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Maternal Diet and Visceral Yolk Sac Function during Mouse Development

Volume 1 of 1

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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Doctor of philosophy

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The visceral yolk sac endoderm (VYSE) has a pivotal role to play in the nutritional support of the developing fetus, particularly in rodents. Maternally-derived proteins are endocytosed by VYSE cells and broken down by acid hydrolysis to release amino acids for fetal growth. I have investigated *in vitro* the endocytic activity of the mouse visceral yolk sac derived from mothers fed *ad libitum* either a control diet or low protein diet (LPD) from the time of conception up specified times in gestation. In addition, I have studied the growth, morphology and biochemistry of the visceral yolk sac in relation to maternal diet.

On the whole maternal diet had minimal effect on the growth and total protein content of the visceral yolk sac during gestation. Overall, non-specific fluid phase endocytosis measured by ^{14}C-sucrose uptake was significantly increased in yolk sacs from LPD fed mothers at 17days ($p=0.012$), suggesting bulk fluid uptake is elevated to compensate for reduced maternal protein availability. This distinction was not apparent at day 12 and day 14 of development. Moreover, ultrastructural analysis of VYSE cells at day 17 from LPD mothers revealed significantly increased numbers of apical translucent endocytic vesicles ($P=0.048$). However, receptor-mediated endocytosis of ^{125}I-bovine serum albumin and subsequent release of iodinated tyrosine by yolk sacs from LPD mothers was significantly reduced at day 17 gestation compared to controls ($P=0.038$), indicating reduced nutritional support for the fetus. Western blotting for candidate proteins involved in receptor-mediated endocytosis and lysosomes showed no significant change in their expression levels between the diets.

These data illustrate that maternal protein undernutrition alters the physiological activity of the mouse yolk sac, thereby undermining nutritional support for the fetus.

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Abbreviations

AFP = Alpha-fetoprotein

APO = Apolipoprotein

BSA = Bovine Serum Albumin

EI = Endocytic Index

FCS = Fetal Calf Serum

FOAD = Fetal origins of adult disease

hCG = Human chorionic gonodatrophin

IgG = Immunoglobulin G

LPD = Low protein diet

PBS = Phosphate buffered saline

PVP = Polyvinylpyrrolidone

RAP = Receptor associated protein

TCA = Trichloroacetic acid

VYSE= Visceral yolk sac endoderm

Chapter 1

General Introduction

1.1.1. Fetal Origins of Adult Disease

Epidemiological studies from distinct populations in the UK have demonstrated an association between low birth weight and adult onset disease, namely hypertension, type 2 diabetes and coronary heart disease; this association appears to be irrespective of lifestyle factors such as smoking, adult weight, social class and alcohol (Drake and Walker 2004). Records kept by health visitors of birth weight and infancy growth in people from Hertfordshire, Preston and Sheffield, presented an association between the size of a baby at birth and its subsequent cause of death in adulthood. Analysis of the Sheffield data showed that a person born at term who was small (less than 2500g) had an increased risk of coronary heart disease in adult life. Further studies showed that the same trend appeared for the risk of developing hypertension and non-insulin dependant diabetes mellitus in adult life. These studies were supported from data in the USA, South India and Sweden in subsequent years (Barker and Clark 1997).

It is reasonable to suggest that the cause for a low birth weight and the increased risk of adult onset diseases could be attributed to factors other than maternal undernutrition. However, statistical analysis of the data accounted for potential confounding factors such as socioeconomic group and smoking, and yet the hypothesis was still supportable; low weight births at term had an increased risk of adult onset disease (Barker 1995).

A developing fetus receives all the nutrients it requires to grow from its mother, either by direct contact with the uterine environment or via the yolk sac and placenta. If the supply of nutrients is abundant, then the human fetus will grow normally having a birth weight of more than 2500g and less than 4000g (Rasmussen 2001). When the supply of nutrients and oxygen is insufficient for normal growth, the fetus initiates several mechanisms in order to combat the environment of under-nutrition (Barker 1995).

Programming occurs when an early stimulus or insult at a critical period in gestation results in a permanent or long-term change to the structure and function of an organ (Szitanyi and Poledne 2003). The main adaptation a fetus initiates is to slow down cell division in order to reduce the size of organs and tissues, thereby reducing the amount of nutrients required by those organs (Barker et al 1997, Barker 1995). The consequential adaptation of the fetal body organs to undernutrition results in long term programming (Figure 1, Szitanyi and Poledne 2003). Fetal programming can cause a smaller proportion of lean muscle tissue, thereby resulting in a low birth weight with increased risk of adult onset disease because muscle is an important site for glucose uptake in response to insulin. A reduction in the amount of muscle available could affect the body's sensitivity to insulin later in life (Singhal et al 2003).

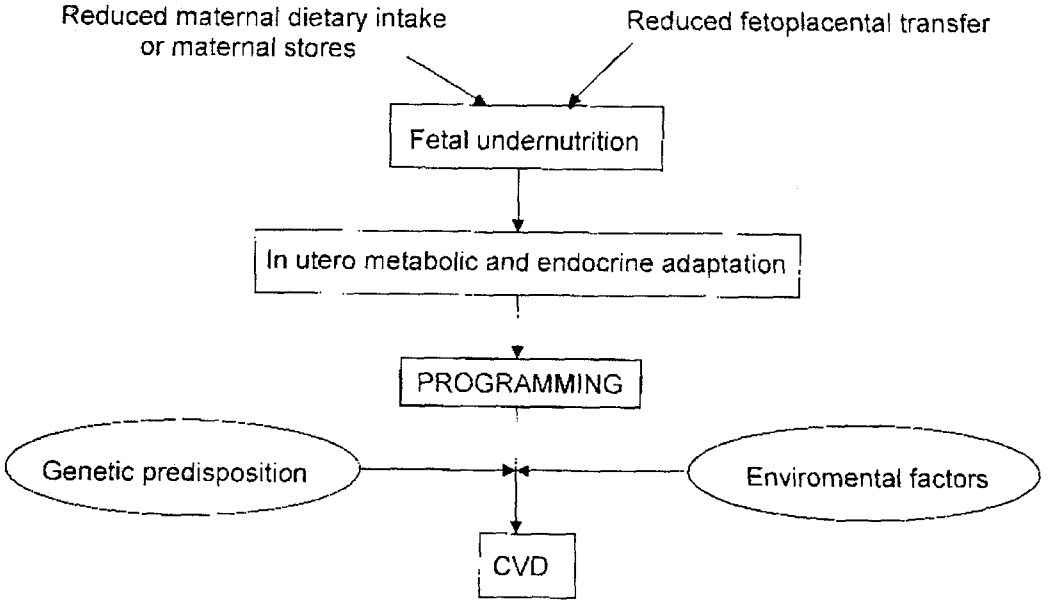


Figure 1: Mechanism of Programming (Szitanyi and Poledne 2003)

The slowing of cell division can occur through a direct effect or through altered concentrations of hormone, such as growth hormone or insulin. These mechanisms can while helping to solve one problem cause another. The response to altered insulin levels may leave the fetus with an impaired glucose tolerance, which in turn can lead to coronary heart disease in later life (Bielinksa 1997). Development of the fetus in poor nutrient conditions will slow down or reduce

any alteration in metabolic activity so that the fetus can make full use of all of the nutrients available (Kwong et al 2000). Studies have clearly shown that undernutrition to the developing fetus at different time points during gestation will cause different types of abnormalities, providing evidence that there are critical time periods for the development of certain systems during gestation (Rasmussen 2001).

Poor conditions for the fetus can originate before conception as has been indicated in human studies such as the Dutch famine of 1944-45 where mothers who were in their first or second trimester and exposed to the famine gave birth to offspring with a low birth weight (Drake et al 2004). In particular, men who were born from these mothers either during or directly after the famine had been exposed to sub-optimal conditions during pregnancy had an increased risk of obesity in adulthood (Langley-Evans et al 1996, B). Animal studies have also investigated poor nutrient conditions during gestation. A poor diet particularly one lacking in protein sustained before and during pregnancy can place the fetus at risk of being born with a low birth weight and therefore more susceptible to developing the adult onset diseases identified in the "Fetal Origins of Adult Disease" (FOAD) hypothesis. This result remains the same even if a normal diet is maintained throughout gestation after poor nutrition before and up to conception (Langley-Evans et al 1996, B).

Undernutrition of the fetus early in gestation will lead to a proportionately small fetus at birth, while undernutrition during mid gestation may cause both a low body mass and the fetus will be thin in proportion to its body mass. From nine weeks after conception in human pregnancy, the fetus undergoes a period of rapid growth, which continues up to and after birth. There are critical time points within this period of rapid growth and cell division with different organs developing at different time points. If alterations in the normal rate of growth occur during a critical period then disproportionality will occur (Barker 1995).

An individual born after mid-gestational undernutrition will have a significantly higher risk of coronary heart disease, hypertension and non-insulin dependant diabetes. Fetal undernutrition late in gestation may lead to the birth of a child that

has a normal body mass but is short in relation to its body mass (Langley-Evans et al 1996, B). Figure 2 outlines these effects of undernutrition at different time points in human gestation. This last anomaly of being short in relation to ones body mass stems from the redistribution of oxygenated blood to the cranium at the expense of trunk organs during development. This occurs in order to sustain metabolism within the brain, which in turn impairs the growth of the liver and may also have an effect on the growth of the yolk sac (Godfrey and Barker 2000). These metabolic changes are part of a concept known as 'programming' (Rasmussen 2001). Programming is the process by which stimuli acting on the fetus during gestation can permanently change tissue structure, metabolic and physiological functions and gene expression (Langley-Evans et al 1999).

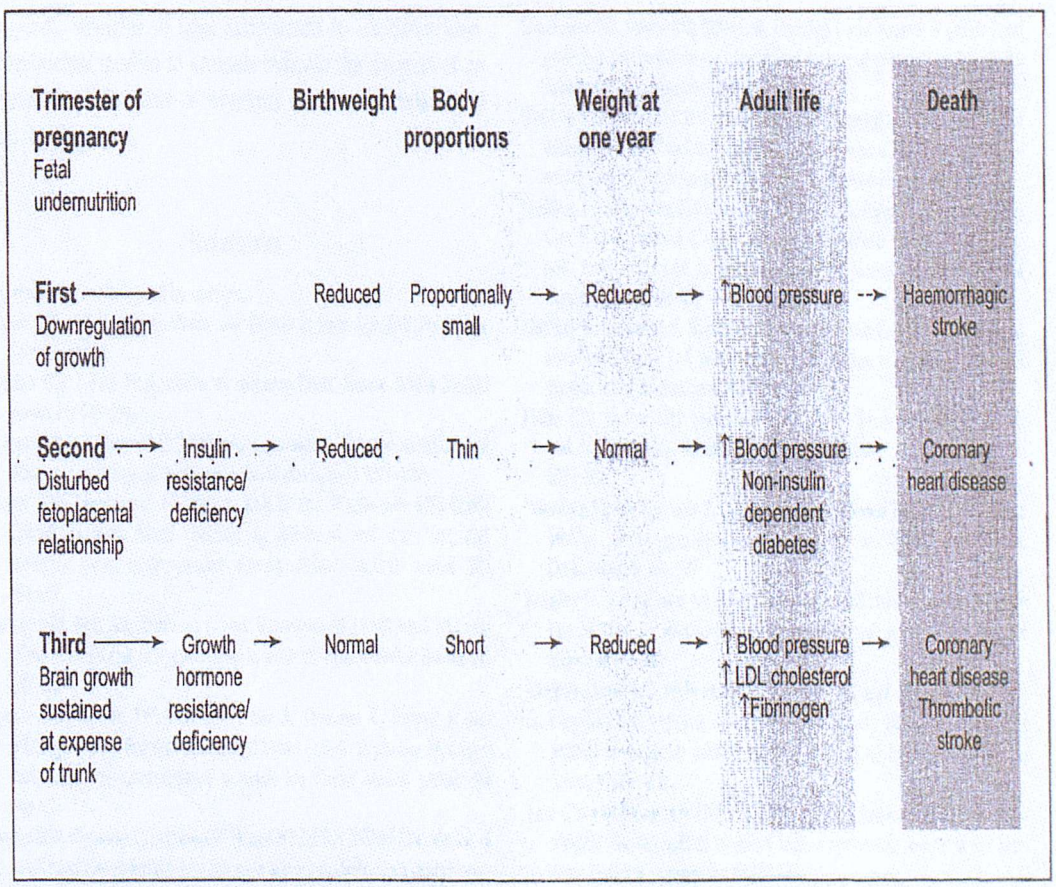


Figure 2: Effects of fetal undernutrition at different stages of gestation. (Barker and Clark 1997)

Although several studies have investigated the effects of under-nutrition, there are other mechanisms surrounding the FOAD hypothesis which may contribute to programming. There is a high correlation between the birth weights of human

siblings which suggests that a genetic influence is acting to regulate growth rate. Thus, changes in the fetus may also be a reflection of altered gene expression in the uterine environment and not solely a direct fetal reaction to under-nutrition. Genetic effects may also be responsible for the susceptibility to adult onset diseases; only 6% of adults suffering from non-insulin dependant diabetes mellitus have a low birth weight at gestational term (Langley-Evans et al 1999). However, some intrapair studies of monozygotic twins have shown that differences can occur in birth weights and lean mass in later life (Singhal et al 2003). This implies that although the FOAD hypothesis may explain the origin of many instances of adult onset diseases it is not responsible for every such case; a healthy adult can be defined by many factors including genetic inheritance, maternal nutrition during gestation, early postnatal nutrition and adult lifestyle (Langley-Evans et al 1999).

1.1.2 Animal models used to study the FOAD hypothesis.

Detailed metabolic and genetic studies and the use of animal models are required to test the FOAD hypothesis proposed from human epidemiological data. Sheep and pig laboratory models have been used for many years to study the effects of maternal undernutrition on the fetus (Rhind et al 2001, Drake et al 2004).

Maternal undernutrition in sheep during gestation and early postnatal life has been shown to induce a reduction in the lifetime reproductive capacity of female offspring. There are clear critical stages of development in the female fetus in sheep, namely between days 35-55 which is a transient period of steroid synthesis in the fetal ovary and around 110 days which is the end of folliculogenesis after which time the number of follicles in the fetal ovary cannot be changed. Male fetuses have less clearly defined windows of critical development affecting their fertility. As yet there has been no relationship between maternal undernutrition and subsequent fertility of offspring in human studies, however there is evidence from the Dutch famine 1944-45 that children born to mothers who were undernourished as fetuses due to the famine have slightly lower birth weight (Rhind et al 2001).

Programming has been linked to excess exposure to glucocorticoids during gestation; the sheep model had been used to study the effect of glucocorticoid injections. Results from theses studies showed postnatal insulin insensitivity and reduced fetal growth, they did not reveal postnatal hypertension in sheep as a direct result of glucocorticoid injections, it was only apparent when accompanied by low birth weight. Administration of synthetic glucocorticoids during the last week of pregnancy in rats causes low birth weight, adult glucose intolerance and increased adult blood pressure in the offspring (Moss et al 2001).

Rodent models are particularly useful given the relatively short gestation period, approximately 21 days depending on species, and reduced length of time after birth needed to collate the growth and physiological data indicative of phenotype. In addition, the plethora of genome sequence information available for the mouse and the extensive published research on post implantation developmental physiology, particularly in the rat, makes the rodent models suitable for analysis of underlying mechanisms.

The rat model has been used extensively to study the effects of altering maternal diet before and during gestation. Primarily the protein content of the diet has been changed, but also the fatty acid composition which influences blood pressure in rats and humans (Langley-Evans 1996). Langley-Evans et al (1996, A) found that rats fed on a low protein diet for 14 days prior to mating and throughout pregnancy had fetuses with an altered body mass and length at day 20 of development and gave birth to offspring with altered birth weight and raised systolic blood pressure throughout life. Significant differences in fetal weight and placental weight were also apparent during gestation. These data reinforce the epidemiological data used to formulate the FOAD hypothesis in humans.

Langley-Evans and his research group also fed a low protein diet for only certain periods of gestation, 0-7 days, 8-14 days and 15-22 days of gestation, without prior habituation to the diet before mating, to study the effects of undernutrition at specific times (Langley-Evans et al 1996, C). This investigation resulted in no change to litter size or maternal food intake during gestation. Gender-specific differences were identified in that females had very little change in birth weight,

however, males had significantly more weight gain after birth in the groups from mothers fed a low protein diet for the first week, second week or throughout gestation compared with females. This research yielded results demonstrating that sufficient nutrition in the first week of gestation was particularly important to ensure the correct development of the fetus and to reduce the risk of hypertension (Langley-Evans et al 1996, C).

Once general undernutrition during gestation had been shown to cause abnormal fetal and postnatal growth and physiology, a second line of investigation considered the effects of differing levels of specific ingredients in the diet. Langley-Evans (2000) investigated the effect of two casein low protein diets when fed from the day of conception. The diets were essentially identical except that one had twice the amount of fat and a higher methionine content in order to avoid sulphur deficiencies than the other diet had. Langley-Evans found that both diets did not affect litter size or maternal food intake during gestation compared to controls, and that both low protein diet (9% casein) caused low birth weight in the pups. The diet with elevated fat and methionine also showed raised blood pressure in the offspring, as has previously been demonstrated (Langley-Evans 2000).

Langley-Evans also investigated the difference between feeding rats a diet containing coconut oil or corn oil as well as incorporating the low protein versus control diet. Coconut oil is made up of saturated fat unlike corn oil which is predominately unsaturated fat, coconut oil also contains less linoleic acid than corn oil which is known to have a hypotensive effect in animals (Langley-Evans 1996). Supplementations with amino acids such as methionine or serine have also been investigated (Langley-Evans 1996). Each different diet was employed to find information about specific mechanisms during gestation.

Overall, the studies using altered maternal diet during gestation have found fetal growth retardation and changes in postnatal blood pressure, increased adiposity and insulin resistance, all of which mirror the findings of the Barker hypothesis in humans (Langley-Evans et al 1996 B).

As well as reinforcement of the basis of the FOAD hypothesis, more

investigations have brought to light more factors that are affected. Long term changes in the hypothalamic-pituitary-adrenal (HPA) axis in both sexes have been shown to be caused by fetal programming and may link fetal growth with adult metabolic disease such as diabetes and hypertension. Alterations in the HPA activity have been investigated in animal models including sheep, guinea pigs and rats. The changes can be caused by prenatal stress, maternal undernutrition or glucocorticoid exposure during gestation, also postnatal manipulations including neonatal handling and modified maternal behavior (Rhind et al 2001, Matthews 2002, Ward et al 2004).

The pathway by which the fetus receives the nutrients it requires is vitally important for proper development. In mouse development during the preimplantation stages (days 0-4), the embryo takes its nutrients from the surrounding uterine environment via absorption mechanisms. After implantation, a structure known as the yolk sac develops and takes on the role of providing all the nutrients that the fetus requires from day 5-10 by which time the placenta has developed and the duties become shared (Bielinska et al 1999).

1.1.3 Yolk Sac Structure and Development

Fertilization in the mouse occurs in the oviduct. The fertilized egg then undergoes a series of cell divisions, known as cleavage, thereby developing from two cells to four cells to eight cells and continues to double in cell number up to a specific size. At the blastocyst stage, the fertilized egg has undergone several asynchronous cell divisions and is now a ball of approximately 32 cells with a cavity in the middle known as the blastocoel (Wild and Fleming 1999). The cells at this stage have segregated into two types, an outer epithelial layer, known as the trophectoderm, which forms the wall of the blastocyst and an inner cluster at one pole of the blastocoel, the inner cell mass (ICM). On the blastocoel surface of the ICM an epithelial layer of cells begins to develop before the embryo implants; this epithelium is known as the primitive endoderm or hypoblast and the remainder of the ICM, as the epiblast or primitive ectoderm (Wild and Fleming 1999).

The primitive endoderm develops to line the cavity of the blastocyst (Jollie 1990,

Wild and Fleming 1999). The primitive endoderm cells that do not remain in contact with the epiblast differentiate into parietal endoderm as they migrate, those cells that remain in contact with the epiblast during migration form the visceral endoderm (Figure 3) (Wild and Fleming 1999, Palis et al 1995, Bielinksa et al 1999). Prior to implantation the primitive endoderm starts to give rise to the visceral yolk sac endoderm (VYSE) (Palis et al 1995). Figure 3 shows in a diagrammatic format the migration of the cells and the development of the embryo from prior to blastocyst implantation to post implantation with the establishment of the visceral yolk sac, egg cylinder and parietal yolk sac (Spyropoulos and Capecchi 1994). After blastocyst morphogenesis, the embryo implants into the wall of the uterus (uterine epithelium) at approximately day 4 in mouse (Wild and Fleming 1999).

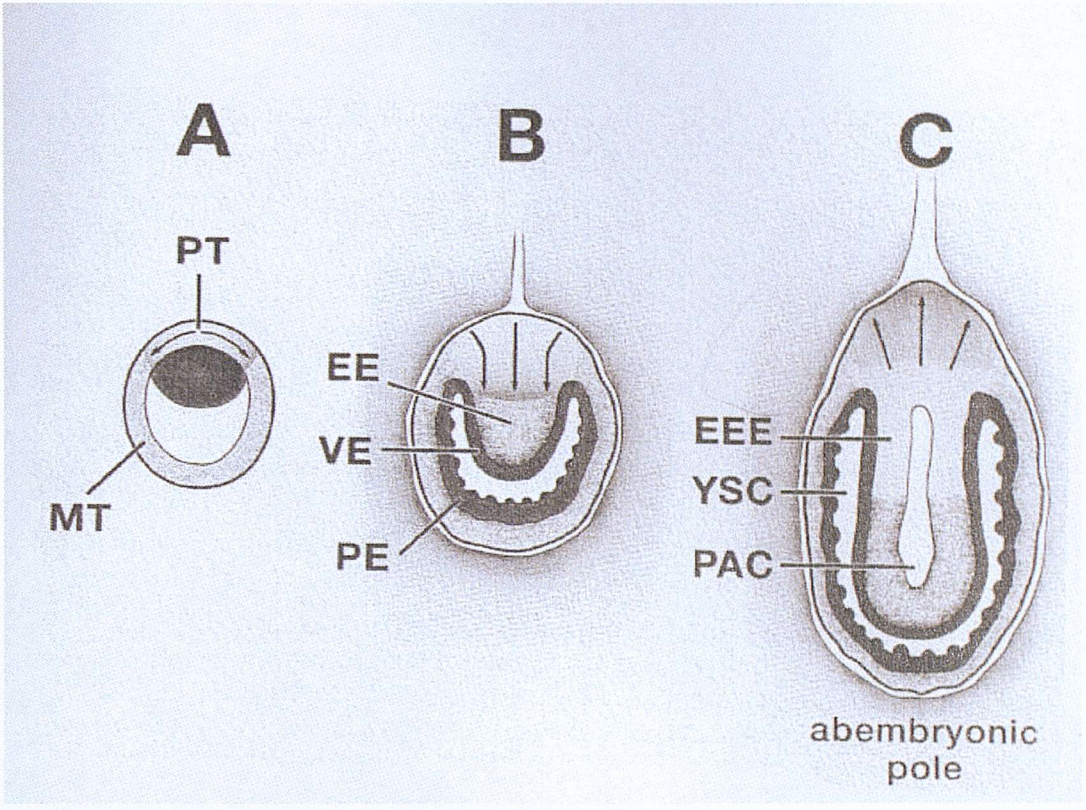


Figure 3: Morphogenesis of conceptus from blastocyst to egg cylinder. (MT) Mural trophectoderm, (PT) polar trophectoderm, (EE) embryonic ectoderm, (VE) visceral endoderm, (PE) parietal endoderm, (EEE) extraembryonic ectoderm, (YSC) yolk sac cavity, (PAC) proamniotic cavity. (from Spyropoulos and Capecchi 1994)

The parietal endoderm develops from the time of implantation and becomes positioned adjacent to the trophectoderm layer (Figure 4) (Palis et al 1995), it synthesizes type IV collagen and laminins which assemble into the Reichert's

membrane; this acts as a passive filter of nutrients from the uterine fluid to the fetus. The cells in the parietal endoderm are terminally differentiated; this means that they do not have the ability to form a different type of cell other than parietal endoderm, unlike the cells of the visceral endoderm. Parietal endoderm cells show no obvious polarity of internal organelles and do not form specialized intercellular junctions (Bielinksa et al 1994).

Gastrulation occurs at day 6.5 of gestation, it results in the formation and segregation of the three germ layers (Wild and Fleming 1999). At this time, cells of the visceral endoderm are displaced by migrating definitive endoderm from the embryonic ectoderm. The movement of cells is a polarized process as the cells move from the distal tip of the egg cylinder to the region overlying the area that will become the anterior region of the embryo (Bielinska et al 1999, Perea-Gomez et al 2001). The migrating cells have an asymmetric gene expression pattern, which suggests a possible mechanism for axial patterning (Bielinska et al 1999). The pattern of expression may help define the axis of the developing embryo.

The visceral endoderm develops at the same time as the ICM continues to develop. It forms a layer of cells that separates the amnion from the parietal endoderm layer (Figure 4), at 7.5 days gestation (Wild and Fleming 1999). The cells of the visceral endoderm retain the ability to differentiate unlike those of the parietal endoderm. They synthesize and secrete a variety of proteins, for example one of the most important proteins is alpha-fetoprotein (AFP) (Huxham and Beck 1984, Bielinska et al 1999, Scohy et al 2000). AFP is important in maintaining both intravascular osmotic pressure and serum transport (Farrington et al 1997). It is the main component of mammalian fetal serum (Lazarevich 2000), and is transcribed at high levels in the visceral yolk sac endoderm (Scohy et al 2000). Morphologically and functionally, the cells of the visceral endoderm resemble those in the gut endoderm. They have numerous microvilli which are coated in glycocalyx (Jollie 1990) and contain a large quantity of phagocytic and pinocytic vesicles, both of which help it to be a very efficient endocytic structure (Bielinska et al 1999, Livesey et al 1979).

The visceral yolk sac has been shown to influence anterior development of the embryo and is required for the correct development of the heart. GATA4 is a transcription factor, required for the formation of visceral endoderm (Farrington et al 1997). It is expressed during development in the visceral endoderm and later in the precardiac mesoderm (Beddington and Robertson 1998). Experiments that investigate a targeted mutation in GATA4 in embryos, have shown that the normal foregut fails to form and the myocardial primordia do not fuse with the ventral midline in the embryo. *Evx1* and HNF-4, a steroid receptor gene, are both required during development for the correct formation and differentiation of the visceral endoderm (Farrington et al 1997).

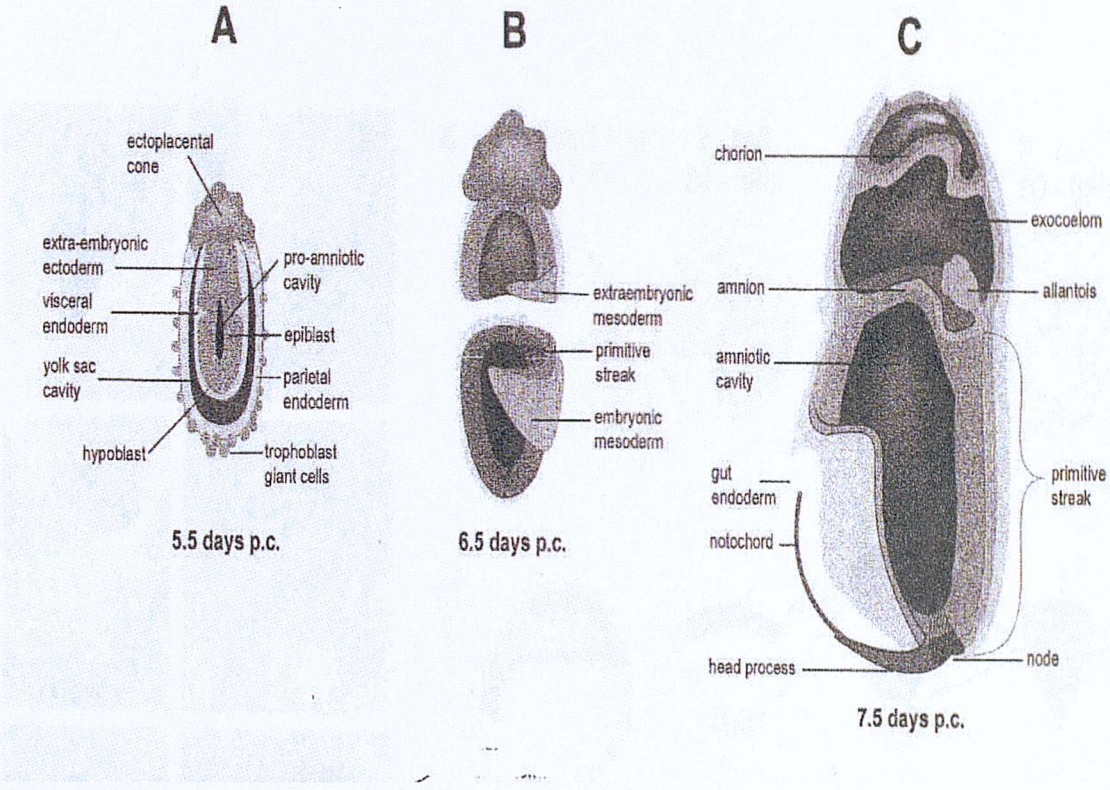


Figure 4: Stages of embryonic development from day 5 gestation to day 7.5. (from Wild and Fleming 1999)

When the embryo reaches approximately day 8 of gestation the visceral endoderm forms a layer that covers the epiblast and extraembryonic ectoderm (Perea-Gomez et al 2001). The cells are not uniform within this layer; those at the distal pole of the egg cylinder have a squamous morphology whilst those at the proximal end are cuboidal in shape. This difference is due to regional differences in gene

expression, for example AFP expression. At day 7.5, AFP is expressed solely in the distal portion of the visceral endoderm covering the epiblast (Bielinska et al 1999). Regional differences are representative of the interactions between the visceral endoderm and the underlying ectoderm. Transplantation experiments have shown that AFP expression is inhibited in the visceral endoderm by the extraembryonic ectoderm (Bielinksa et al 1999). Regional variation within the visceral yolk sac is a continuing attribute of the visceral yolk sac as its functions vary during gestation (Beckman et al 1990, A)

During the period between implantation and approximately day 10 of gestation, the yolk sac inverts. The portion of the membrane lying closest to the fetus inverts so that it surrounds the developing embryo, this membranous layer becomes known as the visceral endoderm. The peripheral portion of the membrane remains bilaminar and is external to the visceral endoderm, this is known as the parietal wall of the yolk sac (Jollie 1990). The parietal wall comprises of parietal endoderm, Reichert's membrane and a trophoblast giant cell layer in that order (Figure 5, Beckman et al 1998, Cockroft 1987).

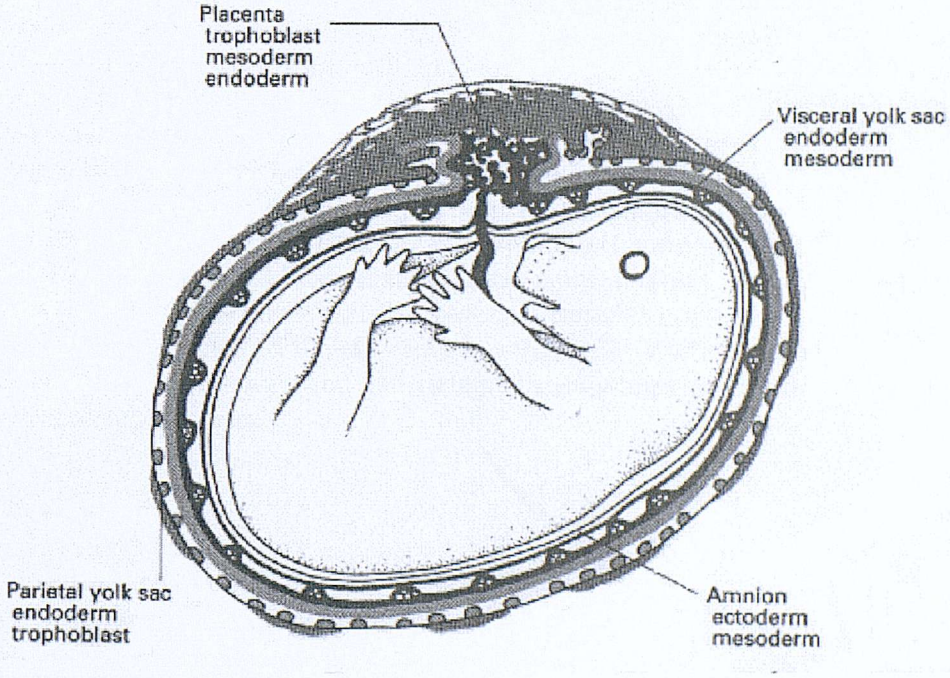


Figure 5: Schematic diagram of 13.5 day mouse conceptus, showing placenta, yolk sac and fetus. (Hogan et al. Manipulating the mouse embryo)

Figure 5 shows the structure of the mouse conceptus at 13.5 days of gestation. It depicts the parietal yolk sac and the visceral yolk sac surrounding the fetus and the connection to the placenta. The visceral yolk sac plus the trophectoderm and the parietal endoderm make up the entire structure of the murine yolk sac at this point in gestation (Bielinksa et al 1999). The parietal endoderm degenerates and the visceral endoderm then lies in direct contact with the uterine environment from approximately day 14. For the rest of gestation, the yolk sac consists purely of the bilaminar visceral endoderm and a mesenchymal layer which houses the vitelline blood vessels (Richardson et al 2000). The ultrastructure of the visceral yolk sac is displayed in the electron microscope micrographs presented in Chapter 5.

1.1.4 Yolk Sac Function

The mouse visceral yolk sac is involved in nutritional, metabolic, endocrine, immunological and haematopoietic functions (Beckman et al 1990, A). In 1947, Noer and Mossman established that the visceral yolk sac's major role in development of the fetus is to provide nutrients, primarily amino acids, to the growing fetus (Beckman et al 1990, A). The proteins from the maternal serum are endocytosed by the visceral yolk sac and digested into amino acids, within lysosomes, which are then passed on to the fetus via the vitelline circulation (Jollie 1990, Freeman and Lloyd 1983 A, Lloyd et al 1998). The vitelline circulation provides a direct connection to the fetus from the visceral yolk sac, therefore proteins that are captured and digested by the visceral yolk sac are transferred directly to the fetus (Beckman et al 1997). In addition to the proteins transported, the visceral yolk sac also transports sodium, calcium and phosphate ions from the maternal circulation to the fetal circulation (Novak and Betteridge 2000). Figure 6 depicts the structure of the visceral yolk sac at day 10 of gestation and shows how the functioning of the visceral yolk sac is carried out during gestation (Moestrup and Verroust 2001).

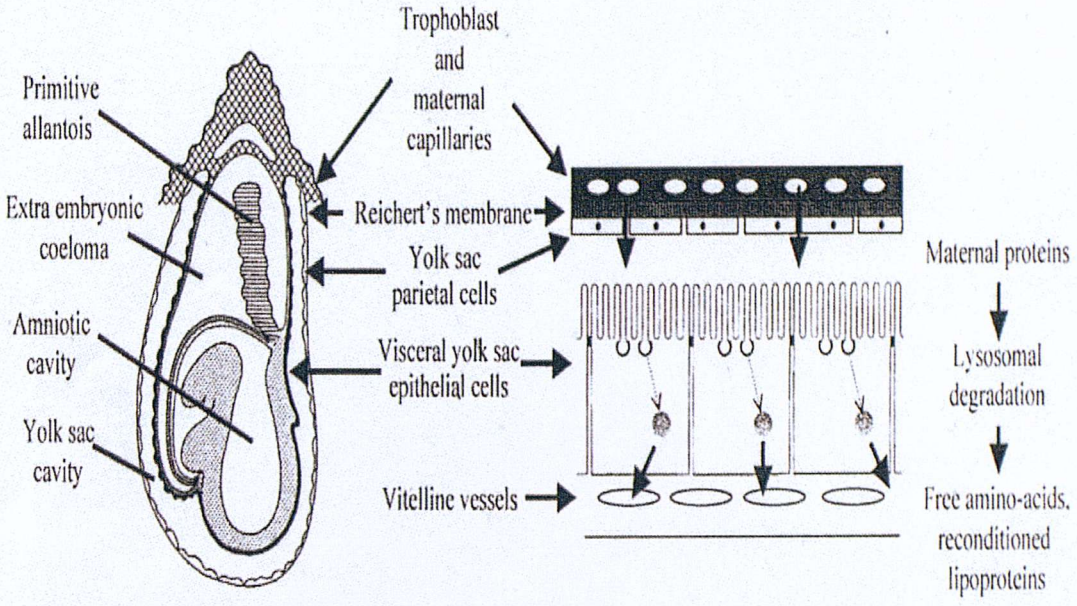


Figure 6: Anatomy and function of the murine visceral yolk sac at Day 10 (from Moestrup et al 2001)

Endocytosis

The visceral yolk sac takes up nutrients from the uterine environment primarily through endocytosis (Jeng and Welch 2001). Endocytosis, as a broad term, is the capture of large molecules i.e. proteins and lipoproteins, from the external environment and their transfer into the cytoplasm of the cell. Smaller substances, for example ions and amino acids, are taken up through fluid phase endocytosis, or channels and transporters directly in the membrane of the epithelial layer (Moestrup et al 2001, Besterman and Low 1983). Channel proteins transport water or specific ions e.g. potassium down a concentration gradient across the plasma membrane. Transporters move one substrate molecule at a time across the membrane from the maternal to the fetal circulation; examples of substrates moved by transporters are sodium, calcium and phosphate (Novak and Betteridge 2000). Integral protein pumps transport sugars, amino acids and ions across the plasma membrane (Conner and Schmid 2003).

Endocytosis encompasses a diverse set of basic cellular transport mechanisms employed by the cell (Apodaca 2001, Besterman and Low 1983). The visceral yolk sac primarily uses two of these mechanisms for the supply of amino acids; fluid phase pinocytosis and receptor-mediated endocytosis. Receptor-mediated

endocytosis involves coated pits which are formed in the plasma membrane (Figure 7); they may be activated and positioned with the help of actin which serves as a possible anchor for scaffold assembly in clathrin-coated pits (Schmidt and Hall 1998, Apodaca 2001, Conner and Schmidt 2003).

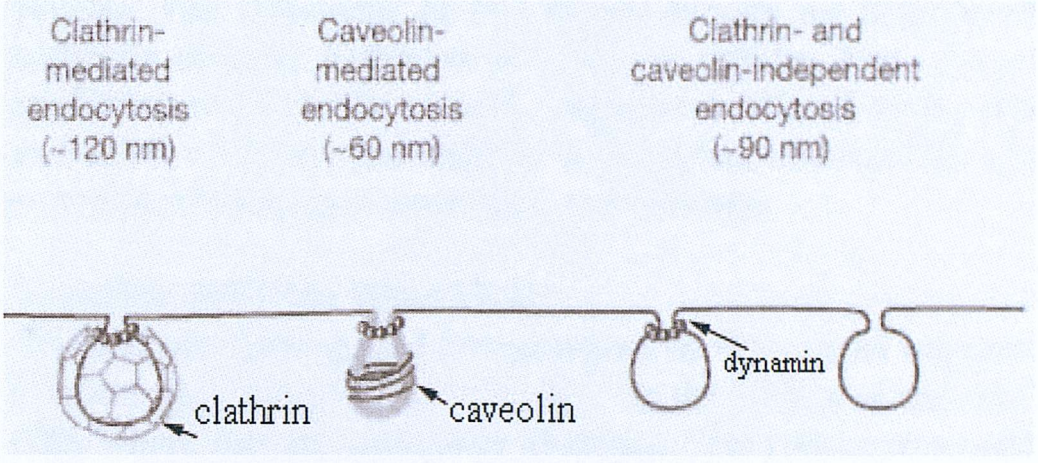


Figure 7: Endocytic pathways (from Conner and Schmid 2003)

Fluid-phase pinocytosis is a non-specific pathway which is normally active at a constant rate internalizing molecules proportional to their concentration. Most substrates internalized through fluid-phase endocytosis are either insoluble particles or low molecular weight solutes. Once they have been internalized, the particles/substrates are commonly transported to lysosomes where they are degraded (Besterman and Low 1983).

Receptor-mediated endocytosis is a selective mechanism which relies upon specific receptors at certain membrane binding sites within coated pits (Besterman and Low 1983). The cytoplasmic lining of the coated plasma membrane invaginations contain a large amount of clathrin (Gruenberg 2001). Figure 8 provides a diagrammatic overview of receptor-mediated endocytosis (Moestrup et al 2001). Ligands (substrates) bind to the receptors on the plasma membrane, the plasma membrane invaginates and then pinches off to form early endocytic vesicles (Besterman and Low 1983, Jeng and Welch 2001). The coated endocytic vesicles deliver the ligand to lysosomes or other intracellular compartments, whilst the clathrin and other receptors are commonly recycled back to the membrane (Besterman and Low 1983, Conner and Schmid 2003). Table 1 lists

some of the ligands that bind to two of the specific membrane receptors found in the visceral yolk sac, namely megalin and cubilin.

Once the receptors have delivered the substrates to the lysosomes, lysosomal proteases degrade the substrates and the derivatives are passed on to the fetus. The major lysosomal proteases in the visceral yolk sac are cathepsins, predominately cathepsin B and L, which are a family of cysteine proteases (Weber et al 1997, Sol-Church et al 1999). Cysteine protease inhibitors have been previously shown to be embryotoxic agents causing malformations in day 10-12 gestation rat fetuses (Sol-Church at al 1999).

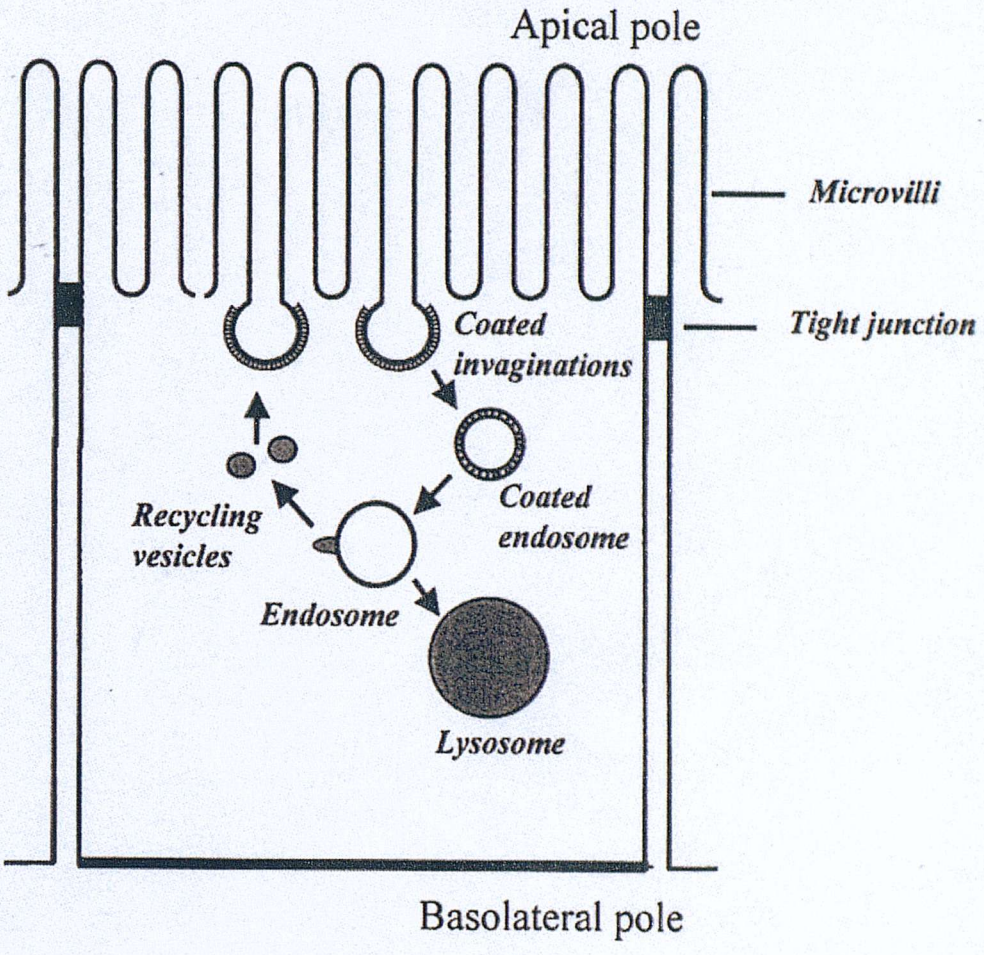


Figure 8: A visceral yolk sac epithelial cell showing an overview of receptor-mediated endocytosis. (Moestrup et al 2001)

Clathrin is a protein made up of three large and three small polypeptide chains which link up to form a three-legged structure known as a triskelion (Figure 9). Numerous triskelions assemble at the cytosolic surface of the membrane to form a

networked basket-like structure (Figure 9). The clathrin network is bound to the surface of the membrane with the aid of adaptin which also binds various transmembrane proteins; it has four different forms, each of them having a specific function in packaging and transporting various proteins (Gruenberg 2001).

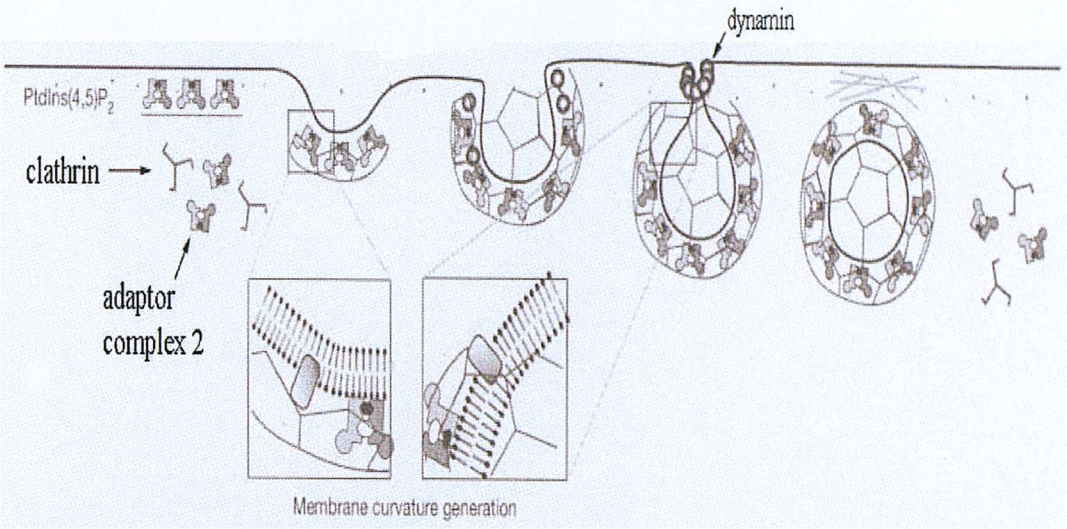


Figure 9: Clathrin-mediated endocytosis. time line showing how the clathrin-coated pit assembles and internalizes substrates. (from Conner and Schmid 2003).

More recently identified in the plasma membrane of various cells, including those of the visceral endoderm, are the receptors megalin (gp330) and cubilin (gp280). Megalin is a 600 kDa transmembrane protein belonging to the Low Density Lipoprotein (LDL) receptor family. It is the largest known single chain receptor and is highly conserved between species. It is located on mouse chromosome 2 (Christensen et al 2002a). Megalin is made up of approximately 4600 amino acids, it has a single transmembrane domain, a short cytoplasmic tail (C-terminal) and a large N-terminal ectodomain containing four ligand binding regions; this can be seen in Figure 10.

Receptor-associated protein (RAP) is a 40 kDa endoplasmic reticulum protein which is important for processing megalin and LDL receptor proteins, chaperoning them from early binding of ligands also synthesized by the cell (Christensen et al 2002b, Moestrup et al 2001). Megalin is synthesized but not activated at the time of synthesis. Maturation or activation of megalin is slow, but is signified by rapid membrane association with RAP located in the membrane,

thereby allowing megalin to arrive at the membrane in an active form. In adult physiology, megalin expression is critical for vitamin homeostasis, it mediates uptake of vitamin complexes including vitamin B_{12}, D3 and A (McCarthy and Argraves 2003). Table 1 lists all the known ligands that megalin and cubilin bind to in their function as plasma membrane receptors.

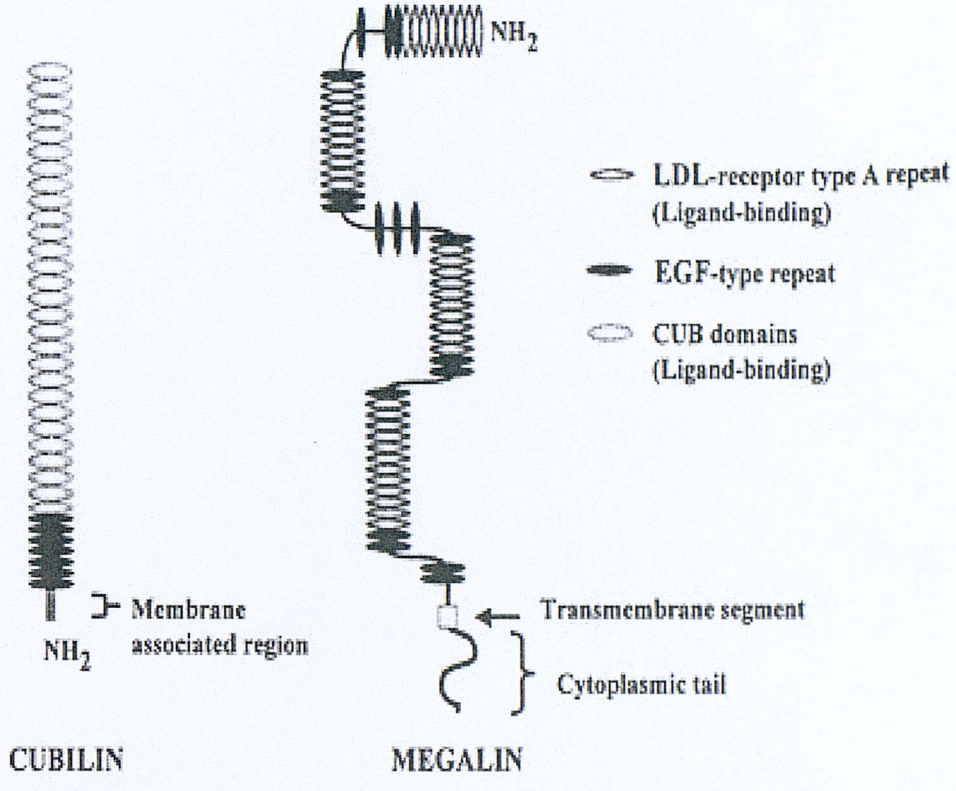


Figure 10: Schematic diagram of Cubilin and Megalin (from Moestrup et al 2001)

Megalin binds to cubilin via the CUB domains positioned 1 and 2 (Christensen et al 2002b). Cubilin is a 460 kDa peripheral membrane glycoprotein of approximately 3600 amino acids (Christensen et al 2002b). It contains 27 CUB domains, which are ligand-binding regions important for multiple protein interactions. (Figure 10) (Moestrup et al 2001, Christensen et al 2002b). It has no classic membrane-spanning segment although the amino terminal has a 100 amino acid region that is important for membrane association. Cubilin mediates the uptake of transferrin through the visceral yolk sac during gestation (Kozyraki et al 2001). Figure 11 shows how megalin and cubilin function during receptor-mediated endocytosis in the visceral yolk sac endoderm cells.

