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The Effects of Maternal Overnutrition on the Developing Embryo

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ABSTRACT

The Effects of Maternal overnutrition on preimplantation embryo development

By Francesca R Lock

The first 4 days of the mouse pregnancy is the preimplantation period. Cells multiply and the first differentiation occurs, establishing distinct stem cell populations; the trophectoderm (TE) which gives rise to the placenta and the inner cell mass (ICM) which gives rise to the embryo proper and yolk sac. Adaptation has been shown to occur at this early stage in response to diet and shows prolonged effects on offspring health (Wakins et al, 2008).

Mice were fed a high fat diet (HF) or high protein (HPD) diet during the preimplantation period to determine if adaptation at this stage was specific in its response to poor diet or whether default adaptation occurred to non-optimal diet. It was found that adaptation was in fact specific to dietary challenge. Maternal causes for these adaptations were investigated, with uterine growth factor expression being found to be altered.

HF embryos were further investigated with pregnancies maintained to a few days prior to birth (E17.5), with HF diet being fed throughout gestation or only for the preimplantation period (emb-HF) and compared to control fed mice. It was found that both the HF and emb-HF fetuses were proportionally larger than their placenta compared to controls, reflecting the proportions of TE and ICM cells observed in the preimplantation embryo. Bone development and glucose tolerance were also affected in these fetuses.

These results suggest that a HF environment experienced during the preimplantation period programmes the growth trajectory of the fetus and placenta. These adaptations may occur in order to establish optimal nutrient supply during gestation in anticipation of nutrient availability. Inappropriate adaptation has been linked with later life diseases such as diabetes and hypertension.

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Contents

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Abbreviations:

4eBP1 – 4 eukaryotic binding protein 1

AHA - L-Azidohomoalanine

AKT – protein kinase B

ART – assisted reproductive technologies

BMI – body mass index

CDX2 - Caudaul related homeobox 2

CVD – cardiovascular disease

DAPI - 4',6-Diamidino-2-phenylindole dihydrochloride

DEPTOR - DEP-domain-containing mTOR-interacting protein

DNA - Deoxyribonucleic acid

DNMT - DNA methyltransferase

dNTP - Deoxyribonucleotide

DOHaD – developmental origins of health and disease

DPC – days post coitum

E4 – embryonic day 4

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

eIF4E - eukaryotic initiation factor 4E

Emb- HF – embryo high fat diet

Emb-HPD – embryo high protein diet

Emb-LPD – embryo low protein diet

EPC – ectoplacental cone

ER – oestrogen receptor

ERK - Extracellular signal-regulated kinases

ExE - extra-embryonic ectoderm

FGF – fibroblast growth factor

FOAD – fetal origins of disease

FSH – follicle stimulating hormone

GLUT - Glucose transporter

GTP - Guanosine-5'-triphosphate

HB-EGF - heparin-binding EGF-like growth factor

HF – high fat diet

HGF - Hepatocyte growth factor

HPD – high protein diet

ICM – inner cell mass

IGF- insulin like growth factor

IRS1 - Insulin receptor substrate 1

IUGR – intrauterine growth retardation

IVF – in vitro fertilisation

LH – leutenizing hormone

LIF – leukemia inhibitory factor

LPD – low protein diet

mLST8 - mammalian lethal with Sec13 protein 8

mSIN1 - mammalian stress-activated protein kinase interacting protein

mTOR – mammalian target of rapamycin

mTORC – mTOR complex

NPD – normal protein diet

OCT – optimal cutting temperature

OCT4 - octomer binding protein 4

PAR – predictive adaptive response

PCR – polymerase chain reaction

PE – primitive endoderm

PI – propridium iodide

PKC – protein kinase C

PPAR - Peroxisome proliferator-activated receptor

PRAS40 - proline-rich AKT substrate 40 kDa

PROTOR-1 - protein observed with Rictor-1

RAPTOR - regulatory-associated protein of mTOR

RICTOR - rapamycin-insensitive companion of mTOR

RNA - Ribonucleic acid

S6K1 – ribosomal protein S6 kinase 1

SDS PAGE – Sodium dodecyl sulphate Polyacrylamide gel electrophoresis

SEM – standard error of the mean

SLC – solute carrier

SOX2 - SRY (sex determining region Y)-box 2

TAMRA – Tetramethylrhodamine

TE – Trophectoderm

TEAD4 - TEA domain family member 4

TNBS - Tetranitrobenzene sulphonic acid

VEGF - Vascular endothelial growth factor

ZGA - Zygotic Genome Activation

DECLARATION OF AUTHORSHIP

I, Francesca Rachel Lock

declare that the thesis entitled

The effects of maternal overnutrition on preimplantation embryo development

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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

Introduction

1.1 The Developmental Origins of Health and Disease Hypothesis

In Britain, the most common adult diseases include cardiovascular disease, obesity and type II diabetes (Hawe et al, 2011). These diseases contribute largely to what is termed metabolic syndrome. Poor diet and lack of exercise are largely credited with the generation of these diseases. It has however also been shown that the origin of these diseases could partially rest in the developmental environment (Bateson, 2001) as outlined by the developmental origins of health and disease (DOHaD) hypothesis. Diet and exercise remain the cornerstone of a healthy lifestyle, however, it appears that an individual's risk of developing cardiovascular disease, diabetes, or becoming obese is variable. The DOHaD hypothesis proposes that the environment faced during gestation and early life could programme the risk of developing these diseases.

The DOHaD hypothesis began in the field of epidemiology. Studies by Barker et al. (1989) initially demonstrated links between development and long term health by associating weight in infancy to later life cardiovascular health. Detailed records kept by midwives in Hertfordshire were used in retrospective cohort studies. These studies showed that men born between 1911 and 1930 weighing less than 8.1 kg at one year old had a three-fold increased risk of death from coronary heart disease (Barker et al, 1989a). Barker also used data from a 1970's British birth survey to demonstrate an inverse relationship between birth weight and systolic blood pressure (1989b).

These lower weight babies from the Hertfordshire studies were also more vulnerable to developing type II diabetes. This is an observation that is also evident amongst twins, with the prevalence of diabetes being greatly increased in the lower birth weight sibling (Poulston et al. 1997). Twins share the same maternal environment, and identical twins even share the same genetic makeup. The inter-twin variation arises largely via the placenta, as there is invariably some inequality in the supply of nutrients to each twin.

It has also been shown that babies that are of high birth weight have an increased prevalence of later life disease, and that the relationship between weight and disease risk is actually U shaped (Huang et al, 2007). Birth weight is a parameter that many of these early studies utilised, and it can be highly indicative of later life metabolic disease but it should be remembered that genetic factors affecting size as well as gestational age make this a variable parameter. Primarily, it serves as an identifying feature within a cohort that growth has been altered by the in utero environment, and as such disease risk is higher.

The DOHaD hypothesis has had several guises, being known as the Barker Hypothesis and the Fetal Origins of Adult Disease (FOAD), it was also not the first to link offspring health to maternal diet (i.e. the fuel mediated teratogenesis hypothesis: Freinkel 1980). It was also originally met with some scepticism (Joseph & Kramer, 1996). The DOHaD hypothesis did however link maternal status to later life disease and since the original contention, the

hypothesis has been extensively demonstrated experimentally and adapted based upon findings. Programming changes are now termed predictive adaptive responses (PARs). The term PAR encompasses the idea that changes in physiology are made to best prepare offspring for the environment in which it will be existing (Gluckman et al, 2005).

Figure 1.1 PAR in butterflies.

An example of PAR, wet-season (left) and dry-season (right) *Precis octavia* butterflies differ wildly in colourings. Picture courtesy of insectnet.com

There are several examples of animals that show differing characteristics depending upon the time of year in which they are born. The *Precis octavia* butterfly is a brown/reddish colour when born in the wet season, or a bright blue when born is the dry season (figure 1.1). It is suggested that this colour change provides a camouflage advantage as well as thermoregulatory advantages (Brakefield & Larsen, 1984). This seasonal adaptation demonstrates that expression changes can be caused developmentally to affect the phenotype of the offspring. This adaptability may have provided evolutionary advantage to those that possessed this capability.

This adaptation may however not always be advantageous. Adult disease can be seen as a result of embryonic environment even in controlled experimental conditions. There is some suggestion that disease will particularly occur in instances of mismatched developmental and postnatal environments. It is found that low birth weight offspring that show juvenile catch up growth have an increased likelihood of developing later life disease (Johnson et al, 2012). This developmental predisposition for later life disease would unfortunately not be an influence on evolutionary fitness as their effects are not seen until post reproductive age.

Pike et al (2007) suggest four broad mechanisms acting in the offspring to cause these disease states; epigenetic process, mitochondrial function, changes in the development of specific organs or tissues and effects on homeostatic control systems.

The term epigenetics refers to heritable modifications to gene function that do not involve changes in DNA sequence. These modifications include deacetylation of histones to increase gene expression and methylation of genes to suppress expression (Jacob & Moley, 2005). Epigenetic modifications can be transient as seen in the regulation of the cell cycle, they can also be permanent, and heritable from mother to daughter cell (Allegrucci et al, 2005).

Epigenetic reprogramming occurs during the preimplantation stage of development and epigenetic patterns may be particularly influenced during this time (see section 1.3). A key influence on methylation is the availability of methyl donors such as folate, largely provided by the diet. In the human population, folic acid consumption is encouraged primarily as a preventative of neural tube defects (Honein et al, 2001). It is found that children born of mothers that consumed recommended doses of folic acid in pregnancy had greater methylation of the insulin like growth factor 2 (IGF2) gene, as well as methylation being inversely related to birth weight (Steegers-Theunissen et al, 2009).

Mitochondria are central to metabolic processes and it is thought that mitochondrial copy number and performance may be affected by environment. These changes may also be led by dietary modification, with maternal high fat diet leading to mitochondrial abnormalities (Taylor et al, 2005). Mitochondria are inherited maternally and provide a mechanism for transgenerational transmission.

Organ size and structure may be altered to spare essential organs from detrimental outcomes or to adapt the offspring to its predicted environment. Nephron number may be reduced to decrease postnatal nutrient demands, or adipose tissue may be increased to provide an adequate buffer in situations of nutritional scarcity (Gluckman & Hanson, 2006; Kuzawa 1998). This may not

be of benefit to the organism, for example a small heart is associated with chronic fatigue (Miwa & Fujita 2008).

Homeostatic control may contribute to the way in which for example diet is responded to in offspring. It is suggested that developmental environment can alter homeostatic set points affecting nutrient handling. Cortisol levels, for example, are found to be higher in babies of low birth weight, along with reduced glucose tolerance (Levitt et al, 2000; Phillips et al, 2000).

The *in utero* environment can be affected by several external influences, but the most common influencing factor for mammals is the maternal diet.

1.2 Maternal Diet

Diet during pregnancy must be adapted to provide adequate resources for the growth of the fetus, placenta and associated maternal tissues. The suitability of maternal environment can affect the development of the embryo and placenta, and impact upon offspring later life health as previously mentioned. Maternal diet is a widely variable parameter, many models of which exist. Simplistically, maternal diet can be in excess or deficit, or a specific nutrient may be altered. The timing and duration by which this insult occurs is also of relevance.

1.2.1 Caloric Challenge

It is suggested that babies subjected to undernutrition during gestation develop what is termed a 'thrifty phenotype'. The thrifty phenotype baby is metabolically programmed for a life without an abundance of resources, or has adaptations that will aid its immediate survival. This may be an evolutionary mechanism to best aid survival of offspring (Gluckman et al. 2007), allowing their metabolic profile to make best use of available food. When the thrifty phenotype baby grows up in an affluent environment, their metabolic programme is inappropriate and they are more likely to develop disease.

An example of the gestational undernutrition in the human population occurred during the Second World War. The Dutch Winter Famine of 1944 saw the development of many pregnancies with maternal undernutrition. During this period women were receiving less than 1,000 kcal per day for six months of pregnancy. Those exposed to the famine during the first months of pregnancy gave birth to babies of significantly lower weight, who then developed in a less restricted environment. These individuals showed reduced glucose tolerance and a higher risk of cardiovascular disease (CVD) (Ravelli et al. 1998 & 1999; Roseboom 2000).

Another famine occurred during this war, during the Siege of Leningrad. This siege resulted in the blockade of food to the city for 870 days, giving citizens a ration of bread equating to around 300 kcal per day (Pavlov 1965). Babies

from this time were born at a lower birth weight, but in contrast to findings from the Dutch Winter Famine, offspring did not exhibit a propensity for later life diseases (Stanner and Yudkin, 2001). There are major differences between this cohort and those affected by the Dutch Winter Famine. The length of exposure to malnourishment in Lenigrad was nearly two and a half years, in a population that was already somewhat undernourished. This population was not subjected to the same switches in diet; undernourishment was commonplace both developmentally and postnatally. These findings reveal that the thrifty phenotype is largely a problem for offspring health when the maternal and fetal lifestyles are mismatched.

Maternal overnutrition is also known to have an impact upon offspring health. Women are recommended to consume an additional 200 kcal per day when first pregnant. In addition to women being likely to consume more food than is required, data show that 34.1 % of normal BMI women gain weight in excess of Institute of Medicine (IOM) recommendations (Wells et al. 2006, see table 1.2). This excessive gain in weight indicates that overnutrition is likely to be a relevant problem in today's pregnancies.

Pre-pregnancy BMI	Optimal weight gain (lbs)	% women gain more	% women gain less
Low (<19.8)	28 – 40		36.2 %
Normal (19.8 to 26.0)	25 – 35	34.1 %	24.1 %
High (>26.0 to 29.0)	15 – 25	61.8 %	18 %
Obese (>29.0)	>15	73.3 %	

Figure 1.2 Weight gain during human pregnancies.

IOM recommended weight gain for the duration of singleton pregnancies, for mothers of differing starting body mass index (BMI) (Olsen et al. 2008), and the percentages of those gaining outside of these limits. Information on the percentage women gaining more than recommended limits is based on a sample in Colorado, USA, studied by Wells et al. (2006) (Column 2). Data regarding inadequate pregnancy weight gain refer to a sample population in Missouri studied by Keil et al. (2007) (Column 3). A high incidence of overnutrition during pregnancy in the human population is seen.

This weight gain is a risk factor for both mother and child. Women who gain more weight during pregnancy are more likely to maintain this additional weight a decade on (Rooney & Schauberger, 2002). This will have ramifications for maternal health as well as any subsequent pregnancies. Obesity in itself is a rapidly growing concern for public health. In 1993, 13.2 and 16.2 % of men and women respectively were overweight. By 2005, these figures had risen to 23.1 % and 24.8 % respectively (NHS, 2006). These figures imply that maternal obesity and again presumably overnutrition is a challenge faced by many pregnancies today. Children born of these overweight mothers are shown to have reduced cognitive function (Casas et

al, 2013), show an increased number of cardiovascular risk factors (Cooper et al, 2013) and be overweight themselves (Kitsantas et al 2010).

Overnutrition and obesity are intrinsically linked but are however different conditions and their separate effects are especially hard to determine in the human population. To address the question of the effects of maternal overnutrition, animals provide a useful and more precise model. Bayol et al. (2005 & 2008) demonstrate the effects of a maternal high calorie, junk food diet during gestation in the rat. This diet model includes a range of palatable food items often consumed by humans and is also high in fat, sugar and salt. Offspring showed increased adiposity, hyperglycemia and hyperinsulinemia as well as impaired muscle development. These effects were exacerbated in females. These results somewhat mirror those seen from maternal undernutrition as discussed, suggesting similar mechanisms can be triggered by different dietary challenges. One of the key questions considered in the current study is of whether the same effects are produced by different overnutrition diets.

Offspring from an undernourished maternal environment that maintained an undernourished lifestyle showed no increase in susceptibility to adult disease (Jimenez-Chillaron et al 2006). Animals exposed to an overnutrition maternal environment, however, show an exacerbated obesogenic response to postnatal overnutrition (Bayol et al. 2007; Khan et al. 2004; Khan et al. 2003; Taylor et al. 2005).

When studying overnutrition in the human population, it is often in conjunction with the confounding effects of obesity. Obese women have been shown to give birth to heavier offspring (Surkan et al. 2004, Ogden et al. 2006). Conversely, obese women can also give birth to lighter babies, under conditions termed intrauterine growth restriction (IUGR). In the case of IUGR, it is believed that placental function is reduced throughout gestation to decrease fetal nutrition (Pardi et al. 2002). Placental function will be further discussed in section 1.6.

As mentioned, even with the use of carefully controlled diets in animal models, it must be considered that a change in caloric value is often achieved by the addition or subtraction of specific nutrients which has the potential to cause these effects rather than simply excess caloric intake.

1.2.2 Specific Macronutrient Challenge

Macronutrients; protein, carbohydrate and fat, are metabolized in differing ways and cause differing metabolic profiles. When food is consumed, it is broken down into its constituent molecules. Carbohydrates are broken down to glucose molecules, proteins to their amino acid constituents, and lipids to fatty acids and glycerol. Once these digested components are absorbed through the intestinal wall, they join the circulation through small capillaries. The consumption of these macronutrients affects the release of several key metabolic regulatory molecules including amongst others insulin

and leptin. These molecules have the potential to alter the uterine environment.

Effects in line with the DOHaD paradigm are seen from specific macronutrient challenges. Protein is a macronutrient that has been most thoroughly investigated for its impact upon offspring health. Increased birth weight, chronic hypertension and aberrant anxiety-related behaviour, to which gender alters susceptibility, are all seen in response to low protein diets in rodent models (Langley Evans et al. 1996, Sayer et al. 2001, Kwong et al. 2000, Watkins et al. 2008).

Protein overnutrition also has an effect on offspring. Daenzer et al. (2001) showed that a high protein diet (40% compared to control of 20% protein) in the rat led to offspring being born of low weight, which underwent a catch up weight gain during suckling. This led to an adult phenotype with increased adiposity, similarly to overnutrition phenotypes (Bayol et al. 2008), and reduced energy expenditure.

Dietary fats are normally consumed in the form of a triglyceride. Triglycerides consist of three fatty acid molecules attached to a glycerol backbone. These fats can have differing levels of saturation, referring to the number of double bonds. Polyunsaturated fats can be further subdivided in to omega-3 and omega-6 polyunsaturates, depending on the position of the first double bond. All fatty acids contain a fatty chain and an acid group. Diets that are high in fat content have also been shown to induce detrimental

changes in the offspring, including increased incidence of obesity, leptin and insulin resistance, poor muscle development and non-alcoholic fatty liver disease (NAFLD) (Bayol et al. 2008, Bruce et al. 2009, Howie et al. 2009).

Carbohydrates in pregnancy are most commonly studied in terms of glucose levels. High sugar levels stimulate the production of insulin by the pancreas leading to the take up and storage of glucose in the form of glycogen. Dis-regulation of homeostatic control of glucose level through insulin is known as diabetes and can occur through a lack of insulin production or by a lack of cellular responsiveness to insulin (types 1 and 2 diabetes mellitus respectively). Gestational diabetes can also occur. The condition is sometimes resolved upon delivery but can also persist in the mother (Alberti & Zimmet, 1998).

Diabetes is not only known to be a feature of poor adult health but is also a major concern in pregnancy. Changes in the ratio between circulating glucose and insulin can be indicative of a developing diabetic phenotype (Tominaga et al, 1999). Raised maternal glucose is associated with elevated weight in children at age three (Deierlein et al, 2010). Obesity, glucose tolerance and hypertension were all observed in murine offspring exposed to high sugar levels (Samuelsson et al, 2013). Gestational diabetes can also affect the development of the fetus in terms of both greater risk of obesity and abnormal glucose metabolism (Metzger, 2007).

With many similar outcomes observed from varying dietary challenges (high/low birth weight, diabetes, CVD, obesity), it remains unclear if offspring are affected by differing challenges via the same pathways. Animals sense specific challenges, or they may respond similarly to different dietary challenges as 'non-optimal'. It seems necessary to further elucidate the nature of these adaptations and their causal mechanisms. The time at which the maternal challenge occurs may also play a role in the way the animal adapts.

1.2.3 The influence of developmental window in dietary challenges

A range of experimental models of maternal dietary challenge have shown that the time of exposure can prove critical for the development of a phenotype. For example, Ellis-Hutchings et al. (2010) fed a 50 % maternal undernutrition diet to Sprague Dawley rats either from day 1 to 15 of gestation, or from day 10 to 21 of gestation. Both undernutrition diets resulted in reduced offspring weight. The study found that undernutrition in the latter half of pregnancy, but not in early pregnancy, resulted in reduced nephron endowment. The time of exposure and the development of a phenotype can depend upon which systems are developing at that time and are therefore most sensitive (Figure 1.3).

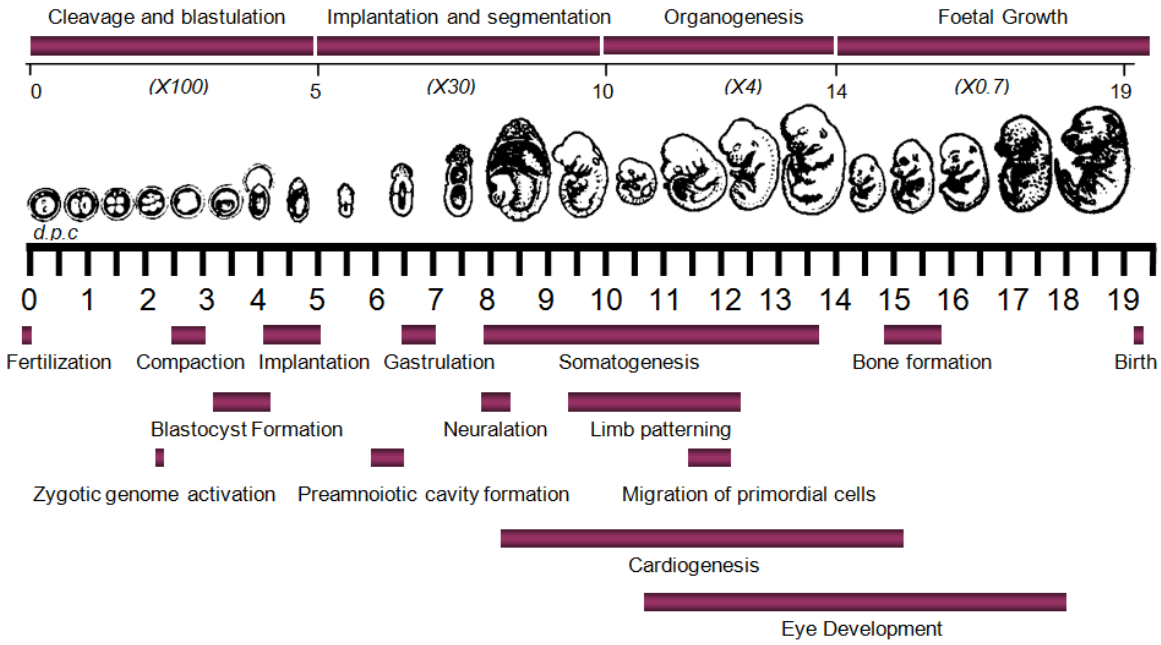


Figure 1.3 Schematic of mouse development

Diagram showing critical periods during mouse development where specific events of development may be disrupted by deleterious environment. Images and information taken from Nagy et al. (2003).

The Dutch Winter Famine of 1944, as mentioned previously, subjected mothers to a short period of undernutrition. Differences in offspring could be observed from birth, with relation to the time of nutritional challenge. Birth weight was increased in those exposed during early gestation and decreased in those exposed in mid-late gestation, demonstrating the importance of the timing of nutritional challenge. Follow up studies of babies born to the Dutch winter famine showed an impact on adult health also differed with the time of exposure. It revealed that babies exposed during early gestation to maternal undernutrition had the most striking later life effects, with a three-fold increase in coronary heart disease (Painter et al. 2005). At age 50, increased

prevalence of obesity, coupled with raised lipid levels, altered clotting and type II diabetes was observed (Painter et al. 2005).

Low birth weight is also a feature of offspring born of assisted reproductive technologies (ART). Studies have found that *in vitro* culture of the early embryo is causative of this effect in both human and animal models (Fernández-Gonzalez et al. 2004, Watkins et al. 2007, Dumoulin et al. 2010). This culture takes place solely in the preimplantation stages of development, and is sufficiently influential as to cause differences in health outcome to the offspring in later life. Lower birth weight was followed by catch up growth in infancy and led to increased peripheral body fat and elevated blood pressure at 8-18 years old in children born of IVF compared to spontaneously conceived to sub fertile parents (Ceelen et al. 2007; Ceelen et al. 2009). It was even found that commercial medias available for human IVF had differing influence on the embryo, with gestational age being advanced when embryos were cultured in Vitrolife media compared to those cultured in Cook media. Embryos cultured in Vitrolife also showed increased head circumference and trans-cerebellar diameter (Neilssen et al, 2013).

Studies have also shown that these adverse conditions can be created *in vivo* by poor maternal diet. In both the mouse and rat model, it has been found that maternal diet change exclusively in the preimplantation period can also cause changes in the adult offspring (Kwong et al. 2000; Watkins et al. 2008). Watkins et al. (2008) showed that maternal low protein diet limited to the

period between conception and implantation (referred to as the embryonic low protein diet; emb-LPD) (Figure 1.4) produced heavier offspring at birth and had effects on behaviour, metabolism and cardiovascular disease in the adult animal. These results are similar to those seen when LPD was fed throughout gestation, indicating that the preimplantation period is critical in the development of PARs.

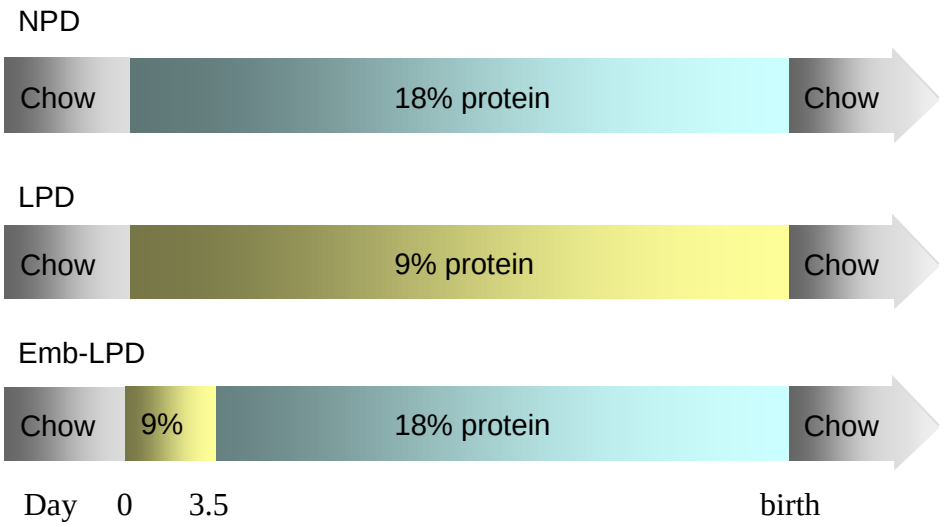


Figure 1.4 NPD and LPD model feeding during gestation.

Feeding regime of normal protein diet (NPD), low protein diet (LPD) and embryonic low protein diet (emb-LPD). Mice were fed either a normal diet throughout gestation of 18% casein, a low protein diet throughout gestation of 9% casein, or were fed the low protein diet simply during the preimplantation period, before continuing with the normal protein diet for the remainder of pregnancy. emb-LPD offspring showed a very similar phenotype to those fed LPD throughout gestation (Watkins et al. 2008).

This result can be replicated in other animal models under differing dietary stress. Maternal undernutrition in sheep during the peri-conception period resulted in offspring with altered vascular function (Torrens et al, 2009).

Maternal protein overnutrition around the time of conception is also associated with poor developmental viability in sheep. An increased birth weight, suggestive of later life disease, is observed under high maternal protein challenge (McEvoy et al. 1997).

It may be suggested that the above experiments targeting nutritional challenge to the periconceptional period do not account for any confounding persistent change in maternal environment resulting from this brief dietary challenge. Differences between offspring due to this short term maternal protein restriction can be reproduced in embryo transfer experiments. Watkins et al. (2008) showed a difference in conceptus weight in late gestation when embryos from LPD and NPD mothers were transferred to the same NPD foster mother at 4 days post coitum (dpc). These results confirm the importance of maternal diet during the preimplantation period for the development of DOHaD effects. Even when the later environment experienced is identical, a difference due to short term diet exposure in the first 4 days of gestation can be observed, and thus enduring differences were induced in the preimplantation period.

In the human, it is unethical to carry out controlled studies altering diet; it is therefore challenging to collect specific data regarding exposure at a specific window of pregnancy. However, it has been shown that maternal characteristics and diet can influence fertility and the embryo at this early stage. Although patients undergoing assisted reproductive technologies (ART)

treatment show sub optimal fertility, this group of patients represent an opportunity to evaluate embryo quality from differing environments. Implantation rates were poorer in obese women compared to those of normal weight. Pregnancy rate and live birth were also decreased by obesity. It was found that each unit of BMI increase worsened these parameters (Bellver et al, 2010). It is assumed that both diet and maternal body condition could contribute to this negative outcome.

It is again hard to decipher the roles of body condition and diet separately in these populations. The importance of a normal diet can somewhat be seen in fertility rates of obese patients. Crosignani et al. (2003) showed that fertility and pregnancy rate were vastly improved in overweight patients that followed a 1200 kcal/day diet and lost around 5% body weight. In a separate study (Tsagareli et al. 2006), patients lost a similar percentage of body weight by following very low caloric intake regime (<500 kcal/day). Patients consuming this severely under-nourishing diet did not see any benefit to their fertility rate, indicating that weight loss alone is not beneficial to fertility but must be in conjunction with good diet.

In animal populations, a short term high energy diet is often given immediately prior to estrus to produce an increased ovulation rate. This is known as a 'flushing' effect, and is shown to also affect reproductive outcomes, with a decrease in embryo survival rate when tested in mice (Pomp & Eisen, 1991). DOHaD-type effects can also be observed in offspring from

maternally challenged environments during this preconception time period. For example, feeding of a low protein diet during oocyte maturation, for 3.5 days prior to ovulation and mating, predisposes offspring to hypertension and abnormal anxiety behaviour (Watkins et al. 2008a &b).

This project will address the effects of diet in the preimplantation period. The preimplantation embryo is highly amenable to developmental plasticity. During this time period, stem cell populations for the entire organism and the placenta are developing and thus have the potential to have a continuing effect on the entire animal. As such, this time period may prove to have the greatest potential for influence on the developing offspring.

1.3 The Preimplantation Embryo

The preimplantation period extends from fertilisation to the time of implantation, which in the mouse takes place over four days (Figure 1.5). This period of development is very similar to that in the human which is almost indistinguishable in appearance and takes place over 5-6 days.

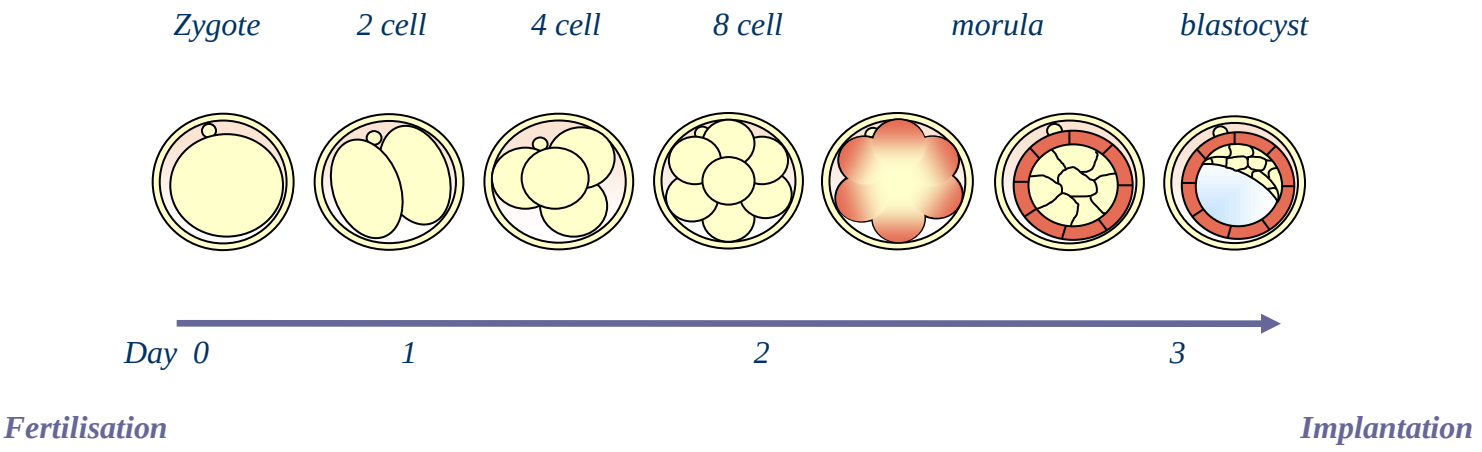


Figure 1.5 Preimplantation development from zygote to blastocyst.

The single cell zygote undergoes several rounds of divisions. At the 8 cell stage, cells begin to polarise. Subsequent divisions create inner and outer cells in the morula. As a cavity called the blastocoele forms, outer cells differentiate to become the trophectoderm whilst the remaining inner cell mass retains pluripotency.

Oogenesis is an extended process beginning during gestation with millions of primary oocytes being developed by the third trimester. By the time of birth, a large atresia event has occurred and less than half of these primary oocytes are present. From birth, the oocyte is in an arrested state in the first prophase of meiosis and will remain so from around 12–50 years in humans. Prior to fertilisation, the oocyte is transcriptionally active with maternal transcripts. Gonadotropin stimulation leads to an increased in the proportion of transcriptionally inactive oocytes and will even develop a stockpile of mRNA and proteins (De La Fuente & Eppig, 2001). Some of these transcrips are specific for the oocyte, for example those utilised for production of the zona pellucida for which transcripts are undetectable just prior to ovulation. Other proteins and transcripts are produced in preparedness for fertilisation.

By the time fertilisation occurs transcription has ceased in the oocyte. Transcripts that are not ready for translation have shorter poly A tails. Adenylation must occur to allow this transcripts to function, as seen by an up to two fold increase in adenylation. This process initiated at uridine-rich sequences called cytoplasmic polyadenylation elements (CPE) which are phosphorylated during development to allow binding of the transcription factor CPE binding protein (CPEB) (Lin, 2010).

The sperm at the point of fertilisation is also at meiosis I and is transcriptionally inactive. Unlike the oocyte, it's contribution to the embryo is predominantly limited to DNA contribution. Fertilization triggers the completion of the II meiotic division of the oocyte.

Fertilisation of the mouse oocyte occurs in the oviduct, from where the embryo will travel to the uterus and implant to establish the placenta that will support it throughout pregnancy (for further discussion of this environment see section 1.4.2) . The newly formed zygote will begin to divide at around 16 – 20 hours post-fertilisation. Cleavage after the 2 cell stage will continue at approximately 12 hour intervals (Marikawa & Alarcón 2009). The individual cells are referred to as blastomeres. Global epigenetic reprogramming occurs following fertilisation.

As mentioned epigenetics refers elements that change the activation of a gene but do not modify DNA. This can include methylation, acetylations, histone

modifications and imprinting. Histones are proteins with which DNA is wound up in, changes to the histone can change the accessability of the DNA. Acetylations are an example of 'active' epigenetic changes. Acetylation removes charges and loosens the histone, making DNA more accessable to transcription factors. The addition of methyl groups to the DNA tends to reduce the transcription of those sites. It occurs mostly at CpG sites, converting cytosine to 5-methylcytosine, which will bind with guanine. Methylated sites tend to attarct DNA methyltransferases (DNMT), which will maintain and encourage methylations. Both histone modifications and methylations can be heritable and are referred to as imprinted genes.

Reprogramming in the embryo occurs by active demethylation of first the paternal and then passive demethylation of the maternal alleles. DNMT1 is usually responsible for the maintenance of methylation; during this demethylation it is restricted to the cytoplasm (Carlson et al., 1992 and Cardoso and Leonhardt, 1999). DNMT1 contains a CPE and a Musashi binding element (MBE), with CPEB1 providing translational control by elongation and shortening of the poly(A) tail in the cytoplasm (Rutledge et al, 2014).

During the initial cell divisions, activation of transcriptional activity by the zygote occurs (Zygotic Genome Activation: ZGA). Before ZGA, the zygote utilises oviductal fluid and transcripts that were produced and stored in the oocyte for cell maintenance and development. The time of embryonic genome activation differs between species; the murine genome is activated at the 2

cell stage whilst in human embryos this occurs in the 4-8 cell stage (Clegg and Piko 1982; Flach et al. 1982; Braude et al. 1988). Cells at this stage are totipotent and have the potential to express any gene. In fact, in the hypomethylated embryo around 10,000 genes are expressed, around half of which are not detected in any other mouse tissue (Ko et al, 2000). Remethylation occurs in the blastocyst led by *de novo* methyltransferases DNMT3a and DNMT3b (Okano et al., 1999). Epigenetic modifications confine gene expression to a lineage and tissue specific pattern. As these modifications have the potential to alter gene expression, and thus phenotype, epigenetic change is largely suggested as an origin of developmentally originating disease (Allegrucci et al. 2005).

The first morphological differences between blastomeres can be observed around the 8 cell stage with the compaction and polarisation of cells. Cells flatten against each other and both cell-cell adhesion and differentiation begin (Fleming & Johnson 1988; Fleming 1992). These adhesions are E-cadherin dependent, which provides orientation for cells to polarise. When these polarised cells divide, they may do so conservatively to give rise to two outer cells, or they can divide differentially to result in an inner and outer daughter cell (Johnson & Ziomek 1981).

Once at least some cells in the embryo have undergone 5 rounds of division, the embryo begins to cavitate. This involves the production of a fluid filled cavity is known as the blastcoele. Basolateral distribution of Na/K^{-}ATPase

allows a sodium ion gradient to form across the outer layer of cells, allowing cavitation to occur by osmosis (Aziz & Alexandre 1991). The outer cells of the blastocyst (represented in red in Figure 1.5) are known as the trophectoderm (TE), whilst the inner cells (represented in Figure 1.5 in yellow) are known as the inner cell mass (ICM). The TE is the first lineage to be established in the embryo and will ultimately be responsible for nutrient exchange between mother and fetus as the progenitor of the placenta. The ICM is a pluripotent cell population, which is the origin of the fetus and extraembryonic endoderm.

1.3.1 Differentiation in the embryo

The differentiation of the TE signifies the first cell lineage decision in the embryo. Several theories exist as to how this event occurs. Many biologists class this cell fate decision as a stochastic event, whilst others are of the opinion that this process is deterministic. Positional information and gene expression have been extensively studied to determine the events that lead to this first differentiation.

The changes in cells during compaction around the 8 cell stage are thought to be important to this lineage decision. This process encompasses formation of E-cadherin dependent cell-cell adhesions and the apical-basal polarisation of blastomeres (Shirayoshi et al. 1983). E-cadherin is linked to the cytoskeleton through α-catenin. Reorganisation of the cytoskeleton is orchestrated by the Rho small GTPases. Molecules known as sec6/8 complex are recruited to the cell adhesion site to form a basolateral domain by providing a site for

basolaterally oriented vesicles (Grindstaff et al. 1998, Hsu et al. 1999). This allows the apical and basal portions of these cells to differ quite significantly. For example, localisation of the partioning defective (Par) gene family (which is largely involved in tight junction formation) differs between apical and basal portions of the cell (see figure 1.6). PAR3 and aPKC are apically distributed, whilst PAR1 is basolaterally located (Pauken and Capco 2000; Thomas et al 2004, Plusa et al. 2005).

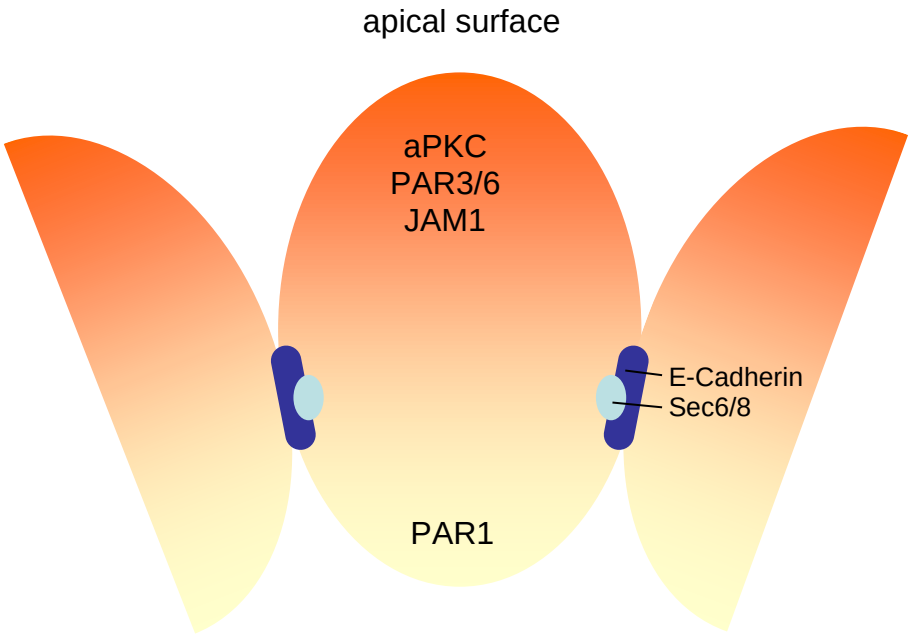


Figure 1.6 Polarity in blastomeres.

E-cadherin is required for cell polarisation. Sec6/8 is further involved with orientating vesicular transport of proteins. Several members of the Par family, JAM1, aPKC and PAR3 become localised in the apical portion of the cell, whereas PAR1 resides in the basolateral portion of the cell. When conservative divisions occur, these proteins are divided equally between the cells. When a differentiative division occurs, the basolateral portion of the cell, including PAR1, takes up an inner cell position, whilst the apical part of the cell including JAM1, aPKC and PAR3 takes an outer position (Pauken and Capco 2000; Thomas et al. 2004, Plusa et al. 2005). A downregulation of these polarity molecules results in the cell

preferentially taking an inside position within the embryo (Plusa et al. 2005). Adapted from a figure by Sutherland (2003).

The inside-outside model of cell fate suggests that this redistribution of proteins during polarisation allows for differentiation. When subsequent divisions of these cells occur conservatively, the cell divides along the medial plane both daughter cells contain proteins specific to the apical plane and the basolateral plane. When these cells divide differentially, apical surface specific proteins are restricted to the outer cell only (Johnson & Ziomek 1981).

An alternative model of cell fate suggests that pre-patterning exists in the oocyte which directs lineage divergence (Dalcq 1957), this model is known to not be entirely correct, as a huge degree of plasticity exists in the embryo. It is however thought by some that some degree of pre-patterning exists which may bias a cell towards a certain lineage if undisturbed (Zernicka-Goetz 2002; Gardener 2007; Torres-Padilla et al. 2007). This model dictates that the transcriptional networks that are required to maintain cell differentiation are established by this pre-patterning.

There are a range of transcription factors known to be essential for the establishment of lineage. At the morula stage when cells take up inside or outside positions, a stable, self-promoting circuit of transcription factors is established that allow cells to resist or promote differentiation in inner and outer cell populations respectively. SRY (sex determining region Y)-box 2 (SOX2), octomer binding protein 4 (OCT4) (also known as POU class 5

homeobox 1 - Pou5F1) and NANOG promote pluripotency in the inner cell population, the ICM. Caudaul related homeobox 2 (CDX2) and eomesdermin (EOMES) proteins are upregulated in the outer cells and ensure the maintenance of the TE (Strumpf et al. 2005). Reciprocal repression occurs to maintain lineage specification. SOX2, OCT4 and NANOG repress TE protein CDX2 and vice versa (see Figure 1.7). Initially cells express both proteins, and the relative concentration of OCT4 to CDX2 establishes the commitment of blastomeres to TE and ICM lineages (Niwa et al. 2005).

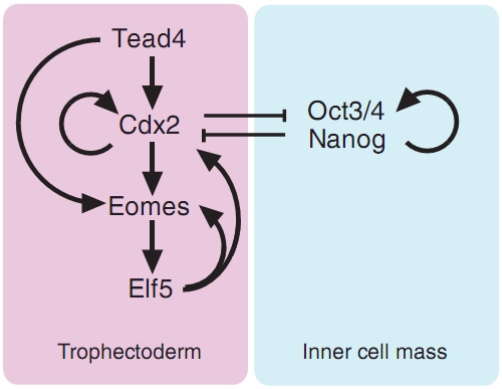


Figure 1.7 Schematic of interaction of gene expression in ICM and TE cells.
Reciprocal repression of Cdx2 and Oct3/4 and Nanog is crucial for the maintenance of the differing cell characteristics of these lineages. Taken from Sasaki et al. 2010.

CDX2 expression enforces a TE cell type by promoting the transcription of *Eomes*, and *elf5*. Repression of TE specific expression by OCT4 in ICM cells is thought to occur by its interaction with a histone H3 Lys 9 (H3K9) methyltransferase known as Eset. Eset binds and represses genes involved in TE fate such as Cdx2 and Tcfap2a (Yuan et al. 2009). CDX2 null embryos are however capable of blastocyst formation, indicating that *Cdx2* is not the initiating signalling event in TE formation. These embryos do however have

poor cell integrity and lack the capability to implant (Strumpf et al. 2005) indicating the essential role of CDX2 in the maintenance of the TE. TEA domain family member 4 (TEAD4) acts upstream of *Cdx2* expression initiating transcription. TEAD4 null embryos fail to form any TE cells (Yagi et al. 2007) demonstrating that this is an essential event in the initiation of TE formation. TEAD4 activity is mediated by the Hippo signaling pathway (see Figure 1.8). TEAD4, together with the coactivator protein Yes-associated protein 1 (YAP) is required for *Cdx2* transcription to take place. Large tumor suppressor (Lats) mediated YAP phosphorylation occurs when the entire cell is subject to cell-cell contact, preventing the formation of the transcription factor complex with TEAD4 in inner cells. In this way *Cdx2* is prevented from being expressed in the inside cells of the embryo and can occur in outside cells of the morula (Nishioka et al. 2009). Yap can be detected in a nuclear localization when unphosphorylated and active. Differences between the inner and outer cells of the morula in terms of Yap can be detected from soon after the 8 cell stage.

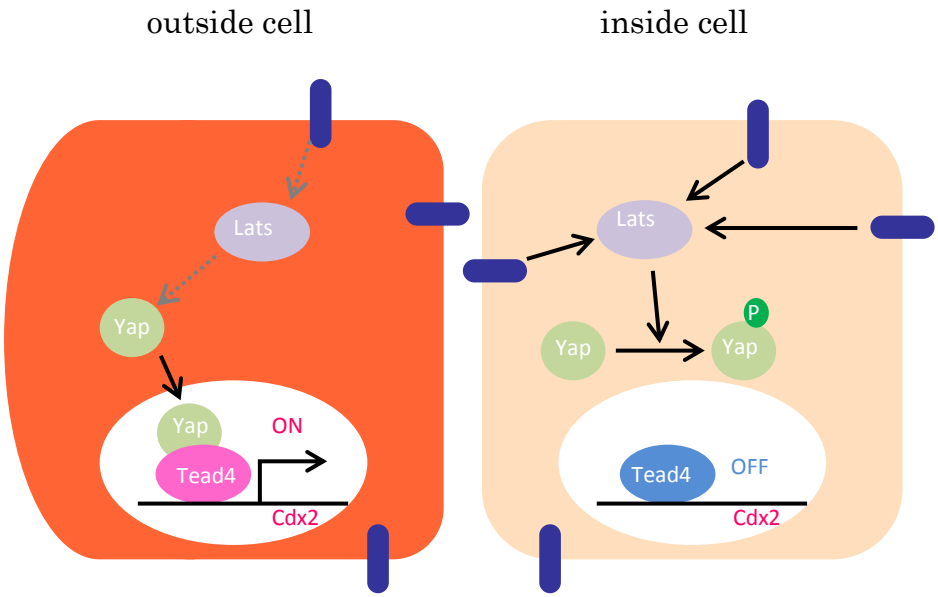


Figure 1.8 Schematic of hippo signaling cascade controlling the transcription of Cdx2.

Under complete cell contact conditions, Lats mediates phosphorylation of Yap, preventing TEAD4 transcription factor complex forming and thus no *Cdx2* expression occurs in inner cells. Adapted from a figure by from Sasaki et al. 2010.

