THE IMPACT OF THE PERICONCEPTIONAL ENVIRONMENT (*IN VIVO* AND *EX VIVO*) ON FETO-PLACENTAL DEVELOPMENT IN THE SHEEP

Severence Michael MacLaughlin B.S. (Hons)

Discipline of Physiology

School of Molecular and Biomedical Science

The University of Adelaide

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Abstract

A range of epidemiological, clinical and experimental studies have demonstrated that exposure of an embryo to a suboptimal environment *in vivo* or *ex vivo* during early embryo development is associated with altered development of cardiovascular, neuroendocrine and metabolic disorders in adult life. A number of perturbations during early embryo development result in developmental adaptations by the embryo to ensure immediate survival, whilst programming the embryo for altered fetal and placental development, resulting in the eventual onset of adult disease. It has been previously shown that maternal nutrient restriction during the periconceptional period results in a hyperactivation of the pituitary – adrenal axis and increased mean arterial blood pressure in twin but not singleton pregnancies.

It was therefore the first aim of this thesis to interrogate the impact of maternal undernutrition during the periconceptional period (defined as from at least 45 days prior until 7 days after conception) on fetal and placental development during early pregnancy at ~ day 55 of pregnancy, which coincides with the period of maximal placental growth. In Chapter 2, it has been demonstrated that there are important relationships between maternal weight gain during the periconceptional period and feto-placental growth during the first ~ 55 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. In singleton pregnancies, periconceptional undernutrition disrupts the relationship between maternal weight

gain during the periconceptional period and utero-placental growth and in twin pregnancies, periconceptional undernutrition results in the emergence of an inverse relationship between maternal weight gain during early pregnancy and uteroplacental growth and in a dependence of fetal growth on placental growth. (Chapter 2)

In order to investigate the origins of the physiological adaptations that lead to the development of hyperactivation of the pituitary – adrenal axis and increased mean arterial blood pressure in late gestational fetuses after exposure as an embryo to periconceptional undernutrition, we investigated the development and steroidogenic capacity of the fetal adrenal gland and development of the fetal heart and kidney at ~ 55 days gestation (Chapter 3 and 4).

The relative weight of the fetal adrenal and adrenal IGF-1, IGF-1R, IGF-2, IGF-2R and CYP 17 mRNA expression were lower in twin compared to singleton fetuses. There was evidence that in control singletons, IGF-2R expression plays an important role in the regulation of adrenal growth and CYP 17 mRNA expression during early pregnancy. In control twins, however, whilst there was a significant positive relationship between adrenal CYP 17 and IGF-2 mRNA expression, adrenal weight was directly related to the level of adrenal IGF-1 mRNA expression. There was no effect of periconceptional undernutrition on the level of expression of any of the placental or adrenal genes in the study. In PCUN ewes, carrying singletons, however, there was a loss of the relationships between either adrenal IGF-2, IGF-2R and IGF-1 mRNA expression and adrenal growth and CYP 17 expression which were present in control singletons. Similarly in

ewes carrying twins, maternal undernutrition during the periconceptional period resulted in the loss of the relationships between adrenal growth and IGF-1 expression and between adrenal CYP 17 and IGF-2 expression which were present in control twin fetuses. Whilst there was no effect of fetal number on fetal heart growth at ~ d55 in twin fetuses, there was a direct relationship between relative fetal heart and adrenal weights, which was present in both the PCUN and control groups. There was also a significant inverse relationship between maternal weight at conception and relative fetal heart weight in PCUN twin, but not PCUN singleton or control fetuses (Chapter 3).

In control pregnancies maternal weight gain during the periconceptional period is inversely related to the relative weight of the fetal kidney at ~55d pregnancy. In this group, relative kidney weight was also directly related to renal IGF-1 mRNA expression. In control twins maternal weight gain was inversely related to fetal kidney weight and this effect was ablated when the effects of maternal cortisol was controlled for in the analysis. In the PCUN group, whilst there was an inverse relationship between maternal weight gain during the periconceptional period and relative kidney weight, it was not possible to separate the independent effects of maternal weight loss during the periconceptional period and the subsequent weight gain during the period of refeeding. Renal IGF-1 mRNA expression was higher and renal IGF-1R and 2R expression were lower in twin fetuses compared to singletons. After exposure to PCUN, renal IGF-1 expression was also higher than in control pregnancies independent of the fetal number (Chapter 4).

Superovulation, artificial insemination, embryo transfer and *in vitro* embryo culture are used in a range of assisted reproductive technologies, and it has been demonstrated that varying the composition of the culture media can result in a change in pre and postnatal development. Culture of sheep embryos in media containing serum is associated with fetal overgrowth which is phenotypic of the Large Offspring Syndrome. It is not known how the combination of superovulation, artificial insemination and embryo transfer alone impacts feto-placental development in late gestation of the sheep. There have been no studies, however, examining the differential impact of superovulation, artificial insemination and embryo transfer with or without *in vitro* embryo culture in the absence or presence of human serum on feto-placental development in singleton and twin pregnancies (Chapter 5).

I have therefore tested the hypothesis that superovulation, artificial insemination and embryo transfer with or without *in vitro* embryo culture in the presence or absence of human serum differentially alters the growth of the placenta, fetus and fetal organs during late gestation when compared to naturally conceived controls and that these effects are different in singleton and twin pregnancies. The fetal weight, CRL and abdominal circumference were significantly larger in IVCHS singleton fetuses. A novel finding in this study was lower fetal weights of twin fetuses in the ET and IVCNS groups compared to NM control twin fetuses. In addition, placental weights were lighter in twin fetuses in the ET, IVCNS and IVCHS treatment groups and this is partially due to a failure to initiate compensatory growth of placentomes in twin pregnancies (Chapter 5).

The results of this thesis therefore highlight the complex interactions between the periconceptional environment (*in vivo* or *ex vivo*) and embryo or fetal number on the programming fetal and placental development. Maternal undernutrition during the periconceptional period and superovulation, artificial insemination and embryo transfer with or without *in vitro* culture in the absence or presence of serum alters fetal development, and I have demonstrated that these changes in fetal growth can be explained by changes in placental growth trajectory. Furthermore, a novel finding of this study is that perturbations of the periconceptional environment affect feto-placental development differently in singleton and twin pregnancies.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Commonly Used Abbreviations

ACTH	adrenocorticotrophic hormone
AI	artificial insemination
ANOVA	analysis of variance
ART	artificial reproductive technology
AT	angiotensin receptor
11-βHSD-2	11 β hydroxyl steroiddehydrogenase
cDNA	complementary deoxyribonucleic acid
CL	corpus luteum
CRL	crown rump length
CYP 17	cytochrome P450 17-hydroxylase
d	day
DM	dry matter
eCG	equine chorionic gonadotropin
ET	embryo transfer
ER	oestrogen receptor
FFA	free fatty acid
FSH	follicle stimulating hormone
GE	glandular epithelium
GH	growth hormone
GLUT	glucose transporter
GnRH	gonadotropin-releasing hormone
HPA	hypothalmo-pituitary-adrenal
HS	human serum

ICM	inner cell mass
IFN-τ	interferon-tau
IGF	insulin-like growth factor
i.m.	intramuscular
IU	international units
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
LH	luteinizing hormone
LOS	Large Offspring Syndrome
LE	luminal epithelium
MAP	mean arterial blood pressure
ME	metabolizable energy
Min	minutes
MOET	multiple ovulation and embryo transfer
mRNA	messenger ribonucleic acid
MZT	maternal zygotic transition
NS	no serum
OPN	osteopontin
OTR	oxytocin receptor
PCUN	periconceptional undernutrition
PGF	prostaglandin F 2 alpha
PM	post mortem
PR	progesterone receptor

SOF	synthetic oviductal fluid
Sv	surface density
TE	trophectoderm
UTMP	uterine milk protein
Vd	volume density

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Chapter 1:
Literature Review

"If everyone is thinking alike, someone isn't thinking"

- General George S. Patton Jr.

1. Literature Review

1.1 The evolution of the "Barker Hypothesis"

The "early" or "fetal" origins of adult disease hypothesis or the "Barker hypothesis" was originally coined by Professor David J.P. Barker and colleagues at the University of Southampton in the United Kingdom during the late 1980's and 1990's. This hypothesis states that factors in the environment of a developing organism, in particular nutrition, may act to "program" resulting pathophysiology such as cardiovascular disease in adult life. To understand how the questions and hypothesises outlined in this thesis were derived it is important to elucidate how the Barker hypothesis was formulated and how this hypothesis has evolved from the "early" origins to its present form of "developmental" origins of adult disease. It is also the purpose of this thesis to extend the hypothesis from the "developmental" origins of adult disease to the "periconceptional" origins of adult disease.

In 1986, Barker and Osmond studied the geographic associations between the mortality rates of ischaemic heart disease in 1968 – 1978 and infant mortality in 1921 – 1925. What resulted from this study were striking positive correlations between geographic locations experiencing high rates of death from ischaemic heart disease and infant mortality some fifty years earlier (Barker & Osmond, 1986). This paper hypothesized that poor living conditions during infancy and early childhood, particularly suboptimal nutrition, resulted in high rates of infant mortality and predisposed individuals to be susceptible to cardiovascular disease

in adult life. Barker and co-workers investigated these epidemiological associations to understand the nascence of the factors that predisposed individuals that experienced an adverse environment in early life to cardiovascular disease and resulting mortality in later life. These investigators were able to determine that individuals born of a low birth weight for gestational age or who were small for their gestational age at birth had significantly higher blood pressure at 10 and 36 years of age and a greater risk of mortality resulting from cardiovascular disease in later life, which was independent of socio-economic variables (Barker *et al.*, 1989a; Barker *et al.*, 1989b). These observations led Barker and co-workers to extend their original hypothesis, to one which expressed that an adverse environment that produces a decrease in fetal and infant growth is linked to an increase in blood pressure in postnatal life and an increased risk of hypertension and cardiovascular disease in adult life. In 1990, this body of work was continued and supported with the findings that individuals born small for gestational age and which had a discordance between placental and birth weight had increased blood pressure in adult life (Barker *et al.*, 1990). These findings were the first to show that the intrauterine environment had a "dominant effect" on the development of blood pressure in postnatal life, again suggesting an association between fetal growth and development and the risk of cardiovascular disease in later life (Barker, 1990; Barker *et al.*, 1990). Continued research into this area determined that restricted fetal growth – as characterized by a low ponderal index or a high ratio of head circumference to length, a "poor maternal physique" and a high placental to fetal weight ratio predicted increased postnatal blood pressure during early childhood and cardiovascular disease in later life (Law *et al.*, 1991; Barker *et al.*, 1992; Barker &

Martyn, 1992; Barker *et al.*, 1993b). These studies led Barker and colleagues to postulate that the origins of cardiovascular disease could be attributed to maternal undernutrition that induced a reduction in fetal growth, possibly very early in gestation, altering the physiology, structure and metabolism of the developing fetal cardiovascular system (Barker *et al.*, 1993b).

Elford and colleagues in the early 1990's challenged the Barker Hypothesis based on causal relationships between "experiences early in life and adult cardiovascular disease". They concluded that there was no consistent dose-response associations between fetal and infant development and the onset of adult cardiovascular disease and that there was no specific hypothesis based in biological mechanisms to explain these correlations (Elford *et al.*, 1991; Elford *et al.*, 1992). The association between low birth weight for gestational age and cardiovascular disease, however, has been supported and confirmed in England (Law *et al.*, 1993), Wales (Frankel *et al.*, 1996), the Netherlands (Roseboom *et al.*, 1999), the United States (Rich-Edwards *et al.*, 1997), India (Stein *et al.*, 1996), and in numerous studies from around the world (Law & Shiell, 1996).

The scientific criticism of the Barker Hypothesis by Elford and colleagues may have helped focus the hypothesis and identify putative biological variables that influence fetal and early neonatal growth, to predispose an individual to pathophysiological development of cardiovascular disease in later life. Barker and colleagues yielded that birth weight was a poor indicator of fetal development, but was a proxy for different phenotypes of retarded fetal growth leading to a similar outcome of low birth weights for gestational age (Barker *et al.*,

1993a). During the mid-1990's Barker and co-workers identified that maternal undernutrition during gestation produced a number of markers that were linked to restricted fetal growth and that might lead to an altered development of the cardiovascular system. These markers included maternal size (Barker *et al.*, 1993a), the stimulation or constraint of placental growth which depended on the timing of a nutritional insult (Barker *et al.*, 1993a), defects in the fetal/neonatal growth hormone and IGF axis (Barker *et al.*, 1993a), effects of increased maternal cortisol or a decreased activity of placental 11-βHSD-2 (Barker *et al.*, 1993a) and timing and duration of maternal undernutrition during gestation (Barker, 1994). Interestingly, a study in Israel has demonstrated that there is a significant relationship between the blood pressure of an individual at 17 years of age and the body weight and body mass index of the mother before pregnancy (Laor *et al.*, 1997).

These findings led Barker and colleagues to phrase the Barker hypothesis in terms of "fetal" origins of adult disease (Barker, 1995), which in essence proposed that the physiological, neuroendocrine, or metabolic adaptations that enable the fetus to adapt to a period of intra-uterine deprivation occurring during critical windows of development result in a permanent programming of the development pattern of proliferation and differentiation events within key fetal tissue and organ systems and have pathological consequences in adult life (Barker, 1992, 1995). This hypothesis is dependent on the terms "programming" and "critical windows of development". Programming is defined by Lucas in 1991 as either the induction, deletion, or impaired development of a permanent somatic structure or the "setting" of a physiological system by an early stimulus or

insult operating at a "sensitive" period, resulting in long-term consequences for function (Lucas, 1991). A "critical window of development" is defined as a point in development at which environmental or nutritional factors can have long-lasting consequences on the growth trajectory and metabolism of an organism (McCance & Widdowson, 1974; Widdowson & McCance, 1975). The term "programming" will be employed in this thesis as an "overarching" term instead of "development plasticity" or "predictive adaptive responses" to include functional adaptations the developing conceptus has made in response to environmental cues that may predispose to an altered development of the cardiovascular system (McMillen & Robinson, 2005).

As discussed above, through the evolution of the Barker Hypothesis, Barker and colleagues have highlighted a number of factors that are important markers of intrauterine growth restriction and have been linked to altered cardiovascular outcomes such as the timing and extent of maternal undernutrition, maternal size, maternal "physique" or body composition before and during pregnancy, the role of the placenta in this phenomenon, the putative actions of the fetal growth hormone and IGF axis and the interplay between maternal cortisol and the level of the activity of the placental enzyme, 11-β hydroxysteroid dehydrogenase 2 (11-β HSD2) which acts to convert cortisol to inactive cortisone. The effects of maternal nutrient status around the time of conception (the periconceptional period) and fetal number are two recent factors that have been postulated to play a role in the programming of cardiovascular disease (Barker, 1997). More recently, further research and understanding of the "fetal" origins of adult disease hypothesis has necessitated another change of name to the "developmental"

origins of health and disease hypothesis in order to recognize a broader range of developmental stages from the maturing oocyte to the growing neonate that may program cardiovascular disease in adult life (Hanson *et al.*, 2004).

1.2 Periconceptional Origins of Adult Health

1.2.1 Periconceptional undernutrition: Human epidemiology studies

There is evidence from a range of epidemiological, clinical and experimental studies that maternal nutrient restriction before or immediately after conception alters fetal and adult health outcomes. The Dutch Winter Famine, which began and ended abruptly, produced a unique historical cohort of individuals resulting from pregnancies that were exposed to maternal nutrient restriction. The famine began in mid-October 1944 and ended on May 7, 1945. The Dutch Winter Famine Study investigated the effects of the 5 month period of malnutrition experienced by pregnant women in Amsterdam on pregnancy outcomes and adult health of individuals, who were either *in utero* or conceived during this time (Stein *et al.*, 1972). Of particular interest is cohort "D2", which are individuals conceived from January to April 1945 and were born between October 1945 to January 1946, because they were exposed to maternal nutrient restriction only during the first trimester (Stein & Susser, 1975a). These mothers were exposed to famine for three to five months before conception, lost an average of 4.3% of body weight (2.6 kg) and gained 10.5% body weight (5.9 kg) when the famine ended and nutrition was restored (Stein & Susser, 1975a). Maximum maternal weight loss occurred in the second month of the famine (December 1944) with no further weight loss observed for the remainder of the famine (Stein & Susser,

1975b), suggesting that maternal body weight adjusted to famine nutrient levels. Maternal nutrient restriction during the first trimester only, did not produce an effect on either the mean birth weight or placental weight of the cohort, however, there was a large number of infants born with very low birth weights and this was attributed to premature delivery (Stein & Susser, 1975a). This anomaly produced a wider standard deviation than observed in other cohorts, but did not result in a significant difference in birth weight and possibly was attributed to a "contraction of the average period of gestation" that could not be attributed to the famine (Stein & Susser, 1975a). These findings are supported by a recent study that has shown that suboptimal first-trimester growth may be associated with premature delivery (Smith *et al.*, 1998). In addition, most famine cohorts produced infants that were thin for length at birth (Stein & Susser, 1975b), which is supported by a report that provides evidence that poor nutrition around the time of conception can influence fetal growth trajectory and weight at birth (Wynn & Wynn, 1988).

Studies investigating the impact of maternal nutrient deprivation on individuals born to the D2 cohort have shown that there is an emergence of cardiovascular and metabolic pathophysiology in adult life at around 50 years of age. Individuals exposed to the famine as either an embryo and/or fetus had an increased prevalence of coronary heart disease (Roseboom *et al.*, 2000b) and increased body mass index (Ravelli *et al.*, 1999; Roseboom *et al.*, 2001a) in later life. These pathophysiological outcomes may be dependent upon altered hepatic function as evidenced by a decrease in factor VII concentrations and a more atherogenic lipid profile (Roseboom *et al.*, 2000a; Roseboom *et al.*, 2000c). These results

show how periconceptional undernutrition during early gestation can be associated with altered cardiovascular and metabolic function during postnatal life. These findings have been supported by a study of Gambian children which showed that individuals exposed to nutrient restriction and maternal weight loss during the first trimester were heavier as children and had higher blood pressure, which was positively related to mothers' weight at six months of gestation, when maternal nutrition levels were improved (Margetts *et al.*, 1991). In addition a study that examined the effects of maternal nutritional status in Jamaican women on the blood pressure on their children determined that children born to women thin and with a low body condition during early gestation were associated with an increased blood pressure at 10 – 12 years of age (Godfrey *et al.*, 1994).

A second historical cohort of individuals who were exposed to a defined period of famine during the Leningrad siege have also been studied. The Leningrad siege study investigated how maternal nutrient restriction (with rations which contained virtually no protein) over ~ 2.25 years (September 1941 – January 1944) affected individuals who were conceived, born, and were infants during the siege. Women lost approximately 16% of their body weight during this period of nutrient restriction, however, there was no association between intrauterine malnutrition and hypertension or cardiovascular disease in adult life (Stanner *et al.*, 1997). Roseboom and colleagues conclude that the differences between the adult health outcomes of individuals exposed to the Dutch Winter Famine and the Leningrad siege *in utero* are due to the circumstances preceding and following the famines. The Dutch Winter Famine started and ended abruptly, lasted only five months and was preceded and followed by relatively good nutrition of the

populace, whilst, the circumstances in Leningrad were more dire. The Leningrad siege lasted more than two years, was preceded and followed by periods of food shortages, and during the period of childhood and adolescent growth after the siege was marked by poverty of Stalin Russia (Roseboom *et al.*, 2000b). Due to the fact that maternal nutrient restriction was followed by a long period of poverty and low level of nutrition during postnatal development, cardiovascular disease may not have developed because there was no mismatch between the prevailing nutrient environment before and after birth (McMillen & Robinson, 2005). The studies summarised above suggest that nutrient restriction during early pregnancy, when the nutrient demands of the early conceptus are minimal, can have specific long-term cardiovascular and metabolic consequences in adult life.

1.2.2 Periconceptional Undernutrition: Animal models and outcomes

There are a range of experimental models that have investigated the periconceptional origins of adult disease, particularly in the area of the programming of cardiovascular disease. It has been shown in the rat model that when pregnant rats were fed a low protein diet during early pregnancy (d 0 – 7) that the resulting offspring had increased body weight at weaning and lower relative kidney weights compared to control animals. This phenotype was associated with increased systolic blood pressure and a 62% decrease in plasma angiotensin II (Langley-Evans *et al.*, 1996). A study by Kwong and co-workers investigated the effects of rat dams fed a low protein diet only during the preimplantation period pregnancy, d 0 – 4.25, and found that resulting blastocysts had decreased cell numbers in the inner cell mass (ICM) and trophectoderm (TE) cell lineages (Kwong *et al.*, 2000). These authors

determined that this decrease in embryo growth was due to a lower cell proliferation and not due to an increase in cell apoptosis and hypothesized that this retarded preimplantation proliferation was caused by high maternal plasma glucose levels, a decrease in insulin and a significant reduction in essential amino acids. The altered periconceptional maternal environment and early embryo development "programmed" a postnatal phenotype characterized by a lower birth weight, followed by a phase of compensatory catch-up growth, increased postnatal systolic blood pressure and increased relative kidney and liver weights compared to control animals (Kwong *et al.*, 2000). It is interesting that maternal low protein diets that are imposed during overlapping windows of development produce offspring that are hypertensive, however, the resulting postnatal phenotype is different in terms of postnatal growth and relative kidney development. Thus, the increased blood pressure in these two phenotypes indicate that imposing a nutrition insult during the critical window of early embryo development and preimplantation causes an increase in relative kidney weight and blood pressure, whilst continuing this maternal protein restriction into early pregnancy results in a decrease in relative kidney growth and an increase in blood pressure. It is interesting to note that maternal nutrient restriction during these two periods affects the postnatal growth of the kidney differently and highlights the possible importance that the renal system plays in the programming of hypertension in these rat models.

Studies investigating the effects of maternal undernutrition during the periconceptional period in sheep have found similar findings in programming alterations in cardiovascular and neuroendocrine development and fetal and

postnatal growth. A severe restriction of maternal nutrition (50% of maintenance) from 18 d prior to conception to d 6 of pregnancy resulted in a mean increase in the number of cells per blastocyst compared to controls, and this was found to be due to an increase in the number of trophectoderm cells (Kakar *et al.*, 2005). Kakar and co-workers further determined that the critical window during which the embryo is vulnerable to maternal undernutrition that results in an increase in trophectoderm cell numbers was from conception to d 6 of pregnancy (Kakar *et al.*, 2005). Intriguingly, this severe nutrient restriction during the periconceptional period led to a delay in myogenesis at d 75 pregnancy, which was due to a 20% decrease in muscle fibers (Quigley *et al.*, 2005). In a model of moderate maternal nutrient restriction (70% of maintenance) from at least 45 d before conception until d 7 of pregnancy, Edwards and co-workers investigated the effects of periconceptional undernutrition on metabolic, cardiovascular and neuroendocrine development of the sheep fetus in late gestation. Ewes that were undernourished in this protocol lost significantly more weight (~2.2kg) before conception and this weight loss was directly related to maternal plasma leptin levels in late gestation (Edwards & McMillen, 2002a; Edwards *et al.*, 2005). Periconceptional undernutrition resulted in an increase in arterial blood pressure, rate pressure product and an increased activation of the fetal hypothalamo-pituitary-adrenal (HPA) axis in twin, but not singleton fetuses during late gestation (Edwards & McMillen, 2002a; McMillen *et al.*, 2004). These authors determined that the increase in mean arterial blood pressure was not dependent upon the activation of the renin-angiotensin system (RAS) (Edwards & McMillen, 2002b). Furthermore the increased activation of the HPA axis was considered to be a consequence of an increase in the corticotropic synthetic and secretory capacity

of the fetal pituitary rather than an alteration of the hypothalamic mechanisms that initiate the prepartum activation of the fetal HPA axis, given that there was an overall increase in plasma ACTH, but not a change in the rate of the prepartum rise in ACTH (Edwards & McMillen, 2002a).

When maternal nutrition is restricted more severely – through restricting intake in order to decrease maternal weight by 10 – 15 % before conception – and for longer periods of time (60 d prior to conception and up to 30 d pregnancy) as in the sheep model developed in Auckland (Harding, 1997; Bloomfield *et al.*, 2003), then there is a reduced fetal growth rate (Harding, 1997; Oliver *et al.*, 2005), a greater reduction in fetal IGF-1 response to an undernutrition challenge (Gallaher *et al.*, 1998) and a larger insulin response to glucose challenge (Oliver *et al.*, 2001) in late gestation. It is hypothesized that exposure of a fetus to periconceptional undernutrition causes a "reprogramming" of the ability of the IGF system to respond to undernutrition challenges in late gestation and that the enhanced insulin response may be due to an early maturation of the β cells of the developing pancreas (Gallaher *et al.*, 1998; Oliver *et al.*, 2001) and this in part may explain the decrease in the growth trajectory of the fetus in late gestation. The hyperactivation of the fetal HPA axis shown by Edwards and colleagues (Edwards & McMillen, 2002a) was confirmed in this study as singleton fetuses had an elevated cortisol baseline in late gestation (Bloomfield *et al.*, 2004) and there was premature delivery in 50% of the pregnancies (Bloomfield *et al.*, 2003). Bloomfield and co-workers have shown that this early delivery was due to an premature activation of the HPA axis resulting in increased fetal plasma ACTH concentrations and an advance in the timing of the prepartum cortisol surge

(Bloomfield *et al.*, 2003; Kumarasamy *et al.*, 2005).

The Nottingham group has also developed a model of severe maternal undernutrition (50% of maintenance) from d 0 to 30 of pregnancy and have shown altered cardiovascular function in lambs of 1 year of age in which there is an increase in pulse pressure product, a decrease in rate pressure product and a leftward shift of the baroreflex curve (Gardner *et al.*, 2004b). Boullin and co-workers have shown that this model of maternal undernutrition results in altered cardiovascular structure in mature sheep at 2.5 years of age (preliminary data Boullin 2005). In addition it was shown that the intrarenal mRNA expression of IGF-2 was decreased in twins, but not singletons exposed to this period and level of maternal nutrient restriction (Brennan *et al.*, 2005).

It is apparent from the different types of sheep models used to investigate the effects of maternal undernutrition during the periconceptional period that such nutritional restriction results in altered cardiovascular, neuroendocrine and metabolic outcomes and that the term "periconceptional" is often used generally to cover broad windows of early development. The duration of maternal undernutrition during the periconceptional period is shown in diagram form (Figure 1) to illustrate the overlapping periods between the three sheep models of periconceptional undernutrition used in Adelaide, Auckland and Nottingham. The Adelaide model is a moderate nutrient restriction (70% of maintenance) that encompasses oocyte maturation, follicular development, conception, and embryo/blastocyst development. The Auckland model is a more severe nutrient restriction (fed to decrease maternal weight by 10 – 15%) and extends the Adelaide model until d 30 of gestation. This model encompasses the

preimplantation development of the embryo and the period of post implantation. The degree of nutritional insult imposed in this model is difficult to gauge as the control animals were fed ad libitum and consequently gained weight. Thus the two groups which are compared include one which lost weight and one which gained weight. These differences, together with the expanded postimplantation phase of maternal undernutrition used in the Auckland model, may explain the differences present between the Adelaide and Auckland models. The Nottingham model utilises a more severe level of maternal undernutrition (50% of maintenance) between d 0 – 30 of gestation, which encompasses embryo/blastocyst development, preimplantation development of the embryo and the period of implantation. The inclusion of the preimplantation and implantation periods may have long term consequences for the subsequent development of adult health outcomes that are distinct and may be unrerelated to effects caused by maternal undernutrition around the time of oocyte and embryo development. Extending maternal undernutrition across this period will alter maternal histotroph and uterine milk production, development of the uterine glands, and the process of placentation. It is thus the opinion of this thesis that the term "periconceptional" should be used to refer to the critical windows of oocyte maturation, follicular development, conception, and embryo/blastocyst growth (from at ~ 45 d before conception to d 7 of pregnancy).

Whilst the models that have been used to investigate the mechanisms of the "Developmental Origins of Health and Disease" may be different in terms of their respective periods of nutrient restriction, it is clear that maternal undernutrition during the periconceptional period results in an altered allocation of cells into trophectoderm and ICM cell lineages during the first cellular differentiation event,

an altered fetal growth trajectory during mid and late gestation, premature activation of the development of the fetal HPA axis and altered physiological development of the renal and cardiovascular systems of the developing fetus and neonate. It is important to understand how altering the nutritional environment of the early embryo can result in changes in physiological systems that may subsequently lead to cardiovascular or metabolic disease. It is interesting to note that twin fetuses in particular seem vulnerable to the effects of periconceptional undernutrition.

1.2.3 Periconceptional environment: Effect of fetal number

Studies investigating how a poor intrauterine environment may program an individual for cardiovascular disease in later life has also highlighted the effects of fetal number during pregnancy and how this may affect the development of adult diseases (Boo Ha & Harding, 2006) and that this may be a challenge to the "Developmental Origins of Adult Disease" hypothesis (Barker, 1997). A Swedish study examined 6612 male twins and determined that there is no basis for an increased mortality due to cardiovascular disease in twins compared to singletons (Vagero & Leon, 1994), and these results have been supported by epidemiological studies in Denmark (Christensen *et al.*, 2001) and in the USA (Hrubec & Neel, 1981). Barker argues that a discordantly smaller twin may have an increased risk of coronary heart disease in later life due to disproportioned growth retardation *in utero* (Barker, 1997), however, there is no epidemiological evidence to support this theory (Davies, 2005). There is one study that has studied birth weight of twins and resulting blood pressure in later life and found an inverse correlation, however, the authors believe that the difference in twin

weights was a consequence of placental dysfunction, rather than inadequate maternal nutrition (Poulter *et al.*, 1999). It has been shown in the human that twins deliver on average three weeks earlier and are lighter at birth when compared to singletons (Bleker *et al.*, 1979) and that the growth rate of twins is down regulated very early in gestation (Wilson, 1974; Leveno *et al.*, 1979; Taylor *et al.*, 1998). These findings are supported by studies in human pregnancies with more than two fetuses and have found that after the number of embryos is reduced to two in the first trimester, the birth weights of the remaining twins were significantly reduced compared to the birth weights of non-reduced twin pregnancies (Sebire *et al.*, 1997), demonstrating that the growth trajectory of the fetus is plastic during early gestation but is also affected by fetal number *in utero*. Interestingly, a study in the sheep model has shown that removing a fetus (fetectomy) and reducing the pregnancy to a singleton one during early gestation altered fetal and placental growth trajectories. Fetectomized fetuses had similar fetal weights in late gestation compared to singleton fetuses, but placental weights were intermediate between singleton and twin pregnancies and this was due to a "plastic" response of hypertrophic growth (Vatnick *et al.*, 1991).

There have been a number of experiments using the sheep model which have shown that the metabolic and hormonal environment is different in twin and singleton pregnancies. In twins, maternal glucose concentrations are decreased (Edwards *et al.*, 2005), fetal plasma micronutrients are decreased (Yildiz *et al.*, 2005), fetal adipose expression of the prolactin receptor is increased (Budge *et al.*, 2003) and there is a direct relationship between maternal and fetal plasma leptin concentrations (Edwards *et al.*, 2005) in contrast to singletons. Thus, the metabolic milieu of both the ewe and fetus appears to be altered by the presence

and nutritional demands of two fetuses *in utero*. Interestingly in normal twin pregnancies in the sheep, there is a delay in the activation of the HPA axis and a blunted adrenocortical responsiveness (Edwards & McMillen, 2002a; Gardner *et al.*, 2004a). It has been speculated that the diminished adrenocortical responsiveness in twin fetuses could possibly be an adaptive response which counters the impact of the potential enhanced intrauterine stress experienced by a twin fetus to thereby reduce the possibility of preterm delivery (McMillen *et al.*, 2004). It has also been shown that in the sheep that in twin pregnancies the normal rise in cortisol is asynchronous between twins, with one twin having an elevated resting plasma cortisol compared to its co-twin (Schwartz & Rose, 1998; Block *et al.*, 1999). This asynchrony is attributable to adrenal responsiveness to ACTH and may be influenced by fetal sex (Schwartz & Rose, 1998). In addition, there is a decrease in relative heart weight of twin fetuses in late gestation (Gardner *et al.*, 2004a) and a direct relationship between a measure of the HPA axis and mean arterial blood pressure in twin but not singleton fetuses (Edwards & McMillen, 2002b; Gardner *et al.*, 2004a). Thus, the presence of two concepti *in utero* alters the growth trajectory of the fetus and placenta, maternal and fetal metabolic milieu and the development of the HPA axis and cardiovascular system.

To understand how maternal undernutrition and the presence of two concepti can alter the development of the embryo, fetus and placenta and program the onset of cardiovascular disease in adult life, it is important to discuss the normal development of the ovine embryo, fetus and placenta during early to mid-gestation.

Figure 1.

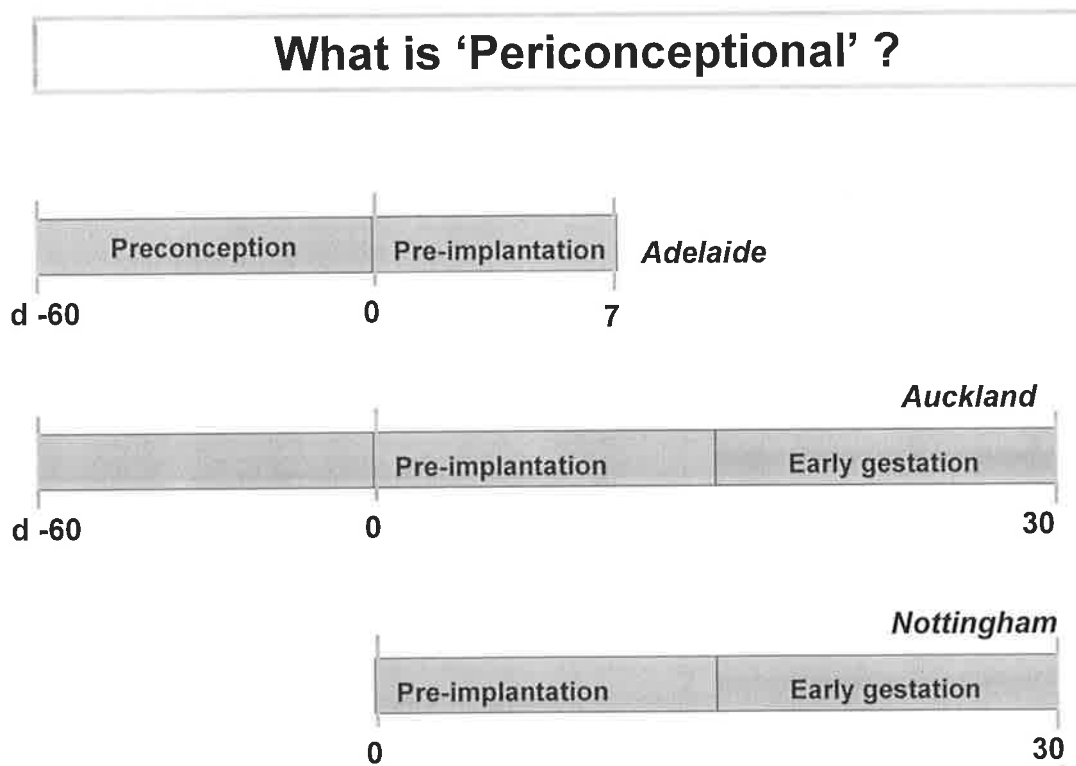

Figure 1.1. Schematic representation of three "periconceptional" models of maternal undernutrition in the sheep

1.3 Early embryo development

Fertilization, occurring in the ampullary section of the oviduct, is a cascade of events beginning with sperm penetration of the corona radiata and terminating with the combination of maternal and paternal chromosomes (Carlson, 1999). Penetration of the unfertilised oocyte by the sperm causes the oocyte to metabolically reactivate and complete meiosis. At the point of development when both the male and female pronuclei are observed the fertilized oocyte is termed an ootid, one of the largest single cells in the body and having a significant volume of cytoplasm, which is important for subsequent cell divisions up to the stage of the hatching blastocyst. When the male and female pronuclei fuse, syngamy, the fertilized oocyte becomes a zygote (Senger, 1999).

After syngamy, the zygote undergoes a number of mitotic divisions, termed cleavage divisions, resulting first in a two-cell embryo of which each cell is termed a blastomere. The two daughter cells are one half the size of the zygote (Senger, 1999). After the two-cell stage, cleavage divisions are asynchronous and continue to divide into 4, 8 and then 16 daughter cells (Carlson, 1999; Senger, 1999). Blastomeres up to the 8 cell stage are believed to be totipotent or having the ability to individually develop into a complete organism and that this developmental ability is lost in the developing blastomere between the 8 and 16 cell stage of development. Due to the fact that the embryo undergoes cleavage divisions within the zona pellucida, of a fixed volume, the cells become progressively smaller and there is no net increase in size of the embryo. In the

sheep the first cleavage division occurs 24 h after ovulation, the embryo reaches the 4-cell stage at 31.2 h after ovulation, and the 8-cell stage is reached 60 h after ovulation, and these events all occur within the oviduct. Between the 16 and 32-cell stage the embryo is termed a morula and begins a process of compaction, which occurs 3 to 4 d after ovulation in the sheep. During the period of morula development and compaction the embryo moves from the oviduct to the uterine lumen (Senger, 1999). Compaction is the process by which blastomeres maximize surface contact with one another. The inner cells of the morula compact more tightly than those on the outer surface and begin to form gap junctions allowing for intercellular and cytoplasmic communication between the inner cells, whilst the outer cell begin to develop tight junctions (Carlson, 1999; Senger, 1999). The development of tight junctions and through the active pumping of sodium into the inner portion of the morula, causing a hypertonic environment, facilitates the diffusion of water into the inner portion of the morula. The accumulation of a fluid filled cavity, the blastocoele, is termed cavitation (Carlson, 1999; Senger, 1999). When a discernable fluid filled cavity occurs, the embryo is termed a blastocyst, which occurs between 5 to 6 d after ovulation in the sheep (Senger, 1999).