Ligands that bind megalin and cubilin	
Megalin	**Cubilin**
Vitamin-binding proteins	
Transcobalamin–vitamin B_{12}	Intrinsic factor–vitamin B_{12}
Vitamin-D-binding protein	Vitamin-D-binding protein
Retinol-binding protein	
Other carrier proteins	
Albumin	Albumin
Lactoferrin	Transferrin
Haemoglobin	Haemoglobin
Odorant-binding protein	
Transthyretin	
Lipoproteins	
Apolipoprotein B	Apolipoprotein A-I
Apolipoprotein E	High-density lipoprotein
Apolipoprotein J/clusterin	
Apolipoprotein H	
Hormones and hormone precursors	
Parathyroid hormone	
Insulin	
Epidermal growth factor	
Prolactin	
Thyroglobulin	
Drugs and toxins	
Aminoglycosides	
Polymyxin B	
Aprotinin	
Trichosanthin	
Enzymes and enzyme inhibitors	
PAI-1	
PAI-1–urokinase	
PAI-1–tPA	
Pro-urokinase	
Lipoprotein lipase	
Plasminogen	
β-amylase	
β_1-microglobulin	
Lysozyme	
Immune- and stress-response-related proteins	
Immunoglobulin light chains	Immunoglobulin light chains
PAP-1	Clara cell secretory protein
β_2-microglobulin	
Others	
RAP	RAP
Ca^{2+}	
Cytochrome c	

Table 1: Ligands that bind Megalin and Cubilin (Christensen et al 2002)

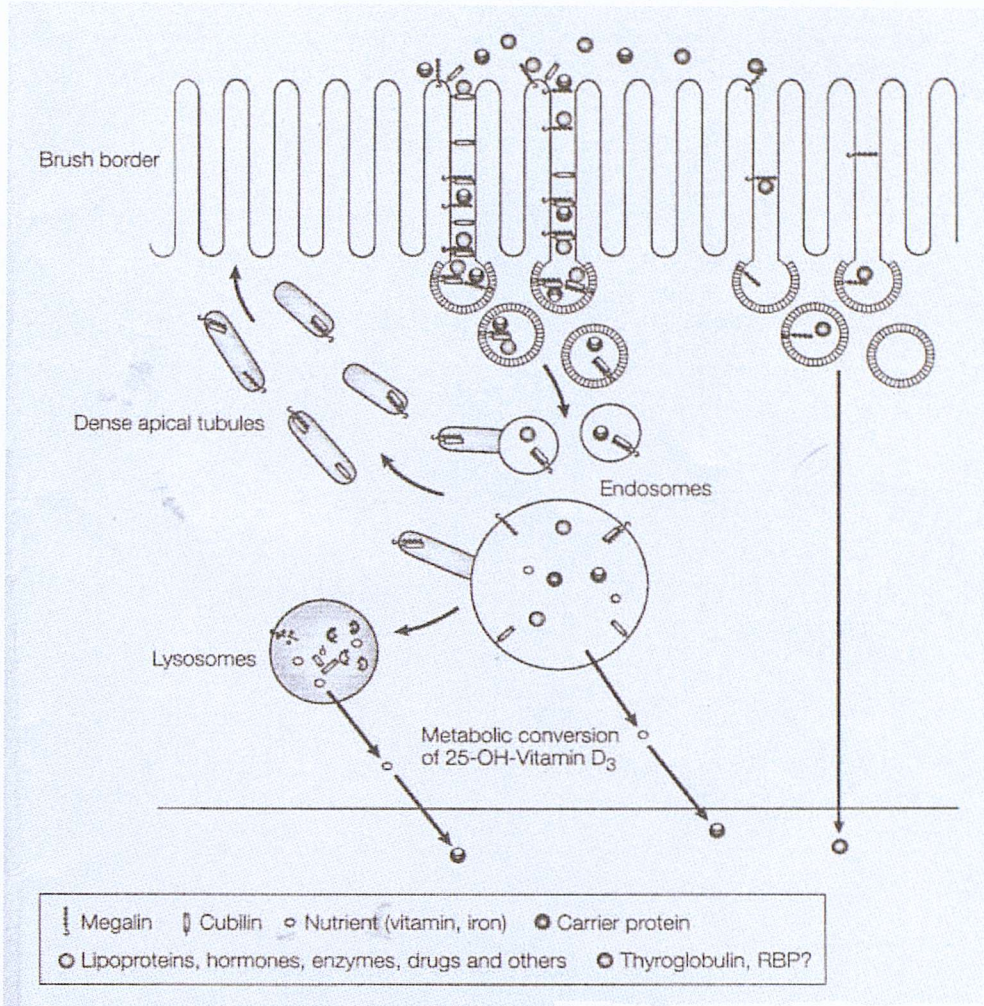


Figure 11: Role of Megalin and Cubilin in endocytosis (Christensen et al 2002)

Also present in the coated invaginations in the plasma membrane are AP-2 adaptor complexes and dynamin GTPase, which regulate the formation of vesicles derived from clathrin-coated pits and caveolae (Apodaca 2001). Cholesterol and signaling molecules are also involved in endocytosis as are some non clathrin-coated membrane invaginations (Figure 7). Caveolae are small flask-shaped vesicles that are static at the plasma membrane and contain the protein caveolin, (Apodaca 2001, Moestrup et al 2001, Conner and Schmid 2003). They are involved in the uptake of extracellular material and in cell signaling (Gruenberg 2001). This mechanism of endocytosis involves a non-specific form of binding to the cell surface, for example cationic ferritin can bind to anionic sites on the membrane (Besterman and Low 1983). Cells that lack caveolin are unable to bind and ingest serum albumin, although this does not prevent albumin from entering the cell via other pathways (Conner and Schmid 2003).

Experimental evidence of visceral yolk sac function.

The visceral yolk sac's function as a nutrient provider has been shown by disruption of the visceral yolk sac endoderm activity by injection of certain chemicals such as trypan blue, suramin, aurothiomalate and antibodies raised against visceral yolk sac endoderm into culture medium containing visceral yolk sacs or directly into the mother during gestation (Williams et al 1976, Freeman and Lloyd 1986, Lerman et al 1986). The latter methods produce a teratogenic effect, including congenital malformations, intrauterine growth retardation or lethality (Freeman and Lloyd 1986, Lerman et al 1986, Beckman et al 1994). Deleting target genes has also demonstrated the nutrient provision role of the visceral yolk sac. For example, apolipoprotein B which is involved in nutrient transport, when deleted through target gene deletion, will result in death of the fetus by day 10 of gestation (Bielinksa et al 1999). A molecule that has been radiolabelled and traced is Immunoglobulin G (IgG), the visceral yolk sac passes IgG to the developing fetus during gestation without digestion, and exocytosis occurs at a faster rate than other pinocytic substrates (Weisbecker et al 1983).

Antibodies to megalin do not disrupt its function sufficiently to cause fetal malformations during gestation; they have a mild affect upon endocytic activity. Antibodies raised against cubilin, however, do have a significant effect upon fetal growth during gestation when they are targeted to the visceral yolk sac (Baricault et al 1995, Sahali et al 1993). This difference in response to the specific antibodies is thought to be due to different biosynthetic pathways. Megalin when newly synthesised is delivered to the plasma membrane in a Golgi processed form. Cubilin, however, is delivered to the plasma membrane in an immature form and is subsequently processed by recycling through the Golgi apparatus (Baricault et al 1995).

Freeman et al (1981) showed, using radioactive-labelled protein, that the rat visceral yolk sac digested the protein intracellularly to amino acids which would then be passed to the fetus via the vitelline circulation. They cultured day 9.5 rat conceptuses in a medium containing a low concentration of free leucine and [^{3}H]leucine-labelled serum proteins and demonstrated that more than 95% of the

leucine in the fetus after culture was derived from protein and not the free leucine in the medium; the same conclusion was drawn for serine with similar experiments (Freeman et al 1981). Therefore, it is probable that the same mechanism applies to all the amino acids passed to the fetus by the visceral yolk sac (Brent et al 1998).

Isolating the yolk sac to study its mechanisms for transporting substances across to the fetus began with the study by Williams et al (1975). Many laboratories since then have studied endocytic activity in the rodent yolk sac (Roberts et al 1977, Duncan et al 1980, Lloyd 1990, Beckman et al 1991, Beckman et al 1994). Experiments that aim to investigate the passing of nutrients to the fetus and subsequent uptake by the fetus primarily use whole conceptus culture, so that radiolabelled molecules and antisera can be followed through uptake and digestion by the yolk sac and subsequent incorporation of labeled molecules by the fetus (Livesey et al 1979, Freeman et al 1982, Freeman and Lloyd 1983 A, Beckman et al 1991).

The visceral yolk sac is the primary source of nutrients for the fetus for days 5-10 of gestation, after which the chorioallantoic placenta takes over from the visceral yolk sac as the predominant structure for nutrient and gas/waste exchange. However, the visceral yolk sac still retains the ability to provide the fetus with amino acids and some vitamins (Pratten and Lloyd 1997), in particular vitamin A and D. Studies of retinol binding protein (RBP) and transthyretin in their role as important transporters for nutrients such as vitamin A have shown synthesis and secretion in the visceral yolk sac endoderm (Soprano et al 1986). RBP is not expressed by the chorioallantoic placenta, therefore, transfer to the fetus throughout gestation of this protein relies entirely on the visceral yolk sac. Retinoic acid is important because it induces some homeobox genes, *Indian hedgehog* gene, GATA 4 and 6 and an extracellular-matrix complex, all of which are essential for normal development of the fetus (Bielinksa et al 1999).

Proteins digested to amino acids are not the only substances to pass across the visceral yolk sac. Others, such as transferrin which carries iron can also be transported (Richardson et al 2000); also alpha fetoprotein (AFP), albumin and

angiotensinogen are synthesized in the visceral yolk sac and secreted. The visceral yolk sac endoderm synthesizes steroid-hormones and some digestive enzymes, for example proteases, phosphatases and gamma-glutamyl transferase (Jollie 1990). Duncan et al (1981) showed that larger molecules (molecular weight 700 000) can be captured by the visceral yolk sac, although it may be at a slower rate than smaller molecules (molecular weight 50 000); they also found that the more tightly compact the structure of the protein the larger in weight it could be whilst still being taken up by the visceral yolk sac. Further studies have also investigated the endocytic index of molecules of various sizes, in particular ^{125}I-polyvinylpyrrolidone, by the rat yolk sac (Pratten et al 1982, Pratten et al 1997).

A developing fetus requires cholesterol for normal development; it acquires the cholesterol either endogenously by making its own or exogenously from the visceral yolk sac (Christensen et al 2002a). The visceral yolk sac has a pivotal role in providing lipids and lipid-soluble nutrients to the developing fetus in the early stages of development around days 7-10 (Terasawa et al 1999). The visceral yolk sac can either synthesize its own cholesterol or transport lipoproteins from the mother to the fetus; the placenta also helps provide enough cholesterol for the fetus (McConihay et al 2000).

Apolipoproteins (APOs) are associated with each class of lipoprotein, they are involved in particle structure, enzyme recognition and cellular recognition. APOs are very important in the development of the embryo (Farese et al 1996). There are several different classes of APOs with varying properties and functions. The visceral yolk sac is a major site of synthesis of APO-A1 and AE between days 10.5 - 18.5 in the mouse (Shi and Heath 1984). APO–A1 synthesis and deposition has been shown to occur in the mouse visceral yolk sac at midgestation stage (Shi and Heath 1984). APO-B is the main structural component needed for synthesis and secretion of triglyceride-rich lipoproteins by the liver and the intestine; it is also highly expressed in the yolk sac visceral endoderm of most mammals and acts as a major part of transporting lipid nutrients to the fetus during gestation (Farese et al 1996). Embryos generated that are deficient in APO-B in the visceral endoderm show accumulation of intracellular lipid droplets, absence of lipoproteins from secretary pathways and a reduced concentration of cholesterol;

embryonic lethality usually occurs in these mice at approximately day 9.5-10.5 (Farese et al 1996).

1.1.5 Blood flow within the uterus and haematopoiesis in the yolk sac

Blood flow within the uterus is important to ensure the correct development of the fetuses. The mouse uterus is composed of two independent horns each with its own cervix and blood supply. Blood flow is bi-directional; in previous studies it has been found that the fetuses in the middle of the uterine horn are smaller than those towards the ovarian or cervical ends. Figure 12 shows a schematic representation of the positions of conceptuses within the horns and the blood vessels surrounding. (Vom Saal and Dhar 1992).

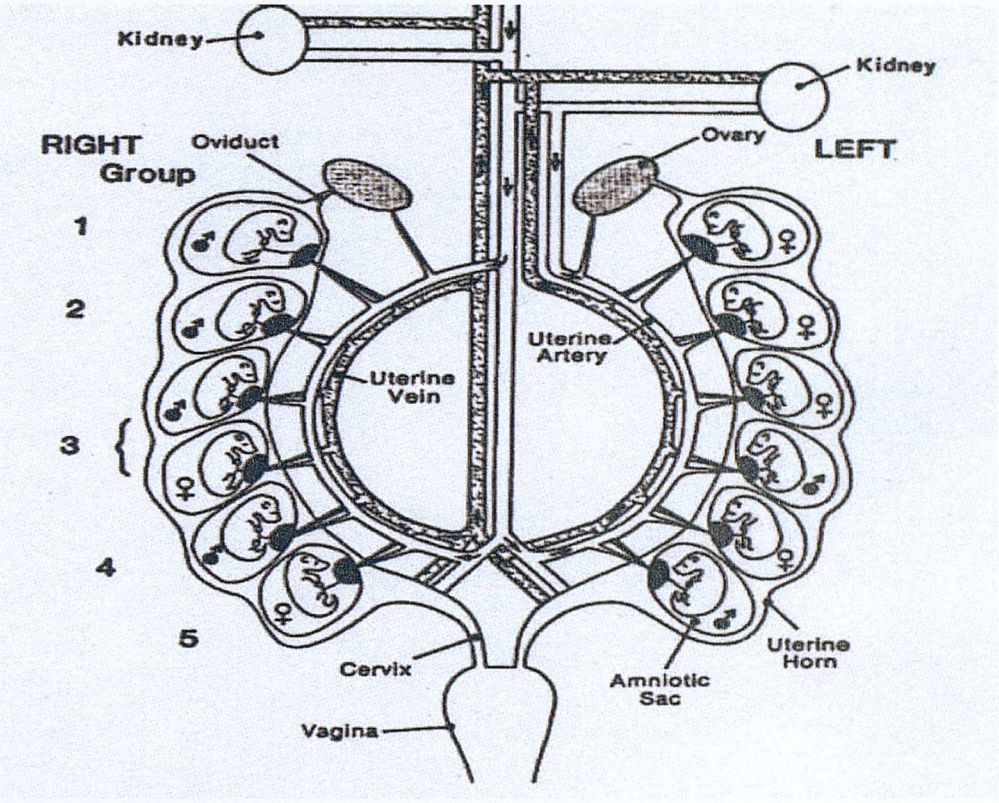


Figure 12: Uterine blood flow in a mouse. (Vom Saal and Dhar 1992)

The visceral yolk sac has an another important role, as the first site of haematopoiesis (Beckman et al 1990, A), the making of blood cells, and of vasculogenesis, the formation of blood vessels, (Auerbach et al 1998).

Vasculogenesis and haematopoiesis occur at the same time in gestation; within this relationship are several interdependent events. The haemangioblast is commonly believed to give rise to both the angioblasts which form blood vessels and haematopoietic stem cells which form blood cells. The visceral yolk sac endothelium is believed to regulate the expansion of haematopoietic stem cells and to control the differentiation of these cells (Auerbach et al 1998)

The mouse visceral yolk sac is haematopoietically active from approximately day 7.5 up to day 12 (Weinberg and Stohlman 1976, Cumano et al 2000). GATA 1 gene expression is essential for haematopoietic cell differentiation and is expressed in a variety of cells important in making blood, for example erythroid cells, megakaryocytes, eosinophils and mast cells; GATA 2 and 3 are also required for the development of blood and blood vessels in the growing embryo (Nishimura et al 2000). Embryonic erythropoiesis is first observed in the yolk sac blood islands (Auerbach et al 1998).

Derivatives of the mesoderm layer form the functional units of the vitelline circulation in vasculogenesis (Farrington et al 1997). Figure 13 shows the development of haematopoiesis during gestation in the mouse (Auerbach et al 1996). At day 8, haematopoietic stem cells arise which are the precursors that will develop into T-cells, B-cells, erythrocytes and different myeloid cell lineages (Figure 13) (Auerbach et al 1997). The lymphoid cells which are formed in the visceral yolk sac mesoderm move to the lymphoid primordium by day 10 (Jollie 1990). These cells are equivalent to approximately less than 0.1% of non adherent cells in the visceral yolk sac (Auerbach et al 1997). Angiogenesis occurs by day 9 and erythropoiesis is active from day 10 (Jollie 1990). At this stage it has been shown that precursor cells that can differentiate into all blood cell types can be found in the visceral yolk sac (Figure 13) (Cumano et al 2000). Studies have shown that at day 11 visceral yolk sac cells can repopulate marrow, spleen, thymus and peripheral lymphoid tissue in irradiated mice (Weinberg and Stohlman 1976).

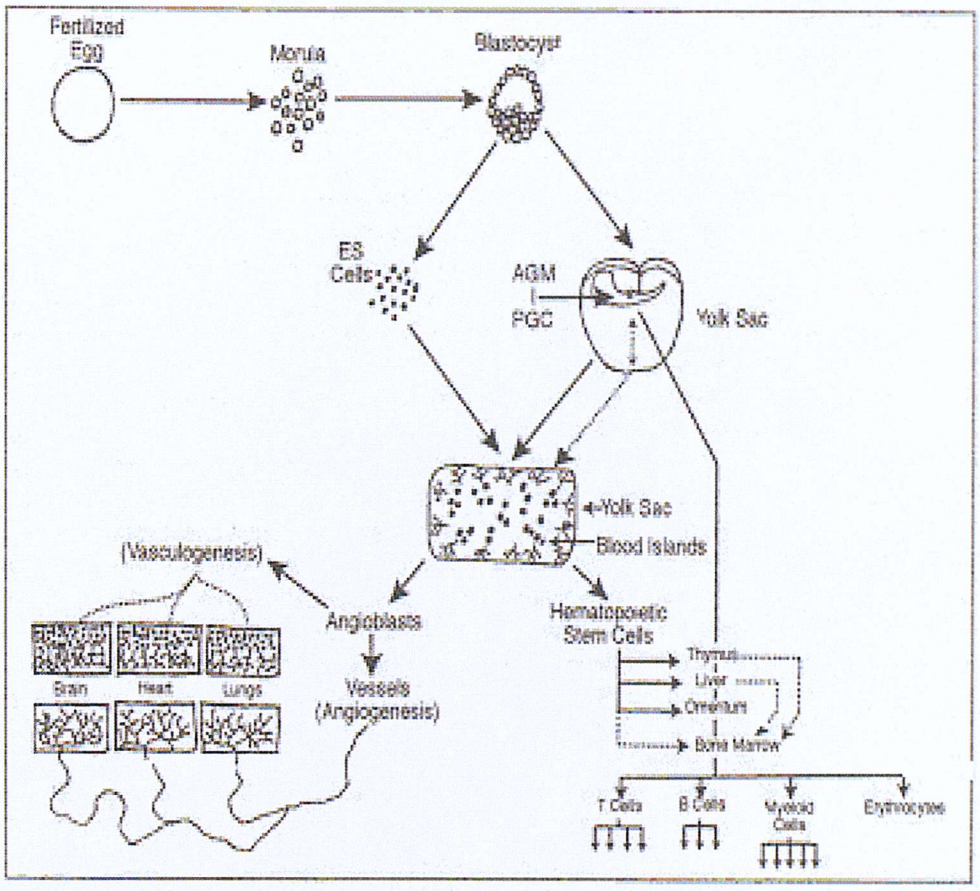


Figure 13: Development of haematopoietic and endothelial cell lineages during mouse embryogenesis. (from Auerbach et al 1996)

1.1.6 Relationship between Mouse Yolk Sac and Human Yolk Sac

The pathway of human gestation has many similarities with rodent gestation, however, it does have some major differences. In the human, pregnancy lasts approximately 9 months whereas in rodents it lasts between 21-24 days, hence the timing of specific events during gestation differs. The visceral yolk sac has been overlooked for many years of being any importance during gestation, it is only through numerous animal studies that the true necessity of the visceral yolk sac for complete successful gestation has been revealed. From 1990 onwards, investigations of the human yolk sac have been completed showing that it is equally as important as that in rodent gestation; particularly as the yolk sac is recognized to be the first site of haematopoiesis, the origin site of primordial germ cells and important for the synthesis of serum proteins (Pereda et al 1994).

As with mouse development during the period prior to implantation, the human fertilized egg is sustained by secretions from the oviduct and then the uterine epithelium. Once implantation has taken place, in the human, the placenta begins to form and provides the main source of nutrients to the developing fetus throughout gestation (Burton et al 2001). In mice, the placenta does not function until day 10 which is half way through gestation. Implantation in the human is far deeper than in the mouse, the conceptus completely embeds into the superficial layer of the endometrium early in gestation, approximately by day 10. At this stage, the conceptus is completely sealed off from the secretions in the uterine lumen, unlike within the mouse where the developing conceptus has contact with the uterine fluid throughout gestation (Burton et al 2001).

The rodent relies heavily upon its yolk sac throughout gestation. However, the human fetus relies on its yolk sac during early gestation, but it is only functional up to week eleven of pregnancy, after which it begins to regress. In both species, the yolk sac synthesizes and secretes a number of proteins. The human yolk sac secretes more proteins to the fetus than the mouse yolk sac; both secrete albumin, AFP and transferrin as previously mentioned. The human yolk sac also secretes Apolipoprotein A1, B and Low Density Lipoproteins and α1-antitrypsin (Beckman et al 1990, A). There are large morphological and positional differences between the two; for example, the yolk sac does not envelop the fetus in the human, as it does in the mouse, but exists as an extraembryonic organ situated close to the embryonic pole (Pereda et al 1994). Figure 14 shows a diagrammatic representation of the early human pregnancy with the yolk sac visible, the numbers indicate the pathway of maternal secretions from the endometrium through to the yolk sac and on to the fetus.

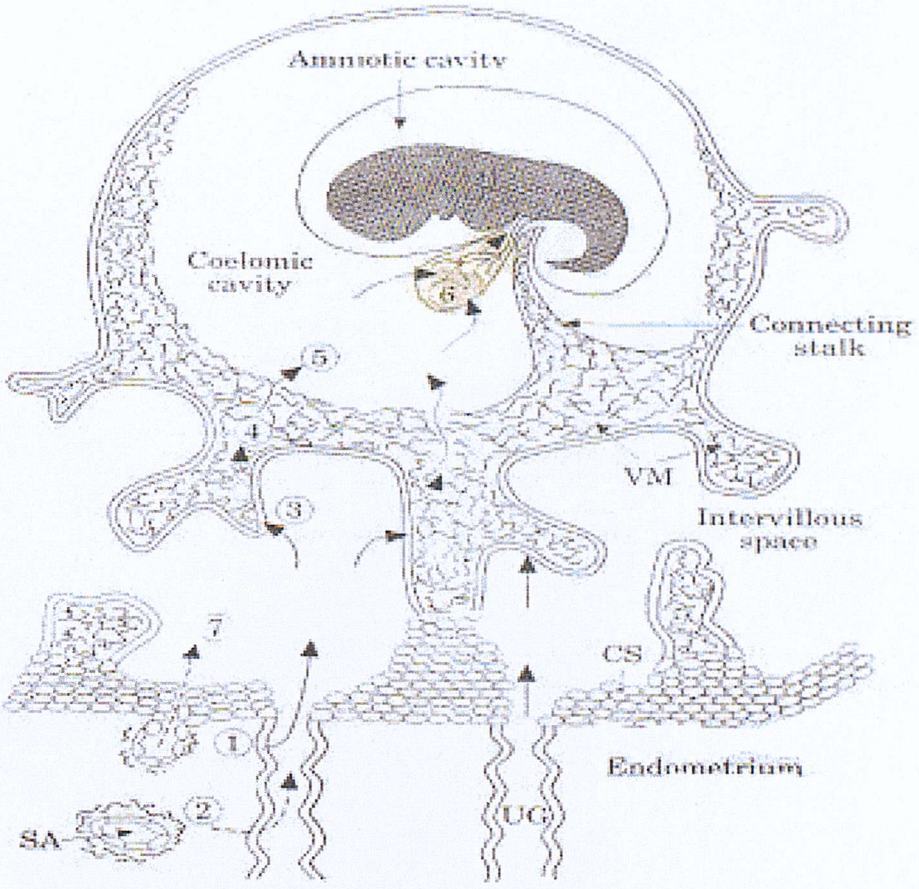


Figure 14: Schematic diagram of early human pregnancy. (from Burton et al 2001). Arrows indicate the histiotrophic pathway in early gestation. 1 = uterine gland secretions, 2 = maternal serum transudate from spiral arteries (SA), 3 = maternal secretions enter the trophoblast, 4 = villous mesenchymal space (VM), 5 = coelomic fluid, 6 = the maternal secretions pass to the yolk sac and are transferred on to the fetus.

In humans, the yolk sac provides folate and vitamin B12, known to be particularly important for DNA synthesis and cell proliferation, at weeks 9-12 of gestation, suggestive of the importance of the yolk sac in transferring nutrients from mother to fetus (Exalto 1995).

The endodermal cells in the mouse and the human yolk sac both have microvilli attached to the apical surface, which aid in the pinocytic activity of the yolk sac (Pereda et al 1994). Experiments have shown that both yolk sacs do provide nutritional aid to the fetus. As yet, evidence for this is mainly circumstantial in the human but studies do suggest that if there is damage induced to the yolk sac, in either mouse or human, abnormalities will occur in the fetus of both. In some cases, the yolk sac may recover from the damage but the fetus will probably not (Beckman 1997).

The yolk sac functions as the first progenitor of haematopoietic cells in both species. As explained earlier, this is the process used by a developing fetus to establish a circulatory system. This pathway in mouse is greatly akin to that of the human, although there are obvious timing differences due to the length of gestation being very different in mouse and human; for example, precursor cells of haematopoiesis arise in the mesodermal layer of the yolk sac in mice at about day 7.5 (Palis and Yoder 2001), in the human yolk sac, haematopoiesis is established by day 30 g.a (gestational age), and blood islands have already formed at this stage (Prindull 1999).

The human yolk sac shortly after implantation has the distal portion cut off leaving a smaller organ termed the secondary yolk sac which floats within the coelomic cavity (Figure 14) (Exalto 1995). It vascularizes before the allantois as happens in all species, the vitelline circulation becomes the initial network for gas and nutrient exchange. Molecules such as immunoglobulin G and T4 have been traced during early pregnancy in humans and have been found to accumulate in the coelomic fluid. Once there, the proteins can pass easily into the yolk sac and then to the fetus. To confirm that the proteins were not simply being made by the yolk sac, hCG was traced and found to be present within the yolk sac although it is known that it is not synthesized by the yolk sac (Burton et al 2001).

1.2 Hypothesis and Aim

The initial aim of my experiments is to investigate the effect of maternal low protein diet upon the metabolic activity of the visceral yolk sac at various time points throughout gestation.

My hypothesis is that under-nutrition of the mothers fed a low protein diet will cause alterations in the mechanisms of growth including changes in metabolism. These changes may result in fewer cells being available from the ICM for the generation of the visceral yolk sac which, therefore, may be smaller in size and less nutritionally competent as a consequence of the low protein diet.

Firstly my study will look at the wet weight of the conceptus and its component parts. Litter size and the position of the conceptus will be assessed, both in their individual affect upon the size of the conceptus and how they are affected by maternal low protein diet. In order to provide a balanced account of metabolic activity, the uptake rate and release rate of nutrients by the visceral yolk sac will be measured. Electron microscopy will be used to examine whether the structural elements of the visceral yolk sac responsible for its physiological activity change in response to diet. Expression levels of key proteins responsible for nutrient support will also be investigated by immunoblotting and densitometry.

Chapter 2

Materials and methods

2.1 Animal treatment and mating:

All animal procedures were carried out in accordance with a Home Office Project License for this work. MF1 strain outbred female mice were used at seven weeks of age (weight range 25-32g) obtained from Biomedical Research Facility at University of Southampton. They were naturally mated with MF1 male studs (age 8-24 weeks) overnight for up to four nights. The females were housed collectively away from the males during the day if found to be plug negative in the morning. Females were plug checked each morning and the presence of a vaginal plug signified day 0 of gestation. Pregnant females were housed singly, at a temperature of 24°C with a 12 hour light cycle and fed *ad libitum* either 18% casein diet (control diet) or 9% casein diet (Low Protein Diet) for up to 17 days from day 0.

2.2 Diet production:

The control diet and low protein diet were first used by Langley and Jackson (1994). Both diets have been specifically designed to provide the same amount of energy, but with the low protein diet having a 50% reduction in protein content. The diets are made by hand using the ingredients and quantities given in Table 2. To bind the mixture together, 1½ litres of distilled water were added to each 5 kg of diet made. The mixture was separated into small lumps and dried overnight in an oven at 60°C.

	18%	9%
Casein	180	90
Corn starch	425	485
Fibre	50	50
Sucrose	213	243
Choline Chloride	2	2
DL-Methionine	5	5
AIN-76 mineral mix	20	20
AIN-76 vitamin mix	5	5
Corn oil	100	100

Table 2: Recipe for control and low protein diets, values are weight in grams per kilogram. (from Langley and Jackson 1994)

2.3 Conceptus and visceral yolk sac collection:

Mice were culled by schedule 1 method of cervical dislocation. The uterus was removed in two pieces, the left and right horns. Conceptuses were removed by carefully cutting through the uterine wall, to expose intact conceptuses and then gently peeling them away from the uterus. The conceptuses were placed in 50 mm Petri dishes (Sterilin) containing 1ml of Medium 199 (Life Sciences, pH 7.4) and 10% Fetal Calf Serum (FCS) on a warming plate. Each conceptus was dabbed on 3 MM chromatography paper (Whatman) to remove excess liquid and weighed by placing on a dish that had previously been tared on the balance. All weighing was carried out wet, using a Sartorius balance, BP61-S. The conceptus was then dissected into its component parts of fetus, placenta and yolk sac. This was achieved by cutting along the join between the yolk sac and placenta and then pushing the fetus out from the surrounding yolk sac. Each component was then weighed separately.

From each conceptus that was dissected, the following information was collated:

1. Wet weight of conceptus, fetus, placenta, yolk sac.
2. Location of conceptus in terms of right or left horn and position within the horn. (Figure 37)
3. Number of conceptuses within each horn within the mother.

Conceptuses identified containing two fetuses with only one placenta were not included in the project.

2.4 Visceral yolk sac endocytic activity experiments

Two methods were used to study the endocytic uptake and release rates of the yolk sac. The first was employed to measure the rate of fluid phase endocytosis and the second to measure receptor-mediated endocytosis by investigating the rate of uptake and release of a specific protein, Bovine Serum Albumin (BSA).

2.4.1 Fluid phase endocytosis

The method for the experiment was adapted from that of Williams et al (1975). At the designated time points (days 12, 14, 17) during gestation, the yolk sacs were dissected and weighed as previously described in Section 2.3, then immediately placed into individual 25 ml glass conical flasks containing 5ml Medium 199, 10% fetal calf serum plus (FCS) ^{14}C-Sucrose (Amersham Biosciences). The use of Medium 199 as a suitable culture medium for culturing yolk sacs has been well established (Ibbotson and Williams 1979, Williams et al 1975 A). The addition of 10% fetal calf serum has been under debate in previous studies; however, at this concentration it was found to aid the reproducibility of results (Ibbotson and Williams 1979, Kooistra et al 1981). The ^{14}C-Sucrose arrived in liquid form and was added to the medium at a concentration of 0.1 mg/ml. The flasks were gassed with 95% oxygen and 5% CO_2 for one minute before the yolk sacs were added. The flasks complete with yolk sac and medium were sealed and placed in a shaking water bath (Clifton) at 37°C for 5 hours. Figure 15 gives a diagrammatic overview of the method.

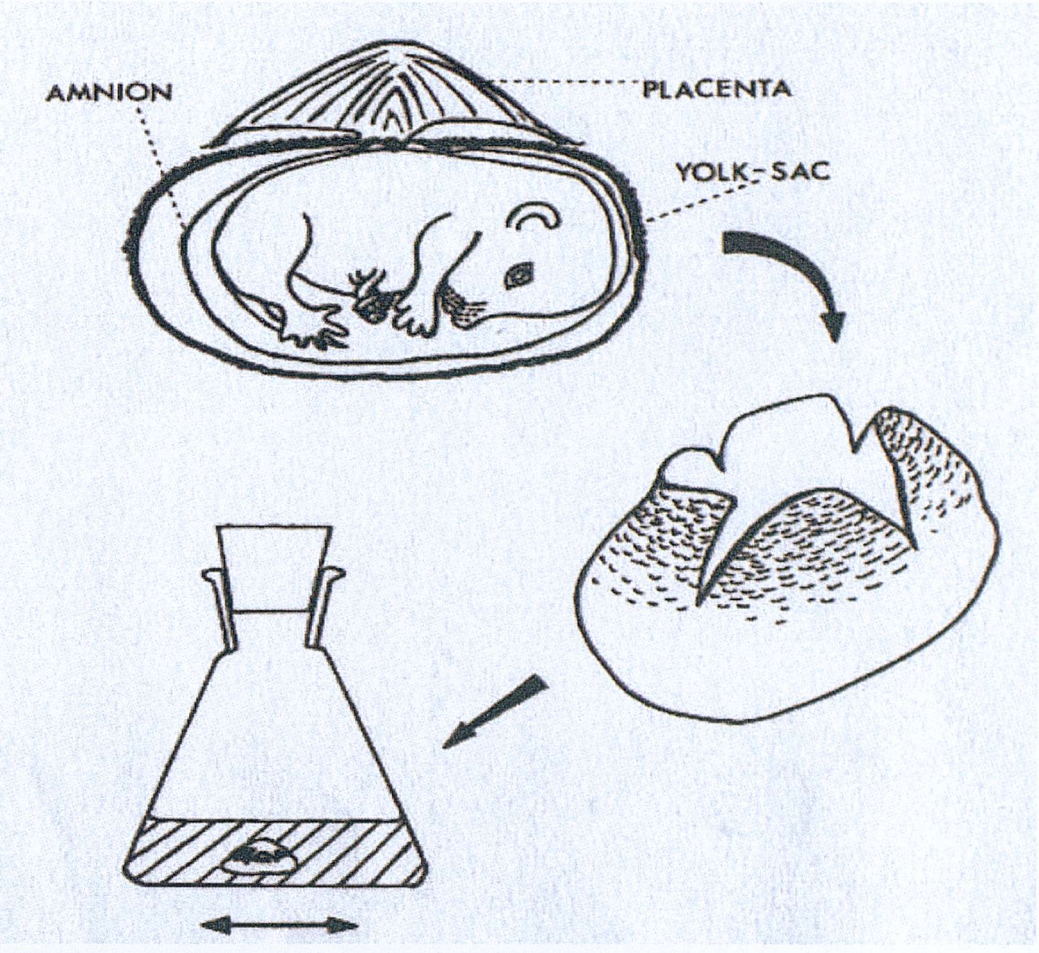


Figure 15: Dissection of a 17 day yolk sac for culture (Williams et al 1975 A)

Following incubation for the required time, the yolk sacs were removed and washed in 2 ml sterile phosphate buffered saline (PBS) (Oxoid see Appendix 1) five times to remove excess medium, and then placed in 1ml 0.25M NaOH in a 37°C incubator overnight to digest to a soluble state. The digested yolk sac was neutralized by addition of 1 ml 0.25M HNO_3 the following morning.

The total protein concentration of each sample was determined using the DC Protein assay kit supplied by Biorad, which is based on the Lowry method (Lowry et al 1951). On every 96 well microtitre plate (Cellstar- Greiner) used in the protein concentration assays a new set of standards was included using dilutions of BSA (Sigma) (range 0.1 mg/ml to 1 mg/ml) in PBS to create a standard curve. Duplicate 5 µl samples were taken from the 2 ml digest of each yolk sac and loaded as shown in Table 3. Each sample was run at two concentrations, neat and diluted 1:1 with distilled water. 25 µl of reagent A was added to each standard and sample, then 200 µl of reagent B. The plate was left for 45 minutes then read

on a spectrophotometer (Dynatech, MR5000) at a wavelength of 720 nm.

	1	2	3	4	5	6	7	8	9	10	11
A	blank	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
B		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
C	S1	S1 d	S2	S2 d	S3	S3 d	S4	S4 d			
D	S1	S1 d	S2	S2 d	S3	S3 d	S4	S4 d			
E											
F											
G											

Table 3: Diagram representing the layout of the 96 well plate used for the protein assays. Rows A and B contain the standards. S = sample S-d = diluted aliquot of sample.

For determination of fluid phase endocytic uptake rate, radioactive samples of the starting culture medium (sample volume 0.5 ml), the medium at the end of the experiment (sample volume 1 ml) and the digested yolk sac (sample volume 0.1 ml) were taken. Each sample was run in duplicate. Samples were mixed with 5 ml Optiphase safe scintillation fluid and run on a scintillation counter (Wallac 1209) for 5 minutes with program mode cpm/dpm and count mode sqp (E).

Using the data gained from the protein assay and the scintillation counter, the uptake rate of fluid phase endocytosis was calculated by the following equation from Williams et al (1975):

$$EI = Y/MP$$

EI is the endocytic index

Y is equal to the amount of radioactivity (^{14}C-Sucrose) in the whole yolk sac (in counts per minute (cpm) corrected for background)

P the total protein content of the yolk sac

M the amount of radioactivity in the medium at the start of the experiment (in cpm corrected for background)

Units are expressed as µl/mg of yolk sac protein.

2.4.2 Receptor-mediated endocytosis

The methodology for this series of experiments was derived from that of Williams et al (1975). The experiments were conducted only at stages 14 days and 17 days of gestation. The radioactive substrate used was ^{125}Iodine labelled Bovine Serum Albumin (BSA).

Iodination of BSA

Ten IODO-beads (Pierce) were washed in 5 ml of 0.2M phosphate buffer (pH 6.4, see appendix 1) for 5 minutes, dried on 3 MM chromatography paper (Whatman), and placed in clean 7 ml Bijous (Sterilin) with 0.5 ml of fresh 0.2M phosphate buffer (pH 6.4 see appendix 1). Two μCi ^{125}Iodine (Amersham). IMS.30) were added to the IODO-beads and left for 5 minutes. One ml of 1 mg/ml BSA (Sigma) in 0.2M phosphate buffer (pH 6.4) was added to the reaction and was left for 15 minutes. At all times, radioactive solutions and their preparation were conducted behind a lead shield facility within a designated fume cupboard. Two 2D-salt dextran columns (Pierce) were washed through with 25 ml 0.2M phosphate buffer (pH 6.4, see appendix 1) then the solution containing labelled ^{125}Iodine and BSA (1.5 ml total) was split evenly between the two columns, 0.75 ml in each. Eight to twelve aliquots of approximately 0.75 ml were collected from the bottom of the columns in 1 ml microcentrifuge tubes (Greiner) and buffer was added to the top of the columns to wash the ^{125}I-BSA through the column.

These aliquots were tested to ascertain which aliquots contained the bulk labelled protein by using the Biorad protein assay kit (Section 2.4.1). The 6 aliquots found to contain labelled protein were concentrated using Centricon, a centrifugal filtration device (Amicon), reducing the total volume from 4.5 ml to approximately 105 μl. The concentrated stock solution was counted on a gamma counter (Wallac) to ascertain the radioactivity level. The stock solution was then diluted with 0.2M phosphate buffer (pH 6.4) to give approximately 1 million counts per minute in 1 μl of stock solution. ^{125}I-BSA was added to the medium 199 at a concentration of 1μl/ml.

Receptor-mediated uptake and release

At the designated time point (days 14, 17) during gestation, the visceral yolk sacs were dissected and weighed as previously described in section 2.3, then immediately placed into individual 25 ml glass conical flasks containing 5 ml Medium 199, 10% FCS and 1 μl ^{125}I-BSA diluted stock solution . The flasks were gassed with 95% oxygen and 5% CO_2 for one minute before the yolk sacs were added. The flasks complete with yolk sac and medium were sealed and placed in a shaking water bath (Clifton) at 37°C for 5 hours. Figure 15 gives a diagrammatic overview of the method.

After the required incubation time, the yolk sacs were removed and washed in 2 ml sterile PBS five times to remove excess medium, and then placed in 1 ml 0.25M NaOH in a 2 ml collection tube (Quiagen), then placed in a 37°C incubator overnight to digest to a soluble state. The digested yolk sac was neutralized by addition of 1 ml 0.25M HNO_3 the following morning.

For determination of receptor-mediated uptake rate, radioactive samples of the starting culture medium (sample volume 1 ml), the medium at the end of the experiment (sample volume 1 ml) and the digested yolk sac (sample volume 0.5 ml) were assayed in duplicate on a Gamma counter (Wallac) for 1 minute. In addition, starting medium (sample volume 1 ml) and medium from the end of culture (sample volume 1 ml) were treated with trichloroacetic acid (TCA) (20% w/vol) and similarly counted. Treatment with TCA allowed the distinction between intact ^{125}I-BSA that had been endocytosed, but not degraded and the break down products, including Iodo-tyrosine, that were present after ^{125}I-BSA had been degraded by intracellular hydrolysis.

The following equation was adapted from Williams et al (1975) and used to determine the rate of receptor-mediated ^{125}I-BSA uptake.

$$EI = Y+5S/MPT \times 1000$$

Y is the total radioactivity in the digested yolk sac (corrected for background)
S is the TCA soluble radioactivity in the medium at the end of the culture period (corrected for background)
M is the TCA insoluble radioactivity in the medium at the end of the culture period (corrected for background)
P is the total protein content in the yolk sac
T is the time in culture.
Units were µl/mg of yolk sac protein per hour

To determine the rate of release of amino acids broken down from the protein endocytosed by the yolk sac during culture, the following equation adapted from Williams et al (1975) was used:

$$\text{Release} = 5S/MPT \times 1000$$

The letters correspond to the same values as those for Endocytic Index.

2.5 Electron microscopy

Yolk sacs at day 17 of gestation were collected from mothers fed a low protein diet (9% casein) and from mothers fed a control diet (18% casein). All yolk sacs were from the right horn at position 4 (4^{th} conceptus from the ovarian end of the uterus). Pieces 1 mm^3 from the villous region (the area closest to the join to the placenta) and the less villous region (the area furthest away from the placenta) (see Figure 16) were dissected and placed immediately in fixative. The tissue pieces were fixed and processed as described below, sectioned and stained by Dr Anton Page at the Biomedical Imaging Unit at Southampton General Hospital.

To fix the yolk sac tissue, the following procedure was used: one hour in primary fixative (3% glutaraldehyde, 4% formaldehyde in 0.1M PIPES buffer at pH 7.2), then rinsed in 0.1M PIPES buffer twice, one hour in post fixative (1% osmium tetroxide in 0.1M PIPES, pH 7.2), then rinsed twice in 0.1M PIPES buffer. The specimens were then placed in increasing concentrations of ethanol for 10-20

minutes each time, before transfer to acetonitrile for 10 minutes, then 50:50 acetonitrile and TAAB epoxy resin (TAAB Laboratories) overnight. The following day, samples were placed in epoxy resin for 6 hours and then embedded in fresh resin and polymerized at 60°C for about 20-24 hours. Specimens were cut on a Leica OMU3 microtome and examined on a Hitachi H7000 transmission electron microscope. The visceral yolk sac samples were double blinded to ensure no bias by the investigator. Photographs were taken at various magnifications (x 2000, x 8000, x 20,000 inclusive of photo magnification). The macropinocytic vesicles and secondary lysosomes were quantified from the micrographs using the 'interactive biological analysis system' software programme (Imaging Associates Ltd).

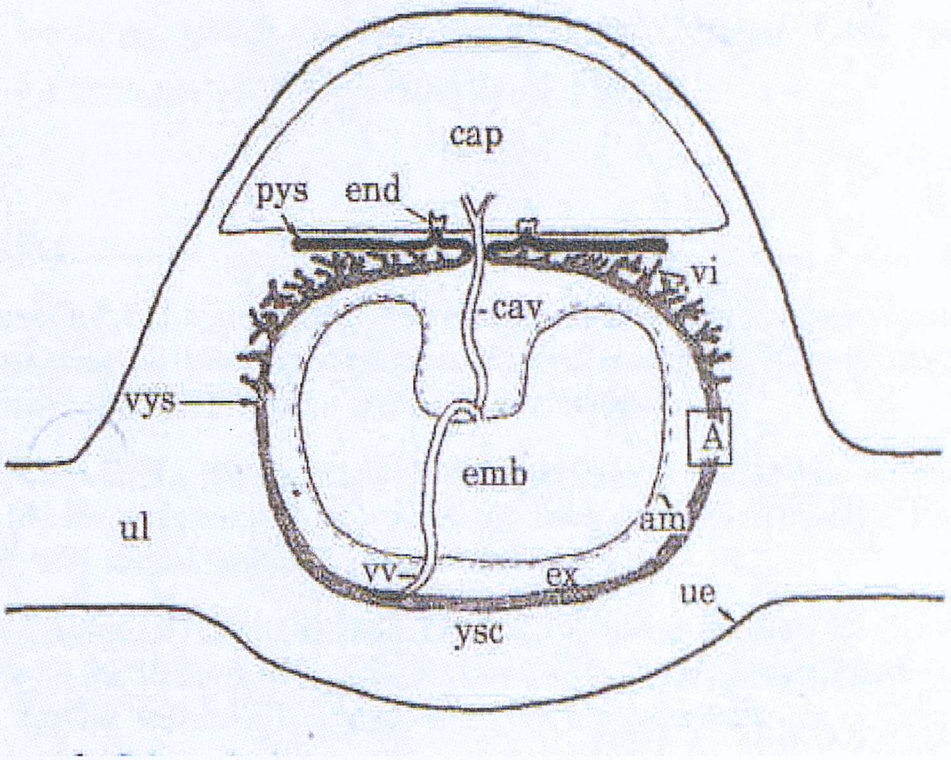


Figure 16: Diagram of conceptus, villous and less villous regions of the visceral yolk sac (vys) are clearly visible. (from Novak and Betteridge 2000). PYS: parietal yolk sac, VYS: visceral yolk sac, cap: chorioallantoic placenta, cav: chorioallantoic vessels; end : endothelium, ul: uterine lumen, ue: uterine epithelium, ysc: yolk sac cavity, emb: embryo, ex: exocoelomic cavity, vv: vitelline vessel, am: amnion.

2.6 Western blotting

2.6.1 Solubilising yolk sacs

Whole yolk sacs following dissection were placed in 500 µl 1% Sodium Dodecyl Sulfate (SDS) (United States Biochemical) at room temperature and immediately grinded using the Plusone sample grinding kit (Amersham), then heated for 10 min at 100°C to solubilise. The solubilised yolk sacs were aliquoted into ten 50 µl samples and stored at -80°C. Each yolk sac was assayed for total protein content using the DC Protein assay kit supplied by Biorad (for protocol see section 2.4.1).

2.6.2 Western Blotting method

Equal protein concentration quantities of solubilised yolk sac were loaded onto pre-cast gels from Invitrogen (see later in this section for specificity of gels used). 1.6 µl DL-Dithiothreitol (0.5M; Sigma) and 4 µl NuPage LDS sample buffer (4x; Invitrogen) were mixed with each sample before loading, and made up to 16 µl with distilled water. This mixture was boiled for 3 minutes then loaded immediately onto the gel; also loaded onto each gel was 5 µl of Seeblue Plus 2 Pre-stained Standard marker (Invitrogen). The gel was run for 1 hour at 200 v and 400 mA in the XCell Surelock Minicell apparatus from Invitrogen.

Gels were transferred overnight onto nitrocellulose membranes (Hybond ECL – Amersham). Cassettes containing the gel and the nitrocellulose were placed in a transfer tank containing transfer buffer (pH 8.3 see appendix 1) with 1% SDS and run overnight at 300 mA.

After transfer, the cassette was unclipped and opened and the nitrocellulose membrane removed using forceps before washing three times in 20 ml PBS. The membrane was then placed in 10% non-fat dried milk powder (Marvel) in 20 ml PBS to block for one hour. After blocking, the membrane was transferred to a sealed bag containing 2 ml PBS plus 10% non-fat dried milk powder and 0.1% Tween 20 plus primary antibody (see below for specificity of antibody). The

sealed bag was placed at 4°C overnight on a rotating turntable. After incubation with the primary antibody, the membrane was washed in 20 ml PBS plus 0.1 % Tween 20 four times, 5 minutes for each wash. The membrane was then placed in 20 ml PBS plus 10% non-fat dried milk powder and 0.1% Tween 20 and secondary antibody (see below for specificity of antibodies used) for 45 minutes. After this incubation, the membrane was washed again four times in 20 ml PBS plus 0.1 % Tween, then washed twice in PBS to remove the Tween 20. The membrane was then air dried by being placed on 3 MM chromatography paper (Whatman) and left on the laboratory bench for 30 minutes. Blots were scanned at the correct wavelength and quantified using the pre-programmed Odyssey infrared imaging system (Licor Biosciences).

Megalin

Samples were run on pre-cast 3-8% tris-Acetate 12 well gels (Invitrogen). The primary antibody was a mouse monoclonal raised in mice against rabbit yolk sac endoderm cells and shown to recognise megalin (Meads and Wild 1993), kindly donated by Dr A.Wild (University of Southampton), used at a concentration of 2 µl per ml of affinity purified antibody. The secondary antibody was a goat anti-mouse immunoglobulin (Rockland) which fluoresced at a wavelength of 720 nm and was used at concentration of 1:10 000.

Cathepsin

Samples were run on 4-12% bis-tris pre-cast 12 well gels (Invitrogen). Primary antibody was an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of Cathepsin L (d-20) of mouse origin (Santa Cruz), used at a concentration of 1:1000; the secondary antibody was a rabbit anti-goat immunoglobulin (Cambridge Bioscience) which fluoresced at a wavelength of 800 nm and was used at a concentration of 1:10 000.

2.7 Statistical analysis

The statistical analysis was carried out in collaboration with Dr Clive Osmond, Medical Research Council Environmental Epidemiology Unit, Southampton General Hospital. The packages used were STATA and Sigmastat. Data was analyzed using a "Random Effects" regression model which is recommended for a hierarchal data set such as this study generates. It ensures the correct integration and consideration of the different variability which occur within and between families and allows the utilization of all the information collected (Kwong et al 2004).

This model has two particular attributes for analyzing the type of data generated in this study; firstly the analysis focuses on the explicit estimation of both parameters and their variances. This steers the analysis away from simple hypothesis testing and towards the reporting of results in terms of parameter estimates with uncertainty expressed in confidence limits. Therefore, the statistics will include within-litter and between-litter estimates. Secondly the "random effects" regression model allows the simple generalizations to more complex data structures, for example allowing the ability to ask if endocytic index is influenced by litter size (Kwong et al 2004).

Chapter 3

Effect of maternal diet on litter size, uterine position, conceptus growth and total protein content.

3.1 Introduction:

Fetal weight gain during gestation increases at a steady rate up until the last trimester in animals and humans, when weight gain dramatically increases. Throughout gestation the placenta and visceral yolk sac must provide an adequate supply of nutrients to the fetus (Matthews et al 1998). The role of the placenta during gestation had been studied in depth in various species, including humans, sheep, and rats (Beckman 1997). Placental free-amino acid transport has been shown to increase in the third trimester in rats. Undernutrition in rats has been associated with a reduced placental blood flow, reduced rates of amino acid transfer and reduced fetal and placental weights (Matthews et al 1998).

Previous studies have shown that in a compromised maternal environment caused by feeding a low protein diet the number of cells in the inner cell mass decreases this also occurs in the trophectoderm lineage in the rat (Kwong et al 2000). Therefore, it is possible that either the amount of cells available to create the visceral yolk sac is diminished or the compromised environment will cause a compensatory change in the metabolic activity of the visceral yolk sac.

Earlier studies, mainly carried out in the rat, investigated the specific weights of the conceptus, fetus and placenta and fetal weight gain after maternal undernutrition. As yet, correlations between the growth of various parts of the conceptus and its positioning and the litter size have not been further investigated. My initial study looked at the effect of litter size, position within the uterus and maternal diet on the weight of the conceptus and its component parts, and the total protein content of the visceral yolk sac.

3.2 Methods:

Mice were mated overnight, the presence of a vaginal plug in the morning designated pregnancy and day 0 (Section 2.1). Pregnant female MF1 mice were fed either a control diet (18% casein) or a low protein diet (9% casein) throughout pregnancy up to either 12, 14 or 17 days gestation (Section 2.3). The mice were culled and the conceptuses and component parts were dissected out and weighed separately (Section 2.3). The exact position within the uterus of every conceptus and hence every yolk sac was recorded during dissection (Section 2.3). Total protein content of each yolk sac was ascertained using the Lowry method (Section 2.4). Statistical analysis using the random effects regression method (Section 2.7) was performed on the data collated in order to ascertain if maternal diet influenced the litter size, position of the conceptus within the uterine horn, the weight of any/all of its constituent parts, and the total protein content.

3.3 Results

3.3.1 Litter Size

The mean litter size in mothers fed either low protein diet (9% casein) or control diet (18% casein) at each of the three stages of development investigated is shown in Table 4. Litter size was not significantly different either between diet treatments or between stages of development.

Days	Diet (% casein)	Mean litter size	Number of litters	P value
12	9	12.87 ± 1.55	8	0.14
	18	11.11 ± 2.84	9	
14	9	11.87 ± 1.46	8	0.26
	18	13.11 ± 2.67	9	
17	9	12.30 ± 2.53	20	0.92
	18	12.38 ± 2.31	21	

Table 4: Mean litter size in relation to diet and stage of development.

The number of conceptuses (litter size) in each uterine horn is not consistent in the mouse, in most of my observations there were more conceptuses in one horn that in the other (Figures 17-19). Figures 17-19 show the difference in number of conceptuses between the right and left horn. The data display a trend for more conceptuses to position in the right horn than in the left. However, this trend was not significantly (P = 0.826) biased to either horn by the mother feeding on a low protein diet throughout gestation. Thus feeding a low protein diet did not alter the positioning of the conceptus within the uterine horn.