Evidence has emerged that *Sox2* is also involved in this pathway. It was previously thought that the role of *Sox2* was limited to maintaining pluripotency as *Sox2* knockout mice are peri-implantation lethal (Avilion et al. 2003). It has been found through siRNA knockdown of *Sox2* that maternally derived transcripts are necessary for TE production. The loss of maternal *Sox2* transcripts leads to a loss of TE specific proteins TEAD4, Yap, CDX2, EOMES, FGFR2 and FGF4, (Keramari et al. 2010).

siRNA knockdown has also been used to show the importance of *Cdx2* transcripts earlier in development, in this case transcripts prove to be essential for correct polarity. siRNA downregulation of *Cdx2* at the 8 cell

stage causes a reduction in aPKC at the apical portion of cells. At the 16 cell stage, these embryos fail to transcribe aPKC, Par1, Par3 and E-cadherin (Jedrusik et al, 2010a). Zygotic knockdown of this gene permits the formation of polarity and developmental arrest occurs post-blastocyst formation (Strumpf et al. 2005).

Upstream signalling also exists for the expression of Oct4. SALL4 has recently been identified as a key regulator of OCT4 (Elling et al. 2006). Embryonic expression of *Sall4* begins around the 8 cell stage. ICM derived lineages do not arise in *Sall4* deficient embryos, whilst TE derived lineages are unaffected.

Cell tracing experiments also point to epigenetic differences in the cells, from before the time of inside-outside positioning, supporting a biased view of cell fate. At the 4 cell stage, histone H3 methylation at specific arginine residues (Arg26 (H3R26me), H3R2me, and H3R17me) is increased in cells that are destined to become ICM or polar TE. This is regulated by the coactivated associated arginine methyl transferase 1 (CARM1) (Torres-Padilla et al. 2007). CARM1 promotes differentiative divisions. Upregulation of CARM1 leads to altered expression of PAR proteins; increasing expression and basolateral localisation of EMK1 (the murine homologue of Par1) whilst simultaneously decreasing Par3 expression and apical localisation. This results in increased ICM cell allocation (Parfitt & Zernicka-Goetz 2010).

Expression of lineage specific markers initially takes a seemingly stochastic distribution (Dietrich & Hiiragi 2007, Rossant & Tam 2009), whilst evidence suggests that lineage can be predicted by examining the much earlier embryo (Torres-Padilla et al. 2007; Parfitt & Zernicka-Goetz 2010). Current opinion suggests cell origin and inter-cell communication as well as geometric and temporal limitations interact to direct fate decisions (Zernicka-Goetz & Huang 2010). Maternal dietary intake of proteins and fats are known to influence lineage allocation in the blastocyst (Kwong et al. 2000, Mitchell et al. 2009, Eckert et al 2012) and raised cellular lipid content has been demonstrated to increase contribution to the inner cell mass in porcine embryos (Kim et al, 2011). It is however unknown how maternal diet interacts with the current model of lineage divergence, and further investigation could well provide additional understanding. Understanding of the events occurring in the preimplantation period may be key to elucidating mechanisms behind changes seen in offspring as a result of very brief dietary challenges at this time

1.4 Interaction of the Blastocyst and the Uterine Environment

1.4.1 The Reproductive tract

As discussed in section 1.2.3., the preimplantation period is capable of eliciting changes in the embryo that will have a continued effect throughout the animal's lifespan (Kwong et al. 2000; Watkins et al. 2008). During this time of development, the embryo is free floating, firstly within the oviduct and then within the uterus. Maternal-embryonic cross-talk is mediated by the constituents of the oviductal and uterine fluid and can influence development. Figure 1.9 shows the gross structure of the murine reproductive tract consisting of the ovary, oviducts and uterus. The ovary is the site of oocyte maturation and ovulation, as well as a site of hormonal production of oestrogen and progesterone. The oviduct is a tightly coiled tubular structure in which the ovulated oocyte is collected. It is sub-divided into three sections; the ampulla

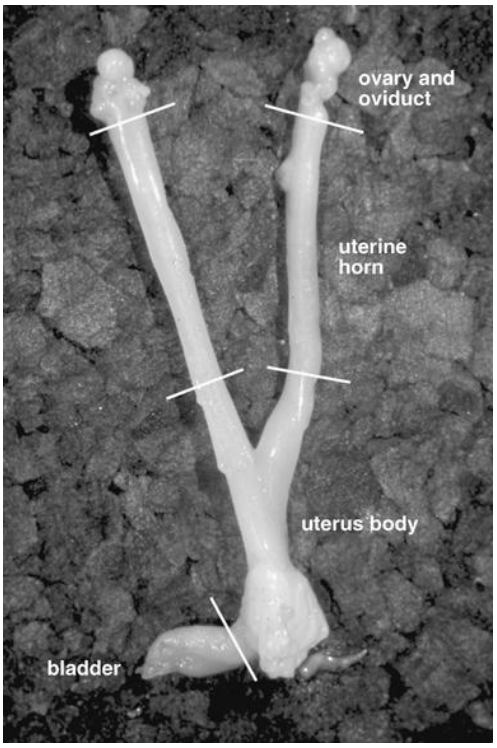


Figure 1.9 The gross structure of the mouse uterus.

(Antal et al. 2007). Fertilisation occurs in the oviduct, the zygote begins cleavage as it travels towards the uterus. From the morula stage, embryos can be found in the uterus.

is the section closest to the ovarian bursa, followed by the tightly-coiled isthmus and finally the intramural portion which resides in the uterine wall. When first described by Gabriele Fallopius, the oviduct was named the "tuba uteri" crediting its structural similarity to the instrument the tuba (Leese, 1988).

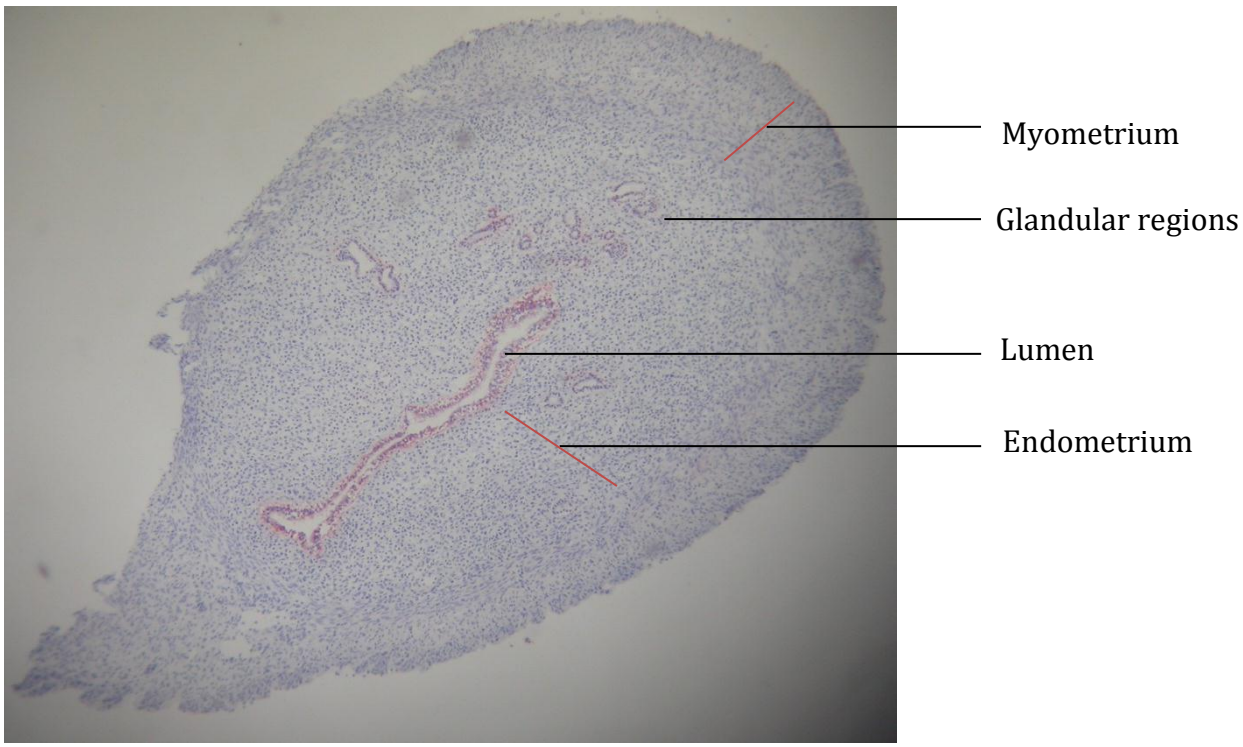


Figure 1.10 Basic histology of the mouse uterus.

Image shows the myometrium, endometrium glandular regions and lumen. Image captured by Grace Evans.

The transition of the fertilized mouse embryo from oviduct to uterus occurs at around 3 dpc in the mouse at the morula stage of development. The uterus is much larger and without convolution compared to the oviduct and consists of

three cell types; myometrium, stroma and epithelium. The epithelium is further subdivided into luminal and glandular cell types (Figure 1.10).

The uterus responds to cyclical hormonal signals known as the menstrual or estrus cycles in human and mammals respectively in the non-pregnant animal. These hormonal signals also synchronise the production of the oocyte with the receptivity of the reproductive tract. The proliferative or follicular phase of the menstrual cycle is characterised by increasing oestrogen production, the follicle develops into an antral follicle under the stimulation of FSH and the endometrial lining thickens. A surge in LH production (produced in the anterior pituitary) signals the onset of ovulation. Following ovulation, LH and oestrogen levels fall whilst progesterone levels rise, originating from the corpus luteum. If an embryo is present it will signal to the maintain progesterone levels via hCG production (Channing et al, 1980).

1.4.2 Nutrients within the reproductive tract

The oviductal and uterine milieus contain all the necessary components to support early pregnancy. In the oviduct this includes glucose, lactate, pyruvate and amino acids, which differ in concentrations observed in the uterine fluid and plasma. Sugars are thought to enter from the circulation by facilitated diffusion (Leese & Jeffries, 1977; Leese, 1983). Prior to compaction of the embryo, pyruvate is the main source of energy required by the embryo (Conaghan et al. 1993, Martin and Leese 1999). After compaction the energy requirements shift from pyruvate to glucose, coinciding with the move from

the low glucose oviduct, to the higher glucose uterine environment (Conaghan et al. 1993, Martin and Leese 1999, Nichol et al. 1998). Prior to compaction, embryos cultured in medias not containing pyruvate will be subject to a developmental block in glycolysis at the level of phosphofructokinase. After compaction this shift in metabolic requirement also involves an increase in the cellular machinery of the embryo to process glucose, with increased expression of GLUT3 and hexokinase, a glucose transporter and an enzyme involved in glycolysis (Pantaleon and Kaye 1998, Houghton et al. 1996). As glucose is a requirement for blastocyst development, it is essential that enough is supplied to the uterus from the blood stream. It is known that the uterus can alter its permeability to glucose, and in normal circumstances a four-fold increase in glucose permeability occurs at the time of blastocyst arrival (Brison and Leese 1991). Maternal serum glucose levels were found to be unaffected by the HF diet used in the present study when fed for the preimplantation period (Dr Sarah Finn-Sell, personal communication). Glucose:insulin ratio was found to be reduced in these animals however, suggesting that there are systemic changes. As mentioned in section 1.2.2, alterations in this ratio can be indicative of the development of diabetic type symptoms and in gestation can lead to developmental conditioning.

The uterine fluid is also required for the supply of amino acids. Particular amino acids are known to affect the development of the embryo. Non-essential amino acids and glutamine are known to increase the rate of cleavage (Lane and Gardener 1997, Devreker et al. 2001) whilst later in development, post

compaction, essential amino acids are implicated in an increased rate of development, particularly in the ICM (Gardener et al. 1994, Lane and Gardener 1997). Amino acids are also known to influence the mTOR (mammalian target of rapamycin) pathway (reviewed by Yang & Guan 2007, see figure 1.11). This energy sensing pathway is known to regulate several key metabolic pathways within the cell such as protein synthesis and cell growth. Two mTOR complexes exist; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 has five components; mTOR, Raptor, mLST8 (also known as GβL), PRAS40 and Deptor. mTORC2 comprises of six proteins; mTOR, Rictor, mSIN1, Protor-1, mLST8 and Deptor (Peterson et al 2009). mTORC1 promotes functions such as protein, lipid and organelle biosynthesis, positively regulating cell growth and proliferation. mTORC1 phosphorylates eukaryotic initiation factor 4E (eIF4E) and ribosomal protein S6 kinase 1 (S6K1) to induce protein synthesis. Phosphorylation of 4E-BP1 is also required to prevent its binding to eIF4E which would prevent its role in initiating translation. S6K1 activity induces protein synthesis through an increase in mRNA biogenesis, translation & elongation and translation of ribosomal proteins such as S6 (Laplante & Sabatini, 2009). mTORC1 also regulates lipid synthesis by positively interacting with SREBP1 and PPARγ. Growth factors can also interact with mTORC1 through insulin and Ras signalling pathways through AKT and ERK1/2. Insulin activates AKT through recruitment of IRS1 and PI3K. Activation of S6K1 by mTORC1 negatively feeds back to repress activation of this pathway in normal cells (Laplante & Sabatini, 2009). mTORC1 function can be inhibited by

rapamycin by binding the FRB domain of mTOR, mTORC2 is thought to be largely unaffected by rapamycin, at least in short term doses (Leone et al, 2006).

mTOR regulates proliferation and protein synthesis in the preimplantation embryo. Disruption of its expression causes proliferation to cease in both lineages (Murakami et al. 2004). Blastocysts treated with rapamycin fail to develop protrusive activity required for implantation (Martin & Sutherland 2001). It has been shown that maternal protein consumption can result in altered uterine fluid amino acid content (Eckert et al, 2012). Branched chain amino acids, valine, isoleucine and leucine are shown to be depleted in the uterine fluid in the emb-LPD model. Leucine is known to stimulate p70 S6Kα phosphorylation, a member of the AMPK pathway, via mTOR (Tokunaga et al. 2004). Altered uterine amino acid levels provides a pathway by which diet influences the developing embryo (Eckert et al 2012). The mTOR pathway could be a target within the maternal tract itself. As nutrient composition in the dam changes and thus circulating amino acids, glucose and growth factors, energy sensing pathways may well lead to changes in uterine expression of growth factors for example. (See figure 1.11)

Methyl donor levels could provide another means by which the uterine environment is altered by diet, and another cue for the embryo to adapt. Production of methyl groups relies upon dietary methyl donors and co-factors. Dietary methyl donors are those that contain methionine, choline and folate,

whilst co factors include vitamins B3 and B6 (Niculescu and Zeisel 2002). The presence of methyl groups influences embryonic histone modifications (Pogribny et al. 2007). As mentioned in section 1.3, the embryo is undergoing epigenetic reprogramming during the preimplantation period and the availability of methyl groups may influence this process. Maloney et al (2013) fed a methyl donor deficient diet to rats during pre and peri-conception periods and found an altered liver proteome in adult offspring indicating an effect on mitochondrial function, as well as poor glucose handling in male offspring (Maloney et al, 2011).

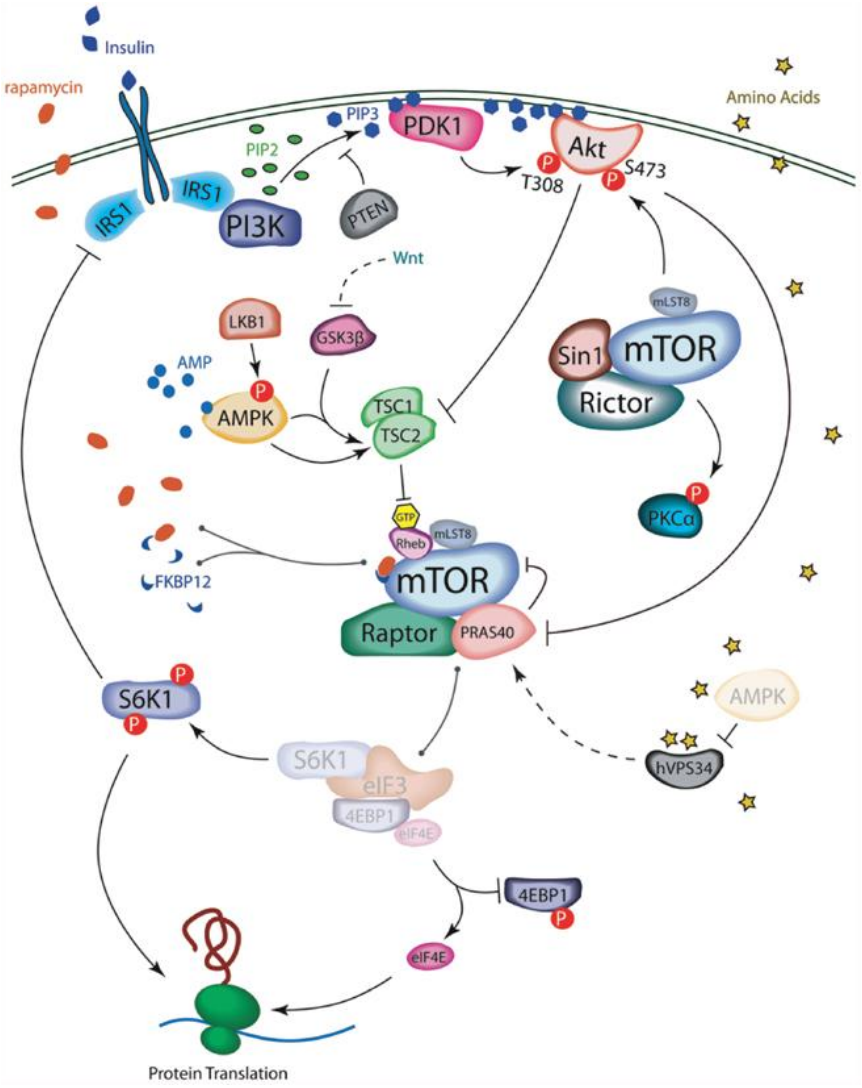


Figure1.11 mTOR pathway.

Showing sections of mTOR pathways, involving insulin signalling (Yang & Guan 2007)

1.4.3 Signalling molecules in the uterine environment

Nutrient availability can also influence signalling molecule expression such as hormones, growth factors and cytokines. Levels of these factors in the uterus must also be correct for receptivity to the embryo for successful implantation to occur. Hormones, growth factors and cytokines are proteins capable of producing cellular growth, proliferation and differentiation. By these actions, these molecules could be key in eliciting the effects in offspring,

also being responsive to circulating nutrients by their effects on the embryo or the uterus itself.

Steroid hormones progesterone and oestrogen are key in synchronising the uterus for implantation. FSH, LH and oestrogen peak sequentially to prime the uterus up to the point of ovulation. Progesterone inhibits the actions of oestrogen to slow down its proliferative effects. Oestrogen is produced primarily by the ovaries in the form of oestrone, oestradiol, and oestriol, with the placenta also producing this hormone in pregnancy in the form of oestetrol. FSH stimulates production of oestrogen in the in the theca cell where cholestrols are converted to androgens. Thecal cells have both FSH receptors and 17,20-lyase and 3β-HSD enzymes required for these first steps of hormone production. Androgens are then transferred to the granulosa cell, which responds to LH stimulation and produces aromatase. Armoatase converts these androgens, from the theca, to oestrogens (Young & McNeilly, 2010).

Steroid hormones are able to diffuse across cell membranes to bind their receptor, ERα and ERß. Dimerisation occurs and these bind to oestrogen receptive elements to cause transcription. The oestrogen receptor is expressed strongly in the uterus indicating its responsiveness to oestrogen signalling (Knapp et al 2013).

In some species delayed implantation can occur, a single injection of oestrogen can initiate the sequence of events required for implantation, demonstrating its role as a crucial co-ordinating factor of implantation (Paria et al, 2002). Many factors which will be further discussed including Leukemia inhibitory factor (Lif), IGF1 and VEGF are responsive to oestrogen to allow implantation to occur (Song et al, 2000; Hewitt et al, 2010; Hyder et al, 2000). Oestrogen levels need to be within a range to allow for successful implantation (See figure 1.12; Ma et al, 2003). This range of receptivity may allow for developmental variation.

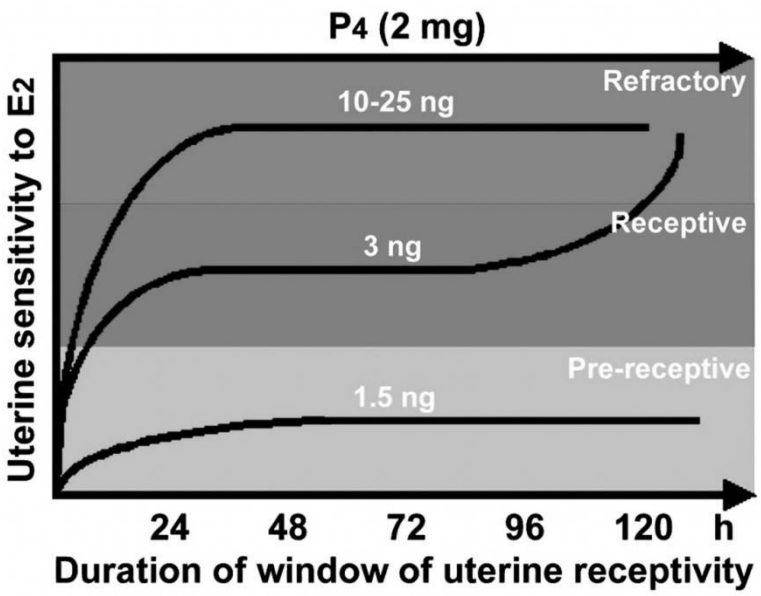


Figure 1.12 Uterine receptivity in response to oestrogen dosing.

From Ma et al, 2003. Diagram depicting uterine receptivity in response to oestrogen dosing. The diagram indicates that low levels of oestrogen lead to non-receptivity, high levels of receptivity lead to premature refractivity whilst mid-range levels of oestrogen allow for uterine receptivity. This study showed that 10 ng of oestrogen administered could still lead to implantation (although a reduced rate) indicating that oestrogen sensitivity is within a range providing the possibility of oestrogen level variation leading to further changes in the uterus.

Insulin like growth factor 1 (IGF1) is an active factor in uterine physiology and is reported to cause proliferation of luminal and glandular epithelial cells (Tong & Pollard, 2002) and may be produced in response to oestrogen stimulation (Walker et al, 2010). Binding at the oestrogen receptor (ERα) will directly cause IGF1 transcription by binding at an oestrogen responsive element, or by activating transcription factors such as AP1 or STAT5 (Hewitt et al, 2010). When IGF1 is produced, a complex of the IGF1 receptor (IGF1R), insulin receptor substrate 1 (IRS-1), and phosphatidylinositol 3-kinase (PI3K) is formed and causes AKT signalling activation (as also mentioned in terms of the mTOR pathway, section 1.3.2). This results in increased progression through the G2/M stage of the cell cycle, associated with cdk1 activity (Walker et al, 2010).

IGF-I is an example of a growth factor known to be present in the uterine environment, to which a receptor is present for on the embryo (Bedzhov et al, 2012) and has been shown to influence proliferation in the embryo, reduce apoptosis, as well as increasing the rate at which embryos develop to the blastocyst stage *in vitro* (Harvey & Kaye 1992; Herrler et al. 1998; Jousan & Hansen 2004; Makarevich & Makkula 2002; Palma et al, 1997; Sirisathien et al. 2003). In human embryos this effect was found to result from reduced apoptosis (Spanos et al, 2000).

The GLUT1 glucose transporter in the embryo is activated by insulin and IGF-I. GLUT1 is predominantly found around the ICM, meaning that in

raised insulin or IGF-I conditions, glucose uptake to the ICM is increased. This increase in energy source leads to increased proliferation in response to insulin or IGF-I (Pantaleon & Kaye 1996). Another insulin responsive glucose transporter, GLUT4 has also been reported in the mouse blastocyst (Tonack et al. 2004). Chi et al. (2000) report that excess IGF-I leads to a down-regulation of its receptors, and a subsequent increase in apoptosis.

PPARδ is expressed in the murine uterus in response oestrogen. It seems that the presence of an active blastocyst is also a requirement for its expression, under delayed implantation no expression of PPARδ is observed (Ding et al, 2003). Prostacyclin acts as an agonist of PPARδ. COX2 null mice show reduced fecundity, due to its role in prostaglandin generation. When COX2 null mice are treated with a PPARδ-selective agonist (L-165041), implantation rates are restored (Lim et al, 1999). Lif expression is also vital for implantation and is expressed in response to oestrogen but not progesterone. Lif is expressed in both the embryo and the uterus, but whilst Lif null dam pregnancies fail at implantation, Lif null embryos are capable of implantation in heterozygous dams indicating that maternal Lif is key at this stage (Stewart et al, 1992). Its uterine expression is also transient around the time of implantation further solidifying its purpose (Kimber et al, 2005).

Vascular endothelial growth factor (VEGF) has also been shown to have an effect on both the embryo and uterus and is responsive to oestrogen. Around the time of implantation, remodelling processes occur in the decidua. There is

evidence to suggest that VEGF and FGF2 interact within the uterus to direct angiogenesis (Laschke et al 2006). VEGF is cyclically regulated in women and is shown to increase along with many other factors mid-way through the secretory phase (the days before menses). It was found that sub fertile women failed to show this increase in VEGF. VEGF is shown to affect endometrial cell behaviour, with increased endometrial epithelial cell adhesion being observed upon the addition of VEGF. The blastocyst is similarly responsive to the addition of VEGF, with enhanced blastocyst outgrowth observed (Hannan et al, 2011). Furthermore VEGF expression in the uterus is increased in the preimplantation stage of pregnancy (Bukowska et al, 2011), probably reflective of its importance as an angiogenic factor. In the rabbit uterus expression is shown to be ubiquitous throughout the uterus at estrus and until day 4 of pregnancy. A shift in expression was observed with endometrial epithelial localization being observed just before and during implantation with particular intensity around implantation sites (Das et al, 1997). In the porcine embryo it has been found that addition of VEGF to culture medium increased the rate of progression to blastocyst stage (Biswas et al, 2008). VEGF has also been reported to be expressed by both mouse (Ingmann et al., 2002) and human (Krussel et al., 2001) blastocysts. It is speculated that its role in the blastocyst is that of angiogenesis at the time of implantation.

The fibroblast growth factor family are potent mitogens and several members have been shown to be present in the uterus. It is thought that they are also primarily involved in angiogenesis. In the cow, FGF2 production is required

to stimulate interferon tau production by the TE, and thus the maternal recognition of pregnancy (Michaels et al, 2006).

These signalling factors, as well as influencing the development of the embryo, provide the means for cross-talk between the embryo and the uterus. When beads of blastocyst size are introduced to the receptive uterus, signalling in line with implantation does not occur. When these beads are pre-soaked with HB-EGF or IGF1 they do initiate implantation like responses (Paria et al, 2001). Teklenburg et al (2010) showed that the endometrial stromal cells do not alter their expression of several growth factors upon the addition of a developing embryo to the culture environment, they do however decrease their expression of IL-1beta, IL-6, IL-10, IL-17, IL-18, eotaxin, and HB-EGF in response to the addition of an arresting embryo. Metabolism of the embryo is also thought to be important, with a range of species showing improved implantation rates when embryos have a low amino acid turnover or low glycolytic rate. This theory is known as the 'quiet embryo' hypothesis which was introduced by Leese (2002).

Cross talk between the embryo and maternal environment continues to be important throughout pregnancy. At 4 dpc the blastocyst will make contact with the uterus and begin to implant, and eventually form the placenta, as a direct means of communication between mother and fetus.

1.5 Implantation and development of the fetal membranes

The success of implantation can determine pregnancy outcome, with improperly implanted embryos more at risk of pre-eclampsia and IUGR (Norwitz 2007). Implantation begins with further differentiation of the TE, establishing the polar TE adjacent to the ICM and the remainder of the TE being known as the mural TE. The ICM at this stage produces FGF4 and due to proximity, the polar TE does not differentiate further, and remains in a proliferative state (Leunda-Casi et al, 2001). The mural TE cells undergo an epithelial-mesenchymal like transition and differentiate first to become primary trophoblast giant cells. These cells attract maternal blood supply by releasing a variety of angiogenic and anticoagulative factors such as VEGF and FGF2. These cells are also highly invasive and motile. Trophoblast cells gain motility by developing protrusions in the apical portion, thought to aid correct positioning within the uterus. Changes in expression of cadherins in trophoblast cells around the time of implantation, and thus stability of cell-cell junctions, also allow for cell movement around one another (Sutherland 2003). Other junctions, such as the desmosome are also important in the stability of cell contact, and thus movement at this time. It is shown that the desmosome changes from a hyper-adhesive form in the preimplantation blastocyst to a weakly adhesive, calcium dependant form in the migrating embryo outgrowth (Kimura et al, 2012). *In vivo*, this protrusive activity aids in phagocytosis of apoptotic decidua, aiding contact between the embryo and the basal lamina (Choy & Manyonda, 1998).

In vitro, attachment can be studied using the outgrowth model. The blastocyst spreads to form a trophoblast cell monolayer with a clump of ICM cells on top on glass or plastic surfaces. Amino acids, leucine and arginine have proved to be vital in this process, with blastocysts failing to develop protrusive activity or formation of outgrowths in their absence (Martin et al, 2003). Outgrowth formation can also be affected by environmental conditioning. As previously mentioned, lineage commitment of the mouse blastocyst can be altered in response to maternal dietary protein manipulation; an increase in TE cells, and total cell number are seen in the LPD blastocyst (Eckert et al, 2012). Sustained differences, of up to four days after removal from the maternal environment, can be observed in these embryos due to maternal challenge in the *in vitro* outgrowth model. Outgrowths of emb-LPD blastocysts show an increase in spreading area. This increased area is not a result of increased proliferation as there is no significant difference in nuclei number in the treatment groups and thus must be a result of each cell occupying a larger area. Further investigation of this phenomena could include study of adherence proteins. These changes are observed in the absence of the continued influence of the maternal environment.

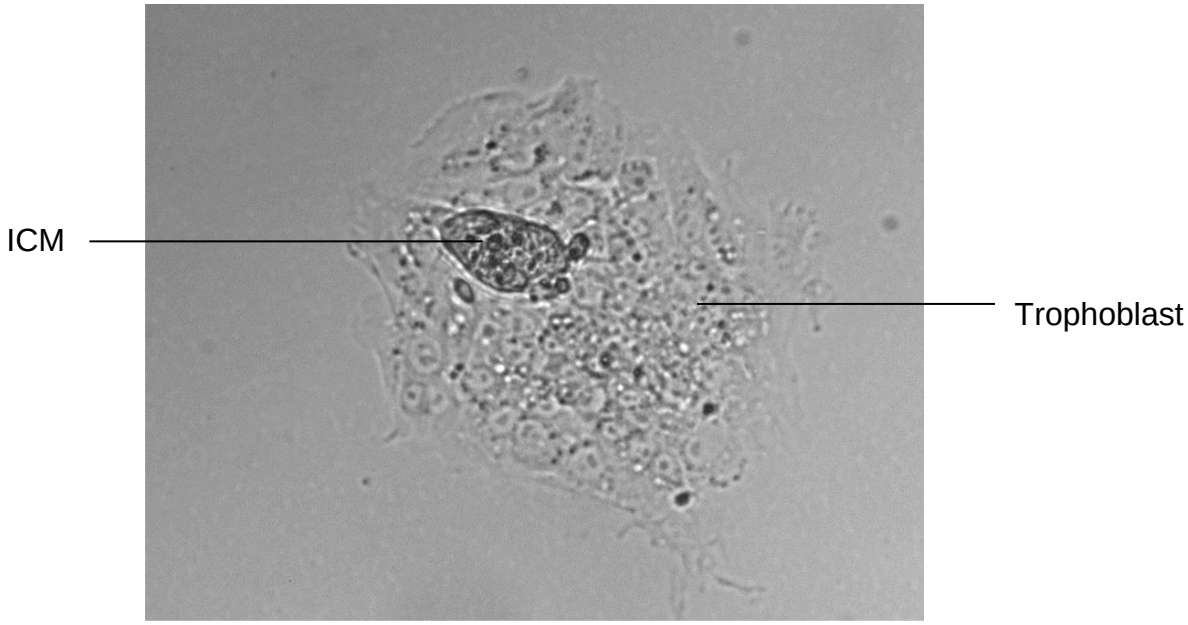


Figure 1.13 A Blastocyst outgrowth.

Blastocyst was collected at E3.5 and put into culture (KSOMaa + 10% BSA) for 48 hours.

Trophoblast giant cells line the entirety of the implantation site and together with cells from the PE form the yolk sac in the mouse embryo (Gardner & Rossant, 1979). The yolk sac mediates maternal-fetal exchange during the early post implantation period. In the human embryo there is no such visceral yolk sac, instead a layer of syncytiotrophoblast cells establishes the border between maternal and fetal tissues, and produces similar anticoagulant molecules (Maruyama et al, 1985). As the embryo develops, an extra-embryonic endoderm and mesoderm layer derived visceral yolk sac is formed continuing nutritional exchange function up until the placenta takes over around E9.5 – 10.5 (Cross, 2003). Those TE cells that remained in contact with the ICM, the polar TE, continue to proliferate after implantation and give rise to the extra-embryonic ectoderm (ExE) and ectoplacental cone (EPC). Around E8, the chorion and yolk sac (allantois) fuse with the EPC. A

trophoblast giant cell subtype emerges from the EPC and establishes the connection to the maternal blood supply at the spiral arteries. This invasion process by these endovascular giant cells links the endothelial cell lined arteries with the hemochorial blood spaces of the placenta (Cross, 2005).

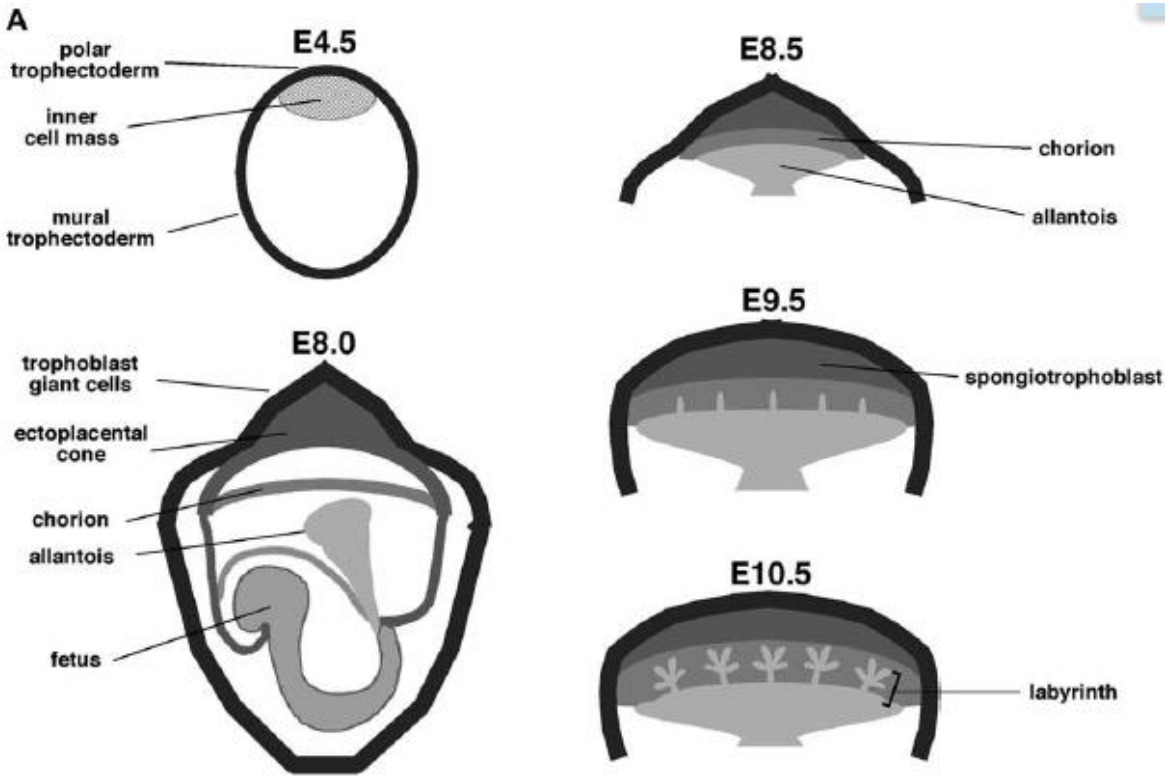


Figure 1.14 Placental development.

This figure demonstrates the development of the murine placenta from E4.5 to E10.5 as further described in section 1.5. Picture from Cross, 2003.

As the placenta develops, the EPC is presumed to establish the spongiotrophoblast layer (also called the junctional zone). Adjacent to this (in the direction of the fetus) is the labyrinth layer, syncitiotrophoblast cells result of the fusion of trophoblast cells and provide the nutrient exchange cellular layer here (Cross, 2003; refer to figure 1.14).

1.6 Placental exchange

The fetal membranes and the placenta continue to grow alongside the fetus, providing nutrient and gas exchange. The primary barrier to nutrient transport in the human is the syncytiotrophoblast. The syncytiotrophoblast has a maternal-facing microvillous plasma membrane (MVM) and a basal plasma membrane (BM) oriented towards the fetal circulation. Nutrient exchange in the murine placenta occurs in the labyrinth zone (see figure 1.14).

The fetus receives nutrition in the form of glucose, amino acids, non esterified fatty acids and triglycerides. Transport of free fatty acids across the placenta is not a passive process and largely relies on transport proteins. The main classes of these carriers are fatty acid transport proteins 1-6 (SLC27A#,) plasma membrane fatty acid binding protein and fatty acid translocase (FAT/CD36) (Duttaroy; 2009). Once taken up by the placenta they may be used as an energy source by the placenta, directly passed on to the fetus, or esterified and transferred to the fetus. Whilst fats remain essential for proper function, excessive quantities of fat have been shown to be detrimental to tissue function (Calder, 2010).

Some fatty acids have been shown to be preferentially transferred to the fetus by the placenta. Arachadonic acid and docosahexaenoic acids are both shown to be bioamplified in the human fetus in comparison to maternal levels. This is in contrast to linoleic acid, which is shown to be transferred to the fetus at

about half the level in the maternal circulation (Crawford, 2000). These fatty acids are particularly important in cell membrane formation.

Similarly to fatty acids, amino acids can be taken up via energy dependent transporters, they can be metabolised by the placenta itself and they can be interconverted in the placenta. At least 15 transport systems for amino acids have been identified in the syncytiotrophoblast of the human placenta, whilst the fetal capillary endothelium is easily crossed without active transport (Jansson, 2001). The serum concentrations of most amino acids are higher in the fetus than the mother (Philipps et al, 1978). Defective amino acid transport has been associated with IUGR (Regnault et al, 2005).

Glucose transfer through the full term human placenta has been shown to be directly proportional between maternal and fetal sides of the placenta (Day et al, 2013). GLUT1 is found in abundance in the syncitiotrophoblast and is thought to be the predominant form of glucose transport (Takata & Hirano, 1997). It is shown that longer exposure to high glucose levels downregulates GLUT1 allowing for regulation somewhat of glucose reaching the fetus (Hahn et al, 1998). GLUT1 expression is shown to be affected by a variety of diets. Increases in GLUT1 are seen in the low protein fed mouse at E16 (Coan et al, 2011), the high fat fed mouse at E18.5 (Jones et al, 2009) and the human diabetic placenta at full term (Jasson et al, 1999). GLUT1 expression is found to be decreased in severe maternal undernutrition at E16 (Coan et al, 2010) and in the human full term placenta when exposed to hypoxia (Zamudio et al.

2006). Changes in expression of several placental transport proteins are reviewed by Sandovici et al (2012). Changes in the placenta can be associated with changes in the offspring. High fat fed mice that showed increased GLUT1 expression were also found to be lareger (Jones et al, 2009).

The placenta has long been known to be important in offspring health, higher fetal placental ratios were associated with a lower apgar score (a crude measure of newborn health) by Molteni et al in 1978.

1.7 Fetal Growth

Following implantation, rapid growth occurs in the embryo. The three primary germ layers; ectoderm, mesoderm and definitive endoderm, are formed from the epiblast in the first 5-10 days as a result of gastrulation. The ectoderm gives rise to tissues such as the skin and nervous system, the mesoderm gives rise to the musculoskeletal and cardiovascular systems whilst the endoderm gives rise to epithelium, liver, pancreas and thymus. Neurulation follows gastrulation, which involves the formation of the neural tube. The notochord sits below the neural tube, adjacent to which the somite pairs develop (Sadler, 2010).

Between 10 and 14 days organogenesis occurs (Hogan et al, 1994). It is suggested that in situations of environmental challenge organogenesis will be altered to best preserve organs of importance. The brain is particularly thought to be protected developmentally. Blood in the fetus may be shunted

from the liver, muscle and gut to protect nutrient supply to the brain (Huagen et al, 2005). The mechanisms mentioned previously in terms of the early embryo will continue to affect the fetus including circulating and uterine growth factors, epigenetic programming and maternal body composition and studies show that maternal diet can affect organ size. Male offspring exposed to a low protein diet during oocyte development show a decreased proportional lung weight (Watkins et al, 2011), whilst protein restricted pig fetuses exhibited reduced kidneys and gastrointestinal tract (Pond et al, 1991). A high fat diet during the entirety of gestation was shown to decrease mineralisation whilst trabecular bone volume increased (Devlin et al, 2013). Altered organ size could contribute to offspring health throughout life and as such fetal heart, lung, kidney, liver and bone mass will be measured in this study.

Growth factors continue to be important to the growth of the fetus. Adiponectin for example is shown to be inversely proportionate to fetal growth. This mechanism works through inhibiting insulin dependant amino acid uptake (Aye et al, 2013).

This study will look at how the developmental process will be affected by the growth trajectory established early on as well as continued exposure to dietary challenge.

1.8 Project Rationale

The prevalence of metabolic diseases is increasing, at a financial cost, but more importantly at cost to quality of life of the population. Previous studies have clearly shown the impact that maternal diet during the preimplantation period of development can have on the continued development and subsequent health of the offspring. The causative mechanisms are not wholly understood, but the understanding of this process may contribute to the prevention and treatment of these diseases.

Two models of overnutrition are addressed here (HF and HPD). These models are likely to be very relevant to many of today's pregnancies and as such may provide useful insight which may be progressed towards developing guidance for the human population. A diagrammatic summary of the study is outlined in figure 1.16.

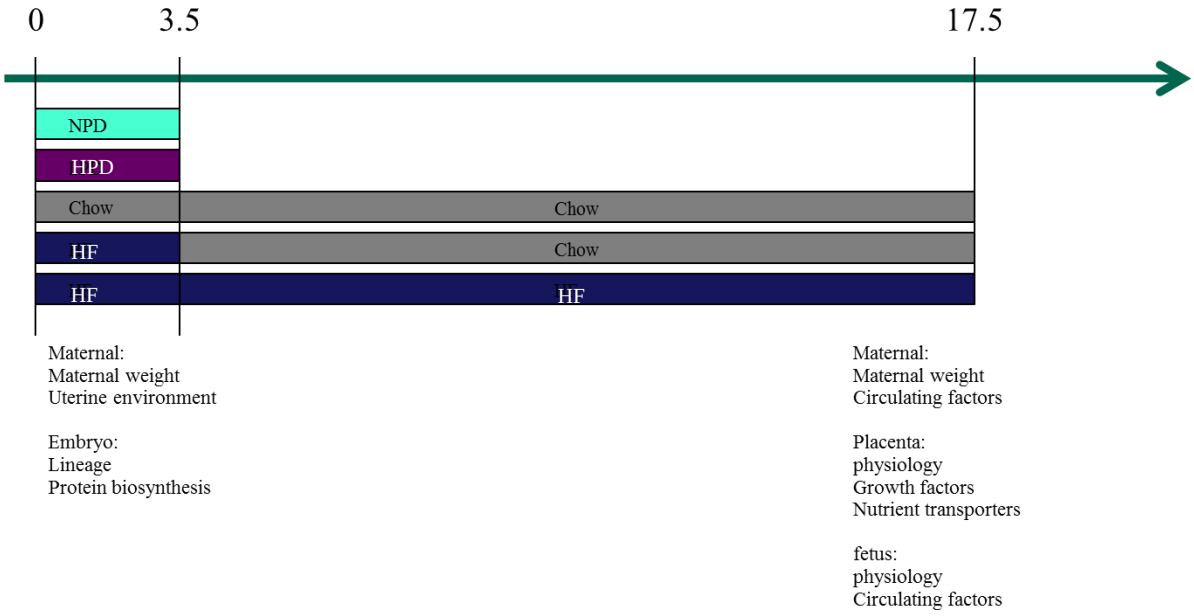


Figure 1.16 Summary of study.

The maternal uterine environment will be studied along with the developing embryo at E3.5 in NPD. HPD, Chow and HF diet models. The HF and Chow embryos will be studied to late gestation when either HF or chow is fed for the remainder of pregnancy. At E17.5 maternal circulating factors; placental physiology, growth factors and nutrient transporters as well as fetal growth including individual organ weight and circulating factors are studied. Techniques used include embryo collection, immunohistochemistry, differential labelling, luminescence assay, protein biosynthesis assay by Click-It chemistry, ELISAs, western blotting, PCR, RTq-PCR, Genorm, physiological study.

This study will address firstly the effect that these short term overnutrition diets have on the dam. Initial studies will determine the diet quantity consumed and the effect this has on the maternal characteristics, in particular it will address changes in the uterine environment that may be causative of an offspring phenotype (Chapter 3).

Maternal protein undernutrition has been studied for its effects on the preimplantation embryo. It is unclear whether the effects seen are specifically due to a reduction in available protein, or simply an adaptation to a poor maternal environment. Studies comparative to those done previously with the LPD will be carried out to investigate the directionality of these effects when a high protein diet challenge is given. Emphasis will be given to the effect that diet has on lineage allocation and metabolic characteristic. It is likely that these early lineage allocations will have impact on later developing lineage and thus may modify growth trajectory of offspring. HPD and HF blastocysts will be compared to establish if excess of differing macronutrients

have the same adaptive effect or if in fact adaptation is more specific to environment (Ch4).

The HF diet will be investigated to the end of pregnancy (17 dpc) where development of organs and serum insulin and glucose will be compared in groups fed a control diet throughout, a HF diet for only the preimplantation period and a HF diet fed throughout pregnancy. This will show both the effect of sustained HF diet in pregnancy and determine the impact of preimplantation HF feeding on the remainder of pregnancy. Parameters studied will give indication to the offspring health trajectory.

It is hoped that by increasing understanding of the changes that occur in the embryo and mother, preventative and palliative measures may be developed.

1.9 Hypothesis

Maternal dietary challenge will alter the uterine environment by change in growth factor expression. This will impact upon blastocyst development. These adaptations may include altered lineage commitment and metabolic activity.

The effect of protein overnutrition on lineage allocation is hypothesised to be opposite to that of the low protein diet. Excess lipid consumption may cause similar effects to protein overnutrition, as it is likely that the embryo will compensate for excesses of different macro nutrients by the same

mechanisms. These changes in lineage are likely to have a continued effect on the offspring, more so when HF diet is maintained. Maternal glucose and insulin levels are likely to be altered when a HF diet is fed throughout pregnancy which will also contribute to the development of the fetus.

Changes observed in the HF embryo will be reflected in the development of the fetus. Preimplantation feeding of HF diet will lead to some of the same changes seen when feeding HF diet throughout pregnancy, indicating the importance of this period in the development of the offspring.

Chapter 2

Methods

2.1 Animals, diet and tissue collection

2.1.1. Housing

MF1 mice were bred in-house (University of Southampton Biomedical Research Facility) under U.K. Home Office license and local ethics approval. Conditions were standardised to a 0700–1900 light cycle, a room temperature of 24 ^{o}C and *ad libitum* feeding of standard chow. Female mice were housed in groups of up to 12. Male studs were housed individually. All mice received standardised environmental enrichment.

2.1.2. Diet

Mice were fed standard laboratory chow. For experimental procedures, mice were given a specific diet to assess the effects of protein or lipid overnutrition (picture 2.1). The high protein diet (HPD) has a 30% protein content and was compared to the normal protein diet (NPD), containing 18% protein. The high fat (HF) diet includes 45% fat, whilst the standard laboratory mouse chow, which served as a control, contained 21% fat (Special Diets Service, Sigma, and ICN Biomedicals). A description of diet composition can be seen in table 2.1.