Blastulation represents the first cellular differentiation event in ovine and mammalian development (Amoroso, 1952). The outer cell lineage, termed the trophoblast, is characterized by having tight junctions and active transport ability, whilst the inner cells is characterized by having gap junctions and is termed the inner cell mass. The trophoblast will develop into the chorion, and the inner cell mass will give rise to the embryo proper, the fetus, the yolk sac, amnion, and

allantois (Carlson, 1999; Senger, 1999). Studies in the embryos of the mouse have created some debate in the literature about whether cleavage divisions and embryo development is “pre-patterned” in the egg (Schatten & Donovan, 2004), influenced by the site of the second meiotic division (Gardner, 2001; Plusa *et al.*, 2002a) or the entry point of the sperm (Piotrowska & Zernicka-Goetz, 2001; Plusa *et al.*, 2002b); however, a recent study aimed at investigating these hypotheses has determined that the cleavage planes of the developing embryo at the 2-cell stage is random and argues against any “pre-patterning” of the embryo before the compactions stage of the morula (Louvet-Vallee *et al.*, 2005).

After blastulation the blastocyst continues to undergo hyperplastic growth, the blastocoele increases in size and the trophoblastic cells produce proteolytic enzymes. The combination of an increase in pressure from the growing blastocoele and digestion of the zona pellucida by trophoblastic enzymes leads to embryonic hatching, when the embryo escapes the zona pellucida and becomes free floating in the uterine lumen. Hatching occurs between 6 and 7 d after ovulation in the sheep (Carlson, 1999; Senger, 1999).

The metabolism of the developing embryo *in vivo* is not completely understood, however, studies of *in vitro* produced embryos have been able to elucidate that pyruvate, not glucose, is the major metabolic energy source during early cleavage cycles. Following the activation of the embryonic genome the utilization of glucose increased dramatically (Robinson & Symonds, 1995).

1.4 Maternal-Zygotic Transition (MZT)

The development of the embryo truly begins during oogenesis when cytoplasm of the developing oocyte accumulates maternal RNA and protein, which directs the first three cleavage and cell cycles (Telford *et al.*, 1999). The transition of the developmental program of the growing embryo from that of the maternal cytoplasm of the oocyte to that of the activation of the embryonic genome is termed the maternal-zygotic transition (MZT) or zygotic gene activation (ZGA) (Schultz, 2002; Ko, 2004). There is evidence that the temporal control of the developmental events of the growing embryo is not chronological and occurs in two stages (early and late zygotic gene activation) (Nothias *et al.*, 1995). It is hypothesized that the control of the growth of the developing embryo is by two separate mechanisms, which are linked to either the early or late zygotic gene activation events. It is proposed that the mechanism controlling cellular differentiation and specific gene activation is determined by the number of cell cycles of DNA replication, however, the early events of morphogenesis is controlled by factors in the embryonic cytoplasm (Satoh, 1982). The critical characteristics of the MZT is the loss or decay of maternal derived mRNA and proteins, activation of embryonic transcription and a significant increase in protein synthesis directed by the translation of the embryonic derived transcripts (Telford *et al.*, 1999). In the sheep the maternal-zygotic transition occurs between the 8 and 16-cell stage of development (Telford *et al.*, 1999) and is associated with the developmental loss of totipotency, however, this association may be causal or coincidental.

1.5 Embryo development from hatching to implantation

The ovine embryo continues to grow in a spherical form after hatching from the zona pellucida. At d 8 of pregnancy the ovine embryo is ~200 μm in diameter and is composed of 300 cells and grows to 400 – 900 μm in diameter and 3000 cells in composition at d 10 of pregnancy (Wintenberger-Torres & Flechon, 1974). After d 10 the embryo grows from its spherical form to slightly tubular shape and then begins to elongate into a tubular and then a filamentous conceptus at d 16 of pregnancy (Spencer *et al.*, 2004b). At d 12 of pregnancy the embryo is 10 – 22 mm long and at d 13 elongation begins to extend into the contralateral area of the uterus (Rowson & Moor, 1966). Development of the embryo up to d 14 occurs without contact with the luminal epithelium of the uterus. The embryo has grown to 10 cm long, become a filamentous body and is immobilised in the lumen of the uterus at d 14 of pregnancy (Guillomot *et al.*, 1993). Between d 14 and 16 binucleate cells begin to differentiate within the trophectoderm and this coincides with the embryo beginning to adhere to the luminal epithelium of the uterus (Spencer *et al.*, 2004b). From d 15 – 20 of pregnancy the trophectoderm develops "finger-like papillae" that invaginate into the uterine glands in the intercaruncular regions of the uterus; these structures vanish after d 20 and are thought to provide a temporary "anchor" for the "periattached" embryo and to absorb histotrophic secretions (Guillomot *et al.*, 1981; Wooding *et al.*, 1982). Histotrophic secretions provide all nutriment to the developing conceptus until implantation. At d 17 of pregnancy the embryo has reached a length of 25 cm and is in the beginning stages of implantation, furthermore, all extraembryonic membranes have formed prior to the start of implantation (Spencer *et al.*, 2004b).

1.6 Maternal recognition of pregnancy

A cascade of signalling between the conceptus and the maternal reproductive tract commences at fertilization and is required for proper nutrition of the developing embryo, endometrial differentiation, uterine receptivity for embryo implantation and for maternal recognition and maintenance of pregnancy. Progesterone plays a major role in the dynamic communication between the mother's womb and the developing conceptus (Spencer & Bazer, 2004a; Spencer *et al.*, 2004a). Maternal recognition of pregnancy is required for the proper establishment of pregnancy, implantation and placentation. Roger Short defined maternal recognition of pregnancy as the physiological process through which the growing embryo communicates to the maternal system of its presence and increases the duration of the lifespan of the corpus luteum (CL) (Short, 1969).

Sheep are spontaneous ovulators and undergo continuous estrous cycles that are dependent upon the uterine endometrium, because the uterus is the source of prostaglandin $F_{2\alpha}$ (PGF), the luteolysin responsible for both functional and structural CL regression and the commencement of the subsequent estrous cycle. The uterine release of PGF is dependent upon the presence of oxytocin (OTR) and ooestrogen receptors (ER) (Senger, 1999). At estrus/ovulation (d 0) ooestrogens from the developing follicles stimulate the expression of ER, OTR, and progesterone receptors (PR) in the luminal and superficial glandular epithelium of the endometrium, however, PGF, is not secreted because there is

no oxytocin stimulus from the corpus hemorrhagicum. During metestrus and diestrus progesterone levels increase and acting through the progesterone receptor (PR) down regulate ER directly and OTR indirectly through ER of LE and superficial glandular epithelial (sGE) cells. Thus during diestrus (d 5 to d 11) there is no ER or OTR expression in LE or sGE cells (Spencer & Bazer, 2004a). Continuous exposure to progesterone for 8 to 10 days results in an auto-down regulation of PR in LE and sGE cells on d 11 to 12 of the estrous cycle, which is followed by a substantial increase in ER expression on d 13 and subsequently an increase in OTR expression on d 14 in LE and sGE cells. Oxytocin is produced by the posterior pituitary and CL from d 9 and signals through its uterine receptor for PGF release. PGF causes luteolysis and a return to estrus on d 17 unless the maternal system recognizes a pregnancy and the CL is saved from luteolysis (Spencer & Bazer, 2004a).

In sheep interferon-tau (IFN-τ) is the antiluteolytic signal produced by the developing embryo, is the signal to the maternal system that she is pregnant, and indirectly results in rescuing the CL from luteolysis and maintenance of pregnancy (Roberts, 1996; Roberts *et al.*, 1996; Senger, 1999; Spencer & Bazer, 2004a). The mononucleated cells of the trophoblast of the filamentous embryo produce IFN-τ between d 10 and 25 of gestation with maximal output between d 14 and 16 in the sheep (Spencer & Bazer, 2004a; Spencer *et al.*, 2004a), and it has been shown that there is cross talk between the conceptus and the uterine epithelium that upregulates IFN-τ expression (Ashworth & Bazer, 1989b). IFN-τ acts through its receptor, Type I IFN receptor, in LE and sGE cells, which directly down regulates the expression of ER and indirectly decreases the expression of

OTR (Spencer & Bazer, 2004a; Spencer *et al.*, 2004a). IFN-τ prevents the regression of the CL by inhibiting the normal expression of OTR and thus the normal secretion of PGF by LE an sGE, which would cause luteolysis (Fleming *et al.*, 2001).

1.7 Uterine preparation for pregnancy

Successful development of the embryo from the zygote stage to the preimplantation stage of development, implantation and placentation requires essential reciprocal communication via paracrine and endocrine signals between the developing conceptus and maternal system that results in time dependent development and maturation of the conceptus and maternal systems (Ashworth & Bazer, 1989b; Spencer *et al.*, 2004a). The interface of this relationship is between the trophoblast and the endometrium (Ashworth & Bazer, 1989b). This dynamic communication between the developing conceptus and the maternal unit commences after fertilization in the oviduct, ensures that the conceptus and the maternal system are developmentally "in synchrony" and communications originating from either the trophectoderm or the maternal unit are developmentally appropriate for the embryonic stage of development and location in the maternal system. The maternal hormones, oestrogen and progesterone, regulate the maturation and communication of the oviductal and uterine epithelium such that the oviduct is communicating with the conceptus during embryonic development within the oviduct and is down regulated after the embryo moves to the uterus and concomitantly the uterine signalling matures as the embryo enters the uterus and not prematurely (Leese *et al.*, 2001; Spencer &

Bazer, 2004a; Spencer *et al.*, 2004a). Oestrogen from the developing follicles prior to ovulation causes a maturation and hypertrophy of the epithelial cells lining the oviduct and causes an increase in ion active transport into the lumen of the oviduct. The resulting hypertonic environment increases fluid secretion into the lumen and is accompanied by a decrease in glucose and lactate concentrations that is significantly lower compared to maternal plasma concentrations. Amino acids are selectively and actively transported into the lumen and are an essential component of oviductal fluid that include methionine, leucine, phenylalanine, lysine, aspartic acid, glycine, alanine, taurine, and tyrosine of which glycine is very important, playing a major role in protecting the developing embryo from inorganic ions (Nancarrow *et al.*, 1992; Leese *et al.*, 2001). Studies *in vitro* have shown that culture conditions that incorporate physiological levels of amino acids of the oviduct improve blastocyst formation, morphology, hatching rates, and produces highly viable embryos (Walker *et al.*, 1996b). In addition to amino acids, oestrogen increases the secretion of glycoproteins that are specific to the oviduct. These glycoproteins bind to the zona pellucida and are thought to protect the microenvironment of the embryo by increasing the viscosity of the luminal fluid and thereby buffering the embryo against deleterious osmotic fluctuations and preventing the dissipation of essential nutrients and ions. This is essential in an environment that has an increase in cilia beating that is upregulated by oestrogen (Nancarrow & Hill, 1995; Leese *et al.*, 2001). Thus, oestrogen prepares the oviduct to nurture and transport the developing embryo through the oviduct.

Uterine secretions or histotroph sustain an "embryotrophic" environment in the uterus before placentation and play a major role in nourishing the conceptus, in

communication between the mother and embryo, protection of the conceptus, attachment, implantation and placentation in both domestic animals (Roberts & Bazer, 1988; Ashworth & Bazer, 1989b) and in human pregnancies (Burton *et al.*, 2002). Studies by Gray and co-workers have shown that uterine gland density and development is associated with conceptus survival and developmental state (Gray *et al.*, 2001). The endometrial glands of the uterus are the source of the histotroph and have been shown to be essential for proper conceptus development beyond blastocyst formation (Gray *et al.*, 2001; Spencer *et al.*, 2004a).

The composition of uterine histotroph is constituted by molecules that are important for adhesion of the conceptus to the uterine LE and communication between the trophoblast and the LE such as uterine milk proteins (UTMPs), osteopontin (OPN), and integrins (Johnson *et al.*, 2003; Spencer & Bazer, 2004a, b; Spencer *et al.*, 2004a; Spencer *et al.*, 2004b). OPN, a glycoprotein, is particularly important for the adhesion of the trophectoderm to the LE and for later placentation by cross-linking integrins on the apical surfaces of the LE and trophectoderm (Johnson *et al.*, 2003). In addition, insulin-like growth factors 1 and 2 (IGF-1, IGF-2) are secreted by both the LE and trophectoderm between d 10 – 16 of gestation, possibly indicating the importance of these factors in mutual regulation of conceptus and uterine epithelial development so early in development (Ko *et al.*, 1991; Watson *et al.*, 1994a).

Spencer and co-workers have been instrumental in elucidating the "servomechanism" whereby the uterine epithelium and stroma is exposed to a

cascade of endocrine signals from the maternal system and developing conceptus that directs uterine endometrial adenogenesis, the process of glandular maturation of the uterus (Spencer & Bazer, 2004b), delivery of nutrients and developmental cues to the conceptus and maintenance of pregnancy. As previously discussed the pregnant uterus of the sheep is sequentially exposed to oestrogen, progesterone and IFN-τ. PR down regulation in GE and LE by continuous exposure to progesterone is required for the commencement of GE secretory capacity (Spencer & Bazer, 2004a, b; Spencer *et al.*, 2004a; Spencer *et al.*, 2004b), and it has been demonstrated the uterine secretions from ~ d 14 – 16 is directly related to progesterone concentrations (Ashworth, 1995). During this period of uterine development only uterine stroma cells express PR, and it has been shown that IFN-τ stimulates the expression of a suite of genes in the uterine stroma. It is thus hypothesized that the combination of progesterone and IFN-τ signalling stimulates the stromal cells of the uterus to express factors that act in a paracrine manner to direct GE histotroph production and functional maturation and development (Ashworth, 1995; Spencer & Bazer, 2004a, b; Spencer *et al.*, 2004a; Gray *et al.*, 2006). In the sheep, uterine glands increase in length by four times and width by ten times between d 15 and 50 of pregnancy (Spencer & Bazer, 2004b; Spencer *et al.*, 2004a). Placental lactogen (PL) begins to be expressed by the binucleate cells of the trophoblast at d 16 of gestation, and this is concomitant with the initiation of UTMP expression and secretion, a marker of GE maturation (Stewart *et al.*, 2000). PL acts through prolactin receptors of the GE and stimulates uterine gland hyperplasia (Noel *et al.*, 2003). Placental growth hormone (GH) is expressed maximally between d 35 and 70 of pregnancy and is directly associated with glandular hypertrophy and maximal

increase in secretion of UTMP and OPN (Spencer & Bazer, 2004b). Histotrophic nutrition is an essential part of nutrient transfer from the maternal system to the placenta in the synepitheliochorial placenta of the sheep, and its importance in post-implantantation development is evidenced by areolae, placental structures of the intercaruncular chorionallantois, that are specialized in absorbing histotroph from the mouth of uterine glands (Spencer & Bazer, 2004b). Thus, glandular development and histotroph production is a precisely controlled part of uterine development that occurs in tandem with conceptus development.

1.8 Asynchrony between embryo and uterine development

As previously discussed it is essential for the developing conceptus and maturing maternal system to communicate for normal embryo and fetal development and that this communication involves developmental cues, however, if the developmental cues between the conceptus and maternal system are not synchronous, then this results in altered embryo and fetal development or failure to establish pregnancy (Barnes, 2000). Experiments in the sheep that have transferred embryos to an advanced environment by 3 days resulted in a failure of implantation and embryo mortality (Wilmut & Sales, 1981; Lawson *et al.*, 1983), however, the difference in synchrony between the conceptus and uterus must be 3 d or greater, because studies transferring embryos +/- 2 d of uterine maturation stage resulted in normal development (Wilmut *et al.*, 1988). These studies have also shown that the failure to establish a pregnancy in an advanced uterine environment was not due to a failure to rescue the CL from luteolysis (Wilmut & Sales, 1981; Lawson *et al.*, 1983), however, the failure to

developmentally progress may be due to the inability of the embryo to signal the uterine epithelium to develop properly, possibly through IFN-τ.

Studies investigating whether exposing an embryo to an asynchronous uterine environment have demonstrated that the maternal environment has the ability to influence the growth rate of the embryo. It has been shown that embryo development can be both enhanced (Wilmut & Sales, 1981; Lawson *et al.*, 1983) or retarded (Sinclair *et al.*, 1998a) by exposing an embryo to an advanced uterine environment of 3 d, and interestingly by exposing an embryo to a premature uterine environment results in a retarded embryo development (Lawson *et al.*, 1983). Thus, the embryo has the ability to alter its growth trajectory to respond to a temporally altered uterine environment, and this may be due to the embryo responding accordingly to the developmental cues of the asynchronous uterus.

Studies have investigated whether exposing an embryo to an asynchronous uterine environment before returning it to a 'synchronous' uterus would alter the normal embryonic growth trajectory. If the embryo is exposed for 3 d to a 'd 6' advanced environment, then it could establish a pregnancy when transferred back to a synchronous uterus (d 6) dependent upon whether it was at a similar developmental state to an embryo which had developed normally to d 6 in a d 6 environment (Wilmut & Sales, 1981; Sinclair *et al.*, 1998a). Interestingly, if the embryo was retarded in development, then pregnancy was not established (Sinclair *et al.*, 1998a). There seems to be a critical window of development, before d 6, when the embryo can respond to advanced environmental cues and develop the capacity to establish a pregnancy in a synchronous uterus. When d

6 embryos are exposed to a 9 d uterus for 3 d there is an initial enhanced development followed by the loss of the ability to establish a pregnancy in a synchronous environment (Wilmut & Sales, 1981).

There is controversy in the literature as to whether embryos, which establish pregnancy after being exposed to an advanced uterine environment, have an altered growth trajectory. Wilmut and co-workers demonstrated that such embryos are larger at d 37 of pregnancy (Wilmut & Sales, 1981) in the sheep, and this is supported by a study in which d 8 embryos were transferred to a d 6 uterus and resulted in an increase in fetal weight at d 34 pregnancy (Wilmut *et al.*, 1988) and similar studies in the pig (Wilson *et al.*, 2001). Sinclair and colleagues in an identical experiment to that reported by Wilmut and co-workers in 1981 did not reproduce these results during early pregnancy or observe advanced fetal development in mid or late gestation (Sinclair *et al.*, 1998a), however, whilst there was no difference in fetal weight in late gestation there was an increase in muscle fibre numbers, indicating an increase in hyperplasia in muscle development (Maxfield *et al.*, 1998). One reason why exposure of an embryo to an advanced uterine environment may alter embryo development in the aforementioned studies is that d 3 embryos were flushed from the oviduct and transferred to the uterus of the recipient, which is not only an advanced environment but a different physiological location resulting in the embryo then being in communication with a physiologically 'incorrect' epithelium. In summary, there is evidence that the maternal environment can alter the growth trajectory of the developing embryo, the embryo can respond to development cues of the maternal system and that there is a critical window during which the embryo can

alter its developmental trajectory and maintain the ability to establish a pregnancy. This critical window includes the loss of totipotency, the maternal zygotic transition, the embryo entering the uterus in the sheep and the rise of maternal progesterone.

1.9 Embryo and uterine synchrony: the role of progesterone

Progesterone is important in the sequential development and maturation of the oviduct and uterus in order to prepare for nuturing and reception of the developing embryo (Leese *et al.*, 2001; Spencer & Bazer, 2004b). It has been hypothesized that progesterone may play a critical role in altering the maternal environment in the form of altering uterine secretions and communication to the embryo and in turn cause abnormal fetal growth by either "accelerating or decelerating" embryo development (Barnes, 2000). Treatment of ewes with exogenous progesterone during the first three to six days of pregnancy has been demonstrated to decrease blastocyst cell numbers by decreasing ICM cell numbers (Hartwich *et al.*, 1995), alter uterine histotroph secretion (Ashworth & Bazer, 1989a), decrease pregnancy rate and increase relative brain and heart weight and fetal weight at d 74 of gestation (Kleemann *et al.*, 1994; Kleemann *et al.*, 2001). Progesterone mediated alterations in development may be attributed to progesterone acting on the maternal system to cause an artificially advanced uterine environment that is asynchronous with the developing conceptus. An interesting study examining the difference in conceptus development between short (~ 15 d) and long (~17 d) estrous cycle ewes provides evidence that progesterone can advance the maturation of the maternal system and development of the conceptus. Short estrous cycle ewes exhibited an earlier

increase in maternal plasma progesterone concentrations compared to long cycle ewes, and these increased progesterone levels were associated with enhanced embryo development and increased IFN-τ production by the conceptus (Nephew *et al.*, 1991). Furthermore, these results have been bolstered by a study in the cow showing that elevated maternal plasma progesterone concentrations were associated with increased conceptus development (Mann *et al.*, 2003). Thus, a premature rise in maternal progesterone, a natural example of an advanced uterine environment, can act to alter uterine maturation and secretions and subsequently enhance embryo development.

Interestingly, it has been reported in the human that elevated progesterone during follicular development is associated with a double ovulation (Gilfillan *et al.*, 1996), and thus the formation of two CLs. Progesterone production and maternal plasma concentrations are directly related the number of CLs formed (Brien *et al.*, 1987; Chagas e Siliva *et al.*, 2003) and litter size during late gestation (Butler *et al.*, 1981). It is tempting to speculate that in twin pregnancies progesterone may be increased prior to ovulation and is elevated after CL formation, which would cause an alteration in both oviductal and uterine communication to the developing embryo. The twin pregnancy may be another example of a natural asynchronous maternal environment, possibly explaining the altered fetal development of the twin fetus. It is also interesting to note that in human twin pregnancies it has been shown that maternal plasma progesterone levels are twice that of singleton pregnancies and progesterone does not fall before parturition (TambyRaja & Ratnam, 1981). These authors speculate that the lack of a fall in progesterone before parturition may indicate a "continuance of placental function", and possibly in twin sheep pregnancies progesterone serves

to act in concert with a delay in the cortisol surge to prevent pre-term delivery.

1.10 Ovine Placentation

1.10.1 Implantation

Implantation in the sheep requires four sequential phases: 1) shedding of the zona pellucida, 2) precontact and blastocyst orientation, 3) apposition and 4) adhesion of the trophoblast to the LE of the uterus and and a formation of a syncytia of LE and trophoblast binucleate cells (Spencer *et al.*, 2004b). The first two phases have been previously discussed during "embryo development hatching to implantation". Apposition begins between d 14 and 16 of pregnancy and is characterized by a down regulation of MUC1, a glycoprotein, that is concomitant with the down regulation of the progesterone receptor (PR). MUC1 and associated proteins of the extracellular matrix of the LE prevent cell-cell contact before this point in development (Spencer *et al.*, 2004b). The apical membranes of the trophoblast, commencing with those cells close to the ICM and extending outwards, and LE come into close contact, there is a reduction in trophoblastic microvilli, interdigitation of the cytoplasmic projections of the two apical membranes, which along with trophoblastic papillae penetrating uterine glands anchor the conceptus onto the LE (Guillomot *et al.*, 1981; Wooding *et al.*, 1982; Wooding, 1984; Guillomot *et al.*, 1993). In addition, the extracellular matrix of both the trophoblast and LE cells play a role in attachment. OPN begins to cross link integrins expressed at the apical membranes of the trophoblast and LE (Johnson *et al.*, 2003), and also during this period of development (~ d 14), progressive morphogenesis of the caruncles begins by becoming edematous, the

apical surface begins to fold, which are the progenitors to maternal crypts where fetal villi will become embedded (Spencer *et al.*, 2004b). The conceptus by d 16 is tightly adhered to the LE, over both the caruncular and intercaruncular areas of the uterus, and implantation is completed at ~ d 22 of pregnancy. Also at d 16 the binucleate cells of the trophoblast have differentiated, begun to migrate from the trophoblast to be situated between the trophoblast and the LE and then fuse with a LE cell to form a trinucleated cell (Spencer *et al.*, 2004b). Continuous fusion of binucleate cells with a trinucleated syncitia occurs until the 20 – 25 nuclei stage. These syncytial plaques develop in caruncular areas and begin the formation of placentomes and a synepitheliochorial placenta (Wooding, 1984).

1.10.2 PLACENTAL DEVELOPMENT: INTRODUCTION

Whilst Barker and colleagues concluded in 1989 birth weight was a poor indicator of *in utero* growth and development, this observation was made in the sheep some fifty years earlier by L. R. Wallace. Wallace concluded that information was "lacking" in terms of understanding fetal development, *in utero* growth did not occur uniformly throughout the fetal body during gestation and the increase in fetal weight over gestation was the summation of the growth rates of the individual organ systems of the fetus that develop during different periods of pregnancy and at different rates of growth (Wallace, 1948). The ovine placenta has been used as a model to investigate placental transport, metabolism, endocrine function, and development; and it is important to understand how this organ develops and grows in order to understand how it serves its purpose as the unique organ of pregnancy. The functions of the placenta include facilitating the

metabolic alterations and exchange of nutrients between the maternal and fetal systems, hormonal regulation of the homeorhetic physiological and metabolic adaptations of both maternal and fetal tissues to pregnancy and immunological protection of the conceptus from the maternal system (Bauman & Currie, 1980; Bell *et al.*, 1989; Bell, 1995; Bell *et al.*, 1999). The placenta is unique because it is a cooperative association between maternal and fetal tissues, it is situated exterior to the fetus, anatomically connected by vascular and connective tissue and through evolution has become a disposable organ with a limited lifespan (Carlson, 1999). In general, ovine placentation is a progressive and cooperative interaction firstly between the LE and trophoblast and then subsequently between maternal vascular, connective and epithelial tissue and fetal vascular, connective and trophoblast tissue (King, 1993).

1.10.3 Placental development: terminology

The ovine placenta is a chorio-allantoic placenta and is classified as a polycotyledonary synepitheliochorial placenta (Wooding, 1992). Historic classification of placentae were determined by the number of tissue types separating the fetal and maternal circulations (Senger, 1999). Historically, the ovine placenta was classified as a syndesmochorial placenta having five tissue layers intermediate between the maternal and fetal blood supplies: maternal vascular and connective tissue and fetal trophectoderm, connective and vascular tissue. It was believed that the LE disappeared during pregnancy at the sites of placentation and that the fetal trophectoderm was in direct apposition to the maternal connective tissue of the uterus. The term “syndesmo” was used to indicate a relation to connective tissue (Wooding, 1992). Ensuing research had

shown that the LE is persistent in pregnancy and subsequently the ovine placenta was termed an epithelialchorial placenta, indicating six tissue types separating the two circulations (Wooding, 1992). Work by Wooding and colleagues in the 1980's and 1990's has demonstrated that the fetomaternal interface is in a continual state of dynamic morphogenesis. It is evident by these studies that the maternal LE persists but is continually modified by fusion of fetal binucleate cells, which forms a fetomaternal syncytium, and this occurs beginning at implantation and continues until term (Wooding, 1982, 1983; Wooding *et al.*, 1986). At the fetomaternal interface a fetomaternal syncytium is formed at placentomal sites that is continuously displaced by regrowth of the LE, and which is continually modified by fetal binucleate cell migration and fusion to form the fetomaternal syncytium (Wathes & Wooding, 1980; Wooding & Wathes, 1980; Wooding, 1984). Thus the ovine placenta is not syndesmochorial, having no LE, nor epitheliochorial with a maternal and fetal epithelium apposed at the fetomaternal interface. Wooding has designated the ovine placenta as a synepitheliochorial placenta, which is a term that accommodates the dynamic changes at the fetomaternal interface, where "syn" emphasizes cellular fusion and "epithelio" designates the fact that there is two epithelial cell layers (Wooding, 1992).

There has been a movement within the literature to differentiate the terms used to describe both the maternal and fetal portions of the placenta that are responsible for the haemotrophic fetomaternal interface. Historically, the term cotyledon was used to refer to the "composite fetomaternal structure", and the fetal and maternal portions were distinguished as the "fetal cotyledon" and the

"maternal cotlyledon". The endometrial thickenings of the ovine uterus that are the site of placentation were termed caruncles, those that were not a site of implantation were referred to "cotyledonary burrs", and the term caruncle was used to refer to both "cotyledonary burrs" and "maternal cotyledons" collectively (Wallace, 1948; Amoroso, 1952; Alexander, 1964a). The confusing nature of the terminology has led to a change in the terms used to refer to the different anatomical portions of the placenta. The composite of fetal and maternal tissues at the sites of implantation are termed "placentomes", and the maternal portion is designated as a "caruncle" and the fetal referred to as a "cotyledon" (Bell, 1984). The term "caruncle" has also come to designate the points of placental attachment in the ovine uterus, replacing "cotyledonary burrs" (Alexander, 1964b; Bell, 1984).

Studies over the past century investigating mid to late gestation developmental changes in the sheep placenta have observed that the placentome morphology is not homogenous with some being termed inverted and some everted (Wallace, 1948; Alexander, 1974, 1978). Vatnick and colleagues devised a system to "type" the heterogeneous population of placentomes into four categories, termed type A, B, C and D (Vatnick *et al.*, 1991). Type A placentomes were designated as inverted placentomes and were characterized by the caruncle surrounding the fetal cotyledon. Type B placentomes were categorized by fetal hemophagous tissue surrounded by the caruncle with a tuft of cotyledonary tissue beginning to grow over the top of the caruncle. Type C placentomes are flat structures with a fetal layer lying atop a maternal layer and resemble "pancakes". Everted placentomes were termed type D, where the fetal cotyledon surrounds the

maternal caruncle. The progression of type A to D indicated a continual eversion of fetal hemophagous tissue (Vatnick *et al.*, 1991). Alexander and co-workers hypothesized that everted placentomes contained more fetal tissue and placentomal eversion was possibly a mechanism of compensatory growth allowing for an increase transport by an increase in the surface area of exchange between the maternal and fetal circulations (Alexander, 1974, 1978). These findings have been recently challenged with evidence that the distribution of the type of placentomes did not have an effect on the rate of nutrient delivery across the placenta (Ward *et al.*, 2006). These investigators also demonstrated that fetal cortisol concentrations have the ability to influence gross morphology of placentomes, decreasing the amount of placentomes exhibiting eversion of the hemophagous zone (types C and D) (Ward *et al.*, 2006).

1.10.4 Placental development: gross morphology

The non-pregnant ovine uterus contains between 60 – 150 caruncles (Alexander, 1964a; Stegeman, 1974). The number of caruncles that are implanted and attached to the chorioallantois and that develop into placentomes varies greatly, however, usually 70 – 80% of these sites are occupied during pregnancy, but is dependent upon a number of factors. Singleton pregnancies usually implant on average 70% of caruncles, whilst twin pregnancies occupy 80% of the caruncles between the two placentas (Wallace, 1948; Alexander, 1964a, 1978). The number of placentomes formed decreases with increasing age of the ewe and is 10% greater in male versus female fetuses (Alexander, 1964a, 1978). The individual weight of each placentome is also variable within pregnancies and between different ewes, and the weight can range from 0.1 to 45 g. The variation

in size of placentomes is not dependent on the sex of the fetus, and placentomes are heavier in pregnancies of older ewes and the weight of placentomes is dependent upon fetal number (Alexander, 1964a, 1978).

There is a great deal of discrepancy in the literature as to when the number of placentomes of an ovine pregnancy is fixed. Experimental evidence from Cloete (Cloete, 1939) and Metcalfe (Metcalfe *et al.*, 1962) have reported that placentome number is fixed by d 50 of pregnancy, whilst evidence from Wallace (Wallace, 1948) suggests that the number is fixed by d 56. Other investigators believe that the number of placentomes is fixed at d 40 of pregnancy (Wimsatt, 1950; Boshier, 1969; King *et al.*, 1982) or as early as d 30 (Stegeman, 1974; Bell, 1984). Vatnick and colleagues give evidence to support the hypothesis that placentome number is fixed prior to d 50. In their experiment they reduced (fetectomy) twin pregnancies to a single fetus per pregnancy at d 50 of gestation, and the number of placentomes of the fetectomized fetus was similar to that present in control twin fetuses (Vatnick *et al.*, 1991). Interestingly, the same laboratory showed a modest trend ($P<0.1$) of an increase in placentome number from d 40 – 60 per fetus, however, the authors caution conclusions drawn from this result (Ehrhardt & Bell, 1995). Thus, it is difficult to ascertain when placentome number is fixed, however, it is clear that this process is confined to early gestation.

1.10.5 PLACENTAL DEVELOPMENT: GROWTH

Whilst histotrophic nutrition is essential for early embryo development in order to provide all nutriment, including ions, carbohydrates, amino acids, and lipids and maternal signalling to the developing conceptus; haemotrophic nutrition is more efficient (Spencer & Bazer, 2004b; Spencer *et al.*, 2004b). It is not known when haemotrophic nutrition becomes the dominant form of nutrient transfer between the maternal system and the conceptus but it is thought that it occurs after implantation once the fetal circulation develops. Placental weight is highly variable as an indicator of placental growth and functional capacity (Mellor, 1983). Placental growth exceeds that of the fetus during early gestation in order to establish the infrastructure needed to support the exponential increase in fetal weight and growth in later gestation (Schneider, 1996). During the first 50 d of pregnancy of the sheep the trophoblast expands rapidly, implantation occurs, and placental growth occurs maximally. The chorioallantois extraembryonic membranes are formed from the fusion of the allantois from the outgrowth of the endodermal diverticulum from the hindgut of the embryo and the chorion membrane. This fusion along with the "ensheathing" mesoderm of the allantois provides vascularization to the placenta (Ehrhardt & Bell, 1995; Carlson, 1999).

Placental growth is maximal between implantation and d 56 of gestation with the placental structure and outline complete by this developmental point with the maternal caruncles forming "cups" surrounding fetal cotyledonary tissue (Wallace, 1948). In addition to maximal placentomal growth at this point in development the chorioallantoic membranes reached their maximum weight at d 56 of pregnancy and were characterized by a "peculiarly thick gelatinous

condition" (Wallace, 1948). Maximal placental growth at ~ d 55 gestation is concomitant with the highest level of angiotensin Type 1 receptor (AT1) in both maternal and fetal portions of the placentome, suggesting that angiotensin II (Ang II) may play a role in placental growth acting through AT1 (Koukoulas *et al.*, 2002). Maximum placental weight is achieved by d 84 of gestation and there is a drop in placental weight thereafter until d 112, when placental weight remains static until term (Barcroft & Kennedy, 1939; Cloete, 1939; Wallace, 1948; Robinson *et al.*, 1977). These historical studies and observations were confirmed by a meticulous study by Erhardt and Bell in 1995, who showed that placental growth was rapid between d 40 to d 75 – 80 with very little change in dry matter content after d 100 of gestation (Ehrhardt & Bell, 1995). Maximum hyperplastic growth was observed at d 55 (Ehrhardt & Bell, 1995) and this period of hyperplastic growth (d 50 – 60) was followed by hypertrophic growth up to d 75 – 80 (Vatnick *et al.*, 1991; Ehrhardt & Bell, 1995). It has been shown that this maximum rate of growth and weight accretion occurring between ~ d 50 and 60 was due to the growth expansion of the fetal villi intertwining with the maternal crypts and an increase in the villi content of "Whartons's Jelly", which is characterised as an essential core component of the developing fetal villi and is composed mainly of hydrophilic glucosaminoglycans (Barcroft & Barron, 1946; Wiley *et al.*, 1989). The decrease in weight observed historically after d 80 is in wet but not dry weight, mainly due to an increase in protein content replacing the "Wharton's Jelly" of the fetal portion of the cotyledon. At d 84 of gestation there is a direct correlation between the weight of the fetal and maternal portions of the placentome, suggesting that there is paracrine regulation of the growth of the placentome between the maternal and fetal tissues (Wallace, 1948), however,

this equal growth seems to alter at d 90 when the fetal portion accounts for 60% of the weight of the placentome (Bell, 1984). After d 80 there is thought to be massive tissue remodelling and little change in growth after d 100 of gestation (Barcroft & Barron, 1946; Stegeman, 1974; Ehrhardt & Bell, 1995).

There is a discrepancy in feto-placental growth during gestation with maximum growth occurring during early to mid-gestation and a shift to fetal growth being the highest in mid to late gestation. To accommodate for this exponential fetal growth in late gestation the placenta increases its transport function, there is an increase in the transplacental gradient of nutrients, and a redistribution of placental use of nutritents (Schneider, 1996). In addition, there is a concomitant increase in blood supply (both uterine and umbilical) and an increase in fetal angiogenesis from ~ d 80 – 120 to support the increased need to deliver more nutrients to the rapidly growing late term fetus (Reynolds, 1995).

1.10.6 PLACENTAL DEVELOPMENT: METABOLISM

There are little data describing the metabolic activities of the ovine placenta during early to mid-gestation. The net metabolism of the gravid uterus accounts for 30 – 50 % of maternal glucose and 80 % of amino acid supply (Bell & Ehrhardt, 1998). The late gestational placenta has a disproportionate metabolic need, consuming 40 – 50 % of oxygen and 60 – 70 % of glucose supplied to the gravid uterus (Schneider, 1996; Bell & Ehrhardt, 1998; Bell *et al.*, 1999). The metabolic cost of supporting the fetus at term per gram of placental tissue is 5 to 10 fold greater than fetal metabolic activity. From mid- to late gestation the placental demand for oxygen and glucose increases 3.4 and 3.5 times respectively, however, the weight of the placenta only increases by a factor of

6.4. Thus, the metabolic demands of the developing placenta during early and mid-gestation is greater than those of the late gestational placenta (Schneider, 1996).