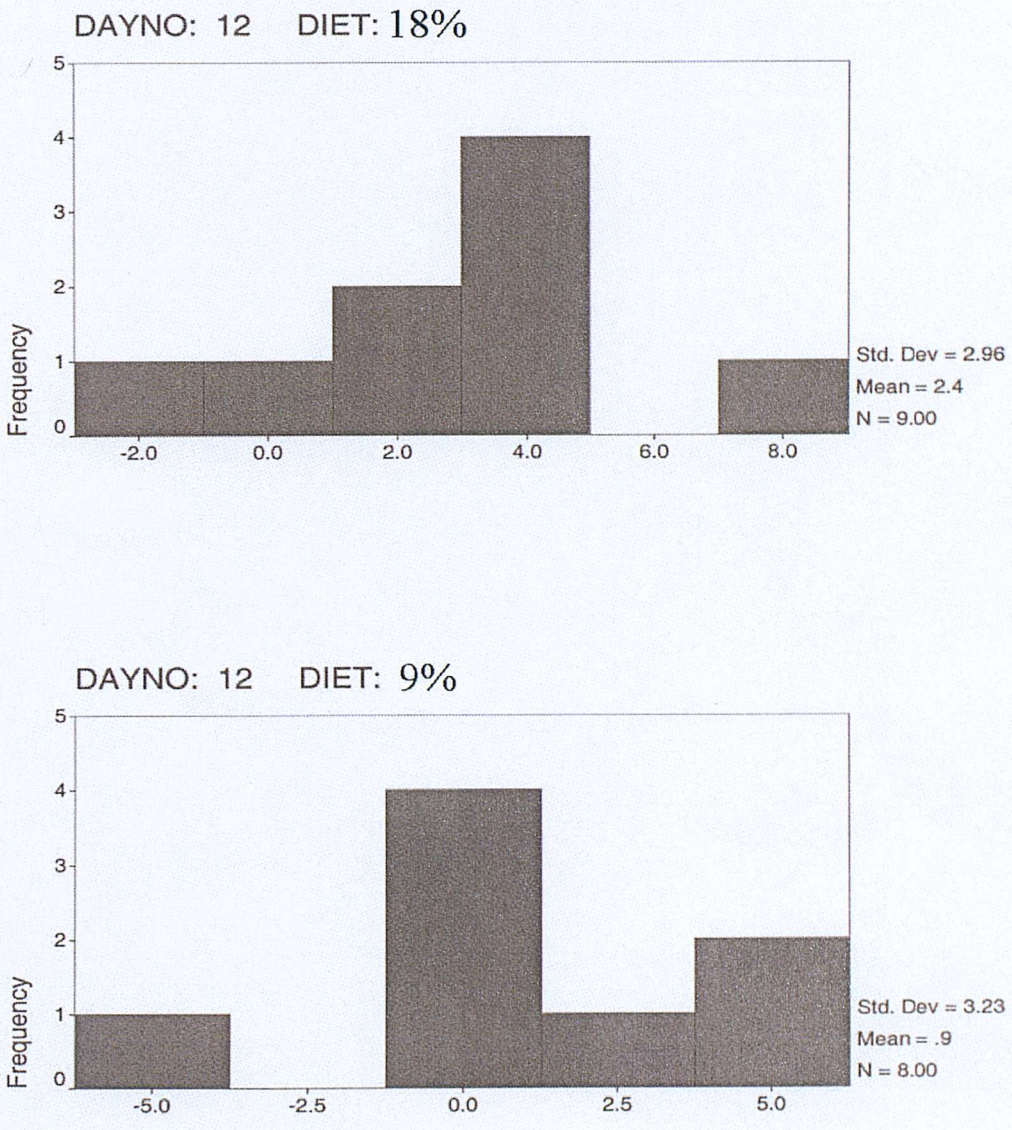
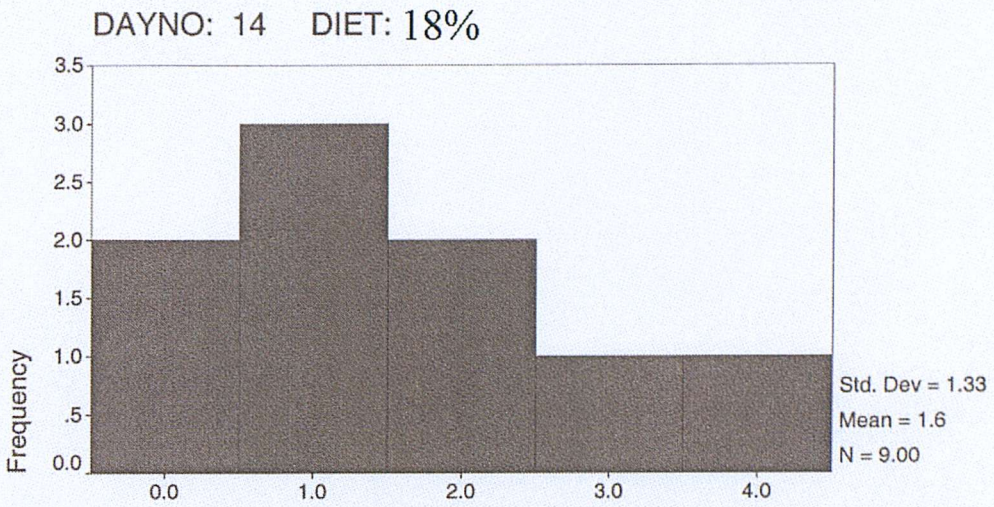


Figure 17: Difference in number of conceptuses between left and right horn of the uterus at Day 12 gestation. 0 value means that there are the same number of conceptuses in each horn, negative values indicate more conceptuses in the left horn than the right and positive values indicate more conceptuses in the right horn than in the left.



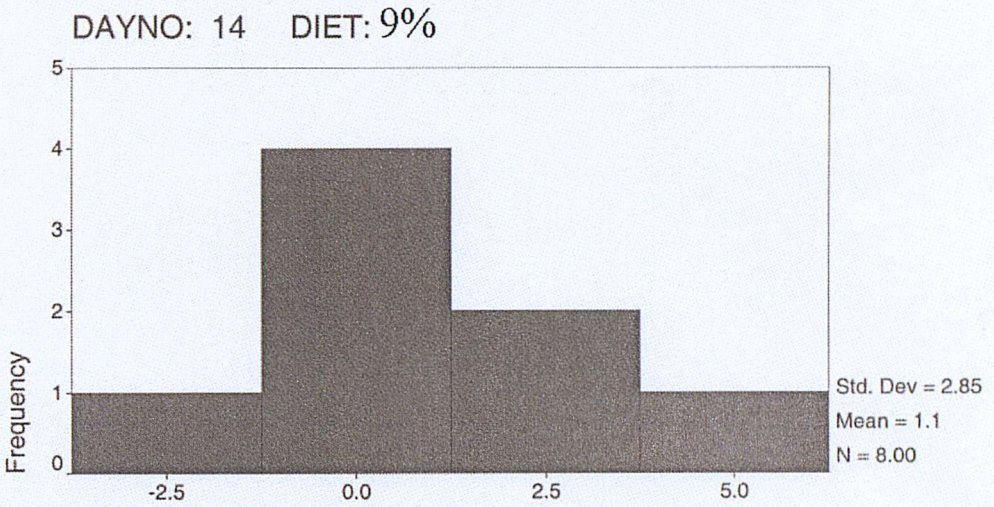
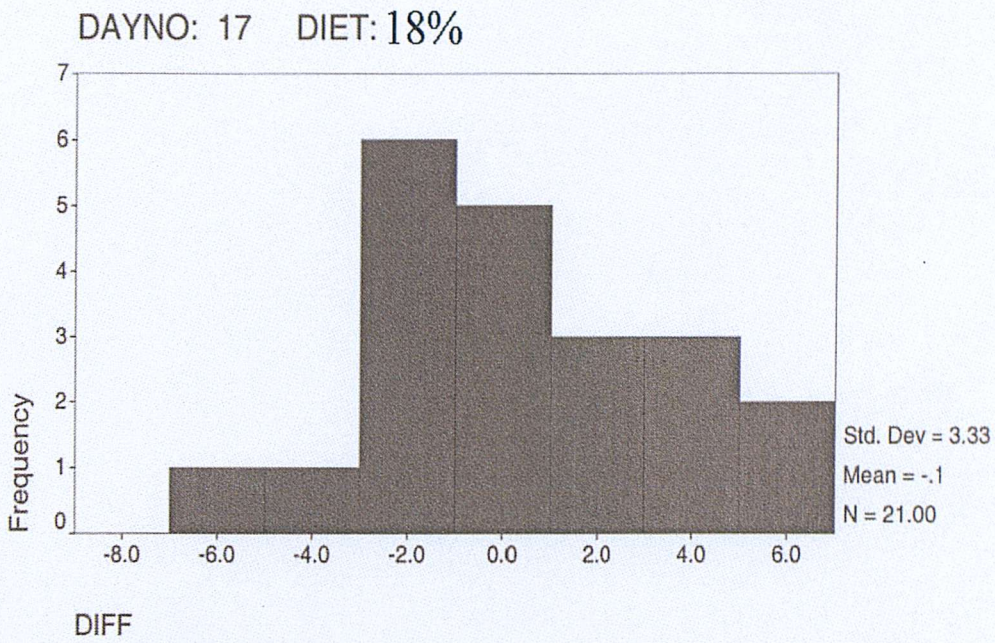


Figure 18: Difference in number of conceptuses between left and right uterine horns at day 14 gestation. 0 value means that there are the same number of conceptuses in each horn, negative values indicate more conceptuses in the left horn than the right and positive values indicate more conceptuses in the right horn than in the left.



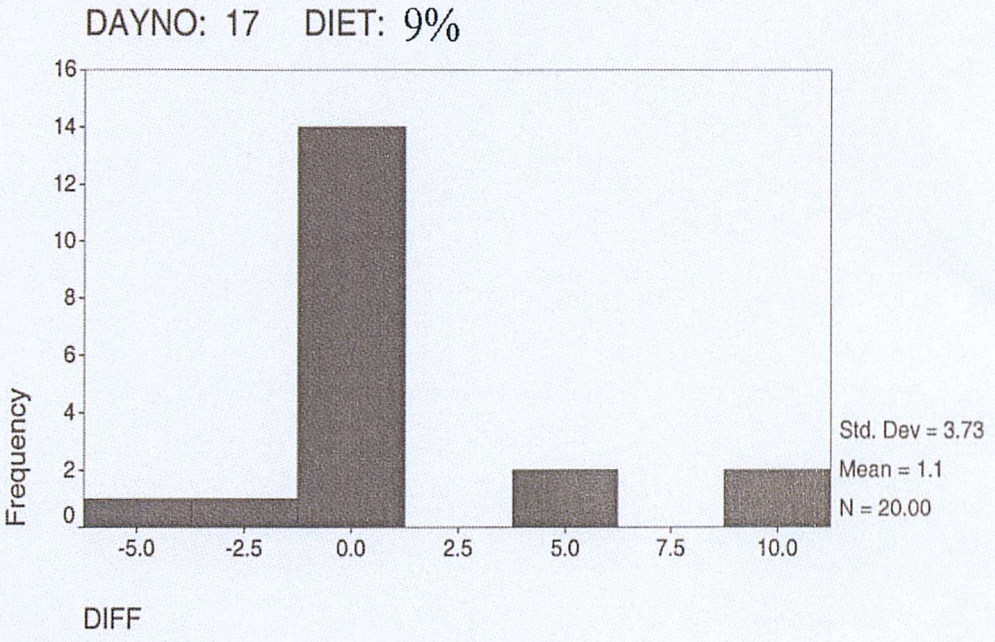


Figure 19: Difference in number of conceptuses between left and right uterine horns at day 17 gestation. 0 value means that there are the same number of conceptuses in each horn, negative values indicate more conceptuses in the left horn than the right and positive values indicate more conceptuses in the right horn in the left.

3.3.2 Conceptus Growth

The mean weights and standard error of means (S.E.M.) of the conceptus, fetus, placenta and visceral yolk sac at each time point investigated in relation to maternal low protein diet or control diet are shown in Tables 5-7. The P values generated show no significant difference between the weights of conceptuses and their component parts from either the low protein diet (9% casein) or the control diet (18% casein) at any of the three time points studied.

12 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean (g)	0.2428	0.2578	0.0772	0.0757	0.0539	0.0585	0.0103	0.0114
S.E.M	0.0051	0.0038	0.0015	0.0014	0.0016	0.0011	0.0004	0.0002
P = value	0.242		0.737		0.299		0.227	
No. of mothers	8	9	8	9	8	9	8	9
No. of conceptuses	103	100	103	100	103	100	103	100

Table 5: Weights of conceptus and component parts at Day 12 of gestation

14 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean (g)	0.5371	0.5359	0.2351	0.2323	0.1071	0.1150	0.0271	0.0251
S.E.M	0.0042	0.0050	0.0026	0.0030	0.0016	0.0020	0.0005	0.0005
P = value	0.848		0.733		0.294		0.105	
No. of mothers	8	9	8	9	8	9	8	9
No. of conceptuses	94	117	94	117	94	117	94	117

Table 6: Weights of conceptus and component parts at day 14 of gestation

17 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean (g)	1.1638	1.2091	0.8745	0.9278	0.1459	0.1427	0.0571	0.0572
S.E.M	0.0094	0.011	0.0097	0.0121	0.0013	0.00169	0.0012	0.00107
P = value	0.170		0.150		0.968		0.838	
No. of mothers	20	21	20	21	20	21	20	21
No. of conceptuses	245	253	245	253	245	253	245	253

Table 7: Weights of conceptus and component parts at day 17 of gestation

Taking the data from Tables 5-7, the average weight gain of the conceptus, fetus, placenta and visceral yolk sac from day 12 to day 17 of gestation is shown in Figures 20-23. The feeding of a low protein diet as opposed to a control diet to the mother during gestation does not affect the weight gain of the conceptus and its component parts (Tables 5-7 statistical analysis).

The largest weight gain occurs in the fetus between day 14 and 17 gestation, Figure 21 clearly shows an increase in weight of almost four times that of the fetus at day 14 gestation. There, also, appears to be a large increase in weight, almost doubling, of the visceral yolk sac between days 14 and 17 gestation (Figure 23). Placental weight gain was largest between days 12 and 14 of gestation, with nearly a doubling in weight occurring (Figure 22).

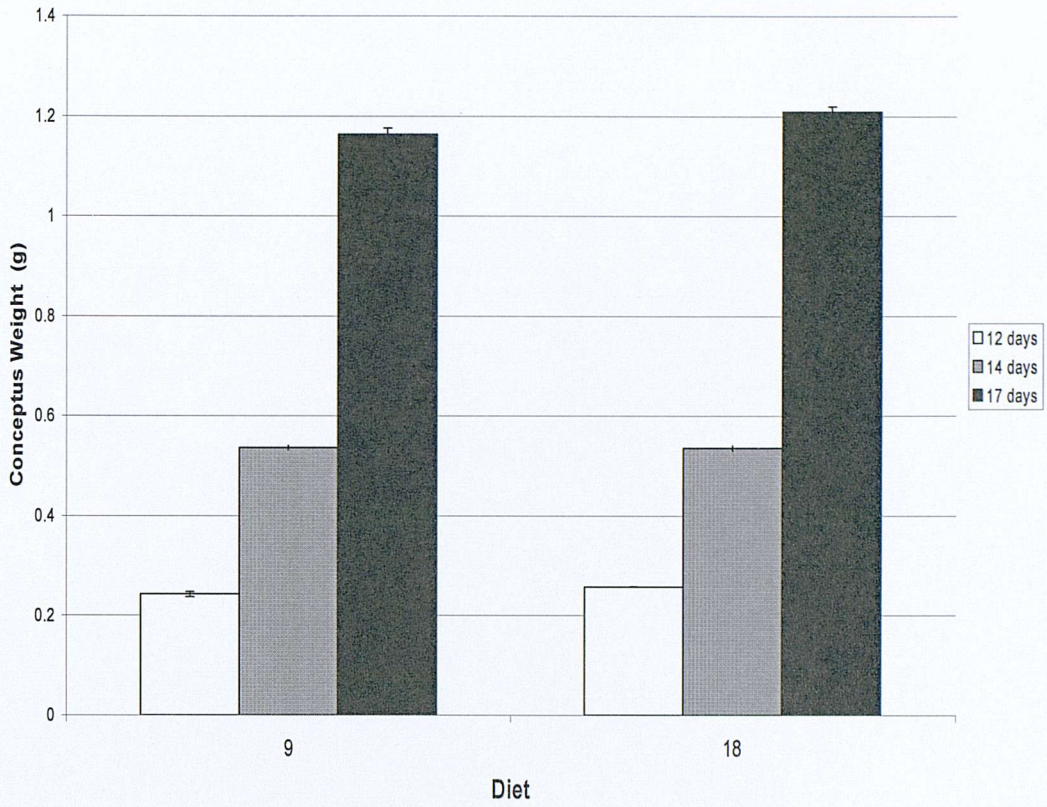


Figure 20: Average conceptus weight gain during gestation, up to day 17.

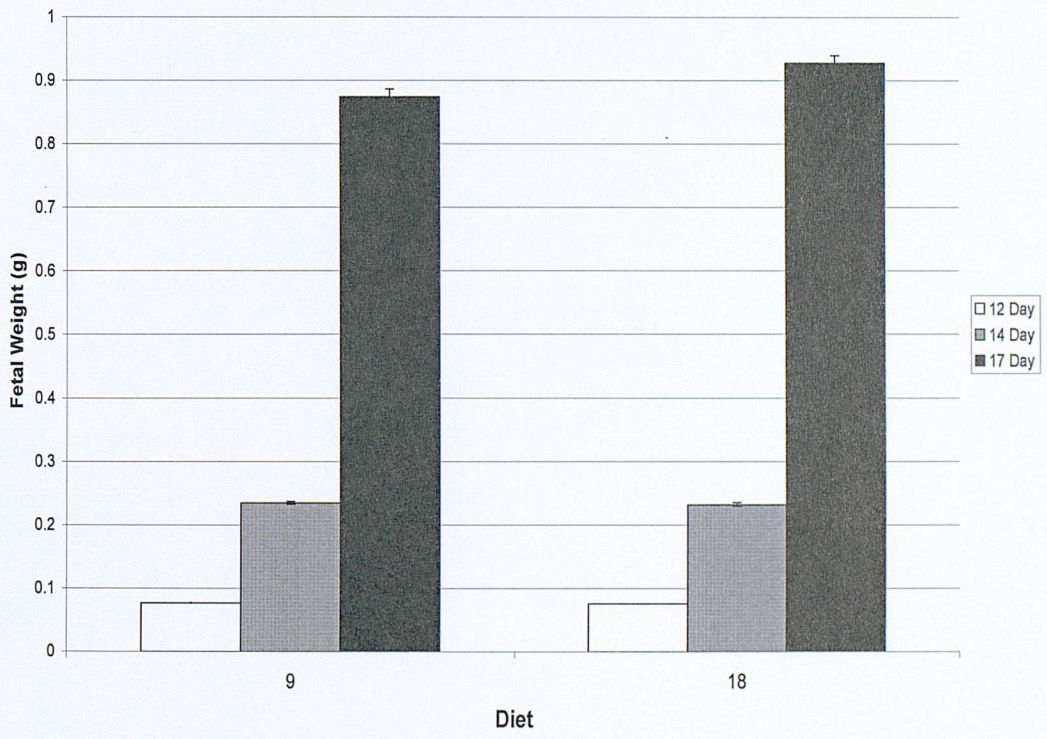


Figure 21: Average fetal weight gain during gestation up to Day 17

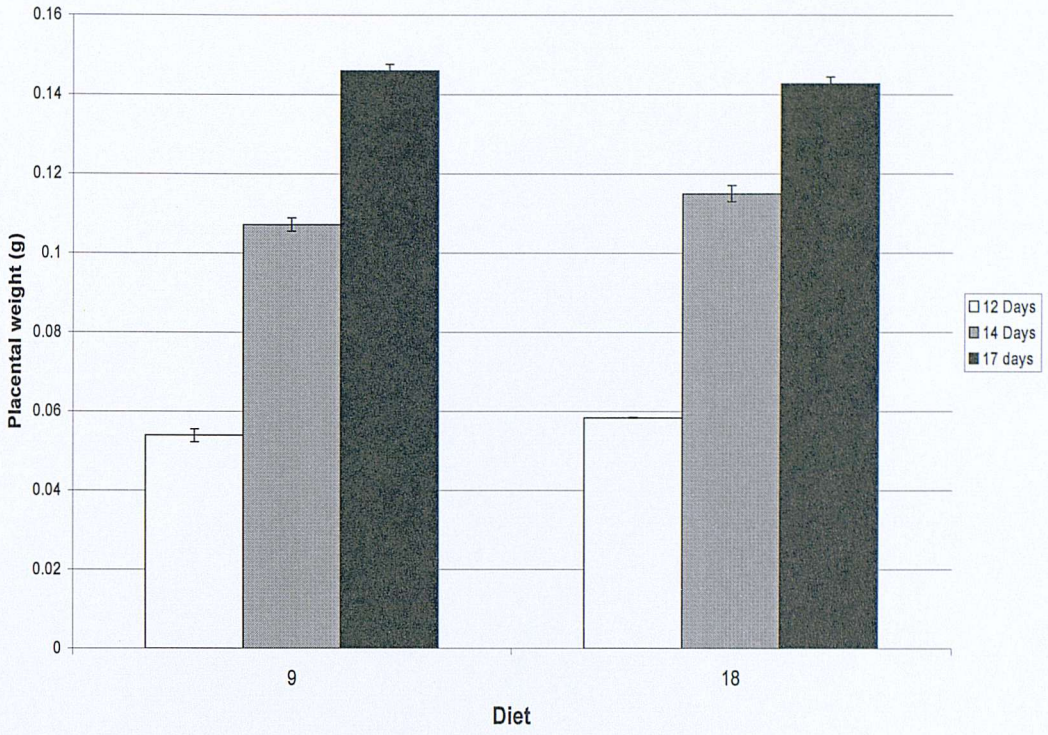


Figure 22: Average placental weight gain during gestation up to Day 17

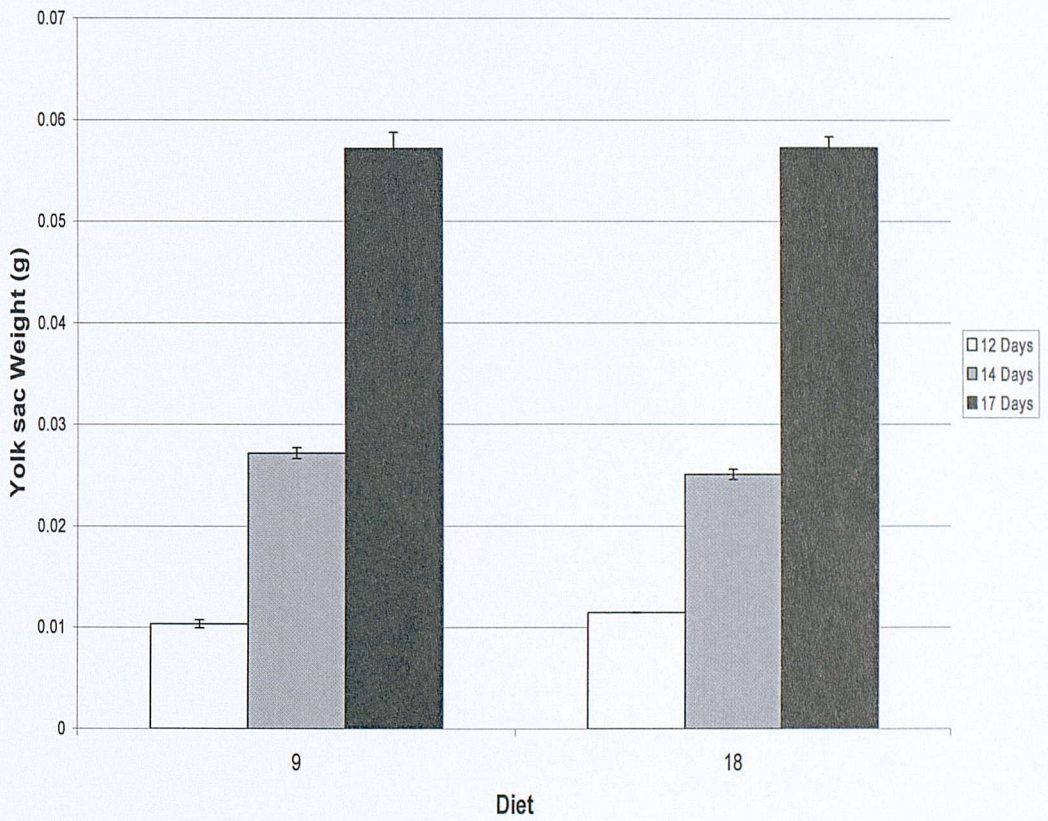


Figure 23: Average visceral yolk sac weight gain during gestation up to Day 17.

From the analysis of conceptus weight, it was apparent that a consistent relationship existed at any one stage of development between the relative weights of component parts. However, this relationship changes as gestation proceeds. Thus, at day 12 the percentage contribution of fetal, placental and visceral yolk sac weight to conceptus weight was 52.0 : 40.2 : 7.8 for controls and 54.6 : 38.1 : 7.3 for low protein diet mothers. Table 8 shows how proportionality changes as gestation proceeds with placental weight having a declining contribution and fetal weight an increasing contribution to conceptus weight with time. The relative proportions of the component parts do not amount to exactly 100% due to there being some loss of fluid from the conceptus during the dissection process.

	Low protein diet			Control diet		
	Conceptus weight	Proportion %		Conceptus weight	Proportion %	
Day 12	0.1414	54.6	Fetus	0.1456	51.99	Fetus
		38.12	Placenta		40.18	Placenta
		7.28	Yolk sac		7.83	Yolk sac
Day 14	0.3639	63.67	Fetus	0.3724	62.38	Fetus
		29.00	Placenta		30.88	Placenta
		7.34	Yolk sac		6.74	Yolk sac
Day 17	1.0775	81.16	Fetus	1.1277	82.27	Fetus
		13.54	Placenta		12.65	Placenta
		5.29	Yolk sac		5.07	Yolk sac

Table 8: Proportionality of fetal, placental and visceral yolk sac tissue weights within conceptus weight during gestation.

To investigate whether proportionality of conceptus growth varied on an individual basis in relation to stage and diet, the relationship between the weight of individual conceptuses and their relevant component parts was analyzed graphically. The data for day 17 gestation are shown in Figures 24-35, while those for earlier stages are in Appendix 2 (Figures 88-99 day 12 gestation, Figures 100-111 day 14 gestation). The R^2 values for the trend line shown on each graph indicate how closely related the two weights plotted on the graph are; the angle of

the trend lines show the positive correlation between these weights per conceptus. Statistical analysis, taking into account variation both within a mother and between mothers, yielded highly significant P values ($P<0.001$) for all of the weight correlations shown in Figures 24-35.

The relationships between the weights of the component parts of the conceptus at day 12 and 14 of gestation show a strong correlation to each other. Almost every relationship displays a statistically highly significant ($P<0.000$) positive correlation. However, at day 12 of gestation in the data from mothers on the control diet (18%), the relationship between visceral yolk sac weight and placental weight is not significant ($P=0.800$), and the correlation in this instance is negative. At day 14 of gestation in mothers from both diet groups the relationship between visceral yolk sac weight and placental weight is again not significant ($P=0.352$ in control diet group, $P=0.147$ in the low protein diet group).

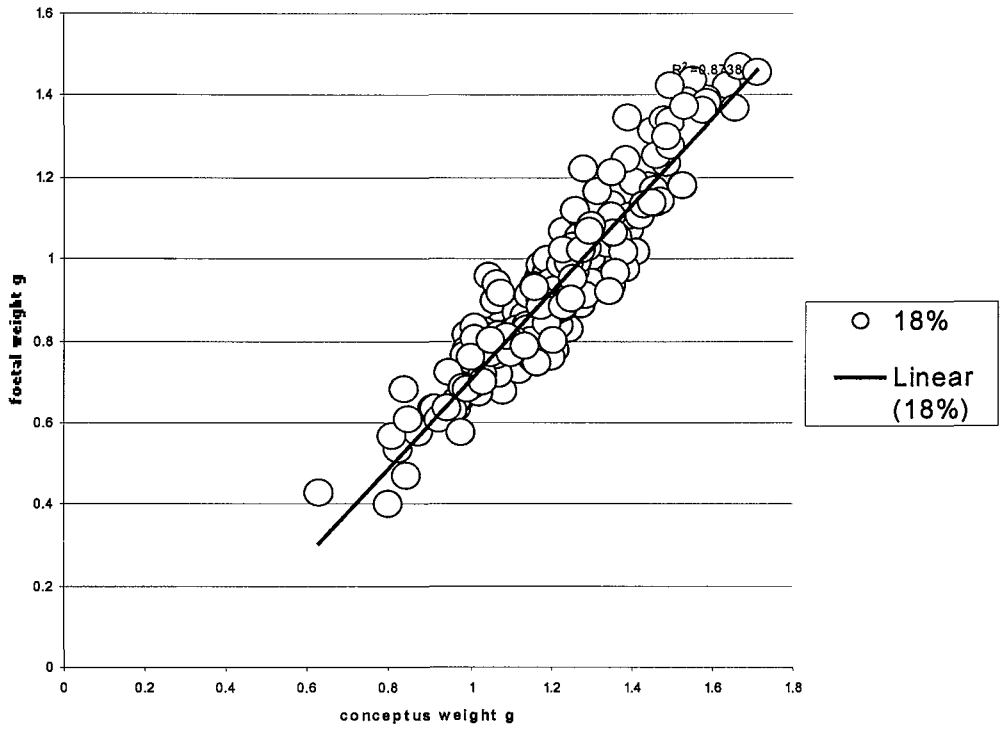


Figure 24: The relationship between Conceptus weight and Fetal weight at 17 days gestation in mice fed 18% diet. R2 = 0.8738 n=253

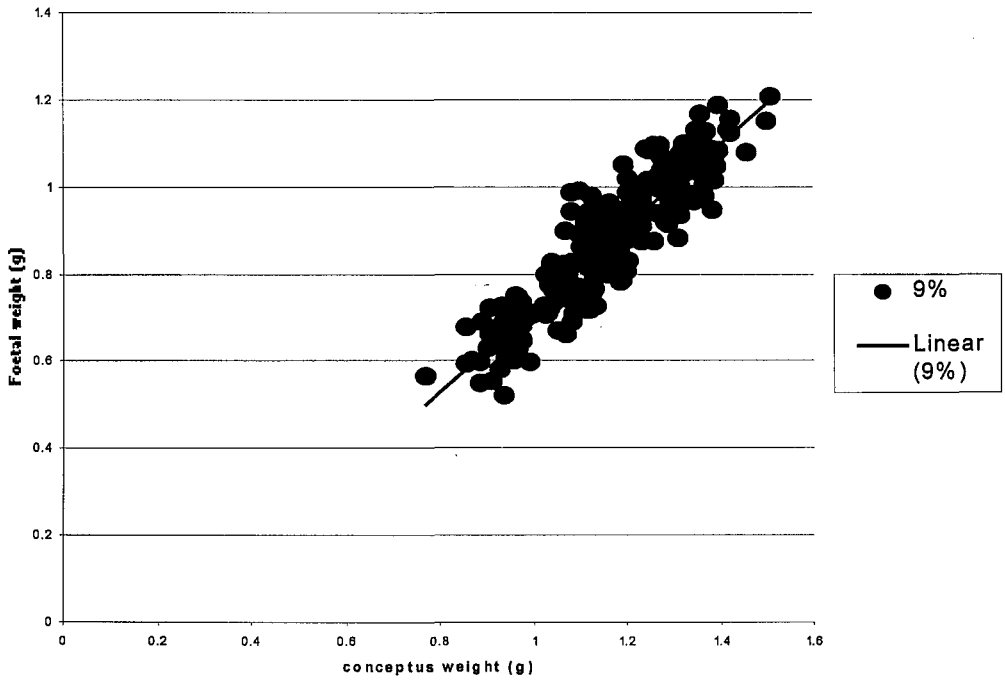


Figure 25: The relationship between Conceptus weight and Fetal weight at 17 days gestation in mice fed 9% diet. R2 = 0.8337 n=245

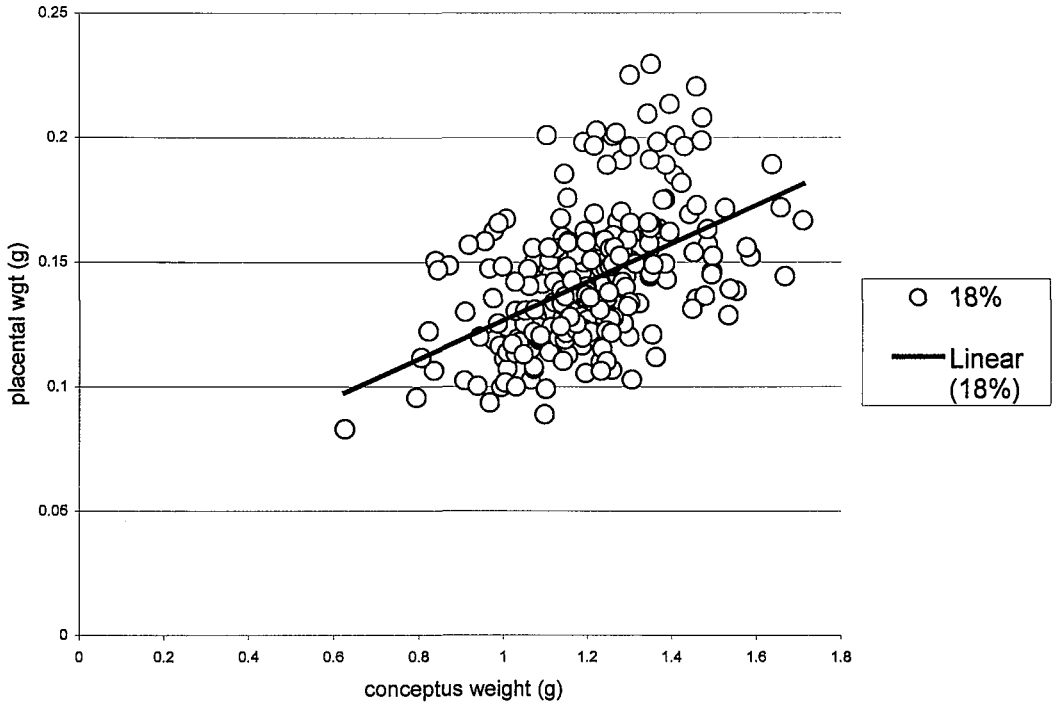


Figure 26: The relationship between Conceptus weight and Placental weight at 17 days gestation in mice fed 18% diet. R2 = 0.2368 n=253

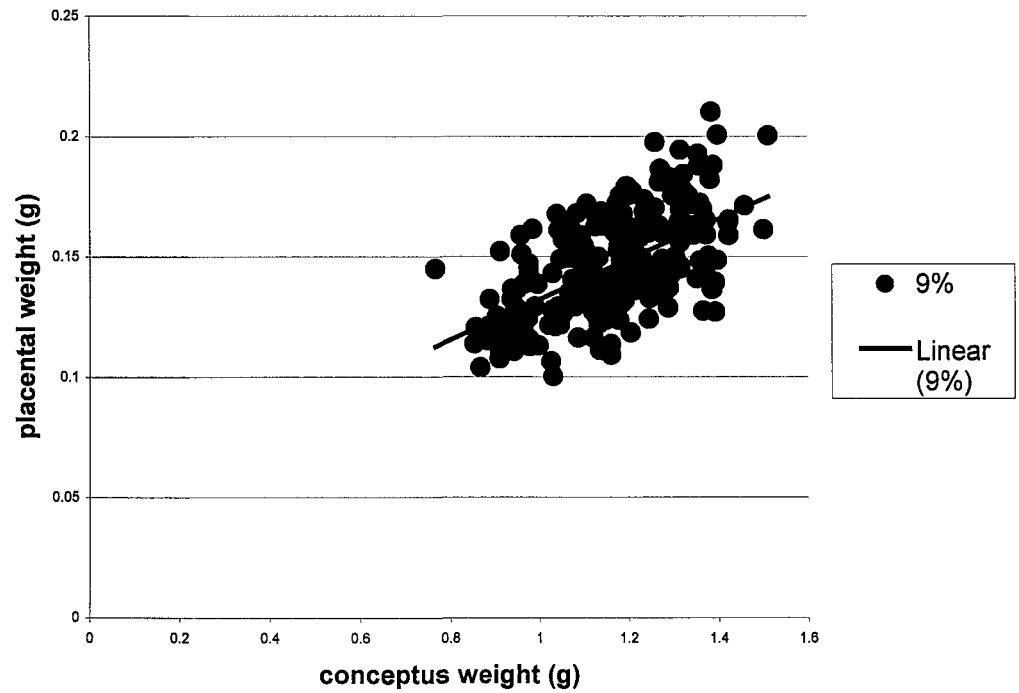


Figure 27: The relationship between Conceptus weight and Placental weight at 17 days gestation in mice fed 9% diet. R2 =0.3503 n=245

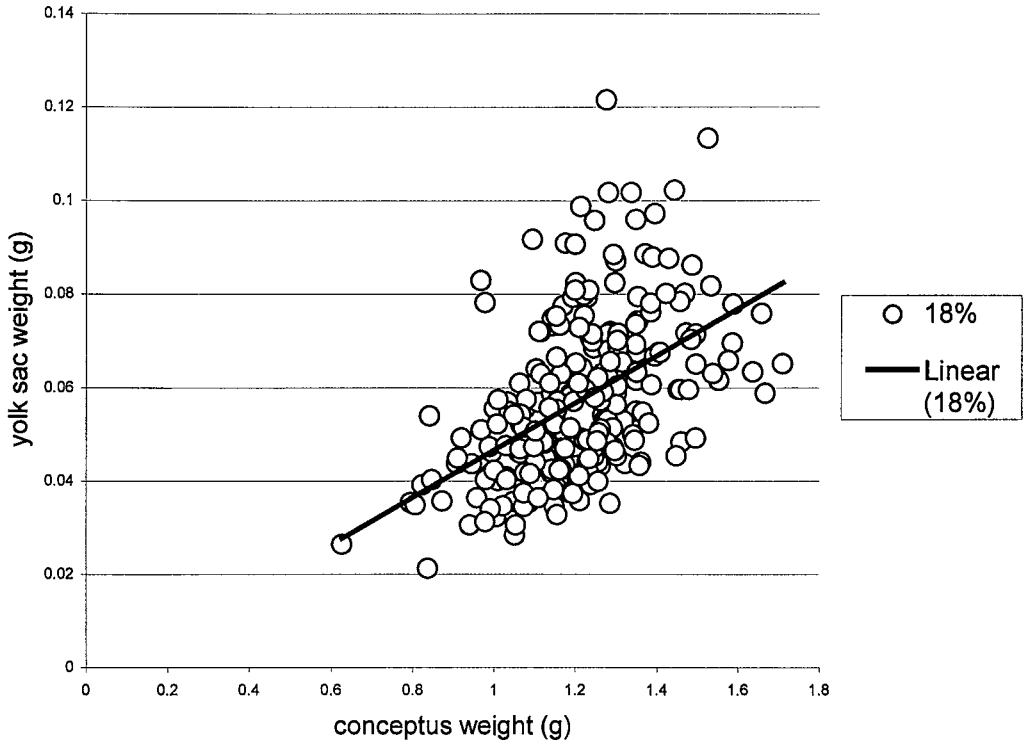


Figure 28: The relationship between Conceptus weight and visceral Yolk sac weight at 17 days gestation in mice fed 18% diet. R2 = 0.2501 n=253

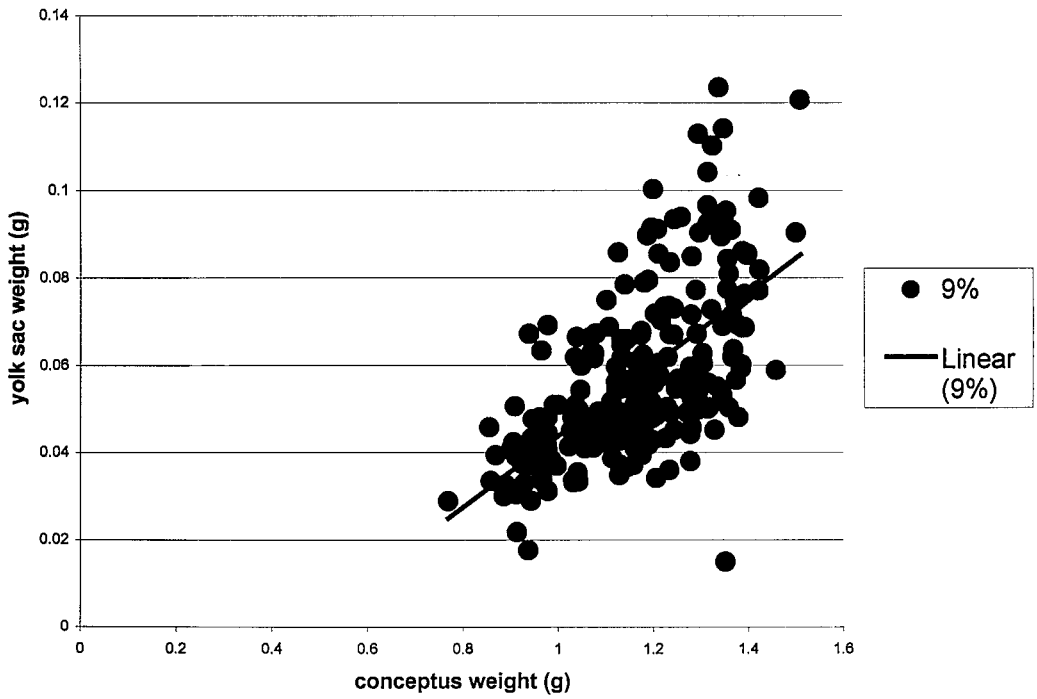


Figure 29: The relationship between conceptus weight and yolk sac weight at 17 days gestation in mice fed 9% diet. R2 = 0.3887 n=245

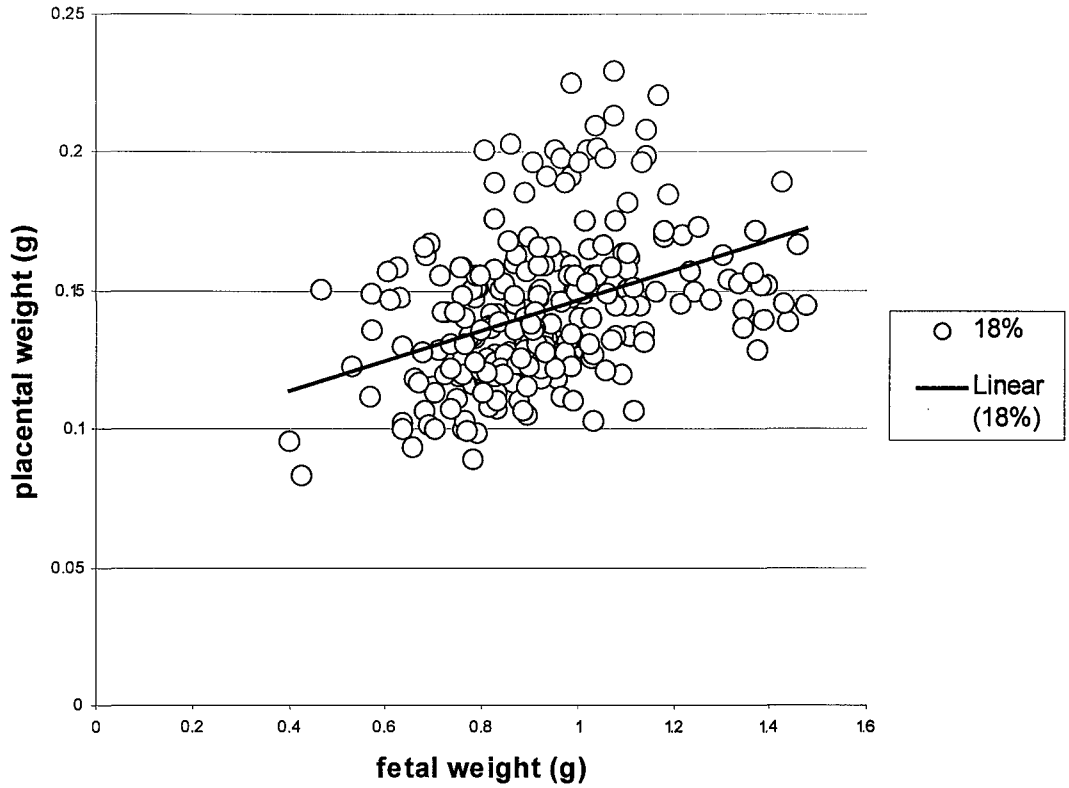


Figure 30: The relationship between fetal weight and placental weight at 17 days gestation in mice fed 18% diet. R2 = 0.1527 n=253

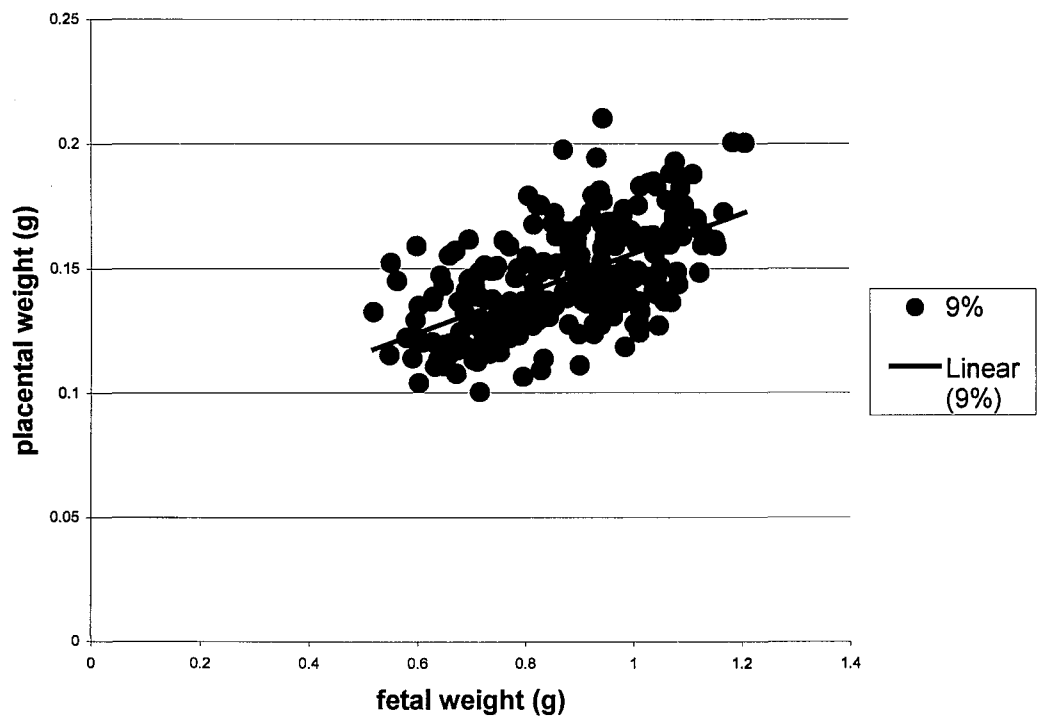


Figure 31: The relationship between fetal weight and placental weight at 17 days gestation in mice fed 9% diet. R2 = 0.3385 n=245

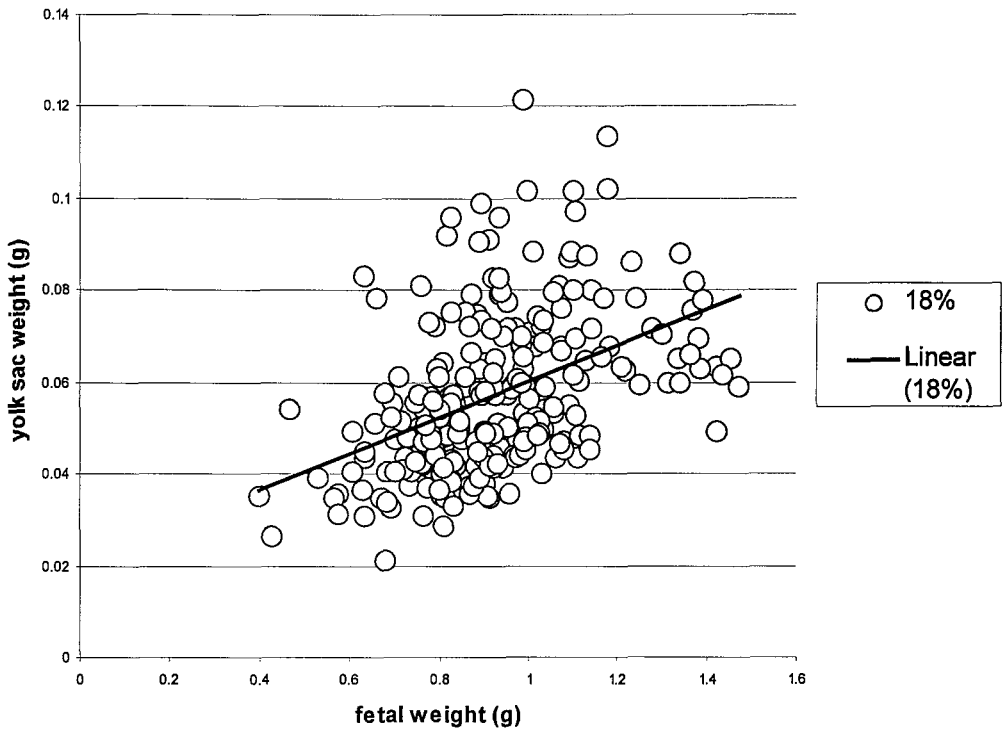


Figure 32: The relationship between fetal weight and visceral yolk sac weight at 17 days gestation in mice fed 18% diet. R2 = 0.1998 n=253

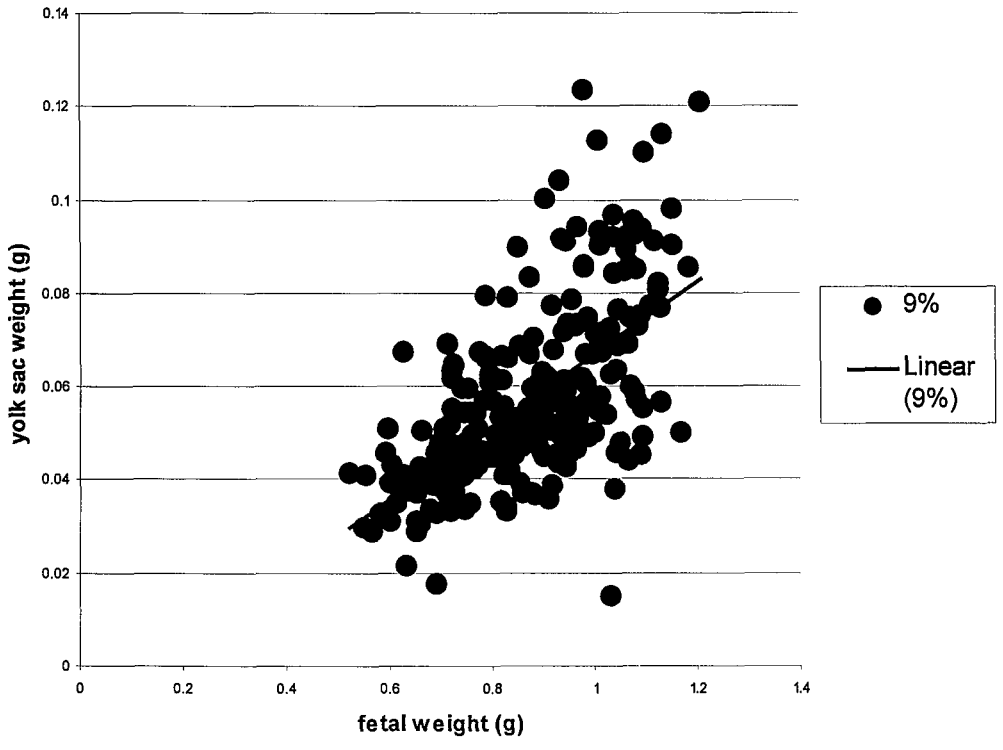


Figure 33: The relationship between fetal weight and visceral yolk sac weight at 17 days gestation in mice fed 9% diet. R2 = 0.372 n=245

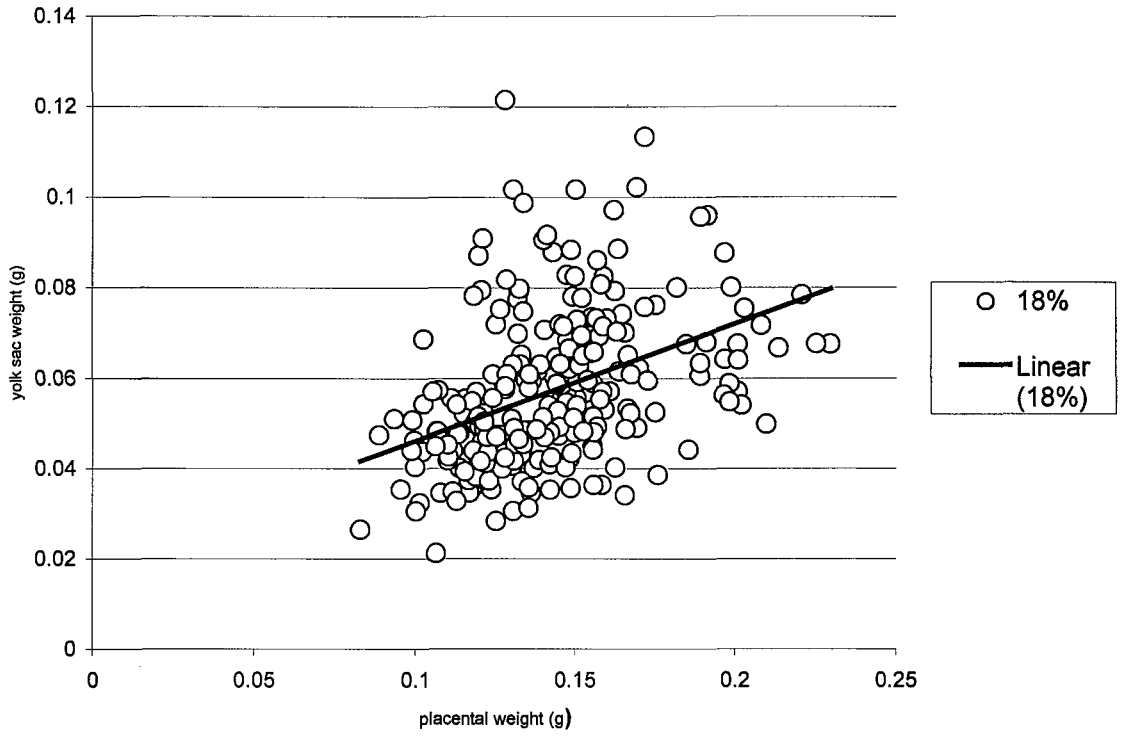


Figure 34: The relationship between placental weight and visceral yolk sac weight at day 17 gestation in mice fed 18% diet. R2 = 0.1674 n=253

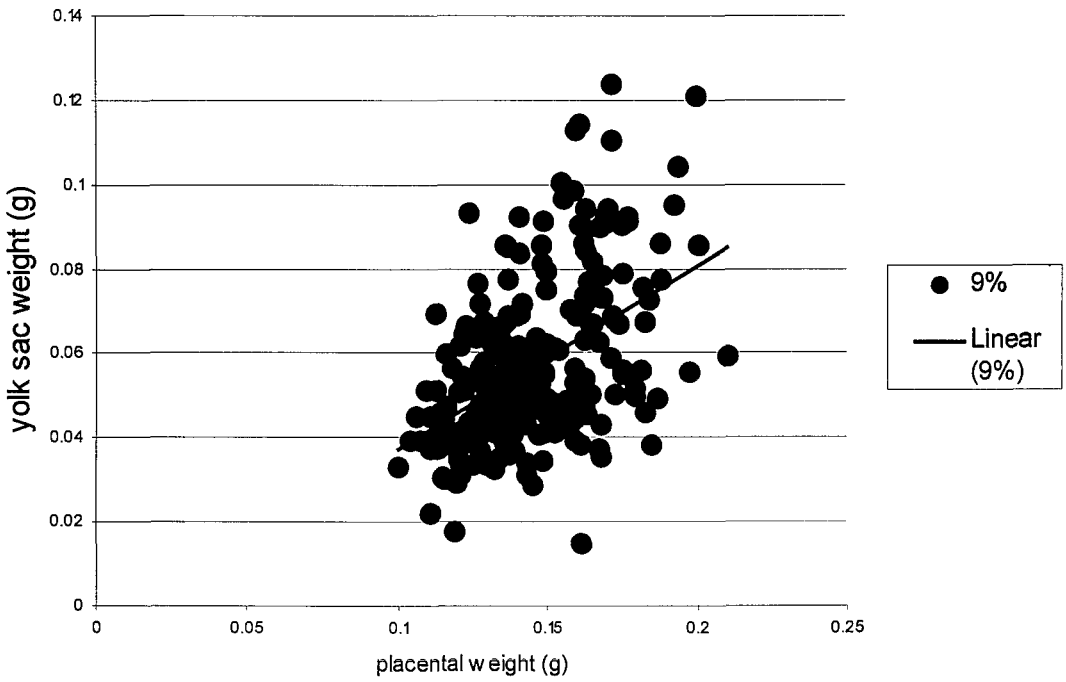


Figure 35: The relationship between placental weight and visceral yolk sac weight at day 17 gestation in mice fed 9% diet. R2 = 0.2212 n=245

The effect of litter size upon the weight of conceptus and its component parts was also assessed for each diet group and developmental stage (Tables 9-11). This statistical analysis shows that there was no significant effect of litter size upon the majority of the weights of the conceptus, fetus, placenta and the visceral yolk sac. There is one exception to this, at day 17 gestation in the group fed a control diet, the conceptus weight is significantly (P = 0.044) influenced by the litter size.