Picture 2.1 MF1 mouse with the HF diet

	NPD	HPD	Chow	HF
Percentage (gm)				
Carbohydrate	68.8	56.8	70.0	49.5
Protein	18.0	30.0	18.0	26.5
Lipid	10.0	10.0	10.0	22.5
Energy (MJ/kg)	16.13	16.00	13.75	17.90

Table 2.1 Dietary composition of diets used throughout this study.

Diets were produced by Special Diet Services, Cambridge, UK. For full dietary data for experimental diets, see appendix

2.1.3. Sacrifice of animals

Mice were sacrificed immediately prior to experimental use by cervical dislocation. This is a schedule one procedure as outlined by Home Office regulations, and as such, was carried out following their guidelines.

2.1.4. Outline of experimental dietary treatments

At 7 to 8 ½ weeks of age, mice were naturally mated overnight with male MF1 studs (Harlen UK Ltd, Bicester, UK). Mating was confirmed by observation of a post copulatory plug, after which mothers were randomly assigned to a dietary treatment group. These groups were a high protein (HPD), normal protein (NPD), high fat diet (HFD) or standard laboratory mouse chow, fed *ad libitum* (Figure 2.2). The first diet model (Figure 2.2) involved analysis just prior to implantation, sampling was carried out at E3.25, E3.5 and E3.75.

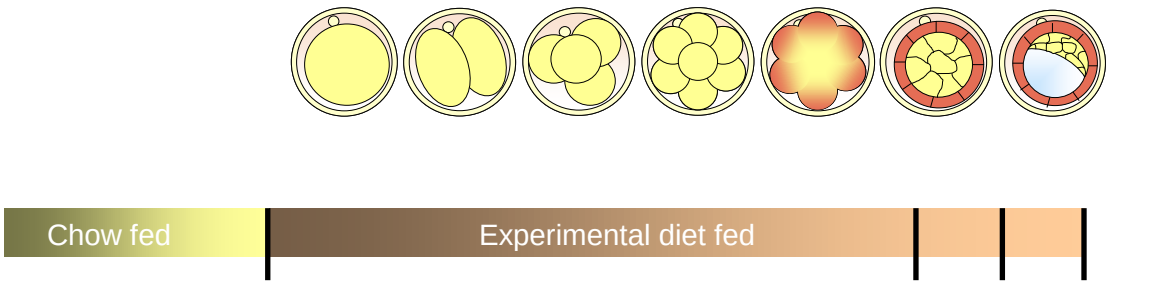


Figure 2.2 Diet model: preimplantation period

Experimental diet (HF/HPD/NPD) was fed from the morning following conception until embryo collection.

In order to assess the effects of HF dietary challenge into late gestation, mice were also randomly assigned to a dietary treatment group upon the observation of a post copulatory plug. The groups were as follows; HF diet fed thoughout pregnancy (HF), chow fed throughout pregnancy (Ch) or HF diet fed solely during the preimplantation period and the remainder of pregnancy maintained on chow (emb-HF) (figure 2.3).

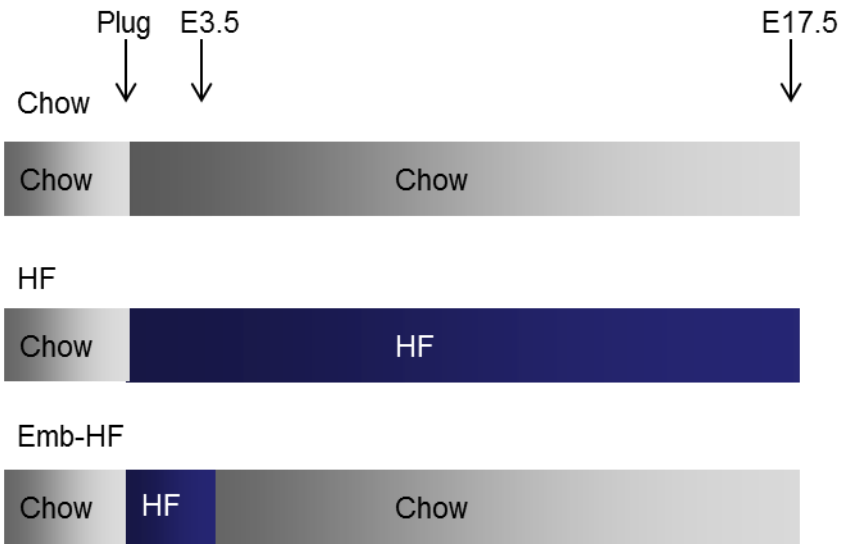


Figure 2.3 Diet model: studies to E17.5

Mice in the control group were fed the same chow diet throughout pregnancy as they had been fed on previously. HF mice were fed a HF diet from the observation of a post copulatory plug and throughout gestation. Emb-HF mice were fed HF diet for the first 3.5 days of pregnancy, at which point they were reverted to the chow diet for the remainder of pregnancy.

2.1.5 Food consumption and weight

Mice were weighed at 24 hr intervals for experiments in diet models one and two. Food was also weighed at this time on a daily basis to assess food consumption in diet model one (please note that for these experiments mice were housed individually). Weighing began from the morning that experimental diet was first given. Dams were also weighed daily throughout pregnancy in diet model 2.

2.1.6. Tissue Collection

Mice were sacrificed at E3.25, E3.5, E3.75 and E17.5. Several maternal tissues were collected for subsequent analysis. Blood was collected by cardiac puncture and allowed to clot on ice for 30 mins before centrifugation at 4 ^{o}C for 10 mins at 10,000 g. Serum was aspirated and frozen on dry ice before storage at -80 ^{o}C. Ovaries and oviducts were dissected with care to remove all fatty tissue and snap frozen in polypropylene tubes on dry ice from dams at E3.5. At this stage, preimplantation embryos were collected by uterine flushing with H6 BSA medium (see section 2.3.1). Following embryo collection, the uterus was also collected and frozen in polypropylene tubes on dry ice.

Serum, heart, lung, liver, kidneys and uterus were collected from dams at E17.5. Each conceptus was weighed before separating the placenta and yolk sac, which were also weighed and frozen in polypropylene tubes in liquid nitrogen. Fetuses were sacrificed by decapitation and blood was collected from the neck by heparinised capillary tube and placed on ice. Blood was transferred into polypropylene tubes and serum was extracted in the same way as described for the dam. The fetal heart, lungs, liver and kidneys were also collected, weighed and frozen in liquid nitrogen. All tissues were stored at -80 ^{o}C until required. Fetuses were numbered as described in figure 2.4

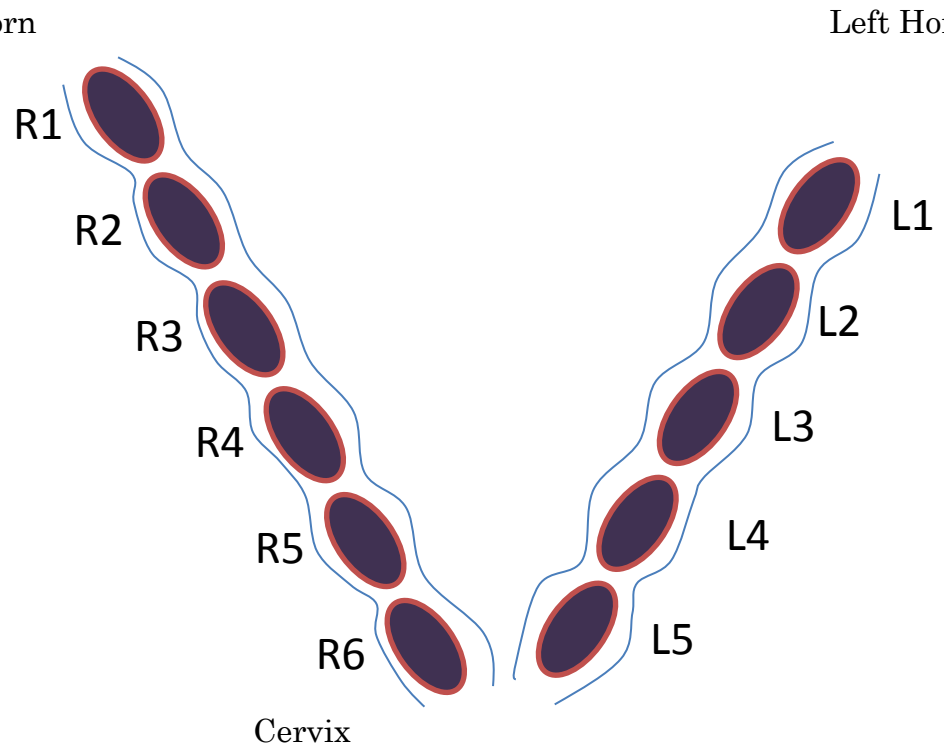


Figure 2.4 Schematic showing labelling system of fetuses during dissections at E17.5.

All fetuses were weighed and dissected. Tissues from the central 4 fetuses from each horn were retained and analysed.

2.1.7 Collection of embryos

Embryos were collected by uterine flushing. The uterus was dissected out and placed into pre-warmed saline for transport to the dissecting microscope. The uterus was placed into a small drop of pre-warmed H6 medium (see appendix) supplemented with BSA (Sigma) (H6 BSA). Embryos were flushed out of the uterus using H6BSA medium; this was achieved by inserting a fine needle into the top of the uterine horn and flushing through the medium with a syringe. Immediately following collection of embryos, experimental procedures were begun.

Embryos collected for PCR analysis were washed through 3 x 25 µl drops of UV irradiated (Spectrolinker XL 1000, Spectronics corporation, New York, USA) DNase and RNase free 0.1% polyvinylacetate (PVA) in phosphate buffered saline (PBS). Embryos were then transferred to polypropylene tubes by mouth pipetting, in a minimal volume of PBS/PVA and frozen in groups of 5 on dry ice.

For the production of experimental standards and optimization purposes, mice underwent superovulation to give maximum number of embryos per mouse used. At either 5 weeks or 8 weeks onwards, an intra-peritoneal injection of pregnant mare's serum (PMS, Intervet) was administered followed 48 hrs later by human chorionic gonadatrophin (hCG, Intervet). Both injections were of 5 IU. In instances where embryos were required, mice were mated following hCG injection Embryos were collected as described for naturally mated animals.

2.2 Analysis of Tissues

2.2.1 Analysis of Protein Content of Tissues

Organs were collected and stored as described in 2.1.6. Proteins were extracted, separated by sodium dodecyl sulphate polyacridimide gel electrophoresis (SDS PAGE) and analysed by western blotting.

2.2.1.1 Protein Extraction

Tissue samples were weighed and homogenised in 0.3 ml protein lysis buffer (See Appendix) using a PowerGen 125 Homogeniser (Fisher Scientific, UK). Homogenates were centrifuged at 10,000 g for 10 mins at 4 ^{o}C. This centrifugation creates a pellet of cell debris, leaving protein in the supernatant. The supernatant was removed to a clean, pre-labelled tube and placed on ice.

Total protein content of lysates was determined by Bio-Rad DC Protein Assay and comparison made to known concentrations of BSA. Serial dilutions of BSA in protein lysis buffer were made at 7.5, 5, 2.5, 1.25, 0.625 and 0 mg/ml. Lysate dilutions of 100, 50, 25 and 12.5 % were made by addition of protein lysis buffer. The Bio-Rad DC protein Assay is based on the Lowry assay (Lowry et al. 1951). This assay was used as described by the manufacturers to cause a colour change relative to the amount of protein present. Absorbance was read at 750 nm on a Dynatec MR 5000 plate reader. Protein concentration of tissue samples was determined by comparison to the BSA standards.

2.2.1.2 SDS PAGE

Samples were prepared for SDS PAGE in volumes of 15 µl per lane, by addition of 1.5 µl 10x dithiothreitol (Sigma) and 3.75 µl 4x NuPAGE LDS sample buffer (Invitrogen, Poole), and dilution to the required protein amount (10 - 25 µg per lane). Samples were heated to 90 ^{o}C, cooled on ice and

centrifuged. Samples were loaded on to premade polyacrylamide gels (4-12% Bis-Tris Gel w/ MOPS, NuPage, Invitrogen) alongside a 1 kb Precision Plus Protein Standard All blue ladder (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) size marker, and a gel control standard protein sample. Gels were run at 120V for 1.5 hrs in running buffers (See appendix).

2.2.1.3 Western Blotting

The separated proteins were transferred to either a Polyvinylidene fluoride membrane (Immobilon FL, Millipore, UK) or nitrocellulose membrane (Amersham Bioscience, Little Chalfont, UK) in cold transfer buffer (See appendix) for 1 to 16 hours at a constant 300 mA. Following protein transfer, the membrane was placed between filter paper to dry for at least 2 hours. The dry membrane was then rehydrated in TBS (See appendix), and blocked in a solution of TBS, 0.1 % Tween and 5 % milk powder (Marvel, Premier Foods, Hemel Hempstead).

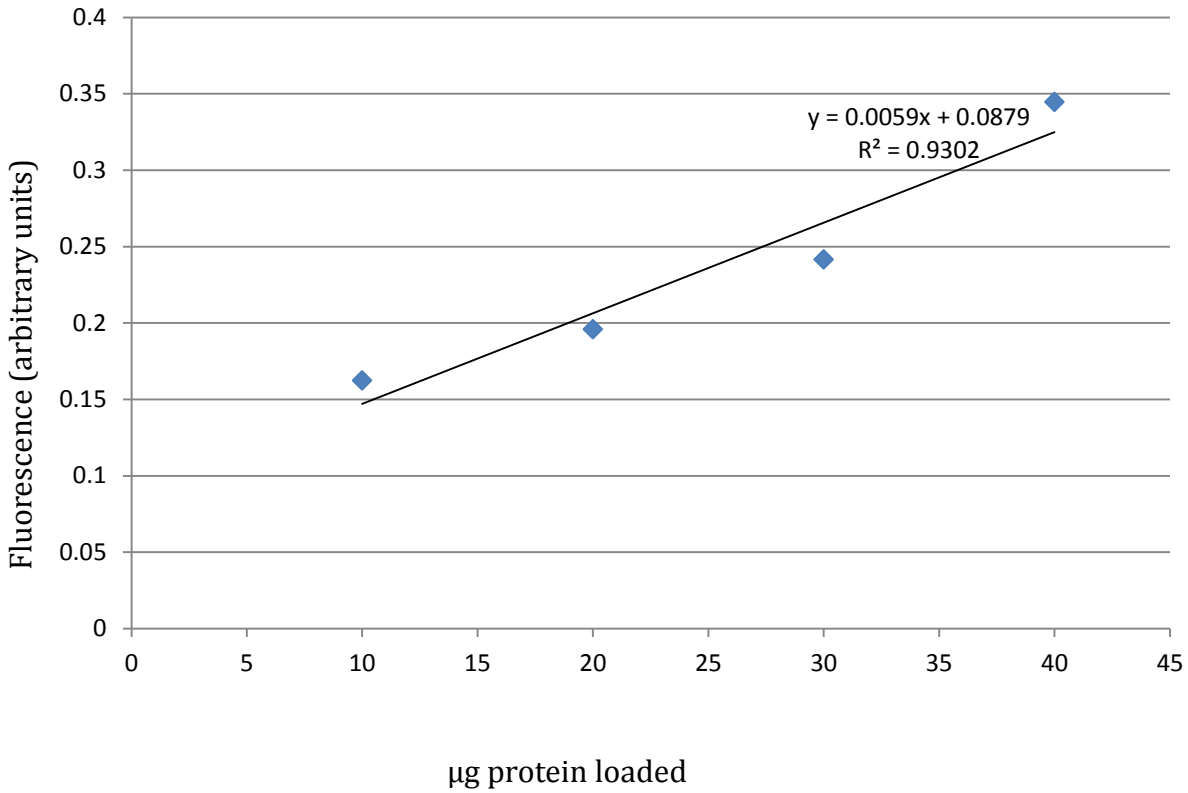
Specific primary antibody was added to the membrane in blocking buffer for one hour at room temperature or overnight at 4 ^{o}C. The membrane was then washed in TBS before addition of a fluorescently conjugated secondary antibody for one hour at room temperature in the dark. Fluorescence was detected and digitally photographed using the Odyssey Infared Imaging System (Li-COR Biosciences, Lincoln, NB, USA). Detection wavelengths of 700 nm or 800 nm were used to measure fluorescence. Analysis was

performed using Odyssey Infared Imaging System Application Software, version 2.1 (Li-COR Biosciences, Lincoln, NB, USA).

2.2.1.4 Validation of Antibodies

Antibodies were validated by western and dot blots. Dilution curves were used to show that fluorescence and protein expression had a linear relationship. It was also necessary to show that in the absence of protein or primary antibody, no fluorescent signal was detected. See figure 2.5.

A)



B)

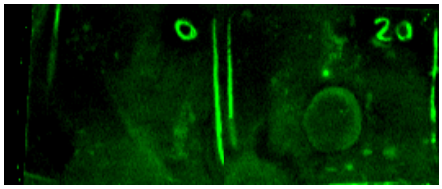


Figure 2.5 Example of antibody validation, FGF2 antibody A) Showing linear expression of FGF2 antibody as tested by western blot. B) Dot blot indicating that no fluorescence is seen in the presence of no protein. 0 and 20 annotations on dot blot indicate the amount in µg of protein added.

2.2.2 Analysis of gene expression

For the analysis of gene expression, RNA was extracted for use in RT PCR experiments.

2.2.2.1 RNA Extraction

An RNeasy Lipid tissue mini kit was used (Qiagen) as per the manufacturer's instructions. Samples of a maximum of 100 mg were used. If the tissue had not been previously stabilised in RNAlater solution, then care was taken to cut tissues on dry ice. 1 ml QIAzol lysis reagent was added to the tissue and homogenised to a uniform lysate. Samples were left at room temperature for 5 minutes before added 200 µl chloroform. Tubes were then shaken vigorously for 15 seconds. Tubes were left to rest again for 2-3 minutes before centrifugation at 12,000 g for 15 mins at 4 ^{o}C. This produces a tri-phase solution. The top layer is the aqueous layer that contains the RNA, this is colourless; the lower phase is reddish and contains DNA; the remaining interphase is white in colour and is the protein. The upper aqueous phase was transferred into a new tube and 600 µl 70 % ethanol was added and mixed by vortex. Up to 700 µl could be transferred into an RNeasy mini spin column sat in a 2 ml collection tube. This was centrifuged for 15 seconds at 10,000 rpm and the flow through was discarded. This was repeated with any remaining solution above the 700 µl threshold. 350 µl of buffer RW1 was added to the column and centrifuged as before. The flow through was again discarded. 10 µl DNase I stock solution was added to 70 µl buffer RDD and added directly to the column membrane. This was left at room temperature for 15 minutes.

Another 350 µl Buffer RW1 was added to the column and centrifuged as before and then any flow through was discarded. This was repeated with 500 µl buffer RPE. A further 500 µl buffer RPE was added and this time centrifuged for 2 minutes at 10,000 rpm. The column was then placed in a fresh collection tube centrifuged for 1 minute at full speed. The column was again placed in a fresh tube and 50 µl RNase-free water was added to the membrane. This was centrifuged for 1 minute at 10,000 rpm leaving the RNA in the tube and ready to use. Quantity and quality were assessed by NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

RNA quality was further addressed by assessment upon gel separation. A 1.5% agarose gel made with TBE running buffer was made including 0.001% ethidium bromide. Water used in this procedure was DEPC treated by addition of 0.1% v/v diethylpyrocarbonate for at least one hour before autoclaving. 1 µl of RNA was added to 6 µl 1x loading buffer along with 1 µl formamide. The gel was submerged in TBE running buffer and electrophoresis was performed at 100V until the loading buffer could be seen to have migrated a suitable distance. RNA was visualised by UV transilluminator and photographed by Alpha imager.

2.2.2.2 cDNA synthesis

cDNA was synthesised from RNA samples using a ImProm-II Reverse Transcription kit (Promega) according to manufacturer's instructions.

1 µg of each RNA sample was mixed with 2 µl random primers and the mixture was made up to 10 µl with nuclease free water. A cDNA blank was also created which contained only water and random primers. Samples were then thermally denatured at 70 °C for 5 minutes and then placed immediately on ice. A master mix was created containing 4 µl 5x sample buffer, 2.4 µl 3mM $MgCl_2$, 1 µl dNTP mix containing 0.5 mM of each dNTP, 0.5 µl (20 units) Recombinant RNasin Ribonuclease Inhibitor, 1µl ImProm II reverse transcriptase and made up to 15 µl with nuclease free water for each sample. 15 µl of the mastermix was added to 1 µl of the RNA/random primers mix of each sample. Another master mix was also created, not containing ImPromII reverse transcriptase to create -RT samples. As before 15 µl of this -RT mastermix was added to 1 µl of the RNA/random primers mix of each sample. Samples were then heated to 25 ^{o}C for 5 minutes to allow annealing of primers, then to 42 ^{o}C for 60 minutes for extention to occur, followed by heating to 70 ^{o}C for 15 minutes to inactivate enzymes. Samples were then diluted to 5 µg/µl with nuclease free water ready for use in PCR and stored at - 20 ^{o}C.

2.2.2.3 Real time PCR

Primers for use in real time PCR were designed using either Roche primer design software or NCBI Primer-BLAST (Primer3 and BLAST software combined). All primers were intron spanning, had a GC content in the range of 40-60 % matched between forward and backward primers, had a maximum of 1 ^{o}C difference in Tm, and were between 18 and 25 nucleotides long.

Primer pairs were checked for specificity using NCBI BLAST software. See figure 2.6 for primer sequences.

Gene Name	Accession number	Primer sequences 5'-3'	
		Forward Primer	Reverse Primer
IGF1	NM_010512.4	gaccgaggggcttttacttc	catccacaatgcctgtctga
VEGF	NM_001025250.3	aacgatgaagccctggagt	caccatcaccaccaacactc
FGF4	NM_010202.5	tacctgctgggcctcaaa	cggagagagctccagaagac
Lif	NM_008501.2	aaacggcctgcatctaagg	agcagcagtaagggcacaat
AdipoR1	NM_028320.3	agcaccggcagacaagag	agtgcatggtgggtacaaca
AdipoR2	NM_197985.3	agagcaggagtgttcgtgggct	ggtggcagccttcaggaaccc
PPAR delta	NM_011145.3	aggcggcagcctcaacatgg	gtccggaagaagcccttg
HGF	NM_010427.4	cacccctttgggagtattgtg	gggacatcagtctcattcacag
Slc38a1	NM_134086.4	aaatccctcatgggagagg	caccatcaccaccaacactc
ERα	NM_007956.4	gctcctaacttgctcctggac	cagcaacatgtcaaagatctcc

Reference genes

Gene Name	Accession number	Primer sequences 5'-3'	
		Forward Primer	Reverse Primer
Tuba1	NM_011653	ctggaacccacggtcatc	gtggccacgagcatagttatt
Ppib	NM_011149	ttcttcataaccacagtcaagacc	accttccgtaccacatccat
Hprt1	NM_013556	tcctcctcagaccgctttt	cctggttcatcatcgctaatc
Pgk1	NM_008828	tacctgctggctggatgg	cacagcctcggcatatttct
Tbp	NM_013684	gggagaatcatggaccagaa	gatgggaattccaggagtca
18S	NR_003278	ctcaacacgggaaacctcac	cgctccaccaactaagaacg
Sdha	NM_023281	tgttcagttccaccccaca	tctccacgacacccttctgt

Table 2.6 Description of primers used in real-time PCR experiments.

For each real time PCR experiment, a mastermix was created containing Precision qPCR MasterMix (Primer Design), the relevant forward and reverse primers and water to make the solution up to 19 µl per sample.

19 µl aliquots were added to wells of a 96 well plate under sterile conditions. cDNA templates for each sample were added in triplicate alongside -RT samples in duplicate, a no template control (no cDNA added) and a cDNA blank (blank created at the time of cDNA synthesis). Standard cycling conditions were as follows:

- Enzyme activation: 10min at 95ºC
- Cycling x40: Denaturation 15s at 95ºC, annealing, extension and data collection: 60s at 60ºC
- Melting Curve

This was altered where required, for example when a temperature gradient was used to show that primers annealed optimally at a different temperature.

Initial PCR involved testing of primers to ensure specificity. A melting curve was assessed for specificity (see figure 2.7). Following PCR, +RT samples were amalgamated and run on a 2% agarose gel containing ethidium bromide alongside blanks. If gels showed a single band of the expected amplicon size amplified in +RT samples and no specific bands in control samples the band was cut out of the gel using a scalpel and gel extraction was undertaken.

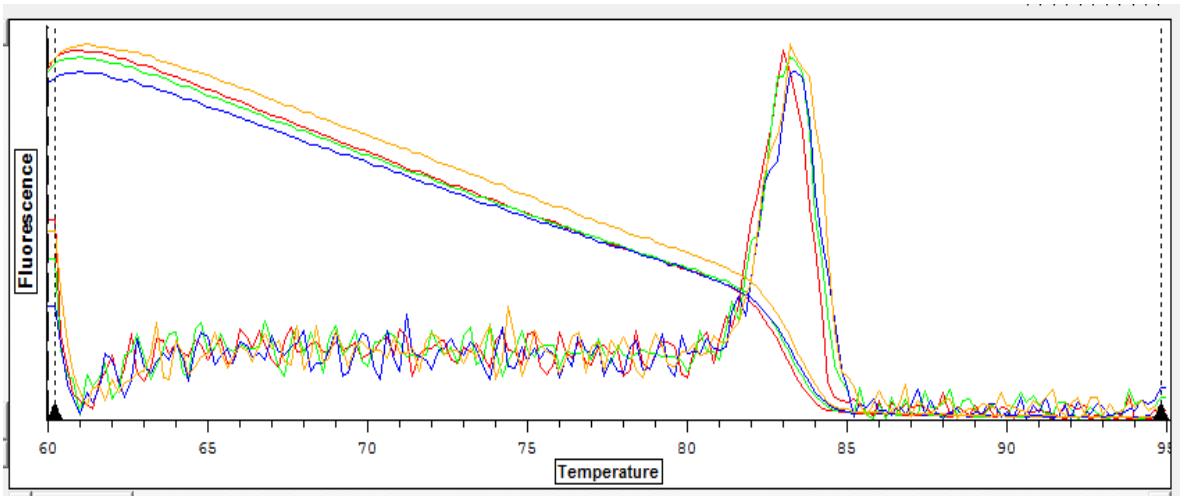
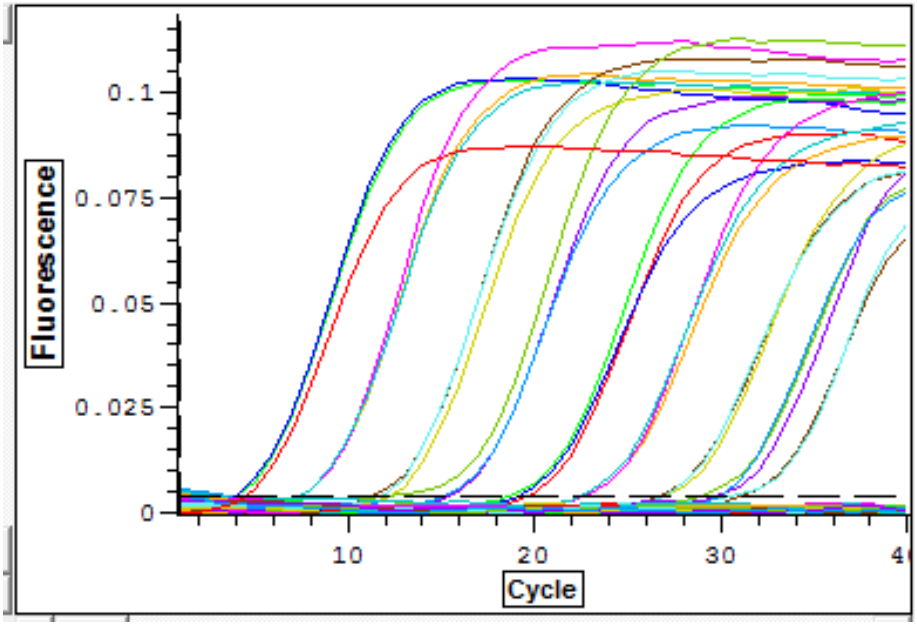


Figure 2.7 Example of melting curve

In this case for PGK1. A single, defined, repeatable curve indicates a lack of genomic or primer dimer contamination.

Gel extraction was carried out using a QIAquick Gel Extraction Kit (Qiagen, Sussex) according to manufacturer's instructions. The gel slice was weighed and 3 volumes of Buffer QC was added per 1 volume of gel (100 mg / gel equates to approximately 100 µl). This was incubated at 50 °C for 10 min or until all the gel was in solution. 1 volume of isopropanol was added and mixed to the sample and placed in a QIAquick spin column in a collection tube. This was centrifuged for 1 min at 13,000 rpm. 0.5 ml of Buffer QG was added to the column followed by addition of 0.75 ml of Buffer PE, each time centrifuging for 1 min before discarding flow through. The QIAquick column was centrifuged for an additional 1 min before placing into a clean 1.5 ml microcentrifuge tube. DNA was eluted by the addition of 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) for 4 minutes before centrifuging for 1 minute. Samples were analysed for concentration by Nanodrop.

Gel extracted cDNA was assessed for concentration by Nanodrop before creating a dilution series ranging from 1 ng to 10 ag. This dilution series was used to assess primer efficiency.



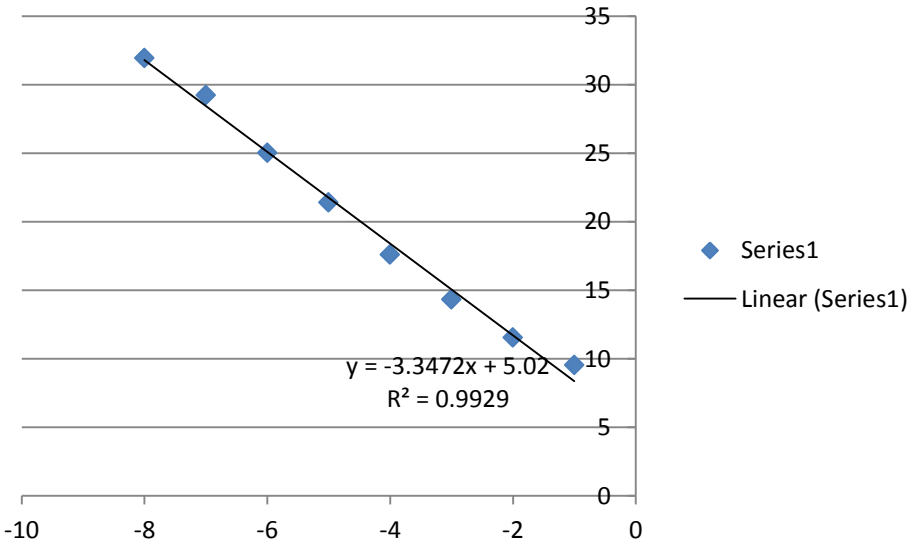


Figure 2.8 rtPCR standard curve created by serial dilution of cDNA

A) Amplification curves from serial dilution of cDNA samples amplified by real time PCR using Slc38a1 primers. B) Quantification of amplification in the exponential range from part A). This figure was used to quantify further results using these primers.

Reference gene selection was performed. SDHA, PGK1, 18s, α-Tubulin, HPRT1, PPIB and Tbp expression was analysed for each sample and stability of expression was confirmed using both GeNorm and NormFinder software. The most stable reference genes were chosen to create relative expression values.

2.2.3 DNA extraction

DNA was extracted from fetal tails for the purpose of sex determination. A simple alcohol salt precipitation method was used. First tails were digested in a lysis buffer containing tris, EDTA, NaCl, proteinase K and water. Each tail was placed in 200 µl of the lysis buffer and heated to 60 ^{o}C for 2 hours – overnight. When the tissue was adequately broken down, samples were centrifuged at 13,000 rpm for 15 minutes. The resulting supernatant was eluted and 30 µl 5M NaCl was added to each sample and left to stand for 5 minutes then centrifuged as before. The supernatant was eluted and added to an equal volume of 100% ethanol. Samples were shaken and left to stand for 10 minutes before centrifuging for 30 minutes at 13,000 rpm. All liquid was removed and 500 µl 70% ethanol was added. Samples were left at room temperature for 15 minutes with regular inversion of tubes. Samples were then centrifuged as previously and ethanol eluted. Pellets were left to air dry before the addition of 50 µl RNase/DNase free water. Purity and concentration were analysed by NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples were then stored at -20^{o}C short term or -80^{o}C for any extended period of time.

2.2.4 Gender determination by endpoint PCR

Previous studies have shown sex differences in the preimplantation embryo, so it is important to be able to segregate results by gender. Primer pairs as described by Greenlee et al (1998) for three loci were used; DXNds on the X chromosome and SRY and ZFY on the Y chromosome (see table 2.9). Unfixed adult liver tissue at 1 ng/µl and 200 pg/µl was used for optimisation.

A Qiagen HotStarTaq™ Kit (QIAGEN Ltd., Sussex, UK) was used for PCR reactions. A mastermix including the outer primer pairs (NDS3, NDS4, ZFY3, ZFY4, SRY2, SRY4) was produced and then aliquoted before addition of 2 µl of the extracted DNA was added to make a total volume of 25 µl per reaction. Magnesium chloride concentrations, Q-solution (Qiagen) and cycling conditions were sequentially altered to obtain the most suitable conditions.

Cycling was carried out in a Biorad thermal cycler. In total the thermal conditions were: 15 min Hotstart at 95 °C, followed by 40 amplification cycles of 1 min denaturation at 94 °C, 30 s annealing at 55 °C and 1 min extension at 72 °C.

Primers sequences

Gene Name	Primer Sequences 5'-3'
SRY	TCTTAAACTCTGAAGAAGAGAC GTCTTGCCTGTATGTGATGG
ZFY 3	AAGATAAGCTTACATAATCACCTGGA CCTATGAAATCCTTTGCTGCACATGT
NDS	GAGTGCCTCATCTATACTTACAG TCTAGTTCATTGTTGATTAGTTGC

Table 2.9 Primer sequences used for gender determination.
Primers are as previously described by Greenlee et al (1998).

Amplified samples were then separated by gel electrophoresis, using a 3 % agarose gel containing ethidium bromide and bands were visualised under UV light and photographed by Alpha imager.

2.2.4 Analysis of fetal bone by micro CT

Upon collection, fetuses were stored in 10 % formalin for 24 hrs. Samples were then dehydrated through graded alcohols (50%, 90%, 100% and 100% ethanol). Micro CT was performed as described previously (Lanham et al, 2010). A Xtek Benchtop 160Xi scanner (Xtek Systems Ltd.,Tring, Hertfordshire, UK) equipped with a Hamamatsu C7943 X-ray flat panel sensor (Hamamatsu Photonics, Welwyn Garden City, Hertfordshire, UK) was used with standardised settings for all scans of 100 kV, 60 μA using a molybdenum target with an exposure time of 534 ms and 4× digital gain. Image resolution of 14 μm was used. Reconstructed volume images were

analysed using VGStudio Max 1.2.1 software (Volume Graphics GmbH, Heidelberg, Germany). Using standards of known density, all the voxels which formed the structure were automatically assigned Hounsfield units. CT scanning was performed by Dr Stuart Lanham. See figure 2.10 for example images.

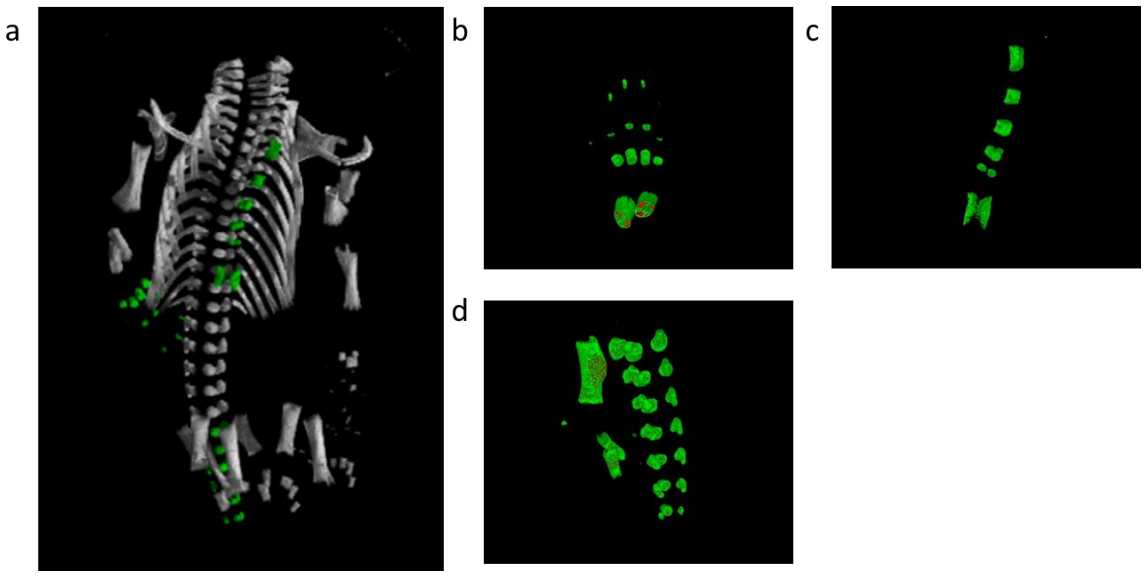


Figure 2.10. Examples of microCT scans of fetal mouse skeletons.

Highlighted in green are b) paw, c) sternum and d) tail region.

2.2.5 Serum analysis

Serum glucose levels were assessed using an enzymatic-colorimetric assay using Glucose Liquid reagent (Sentinel diagnostics - 17630) according to manufacturer's instructions. Standards of 200 mM to 0.016 mM glucose were tested alongside samples, each was loaded at 5 µl in triplicate. 200 µl glucose testing liquid was added to each well. Samples were incubated at 37 ^{o}C for 10 mins under constant agitation. Colour change was measured using an

ultiskan EX plate reader (Thermo Labsystems) utilising the 490 nm filter. Results were determined using a cubic spline regression.

An ELISA assay (Mercodia, 10-1247-01) was used to assess insulin levels in maternal and fetal serum. The assay was performed to manufacturer's instructions. Briefly, samples or calibrators were added to the plate at a volume of 10 µl, in duplicate. 100 µl of 1x enzyme conjugate was added before incubation for 2 hours at room temperature under constant agitation. Each well was washed 6 times with the 1x wash solution. 200 µl substrate TMB was added to each well and incubated for 15 minutes at room temperature. 50 µl stop solution was added and the plate briefly mixed before reading optical density at 450 nm on a plate reader.

2.2.6 Staining Protocols

All staining protocols were carried out with relevant controls, for example 'no primary' controls were required to show no staining.

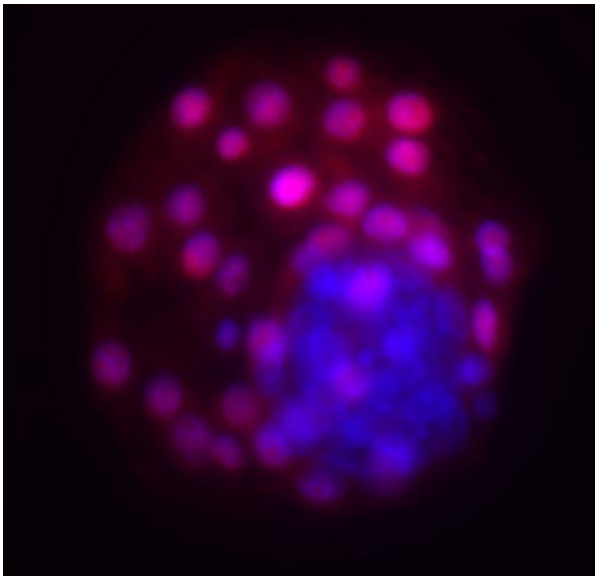
2.2.6.1 Tissue histology

Uteri were set in OCT matrix (TissueTek) and cut into 8 µm thick sections on a cryostat and collected on Superfrost glass slides. Sections were stained for lipid accumulation using oil-red O (Sigma). A stock solution was produced by dissolving 0.5 g oil red O in 100 ml isopropanol overnight and filtering through Whatman filter paper. A working solution was made by the addition of 30 ml of the stock stain to 20 ml of distilled water. This solution was left to

stand for 10 mins before filtering. Frozen sections were thawed and fixed in 2% formalin, before washing with tap water and 60% isopropanol. Freshly prepared Oil Red O working solution was added to slides for 15 mins before rinsing with 60% isopropanol. Cells were lightly stained with Mayer's haematoxylin by a 1 min incubation before allowing to blue in tap water and rinsing with distilled water. Glass coverslips were then applied with an aqueous mountant.

2.2.6.2 Differential nuclear labelling of blastocysts

Immediately after collection, zona pellucidae of embryos were removed by brief incubation in acid Tyrodes solution (Sigma, UK) warmed to 37 ^{o}C. Embryos then underwent differential labelling, a technique modified from that previously described by Handyside & Hunter (1984) to show TE and ICM lineages. Firstly embryos were placed in 10% Tetranitrobenzene sulphonic acid (TNBS) (Sigma, UK) in H6 media supplemented with PVA for 10 mins. TNBS adheres to blastomeres at the periphery of the embryo. This was followed by 3 washes in H6 BSA medium. Embryos were then incubated in 0.4 mg/ml rabbit anti-Dinitrophenyl (anti-DNP) antibody (Sigma, UK) diluted in H6 BSA for 10 mins, followed by 3 washes in H6 BSA medium. Incubation in a 25 µl drop of low-tox guinea pig complement (1ml lyophilized powder reconstituted in 1ml water and diluted 1:10, Cedarlane labs, Canada) with 2 µl propidium iodide (PI) solution (1mg/ml) was then carried out for 10 mins at 37 ^{o}C. This serves to lyse the outer trophectoderm cell of the blastocyst, making them accessible to PI.



Embryos underwent 3 washes in H6 BSA medium and were then fixed in ethanol at 4 ^{o}C to which 1% Bisbenzimide (Sigma) was added. Bisbenzimide is able to permeate the cell membrane and thus is able to label all cells, including non-lysed ICM cells blue where PI could not.

Figure 2.11 Blastocyst stained by the differential labelling technique.

TE cells are seen in red, and ICM are blue.

The following day, embryos were washed in fresh ethanol before mounting with glycerol on a glass slide. The slide was first cleaned with ethanol and a circle was drawn on the underside to aid in locating the embryo. A drop of glycerol of about 15 µl was placed adjacent to the marked circle. The embryo was placed into the spot and the coverslip was lowered so that the glycerol covered the embryo. The coverslip was then gently pressed to disperse cells for counting purposes. Embryos were visualised by fluorescence microscopy. PI and bisbenzimide have widely differing fluorescent spectra and therefore by exiting the fluorochromes with UV and using the appropriate filter sets, nuclei labelled with PI and bisbenzimide will appear pink and nuclei labelled with bisbenzimide will appear blue (see Figure 2.11). Photographs were taken in Z-series on a Zeiss Axiophot upright epifluorescent microscope, using

Metamoph software (Version 6.2r6). Cells were manually counted using Metamorph software. This was carried out at both 3.5 and 3.75 d.p.c.

Embryos collected at the morula stage were not subjected to the differential labelling technique. Embryos were instead fixed in ethanol and labelled with bisbenzimide as described above to allow nuclei counting.

2.2.6.3 Immunocytochemistry

Immunocytochemistry was used for the detection of specific proteins in the embryo. Localisation of transcription factors, SOX2, CDX2 and GATA4 was used to assess whether positional data obtained by differential labelling was reflected in the different localisation of transcription factors in the embryo.

Blastocysts and morulae were collected at E3.25 and E3.5 and immediately the zona pellucida was removed by brief incubation in warm acid Tyrodes solution. Embryos were allowed to recover in H6BSA for 20 mins before fixation. Fixation was at 1-2 % formaldehyde or 4% paraformaldehyde (PFA) for 10 to 30 mins dependant on the antibody used. Embryos were then permeabilised with 0.25% triton X-100 in PBS (Sigma, UK) treatment for 15 mins, then the formaldehyde fixation was neutralised with 0.026 % ammonium chloride in PBS treatment for 10 min. Primary antibodies were diluted in 0.1% Tween-20 (Sigma, UK) solution in PBS (PBST) at the concentration indicated in optimisation. Primary antibodies (Figure 2.12) were applied and left at 4^{o}C overnight. The following morning the antibody

was washed off by 3 x 10 mins incubations in PBST solution. Relevant secondary antibodies were diluted in PBST at a 1 in 300 and applied to embryos for 1 hour at room temperature. For primary antibodies raised in rabbit a donkey anti-rabbit Alexafluor 488 secondary antibody (Invitrogen) was used; for those raised in mouse, a donkey anti-mouse Alexafluor 647 antibody (Invitrogen) was used; and for detection of antibodies raised in goat a donkey, anti-goat Alexafluor 546 antibody (Invitrogen) was used. After this incubation period, embryos were washed in PBST 3 times. The first wash was for 10 mins, the second wash included 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) counterstain at a concentration of 5 μg/ml, and applied for 40 mins, this was followed by a further 10 min wash in PBS Tween.

		Fixation Conditions	
Antibody	Catalogue Number	% fixation	Duration
Anti Sox2 (Millipore)	AB5603	2% Formalin	20 mins
		4% PFA	30 mins
Anti Cdx2 (Biogenex)	MU392A-UC	2% Formalin	20 mins
		4% PFA	30 mins
Anti Gata4 (Santa Cruz)	sc-1237	4% PFA	30 mins

Figure 2.12 Conditions for primary antibodies

Labelling was visualised on a Leica confocal microscope (Leica TCS SP2), using consistent parameters for all images obtained. The following mirror

settings were used for all image acquisition; Alexafluor 488: 499-543 nm and Alexafluor 555: 573-694 nm

2.2.6.4 Quantification

Metamorph software (Version 6.2r6) was used to assess immunocytochemical labelling. The number of positive and negative nuclei was counted in each embryo.

2.3. Protein Biosynthesis Assay

2.3.1. Incorporation of AHA

Blastocysts from different maternal dietary environments were collected as previously described at E3.5, and cultured overnight in 50 µl drops of modified KSOMaa under oil. The modified KSOMaa was made without methionine (see appendix), and instead contained a methionine analogue L-Azidohomoalanine at a concentration of 0.5% (AHA, Invitrogen). During the overnight incubation, new proteins synthesised will incorporate AHA. The following morning, embryos were washed in 0.1% PVA in PBS solution and frozen on dry ice in 4 µl lysis buffer (Complete Protease inhibitor cocktail, Roche) in groups of 5. Embryos were stored at -80^{o}C until required.

2.3.2 TAMRA reaction

Tetramethylrhodamine (TAMRA) is composed of both an alkyne capable of undergoing a Azide-Alkyne Huisgen Cycloaddition reaction, also known as a Click reaction and a fluorochome. TAMRA is added to the incorporated AHA molecule by a process known as Huisgen cycloaddition or Click chemistry. This involves a reaction between the azide (AHA) and the alkyne (TAMRA) under the catalysis of copper sulphate (for a review of the click process see Kolb et al. 2001). This process was achieved using a TAMRA protein analysis detection kit (Invitrogen) according to manufacturer instructions, at a 10^{th} of the recommended volume due to small sample size.

In brief, 6.25 µl of alkyne solution (Component A) and 6.25 µl of reaction buffer (Component B), along with 1.25 µl ultrapure water were added to each sample and vortexed thoroughly. 1.25 µl of a copper sulphate catalyst (Component C) plus 1.25 µl of reaction buffer additive (Component D) was then added and samples were left to stand for 2-3 mins. Finally, 2.5 µl reaction buffer additive (Component E) was added before samples were gently mixed for 20 mins to complete the TAMRA incorporation reaction.

2.3.3 Precipitation of AHA-TAMRA labelled proteins

Once the TAMRA was added to AHA molecules, all protein was precipitated by a chloroform – methanol extraction method. Methanol and chloroform at a ratio of 4:1 were added to samples along with ultrapure water (75 µl methanol, 18.75 µl chloroform, 50 µl water). Samples were centrifuged for 5

mins at 14,000 g. This separated the sample into 3 phases: a top aqueous phase, which was discarded, and a protein interphase above a lower inorganic phase. 56.25 µl methanol was then added and the sample was centrifuged as before to pellet the protein. Supernatant was discarded to leave isolated protein, containing any incorporated AHA-TAMRA complexes. These samples were then stored at -20 ^{o}C.

2.3.2 Detection of TAMRA labelled proteins

Following fluorescence quantification of TAMRA labelled protein, loading dye was added to samples which were separated by SDS PAGE (see section 2.2.3.2) and transferred to nitrocellulose membrane (see section 2.2.3.3).