1.10.7 PLACENTAL DEVELOPMENT: TRANSPORT

There is not a great deal known about the functional transport of the ovine placenta during early gestation. Bell uses "clearance" as an appropriate term to describe the net transfer of a nutrient from the maternal to the fetal circulation and is defined as "the transfer rate over materno-foetal arterial concentration difference" (Bell *et al.*, 1999). There is a 5 to 10 fold increase in glucose transport from the maternal to fetal circulation between mid to late gestation (Molina *et al.*, 1991). The placenta's ability to increase its glucose transport capacity is dependent upon increase in blood flow (uterine and umbilical), increase in the glucose concentration gradient between maternal and fetal circulations, and a gestational increase in glucose transporter protein (GLUT) expression from mid to late gestation (Ehrhardt & Bell, 1997; Bell & Ehrhardt, 1998; Bell *et al.*, 1999). From d 75 to 140 gestation there is an increase in GLUT 1 and 3. GLUT 1 plays a more important role during early and mid gestation, however, GLUT 3 is expressed relatively more in late gestation than GLUT 1 and plays a more critical role in glucose transport because of its high K_d value for glucose (Ehrhardt & Bell, 1997). Amino acid transport to the fetal circulation occurs against the fetomaternal concentration gradient and is facilitated by active transport (Hay, 1998; Bell *et al.*, 1999).

1.10.8 PLACENTAL DEVELOPMENT: TWIN PREGNANCIES

There is evidence that the placentas of twin pregnancies develop differently than those of singletons. In twin pregnancies it has been shown that the ewe gains more weight (~ 50% more) in the first four months of pregnancy compared to ewes carrying singleton pregnancies and much of this increased weight is thought to be in fetoplacental fluids (Wallace, 1948). In singleton placentas, implantation and attachment occurs in both horns of the ovine uterus, however, placentomes on the non-pregnant side are smaller and less developed, which is thought to be due to lower blood flow to that side of the uterus (Wallace, 1948). In twin pregnancies, each placenta is not confined to the horn that carries the fetus, and it is usually the case that the placenta of at least one fetus will invade the opposite horn for attachment to caruncles. This may occur because one fetus' trophoblast develops faster during early development and accounts for the wide variability in the number of placentomes between litter mates in twin pregnancies (Wallace, 1948). On average twin pregnancies occupy 80% of caruncles, have more placentomes per pregnancy than singletons, but less placentomes per fetus (Wallace, 1948; Alexander, 1978). Placentomes of twin pregnancies are heavier than singleton pregnancies (by ~ 30%) (Wallace, 1948; Alexander, 1964b, 1978; Vatnick *et al.*, 1991) and this is due to compensatory growth. Compensatory placental growth is the ability to increase the size of the placentome in order to meet the future needs of the developing fetus(es) and occurs in the form of hypertrophy (Alexander, 1964b; Vatnick *et al.*, 1991).

It is interesting that fetal weight is not different between singleton and twin

fetuses until the last month of gestation and that placental tissue and transport is not limiting until after d 100 of gestation (Wallace, 1948; Alexander, 1964b). Placental size is thought to relate to functional capacity to support the developing fetus and is thus a good indicator of fetal development and size. This hypothesis is supported by evidence in the sheep that variation in placental weight explains 4 – 50% of fetal weight between d 60 – 70 gestation and ~70 – 90% of fetal weight after d 130 of gestation (Wallace, 1948; Alexander, 1964a, b, 1978; Dingwall *et al.*, 1987; Vatnick *et al.*, 1991; Greenwood *et al.*, 2000; Kleemann *et al.*, 2001; Osgerby *et al.*, 2003a).

1.11 Fetal growth and development

After hatching, during implantation and trophoblast expansion the process of organogenesis occurs within the ICM or the embryo proper. By d 40 of gestation the embryo proper has become a distinct fetus and organogenesis has laid the foundations of all of the major organ systems (Carlson, 1999; Senger, 1999). At d 28 of gestation the fetus represents less than 1% of the weight of the gravid uterus and weighs less than half a gram (Wallace, 1948). Ovine fetal growth follows a "sigmoid-shaped curve" trajectory, beginning initially with a low rate of growth, leading to an exponential period of growth harnessing the placental infrastructure that preceded the fetus in growth and finally the growth trajectory plateaus during late gestation (Owens & Robinson, 1988). From mid to late gestation the fetus increases in mass by 6 to 7 times (Molina *et al.*, 1991). The window of exponential fetal growth has been determined to bridge mid to late gestation from d 56 – 84 (Wallace, 1948) or d 40 – 100 (Bell, 1992; Ehrhardt &

Bell, 1995). As the fetus continues to grow during this critical window of development the relative rate of growth decreases from 15% to 6% per day at d 100 (Bell, 1992). The fetus increases in weight from less than a gram at d 28 to 500 g -- 1.5 kg at d 100 of pregnancy (Wallace, 1948; Alexander, 1974). The fetus becomes the largest portion of the gravid uterus around d 90 of gestation (Ehrhardt & Bell, 1995), and it has been shown that a minimal amount of placental tissue is needed to support growth until d 100, when fetal growth is not relatively high but the demands due to the increase in mass necessitate adequate placental supply (Alexander, 1974). Ferrell and colleagues have determined that in the cow, genotype is the greatest influence on fetal growth during early to mid-gestation and that environmental factors have a larger impact during late gestational growth (Ferrell, 1991a).

The switch from exponential growth of the fetus to a "plateau" effect on the growth trajectory in late gestation (after d 100 of gestation) has been shown to be due to placental constraint and the inability of the placenta to transfer nutrients such as glucose to the fetus in amounts that would sustain exponential growth (Alexander, 1974; Bell, 1992). The decrease in glucose transport to meet the metabolic demands of the fetus is reflected in a decrease in IGF-1 expression in the growing fetal tissues in late gestation compared to earlier windows of growth (Stevens *et al.*, 1990; Bell, 1992).

Alexander reported that fetal growth was sensitive to many influences and found that male fetuses were larger than female fetuses, singleton fetuses were larger than twin fetuses and male twin fetuses were larger than female twin fetuses (Alexander, 1974).

1.11.1 Fetal growth and development: Twins

Whilst singleton fetuses were larger than twin fetuses, the entire fetal mass of a twin pregnancy is larger than a singleton fetus (Wallace, 1948; Alexander, 1974), however, the difference in growth trajectories between singleton and twin fetuses has been found to only occur in late gestation after d 112 of pregnancy (Wallace, 1948). There is some debate in the literature whether the growth trajectory of the twin is different from that of a singleton earlier in gestation. Robinson and co-workers reported that an increasing number of fetuses per pregnancy in associated with a decrease in fetal growth rate and this reduction in fetal growth trajectory increases as pregnancy progresses towards term (Robinson *et al.*, 1977). Dingwall and colleagues report differences in growth trajectories between singleton, twin and triplet pregnancies as early as d 34 and 55 gestation (Dingwall *et al.*, 1987). Increased fetal numbers *in utero* may alter the growth trajectories of the respective fetuses very early in gestation, however, it has been shown in fetectomy experiments in which a twin pregnancy is reduced to a singleton around d 50 of gestation that the remaining fetus' growth trajectory resembles that of a singleton for the remainder of gestation and fetal weight at d 136 is not different than a control singleton (Vatnick *et al.*, 1991).

1.11.2 Fetal growth and development: Organ systems

The allometric growth of fetal organ systems does not occur symmetrically. Neural tissue develops and matures first, followed by skeletal tissue, and finally by soft tissue accretion and growth and these tissues are formed well before d 55

of gestation (Wallace, 1948). In fetuses that experience intrauterine growth restriction and are small for gestational age the brain, heart, kidneys and adrenal glands are relatively larger than in control fetuses (Alexander, 1974), which represents physiological alterations in cardiac output to these key organs so growth is maximized at the cost of peripheral tissues (McMillen & Robinson, 2005).

Heart growth: Heart growth is steady throughout gestation with the largest amount of accretion occurring between d 90 and term (Wallace, 1948). Relative heart weight is greatest at ~ d 55 pregnancy, decreases in relative weight between d 55 and d 84 of gestation and remains relatively static until term (Wallace, 1948). In terms of function the fetal heart rate is significantly greater during mid-gestation compared to late gestation (Bell *et al.*, 1986).

Kidney growth: In the sheep, the fetal kidney begins to develop in the form of the mesonephros as early as d 17 of pregnancy and the metanephros, the functional kidney in mammals, develops at around d 27 of pregnancy (Moritz & Wintour, 1999). It has also been established that the fetal sheep kidney is functional at d 20 of pregnancy producing hypotonic urine, which is essential in the expansion of the allantois and normal placentation in the sheep (Moritz & Wintour, 1999). Fetal kidney development is relatively highest at ~ d 55 gestation (Wallace, 1948).

Adrenal growth: Adrenal growth occurs throughout gestation and as gestation progresses the relative weight of the gland decreases, thus being relatively

largest during early gestation (Wallace, 1948). During early gestation (~d 40 – 60), the fetal adrenal gland of the sheep undergoes hyperplastic growth and a phase of increased steroidogenic activity (Wintour *et al.*, 1975; Boshier & Holloway, 1989; Coulter *et al.*, 2002). Insulin like growth factors (IGFs) have been implicated in the regulation of adrenal growth and steroidogenesis in the fetal sheep (Han *et al.*, 1992). IGF-2, a paternally imprinted gene (Young *et al.*, 2000), is maximally expressed in a range of fetal tissues including the adrenal during early gestation (van Dijk *et al.*, 1988; Han *et al.*, 1992), where it is present in adrenocortical steroidogenic cells (Han *et al.*, 1992). Wintour and co-workers have shown that the fetal adrenal gland is responsive to ACTH and produces more cortisol relative to body weight at d 40 to 60 than any other point in gestation (Wintour *et al.*, 1975; Tangalakis *et al.*, 1994).

1.11.3 FETAL GROWTH AND DEVELOPMENT: ENDOCRINE CONTROL

Endocrine control of fetal development directs both tissue accretion, growth and differentiation, allowing for an orderly pattern of allometric development and different organ systems to grow during precise critical windows of development. IGF-1 and IGF-2 are important mediators of fetal growth through metabolic, mitogenic and differentiative actions. IGF-2 is expressed more abundantly than IGF-1 in fetal tissues compared to postnatal levels (Owens, 1991; Fowden, 1995). Nutrition, in particular glucose availability, modulates IGF actions in fetal growth (Owens, 1991). A decreased transfer of glucose to the fetus during late gestation may cause a decrease in IGF expression and thus may explain the plateau in fetal growth in late gestation. Cortisol causes the maturation and differentiation of fetal tissue growth and is important during late gestation to

prepare for the transition from fetal to postnatal life. Cortisol also has the ability to alter the expression of fetal growth factors such as the IGFs *in utero* (Fowden, 1995).

1.11.4 Fetal growth and development: metabolism

There is not a great deal of literature describing the metabolic demands of the fetus during early gestation, however, the metabolic demands per gram tissue of the mid-gestational fetus (~ d 75) is significantly greater than during late gestation (Bell, 1992). Oxygen consumption of the mid-gestation fetus is 40% greater per gram of wet weight and 2.5 times greater per dry weight than the late gestational fetus, and glucose uptake by the mid-gestational fetus was double that of the fetus during late gestation (Bell *et al.*, 1986). Amino acid uptake and utilization is also maximal during mid-gestation per gram of fetal tissue (Bell *et al.*, 1989). The reasons for an elevated level of oxidative metabolism during mid-gestation have not been elucidated, however, Bell and colleagues postulate that the growth and "preponderance of metabolically active organs" such as the heart and kidney during this period of development compared to muscle and "other skeletal tissues" may cause the metabolic level of the fetus during mid-gestation to be highest (Bell *et al.*, 1986).

1.12 Fetoplacental growth and development: maternal influences

The maternal environment has been credited with accounting for a significant amount of total variation observed in fetal and placental weight during mid to late gestation (Ehrhardt & Bell, 1995), however, these influences and their underlying

mechanisms by which they influence feto-placental development have not been fully elucidated. Maternal attributes that have been determined to influence feto-placental development are maternal frame size, the interaction between maternal and fetal genotypes during pregnancy and maternal weight, weight gain, and body condition during the periconceptional period. In addition it has been shown in the sheep that fetal weight increases with increasing parity and age of the ewe (Alexander, 1964a).

1.12.1 MATERNAL FRAME SIZE

Maternal frame size is positively correlated with fetal and postnatal development (Walton & Hammond, 1938; Wallace, 1948; Russel *et al.*, 1981; Kemp *et al.*, 1988; Allen *et al.*, 2002b; Scheaffer *et al.*, 2004). It has been demonstrated in a number of domestic animal models: the horse (Walton & Hammond, 1938; Allen *et al.*, 2002b), cow (Ferrell, 1991a), and the sheep (Wallace, 1948; Russel *et al.*, 1981; Scheaffer *et al.*, 2004) that during pregnancy the fetus and placenta and offspring are proportional to maternal frame size and that these influences continue to influence postnatal development through to adulthood (Walton & Hammond, 1938; Kemp *et al.*, 1988; Allen *et al.*, 2002b). Examining birth weight across a species has demonstrated that a newborn's birth weight relative to maternal size is conserved and not significantly different, for example the relative birth weight to maternal weight is similar between Shire horses and Shetland ponies (Walton & Hammond, 1938).

The question begs to be asked as to how this relationship is maintained across species. It is hypothesized that the maternal environment is responsible for

constraining fetal growth, and thus a larger maternal size would allow for a conceptus to more fully realize its genetic growth potential. Studies in the cow have shown that larger framed cows have the ability to deliver more nutrients to the developing fetoplacental unit with increased uterine blood flows compared to smaller individuals (Ferrell, 1991b). It is now evident from a number of cross breeding and embryo transfer studies that the influence of maternal size interacts with fetal characteristics to determine fetal and placental growth.

1.12.2 Interaction of Maternal and Fetal Genotypes

The classic and historical study by Walton and Hammond in which they reciprocally crossbred Shetland ponies and Shire horses further investigated the interactions between maternal variables and fetal growth (Walton & Hammond, 1938). This study showed that a crossbred fetus in a shire mare resulted in a foal three times the size of a crossbred in a Shetland mare and demonstrated the ability of maternal frame size to either restrict or allow a conceptus to realize its genetic growth potential (Walton & Hammond, 1938). Whilst it could be argued that using a crossbred conceptus does not allow for the study of the interactions between genetically different maternal and fetal genotypes, the advent of embryo transfer technology has allowed this experimental design to be performed by transferring an embryo of small genotype into a maternal system of a large genotype and vice versa in the horse (Allen *et al.*, 2002b) the cow (Ferrell, 1991a) and the sheep (Scheaffer *et al.*, 2004). These transfer experiments showed that the transferred embryos resulted in pregnancies that produced intermediate fetal weight, placental weight, gestational length and birth weight between the large and small breeds (Ferrell, 1991a; Allen *et al.*, 2002b; Scheaffer

et al., 2004). In the cow the large fetal genotype was larger than either the small genotype transferred to the large maternal genotype or the small fetal genotype conceived in the small genotype cow, which was the opposite in the horse where the small fetal genotype in the large maternal genotype was larger than the large fetal genotype in the small maternal genotype and the small fetal and maternal genotype control (Ferrell, 1991a; Allen *et al.*, 2002b). This suggests different fetal interactions with the maternal genotype *in utero*. It was shown that in the cow regardless of maternal genotype, the umbilical blood flow of the large fetal genotype was greater than the small fetal genotype resulting in a more efficient extraction of nutrients from the maternal system (Ferrell, 1991b). In the horse, the gestation length of the large fetal genotype in the small maternal genotype was significantly reduced compared to a natural large fetal and maternal genotype pregnancy, which indicates maternal constraint of fetal growth (Allen *et al.*, 2002b). Interestingly, these fetuses also exhibited increased plasma progestagen and premature maturation of the adrenal gland that is characteristic of fetal stress (Allen *et al.*, 2002a), however, the elevated progestagens could possibly be a mechanism by which premature delivery was avoided. These authors also found that maternal weight prior to pregnancy positively correlated with birth weight (Allen *et al.*, 2002b).

1.12.3 Maternal weight at conception

There is evidence in the sheep that maternal weight, a measure of nutritional status, around the time of conception can influence reproduction in humans and in domestic species. An increased weight, heavier ewes, at conception has been

associated with an increase in ovulation rate in the sheep (Bramley *et al.*, 1976; Nawaz & Meyer, 1991; West *et al.*, 1991; Kleemann & Walker, 2005) and in the pig (Aswhorth1991). It has been demonstrated that maternal live weight at conception accounts for 40% of the variation in ovulation rate (Kleemann & Walker, 2005) and that for an increase in maternal weight of 10% ovulation rate is increased between 5 – 9% (Nawaz & Meyer, 1991). Heavier ewes at mating have increased conception rates (Kleemann & Walker, 2005), rates of multifetal pregnancies (Nawaz & Meyer, 1991) and pregnancy rates (Bramley *et al.*, 1976).

In the human it has been shown that women that are heavier before conception have larger placentas and fetal capillary surface area and that maternal weight gain during early gestation positively influences the density of fetal capillaries (Stevens-Simon *et al.*, 1995), however, low prepregnancy weight in women is associated with a decrease in birth weights of infants (Wynn & Wynn, 1988). Robinson and co-workers investigated the influence of maternal weight at conception and determined that a 10 kg increase in maternal weight corresponded to a 4.7% increase in fetal weight (Robinson *et al.*, 1977). Ewes that are smaller at conception and during early gestation have smaller placentas (De Barro *et al.*, 1992; Clarke *et al.*, 1997; Greenwood *et al.*, 2000), which is associated with smaller fetuses at d 130 of pregnancy (Greenwood *et al.*, 2000) and at term (Clarke *et al.*, 1997). DeBarro and co-workers found a positive influence of maternal weight at conception on fetal weight in both singleton and twin fetuses, however, maternal weight positively influenced placental weight at d 90 gestation in only twin pregnancies (De Barro *et al.*, 1992). In addition, a lower maternal weight at conception was associated with increased susceptabilty to

neonatal morbidity and an increase in relative weight of the heart (Clarke *et al.*, 1997).

1.12.4 Maternal body condition at conception

Maternal body condition is another indicator of nutritional status of the ewe, and a number of studies have linked body condition of the ewe at conception to reproductive performance and lamb survival (Bramley *et al.*, 1976; West *et al.*, 1991; Kleemann & Walker, 2005). Increased body condition at conception, indicating a good level of nutrition, has been shown to increase ovulation rates in the sheep (Bramley *et al.*, 1976; West *et al.*, 1991) and has been demonstrated to explain 12.7% of variation in ovulation rate in a homogenous flock (Kleemann & Walker, 2005). McNeilly and co-workers have determined that one mechanism by which body condition before conception affects ovulation rate is through modulation of circulating plasma levels of FSH and have shown that low body condition during the time of follicular development suppresses FSH stimulation of the ovary and concomitantly decreases the number of ovulatory follicles (McNeilly *et al.*, 1987). In addition to decreasing the number of ovum ovulated a low body condition has been associated with a decrease in conception rates, uterine efficiency, pregnancy rates and litter size (Bramley *et al.*, 1976; West *et al.*, 1991). In an interesting set of experiments Osgerby and co-authors investigated the effect of body condition at conception on placental and fetal development during mid-gestation and determined that placental weight, mean placentome weight, caruncular IGFBP expression and fetal weight were increased in ewes having high body condition at conception and higher levels of circulating plasma glucose, insulin and IGF-1 concentrations during mid-gestation (Osgerby *et al.*,

2003b, a). Interestingly, pregnancies of ewes in moderate body condition at conception generated the smallest fetuses and placentae but had the highest levels of placental IGF-2 expression, however, the transport efficiency in these pregnancies was the greatest compared to ewes of high or low body condition (Osgerby *et al.*, 2003a). In this study the authors hypothesize that fetal growth was promoted and more efficient in ewes of low body condition due to an increase in histotroph and UTMP secretory capacity by the uterine glands (Osgerby *et al.*, 2003a). Body condition during early gestation has been shown to negatively influence fetal and placental development in late gestation (Greenwood *et al.*, 2000).

Nutritional status of the ewe during the periconceptional period and early pregnancy has been determined to be associated with an increased number of placentomes, indicating that maternal nutrition during early development can affect the number of sites of attachment during implantation and subsequently influence the nutrient transport capacity to the fetus in late gestation (Greenwood *et al.*, 2000; Osgerby *et al.*, 2003b). It is thus clear that maternal weight and body condition at conception influences the successive development of the conceptus from ovulation until birth. It is of interest to note that there is evidence that the maternal weight at mating has an effect on cardiovascular development and that the placentas of twin pregnancies is influenced differently than singletons.

Therefore the hypothesis that there are direct relationships between maternal weight or body condition at the time of conception and feto-placental growth during early gestation was investigated in Chapter 2.

1.13 Maternal undernutrition during early and mid-pregnancy: effects on conceptus growth and development

Historically, studies investigating the effects of maternal undernutrition have focused on the period of late gestation that coincides with the window of development when the fetus maximally increases in absolute weight rather than earlier in development. It has become clear that maternal undernutrition during earlier critical windows of development can have a lasting “legacy of growth and development” that can affect fetal development in late gestation and during postnatal development (Robinson *et al.*, 1999). The shift to examining how maternal nutrient restriction can affect oocyte and follicular development, embryo development, placental development during the period of maximum hyperplastic and hypertrophic growth and fetal development during the period of organogenesis is indicative of investigating how the developmental infrastructure of the placenta and fetus is changed, which leads to the augmentation in gross fetal growth during late gestation, postnatal pathophysiology and adult health consequences such as coronary heart disease. Thus, it is important to understand how maternal undernutrition during putative windows of development when the “absolute nutrient requirements” of the fetus are minimal but specific metabolic performance and growth of the conceptus are relatively high impacts feto-placental and postnatal growth (Robinson *et al.*, 1999). It is also important to review how maternal influences such as frame size, nutritional status and genotype interact with nutrient restriction to augment conceptus growth during these critical windows of development.

1.13.1 Maternal undernutrition during follicular, oocyte and embryo development

Early embryo mortality, reproductive wastage and failure of implantation and establishment of pregnancy is a significant cause of reproductive failure in ruminants, and reproductive wastage early in gestation is particularly sensitive to maternal nutrition (Boland *et al.*, 2001). It has been demonstrated that maternal nutrition during the periconceptional period and early development has a significant impact on follicular development, oocyte growth and maturation and early embryo development (Bassett, 1986; Robinson *et al.*, 1999).

In the cow, the effect of maternal nutrition on follicular development has been studied and results demonstrate that moderate maternal undernutrition leads to a decrease in the size of the ovulatory follicle (Murphy *et al.*, 1991), whilst a high plane of nutrition was associated with an increase in the growth of the dominant follicle (Armstrong *et al.*, 2001). Further studies have demonstrated that this is associated with an increase in bioavailability of intrafollicular and plasma IGF-1 concentrations, which increases follicular sensitivity to FSH (Armstrong *et al.*, 2001). In addition, in the sheep it has been shown that an increase in maternal weight by an elevation in nutrition leads to an increase in the number of ovulatory follicles and this is associated with increased ovarian estradiol and inhibin content (Boukhliq *et al.*, 1996). Nutrient restriction during follicular development and as early as six months prior to ovulation has been shown to decrease ovulation rates in the sheep (Rhind *et al.*, 1989; Nottle *et al.*, 1997), however, supplementation of ewes on a low plane of nutrition with increased energy 10 d prior to ovulation has been shown to increase these rates to those of well nourished ewes (Nottle *et al.*,

1997). It has been shown that both undernutrition and overnutrition during follicular development and oocyte maturation produce oocytes that are of low quality and developmental competency in cows and sheep (O'Callaghan & Boland, 1999; Adamiak *et al.*, 2005); and feeding of a high plane of nutrition to heifers in low body condition increased oocyte quality while decreasing oocyte quality in high condition animals (Adamiak *et al.*, 2005). The adverse effects of impaired oocyte quality by well feeding heifers of high body condition was determined to be associated with maternal hyperinsulinaemia (Adamiak *et al.*, 2005). Interestingly, in the sheep fertilization rate is decreased in oocytes derived from nutrient restricted ewes (Lozano *et al.*, 2003).

An inventive study in the sheep model that investigated the importance of the maternal message pool and oocyte cytoplasm on conceptus development was performed by Peura and co-workers. These investigators harvested donor oocytes from either ewes on a high or low plane of nutrition for ~ 120 d and were used as oocyte hosts for nuclear transfer. It was determined that ooplasm donated from well fed ewes significantly increased pregnancy rates, indicating the importance of ooplasma development during oocyte maturation (Peura *et al.*, 2003). Maternal nutrient restriction during mid-gestation has been shown to affect the development of the fetal ovary and developing oogonia, resulting in lower blastocyst rates from oocytes derived from ewes undernourished *in utero* (Kelly *et al.*, 2005a). Murdoch and co-workers hypothesize that during this period of maternal undernutrition the DNA of the interphase fetal oogonia are damaged and attribute to the results of Kelly and co-authors (Murdoch *et al.*, 2003).

Maternal nutrient restriction during follicular development and early gestation has also been shown to retard embryo development in the sheep before implantation (Parr *et al.*, 1982; Rhind *et al.*, 1989; Abecia *et al.*, 1997; Lozano *et al.*, 2003). The resulting decrease in embryo development may be due to altered communication and/or synchronizing mechanisms between the conceptus and the maternal system. Ewes that are undernourished during follicular and early embryo development have been shown to have an increase in uterine ER and PG expression and a decrease in conceptive derived IFN-τ signalling, which collectively predicts a failure in signalling maternal recognition of pregnancy (Boukhliq *et al.*, 1996; Abecia *et al.*, 1999; Lozano *et al.*, 2003). Failure for synchronous development of the conceptus and maternal system after maternal restriction may be due to alterations in maternal plasma progesterone concentrations.

1.13.2 MATERNAL NUTRITION AND PLASMA PROGESTERONE CONCENTRATIONS

Maternal undernutrition in the sheep around the time of mating and early embryo development has been demonstrated to increase peripheral plasma progesterone concentrations during the luteal phase of the estrous cycle (Parr *et al.*, 1982; Lozano *et al.*, 1998; Kiyma *et al.*, 2004), however, ewes that are overfed during this period have a decrease in peripheral progesterone levels (Brien *et al.*, 1987; Parr *et al.*, 1987). In general maternal progesterone concentrations in the peripheral circulation were inversely related to the level of maternal nutrition (Parr, 1992), however, there is no difference in ovarian or uterine venous levels of progesterone in ewes fed a high or low plane of nutrition, but the concentration of endometrial progesterone content was decreased at d 5

of gestation (Lozano *et al.*, 1998). The mechanism by which progesterone levels are decreased in well fed ewes has been hypothesized by Parr and co-workers to be attributed to an increase in hepatic blood flow and hepatic function, where progesterone is efficiently removed from circulation (Parr, 1992). Brien and co-authors hypothesize the decrease in pregnancy rates in ewes overfed may be attributed to the differences in peripheral progesterone concentrations (Brien *et al.*, 1987). Thus, overnutrition causes a decrease in maternal peripheral progesterone concentrations and possibly a retarded asynchronous uterine environment, but undernutrition leads to an increase in maternal peripheral progesterone concentrations and an advanced asynchronous uterine environment, where both endocrine primed environments would be deleterious to successful embryo development and the establishment of the correct "servomechanism" of conceptus-maternal communication.

1.13.3 Maternal nutrition and plasma cortisol concentration

Whilst alterations in maternal plasma concentrations of progesterone have been shown to alter follicular, oocyte and conceptus development, cortisol has also been implicated in decreasing reproductive efficiency in sheep. In the human, glucocorticoids play an essential part in the normal antiinfamatory function of ovulation (Yong *et al.*, 2000). In the sheep maternal nutrient restriction has been shown to elevate maternal plasma cortisol concentrations during early and late gestation (Edwards & McMillen, 2001; Kiyma *et al.*, 2004). Stress-like cortisol levels resulting from maternal nutrient restriction have been shown to alter follicular phase development of the oocyte and follicle by suppressing normal luteinizing hormone (LH) pulses and interfering with the normal preovulatory

surge of oestrogen, FSH and LH (Daly *et al.*, 1999; MacFarlane *et al.*, 2000; Breen *et al.*, 2005). In addition to suppressing normal ovarian function during the follicular phase of ovarian development augmenting the preovulatory surges of oestrogen, FSH and LH may alter oviductal synchrony, transport and secretory capacity. That elevated cortisol concentrations can alter the oviductal environment is supported by work in the pig that has demonstrated where maternal undernutrition for 48 h around the time of ovulation is associated with retarded embryo development and increased plasma concentrations of cortisol, progesterone and PGs (Mburu *et al.*, 1998). Furthermore exogenous elevation of maternal cortisol levels by ACTH stimulation for the same period of time results in a decreased embryo development (Razdan *et al.*, 2002).

1.13.4 Maternal undernutrition during early and mid-gestation

1.13.4.1 Placental development

The close association between placental weight and fetal weight throughout gestation indicates that a nutritional insult that alters the growth trajectory of the placenta will indirectly affect the growth of the fetus. Placental size is a measure of functionality and it has been demonstrated that a decrease in placental growth affects nutrient transfer to the fetus by altering placental blood supply, morphology and transporter capability (Fowden *et al.*, 2006). Nutritional restriction or enhancement of placental growth has been studied in early and mid-gestation. Whilst maternal undernutrition during this period of development (prior to d 40) has significant effects on conceptus growth through decreasing embryonic growth and negatively affecting implantion, maternal undernutrition

during early gestation has not been shown to affect placental growth during mid and late gestation (Kelly, 1992). Maternal nutrient restriction during mid-gestation, the period of maximal placental growth (Ehrhardt & Bell, 1995), has been shown to either enhance or restrict placental growth during mid and late gestation in the sheep, however, the extent and severity to which placental development is altered is dependent upon the timing, duration and acuteness of the nutritional insult (Heasman *et al.*, 1999; Fowden *et al.*, 2006).

There is discrepancy in the literature as to whether maternal undernutrition during early to mid-gestation (~ d 28 – 100) either enhances or restricts placental development in the sheep and these studies have predominantly been preformed in singleton pregnancies. A number of studies have demonstrated that moderate to severe maternal nutrient restriction (85 to 50% of maintenance) results in an increase in placental growth in terms of weight in both singleton (Faichney & White, 1987; Rasby *et al.*, 1990; McCrabb *et al.*, 1991; Heasman *et al.*, 1998; Dandrea *et al.*, 2001) and twin (Steyn *et al.*, 2001) pregnancies and an increase in the proportion of everted placentomes (Type C and D) (Heasman *et al.*, 1998; Osgerby *et al.*, 2004; McMullen *et al.*, 2005) from mid to late gestation in the sheep. Contrary to these findings severe to moderate maternal undernutrition during the same period of development resulted in a decrease in placental weight (Holst *et al.*, 1992; Heasman *et al.*, 1999; McMullen *et al.*, 2005) and association with an increase in small placentomes (Clarke *et al.*, 1998) during mid to late gestation. Kelly hypothesizes that the disparity observed in placental growth as a result of undernutrition during mid-gestation may be due to an influence of maternal weight and body condition, as a measure of maternal nutritional status

and latent body reserves, at mating (Kelly, 1992). An interesting study by McCrabb and co-workers provides evidence to support this hypothesis. In these two similar cohorts of animals subjected to identical nutrient restriction and experiment conditions resulted in one cohort of an increased placental weight and decreased placental weight in the second cohort when compared to control animals. The only difference between these experimental groups was their mating weights. The group that produced the larger placentae as a result of maternal nutrient restriction were 10 kg heavier at mating (McCrabb *et al.*, 1992). This has led to the hypothesis that there is an interaction between the nutritional status of the ewe at mating and maternal undernutrition during mid-gestation, where ewes in good condition have the ability to draw on maternal body reserves to provide substrates for placental growth that is undergoing a "compensatory response" in order to prepare for a period of undernutrition during late gestation and compensatory growth is not able to be supported in ewes in a low nutritional state (Kelly, 1992; McCrabb *et al.*, 1992). Thus, the placenta has the ability to undergo compensatory growth in response to maternal nutrient restriction during mid-gestation in order to grow a larger placental infrastructure to meet the nutrient demands of the late gestational fetus.

Similarly, in the cow a low protein diet during early to mid-gestation resulted in an increase in placental weight and an increase in trophectoderm volume density in late gestation (Perry *et al.*, 1999). In the sheep, the surface area of exchange of the placenta increases by 5 to 15 fold from mid to late gestation and is accompanied by an increase in fetal villi branching and elongation and a decrease in the barrier thickness of exchange in order to increase efficiency of

exchange at the feto-maternal interface (Fowden *et al.*, 2006). Compensatory increase in placental growth does not necessarily translate into an increase in the efficiency of the placenta. A study in the guinea pig investigating a moderate level of undernutrition during early to mid-gestation found that there was an overall decrease in placental transfer capability characterized by a decrease in the surface area of exchange and an increase in the barrier thickness of exchange (Roberts *et al.*, 2001).

1.13.4.2 Fetal development

It is well accepted that maternal nutrition during pregnancy is one of the key environmental factors that has the ability to allow a developing conceptus to achieve or constrain its growth potential (Wu *et al.*, 2004). During late gestation maternal nutrient restriction has the ability to severely diminish the growth trajectory of the fetus as much as 30 to 70%, and if the insult occurs over 21 days the ability of the fetus to recover is marginalized (Mellor, 1983). Maternal undernutrition during early gestation has been shown to increase embryo/fetal wastage and morbidity and to decrease embryo growth (Rhind *et al.*, 1989). The effects of maternal nutrient restriction during mid-gestation on fetal growth *in utero* and during postnatal development have shown a similar discrepancy in results as placental development. Studies have shown that severe to moderate maternal restriction during mid-gestation has led to an increase in indices of fetal growth such as fetal weight, thoracic girth, and crown rump length (CRL) in the mid and late gestational fetus (Faichney & White, 1987; Heasman *et al.*, 1998; Dandrea *et al.*, 2001; Whorwood *et al.*, 2001; Osgerby *et al.*, 2002). Equally, other studies have shown that a similar nutritional regime has led to a decrease

in these indices of fetal growth in mid to late gestation (Everitt, 1964; Parr *et al.*, 1986; Nordby *et al.*, 1987; Osgerby *et al.*, 2002; Vonnahme *et al.*, 2003; McMullen *et al.*, 2005) and in postnatal growth potential (Kemp *et al.*, 1988). Similar to placental development it has been demonstrated that the prior nutritional status of the mother around the time of conception or during early gestation has the ability to influence fetal growth during periods of nutrient restriction in mid-gestation. Ewes of high body weight, condition or nutritional status subjected to nutrient restriction during mid-gestation produce larger fetuses (Robinson *et al.*, 1977; Russel *et al.*, 1981). There are also a number of studies that have shown that fetal weight was not affected by maternal undernutrition during mid-gestation (McCrabb *et al.*, 1991; McCrabb *et al.*, 1992; Steyn *et al.*, 2001). So why are there so many different fetal responses to maternal undernutrition during mid-gestation?

It is well understood that the plane of maternal nutrition during pregnancy and the size of the placenta are major determinants of fetal growth and that these factors act in concert or the effects of which may be confounded due to one factor compensating for the other or vice versa (Mellor, 1983). Ewes that are in high nutritional status, as indexed by high body weight or condition around the time of mating, have the ability to draw on latent reserves to provide the needed nutrients for the developing placenta and thus produce a larger placenta that has the ability to meet the demands of the developing fetus to either maintain fetal growth trajectory or to enhance it.

Whilst interactions between the effects of maternal body weight or condition at

mating and maternal undernutrition during early-mid pregnancy on the growth of the placenta and fetus have been investigated (McCrabb *et al.*, 1992), there have been no studies that have investigated the interactions between maternal body weight or condition at the start of pregnancy and the level of periconceptional nutrition on feto-placental growth and morphology during early pregnancy.

Therefore the hypothesis that there are relationships between maternal weight or body condition at the time of conception and feto-placental growth and morphology during the first ~ 55 days of pregnancy, and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies has been investigated in Chapter 2.

1.13.4.3 Heart development

Maternal undernutrition during early to mid-gestation has been shown to alter the pattern of fetal heart growth. Osgerby and co-workers have shown that a 30% reduction in maternal nutrition from d 22 of pregnancy did not alter fetal heart weight by day 45 or 90 of pregnancy but did result in a decrease in the absolute and relative weight of the fetal heart by day 135 (Osgerby *et al.*, 2002). In contrast a more severe maternal undernutrition (50% reduction) imposed between d 28 and 78 of gestation resulted in an increase in the weights of the right and left ventricles of the fetal heart and in altered expression of a range of genes [cardiac α-actin, cyclin G1, stathmin, NADH dehydrogenase subunit 2, titin, prostatic binding protein, caveolin, cardiac ankyrin repeat protein (CARP),

and cardiac-specific RNA-helicase activated by MEF2C (CHAMP)] that have been implicated in cardiac hypertrophy or in the inhibition of the remodelling of cardiac hypertrophic tissue by d 78 gestation (Vonnahme *et al.*, 2003; Hun *et al.*, 2004). Moderate maternal undernutrition during early to mid-gestation (85% of maintenance) has been shown to alter the development of the cardiovascular system in late gestation in terms of a lower operating point of the fetal baroreflex, lower mean arterial blood pressure, altered chemoreflex response to hypoxia and a decreased cortisol response to corticotropin releasing hormone (CRH) and arginine vasopressin (AVP); and during postnatal life development of the cardiovascular system resulted in an increase in mean arterial blood pressure (MAP) and an enhanced ACTH and cortisol response to CRH and AVP challenges (Hoet & Hanson, 1999; Hawkins *et al.*, 2000a; Hawkins *et al.*, 2000b). These authors postulate that maternal undernutrition during early to mid-gestation may act through the HPA axis to alter fetal and postnatal cardiovascular development.

