting, lysosomal 70kD, V1 subunit A, isoform 1 (Atp6v1a1), mRNA	0.21	0.21	H3062F11 (l)	Similar to: *Mus musculus* similar to hypothetical protein MGC955 [*Homo sapiens*] (BC035522), mRNA	0.27	0.21
H3077G09 (l)	*Mus musculus* Williams-Beuren syndrome chromosome region 16 homolog (human) (Wbscr16), mRNA	0.33	0.42	H3059B04 (l)	hypothetical Armadillo/plakoglobin ARM repeat profile containing protein	0.45	0.56
H3054F07 (l)	*Mus musculus* hepatoma up-regulated protein (Hurp-pending), mRNA	1.33	2.50	H3056F03 (l)	FUSED	0.51	0.38
H3024G12 (l)	*Mus musculus* heparan sulfate (glucosamine) 3-O-sulfotransferase 3B (Hs3st3b), mRNA	0.29	0.21	H3060H11 (l)	*Mus musculus* helicase, mus308-like (Drosophila) (Hel308-pending), mRNA	0.23	0.14
H3080F05 (l)	*Mus musculus* fetal liver zinc finger 1 (Fliz1-pending), mRNA	0.17	0.27	H3077E09 (l)	*Mus musculus* gene trap locus 3 (Gtl3), mRNA	0.48	0.28
H3002E02 (l)	DNA SEGMENT, CHR 16, WAYNE STATE UNIVERSITY 83, EXPRESSED.	0.52	0.35	H3055A02 (l)	hypothetical Eukaryotic protein kinase/Doublecortin containing protein	0.38	0.36
H3055E01 (l)	*Mus musculus* DNA segment, Chr 16, ERATO Doi 454, expressed (D16Ertd454e), mRNA	0.15	0.17	H3037H02 (l)	COFACTOR	1.72	1.61
H3076G12 (l)	Similar to: *Mus musculus*, Similar to hypothetical protein MGC40397, clone IMAGE:4234918, mRNA	0.27	0.16	H3077H05 (l)	Similar to: *Mus musculus* RIKEN cDNA 2610205H19 gene	0.61	0.56
H3021H12 (l)	*Mus musculus* expressed sequence AL022848	0.42	0.24	H3076H09 (l)	*Mus musculus* hypothetical protein MGC40770 (MGC40770), mRNA	0.44	0.25
H3059G05 (l)	*Mus musculus* hypothetical protein MGC30493 (MGC30493), mRNA	0.31	0.15	H3024C12 (l)	*Mus musculus* hypothetical protein MGC28623 (MGC28623), mRNA	0.32	0.27
H3021G12 (l)	*Mus musculus* RIKEN cDNA 2810409H07	0.46	0.41	H3059H01 (l)	*Mus musculus* RIKEN cDNA 2810452K22	0.29	0.29
H3030A01 (l)	RIKEN cDNA 2210012C09 gene	0.25	0.22	H3024A12 (l)	*Mus musculus* RIKEN cDNA 3300001P08	0.36	0.26
H3059H12 (l)	*Mus musculus* LOC225922	0.25	0.21	H3059E12 (l)	RIKEN cDNA 2210021E03 gene	0.60	0.49
H3053G06 (l)	MG53D08.R1 (FRAGMENT)	1.82	1.76	H3072H04 (l)	*Mus musculus* LOC212249 mRNA	0.23	0.17

Table 7.3d: List of unknown genes exhibiting a significant difference in expression between dietary treatments on day 3.5 of pregnancy.

Clone Name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change
H3012G01	0.49	H3071H04	0.24	H3059B09	0.59	H3055F07	0.46	H3072H03	0.32
H3043H07	0.44	H3019D06	0.14	H3062C07	0.62	H3075E10	0.20	H3031A01	1.56
H3048H09	0.35	H3059E01	0.34	H3070H01	0.36	H3059C07	0.60	H3079D10	0.61
H3069B01	0.53	H3055F08	0.53	H3066H05	0.50	H3059C03	0.45	H3073D06	0.51
H3062F03	0.26	H3034B12	0.24	H3062H01	0.53	H3070A08	0.56	H3066C04	0.49
H3052B12	1.85	H3021E12	0.15	H3038C06	2.70	H3034G07	1.75	H3073A08	0.50
H3080C03	0.53	H3045G07	1.72	H3030D07	2.00	H3030D08	1.56	H3062E01	0.40
H3067F04	1.72	H3011F02	1.59	H3062A06	0.70	H3058G04	0.56	H3046E06	1.79
H3033B06	1.89	H3005E03	0.37	H3060B04	0.63	H3022A08	0.49	H3063B01	0.61
H3072E01	0.48	H3080G12	0.48	H3063C12	0.72	H3062F07	0.42	H3055H09	0.14
H3060G03	0.22	H3069H03	0.48	H3047C02	1.82	H3056F04	0.66	H3039G08	1.33
H3021H10	0.20	H3058E03	1.49	H3070A01	0.63	H3071H11	0.11	H3067F01	1.41
H3067D11	1.39	H3026D01	1.56	H3044D07	1.75	H3072H02	0.16	H3031B05	2.17
H3061H01	0.43	H3012D05	1.69	H3059A08	0.74	H3079C01	0.60	H3021A04	0.49
H3062F04	0.23	H3019D07	2.38	H3070G03	0.53	H3079F07	0.73	H3063H03	0.54
H3051A01	2.22	H3033C03	0.63	H3060H09	0.29	H3075D07	0.74	H3072F10	0.60
H3062G07	0.65	H3060H10	0.58	H3063H05	0.62				

Table 7.3e: List of unknown genes exhibiting a significant difference in expression between dietary treatments on day 4.5 of pregnancy.