TAMRA labelled protein was detected with a polyclonal primary antibody, anti-tetramethylrhodamine, rabbit IgG antibody (A-6397, Invitrogen, Poole). This primary antibody was added to the membrane at a dilution of 1:5000 at 4 ^{o}C for 16 hours. The membrane was washed in three washes of 0.1% Tween-20 in TBS. This was followed by 1 hour incubation with goat anti-rabbit IgG IRDye 800 conjugate secondary antibody (Cat# 611132122, Rockland Immunochemicals Inc., Gilbertsville, PA, USA) at a dilution of 1:15,000.

2.4 Statistical Analysis

SPSS software (Version 17) was used for statistical analyses. The normality of data was investigated by the Kolmogorov–Smirnov test, and box plotting

was used to identify any extreme outliers (as determined by SPSS software), which would be removed from the dataset if determined the data point was recorded in error.

For non-nested data, normally distributed data was analysed by student T-test. Non-parametric data were analysed by Mann-Whitney-U test. Grouped percentages were analysed by z-test.

A multilevel random effects regression was used to account for the hierarchical structure of datasets that included groups of embryos from different mothers. Maternal and offspring effects were estimated simultaneously, incorporating between-mother and within-mother variation and different parameters measured from individual animals. Results are presented as ±SEM.

Chapter 3

Effect of Dietary Challenge on Maternal Characteristics

3.1 Introduction

Food consumption has been shown to exhibit a steady increase throughout pregnancy under normal conditions. (Markarova et al. 2010) (see Figure 3.1).

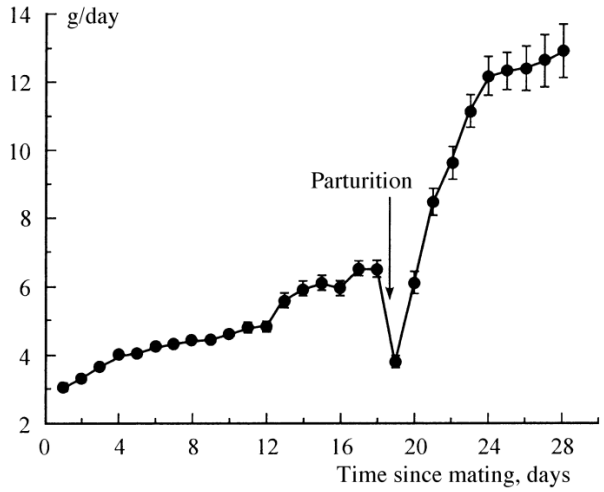


Figure 3.1 Food consumption in the mouse during pregnancy (Markarova et al. 2010). A general upwards trend is seen in food consumption from the point of conception.

Unpublished results (Dr Richard Porter, thesis 2005) demonstrate that the short term exposure to dietary challenge is capable of eliciting an effect on maternal weight gain in the first few days of pregnancy. This study considered a low protein diet (9% protein LPD compared to 18% protein control NPD), and showed that weight gain was slower in low protein fed dams from conception to E3.5. LPD fed animals actually consumed significantly greater quantity of food, suggesting differences in weight are a result of metabolic alteration. It has been shown that protein has greater thermogenic effect than other dietary components, which may explain these observations (Giordano & Castellino 1997).

The numerous studies of different diets with differing protein content have demonstrated that protein content has a non-linear relationship with appetite. Du et al. (2000) fed a

range of low protein diets, from 2% to 15% protein content to rats. This research found a quasi bell-shaped response, with the highest consumption occurring in animals fed 8-10% protein diets. Kulha et al. (2010) studied the impact of increased dietary protein on consumption. In this model the high protein diet contains 42% crude protein, whilst the control diet contains 24.8% protein. No difference in the amount of food consumed was observed in the pregnant mouse, weight was not addressed. Mitchell et al. (2009) compared the weights of mice fed a diet of higher protein content for three weeks and found no difference in weight. This high protein diet is however a very mild protein overnutrition, of only 21.7 % protein compared to the control diet of 12.7% protein.

High fat diets consumed over a sustained period of time are commonly associated with weight gain and metabolic syndrome. Winzell & Ahren (2004) showed a HF diet (58% Kcal from fat compared to 11.4%) caused an increase in weight in just a week, an increased production of glucose and insulin was also found. The HF diet used in this study was previously shown to have no effect on serum glucose levels but to cause a significant increase in insulin levels at E3.5 (Dr Sarah Finn-Sell, personal communication). This suggests that the short term feeding of this diet affects glucose sensitivity.

Mice are known to be adept at modifying their energy intake. As the HF diet is of greater calorific content, mice could be predicted to reduce their consumption of this diet. The varying effects of individual diets mean that validation of the intake and effects of the diets used in the current study is necessary. Four diets were used in this project (2.14). Experimental diets are likely to have varied effects on levels of consumption and maternal weight; factors that may need to be considered in later analysis. Further to

changes in food consumption and weight, changes are expected in uterine physiology. Gene and protein expression in the dam, as well as histological examination of the uterus will be studied to assess the environment in which the embryo is developing.

Fibroblast growth factor 2 (FGF2) has angiogenic properties, and when released in the uterus by macrophages and extracellular matrix (ECM) will contribute to placental development. High levels of FGF2 are associated with a reduction in blastocyst formation rate in mouse embryos (Seri et al. 1999). Particular structures may be sensitive to FGF2, in the rat culturing with anti-FGF2 for 48 hrs at E9.5 resulted in retardation of the yolk sac (Unur et al. 2009). This, along with other growth factors may influence embryo development. FGF2 levels in the uterus will be studied around E3.5. A previous unpublished study (Dr Richard Porter, thesis 2005) demonstrated LPD challenge at this time led to a rise in FGF2 levels. This established a precedent that diet can influence the production of this protein in the uterus. VEGF is another angiogenic factor, whilst also increasing the permeability of vessels causing localised oedema. Its expression is steadily increased in the first few days of pregnancy (Chakraborty et al 1994, Das et al 1997).

Several factors are upregulated in the uterus in response to oestrogen, the expression of the receptor (ERα) will be studied along with several of its induced factors. Lif expression is induced by oestrogen, it has biphasic action, firstly in the induction of uterine receptivity, and then aiding implantation (Aghajanova 2004). Its receptor is expressed in the embryo, suggesting a mechanism of cross talk. In stem cells and embryoid bodies it is reported to prevent ectodermal differentiation, but not endodermal differentiation (Shen & Leder 1994, Murray & Edgar 2001) It also exhibits paracrine effects on the uterus, upregulating junctional adhesion molecule 2 (JAM2), aiding adhesion of the embryo (Su

et al 2012). EGF expression fails to occur without induction by oestrogen and Lif (Cai et al 2003) and is also essential for implantation. Hgf is similary induced by oestrogen, and also progesterone (Zhang 2012). In vitro is has been shown to cause expansion of trophoblast populations and is suggested to induce invasion of the embryo (Patel et al 2000, Kauma 1999). IGF1 is also responsive to oestrogen expression, and is known to have an impact on embryo development, specifically in terms of lineage, inducing an enlarged ICM (Harvey & Kaye 1992, Lighten et al, 1988).

Adiponectin is expressed by adipocytes in a negative proportion to fat content. Its receptors allow response of tissues by altering insulin sensitivity (Kim et al 2011). It should be noted that insulin was found to be increased at this stage of development in HF mice Dr Sarah Finn-Sell, personal communication). Pparδ is also readily expressed at the site of implantation and is upregulated in response to lipid accumulation (Lim et al 1999, Luquet et al 2005). Snat1 (slc38a1) is an amino acid transporter, whilst slc27a1 is a fatty acid transporter. These will be studied as uterine adaptation may occur in response to altered dietary intake. The uterus will also be assessed histologically for changes in lipid content.

This chapter will help to characterise the effects that diet is having on the dam around the preimplantation stage, which may aid in interpretation of adaptions of the embryo and subsequent fetus.

3.2 Methods

MF1 mice were naturally mated overnight and pregnancy was confirmed by the observation of a post copulatory plug. Upon the confirmation of pregnancy mice were weighed and allocated randomly to dietary treatment groups; NPD or HPD and HF or chow. Measurement of weight was taken at 10 am on consecutive days until the day of sacrifice for embryo / fetus collection. Weighing mice at the same time of day was done to reduce error associated with metabolic and excretory activity throughout the day. Food consumption was also measured at the time of maternal weight measurement from E0.5 to E3.5. Food was collected manually and weighed to assess the reduction in weight from eating. The percentage composition of each diet was used to calculate specific nutrient consumption (see Figure 2.1.2).

The maternal environment was further analysed in HF dams. Protein content was analysed in the uterus. Protein in the uterus was further analysed by western blot for FGF2, eIF2α, eEF2, S6, S6 kinase and 4EBP1 (section 2.2.1). mRNA expression in the uterus was studied by real time PCR (section 2.2.2). GeNorm was used to determine the most suitable reference genes and expression of *Lif, scl38a1, ERα, Vegf, Igf1, Pparδ, Hgf, AdipoR1 and AdipoR2* were analysed. Frozen sections were made and analysed for lipid content by staining with oil-red-O (section 2.26). The luminal epithelium and glandular regions were studied (Figure 3.2)

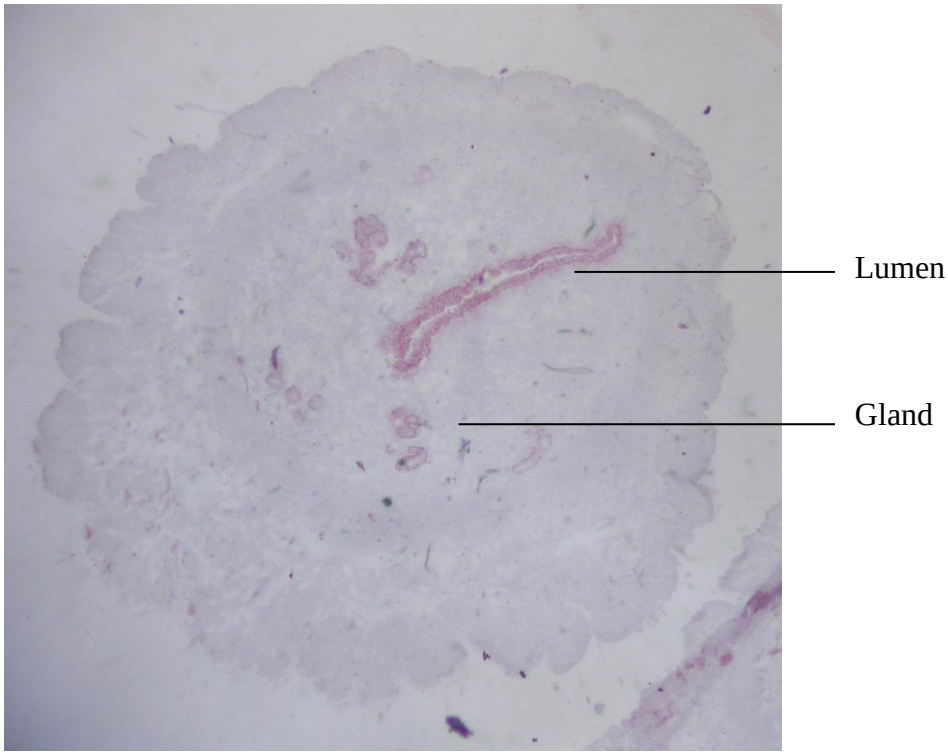


Figure 3.2 Section of uterus

E3.5 stained with haematoxylin and oil-red-O to distinguish cells and lipid. Lumen and glandular regions are indicated.

3.3 Results

3.3.1 Weight change during the preimplantation period

Weight change was measured on a daily basis in mice fed different dietary challenges. HPD was compared to NPD as shown in Figure 3.3, whilst HF was compared to chow as shown in Figure 3.4. NPD mice were the only group to show an average weight gain during the first day of pregnancy, which was significantly more than that of the HPD mice. No difference was observed between the two diets on the second day of pregnancy. On the third day of pregnancy, mice fed HPD gained significantly more weight than NPD controls. The sum of weight change across these three days was not significantly different between diet treatments. Mice fed HF diet showed no significant difference in weight to chow fed controls. Differences remained when data were analysed as a percentage of starting body weight.

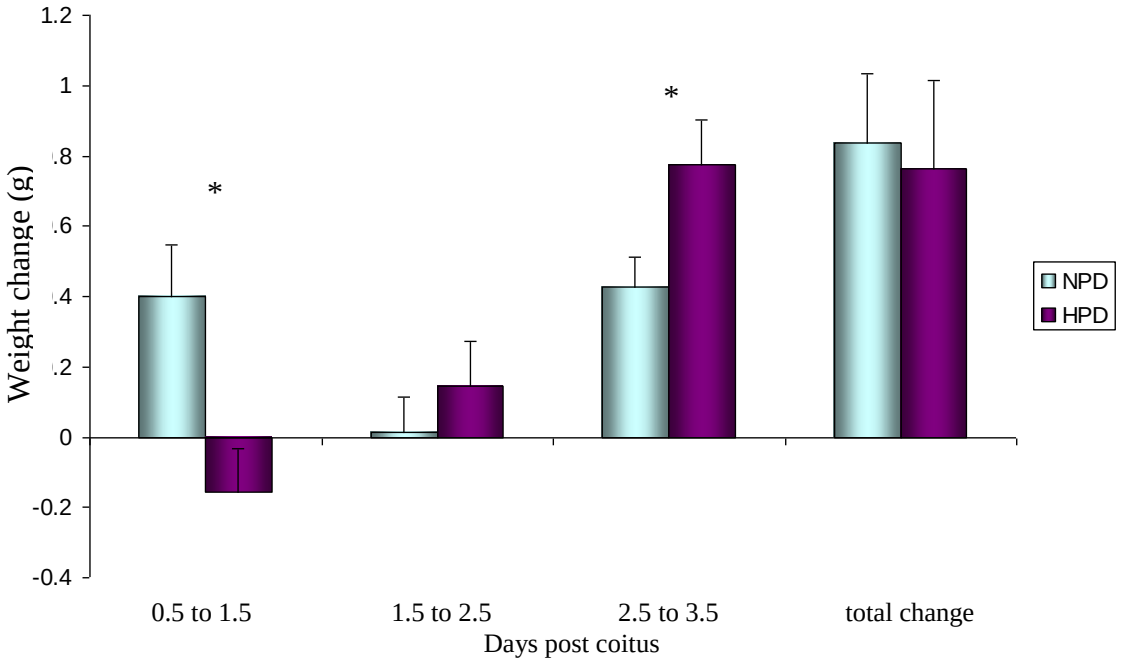


Figure 3.3 Weight change during the period from conception to E3.25 in mice fed HPD or NPD

Average weight change of mice fed NPD and HPD during the first 3 days of pregnancy. NPD mice gained significantly more weight on the first day of pregnancy compared to HPD mice. This was reversed between 2.25 and E3.25 with HPD mice gaining more weight than controls. Overall there was no difference in

weight change during this time between diets. NPD n= 24, HPD n=21. Error bars ±SEM. Statistical analysis by student T-test *P<0.05.

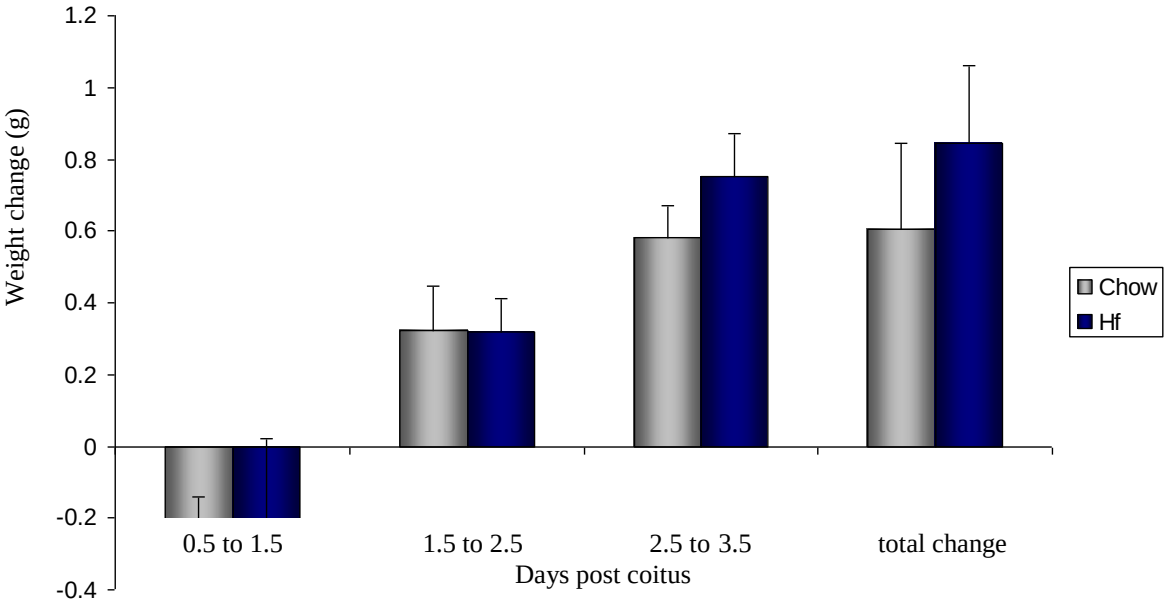


Figure 3.4 Weight change during the period from conception to E3.25 in mice fed HF or Chow
Weight change in pregnant mice fed HF or chow diets. On each day and overall there was no significant difference in weight in these dams during these first days of pregnancy. Chow n= 22, HF n=20. Error bars ±SEM. Statistical analysis by student T-test.

3.3.2 Consumption of modified diets

Food was weighed daily, to determine consumption. No difference in food weight consumed was observed between NPD and HPD (Figure 3.5). The amount of protein consumed was significantly greater on all days measured with feeding of the HPD (Figure 3.6). Other macronutrients were unaltered.

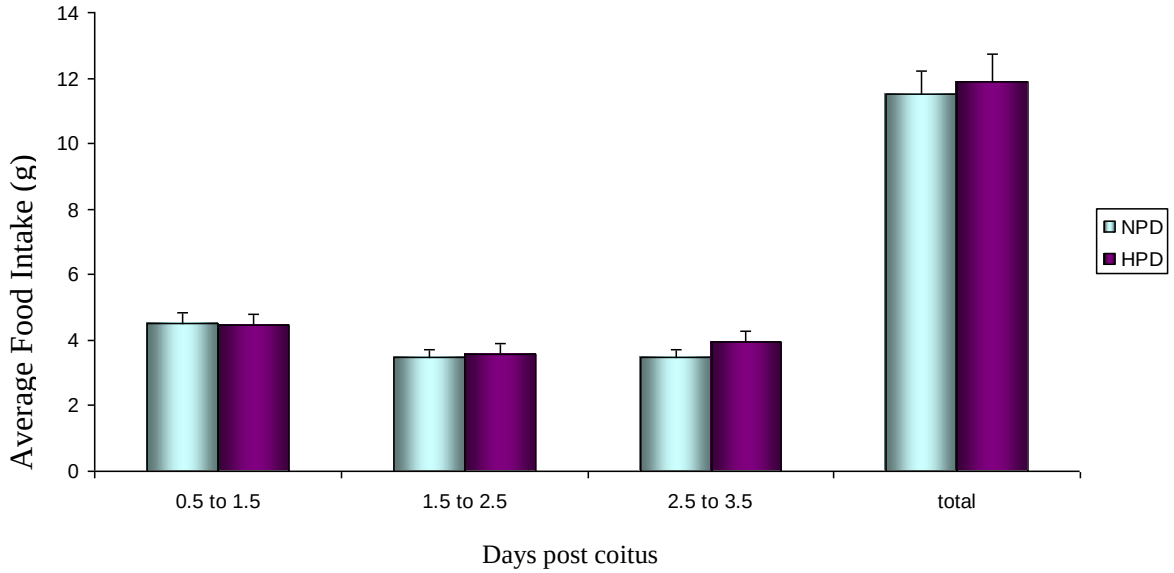


Figure 3.5 Consumption of NPD and HPD during the preimplantation period of pregnancy
Average consumption of NPD and HPD by pregnant mice from observation of post copulatory plug to E3.5. No significant differences were observed in the consumption of these diets. NPD n= 24 HPD n=21. Error bars ±SEM. Statistical analysis by student T-test.

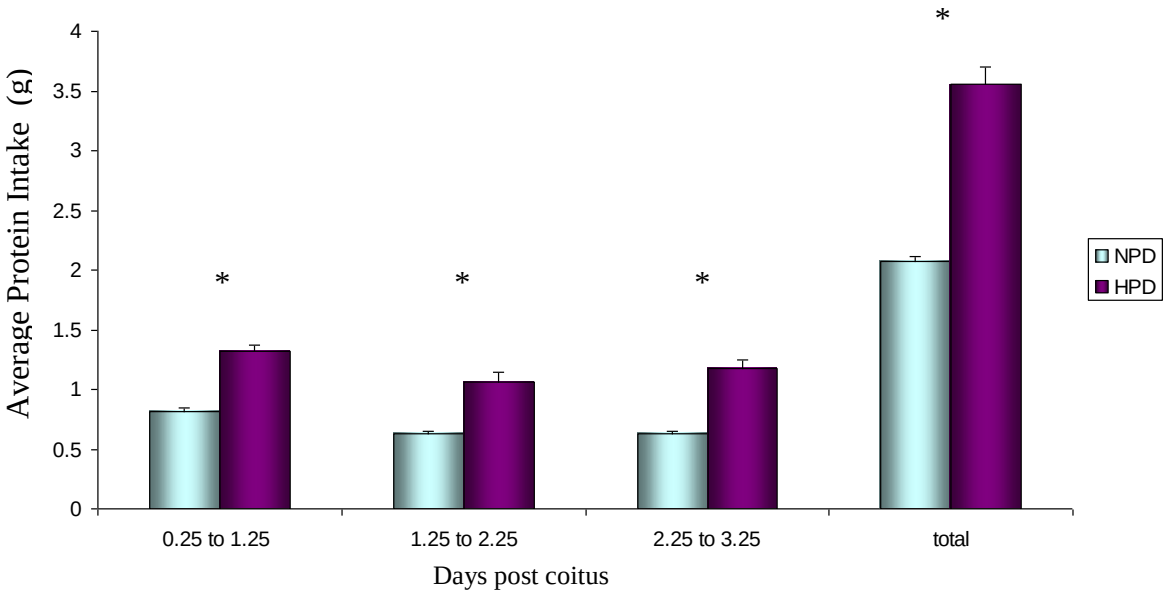


Figure 3.6 Protein intake by mice fed NPD and HPD during the preimplantation period of pregnancy

Average protein intake per dam fed either NPD or HPD from the time of observation of post copulatory plug to E3.5. Mice fed HPD consumed significantly more protein at all times studied than those fed NPD. NPD n= 24 HPD n=21. Error bars ±SEM. *P<0.05. Statistical analysis by student T-test.

A significant reduction was seen in the consumption of the HF diet compared to chow on all days (Figure 3.7). Despite this reduction in intake of the HF diet, the intake of fat remained significantly increased (Figure 3.8). The amount of carbohydrate consumed was reduced in those mice fed HF diet compared to chow (Figure 3.9). Protein consumption was unaffected between HF and chow diet treatments (Figure 3.10). The caloric intake was also unaffected between these diets (Figure 3.11).

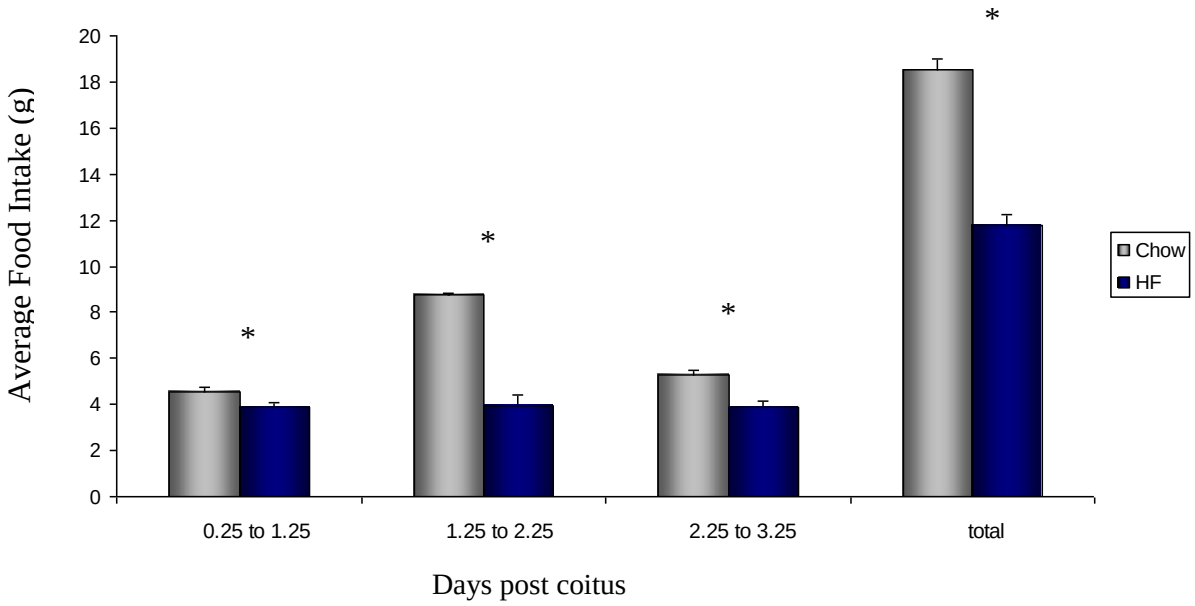


Figure 3.7 Consumption of chow and HF diet during the preimplantation period of pregnancy
Average consumption of HF and chow diets in pregnant dams during the first 3 days post coitum. Mice eat a significantly greater weight of chow on all days observed. The total weight of chow eaten during this period is significantly greater than that of the HF diet. Chow n= 22, HF n=20. Error bars ±SEM. *P<0.05 Statistical analysis by student T-test.

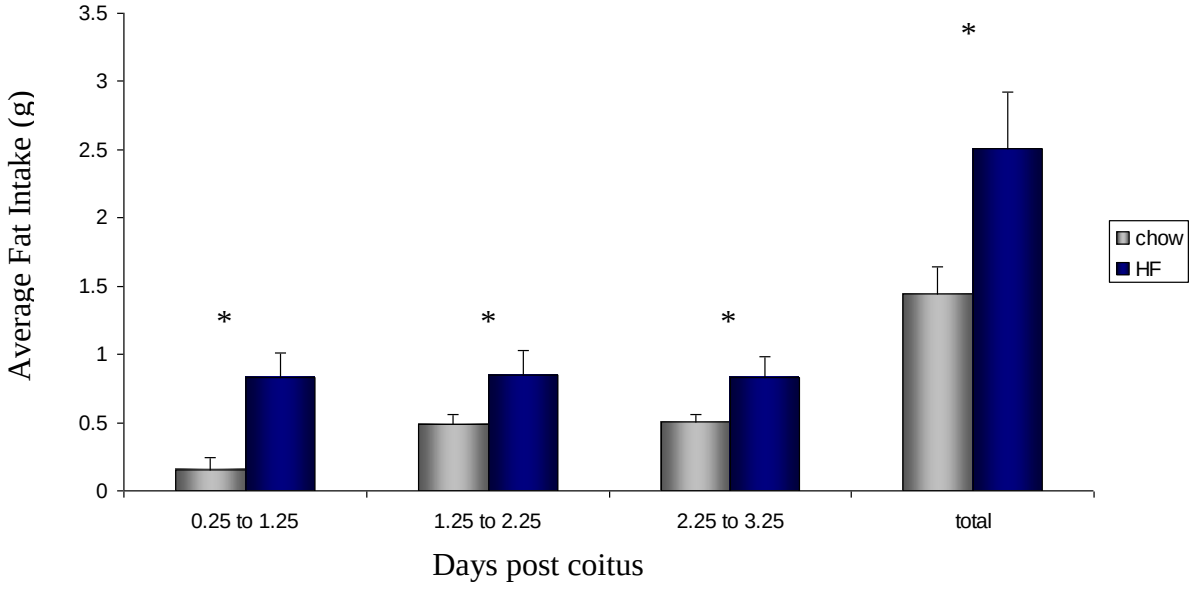


Figure 3.8 Average fat consumption per mouse

The average consumption of fat on the chow and HF diets. Although a greater amount of food is consumed on the chow diet, the fat intake following a HF diet remains significantly increased on each day studied. Chow n= 22, HF n=20. Error bars ±SEM. *P<0.05. Statistical analysis by student T-test.

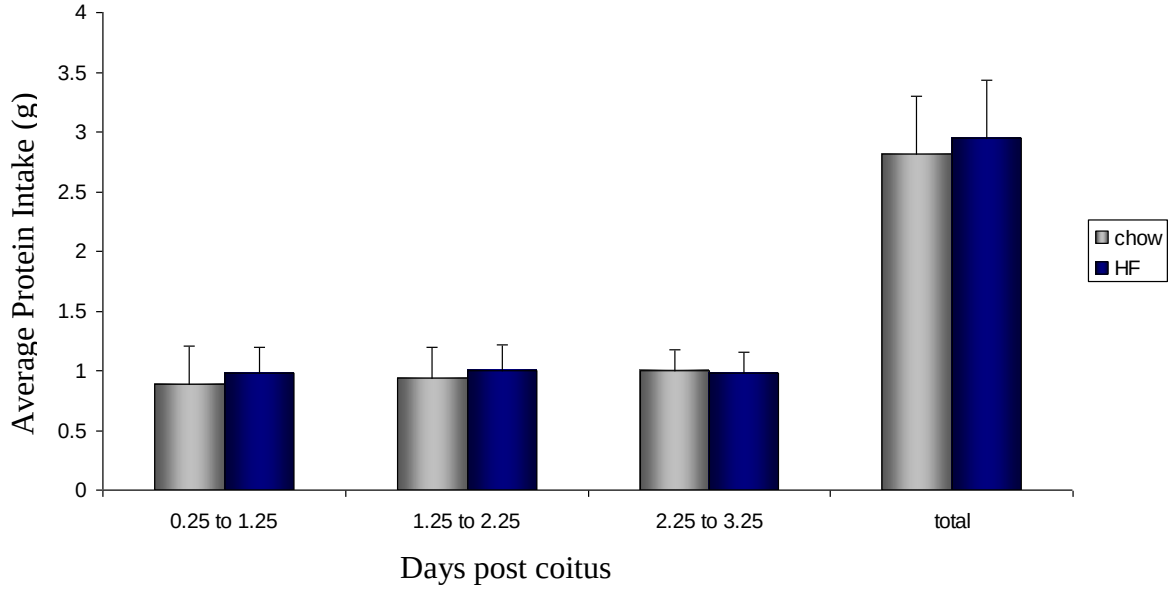


Figure 3.9 Average protein consumption per mouse

The average consumption of protein on the chow and HF diets. Although a greater amount of food is consumed on the chow diet, the protein intake following a HF diet remains stable between diets throughout the study period. Chow n= 22, HF n=20. Error bars ±SEM. Statistical analysis by student T-test.

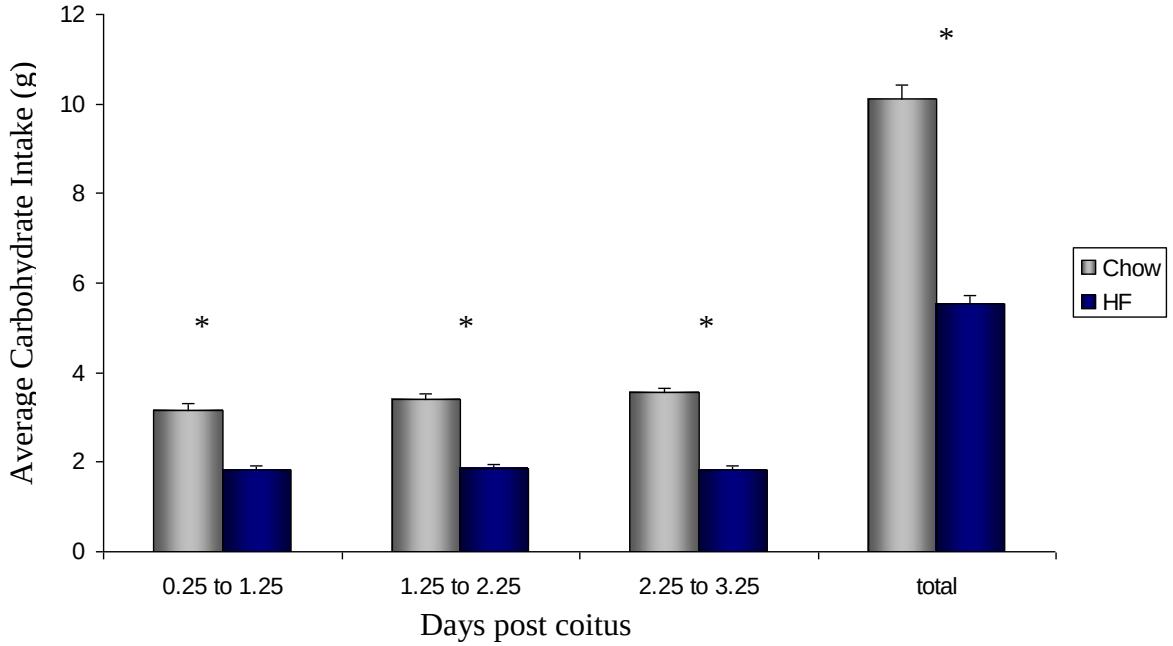


Figure 3.10 Average carbohydrate consumption per mouse

The average consumption of carbohydrate on the chow and HF diets. Dams consuming the chow diet have a raised carbohydrate intake throughout the study compared to those eating the HF diet. Chow n= 22, HF n=20. Error bars ±SEM. *P<0.05. Statistical analysis by student T-test.

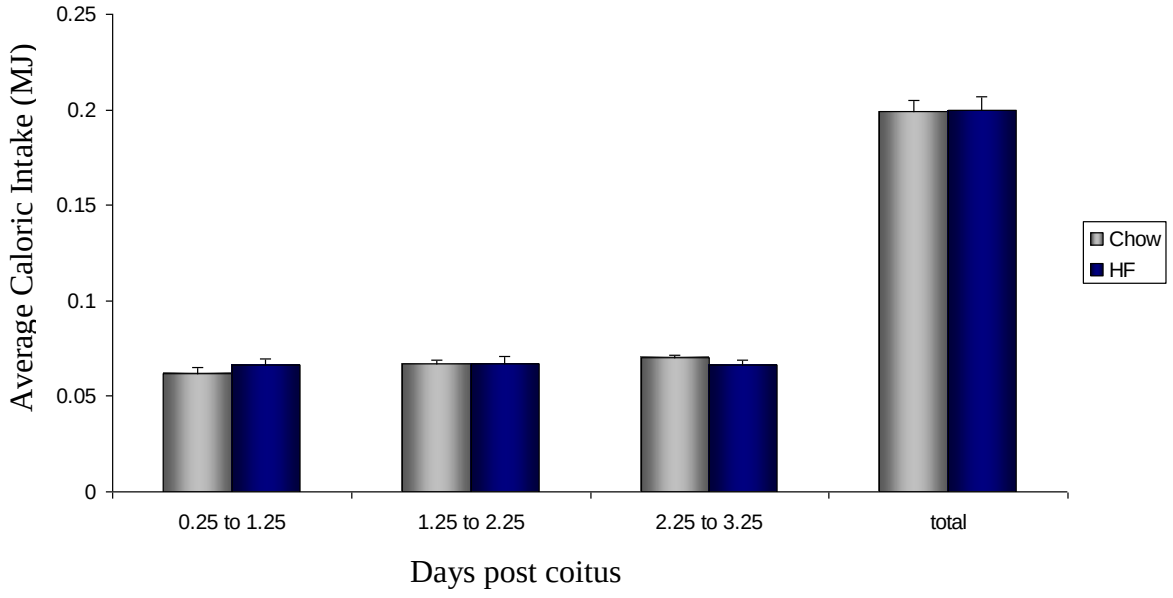


Figure 3.11 Average caloric intake per mouse

The average caloric intake per mouse on the chow and HF diets. Although a greater amount of food is consumed on the chow diet, the caloric intake remains stable between diets throughout the study period. Chow n= 22, HF n=20. Error bars ±SEM. Statistical analysis by student T-test.

3.3.3 Maternal environment

The maternal environment was further analysed in HF dams. Protein content was analysed in the uterus. It was found that total protein content was not affected by dietary treatment (Figure 3.12). Protein in the uterus was further analysed by western blot and antibody detection. Alpha tubulin was used for normalisation, no significant difference was observed between diets when loaded in the same concentration (P=0.14, figure 3.3). Levels of FGF2, an angiogenic molecule, were found to be increased in HF challenged dams (Figure 3.13). No significant differences were observed in the total amounts, or phosphorylated forms of eEF2, p70 S6K, eIF2 or S6 ribosomal protein between uteri from HF and chow fed dams. The ratio between the total and phosphorylated versions of proteins were also analysed, and no significant differences were observed. A trend was observed towards increased levels of the phosphorylated version of the two larger forms (18, 20 KD) of 4eBP1 in HF fed dams, also resulting in a trend towards an increased ratio of the total of the phosphorylated versions of these protein in relation to the total amount expressed (figure 3.14 and 3.15) .

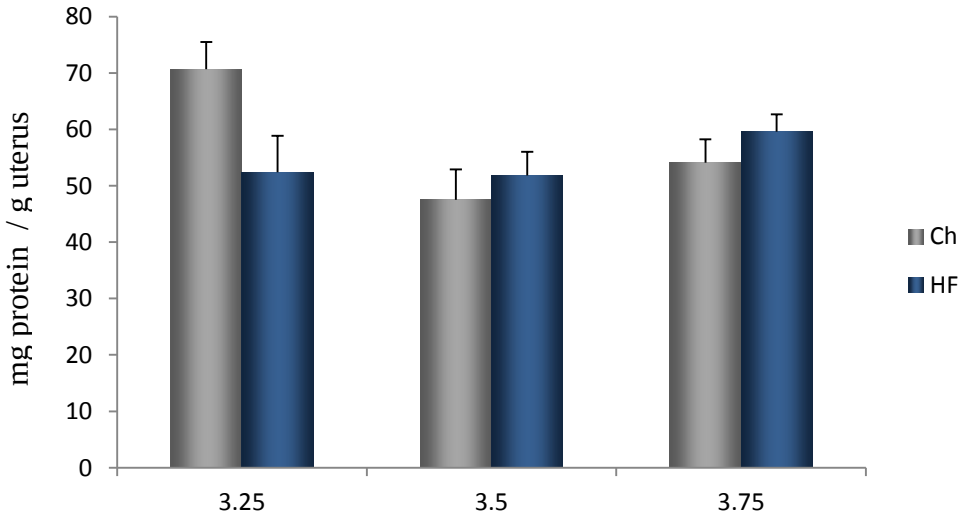
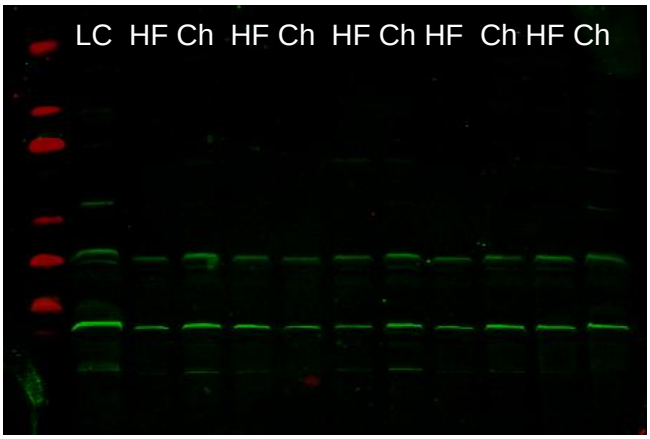


Figure 3.12 Protein content of the uterus

The average protein extracted from uteri of chow and HF challenged dams. No significant difference was observed between diet groups at any time point. E3.5: Chow n= 12, HF n=11, 3.25 and E3.75 Chow n=5, HF n=5 Error bars ±SEM. Statistical analysis by student T-test.

Figure 3.13 FGF2 expression in the uterus

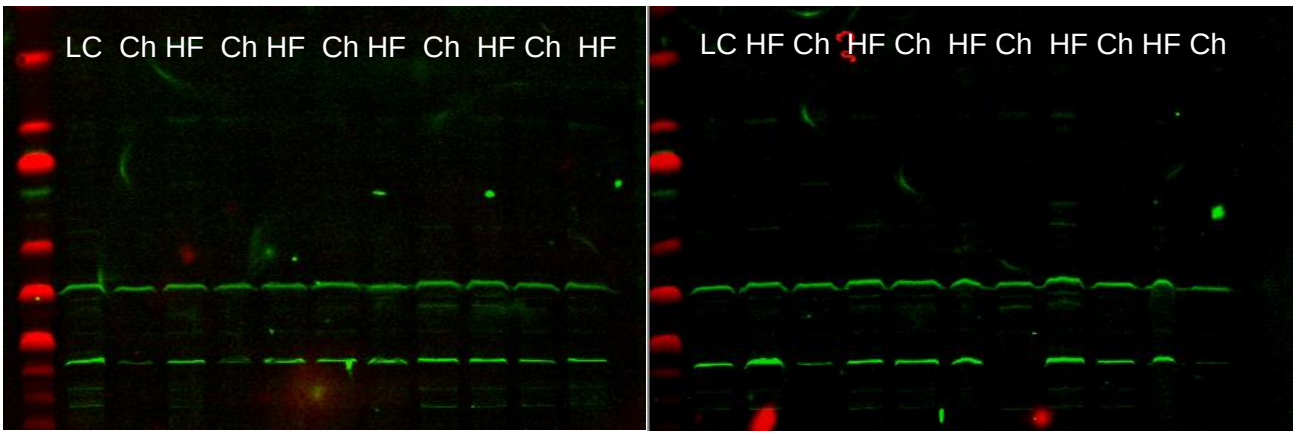
a)



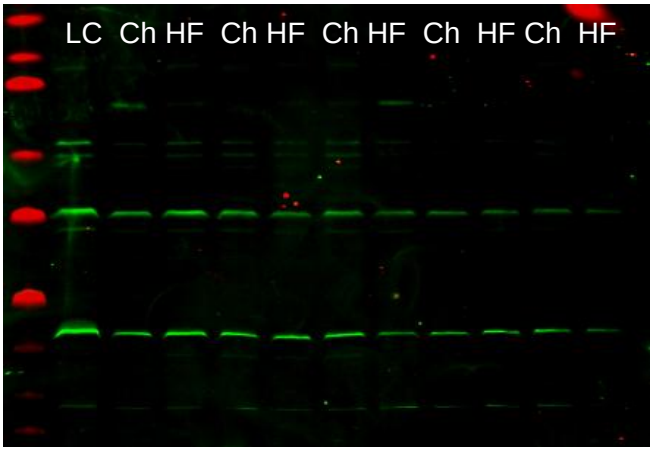
Alpha tubulin

FGF2

b)

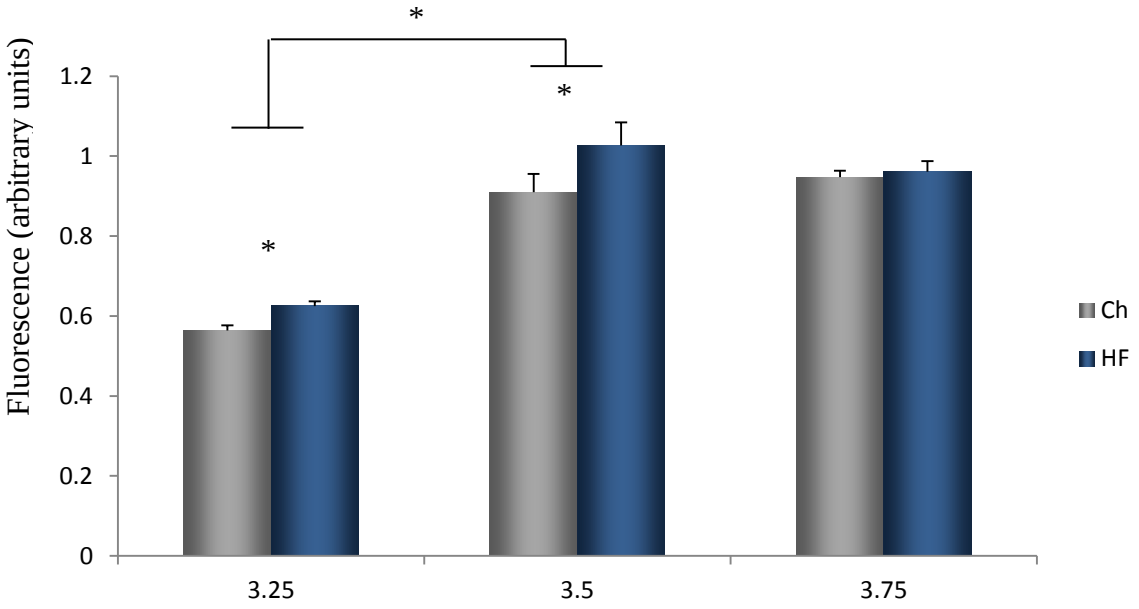


c)

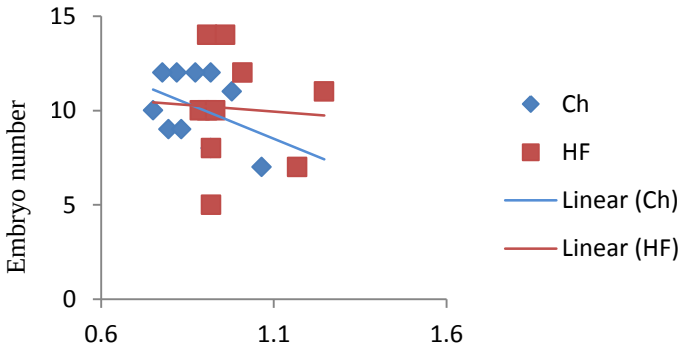


LC: Loading control, Ch: chow fed, HF: HF fed

Figure continued overleaf.



d) Average FGF2 content in the uterus at from E3.25 to E3.75



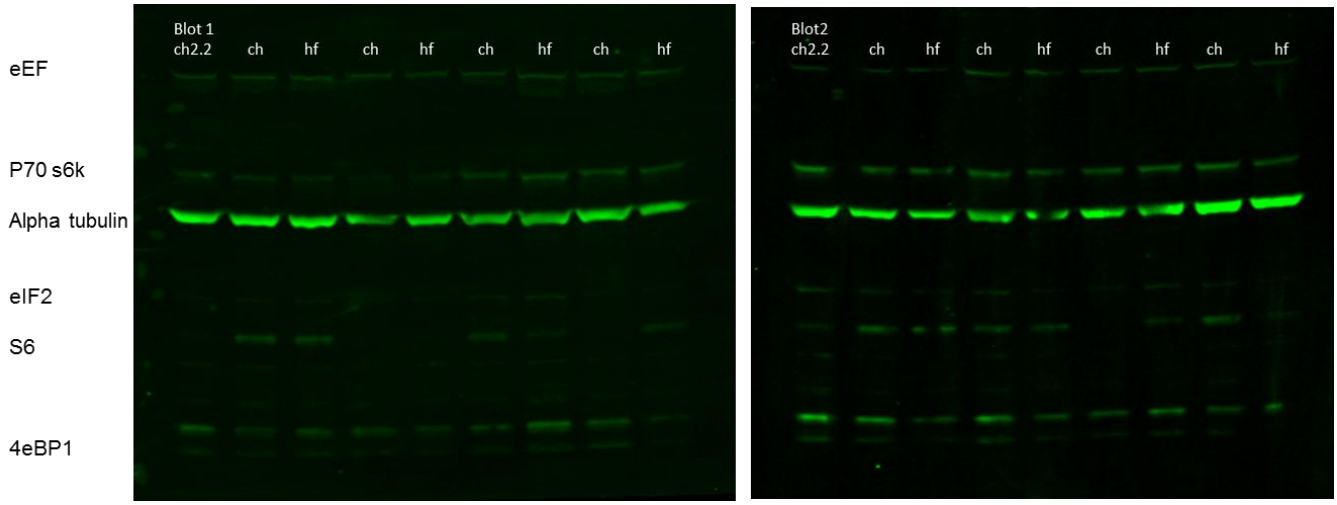
FGF2 fluorescence

e) Correlation between embryo number and FGF2 levels

a-c) western blots of uterus tissue at a) E3.25, b) E3.5 and c) E3.75, upper bands (~40KD) is alpha tubulin, used as a reference, lower band at 22 kD is FGF2. LC denotes loading control, used to normalize between blots. d) FGF2 expression, as determined by western blot, in the uteri of chow and HF fed dams. A significant increase in FGF2 expression was observed in HF fed mice at E3.25 (P= 0.028) (Chow n= 5, HF n=5). A significant increase in FGF2 expression was also observed in HF fed mice at 3.5 (P= 0.035) (Chow n= 10, HF n=9). No significant difference was observed between diets at E3.75 (P= 0.336) (Chow n= 5, HF n=5). A significant increase over time was observed in FGF2 expression in both Ch and HF groups between E3.25 and E3.5 (P= 0.000, and P=0.000 respectively). No differences were seen between groups from E3.5 to E3.75. e) No correlation was observed between the total number of embryos recovered and FGF2 levels. Chow p= 0.263 HF P=0.871. Error bars ±SEM. Statistical analysis by student T-test.