12 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean weight	0.2428	0.2578	0.0772	0.0757	0.0539	0.0585	0.0103	0.0114
S.E.M	0.0051	0.0038	0.0015	0.0014	0.0016	0.0011	0.0004	0.0002
No. of mothers	8	9	8	9	8	9	8	9
No. of conceptuses	103	100	103	100	103	100	103	100
P value for effect of litter size	0.581	0.591	0.753	0.692	0.842	0.733	0.291	0.151

Table 9: Statistical effect of litter size at Day 12 upon weights of the conceptus and its component parts and mean weights. Mean litter size 18%:11.1, 9%: 12.8

14 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean Weight	0.5371	0.5359	0.2351	0.2323	0.1071	0.1150	0.0271	0.0251
S.E.M	0.0042	0.0050	0.0026	0.0030	0.0016	0.0020	0.0005	0.0005
No. of mothers	8	9	8	9	8	9	8	9
No. of conceptuses	94	117	94	117	94	117	94	117
P value for effect of litter size	0.587	0.551	0.409	0.369	0.584	0.561	0.846	0.887

Table 10: Effect of litter size upon weights of the conceptus and its component parts at day 14 gestation. Mean litter size 18%: 13.1, 9%:11.8

17 Day	Conceptus		Fetus		Placenta		Yolk sac	
Diet %	9	18	9	18	9	18	9	18
Mean Weight	1.1638	1.2091	0.8745	0.9278	0.1459	0.1427	0.0571	0.0572
S.E.M	0.0094	0.011	0.0097	0.0121	0.0013	0.00169	0.0012	0.00107
No. of mothers	20	21	20	21	20	21	20	21
No. of conceptuses	245	253	245	253	245	253	245	253
P value for effect of litter size	0.529	0.044	0.208	0.244	0.271	0.068	0.370	0.731

Table 11: Effect of litter size upon the weights of the conceptus and component parts at day 17 gestation. Mean litter size 18%: 12.3, 9%:12.3

3.3.3 Total Protein Content

Total protein content of the visceral yolk sac was calculated in micrograms per whole tissue as opposed to per unit weight. The total protein content of visceral yolk sacs at each stage of development is shown in Figure 36. From Figure 36, it can be seen that total protein content increases as gestation proceeds, similar to visceral yolk sac weight as discussed above (Tables 5-7, Figure 23). At day 12 gestation, there was a significantly (P=0.022) higher total protein content in the visceral yolk sacs from mothers fed a control diet (18% casein) compared to visceral yolk sacs from mothers fed a low protein diet (9% casein). However, at day 14 (P = 0.194) or day 17 (P = 0.094), no significant differences were apparent in visceral yolk sac total protein content between diets, although a trend for increased total protein content was apparent in the low protein diet group at day 17 (Figure 36).

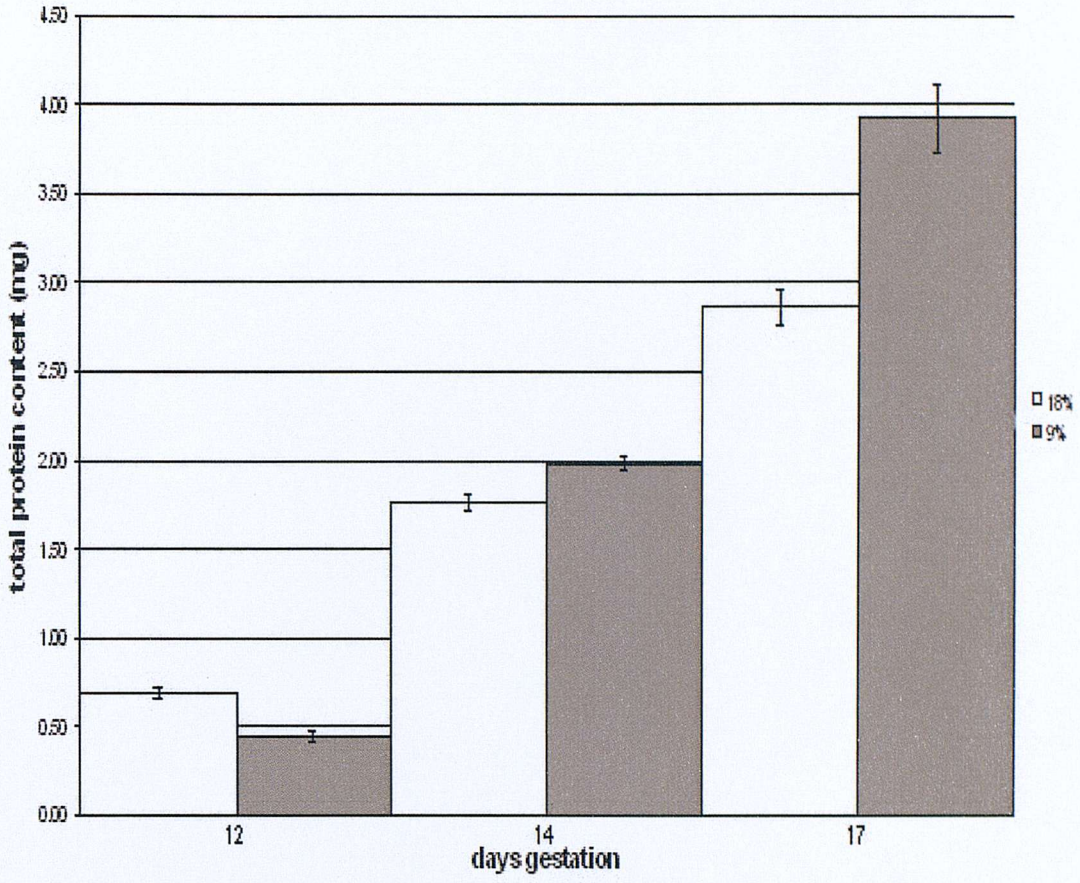


Figure 36: Total protein content of visceral yolk sac through gestation. Day 12 P = 0.022, Day 14 P = 0.194, day 17 P = 0.094

Total protein content of the visceral yolk sac was not significantly affected by litter size at any of the three time points studied (Table 12).

Days gestation	12		14		17	
Diet	9	18	9	18	9	18
Mean	0.444	0.693	1.986	1.7619	3.926	2.862
S.E.M	0.027	0.030	0.040	0.041	0.190	0.098
P value for litter size	0.514	0.894	0.310	0.054	0.536	0.859
Number of litters	8	9	8	9	20	21
Number of yolk sacs	88	78	94	117	103	152

Table 12: Effect of litter size upon total protein content of visceral yolk sac at each time point, including mean and SEM

3.3.4 Position in the Uterine Horn

The weight of the conceptus and its component parts at each developmental stage and in relation to maternal diet was also assessed with respect to conceptus position within the uterine horn. Conceptus position was recorded from distal position 1 (ovarian end) to proximal locations (up to 12, cervical end) (Figure 37). Overall conceptus weight and that of its component parts was not affected with respect to conceptus position for nearly all analyses (P = 0.826, Figures 38 - 45 show data for day 17, data for day 14 and 12 are shown in Appendix 3 Figures 112-127). On two occasions, there is a significant effect of position upon the weights; firstly, at day 12 gestation in conceptuses harvested from mothers fed a control diet throughout gestation there was a significant (P = 0.016, Figure 113) increase in whole conceptus weight as the position of the conceptus moves down the uterine horn from the ovarian to the cervical end. Secondly, also at day 12 gestation, there is a significant (P = 0.003, Figure 116) decrease in the weight of the placenta as the position of the conceptus moves down the uterine horn from the ovarian to cervical end.

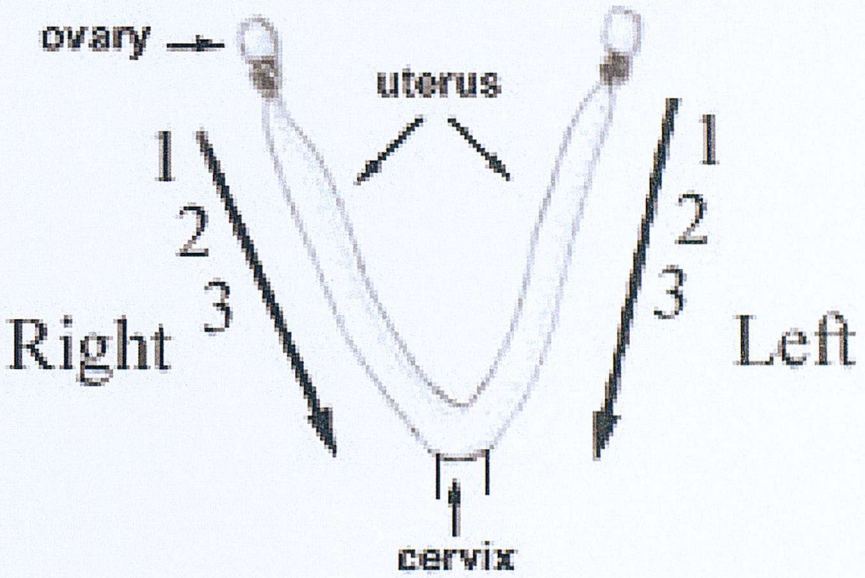


Figure 37: Schematic diagram of the mouse uterus

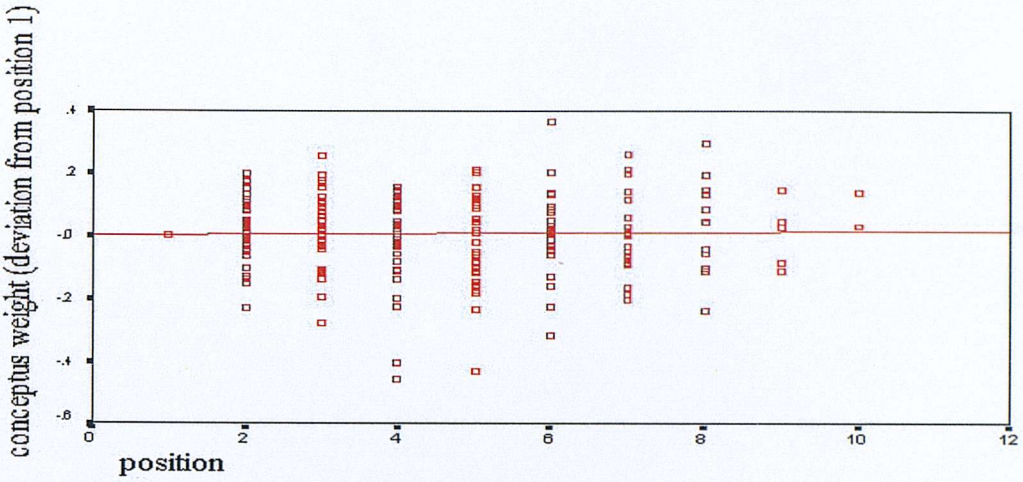


Figure 38: Deviation in conceptus weight from mothers fed a control diet up to day 17 gestation in relation to position along the uterine horn (1,ovarian end; 12,cervical end). P = 0.339 N = 253

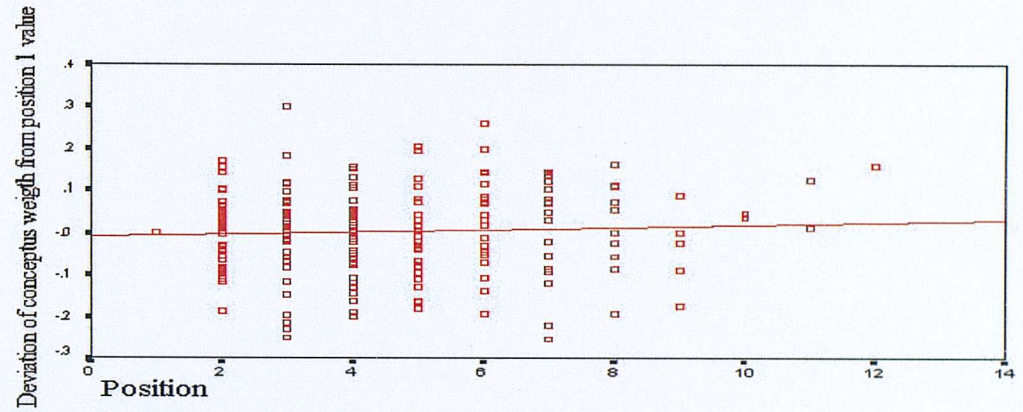


Figure 39: Deviation in conceptus weight from mothers fed low protein diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12,cervical end). P = 0.769 N = 245

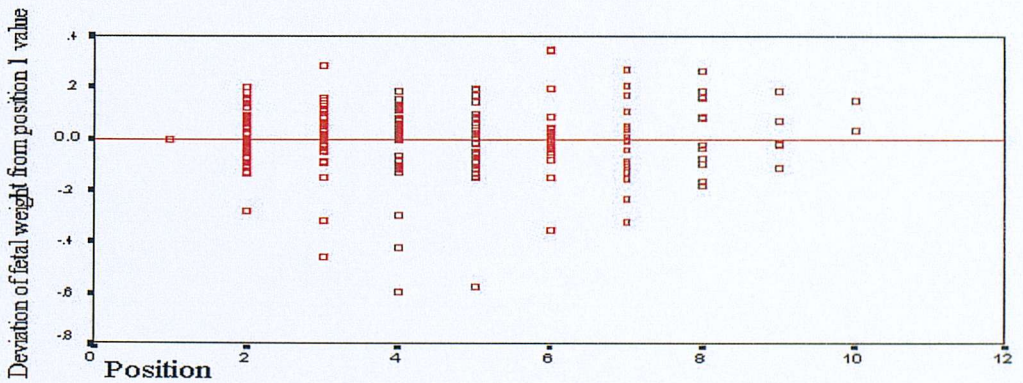


Figure 40: Deviation in fetus weight from mothers fed control diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12,cervical end). P = 0.101 N = 253

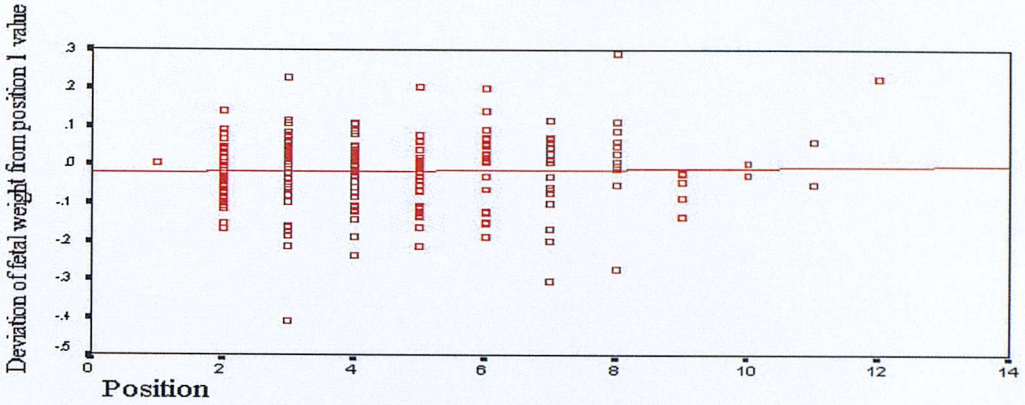


Figure 41: Deviation in fetus weight from mothers fed low protein diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.552 N = 245

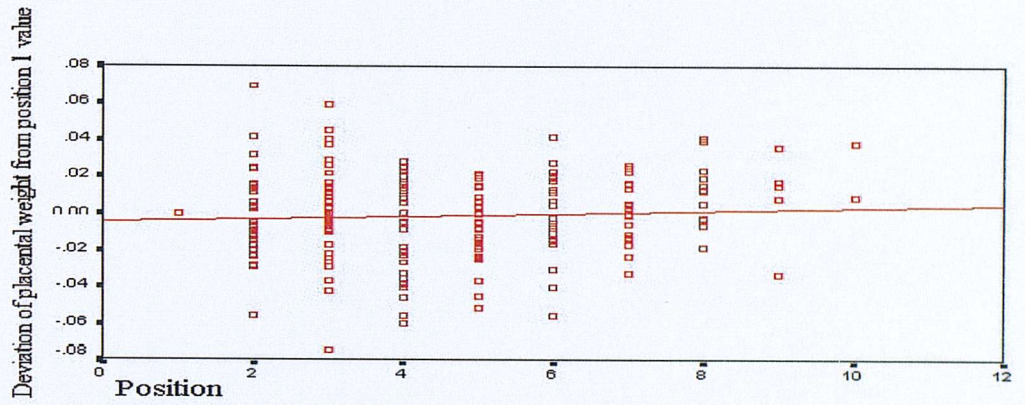


Figure 42: Deviation in placenta weight from mothers fed control diet up to 17 days gestation in relation to position in uterine horn (1, ovarian end; 12, cervical end). P = 0.328 N =253

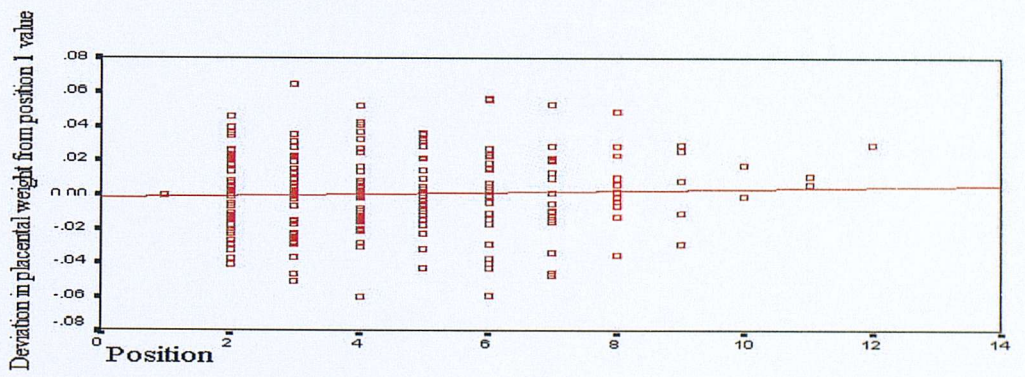


Figure 43: Deviation in placenta weight from mothers fed low protein diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.156 N = 245

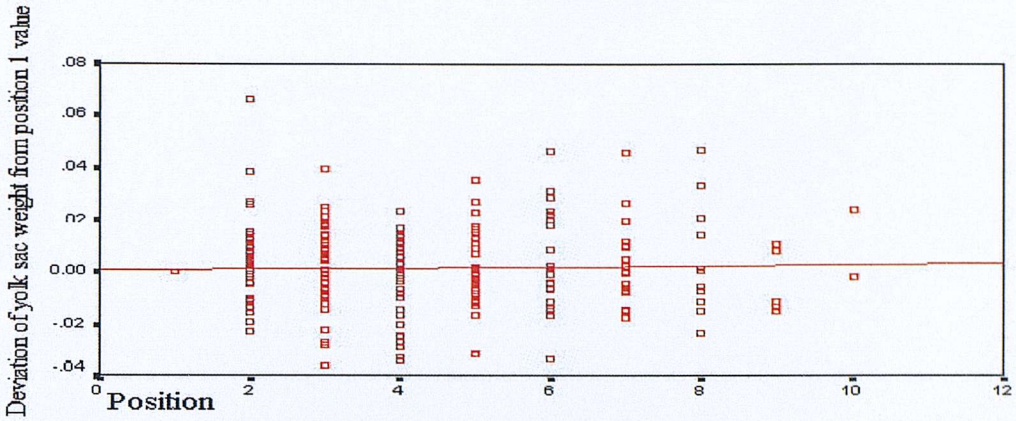


Figure 44: Deviation in visceral yolk sac weight from mothers fed control diet up to 17 days gestation in relation to position in uterine horn (1, ovarian end; 12, cervical end). P = 0.184 N = 253

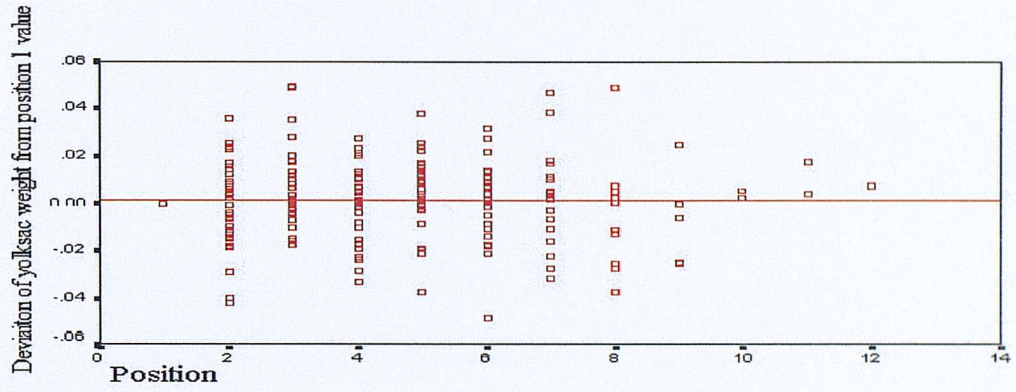


Figure 45: Deviation in visceral yolk sac weight from mothers fed low protein diet up to 17 days gestation in relation to position in uterine horn (1, ovarian end; 12, cervical end). P = 0.483 N = 245

The effect of conceptus position within the uterine horn on the total protein content in visceral yolk sacs was also assessed at all three time points studied (Figures 46-51). Overall, conceptus position had no effect on visceral yolk sac total protein content with one exception; visceral yolk sacs from mothers fed a control diet up to day 17 gestation show a statistically significant (P = 0.043, Figure 50) increase in total protein content as position of the conceptus moves down the uterine horn from the ovarian to cervical end.

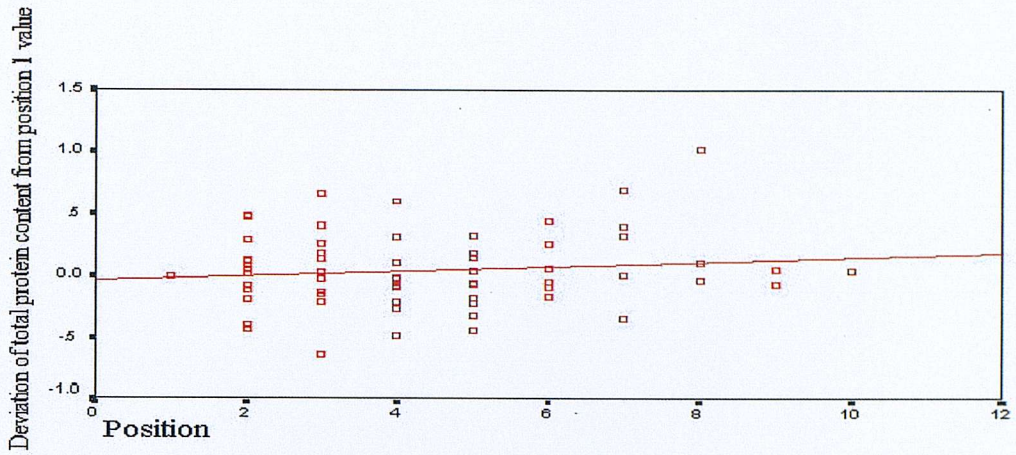


Figure 46: Deviation in total protein content of visceral yolk sacs from mothers fed control diet up to 12 days gestation in relation to position in uterine horn (1, ovarian end; 12,cervical end). P = 0.859 N = 78

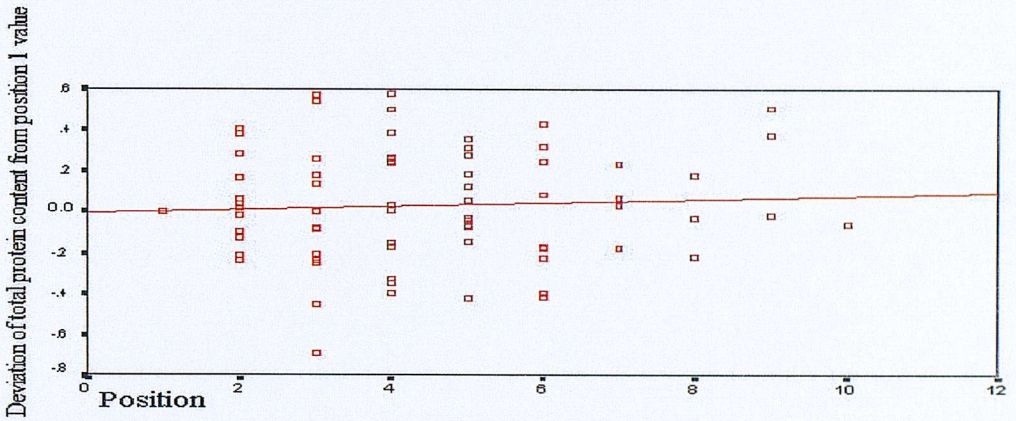


Figure 47: Deviation in total protein content of visceral yolk sacs from mothers fed low protein diet up to 12 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.497 N = 88

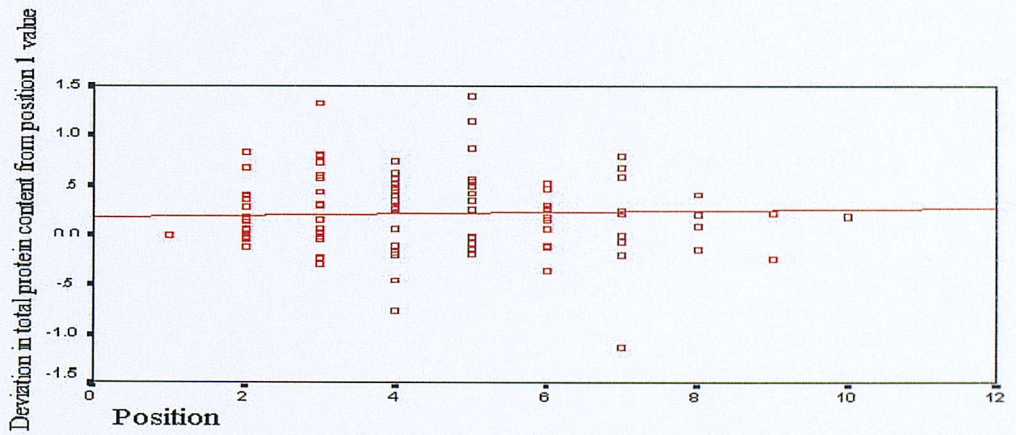


Figure 48: Deviation in total protein content of visceral yolk sacs from mothers fed control diet up to 14 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.781 N = 117

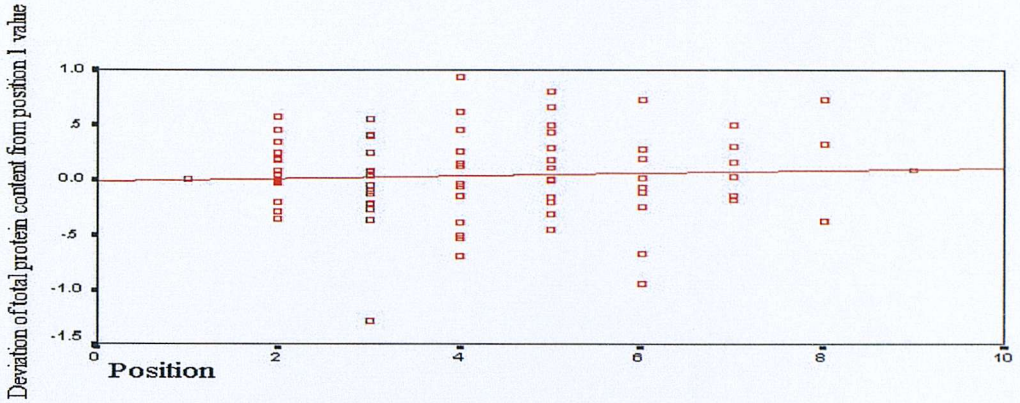


Figure 49: Deviation in total protein content of visceral yolk sacs from mothers fed low protein diet up to 14 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.627 N = 94

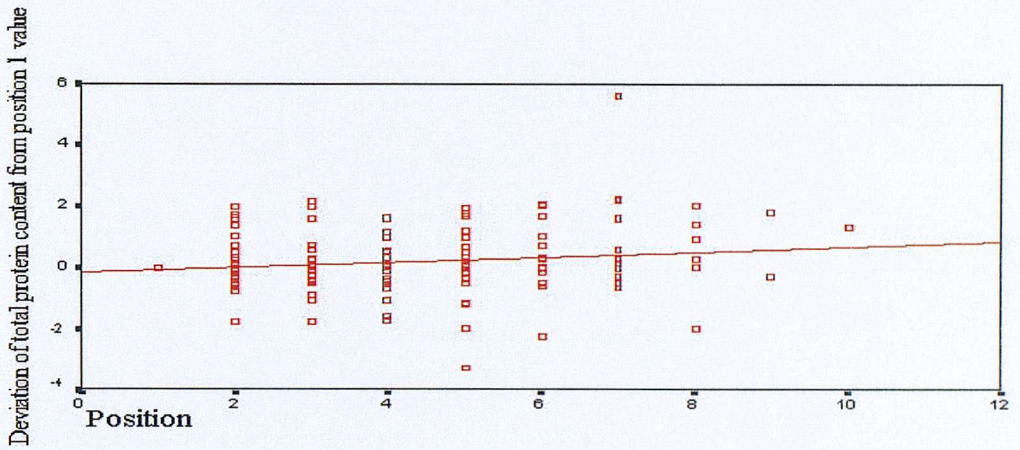


Figure 50: Deviation in total protein content of visceral yolk sacs from mothers fed control diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12, cervical end). P = 0.043 N = 152

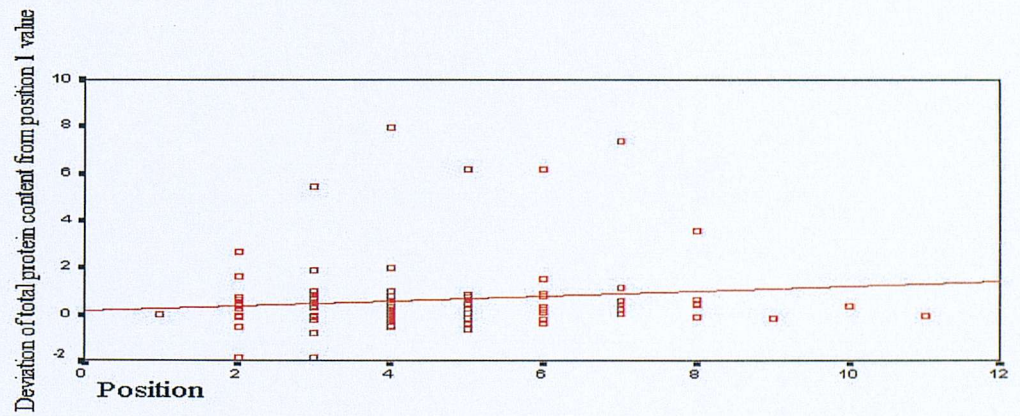


Figure 51: Deviation in total protein content visceral yolk sacs from mothers fed low protein diet up to 17 days gestation in relation to position in uterine horn (1,ovarian end; 12,cervical end). P = 0.250 N = 103

3.4 Discussion:

In this chapter, I have investigated the effect of maternal low protein diet on litter size, conceptus weight and that of its component parts, and on visceral yolk sac total protein content at three stages of fetal development. The analysis has also included the effect of conceptus position within the uterine horn on these parameters. Overall, maternal diet had little effect.

Litter size has been a factor of consideration in past studies investigating dietary effects in rodents. Langley-Evans et al (1996,b,c) in his numerous studies found no change in litter size in rats when fed a low protein diet, either when the mothers had been habituated to the diet before pregnancy and then fed the low protein diet throughout gestation, or when the diet was fed from the day of conception to the required time point in gestation (Langley-Evans et al 1996 b,c, Langley-Evans 2000). My study did not find any effect of maternal low protein diet on litter size at any of the three time points investigated.

My study has not shown a significant difference between the weight of any part of the conceptus and the conceptus as a whole in relation to diet at 12, 14 or 17 days gestation. Slight but insignificant differences are apparent, however, in that the components of the conceptus are marginally lower in weight in those mothers fed a low protein diet than those mothers on control diet. It is apparent in Figures 18-21 that as the weight of the conceptus increases so does the weight of individual parts. The correlation of the weight of each part of the conceptus with another part of the same conceptus was statistically significant in most instances except the weight correlation of the placenta and the visceral yolk sac at day 14 gestation; in both diet groups, these two weights were not significantly correlated. Also, at day 12 gestation in the group from mothers fed a control diet, there was no significant correlation. The predominant structure in the conceptus is the fetus and thus a significant correlation between their weights would be expected, as would the correlation between placental and fetal weight as they increase in weight through gestation.

In the current investigation fetal weight was unaffected by diet at any of the three

time points studied, as is clearly shown in Tables 5-7. Previous studies, predominately in rats, have shown that feeding a low protein diet throughout gestation can lead to reduced fetal weight and low birth weight in the offspring (Langley-Evans 2000, Langley-Evans et al 1996 b). My result is not an unexpected result because numerous other studies investigating the effect of diet have shown that an altered fetal weight is not always apparent. Langley-Evans et al (1996, b) fed rats on a low protein diet for 14 days prior to pregnancy and then throughout pregnancy up to day 20 (approximately equivalent to day 17 gestation in mouse) and found that fetal weight was significantly reduced. However, another study published by the same author in the same year showed that habituation to a low protein diet before pregnancy for 14 days and feeding the diet throughout gestation yielded increased fetal weight at day 18 and 20, but at term the offspring from mothers fed a low protein diet had a low birth weight in comparison to the controls (Langley-Evans et al 1996 b). A complex relationship appears to exist, therefore, between rodent diet and fetal growth rate at different periods of gestation.

In my experiments there was no significant change in the placental weight from mothers fed a low protein diet (9% casein) compared to those from a control diet (18% casein). Previous studies (Langley-Evans et al 1996 b) have shown that feeding a low protein diet (9% casein) to pregnant rats can cause an increase in placental weight in the conceptus, whilst feeding a more severe protein restricted diet (6% casein) causes a reduction in placental growth. It does not appear that the relationship between fetal, placental, and visceral yolk sac weight and litter size has been addressed previously. My study found no effect of litter size at day 12 and 14 gestation in either diet group, however, at day 17 gestation the weight of the conceptus was significantly affected.

This study investigated the total protein content of the visceral yolk sac at 3 time points during gestation, I found that total protein content was not affected by diet or litter size at 14 days gestation or 17 days. However, at day 12 gestation there was significantly more total protein in visceral yolk sacs from mothers fed a control diet. At this stage of gestation the placenta has only just been established and begun to function by providing the majority of nutrients, also the adsorptive

surface of the visceral yolk sac increases with gestational age therefore it may be expected that there is a reduction in the amount of total protein in the visceral yolk sac due to the reduced protein available from the mother (Beckman et al 1994).

There was no significant effect of position on the conceptus weight and its component parts at days 17 and 14 gestation found in my study. There was no significant bias of positioning of the conceptus to either uterine horn found in this study. However, at day 12 gestation the conceptus weight significantly increased as the position of the conceptus changed from the ovarian to cervical end of the uterine horn, the placental weight decreased when the positioning changed as for the conceptus. Previous studies have found differences; in particular heavier conceptuses were noted positioned nearer the ovarian and cervical ends of the uterine horn (Vom Saal and Dhar 1992). The effects of position identified in previous studies may be due to the steroids known to pass between adjacent fetuses during gestation. Testosterone and oestradiol, being passed through amniotic fluid across fetal membranes between fetuses, are known to impact upon the development of the fetus (Vom Saal and Dhar 1992).

There is a trend for increasing total protein content of the visceral yolk sac as the positioning progresses from the ovarian to cervical ends of the uterine horn. This effect of uterine position upon the total protein content of the visceral yolk sac suggests that the position of the conceptus within the uterine horn may influence protein synthesis rates within the developing fetus during gestation. Blood flow in the mouse uterus is bidirectional in the uterine loop artery and vein. Dependant on where the conceptus is positioned within the uterine horn, the direction of blood flow out of the placentae changes. Blood flows at a higher rate in the ovarian and cervical ends of the uterine horns as compared to the middle region (Vom Saal and Dhar 1992). The effect of position upon the weight of the conceptus and its component parts and the total protein content may relate to the maternal blood supply to the uterus during gestation.

Chapter 4

Effect of maternal diet on endocytic activity in the visceral yolk sac

4.1 Introduction:

The developing fetus must acquire nutrients throughout gestation in order to grow and mature properly. During the preimplantation stage of development, the embryo receives its nutrients from the uterine environment via absorption. Once the embryo has implanted, in mice this is during day 4 of gestation, the yolk sac begins to develop surrounding the embryo. The yolk sac takes on the role of providing all the nutrients, via endocytosis primarily, required by the embryo until the placenta develops from about day 10 and becomes functional in providing nutrients (Freeman et al 1981, Lloyd et al 1996). The visceral yolk sac continues to play an important role in providing the growing fetus with amino acids throughout gestation, after the placenta has begun to function (Pratten et al 1997).

Previous studies have investigated the effect of maternal undernutrition upon the placenta during different periods in gestation using the rat model. These studies have shown an increase in the size of the placenta in correlation with a low birth weight (Langley-Evans et al 1996). Studies have concluded that the capacity of the yolk sac to take up proteins and break them down to amino acids is far greater than that which is required by the yolk sac itself; it has, therefore, been suggested with suitable evidence that the yolk sac's capacity for providing amino acids is sufficient to meet the amino acid needs of the developing fetus (Beckman et al 1994, Lloyd et al 1996).

Quantification of fluid-phase endocytosis by the visceral yolk sac has been carried out in previous studies using mainly radiolabelled sucrose, insulin and polyvinylpyrrolidone (Freeman et al 1981). Accumulation of marker is directly proportional to the concentration of marker in the culture medium (Besterman and Low 1983). To measure the receptor-mediated form of endocytosis, radio-labeled

BSA and amino acids, primarily leucine and methionine (Beckman et al 1990 B, Lloyd et al 1996, Beckman 1997), have been used by different laboratories. These molecules are taken up heterogeneously via fluid phase and receptor-mediated endocytosis with the majority of the protein taken up through the latter mechanism (Williams et al 1975 A). BSA is endocytosed and broken down to amino acids before being released to the fetus via the vitelline circulatory system.

The experiments described here investigate the effect of a maternal low protein diet fed up to day 17 gestation upon the visceral yolk sac fluid-phase and receptor-mediated endocytic activity, thereby indicating if environmental undernutrition conditions have an effect upon the developing fetus through the visceral yolk sac activity during gestation.

^{14}C-Sucrose was used as the fluid-phase uptake marker because there is no sucrase in the visceral yolk sac, therefore, it can be reasonably assumed that sucrose will not be broken down once it has entered the visceral yolk sac (Weisbecker et al 1983). Thus, the endocytic index results will be representative of all the sucrose that is taken up, and will not be affected by breakdown and release of this molecule (Besterman and Low 1983). ^{125}I-BSA was used as a radiolabelled marker to quantify receptor-mediated endocytosis in the visceral yolk sac at 14 and 17 days gestation. ^{125}Iodotyrosine, the break down product of digestion of BSA cannot be used for protein synthesis and is, therefore, not used by the visceral yolk sac during culture and is a definitive measure of the amount of BSA digested and released (Freeman et al 1981, Lloyd et al 1975).

4.2 Methods

Mice were mated overnight, the presence of a vaginal plug in the morning designated pregnancy and day 0 (Section 2.1). Pregnant female MF1 mice were fed either a control diet (18% casein) or a low protein diet (9% casein) throughout pregnancy up to either 12, 14 or 17 days gestation (Section 2.3). Visceral yolk sacs were dissected out and weighed (Section 2.3), then placed in 25 ml flasks with Medium 199 and the appropriate radiolabelled marker (section 2.4.1 and

2.4.2). after 5 hours culture visceral yolk sacs were removed washed in PBS and digested overnight (Section 2.4.1). Total protein content and radioactivity levels were assayed (Section 2.4.1 and 2.4.2). the endocytic index values were calculated using the equation from Williams et al (1975) (Section 2.4.1 and 2.4.2).

4.3 Results

4.3.1.1 Fluid-phase Endocytosis.

In initial experiments, a time course of ^{14}C-Sucrose uptake was undertaken using two yolk sacs from each of two mothers (N = 4 for each time point) at 17 days gestation for hourly intervals up to 5 hours incubation (Figure 52). The results show a linear increase in sucrose uptake rate with respect to time.

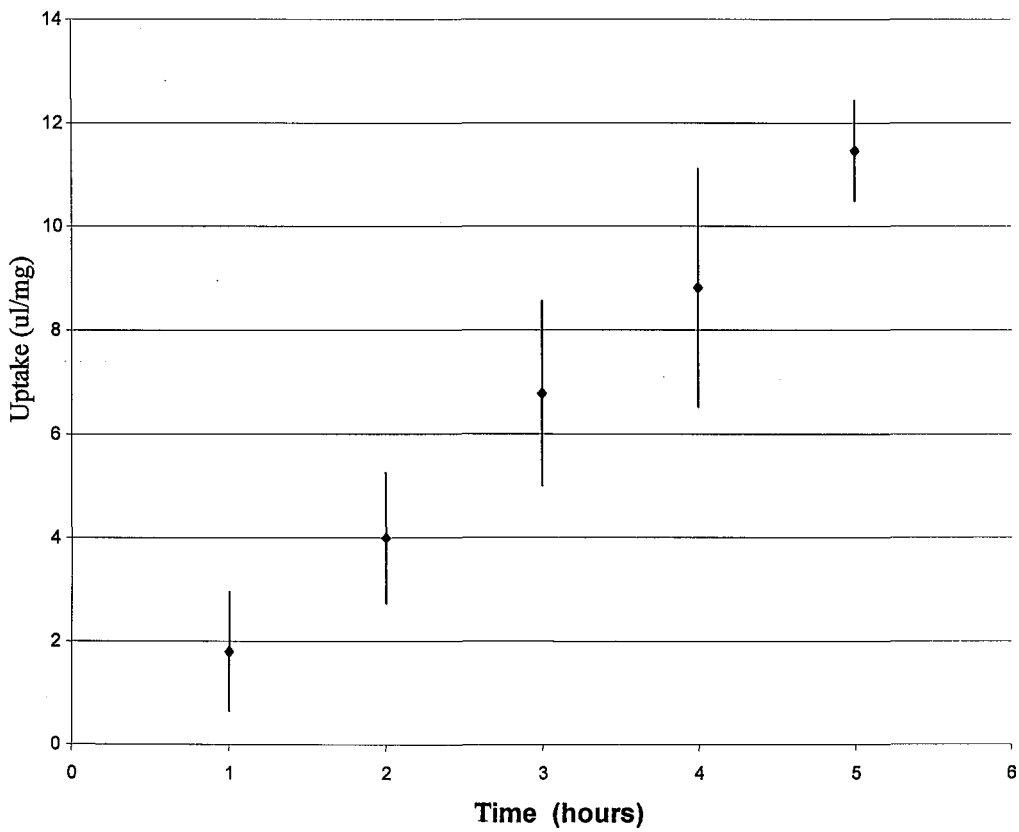


Figure 52: Time course for ^{14}C-Sucrose fluid phase endocytosis in yolk sacs from mothers fed a control diet up to 17 days gestation

Endocytic Index is widely accepted as a unit representative of clearance rates of pinocytic uptake in experiments. It is defined as the volume of culture medium whose substrate content has been captured by a defined amount of living tissue in a given time (Lloyd, J.B.). The endocytic index (EI) of visceral yolk sacs grouped according to mothers and comparing the two diets at different time points of gestation are shown in Figures 53-55. The numbers on the X axis are arbitrary numbers indicating the reference number for the mother from which the visceral yolk sacs were derived. The star indicates the mean of the visceral yolk sacs EI for each mother and the dashed line shows the mean of all the values for either diet. These Figures illustrate the influence of maternal origin on visceral yolk sac endocytic activity and hence the need to assess 'within mother' effects when applying statistical analysis.

This study revealed a significant increase ($P = 0.012$) in the mean endocytic index values of visceral yolk sacs from mothers fed a low protein diet (9%) compared with those fed a control diet (18%) at day 17 of gestation (Figure 55). However, this difference in visceral yolk sac endocytic index values between dietary groups is not evident at day 12 and day 14 of gestation ($P=0.23$ and $P=0.66$ respectively) (Figures 53 & 54).

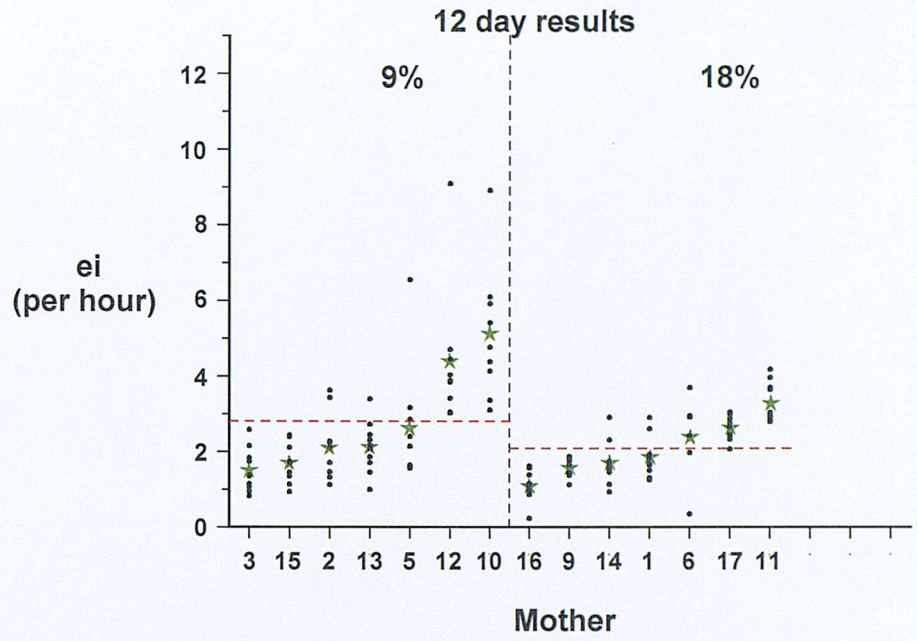


Figure 523: The endocytic index of ^{14}C-Sucrose endocytosis by visceral yolk sacs at day 12 gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

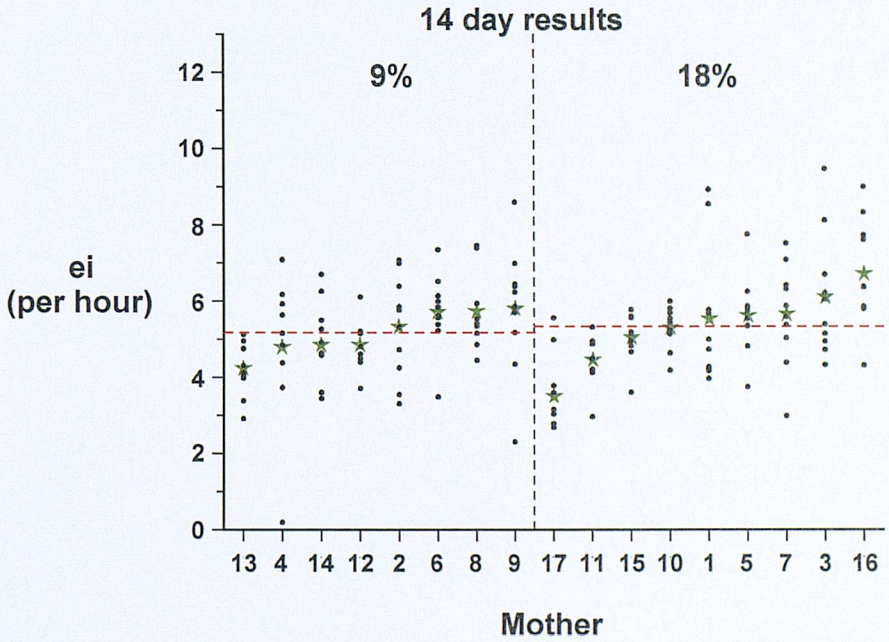


Figure 534: The endocytic index of ^{14}C-Sucrose endocytosis by visceral yolk sacs at day 14 gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

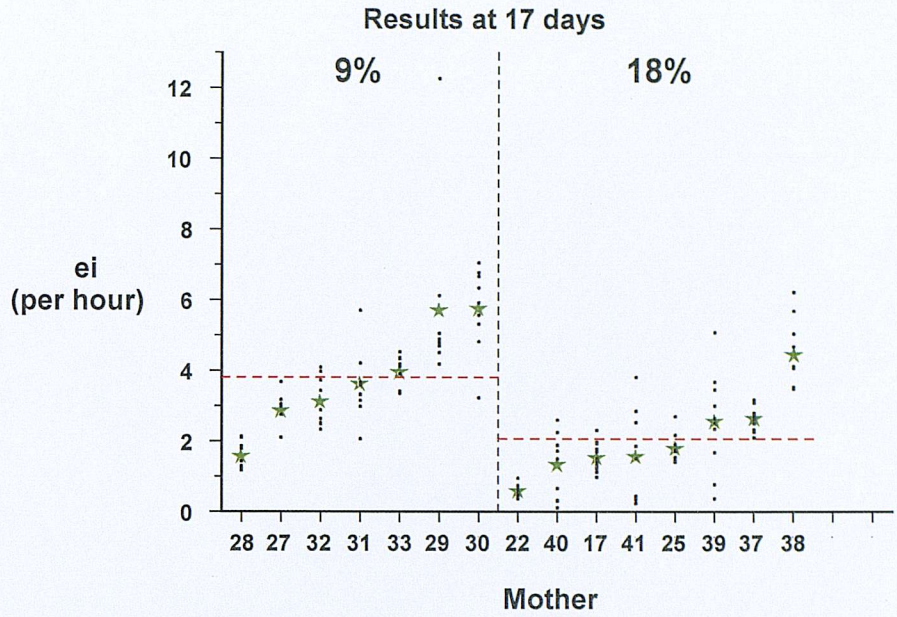


Figure 55: The endocytic index of ^{14}C-Sucrose endocytosis by visceral yolk sacs at day 17 gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

4.3.1.2 Effect of litter size and position

The effect of litter size and the position of the visceral yolk sac within the uterine horn upon the ^{14}C-Sucrose endocytic index at each of the three time points studied was investigated. The EI values were found not to be affected by either parameter.

4.3.2 Receptor Mediated endocytosis

The time course of ^{125}I-BSA uptake and release by visceral yolk sacs recovered from day 17 control animals was assessed over 1-24 hours. For each time point (n=4), four visceral yolk sacs from a total of three mice were assessed. The mean EI ± SEM for ^{125}I-BSA uptake and I-tyrosine release over the time course are shown in Figure 56.

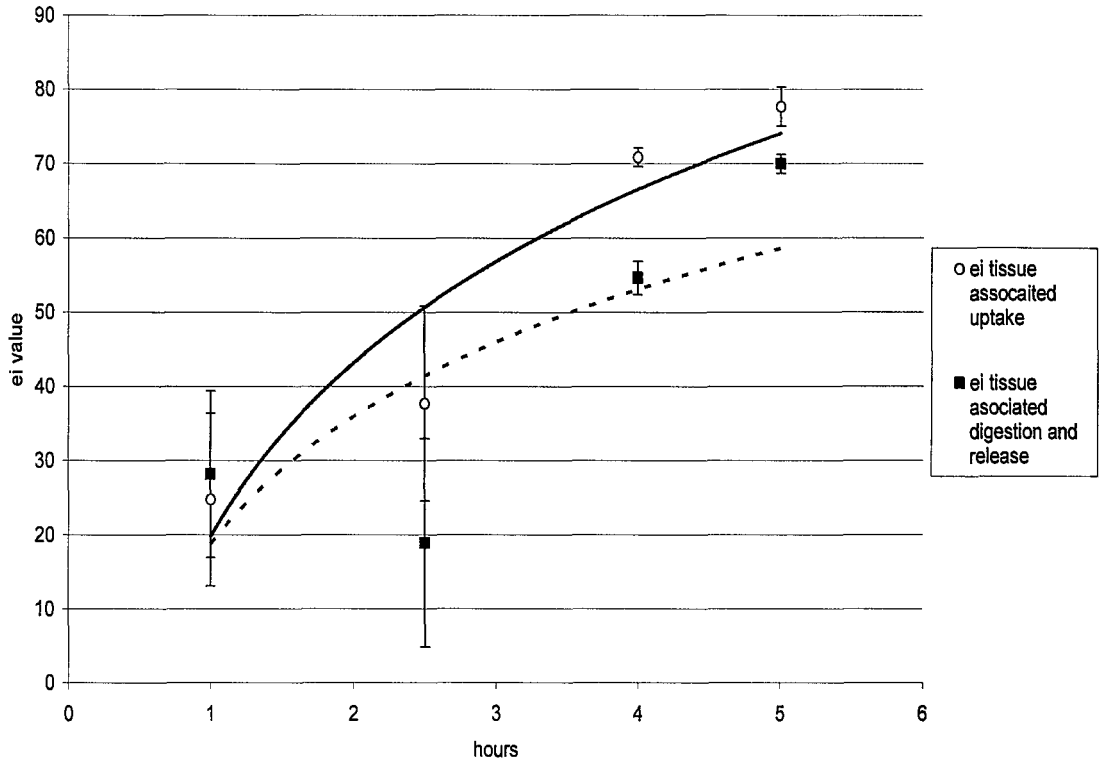


Figure 56: Time course for receptor-mediated uptake and release of ^{125}I-BSA in visceral yolk sacs from mothers fed a control diet up to day 17 gestation

4.3.2.1 Endocytic uptake rate:

The endocytic index values for uptake of ^{125}I-BSA in visceral yolk sacs from mothers fed either a low protein diet (9% casein) or a control diet (18% casein) at 17 days gestation are shown in Figure 57. The graph is presented as those for the fluid–phase endocytosis experiments (Section 4.3). Thus, each dot is a yolk sac and they are grouped within each mother, the star indicates the mean of the visceral yolk sacs within the mother and the dotted line indicates the mean of all the visceral yolk sacs in the diet group. The majority of the mothers produce visceral yolk sacs with very similar endocytic index values, this emphasizes the need to incorporate the mother-effect into the statistical analysis. The x axis is arbitrary, merely denoting the mother from which the visceral yolk sacs were derived. Each graph shows one to two mothers that appear to have visceral yolk sacs that have markedly different endocytic index values than the other mothers within the group; this may be due to partial denaturing of the I-BSA or a variation in batches of I-BSA that were made.