Clone name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change
H3048B08	2.08	H3011A08	2.33	H3055B12	0.63	H3040E07	1.56	H3035F12	2.38
H3034G10	2.50	H3060H06	0.13	H3031C03	1.59	H3034H05	2.04	H3036H08	1.61
H3037D08	2.22	H3064D12	1.96	H3037G01	2.00	H3055H06	0.50	H3052F07	1.72
H3045H05	1.12	H3037D07	1.69	H3034E12	0.36	H3037B05	2.17	H3039G11	2.86
H3032F01	0.26	H3034G04	2.17	H3021A09	0.45	H3037B07	1.69	H3045D01	2.04
H3004B05	2.63	H3046H07	2.50	H3060A02	0.55	H3057C06	1.41	H3033D06	1.85
H3038E04	1.72	H3041E05	2.78	H3005H04	1.67	H3062B04	0.53	H3066F09	0.29
H3042E04	2.94	H3001H12	0.26	H3046C12	2.00	H3080F07	0.63	H3068F07	2.33
H3058G06	0.53	H3013A02	0.16	H3036B08	1.96	H3029A06	0.54	H3013D12	1.79
H3069H12	0.34	H3036F07	2.50	H3013D06	2.22	H3004A08	0.62	H3032G08	1.43
H3052D07	3.13	H3014G06	3.13	H3047D11	1.52	H3059G01	0.17	H3047B12	1.79
H3034G06	2.27	H3037H12	3.33	H3052B02	2.00	H3041F11	1.61	H3080F04	0.27
H3077E11	0.28	H3070G12	0.51	H3072B06	1.45	H3067D05	1.52	H3037A12	0.44
H3039H07	1.64	H3062A10	0.37	H3007H07	1.41	H3033F08	2.00	H3061E09	0.51
H3035A07	2.50	H3040A07	1.96	H3062H02	0.52	H3034G08	2.00	H3057A09	0.62
H3033D11	1.61	H3037E06	1.96	H3057A06	0.55	H3041E06	2.70	H3037H03	1.41
H3038D01	3.13	H3058G06	0.51	H3072H11	0.34	H3002F05	0.48	H3004F03	2.00
H3073A01	0.64	H3071A01	0.33	H3052H10	2.00	H3034G12	0.28	H3077E06	0.16
H3071C10	0.20	H3044A07	2.44	H3055B06	0.33	H3033B07	1.61	H3034C04	1.47
H3062C06	0.60	H3038C11	2.22	H3072F07	0.67	H3039D08	2.33	H3053D11	1.61
H3055C06	0.50	H3012F02	1.79	H3066C10	0.48	H3063H11	0.69	H3078E06	0.57
H3042D07	1.85	H3071H06	0.13	H3051E04	2.13	H3070H05	0.85	H3051F06	2.56
H3001C01	0.28	H3011E12	0.31	H3072H09	0.30	H3055E04	0.39	H3036C02	1.64
H3038B05	2.17	H3020E07	2.04	H3047H07	1.96	H3036B07	3.33	H3051D10	1.79
H3059H06	0.42	H3055E03	0.34	H3036A11	0.24	H3046F09	2.08	H3033H06	2.38
H3075H12	0.41	H3018C05	2.04	H3015B08	1.49	H3039C07	2.04	H3071H10	0.36
H3036B10	1.92	H3038C07	2.63	H3060B05	0.51	H3034B03	1.49	H3072B01	0.50
H3015D03	1.54	H3037B06	2.94	H3039B10	1.69	H3063D02	0.54	H3009F12	2.50
H3047E07	3.45	H3063F12	0.39	H3038G06	1.59	H3044A01	1.32	H3077F01	0.60
H3073C06	0.45	H3071G12	0.43	H3042F06	2.56	H3063C06	0.58	H3042F08	1.30
H3060G02	0.53	H3003B11	2.33	H3007H08	1.45	H3048A07	2.04	H3056H11	0.64
H3044B10	2.00	H3067H12	0.50	H3035E07	1.64	H3064G07	1.47	H3071F12	0.42
H3056A04	0.35	H3007B01	1.39	H3060F02	0.61	H3069A05	0.57	H3039A12	0.71
H3055H01	0.14	H3069F01	0.53	H3074B07	1.45	H3056F02	0.44	H3064B08	1.61
H3046E06	2.13	H3048F08	1.79	H3036D06	1.69	H3035H01	1.79	H3040D12	0.60
H3040H02	1.69	H3056F05	0.45	H3014F12	0.35	H3033B03	1.47	H3060G11	0.59
H3056A12	0.48	H3045D02	1.52	H3064A06	1.69	H3041C11	1.85	H3042G01	2.08
H3056H02	0.55	H3041C12	2.08	H3062A11	0.53	H3070E12	0.85	H3044E07	2.63
H3042D03	1.96	H3034E07	2.33	H3062B10	1.96	H3068C07	1.49	H3035F07	1.59
H3066H12	0.40	H3021G06	0.45	H3048D10	1.96	H3052F01	1.75	H3071H07	0.43
H3062F06	0.41	H3029H05	2.13	H3039F08	1.69	H3011G05	1.82	H3039B12	1.85
H3041H05	1.32	H3033B09	1.92	H3039A01	1.89	H3063D09	2.33	H3037A06	0.41

Table 7.3e: continued

Clone name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change	Clone name	Fold change
H3056F12	0.72	H3042A10	2.50	H3058G11	0.68	H3056E06	0.54	H3026H07	1.45
H3071E04	0.42	H3071C12	0.76	H3073A08	0.39	H3057A09	0.57	H3046A10	1.59
H3014H01	2.22	H3035H08	1.59	H3056H03	0.57	H3052E01	0.38	H3008E06	1.75
H3053H01	0.54	H3034C01	2.00	H3033H05	1.61	H3019F10	1.79	H3038F05	2.56
H3046G04	1.92	H3056A06	0.45	H3062A03	0.65	H3053A03	0.37	H3068H05	0.51
H3035F03	2.17	H3037F07	1.85	H3059E08	0.49	H3064D01	1.22	H3069H08	0.52
H3035F01	2.44	H3039B07	1.61	H3033G09	0.41	H3042D11	1.69	H3044C04	1.89
H3041G01	0.21	H3065H12	1.92	H3054G06	2.63	H3066H07	0.35	H3061A12	0.67
H3057A06	0.56	H3041H08	2.08	H3037F09	1.43	H3060A07	0.61	H3034C07	2.33
H3044F12	2.38	H3055H07	0.17	H3051G10	1.89	H3039E03	1.67	H3045D05	2.08
H3007F10	1.47	H3040C03	1.92	H3038D04	1.39	H3004C10	2.13	H3071G06	0.71
H3060C04	0.64	H3071H11	0.12	H3033H11	1.69	H3065G01	1.35	H3003C10	1.61
H3047C11	2.38	H3045H06	3.03	H3048C06	2.17	H3032H12	0.47	H3046E10	1.85

Table 7.3f: List of unknown genes exhibiting a significant difference in expression between dietary treatments on day 3.5 and 4.5 of pregnancy

Clone name	Fold change day 3.5	Fold change day 4.5	Clone name	Fold change day 3.5	Fold change day 4.5	Clone name	Fold change day 3.5	Fold change day 4.5
H3062B05	0.48	0.47	H3043H08	0.33	0.41	H3063D01	0.50	0.38
H3077B04	0.59	0.40	H3063C04	0.47	0.37	H3056H07	0.22	0.17
H3056E02	0.16	0.17	H3077H10	0.43	0.34	H3077F02	0.52	0.40
H3077E01	0.50	0.43	H3062D12	0.50	0.44	H3043H04	0.48	0.35
H3059D05	0.45	0.43	H3077G01	0.65	0.62	H3071H02	0.23	0.27
H3063C01	0.40	0.24	H3059G07	0.43	0.45	H3048F07	1.89	2.63
H3077E10	0.28	0.26	H3071C11	0.48	0.48	H3063C02	0.36	0.15
H3055C12	0.52	0.45	H3062F10	0.23	0.18	H3055A11	0.56	0.49
H3066D02	0.43	0.30	H3056F11	0.53	0.34	H3056E12	0.18	0.20
H3048G01	0.33	0.25	H3062A02	0.37	0.31	H3058G07	0.65	0.40
H3072F01	0.29	0.36	H3060H12	0.16	0.20	H3048H07	0.35	0.49
H3060G01	0.18	0.18	H3066A02	0.27	0.29	H3076H05	0.27	0.17
H3063D12	0.28	0.11	H3060E06	0.56	0.53	H3043H06	0.46	0.44
H3041G10	0.25	0.11	H3055F01	0.15	0.18	H3055H04	0.22	0.12
H3073G05	0.24	0.17	H3060H02	0.16	0.16	H3075A07	0.68	0.58
H3038F01	1.69	2.00	H3062E12	0.40	0.34	H3077G05	0.29	0.45
H3066F02	0.27	0.26	H3076H02	0.22	0.20	H3062A01	0.41	0.49
H3040D06	1.89	2.50	H3066D11	0.50	0.42	H3058E04	0.45	0.38
H3071H05	0.23	0.20	H3072H05	0.20	0.11	H3069G02	0.53	0.41
H3062F12	0.15	0.13	H3077H01	0.55	0.58	H3071D01	0.34	0.32
H3055H02	0.11	0.13	H3059H02	0.34	0.32	H3019E07	0.46	0.36
H3060H08	0.22	0.22	H3019D06	0.14	0.13	H3021G01	0.36	0.32
H3059G02	0.17	0.22	H3069H01	0.21	0.18	H3058C02	0.43	0.43
H3071H01	0.18	0.14	H3060E01	0.30	0.30	H3034B12	0.24	0.29