Figure 3.14 Western blotting of members of the mTOR pathway in the uterus at E3.5

a)



b) Phosphorylated forms

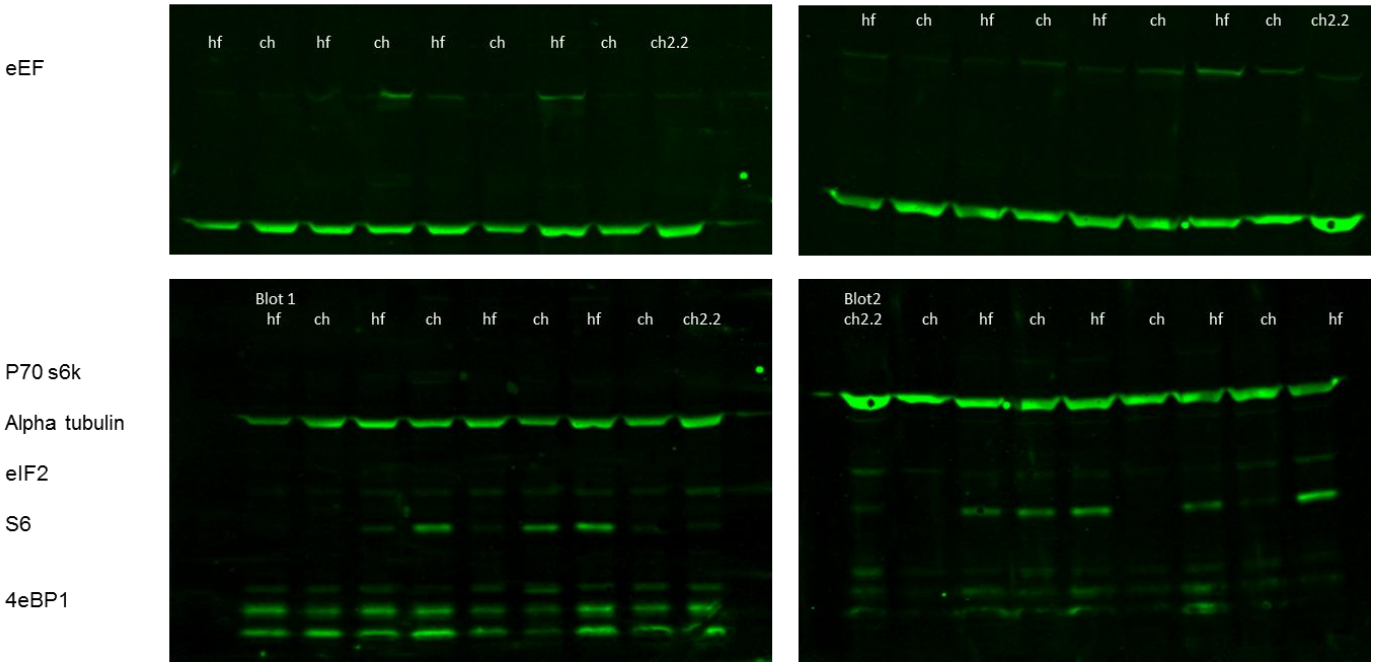
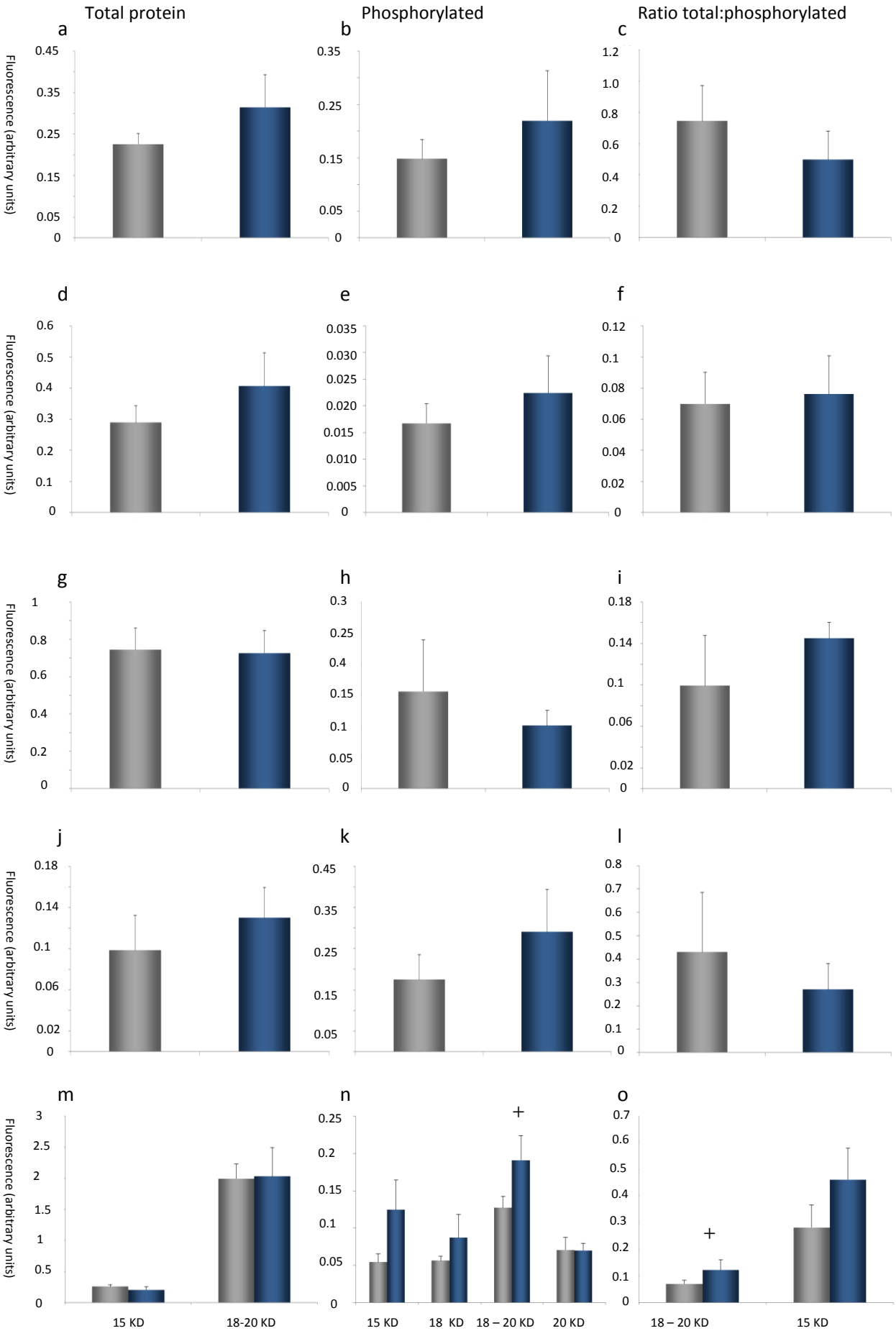


Figure 3.14. Western blots showing a) total expression of eEF, p70 S6k, alpha tubulin (for normalisation purposes), eIF2, S6 ribosomal protein and 4EBP1 and b) the phosphorylated version of these proteins.
Figure 3.15 (right) Expression of proteins involved in the mTOR pathway - eEF (a), P70 S6 (d) , eIF2α (g), S6 (j) and 4eBP1(two isoforms) (m), their phosphorylated forms - eEF (b), P70 S6 (e) , eIF2α (h), S6 (k) and 4eBP1(n) and the ratio between the total expression and phosphorylated forms - eEF (c), P70 S6 (f) , eIF2α (i), S6 (l) and 4eBP1(three isoforms) (o) No significant differences were observed in the total amounts, or phosphorylated forms of eEF2, p70 S6K, eIF2 or S6 ribosomal protein between uteri from HF and chow fed dams. The ratio between the total and phosphorylated versions of proteins were also analysed, and no significant differences were observed. A trend was observed towards increased levels of the phosphorylated version of the two larger forms (18, 20 KD) of 4eBP1 in HF fed dams, also resulting in a trend towards an increased ratio of phosphorylated versions of these protein in relation to the total amount expressed. Statistical analysis by student T-test.

Figure 3.15


Total protein
Phosphorylated
Ratio total:phosphorylated
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
Fluorescence (arbitrary units)
15 KD
18-20 KD
18 KD
18 – 20 KD
20 KD
+

3.3.4 Gene expression in the uterus

Reference gene suitability was first assessed by GeNorm and NormFinder expression analysis. Both these techniques found that *Shda* and P*gk1* were the most suitable pair of genes for normalisation (figure 3.16). *Lif*, *Scl38a1, ERα, Vegf, Igf1, Pparδ, Hgf, AdipoR1* and *adipoR2* expression were examined. A trend towards increased *Lif* expression in HF uteri was observed (figure 3.17).

Genorm	***NormFinder***	
Sdha-Pgk1	*Pgk1*	**Best Pair: *Pgk1-Sdha***
Tuba1	*Sdha*	
Ppib	*Hprt1*	
Hprt1	*Ppib*	
Tbp	*Tuba1*	
18S	*Tbp*	
	18S	

Figure 3.16 Stability of gene expression between Hf and chow uteri was determined by GeNorm and NormFinder software. Both found Sdha and Pgk1 to be the most stable pair of genes and thus were used for normalisation on real time PCR experiments.

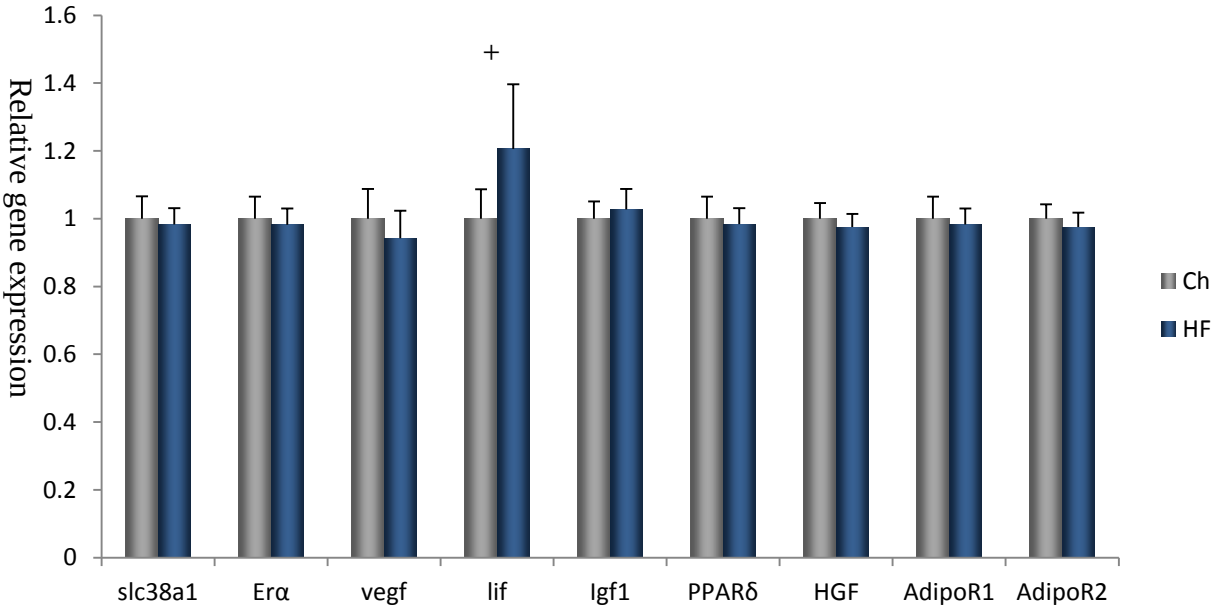


Figure 3.17 Relative Gene Expression in the Uterus at E3.5

Relative gene expression of *Scl38a1, Erα, Vegf, Igf1, Pparδ, Hgf, AdipoR1* and *AdipoR2* were not found to be differentially expressed between HF and Chow uteri. A trend towards increased expression of *Lif* was observed in HF uteri..n= 10 dams per diet. + P<0.1 Statistical analysis by student T-test.

3.3.5 Lipid Content in the uterus

Histological analysis of the uterus at E3.5 for lipid content was performed. A preliminary qualitative assessment of lipid content indicate that lipid droplets covered a greater area and were thus larger or in greater quantity in HF uteri, particularly in the glandular regions (3.18 and 3.19). Further development of analysis of this data is required.

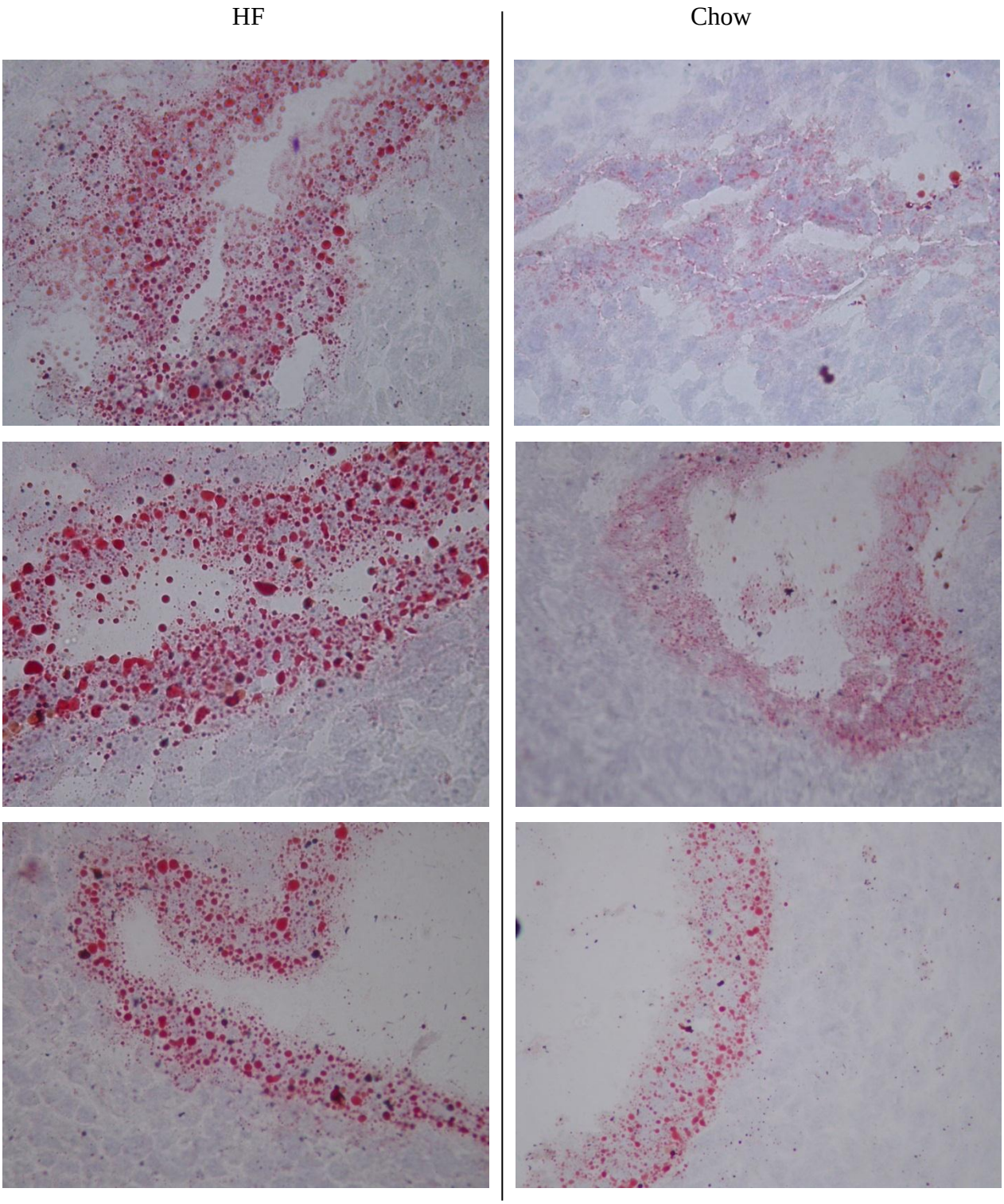


Figure 3.18 Lipid content of the luminal epithelium x20 magnification.
Representative images of uterine lumen stained for lipid content (red) in HF (left) and chow (right) mice. Chow n=7, HF n=8.

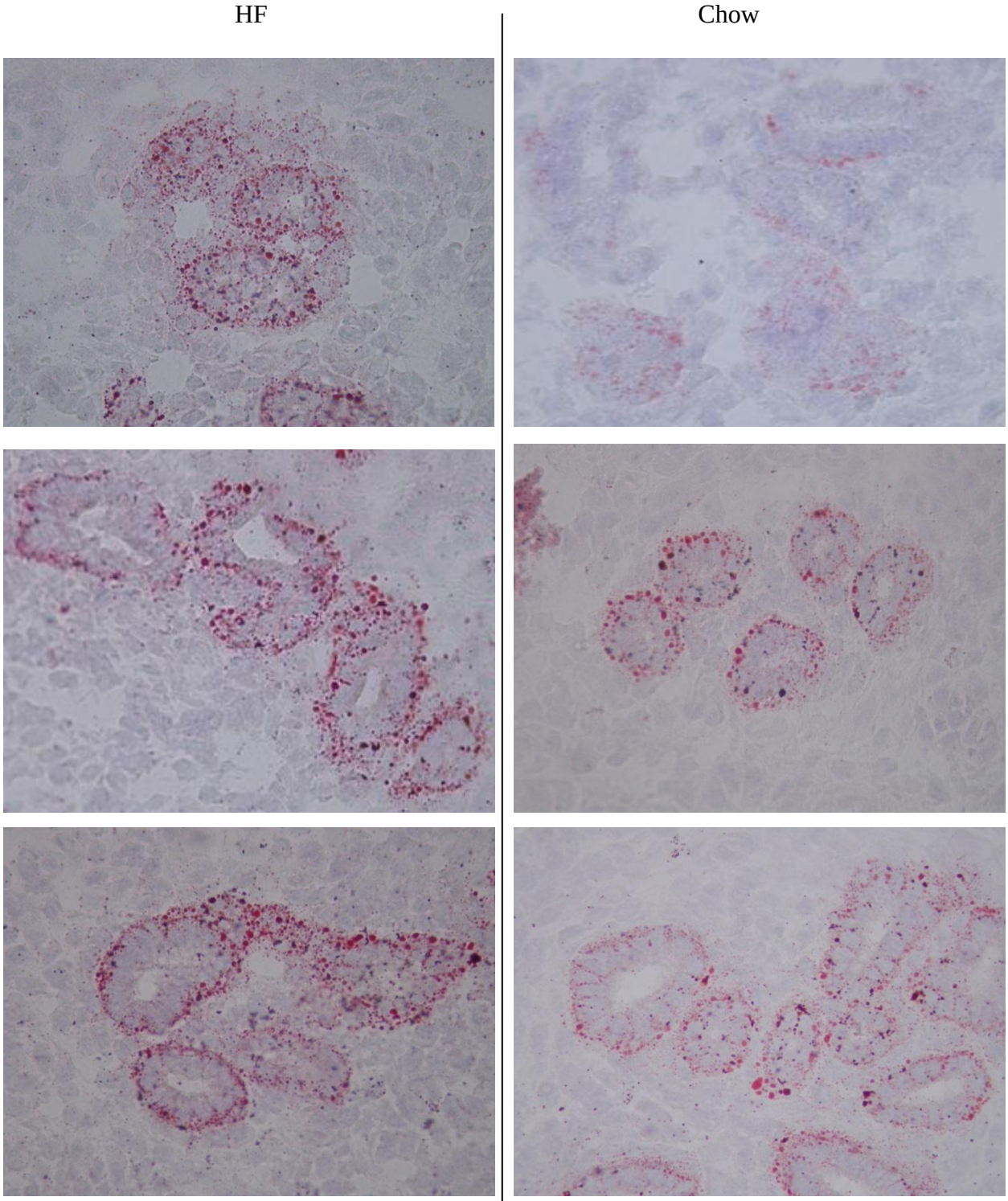


Figure 3.19 Lipid content of the glandular regions x20 magnification
Representative images of uterine glands stained for lipid content (red) in HF (left) and chow (right) mice. Chow n=7, HF n=8.

3.4 Conclusions

Previous studies have shown that maternal dietary composition can influence the development of the embryo. The aim of this chapter is to characterize the maternal environment induced by the diets used in this study that will act as an intermediary step in the establishment of any altered growth trajectory.

Previous data indicate that once pregnant, a mouse's weight should have a steadily increasing upwards trend (Makarova et al 2010). Over the days studied, the resultant weight change was positive in all dietary treatments. However, when the trends in weight are evaluated on a day to day basis, the current data do not concur with the findings of Makarova et al. HPD, HF and chow diet fed mice all lost weight on average on the first day of feeding. This could to be due to stress factors including mating and change from group housing to singular housing. Interestingly, NPD fed mice did not follow this trend and instead showed an increase in weight on the first day of feeding. The NPD group did not follow the increasing weight trend expected across the three days studied, and instead showed a decrease in weight between 1.25 and 2.25 dpc followed by an increase in weight between 2.25 and 3.25 dpc. This suggests that the NPD interacts differently from the other diets with the metabolism of the mouse and may be worth further investigation.

As mice are fed *ad libitum*, consumption of chow and HF diets varied significantly. Food consumption during the preimplantation period of pregnancy is reduced by feeding of the HF diet in comparison to chow. There are several possibilities for this anorexia. The palatability may be poor, or the level of satiation may differ. Rodents are also known to regulate dietary intake according to the nutritional content (Musten et al, 1974). This regulation however is not precise and the intake of the HF and chow diets still results in

differing levels of nutrition gained by consuming these different diets. The adjustment in consumption made by mice resulted in the caloric intake of the HF and chow diets being equal. The fat consumption is however still greatly increased. There is also a significant reduction in the carbohydrate intake with consumption of the HF diet, whilst protein intake remains equal between dietary treatments. This may be an important point to consider.

There are several regulatory mechanisms of appetite that may contribute to the altered consumption of the HF and chow diets. Neuropeptide Y (NPY) is known to be a potent stimulator of food intake and encourage fat accumulation (Kalara et al 1991). NPY is produced in the hypothalamus in response to extrinsic signaling. NPYergic signals originate in the arcuate nucleus (ARC) and terminate at the paraventricular nucleus (PVN). Ghrelin, produced by the stomach provides positive stimulus to the system, and leptin, a hormone produced by adipose tissue can create a positive or negative signal to regulate NPY production (see Figure 3.20). 5-hydroxythryptamine (5-HT) also acts upon NPY neurons (Guy et al 1988).

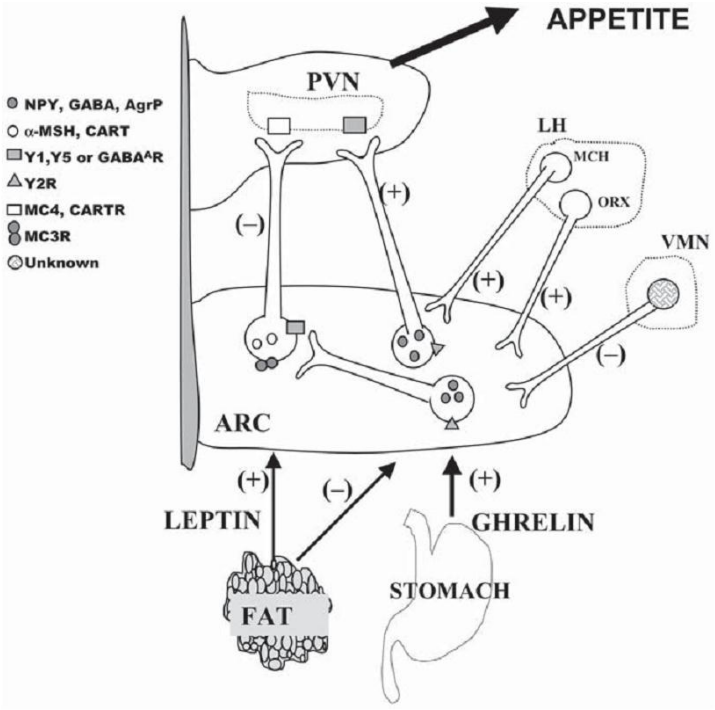


Figure 3.20 Control of appetite, diagram taken from Kalra & Kalra (2003)

The melanocortin system is also involved in the regulation of appetite. Activation of the melanocortin receptor (MC4R) by melanocortin drives satiety, and is indirectly regulated by 5-HT. A subpopulation of neurons in the ARC expresses an endogenous melanocortin precursor (POMC) from which melanocortin is produced. Agouti-related peptide (AgRP) is the endogenous MC4R antagonist. 5-HT is responsible for both activation of POMC expressing neurons and the simultaneous inhibition of AgRP expressing neurons (Heisler et al 2006).

5-HT production is known to be upregulated by consumption of carbohydrates, via the upregulation of insulin (Wurtman & Wurtman 1986). It is a possibility that the decreased consumption of the HF diet in comparison to the chow diet is driven partially by increased insulin in the HF diet (Dr Sarah Finn-Sell, personal communication), leading to

5 H-T mediated decrease in appetite, although increased insulin levels observed seem unlikely to be linked to carbohydrate consumption (being less than in the chow diet).

NPD has a greater carbohydrate content than HPD, but the effect of increased consumption was not observed. However, the difference in carbohydrate content in these diets is far less drastic with the increased weight percentage of carbohydrate being around double that in the HF/chow diets compared to HPD/NPD. It may be that this diet does not cause a physiologically significant increase in insulin to produce this effect.

High concentrations of sugars in the blood stimulate the production of insulin by beta cells of the pancreas. Cells of the liver and muscle take up glucose from the blood and store it as glycogen in response to insulin production. Leptin is another important molecule involved in nutritional status sensing. It is released by adipocytes, and as such, an increased adiposity leads to an increase in circulating leptin. Leptin suppresses both neuropeptide Y and agouti like protein expression, thus reducing appetite (Jequier 2002). In this study it was observed that the diet high in fat led to a reduced appetite. It is likely that circulating leptin is increased to cause this effect and may be an area of investigation to pursue.

No dietary treatment resulted in an overall difference in weight change, despite differing nutritional intake. This could be in part due to the isocaloric nature of these diets, or simply a result of the short term exposure. Research has shown the potential of alteration in dietary composition to cause weight gain or loss (Stern et al, 2004). Low carbohydrate diets are particularly popular currently as a method of weight loss such as the Atkins diet. These diets are thought to occur by alteration in metabolic status (Boden et al 2005).

Increased carbohydrate leads to insulin sensitivity and increased storage of fat (Santos et al, 2012).

Although no differences in weight are seen in this study population, it is likely that the local hormonal status, including known changes to insulin, is affected. A change in circulating molecules is likely to result in altered uterine fluid concentrations, and could thus provide a mechanism for establishing differences in the embryo. It is established that a short term low protein diet leads to a reduction in amino acid concentration in the uterine fluid (Eckert el at, 2012), creating a precedent that short term diet can induce uterine fluid composition.

Changes to the maternal uterine environment were also studied. The total protein content was found to be unaffected by HF dietary challenge. This may not be surprising due to the short term nature of the dietary insult. Levels of a particular protein, FGF2 however are demonstrated to be affected by diet. FGF2, a growth factor and molecule involved in angiogenesis, is shown to be upregulated in response to maternal HF dietary challenge. Its expression is shown to be temporally advanced, as demonstrated by the time course of experiments. FGF2 is a growth factor, and as mentioned in section 3.1 and 1.4.3 is involved in uterine receptivity, angiogenesis and can influence the developing embryo.

It has been shown that FGF2 can be produced downstream of glucocorticoids (GC) (Pavia et al. 2011). GC production acts to increase angiotensin converting enzyme, catalysing the conversion of ANGI to ANGII. ANGII binds to the AT 1 receptor and stimulates the production of FGF2 (Skaletz-Rorowski et al 2004). GCs act generally to regulate macronutrient metabolism by binding to glucocorticoid response elements (GREs) in the

regulatory regions of target genes to alter expression. A raised glucocorticoid expression is associated with insulin resistance (Ferris & Kahn, 2012), an observation found in response to HF diet. Exogenous GC administration has been shown to induce insulin sensitivity clearly within two weeks (Beaudry et al, 2013), it is plausible that this effect could be inducted within the current model. Investigation of glucocorticoid levels in the maternal serum may provide evidence of this occurring in the HF mouse.

FGF2 is shown to be present at implantation sites and this increased incidence may be reflective of cross-talk between the uterus and the embryo at this time. This increase is suggestive of increased implantation sites. Although no correlation was observed between the amount of FGF2 and embryo number, this may not be reflective of the stage of development. Chapter 4 will further discuss indicators that blastocysts are advanced in development and this may link the information gained in this study to the emerging story. However this elevation in FGF2 occurs, it indicates an adaptation in uterine physiology that has the potential to impact upon the developing blastocyst, and should be further investigated.

Protein analysis in the uterus also revealed a trend towards increased 4eBP1 phosphorylation. 4eBP1 is upregulated in response to growth factors and cytokines and is required for cap-dependent initiation of translation. It also inhibits eIF2α in its unphosphorylated state, which was not shown in this study to be affected. As mentioned, this HF diet model shows a trend towards increased insulin levels (Dr Sarah Finn-Sell, personal communication). Insulin is known to affect the mTOR pathway by 4EBP1 phosphorylation (Wang et al, 2006). The trend observed in the present study may be representative of this effect. These results are at a trend level and may therefore require

more statistical power in the model, or perhaps this is representative of this effect being in its early (or even late) stages. Study of this expression at another time point may add weight to this observation. 4EBP1 exists as part of a feedback loop by which phosphorylation of S6K1 can inhibit IRS1 and thus inhibit sensitivity to insulin (Laplante & Sabatini, 2009). Study of this pathway through various timepoints may also reveal this action. Other members of the mTOR pathway in this insulin sensitive pathway such as mSLT8 and raptor may further strengthen this observation.

Gene expression was predominantly unchanged in the real time PCR analysis of the uterus. A trend towards increased Lif expression was seen in HF uteri. This could indicate advancement in the receptivity of the uterus, a correlation with those results observed in FGF2 expression seen here. This will be further discussed in terms of the embryo in chapter 4. PPARδ and HGF, similarly to Lif, is found at implantation sites (Lim et al, 1999; Patel et al, 2000) and therefore may be expected to show similar expression to that of Lif. The absence of this correlation may indicate a deregulation of the signalling process at the time of implantation. A time course study of these different factors may be necessary to further understand if the timing of the expression of these factors is affected by diet. Cross talk between uterine signalling and the embryo may contribute to changes observed. Adiponectin receptors have been shown to be responsive to insulin in several tissues (Sattar & Sattar, 2012), no change in expression of these receptors was observed here. This may be due to differential tissue expression pattern.

Lipid accumulation in cells of the uterus appears greater in the HF dam in both the luminal and glandular regions. This may affect functionality of cells and induce lipid related signalling. Further investigation of this should be undertaken, particularly in terms

of accurate quantification. It may have been predicted that an alteration in lipid accumulation would have had an effect of PPAR expression (Luquet et al, 200), real time PCR results did not find any such correlation. This may be simply due to an insufficient length of exposure to the dietary challenge. It could be that some adaptation occurs that prevents these pathways being activated. It must also be remembered that mRNA expression is not always indicative of response, post translational modification may occur as an example.

Overall, this chapter has established that the maternal environment is altered by diet at E3.5. This is in response to only 3.5 days of exposure to dietary challenge. In particular results indicate that HF diet causes the uterus be in an advanced stage of development. Some indications observed may require further investigation. The following chapter will address how the embryo is affected in relation to this, whilst chapter 5 will determine the effects of this in later gestation.

Chapter 4

Effects of Maternal Dietary Challenge on Preimplantation Embryo Physiology

4.1 Introduction

Dietary challenge fed to the mother solely during the preimplantation period is known to be capable of causing detrimental health outcomes in offspring (Watkins et al, 2008). This chapter looks at changes occurring in the preimplantation embryo in response to HF and HPD feeding.

A major event of the preimplantation period is the first lineage decision, establishing the TE; progenitor of the placenta and the ICM; progenitor of the embryo proper and yolk sac (see also section 1.3.1). Epidemiological studies have shown that in the human population the relative size of the placenta has correlations with blood pressure. Barker et al. (1990) showed that babies that were small with a large placenta were most hypertensive in adulthood, whilst large babies with a small placenta had the lowest adult blood pressure. Godfrey and Robinson (1997) state that fetuses with either a disproportionately small or large placenta have increased standardized mortality ratios. This effect may arise through early adaptations to progenitor cell populations of the placenta (TE) and fetus (ICM).

As shown in table 4.1, there is variation in the effects different diets can have on lineage allocation. Mice fed a low protein diet solely during the preimplantation period show an increased number of cells committed to the TE lineage (Eckert et al, 2012. Mitchell et al (2009) also studied the effects of a low protein diet in mice finding differing results of reduced ICM and no effect on the TE. In this case the low protein diet was fed for four weeks prior to gestation, possibly explaining the differing results. Another confounding factor in this experiment not included in the Southampton experimental design is the superovulation of mice which has previously been shown to be deleterious to proliferation of the blastocyst (Carney & Foot 1990). It is also apparent that the effect of diet is species

specific, with rats undertaking this same LPD feeding regime as used in the Southampton study producing embryos with reduced cell numbers, primarily within the ICM (Kwong et al, 2000).

Diet	Species	ICM	TE	Total cells	Reference
Undernutrition	Sheep	NC	↑	↑	Kakar et al 2005
Overnutrition	Sheep	NC	NC	NC	Kakar et al 2005
Low Protein	Mouse	NC	↑	↑	Eckert, et al, 2012
	Mouse	↓	NC	NC	Mitchell et al 2009
	Rat	NC	↓	↓	Kwong et al 2000
High Protein	Mouse	↓	↑	NC	Mitchell et al 2009
High Fat	Mouse	NC	↑		Minge et al 2008
	Cow	↑	↑	↑	Fouladi-Nashta 2007
Paternal obesity	Mouse	↑	↑	↑	Mitchell et al 2011

Table 4.1 NC- No Change. Summary of studies investigating the effect of diet on lineage allocation. It should be highlighted that conditions of dietary challenge vary between studies. Karkar et al fed ewes 18 days prior to pregnancy until E6. Mitchell et al fed prior to conception and also superovulated dams. Minge et al fed a high fat diet for 16 weeks prior to mating, mice were also obese by this time. Fouladi-Nashta et al fed a high fat supplement (controls 0.87 kg/day, high fat 1.15 kg/day) and collected oocytes which were subjected to in vitro culture to the blastocyst stage. Studies here in Southampton (Eckert and Kwong) used the diet model outlined in section 2.1.4 in which diet was fed only from conception to naturally mated mice.

These previous studies demonstrate the potential of maternal diet to influence lineage allocation in the embryo and the variability of results from different treatments. Here we aim to directly compare the effects of different diets in a standardized model system (figure 2.2 chapter 2: diet model one) to determine the specificity of response by the embryo to dietary treatment. Effects may be dose dependent in response to maternal protein, this will be investigated by comparing HPD embryos to previous results from our lab studying LPD embryos. Embryos may respond to overnutrition in a standardised manner which will be studied by comparison of HPD and HF embryos. Alternatively,

adaptation by the embryo may be standard in response to any poor diet, in which case we would expect to find the same embryo phenotype under all dietary treatments.

HF embryo physiology was further investigated with the use of immunohistochemistry to examine lineage allocation, and methionine analogue incorporation to investigate protein biosynthesis.

Immunohistochemistry will allow us to study lineage on the basis of transcription, rather than simply cell position. It will also allow for study of the next lineage allocation of the primitive endoderm; which will go on to form the yolk sac (Yamanaka, 2011). The yolk sac is heavily involved in fetal circulation (Palis & Yoder, 2001) and has been previously implicated in adaptations in response to maternal diet (Watkins et al, 2008).

Protein synthesis increases around the morula-blastocyst stage (Brinster et al 1976). Much of protein turnover, around 90%, occurs in the TE, as determined by amino acid and oxygen consumption (Houghton 2006). During these first few days of development amino acid turnover has been shown to be a predictor of viability, Houghton et al (2002) demonstrated that those within the lower turnover ranges are more likely to develop to the blastocyst stage. This has been previously studied in the LPD and HPD with no difference in synthesis seen in the LPD embryos, but a significant increase in the HPD embryos (Dr Judith Eckert, personal communication).

4.2 Methods

MF1 mice were naturally mated and pregnancy was confirmed by the presence of a post copulatory plug. Pregnant mice were randomly allocated to an experimental diet; NPD, HPD, Chow or HF. Mice were sacrificed by cervical dislocation at either E3.25 and E3.5 for HF/chow diets or E3.5 and E3.75 for HPD/NPD diets (section 2.1.1 to 2.1.3). Embryos were collected by uterine flushing. The number of embryos and their stage of development was recorded (section 2.3.1).

Embryos collected at E3.25 were predominantly of the morula or early blastocyst stage. In these embryos, total cell number was assessed by measurement of nuclei number. Embryos collected at either E3.5 or E3.75 were processed for differential nuclear labelling to determine cell number in both TE and ICM lineages (section 2.3.3).

Further cohorts of HF embryos were collected. Embryos collected at E3.25 and E3.5 were processed for immunohistochemistry. Embryos were fixed in 2% formalin for 20 mins and processed as previously described. SOX2 expression was studied in E3.25 embryos and SOX2, CDX2 and GATA4 expression was investigated at E3.5 (section 2.3.4).

Protein synthesis in the HF/Chow was measured in the blastocyst collected at E3.5 by Click-iT TAMRA protein synthesis kit. An analogue of methionine, AHA, was included in media during overnight incubation of the blastocyst. New proteins will incorporate the AHA molecule, which can be detected by Click-iT reaction of AHA with a TAMRA molecule. Protein was precipitated and analysed by western blot. Intensity of staining was measured by Odyssey software (section 2.5).

4.4 Results

4.4.1 The effects of dietary challenge on embryo number and stage of development

At collection, the number of embryos and their stage of development were recorded. This may provide an initial insight into the effects of diet on the developmental rate and the suitability of the uterine environment. No significant differences were observed in the number of embryos collected or their stage of development at either of the time points studied between NPD and HPD (Figures 4.1 & 4.2). A significant reduction in both the number of embryos collected, and the number of blastocysts collected was observed in HF embryos at E3.25. There was no significant difference in the number of morulae recovered (Figure 4.3). At E3.5, no significant differences were found in the number of embryos, or their stage of development (Figure 4.4). No significant differences were observed in the numbers of mice which were flushed due to observation of a post copulatory plug, but in which no embryos were obtained (data not shown).

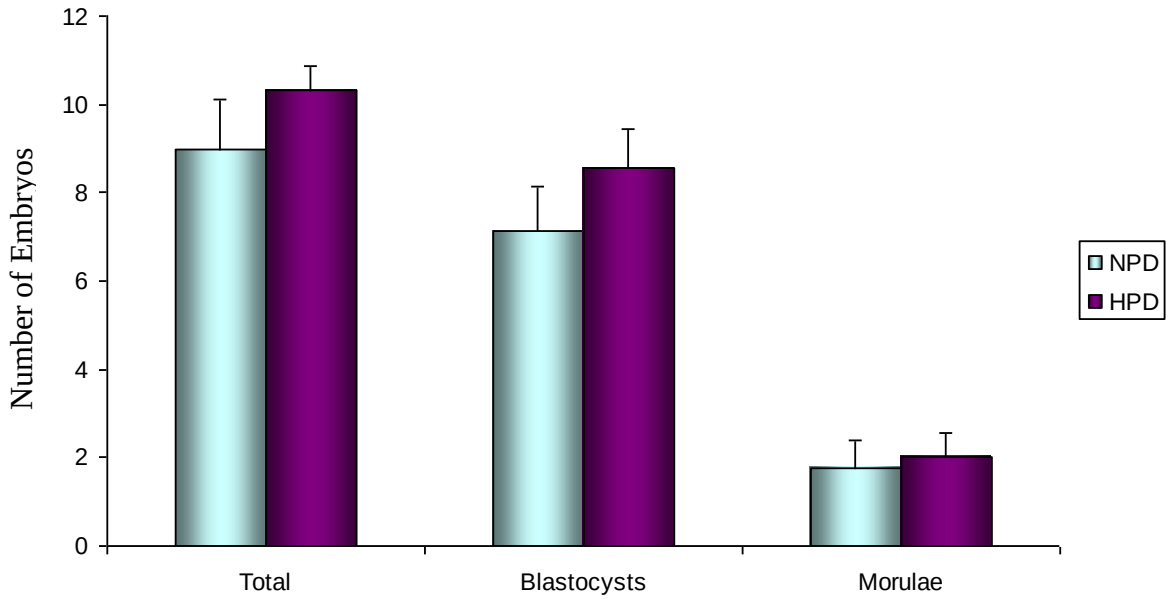


Figure 4.1 Embryo number and stage collected at E3.5 after protein challenge

The average total number of embryos collected per dam and the number of which were at the blastocyst and morula stage at E3.5. No significant differences were seen between dietary treatments in either total number or the stage of development. HPD n=25, NPD n=25. Error bars ±SEM. Statistical analysis by student T-test.

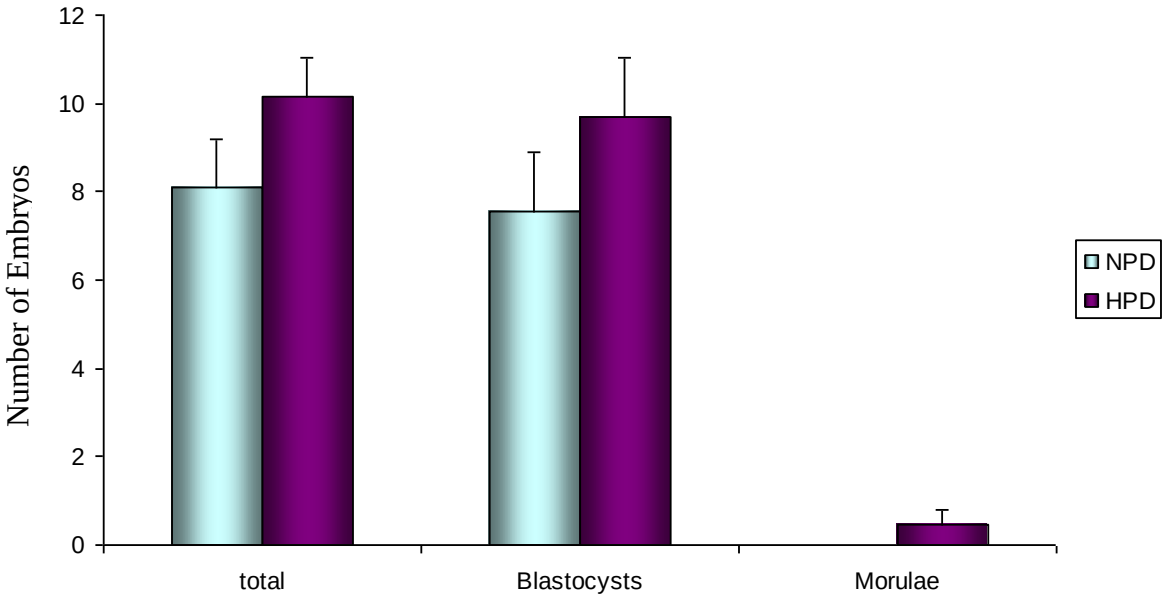


Figure 4.2 Embryo number and stage collected at E3.75 after protein challenge

The average total number of embryos collected per dam and the number of which were at the blastocyst and morula stage at E3.75. HPD n=25, NPD n=25. Error bars ±SEM. Statistical analysis by student T-test.

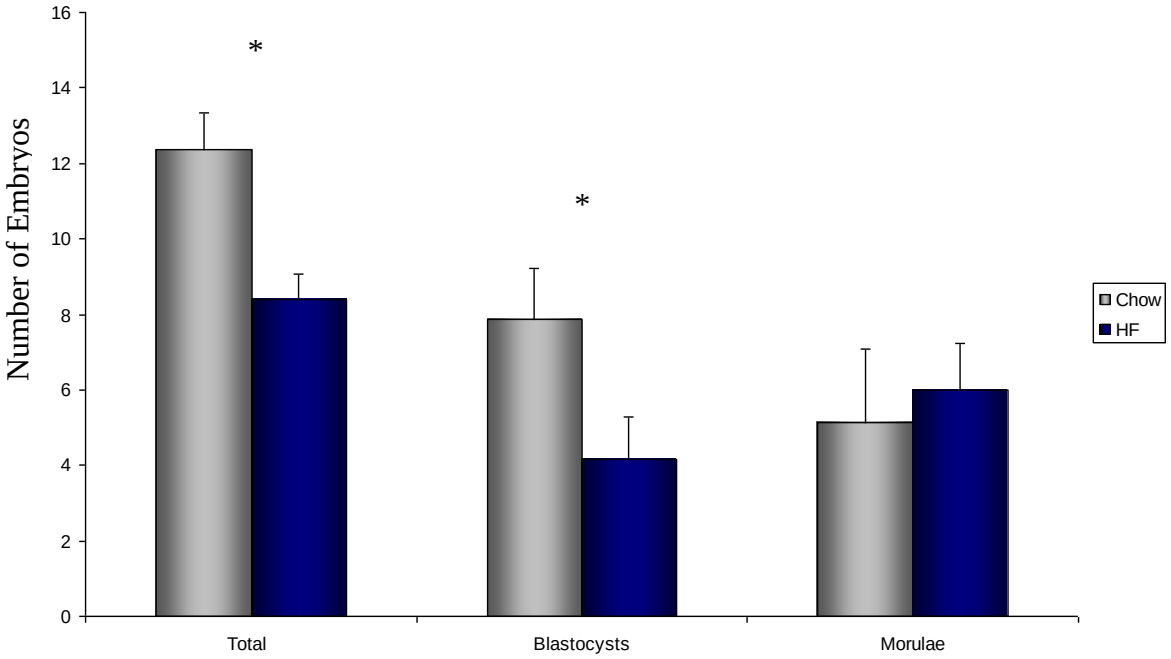


Figure 4.3 Embryo number and stage collected at E3.25 following lipid challenge

The average total number of embryos collected per dam and the number of which were at the blastocyst and morula stage at E3.25. A significant reduction in the total number of embryos and the number of blastocysts collected was observed. HF n=25, Chow n=21. Error bars ±SEM. *P<0.05. Statistical analysis by student T-test.