1.13.4.4 Adrenal development

Alteration of the early embryo environment has been shown to alter the composition of pituitary cells that secrete ACTH in the late gestational fetus (Butler *et al.*, 2002). A moderate maternal restriction during early to mid-gestation has been shown to result in a decreased cortisol response to corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) in the late gestation fetus and an enhanced ACTH and cortisol response to CRH and AVP challenges during postnatal development (Hawkins *et al.*, 2000a; Hawkins *et al.*, 2000b). It was concluded from these experiments that the diminution of HPA

activity in conceptuses in this experimental design was partly explained by a decrease expression of CRH mRNA in the hypothalamic paraventricular nucleus and expression of glucocorticoid receptor mRNA in the anterior pituitary (Hawkins *et al.*, 2001).

Thus, I have tested the hypothesis in Chapter 3, that periconceptional undernutrition increases expression of the steroidogenic enzyme 17 alpha hydroxylase (CYP 17) and the intradrenal growth factor, IGF2 in the adrenals of twin, but not singleton, fetal sheep. Furthermore I have also determined whether there are direct relationships between the growth of the fetal adrenal gland and heart during the first ~ 55 days of pregnancy and that these relationships are differentially altered in singleton and twin fetuses by periconceptional undernutrition.

1.13.4.5 Kidney development

Maternal plasma cortisol concentrations have been demonstrated to be elevated by maternal undernutrition in the pregnant sheep (Edwards & McMillen, 2001). The administration of physiologic levels of glucocorticoids, both dexamethasone and cortisol, at the end of the first month of pregnancy (~ d 27) for 48 hours in the sheep has been demonstrated to cause hypertension and alterations in cardiovascular development in postnatal life (Dodic *et al.*, 1998; Dodic *et al.*, 1999; Moritz & Wintour, 1999; Dodic *et al.*, 2001; Dodic *et al.*, 2002a; Dodic *et al.*, 2002b; Wintour *et al.*, 2003b). A series of studies has also demonstrated that exposure to glucocorticoids at ~ d 27 pregnancy causes structural and functional changes in the developing metanephros leading to a 40% decrease in nephron numbers, resulting in hypertension in postnatal life (Moritz & Wintour, 1999;

Wintour *et al.*, 2003a; Wintour *et al.*, 2003b), and that these programming effects are not observed when glucorticoids are administered during mid – gestation (~ d 64) or over a longer period in early pregnancy (d 25 – 45) (Dodic *et al.*, 1999; Moritz *et al.*, 2002a; Dodic *et al.*, 2003). These findings have resulted in the hypothesis that an insult to the developing fetal kidney during active nephrogenesis will result in a renal deficit which is associated with the development of hypertension in postnatal life. The mechanisms by which such early exposure to glucocorticoids perturb nephrogenesis during this early period of development have not, however, been elucidated (Wintour *et al.*, 2003a).

Given the impact of exposure to cortisol on renal development during early gestation, in the present study, I investigated the hypothesis in Chapter 4 that there are relationships between maternal weight change during the periconceptional period, maternal plasma cortisol concentrations, and relative kidney weight at ~ d 55 pregnancy which are different in singleton and twin fetuses and which are altered by periconceptional undernutrition.

In the late gestation sheep fetus it has also been shown that IGF-2 mRNA is highly expressed in the fetal kidney (Kind *et al.*, 1995) and that intrafetal infusion of a long acting analogue of IGF-1 stimulates kidney growth in late gestation (Lok *et al.*, 1996; Marsh *et al.*, 2001). Interestingly, severe maternal nutrient restriction (to 50% of maintenance levels) during early gestation results in decreased IGF-2 mRNA expression in the kidney of twin, but not singleton fetuses during late gestation (Brennan *et al.*, 2005).

Therefore, I also tested the hypotheses in Chapter 4 that embryo number and/or maternal undernutrition during the periconceptional period alters the growth trajectory of the fetal kidney during early pregnancy and the mRNA expression of intrarenal growth factors, IGF-1, IGF-2, and their receptors, IGF-1R and IGF-2R.

1.14 *Ex vivo* nutrition of the embryo: effects of *in vitro* culture on development

With the advent of artificial reproductive technologies (ARTs) over the past four decades the ability to increase the reproductive efficiencies of subfertile individuals in humans and mammals has been increased. ARTs were developed in order to surmount poor fertility and reproductive challenges in the human, to increase the number of offspring from genetically superior females in farm species and to decrease the generational interval in genetic gain of economically viable characteristics in domestic production species (Boerjan *et al.*, 2000). *In vitro* production of embryos may include *in vitro* maturation of oocytes, *in vitro* fertilization, and *in vitro* culture of embryos (IVC) from the zygote to the blastocyst stage. *In vitro* culture is an essential and nascent step in most ARTs such as *in vitro* fertilization, cloning, intracytoplasmic sperm injection and zygote intrafollopian transfer (McEvoy *et al.*, 2001). It has been demonstrated that *in vitro* culture in the human, mouse and domestic ruminant species during zygote and early embryo development is associated with altered fetal and postnatal development (Biggers *et al.*, 1965; Bowman & McLaren, 1970; Walker *et al.*,

1996a; Young *et al.*, 1998; Hansen *et al.*, 2005). The future health effects of exposing the preimplantation embryo to the *ex vivo* environment of *in vitro* culture have not been elucidated. Louise Joy Brown, the first IVF baby, was born in July 1978 and thus the resulting children of *in vitro* culture and associated ARTs have not reached a mature enough age to evaluate the "adult health outcomes" of this embryonic exposure (van der Lende *et al.*, 2000).

1.14.1 *IN VITRO* CULTURE: DEVELOPMENT IN MAN AND MOUSE

Between 1996 and 2002 there was a 120% increase in the number of live babies born in the US which were conceived by the use of ARTs (Stroup *et al.*, 2002). *In vitro* fertilization and intracytoplasmic sperm injection both utilize the process of IVC, and these ARTs have now been shown to be extensively associated with increased pregnancy loss, low birth weight, babies small for gestational age and very small for gestational age, preterm birth intrauterine growth restriction and perinatal mortality (Doyle *et al.*, 1992; Tan *et al.*, 1992; McFaul *et al.*, 1993; Wang *et al.*, 1994; van Wagtendonk-de Leeuw *et al.*, 2000; Koivurova *et al.*, 2002a; Koivurova *et al.*, 2002b). Low birth weight for gestational age after conception via ARTs has been attributed to the higher rate of multi-fetal pregnancies (1990), however, a recent study has shown that singletons conceived by ARTs are also at an increased risk of low birth weight at term compared to spontaneously conceived singleton babies (Schieve *et al.*, 2002). In addition, IVC and ARTs in humans have been demonstrated to increase the rates of obstetric problems during pregnancy such as increased vaginal bleeding and increased rates of caesarean births (Tan *et al.*, 1992; Koivurova *et al.*, 2002b). Hansen and co-workers have estimated that babies conceived from ARTs have an increased rate

(25 – 200%) of birth defects and malformations compared to naturally conceived infants (Hansen *et al.*, 2002; Hansen *et al.*, 2005) and more specifically within the cardiovascular system (Anthony *et al.*, 2002). In terms of postnatal development, children conceived from ARTs have increased prevalence of neurological sequelae such as cerebal palsy (Stromberg *et al.*, 2002) and a significantly decreased growth rate for the first three years of life compared to children conceived spontaneously (Koivurova *et al.*, 2003).

In the mouse it has been observed that culture of embryos from the pre-morula to the blastocyst stage of development resulted in a decrease in implantation rates and fetal weight on d 17 of gestation after transfer (Biggers *et al.*, 1965; Bowman & McLaren, 1970). Further investigation into the effects of culture conditions on fetal development determined that protein free culture conditions increased embryo and fetal viability, however, culture conditions containing sera were associated with a decrease in fetal weight (Caro & Trounson, 1984; Khosla *et al.*, 2001a) and an increase in fetal death (Arny *et al.*, 1987; Van der Auwera *et al.*, 1999; Khosla *et al.*, 2001a) compared to *in vivo* derived control fetuses. In addition to decreasing embryo and fetal development, culture conditions containing sera as a protein source have been shown to influence the expression of growth related genes that are imprinted (Khosla *et al.*, 2001a; Khosla *et al.*, 2001b). The deleterious effects of a protein source in murine embryo culture may not be due to the sera or protein itself but the build up of embryo derived ammonium as waste (Lane & Gardner, 1994) or that the protein is not presented in culture in a synchronous manner as in oviductal and uterine phases of the maternal reproductive tract (Van Winkle & Dickinson, 1995). Consequently it has

been shown that mouse embryos cultured with a protein source during the preimplantation period positively affects development during the postimplantation period (Lane & Gardner, 1994).

Studies in the sheep employing a serum free synthetic oviductal fluid (SOF) media showed a retardation in embryo development (Tervit & Rowson, 1974), which was similar to observations by Bowman & McLaren in the mouse (Bowman & McLaren, 1970). There is evidence that mice and ruminants utilize amino acid and protein differently during embryo development which may explain the difference responses to the presence of a protein source in culture (Gardner *et al.*, 1994). The decrease in embryo and fetal growth of human and murine embryos contrasts with the increase in embryonic and fetal growth more commonly reported after *in vitro* culture of the ovine or bovine embryo (Kruip *et al.*, 2000).

1.14.2 *IN VITRO* CULTURE: SHEEP AND COWS

The ability for embryos to develop in a harsh *ex vivo* environment, which does not reflect the physiological or homeostatic environment of the oviduct and uterus would at first appear to indicate that the embryo is relatively robust. Observations, however, of increased fetal wastage and the generation of abnormally large offspring as a result of the commercial use of ARTs in breeding programs when compared to the use of artificial insemination alone indicate that the embryo may have had to adapt to ensure its immediate survival while in culture, thus altering its future fetal and postnatal growth trajectory (Walker *et al.*, 1992a).

During the early 1990's there was a great deal of communication amongst commercial animal breeding experts about the production of unusually large offspring as a result of ARTs and *in vitro* culture, and these discussions were addressed at the 1992 symposium of the International Embryo Transfer Society Congress (Bertolini & Anderson, 2002) and were first reported in the literature by Willadsen and colleagues in 1991 (Willadsen *et al.*, 1991). *In vitro* culture of ruminant embryos containing undefined sources of protein and growth factors such as serum or co-culture with support cells have led to a proportion of the resulting offspring having a number of characteristic abnormalities of which the enhanced size of the lamb or calf is the most recognizable (Walker *et al.*, 1996a; Walker *et al.*, 1998; Young *et al.*, 1998) and this has led to employing the term "Large Offspring Syndrome" (LOS) as an overarching term to describe the deleterious effects of exposing ruminant species to an *ex vivo* environment during early embryo development (Young *et al.*, 1998).

1.14.2.1 Large Offspring Syndrome: fetal effects

The "Large Offspring Syndrome" (LOS) is characterized by an increase in fetal size during early and late gestation, which varies from treatment to treatment but can be as large as five fold greater than mean control birth weights, altered allocations of cells during blastulation, decreased pregnancy rates, decreased survival during embryo development, early gestation, late gestation and during the perinatal period, altered growth trajectory of key organs such as the liver and heart, organomegaly, increase in crown rump length, skeletal growth, physical deformities and body temperature, altered fetal and perinatal blood chemistry and plasma nutrient concentrations, increased rates of abortion and dystocia –

sometimes requiring surgical intervention – and gestation length is significantly increased (Willadsen *et al.*, 1991; Walker *et al.*, 1992b; Hasler *et al.*, 1995; Thompson *et al.*, 1995; Holm *et al.*, 1996; Walker *et al.*, 1996a; Sinclair *et al.*, 1997; Walker *et al.*, 1998; Young *et al.*, 1998; Sinclair *et al.*, 1999; Sangild *et al.*, 2000; Bertolini & Anderson, 2002; Bertolini *et al.*, 2002; Miles *et al.*, 2004). Whilst gestational length is increased, it does not explain the enhanced fetal growth (Walker *et al.*, 1992b). The growth trajectory of large offspring has been demonstrated to be set early in gestation in both the sheep (~ d 60 – 75) (Walker *et al.*, 1998; Sinclair *et al.*, 1999) and the cow (~ d 70) (Farin *et al.*, 1997). In the cow there is evidence, however, that the resulting enhancement of fetal development is a result of biphasic growth in which there is actually a decrease in the growth of the embryonic disc during embryogenesis and fetal growth during early gestation compared to “control” fetuses, and then a subsequent increase of fetal growth and development in late gestation (Bertolini *et al.*, 2002).

LOS offspring are very susceptible to perinatal morbidity and this is associated with difficult breathing and reluctance to suckle (Walker *et al.*, 1996a). Although neonates exhibiting LOS are large at birth there is discrepancy as to whether this enhanced growth trajectory continues in postnatal development. In sheep, *in vitro* produced lambs had an increased body weight gain and growth rate during the first six months of life than *in vivo* derived lambs of the same sire (Walker *et al.*, 1998), however, it has also been shown that at one year of age LOS lambs were of similar weight as control animals (Walker *et al.*, 1996a). Similarly, in cows, exhibiting LOS at birth, slaughter weights and lifetime growth rates were not different from those of control individuals (McEvoy *et al.*, 1998). Interestingly,

the growth and development of the heart and the cardiovascular system seems to be particularly affected by *in vitro* culture during *in utero* development (Willadsen *et al.*, 1991; Sinclair *et al.*, 1999; Young *et al.*, 2001; Bertolini *et al.*, 2002) and the increased growth of the heart at birth persists in postnatal life (McEvoy *et al.*, 1998).

In vitro culture has also been shown to affect the maternal system that carries concepti exhibiting characteristics of LOS by decreasing signs of parturition and mammary development (McEvoy *et al.*, 2001), which may explain the decrease in drive in these neonates to suckle. In addition, the suppression of signs of parturition and mammary development may be placental in origin. The Aberdeen group have shown that culture of ovine embryos in co-culture and culture with serum systems lead to fetal overgrowth during late gestation, however, there was no observation in altered placental growth (Sinclair *et al.*, 1999), and this has lead these authors to speculate that the increase in conceptus size is "driven by the fetus and not by the placenta" (Young *et al.*, 1998). In contrast, other studies have shown an association in aberrant fetal and placental growth (Bertolini & Anderson, 2002), which may be due to augmentation of the blastocyst development during culture. Culture conditions known to enhance fetal growth have also been shown to alter the allocation of cells between the ICM and trophectoderm with a preference to increase trophectoderm to ICM cells (Walker *et al.*, 1996a; Walker *et al.*, 1998; McEvoy *et al.*, 2001).

1.14.2.2 Large Offspring Syndrome: placental effects

Whilst there is a substantial amount of literature describing how *in vitro* culture can alter the growth trajectory of the fetus and neonate in both sheep and cows, the literature is sparse in describing the effects of culture on the development of the placenta. In the sheep, there was no increase in size of the placenta in early or late gestation in association with the observed increase in fetal size (Sinclair *et al.*, 1998b; Young *et al.*, 1998). There is evidence in the cow that *in vitro* culture causes an increase in placental size during early (Miles *et al.*, 2005) and late gestation (Bertolini *et al.*, 2002; Bertolini *et al.*, 2004; Miles *et al.*, 2004), however, the growth trajectory during early gestation is disputed. Studies by Miles and co-workers have shown that there is an increase in placental weight in early gestation resulting from embryo culture in a defined media, but this is associated with a decrease in placentome number (Miles *et al.*, 2005), whilst Bertolini and co-authors, using an undefined co-culture method, hypothesize that there is a biphasic growth pattern resulting in an increase in fetal and placental size in late gestation where there is a retardation of placental growth during early gestation (Bertolini *et al.*, 2002). The differences observed in these two experiments may be dependent upon the culture system used to generate the experimental groups.

Interestingly, *in vitro* embryo culture, in addition to increasing placental weight in the cow, alters placentome morphology (Bertolini *et al.*, 2002; Farin *et al.*, 2006) and increases the diameter of these placentomes (Bertolini *et al.*, 2002) indicating a shift in placentome population to those more everted (equivalent to type C and D in the sheep). There is also discrepancy in the literature as to

whether these observed changes after exposure of an embryo to culture are a measure of dysfunction or compensatory growth to meet fetal demands or drive fetal growth. There is evidence that *in vitro* culture decreases placental function and efficiency (Miles *et al.*, 2005), which could be mediated through the observed decrease in density of fetal villi and binucleate cells (Miles *et al.*, 2004), however, if this is the case there are maternal compensatory mechanisms such as increase in maternal caruncular vasculature and surface area of exchange that are implicated in maintaining nutrient transport to the fetus (Miles *et al.*, 2004). In contrast there is evidence that the placenta increases its efficiency in nutrient transfer to meet or drive the growth demands of larger fetuses by increasing placental gross surface area of exchange, which results in higher fetal plasma concentrations of glucose and fructose without altering the expression of the glucose transport system (Bertolini *et al.*, 2004).

In vitro culture is widely reported to cause polyhdramnios, hydramnios and hydrallantois in pregnancies even if a large offspring is not generated (Willadsen *et al.*, 1991; Walker *et al.*, 1996a; Young *et al.*, 1998; Miles *et al.*, 2004; Farin *et al.*, 2006). There is a "failure" of normal development of the chorioallantoic membranes and blood vessels in *in vitro* produced pregnancies (Farin *et al.*, 2006) and this may be the physiological cause to the increase in utero-placental fluids.

There is no explanation for the variation of abnormalities across the number of experiments examining the effects of *in vitro* culture on fetal and placental development. One possible explanation for the differences reported is the

various types of culture conditions used to produce the experimental offspring. Currently *in vitro* production of embryos is constituted by or in part by oocyte collection, *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF), *in vitro* culture to the blastocyst stage (IVC) and embryo transfer (ET) to recipients. Currently in the cow, the efficiency of IVP is 85% of immature oocytes undergo nuclear maturation during IVM (this may be further depressed if oocytes are collected from abattoir ovaries), 80% are fertilized during IVF, 30 – 40 % blastulate, and of embryos transferred 50 % will result in a maintained pregnancy (Wrenzycki *et al.*, 2004). It is thus clear that whilst embryos of ruminants are resilient to an *ex vivo* environment that this environment is not as efficient as the maternal reproductive tract in facilitating the normal development of offspring (Walker *et al.*, 1992a). *In vitro* culture is, in principle, a biochemical manipulation of the embryo and during the early stages of this technology was based on normal cell culture systems and "empircalism rather than precise" scientific knowledge (McEvoy *et al.*, 2001). As the understanding of the technology of *in vitro* culture of embryos advanced it was understood that the culture media needed to temporally reflect the conditions of the reproductive tract (ie the secretions of the oviduct or uterus depending on embryo stage of development) for normal development to occur (Walker *et al.*, 1998).

1.14.3 LARGE OFFSPRING SYNDROME: CULTURE CONDITIONS

The female reproductive tract nurtures and communicates to the embryo and maintains its environment in a precise homeostatic fashion, however, the environment of *in vitro* culture systems are usually static and various components such as metabolites, nutrients and cellular waste change with the growth and

metabolism of the embryo (Walker *et al.*, 1998). A number of different culture systems have been used to culture embryos and include both defined and undefined media. The difference between defined and undefined media is basically the source of protein for embryo metabolism and development.

1.14.3.1 IVC undefined: co-culture

IVC co-culture methodologies culture prepared mammalian cells with the developing embryos in order to produce the needed nutrients, growth factors, maintain the embryonic environment and mimic the maternal reproductive tract of the growing embryo (Bavister, 1988; Walker *et al.*, 1992a). Co-culture systems use a range of cell types, which are usually from the reproductive tract such as cumulus, granulosa and oviductal epithelial cells (Holm *et al.*, 1996; Sinclair *et al.*, 1999; Bertolini *et al.*, 2004), however, some culture systems use other types of cells from other species such as Buffalo Rat Liver cells (Hasler *et al.*, 1995). The addition of serum to co-culture systems is usually necessary to help maintain the "feeder" cells for the embryo. Co-culture of ovine and bovine embryos has resulted in accelerated embryo growth and blastulation rates (Watson *et al.*, 1994b; Hasler *et al.*, 1995) and decreased growth rates in the sheep (Holm *et al.*, 1996). Co-culture systems reliably increase fetal and birth weights, increase rates of abortion and dystocia in the sheep and cow (Hasler *et al.*, 1995; Holm *et al.*, 1996; Sinclair *et al.*, 1999; Bertolini *et al.*, 2002; Bertolini *et al.*, 2004), increase placental weight and alter the morphology of placentomes – specifically in the cow (Bertolini *et al.*, 2002; Bertolini *et al.*, 2004). There is evidence that there may be an interaction with the media type and the use of co-culture as the source of fetal overgrowth (Hasler *et al.*, 1995; Van Soom *et al.*, 1996). Co-

culture of embryos has also been shown to produce effects that are greater than culturing embryos in a defined culture media with added serum as a protein source (Sinclair *et al.*, 1999), however, the extent to which the characteristics of the LOS are observed may be dependent upon the type of media being used.

1.14.3.2 IVC undefined: serum as a protein source

The use of serum as a protein source in culture systems is one variable that has been associated with the LOS (van Wagtendonk-de Leeuw *et al.*, 2000). An undefined culture system usually consists of synthetic oviductal fluid that reflects the fluid composition of the oviduct and is supplemented with a proteins source, commonly serum or bovine serum albumin (Walker *et al.*, 1992a; Walker *et al.*, 1998). Serum has been shown to be superior to BSA in promoting embryo growth and health (Walker *et al.*, 1992b), and human serum that is heat inactivated at a 20%/vv is the optimal serum supplement for positively influencing embryo growth and blastulation (Walker *et al.*, 1992a; Walker *et al.*, 1992b; Walker *et al.*, 1998). Serum includes a variety of components such as hormones, proteins and growth factors, which presents a “rich environment” for embryonic development (van Wagtendonk-de Leeuw *et al.*, 2000). Serum, due to its undefined nature, is unpredictable in affecting embryo and *in utero* development, and this is thought to be due to different serum batches or sources (Batt & Miler, 1988). Culture systems using serum as a protein supplement increases embryo growth and blastulation rates but decrease ICM cell numbers of the blastocyst (Walker *et al.*, 1992a; Walker *et al.*, 1998). Interestingly, while embryo growth is promoted by the presence of serum, morphology is changed by the excess accumulation of lipid droplets (Thompson *et al.*, 1995), which may explain the

observed increase in fragmentation of embryo cells, altered gene expression and decreased viability after transfer (Walker *et al.*, 1992a; Walker *et al.*, 1998). Viability of transferred embryos produced in culture systems using serum have been demonstrated to be similar to *in vivo* embryos up to d 14 of pregnancy, then fetal wastage occurs until d 50 and a subsequent higher rate of fetal death between d 50 and term in the sheep (Walker *et al.*, 1992a; Walker *et al.*, 1992b; Walker *et al.*, 1998). Undefined culture systems using serum as a protein source reliably produce concepti exhibiting characteristics of LOS (Thompson *et al.*, 1995; Walker *et al.*, 1998; Sinclair *et al.*, 1999) and in the cow increases placental weight and functional capacity (Miles *et al.*, 2004). Thus, Young and co-workers logically postulated that serum may contain factors such as growth factors or free radicals that may cause the alteration in embryo physiology that leads to LOS (Young *et al.*, 1998). It is important to note that co-culture systems sometimes contain serum and have greater effects of LOS than non-coculture sytstems supplemented with serum (Sinclair *et al.*, 1999).

1.14.3.3 IVC defined

Defined medium used in embryo culture systems are composed of synthetic oviductal fluid and known quantities of amino acids that reflect the physiological concentrations of oviductal fluid (Walker *et al.*, 1992a). The use of defined media in culture systems in the sheep has proven to decrease the incidence of characteristics of LOS (Thompson *et al.*, 1995; van Wagtendonk-de Leeuw *et al.*, 2000), whilst facilitating a high level of blastulation, improving embryo health and morphology – significantly decreasing embryo lipid accumulation and fragmentation, an increase in hatching rates and improving embryo/fetal viability

after transfer (Walker *et al.*, 1998). In contrast there is evidence that defined medium in bovine embryo culture continues to alter placental development (Miles *et al.*, 2005) and embryonic metabolism (Eckert *et al.*, 1998).

1.14.3.4 Effects of embryo transfer

Many researchers have used *in vivo* matured embryos that are subsequently transferred to final recipients as “control” pregnancies or animals to compare *in vitro* cultured embryos, however, there are data in both the sheep and cow that suggests that *in vivo* culture and/or embryo transfer affects the development of the fetus. *In vivo* maturation or culture of embryos involves superovulating the dam and collecting the embryos approximately 6 to 8 days after fertilization. The birth weight of lambs produced by multiple ovulation and embryo transfer (MOET) has been shown to be increased compared with naturally mated animals in a control flock, however, the increase in weight is not as dramatic as those derived from embryo culture (Walker *et al.*, 1996a). In the cow, fetuses derived from MOET have increased gestation length, birth weights, dystocia and decreased pregnancy rates when compare to controls derived from artificial insemination (van Wagtendonk-de Leeuw *et al.*, 2000). It has been postulated that the reason that MOET alone can affect fetal development is superovulation. The increased number of follicles developing and the subsequent CLs would produce physiologically larger amounts of oestrogen and progesterone (van Wagtendonk-de Leeuw *et al.*, 2000), which would lead to an altered development of the oviductal and uterine epithelium and secretions (Barnes, 2000). In addition synchrony of the recipient could have developmental effects (Boerjan *et al.*, 2000).

A significant problem in evaluating the extent to which *in vitro* embryo culture and ARTs cause differences in fetal, placental and postnatal development compared to spontaneously conceived offspring *in vivo* is that many experimental protocols use either MOET or artificially inseminated (AI) derived control animals (Young *et al.*, 1998). It has been shown that both MOET and AI fetuses, pregnancies and offspring are not the "gold standard" and develop differently than those spontaneously conceived *in vivo* (van Wagtendonk-de Leeuw *et al.*, 2000). Van Wagtendonk-de Leeuw and co-workers postulate that the true "gold standard" to measure the effects of ARTs are naturally conceived controls (van Wagtendonk-de Leeuw *et al.*, 2000). Another significant problem in the field is that a great deal of scientific findings about the effects of ARTs and *in vitro* culture are presented in conference abstracts and are not published fully, whilst many authors quote these proceedings one must wonder if the results presented have been validated.

1.14.4 LARGE OFFSPRING SYNDROME: JUST A PHENOTYPE?

The features and characteristics ascribed to LOS do not occur consistently from all embryo culture systems, and the incidence of LOS is highly variable from no observable phenotype to 100% (Young *et al.*, 1998). Young and colleagues also question whether the observed outcomes from different culture systems and ARTs are the same phenotype or a combination of many (Young *et al.*, 1998). Additionaly, it is possible that many of the "normally" born individuals resulting from IVC and ARTs may present a less severe phenotype that is physiologically active at the cellular or system level, but does not cause observable characteristics such as enhanced growth (Farin *et al.*, 2006). These phenotypes

may prove to be more severe with increasing age or “program” an individual for adult diseases. In addition, the high rate of embryo and fetal wastage after the blastocyst stage of development may indicate more severe phenotypes whose physiology was so augmented that they were not sustainable *in utero* (Farin *et al.*, 2006). Due to these questions Farin and co-authors have discussed that the term of Large Offspring Syndrome be used to refer to individuals with enhanced growth and that the term “Abnormal Offspring Syndrome” be adapted as an overarching term to refer to all altered phenotypes as a result of IVC and ARTs (Farin *et al.*, 2006).

1.14.5 *In vitro* culture: fetal number?

The increased rates of decreased fetal growth, low birth weights and preterm delivery in both man and mouse resulting from ARTs have been hypothesized to be due in part to multifetal pregnancies. However, in ruminants fetal growth is enhanced and the majority of studies have only investigated singleton pregnancies. The literature describing the effects of *in vitro* culture on twin pregnancies is sparse. In the sheep there was no difference in fetal or placental measurements in twin *in vitro* produced pregnancies compared to control animals (Walker *et al.*, 1992b; Holm *et al.*, 1996), however, twin fetuses resulting from IVC and microinjection were larger than *in vivo* derived fetuses (Walker *et al.*, 1992b). In the cow, there was no difference in fetal growth parameters in twins derived from IVC, but there was a similar effect on placentome size with a trend towards more everted placentomes (Bertolini *et al.*, 2002).

In Chapter 5, I have therefore tested the hypothesis that superovulation, artificial insemination and embryo transfer with or without *in vitro* culture in the presence or absence of human serum differentially alters the growth of the placenta, fetus and fetal organs during late gestation when compared to naturally conceived controls.

Chapter 2:

Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep

"Go for gold, silver is first to lose!"
- Sister Carol Anne

"Lead me, follow me or get out of my way"
- General George S. Patton Jr.

2. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep

2.1 SUMMARY

Recent studies in the sheep have shown that maternal undernutrition during the periconceptional period, when the nutrient demands of the embryo are minimal, can alter the subsequent development of the metabolic, endocrine and cardiovascular systems and that these effects may, in part, depend on embryo number. We have tested the hypotheses that there are relationships between maternal weight or body condition at the time of conception and feto-placental growth during the first 55 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. We have investigated the effect of periconceptional undernutrition in the ewe (control n= 24, restricted at 70% of control feed allowance, PCUN n=21) from 45 days prior to mating until 7 days after mating on placental and fetal weight and on placental histology in singleton and twin pregnancies at d 53-56 gestation ie during the period of maximal placental growth. In control, but not PCUN ewes carrying singleton pregnancies, there were direct relationships between maternal weight gain during the periconceptional period and uteroplacental weights at d 53-56 gestation. There were direct relationships, however between placental and fetal weights in both control and PCUN singleton pregnancies. In contrast to the singletons, in control twin pregnancies, there was no effect of maternal

weight gain in the periconceptional period on any measure of uteroplacental growth and there was also no relationship between placental and fetal weight. This lack of a relationship may be related to the increased uteroplacental weight and mean placentome weight in the twin pregnancies (control singletons: 2.45 ± 0.18g; control twins: 4.10 ± 0.62g). In the PCUN group, however, a greater weight loss between the start of the feeding regime and post mortem at ~ d 55, was associated with a larger placenta and fetus and the direct relationship between placental and fetal mass was restored. In summary, the present study has demonstrated that there are important relationships between maternal weight gain during the periconceptional period and feto-placental growth during the first 56 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. In singleton pregnancies, periconceptional undernutrition disrupts the relationship between maternal weight gain during the periconceptional period and uteroplacental growth and in twin pregnancies, periconceptional undernutrition results in the emergence of an inverse relationship between maternal weight gain during early pregnancy and uteroplacental growth and in a dependence of fetal growth on placental growth. These changes highlight the importance of the periconceptional environment in setting the placental and fetal growth trajectories and have implications for the programmed development of the metabolic, cardiovascular and endocrine systems of the fetus and adult.

2.2 INTRODUCTION

There is evidence from a range of epidemiological, clinical and experimental studies that maternal nutrient restriction before or immediately after conception alters fetal and adult health outcomes. The Dutch Famine Winter Study investigated the effects of the 5 month period of malnutrition experienced by pregnant women in Amsterdam during 1944-1945 and found that individuals exposed as an embryo and fetus to the maternal malnutrition of the famine during the first trimester had an increased prevalence of coronary heart disease and a higher body mass index in adult life (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000b; Roseboom *et al.*, 2001b). These findings suggest that nutrient restriction during early pregnancy, when the nutrient demands of the early conceptus are minimal, can have specific long-term consequences. It has also been shown that when pregnant rats were fed a low protein diet for the first 4.25 days after conception, there was a decrease in the cell numbers in the blastocyst, a decrease in birth weight and an increase in systolic blood pressure in postnatal life (Kwong *et al.*, 2000). In the sheep, a 30% reduction in maternal nutrition from at least 45 days before until 7 days after conception resulted in an increase, in late gestation, in arterial blood pressure and in the activation of the fetal pituitary-adrenal axis in twin but not singleton fetuses (Edwards & McMillen, 2002a, b). When maternal nutrition is restricted more severely and for longer periods (up to 30 days after conception) then there is enhanced activation of the fetal pituitary-adrenal axis (Bloomfield *et al.*, 2003) and altered cardiovascular function in postnatal life in singletons (Gardner *et al.*, 2004b). The reason for the differential effects of periconceptional undernutrition in twin and singleton

pregnancies is not clear. Studies in human pregnancies with more than 2 fetuses have found that after the number of embryos is reduced to two in the first trimester, the birth weights of the remaining twins were significantly reduced compared with the birth weights in the non-reduced twin pregnancies (Sebire *et al.*, 1997). Thus the fetal growth trajectory in early pregnancy is related to fetal number and this may be important in determining the differential impact of periconceptional undernutrition on singletons and twins.

It also appears that maternal weight or body condition at the start of pregnancy may play a role in feto-placental growth in early pregnancy and that this role differs in multi-fetal and singleton pregnancies. In ewes of a prolific genotype, Greenwood and colleagues (Greenwood *et al.*, 2000) found that there was no relationship between fetal and placental weight at d 85, although there was a significant relationship at d 130 (term = ~147). In that study, the weight of the ewe in early pregnancy was positively related to placental and fetal weights at d130, whereas ewe fatness in early pregnancy was inversely related. In contrast, Osgerby and colleagues (Osgerby *et al.*, 2003b) found that the weight of singleton fetuses at d 65 was greater in fat ewes than in ewes of moderate condition and it was suggested that the increased placental weight in these ewes may have contributed to the increase in fetal growth in early pregnancy.

Whilst interactions between the effects of maternal body weight or condition at mating and maternal undernutrition during early-mid pregnancy on the growth of the placenta and fetus have been investigated (McCrabb *et al.*,

1992), there have been no studies that have investigated the interactions between maternal body weight or condition at the start of pregnancy and the level of periconceptional nutrition on placental and fetal growth during early pregnancy. In the sheep, the number of placentomes in the placenta is fixed by d 56 (Wallace, 1948), placental cellular proliferation peaks at d 50 – 60 and placental weight is maximum around d 80 of pregnancy (Ehrhardt & Bell, 1995). We have therefore tested the hypotheses that there are relationships between maternal weight or body condition at the time of conception and feto-placental growth during the first 55 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies.

2.3 MATERIALS AND METHODS

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee.

Forty-five South Australian Merino ewes were used in this study. Ewes were moved into an enclosed shed and housed in pens 2 weeks before the start of the feeding regime. All ewes were weighed and a body condition score assessed employing a 1-5 scale with 0.5 intervals by an experienced assessor (Russel *et al.*, 1969; Greenwood *et al.*, 2000). A body condition score of 1 represents an extremely emaciated animal and a body condition score of 5 represents an extremely obese animal. During this 2 week period,

ewes were acclimatized to a pelleted diet containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime and molasses (Johnsons & Sons Pty Ltd, Kapunda, South Australia, Australia). The pellets provided 9.5 MJ/kg of metabolizable energy and 120 g/kg of crude protein and contained 90.6% dry matter. All ewes received 100% of nutritional requirements (7.6 MJ/ day for the maintenance of a 64 kg non pregnant ewe) as defined by the Agricultural and Food Research Council . At the end of this acclimatization period, ewes were randomly assigned to one of two feeding regimes, a control regime (C, n = 24), in which ewes received 100% of nutritional requirements or a restricted regime (PCUN, n = 21), in which ewes received 70% of the control allowance. All of the dietary components were reduced by an equal amount in the restricted diet. Ewes were maintained on these respective diets for at least 45 days before mating. Control ewes were maintained on the control diet for 62 ± 5 days and the PCUN ewes were maintained on the 70% diet for 55 ± 2 days prior to conception. The periconceptional undernutrition regime (PCUN) is defined as restriction for at least 45 days prior to mating and for 7 days post conception (Figure 1).

Ewes were then released in a group every evening at 1600h with two intact rams of proven fertility that were fitted with harnesses and marker crayons. Ewes were individually penned the following morning at 0800h, and the occurrence of mating was confirmed by the presence of a crayon mark on the ewe's rump. Pregnancy was diagnosed and fetal number estimated by ultrasound at d 45 of pregnancy. The day of mating was defined as d 0. From 7 days after mating (d 7 of pregnancy), all ewes were fed a control diet

(100% of requirements) until post mortem (PM) at d 53-56 pregnancy (Figure 1). Ewes were weighed and their body condition was assessed and scored approximately every two weeks after commencing the feeding regime until post mortem at d53-56 of pregnancy. Whilst 36 of the 45 ewes were weighed within the first week after mating, due to logistics a small group were weighed outside this week up to d 10 after mating. We have therefore defined the weight change during the periconceptional period as up to d 10 after mating. The number of fetuses carried by each ewe was confirmed at PM generating four treatment groups: control singleton pregnancies (n = 18), PCUN singleton pregnancies (n = 16), control twin pregnancies (n = 6), and PCUN twin pregnancies (n = 5) (Figure 1).

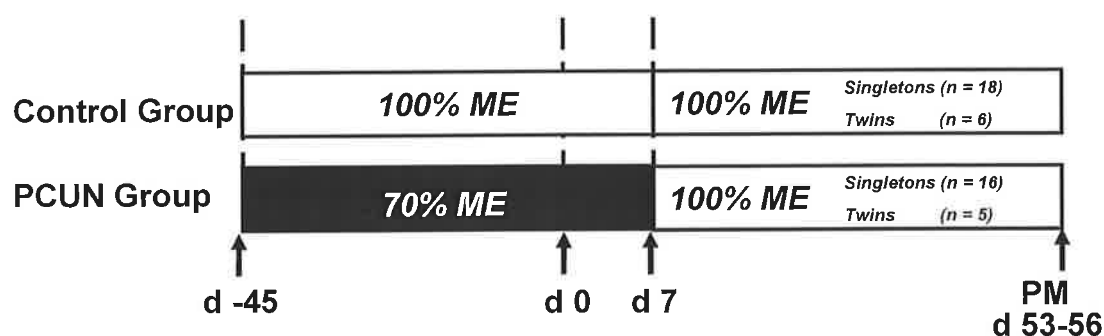

Figure 2.1 Schematic representation of nutritional regime during the periconceptional period.