Table 7.3g: Key to EST functional classifications in Tables 7.3a-c. Classifications are based upon the EST descriptions at **http://lgsun.grc.nia.nih.gov/cDNA/15k.html** adapted from systems used by Kargul *et al* (2001) and Mu *et al* (2001)

Code	Functional classification	Functional subclasses
a	Apoptosis	Apoptosis
b	Cell cycle/differentiation	Mitogens Cell cycle proteins
c	Signalling	Receptors Kinases Hormone regulation growth factors
d	Transcription/chromatin	Transcription factors DNA repair Chromatin remodeling
e	Translstion/protein synthesis	Ribosomal proteins Protein folding
f	Protein degredation	Proteases Ubiqitination factors Protein turnover
g	Heatshock/stress	Heatshock/stress
h	Matrix/structrual proteins	Cell adhesion Cytoskelital components Extracellular matrix
i	General metabolism	General metabolism
j	Protein function	Post-translational modifications
k	Transport	Protein trafficking Importins Secretins
l	Unclasified EST's	Unclasified EST's

7.4 Discussion

Traditionally "differences" in expression levels of EST's in microarray analysis have been assessed by using a system of ratios of values (for example, an arbitrary 2- or 10-fold expression difference might be considered to represent a significant difference) obtained from a single determination. However, as is becoming comman place, this study used multiple determinations to assess gene differences (Tanaka *et al*, 2000; Small

et al, 2005). As a result, this investigation used several indipendant determinations, which permited an analysis satisfing conservative statistical criteria. Expression differences of under 2-fold have historically been considered to be the limit of detection in previous mircroarray analyses. While many of the 690 EST's found to alter significantly in relation to dietary treatment did alter by more than 2-fold, the application of statistical criteria allowed significant differences to be observed in EST's where fold change was as little as 1.15-fold between dietary treatments (Fig. 7.3b).

690 EST's were found to alter significantly in relation to dietary treatment. Of these the vast majority on both day 3.5 and 4.5 of pregnancy were novel or unclassified EST's. Of those EST's of known function found to alter significantly in relation to diet, genes for proteins involved in intra and extra cellular signaling, transcription, translation and general metabolism were the most abundant. While numbers of genes involved in stress responses, cellular structure, protein function, apoptosis and cellular differentiation were altered in lower numbers. This pattern of gene expression differences is perhaps unsurprising as it is changes within signaling transcription and translation would form the basis of any other adaptations made to accomadate dietary stress. While all the EST's found to significantly alter in relation to dietary treatment are potentially interesting, it is well beyond the scope of this investigation to discuss the relevance to metabolic programming of each individual EST found to exhibit a significant difference. Instead four genes of particular interest to this investigation have been selected for discussion.

The aspartyl protease renin catalyzes the cleavage of angiotensinogen to the decapeptide angiotensin-I (ANG-I) (reviewed by Nicholls *et al*, 2001). As described in section 5.4, ANG-I is then further processed by angiotensin converting enzyme (ACE) to ANG-II. ANG-II is the final product of the renin-angiotensin system (RAS) and is a potent mediator of blood pressure and vascular differentiation. In section 5.3.6 it was shown that ACE activity was significantly increased in uterine tissue in response to treatment with a 9% LPD. This increase in ACE activity was suggested to be responsible increased expression of FGF-II and VEGF in uterine tissues, which in turn could potentially lead to increased uterine vascularisation. As a result, it is of particular

interest that a significant 1.61-fold up regulation of an EST identified as renin binding protein (RnBP) was observed on day 3.5 of pregnancy in response to treatment with a 9% LPD (Fig. 7.3d Table 7.3a).

RnBP itself was first isolated from porcine livers by Murakami et al (1980 and was shortly after shown to inhibit renin function (Ueno et al, 1981). The RnBP gene is well conserved amoung animals (Takahashi, 1997), suggesting it is biologically essential. In the rat, RnBP exists as a homodimer that forms a heterodimer with rat renin to extensively inhibit renin activity. Indeed, in rats intravenous injection of the RnBP results in a rapid and strong inhibition of plasma renin activity, which can persist for at least for 2 h, however, it should be noted that RnBP is not found in plasma (Tada et al, 1992). RnBP mRNA has been isolated in many tissues and simmilar to ACE there is evidence to suggest that RnPB levels can be locally controlled (Tada et al, 1992).

Conversion of angiotensinogen to ANG-I by renin is a major rate-limiting step in initiating the endocrine cascade of the RAS which may lead to increased FGF-II levels. It is possible that increases in local uterine levels of RnBP in response to LPD treatment could infact be reducing RAS signaling overall even if ACE activity is elevated in response to LPD treatment. Alternatively, ACE activity may be elevated to compensate for reduced conversion of angiotensinogen to ANG-I by renin. A third, perhaps surprising, hypothesis is that the increase observed in RnBP levels in response to LPD treatment may be completely irrelevant to the previously discussed bypothesies concerning ACE, FGF-II , uterine vascularisation or any other component of the RAS signaling pathway. In fact several investigators have argued against a role of RnBP in RAS regulation and there is strong evidence to support this. As already mentioned above, while RnPB can inhibit renen when injected into the bloodstream RnBP is not normally located there. In addition, renin and RnBP have been shown not to co-localise in renal tissues (Schmitz *et al*, 2000). Schmitz *et al* (2000) have also demonstrated that RnBP knock out mice not only healthy and normotensive but also and that lack of RnBP does not affect expression or activity of renin under normal physiological conditions. Finally, RnBP has also been shown to have precede sequence homology with *N*-acyl-D-glucosamine 2-epimerase (GlcNAc 2-epimerase), a protein that interconverts GlcNAc

and *N*-acetylmannosamine (Maru *et al*, 1996). Indeed, it may be that while RnBP can bind and inhibit the action of renin it may function in carbohydrate metabolism rather than in the RAS *in vivo*.