The results show that at day 17 of gestation, the endocytic index of ^{125}I-BSA uptake in the low protein diet group is significantly lower (P= 0.038 n=128) by almost 50% than that of the control diet group (Figure 57). At 14 days gestation there is no significant difference (P=0.607 n=85) between the endocytic index value for ^{125}I-BSA uptake by visceral yolk sacs from mothers fed a low protein diet compared with those from mothers fed a control diet (Figure 58).

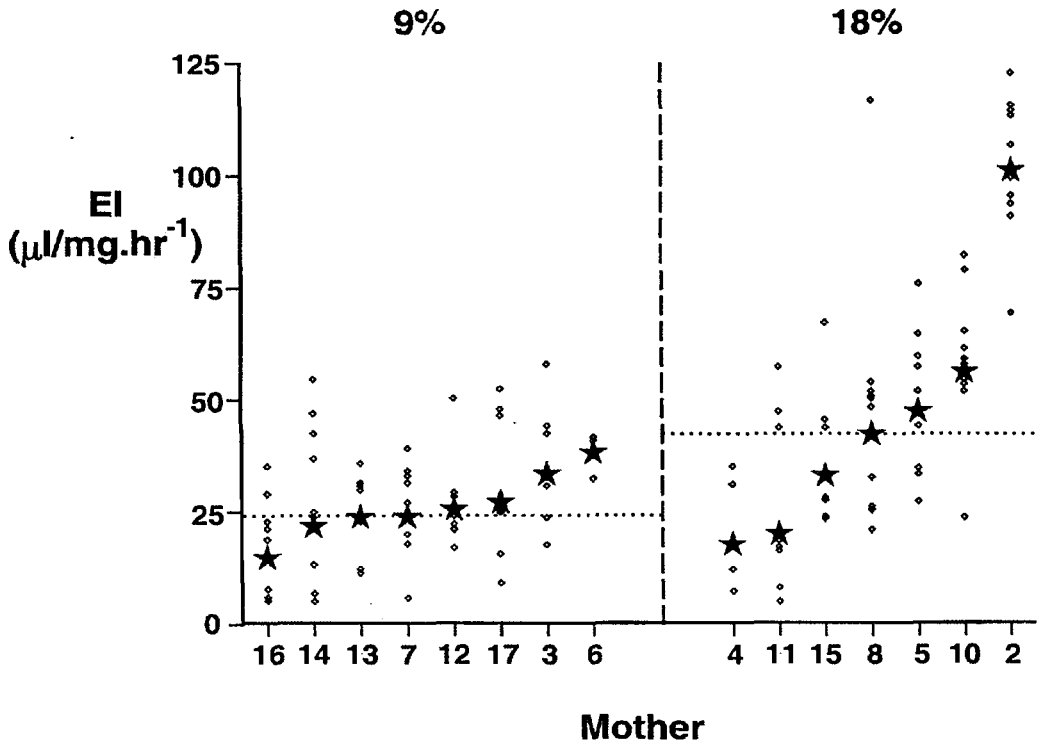


Figure 547: Graph showing Endocytic index values for uptake of ^{125}I-BSA at 17 day. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

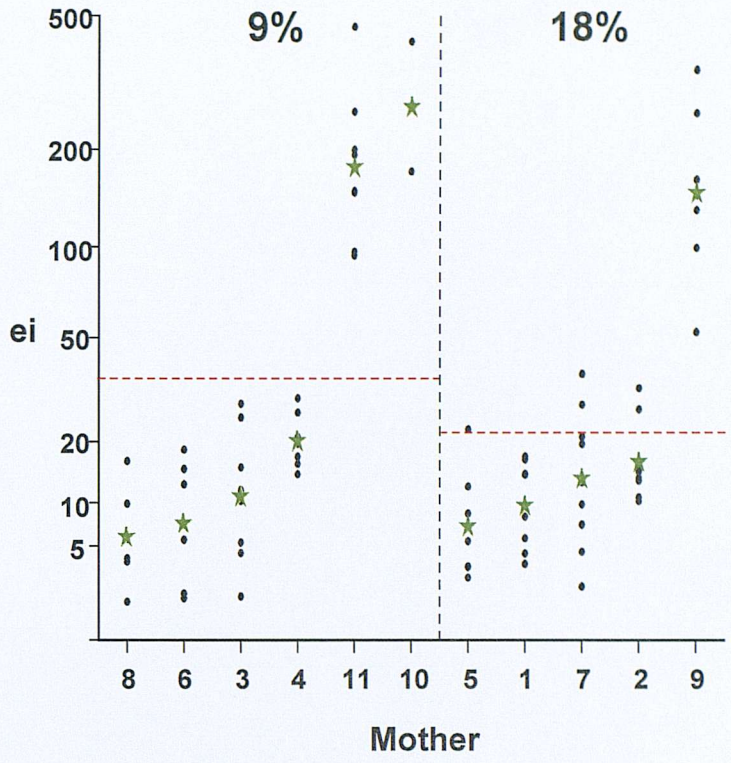


Figure 58: Endocytic index uptake rate at 14 days gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

4.3.2.2 Endocytic release rate:

Release of radiolabelled Iodo-tyrosine from the yolk sac after the ^{125}I-BSA has been digested was assessed at 14 and 17 days gestation (Figure 59, 60). A significant (P=0.019, n=128, Figure 60) decrease in the release of iodo-tyrosine was evident in the visceral yolk sacs from mothers fed a low protein diet up to 17 days gestation, while no difference in release was found at 14 days (P = 0.549, n = 85, Figure 59)

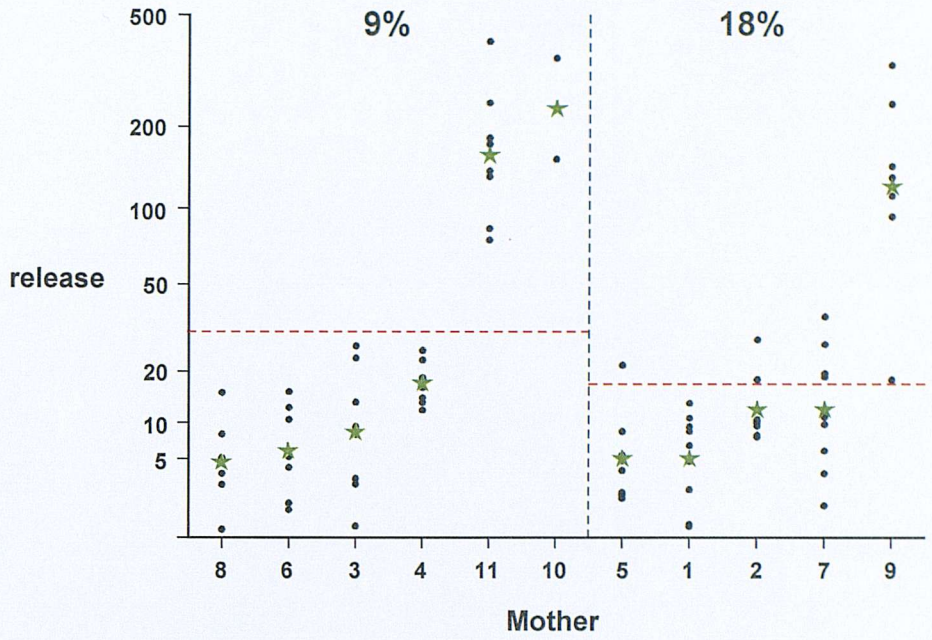


Figure 559: Endocytic index release rate of iodo-tyrosine at day 14 gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

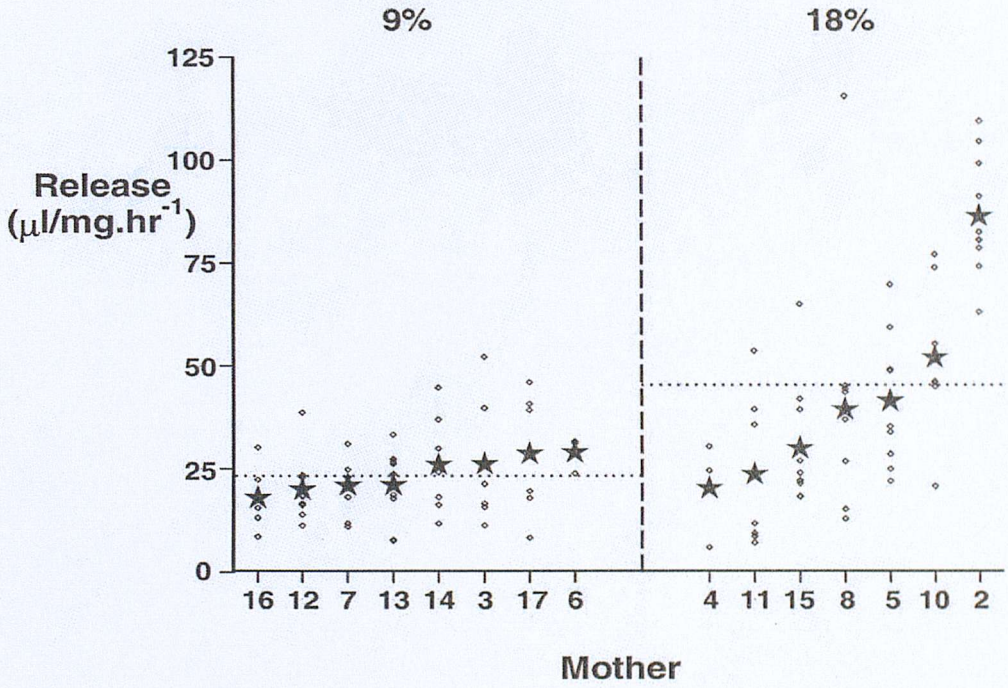


Figure 6056: Endocytic index release rate of iodo-tyrosine at 17 days gestation. ei = endocytic index. Dots are individual visceral yolk sacs, stars are the mean of the visceral yolk sac EI for that mother and the dashed line is the mean for the EI of all the visceral yolk sacs within the diet group.

4.4 Discussion

My study found a significant increase in fluid-phase endocytosis of ^{14}C-Sucrose in visceral yolk sacs from mothers fed a low protein diet up to day 17 gestation. At 12 days gestation there was a slight increase in the fluid-phase endocytic rate in visceral yolk sacs from the low protein diet group, although this was not significant; at 14 days gestation the rate of fluid-phase endocytosis was approximately equal in visceral yolk sacs from both diet treatments. Position within the uterus does not significantly affect the endocytic activity of the yolk sac measured by endocytic index at 12, 14 or 17 days gestation. Also litter size had no significant effect upon the endocytic index at any of the time points studied. My investigation of how maternal low protein diet affects receptor-mediated endocytosis shows that the rate of receptor-mediated uptake and release are significantly reduced in visceral yolk sacs from mothers fed a low protein diet up to day 17 gestation. At 14 days gestation the receptor-mediated endocytosis uptake and release rates were higher, although not significantly, in the group of visceral yolk sacs from the low protein diet group compared to the control group.

The uptake of ^{14}C-Sucrose by the visceral yolk sac is through fluid-phase non-adsorptive endocytosis (Freeman et al 1981). Roberts et al (1977) used three different radiolabelled markers to study pinocytosis in the rat yolk sac at day 17.5 gestation, one of which was ^{14}C-Sucrose. They fed a normal chow diet and cultured the yolk sacs taken from one mother in Medium 199 with 10% FCS. They reported similar endocytic index values in their paper to those found in mouse visceral yolk sacs from mothers fed the control (18%) diet in the experiments reported here. Roberts et al (1977) demonstrated a low rate of substrate release of ^{14}C-Sucrose when visceral yolk sacs were reincubated in tracer-free medium after primary incubation in Medium containing ^{14}C-Sucrose. This experiment showed that sucrose is taken up by pinocytic ingestion as opposed to reversible surface adsorption (Roberts et al 1977).

Beckman et al (1994) studied endocytic rates in rat yolk sacs at various stages throughout gestation. They found a decrease in the fluid-phase endocytic activity as gestation progressed, this was not found in my study. At 12 days gestation and

17 days the endocytic activity is approximately the same, at 14 days it is much higher in the visceral yolk sacs from the control diet, the visceral yolk sacs from a low protein diet have a slightly higher level of endocytic activity. This may suggest that maternal low protein diet induces maintenance of mid-gestation endocytic uptake rate through to late gestation, thereby attempting to compensate for the reduced amount of protein available to the fetus from the uterine environment.

Albumin is ingested by the visceral yolk sac through receptor-mediated endocytosis. Livesey et al (1979) demonstrated that ^{125}I-BSA is taken up by the visceral yolk sac and undergoes intralysosomal hydrolysis. The experiments reported here are in agreement with this earlier study, ^{125}I-BSA was taken up via receptor-mediated endocytosis and degraded and then released as acid soluble Iodo-tyrosine, Lloyd et al (1975) showed this when they cultured rat yolk sacs at day 17.5 gestation in ^{125}I-labelled albumin.

There is no progressive accumulation of the ^{125}I-BSA in the visceral yolk sac tissue and there is a steady increase in the amount of radioactivity in the culture medium, therefore a balance is established between uptake and release (Lloyd et al 1975, Lloyd 1990). ^{125}I-BSA breaks down to ^{125}Iodo-tyrosine, this is not used for protein synthesis by either the fetus or the visceral yolk sac as it cannot be incorporated into newly formed protein so provides a good marker for the release rate of the visceral yolk sac (Lloyd et al 1975, Freeman et al 1981, McArdle and Priscott 1984).

Livesey et al (1979) showed that after the initial first hour of incubation in medium containing radiolabelled tracer, the radioactivity associated with the visceral yolk sac remained constant, uptake equaling release. This is also seen in the data presented here; at day 17 of gestation after the 5 hour incubation period the rate of uptake and the rate of release are the same within the specific diet group. At 14 days gestation the release rate in the visceral yolk sacs from mothers fed a low protein diet was found to be slightly less than the uptake rate.

Maternal low protein diet does not alter the overall wet weight of the visceral yolk

sac as originally hypothesized, however, it does appear to increase fluid-phase endocytosis possibly in a compensatory reaction to the low levels of protein available from the uterine environment. Maternal low protein diet also affects receptor-mediated endocytosis rates of uptake and release, however, unlike the effect on fluid-phase endocytosis, receptor-mediated endocytosis rates are reduced. This may indicate a change in expression levels of receptors at the plasma membrane of the visceral endoderm cells, or changes in the lysosomal activity within the visceral endoderm cells. Both receptor-mediated uptake and release rates are affected by maternal low protein diet, thus changes to the receptor expression levels and the lysosomal activity may occur in unison. The changes in fluid-phase endocytosis and receptor-mediated endocytosis maybe a factor of the complexity of endocytosis within a cell to control entry and exit from the cell, which help to control the cellular response to its environment (Conner and Schmidt 2003).

Chapter 5

Effect of maternal diet on visceral yolk sac ultrastructure

5.1 Introduction

The ultrastructure of the visceral yolk sac in laboratory animals, mainly rats, mice and guinea pigs, has been studied, at various gestational stages over the past 40 years (Sorokin and Padykula 1964, Calarco and Moyer 1966, Clark et al 1975, Jollie 1984). The fine structure of the visceral yolk sac was found to be similar in most rodents unlike the parietal endoderm and the placenta which have differences between species (Jollie 1990).

The visceral yolk sac, in mice, has an important role in supplying nutrients to the developing fetus throughout gestation (Jollie 1984). Nutrients are taken up through endocytosis in the cells of the visceral yolk sac and degraded before being transported to the fetus via the vitelline circulation (Beckman et al 1990 A, Lloyd et al 1998).

The aim of the experiments in this chapter was to ascertain if the changes seen in the endocytic activity and reported in chapter 4 are supported by identifiable changes in the visceral yolk sac morphology.

5.2 Methods

Pregnant mice were fed either a low protein diet (9%) or a control diet (18%) from day 0 of gestation to day 17 (section 2.1). The yolk sac tissue was dissected away from the conceptus (section 2.3) and 1 mm sections of visceral yolk sac tissue were taken:

a) from the highly villous region near to where the placenta joins the visceral yolk sac (Figure 16).

b) from the less villous regions (Figure 16).

Tissues were fixed and processed for transmission electron microscopy as in

section 2.5. All the yolk sacs used were taken from a conceptus in the same position of the uterus in each dam (4^{th} from the ovarian end in the right horn, Figure 37, Chapter 3) in order to prevent any positional effect which may have been present in the uterus (n = 15). Micrographs at x 2K magnification were used to quantify the number of specific vesicles (macropinocytic vesicles and secondary lysosomes), the total area of the cell, the perimeter and the apical length of the cell using IBAS (Section 2.5). Only cells that had a clear basement membrane, nucleus and apical microvillous border were used in the quantitation analysis.

5.3 Results

Micrographs of the visceral yolk sac at Day 17 of gestation are presented in Figures 63-74. Figures 64, 65, 67, 68, 70, 71, 73 and 74 have annotations labeling the internal structures of the mouse visceral yolk sac at day 17 gestation (Jollie 1990, Calarco and Moyer 1966). Facing the uterine environment at this stage of gestation, the visceral yolk sac has numerous microvilli to capture nutrients, in particular protein (Figure 70). The microvilli are at the apical extremity of the visceral endoderm cells and lie upon a visceral basement membrane. Beneath this is the mesenchymal layer which surrounds and forms the vitelline vessels. This mesenchymal layer overlies the serosal basement membrane and the mesothelium (Figure 64) (Sorokin and Padykula 1964, Novak and Betteridge 2000). Within the endodermal cells there is a nucleus, several mitochondria, rough endoplasmic reticulum, lysosomes, an apical canaliculi system and storage vacuoles (Figure 67) (Calarco and Moyer 1966).

My investigation measured the surface area, the cell membrane perimeter and the apical cell membrane length of the visceral yolk sac endoderm cells individually; I also measured the number of macropinocytic vesicles (translucent vesicles apically situated) per cell and the number of secondary lysosomes (dense black vesicles basally located, labeled phagolysosomes). Organelles with this morphology and recognized in my micrographs have been shown previously to accumulate sequentially endocytic tracers at the transmission electron microscope level (Sorokin and Padykula 1964, Calarco and Moyer 1966, Jollie 1990). Tables

13-15 show the mean and SEM of the surface area, perimeter length and apical length of the visceral yolk sac cells quantified. The Tables also show the effect of diet upon the surface area, perimeter and apical length. In neither region, villous or less villous, did maternal low protein diet compared to control diet have a significant effect upon the surface area, perimeter and apical length.

	Diet	Mean	SEM	P = value	Number of cells	Number of visceral yolk sacs
Villous region	18%	724.35	48.68	0.465	39	8
	9%	956.85	89.02		25	7
Less Villous region	18%	487.76	31.12	0.187	17	7
	9%	433.07	23.46		34	6

Table 13: Effect of diet upon the surface area of visceral endoderm cells. Measurements are in pixels

	Diet	Mean	SEM	P = value	Number of cells	Number of visceral yolk sacs
Villous region	18%	141.65	10.36	0.347	39	8
	9%	207.06	18.66		25	7
Less Villous region	18%	116.95	12.15	0.283	17	7
	9%	129.12	7.83		34	6

Table 144: Effect of diet upon the perimeter length of the cell membrane of visceral endoderm cells. Measurements are in pixels.

	Diet	Mean	SEM	P = value	Number of cells	Number of visceral yolk sacs
Villous region	18%	42.43	2.39	0.461	39	8
	9%	45.01	3.42		25	7
Less Villous region	18%	32.76	2.47	0.710	17	7
	9%	33.52	1.56		34	6

Table 15: Effect of diet upon the apical membrane length of visceral endoderm cells. Measurements are in pixels.

Overall as surface area, membrane perimeter and apical membrane length increased the number of macropinocytic vesicles and the number of secondary lysosomes increased significantly ($P < 0.000$) in visceral yolk sacs from both diet groups, irrespective of the region under scrutiny being villous or less villous. The micrographs (Figures 63-68) of the visceral yolk sacs from mothers fed a control diet (18% casein) show a greater number of secondary lysosomes, and fewer macropinocytic vesicles than the micrographs (Figures 69-74) displaying the visceral yolk sac tissue sections from mothers fed a low protein diet (9% casein). Statistical analysis showed that the number of macropinocytic vesicles per cell and secondary lysosomes per cell in the villous regions was not significantly affected by either diet ($P = 0.428$, and $P = 0.225$, Figures 61 and 62). Maternal low protein diet significantly ($P = 0.033$, Figure 61) increased the number of macropinocytic vesicles in the less villous region of the visceral yolk sac compared with the less villous region of visceral yolk sacs from control diet and significantly decreased the number of secondary lysosomes per cell ($P = 0.041$, Figure 62) in this same area.

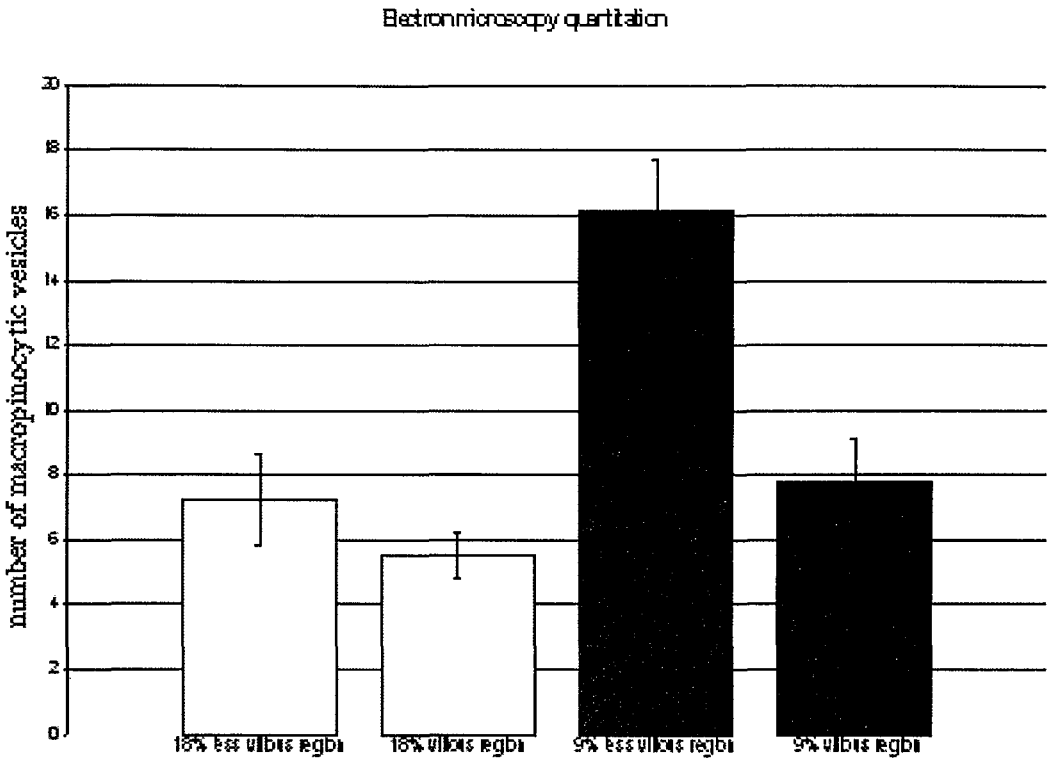


Figure 61: Mean number of macropinocytic vesicles per cell in visceral yolk sac endoderm cells from mothers fed either a low protein diet (9%) or control diet (18%).

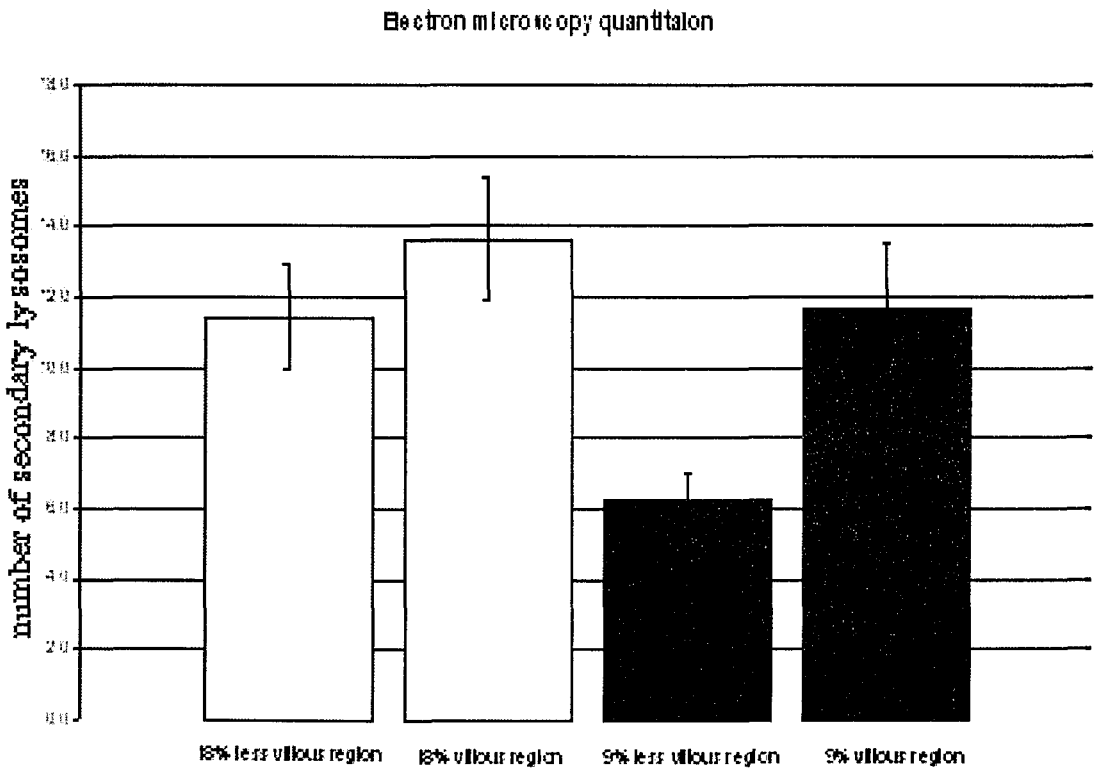


Figure 62: Mean number of secondary lysosomes per cell in visceral yolk sac endoderm cells from mothers fed either a low protein diet (9%) or control diet (18%).

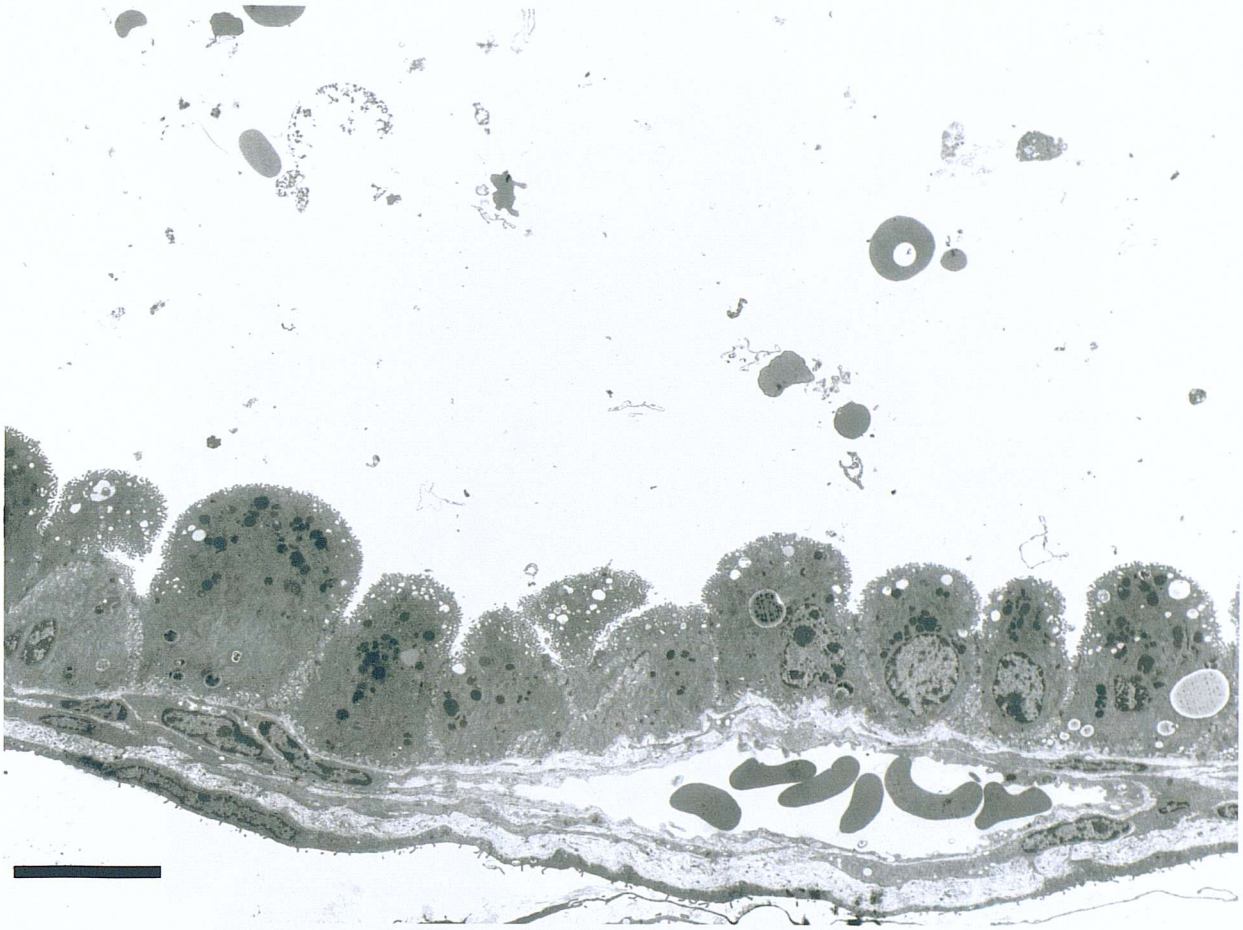


Figure 63: Mouse visceral yolk sac less villous region, from a mother fed control diet (18% casein) up to Day 17 gestation.
scalebar = 10 microns

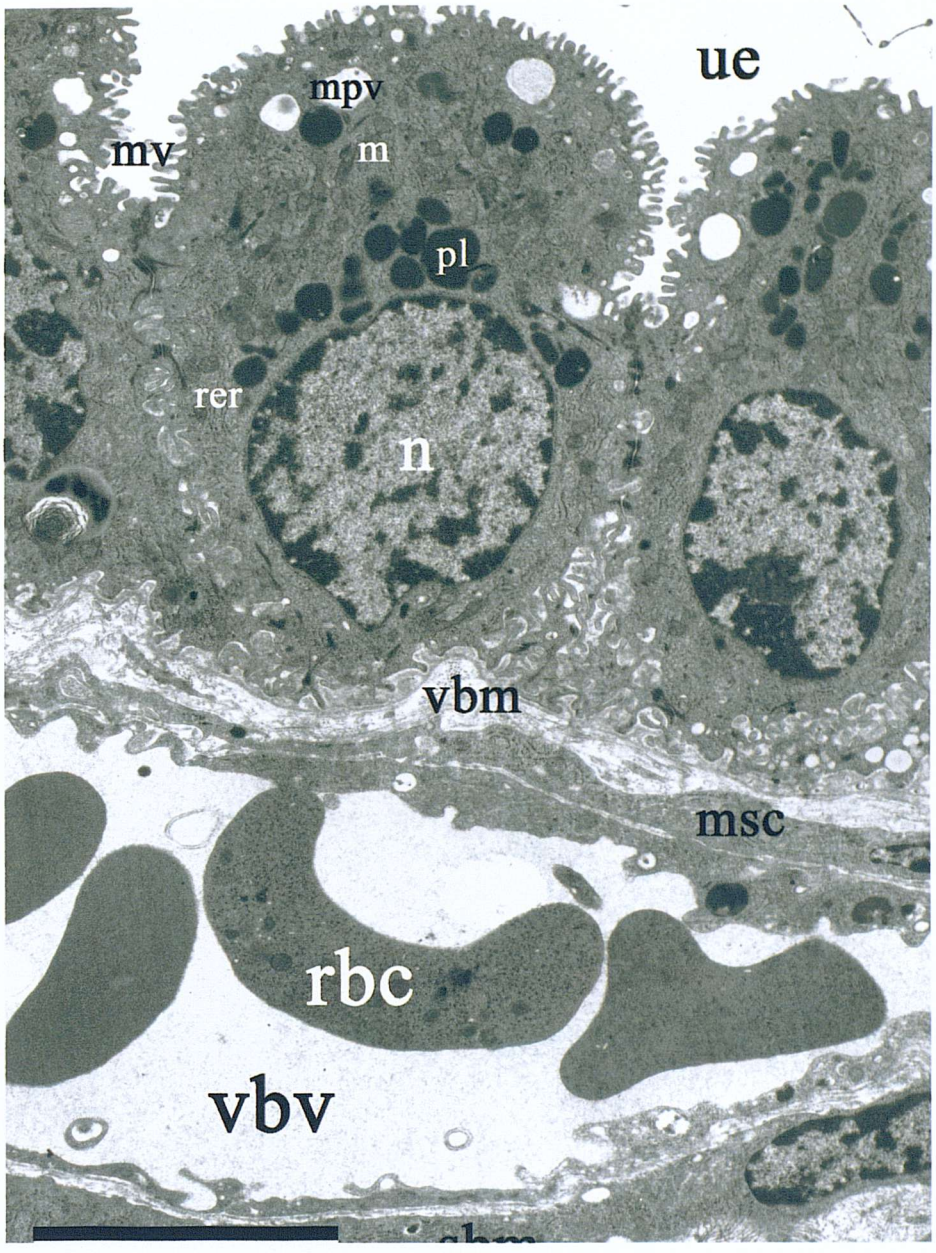


Figure 64: Mouse visceral yolk sac less villous region, from a mother fed control diet (18% casein) up to day 17 gestation.
scalebar = 5 microns
vbm = visceral basement membrane, vbv = vitelline blood vessel, rbc = red blood cell, n = nucleus, m = mitochondria, mpv = macropinocytic vesicle, msc = mesenchyme, mtm = mesothelium, mv = microvilli, pl = phagolysosome, rer = rough endoplasmic reticulum, sbm = serosal basement membrane, ue = uterine epithelium.

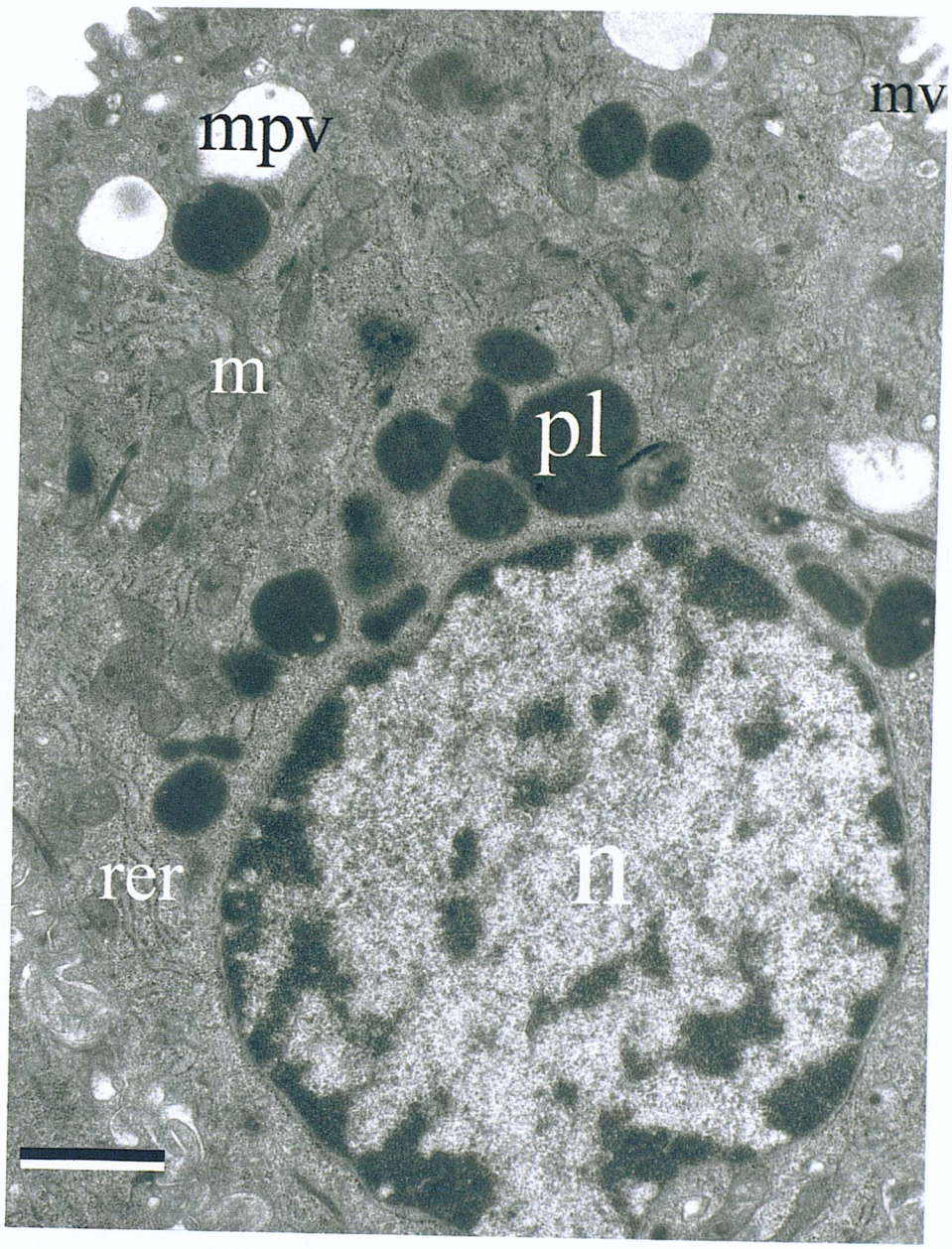


Figure 65: Mouse visceral yolk sac from a mother fed control diet (18% casein) up to Day 17 gestation.
scalebar = 1 micron
m= mitochodria, mv = microvilli, mpv = macropinocytic vesicle, n = nucleus, pl = phagolysosome, rer = rough endoplasmic reticulum.

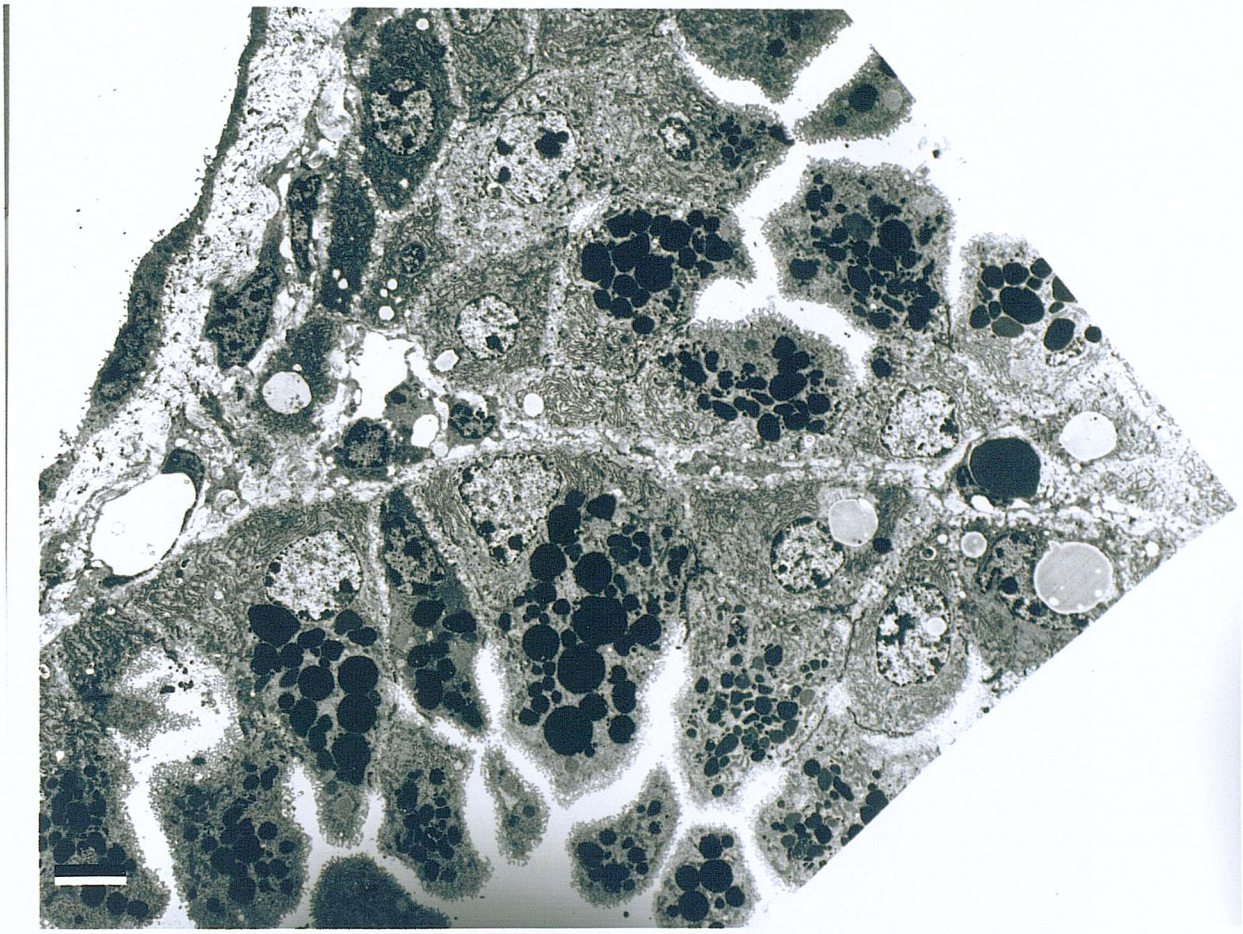


Figure 66: Mouse visceral yolk sac villous region from a mother fed control diet (18% casein) up to Day 17 gestation.
Scalebar = 10 microns

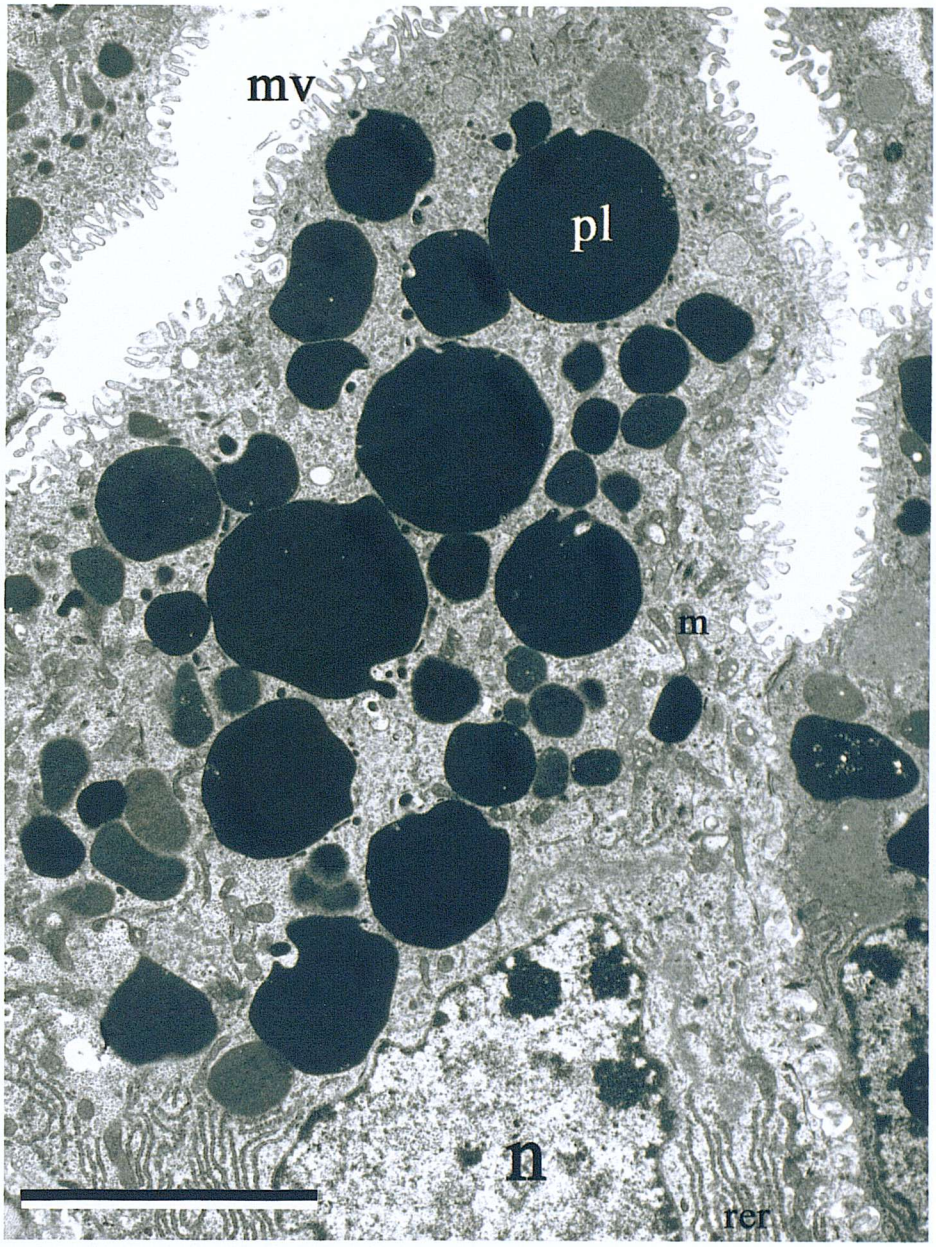


Figure 67: Mouse visceral yolk sac villous region from a mother fed control diet (18% casein) up to Day 17 gestation.
Scalebar = 5 microns
m = mitochondria, mv = microvilli, n = nucleus, pl = phagolysosomes, rer = rough endoplasmic reticulum.

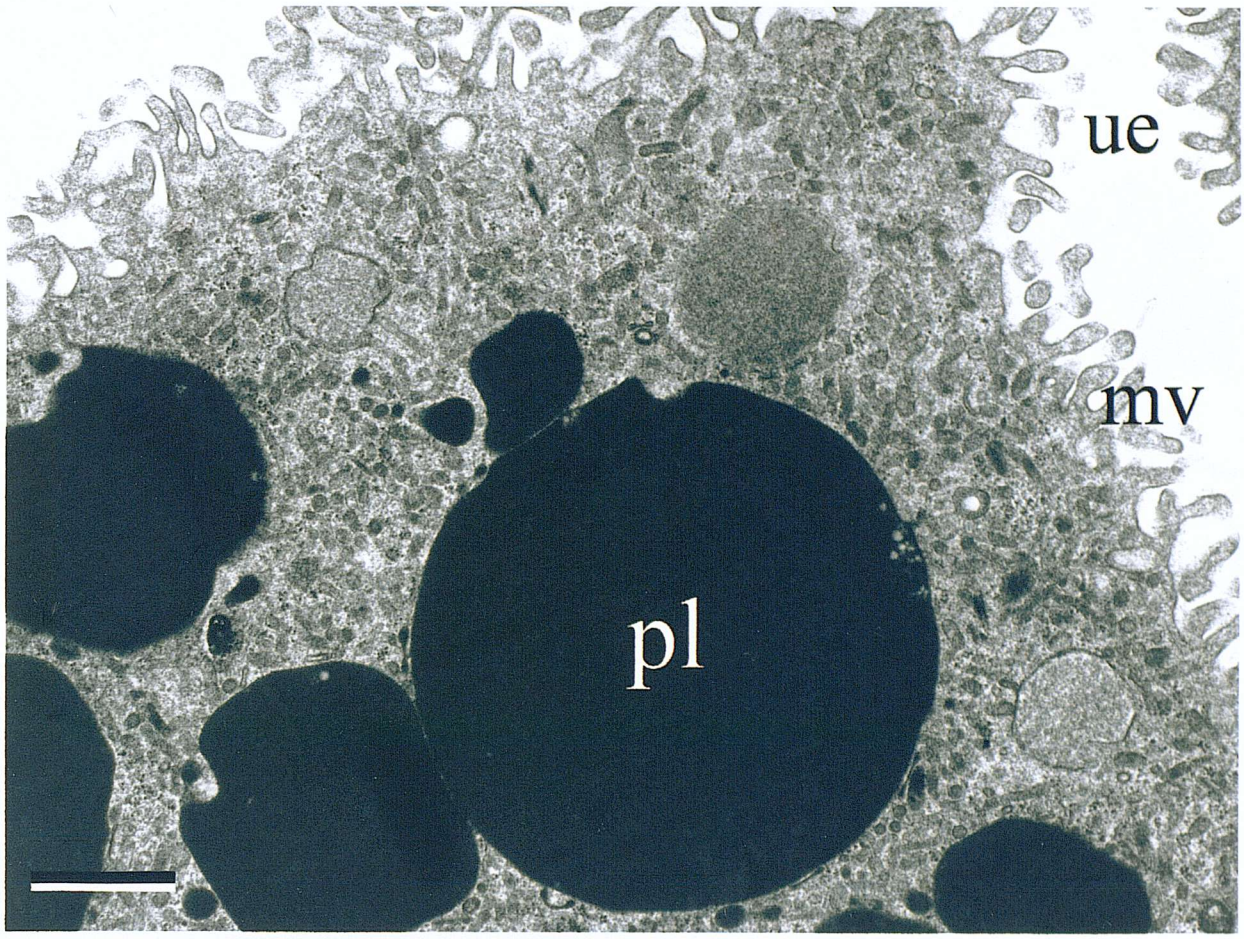


Figure 68: Mouse visceral yolk sac villous region from a mother fed control diet (18% casein) up to Day 17 gestation.
Scalebar = 1 micron
mv = microvilli, ue = uterine epithelium, pl = phagolysosomes

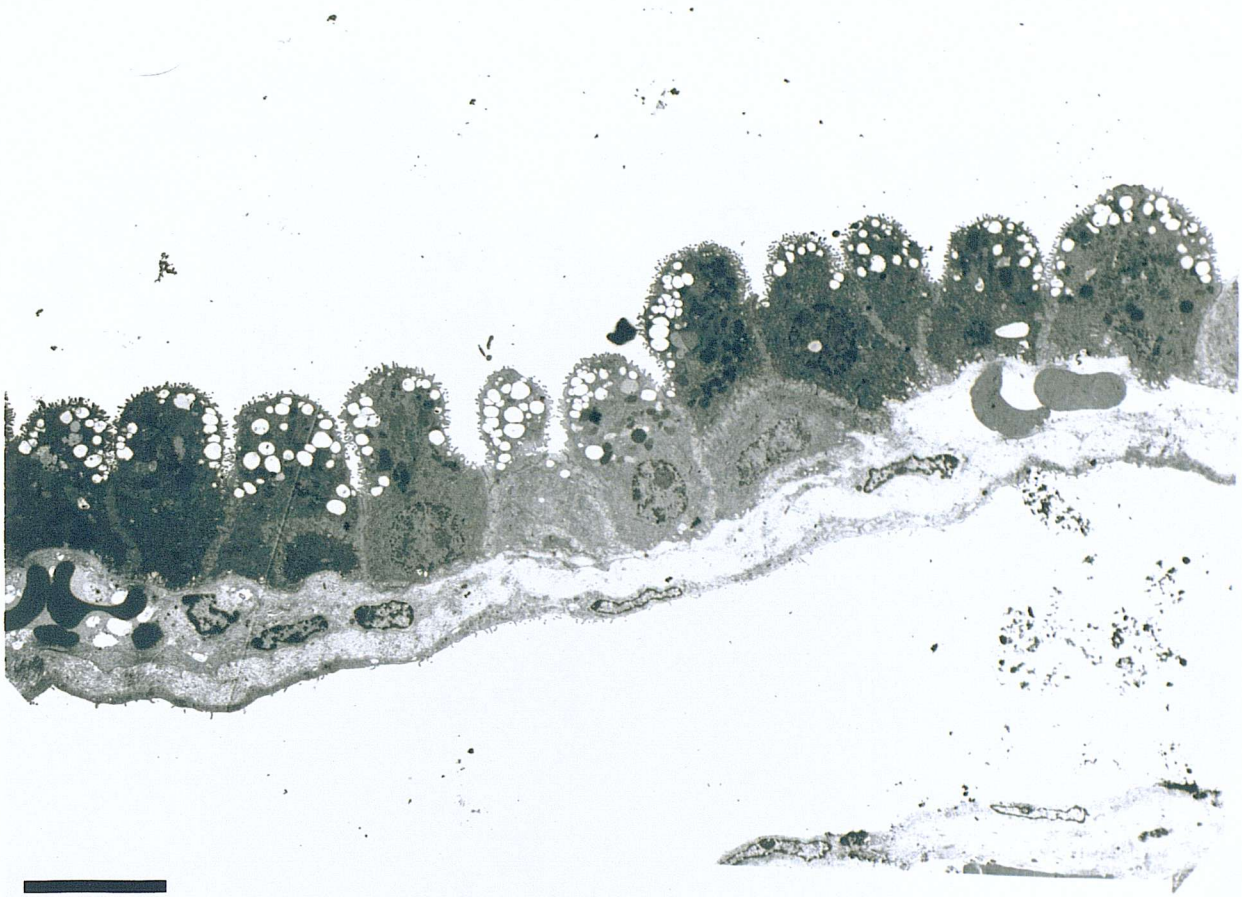


Figure 69: Mouse visceral yolk sac less villous region from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scalebar = 10 microns

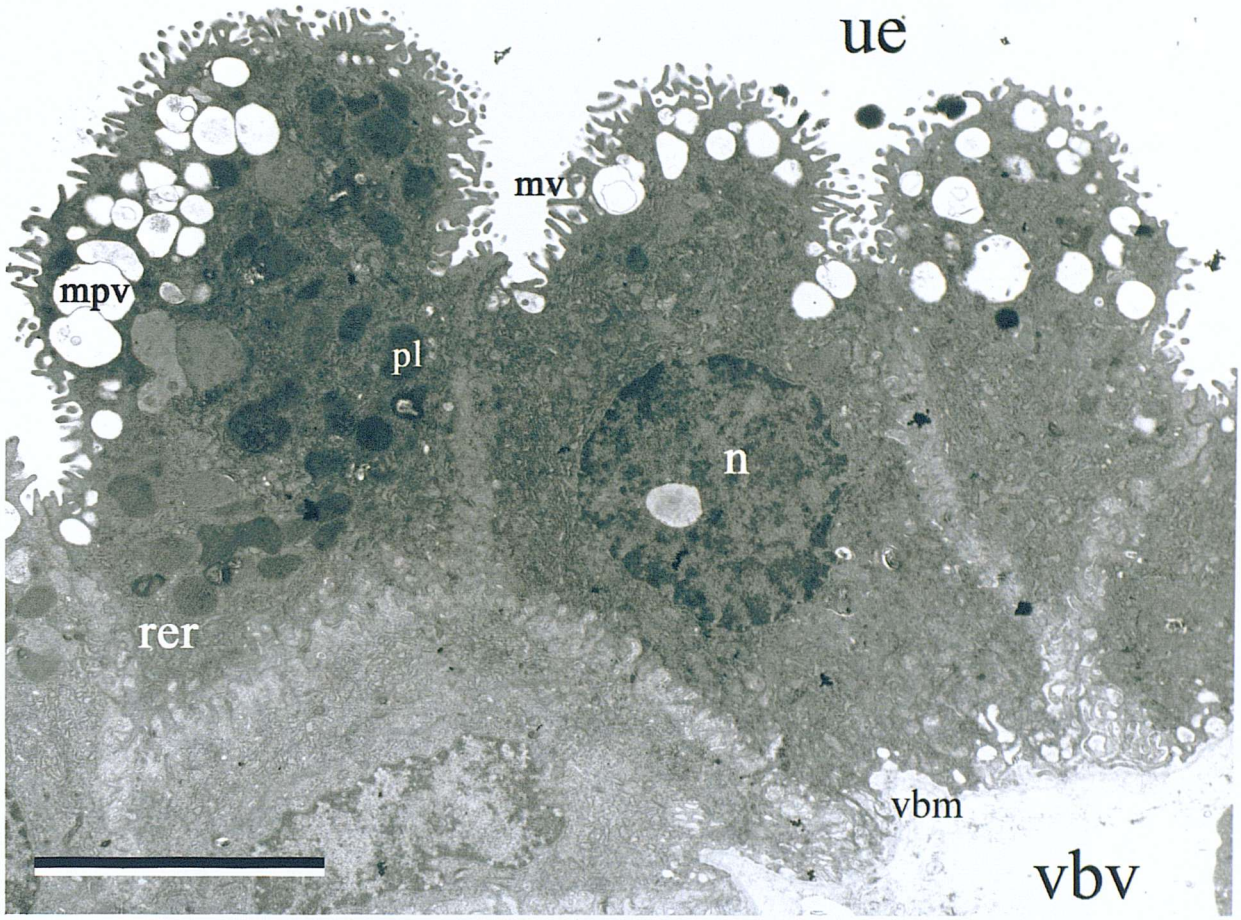


Figure 70: Mouse visceral yolk sac less villous region, from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scalebar = 5 microns
mpv = macropinocytic vesicle, mv = microvilli, n = nucleus, pl = phagolysosome, rer = rough endoplasmic reticulum, vbm = visceral basement membrane, vbv = vitelline blood vessel, ue = uterine epithelium.