2.3.1 COLLECTION OF TISSUES

Ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) between d 53 and d 56 of pregnancy (term = 150 ± 3 days gestation), and the utero-placental unit was delivered by hysterotomy. The placenta was immediately dissected and the placentomes were individually weighed and counted. Between 2 and 4 placentomes from each placenta were immersed in 4% paraformaldehyde/0.1M phosphate buffer for a maximum of 24 h for histological analysis. Fetal membranes and the uterus were trimmed of excess fluid and weighed and fetuses were also weighed.

2.3.2 PLACENTAL HISTOLOGY AND MORPHOMETRY

The day after PM, tissues were briefly rinsed in phosphate buffered saline (0.05M PBS) and then immersed into 70% ethanol for 2 d, prior to processing and embedding in paraffin wax. Sagittal sections (7 μm) were cut and stained with Masson's trichrome using standard methods. Sections were examined with a 10x objective lens and a 2.5x ocular lens on an Olympus BH2 microscope using a Video Image Analysis system and Video Pro software (Leading Edge, Australia). The proportions of placental trophoblast, fetal capillaries, fetal connective tissue, maternal epithelium, maternal capillaries, maternal connective tissue and 'other' tissue were quantified using point counting with an isotropic L-36 Merz transparent grid placed on the monitor screen. A random systematic field selection method was utilized. Ten fields (360 points) were counted in each section. The first field was selected in the

zona intima at random and subsequent sections were systematically selected 1 mm apart with the aid of the stage micrometer. The volume density of each of the specified components of the placenta was calculated using the following formula, Volume Density, $V_d = P_a/P_T$, where P_a is the total number of points falling on that component and P_T is the total number of points in the section (Weibel, 1979; Roberts *et al.*, 2001). The estimated weight of each of the specified components in each individual placentome or each placenta was calculated by multiplying the volume density of each component by the weight of the individual placentome or placenta.

Intercept counting on the same grid on the same fields was utilised to calculate the surface density (surface area per gram of placentome or placenta) of trophoblast, taking into account the total magnification on the monitor screen by using the formula, Surface Density, $S_v = 2 \times I_a/L_T$, where I_a is the number of intercepts with the line and L_T is the total length of the lines applied (Weibel, 1979; Roberts *et al.*, 2001). Total surface area for the total placenta and placentome from which the section was cut was calculated by multiplying the surface density by total placental weight or placentome weight. The arithmetic mean barrier of trophoblast to diffusion was calculated using the formula, Barrier Thickness, $B_T = V_d/S_V$, where V_d is the volume density of trophoblast and S_v is the surface density of trophoblast (Weibel, 1979; Roberts *et al.*, 2001). The reproducibility of the method was determined by repeating the observations on one section seven times. The variation between each was less than 5%.

2.3.3 Statistical Analysis

Data are presented as the mean ± SEM. The effects of periconceptional undernutrition on ewe weight or body condition score and on the change in either weight or body condition score between –d 45 and d 10 (as an estimate of change during the periconceptional period), between d 10 and d 56 and between –d 45 and d 56 (the period before post mortem) were determined using a 2 way Analysis of Variance (ANOVA). The effects of periconceptional undernutrition and fetal number on uterine weight, fetal membrane weight, placentome number, mean placentome weight, total placental weight, individual fetal weight or total fetal weight (twins) were also determined by a 2 way ANOVA using the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). Relationships between variables were assessed separately in singleton and twin pregnancies by linear regression using Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA). Partial correlational analysis was used in order to determine whether the effects of maternal weight or maternal weight changes on fetal weight were present when the effects of placental weight were controlled for in the analysis. A probability level of 5% ($P<0.05$) was assumed to be significant.

2.4 RESULTS

2.4.1 SINGLETON PREGNANCIES

2.4.1.1 PCUN, maternal weight and condition

The weights of the non pregnant ewes assigned to the control (65.3 ± 1.4 kg, n = 18) or PCUN (62.9 ± 1.6 kg, n = 16) groups were not different at the start of the feeding regime. The mean body condition score of the non pregnant ewes assigned to the control group (3.7 ± 0.1) was higher than in the PCUN group (3.3 ± 0.1, $P < 0.01$) at the start of the feeding regime. Ewes in the PCUN group lost significantly more weight ($P < 0.05$) and body condition score ($P < 0.05$) than those in the control group during the periconceptional period (Table 1 and Figure 2A and B). The PCUN ewes also gained less weight ($P < 0.05$, Table 1) and body condition score than control ewes between the start of the feeding regime and PM at d 53 – 56 pregnancy. There was no difference, however, in the changes in maternal weight and body condition score between d 10 and PM between the two feeding groups (Table 1).

Table 2.1: Effect of fetal number and PCUN on maternal weight changes

	Maternal weight change between the start of the feeding regime and d 10 after mating (kg)	Maternal weight change from d 10 to post-mortem at d 53-56 (kg)	Total maternal weight change between the start of the feeding regime and post-mortem at d 53-56 (kg)
Control singles	0.44 ± 0.76	3.22 ± 0.53	3.67 ± 0.67
PCUN singles	-5.3 ± 0.78*	5.34 ± 0.90	0.94 ± 0.59*
Control twins	0.83 ± 1.44	2.08 ± 0.87	2.92 ± 1.46
PCUN twins	-2.20 ± 2.38#	4.20 ± 2.38	2.00 ± 2.01#

*Denotes significant differences between control and PCUN singles ($P < 0.05$)
#Denotes significant differences between control and PCUN twins ($P < 0.05$)

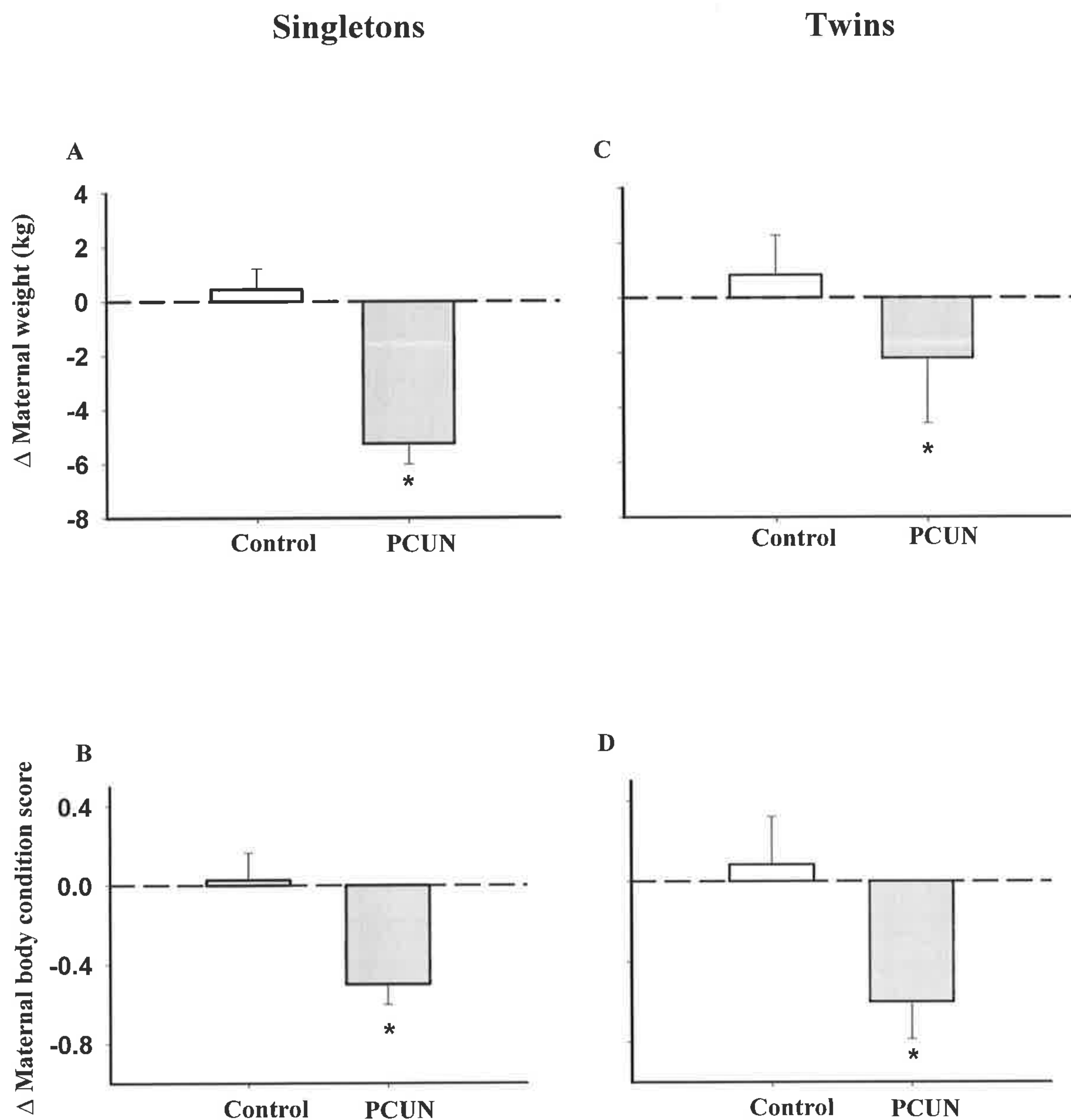

Figure 2.2 Change in maternal weight during the periconceptional period

Ewes carrying singleton pregnancies in the PCUN group (n = 16) lost significantly more weight (A) and body condition score (B) during the periconceptional period than ewes carrying singletons in the control group (n=18). Ewes carrying twins in the PCUN group (n = 5) also lost significantly more weight (C) and body condition score (D) (P<0.002) during the periconceptional period than ewes carrying twins in the control group (n = 6). Δ = Change. * denotes a difference (P<0.05) between control and PCUN groups.

2.4.1.2 PCUN, changes in maternal weight during the periconceptional period and uteroplacental and fetal growth

In singleton pregnancies, there was no effect of PCUN on uterine weight, the weight of the fetal membranes, placentome number, mean placentome weight or total placental weight at d 53-56 pregnancy (Table 2). There were significant relationships, however, between maternal weight at conception and either uterine weight, or the weight of the fetal membranes in the control but not the PCUN group (Table 3). There were also significant relationships between maternal weight gain during the periconceptional period and fetal membrane weight, mean placentome weight and total placental weight in ewes in the control but not the PCUN group (Table 3 and Figure 4A and D).

There was no difference between the weights of male and female fetuses in either the Control or PCUN groups (Table 4). There was also no effect of PCUN on either fetal weight or fetal ponderal index at d 53-56 (Figure 3A and B). There was, however, a significant relationship between maternal weight gain during the periconceptional period and fetal weight in control but not PCUN ewes (Table 3 and Figure 4B and E). There was also a significant relationship between placental weight and fetal weight in both control ($y = 0.05x + 17.56$, $r = 0.68$, $n = 18$, $P < 0.002$, Figure 4C) and PCUN groups ($y = 0.03x + 22.22$, $r = 0.61$, $n = 16$, $P < 0.02$, Figure 4F). When a partial correlational analysis was performed, it was determined that the relationship between maternal weight gain during the periconceptional period and fetal weight in control pregnancies was no longer significant ($r=0.19$, $P=0.47$) when the effects of placental weight were controlled for in the analysis.

Table 2.2: Effect of fetal number and PCUN on uteroplacental weights

	Singles		Twins	
Tissue	*Control (n=18)*	*PCUN (n=16)*	*Control (n=6)*	PCUN *(n=5)*
Uterine weight (g)	213.8±11.4	198.4±8.1	288.6±40.0*	286.5±32.9*
Fetal membrane weight (g)	127.1±15.9	127.4±13.5	223.3±57.4*	259.2±53.9*
Placentome number	66.7±3.9	73.4±5.3	71.8±4.3	86.0±5.5
Mean placentome weight (g)	2.45±0.18	2.72±0.26	4.10±0.62*	3.91±0.43*
Total placental weight (g)	165.3±16.1	191.8±15.5	295.2±47.4*	334.4±38.3*

* Denotes significant differences between twin and singleton pregnancies ($P < 0.0001$).

Table 2.3 Relationships between maternal weight at conception or maternal weight change during the periconceptional period and uteroplacental and fetal weights in singleton pregnancies.

	Control Singles		PCUN Singles	
Tissue	***Maternal weight at conception***	***Maternal weight change during the periconceptional period***	***Maternal weight at conception***	***Maternal weight change during the periconceptional period***
Uterine weight	y=4.12x – 57.18 (r=0.54, n = 18, P< 0.03)	NS	NS	NS
Membrane weight	y=6.36x – 291.23 (r=0.60, n=18, P< 0.01)	y=10.76x +122.30 (r=0.52, n=18, P < 0.03)	NS	NS
Total Placental Weight	NS	y = 11.98x + 159.9 (r = 0.57, n = 18, P < 0.02)	NS	NS
Mean placentome weight	NS	y = 0.12x + 2.4 (r=0.50, n=18, P < 0.04)	NS	NS
Fetal weight	NS	y = 0.75x + 25.92, (r = 0.46, n = 18, P = 0.05)	NS	NS

NS denotes relationships which were not significant

Table 2.4: Effect of fetal number, sex and PCUN on fetal weight

	Male	**Female**
Experimental *Group*	*Fetal weight (g)*	*Fetal weight (g)*
Control Singles	25.87±2.37 (n= 8)	26.57±1.32 (n=10)
Control Twins	29.53±1.33 (n=7)	26.94±0.76 (n=5)
PCUN Singles	28.07±1.21 (n=8)	28.15±1.07 (n=8)
PCUN Twins	26.58±2.31 (n=6)	24.85±3.51 (n=4)

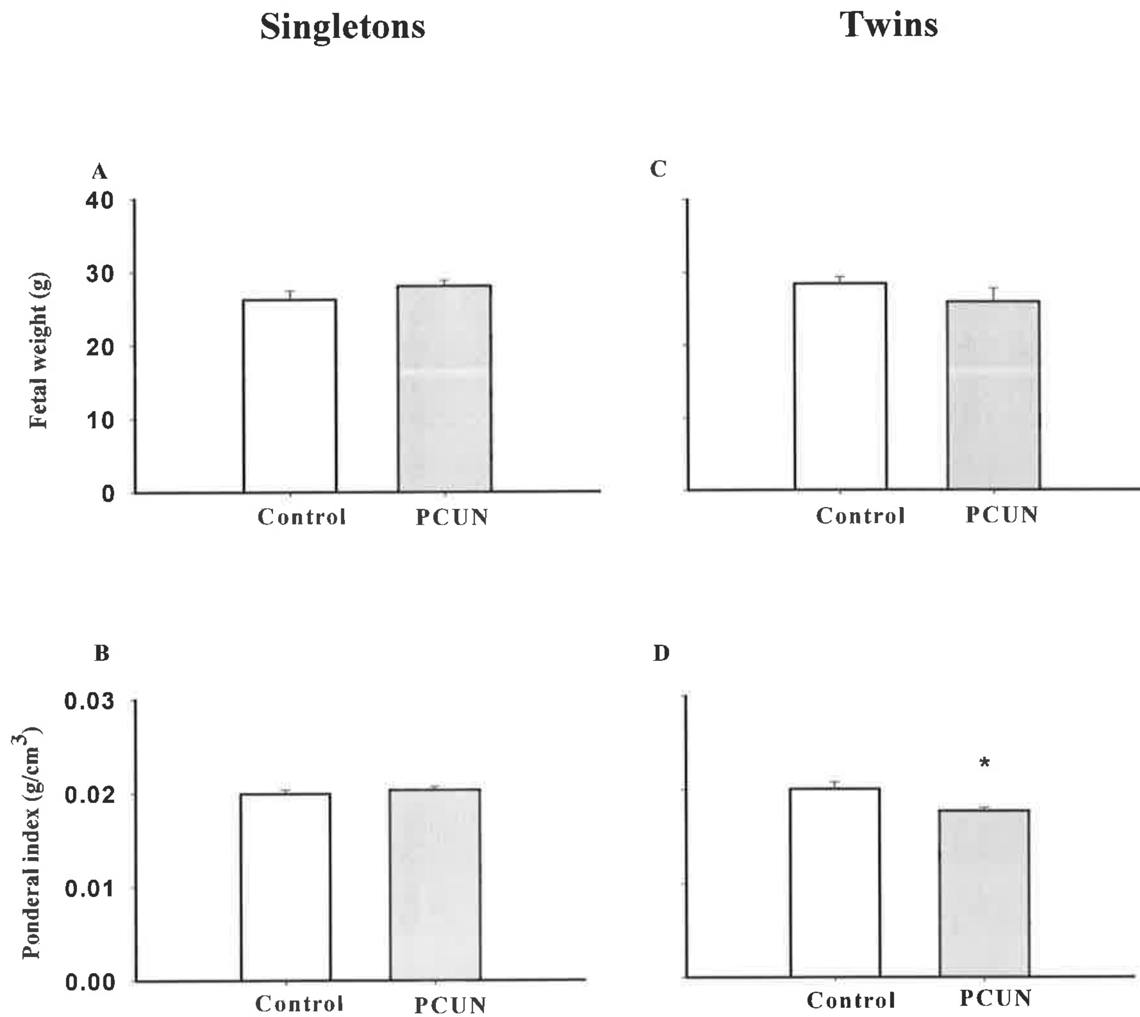

Figure 2.3 The effect of PCUN on fetal growth

There was no effect of PCUN on the mean weight (A) or ponderal index (B) of singleton fetuses. There was no effect of PCUN on individual weight (C) of twin fetuses. Fetal ponderal index (D) was lower in twins in the PCUN group (* denotes a difference ($P<0.05$) between control and PCUN groups).

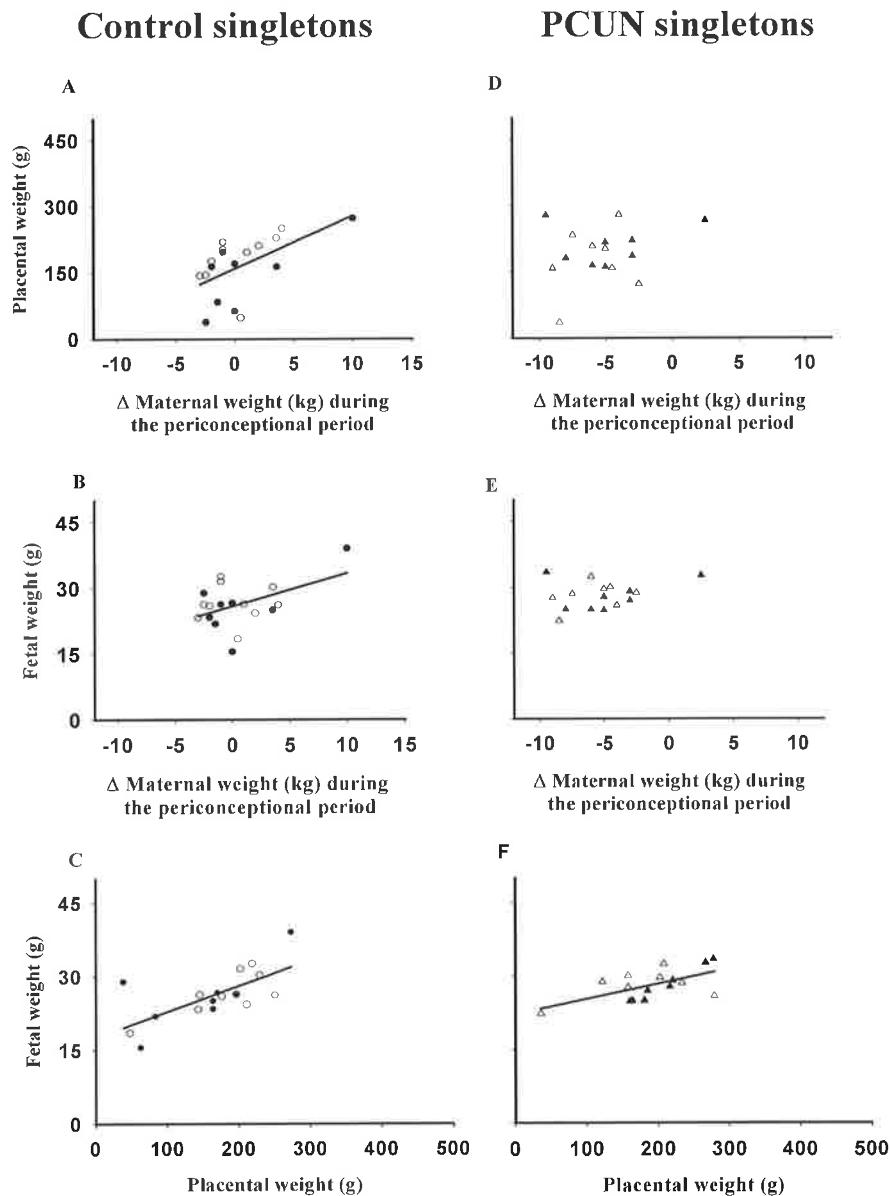

Figure 2.4 Singletons: effect of change in maternal weight during periconceptional period on fetal and placental weight

There was a significant positive relationship between total placental weight and the change in maternal weight during the periconceptional period in control (males closed circles, females open circles A), but not PCUN singleton pregnancies (males closed triangles, females open triangles D). B) There was a significant positive relationship between fetal weight and maternal weight change during the periconceptional period in control (B) but not PCUN (E) singleton pregnancies. There was a significant positive relationship between fetal weight and placental weight in control singleton pregnancies (C) and in PCUN (F) singleton pregnancies.

2.4.1.3 PCUN, maternal weight and body condition at d 53-56 and uteroplacental and fetal growth

There were no relationships between maternal weight or body condition score at d 53-56 and total placental weight, mean placentome weight or fetal weight. There was a relationship between maternal weight at post mortem and the weight of the fetal membranes in the control ($y = 6.00x - 287.31$, $r = 0.51$, $n = 18$, $P < 0.04$) but not PCUN group. There was also a positive relationship between the total maternal weight gain up to d 53-56 pregnancy and the mean placentome weight in the control ($y = 1.02x + 2.22$, $r = 0.71$, $n = 18$, $P < 0.001$) but not the PCUN group.

2.4.2 Twin Pregnancies

2.4.2.1 PCUN, maternal weight and condition

There were no differences before the start of the feeding regime between the weights of the ewes which went on to carry either singleton or twin pregnancies. The weights of the non pregnant ewes which subsequently carried twin pregnancies and which were assigned to the control (66.3 ± 1.9 kg) or PCUN (60.6 ± 2.5 kg) feeding regime were also not significantly different at the start of the feeding regime. The body condition score of the non pregnant ewes which were assigned to the control group (4.2 ± 0.2) was higher ($P < 0.01$) than in the PCUN group (3.5 ± 0.2) at the start of the feeding regime. Ewes in the PCUN group lost significantly more weight ($P < 0.05$) (Table 1) and body condition score ($P < 0.05$) during the periconceptional period than ewes in the control group (Figure 2C and D).

Consequently ewes in the PCUN group gained less weight ($P < 0.04$) than control ewes between the start of the feeding regime and PM (Table 1). Ewes in the PCUN group gained more body condition (0.60 ± 0.37, $P < 0.03$) than control ewes (-0.42 ± 0.15) between d 10 and PM and therefore, there was no overall difference in the change in body condition score between the start of the feeding regime and PM between the two feeding groups.

2.4.2.2 PCUN, uteroplacental and fetal growth

Uterine ($P < 0.0001$), fetal membrane ($P < 0.0001$), mean placentome ($P < 0.0001$), total placental ($P < 0.0001$) and total fetal ($P < 0.0001$) weights were all greater in twin when compared to singleton pregnancies, but were not affected by PCUN (Table 2).

There was no difference between the weights of male and female fetuses in either the Control or PCUN groups (Table 4). There was also no effect of PCUN on fetal weight (Figure 3C). There was, however, an interaction ($P < 0.02$) between the effects of PCUN and fetal number on the fetal ponderal index at d 53-56 pregnancy. The ponderal index of twin, but not singleton fetuses was lower in the PCUN group when compared with controls (Figure 3D).

2.4.2.3 PCUN, maternal weight changes during the periconceptional period and uteroplacental and fetal growth

There was no relationship between either maternal weight or condition at conception or the change in maternal weight or condition during the periconceptional period and uterine weight, fetal membrane weight, mean placentome weight, or total placental weight at d 53-56 pregnancy in either the control or PCUN groups (Table 5). There was, however, a positive relationship between the change in maternal body condition score during the periconceptional period and placentome number in PCUN twin pregnancies ($y = 27.86x + 102.71$, $r = 0.94$, $n = 5$, $P < 0.02$).

In the control, but not the PCUN group, there were positive relationships between either maternal weight at conception and individual fetal weight (Table 5) or maternal weight gain during the periconceptional period and either individual or total fetal weight (Table 5 and Figure 5).

2.4.2.4 PCUN, maternal weight or maternal weight gain up to d 53-56 pregnancy and placental and fetal growth

In the control group, there was a direct relationship between maternal weight at d 53-56 pregnancy and either the individual or total fetal weight (Table 5). In the PCUN group, however, there were inverse relationships between the maternal weight gain that occurred up to d 53-56 and the total placental weight, the mean placentome weight and either individual or total fetal weight (Table 5 and Figure 6A and B). In the PCUN group, there was a direct relationship between total placental and fetal weight ($y = 0.13x + 9.21$, $r =$

0.88, n = 5, $P < 0.05$, Figure 6C), which was not present in the control group. When a partial correlation analysis was performed, the relationship between the maternal weight gain up to d 53-56 and fetal weight in PCUN pregnancies was no longer significant ($r=0.66$, $P=0.21$) when the effects of placental weight were controlled for in the analysis.

Table 2.5: Relationships between maternal weight and weight changes during the experimental protocol and uteroplacental and fetal weights in twin pregnancies.

	Control Twins			PCUN Twins
Tissue	*Maternal weight at conception*	*Maternal weight change during the periconceptional period*	*Maternal weight at d 53-56*	*Maternal weight change between the start of the feeding regime and d 53-56*
Total Placental Weight	NS	NS	NS	y = -17.70x + 369 (r = 0.93, n = 5, P < 0.03)
Mean placentome weight	NS	NS	NS	y = -0.20x + 4.3 (r = 0.93, n = 5, P < 0.03)
Individual Fetal Weight	y = 0.30x + 8.6 (r = 0.62, n = 12, P < 0.04)	y = 0.54x + 28.0 (r = 0.59, n = 12, P < 0.05)	y = 0.36x + 3.5 (r = 0.66, n = 12, P < 0.02)	y = -1.32x + 28.5 (r = 0.94, n = 10, P < 0.0002)
Total Fetal Weight	NS	y = 1.08x + 56,(r = 0.79, n = 6, P = 0.06)	y = 0.72x + 6.9 (r = 0.88, n = 6, P< 0.03)	y = -2.64x + 57.1 (r = 0.96, n = 5, P < 0.02)

NS denotes relationships which were not significant

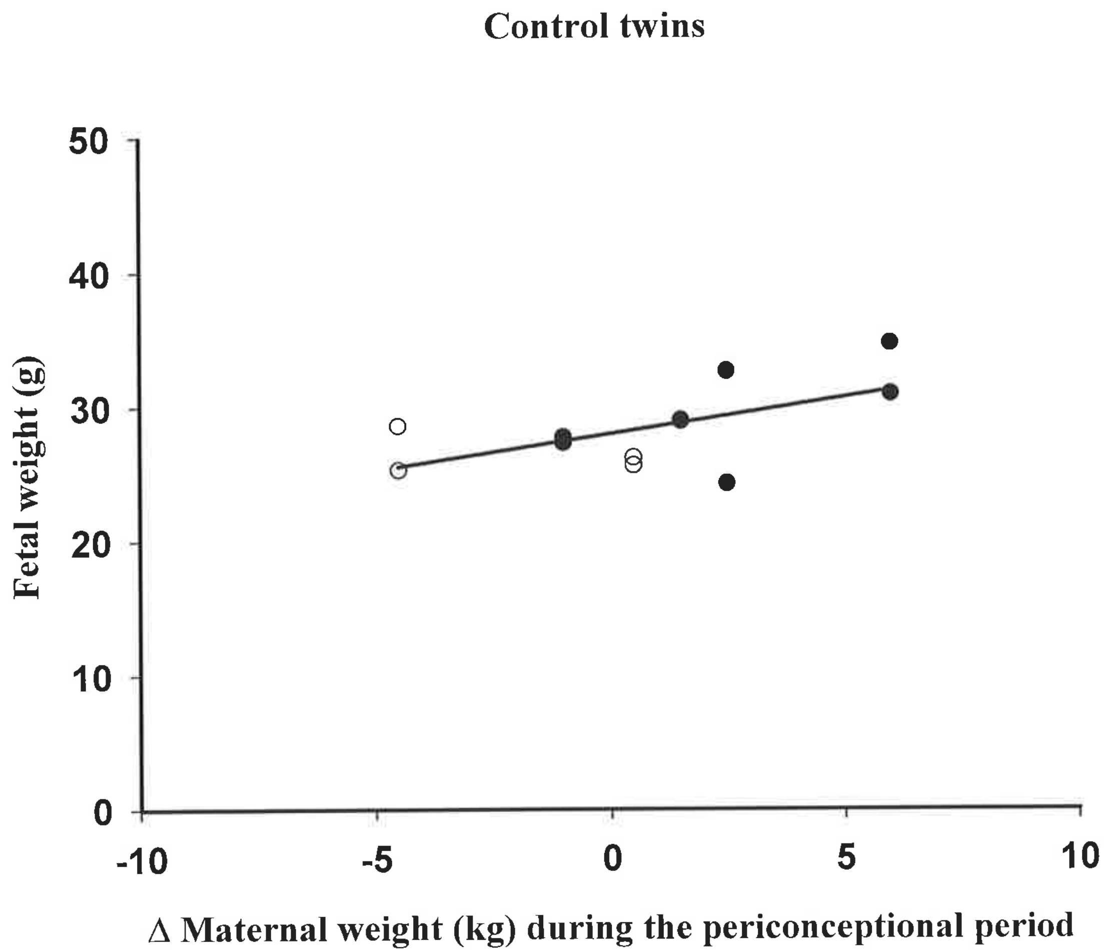

Figure 2.5. Control twins: Relationship between change in maternal weight during the periconceptional period and fetal weight

There was a positive relationship between fetal weight and maternal weight change during the periconceptional period in control twins (males closed circles and females open circles).

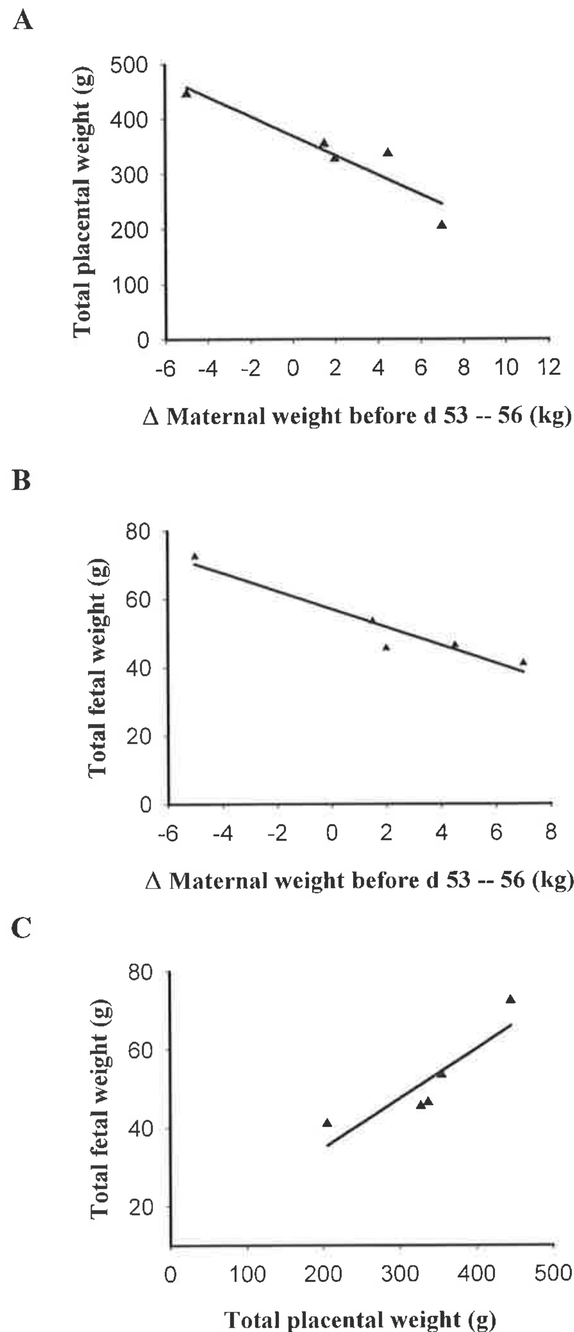

Figure 2.6 PCUN twins: relationship between change in maternal weight and fetal and placental weight

There was a significant inverse relationship between total placental weight and maternal weight change between ~45 d before mating and d 53 – 56 gestation in PCUN twin pregnancies (closed triangles A). There was a significant negative relationship between total fetal weight and maternal weight change between ~45 d before mating and d 53 – 56 gestation in PCUN twin pregnancies (B). There was a significant positive relationship between total fetal weight and total placental weight in PCUN twin pregnancies (C).

2.4.3 Effects of PCUN and fetal number on the histological development of the placenta in early pregnancy

There was no effect of fetal number or PCUN on the volume density and weight of the exchange surfaces of either the fetal (Table 6) or maternal (Table 7) portions of the placentome, the trophoblast and maternal epithelium, respectively. The volume density of the fetal capillaries ($P < 0.03$), the fetal capillary volume of the placenta ($P < 0.03$) and the volume of the fetal connective tissue portion of the placenta ($P < 0.008$) were higher in twin compared to singleton pregnancies (Table 6 and Figure 7) and there were no differences in these measures between the PCUN and control groups. Similarly, the volume of maternal capillary tissue ($P < 0.03$) and maternal connective tissue ($P < 0.05$) in the placenta were also higher in twin compared to singleton pregnancies (Table 7 and Figure 7) and again, there was no effect of PCUN on these measures (Table 7).

Neither PCUN nor fetal number altered the surface density, surface area per placentome or total placental surface area of trophoblast tissue nor did they alter the arithmetic mean barrier thickness of the trophoblast for diffusion in early gestation (Table 8).

Table 2.6: Effect of fetal number and PCUN on the volume density (Vd) and weight of fetal placental tissues.

	Singleton Pregnancies		Twin Pregnancies	
Placental tissue	***Control (n=6)***	***PCUN (n=5)***	***Control (n=4)***	***PCUN (n=5)***
Trophectoderm				
Vd	0.302± 0.056	0.282±0.026	0.231±0.033	0.235±0.036
Volume placentome (cm^3)	1.56±0.20	2.40±0.60	1.84±0.50	2.03±0.62
Volume placenta (cm^3)	63.9±17.5	49.7±5.9	76.7±17.2	75.5±11.3
Fetal capillaries				
Vd	0.001±0.001	0.002±0.001	0.004±0.002*	0.009±0.003*
Volume placentome (cm^3)	0.017±0.017	0.021±0.010	0.027±0.010	0.065±0.016
Volume placenta (cm^3)	0.31±0.31	0.37±0.17	1.26±0.58*	3.22±1.14*
Fetal connective tissue				
Vd	0.303±0.066	0.337±0.079	0.485±0.101	0.391±0.093
Volume placentome (cm^3)	2.24±0.75	3.59±1.93	3.60±1.18	3.18±1.09
Volume placenta (cm^3)	58.6±14.5	59.1±14.5	155.2±36.4**	135.2±41.6**

* Denotes significant differences between twin and singleton pregnancies * (P < .05) ** (P < 0.01)

Table 2.7: Effect of fetal number and PCUN on the volume density (Vd) and weight of maternal placental tissues.

Placental tissue	Singleton Pregnancies		Twin Pregnancies	
	Control (n=6)	*PCUN (n=5)*	*Control (n=4)*	*PCUN (n=5)*
		Maternal epithelium		
Vd	0.134±0.027	0.154±0.027	0.092±0.030	0.092±0.021
Volume placentome (cm^3)	0.70±0.14	1.16±0.19	0.68±0.21	0.70±0.22
Volume placenta (cm^3)	26.76±5.76	26.55±4.33	31.59±13.26	30.16±7.28
		Maternal capillaries		
Vd	0.007±0.003	0.006±0.005	0.010±0.004	0.015±0.003
Volume placentome (cm^3)	0.047±0.027	0.054±0.051	0.070±0.027	0.124±0.044
Volume placenta (cm^3)	1.63±0.66	1.13±1.02	3.48±1.77*	4.62±1.07*
		Maternal connective tissue		
Vd	0.247±0.026	0.208±0.041	0.176±0.037	0.257±0.042
Volume placentome (cm^3)	1.62±0.53	1.74±0.53	1.42±0.42	2.19±0.73
Volume placenta (cm^3)	50.70±7.67	37.334±9.50	59.77±17.72*	85.41±16.53*

*Denotes significant differences between twin and singleton pregnancies ($P < 0.05$).