This study has also found that expression of another protein related to RAS signaling, tissue plasminogen activator (PLAT), is reduced in response to LPD treatment on day 3.5 and 4.5 of pregnancy. In this model, the most likely explanation for reduced PLAT expression is probably due to the observed increase in uterine ACE activity (Fig 7.4). ACE acts to breakdown bradykinin an enhaner of PLAT expression, thereby reducing PLAT transcription (reviewed by Vaughan, 1997) (Fig. 7.4). Indeed, it has been shown in human trials, the effect of bradykinin on PLAT upregulation can be enhaned by the addition of ACE inhibitors (Minai *et al*, 2001). Furthermore, ACE activity is also positively correlated with expression of PLAT's repressor, PLAT inhibitor-1. PLAT activity in vascular and extracellular spaces is modulated by PLAT inhibitor-1, a glycoprotein of the serine protease inhibitor family, that covalently binds to and inhibit PLAT activity (Loskutoff, *et al*, 1986; Wun and Reich, 1987). Expression of PLAT inhibitor-1 has been demonstrated to be controlled by ANG-II in both rats (van Leeuwen *et al*, 1994) and humans (Ridker *et al*, 1993). In addition, ACE inhibitors have been shown to decrease expression of PLAT inhibitor-1 in spontaneously hypertensive rats (Mitsui *et al*, 1999) and in rats with balloon injured aortas (Hamden *et al*, 1996). It is of interest to note that it is not through ANG-II binding to the AT-I or AT-II receptors that PLAT inhibitor-1 expression is increased. In fact, for PLAT inhibitor-1 expression is increased, ANG-II must be converted to ANG-III by aminopeptidase A then to ANG-IV by aminopeptidase M which in turn binds to the AT-IV receptor (reviewed by Vaughan, 1997).

PLAT has been detected in both human (Rijken *et al*, 1981) and murine (Danglot *et al*, 1986) uterine tissues. In mice, trophoblast production of PLAT has been shown to temporally correlate with blastocyst invasion (Sherman *et al*, 1976; Strickland *et al*, 1976). Moreover, implantation-defective mouse embryos express reduced amounts of PLAT (Axelrod, 1985). It is possible that reduced uterine PLAT expression could reduce the ability of the embryo to implant as trophoblast invasiveness could be

impaired. In addition, if increased ACE activity is responsible for the observed reduction in PLAT expression then it is possible that PLAT inhibitor-1 could be further impairing trophoblast invasion through further repression of PLAT. This situation presents a fascinating conundrum, because on one hand, as hypothesised in Chapter 5, increased ACE activity drives higher expression of FGF-II to aid embryo implantation. But on the other hand, increased ACE activity could also be reducing the endometrums receptivity to embryo implantation.

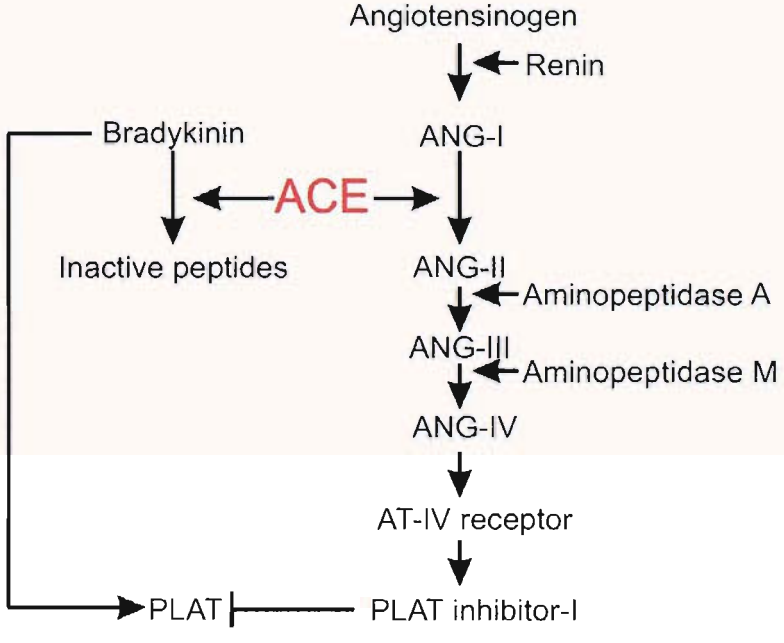

Fig. 7.4: ACE plays a pivotal role in controling not only the expression of PLAT but also the expression of its repressor PLAT inhibitor-I Adapted from (Vaughan, 1997)

In Section 6.4 it was suggested that CCAAT/enhancer binding protein α (C/EBP) could be involved in the significant increse in serum total protein observed on day 2.5 of pregnancy. In short, based on the observations of Ogawa *et al* (1999), it was hypothesised that the increase in serum total protein observed on day 2.5 was due to a transient increase in the amount of HNF-1 and/or C/EBP acting on an enhancer in the albumin gene, the most prevalent protein in serum. While no microarrays were conducted on uterine tissue on day 2.5 of pregnancy in this study it was also noted in section 6.4 that Ogawa *et al* (1999) observed a significant decrease in C/EBP mRNA after 3 days of starvation. This present study has also found that an EST identified to be C/EBP is subject to an aproximately 2-fold decrease in expression of days 3.5 and 4.5 of

pregnancy (Fig.7.3d; Table 7.3c). Additionally, this study has also demonstrated that an EST identified as CBP/p300 has reduced expression in relation to treatment with an LPD on day 4.5 of pregnancy. It is speculated that HNF-1 recruits CBP/p300, and C/EBP interacts with SWI/SNF for expression of the albumin gene in hepatocytes (Takeaki et al, 2004). While this study has not demonstrated a reduction in total protein in the serum in relation to dietary treatment concomitant with decreased C/EBP or CBP/p300 mRNA, it is none the less interesting that our model of mild dietary peotein restriction has elisieted a similar ternds to those observed by Ogawa *et al* (1999). It should also be noted that while albumin production is classically described as a function of the liver and there are as yet no papers to suggest that albumin is produced in the murine uterus, Shamay *et al*, (2005) have shown albumin mRNA to be present in the bovine uterus.

This chapter has used micro array technology to identify a large number of EST's that significantly alter expression in relation to dietary treatment. Among those genes found to change are several that are involved in biological pathways previously discussed in this investigation; these include RBP, C/EBP, CBP/p300 and PLAT. Nevertheless, it should be remembered that microarrays are primarily an exploitory hypohesis generating tool. As such microarrays effectively form the basis of future experiments. Indeed, where the primary thrust of an investigation is to examine differential gene expression the traditional techniques of RT–PCR or northern blots are nessersary to validate microarray results (Chaib *et* al, 2001; Rajeevan *et al*, 2001). Unfortunately, time constraints have prevented the verification of the microarray data contained in this chapter. Nevertheless, several interesting questions are raised and discussed with respect to the possible genetic control of metabolic programming of Predictive adaptive responces.