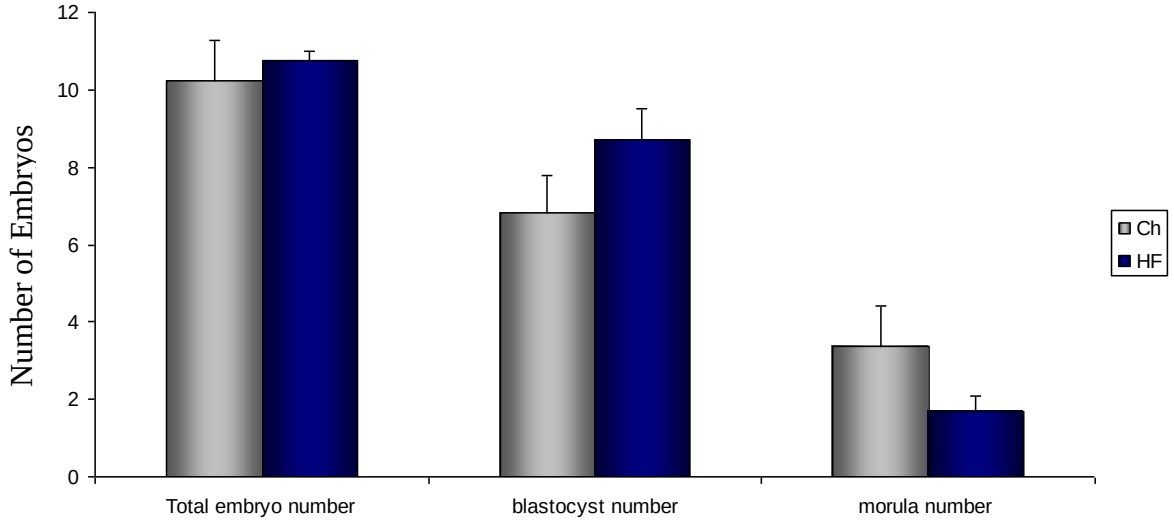


Figure 4.4 Embryo number and stage collected at E3.5 following lipid challenge

The average total number of embryos collected per dam and the number of which were at the blastocyst and morula stage at E3.5. Dietary treatment made no significant alterations to either total number of embryos or their stage of development. HF n=25, Chow n=21. Error bars ±SEM. *P<0.05. Statistical analysis by student T-test.

4.4.2 The effects of dietary challenge on cell number and lineage allocation

Cell number was determined by nuclear labelling with Hoechst, whilst the differential labelling technique was utilised to indicate the ICM and TE lineages by position in the embryo. Immunohistochemistry was used to determine lineage based on lineage marker expression.

No significant differences were observed in the number of cells in blastocysts collected at E3.5 between NPD and HPD embryos (Figure 4.5). At E3.75, a significant reduction in the number of cells in the ICM was observed (Figure 4.6). This significantly reduced the ratio of ICM to TE, causing a preference to the TE lineage (Figure 4.7).

No significant differences were observed in the total number of cells in the blastocyst or morula at E3.25 between HF and chow diets as determined by differential labelling

(Figures 4.7 & 4.8). At E3.5, a significant increase in proliferation can be observed in both lineages in the HF blastocyst. This causes an increase in the ratio of ICM to TE cells, with a preference for the ICM lineage in HF embryos (Figure 4.10).

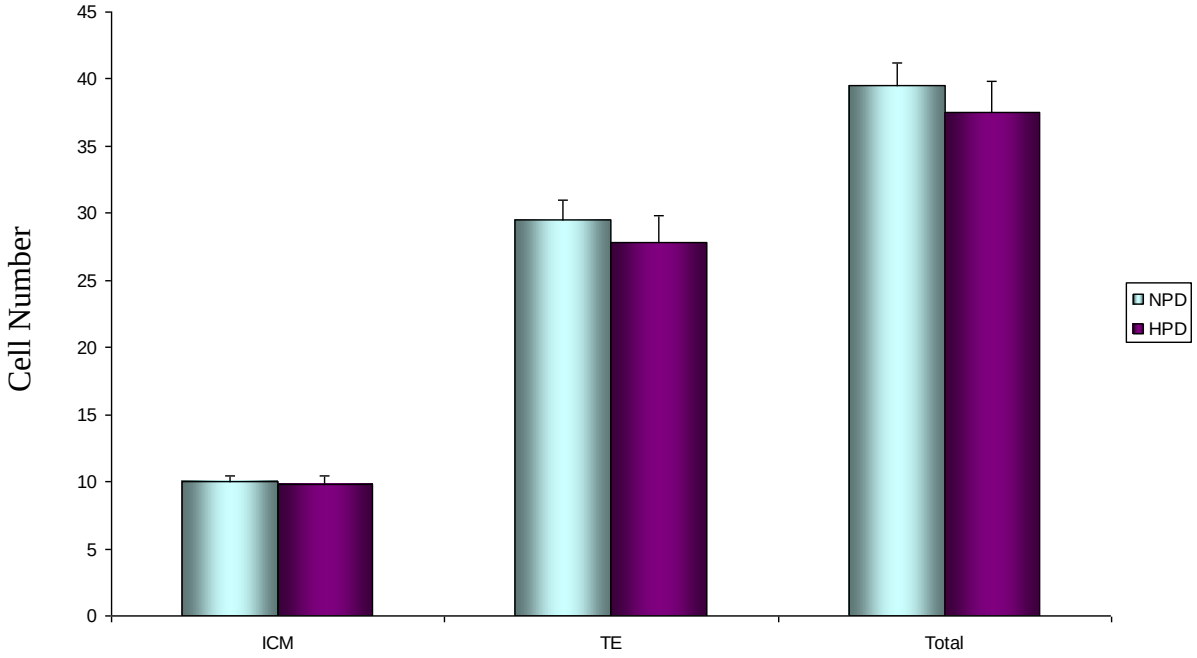


Figure 4.5 Cell number and lineage allocation in the blastocyst collected at E3.5

Cell number and lineage allocation in the embryo at E3.5 as determined by differential labelling from mice fed either NPD or HPD from conception. NPD: dams n=11 embryos n=40, HPD: dams n=10 embryos n=48. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

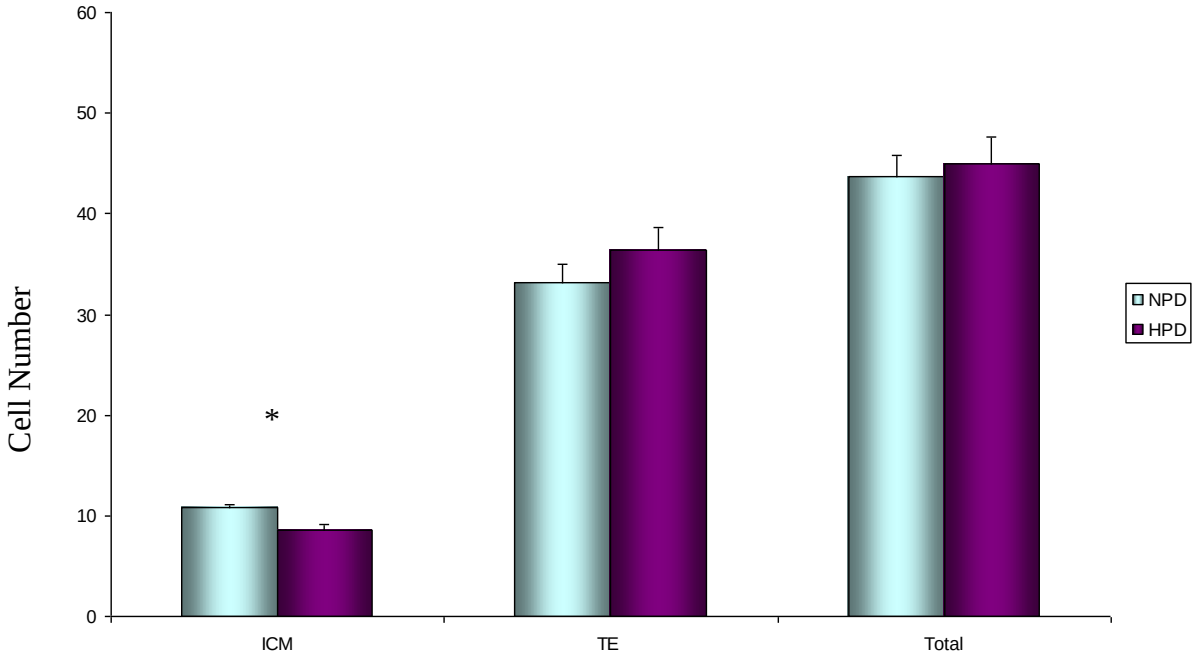


Figure 4.6 Cell number and lineage allocation in the blastocyst collected at E3.75

The average cell number in ICM and TE lineages, and total cell number in embryos collected at E3.75 from mice fed either NPD or HPD as determined by differential labelling. A significant reduction was observed in the number of cells in the ICM lineage of embryos from a HPD maternal environment compare. NPD: dams n=12 embryos n=43, HPD: dams n=14 embryos n=79. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

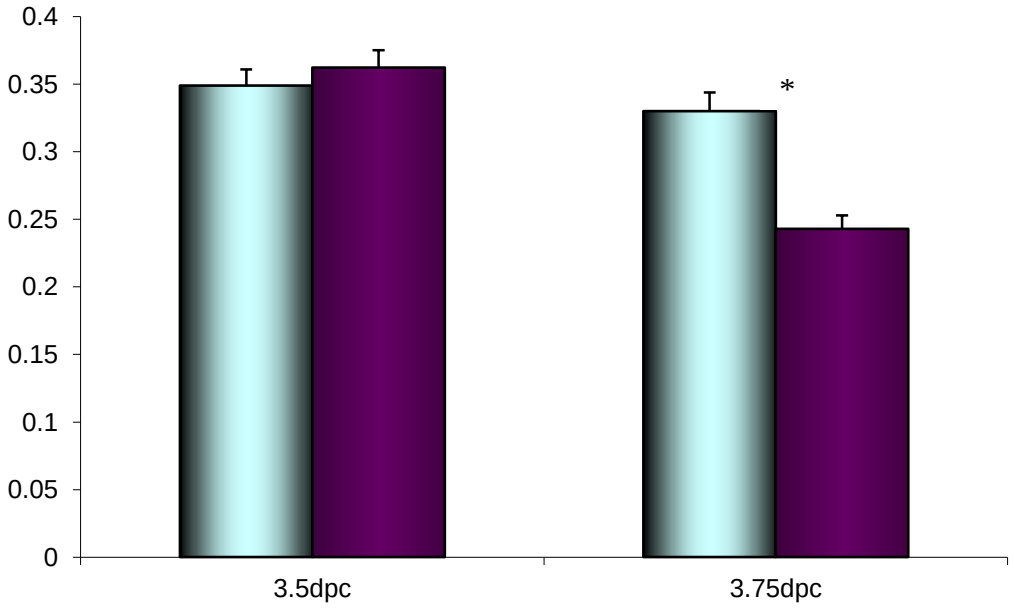


Figure 4.7 The ratio of ICM to TE

The ratio of ICM/TE as determined by differential labeling in blastocysts collected from NPD/HPD maternal environments, at E3.5 or E3.75. At E3.75, HPD embryos have a significantly greater proportion of

TE cells than NPD embryos. N numbers are as stated previously. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

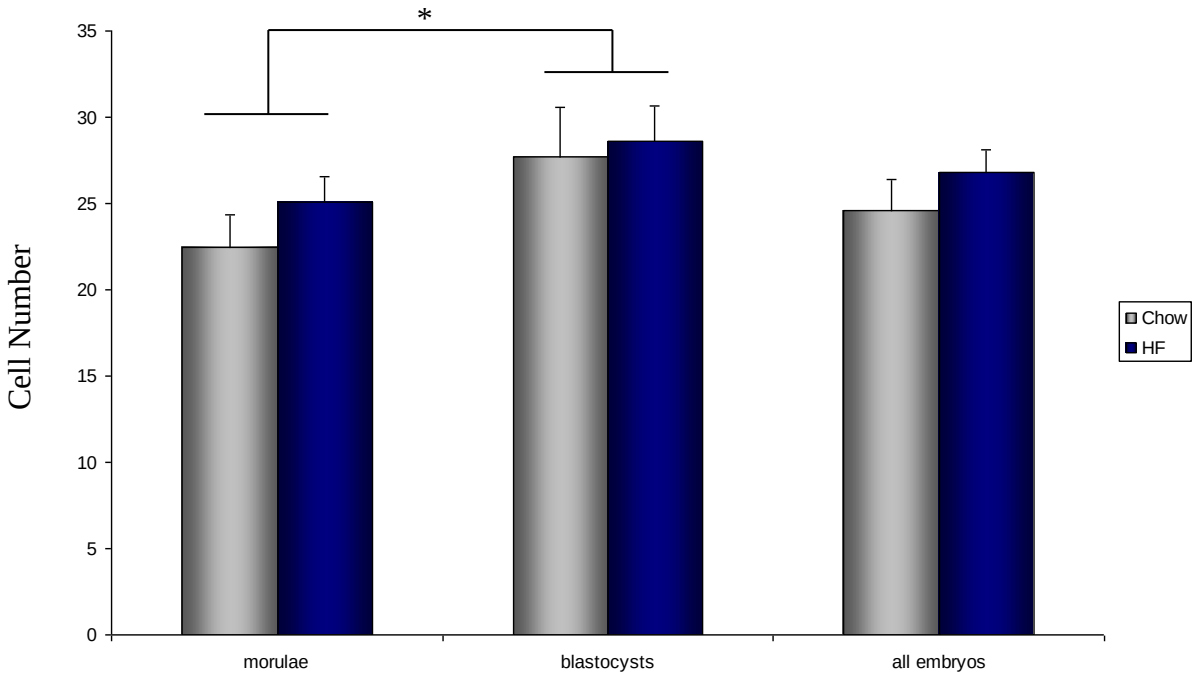


Figure 4.8 Cell number in morulae and blastocysts collected at E3.25

Average cell number at E3.25 after feeding of Chow and HF diets from conception. Error bars ±SEM. *P<0.05. No significant differences were observed between dietary treatments. Blastocysts had significantly more cells compared to morulae in both dietary treatments. Chow: dams n=9 embryos n=45, HF: dams n=7 embryos n=37. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

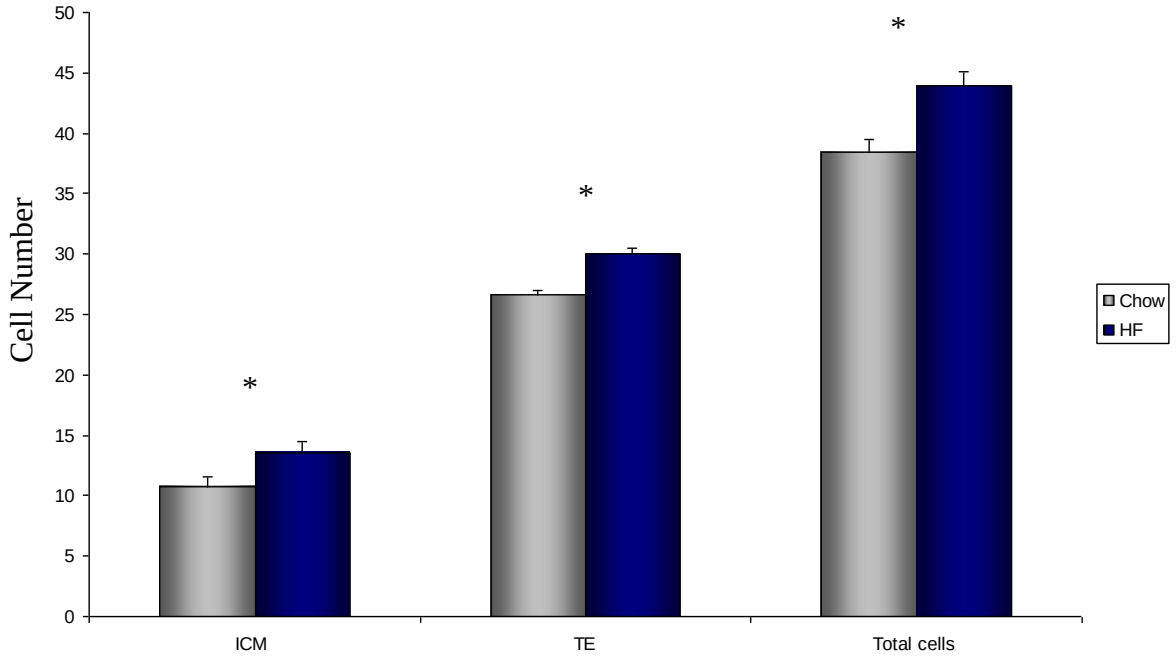


Figure 4.9 Cell number and lineage allocation in the blastocyst collected at E3.5

Cell number and lineage allocation in the embryo at E3.5 from dams fed HF or chow diets as determined by differential labelling. A significant increase was observed in the number of cells in both ICM and TE lineages in HF embryos. This also resulted in a significant increase in the total number of cells in the HF blastocyst. Chow: dams n=9 embryos n=80, HF: dams n=10 embryos n=88. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

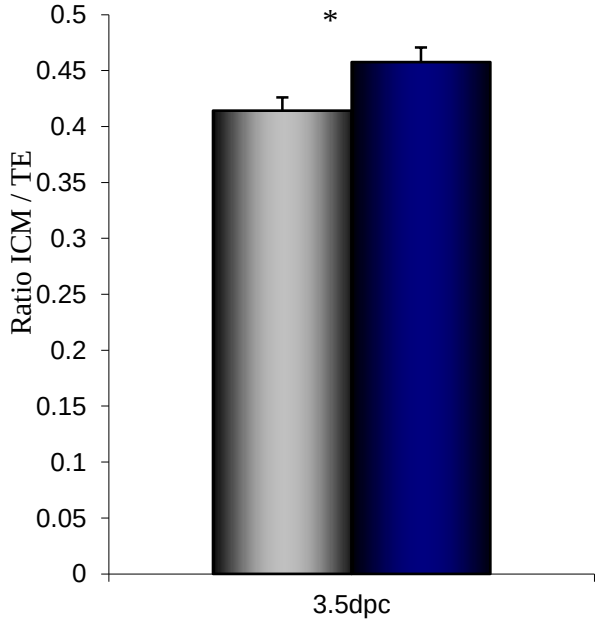


Figure 4.10 The ratio of ICM to TE

The ratio of ICM/TE as determined by differential labeling in blastocysts collected from HF/chow maternal environments at E3.5. HF embryos have a significantly greater proportion of ICM cells than chow embryos. N numbers are as stated previously. Error bars ±SEM. *P<0.05. Statistical analysis was performed by multilevel random effects regression

Lineage commitment at E3.25, as shown by expression of SOX2, was affected in blastocysts. A trend towards a reduced number of SOX2 expressing cells, along with a significant decrease in percentage of SOX2 positive cells was observed in the HF blastocyst (Figure 4.11).

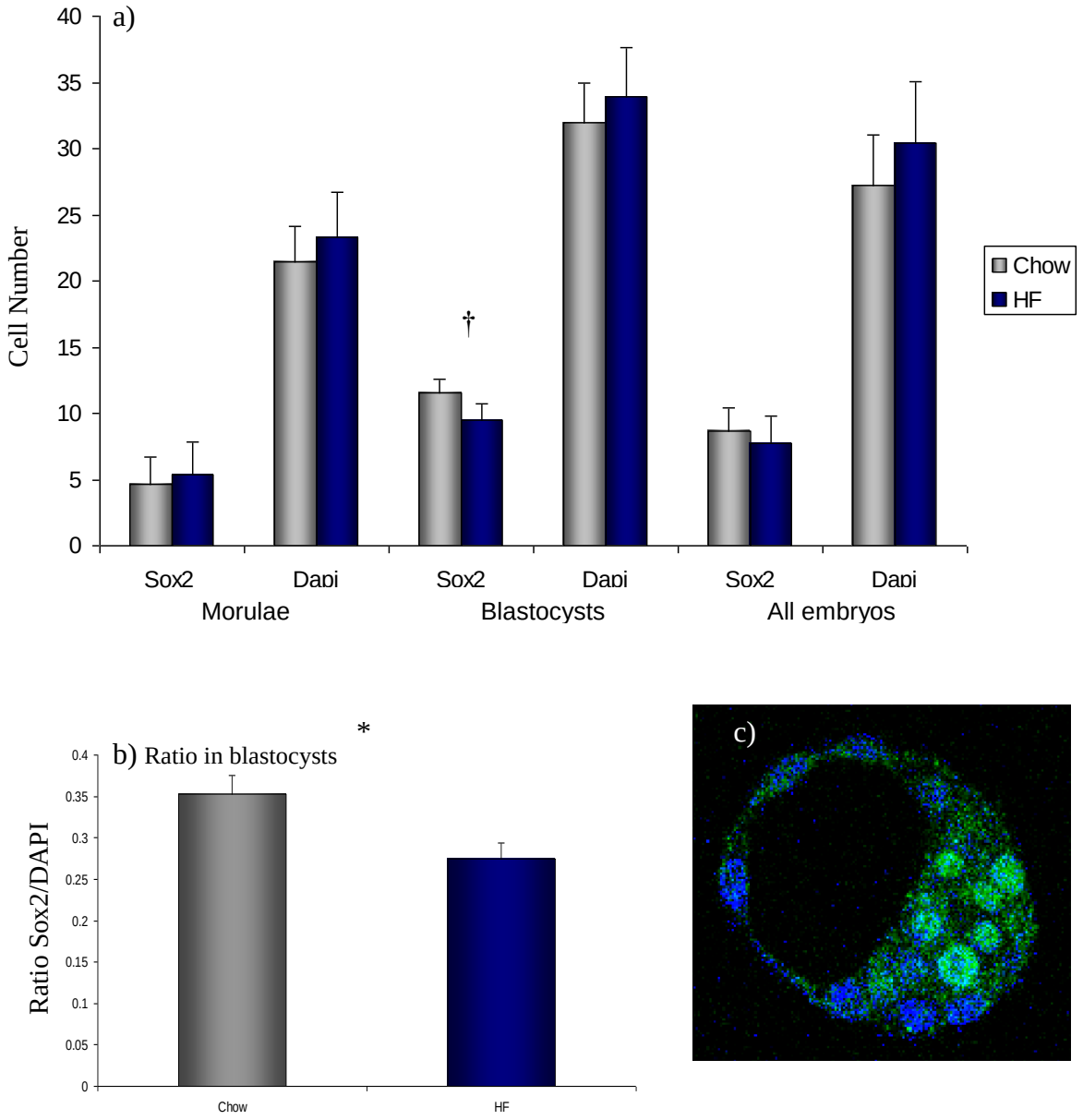


Figure 4.11 Cell number and lineage allocation in embryos collected at E3.25

a) Average expression of SOX2, a marker of ICM cell characteristics, at E3.25 after feeding of Chow and HF diets from conception. Average nuclei number was determined by DAPI labelling. Data is separated by stage of development. A trend towards decreased SOX2 expression in HF embryos was observed. b) The ratio of SOX2:DAPI labelling in HF embryos is significantly reduced.in blastocysts (P=0.01). c) Immunofluorescently labelled blastocyst. All nuclei are labelled with DAPI and shown in the blue channel. SOX2 positive nuclei were labelled with an Alexaflor 488 secondary antibody, and are shown here in the green channel. Morulae Ch n=11 dams n=5 HF n=12 dams n=4, blastocysts Ch n=15 dams n =4, HF n=28 dams n=7, all embryos Ch n=26 dams n=7, HF n=40 dams n=7. Error bars ±SEM. †P<0.1, *P<0.05. Statistical analysis was performed by multilevel random effects regression

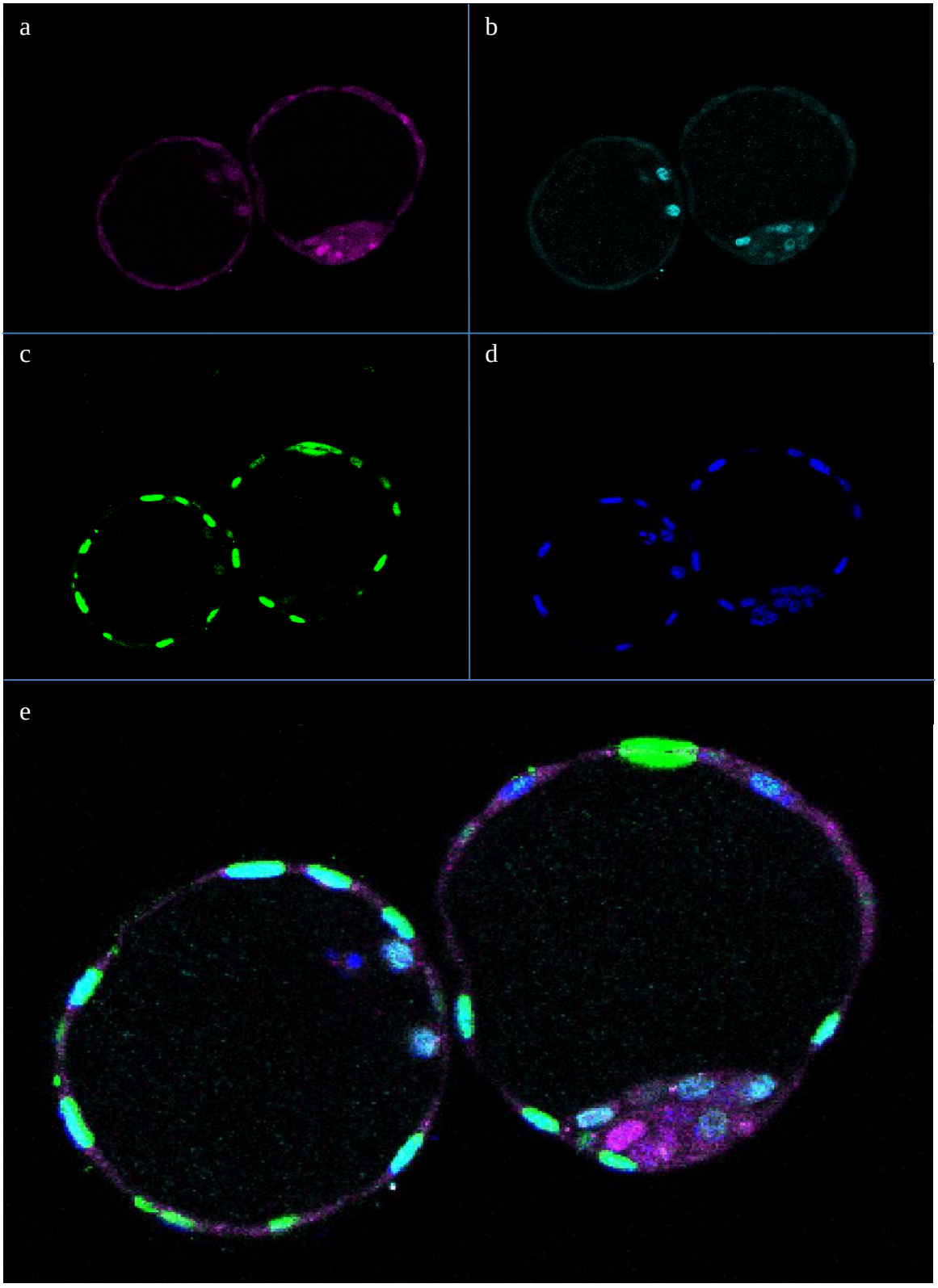


Figure 4.12. Representative image of immunohistochemical labelling for SOX2 (a), GATA4 (b), CDX2 (c) and DAPI labelling (d) along with an overlay of all channels (e)

CDX2, SOX2 and GATA4 expression was studied in HF embryos at E3.5. HF showed an increase in total cell number and a strong trend towards increased numbers of CDX2 expressing cells (figure 4.13). Proportionally, HF embryos expressed significantly more GATA4 (figure 4.14). These changes led to a significant shift in the ICM:TE proportions of the embryo as determined by the addition of SOX2 and GATA4 expression to indicate the ICM, with CDX2 expression representing the TE (figure 4.15). Embryos had proportionally larger ICMs, as was also seen in differentially labelled embryos.

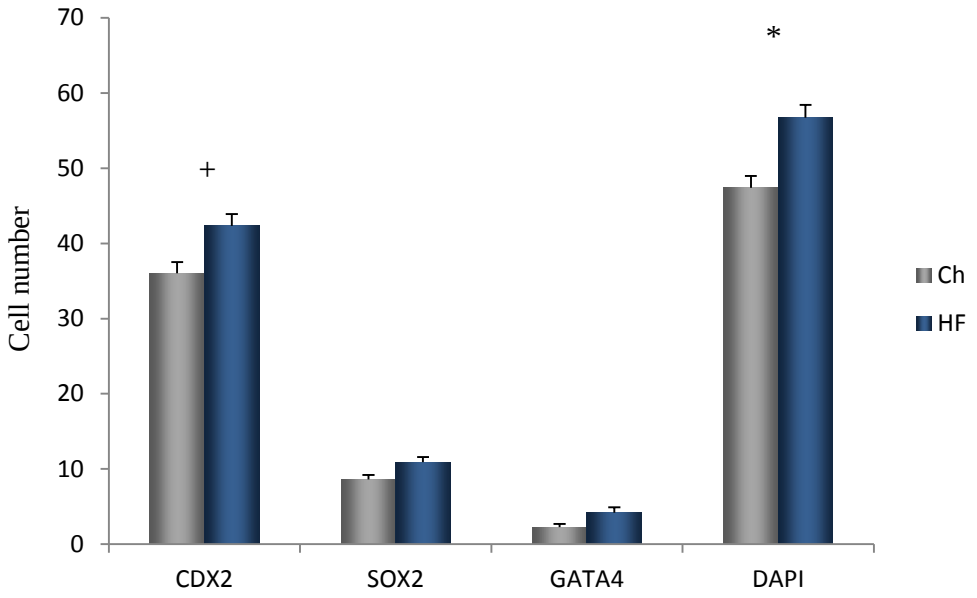


Figure 4.13 Lineage allocation by transcription factor in HF embryos at E3.5

Cells positive for lineage markers CDX2, SOX2 and GATA4 along with total nuclei number determined by DAPI. DAPI P=0.020, CDX2 P=0.056. Embryos Ch n= 46, HF n=40 Dams n=9 per diet. Statistical analysis was performed by multilevel random effects regression

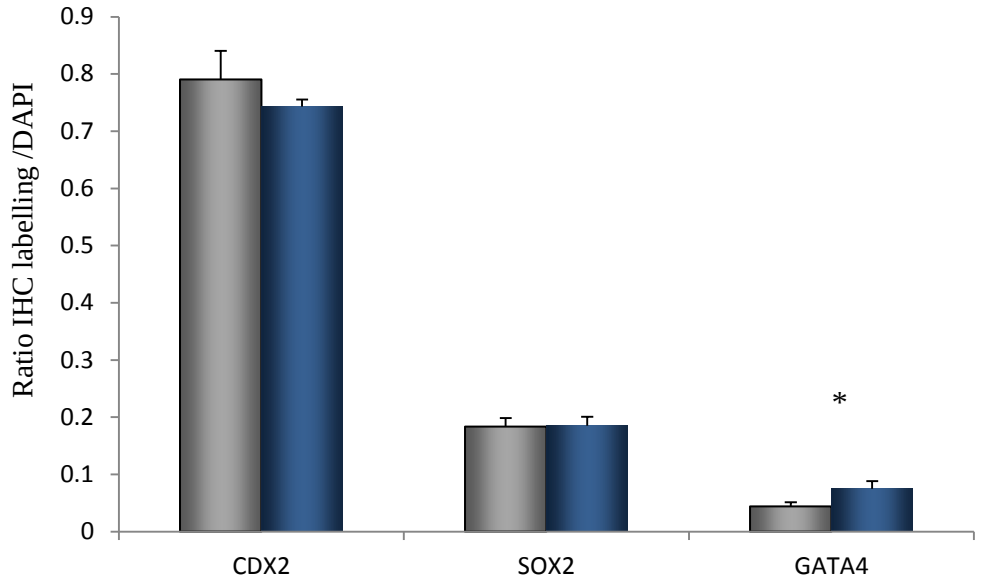


Figure 4.14 Proportion of transcription factor in the HF embryo at E3.5

Ratio of cells positive for lineage markers CDX2, SOX2 and GATA4 to total nuclei number determined by DAPI. GATA4/DAPI P=0.011. Embryos Ch n= 46, HF n=40 Dams n=9 per diet. Statistical analysis was performed by multilevel random effects regression

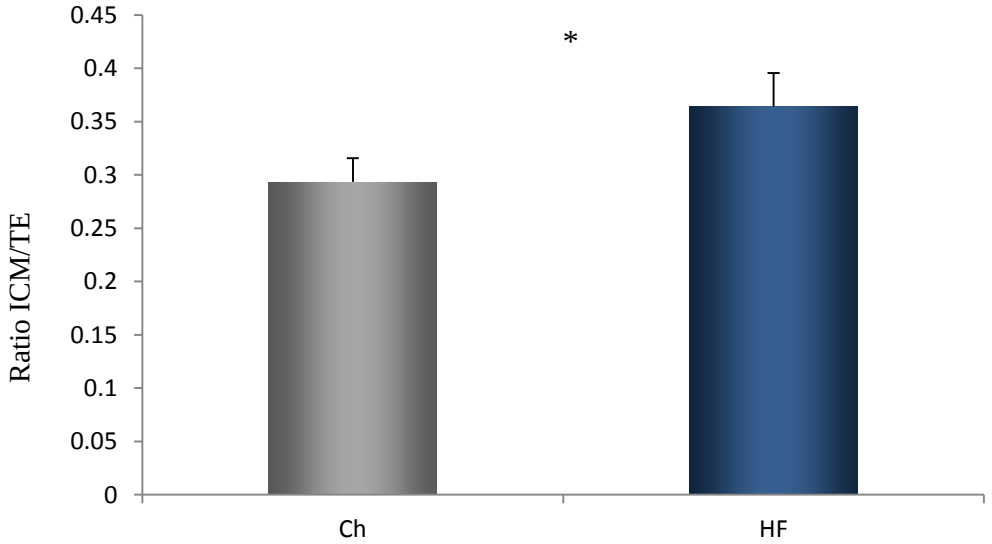


Figure 4.15. Ratio of TE:ICM determined by transcription factor expression

ICM / TE by IHC. ICM was determined by addition of SOX2 and GATA4 positive cells. CDX2 was used to determine the TE. P=0.027. Embryos Ch n= 46, HF n=40 Dams n=9 per diet. Statistical analysis was performed by multilevel random effects regression

4.4.3 Protein Biosynthesis in the Blastocyst

For use of the Click-iT AHA kit with small groups of embryos, validation of the procedure was required. Various sized groups of embryos were processed by the Click-iT TAMRA technique, from single embryos to groups of 10.

a) b)

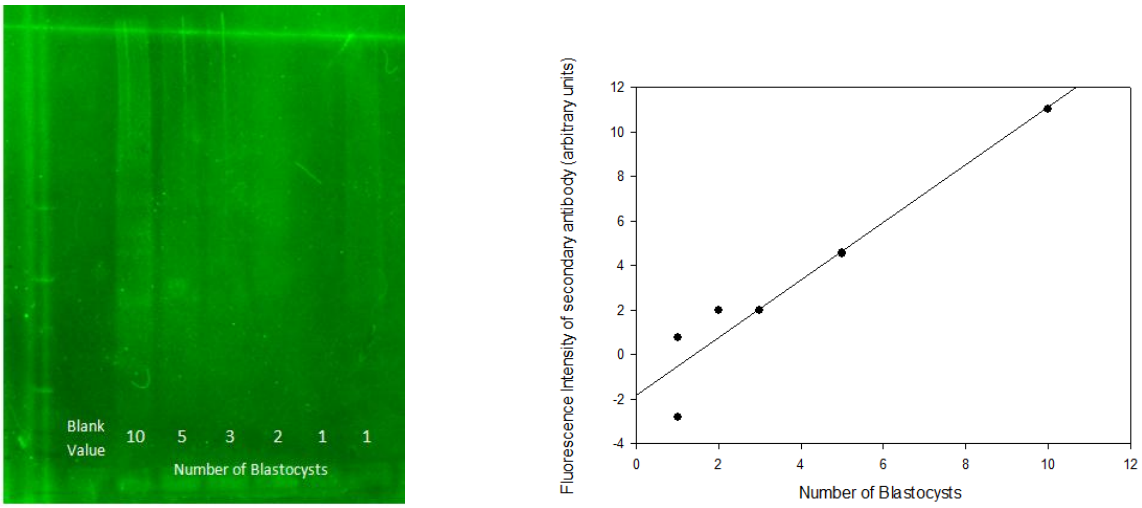
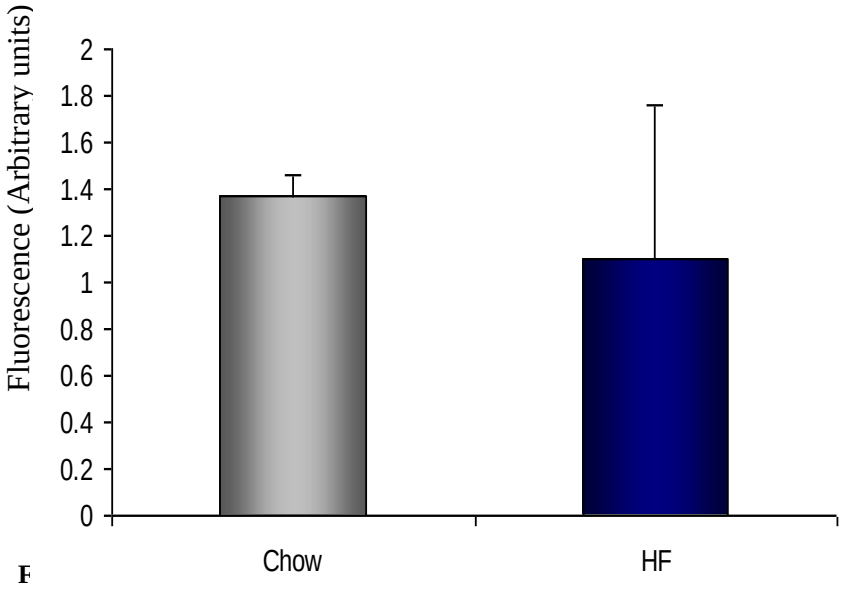


Figure 4.16 Western blotting of TAMRA labelled proteins

a) AHA-TAMRA labelled proteins were separated and blotted onto nitrocellulose. TAMRA molecules were detected by antibody conjugation. b) Fluorescence intensity of secondary antibody was detected using LiCor Odyssey software, averaged and plotted in a graph to determine the limit of detection (r=0.960) n=2, 1 representative blot shown.



F ry environment

Average fluorescence intensity from western blot. Anti TAMRA antibody fluorescence is detected, which is bound to the AHA-TAMRA complex. AHA was firstly incorporated overnight by embryos from differing maternal environments. The incorporation of AHA shows the rate of protein biosynthesis. No significant differences were detected. n=5 groups of embryos from 5 dams per diet (25 embryos total per diet). Statistical analysis was performed by student T-test.

Detection of fluorescence signal from a group of 5 embryos was found to be in the linear range. No significant differences were observed in the rate of protein biosynthesis in a 12 hour period between dietary groups.

4.4 Conclusions

It is known that maternal dietary challenge can influence the preimplantation embryo and lead to poor offspring health outcomes (Kwong et al. 2000, Watkins et al. 2008), making the understanding of embryo sensitivity at this stage highly relevant.

As discussed in the introduction to this chapter, several studies have looked at the preimplantation embryo in response to diet. A major strength of this study in determining the effect of diet is the limited exposure period. In several previous studies, dietary challenge is given prior to conception or in combination with other confounding factors such as superovulation, obesity or *in vitro* culture (Mitchell et al. 2009, Minge et al. 2008, Fouladi-Nashta et al 2007).

This chapter aims to characterise the differences in the early embryo arising from HPD and HF diet in terms of rate of production, morphology and metabolic characteristics, and addresses the sensitivity of embryos to specific diets.

4.4.1 Litter Size

In this study, HPD was shown to have no significant effect on the number of embryos recovered by uterine flushing. Similarly, LPD does not incur any increased pregnancy losses at this time, or indeed throughout pregnancy (Watkins et al. 2008). It is likely that dietary protein challenge, which is a mild challenge, does not cause drastic changes that would lead to abortion. Protein content can be reduced more drastically without leading to abortion, for example a 6% protein diet used by Langley-Evans et al (2011) did not affect litter size. Instead, the embryo adapts its physiology, presumably as a mechanism to

counteract the suboptimal conditions; a PAR (as discussed in section 1.1: The Developmental Origins of Health and Disease Hypothesis).

Feeding a HF diet, on the other hand, did have an impact on embryo development. The total number of embryos collected at 3.5 days was unaffected by HF feeding. At E3.25 however, a reduction in total number of embryos and blastocysts recovered was observed. As embryos are collected by uterine flushing, it is suggested that some embryos at E3.25 in this study may be less developed and are still residing within the oviduct. Although no differences were observed in the current dataset at E3.5, this same observation of a reduction of total blastocysts collected has been observed in our laboratory (Dr Sarah Finn-Sell, Dr Roger Leandri, personal communication), suggesting the potential for delayed development at E3.5. In previous studies, mixed results are seen in the effect of diet on blastocyst rate. In sheep it was found that a low fat diet resulted in a reduced blastocyst rate compared to a high fat diet (Fouladi-Nashta et al. 2007). Adamiak et al. (2005) found that in heifers of good body condition, a twice maintainance diet had a detrimental effect of blastocyst rate.

4.4.2 Lineage Allocation

Previous experimental evidence has shown that maternal dietary challenge of LPD from conception in the mouse can affect cell number and lineage allocation. An increase in cell number, resulting from increased TE cells was seen in embryos from a maternal LPD environment (Eckert et al, 2012). This difference in cell number can be observed from E3.75. HPD also affects lineage allocation in the blastocyst, with both similarities and differences to the results observed in LPD embryos. The proportions of the embryo are shifted in HPD blastocysts, again with preference to the TE, however this results from a

significant reduction in the number of cells in the ICM. Again, although at E3.5 no differences in the blastocyst can be observed, by E3.75 differences could be observed.

Whilst there are similarities between the LPD and HPD embryos, it may have been expected that embryos would adapt in the same way to inappropriate levels of protein, or a dose dependent effect upon the same lineage commitment would occur. Importantly these data show that the adaptive response of the embryo is not simply a 'stress' response, but is a specific adaptation to maternal dietary conditions. The similarities observed between LPD and HPD embryos (shift towards TE observable from E3.75) may implicate a common mechanism. The phenotype is not however identical suggesting that additional adaptive mechanism is activated in either HPD or LPD embryos to lead to differing rates of proliferation.

This difference in lineage allocation is likely to occur either by a decrease in the rate of proliferation at this stage, or by an increase in apoptotic cells. Previous data in the rat preimplantation embryo found no differences in the rate of apoptosis between LPD and NPD embryos (Kwong et al. 2000). Overnutrition in sheep was shown to reduce PCNA (a marker of proliferation) and increase Bax expression (a marker of apoptosis) in the trophectoderm specifically (Lea et al, 2003).

The ratio of cells in the HPD embryo is with a preference for the TE, suggesting that adaptation towards increased relative placental size is occurring. This will regulate the nutrition of the fetus throughout gestation. As previously mentioned, epidemiological studies have shown that placenta to infant size is indicative of health outcome. Chapter 5

will address how lineage is affected at the end of pregnancy and will further discuss this effect.

As shown in table 4.1, Mitchell et al (2009) also studied the effects of a high protein diet on lineage allocation. Although this study used only 21% protein in their 'high' protein diet (in the present study the high protein diet includes 30% protein) and included superovulation in their methodology, both our studies found a similar reduction in the number of cells in the ICM. Mitchell et al also found an increase in TE cells, which the present study did not. Their study also found that a low protein diet decreased cell number in the ICM. It appears that studies from both this group and our own agree that in the mouse alterations in maternal protein levels, either too much or too little, cause a shift in proportions in the blastocyst, favouring increased TE in comparison to ICM.

HF diet was shown to increase cell number in both ICM and TE lineages, as well as overall cell number from E3.5. There was also an observed difference in the ratio between ICM and TE, with an increased proportion of ICM cells compared to controls. These results are in vast contrast to those seen in protein challenged embryos, in terms of morphology and the time at which these changes develop. This again suggests a specific adaptation to dietary environment, as opposed to a generalized stress response to non-optimal diets.

Total cell number, as determined by Hoechst labelling, revealed no differences at E3.25. When lineage was assessed by SOX2 immunolabelling at E3.25 in blastocysts, a trend ($P=0.08$) towards a reduced number of pluripotent cells in HF embryos was found. The ratio of SOX2 positive cells to total cells was significantly decreased in these embryos.

When interpreting the data gained from differential labelling, it must be considered that ICM cell number is determined by position only and not by pluripotency gene expression pattern. The increased ICM cell number observed in HF embryos at E3.5 may be due to an increased number of primitive endoderm cells. Whereas differential labelling suggests that ICM cell number is increased at E3.5, SOX2 labelling confirms a decreased proportion of these embryos has full pluripotent gene expression patterns. The reduced number of pluripotent cells could suggest that although the embryo is of greater cell number, which would classically suggest better quality, transcriptionally the HF embryo is of poorer quality (in terms of a reduced ICM). Further study of the HF embryo at E3.5 shows that the total number of cells in the embryo was significantly increased and the number of CDX2 positive cells was increased at a trend level, in agreement with conclusions made through differential labelling. There were also found to be proportionally more GATA4 positive cells in the embryo. This may go some way towards explaining the reduced number of SOX2 positive cells at E3.25 particularly in the blastocyst; that differentiation of the primitive endoderm in the HF blastocyst is beginning earlier. This data does again suggest that HF blastocysts studied were more developed than controls. As discussed in section 1.3.1, a series of molecular events occur in the development of the two lineages. This study did not extend to include expression patterns of NANOG or OCT4 for example, which are both key to pluripotency. GATA6 expression may also have been interesting to study due to its slight advancement in expression in comparison to GATA4.

It could be hypothesised that embryos within a litter respond differentially to HF diet, those that are responsive develop faster, with others being un-affected. The reduction in blastocyst number seen at E3.25 in this study and by others at E3.5 may support this

hypothesis and is also in accordance with other models demonstrating differential sensitivity to environment, such as in an obese mouse almost half were unable to breed, whilst others were fertile (Bermejo-Alvarez et al 2012).

There are several candidate molecules that may be responsible for changes to lineage commitment. *In vitro* studies have shown that low concentrations of essential amino acids in culture media leads to an increased cell number in the ICM (Lane et al. 2001). This response may be mediated by the amino acid sensing mTOR pathway. Disruption of this pathway leads to a complete loss of proliferation in the embryo (Murakami et al. 2004). Current results show that increased protein actually leads to a decrease in ICM. This indicates that essential amino acid concentration is not the determining factor in this outcome for changes seen in HPD embryos. ICM reduction is a feature observed by Gardener et al. (2004) who suggest that the causative change to HPD embryo environment is actually an increased concentration of ammonia. *In vitro* studies show that insulin and IGF-I levels are likely to cause increased blastocyst proliferation, particularly in the ICM (Pantaleon & Kaye 1996). These effects are seen in the HF embryo, suggesting that insulin levels may be modulated by the HF diet. Cellular composition has also shown to be a factor in lineage allocation in porcine embryos, with lipid rich blastomeres being more likely to contribute to the embryonic portion of the embryo (Kim et al 2012). This study also found that lipid rich cells expressed higher levels of Oct4 and Carm1 (pluripotency factors) at the 2 cell stage.

The changes in lineage observed could possibly be a transient effect that may be rectified by the plastic nature of embryo development. Further chapters will discuss the continued

development of these lineages which may indicate how likely they are to cause later life disease.

4.4.3 Protein Biosynthesis

An increase in cell number was observed in the HF embryo. This suggests that the overall rate of proliferation in the embryo is increased. It would be expected that more proliferation would lead to an increased rate of protein synthesis per embryo. However this observation was not made, indicating that metabolism of each individual blastomere may be decreased in HF embryos. A low metabolic rate has been linked to improved pregnancy outcome (discussed in section 1.4.3), it is not however known how low a metabolic rate is beneficial. Further investigation of this phenomenon would be productive. No corresponding increase in embryo number or litter size (Chapter 5) was observed as may be expected as seen in studies of lower metabolic rate blastocysts. This could be due to other interactions or that the low metabolic rate observed here is excessively low demonstrating that embryos are of poor quality.

There was a great degree of variation in protein synthesis in the HF group. This may suggest that the population of embryos is diverse. Other results may also contribute to a hypothesis of greater variation amongst HF embryos, for example blastocyst number is reduced but those embryos that are at the blastocyst stage are of greater cell number, suggesting they are more advanced.

This work potentiates further research, raising further questions about how these changes arise. An effect on cell number was seen in both dietary treatments, this difference may have come about by changes in cell cycling rate. Investigation into regulatory molecules

of the cell cycle may be enlightening. Another remaining question is where the differences arise from. Signalling pathways establishing lineage may well be involved. TEAD4 and Sall4 may be good markers to identify any preceding lineage commitments.

4.4.4 Summary

This chapter demonstrates that lineage adaptations in the blastocyst are specific to diet, not simply a stress response. Evidence also suggests an advancement of HF embryos at the blastocyst stage. This, seen in combination with some slower developing embryos as determined by morphological stage and flushing recovery, suggests that embryos within a litter are differentially responsive to diet. The further development of the HF embryo will be the focus of chapter 5.