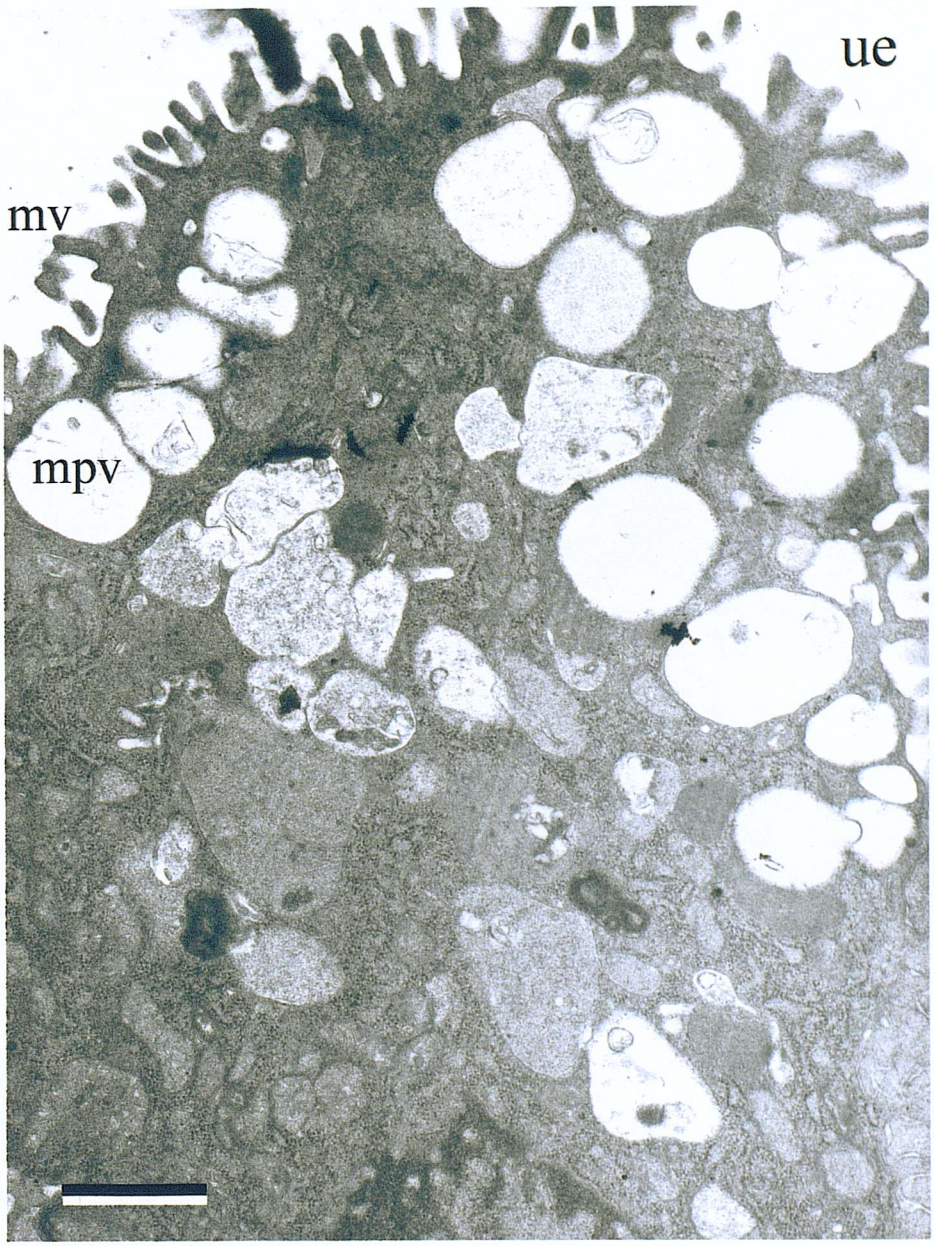


Figure 71: Mouse visceral yolk sac less villous region, from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scalebar = 1 micron
mpv = macropinocytic vesicle, mv = microvilli, ue = uterine epithelium.

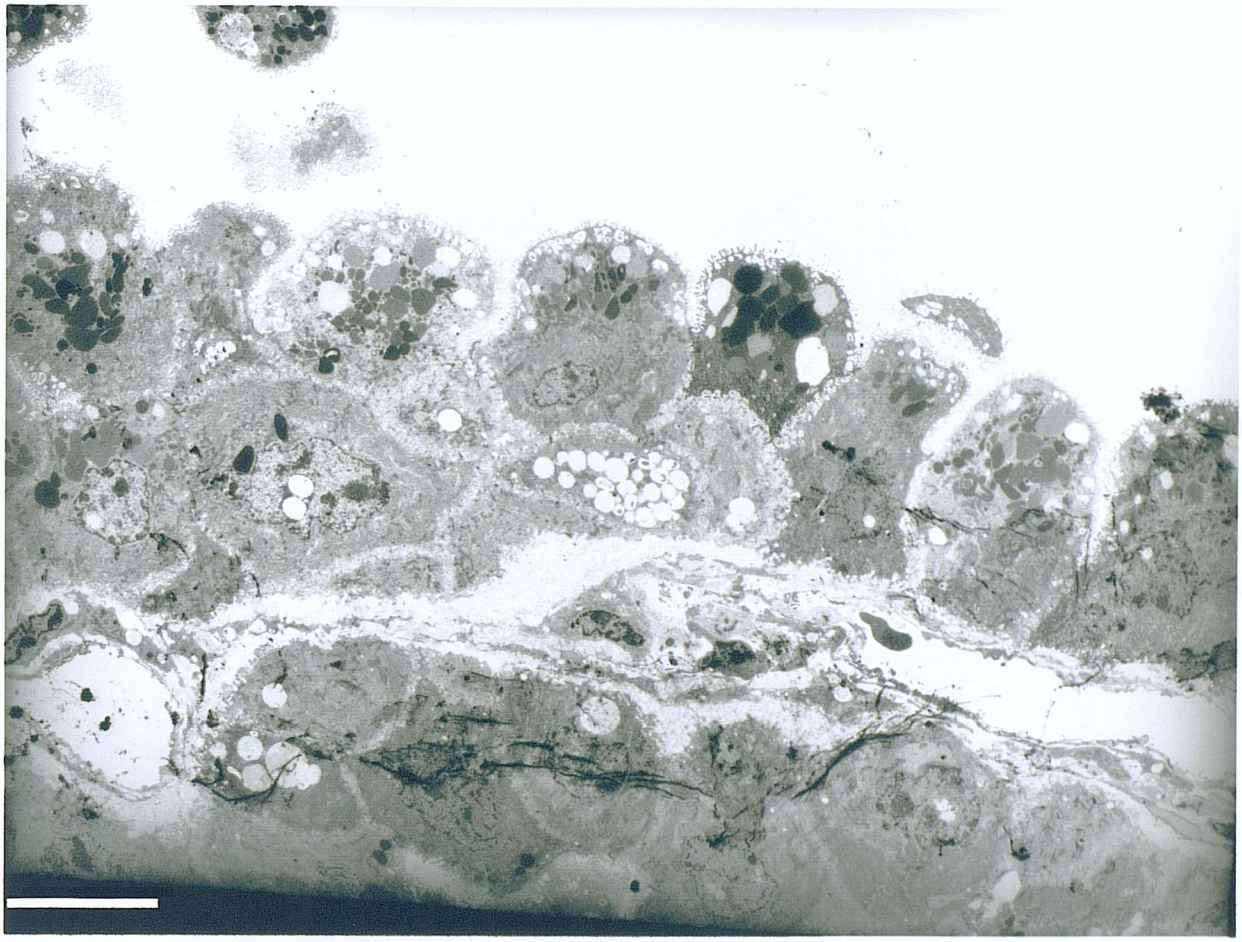


Figure 72: Mouse visceral yolk sac villous region from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scalebar = 10 microns

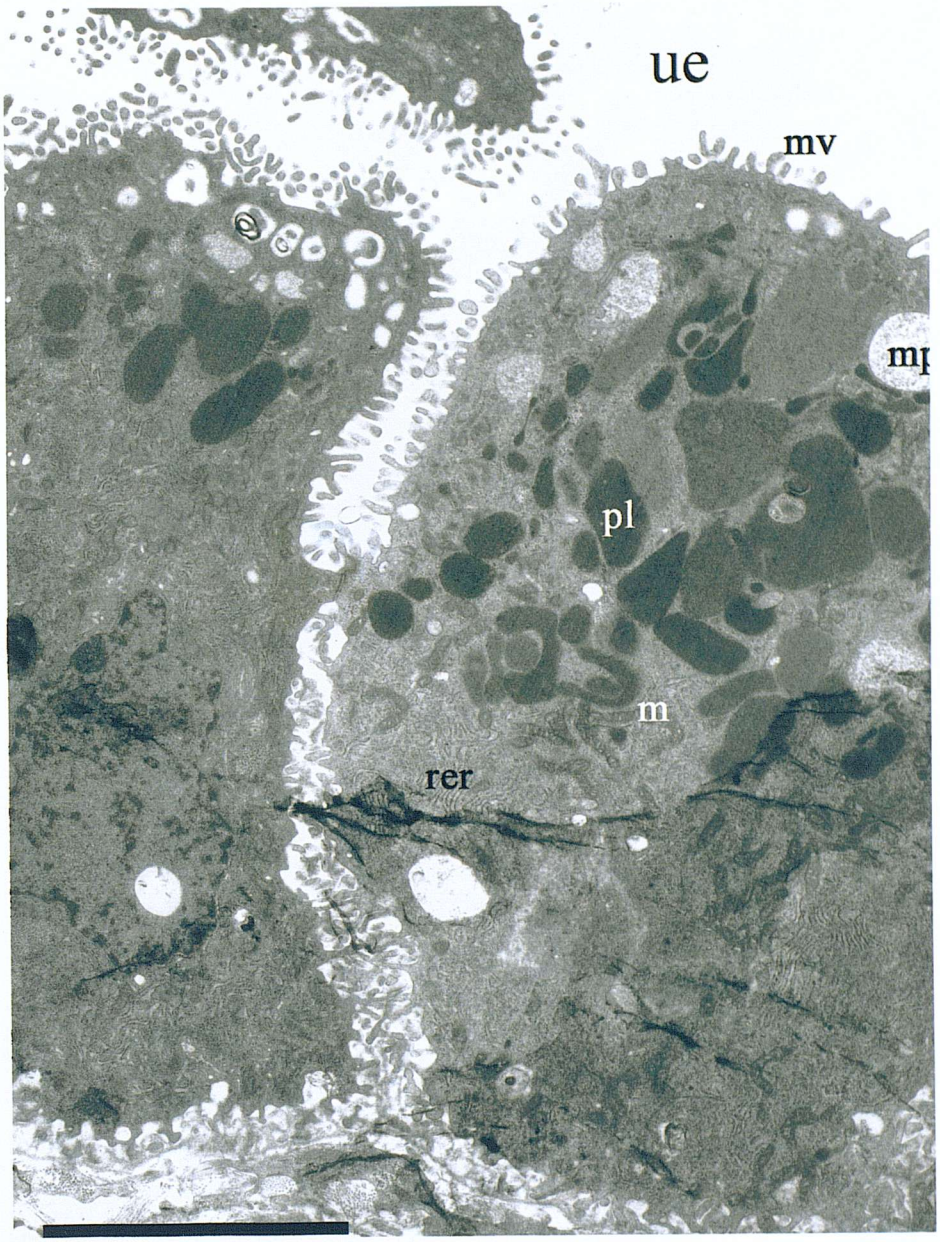


Figure 73: Mouse visceral yolk sac villous region from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scale bar = 5 microns
m = mitochondria, mpv = macropinocytic vesicle, mv = microvilli, pl = phagolysosome, rer = rough endoplasmic reticulum, ue = uterine epithelium.

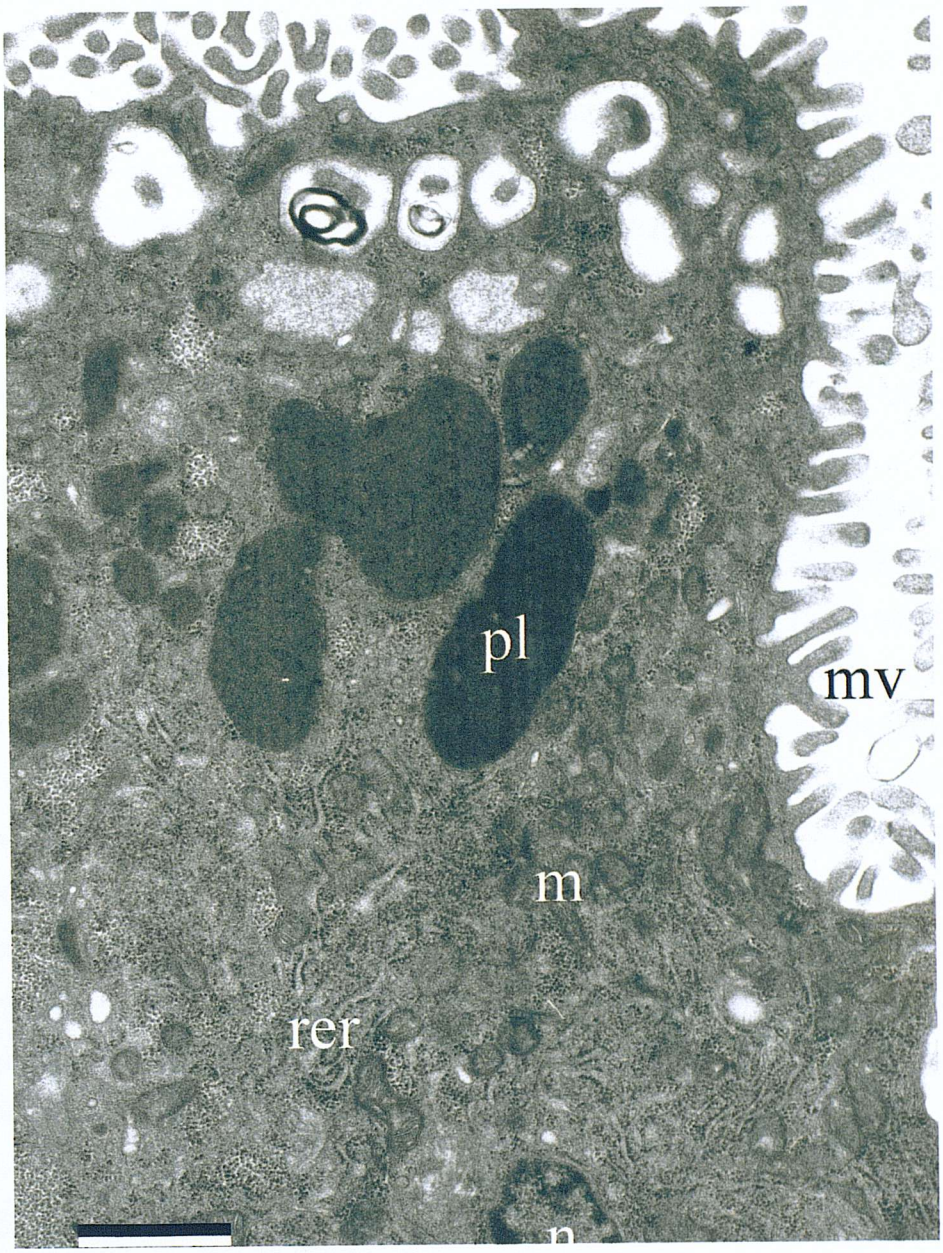


Figure 74: Mouse visceral yolk sac villous region from a mother fed low protein diet (9% casein) up to Day 17 gestation.
Scale bar = 1 micron
m= mitochodria, mv = microvilli, n = nucleus, pl = phagolysosome, rer = rough endoplasmic reticulum.

5.4 Discussion

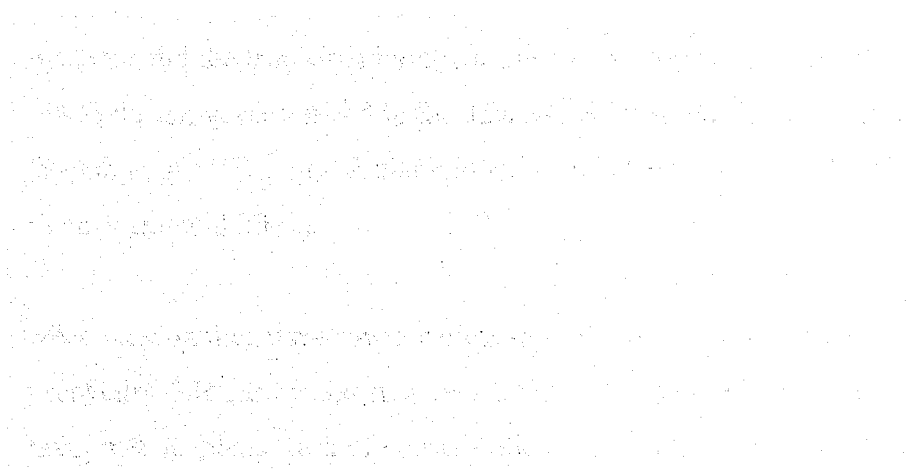
This investigation showed that maternal low protein diet fed during gestation up to day 17 significantly increased the number of macropinocytic vesicles per cell present in the less villous region of the visceral yolk sac and significantly decreased the number of secondary lysosomes per cell in the same region. There was no significant effect of low protein diet compared to a control diet on the number of macropinocytic vesicles per cell and the number secondary lysosomes per cell in the villous region of the visceral yolk sac. Maternal low protein diet did not affect the overall size of the endodermal cells in the visceral yolk sac.

The results shown in this chapter are in agreement with previous electron microscope studies of rodent visceral yolk sac (Jollie 1984, 1986, Calarco and Moyer 1966), clearly showing the endocytic role of the visceral yolk sac during gestation. Nutrients are captured by the apical microvillous surface of the visceral yolk sac in either the clathrin coated pits or the non-coated pits in the plasma membrane of the cells. They are transported through the sub-apical tubular canilicular system (Jollie 1984, 1986, Lloyd et al 1975), the small translucent vesicles at the apical edge of the membrane are part of this system. Chapter 4 demonstrated a significant increase in fluid-phase endocytosis in visceral yolk sacs from mothers fed low protein diet. The significant increase in macropinocytic vesicles per cell seen here in the micrographs of visceral yolk sacs from the same diet group therefore supports the fluid-phase endocytic activity data.

Molecules are transferred from the macropinocytic vesicles to phagolysosomes where they are hydrolysed by proteolytic enzymes, then moved into the basement membrane and passed into the vitelline membrane which facilitates the movement of digested proteins to the fetus (Jollie 1984). The mitochondria provide much needed energy for these processes, and the rough endoplasmic reticulum synthesizes proteins and steroids made by the yolk sac (Sorokin and Padykula 1964). The dense apical tubules of the apical tubular canilicular system constitute the membrane-recycling compartment of the visceral yolk sac cells (Christensen et al 2002b). My study of receptor-mediated endocytosis described in chapter 4 showed a significant effect on the endocytic activity of the visceral yolk sac due to maternal low protein diet. This study of the morphology of the visceral yolk sac

from mothers fed a low protein diet showed a significantly reduced number of secondary lysosomes in the less villous region of the visceral yolk sac which may support the significant reduction in receptor mediated endocytic uptake and release also seen in visceral yolk sacs from mothers fed low protein diet up to day 17 gestation.

The role of the visceral yolk sac has been shown in previous studies to be the uptake and degradation of proteins and other nutrients from the uterine environment and their delivery to the developing fetus (Jollie 1984, Calarco et al 1996). The villous section of the yolk sac functions as the placenta does during gestation (Jollie 1986 B). Maternal undernutrition during gestation up to day 17 appears to influence significantly number of specific vesicles within the visceral yolk sac, clearly evident in the electron microscope micrographs, although it does not alter the size of the endodermal cells as initially hypothesized.



Chapter 6

Effect of maternal diet on Megalin and Cathepsin L expression in the visceral yolk sac.

6.1 Introduction

Receptor-mediated endocytosis is facilitated, primarily, by coated pits within the membrane of the cell; in particular, these coated pits contain clathrin and receptors that are specific for certain molecules (Apodaca 2001). Two of these receptors are megalin and cubilin which work together to capture and internalize several proteins, including albumin (Le Panse et al 1995, Farquhar et al 1995 Zhai et al 2000). These two receptors are also detectable in non-clathrin coated areas of the membrane, although not as concentrated as in the clathrin coated pits (Le Panse et al 1997).

Megalin is a 600 kDa transmembrane receptor (Figure 10) (Caplan 2003), it has a large amino-terminal extracellular domain, a single transmembrane domain and a short carboxy-terminal cytoplasmic tail (Christensen et al 2002b). It is found expressed at the apical surface of the plasma membrane in the visceral yolk sac, placenta and the trophectoderm (McCarthy and Argraves 2003, Moestrup et al 1996) during gestation and in the adult renal brush border of the proximal tubule (Sahali et al 1993), glomerular epithelial cells (Baricault et al 1995) and intestine (Yammani et al 2001).

Mice lacking megalin show a variety of embryonic abnormalities, most fetuses die prenatally (McCarthy and Argraves 2003). Megalin-deficient mice are born with holoprosencephaly, lack of corpus callosum, anopthalmia and abnormal development of the forebrain, thus, showing that megalin is important for transport of nutrients into the developing brain (Christensen et al 2002a).

Cathepsins are lysosomal cysteine proteases employed in the cell (Ohashi et al 2003). They have numerous functions within the cell predominantly to break

down proteins into amino acids in the lysosomes, but are also involved in antigen presentation, prohormone processing, turnover of ß-amyloid in Alzheimer's disease, tumour invasion and angiogenesis, and apoptosis (Felbor et al 2002). In this context, they have been shown to break down extracellular matrix components (Deussing et al 1997). Cathepsins can also be secreted outside of the cell either in their proenzyme or mature form (Ohashi et al 2003).

Some of the cathepsin family, namely B, H and L, have specific functions employed during gestation. All three of these cathepsins have been found to be expressed in the visceral yolk sac (Sol-Church et al 1999) throughout gestation and in the trophoblast giant cells and the placenta, although at a lower amount than in the visceral yolk sac. During early gestation, at implantation (Day 3.5 in mouse) and placentation (Day 10/11 in mouse), there is up regulation of cathepsins B, D and L in the trophoblast giant cells (Afonso et al 1999). The activity of these cathepsins during gestation is important in order to provide the fetus with amino acids that are derived from endocytosed maternal protein. Thus, inhibition of these three cathepsins prevents normal yolk sac function (Beck and Lowy 1982). These cathepsins are also required after birth for the maturation and integrity of the postnatal central nervous system (Felbor et al 2002).

Cathepsin L has a prepro sequence in front of the mature enzyme. This prepro form is processed into a proenzyme form in the rough endoplasmic reticulum within the cell, the enzyme is then localised in lysosomes (Ishidoh and Kominami 1994). Cathepsin L is the most potent cysteine protease and plays a major role in lysosomal proteolysis (Ishidoh and Kominami 1994), quantitatively it is the most significant of the cysteine proteases present in lysosomes (Freeman and Lloyd 1983 B).

The aim of this chapter was to investigate, at a molecular level, changes that may occur in the visceral yolk sac endoderm pathways involved in endocytosis due to a maternal low protein diet. Chapter 4 and 5 identified significant alterations in endocytic rates and morphology of the visceral yolk sac, this chapter looks at the expression levels of a receptor, megalin involved in binding albumin, and a lysosomal protease, cathepsin L involved in breaking down proteins, in relation to

maternal diet.

6.2 Methods

Dams were mated and fed a low protein (9% casein) or control diet (18% casein) from day 0 to day 17 gestation (section 2.1). These mice were culled at day 17 and the visceral yolk sacs were harvested (section 2.3). Visceral yolk sacs were digested in 1% SDS and the total protein content assayed (section 2.4.1). Samples were coded to enable blinding of the samples until quantitation had been completed. Digested visceral yolk sac samples were loaded onto pre-cast gels and electrophoresed prior to western blotting as described in section 2.6.2. A standard was run on every gel; this was a visceral yolk sac lysate from a mother fed a control diet. Specific antibodies to detect megalin, cathepsin L and actin were used with 25 mg total protein loaded for megalin and 30 mg for cathepsin L (section 2.6.2). Samples were assessed quantitatively by fluorescence intensity using the Odyssey infrared imaging system (section 2.6.2). An identical sized box was placed around each band to measure the intensity within that box, in this way the quantitation of intensity was kept as unbiased as possible. The statistical analysis incorporated adjustments for gel effects, as duplicates of each sample were run on different gels. Statistical analysis was carried out on the most prominent band seen for megalin expression, 380kDa and for cathepsin L expression analysis was carried out on the mature single-chain form, 30 kDa.

6.3 Results

6.3.1 Megalin expression

To determine optimal conditions for western blotting of megalin, four different protein concentrations (20 mg, 25 mg, 30 mg, 35 mg) were tested (Figure 75). Figure 76 shows the signal intensity for these protein concentrations; 25 mg was chosen as the optimal protein concentration as saturation was reached at approximately 35 mg.

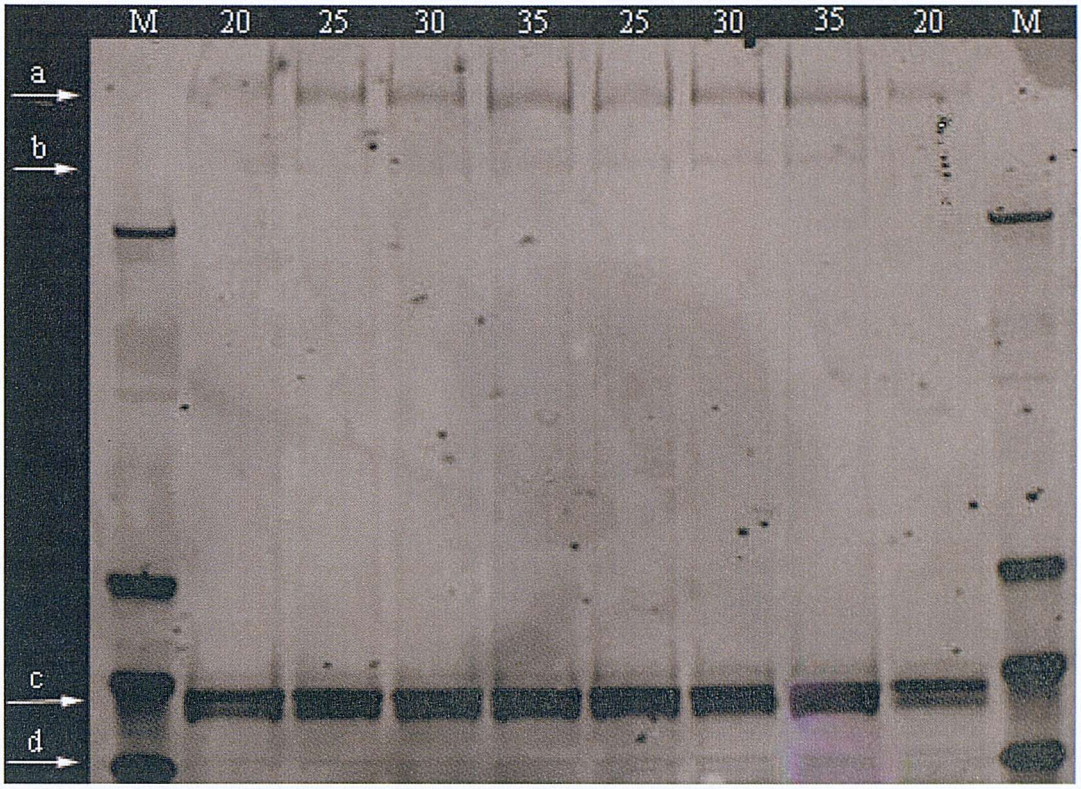


Figure 585: Megalin expression in mouse visceral yolk sac, using different sample total protein concentrations. arrows: a = megalin (380 kDa) , b = megalin (330 kDa) , c = mouse IgG heavy chain, d = actin

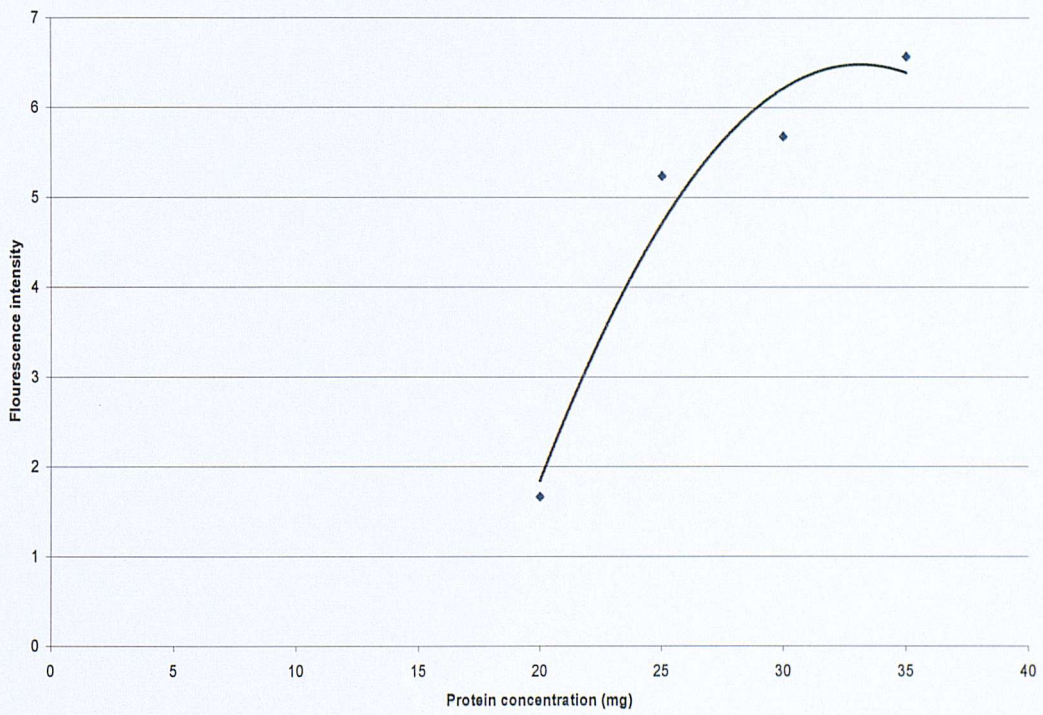


Figure 76: Mean intensity of megalin expression in mouse visceral yolk sac, using different sample total protein concentrations.

Three different primary antibody concentrations (1µl/ml, 2 µl/ml, 10 µl/ml) were used in western blotting to ascertain the optimal antibody concentration (Figure 77). The optimal primary antibody concentration for detecting megalin expression in mouse visceral yolk sac was 2 µl/ml. Specificity of primary antibody was demonstrated in blots which had only been probed with secondary antibody (Figure 78), they showed only one band at a molecular weight concurrent with mouse IgG heavy chain. This assay determined that the bands seen in the western blots at 380 kDa and 330 kDa after megalin antibody treatment are not the result of the secondary antibody binding non-specifically.

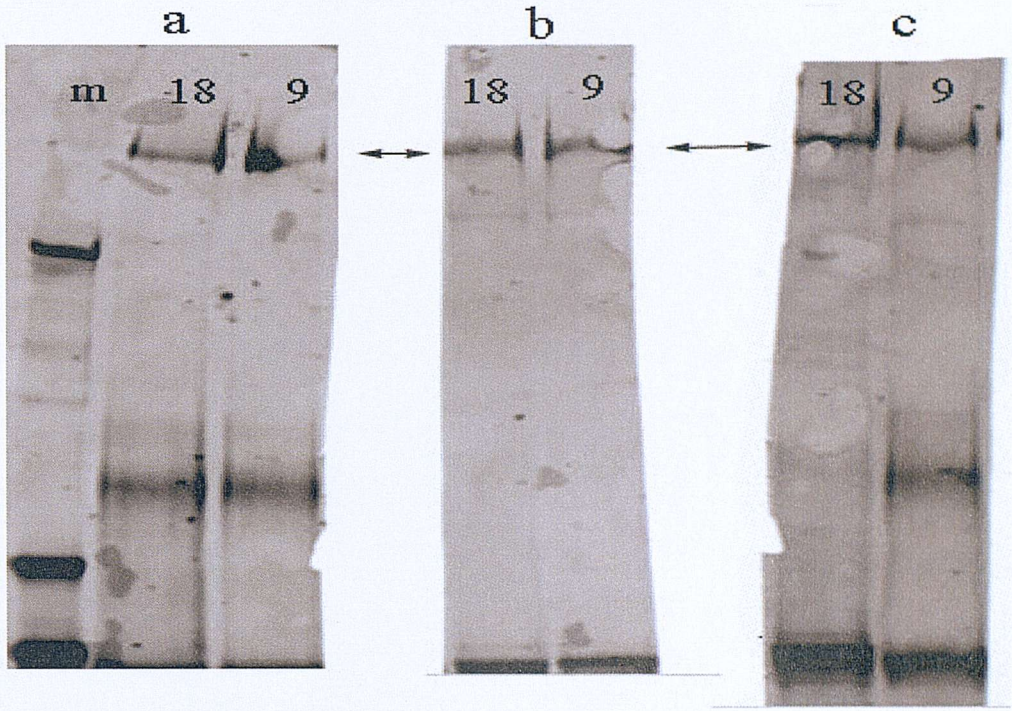


Figure 77: Megalin expression (arrows). Primary antibody concentration experiment. a = 1:1000, b = 1:500, c = 1:100. m = marker, 18 = control diet, 9 = low protein diet.

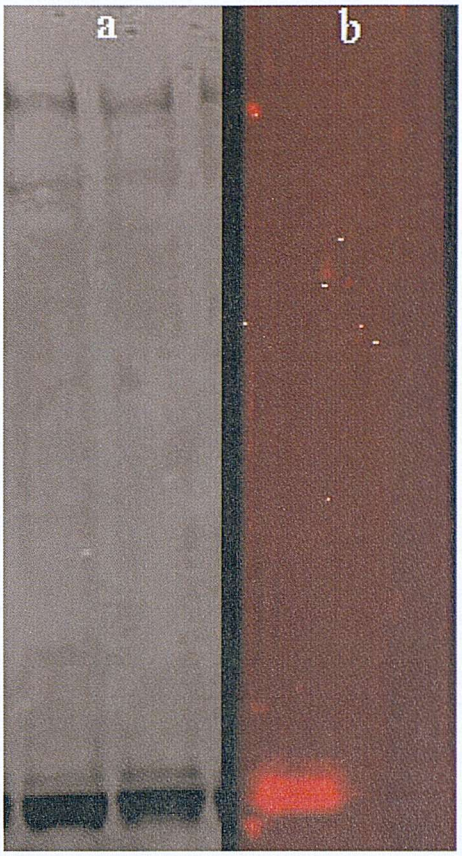


Figure 78: Megalin expression in mouse visceral yolk sac. Membrane A was probed with primary and secondary antibodies, membrane B was probed with secondary antibody only. The single band visible in blot B is synonymous with immunoglobulin heavy chain.

Immunoblotting of the visceral yolk sac lysates for megalin consistently detected a band at 380 kDa (arrow a, Figure 79) and a second band at 330 kDa (arrow b, Figure 79) on each gel. Actin expression was detected at 44 kDa and can be seen at the bottom of the gel in Figure 79 (arrow D). Fifteen visceral yolk sacs were assayed from each diet group, in total thirty visceral yolk sacs were used each from a different mother. Figure 80 shows the mean signal intensity of fluorescence of megalin expression in visceral yolk sacs at Day 17 gestation, Figure 81 shows the mean signal intensity of actin expression in visceral yolk sacs, also, at day 17 gestation.

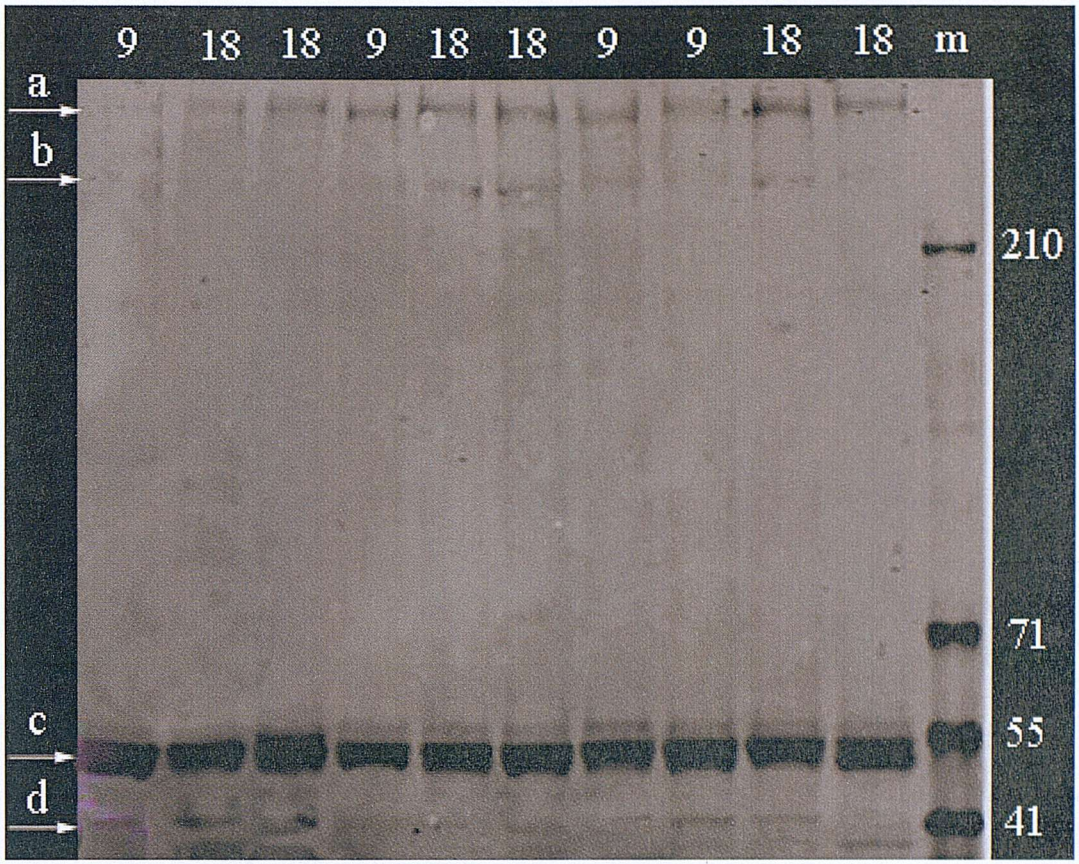


Figure 79: Expression of megalin in the mouse visceral yolk sac at day 17 gestation analyzed by western blotting. A=Megalin (380kDa), B=Megalin (330kDa), C = IgG heavy chain, D= Actin.

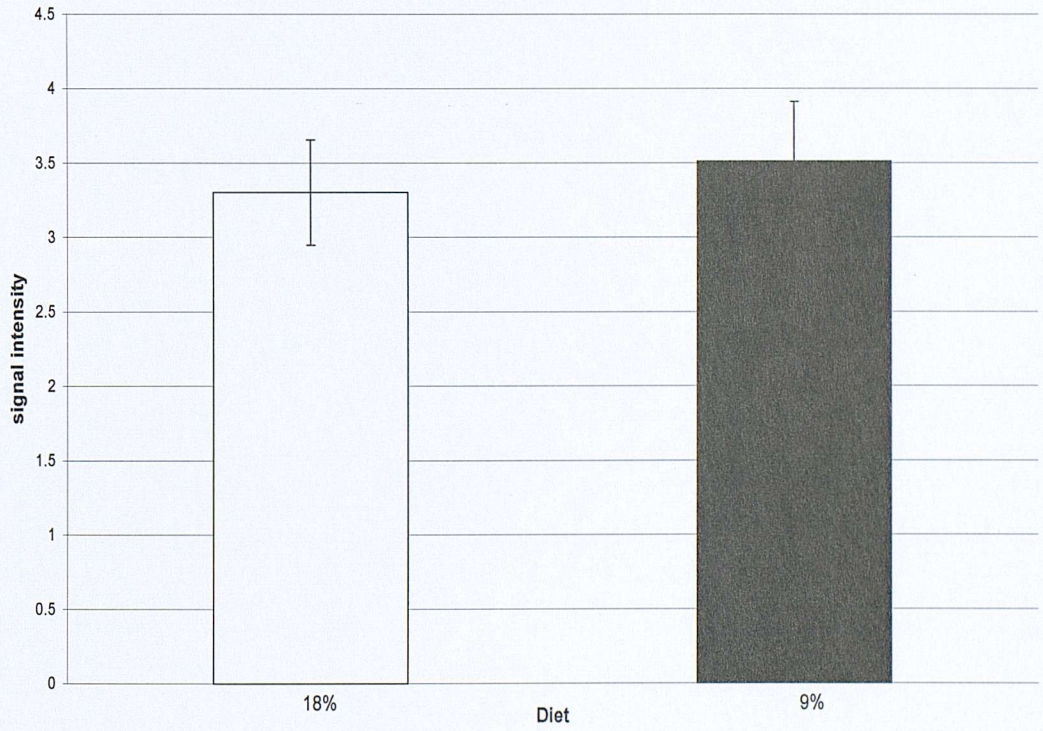


Figure 80: Mean signal intensity of megalin expression in mouse visceral yolk sac. P = 0.665, N = 30.

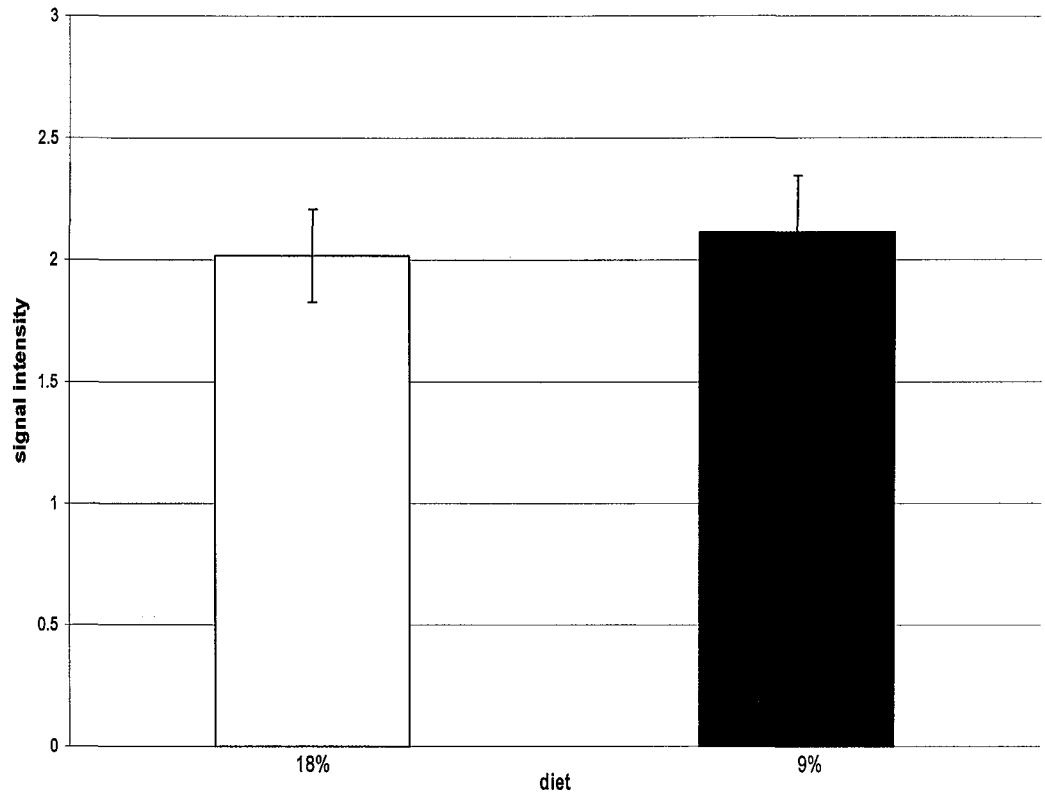


Figure 81: Mean signal intensity for actin expression in mouse visceral yolk sac obtained in western blotting for megalin expression. P = 0. 805. N = 30.

Statistical analysis of the expression levels of megalin (Figure 80) show that visceral yolk sacs from mothers fed a low protein diet (9% casein) had slightly increased levels of expression compared to visceral yolk sacs from mothers fed a control diet (18% casein) up to day 17 gestation, although this is not statistically significant (P=0.665). Levels of actin expression (Figure 81) were not altered significantly (P=0.805) between yolk sacs from mothers fed on either diet.

6.3.2 Cathepsin L expression

To optimise western blotting conditions, visceral yolk sac lysates were run on gels at different total protein concentrations and with different antibody concentrations. Figure 82 shows a membrane using different total protein concentrations, and in Figure 83 the signal intensity detected in relation to protein concentration is shown. This analysis indicated that the optimal total protein concentration for investigating cathepsin L expression was found to be 30 mg.

Figure 84 shows membranes run using different primary antibody concentrations; from these experiments the optimal primary antibody concentration was found to be 1 µl/ml. Western blotting using only secondary antibodies showed a band concurrent with mouse IgG heavy chain (Figure 85). This assay confirmed that western blotting for cathepsin L expression giving bands at 30 kDa and 25 kDa are not a result of the secondary antibody binding non-specifically.