Chapter 8

General discussion and future work

8.1 General discussion

Over 2300 years ago Plato proposed that the "form" of an object was all-important not its component matter and that if all the appendages of change were stripped away (e.g. colour and age) you would only be left with the said "form". His pupil Aristotle was critical of Plato's reductionist approach and he instead concentrated on the "causes" of objects realising that objects arose from a combination of nature, matter and form, causation, potentiality and actuality. Aristotle's philosophy enabled him not only to appreciate there were different forms of objects, but that an object's environment and its component parts were integral to that object's character. Today, in a rather Platonic approach, genetic determinists see the genome as the key to biological processes, and that by stripping away the appendages of change down to sets of nucleotides one will be able to comprehend all the facets of an organism. While the genome is an important tool for understanding an organism, dependence on it shadows the concept of causes and reduces our ability to effectively assess all the available information. A more enlightened approach is to view the genome as the determinant of an organisms general form and potentiality, and the environment as the determinant or cause of an organisms ultimate realisation of its genetic potential.

The Developmental origins of Health and Adult Disease (DOHAD) hypothesis is one such example of an environment acting on an organism to determine realisation of genetic potential. DOHAD proposes that the fetus makes predictive adaptative responses (PAR's) to compromised nutrient supply *in utero* in an attempt to produce a phenotype for optimal postnatal fitness. However, when a postnatal mismatch exists between PAR to a specific environment and the actual environment then the organism becomes predisposed to Syndrome X risk factors. These include diabetes type II, glucose intolerance, insulin resistance, obesity, hypertension, hyperinsulinemia and other CVDs. While nutritional insult during embryonic growth is linked to adult diseases, these afflictions often only manifest in chronic forms post sexual peak and thus reproductive fitness is not necessarily adversely affected. Hence, as humans live longer and contribute to society long after having children, nutritionally programmed disease has

profound implications for an aging population, especially those in countries experiencing vast demographic change.

Several previous studies have shown that even mild dietary protein restriction during pregnancy can induce metabolic programming of disorders such as hypertension and glucose intolerance (Langley and Jackson, 1994; Langley-Evans *et al*, 1996; Sayer *et al*, 2001; Vehaskari *et al*, 2001). In these investigations, which have focused on post-implantation development, there is a significant amount of interplay between dietary restriction and the nutrient demands of the fetus. Pre-implantation development poses a very small maternal burden as the nutrient demands of the embryo are negligible at this time. As a result, the discovery that adult disorders could be programmed by dietary protein restriction confined to the pre-implantation period by Kwong *et al* (2000) was in itself quite remarkable. Metabolic programming of the peri-implantation embryo is probably associated with subtle alterations in maternal metabolism and nutrient allocation. This study primarily sought to characterise the maternal factors that may underpin metabolic programming of the peri-implantation embryo. However, this study has done much more than that; it has also accurately measured amino acid concentrations in uterine luminal fluid, identified changes in protein expression in uterine tissue and sought to define the pathways responsible; and described global responses of mice to treatment with a LPD for up to 4.5 days of pregnancy.

This study found no significant differences between the weight gained by mice treated with the LPD compared to those fed the NPD control diet. Both mice treated with the NPD and the LPD gained weight at a comparable rate (Section 3.3.1). However, a significant difference was observed in the amount of food consumed between treatment groups. Mice fed the LPD consumed significantly more food than those fed the NPD (Section 3.3.2). This accounted in real terms for an elevation in food intake of approximately 14% in the LPD treatment group. It is hypothesised that this increase in food intake could be linked to the observation that serum insulin levels are decreased on day 3.5 in mice treated with a LPD (Section 6.3.3). Insulin has been shown to inhibit the action of NPY (Liebowitz, 1997), a potent regulator of food intake and body fat accumulation (Kalara *et al*, 1991). Expression of the NPY gene itself has also

been shown to be significantly elevated in the basomedial hypothalamus in response to LPD treatment (White *et al*, 1994; White *et al*, 1998). While elevations in circulating GCs have been linked to increased NPY expression (Strack *et al*, 1995), it is unlikely that this is the case in this model due to the fact this study has failed to demonstrate a rise in serum corticosterone (section 6.3.4) or an increase in GR expression (Section 5.2.3).

This study also sought to examine how nutrients may be altered in murine uterine luminal fluid in response to LPD treatment and how this may impact upon developmental plasticity of the embryo. In accord with previously published data on oviduct amino acid balance (Dumoulin *et al*, 1992; Guerin *et al*, 1995) the novel sampling methods employed have demonstrated that taurine is the most prevalent amino acid in uterine luminal fluid and that glutamate, glutamic acid, glycine and alanine also contributed significantly to the uterine amino acid milieu (Sections 4.3.2 and 4.3.4). Moreover, using these novel methods to sample uterine fluid directly this study has, for the first time, been able to measure precisely the concentrations of amino acids in murine uterine luminal fluid. Furthermore, this study has demonstrated that there are significant modifications to the amino acid composition of uterine fluid in response to treatment with a LPD. The concentrations of the amino acids valine, isoleucine and leucine have shown to be depleted in uterine luminal fluid of mice treated with a LPD on days 3.5 and 4.5 of pregnancy (section 4.3.4).

Previous publications have shown that supplementation of embryo culture media with branch chain amino acids can increase cell numbers (Biggers *et al*, 2000; Ho *et al*, 1996; Summers *et al*, 2000) and aid hatching (Biggers *et al*, 2000; Ho *et al*, 1996; Summers *et al*, 2000). Leucine has been shown to be important for stimulation of trophoblast outgrowths (Gwatkin 1966; Martin and Sutherland, 2001; Naeslund, 1979). In the developing embryo, it is probable that these effects are mediated through the mTOR pathway. The branched chain amino acids valine, isoleucine and especially leucine are powerful mediators of the mTOR pathway (Hara *et al*, 1998; Peyrollier *et al*, 2000; Xu *et al*, 2001) (Fig.4.4). mTOR ultimately regulates the downstream targets p70 S6 and 4E-BP1 which in turn regulate preferential translation of translational machinery and overall translational efficiency (Gingras *et al*, 1999; Proud, 2002). Indeed studies

have shown that leucine-dependant trophoblast outgrowth could be ablated by administration of the mTOR inhibitor rapamycin (Martin and Sutherland, 2001). The observed reduction in branched-chain amino acids in the uterine luminal fluids of mice fed the 9% LPD could well be causing reduced signaling through the mTOR pathway and be causing embryos to undergo more catabolic rather than anabolic processes. Indeed the observation by Kwong *et al* 2000 that there is a reduction in blastocyst cell number in embryos collected from dams treated with LPD could well associate with the reductions in branched-chain amino acids observed here and the observations of Biggers *et al* (2000), Ho *et al*, (1996) and Summers *et al*, (2000) that these amino acids can stimulate embryo growth. Alterations in amino acid signalling and decreases in blastocyst cell numbers could possibly be some of the earliest observable PAR's.