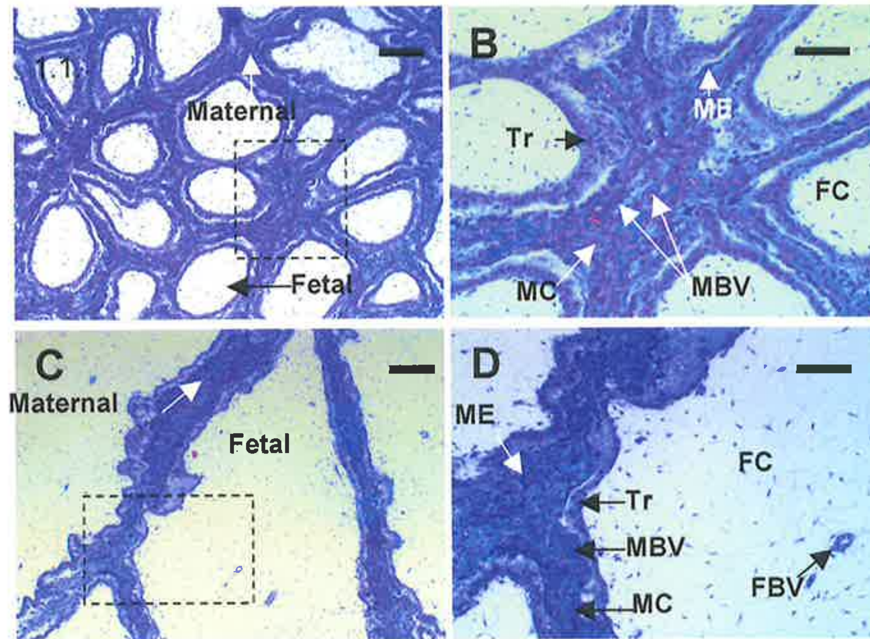

Figure 2.7 Representative fields of d 55 gestation control placentomes stained with Masson's trichrome used for point and intercept counting.

Panel A and B are control singleton placentome fields, 4x and 10x respectively. Panel C and D are control twin placentoma fields, 4x and 10x respectively. In Panels A and C, maternal crypts are much more dense than the fetal villi. Panels B and D represent higher magnification (10x) of the boxed areas in A and C. Trophectoderm (Tr), fetal connective (FC), fetal blood vessel (FBV), maternal epithelium (ME), maternal connective (MC) and maternal blood vessel (MBV) tissues are labelled. Fetal connective tissue is more abundant in twin placentomes (C and D) than singleton placentomes (A and B). Scale bars represent (A and C) 200μm and (B and D) 100μm.

Table 2.8: Effect of fetal number and PCUN on trophoblast exchange surface at d 53-56 of pregnancy

	Singleton Pregnancies		Twin Pregnancies	
Placental tissue	***Control (n=6)***	***PCUN (n=5)***	***Control (n=4)***	***PCUN (n=5)***
Surface density (cm^2/g)	133.0±23.1	123.4±20.6	91.6±18.1	99.7±17.4
Surface area placentome (cm^2)	200.8±35.0	287.3±83.7	178.9±55.4	220.6±95.2
Surface area placenta (m^2)	1.03±0.47	0.65±0.17	0.78±0.33	0.82±0.24
Barrier thickness (μm)	20.0±1.0	20.0±3.0	30.0±1.0	20.0±3.0

2.5 DISCUSSION

The objective of this study was to investigate whether the plane of nutrition of the ewe before conception and during early embryo development is a determinant of the growth trajectory of the placenta and fetus during the period of maximal placental development in singleton and twin pregnancies. We have demonstrated for the first time that there are important relationships between maternal weight gain during the periconceptional period and feto-placental growth during the first 56 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. Our results are relevant in the context of the series of epidemiologic and clinical studies that show that either restriction of maternal nutrient intake or low rate of fetal growth during early pregnancy is associated with changes in gestation length and birth weight and specific adverse health outcomes in later life (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000b; Roseboom *et al.*, 2001b).

There was no significant difference at the beginning of the feeding regime between the body weight of ewes allocated to the PCUN or control groups. The initial body condition score of ewes in the control group carrying either singletons or twins was marginally higher (~ 0.7 difference) than in the PCUN ewes. In light of the marked difference in the impact of restricted periconceptional nutrition in singleton and twin pregnancies, this difference in initial body condition did not appear to contribute to the major findings of the study. As expected, ewes in the PCUN group carrying singleton fetuses lost more weight and body condition than control ewes during the period of restricted periconceptional nutrition. Once the plane of nutrition was restored, there was no difference between the PCUN and

control ewes in the changes in either body weight or condition up until ~d 56 pregnancy. Similarly in ewes carrying twins, ewes in the PCUN group lost more body weight and condition than ewes in the control group during the period of nutritional restriction. Whilst there was no difference in the change in body weight between the PCUN and control groups after the plane of nutrition was restored at the end of the first week of pregnancy, the increase in body condition was marginally higher in the PCUN, than in the control groups before post mortem.

The findings of the present study in control ewes carrying singletons agree with previous reports that the variation in placental weight explains between 4-50% of fetal weight at 60-76 days gestation and ~70-90% of fetal weight after 130 days gestation (Dingwall *et al.*, 1987; Vatnick *et al.*, 1991; Greenwood *et al.*, 2000; Kleemann *et al.*, 2001; Osgerby *et al.*, 2003b). Two prior studies (McCrabb *et al.*, 1992; Osgerby *et al.*, 2003b) did not find a relationship between maternal weight at mating and fetal weight in later gestation, although the relationship between maternal weight gain during the periconceptional period and fetal weight was not investigated in these studies. Osgerby and co-workers did report, however, that a high maternal body condition score before conception was associated with an increase in placental and fetal weight at d 65 gestation (Osgerby *et al.*, 2003b). In the present study we also found that in control singleton pregnancies, there were direct relationships between maternal weight at conception and the weights of either the uterus or fetal membranes and between maternal weight gain during the periconceptional period and either the total weight of the placenta or the mean placentome weight. Interestingly whilst there was a relationship between maternal weight gain during the periconceptional period and fetal weight at ~d55

gestation, this relationship was no longer significant when placental weight was controlled for in the analysis.

Interestingly, in the ewes which experienced periconceptional undernutrition, there were no relationships between either maternal weight gain during the periconceptional period or in the period up to post mortem and any measure of uteroplacental or fetal growth in singleton pregnancies. Nevertheless, there was still a significant relationship between placental and fetal weights. Thus periconceptional undernutrition specifically disrupts any influence of maternal weight or weight gain exerted before and within 10 days after conception on uteroplacental growth. There are a number of candidate mechanisms that may be associated with maternal weight gain during the periovular and preimplantation periods and enhanced uteroplacental growth, which in turn may be sensitive to the impact of periconceptional undernutrition. A recent study has demonstrated that periconceptional undernutrition results in an increase in progesterone concentrations in the oviductal fluid (Kakar *et al.*, 2005) and it has been shown that progesterone priming during the first 3 days of pregnancy is associated with an increase in fetal weight at d 76 gestation (Kleemann *et al.*, 1994). In the current study, however, periconceptional undernutrition did not result in a change in fetal weight at ~d 55 gestation.

A novel finding of the current study was the differential impact of maternal weight changes in the periconceptional period on uteroplacental growth between singleton and twin pregnancies. In contrast to the singletons, there was no effect of either maternal weight at conception or maternal weight gain in the

periconceptional period on any measure of uteroplacental growth in the control twins. There was also no relationship between total placental weight and fetal weight in the control twin pregnancies at ~ d 55 gestation. In part these findings may be related to the increased uteroplacental weight and mean placentome weight which is already present in the twin pregnancies at this stage of early gestation. There was also an increase in the volume density of fetal capillaries within the placentomes and in the placental volume of maternal vascular and connective tissue and fetal connective tissue, although there was no difference in the trophoblast exchange surface area or in the thickness of the barrier to exchange between the maternal and fetal components of the placenta in twins compared with singletons. The increase in the mean placentome weight and the lack of a relationship between total placental and fetal weight at ~d 55 gestation suggests that the presence of twins (either 2 corpora lutea or 2 embryos) induces a 'predictive' uteroplacental growth response in preparation for the increased growth demands of twin fetuses in late gestation. It has been shown that the mean placentome weight is greater in twins than singleton lambs at d 136 (Vatnick *et al.*, 1991) and it appears from the present study that this increase occurs from early in pregnancy. Whilst maternal weight gain during the periconceptional period did not influence uteroplacental growth, it had a direct effect on fetal weight in control twin pregnancies. In twins, there was also a direct influence of maternal weight at ~d 55 pregnancy on total fetal weight which suggests that the maternal metabolic or endocrine environment in early pregnancy plays a significant role in fetal growth in twins as well as singletons, but that in contrast to singletons, this effect continues beyond the first 10 days after conception and is not mediated indirectly through placental growth.

Periconceptional undernutrition had a striking impact on twin pregnancies. Following periconceptional undernutrition, there was no relationship between maternal weight change during the periconceptional period and fetal weight. There was, however, the emergence of a strong inverse relationship between maternal weight gain during the first 55 days of pregnancy and either total placental weight, mean placentome weight or total fetal weight. Thus, in ewes exposed to undernutrition from before pregnancy and up to the end of the first week after conception, a greater weight loss between the start of the feeding regime and post mortem at ~d 55, was associated with a larger placenta and total fetal mass and the direct relationship between placental and fetal mass was restored. There were no specific changes, however, at ~ 55 days gestation in the histology of the twin placenta after maternal periconceptional undernutrition, although there may be specific changes in the expression of a range of vascular or placental growth factors which were not determined in the present study. The inverse association between maternal weight gain and placental weight may represent a 'predictive' or compensatory response to maintain fetal growth. One possible mechanism to explain this association may be an impact of maternal nutrition on uterine histotroph secretion which in turn may play a role in the pattern of early placental growth and development. Whilst no prior studies have investigated the impact of periconceptional undernutrition on placental growth during early pregnancy, a number of previous studies have found variable effects of maternal undernutrition imposed between 30 and around 90 days gestation on placental weight in later gestation (Faichney & White, 1987; McCrabb *et al.*, 1991; Holst *et al.*, 1992; McCrabb *et al.*, 1992). In part, these inconsistent effects

may be due to differences in initial maternal body weight and to the compensatory increase in maternal nutrition over the remainder of pregnancy following the protracted period of undernutrition (Heasman *et al.*, 1999).

In contrast to the singletons, periconceptional undernutrition resulted in a reduced ponderal index in twin fetuses compared with controls. This decrease in fetal ponderal index may indicate that the compensatory changes within the PCUN twin placenta results in some limitation of nutrient supply to the fetus during early pregnancy to impact on the proportional growth of the fetus. Thus in singleton pregnancies, periconceptional undernutrition disrupts the direct relationship between maternal weight gain during the periconceptional period and uteroplacental growth and in twin pregnancies, periconceptional undernutrition results in the emergence of a new and inverse relationship between maternal weight gain during early pregnancy and uteroplacental growth and in a dependence of fetal growth on placental growth. Whether the changes induced by periconceptional undernutrition in the relationships between placental and fetal growth in early pregnancy are important in determining the subsequent functional development of the fetus is not well understood. It has been previously reported that as in the present study, undernutrition during the periconceptional period has a differential impact on activation of the fetal pituitary-adrenal axis and on the cardiovascular system in twin fetuses compared with singletons in later pregnancy (Edwards & McMillen, 2002a, b). After exposure to moderate periconceptional undernutrition (30% reduction), there was an increase in circulating ACTH concentrations, an increase in adrenocortical responsiveness to ACTH and an increase in fetal arterial blood pressure in twin but not singleton

fetuses in late gestation (Edwards & McMillen, 2002a, b). These programmed changes may be an independent consequence of the impact of periconceptional undernutrition on embryonic development, or may be dependent on the changes induced by periconceptional undernutrition in placental and fetal growth and in the fetal growth trajectory. More recently it has been reported that exposure of the embryo to a severe level of maternal undernutrition (a 50% reduction) and for up to 30 days after conception results in an earlier activation of the fetal pituitary-adrenal axis in singleton fetuses (Bloomfield *et al.*, 2003). Similarly, a 50% reduction in maternal nutrient intake for the first 30 days after conception in ewes carrying singletons resulted in an increased pulse pressure, a leftward shift in the baroreflex function curve and the blunting of the baroreflex sensitivity during angiotensin II infusion in one-year old offspring (Gardner *et al.*, 2004b). It remains to be determined whether the effects of maternal periconceptional undernutrition in singletons is dependent on an increased severity of nutrient restriction or on the extension of the period of maternal undernutrition beyond the period of implantation.

The findings of the present study and previous studies on the impact of periconceptional undernutrition in the ewe and her fetus are important in the context of the impact of undernutrition immediately before and after conception in the pregnant woman. In infants born during the Dutch Famine Winter of 1944-1945, there was an increase in placental weight but not birth weight in those infants whose mothers' nutrition was compromised around conception or in the first trimester of pregnancy (Lumey, 1998). First trimester undernutrition was also associated with increased obesity in adult men and women (Ravelli *et al.*, 1976;

Ravelli *et al.*, 1999) highlighting the importance of the nutritional environment during early gestation when the nutritional requirements of the embryo and fetus are minimal.

In summary, the present study has demonstrated that there are important relationships between maternal weight gain during the periconceptional period and feto-placental growth during the first 56 days of pregnancy and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. In singleton pregnancies, periconceptional undernutrition disrupts the direct relationship between maternal weight gain during the periconceptional period and uteroplacental growth. In twin pregnancies, periconceptional undernutrition results in the emergence of an inverse relationship between maternal weight gain during early pregnancy and uteroplacental growth and in a dependence of fetal growth on placental growth. These changes highlight the importance of the periconceptional environment in setting the placental and fetal growth trajectories and for the programmed development of the metabolic and cardiovascular systems of the fetus and adult.

Chapter 3:

Impact of maternal undernutrition during the periconceptional period and fetal number on heart and adrenal growth and on adrenal IGF and steroidogenic enzyme expression in the sheep fetus during early pregnancy

"Speak softly and carry a big stick; you will go far"

- President Theodore Roosevelt

-

"Never tell people how to do things. Tell them what to do and they will surprise you with their ingenuity"

- General George S. Patton Jr.

3. Impact of maternal undernutrition during the periconceptional period and fetal number on heart and adrenal growth and on adrenal IGF and steroidogenic enzyme expression in the sheep fetus during early pregnancy

3.1 Summary

Recent studies in the sheep have shown that maternal undernutrition during the periconceptional period, when the nutrient demands of the embryo are minimal, can alter the subsequent development of the pituitary-adrenal axis and the cardiovascular system and that these effects may, in part, depend on embryo number. I have tested the hypotheses that the growth and functional development of the fetal sheep adrenal is delayed in twin pregnancies from as early as ~55 days (d) gestation and that periconceptional undernutrition accelerates adrenal growth and increases the expression of the intraadrenal insulin like growth factors, IGF-1 and -2 and the steroiodgenic enzyme, cytochrome P450 17-hydroxylase (CYP 17) in early gestation. I have investigated the effect of periconceptional undernutrition in the ewe (PCUN, restricted at 70 % of control feed allowance, n = 21; Control, n=24) from at least 45 d prior to mating until d 7 after mating on maternal plasma free fatty acid, cortisol and progesterone concentrations, fetal adrenal weight, adrenal IGF-1, IGF-1R, IGF-2, IGF-2R and CYP 17 mRNA expression and placental 11-βhydroxysteroid dehydrogenase -2 (11-βHSD-2) mRNA expression at d 53-56 pregnancy. I have also tested the hypotheses that there are relationships between the growth of the fetal adrenal gland and heart during the first 55 days of pregnancy and that these relationships are altered by periconceptional undernutrition in twin, but not

singleton fetuses. Placental 11-βHSD-2 mRNA levels were higher in twin pregnancies, independent of the level of maternal nutrition before conception. The relative weight of the fetal adrenal and adrenal IGF-1, IGF-1R, IGF-2, IGF-2R and CYP 17 mRNA expression were lower in twin compared to singleton fetuses. There was evidence that in control singletons, IGF-2R expression plays an important role in the regulation of adrenal growth and CYP 17 mRNA expression during early pregnancy. In control twins, however, whilst there was a significant positive relationship between adrenal CYP 17 and IGF-2 mRNA expression, adrenal weight was directly related to the level of adrenal IGF-1 mRNA expression. There was no effect of periconceptional undernutrition on the level of expression of any of the placental or adrenal genes in the study. In PCUN ewes, carrying singletons, however, there was a loss of the relationships between either adrenal IGF-2, IGF-2R and IGF-1 mRNA expression and adrenal growth and CYP 17 expression which were present in control singletons. Similarly in ewes carrying twins, maternal undernutrition during the periconceptional period resulted in the loss of the relationships between adrenal growth and IGF-1 expression and between adrenal CYP 17 and IGF-2 expression which were present in control twin fetuses. In twin fetuses there was also a direct relationship between relative fetal heart and adrenal weights, which was present in both the PCUN and control groups. There was a significant and direct relationship between relative heart weight and maternal plasma cortisol at ~ d 55 pregnancy in control twin but not control singleton or PCUN fetuses. There was also a significant inverse relationship between maternal weight at conception and relative fetal heart weight in PCUN twin, but not PCUN singleton or control fetuses. These findings highlight the importance of the periconceptional

environment in setting the growth trajectories of the fetal heart and adrenal gland during early development, and have implications for the parallel changes which occur in the development of the cardiovascular and neuroendocrine systems of the fetus and adult following a period of periconceptional undernutrition.

3.2 INTRODUCTION

A range of epidemiological, clinical and experimental studies have demonstrated that exposure to poor maternal nutrition either before or around the time of conception is associated with altered development of the cardiovascular system and an increased risk of hypertension and cardiovascular disease in adult life (Kwong *et al.*, 2000; Roseboom *et al.*, 2000b; Roseboom *et al.*, 2001b; Edwards & McMillen, 2002b). The Dutch Famine Winter Study investigated the long term effects of a 5 month period of malnutrition experienced by pregnant women in Amsterdam during 1944-1945 and found that individuals exposed to maternal malnutrition as an embryo and fetus during the first trimester of pregnancy had an increased prevalence of coronary heart disease in adult life (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000b; Roseboom *et al.*, 2001b). It has also been shown that when rat dams were fed a low protein diet for the first 4.25 days after conception, the offspring had a low birth weight and raised systolic blood pressure in postnatal life (Kwong *et al.*, 2000). Similarly in the sheep, a 30% reduction in maternal nutrition for at least 45 days before until 7 days after conception resulted in an increase in arterial blood pressure and a concomitant increase in the activation of the pituitary–adrenal axis in twin, but not singleton fetuses during late gestation (Edwards & McMillen, 2002a, b). When maternal nutrition was restricted more severely for up to 30 days after conception in the

sheep, there was enhanced activation of the fetal pituitary-adrenal axis in singleton pregnancies (Bloomfield *et al.*, 2003). Furthermore, maternal undernutrition for 30 days after conception also resulted in the blunting of the baroreflex sensitivity during angiotensin II infusion in one-year old singleton offspring (Gardner *et al.*, 2004b).

It is not clear why periconceptional undernutrition has differential effects on cardiovascular and pituitary-adrenal development in twin and singleton pregnancies. Studies in human pregnancies with more than 2 fetuses have found that after the number of embryos is reduced to two in the first trimester, the birth weights of the remaining twins were significantly reduced compared with the birth weights in the non-reduced twin pregnancies indicating that the fetal growth trajectory may be set early in pregnancy (Sebire *et al.*, 1997). In sheep, the growth trajectory of the fetus and placenta are also set early in pregnancy, and as shown in Chapter 2 periconceptional undernutrition has a different effect on fetal and placental growth in singleton and twin pregnancies.

Studies in the sheep have also demonstrated that the maturation of the fetal hypothalamic-pituitary-adrenal axis appears to be delayed or suppressed in twins compared to singletons during late gestation. Fetal plasma ACTH concentrations are lower and the prepartum cortisol surge occurs later in twin compared to singleton fetuses (Edwards & McMillen, 2002a; Gardner *et al.*, 2004a). There is also a significant relationship between mean arterial blood pressure and plasma cortisol concentrations in twin but not singleton fetuses during late gestation (Edwards & McMillen, 2002b; Gardner *et al.*, 2004a), and it has been reported

that heart growth is decreased and adrenocortical responsiveness to ACTH stimulation is 'blunted' in twin relative to singleton fetuses (Gardner *et al.*, 2004a).

During early gestation (~d 40 – 60), the fetal adrenal gland of the sheep undergoes hyperplastic growth and a phase of increased steroidogenic activity (Wintour *et al.*, 1975; Boshier & Holloway, 1989; Coulter *et al.*, 2002). Insulin like growth factors (IGFs) have been implicated in the regulation of adrenal growth and steroidogenesis in the fetal sheep (Han *et al.*, 1992). IGF-2, a paternally imprinted gene (Young 2000), is maximally expressed in a range of fetal tissues including the adrenal during early gestation (van Dijk *et al.*, 1988; Han *et al.*, 1992), where it is present in adrenocortical steroidogenic cells (Han *et al.*, 1992). Wintour and co-workers have shown that the fetal adrenal gland is responsive to ACTH and produces more cortisol relative to body weight at d 40 to 60 than any other point in gestation (Wintour *et al.*, 1975; Tangalakis *et al.*, 1994). It is not known whether fetal adrenal growth and functional development are different in twin and singleton fetuses at this early stage in gestation.

One possibility is that fetal adrenal growth and steroidogenic capacity is reduced in twin, compared to singleton fetuses as early as ~ d 55 gestation and that periconceptional undernutrition alters the functional development of the adrenal and heart of twin fetuses at this stage resulting in a parallel increase in adrenocortical responsiveness and fetal blood pressure in later gestation (Edwards & McMillen, 2002a, b).

An alternative mechanism is that the expression of the placental enzyme, 11-βHSD-2, which operates to protect the fetus from excess exposure to transplacental cortisol is lower in twin pregnancies, resulting in an increased exposure of the fetal pituitary-adrenal axis to the negative feedback actions of maternal cortisol. I have, therefore, tested the hypotheses that there is a decreased expression of placental 11-βHSD-2 expression, relative adrenal weight and adrenal IGF-2 and CYP 17 mRNA levels in twin, but not singleton, fetuses in early pregnancy.

I have therefore determined whether periconceptional undernutrition alters maternal cortisol concentrations, placental 11-βHSD-2 mRNA expression, fetal adrenal and heart weights and the mRNA levels of the insulin like growth factors-1 and 2 and their receptors and the expression of CYP 17 in the fetal adrenal during the phase of maximal hyperplastic growth of the adrenal at d 55 gestation.

3.3 MATERIALS AND METHODS

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee.

As discussed in the preceding chapter, forty-five South Australian Merino ewes were used in this study and the feeding and breeding protocols were as previously reported (Chapter 2). Briefly, ewes were moved into an enclosed shed and housed in pens 2 weeks before the start of the feeding regime. All ewes

were weighed and a body condition score assessed employing a 1-5 scale with 0.5 intervals by an experienced assessor (Russel *et al.*, 1969; Greenwood *et al.*, 2000). Using this scale, a body condition score of 1 represents an extremely emaciated animal and a body condition score of 5 represents an extremely obese animal. During this 2 week period, ewes were acclimatized to a pelleted diet containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime and molasses (Johnsons & Sons Pty Ltd, Kapunda, South Australia, Australia). The pellets provided 9.5 MJ/kg of metabolizable energy and 120 g/kg of crude protein and contained 90.6% dry matter. All ewes received 100% of nutritional requirements (7.6 MJ/ day for the maintenance of a 64 kg non pregnant ewe) as defined by the Agricultural and Food Research Council . At the end of this acclimatization period, ewes were randomly assigned to one of two feeding regimes, a control regime (C, n = 24), in which ewes received 100% of nutritional requirements or a periconceptional restricted regime (PCUN, n = 21), in which ewes received 70% of the control allowance. All of the dietary components were reduced by an equal amount in the restricted diet. Ewes were maintained on these respective diets for at least 45 days before mating. Control ewes were maintained on the control diet for 62 ± 5 days and the ewes in the PCUN group were maintained on the 70% diet for 55 ± 2 days prior to conception. The starting weights were not different between ewes that were allocated to the control group (65.6 ± 1.2 kg) or the PCUN group (62.4 ± 1.3 kg).

Ewes were released in a group every evening at 1600h with two intact rams of proven fertility that were fitted with harnesses and marker crayons. Ewes were individually penned the following morning at 0800h, and the occurrence of mating

was confirmed by the presence of a crayon mark on the ewe's rump. The day of mating was defined as d 0. Ewes in the PCUN group were maintained on the 70% restricted diet for 7 d after conception. From 7 days after mating (d 7 of pregnancy), all ewes were fed a control diet (100% of requirements) until post mortem (PM) at d 53-56 pregnancy. Ewes were weighed and their body condition was assessed and scored approximately every two weeks after commencing the feeding regime until post mortem at d 53-56 of pregnancy. Ewes in the PCUN group lost more weight (-4.52 ± 0.82 kg, $P < 0.0001$) than control ewes (0.54 ± 0.66 kg) between the start of the feeding regime and d 10 of pregnancy. Pregnancy was diagnosed and fetal number estimated by ultrasound at d 45 of pregnancy. The number of fetuses carried by each ewe was confirmed at PM generating four treatment groups: control singleton pregnancies ($n = 18$), PCUN singleton pregnancies ($n = 16$), control twin pregnancies ($n = 6$), and PCUN twin pregnancies ($n = 5$).

3.3.1 COLLECTION OF TISSUES

Ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) between d 53 and 56 of pregnancy (term = 150 ± 3 days gestation), and the utero-placental unit was delivered by hysterotomy. Fetal organs, including the heart and adrenal glands, were dissected from the fetus. The fetal hearts were weighed, and because of their small size, the fetal adrenals were snap frozen immediately in liquid nitrogen and weighed subsequently on a microbalance before RNA extraction. The placenta was immediately dissected and the placentomes were individually weighed and counted. Between two and

four placentomes from each placenta were snap frozen immediately in liquid nitrogen and stored at –80°C for further analysis.

3.3.2 Maternal blood samples

Maternal blood samples were collected from the jugular vein by venepuncture from all ewes on the 12th day after the start of the feeding regime (Control, 50 ± 5 days before conception: PCUN, 43 ± 2 days before conception), on the 40th day after the start of the feeding regime (Control, 22 ± 5 days before conception; PCUN, 19 ± 2 days before conception) and at post mortem between d 53 and 56 pregnancy. Blood samples (10 ml) were collected into chilled heparinized tubes. All samples were centrifuged at 1500g for 10 min and plasma separated into aliquots and stored at -20°C for the measurement of non-esterified free fatty acids (FFA) and maternal plasma progesterone and cortisol.

3.3.3 FFA Assay

As a measurement of maternal nutrient restriction, maternal plasma FFA concentrations were measured in all samples in one assay by an *in vitro* enzymatic colorimetric method (Wako Pure Chemicals Industries Ltd, Osaka, Japan). The sensitivity of the assay was 0.25 mEq/l and the intra- assay coefficient of variation was < 5%.

3.3.4 Progesterone Radioimmunoassay

Progesterone was measured in maternal plasma using a radioimmunoassay (Diagnostic Systems Laboratories Inc, Texas, USA). The sensitivity of the assay was <0.3 ng/ml and the cross reactivity of the progesterone antiserum was <0.1%

with cortisol, pregnenolone and estradiol. All maternal samples were measured within the one assay and the intra- assay coefficient of variation was < 5%.

3.3.5 CORTISOL RADIOIMMUNOASSAY

Maternal plasma samples were extracted and assayed in duplicate. Briefly, cortisol was extracted from the plasma using dichloromethane (Bocking *et al.*, 1986), using an assay previously validated for use in sheep plasma (Warnes *et al.*, 2003). The efficiency of the recovery was > 85%. Samples were then reconstituted in assay buffer (Tris hydrochloride: bovine serum albumin; sodium azide). Standards were serially diluted in assay buffer, from a stock (1000nmol l^{-1}) solution (range 0.78-100nmol l^{-1}). Anti-cortisol (100μl; 1:15 dilution; Orion Diagnostica, Turku, Finland) was added followed by ^{125}I-labelled cortisol (100μl; Amersham Pharmacia Biotech, UK). Tubes were vortexed and incubated at 37°C for 1 h before the addition of goat anti-rabbit serum (initial dilution 1:30; 100μl) and polyethylene glycol (1ml; 20%; BDH Laboratory Supplies). Tubes were vortexed before centrifugation at 3700g and 4°C for 30 min. The supernatant was aspirated and the precipitate counted on a gamma counter (Packard, Downers Grove, IL, USA). The sensitivity of the assay was 0.2 nmol l^{-1}. The intra- and inter-assay coefficients of variation were < 15%.

3.3.6 RNA EXTRACTION AND cDNA SYNTHESIS

RNA was isolated from frozen adrenal and placental tissues samples using Trizol reagent (Invitrogen, The Netherlands) and purified using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland). Genomic DNA contamination was minimised by treating each sample with DNase 1 (Ambion, Austin, Texas, USA), and RNA was

quantified by spectrophotometric measurements at 260nm and 280nm. cDNA was synthesised from 5µg RNA using Superscript III (Invitrogen, The Netherlands) by reverse transcription. Controls containing no RNA transcript or no superscript were used to test for DNA contamination.

3.3.7 Quantitative Real Time Reverse Transcription PCR

The relative abundance of IGF-1, IGF-2, IGF-1R, IGF-2R, and CYP 17 mRNA transcripts in fetal adrenal tissue were measured by quantitative real time reverse transcription PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each qRT-PCR well contained 5µl Sybr Green Master Mix (Applied Biosystems), 1µl each of forward and reverse primer (GeneWorks, SA, Australia) for the appropriate gene (Table 1), water (2µl), and 50ng/µl cDNA (1µl) to give a total volume of 10µl. Controls for each primer set containing no cDNA were included on each plate. Three replicates of cDNA from each pair of fetal adrenal glands were performed for each gene on each plate, and each plate was repeated three times to ensure a consistent result. Amplification efficiencies were determined, from the slope of a plot of Ct (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (ranging from 1 to 100ng/µL). The abundance of each transcript relative to the abundance of the reference gene, ribosomal protein P0 (RpP0), was calculated using Q-Gene analysis software (Muller *et al.*, 2002).

The relative abundance of 11-βHSD-2 mRNA transcripts in placental tissue was also measured by qRT-PCR using the Sybr Green system in an ABI Prism 7000

Sequence Detection System as detailed above using primers previously validated in the sheep (Dodic *et al.*, 2002b). The abundance of the 11-βHSD-2 mRNA was determined relative to the abundance of the reference gene, cyclophilin, using Q-gene analysis software.

3.3.8 Statistical Analysis

Data are presented as the mean ± SEM. The effects of periconceptional undernutrition and fetal number on maternal FFA, progesterone and cortisol concentrations, absolute and relative fetal heart and adrenal weight, fetal adrenal expression of IGF-1, IGF-1R, IGF-2, IGF-2R, and CYP 17 and placental expression of 11-βHSD-2 were determined using a 2 way Analysis of Variance (ANOVA) using the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). Relationships between variables were assessed by linear regression using Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA) and partial correlation analyses were used where appropriate. A probability level of 5% ($P<0.05$) was assumed to be significant.

Table 3.1: Primer sequences for qRT PCR

Primer Name	5'/3'	Sequence
IGF-1 Fwd	5'→3'	TTG GTG GAT GCT CTC GAG TTC
IGF-1 Rev	5'→3'	AGC AGC ACT CAT CCA CGA TTC
IGF-1R Fwd	5'→3'	AAG AAC CAT GCC TGC AGA AGG
IGF-1R Rev	5'→3'	GGA TTC TCA GGT TCT GGG CAT T
IGF-2 Fwd	5'→3'	GCT TCT TGC CTT CTT GGC CTT
IGF-2 Rev	5'→3'	TCG GTT TAT GCG GCT GGA T
IGF-2R Fwd	5'→3'	GAT GAA GGA GGC TGC AAG GAT
IGF-2R Rev	5'→3'	CCT GAT GCC TGT AGT CCA GCT T
CYP 17 Fwd	5'→3'	CCC CCA CAA GGC TAT CAT TGA
CYP 17 Rev	5'→3'	CTG CTG CCA CTC CTT CTC ATT
11βHSD-2 Fwd	5'→3'	AGC AGG AGA CAT GCC GTT TC
11βHSD-2 Rev	5'→3'	GCA ATG CCA AGG CTG CTT
Cyclophilin Fwd	5'→3'	CCT GCT TTC ACA GAA TAA TTC CAG
Cyclophilin Rev	5'→3'	CAT TTG CCA TGG ACA AGA TGC CAG
RpP0 Fwd	5'→3'	CAA CCC TGA AGT GCT TGA CAT
RpP0 Rev	5'→3'	AGG CAG ATG GAT CAG CCA

3.4 RESULTS

PCUN and maternal plasma FFA

Plasma FFA concentrations were significantly higher in the PCUN group than in the control group on d 12 (PCUN, 0.191 ± 0.027 meq/l, n = 19: control, 0.081 ± 0.102 meq/l, n = 22; P = 0.001) and on d 40 of the feeding regime (PCUN, 0.177 ± 0.028 meq/l, n = 20, control, 0.108 ± 0.02 meq/l, n = 24, P < 0.02). There was no difference, however, in plasma FFA concentrations between the PCUN and control groups at d 53 – 56 pregnancy after the restoration of maintenance nutrition in either singleton or twin pregnancies.

3.4.1 PCUN AND MATERNAL PLASMA PROGESTERONE AND CORTISOL

3.4.1.1 Progesterone:

There was no difference between maternal plasma progesterone concentrations at either d 12 or d 40 after the start of the feeding regime in either the control and PCUN groups or in the singletons or twins groups (Table 2). Maternal plasma progesterone concentrations were significantly higher, however, in ewes carrying twin fetuses at d 53 – 56 gestation and this occurred independently of the level of periconceptional nutrition (P < 0.0001, Table 2). There was a significant relationship between plasma progesterone concentrations at post mortem (y) and total placental weight in both control pregnancies ($y = 0.0145x + 4.5544$, r = 0.56, P = 0.0055) and PCUN pregnancies ($y = 0.0172x + 3.3294$, r = 0.60, P = 0.004).

3.4.1.2 Cortisol:

Singleton pregnancies: Whilst there was no difference in plasma cortisol concentrations between the control and PCUN groups on d 12 of the feeding regime (31.6 ± 5.4 nmol/l vs 22.7 ± 3.4 nmol/l, respectively, Table 2), plasma cortisol concentrations were lower in the PCUN group than in controls (11.1 ± 2.1 nmol/l vs 26.9 ± 6.6 nmol/l, $P < 0.04$, Table 2) on d 40 of the feeding regime (Table 2). Plasma cortisol concentrations were not different, however, between the control and PCUN groups at d 53 – 56 pregnancy (13.3 ± 2.4 nmol/l vs 14.9 ± 3.4 nmol/l, respectively, Table 2).

Twin pregnancies: On d 12 of the feeding regime, plasma cortisol concentrations were higher in the PCUN than control ewes (37.3 ± 10.6 nmol/l vs 7.7 ± 0.4 nmol/l $P < 0.02$, Table 2), but there was no difference in plasma cortisol between the 2 feeding groups on either d 40 of feeding (15.6 ± 3.0 nmol/l vs 26.6 ± 7.4 nmol/l, respectively, Table 2) or d 53 – 56 pregnancy (18.8 ± 4.2 nmol/l vs 10.6 ± 2.4 nmol/l, respectively, Table 2).

Table 3.2: Effect of fetal number and PCUN on maternal plasma cortisol and progesterone concentrations (nmol/l) during the periconceptional period and early pregnancy.

		Singleton Control (n=18)	*Singleton PCUN (n=16)*	*Twin Control (n=6)*	*Twin PCUN (n=5)*
d 12 of feeding regime	**Cortisol (nmol/L)**	31.6 ± 5.4	22.7 ± 3.4	7.7 ± 0.4$	37.3 ± 10.6*
	Progesterone (nmol/L)	3.2 ± 0.5	3.4 ± 0.6	2.7 ± 0.6	1.4 ± 0.2
d 40 of feeding regime	**Cortisol (nmol/L)**	26.9 ± 6.6	11.1 ± 2.1*	15.6 ± 3.0	26.6 ± 7.4
	Progesterone (nmol/L)	3.2 ± 0.5	3.2 ± 0.5	3.1 ± 1.2	5.9 ± 1.2
~d 55 of pregnancy	**Cortisol (nmol/L)**	13.3 ± 2.4	14.9 ± 3.4	18.8 ± 4.2	10.6 ± 2.4
	Progesterone (nmol/L)	6.8 ± 0.3	6.3 ± 0.6	11.0 ± 2.1#	10.1 ± 0.6#

*denotes a significant difference between control and PCUN nutrition groups within either singleton or twin pregnancies ($P < 0.05$); # denotes a significant difference between singleton and twin pregnancies ($P < 0.0001$); and $ denotes a significant difference between singleton and twin pregnancies within the control group ($P < 0.05$).

3.4.2 PCUN, FETAL NUMBER AND PLACENTAL 11-βHSD-2 MRNA EXPRESSION

Placental expression of 11-βHSD-2 mRNA was significantly higher in twin compared to singleton pregnancies ($P < 0.01$, Figure 1) and this was independent of the level of maternal nutrition during the periconceptional period. There was no specific effect of PCUN on placental expression of 11-βHSD-2 mRNA in either singleton or twin pregnancies. There was, however a direct relationship between the level of 11-βHSD-2 mRNA expression (y) in the placenta and the amount of weight gained in the periconceptional period in the PCUN, but not control pregnancies ($y = 0.0011x + 0.0269$, $r = 0.71$, $P = 0.001$) and this relationship remained significant when the effects of progesterone were controlled for in a partial correlational analysis.

3.4.3 PCUN, FETAL NUMBER AND FETAL ADRENAL WEIGHT AT D 53 – 56

There was no effect of PCUN on the weights of singleton and twin fetuses. The absolute and relative adrenal weights were each significantly lower ($P < 0.0001$), however, in twin compared to singleton fetuses in both the PCUN and control groups (Table 3). There was no effect of PCUN on either the absolute or relative fetal adrenal weight at d 53 – 56 in either singleton or twin fetuses (Table 3).