Chapter 5

The effects of HF diet during pregnancy at E17.5

5.1 Introduction

The previous chapters have demonstrated that maternal HF diet in preimplantation stages of development affects both the maternal environment and the developing blastocyst. This chapter addresses how these changes affect the ongoing pregnancy when maintained on a HF diet, or when a normal diet is resumed for the remainder of pregnancy (emb-HF). A precedent for maternal diet fed only during the preimplantation period having an effect upon offspring was observed in the low protein diet. Mice fed 9% protein for the 3.5 days of pregnancy before resuming a normal 18% protein diet during pregnancy (emb-LPD) were heavier at birth than both their control counterparts (NPD) and those fed 9% protein throughout gestation (LPD) (Watkins et al 2008). LPD and emb-LPD mice were shown to have raised systolic blood pressure, and upon termination of the experiment were found to have proportionally smaller hearts. This study will also adopt a short term dietary challenge alongside maintained and control diets to determine the impact of the very early exposure to HF diet. It is unknown whether the effects induced in the preimplantation period by this particular diet (observed in Chapter 3) will be transient or continued.

The extent to which diet affects pups will be mediated by the effect the diet has on the dam. At this stage of the study this will be addressed in terms of weight, as well as serum glucose and insulin levels. A degree of hyperinsulinemia is a normal feature of pregnancy, peaking in the third trimester and returning to normal levels postpartum (Buchanan et al. 1990). However insulin resistance during pregnancy, termed gestational diabetes when glucose levels are also raised, can lead to serious complications (Barden & Knowles, 1981). This occurs in 3-5% of human pregnancies, and is heavily linked with poor health outcomes such as pre-eclampsia or increased probability of requiring a caesarean delivery (Bellamy et al 2009, Norman & Reynolds 2011). As mice are not

fasted in this study, glucose:insulin ratio is the most appropriate way of studying insulin sensitivity. Whilst a previous study at E3.5 showed a level of insulin resistance from HF consumption solely from the point of conception (Dr Sarah Finn-Sell, personal communication), this study will demonstrate how the dam and fetus are affected by continuing HF diet as well as returning to a normal chow diet. Serum glucose and insulin levels will also be measured in the fetus. Maternal high fat diet has previously been linked to increased glucose and insulin levels in offspring (Samuelsson et al 2008), but the effects of maternal glucose: insulin is largely understudied in the fetus (see also section 1.2.2.).

Conceptuses will be weighed, as well as the composing organs; placenta, yolk sac, whole fetus, lung, liver, heart and kidneys. Studies in rodents report increases (Jones et al 2009, Strakovsky & Pan 2012), no change (Zhang et al 2009) or reductions (Howie et al 2009) to fetal and birth weights as a result of a high fat diet. These diets vary in composition suggesting, as previously observed, that specific diet is likely to dictate a specific adaptation. HF diet made no difference to weight in the first 3.5 days of pregnancy (chapter 3), and it was also found that diet was consumed isocalorically to the control diet, which may mean that no difference in body weight will be observed at the end of pregnancy. Thus any effects observed in this study will be a result of only a change in macronutrient composition and will exclude obesity as a confounding factor.

As identified in chapter 4, HF diet causes changes in the blastocyst including a shift in lineage allocation, to produce a proportionally smaller TE – the progenitor of the placenta. We will investigate fetal and placental growth to determine the implications of these early adaptations. A higher placenta/fetal weight ratio is associated with increased

later life disease risk, such as hypertension, cardiovascular mortality and glucose intolerance (Barker et al, 1992, Risnes et al 2009, Phipps et al 1993). Shehata et al (2011) found that higher placenta/fetal weight ratio was more common amongst higher BMI patients, suggesting a link to high energy diet. It is thought that larger babies with a small efficient placenta are those least at risk of developing heart disease (Fowden et al 2009)

Whilst fetal placental size and ratio are an important indicator of fetal health, gene expression studies should provide a more useful insight into placental function. The placenta regulates transport of nutrients to the fetus, as discussed previously (section 1.5) there are various transporters that facilitate this process. Other maternal dietary interventions have demonstrated that these transporters respond to the changes in nutrition to regulate fetal growth. A high fat maternal diet throughout gestation has previously been shown to cause an increase in fetal growth in mice coupled with increases in placental GLUT1 and SNAT2 expression and resulting glucose and amino acid transport (Jones et al 2009). Snat2 has also been shown to be sensitive to protein levels with a decrease in Snat2 expression in response to a low protein diet, which is seen alongside reduced maternal insulin (Jansson et al 2006). Meanwhile, obesity has been shown to cause increased Scl27a1, lipin1 and lipin3 expression in the placenta (Strakovsky & Pan, 2012). Scl27a1 is a fatty acid transporter, whilst lipin 1 and 3 are involved in the metabolism of lipid within tissues. This study showed an accumulation of lipid in the placenta.

Other regulators of conceptus growth, IGF2 and adiponectin receptors, will be investigated. Cord adiponectin has been previously associated with fetal placental ratio (Kadowaki et al 2006). Adiponectin has also been linked to the regulation of amino acid

transporter activity in the placenta (Rosario et al 2012). IGF2 is paternally expressed, its knockout results in growth retardation of first the placenta and then the fetus. Interestingly the mutant placenta will show increased expression of active amino acid transporters, presumably in attempt to compensate for the reduced placental size (Constância et al 2002). There is also precedent for alterations in placental IGF2 levels in response to diet, for example a high protein diet in mice has been shown to lead to lower IGF2 expression (Coan et al, 2011). Like IGF2, IGF1 is expressed in the placenta and is involved in growth. An infusion of IGF1 has been shown to increase fetal weight in the guinea pig (Sferruzzi-Perri et al 2006)

Organ allometry will also be investigated. A low protein diet in rats has previously been shown to increase kidney size and a decrease in liver size in male rat offspring (Kwong et al 2000). A high fat diet has similarly been observed to reduce liver size in offspring (Dudley et al, 2011). In the mouse LPD and emb-LPD model, no differences were observed in organ allometry although LPD females were shown to have a reduced fat pad weight (Watkins et al, 2011)

Finally bone mineralisation of fetuses will be measured by micro CT scan which will indicate the stage of skeletal development. In the human population studies have demonstrated that maternal diet can affect the child's bone density in adolescence (Yin et al, 2010). The authors cite maternal fat as a key predictor of bone density suggesting a programming event, which may well be relevant in the current study.

In summary, this chapter will observe a range of indicators of the effects of a maternal HF diet during pregnancy on the fetus. Of particular interest are the effects that will be observed in response to this HF diet being fed solely during the preimplantation period.

5.2 Methods

MF1 mice were naturally mated and pregnancy was confirmed by the presence of a post copulatory plug. Pregnant mice were randomly allocated to an experimental diet; Chow, HF or emb-HF. Mice were sacrificed by cervical dislocation at E17.5 (section 2.1.1 to 2.1.3). Serum was collected from dams (section 2.1.6). Conceptuses were collected and weighed before separation of the placenta and yolk sac which were weighed. The fetus was also weighed before sacrifice by decapitation. Fetal blood was collected by capillary tube and the lungs, heart, kidneys and liver were weighed. Organs from the four centrally located fetuses in the right and left horns were kept for further analysis either stored in RNA later (Qiagen) or snap frozen (section 2.1.6). Gender was determined in the 4 central fetuses of each horn. Maternal and fetal serum was analysed for insulin and glucose concentrations (section 2.2.3). Bone development was analysed by CT scan. Bone volume to surface ratio and developmental stage in the hand, sternum and rib were analysed (section 2.2.4).

5.3 Results

5.3.1 Maternal Characteristics

No significant differences were observed in total weight change at E17.5 between HF emb-HF and chow groups. Differences in percentage weight gain were seen on particular days. The percentage weight gain at E3 was significantly greater in the HF group compared to controls (P=0.007), and in emb-HF compared to controls (P=0.026) in this group of animals. At E4 HF mice gained more than emb-HF mice (P=0.022). A trend towards less weight gain in emb-HF mice compared to HF mice was observed at E7 (P=0.098). At E13 emb-HF dams gained significantly less weight compared to chow fed dams (P=0.03). A trend was again observed of a lesser gain in weight in emb-HF compared to HF at E15 (P=0.067).

Serum glucose and insulin were measured in E17.5 dams. No differences were observed in glucose levels between dietary treatment (figure 5.2). Insulin levels were found to be raised in HF dams (figure 5.3), leading to an increased glucose:insulin ratio (figure 5.4).

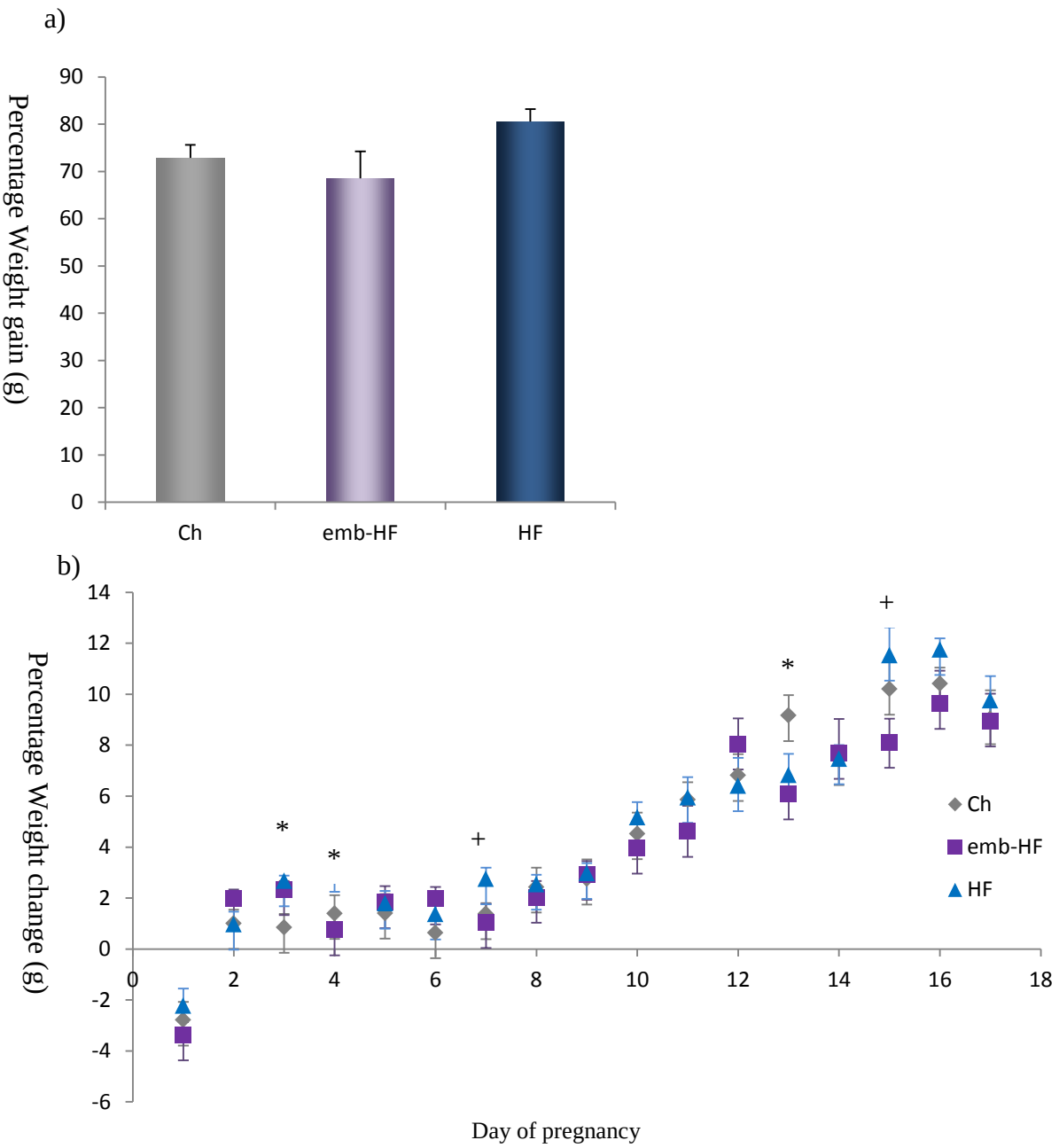


Figure 5.1 Maternal Weight gain at E17.5

Maternal weight change was monitored throughout pregnancy. There were no significant differences in the total percentage weight gain throughout pregnancy between the 3 diet groups (a). Day by day percentage weight gain is shown in graph b. The percentage weight gain at E3 was significantly greater in the HF group compared to controls (P=0.007), and in emb-HF compared to controls (P=0.026). At E4 HF mice gained more than emb-HF mice (P=0.022). A trend towards less weight gain in emb-HF mice compared to HF mice was observed at E7 (P=0.098). At E13 emb-HF dams gained significantly less weight compared to chow fed dams (P=0.03). A trend was again observed of a lesser gain in weight in emb-HF compared to HF at E15 (P=0.067). Error bars ±SEM.n= 10 per diet. Statistical analysis was performed by student T-test.

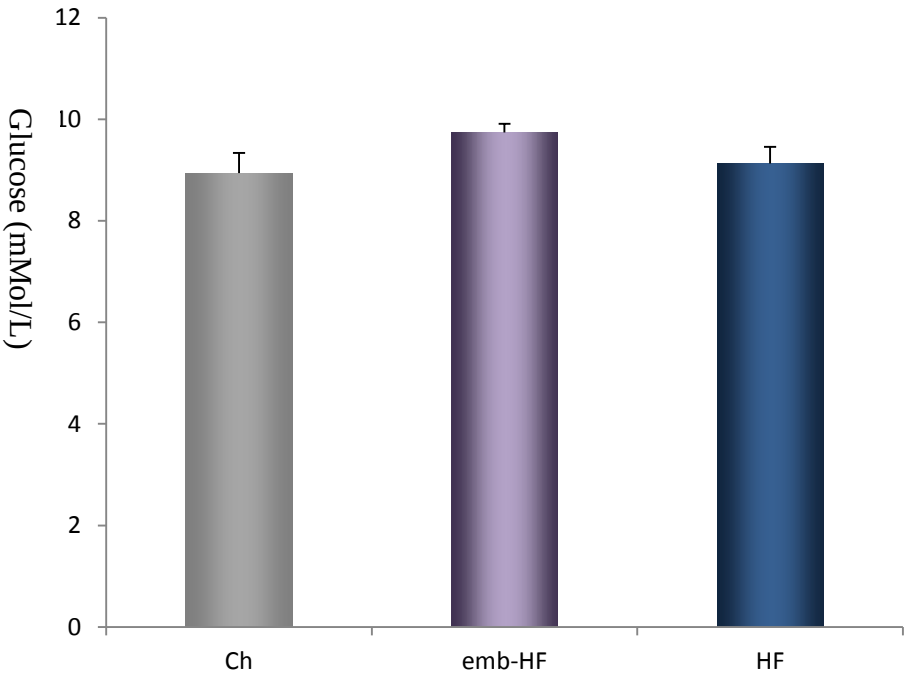


Figure 5.2 Maternal serum glucose

Serum glucose levels were measured in dams fed HF, emb-HF and Chow diets up to E17.5. No significant differences were observed. Chow n=7, emb-HF n=6, HF n=9. Error bars ±SEM. Statistical analysis was performed by student T-test.

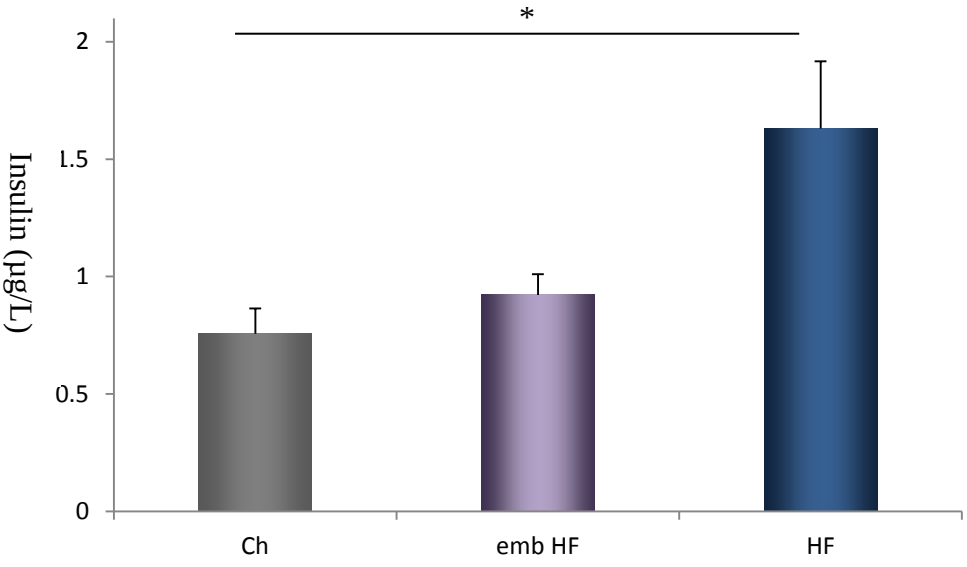


Figure 5.3 Maternal serum insulin

Serum insulin levels were measured in dams fed HF, emb-HF and Chow diets up to E17.5. HF dams had significantly raised insulin levels compared to controls. Chow n=7 , emb-HF n=6, HF n=9. *P<0.05. Error bars ±SEM. Statistical analysis was performed by student T-test.

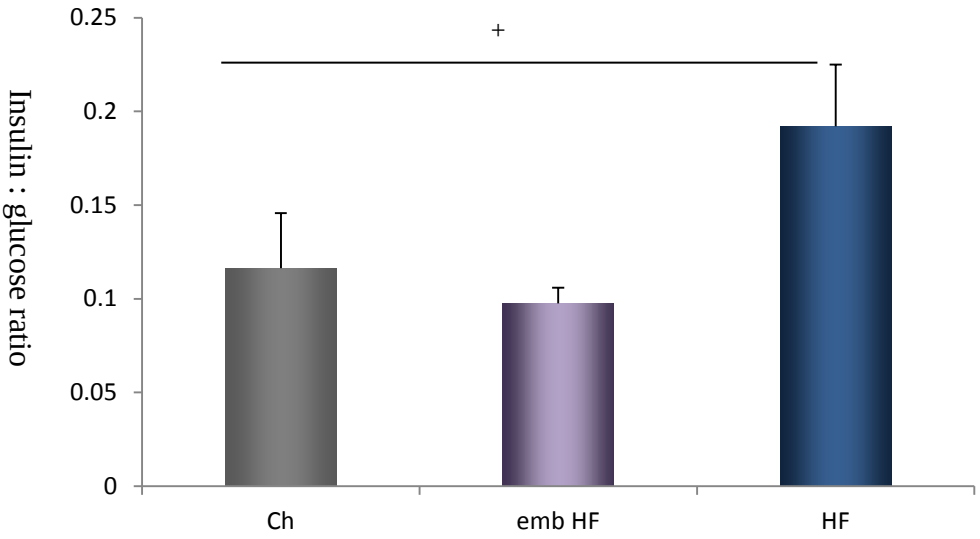


Figure 5.4 Maternal serum glucose : insulin ratio

Serum glucose : insulin ratios were determined in dams fed HF, emb-HF and Chow diets up to E17.5. A trend was observed in HF dams towards an increased serum glucose:insulin ratio compared to controls. Chow n=7 , emb-HF n=6, HF n=9. +P<0.1. Error bars ±SEM. Statistical analysis was performed by student T-test.

5.3.2 Fetal Characteristics

No differences were observed in litter size or gender distribution between diets (figure 5.5 & 5.6). An increase in fetal weight was observed in male emb-HF fetuses. This increase did not reach significance in the females only or combined male and females groups (figure 5.7). A significant decrease in placental weight was found in the HF group for both females and males, as well as when groups were combined (figure 5.8). The fetal: placental ratio was increased in both genders in both emb-HF and HF groups (figure 5.9).

Organ allometry was studied, with no significant differences found in the yolk sac, heart, lung or liver (figures 5.10-5.13) HF females and male and female combined groups showed a significant increase in left kidney proportion. This was also a significant increase for emb-HF females and increased to a trend level in combined males and females emb-HF (figure 5.14).

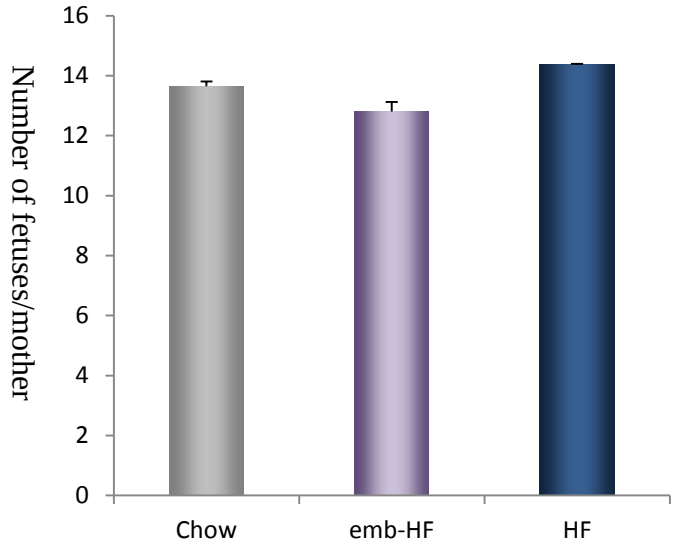


Figure 5.5 Litter Size at E17.5 No significant differences were observed in litter sizes produced from HF emb-HF and chow dams. Error bars ±SEM. Statistical analysis was performed by student T-test.

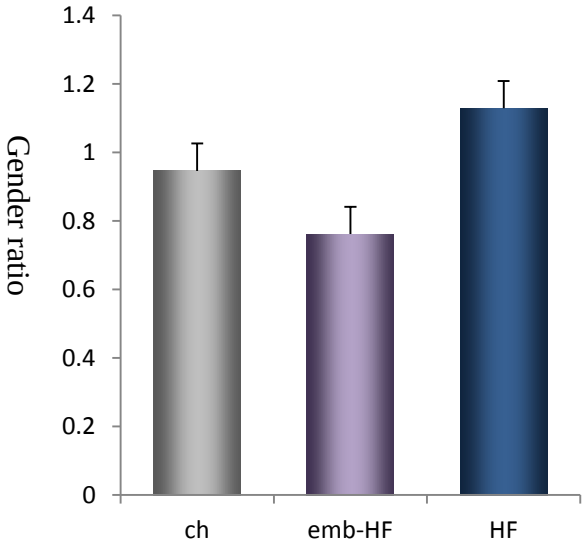


Figure 5.6 Gender Ratio

No significant differences in the ratio between males and females was observed between diets. Statistical analysis was performed by student T-test.

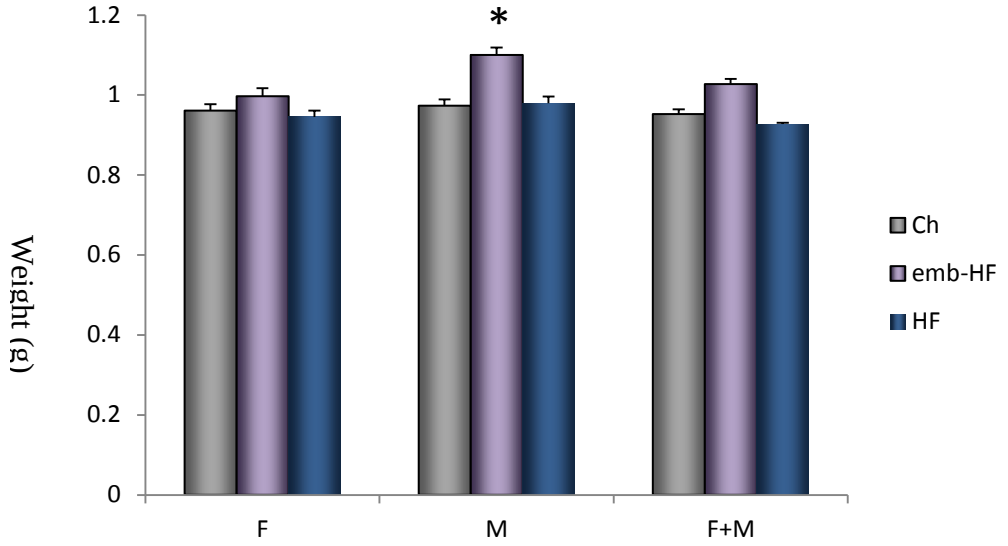


Figure 5.7 Fetal weight at E17.5.

A significant increase was observed in emb-HF male fetuses. No other changes were statistically significant. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. *P=<0.05 Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

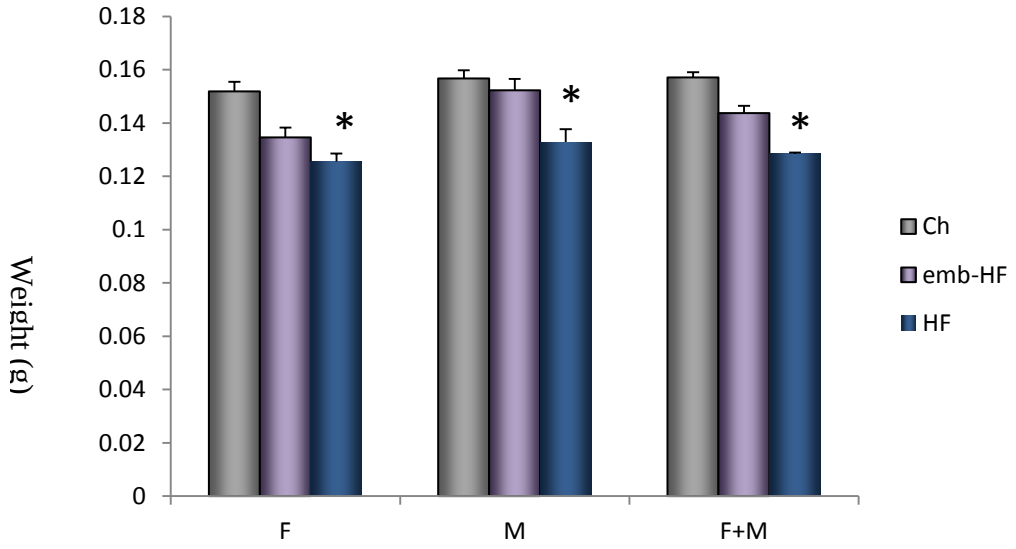


Figure 5.8 Placenta weight at E17.5.

A significant reduction in placental weight was observed in HF conceptuses. This effect was present in both males and females. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. *P=<0.05 Error bars ±SEM._Statistical analysis was performed by multilevel random effects regression

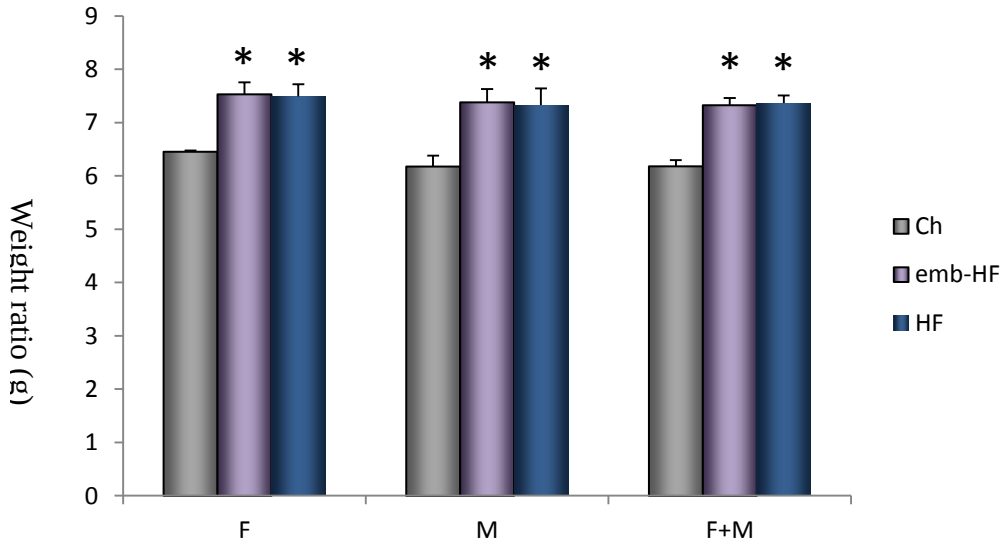


Figure 5.9 Fetal to placental ratio at E17.5.

Both emb-HF and HF fetuses, both males and females, displayed an increased fetal placental ratio. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. *P=<0.05 Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

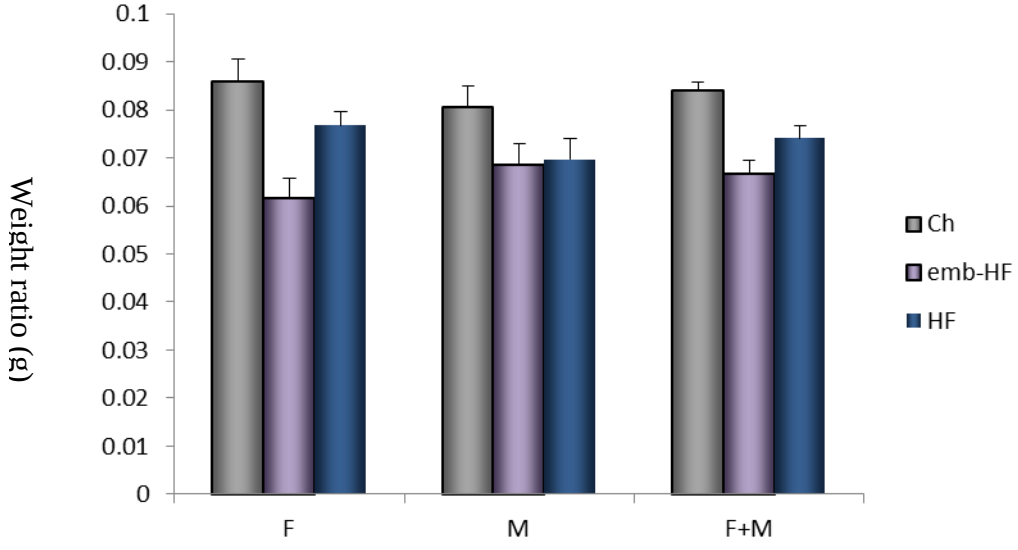


Figure 5.10 The ratio of yolk Sac to fetal tissue at E17.5.

No significant differences were observed between diets. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

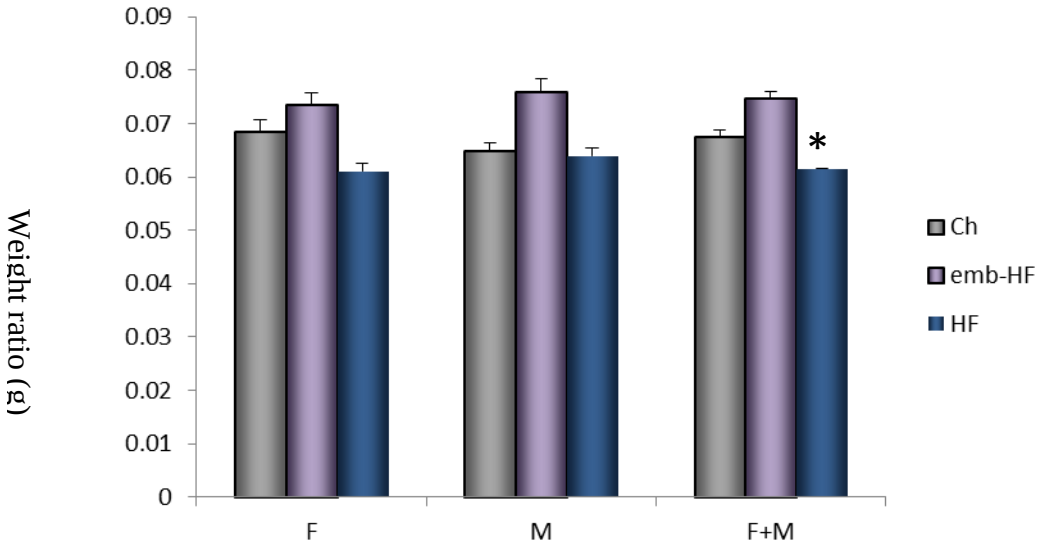


Figure 5.11 Liver:fetus weight ratio at E17.5.

A significant decrease was observed in HF males and females grouped together. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. *P=<0.05 Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

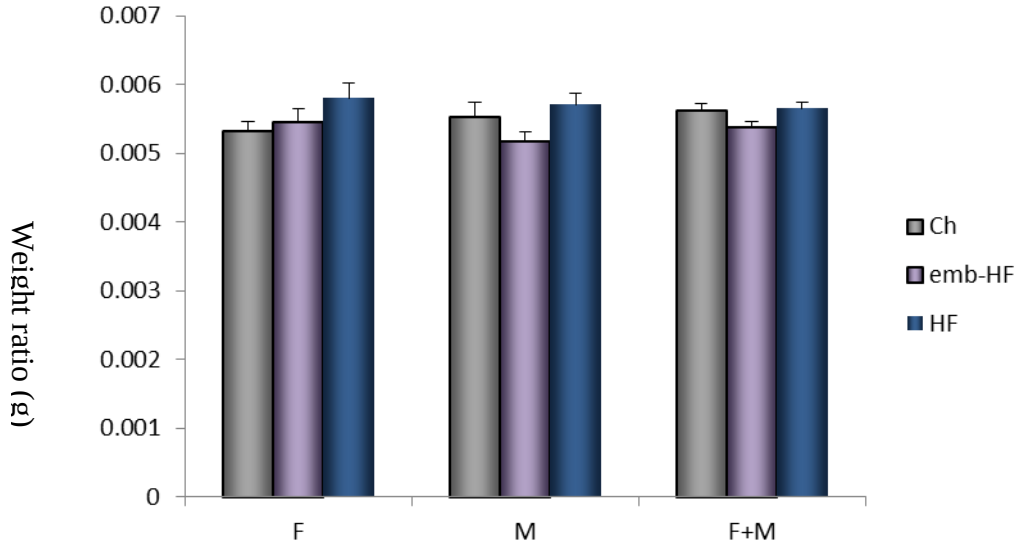


Figure 5.12 Heart:fetus weight ratio at E17.5.

No significant differences were observed in these results. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

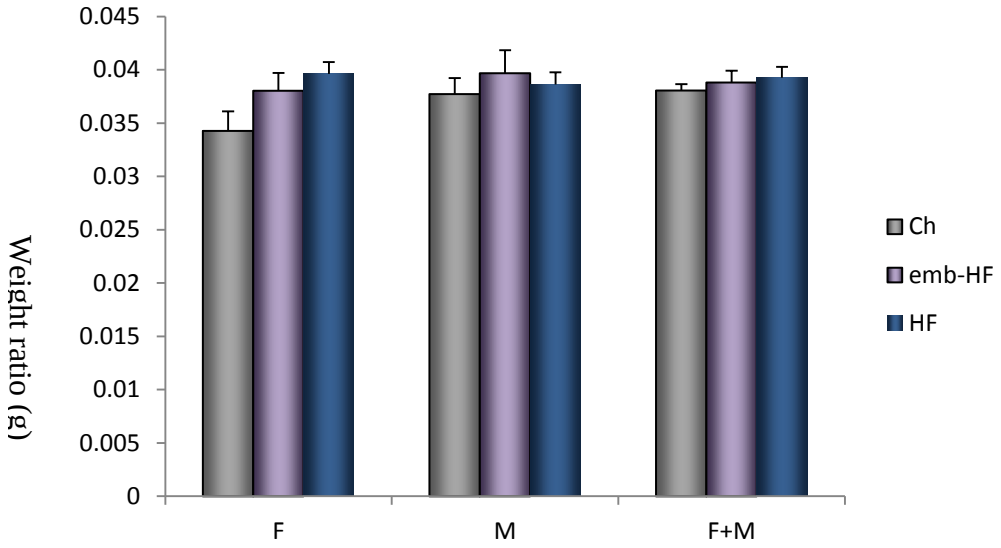


Figure 5.13 Lung:fetal ratio

Lung:fetus weight ratio at E17.5 was not observed to change in response to maternal diet. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

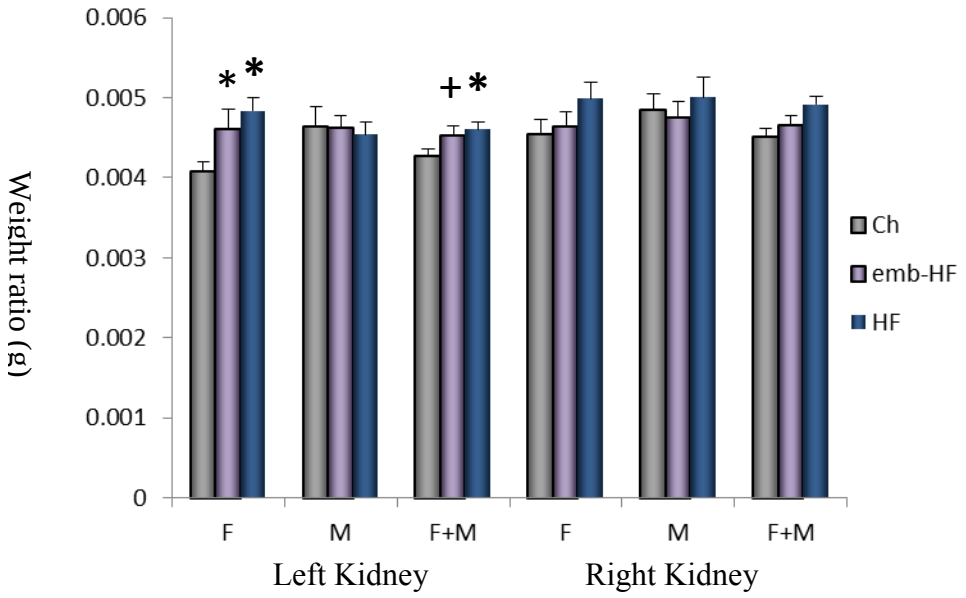


Figure 5.14 Kidneys:fetus weight ratio at E17.5.

In the left kidney significant increases were seen in this ratio in female emb-Hf and HF groups. In the female and male group results, this was found to be at trend level in the emb-HF group and significant in the HF group. Chow F n=35 M n=37 F+M n=120, emb-HF F n=31 M n=28 F+M n= 94, HF F n=30 M n=40 F+M n=133. Dams n=10 per diet. +P=<0.1, *P=<0.05. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

5.3.2.1 Fetal insulin

HF fetuses had significantly raised insulin levels compared to controls irrespective of gender. A trend towards increased insulin in HF fetuses compared to emb-HF fetuses was observed in females alone. This difference was significantly different when males and females were combined (figure 5.15).

When studied as a ratio to maternal insulin or glucose (Figure 5.16 and 5.17), it was found that HF fetuses had an increased insulin level in comparison to maternal levels. The same was true of the proportion of fetal insulin to maternal glucose. A trend towards this effect was also seen in emb-HF fetuses compared to maternal glucose.

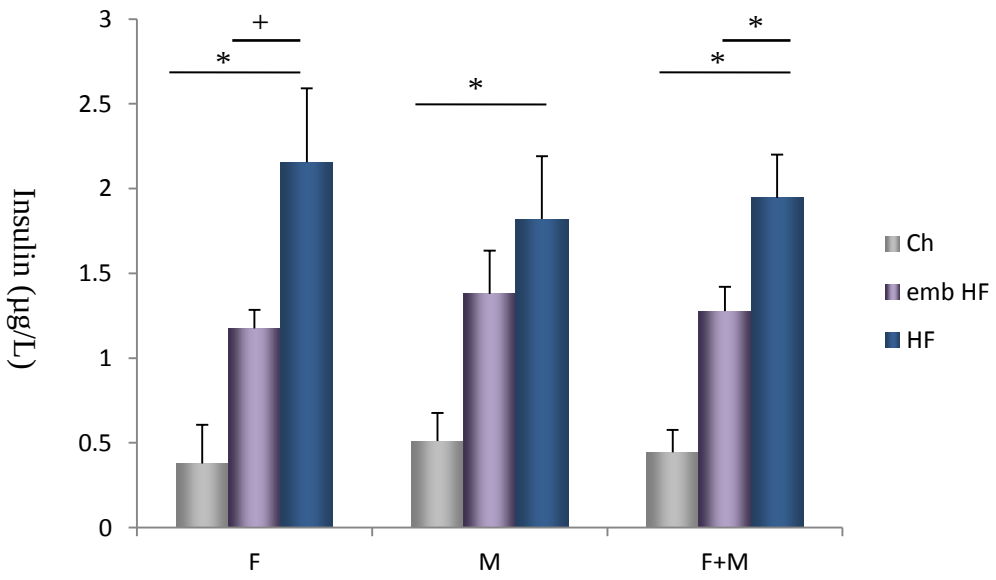


Figure 5.15 Fetal insulin

Serum insulin levels were measured in E17.5 fetuses from dams fed HF, emb-HF and Chow diets. HF fetuses had significantly raised insulin levels compared to controls irrespective of gender. A trend towards increased insulin in HF fetuses compared to emb-HF fetuses was observed in females alone. This difference was significantly different when males and females were combined. Fetuses per gender Chow n=6, emb-HF n=6, HF n=6. *P<0.05. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

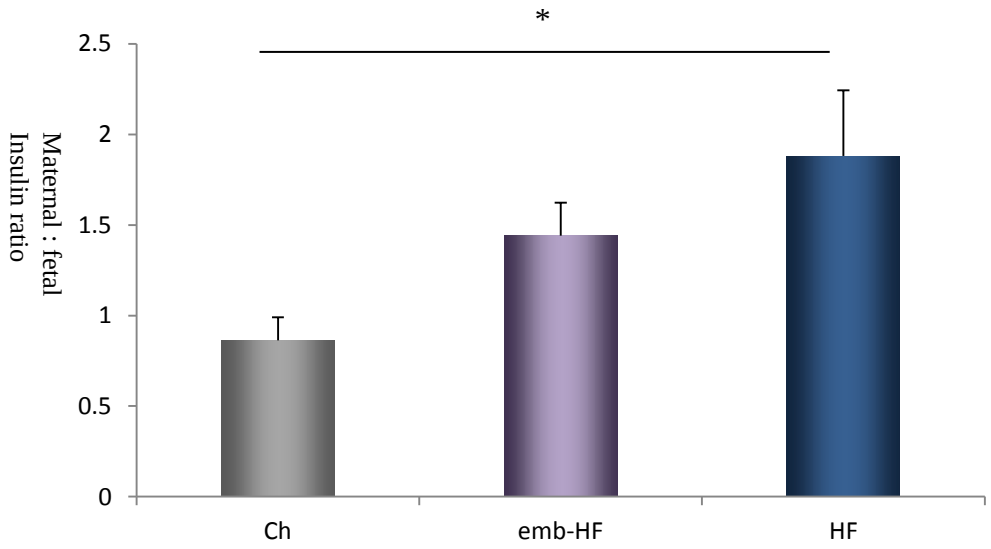


Figure 5.16 maternal to fetal insulin ratio.

The ratio of maternal to fetal insulin was increased in the HF group, indicating a comparative raise in fetal insulin. Chow n=6, emb-HF n=6, HF n=6. *P<0.05. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

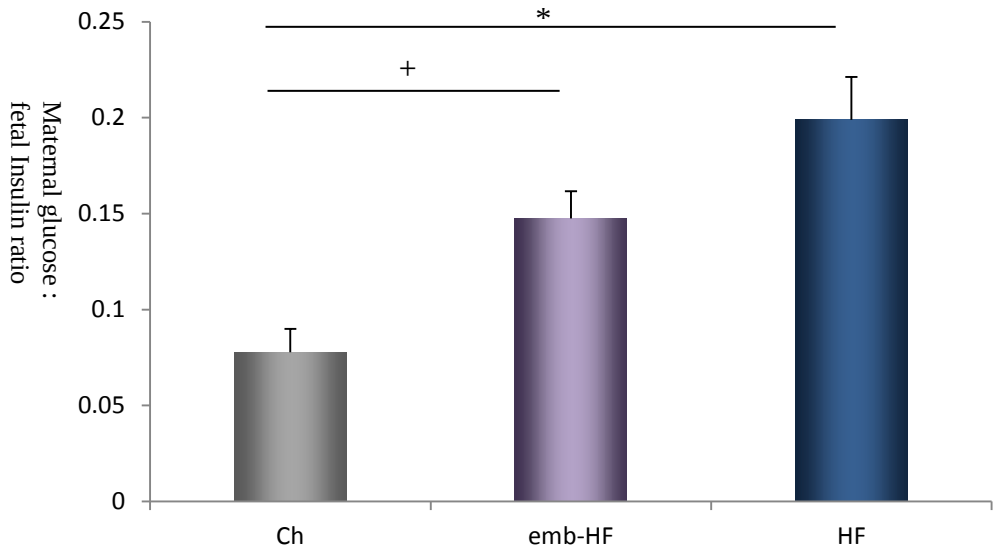


Figure 5.17 The ratio of fetal insulin to maternal glucose was found to be raised in HF fetuses to a significant level and in emb-HF fetuses to a trend level compared to controls. Chow n=6, emb-HF n=6, HF n=6. *P<0.05. Error bars ±SEM. Statistical analysis was performed by multilevel random effects regression

5.3.3 Bone development

Bone development was assessed in fetuses firstly by developmental index, which refers to the extent of ossification in the paw, sternum and tail (method detailed in section 2.2.4). Increased developmental indices were observed in the sum of developmental indices in the HF fetuses and to trend level in the emb-HF group (Figure 5.18) in the male and female combined group. This result was mirrored in female fetuses, and the same pattern observed in males including to a significant level in emb-HF fetuses. When each developmental index was studied individually (figure 5.19-5.22), the sternum, paw and vertebrae were found to be advanced in the HF group. An increase in developmental index was observed in the sternum of the male and female combined group HF fetuses, this difference was observed at a trend level in female fetuses. In the vertebrae an increase in developmental index was identified in the vertebrae of HF fetuses (male and female and male combined group) as well as an increase in emb-HF fetuses (female and male group, and to a trend level in males) compared to controls. An increase in developmental index of the paw was observed in the paw of HF fetuses compared to chow fed fetuses (all gender groups) and emb-HF fetuses (female and female and male combined groups only).

A trend towards increased femur length was detected in male emb-HF fetuses (figure 5.23). A significant increase was observed in total bone volume in HF fetuses (female and male group) as well as a trend towards this increase in emb-HF fetuses (female and male group) (figure 5.24). No differences were observed in surface to volume ratio (figure 5.25).