This study also measured amino acid levels in maternal serum on day 3.5 of pregnancy and observed a similar pattern in amino acid balance (Section 6.3.7); that is taurine was the most prevalent amino acid and that glutamate, glutamic acid, glycine and alanine also contributed significantly to the serum amino acid composition. However, this is where the similarities end as the overall concentration of uterine fluid amino acids was found to be almost six times more than that of maternal serum (≈30 mM vs. ≈6mM). This large difference in amino acid concentration was due to significant elevations of the amino acids aspartic acid, asparagine, glutamic acid, serine, histidine, glutamine, glycine, threonine, taurine, and alanine, tyrosine, valine, phenylalanine, isoleucine and leucine in uterine luminal fluid (Fig. 4.3.4d). In contrast, the concentrations of arginine and methionine were not significantly altered and trypsin and lysine were significantly reduced in concentration in uterine fluid compared to maternal serum. These differences in amino acid concentration equate to the essential amino acids comprising approximately 17% of total measurable amino acids in uterine luminal fluid but nearly 45% in maternal serum.

This concentration differential of amino acids between maternal serum and uterine luminal fluid suggests that active transport of amino acids is occurring across the uterine epithelium. The branch-chain amino acids were significantly depleted in both maternal uterine fluid and maternal serum and while concentrations were significantly

elevated in uterine fluid the observed elevations were much less than many of the non-essential amino acids. Conversely, amino acid alanine is significantly depleted in maternal serum but not uterine fluid; this is possibly because it is of a much higher concentration in uterine fluid due to greater active transport across the uterine epithelium buffering its concentration. The fact that the branch-chain amino acids are of a more similar concentration in uterine fluid and maternal serum suggests that there is less active transport of these amino acids across the uterine epithelium. This comparatively low rate of active transport is possibly insufficient to buffer branch chain amino acid concentrations in uterine fluid against alterations in circulating amino acid balance due to LPD treatment. Additionally, data from studies by Jozwick *et al* (1999) and Patti *et al* (2000) in pregnant sheep have suggested that transplacental transfer rates of the branched-chain amino acids are directly dependent on their concentrations in the maternal plasma. This study also found that methinine concentration increased in uterine fluid but decreased in maternal serum on day 4.5 of pregnancy. Jozwick *et al* (2001) have demonstrated in sheep that umbilical uptake of methionine can be inhibited by branch chain amino acids. It is possible that the significant depletions in valine, isoleucine and leucine concentration allow for a greater rate of methinine transportation into the uterine luminal fluid. Regardless, in experimental terms, these differences in serum and luminal fluid amino acid cpncentrations suggest, quite reassuringly, that using the direct collection method I are indeed measuring a biological fluid quite distinct from maternal blood.

Despite problems sampleing uterine luminal glucose levels on day 2.5 and 3.5 of pregnancy it was found that glucose levels increased throughout the preimplantation period. Also, glucose levels in uterine luminal fluid were not found to be significantly altered at any time point in this experiment in mice administered the LPD (Section 4.3.5). However, unlike amino acids, the concentration of glucose in uterine luminal fluid on day 4.5 of pregnancy was about half that of maternal serum ($\approx$6 mmol l^{-1} vs. $\approx$12 mmol l^{1}). Furthermore, this study has found serum glucose levels to be significantly elevated (Section 6.2.6) on day 3.5 of pregnancy and this occurs in concert with reduced serum insulin levels. These results are in accord with the work of Kwong *et al* (2000) who also reported that administration of a LPD for the first 4 days of pregnancy caused a

decrease in serum insulin and increase in serum glucose levels. It is possible that this increase in serum glucose is a result of the decrease in serum insulin due to lowered insulin:glucagon ratio. For example, weanling rats fed a LPD for 3 weeks had significantly lowered serum insulin:glucagon ratio compared to controls (Claeyssens *et al* 1992). Interestingly, leucine has been shown to be a stimulator of insulin release (Gao *et al*, 2003; Li *et al*, 2003) and LPD treatment can impair leucine mediated insulin secretion (Reis *et al*, 1997) As already discussed leucine is significantly reduced in the serum of mice treated with a LPD during early pregnancy in this and other studies (Kwong *et al*, 2000; Petrie *et al*, 2002). This study has also shown serum estrogen to be significantly elevated on day 4.5 of pregnancy. Estrogen in combination with progesterone is a suppressor of insulin secretion by β-cells (Sorenson *et al*, 1993). While insulin is not significantly depleted on day 4.5 of pregnancy this result may also help to explain the apparent drop in circulating insulin. Partly because of this I will shortly begin to investigate circulating progesterone levels in response to maternal LPD treatment.

Alternatively the depletion in serum insulin and hence the elevation in serum glucose could be due to poor insulin secretion mediated by altered gene expression of PKCα, PLCβ1 (reviewed by Zawalich and Zawalich, 1996) or voltage-gated K^+ channels (Delghingaro-Augusto *et al*, 2004). PKCα, PLCβ1 (Ferreira *et al*, 2003) and voltage-gated K^+ channel (Delghingaro-Augusto *et al*, 2004) mRNA expression can be lowered in response to LPD administration. It is possible that reduced circulating leucine and or altered gene expression in pancreatic islets may be responsible for the lower insulin levels observed in this study.

This study has also found uterine FGF-II levels to be increased in response to LPD treatment (Sections 5.3.1 and 5.3.2). This rise in uterine FGF-II levels is hypothesised to be due to a response to aid implantation and/or nutrient supply to the embryo. It seems likely that the observed rise in FGF-II levels is a result of increased activity of ACE (Section 5.3.6) in uterine tissue (Fig 5.4c). This increase in ACE activity *in vivo* could cause an increase in the conversion of angiotensin I (ANGI) to ANGII and in turn lead to increased binding of ANGII to the AT 1 receptor then causing,

through intracellular signalling, FGF-II production to be increased (Peifley and Winkles 1998; Peng *et al*, 2001). It would seem that this increase in the activity of ACE is restricted to the local uterine RAS as circulating ACE activity in not increased (Section 6.3.2). It was also hypothesised that any rise in ACE activity would be due to increased protein levels due to increased circulating corticosterone (Fishel *et al*, 1995; Barreto-Chaves *et al*, 2000, Barreto-Chaves *et al*, 2001), however, this study has failed to find any significant increase in circulating glucocorticoid levels (section 6.3.4). As a result, I am currently attempting to investigate glucocorticoid receptor expression in uterine tissue as reduction in the quantity of this protein may have much the same effect as reduced circulating corticosterone. It is also possible that the observed alterations in uterine ACE activity to LPD treatment may have some significance other than attenuation of FGF-II. ACE has been suggested to play a role in rodent decidualisation (Squires and Kennedy 1992) and uterine blood flow (Hagemann *et al*, 1994). Indeed it has been demonstrated that there is a small yet detectable quantity of ACE within uterine tissues (Cushman and Cheung, 1971) which is localised within the uterine blood vessels (Moeller *et al*, 1992).