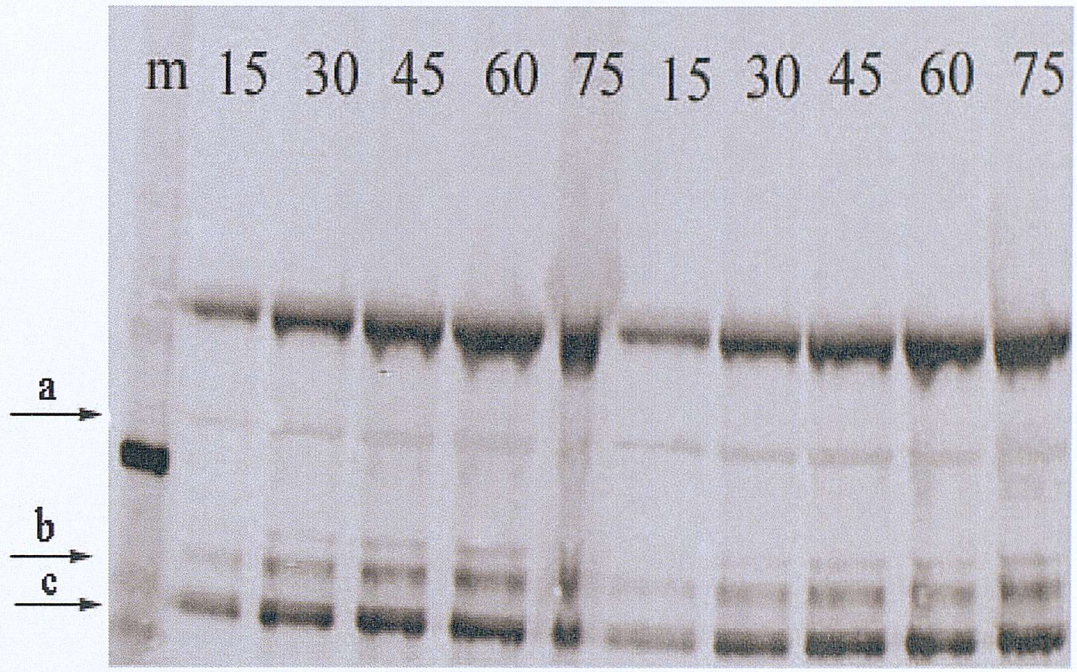


Figure 82: Cathepsin L expression. varying protein concentrations. arrows: a = actin. b = Cathepsin L (30 kDa) c = cathepsin L (25 kDa)

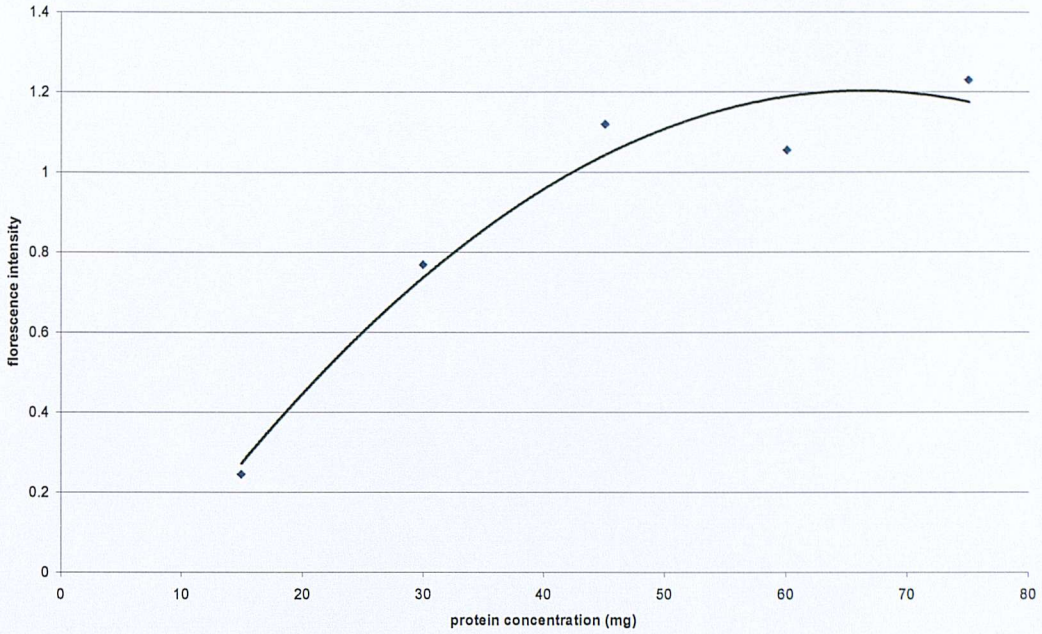


Figure 83: Mean intensity of cathepsin L expression using different protein concentrations

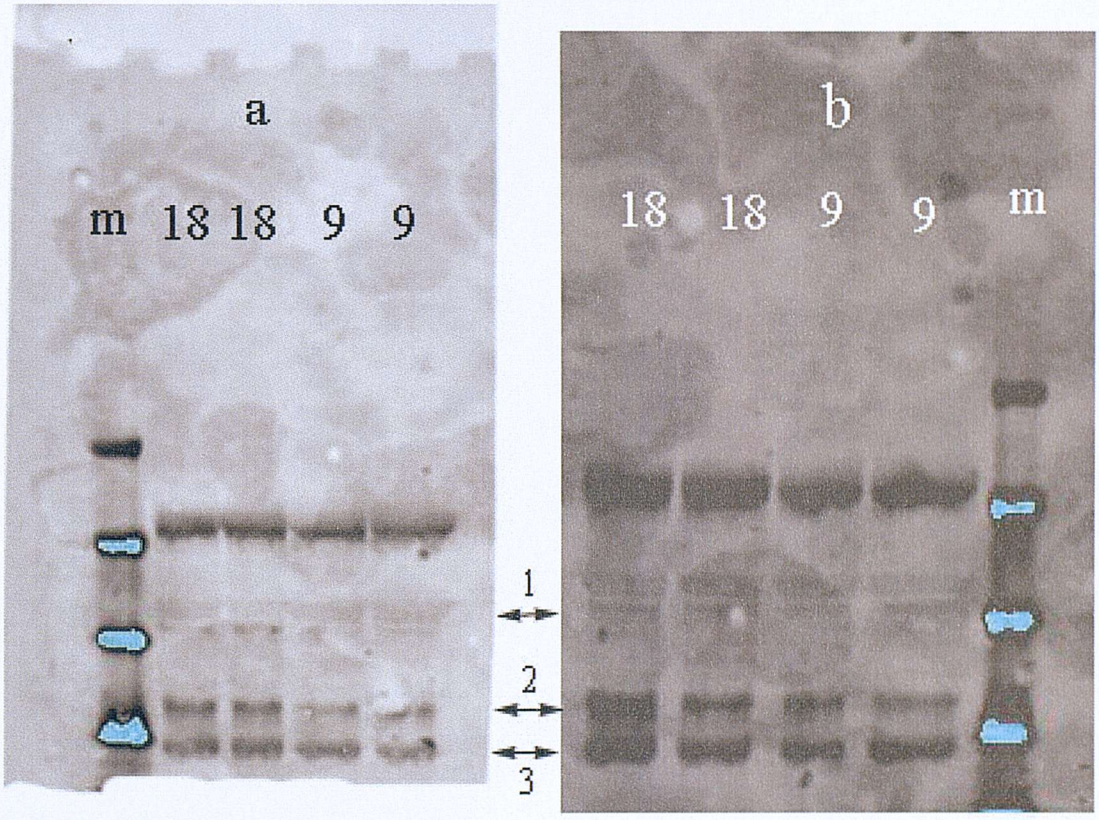


Figure 84: Cathepsin L expression using different primary antibody concentrations. a = 1:500, b = 1:1000. m = marker, 18 = control diet, 9 = low protein diet. arrows: 1 = actin, 2 = cathepsin l (30 kDa) 3 = cathepsin L (25 kDa)

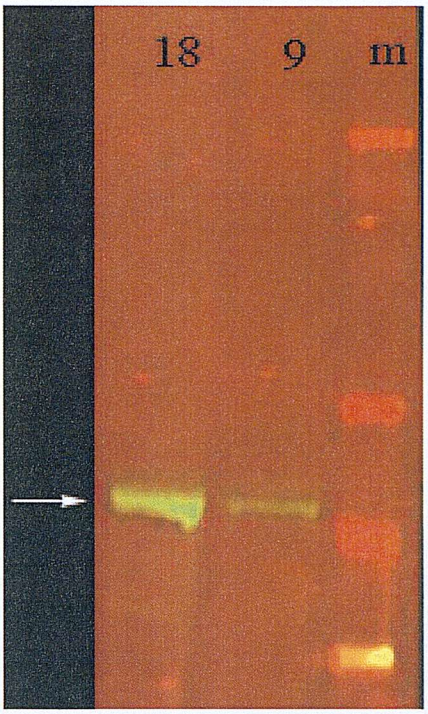


Figure 85: Secondary antibody only used for blotting cathepsin L. arrow = mouse IgG heavy chain. m = marker, 18 = control diet, 9 = low protein diet

Cathepsin L is synthesised as a proenzyme and becomes activated when it reaches the endosomes or lysosomes (Fiebiger at al 2002, Sol-Church et al 1999). Western blotting of visceral yolk sac samples revealed the presence of more than one band (Figure 86), detected at 30 kDa and 25 kDa. These may reflect the different forms of cathepsin L present in cells (Fiebiger at al 2002, Sol-Church et al 1999). Fluorescence intensity analysis of cathepsin L expression level was carried out on the mature single-chain form of cathepsin L (Figure 86 arrow b, Figure 87) which blots at 30 kDa (Ishidoh and Kominami 1994, Felbor et al 2000, Fiebiger et al 2002). The second band at 25kDa (Figure 86 arrow a) is part of the two-chain form of cathepsin L which is linked to a 5 kDa chain (not seen on the gels, Figure 86) by disulphide bonds (Fiebiger et al 2002). One visceral yolk sac was used from each mother; for each diet group fifteen mothers were used; in total thirty visceral yolk sacs were assayed each from different mothers.

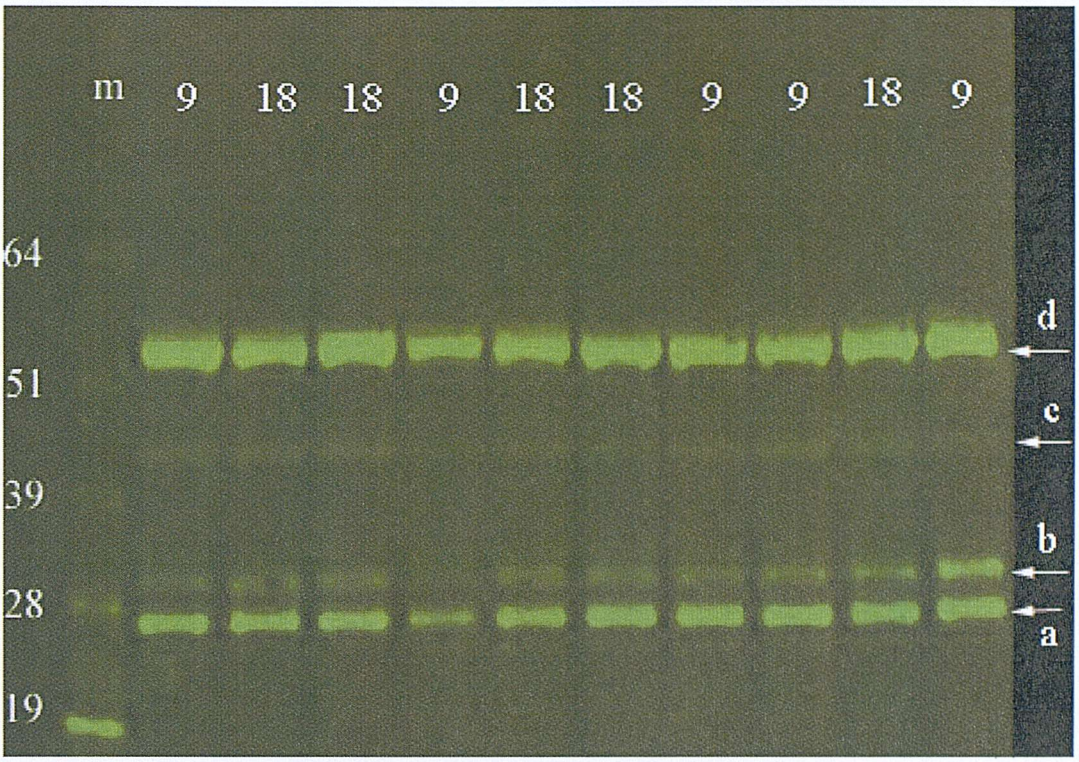


Figure 86: Western blotted membrane showing cathepsin L (30, 25 kDa) expression and actin (44kDa) expression in visceral yolk sac at day 17 gestation. M= marker, arrows = a – cathepsin L 25kDa, b – cathepsin L 30 kDa, c – actin, d – mouse IgG heavy chain

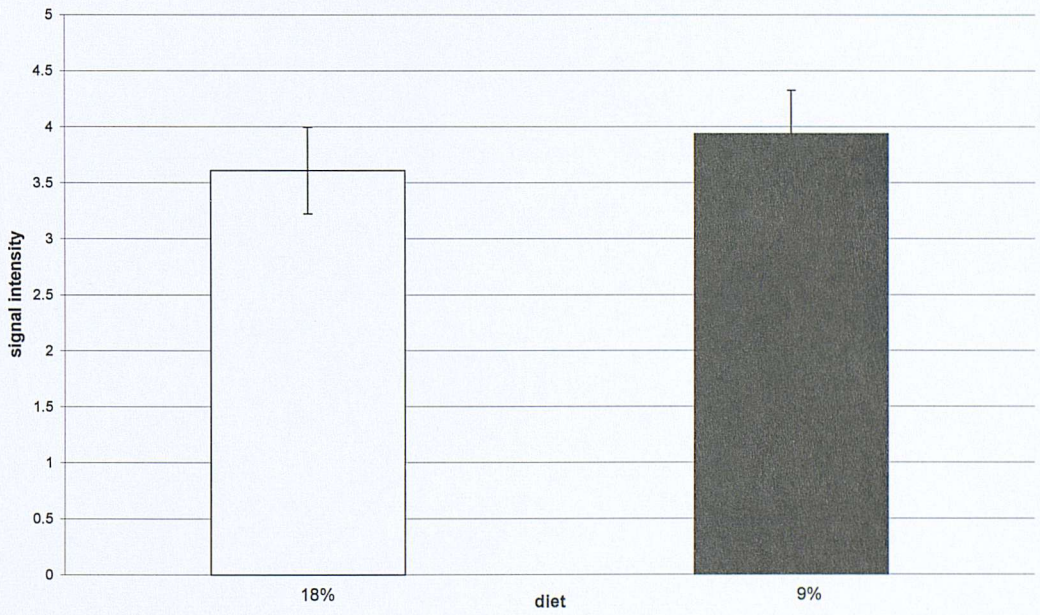


Figure 87: Mean cathepsin L expression signal intensity. P = 0.526. N = 30.

The cathepsin L expression level in visceral yolk sacs from mothers fed a low protein diet (9% casein) was compared to that of visceral yolk sacs from mothers fed a control diet (18% casein) up to day 17 gestation (Figure 87); it was found to

be slightly higher although this is not statistically significant (p=0.526).

6.4 Discussion

Megalin is a member of the low-density lipoprotein receptor family, it is capable of binding several ligands during receptor-mediated endocytosis. It is first detectable during gestation at the 16-cell stage in the trophectoderm lineage and remains actively expressed throughout gestation (Christensen et al 2002a).

The current experiments show megalin expression in the mouse visceral yolk sac at day 17 gestation, the results are similar to those gained by Meads et al (1993) studying rabbit visceral yolk sac. Both studies show the presence of two bands following megalin immunoblotting. Maternal diet did not have a significant effect on the expression level of megalin or actin at day 17 gestation in mice.

Cubilin is an endocytic receptor like megalin, and works in unison with megalin to internalise ligands, in particular they work together to internalise albumin. Megalin and cubilin can be found expressed in the dense apical tubules of the visceral yolk sac and it is at this site that they are recycled through the apical network back to the plasma membrane (Verroust and Christensen 2002 , Baricault et al 1995). Cubilin is not as widely expressed as megalin within the developing conceptus (Christensen et al 2002b), however, it would be a suitable molecule to further investigate the effect of maternal diet on visceral yolk sac endocytic activity.

The majority of amino acids used to synthesize embryonic protein are hydrolysed within the visceral yolk sac (Sol-Church et al 1999). Exogenous albumin is degraded exclusively within the lysosomal system of the visceral yolk sac (Knowles et al 1981). The mature single chain form of cathepsin L is located in the lysosomes of cells in the visceral yolk sac (Ishidoh and Kominami 1994). It has a major role in degradation of proteins in the yolk sac (Sol-Church et al 1999). Its location and abundance (Freeman and Lloyd 1983 B) in comparison to other cathepsins in the yolk sac makes cathepsin L a good candidate to use to identify any changes that might occur at a cellular level within the visceral yolk sac when

mice are placed under conditions of low protein diet during gestation.

Activity of cathepsin B and L increases as gestation increases (Beckman et al 1994, Sol-Church et al 1999). Cathepsin B has a general house-keeping role and has expression levels that are the same in adult and fetal tissue (Sol-Church et al 1999). Use of cathepsin L inhibitors such as leupeptin and chymostatin have shown that cathepsin L may be a rate-limiting step in the degradation of exogenous albumin, as could cathepsin B (Knowles et al 1981). High levels of cathepsin L will increase the capacity of the visceral yolk sac for proteolysis to provide amino acids for the fetus (Sol-Church et al 1999).

This study found no significant increase or decrease in the expression level of cathepsin L in the visceral yolk sacs from mothers fed a low protein diet (9% casein) up to day 17 gestation. There was a slight increase in the expression level, however, this is not sufficient to state that feeding a low protein diet during gestation in mice has a significant effect upon this specific protease. However, maternal low protein diet during gestation may have a significant effect upon other enzymes, for example cathepsin B, within the lysosomes of the visceral yolk sac.

Previous studies using targeted disruption of cathepsin B, D and L have shown that inhibition of one cathepsin and not the others is not sufficient to impair fetal growth and development, however, disruption of more than one cathepsin may cause injuries to fetal development (Sol-Church et al 1999). Therefore, although my experiments show no significant change in cathepsin L expression, analysis of more than one cathepsin may present a different picture, and may help explain the significant changes seen in receptor-mediated endocytosis in visceral yolk sacs from mothers fed a low protein diet throughout gestation.

Chapter 7

General Discussion

The original aims of this project hypothesized changes in yolk sac weight due to reduced inner cell mass cell number seen in a previous study (Kwong et al 2000), the yolk sac develops from a layer of cells originating in the inner cell mass. It is probable from the results found in this study that the cell number of the inner cell mass is unlikely to be associated with the size of the yolk sac, as originally proposed.

Maternal low protein diet during gestation was found not to have an effect upon the litter size at 12 days, 14 days and 17 days gestation. The effect on litter size caused by maternal diet during gestation has been studied previously by Langley-Evans et al (1996,b ,c, 2000); he found no change in litter size when mothers were fed a low protein diet from conception throughout gestation and if they had been previously habituated to the low protein diet for 14 days prior to conception.

Low protein diet has been shown to programme changes during gestation (Rasmussen 2001). It was, therefore, thought that a change in the size of the fetus would be seen and possibly also a change in the placental and yolk sac size in this study. Studies from the laboratory of Langley-Evans and others have showed changes in the fetal size both in weight and body length in rats when mothers were fed a low protein diet (Langley- Evans et al 1996). The wet weight was measured in the experiments I carried out, which although will not identify very small changes in weight, should be sensitive enough to indicate any larger differences, particularly by day 17 of gestation. No significant differences were identified in the weight of the conceptus and its component parts measured, between the low protein diet and control diet treatments at any of the three time points examined in my study. This is in contrast to the findings of others who found significant changes when using a rat model to study the effects of a maternal low protein diet (Langley-Evans et al 1996).

It was possible to identify statistically significant correlations between weights at

the three time points studied, days 12, 14 and 17 during gestation, of the conceptus and its component parts. Figures 19 to 30 show that there is a strong relationship between the weights of the different parts of the conceptus and the weight of the conceptus as a whole. The R^2 values indicate the strongest correlation of weight is between the conceptus and fetus which is expected as the majority of the conceptus weight is made up of the fetus. However, these values also show that the weights from mice fed on low protein diet (9% casein) have a higher correlation than the control diet (18% casein) fed mice. The heavier the conceptus the heavier each component part will be; this trend is almost twice as likely to occur in low protein diet fed mice than in control diet fed mice. These data indicate that maternal nutrition, particularly the response to low protein diet, contributes proportionally to the growth of all three components of the conceptus during development.

Visceral yolk sacs at all three time points did not show an alteration in total protein content when they develop in a maternal protein deficient environment. As would be expected, the amount of total protein present in the visceral yolk sac increases with gestational age (Beckman et al 1994). However, the amount of protein present in the visceral yolk sac changes significantly between conceptuses positioned closest to the ovary compared with those closest to the cervix (Figure 50); this phenomenon is not apparent at the earlier stages of gestation, only at day 17 gestation. A possible explanation for the increasing visceral yolk sac total protein content at day 17 gestation at the cervical region could be an enriched blood supply to the part of the uterus closest to the cervix. However, as previously explained, the flow of blood in the uterus is bi-directional and would suggest that the total protein content should be lowest in the middle of the uterine horn. It has been reported previously that fetal weight from the middle of the uterine horn is reduced in comparison to those nearer the ovarian or cervical ends (Vom Saal and Dhar 1992). Although the variation in blood flow within the uterus does not explain why the observed change in total protein content is not obvious at earlier stages, this may be due to the vitelline vessels not being fully matured at earlier stages.

Position of the conceptus within the uterine horn did not have any effect other

than that stated above. The data was analyzed for differences within each horn of the uterus and between the two horns of the uterus. Vom Saal et al's paper (1992) supports our findings that there should be no effect between the two horns as they are physiologically independent, each having it's own cervix and blood supply, although local (within the horn) differences may have been expected.

One of the aims of this study was to ascertain what effects maternal dietary restriction would have upon the nutrient-providing function of the mouse visceral yolk sac. Metabolic studies of rodent yolk sacs have been carried out over a number of years, each study using a slightly different method to identify mechanisms involved in the supply of amino acids to the growing fetus by the yolk sac during gestation, as well as measuring the endocytic activity. The most common marker substrates used were radiolabelled PVP (Duncan et al 1981, Ibbotson and Williams 1979), ^{14}C-Sucrose (Beckman et al 1994, Ibbotson and Williams 1979), ^{125}I-BSA (Ibbotson and Williams 1979, Kooistra et al 1981), tritiated amino acids such as serine, leucine and methionine (Lloyd et al 1996, Beckman 1997) and radiolabelled IgG (Weisbecker et al 1983).

Substrates such as PVP and sucrose are used to measure only the fluid phase endocytic uptake rate of the yolk sac, there are no enzymes such as sucrase in the visceral yolk sac to digest these two molecules further, therefore, they accumulate in the visceral yolk sac, and uptake can be directly measured without concern about loss of marker by release back into the culture medium (Williams et al 1975 B, Besterman and Low 1983). Some studies have presented their results in the form of Endocytic Index (EI), this is "the rate of pinocytic accumulation of a substrate" (Lloyd 1990); others gave clearance values, which is "the volume of culture medium whose content of radiolabelled substrate has been captured" (Lloyd 1990). Both fluid phase and receptor-mediated methods of endocytosis can be measured with either clearance values or endocytic index. The study described is Chapter 4 uses EI as the means of measuring endocytic rates.

The results presented in Chapter 4 are similar to other studies that have been carried out previously. Beckman et al (1994) used ^{14}C-Sucrose in rat yolk sacs at five time points during gestation and expressed their results as EI. The mean EI

for a rat yolk sac at Day 17.5 was 2.02 per hour, the equivalent value in the study described here for a mouse yolk sac fed on control diet (18% casein) at day 17 of gestation in slightly lower at approximately 1.5. This is not directly comparable as the gestation length in mouse is slightly less than in rat, therefore 17 days in mouse is marginally ahead in its developmental stage than 17 days in rat. The study carried out by Beckman et al (1994) also found that the EI decreased as gestation continued, which conflicts with the data from my fluid phase ^{14}C-sucrose culture experiments. It is clear from Figures 53-55 that the EI increases from day 12 to 14 then decreases by day 17. This anomaly may indicate a difference between the endocytic activity level of the mouse yolk sac compared with the rat yolk sac.

The results show a statistically significant difference in the endocytic index level at day 17 between the two diets. At this later stage of gestation the results of the experiment appear to indicate that there is a higher rate of fluid phase endocytosis occurring in the yolk sacs derived from mothers fed a low protein diet. This may suggest that in order to compensate for the lack of protein available to the fetus, the visceral yolk sac must increase its fluid-phase endocytic activity.

Visceral yolk sacs from the low protein diet group at day 17 gestation had significantly lower rates of endocytic uptake activity when cultured with ^{125}I-BSA, a receptor-mediated and fluid phase marker, and equally significantly lower amino acid release back into the culture medium. BSA is taken up predominately through receptor-mediated endocytosis. Receptors such as megalin and cubilin work together in the coated invaginations of the plasma membrane to bind BSA and incorporate it into the cell. They are then recycled back to the membrane and the BSA passes through the lysosomal pathway. Exogenous albumin is exclusively degraded in the lysosomal system of the yolk sac (Knowles et al 1981). The albumin is digested into amino acids, these are then passed on to the fetus.

Fluid-phase and receptor-mediated endocytosis rates have been shown to vary independently from each other (Jollie 1986 A). Fluid-phase ^{14}C-Sucrose culture experiments do not follow the same pattern of alteration as the receptor-mediated

^{125}I-BSA experiments when the visceral yolk sac is placed under conditions of maternal dietary restriction. However, the fluid-phase endocytosis results do support the view that a maternally restricted diet has metabolic effects upon the endocytic activity of the visceral yolk sac during gestation causing a significant increase of fluid phase activity. This indicates that the visceral yolk sac is trying to compensate for the low maternal protein level available for the developing fetus. Receptor-mediated ^{125}I-BSA endocytosis in the mouse visceral yolk sac showed a significant reduction in rate when the mother was placed in conditions of undernutrition during gestation, which may indicate changes in a less mature endocytic system in the visceral yolk sacs from mothers fed a low protein diet or a reduction in the expression levels of key receptors in the plasma membrane of the visceral yolk sac endoderm cells.

There are other factors that have been identified which cause a change in the endocytic index of the rat yolk sac. Lerman et al (1986) found that culturing the rat yolk sac with anti-rat yolk sac antiserum was teratogenic and caused a reduction in its pinocytic function by up to 40%. Ibbotson et al (1979) found that culturing the yolk sac without serum proteins caused a small increase in the rates of both fluid phase endocytosis and adsorptive endocytosis. Serum proteins would no longer compete with substrate such as ^{127}I-BSA, therefore, more receptors were free to bind BSA and increase its rate of uptake. It is possible that this competition between serum proteins and ^{125}I-BSA is a contributing factor in the reduction in receptor-mediated endocytosis identified in the visceral yolk sacs from the low protein diet group.

Although other studies have shown that the endocytic activity of the visceral yolk sac can be altered in response to availability of various substrates in the culture medium, the effect of changing the physiological conditions of the visceral yolk sac in vivo has not been analyzed previously. My studies may be the first to change the in vivo environment of the visceral yolk sac before analyzing effects on endocytic activity, and do indicate that maternal diet may be a significant regulator of endocytic activity within the mouse visceral yolk sac.

These experiments have shown that feeding a low protein diet to female mice

during pregnancy has an effect upon the metabolic activity of the yolk sac at later stages of gestation. It is clear from Figures 53-55 that the level of fluid-phase endocytic activity rises from day 12 to 14 and then decreases at day 17. This may be because the endocytic role of the visceral yolk sac, although extremely important throughout gestation, begins to slow down as the time of parturition approaches; this has not been seen in the rat visceral yolk sac (Sorokin and Padykula 1964, Beckman et al 1994).

The results presented in Chapter 4 from culturing visceral yolk sacs with two different marker substrates *in vitro* at 17 days gestation, show that when visceral yolk sacs are exposed to a maternal low protein diet they react with a reduction in the receptor-mediated uptake and release of protein to the fetus, despite a compensatory mechanism increasing the amount of fluid phase activity that occurs.

Initial electron microscope studies of the rodent yolk sac were carried out in the early 1900s, Brunschwig (1927) and Everett (1933) were the first to identify the rat yolk sac as "an organ of exchange whose importance is not secondary to that of the chorioallantoic placenta" (Everett, 1933) (Jollie 1986 A). It was not until Dempsey (1953), nearly 20 years later, that the fine structure of the rodent (guinea pig) visceral yolk sac was studied in more depth (Jollie 1986 A). Further studies have continued to establish the visceral yolk sac as highly endocytic and adaptive organ (Sorokin and Padykula 1964, Calarco and Moyer 1966). My investigation thus incorporated an electron microscope study of the visceral yolk sac with the aim of investigating any morphological changes that may occur due to maternal undernutrition during gestation.

My investigation into the effects of maternal dietary restriction upon the mouse visceral yolk sac is supported by the morphology displayed in the electron micrographs (Figures 63-74). Analytical studies of the micrographs produced showed a difference in the fine structure of the visceral yolk sac between the two diet groups; changes that although are not apparent at the level of changes in weight become more visible at the cellular level. The results of the analysis revealed significantly more macropinocytic vesicles per cell in the less villous

region of visceral yolk sacs from mothers fed a low protein diets compared with those from a control diet. There was also a trend showing less secondary lysosomes in the less villous region of visceral yolk sacs from low protein diet fed mothers compared to visceral yolk sacs from mothers fed a control diet up to 17 days gestation. There was no significant difference in the size of the endodermal cells of the visceral yolk sacs between the two diets although as the cell size increased the number of macropinocytic or secondary lysosomes increased significantly.

The visceral yolk sacs from mothers fed a low protein diet appear to have increased numbers of apically situated translucent vesicles which correlate with the increased rate of fluid-phase endocytic activity. The visceral yolk sacs from mothers fed a control diet (18% casein) throughout gestation appear to have an increased number of dense basally situated lysosomes which is concurrent with a higher level of receptor-mediated endocytosis compared to visceral yolk sacs from the low protein diet (9% casein) group.

The large number of phagolysosomes and macropinocytic vesicles apparent in the visceral endoderm of the mouse yolk sac is indicative of the highly endocytic nature of this structure, as is the presence of numerous mitochondria and the vast quantity of rough endoplasmic reticulum that can be easily identified in the micrographs displayed in the Chapter 5 (Calarco and Moyer 1966, Jollie 1986 A). The observations from the micrographs in Chapter 5 may relate directly to the dietary restriction imposed. When linked with the metabolic data displayed in Chapter 4 concerning the receptor-mediated ^{125}I-BSA culture experiments (Figure 57-60), which show that overall less protein was digested and released into the medium during culture by the visceral yolk sacs from mothers fed a low protein diet during gestation than by those fed a control diet. Therefore, the idea can be proposed that maybe there are less proteases available in the low protein diet visceral yolk sacs to digest the protein taken up so it is simple released undigested back into the culture medium.

Jollie's review article (1986) explains how tracers used to show fluid phase endocytosis in polarized cellular systems such as the visceral yolk sac were found

localized to the apical surface of the cells within minutes of exposure; whereas tracers for receptor-mediated endocytosis were seen closely associated with the plasma membrane and bound to invaginating pits of the membrane then in dense lysosomal bodies found more basally located within the cells. This evidence supports what is seen in the micrographs in Chapter 5 regarding the changes in the visceral yolk sac ultrastructure caused by maternal undernutrition during gestation (Figure 63-74).

My study progressed to investigate the effect of maternal diet on megalin and cathepsin L. Megalin is expressed in clathrin-coated membrane areas of the visceral yolk sac (Le Panse et al 1995), it is expressed in the embryo from the 16 cell stage and throughout gestation (Gueth-Hallonet et al 1994). Megalin is recycled through the network of dense apical tubules in the visceral yolk sac (Baricault et al 1995). Since changes have been seen in the morphology of the visceral yolk sac in response to maternal undernutrition, it would be possible that changes may occur in the expression level of megalin in this context. There was a slight elevation in the expression level of megalin although not statistically significant. Further studies should investigate cubilin which acts in association with megalin in the plasma membrane to internalize ligands (Christensen et al 2002a,b, Moestrup et al 2001) .

Cathepsins are a group of lysosomal cysteine proteases employed in the cell to accomplish numerous functions including antigen presentation, prohormone processing, tumour invasion and angiogenesis. Primarily, the function of cathepsins is to break down proteins into amino acids within lysosomes (Felbor et al 2002). Cathepsins B, H and L are expressed in the visceral yolk sac, the placenta and trophoblast giant cells during gestation, they are important in order to provide the growing fetus with the amino acids that it requires (Beck and Lowy 1982, Sol-Church et al 1999). My study showed that there was a slight although not significant increase in cathepsin L expression in visceral yolk sacs from mothers fed a low protein diet compared to those from mothers fed a control diet up to day 17 gestation. It has been previously shown that increased levels of cathepsin L expression increase the capacity of the visceral yolk sac for proteolysis thereby providing amino acids for embryonic nutrition (Sol-Church et

al 1999), this may therefore explain the slight increase in expression that I identified in my study.

Summary

Low protein diet has been shown to lead to altered birth weight, raised systolic blood pressure throughout life, increased adiposity and insulin resistance (Langley-Evans et al 1996, Matthews 2002). My thesis concentrated upon the function of the visceral yolk sac during gestation, using a mouse model to investigate how maternal low protein diet may affect the role of the visceral yolk sac as a nutrient provision and how conceptus growth may be affected.

Maternal dietary restriction during gestation did not alter the growth of the conceptus, fetus, placenta and visceral yolk sac at day 12, 14 and 17 gestation. The litter size from the mother and the position of the conceptus within the uterus did not significantly affect most of the variables investigated. There were three occasions upon which the position of the conceptus did significantly affect either the weight or the total protein content.

The fluid phase endocytic activity of the mouse visceral yolk sac significantly increased at day 17 gestation in response to low protein diet. No difference in fluid phase endocytosis activity was apparent at day 12 or day 14 gestation. The elevation in endocytic activity at day 17 in response to maternal low protein diet may represent a 'compensatory' mechanism. Receptor-mediated endocytosis significantly decreased in visceral yolk sacs at day 17 gestation from the low protein diet group, suggesting a possible reduction in the efficiency of the receptor activity in the plasma membrane or in the lysosomal pathway.

Electron microscope investigations support the endocytic activity data gained form the endocytic activity culture experiments. A significant increase in the quantity of apically-situated translucent macropinocytic vesicles was observed in the visceral yolk sacs from mothers fed on low protein diet to 17 days gestation, concurrent with an increase in fluid phase endocytic activity. The visceral yolk sacs from mothers fed a control diet up to 17 days gestation, showed an increase

in basally-situated dense phagolysosomes compared with visceral yolk sacs from low protein diet group. This may be indicative of the decrease in receptor-mediated endocytosis seen in the visceral yolk sacs from mothers fed a low protein diet up to 17 days gestation.

Immunoblotting revealed a slight, though not significant, increase in expression levels of megalin and cathepsin L in visceral yolk sacs from mothers fed a low protein diet up to day 17 gestation. This result is not statistically significant therefore it is not robust enough to state that maternal undernutrition affects expression of these particular receptors at the plasma membrane and lysosomes within the cells. Thus it would be advisable to conduct further experiments on these and alternative candidates.

My study has shown that maternal undernutrition during gestation does have a significant effect upon the visceral yolk sac and specific mechanisms within it. Overall there were no significant changes in weight of the conceptus, fetus placenta and visceral yolk sac due to maternal low protein diet. Endocytic activity, both fluid-phase and receptor-mediated, alters under the influence of maternal low protein diet. This change was supported with changes in the morphology of the visceral yolk sac from mothers fed a low protein diet. Together they may be indicative of some of the programming changes which will go on to cause adult onset diseases. Investigations at a molecular level for changes in the receptor megalin expression and changes in the cysteine lysosomal protease cathepsin L did not show significant alterations caused by maternal low protein diet. However, further study of other candidate receptors and lysosomes may yield significant results.

Appendix 1

1. Phosphate buffer:

Stock A= 0.2M sodium dihydrogen orthophosphate (MW 156)

3.12 g in 100cm3 distilled water

Stock B = 0.2M disodium hydrogen orthophosphate (MW 177)

3.55 g in 100cm3 distilled water.

To make the buffer at pH 6.4, add 36.7 ml of stock A to 13.3 ml of stock B then make up to 100 ml with distilled water.

2. Transfer Buffer (pH 8.3)

6.06 g Tris (25 mM)

22.5 g Glycine (192 mM)

2.0 g SDS

400 ml Methanol (20%)

- make up 2 litres using distilled water.

Appendix 2

Day 12 of gestation weight correlation graphs

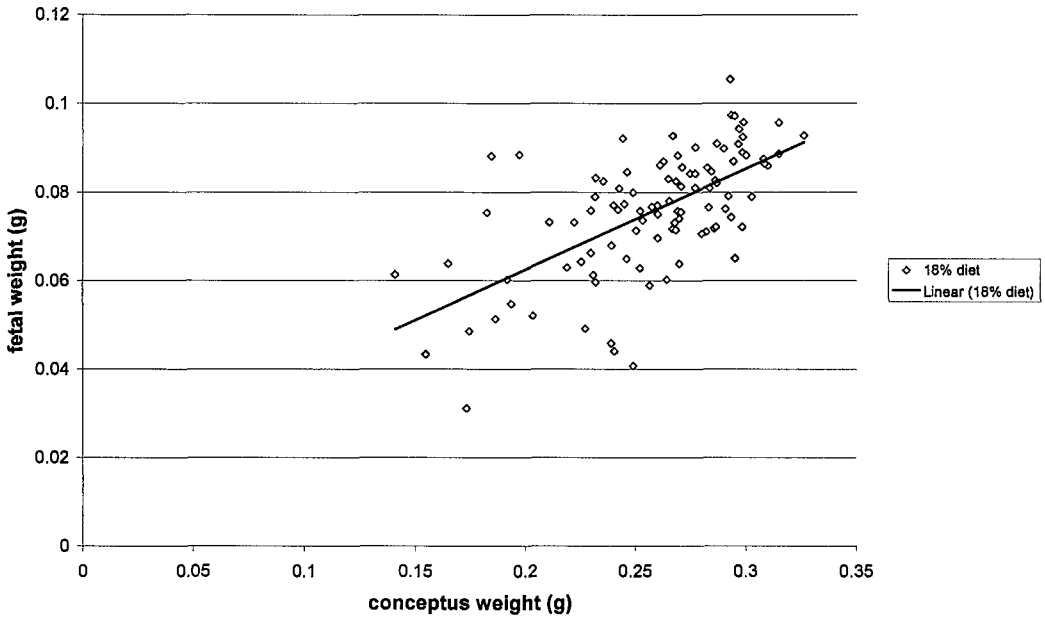


Figure 88: Correlation of Conceptus weight and Fetal weight in control diet fed mice at day 12 gestation. n=100

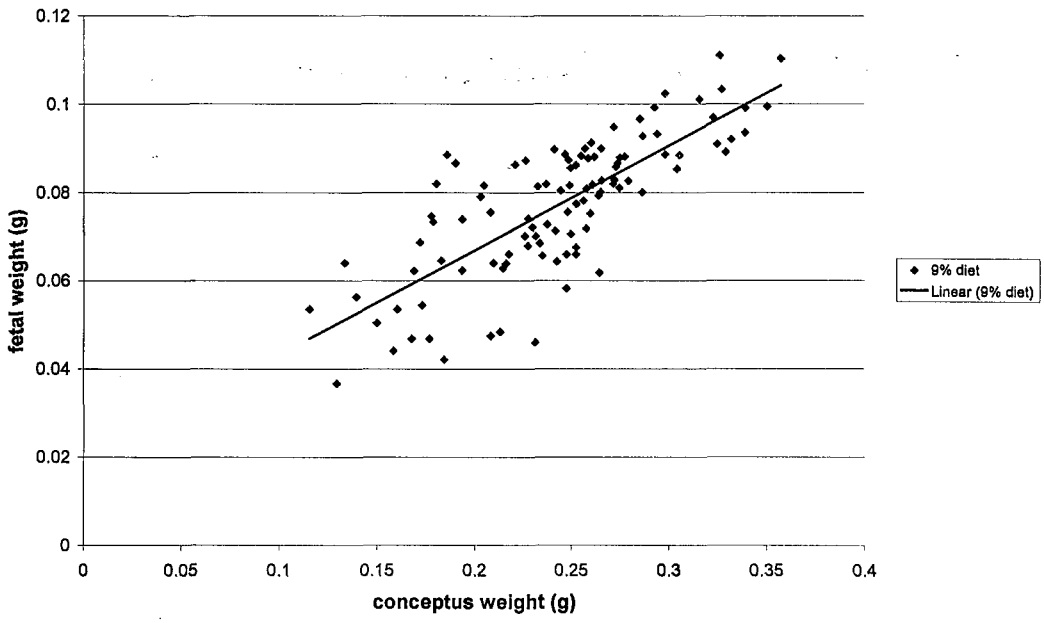


Figure 89: Correlation of Conceptus weight and fetal weight from mice fed a low protein diet up to day 12 gestation. n=103

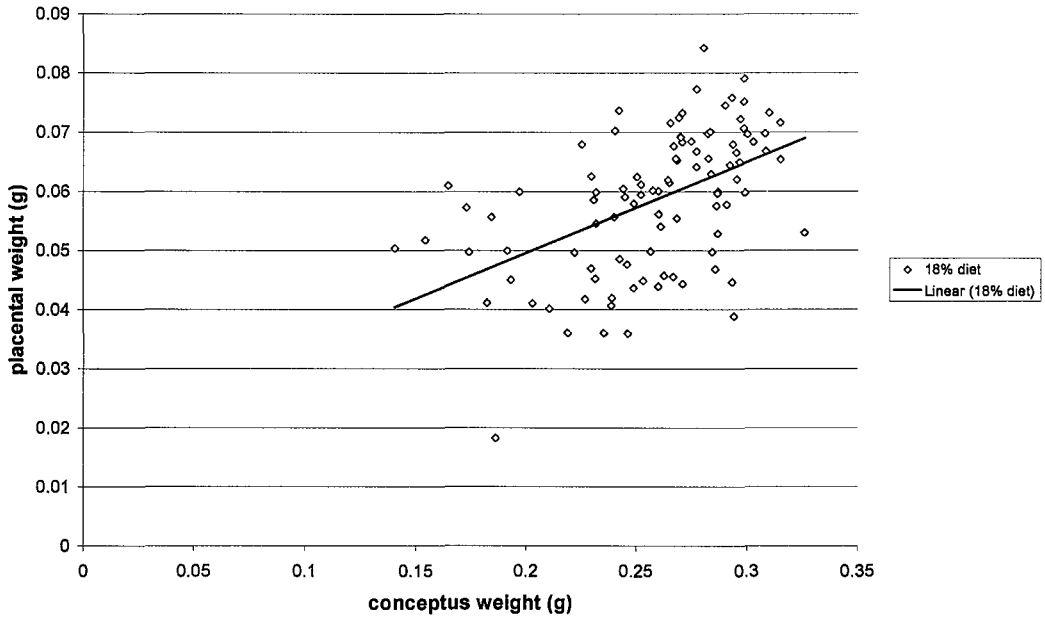


Figure 90: Correlation of Conceptus weight and Placental weight in mice fed a control diet up to day 12 gestation. n=100

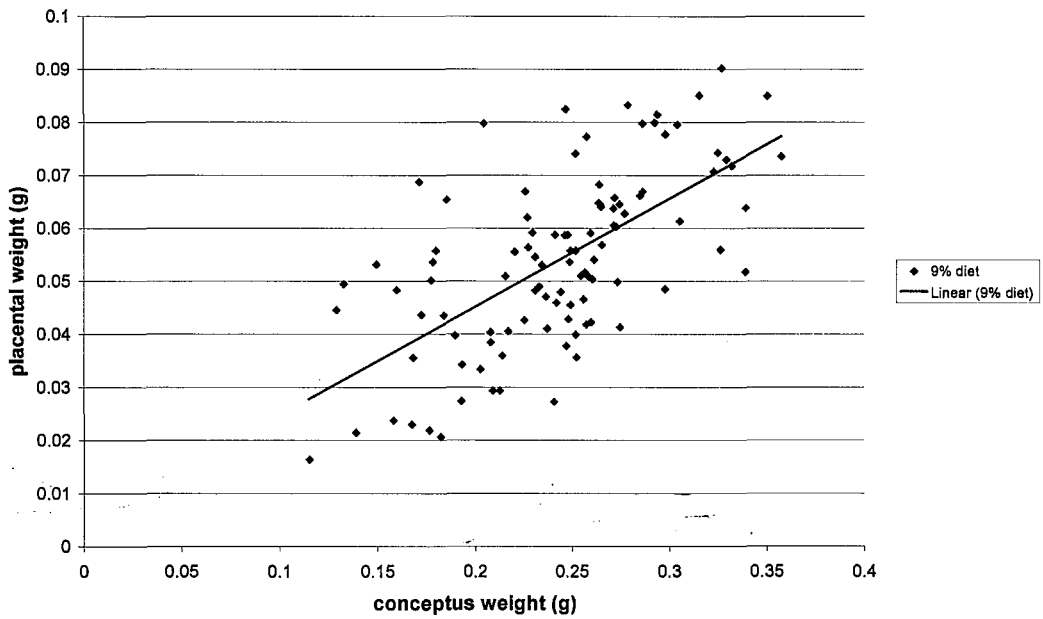


Figure 91: Correlation of Conceptus weight and Placental weight in mice fed a low protein diet up to day 12 gestation. n=103

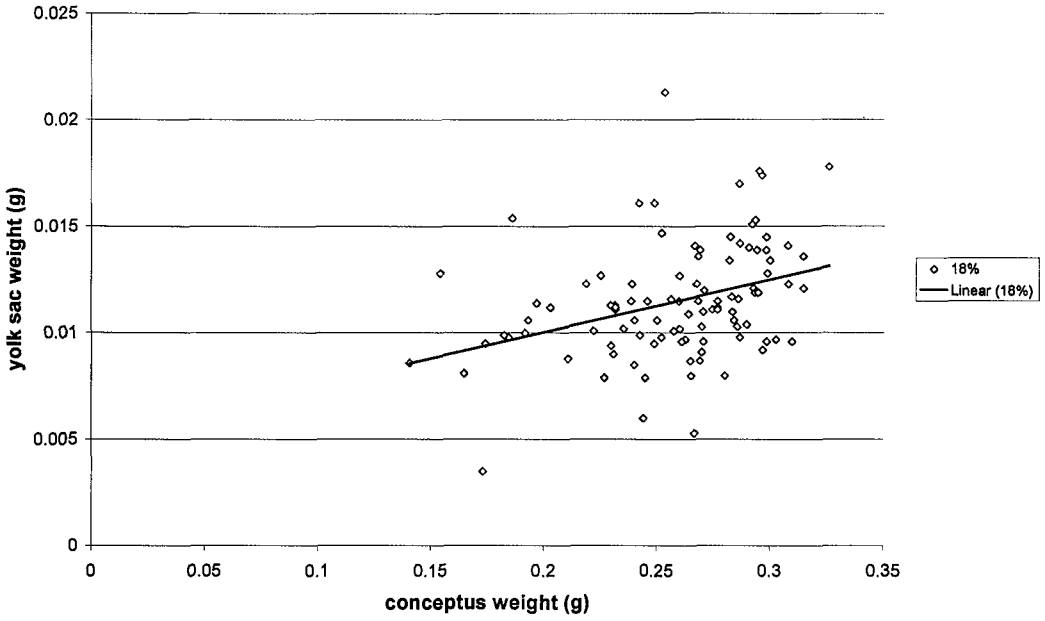


Figure 92: Correlation of Conceptus weight and yolk sac weight in mice fed a control diet up to day 12 gestation. n=100

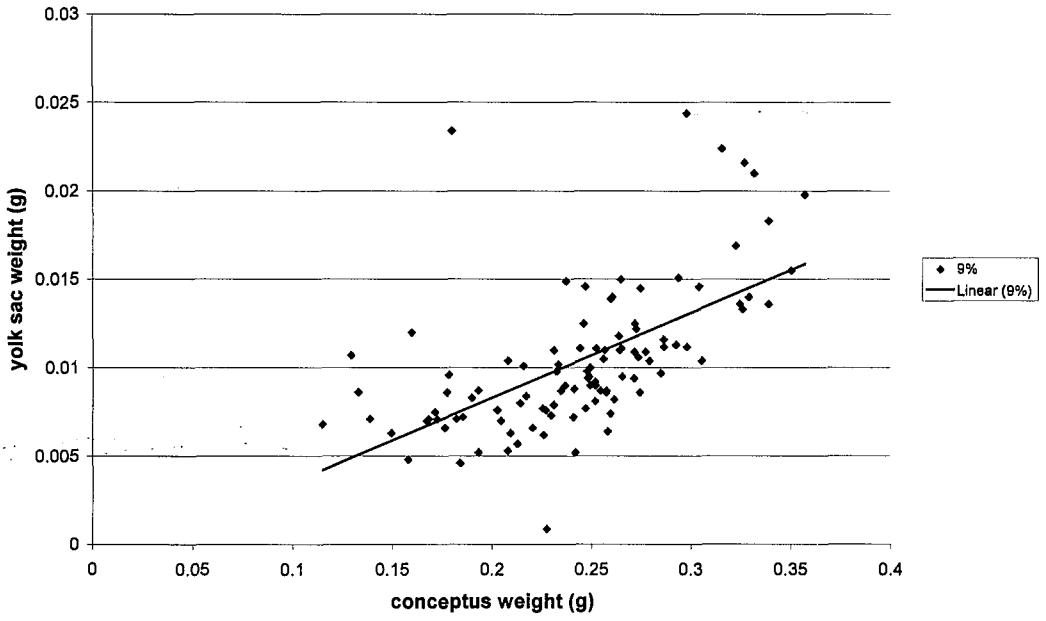


Figure 93: Correlation of Conceptus weight and yolk sac weight in mice fed a low protein diet up to day 12 gestation. n=103

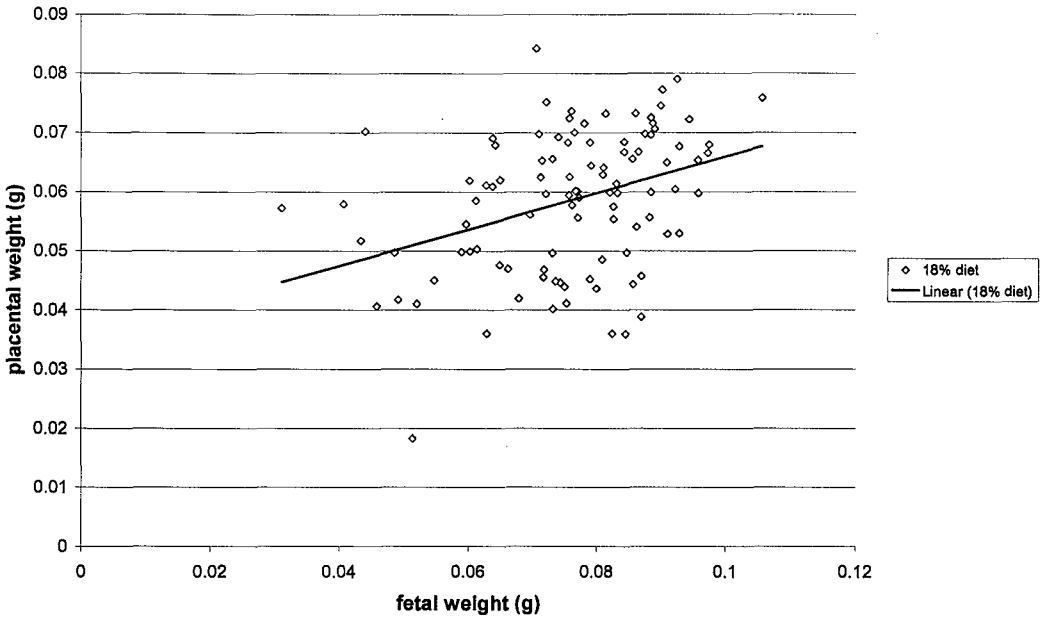


Figure 94: Correlation of Fetal weight and Placental weight in mice fed a control diet up to day 12 gestation. n=100

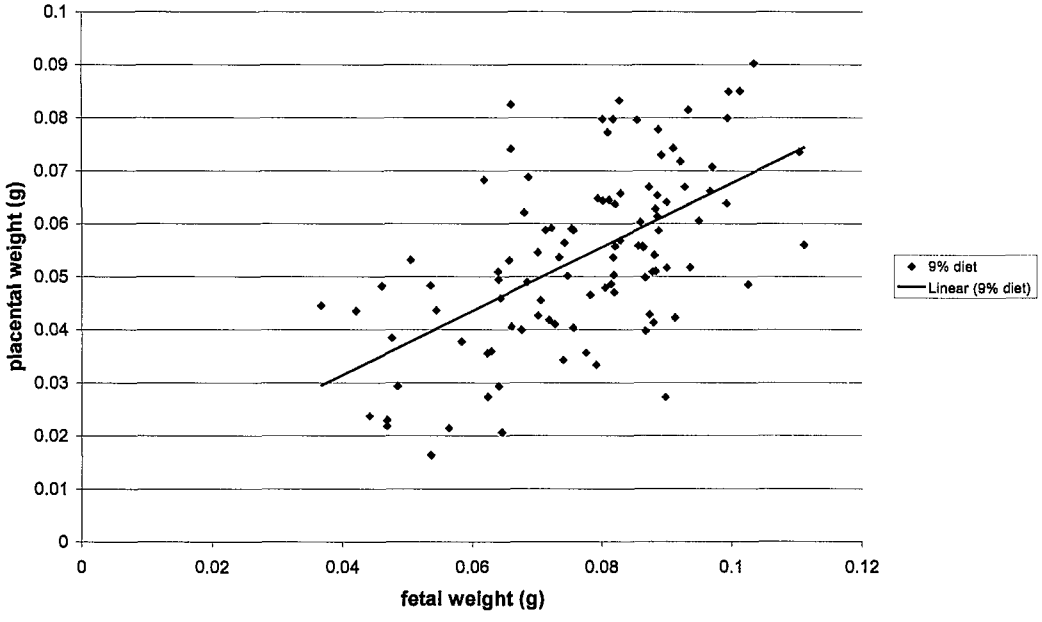


Figure 95: Correlation of fetal weight and Placental weight in mice fed a low protein diet up to day 12 gestation. n=103

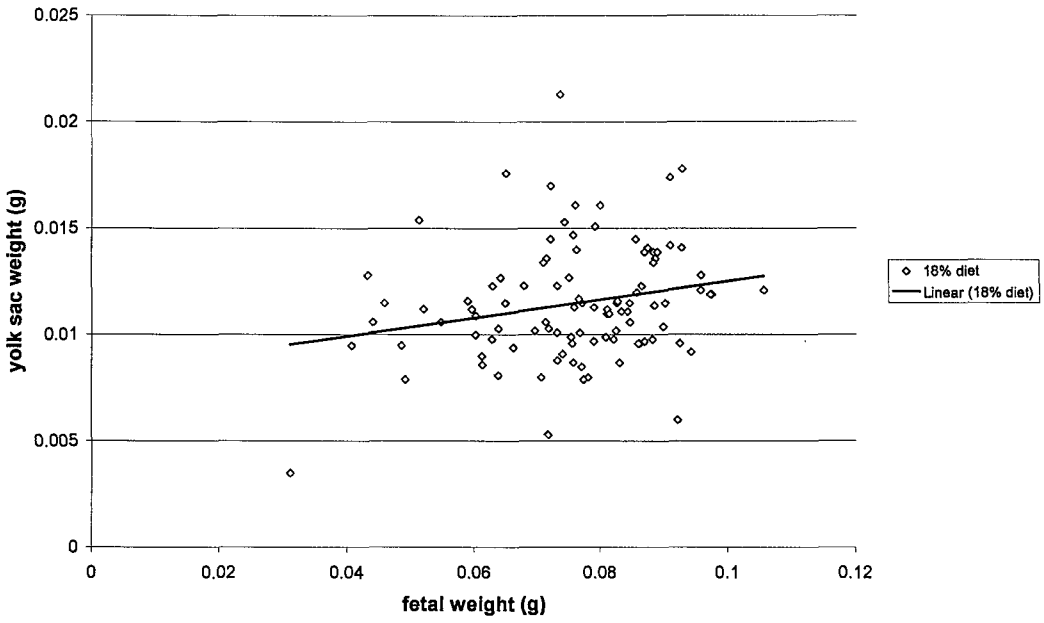


Figure 96: Correlation of fetal weight and yolk sac weight in mice fed a control diet up to day 12 gestation. n=100

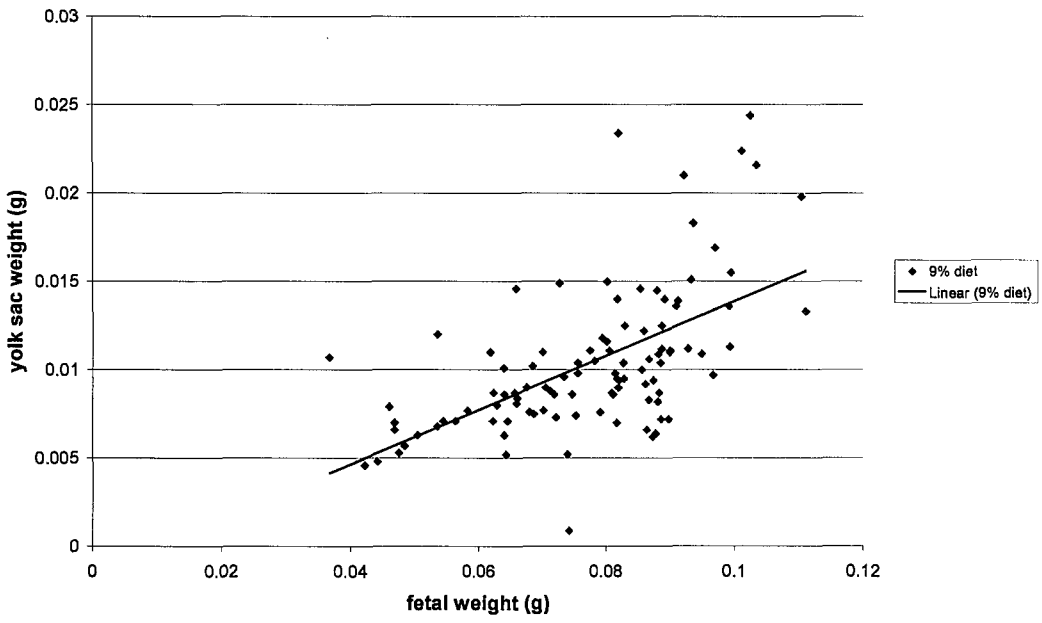


Figure 97: Correlation of fetal weight and yolk sac weight in mice fed a low protein diet up to day 12 gestation. n=103