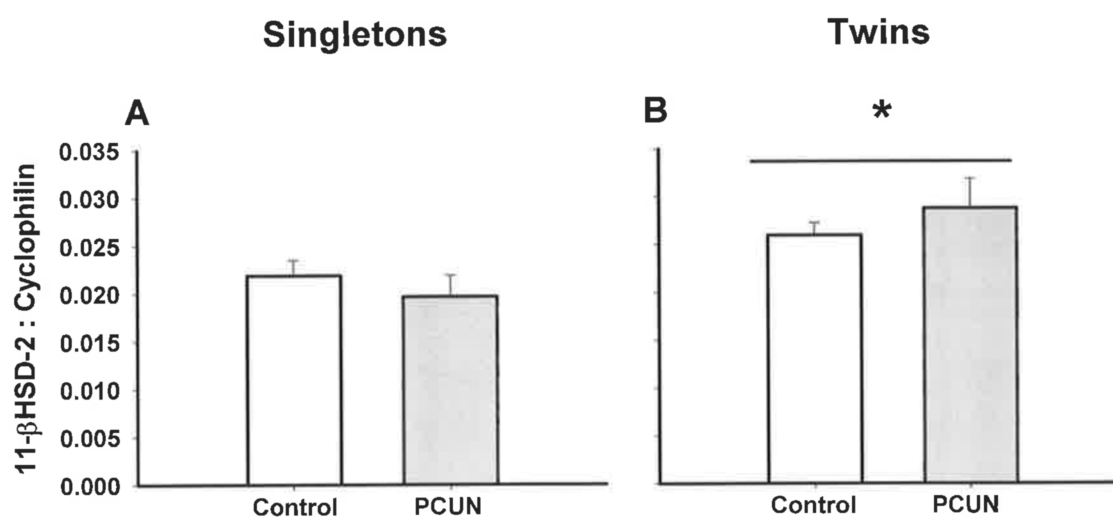

Figure 3.1 Placental 11-βHSD-2 expression at ~ d 55 pregnancy

Placental 11-βHSD-2 expression in either singleton (A) or twin (B) fetuses.

* denotes a difference ($P < 0.01$) between singleton and twin fetuses.

Table 3.3: Effect of fetal number and PCUN on absolute and relative adrenal gland and heart weights

	Singletons		Twins	
Organ weight	*Control (n=17)*	*PCUN (n=13—16)*	*Control (n=12)*	*PCUN (n=10)*
Adrenal weight (g)	0.046±0.0025	0.044±0.0036	0.032±0.0027*	0.033±0.0024*
Relative adrenal weight (g/g)	0.0018±0.00012	0.0016±0.0001	0.0011±0.00009*	0.0013±0.00011*
Heart weight (g)	0.287±0.015	0.294±0.014	0.311±0.017	0.310±0.026
Relative heart weight ((g/g)	0.0110±0.0004	0.0105±0.0004	0.0109±0.0005	0.0120±0.0007

*denotes a significant difference between singletons and twins (P < 0.0001).

3.4.4 PCUN AND ADRENAL IGF-1, IGF-2, IGF-1R, IGF-2R AND CYP 17 MRNA EXPRESSION IN SINGLETON AND TWIN FETUSES

Adrenal expression of IGF-1, IGF-1R, IGF-2, IGF-2R, and CYP 17 mRNA was significantly lower in twin compared to singleton fetuses ($P < 0.0001$, Figure 2 – 4). The ratio of the expression of adrenal IGF-2 : IGF-2R mRNA was higher, however, in adrenals from twin compared to singleton fetuses in both control and PCUN groups (Singletons: Control, 29.8 ± 2.1; PCUN, 29.3 ± 2.4; Twins: Control, 39.2 ± 5.6; PCUN, 40.4 ± 4.7, $P < 0.01$). There was no effect of PCUN on adrenal expression of either IGF-1, IGF-1R, IGF-2, IGF-2R, or CYP 17 mRNA at d 53 – 56 gestation in either singleton or twin fetuses.

Singletons: In control, but not PCUN singletons, there was a direct relationship between adrenal IGF-2R (y) and IGF-2 (x) mRNA expression (Table 4). There was also a significant inverse relationship between the relative weight of the fetal adrenal (y) and adrenal IGF-2 mRNA expression (x) at d 53 – 56 ($y = -0.0002x + 0.0027$, $r = 0.57$, $n = 17$, $P < 0.02$) in control, but not PCUN singletons. The significance of this relationship was reduced, however, when the effects of adrenal IGF-2R expression ($r = -0.48$, $P = 0.06$) were controlled for in a partial correlation analysis. There was also a significant inverse relationship between adrenal CYP 17 mRNA (y) and IGF-2R mRNA (x) expression in control (Figure 5A), but not PCUN fetuses. In control fetuses, there was also a positive relationship between adrenal CYP 17 expression (y) and the ratio of expression of IGF-2 : IGF-2R mRNA (x) (Figure 5B) and between the adrenal expression of CYP 17 mRNA (y) and IGF-1R mRNA (x) (Figure 5C). These relationships were not present in the PCUN group.

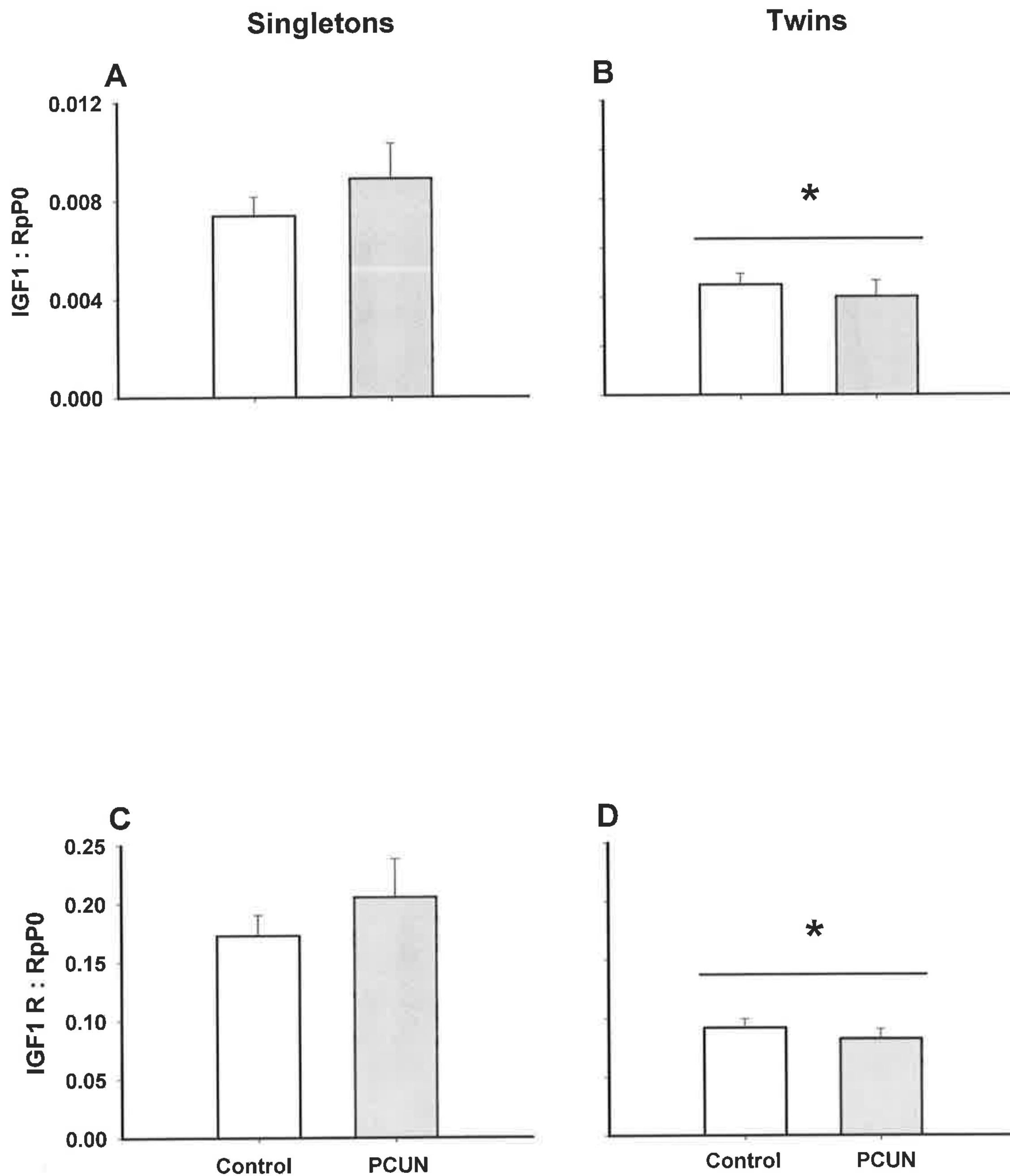

Figure 3.2 Adrenal IGF-1 and 1R expression at ~ d 55 of pregnancy

Adrenal IGF-1 expression in either singleton (A) or twin (B) fetuses and adrenal IGF-1R expression in either singleton (C) or twin (D) fetuses in the control and periconceptional undernutrition groups.

* denotes a difference ($P < 0.0001$) between singleton and twin fetuses.

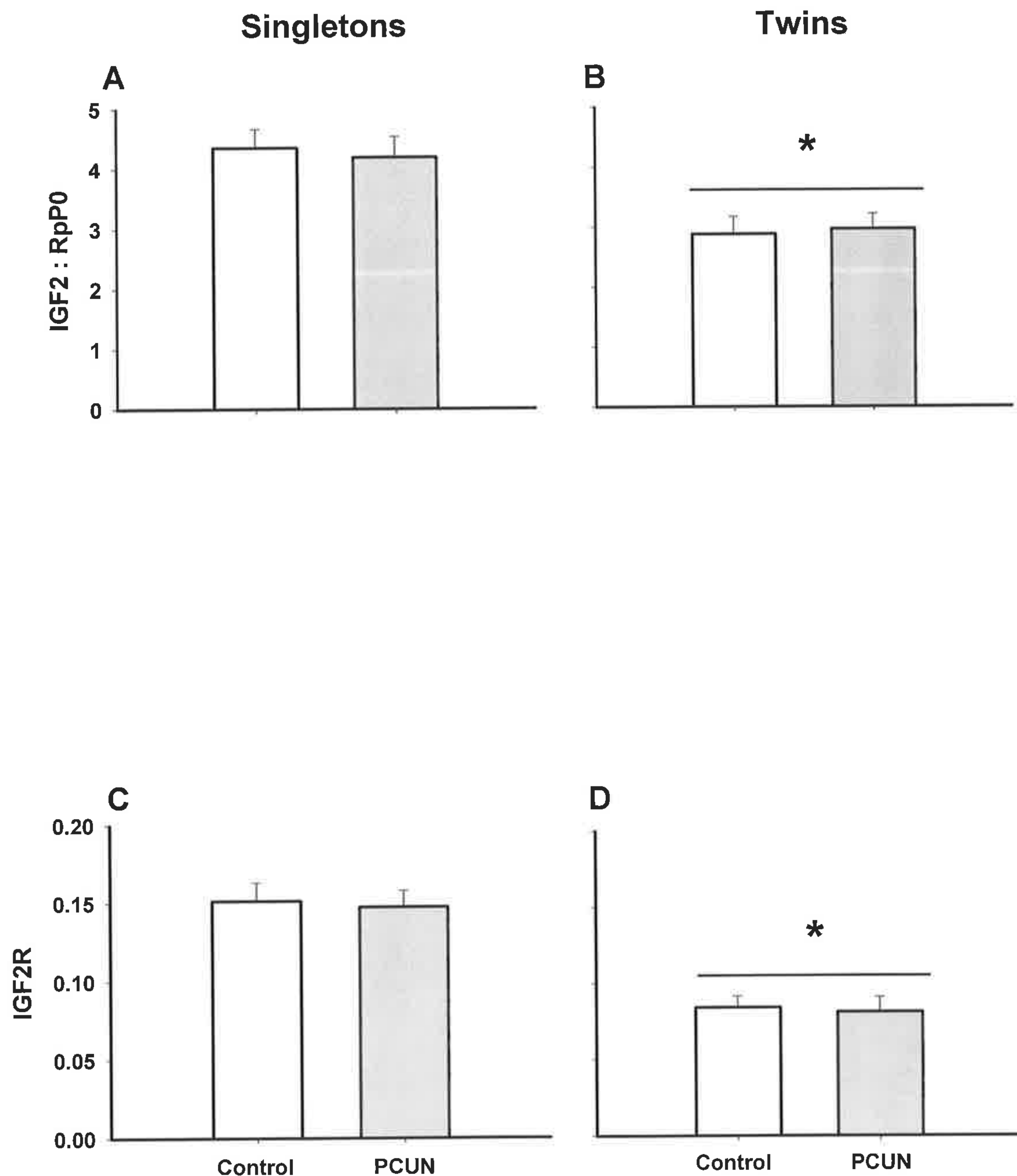

Figure 3.3 Adrenal expression of IGF-2 at ~ d 55 gestation

Adrenal IGF-2 expression in either singleton (A) or twin (B) fetuses and adrenal IGF-2R expression in either singleton (C) or twin (D) fetuses in the control and periconceptional undernutrition groups.

* denotes a difference ($P < 0.0001$) between singleton and twin fetuses.

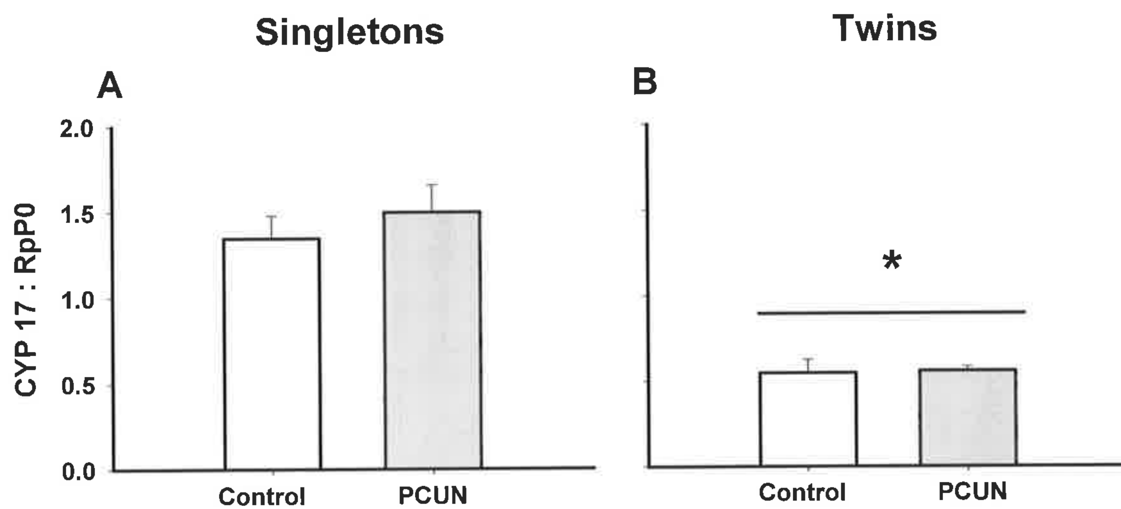

Figure 3.4 Adrenal CYP 17 expression at ~ d 55 gestation

Adrenal CYP 17 expression in either singleton (A) or twin (B) fetuses in the control and periconceptional undernutrition groups.

* denotes a difference ($P < 0.0001$) between singleton and twin fetuses.

Table 3.4: Relationships between adrenal IGF-1, IGF-1R, IGF-2, IGF-2R or CYP 17 mRNA expression in singleton fetuses at ~ d 55 pregnancy.

	IGF-1R		IGF-2R	
	Control	***PCUN***	***Control***	***PCUN***
IGF-1	y = 13.94x + 0.07 r = 0.62, n = 17, P < 0.01	y = 12.46x + 0.09 r = 0.55, n = 13, P = 0.05	NS	y = 5.07x + 0.10 r = 0.69, n = 13, P < 0.01
IGF-1R	--	--	NS	y = 0.27x + 0.09 r = 0.83, n = 13, P < 0.001
IGF-2	NS	NS	y = 0.022x + 0.056 r = 0.57, n = 17, P < 0.02	NS

NS denotes relationships that were not significant.

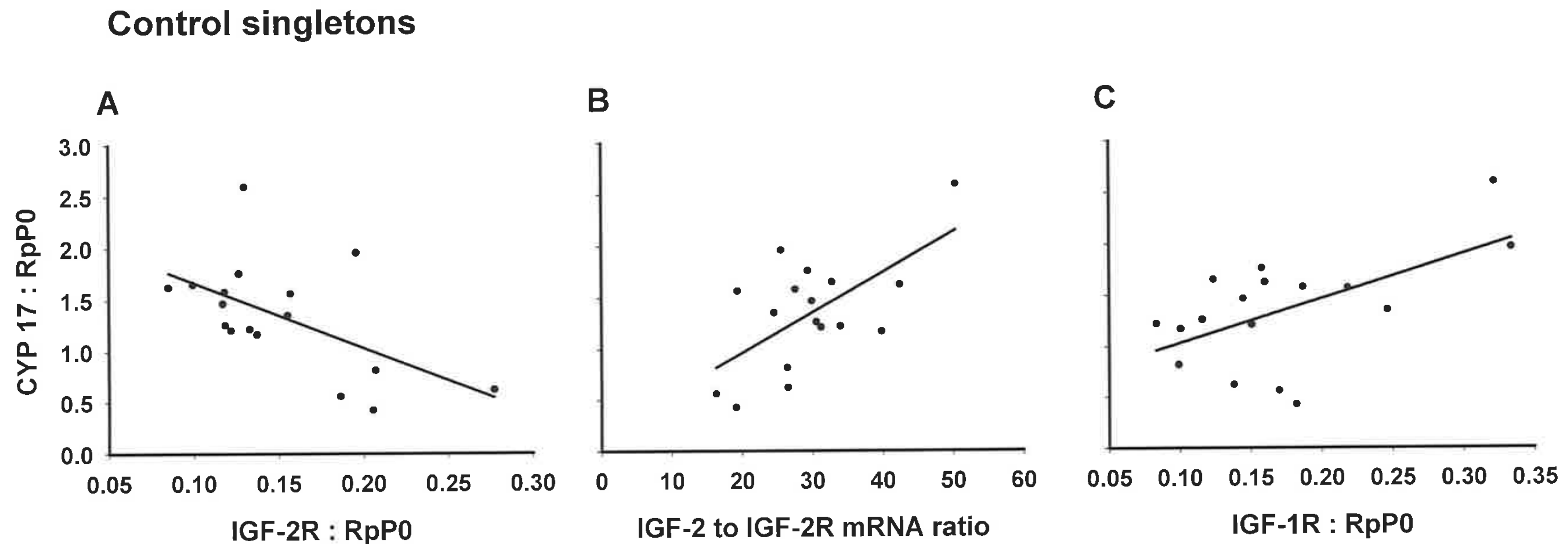

Figure 3.5 Control singletons: relationships between IGF and CYP 17 expression at ~ d 55 gestation

There was a significant inverse relationship (A) between the fetal adrenal CYP 17 (y) and IGF-2R (x) mRNA expression at d 53 – 56 gestation [$y = -6.35x + 2.31$, $r = 0.57$, $n = 17$, $P < 0.02$] in control singleton fetuses (closed circles). There was a significant relationship (B) between the fetal adrenal CYP 17 mRNA expression (y) and the ratio of the expression of IGF-2 to IGF-2R (x) at d 53 – 56 gestation [$y = 0.04x + 0.18$, $r = 0.62$, $n = 17$, $P < 0.01$] in control singleton fetuses. There was a significant relationship (C) between the fetal adrenal CYP 17 (y) and IGF-1 (x) mRNA expression at d 53 – 56 [$y = 0.08x + 0.07$, $r = 0.57$, $n = 17$, $P < 0.02$] in control singleton fetuses

Adrenal expression of IGF-1R (y) and IGF-1 (x) were directly related in adrenals in both the control and PCUN groups (Table 4). In PCUN, but not control fetuses there were significant relationships between fetal adrenal expression of IGF-2R (y) and either IGF-1 (x) or IGF-1R (x) (Table 4).

Twins: In contrast to control singleton fetuses, there was no relationship between adrenal weight and either adrenal IGF-2 or IGF-2R mRNA expression in control twins, but there was a significant relationship between relative adrenal weight (y) and adrenal IGF-1 mRNA expression (x) (Figure 6A) in control fetal sheep which was not present in the PCUN group. In control, but not PCUN twins, however, there was a positive relationship between adrenal CYP 17 (y) and IGF-2 (x) expression (Figure 6B).

3.4.5 PCUN, AND FETAL HEART WEIGHT AT D 53 – 56 GESTATION

Fetal heart weight: There was no effect of either PCUN or fetal number on the absolute or relative fetal heart weight at d 53 – 56 gestation (Table 3). In singleton fetuses there was no relationship between the relative heart and adrenal weights (Figure 7A, Table 5). In twin fetuses, however, there was a significant positive relationship between the relative heart (y) and adrenal (x) weights (Figure 7B), which was present in each of the PCUN and control groups (Table 5). There was, also, a significant inverse relationship between the relative heart weight (y) and adrenal CYP 17 mRNA expression (x) (Figure 8) in PCUN twin, but not control twin or singleton fetuses.

Maternal cortisol and weight at conception: There was a direct relationship between the relative weight of the fetal heart (y) and maternal plasma cortisol

concentrations (x) measured on the day of post mortem in control twin, but not PCUN twin or singleton fetuses (Table 5). There was, also, a significant inverse relationship between fetal relative heart weight (y) and maternal weight at conception (x) in PCUN twin (Figure 9B, Table 5), but not PCUN singleton or control fetuses (Figure 9A, Table 5). This relationship was present when either the mean or total fetal heart weight per pregnancy was used to remove the effect of twinning. The significance of this relationship was reduced when the effects of adrenal CYP 17 expression ($r = -0.62$, $P = 0.074$) or maternal cortisol concentrations ($r = -0.64$, $P = 0.06$) at d 53 – 56 gestation were controlled for in the analysis.

Table 3.5: Relationships between relative fetal heart weight (y) and maternal plasma cortisol, maternal weight at conception or relative fetal adrenal weight (x) at ~ d 55 pregnancy.

	Control		PCUN	
	Singletons	*Twins*	*Singletons*	*Twins*
Relative adrenal weight (g/g)	NS	$y = 3.85x + 0.0065$ $r = 0.72$, $n = 12$, $P < 0.009$	NS	$y = 7.56x + 0.0033$ $r = 0.80$, $n = 8$, $P < 0.018$
Maternal plasma cortisol (nmol/l)	NS	$y = 0.0001x + 0.0087$ $r = 0.73$, $n = 12$, $P < 0.007$	NS	NS
Maternal weight at conception (kg)	NS	NS	NS	$y = -0.0003x + 0.0268$ $r = 0.68$, $n = 10$, $P < 0.03$

NS denotes relationships that were not significant

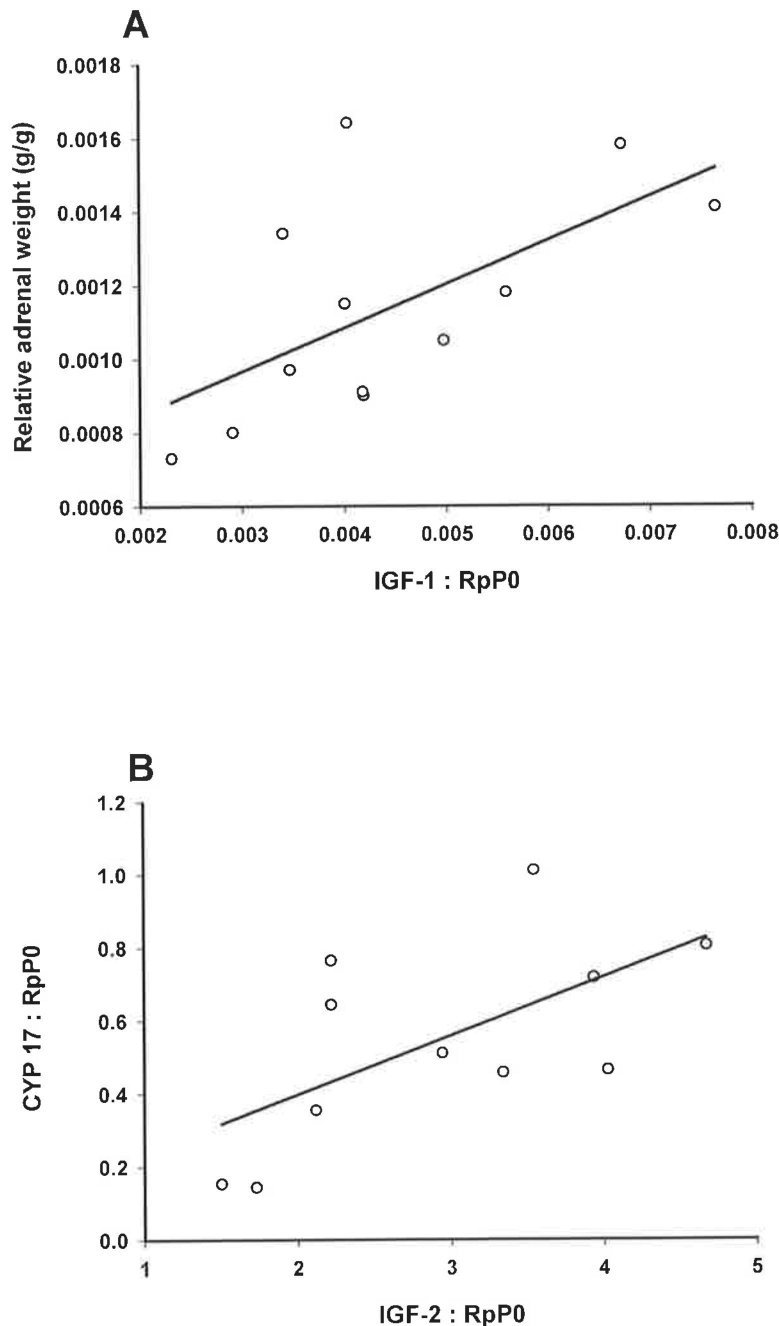

Figure 3.6 Control twins: relationships between with either relative adrenal weight or CYP 17 expression

There was a significant relationship (A) between relative adrenal weight (y) and the fetal adrenal IGF-1 mRNA expression (x) at d 53 – 56 gestation [$y = 0.11x + 0.0006$, $r = 0.61$, $n = 12$, $P < 0.05$] in control twin fetuses (open circles). There was a significant relationship (B) between the fetal adrenal CYP 17 (y) and IGF-2 (x) mRNA expression at d 53 – 56 gestation [$y = 0.16x + 0.08$, $r = 0.62$, $n = 11$, $P < 0.05$] in control twin fetuses.

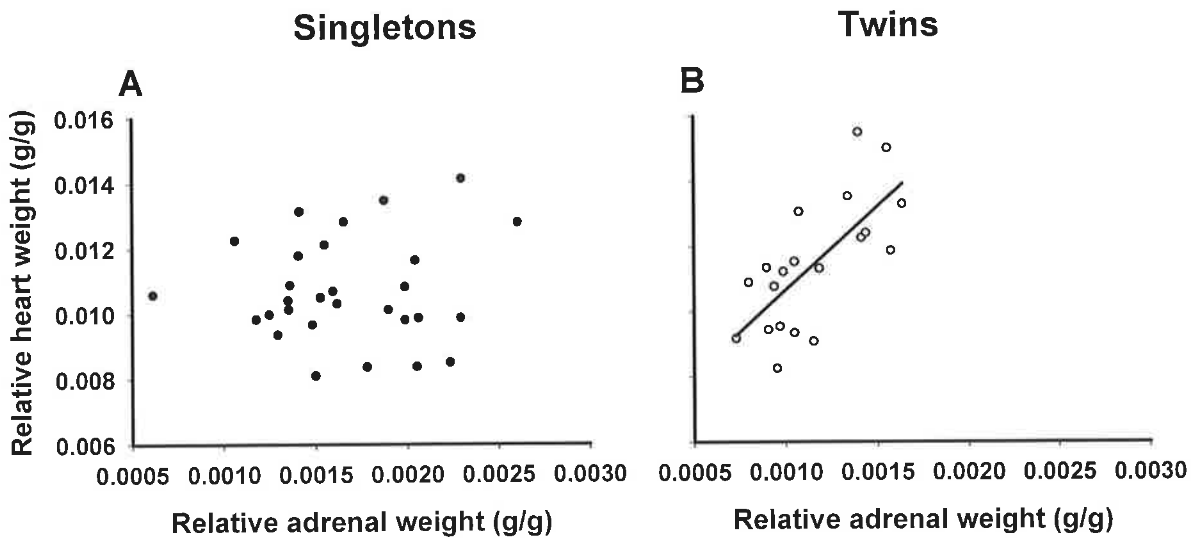

Figure 3.7 Relationship between relative heart and adrenal weight in control fetuses

There was no relationship between relative fetal heart weight and relative fetal adrenal weight at d 53 – 56 pregnancy in singleton pregnancies (closed circles, A). There was, however, a significant relationship in twin pregnancies (open circles) between relative fetal heart weight (y) and relative fetal adrenal weight (x) [y = 5.11x + 0.0055, r = 0.70, n = 20, P < 0.001] at d 53 – 56 pregnancy (B).

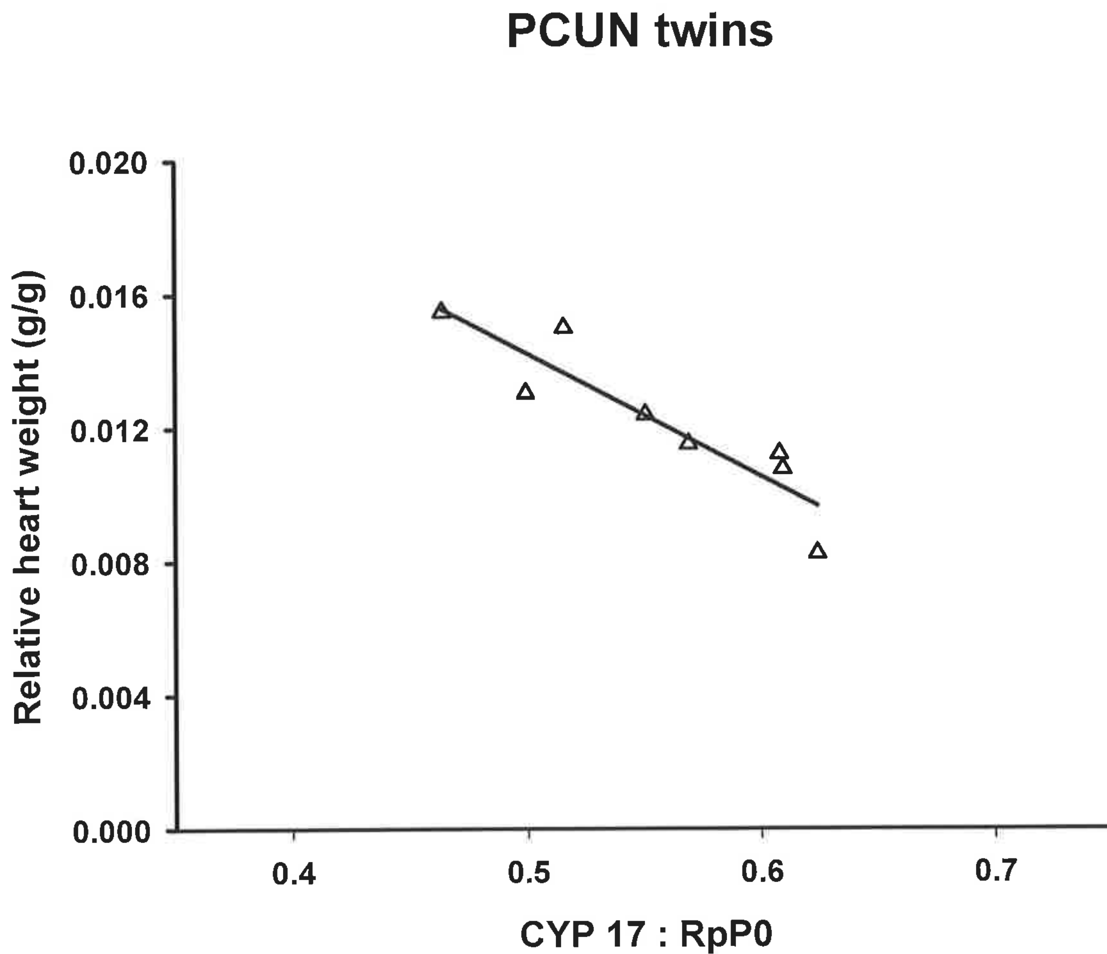

Figure 3.8 PCUN twins: relationship between relative heart weight and CYP 17 expression

There was a significant inverse relationship between relative heart weight (y) and the fetal adrenal CYP 17 mRNA expression (x) at d 53 – 56 gestation [y = -0.04x + 0.03, r = 0.91, n = 8, P < 0.002] in PCUN twin fetuses (open triangles).

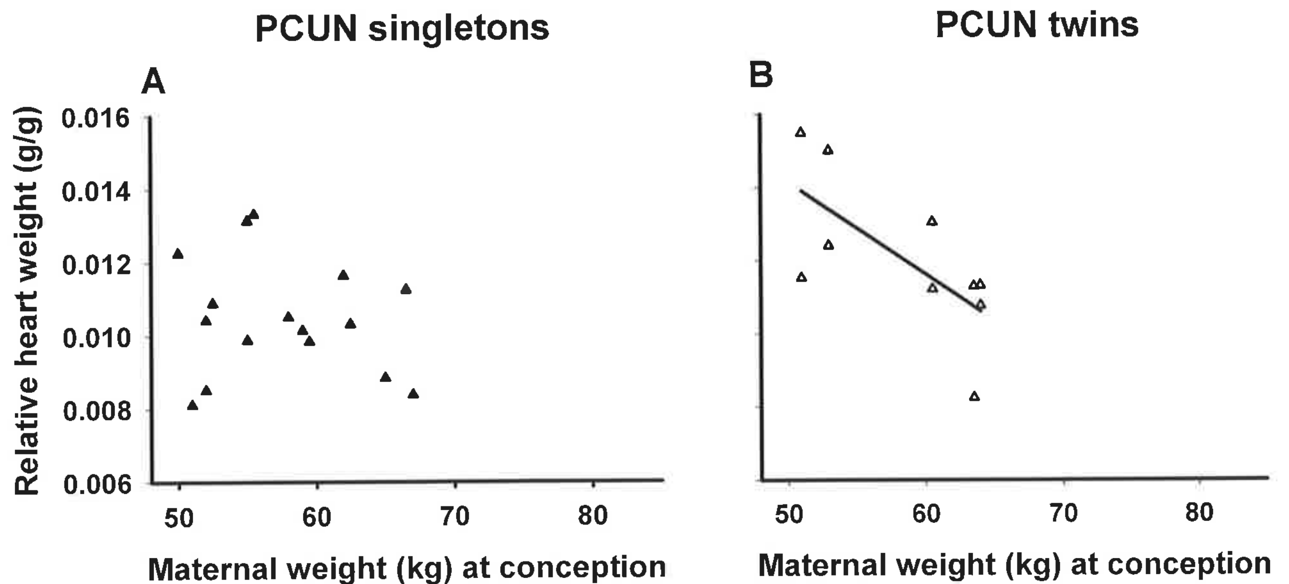

Figure 3.9 PCUN group: relationship between maternal weight at conception and relative heart weight at d 55

There was no relationship between relative fetal heart weight at d 53 – 56 pregnancy and maternal weight at conception in PCUN singleton fetuses (closed triangles, A). There was a significant inverse relationship between relative fetal heart weight (y) and maternal weight at conception (x) [$y = -0.0003x + 0.0268$, $r = 0.68$, $n = 10$, $P < 0.03$] in PCUN twin fetuses at d53-56 pregnancy (open triangles, B).

3.5 DISCUSSION

The objective of this study was to investigate whether embryo number and/or periconceptional nutrition are important determinants of adrenal growth during early pregnancy. A novel finding of the current study was that fetal adrenal weights were lower in twin compared to singleton fetuses at ~ d 55 gestation, independent of the level of maternal nutrition during the periconceptional period. It has previously been shown that fetal plasma ACTH concentrations are lower, the pre-partum surge of cortisol occurs later, and adrenocortical responsiveness to ACTH is blunted in twin compared to singleton sheep fetuses in late gestation (Edwards & McMillen, 2002a; Gardner *et al.*, 2004a). It has been speculated that a diminished adrenocortical responsiveness in the twin sheep fetus during late gestation may be an adaptive response induced early in development designed to counter the impact of the potential exposure of a twin fetus to increased intrauterine stress and thereby reduce the possibility of preterm delivery (Gardner *et al.*, 2004a; McMillen *et al.*, 2004). Our finding of a lower fetal adrenal weight and concomitant lower adrenal expression of IGF-1, IGF-1R, IGF-2, IGF-2R, and CYP 17 mRNA in twin, compared to singleton fetuses in early pregnancy indicates that a delay in the development of the pituitary-adrenal axis and adrenal growth in twins is present from as early as day 55 of pregnancy. It has been previously demonstrated that the fetal sheep adrenal is responsive to ACTH at this early stage in gestation (Wintour *et al.*, 1975; Tangalakis *et al.*, 1994), and thus the decrease in adrenal growth and CYP 17 mRNA expression in the twin fetal sheep may be a result of the decrease in either pituitary ACTH secretion and/or the decrease in the expression of intra-adrenal IGF expression or

bioavailability. One possible mechanism which would result in a decrease in hypothalamo-pituitary stimulation of the fetal adrenal in twin pregnancies would be an increase in maternal cortisol concentrations coupled with an increase in transfer of maternal cortisol across the placenta.