Expression of, tissue plasminogen activator (PLAT), was found to be reduced in microarrays in response to LPD treatment on day 3.5 and 4.5 of pregnancy (Chapter 7). It was hypothesised that reduced PLAT expression was due to the the increase in uterine ACE activity previously discussed. In this system ACE acts in a biphasic fashion to repress the funtion of PLAT. Firstly, ACE breaks down PLAT's enhancer bradykinin reducing PLAT transcription. Secondy ACE activity is positively correlated with PLAT inhibitor 1 expression, a glycoprotein of the serine protease inhibitor family, which covalently binds to and inhibits PLAT activity. PLAT itself has been localised within the mammalian uterus (Rijken *et al*, 1981; Danglot *et al*, 1986) and been demonstrated to be involved in trophoblast invasion (Sherman *et al*, 1976; Strickland *et al*, 1976; Axelrod, 1985). Reduced PLAT expression and possible increased PLAT inhibition in the uterus could well reduce the ability of the embryo to implant due to impaired trophoblast invasion. This model does however introduce an interesting issue, because on the one hand, increased ACE activity could also be driving higher expression of FGF-

II to aid embryo implantation. But on the other hand, reduces the endometrium's receptivity to embryo implantation by reduced expression and inhibition of PLAT.

Continuing in the theme of the increased uterine vascularity and receptivity question serum VEGF was found to be significanly elevlated on day 4.5 of pregnancy (Section 6.2.7). VEGF has been shown to be a principle factor responsible for increased endometrial vascular permeability up to implantation (Rabbani and Rogers, 2001). VEGF and FGF-II are co-localised in epithelial cells, stromal cells, and blood vessels in the endometrium during the menstrual cycle and early pregnancy of the rhesus monkey (Wei *et al*, 2004). The relationship between co-expression VEGF and FGF-II appears less to be the case of one causing the expression of the other and more the case that expression of these two mitogens is driven by some diverse and some common factors leading to synergistic expression and function during vascularisation. Similar to FGF-II, VEGF expression can be enhanced by ANG-II (Chua *et al*, 1998). Additionaly VEGF expression can be stimulated by estrogen, which has also been shown to be elevated in serum on day 4.5 of pregnancy in relation to treatment with an LPD (Hyder *et al*, 2000) (Section 6.3.5). Interestingly, estrogen has also been shown to promote the expression of FGFR1IIIc in the porcine endometrium (Welter *et al*, 2004) and FGFR1 splice variants have been located in the rodent uterine stroma (Rider *et al*, 1995).

In this study, serum total protein was found to be significantly elevated in response to LPD treatment (Section 6.3.1). Ogawa *et al* (1999) found that liver mRNA for CCAAT/enhancer binding protein α (C/EBP), an albumin promoter, was increased after 1 day of starvation but decreased after 3 days of starvation (Section 6.4). While no microarrays were conducted on uterine tissue on day 2.5 of pregnancy this study has also found that an EST identified to be C/EBP is subject to an aproximately 2-fold decrease in expression of days 3.5 and 4.5 of pregnancy (Fig.7.3d; Table 7.3c). Additionally, this study has also demonstrated that an EST identified as CBP/p300, also involved in albumin expression (Takeaki et al, 2004), has reduced expression in relation to treatment with an LPD on day 4.5 of pregnancy. While this study has not demonstrated a reduction in total protein in the serum in relation to dietary treatment concomitant with decreased C/EBP or CBP/p300 mRNA, it is none the less interesting that our model of

mild dietary peotein restriction has found similar trends to those observed by Ogawa *et al* (1999).

In summary, this study has identified significant alterations not only to direct environment of the peri-implantational embryo, uterine luminal fluid, but also alterations in protein expression within uterine tissue in response to LPD treatment. Specifically, this investigation has highlighted alterations in uterine fluid amino acids, particularly reductions in the concentration of branched chain amino acids and methionine, which may result in altered embryo metabolism via the mTOR system or even altered embryo DNA methylation. Microarray analysis of uterine tissue identified 690 EST's that altered expression with respect to dietary treatment. Several of these EST's further highlighted biological pathways identified by traditional methodologies. Perhaps most interesting with regards to uterine tissue and maternal serum were alterations in levels of FGF-II, ACE, VEGF, and estrogen, which have all been suggested to have a role in uterine vascularisation and previously published literature seems to suggest that their modes of action are linked. These data adds credence to the hypothesis that the alterations observed in uterine tissue may represent maternal responses that compensate for impaired or sub-optimal embryo development and in the short term help to maintain a healthy viable pregnancy. In the long term however it may be that these alterations affect developmental plasticity in such a way that a PAR geared to a specific environment is incorrect and as a result Syndrome X phenotypes become apparent post-reproductive peak.

8.2 Synopsis of project objectives

The primary objective of this study was to characterise changes in both the uterine environment. To examine the contents of uterine luminal fluid two novel uterine fluid methods of collection were developed. This part of the project presented perhaps the largest challenge of the entire investigation due to the minute amounts of fluid found within the uterus during the peri-implantation period. In particular, the *direct collection method* (Section 4.3.4) provided superbly accurate and repeatable measurements of

amino acid concentrations in uterine luminal fluid on days 2.5-4.5. Indeed, it was not only possible to precisely measure amino acid concentrations in the murine uterus for the first time it was also possible to statistically measure differences in individual amino acid concentrations between dietary treatments and between days of pregnancy. Reassuringly the branch chain amino acids were found to be reduced significantly as a percentage of total amino acids both in the *perfusion method* and in the *direct collection method.*

Despite the overwhelming success in measurement of amino acids in uterine luminal fluid measurement of glucose (Section 4.3.5) and insulin (Section 4.3.6) levels proved to be much more challenging. While glucose levels in uterine luminal fluid were relatively simple to assess on day 4.5 of pregnancy, on day 2.5 and 3.5 of pregnancy glucose measurements were below the detection limits of the Cobas Mira; this was probably due to sub optimal dilutions. Nonetheless, it was possible to statistically analyse the data after normalisation. Unfortunately, it was found to be impossible to measure insulin concentrations in uterine luminal fluid due to a lack of sensitivity in the ELISA kit used. Nevertheless, this investigation has made important advances in the understanding of how the uterine environment may effect embryo development.

The more traditional SDS-PAGE methodology provided excellent semi quantative measurements of growth factors within the uterus in relation to dietary treatment (Chapter 5). This was especially true in the case of FGF-II as splice variants of its gene were found to be reduced in the uterine tissue of mice treated with the LPD using both classical chemiluminescence (Section 5.3.1) and contemporary (Section 5.3.2) infra-red technologies. These findings, using 2 distinct methods, not only provide evidence that the observed reduction in FGF-II is indeed real but also help to validate the integrity of the Licor Odyssey infrared protein imaging system. Additionally an extremely cheap, simple and effective method was also used to analyse ACE activity in day 4.5 uterine tissue (Section 5.3.6) and serum (Section 6.3.2).

The results of ELISA's and RIA's of components of maternal serum allowed the characterisation of maternal hormonal and metabolic status and also allowed the investigation of distinct components of common biological pathways (Chapter 6). In

particular, components of the RAS (Section 6.3.2) and linked pathways such as those for estrogen (Section 6.3.5) and VEGF (Section 6.3.7) were simple to charaterise using relatively cheap, straightforward and swift established technologies. Perhaps the most time consuming part of these experiments was the collection and aliquoting of the samples themselves. Indeed, experiments involving ELISA's and RIA's were often a welcome distraction from the analysis of HPLC data as they could be completed in an afternoon rather than over several weeks. In terms of measurement of glucose and amino acids one of the most challenging parts of the investigation was probably establishing sufficient dilutions due to the high sensitivity of the HPLC and Cobas Mira at the University of York.