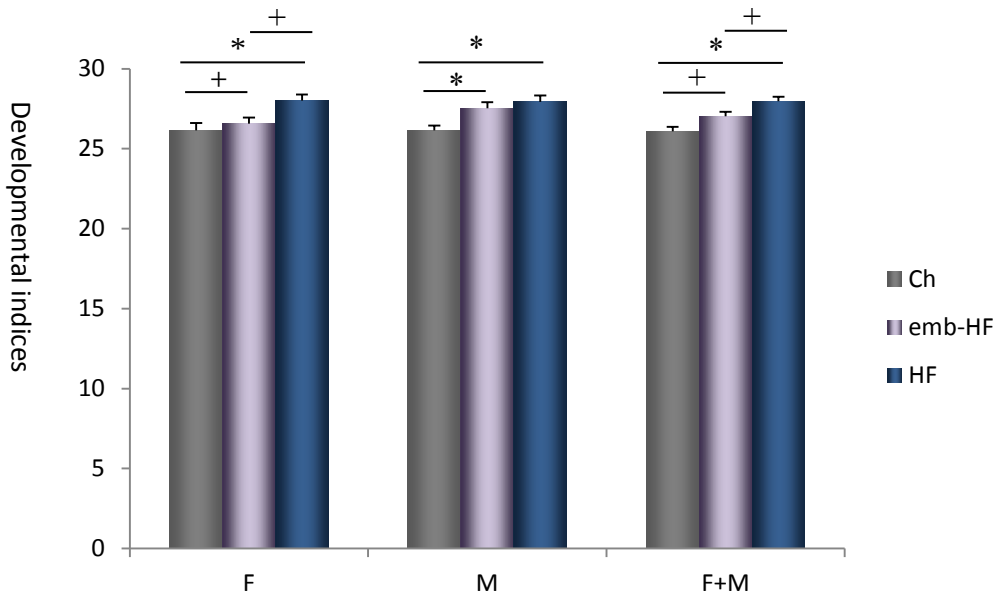


Figure 5.18 Total of developmental measures

A significant increase in developmental indices (combined measures of paw, rib, vertebrae and sternum) in HF fetuses was seen compared to controls in M, F and M+F. This was also increased in emb-HF fetuses compared to controls at a significant level in M, and to a trend level in F and F+M. A trend was also observed in F and F+M fetuses of an increase between emb-HF and HF. Error bars ±SEM. *P<0.05, + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

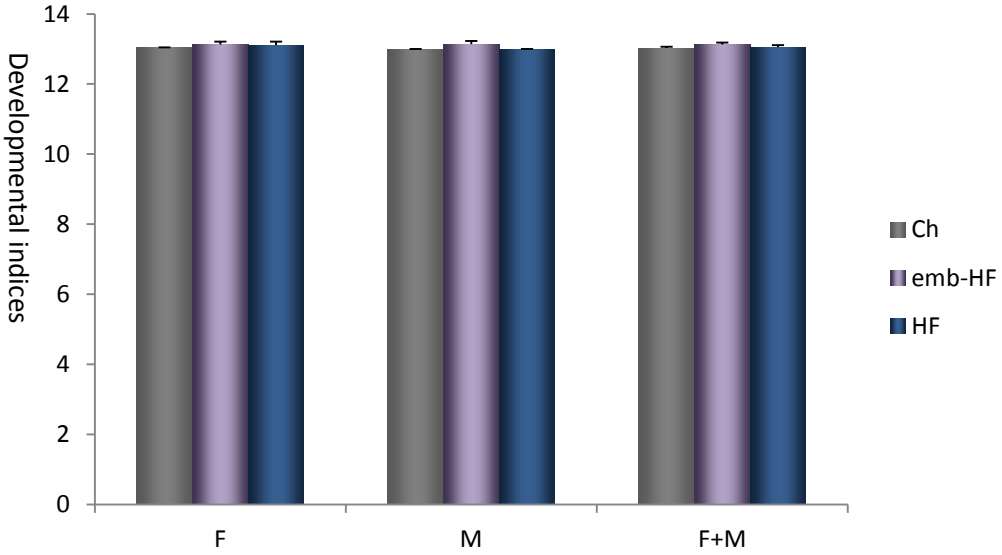


Figure 5.19 Developmental index: Ribs

There was no observable differences between diet in the development of the ribs Error bars ±SEM. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

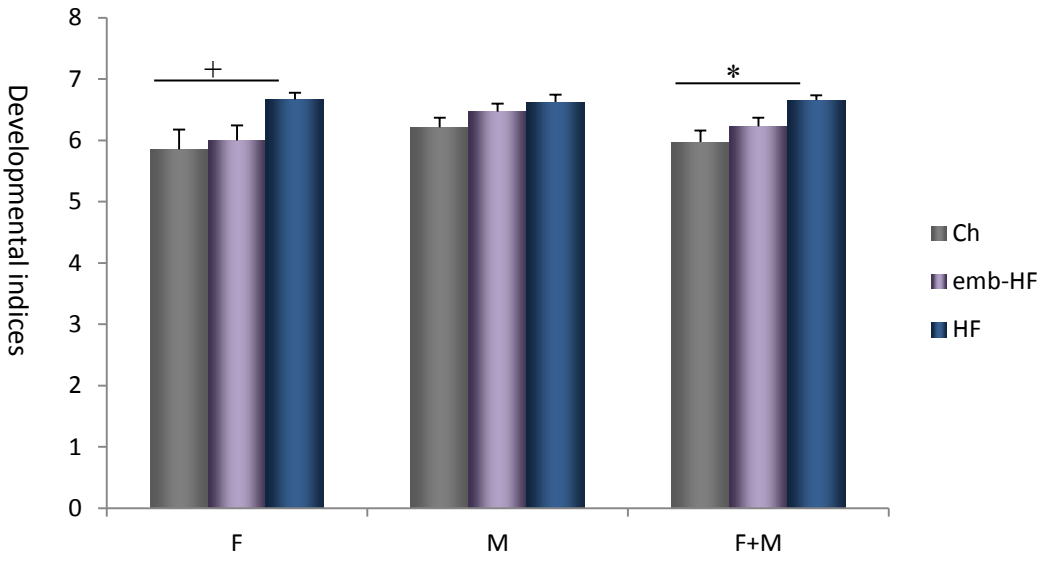


Figure 5.20 Developmental index: Sternum

An increase in developmental index was observed in the sternum of M+F HF fetuses, this difference was observed at a trend level in F fetuses. Error bars ±SEM. *P<0.05, + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

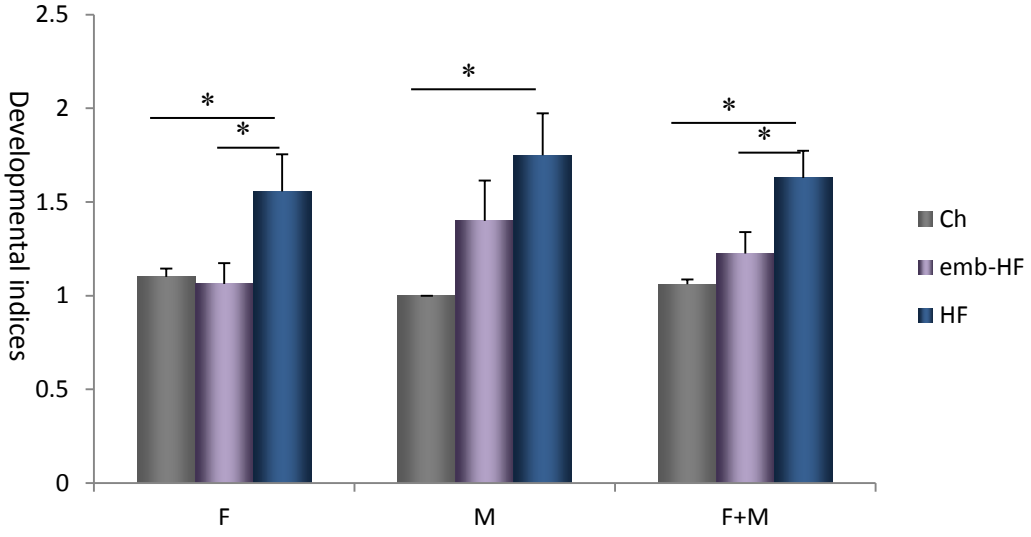


Figure 5.21 Developmental index: Paw

An increase in developmental index was observed in the paw of HF fetuses compared to chow fed fetuses (F, M and F+M) and emb-HF fetuses (F and F+M only). Error bars ±SEM. *P<0.05, + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

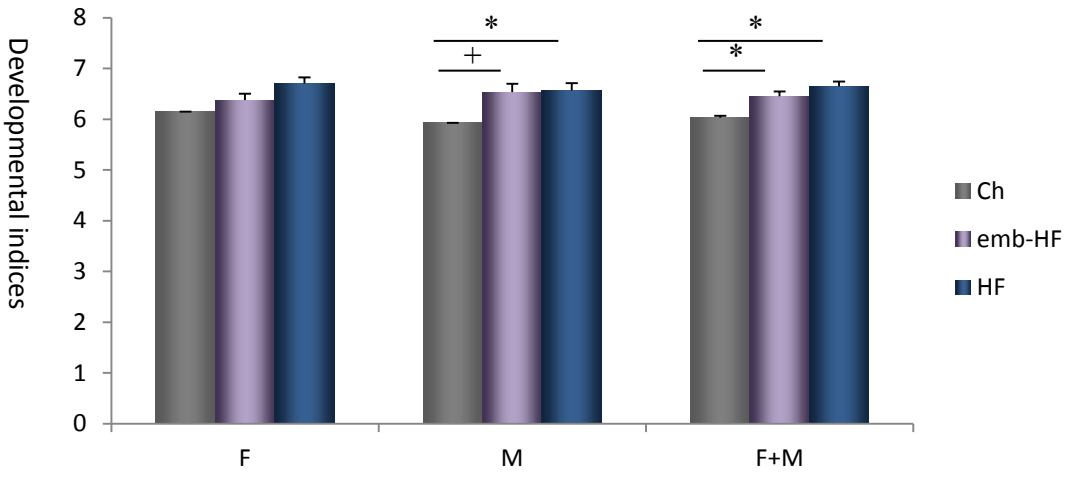


Figure 5.22 Developmental index: Vertebrae

An increase in developmental index was identified in the vertabrae of HF fetuses (M and F+M) as well as an increase in emb-HF fetuses (F+M, and to a trend level in M) compared to controls. Error bars ±SEM. *P<0.05, + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

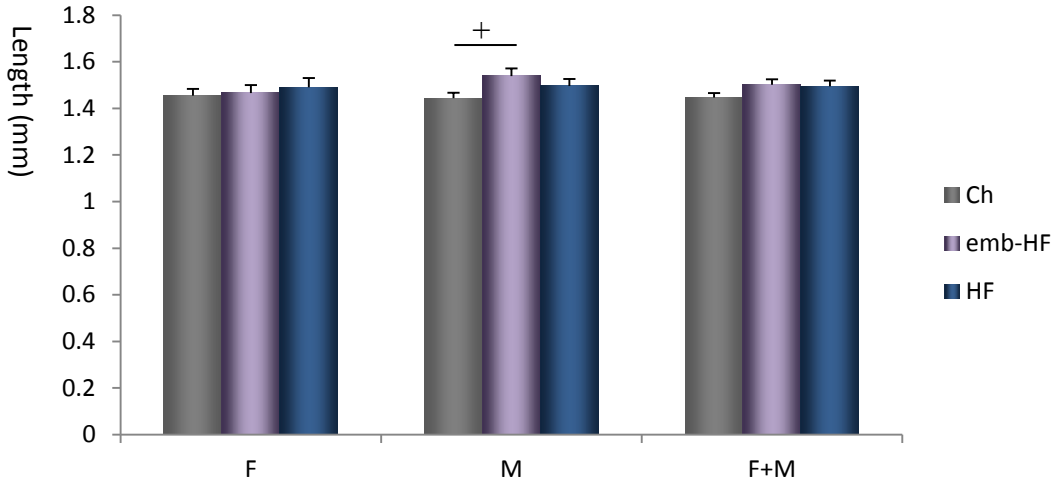
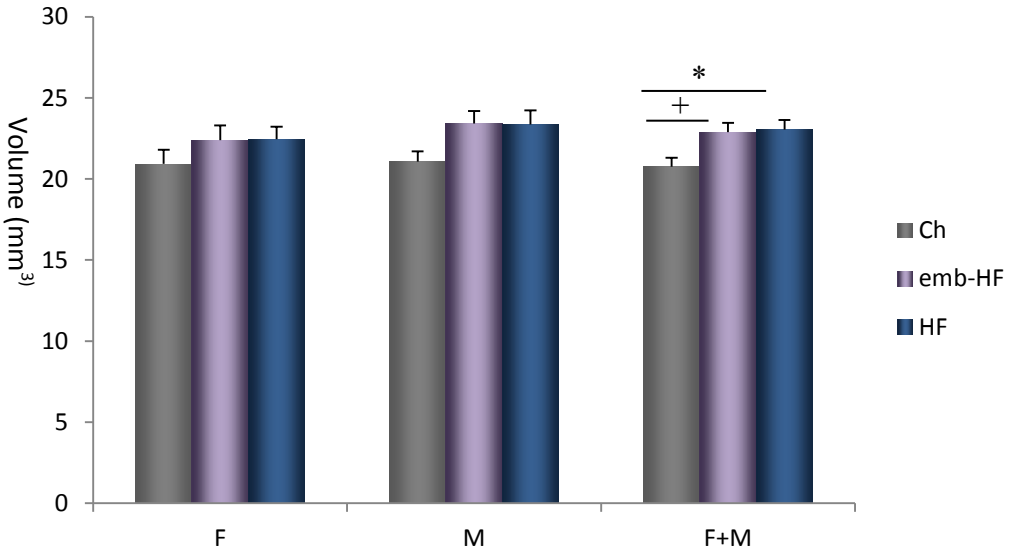


Figure 5.23 Femur length

A trend towards increased femur length was detected in M emb-HF fetuses. Error bars ±SEM. + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17 Statistical analysis was performed by multilevel random effects regression

a) Bone volume



b) bome volume ratio to fetal weight

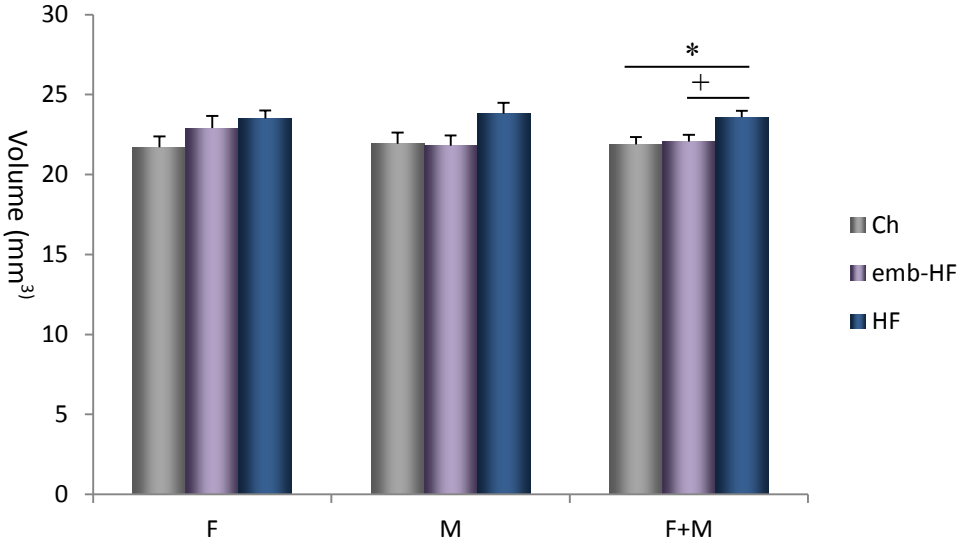


Figure 5.24 Bone Volume

a) A significant increase was observed in total bone volume in HF fetuses (F+M) as well as a trend towards this increase in emb-HF fetuses (F+M). b) A significant increase was observed in total bone volume to fetal weight ratio in HF fetuses compared to controls (F+M) as well as a trend towards this increase compared to emb-HF fetuses (F+M). Error bars ±SEM. *P<0.05, + P<0.1. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

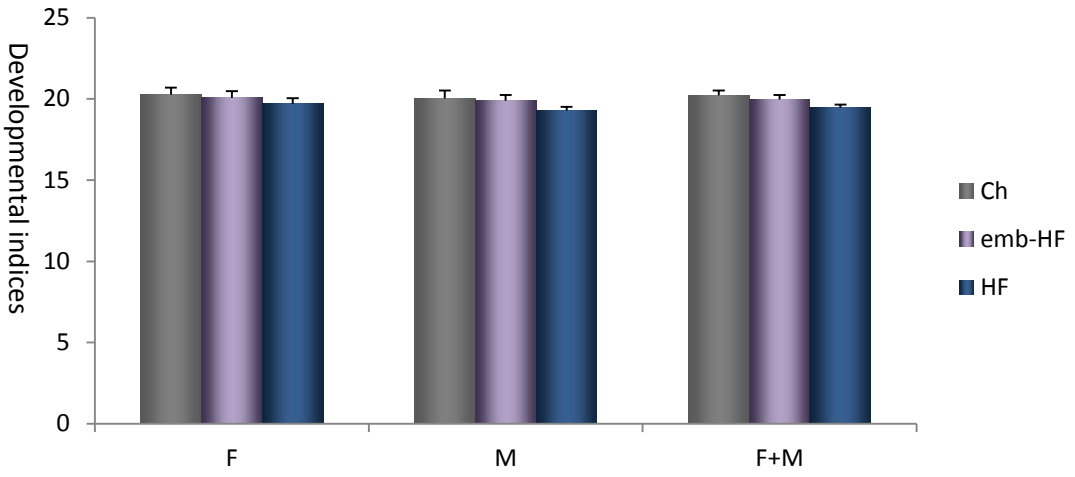


Figure 5.25 Surface to volume ratio

No differences were seen in bone surface are to volume ratio between diets. Error bars ±SEM. M+F Ch n=33, emb-HF n=32, HF n=37; F Ch n=20, emb-HF n=17, HF n=20 ;M Ch n=13, emb-HF n=15, HF n=17. Statistical analysis was performed by multilevel random effects regression

5.3.4 Placental characteristics

Placentas were studied for gene expression. GeNorm and NormFinder algorithms were used to determine which reference genes were most stable between the experimental groups (figure 5.26). *Sdha* expression was found to be most stable by both techniques across all gender groups. All reference genes were found to be of good stability. A second reference gene as determined by gender under GeNorm analysis was used; *Pgk1* for females, *Tuba1* for males.

Expression of *Lipin1* and *Slc27a1* were affected by dietary challenge (figure 5.27). A significant decrease was observed in *Slc27a1* expression in male HF placentas. A trend towards an increase in S*lc27a1* expression was seen in both emb-HF and HF female placentas. A trend towards decreased *Lipin1* expression was observed in HF and emb-HF males placentas. A significant increase in *Lipin1* was observed in female emb-HF as well

as a trend towards a similar increase in HF female placentas. These gender different effects were evident within males and females from the same litters (figure 5.28).

	GeNorm	NormFinder	
Females	***Sdha-Pgk1***	*Sdha*	***Best Pair: Sdha-Ppib***
	18S	*Tuba1*	
	Ppib	*Pgk1*	
	Tuba1	*Ppib*	
	Tbp	*18S*	
	Hprt1	*Tbp*	
		Hprt1	
Males	***Sdha-Tuba1***	*Sdha*	***Best Pair: Sdha-Tuba1***
	Tbp	*Tuba1*	
	Hprt1	*Hprt1*	
	Ppib	*Ppib*	
	18S	*Tbp*	
	Pgk1	*18S*	
		Pgk1	
Females and Males	***Sdha-Ppib***	*Sdha*	***Best Pair: Sdha-Tuba1***
	Pgk1	*18S*	
	18S	*Tuba1*	
	Tbp	*Ppib*	
	Tuba1	*Tbp*	
	Hprt1	*Pgk1*	
		Hprt1	

Table 5.26 Placental reference genes

Stability of reference genes in the ch/emb-HF/HF placenta as determined by Genorm and NormFinder techniques.

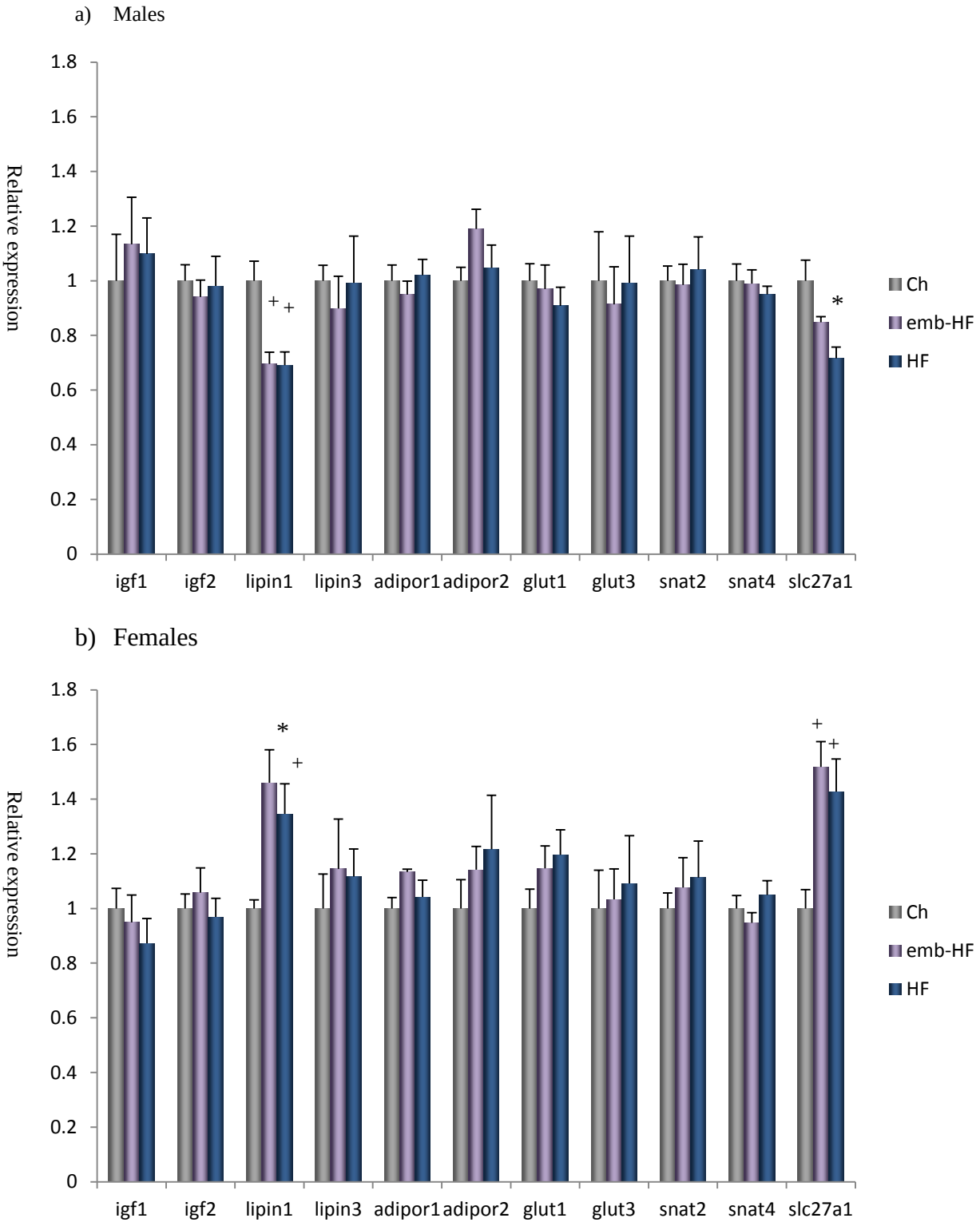
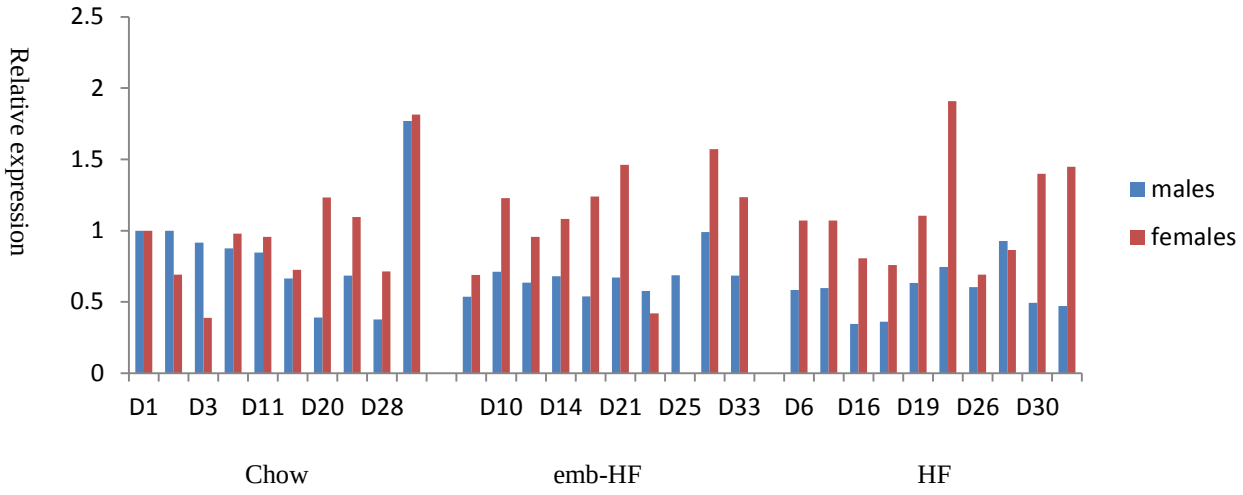


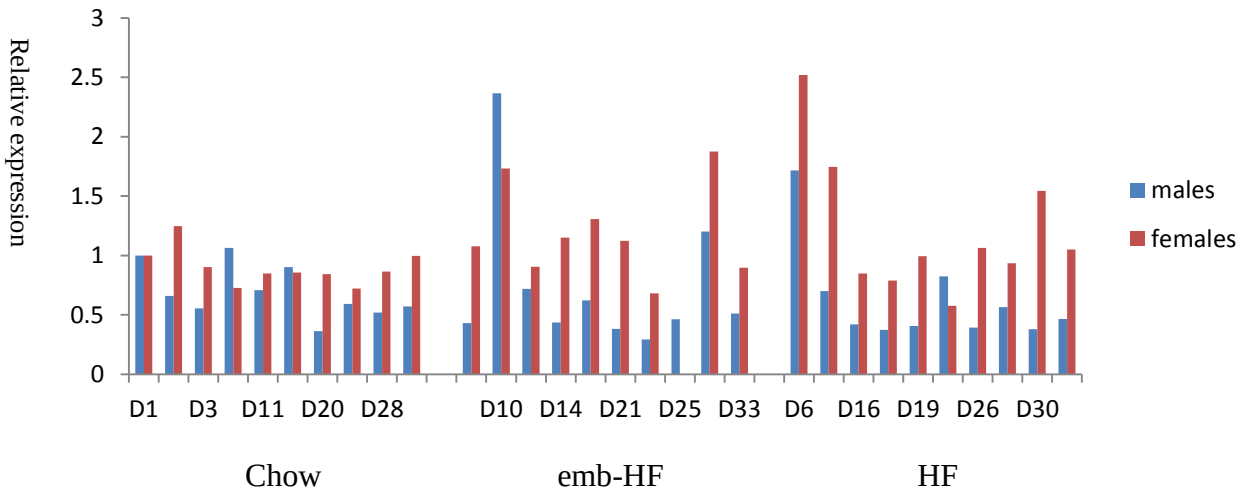
Figure 5.27 Gene Expression in the E17.5 placenta

Fold change in gene expression as determined by real time PCR analysis in a) male and b) female placentas of E17.5 mice from different dietary treatments. A significant decrease was observed in *slc27a1* expression in male HF placentas. A trend towards an increase in slc27a1 expression was seen in both emb-HF and HF female placentas. A trend towards decreased *lipin1* expression was observed in HF and emb-HF males placentas. A significant increase in lipin1 was observed in male emb-HF as well as a trend towards a similar increase in HF female placentas. Statistical analyis was performed by student t-test.

a) *Slc27a1*expression per dam



b) *Lipin1* expression per dam



Average difference between male and female per litter in *lipin1*and *Slc27a1* expression

c) d)

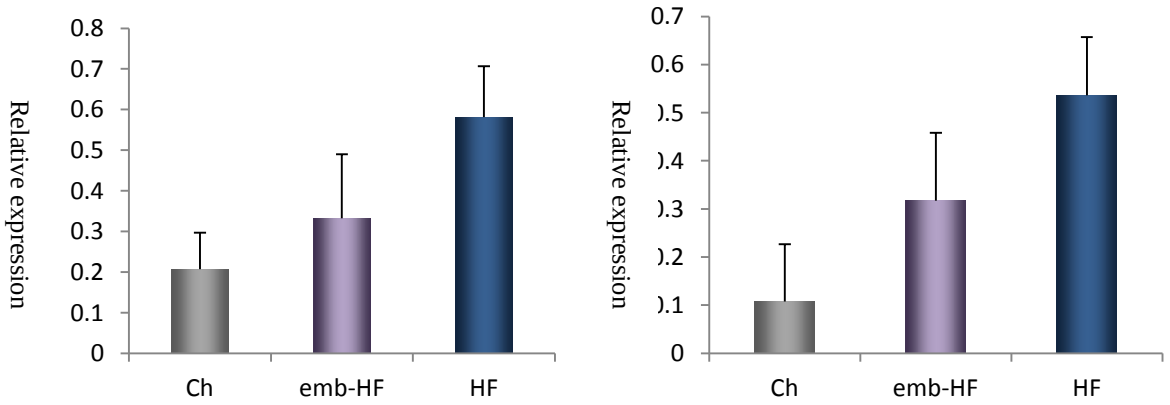


Figure 5.28 Normalised ΔΔCt values (to reference gene and to D1 as arbitary value 1) for a) slc27a1 and b) lipin1 expression by gender, and the average difference between expression of these genes in males and females within the litter for c) slc27a1 and d) lipin1 expression , demonstrating that even within mothers a differential gender effect of diet is seen in the expression of these genes. Statistical analyis was performed by student t-test.

Conclusions

In the present study mice were exposed to dietary HF challenge from conception to E3.5 or throughout pregnancy and compared to chow fed controls.

Firstly, maternal characteristics were investigated. No significant differences in weight gain were observed by the end of pregnancy, with only mild fluctuations being observed on a day to day basis between dietary treatments. From this we can conclude that the HF diet in this study is insufficient for significant weight gain. This is an important feature of this experiment as many other studies combine a fat dietary challenge alongside obesity, providing a confounding factor when studying these two conditions (Howie et al 2009, Bayol et al 2008). It was shown in chapter 3 that although this diet was more calorific than the control chow diet, the mice ate less of this diet. This regulation of caloric intake may be sustained and thus contribute to the cause of appropriate weight gain.

Dams were found not to differ in glucose levels between diets. It should be noted that these are non-fasted animals and therefore dietary intake could obscure any difference in glucose levels that would be otherwise seen. Insulin levels and thus insulin:glucose levels were found to be elevated in HF dams suggesting that the mouse is having to produce greater levels of insulin to maintain a stable glucose level. Dietary glucose is not increased in the HF diet which could indicate that the HF diet makes the dams somewhat insulin insensitive. Alternatively, the higher insulin levels observed may be yet to have caused an effect on glucose levels. As expected, switching back to a chow diet restores control values of insulin in the dam. Insulin levels were measured in offspring and also found to be elevated in the HF group. Interestingly this also occurs in emb-HF fetuses, those that were exposed to maternal HF diet for only 3.5 days, and in which the dams

have normal glucose: insulin ratios. This suggests that programming of these fetuses occurs early in development. This is a unique finding, that this brief exposure causes insulin tolerance reprogramming. Other studies have suggested epigenetic reprogramming as a cause of insulin intolerance. Masuyama & Hiramatsu (2012) showed a maternal high fat diet caused raised triglyceride and leptin levels and lowered adiponectin levels. It was found in this example that acetylation was reduced and methylation levels increased at histone H3 at lysine 9 of the promoter of adiponectin whilst methylation of histone 4 at lysine 20 in the leptin promoter was significantly higher. Short term exposure to poor diet at this crucial time in epigenetic reprogramming could perhaps be the cause of the insulin resistance observed in the current model. Insulin resistance is more commonly associated in the human population with low birth weight (Horáková et al 2005), a feature that was not observed here.

No differences were observed in litter size in the present study. This is in contrast to results observed by Dr Kim Bruce, who reported a reduction in litter size from mice fed the same HF diet prior to conception and throughout pregnancy (Personal communication), There may be several reasons for the discrepancy between studies. Firstly, in Dr Bruce's study animals were fed HF diet prior to conception. It is well documented that oocyte quality can be affected by diet and it may be a reduction in quality at this stage that causes reduction in litter number. It may also be that the maternal characteristics have longer to establish themselves prior to pregnancy and therefore provide an unsuitable embryo environment. C57 BL6J mice were used in Bruce's study as opposed to the MF1 used in the present study. An alternate explanation may be that in fact litter size was not different in Dr Bruce's study, but as dams were not observed at the time of parturition, so pups may have been consumed as is common in mice under stress.

For the present study this reduces the chance that differences in weight being due to dilution of nutrient supply.

Weight differences were observed at E17. Conceptus weight was found to be significantly reduced in HF fed mice. When the component parts, fetus, placenta and yolk sac were weighed, it was found that the placenta of HF pups were significantly smaller. In the emb-HF group a trend towards larger fetuses was seen, although this was not significant except in the males only group. Placental:fetal weight was significantly reduced in both the emb-HF and HF groups. This means that there was reduced placenta in proportion to the fetus in these groups, suggesting increased placental efficiency. Interestingly this patterning comes about differently by diet; in the HF mouse, a decreased placental size was seen (both males and females), whereas in the emb-HF mouse this change occurs by an increased fetal size (significant only in males). More interestingly still is that this mirrors the proportions seen in the blastocyst, in which a shift in proportion towards the ICM is observed, suggesting this early modification in stem cell populations programmes growth trajectory. These results are partially in agreement with those found by Luzzo et al (2012). A similar HF diet was fed to ICF mice for 4 weeks up to the blastocyst stage of development before transfer to pseudopregnant dams. They found a reduction in both placental size and fetus by size measurement. Brain development was also found to be altered in that study. These differences may be due to differences in strain or perhaps the difference in developmental window. Hormonal induction of ovulation was also used there, which may prove to be unrepresentative of normal conditions.

Generally, no significant differences were seen between organ weights from the different groups. Proportionally the left kidney was enlarged a significant level in emb-HF and HF

females, HF males and females combined, and at a trend in emb-HF male and female fetuses. Congential abnormality is known to be more often found in the left kidney, no reason has been determined but suggestions include links with vascular development (Schreuder 2011).

Measures of developmental progression demonstrated that HF fetuses were generally advanced in terms of bone development. It has been shown under in vitro conditions that insulin can increase the rate of bone mineralisation (Yano et al 1994). The increase in bone development here in HF fetuses may well arise from the increased insulin levels seen. Chondrocyte proliferation, matrix synthesis, and increases in chondrocyte size determine bone length, suggesting the effect is upon these components. Further measures could be undertaken here; crown to rump length is a commonly used measure of skeletal development that we have not addressed here.

Gernorm and Normfinder found placentas to express *Sdha* most steadily across both genders. All reference genes studied were stable to an acceptable level for comparison with M values less than 0.5. It was determined that male and female specific normalisation was most suitable, and GeNorm derived gene combinations were used for analysis.

A previous study (Strakovsky & Pan, 2012) of obese mice found that placental lipid accumulation occurred in combination with increases in a range of genes including *glut1*, *slc27a1*, *lipin1* and *lipin3.* Results in the current study also found that *slc27a1* and *lipin1* were affected in the case of HF feeding, *lipin3* and *glut1*were not however found to be affected. *Slc27a1* is a fatty acid transporter whilst the lipins are involved in glycerolipid

biosynthesis, control of lipid metabolism genes (Reue 2009; Martin et al, 2000). These changes in expression are indicative of the HF diet influencing placental function. HF and emb-HF females were found to increase expression of these genes, whereas decreases were observed in the males. Real time PCR gives a snapshot of events. These differential expression patterns may be a result of a time course of adaptation by the placenta to the HF conditions.

The differential response of the genders appears to be true even within the same dam signifying that the fetus is responding differentially to the same maternal cues dependent on sex. The mechanisms behind this gender specific response would be a matter of further study. Importantly for future developments of interventions in human pregnancies, it must be considered that these must be gender relevant.

In summary the proportions between lineages observed in the blastocyst were maintained in emb-HF and HF conceptuses at E17.5 signifying a programming of embryos at the early stage for growth trajectory. This also affected bone development particularly in HF fetuses with a general advancement observed in development. Gender specific responses were heavily observed in this cohort.

Chapter 6

Discussion

The current study proposed to investigate the effects of maternal overnutrition on preimplantation embryo development and the effects this may have on continued development. HF and HPD were fed to mice from conception and the dam and embryo were observed at E3.5. The HF model was chosen to investigate further into gestation, where the dam and fetus were studied at E17.5, when the HF diet was fed for the entirety of gestation or for the preimplantation period only and compared to a chow fed control. Several key findings have been observed.

6.1 Embryos adapt to specific dietary conditions

One of the initial questions considered in this project was that with observations from many different diet models having similar hallmarks, would a very simple model (the differentiation of the first lineages) be responsive to specific dietary challenges or would a generalised response be taken to any non-optimal diet? Chapter 4 addressed changes in the blastocyst in response to HPD and HF diets. The short term challenged blastocyst provides a good model to address this question due to its simplicity.

Comparison between the LPD and HPD models indicate how changes in level of the same macronutrient might affect the preimplantation embryo. Comparison between HPD and HF diets indicates how overnutrition of different macronutrients differ. The LPD mouse model had previously shown that the blastocyst responds to short term maternal nutritional challenge. In this model the total cell number was increased, arising from an increased TE

(Eckert et al, 2012). Similar proportions were observed in the HPD embryo, but in this case as a result of a smaller ICM. A completely different phenotype was observed in the HF embryo; total cell number was increased as a result of an increased ICM, opposing the proportions observed in the protein challenged embryos.

In terms of protein biosynthesis, LPD embryos were shown not to change their protein synthesis rate, whereas HPD embryos show an increase protein biosynthesis rate (Dr Judith Eckert, personal communication). HF embryos were here found not to alter their protein biosynthesis rate. These results may be indicative of the availability of substrate as a result of these diets. Variation in the current data set was high, so increased replicates may be useful as well as development of a more sensitive assay which could look at these results on an individual embryo basis rather than the grouped results here. However, these results all contribute to conclude that embryos respond differentially to different diets and not simply as a standardised response to a non-optimal diet.

6.2 Patterning at the preimplantation stage has an enduring effect on development.

The ratio of TE and ICM cells was found to be altered in the blastocyst by both HF and HPD diets. This difference in patterning was found to be maintained into late pregnancy, with HF fetuses having a proportionally smaller placenta. Interestingly this is also observed in the emb-HF group,

indicating that the patterning of the preimplantation embryo establishes placental: fetal ratio independent of diet in later pregnancy. This study is unique in this link between stem cell populations in the blastocyst providing a patterning effect into late pregnancy. Further experimentation into 'rescue' procedures could be undertaken, to indicate whether this effect can be repaired.

6.3 HF diet causes an increased rate of development

It was observed that HF embryos show a greater cell number compared to their chow counterparts. They also have significantly more cells expressing GATA4 at E3.5. GATA4 is a marker of primitive endoderm, indicating that differentiation to the next cell lineage begins fractionally earlier in the HF embryos. Uterine expression of implantation markers FGF2 and *Lif* were found to be increased in the HF uterus. Particularly revealing is when the time course of FGF2 expression was studied, the pattern of expression seemed to be unchanged, just that the elevation of its expression was earlier.

In contrast to the observations here, Howie et al (2009) report that their HF diet causes an increase in gestation by one day in their HF model. It may be pertinent to study this model to birth (and of course beyond) to see if this observed increased rate of development extends to the end of gestation.

Information gathered at E17.5 may also being to indicate that development is advanced. Some parameters of bone development measured may indicate that

HF diet causes an advancement of development. The paw, sternum and vertebrae were on average more mineralised, an indicator that these fetuses are more advanced. This is not a conclusive picture however as for example femur length was largely unaffected with only male fetuses in the emb-HF group showing a trend towards an increased length. This may however be a result of localised signalling and not a generalised increased rate of development.

6.4 Insulin levels are affected

Insulin appears to play a key role in the development of this HF phenotype. Dr Sarah Finn-Sell demonstrated the effects of HF diet on glucose and insulin in this preimplantation HF model at E3.5, finding that insulin levels were increased. This could potentially be linked to results seen here in the proliferation of the blastocyst. *In vitro* studies show that raised insulin causes increased blastocyst proliferation, particularly in the ICM (Pantaleon & Kaye 1996). Other indicators of insulin elevation were observed in the dam at E3.5, with insulin responsive 4EBP1 and FGF2 pathways being activated at E3.5 (Laplante & Sabatini, 2009, Beaudry et al, 2013), demonstrating that even this short exposure is having physiological effects.

At E17.5 maternal insulin was increased in HF dams, but not in those who had been exposed to HF diet only for the 3.5 days of the preimplantation period. As mentioned, bone mineralisation is shown to be largely increased

by HF diet. Bone mineralisation has previously be shown to be increased by insulin (Yano et al 1994), providing a potential mechanism for this effect.
Fetal insulin levels were increased by maternal HF diet, they were also increased even if the mother had only been exposed to HF diet for the preimplantation period only. This is a key result and most certainly warrants more investigation due to the impact this could have on offspring. Due to the small amount of serum collectable from a mouse fetus, glucose levels were not measured in these fetuses, further study would ideally address this. Glut1 expression was looked at in the placenta and found to be unaffected.

The term PAR suggests that diet optimizes offspring for the environment in which they are to develop. Evidence may indicate this to be true in models such as the low protein diet, but most evidence points to maternal high fat diets not providing any protection for offspring against a high fat diet in later life. In fact, experiments in this study have shown that a very short term exposure to maternal high fat causes programming changes that are non-rectifiable throughout pregnancy, and suggest that offspring will be affected throughout life.

The author questions the premise of the PAR. It has been the assumption that these PARs allow for adaptation to best suit the environment in which the animal is found. Whilst in the case of undernourishment it is found that adverse effects are not seen in animals whose gestational environment matches their postnatal environment, other interventions are not privilege to

this effect. If this is considered on an evolutionary scale, perhaps this protection only occurs in the case of undernourishment as overnourishment was far less likely to occur in an environment where food would have to be obtained by oneself, rather than with the convenience of modern life. In cases where food was eaten in excess, this surely would be coupled with additional exercise due to the efforts involved in obtaining it.

6.5 The relevance of the study to society

The data here suggest that women should be very conscious of the diet they are consuming at reproductive age. A common opinion within society is that poor food is considered to be acceptable when consumed infrequently, but the effects of a small breach to good diet could have a profound long-term consequence if timed with the onset of pregnancy.

Poor quality, palatable foods are often high in fat and it is known that these food stuffs are not conducive to good health. These foods are however profitable and of an addictive nature thus people find them hard to resist. A concerted effort is required to reduce the sale of high fat, but also high sugar, high salt, foodstuffs and make good nutrition an affordable and socially normal lifestyle. Broader education needs to occur to reduce the perpetuation of this problem. It is shown that poor diet and DOHaD type effects are showing a greater increase within poorer populations, such as Hispanic groups in America (Rokholm et al, 2010). The current system is failing people, allowing them to put not only themselves but their children and

grandchildren at greater risk of developing disease. This makes for great expense to the health system, reduction in work capability but more importantly imposes upon quality of life. Education and improvement of diet within the general population is a huge task and a change that is not going to happen overnight. There are indicators to suggest that some campaigning is beginning to make a difference, such as a leveling off of the increase of obesity (Rokholm et al, 2010).

A recent study by the Josephine Rowntree foundation (2012) suggests that in Britain, for a person to live at an acceptable standard of living, to include a healthy diet, a single person needs to earn £193 a week, not including rent (from approximately £80 per week). This equates to £10,036 per year, still excluding rent. The average income in many areas is well below this threshold. The Meadows estate in Nottingham currently has an average income of around £10,000 per person, a figure from which other expenses must be paid. These areas of deprivation clearly risk the propagation of DOHaD type phenotypes and should perhaps be given greater attention so as to not create a trend for illness in this already deprived group.

Just as in the non-pregnant state, recommendations also point to exercise as being an important factor in maternal wellbeing (reduced weight gain, improved psychological state, reduction in pain) as well as improved fetal outcomes (lower body fat, improved stress tolerance, and advanced neurobehavioural maturation)

6.6 Future work

All chapters of this thesis have indicated that further research is warranted on this subject.

Many results were seen at a trend level. An increase in statistical power may help validate these results. The model of mouse used throughout was the MF1, this is an outbred strain. Being outbred has the benefit of being a more realistic representation of genetic variation within the human population which is also outbred. This also however does mean that there is always more variation in results and may account for some results not reaching definite levels of statistical significance. Results could be further strengthened by looking at other aspects of the same question, for example other members of the same signalling pathways. Results in agreement from different studies add great support to developing hypotheses. Of course this study was performed in the mouse, which although has many benefits as a model, can be somewhat representative. Establishment of these effects in larger animal models or in human examples such as IVF where ethical will help to confirm accurate advice for women during pregnancy.

Preliminary investigation was begun into lipid accumulation in the uterus, placenta and embryo. It is predicted that a HF diet would cause an accumulation of lipid droplets in these tissues which may affect cell function. Chapter 5 shows images of lipid droplets in the uterus, qualitative assessment would suggest that lipid accumulation occurs in HF uteri. Early

computerised assessment of these images by area in student projects has suggested that the droplets in the HF uteri actually cover a smaller area. A similar observation was found in placentas from this study, along with reduced area of the junctional zone (Dr Judith Eckert, personal communication). These preliminary observations are contradictory to the hypothesis. In a study of obese prone mice it was found that lipid accumulation was a factor in the placenta, but that the decidual and junctional zones were reduced (Strakovsky &Yuan-Xiang Pan, 2011). Further development of these indications are required, perhaps to include measurement of droplet size. The same technique was found to be viable (but technically demanding) to assess lipid droplets in the blastocyst (see figure 6.1). This would be an interesting angle to develop.

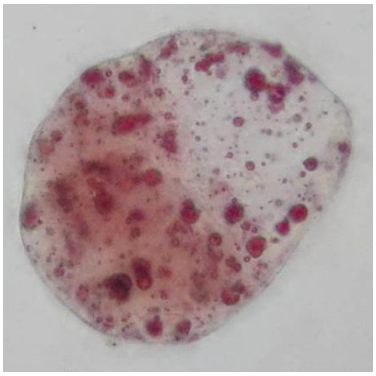


Figure 6.1 blastocyst stained with oil-red-O

A potential future of this work could be the development of a biomarker of poor metabolic state. This could be useful for women trying to get pregnant, whereby they could aim to improve their metabolic state in preparation for pregnancy. There are of course huge implications both ethically and in terms of determining a suitable marker. The current study has begun to implicate insulin responsive pathways are activated in this model, whilst work by

Sarah Finn-Sell has shown a trend towards increases in serum insulin. Further work to validate this observation could be valuable. Although insulin measurement is currently a mildly invasive procedure, those thought to be in risk categories could opt to undertake such monitoring. An ideal situation would be to find a marker that could be monitored easily within the home, perhaps by saliva or urine test.

6.7 Concluding Remarks

Maternal diet during the preimplantation period is shown to have an effect on the blastocyst which is specific to dietary challenge. These affects are enduring throughout pregnancy. Included in these effects are the potential for detrimental outcome (raised insulin levels). This may indicate that in the human population more care should be taken in regulating diet from the very beginning of pregnancy to reduce the potential for detrimental programming of offspring health. Education of the general public should be provided to aid in prevention of offspring poor health.

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Appendix

H6 medium recipe

Stock solution	**volume added**
F	**1 ml**
B	**1 ml**
G	**0.1 ml**
H	**0.1 ml**
E	**0.84 ml**
Embryo culture tested water	**7.8 ml**
20% NaCl	**60 µl**
BSA / PVA	**40 mg**

Adjust to mOsm 255

Stock F (per 100 ml)	
Sodium Chloride	4.720g
Potassium Chloride	0.11g
Sodium dihydrogen orthophosphate	0.06g
Magnesium chloride	0.1g
D-glucose	1.0g
DL-Lactic acid	3.4 ml
Stock B (per 10 ml)	
Sodium hydrogen carbonate	0.2106g
Stock G (per 10 ml)	
Pyruvic acid	0.03g
Penicillin	0.06g
Streptomycin	0.05g
Stock H (per 10ml)	
Calcium chloride 2-hydrate	0.26g
Stock E (per 50ml)	
Hepes	2.9765g

KSOMaa medium recipe

To make 100 ml of 2X stock:

(Storable at -80 ^{o}C for 2 months)

Mix the following:

	Amount	Final Concentration
Sodium Cloride	1.110 g	190 mM
Potassium Chloride	0.0373 g	5.0 mM
Potassium Dihydrogen Orthophosphate	0.0095 g	0.7 mM
Magnesium Sulphate 7-hydrate	0.0099 g	0.4 mM
Lactic Acid (5.5 M stock)	0.362 ml	20 mM
Sodium Pyruvate	0.0044 g	0.4 mM
Glucose	0.0072 g	0.4 mM

Sodium Bicarbonate	0.420 g	50 mM
EDTA (0.5 mM stock)	4 ml	0.02 mM

Penicillin 0.01256 g

Streptomycin Sulphate (0.1 g/ml stock) 1 ml

Make up to 100 ml with tissue culture certified water. Aliquot and store at -80 ^{o}C

To make up 10 ml of 1X KSOM:

Thaw 5 ml of 2X stock and add the following:

$CaCl_2$	100 µl
Non essential amino acids	50 µl
Essential amino acids	100 µl
L-Glutamine	50 µl
Sterile H_20	4.75 ml
BSA	0.040 g

Filter through a 0.2 µm filter

Check osmolarity is between 255 +/- 5 mOsm. Adjust with NaCl

Aliquot and store at 4-8 ^{o}