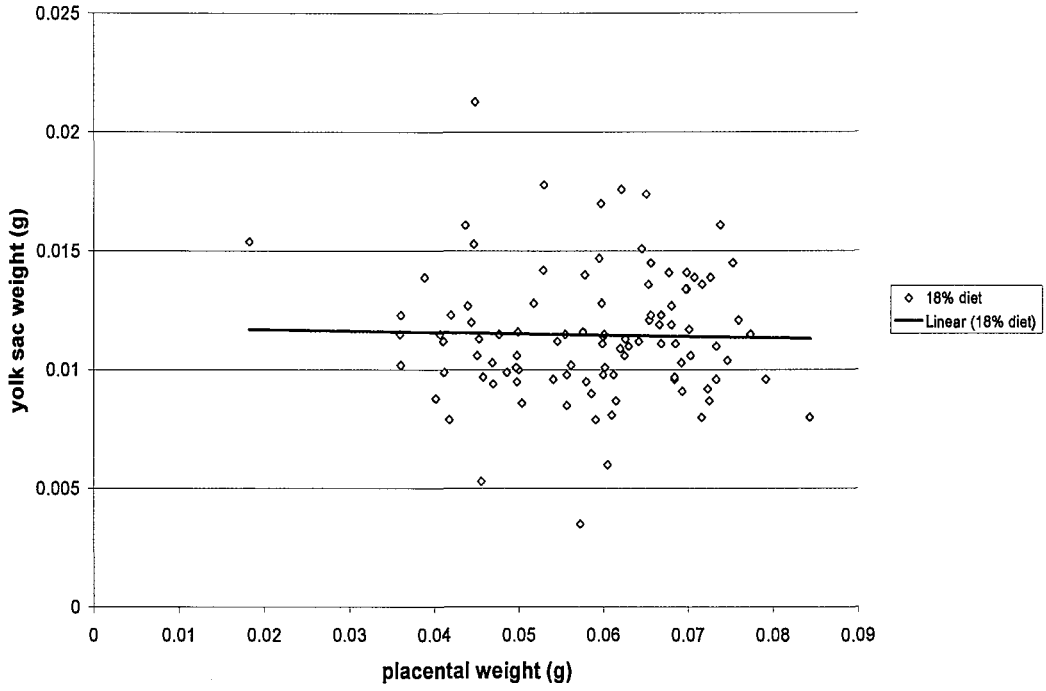


Figure 98: Correlation of placental weight and yolk sac weight in mice fed a control diet up to day 12 gestation. n=100

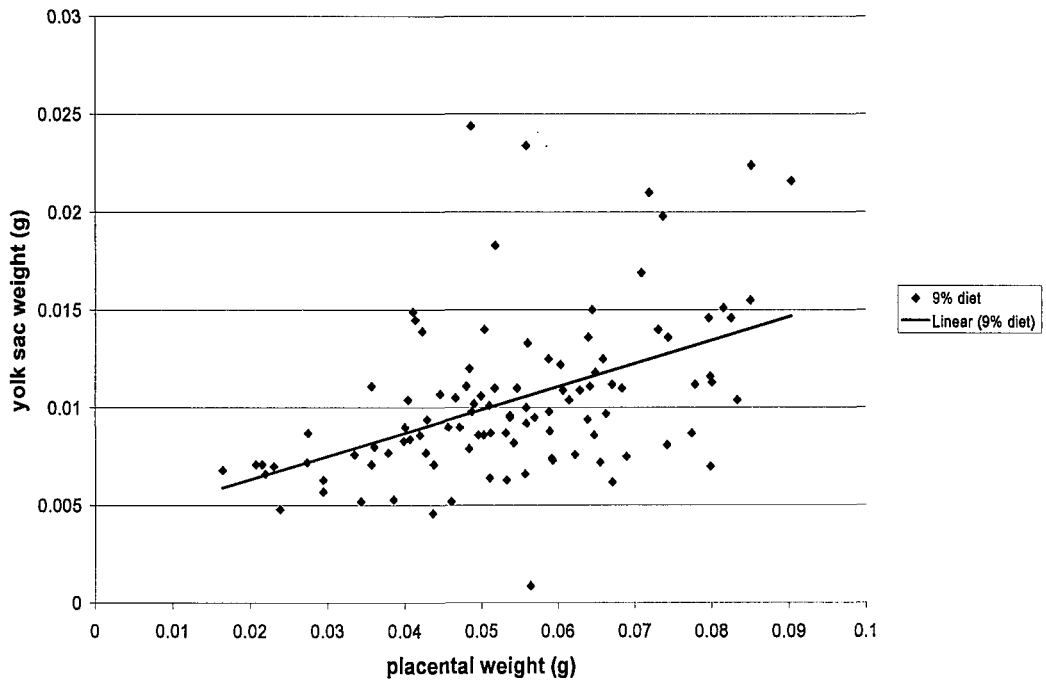


Figure 99: Correlation of placental weight and yolk sac weight in mice fed a low protein diet up to day 12 gestation. n=103

Day 14 of gestation, weight correlation graphs

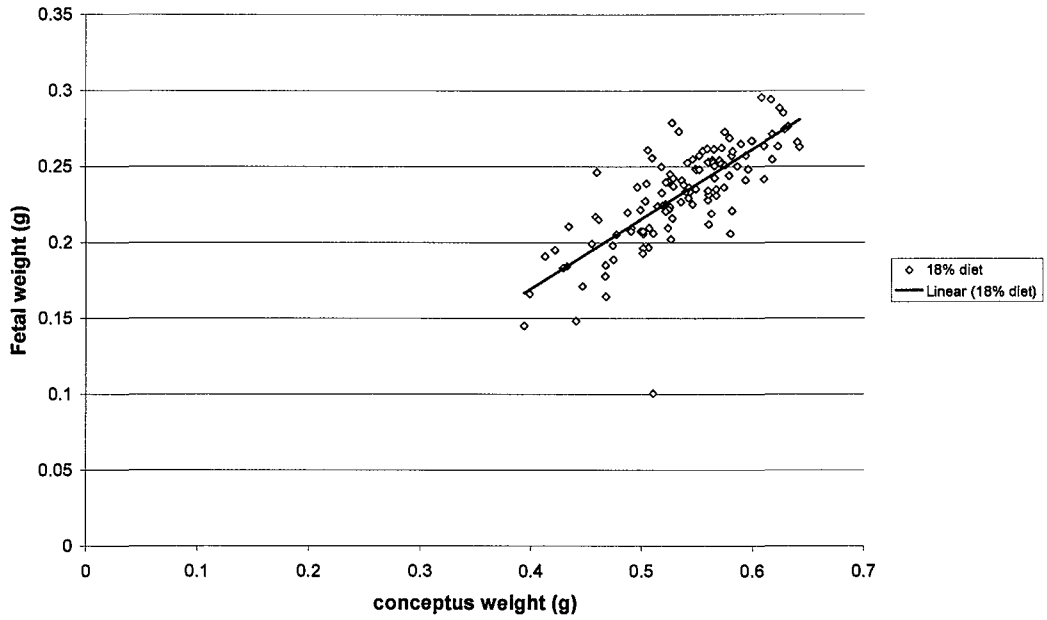


Figure100: Correlation of Conceptus weight and fetal weight in mice fed a control diet up to day 14 gestation. n=117

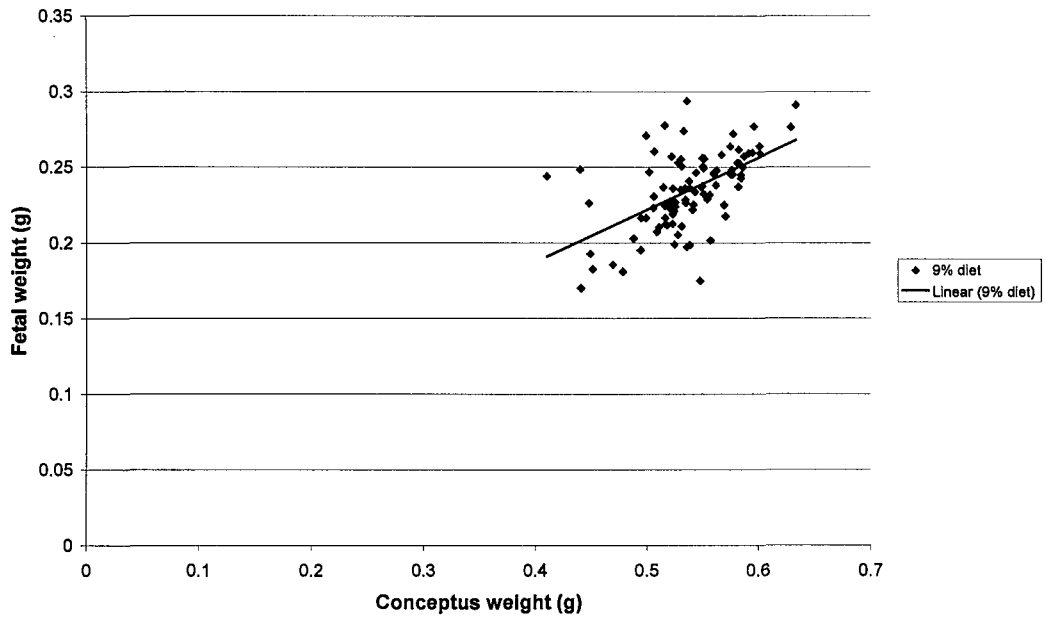


Figure 591: Correlation of Conceptus weight and fetal weight in mice fed a low protein diet up to day 14 gestation. n=94

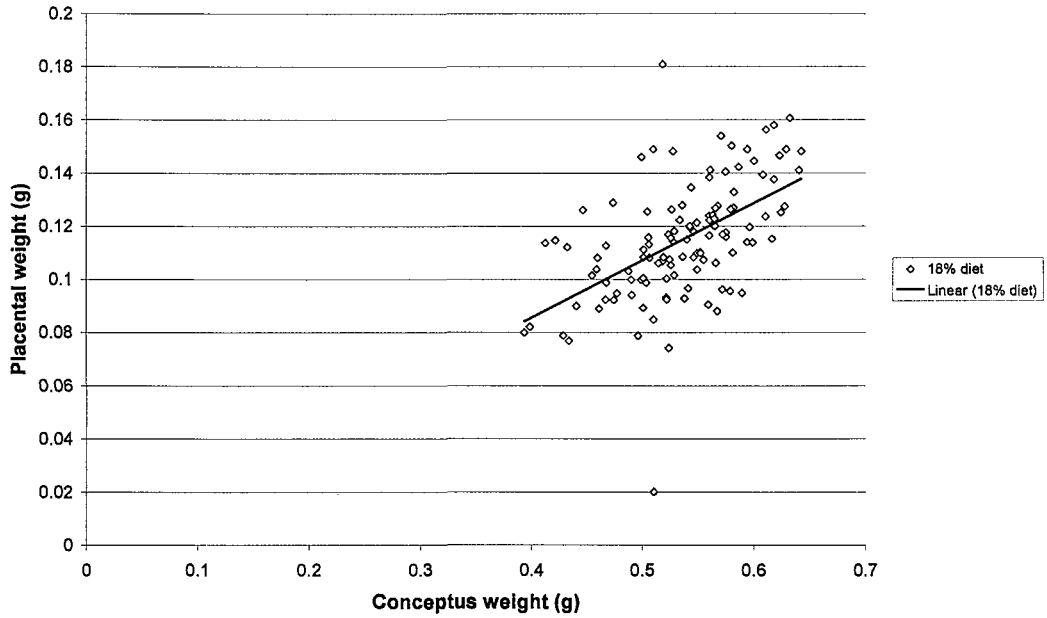


Figure 102: Correlation of Conceptus weight and placental weight in mice fed a control diet up to day 14 gestation. n=117

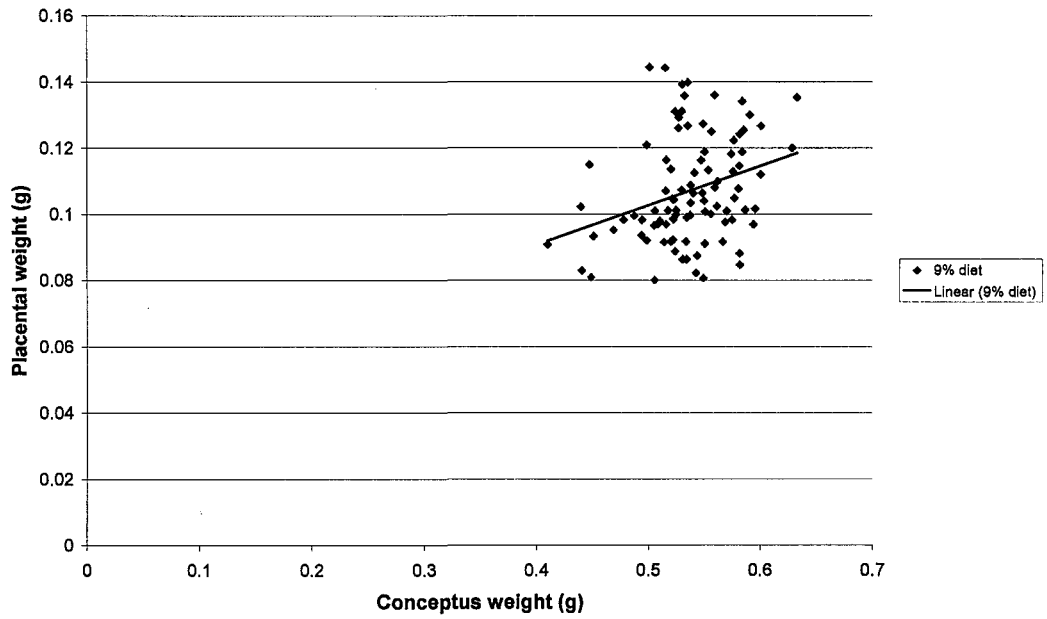


Figure 103: Correlation of Conceptus weight and placental weight in mice fed a low protein diet up to day 14 gestation. n=94

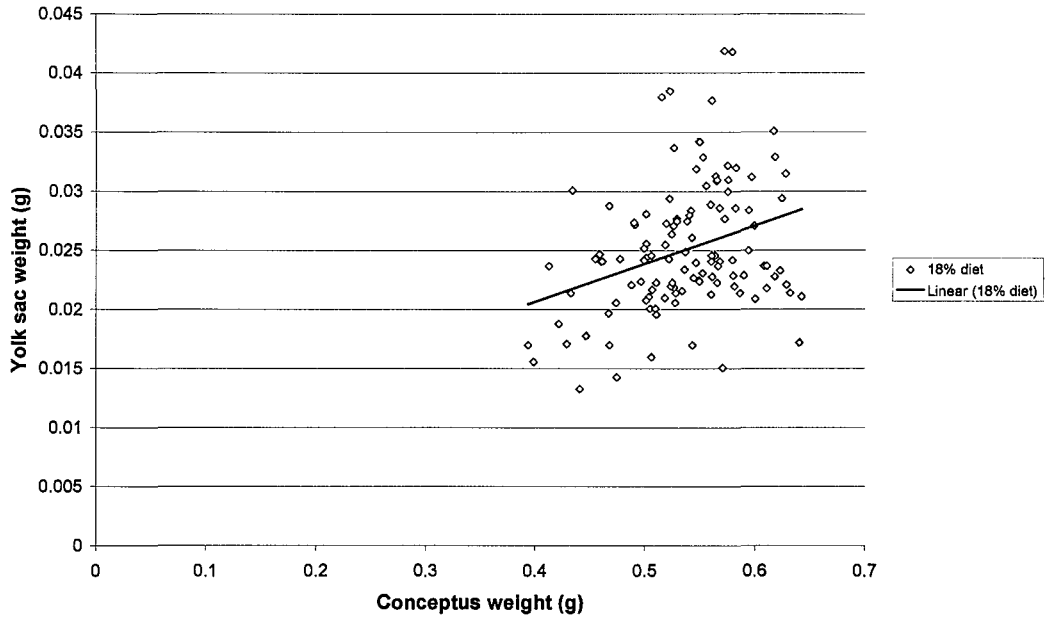


Figure 104: Correlation of Conceptus weight and visceral yolk sac weight in mice fed a control diet up to day 14 gestation. n=117

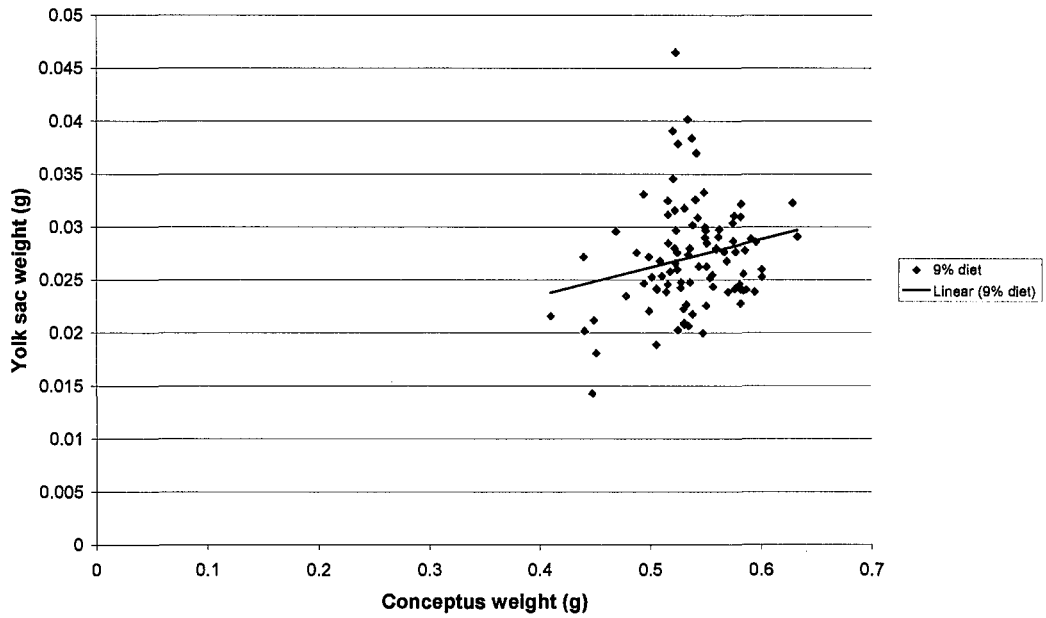


Figure 105: Correlation of Conceptus weight and visceral yolk sac weight in mice fed a low protein diet up to day 14 gestation. n=94

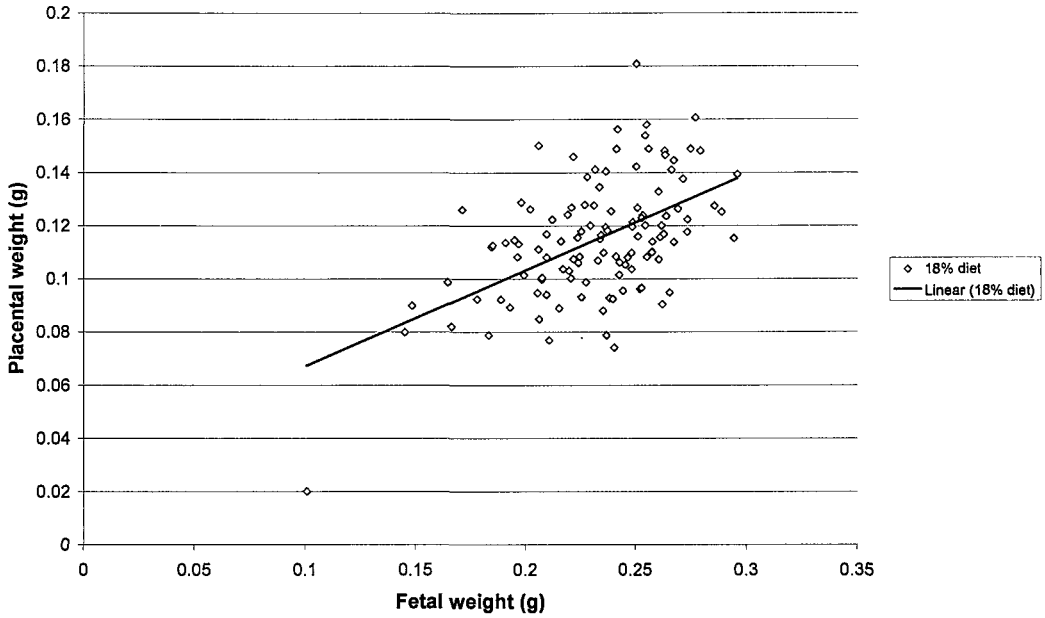


Figure 606: Correlation of fetal weight and placental weight in mice fed a control diet up to day 14 gestation. n=117

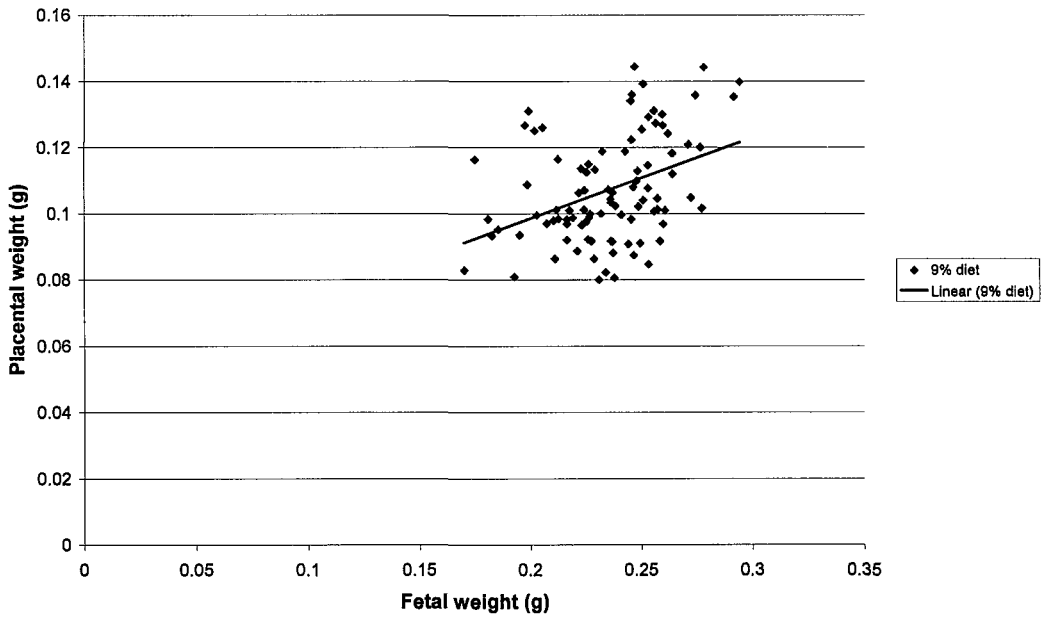


Figure 107: Correlation of fetal weight and placental weight in mice fed a low protein diet up to day 14 gestation. n=94

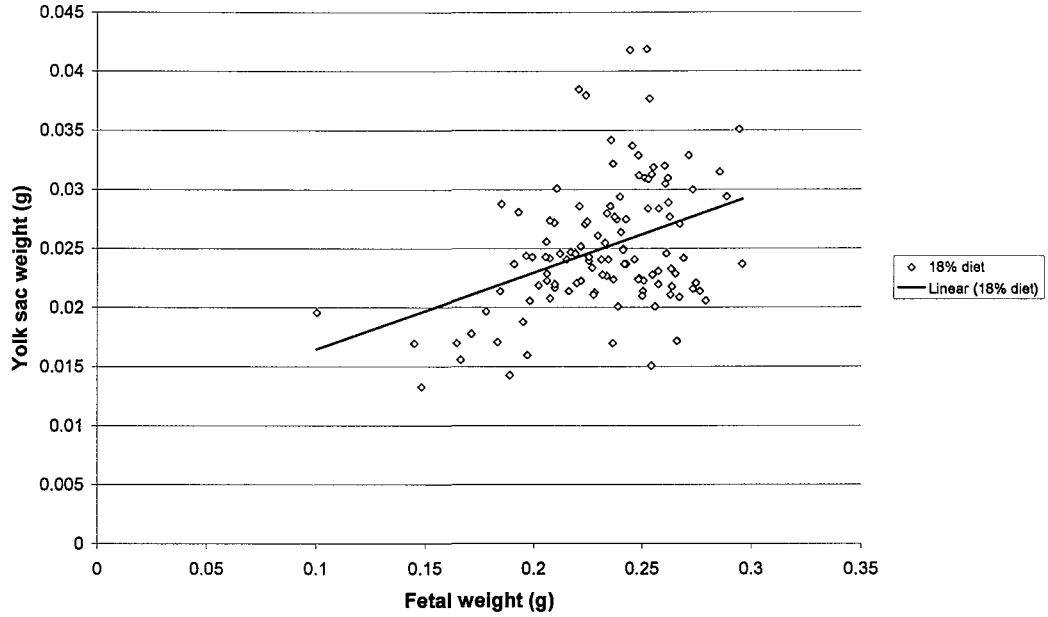


Figure 108: Correlation of fetal weight and yolk sac weight in mice fed a control diet up to day 14 gestation. n=117

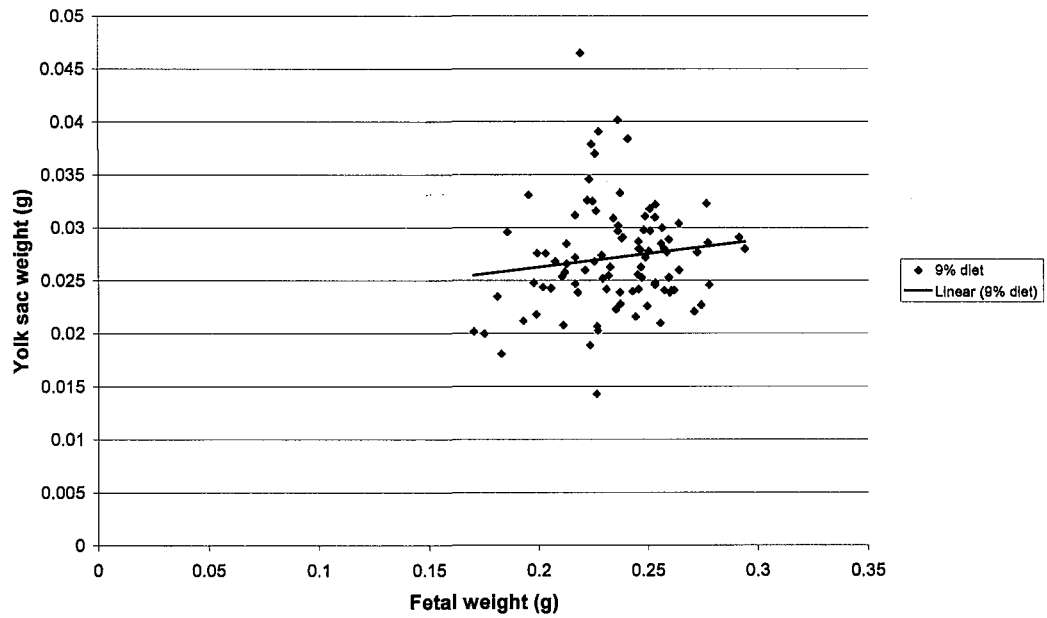


Figure 109: Correlation of fetal weight and yolk sac weight in mice fed a low protein diet up to day 14 gestation. n=94

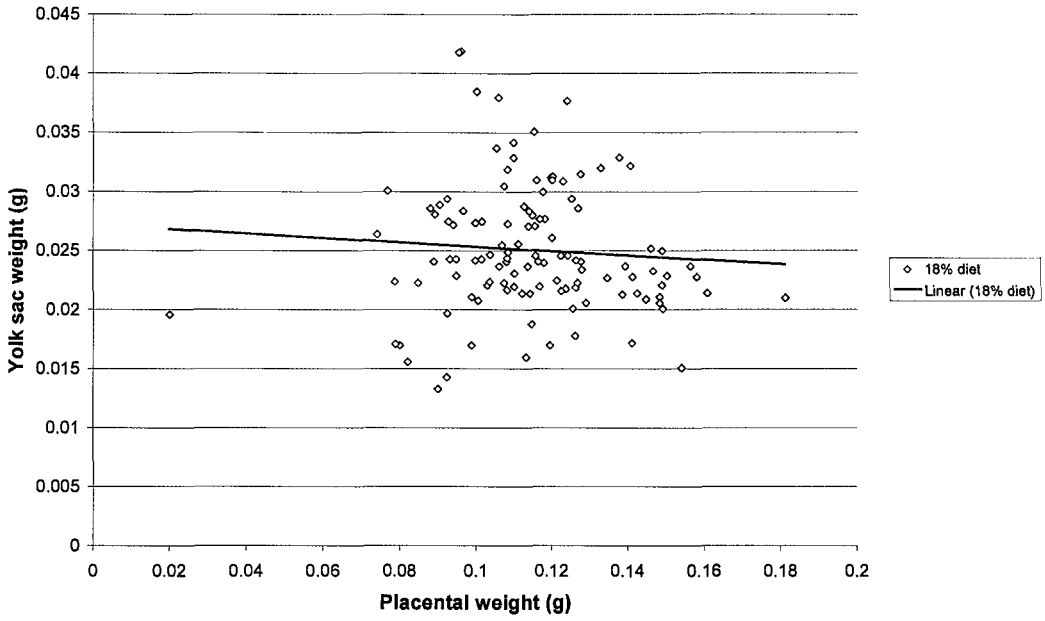


Figure 110: Correlation of placental weight and visceral yolk sac weight in mice fed a control diet up to day 14 gestation. n=117

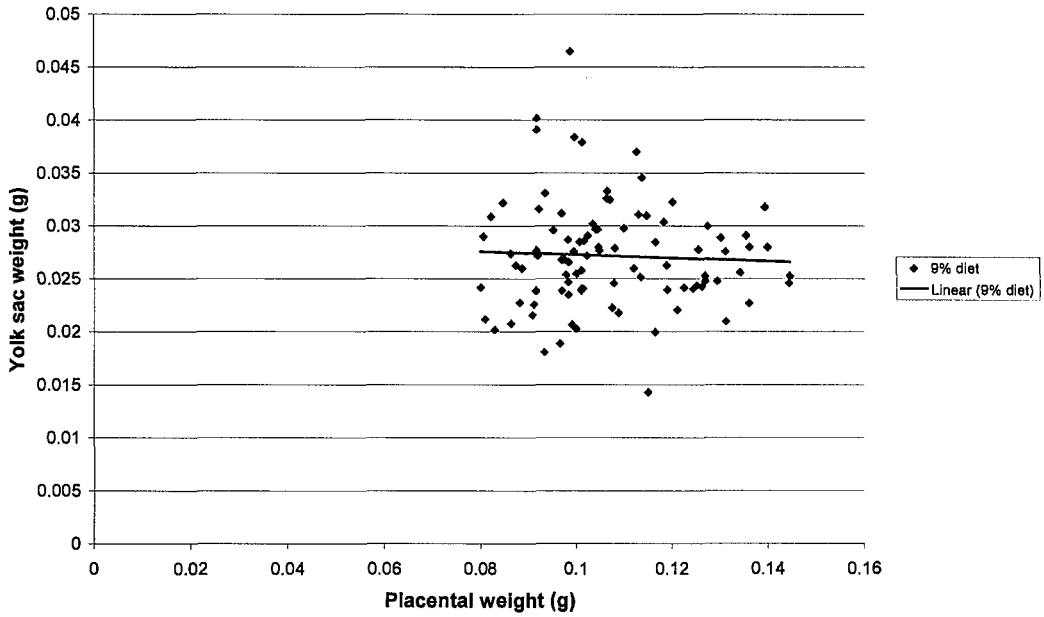


Figure 111: Correlation of placental weight and visceral yolk sac weight in mice fed a low protein diet up to day 14 gestation. n=94

Appendix 3

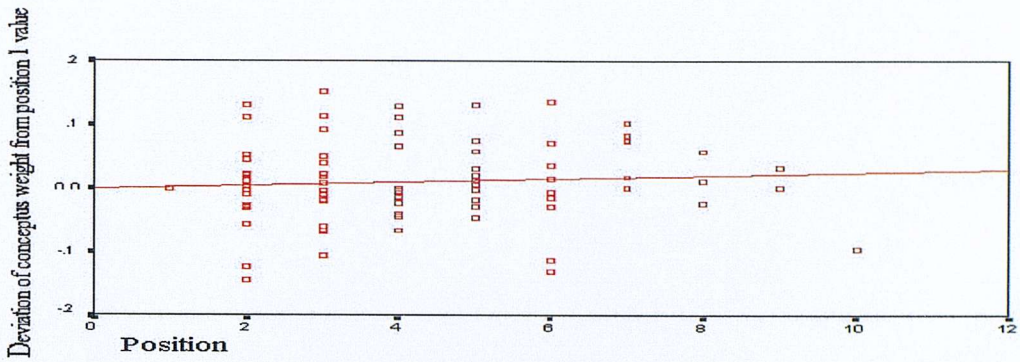


Figure 112: Deviation in conceptus weight from mothers fed control diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.343 N = 100

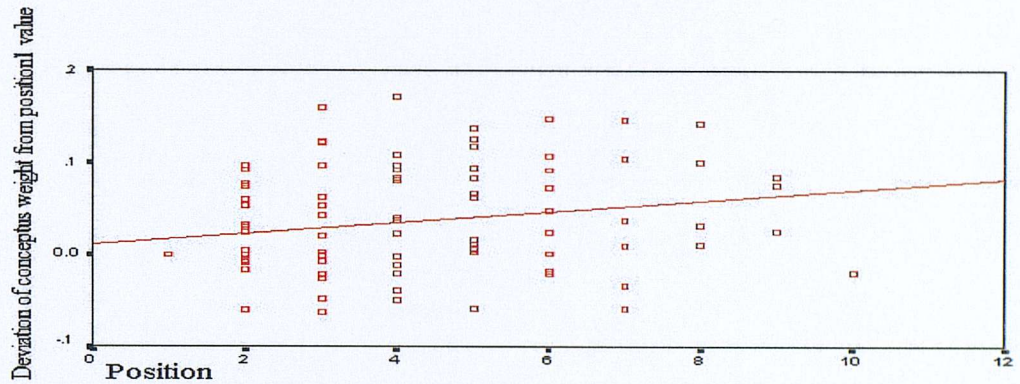


Figure 113: Deviation in conceptus weight from mothers fed low protein diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.016 N = 103

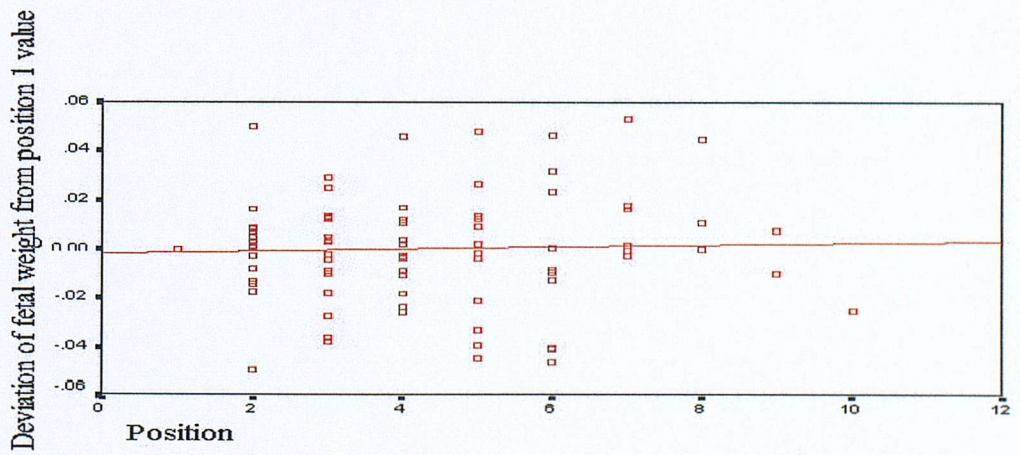


Figure 114: Deviation in fetus weight from mothers fed control diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.124 N = 100

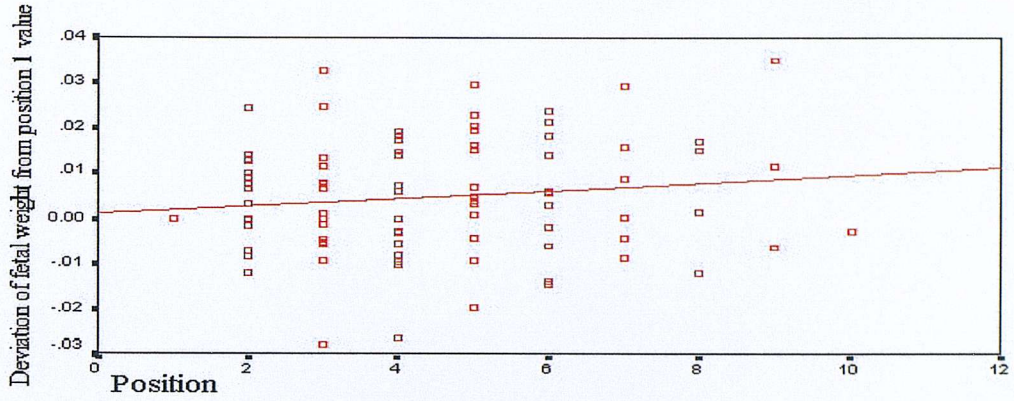


Figure 115: Deviation in fetal weight from mothers fed low protein diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.521 N = 103

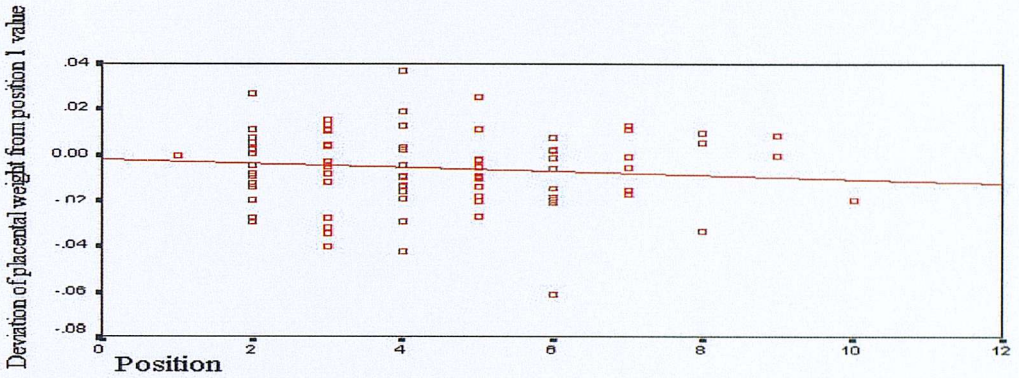


Figure 116: Deviation in placental weight from mothers fed control diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.003 N = 100

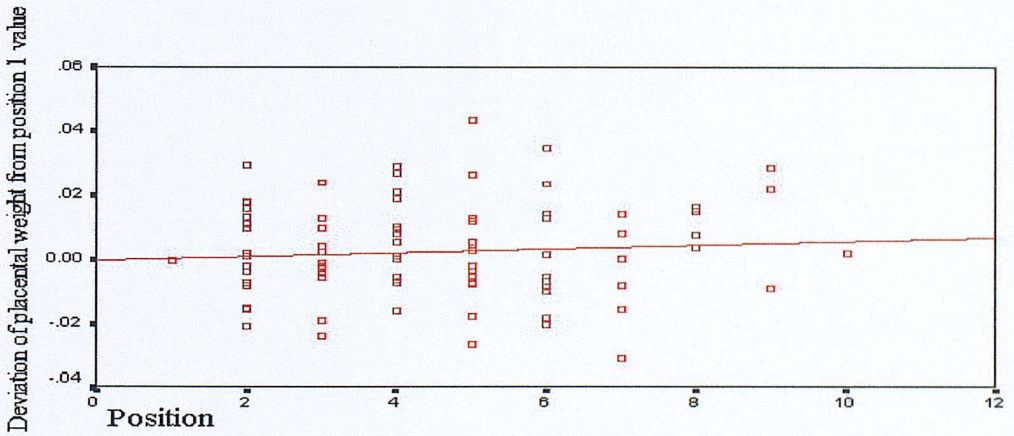


Figure 117: Deviation in placenta weight from mothers fed low protein diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.778 N = 103

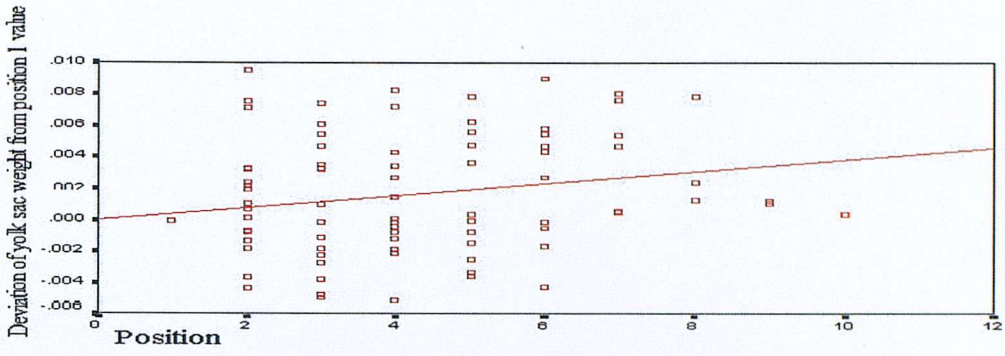


Figure 118: Deviation in visceral yolk sac weight from mothers fed control diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.388 N = 100

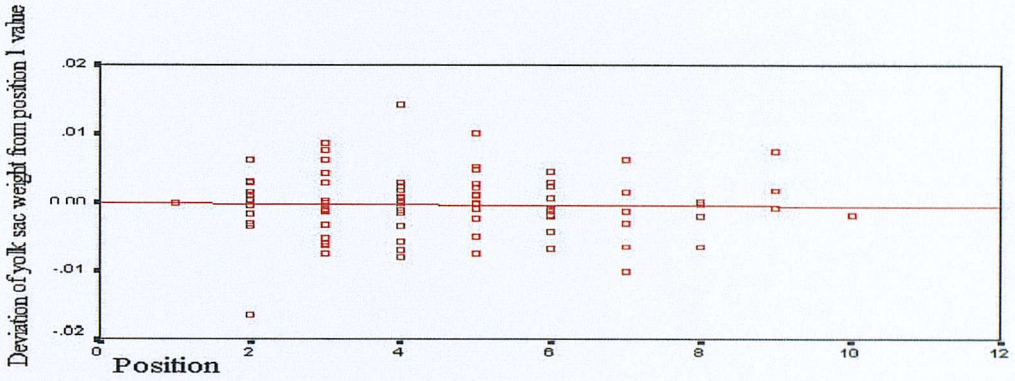


Figure 119: Deviation in visceral yolk sac weight from mothers fed low protein diet up to 12 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.761 N = 103

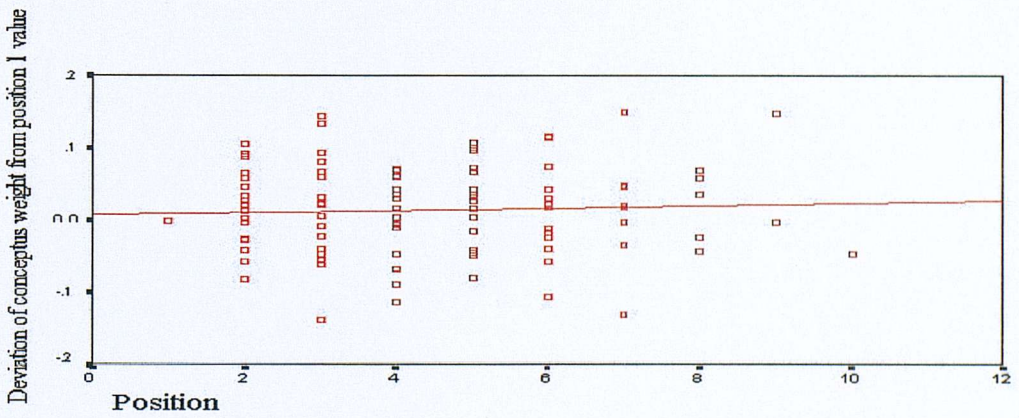


Figure 120: Deviation in conceptus weight from mothers fed control diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.602 N = 117

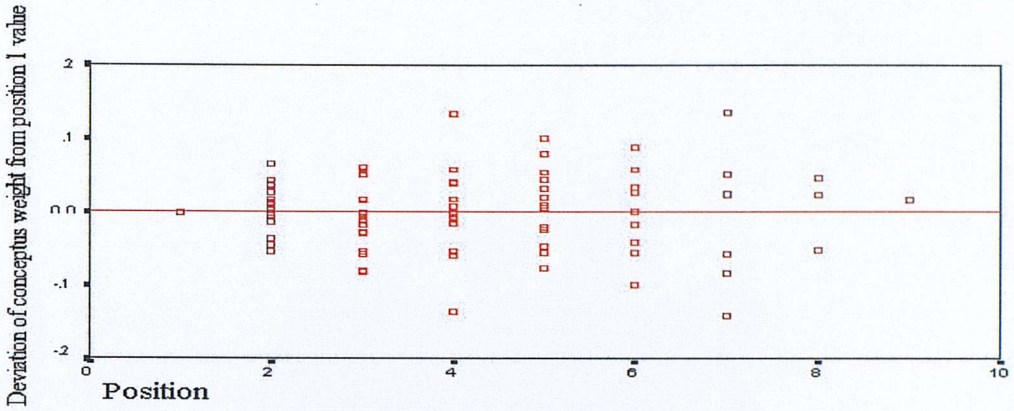


Figure 121: Deviation in conceptus weight from mothers fed low protein diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). 0.775 N = 94

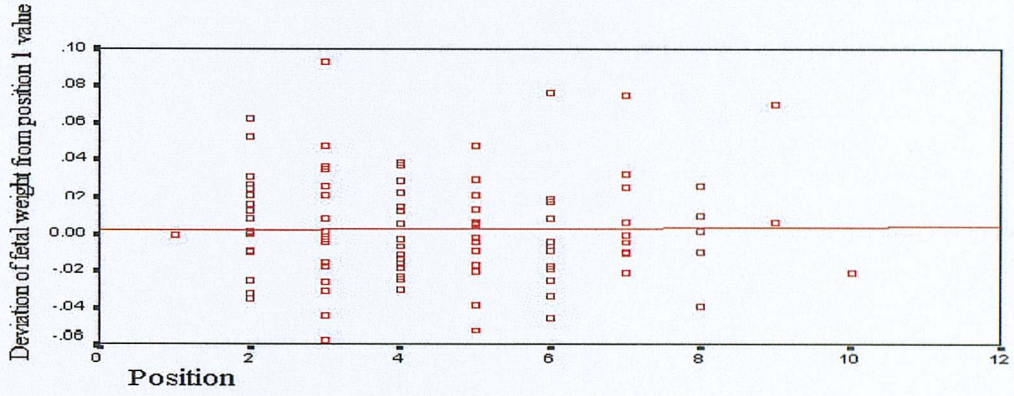


Figure 122: Deviation in fetal weight from mothers fed control diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.735 N = 117

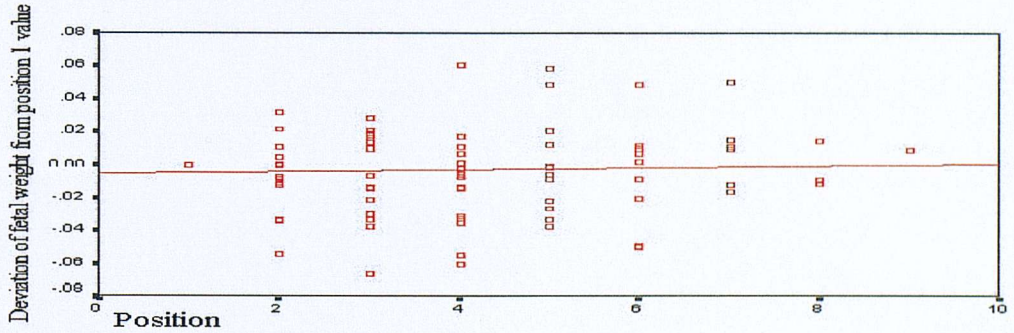


Figure 123: Deviation in fetal weight from mothers fed low protein diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.748 N = 94

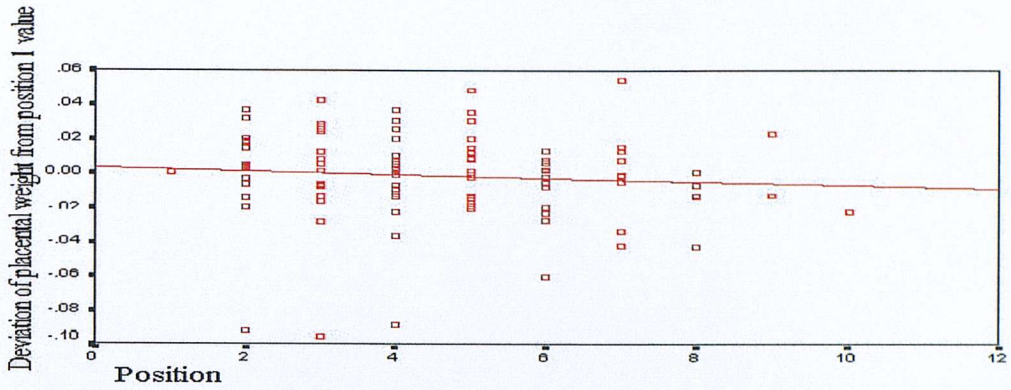


Figure 124: Deviation in placental weight from mothers fed control diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.150 N = 117

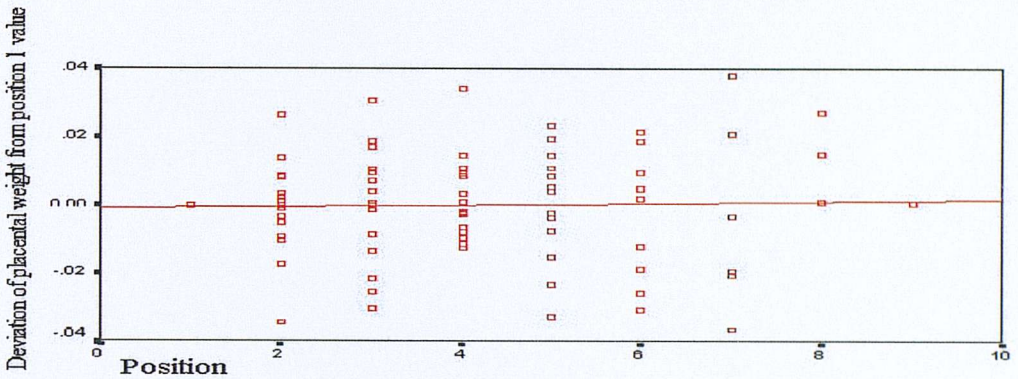


Figure 125: Deviation in placental weight from mothers fed low protein diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.675 N = 94

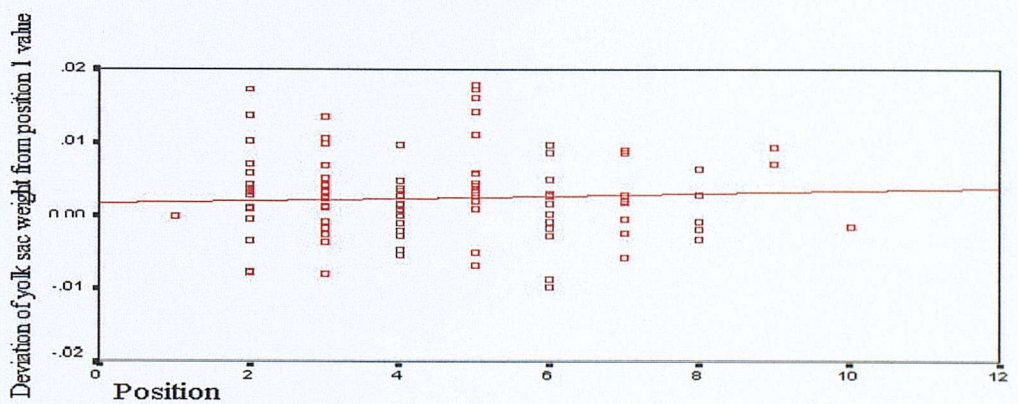


Figure 126: Deviation in visceral yolk sac weight from mothers fed control diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.581 N = 117

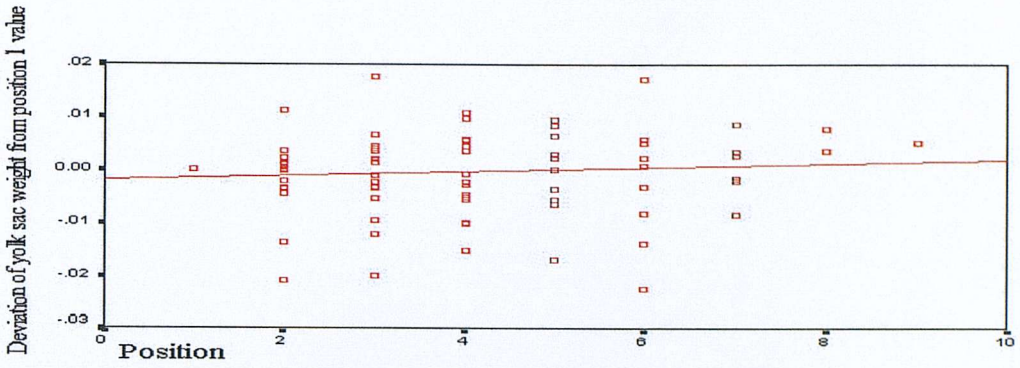


Figure 127: Deviation in visceral yolk sac weight from mothers fed low protein diet up to 14 days gestation in relation to position in uterine horn from position 1 (ovarian end) to position 12 (cervical end). P = 0.618 N = 94

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