Finally, microarray analysis of day 3.5 and 4.5 uterine tissue has allowed for a huge amount of information to be collected in a relatively short time span (Chapter 7). Over 260 previously described EST's were found to alter in relation to dietary treatment (Section 7.3). However, the sheer volume of information collected using microarray analysis is both this systems greatest strength and greatest weakness. Not only in this study but in many other investigations hundreds of EST's are found to alter in relation to experimental treatment but only a handful can be effectively described. In this investigation I chose four genes that almost immediately struck me to be relevant to this investigation; however, there are probably many other genes that are in Tables 7.3a-c that are of equal or even greater importance in relation to dietary treatment, embryo environment uterine receptivity and general metabolism, one just needs more time to identify them. Also, one should not forget that identification of genes found to alter in a microarray experiment also require verification using more traditional methodologies.

8.3 Future work

Perhaps one of the most interesting challenges for any investigator wishing to further advance data from this study would be the quantification of the exact quatities of glucose and insulin on days 2.5-4.5 in murine luminal fluid in relation to dietary treatment. For glucose this could be a relatively simple task of reducing the dilution

factors used or even by pooling fluid collected from 2 separate mice uteri. Infact I believe a combination of these approaches could provide accurate measuemts in a short about of time. Indeed the measurements of glucose concentration obtained in this investigation could be used to validate any results gained using a modified methodology. As always, the most time consuming factor would be collection of the samples themselves. For insulin however, measurement of its concentrations in uterine luminal fluid could be a much more daunting task. Indeed from the experiments in this study it is not even clear whether I was indeed close to obtaining a reading for insulin concentration above background levels. The simplest solution to this problem would be to pool more samples of uterine luminal fluid for analysis. However, the major problem here is that it is a quite time consuming task to collect the uterine luminal fluid samples even using the *direct collection method.* While it is possible to do up to about 10 collections in a single day there is no guarantee you will get fluid from every mouse and the logistics of mating a large numbers of mice for such an experiment would be quite overwhelming. Even if one were to pool samples from many mice there is no guarantee that insulin measurements could be accurately measured using the ELISA technology employed in this study. Nonetheless, the potential benefits of such an experiment are obvious. Perhaps a better approach to this problem would be to pool less uterine fluid samples and attempt a different method of measuring insulin. It may be possible to find a more sensitve ELISA system or even perhaps use the relatively new technology of isotope coded affinity tag (ICAT) to assay not only insulin expression but similar to microarray, also expression of large number of other proteins in a single experiment (Smolka *et al*, 2001). Such experimental methodology may allow one to measure FGF-II and VEGF levels in uterine luminal fluid.

This investigation, as already discussed in some detail, has investigated components of the RAS and its affiliated signaling pathways. While I have shown that local uterine ACE activity is significantly elevated on day 4.5 of pregnancy and speculated quite widely about the possible consequences of this, I have not conclusively shown that ANG-II levels are indeed increased. In fact it should be remembered that the ACE activity assay used in this investigation does not measure conversion of ANG-I to ANG-II. The obvious solution here is to actually measure not only ANG-II protein

levels in relation to dietary treatment in uterine tissue but also to measure the levels of ACE protein to discover whether the observed increase hip-his-leu breakdown is due to greater ACE activity or greater amounts of ACE protein.

Other proteins found to alter in relation to dietary treatment related to RAS signalling included FGF-II and VEGF. This investigation hypothesised that any increases in the observed expression of FGF-II and VEGF were probably due to common factors acting on their enhancers. Indeed it should be interesting to further dissect these signaling pathways by investigating expression of more of their intermediate signaling molecules. One such molecule is SP1 as its expression precedes the increased synthesis of FGF-II and VEGF during the healing of duodenal ulcers (Szabo *et al*, 2000).

This study has widely hypothesised that many of the observed changes in growth factors observed in the peri-implantation uterus in relation to dietary treatment are to increase vascularisation and aid embryo implantation. It would be informative to design an experiment that would measure end markers of uterine vascularisation in relation to diet; an example of such an experiment would be measurement of numbers of blood vessels in the endometrium of uterine sections.

While not central to this investigation it was interesting to find that serum total protein levels were transiently elevated in response to LPD treatment on day 2.5 of pregnancy. It was hypothesised that this increase in serum total protein was due to an increase in serum albumin levels. It would be relatively simple for an ELISA kit to measure serum albumin levels in respose to dietary treatment and perhaps provide a definitive answer as to why maternal serums total protein levels increase on day 2.5 of pregnancy.

In Chapter 3 it was found that mice treated with the 9% LPD actually consumed significantly more food than control mice. These data led to the hypothesis that the reduced protein levels of the LPD were actually causing an increase in levels of neuro peptide Y (NPY) gene expression in the basomedial hypothalamus. As previously discussed NPY is a potent mediator of appetite. It may be of some interest to measure

NPY levels in the hypothalami of mice treated with the LPD and NPD to investigate if NPY is indeed increasing appetite in mice treated with the LPD.

In terms of the microarray investigation and as discussed in Chapter 7, microarrays are primarily an exploitory hypohesis generating tool and their results require validation using either RT-PCR or northern blotting. The four EST's I have described in detail in Chapter 7 that significantly alter in relation to dietary treatment would definitely require validation before publication. However, it would be prudent to further define many more of the EST's functions that were found to alter in relation to dietary treatment so that hypothesis can be generated as to their possible functioning in metabolic programming, generation of PAR's or in general metabolism. Of course any more EST's that are defined would also require validation.

While all of the possible experiments discussed above would provide valuble information to complment that found in this investigation perhaps the most interesting future experiments would involve exposing embryos to the altered uterine environments dissussed in this investigation. Most obviously, embryos could be cultured in media containing reduced quatities of branch chain amino acids to assess not only traditional markers such as cell number but also other factors such as components of the mTOR system or embryonic amino acid turnover/concentrations. It may even be possible to conduct a microarray analysis to determine gene expression levels in embryos cultured in such media. However it may be interesting to measure such markers in relation to culture media with varying concentrations of growth factors like FGF-II or VEGF. Not only could one measure pre-implantation markers that may control early PAR's but also one could measure traditional markers of blood pressure, glucose tolerance or even behavioural aspects post partuition in offspring from transferred embryos. These experiments would allow a better understanding of how maternal environment in relation to diet may control metabolic programming.

In totality, while this investigation provides many answers to the questions posed in Section 1.8 it also raises many more and opens up new avenues of investigation for cohorts of undergraduate project students, PhD students and post docs. I now pass the

torch and leave it with you, the reader, to design the next generation of experiments for investigating *the maternal factors influencing metabolic programming of the blastocyst within the peri-implatation uterine environment.*

Now go forth and find the funding!!

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