3.5.1 Maternal progesterone and cortisol concentrations and placental 11-βHSD-2 mRNA expression in twin pregnancies

Whilst maternal progesterone concentrations were higher, and related to placental weight, as expected in twin pregnancies, there were no differences in circulating cortisol concentrations between singleton and twin pregnancies at d 53-56 gestation. Interestingly, the expression of placental 11-βHSD-2 mRNA was higher in twin compared to singleton pregnancies, which would suggest that materno-fetal transfer of cortisol in these pregnancies would be lower, rather than higher, resulting in less negative feedback on the fetal pituitary-adrenal axis. It is not clear what factors related to the mother, embryo or placenta act to regulate the expression of this enzyme which rapidly dehydrogenates cortisol in the placenta. Clearly further studies are required to determine at what stage of pregnancy the higher placental expression of 11-βHSD-2 mRNA expression occurs and for how long this this persists in twin pregnancies. It does not appear, however that the decreased adrenal growth and CYP17 expression in twin pregnancies, is a direct consequence of enhanced materno-fetal transfer of cortisol and increased negative feedback actions of cortisol on the twin pituitary-adrenal axis.

In this context it is interesting that we have demonstrated that there are different relationships between adrenal weight and the expression of CYP 17 mRNA with the expression of the intraadrenal IGFs and their receptors in control singleton and twin fetal sheep.

3.5.2 ADRENAL GROWTH, IGFS AND CYP 17 EXPRESSION IN SINGLETON AND TWIN FETUSES

In control singletons, there was an inverse, rather than a positive correlation between relative adrenal weight and IGF-2 expression. This relationship was not significant, however, once the effects of IGF-2R expression were controlled for in the analysis. Given the direct relationship between adrenal expression IGF-2 and IGF-2R, it seems likely that low adrenal IGF-2R expression results in a decrease in IGF-2 clearance and an increase in IGF-2 bioavailability in the adrenal which would then act through the IGF-1 receptor to promote adrenal growth. This is supported by the presence of an inverse relationship between adrenal CYP 17 and IGF-2R mRNA expression and a positive relationship between CYP 17 expression and the ratio of expression of IGF-2 : IGF-2R mRNA expression in control singletons. IGF-2 is co-localized to steroidogenic cells of the adrenal gland at this stage of gestation (Han *et al.*, 1992), and one possibility is that IGF-2 acts through the IGF-1 receptor to up regulate adrenal CYP 17 mRNA expression at ~ d 55 gestation. Thus in the control singleton fetus in early pregnancy, it appears that the clearance receptor, IGF2-R plays a key role in determining the bioavailability of IGF-2 in the adrenal and that IGF-2 may be an important determinant of early adrenal growth and steroidogenic activity.

Whilst adrenal IGF-2 expression was lower in control twin compared to singleton fetuses, the ratio of expression of adrenal IGF-2:IGF-2R mRNA, was higher than in the singleton fetuses. Despite this relative increase in the expression of the growth factor over the clearance receptor, there was no relationship between adrenal weight and either adrenal IGF-2 or IGF-2 : IGF-2R mRNA expression in control twins. In twins, there was, however the emergence of a positive relationship between adrenal weight and adrenal IGF-1 expression. Thus, in twin fetuses it appears that when IGF-2 expression levels are not above the threshold levels attained in the singleton fetal adrenal, IGF-1 becomes the major determinant of adrenal growth. Whilst adrenal growth was not related to IGF-2 or IGF-2R expression in twins, there was, however, a direct relationship between adrenal CYP 17 and IGF-2 mRNA expression in twin fetuses. Thus the cellular processes which regulate adrenal steroidogenesis appear to be activated at lower levels of IGF-2 expression compared to those regulating adrenal growth in twin fetuses at ~d 55 gestation.

Thus in summary, in control singletons, IGF-2R expression plays an important role in the regulation of the intraadrenal bioavailability of IGF-2 and thus in the regulation of adrenal growth and CYP 17 mRNA expression during early pregnancy. In control twins, however, whilst the ratio of IGF-2 : IGF-2R mRNA expression is higher than in singletons and adrenal CYP 17 and IGF-2 expression are related, adrenal growth appears to be predominantly regulated by IGF-1.

The lower adrenal IGF-1, IGF-2, and CYP 17 mRNA expression clearly may contribute to a decrease in adrenal growth and function in twin fetuses in early gestation. It is unclear, however, whether adrenal IGF-1 and -2 and CYP 17 expression are decreased in twin fetuses because of a decrease in the secretion of ACTH or other pituitary growth factors in the twin. Whilst it is intriguing to speculate that the early environment of the twin embryo may act through epigenetic mechanisms to down regulate the expression of the imprinted genes, IGF-2 and IGF-2R, it is the case that the expression of the non-imprinted genes, IGF-1 and IGF-1R were also down regulated in the adrenal of the twin fetus. Independent of the mechanisms involved, I have demonstrated that the growth trajectory of the fetal adrenal in the twin is different from to that of the singleton sheep fetus from early in pregnancy and that therefore the delay in the prepartum activation of the pituitary-adrenal axis in the twin fetus is programmed early in pregnancy.

3.5.3 IMPACT OF PCUN ON PLACENTAL AND ADRENAL GROWTH AND DEVELOPMENT

Whilst there was no effect of periconceptional undernutrition on the mean level of placental 11-βHSD-2 mRNA expression, there was a relationship between maternal weight change during the periconceptional period and placental 11-βHSD-2 mRNA levels in the undernourished, but not control group of ewes. Thus ewes that lost more weight during the periconceptional period had lower levels of placental 11-βHSD-2 mRNA expression. There is evidence from studies in both rats and humans that there is considerable natural variation in placental 11-βHSD-2, and that the level of placental activity of this enzyme correlates with birth weight. Moreover, inhibition of feto-placental 11-βHSD-2 in the rat reduces

birth weight and produces hypertensive and hyperglycaemic adult offspring, many months after prenatal treatment; and maternal protein restriction during pregnancy also selectively attenuates placental 11β-HSD2 activity and produces hypertensive offspring (Seckl, 1997). Our data suggest that metabolic or hormonal cues associated with maternal weight loss in the periconceptional period results in persistent changes in placental 11-β-HSD-2 mRNA expression. These data are consistent in part with recent studies which have found that periods of more severe maternal undernutrition which extend beyond implantation and into early pregnancy (Jaquiery *et al.*, 2006) or during early-mid gestation (between d 28-78d) (Whorwood *et al.*, 2001) result in a decrease in placental 11-βHSD-2 mRNA expression. It has been reported that the decrease in placental 11-βHSD-2 activity associated with relatively severe maternal undernutrition extending beyond the periconceptional period did not result in a change in fetal cord blood cortisol concentrations sampled at 50 d gestation (Jaquiery *et al.*, 2006). It may be, however, that changes in the level of this placental enzyme when coupled with either a persistent maternal nutritional stress or episodes of maternal stress during pregnancy may then predispose the fetus to increased exposure to maternal cortisol with consequent long term consequences (McMillen & Robinson, 2005).

In ewes, carrying singletons, which were exposed to undernutrition during the periconceptional period we found that there was a loss of the relationships between adrenal IGF-2 mRNA expression and either adrenal growth or CYP 17 expression and between adrenal CYP 17 and IGF-1 expression which were present in control singletons. In the singletons, there was also the emergence of

a positive relationship between adrenal IGF-2R and adrenal IGF-1 and IGF-1R expression. In ewes carrying twins, maternal undernutrition during the periconceptional period resulted in the loss of the relationships between adrenal growth and IGF-1 expression and between adrenal CYP 17 and IGF-2 expression which were present in control twin fetuses. It is not clear at this stage, why maternal undernutrition during the periconceptional period ablates intraadrenal relationships between the expression of intraadrenal growth factors and adrenal growth and CYP 17 mRNA expression, but these data highlight that the regulation of adrenal growth and functional development is different in both singleton and twin fetal sheep after a period of periconceptional undernutrition, which only extends up to d 7 after mating. This highlights the preconceptional period and first week after conception as critical windows within which exposure of the embryo to relatively subtle changes in maternal nutrition result in changes in neuroendocrine development and in the level and timing of the prepartum activation of the fetal pituitary-adrenal axis and on cardiovascular development (Edwards & McMillen, 2002a, b). It remains to be determined whether the effects of maternal undernutrition which extends beyond the preimplantation period to include early placental formation (Bloomfield et al 2003), on early adrenal development are similar to those described above or include both a periconceptional and early gestational component.

3.5.4 PCUN, FETAL HEART GROWTH AND THE INFLUENCE OF THE DEVELOPING HPA AXIS

There was a direct relationship between fetal heart and adrenal weight at ~55d gestation in twin, but not singleton fetuses and this relationship occurred

independently of the level of maternal nutrition during the periconceptional period. One possible explanation of the relationship between fetal adrenal and heart growth in twins is that heart growth may be regulated by exposure to fetal cortisol derived from the fetal adrenal during early pregnancy in twins. Administration of the synthetic glucocorticoid, dexamethasone, at 26-28 d gestation results in an increased cardiac output, left ventricular hypertrophy and hypertension in the adult sheep (Dodic *et al.*, 1998; Dodic *et al.*, 1999; Dodic *et al.*, 2001; Roghair *et al.*, 2005). It has also been shown that in twin, but not singleton fetuses, there is a significant and positive relationship between arterial blood pressure and plasma cortisol concentrations in late gestation (Edwards & McMillen, 2002b; Gardner *et al.*, 2004a). Whilst it is possible that fetal adrenal glucocorticoid output stimulates heart growth in twin fetuses, it is not clear why this would occur in twin and not singleton fetuses, given that we have provided evidence that adrenal growth and CYP 17 mRNA are lower in twins. It is also the case that fetal heart weights were not different between singleton and twin fetuses.

In the PCUN twins, however, there was the emergence of an inverse relationship between relative heart weight and adrenal CYP 17 expression and between relative heart weight and maternal weight at conception. This inverse relationship occurred in the presence of a strongly positive relationship between relative heart weight and adrenal weight in the PCUN twins. It is therefore possible that CYP 17 mRNA expression is regulated by factors additional to glucocorticoids in the PCUN twins. Hence, fetal heart and adrenal weights are relatively increased when adrenal CYP 17 expression is low. In this context, it is intriguing that there

was an inverse relationship between relative fetal heart weight and maternal weight loss at conception and that this relationship was absent when the effects of adrenal CYP 17 mRNA were controlled for in the analysis.

3.5.5 SUMMARY

In summary, the present study has demonstrated that fetal adrenal growth, and CYP 17 mRNA expression was reduced in twin fetuses, and we hypothesize that these changes are in part mediated by a decrease in adrenal IGF-2, IGF-2R, IGF-1, and IGF-1R mRNA expression. In addition, periconceptional undernutrition ablated the relationships between adrenal IGF mRNA expression and adrenal growth and CYP 17 mRNA expression, which were present in control singleton and twin fetuses. A novel finding in the present study is the direct relationship between fetal adrenal and heart growth in twin fetuses that is not present in singletons during early pregnancy. Furthermore in ewes carrying twins, which were undernourished during the periconceptional period, there were significant inverse relationships between relative fetal heart weight and either maternal weight at conception or fetal adrenal CYP 17 expression. Whilst it is not yet possible to determine the specific mechanisms which underlie the effects of undernutrition during the periconceptional period on the subsequent functional development of the heart and adrenal gland, these findings highlight the importance of the interaction between the periconceptional environment and embryo number in setting the growth trajectories of the fetal adrenal and heart during early pregnancy and suggest that the periconceptional period is a critical window for the programming of the subsequent development of the neuroendocrine and cardiovascular systems of the fetus and adult.

Chapter 4:

Impact of maternal undernutrition during the periconceptional period and fetal number on kidney growth and on kidney IGF expression in the sheep fetus during early pregnancy

"Success is how high you bounce when you hit rock bottom"

- General George S. Patton Jr.

4. Impact of maternal undernutrition during the periconceptional period and fetal number on kidney growth and on kidney IGF expression in the sheep fetus during early pregnancy

4.1 Summary

Recent studies in the sheep have shown that maternal undernutrition during the periconceptional period, when the nutrient demands of the embryo are minimal, can alter the subsequent development of the metabolic, endocrine and cardiovascular systems and that these effects may, in part, depend on embryo number. I have tested the hypotheses that there are relationships between maternal weight change during the periconceptional period, maternal plasma cortisol concentrations, and relative kidney weight in early gestation. I have also tested the hypothesis that embryo number and/or maternal undernutrition during the periconceptional period alters the mRNA expression of IGF-1 and 2 and the IGF receptors, IGF-1R and 2R. We have investigated the effect of periconceptional undernutrition in the ewe (control n = 24, restricted at 70 % of control feed allowance, PCUN n = 21) from 45 days prior to mating until 7 days after mating on fetal kidney weight, maternal plasma cortisol levels and renal expression of IGF-1, IGF-2, IGF-1R and IGF-2R mRNA at ~ d 55 pregnancy. In control pregnancies maternal weight gain during the periconceptional period is inversely related to the relative weight of the fetal kidney at ~55d pregnancy. In this group, relative kidney weight was also directly related to renal IGF-1 mRNA expression. In control twins maternal weight gain was inversely related to fetal kidney weight and this effect was ablated when the effects of maternal cortisol

was controlled for in the analysis. In the PCUN group, whilst there was an inverse relationship between maternal weight gain during the periconceptional period and relative kidney weight, it was not possible to separate the independent effects of materna weight loss during the periconceptional period and the subsequent weight gain during the period of refeeding. Renal IGF-1 mRNA expression was higher and renal IGF-1R and 2R expression were lower in twin fetuses compared to singletons. After exposure to PCUN, renal IGF-1 expression was also higher than in control pregnancies independent of fetal number. These changes highlight the importance of the periconceptional environment in setting the fetal kidney growth trajectory, and have implications for the programmed development of the renal, cardiovascular, and endocrine systems of the fetus and adult.

4.2 INTRODUCTION

As discussed in Chapters 2 and 3, a range of epidemiological, clinical and experimental studies have demonstrated that exposure to poor maternal nutrition before or around the time of conception is associated with altered development of the cardiovascular system which may in turn result in hypertension or cardiovascular disease in adult life (Kwong *et al.*, 2000; Roseboom *et al.*, 2000b; Roseboom *et al.*, 2001b; Edwards & McMillen, 2002b; Gardner *et al.*, 2004b). The Dutch Winter Hunger Famine Study investigated the effects of a 5 month period of malnutrition experienced by pregnant women in Amsterdam during 1944 – 1945 and found that individuals exposed as an embryo and fetus to maternal undernutrition during the period of the famine in the first trimester had an increased prevalence of coronary heart disease and a higher body mass index

in adult life (Ravelli 1976, Ravelli 1999, Roseboom 2000, Roseboom 2001). In pregnant rats fed a low protein diet for the first 4.25 days after conception, the offspring had a low birth weight, an increased relative kidney weight and raised blood pressure in postnatal life (Kwong *et al.*, 2000). Similarly in the sheep a 30% reduction in maternal nutrition from ~ 45 days before until 7 days after conception resulted in an altered growth trajectory of the fetus and placenta in early pregnancy (Chapter 2), and an increase in arterial blood pressure and in the activation of the fetal pituitary–adrenal axis in twin but not singleton fetuses during late gestation (Edwards & McMillen, 2002a, b). As reviewed earlier in this thesis, these findings suggest that a period of maternal undernutrition during early pregnancy, when the nutrient demands of the early conceptus are minimal, can have specific long-term consequences for the development of the systems that regulate arterial blood pressure.

In the sheep, the fetal kidney begins to develop in the form of the mesonephros as early as d 17 of pregnancy and the metanephros, the functional kidney in mammals, develops at around d 27 of pregnancy (Moritz & Wintour, 1999). It has also been established that the fetal sheep kidney is functional at d 20 of pregnancy producing hypotonic urine, which is essential in the expansion of the allantois and normal placentation in the sheep (Moritz & Wintour, 1999). The administration of physiologic levels of glucocorticoids, both dexamethasone and cortisol, at the end of the first month of pregnancy (~ d 27) for 48 hours in the sheep has been demonstrated to cause hypertension and alterations in cardiovascular development in postnatal life (Dodic *et al.*, 1999; Dodic *et al.*, 2001; Dodic *et al.*, 2002a; Dodic *et al.*, 2002b; Moritz *et al.*, 2003; Wintour *et al.*,

2003b). A series of studies has also demonstrated that exposure to glucocorticoids at ~ d 27 pregnancy causes structural and functional changes in the developing metanephros leading to a 40% decrease in nephron numbers and resulting in hypertension in postnatal life (Moritz & Wintour, 1999; Wintour *et al.*, 2003a; Wintour *et al.*, 2003b), and that these programming effects are not observed when glucorticoids are administered in mid – gestation (~ d 64) or over a longer period in early pregnancy (d 25 – 45) (Dodic *et al.*, 1999; Moritz *et al.*, 2002a; Dodic *et al.*, 2003). These findings have resulted in the hypothesis that an insult to the developing fetal kidney during active nephrogenesis will result in a renal deficit which is associated with the development of hypertension in postnatal life. The mechanisms by which such early exposure to glucocorticoids perturb nephrogenesis during this early period of development have not, however, been elucidated (Wintour *et al.*, 2003b).

Given the impact of exposure to cortisol on renal development during early gestation, in the present study, we have investigated whether there are relationships between maternal weight change during the periconceptional period, maternal plasma cortisol concentrations, and relative kidney weight at ~ d 55 pregnancy.

In the late gestation sheep fetus it has also been shown that IGF-2 mRNA is highly expressed in the fetal kidney (Kind *et al.*, 1995) and that intrafetal infusion of a long acting analogue of IGF-1 stimulates kidney growth in late gestation (Lok *et al.*, 1996; Marsh *et al.*, 2001). Interestingly, severe maternal nutrient restriction (to 50% of maintenance levels) during early gestation results in decreased IGF-2

mRNA expression in the kidney of twin, but not singleton fetuses during late gestation (Brennan *et al.*, 2005).

I have, therefore, tested the hypotheses that embryo number and/or maternal undernutrition during the periconceptional period alters the growth trajectory of the fetal kidney during early pregnancy and the mRNA expression of intrarenal growth factors, IGF-1, IGF-2, and their receptors, IGF-1R and IGF-2R.

4.3 MATERIALS AND METHODS

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee.

As discussed in Chapter 2 and 3, forty-five South Australian Merino ewes were used in this study and the feeding and breeding protocols were as previously reported (Chapter 2 and 3).

4.3.1 COLLECTION OF TISSUES

Ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) between d 53 and 56 of pregnancy (term = 150 ± 3 d gestation), and the utero-placental unit was delivered by hysterotomy. Fetal organs, including the kidneys, were dissected, weighed and snap frozen until further analysis.

4.3.2 Maternal blood samples

As described in Chapter 3 maternal blood samples were collected from the jugular vein by venepuncture for cortisol assay from all ewes at post mortem on either d 53 or 55 pregnancy.

4.3.3 Cortisol radioimmunoassay

Maternal plasma samples were extracted and assayed in duplicate as described in Chapter 3.

4.3.4 RNA Isolation and cDNA Synthesis

RNA was isolated from frozen kidney tissue samples using Trizol reagent (Invitrogen, The Netherlands) and purified using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland). Genomic DNA contamination was minimised by treating each sample with DNase 1 (Ambion, Austin, Texas, USA), and RNA was quantified by spectrophotometric measurements at 260nm and 280nm. cDNA was synthesised from 5µg RNA using Superscript III (Invitrogen, The Netherlands) reverse transcription. Controls containing no RNA transcript or no superscript were used to test for DNA contamination.

4.3.5 Quantitative Real Time Reverse Transcription PCR

As described in Chapter 3, the relative quantities of IGF-1, IGF-2, IGF-1R, and IGF-2R mRNA transcripts in fetal kidney tissue were measured by quantitative real time reverse transcription PCR (qRT-PCR) using the Sybr Green system in

an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each qRT-PCR well contained 5µl Sybr Green Master Mix (Applied Biosystems), 1µl each of forward and reverse primer (GeneWorks, SA, Australia) for the appropriate gene (Table 1, Chapter 3), water (2µl), and 50ng/µl cDNA (1µl) to give a total volume of 10µl. Controls for each primer set containing no cDNA were included on each plate. Three replicates of cDNA from kidney tissue were performed for each gene on each plate, and each plate was repeated three times to ensure a consistent result. Amplification efficiencies were determined, from the slope of a plot of Ct (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (ranging from 1 to 100ng/µL). The abundance of each transcript relative to the abundance of the reference gene, ribosomal protein P0 (RpP0), was calculated using Q-Gene analysis software(Muller *et al.*, 2002).

4.3.6 Statistical Analysis

Data are presented as the mean ± SEM. The effects of periconceptional undernutrition on maternal weight loss were analysed using multifactorial analysis of variance (MANOVA) with repeated measures using the Statistical Package for Social Sicences (SPSSX, SPSS Inc., Chicago, IL, USA). The effects of periconceptional undernutrition and fetal number on maternal plasma cortisol concentrations, fetal weight, and absolute and relative fetal kidney weight, and fetal kidney expression of IGF-1, IGF-1R, IGF-2, and IGF-2R mRNA were determined using a 2 way Analysis of Variance (ANOVA) using the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). Relationships between variables were assessed by linear

regression using Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA) and partial correlational analyses were used where appropriate. A probability level of 5% ($P<0.05$) was taken to be significant.

4.4 RESULTS

4.4.1 PCUN, FETAL OUTCOMES AND FETAL KIDNEY GROWTH

As described in Chapters 2 and 3, ewes in the PCUN group lost significantly more weight (-4.54 ± 0.84 kg, P <0.0001) than control ewes (0.04 ± 0.48 kg) between the start of the feeding regime and by d 10 after conception (Figure 1). There was no effect of PCUN or fetal number on fetal weight, absolute or relative kidney weight at ~ d 55 pregnancy (Table 1).

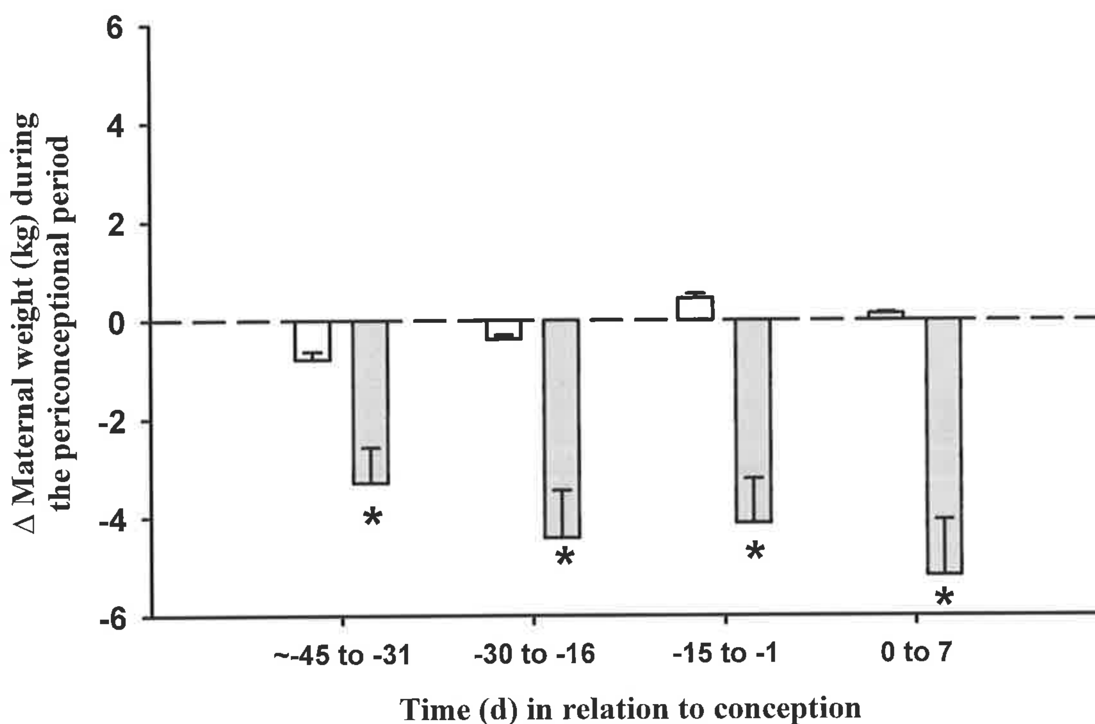

Figure 4.1 Maternal weight loss over time during the periconptional period

Ewes in the PCUN group (n = 21, shaded bars) lost significantly more weight (-4.54 ± 0.84 kg) during the periconceptional period than ewes in the control group (0.04 ± 0.48 kg, n = 24, open bars) from the start of the feeding regime to d 10 of pregnancy. Δ = Change.

* denotes a significant difference (P < 0.0001) between control and PCUN groups.

Table 4.1: Effect of fetal number and PCUN on fetal weight and absolute and relative kidney weights

	Singles		**Twins**	
Organ weight	***Control***	***PCUN***	***Control***	***PCUN***
Kidney weight (g)	0.329±0.018 (n=17)	0.362±0.031 (n=16)	0.344±0.022 (n=11)	0.302±0.029 (n=9)
Fetal weight (g)	26.26±1.24 (n=18)	28.11±0.78 (n=16)	28.45±0.89 (n=12)	25.89±1.87 (n=10)
Relative kidney weight (g/g)	0.0126±0.0005 (n=17)	0.0128±0.0009 (n=16)	0.0121±0.0008 (n=11)	0.0115±0.0009 (n=9)

4.4.2 FETAL KIDNEY GROWTH, MATERNAL WEIGHT CHANGE AND MATERNAL CORTISOL CONCENTRATIONS

4.4.2.1 Control Group:

There was an inverse relationship between relative kidney weight at ~ d 55 (y) and maternal weight gain during the periconceptional period (x) in control twin (Figure 2D) but not singleton (Figure 2A) fetuses. There were no relationships, however, between relative kidney weight and the change in maternal weight between d 10 and ~ d 55 pregnancy in either control singleton (Figure 2B) or twin (Figure 2E) fetuses. There was a significant inverse relationship between relative kidney weight (y) and the total change in maternal weight from the start of the feeding regime until ~ d 55 pregnancy (x) in both control singleton (Figure 2C) and twin (Figure 2F) fetuses. In control twins, this relationship was dependent on the maternal weight loss during the periconceptional period as the relationship between relative kidney weight and total maternal weight loss before PM was no longer significant (r = -0.54, P = 0.108) when the effects of maternal weight loss during the periconceptional period was controlled for in the analysis. There was also a significant inverse relationship between plasma cortisol concentrations on the day of post-mortem (y) and either maternal weight or maternal weight gain before postmortem (x) in control twin (Table 2), but not in control singleton pregnancies. In control singletons, however, the relationship between relative kidney weight and maternal weight loss before PM was no longer significant (r = -0.34, P = 0.180) when the effects of maternal plasma cortisol at ~ d 55 pregnancy were controlled for in a partial correlation analysis.

Whilst there was a positive relationship between relative kidney weight (y) and maternal plasma cortisol levels at ~ d 55 (x) in control twin pregnancies (Figure 4A), it was not possible to separate out the independent effects of maternal cortisol from those of maternal weight gain on fetal kidney weight in partial correlation analyses, but this relationship was no longer significant when the effects of maternal weight change before PM ($r = 0.23$, $P = 0.53$) were controlled for in the analysis.

Table 4.2: Relationships between maternal plasma cortisol concentrations (y) and either maternal weight at PM or change in maternal weight before PM(x).

	Singletons		Twins	
	Control	***PCUN***	***Control***	***PCUN***
Maternal weight at PM	NS	NS	$y = -1.13x + 97.02$, $r = 0.65$, $n = 6$, $P < 0.03$	$y = 0.72x + -34.63$, $r = 0.72$, $n = 5$, $P < 0.02$
Change in maternal weight before PM	NS	NS	$y = -2.06x + 24.81$, $r = 0.71$, $n = 6$, $P < 0.01$	NS

NS denotes relationships that were not significant.

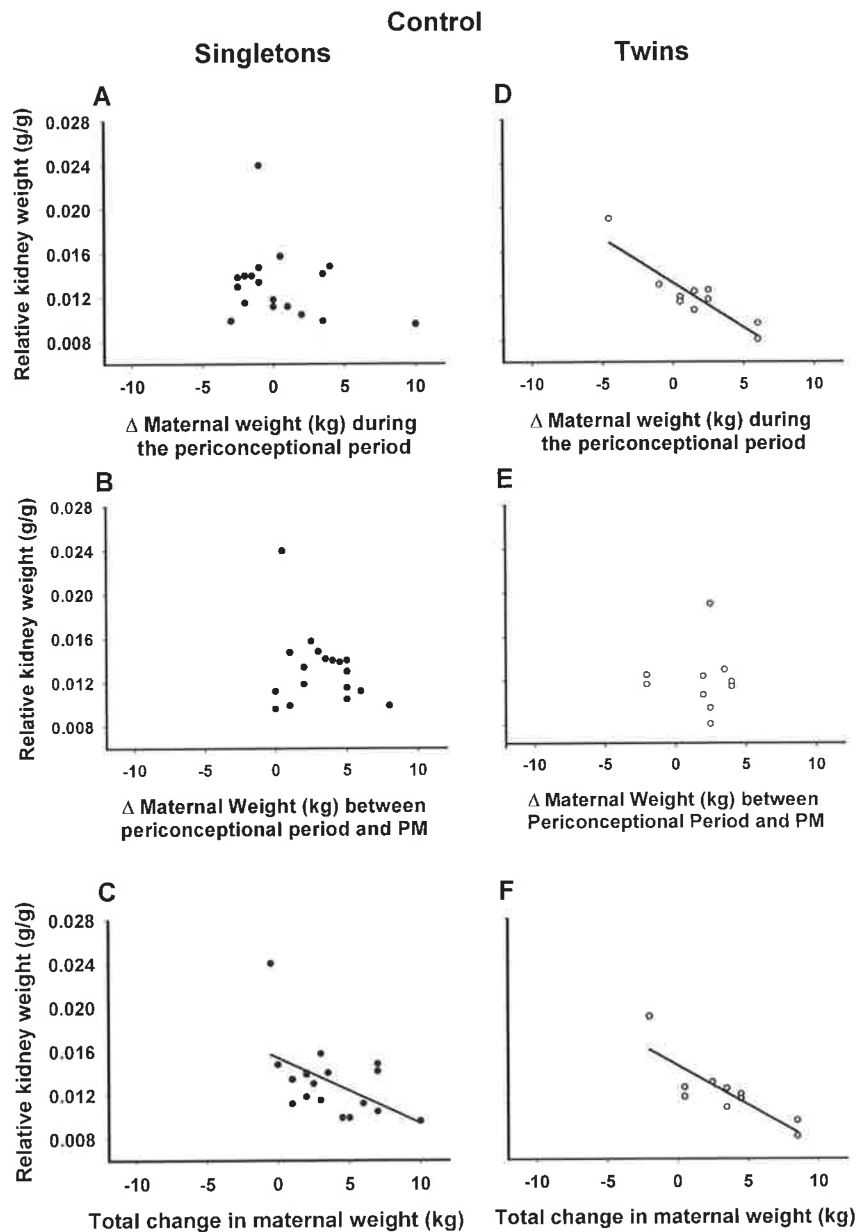

Figure 4.2 Impact of maternal weight change on renal development during early gestation in the control treatment group

There was a significant inverse relationship between relative kidney weight at d 53 – 56 pregnancy (y) and the change in maternal weight during the periconceptional period (x) [y = -0.0008x + 0.0132, r = 0.90, n = 11, P = 0.0002] in control twins (open circles, D), but not control singletons (closed circles, A). There were no relationships between relative kidney weight at d 53 – 56 pregnancy and the change in maternal weight between the periconceptional period and PM in either control singletons (B) or twins (E). There was a significant inverse relationships between relative kidney weight at d 53 – 56 pregnancy (y) and the change in maternal weight before PM at d 53 – 56 pregnancy (x) in both control singletons [y = -0.0006x + 0.0154, r = 0.51, n = 18, P < 0.03, C] and twins [y = -0.0007x + 0.0145, r = 0.84, n = 11, P < 0.002, F].

4.4.2.2 PCUN Group:

There were significant inverse relationships between relative kidney weight at ~ d 55 (y) and maternal weight gain during the periconceptional period in PCUN singleton (x) (Figure 3A) and PCUN twin (Figure 3D) fetuses. These relationships were no longer significant when the effect of maternal weight gain between d 10 and ~ d 55 pregnancy was controlled for in the analysis (singletons, $r = -0.42$, $P = 0.117$; twins, $r = -0.39$, $P = 0.336$). There were also significant positive relationships between relative kidney weight at ~ d 55 (y) and the change in maternal weight between d 10 and ~ d 55 pregnancy (x) in both PCUN singleton (Figure 3B) and twin (Figure 3E) fetuses. These relationships were also no longer significant when the effect of maternal weight change during the periconceptional period was controlled for in the analysis (singletons, $r = 0.09$, $P = 0.747$; twins, $r = 0.70$, $P = 0.055$). There were no relationships between relative kidney weight and the total change in maternal weight before ~ d 55 in either PCUN singleton (Figure 3C) or twin (Figure 3F) fetuses. There was a significant positive relationship between plasma cortisol concentrations (y) and maternal weight (x) on the day of post-mortem in PCUN twin, but not singleton pregnancies (Table 2). In contrast to the control pregnancies, there were no relationships between relative kidney weight and maternal plasma cortisol concentrations in the PCUN group (Figure 4B) and the relationship between relative kidney weight and maternal weight change during the periconceptional period in PCUN twins was not altered when the effects of maternal plasma cortisol concentrations at d 55 were controlled for in the analysis.

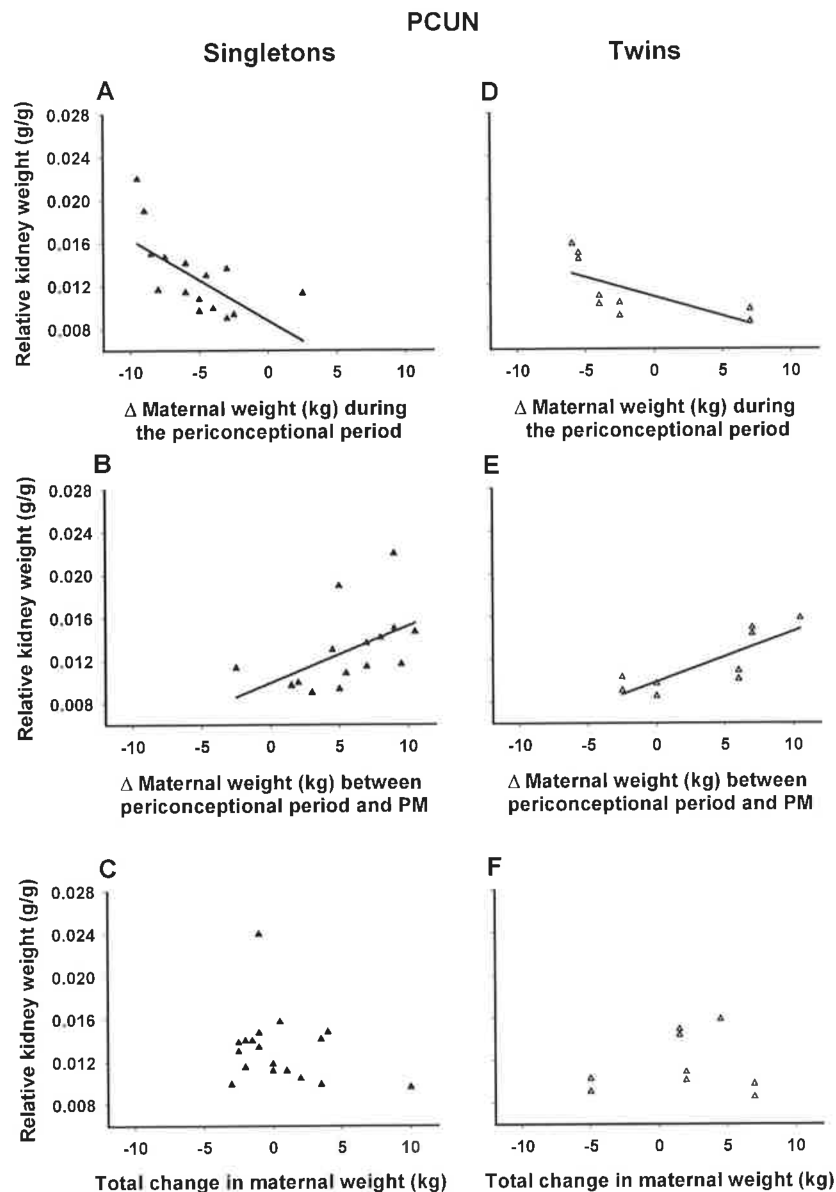

Figure 4.3 Impact of maternal weight change on renal development during early gestation in PCUN treatment groups

There were significant inverse relationships between relative kidney weight at d 53 – 56 pregnancy (y) and the change in maternal weight during the periconceptional period (x) in both PCUN singletons [y = -0.0008x + 0.0088, r = 0.63, n = 16, P < 0.009, closed triangles, A] and twins [y = -0.0004x + 0.0109, r = 0.68, n = 9, P < 0.05, open triangles, D]. There were significant positive relationships between relative kidney weight at d 53 – 56 pregnancy (y) and the change in maternal weight between the periconceptional period and PM (x) in both PCUN singletons [y = 0.0005x + 0.0099, r = 0.52, n = 16, P < 0.04, B] and twins [y = 0.0005x + 0.0099, r = 0.82, n = 9, P < 0.007, E]. There were no relationships between relative kidney weight and the change in maternal weight before PM at d 53 – 56 pregnancy in either PCUN singletons (C) or twins (F).

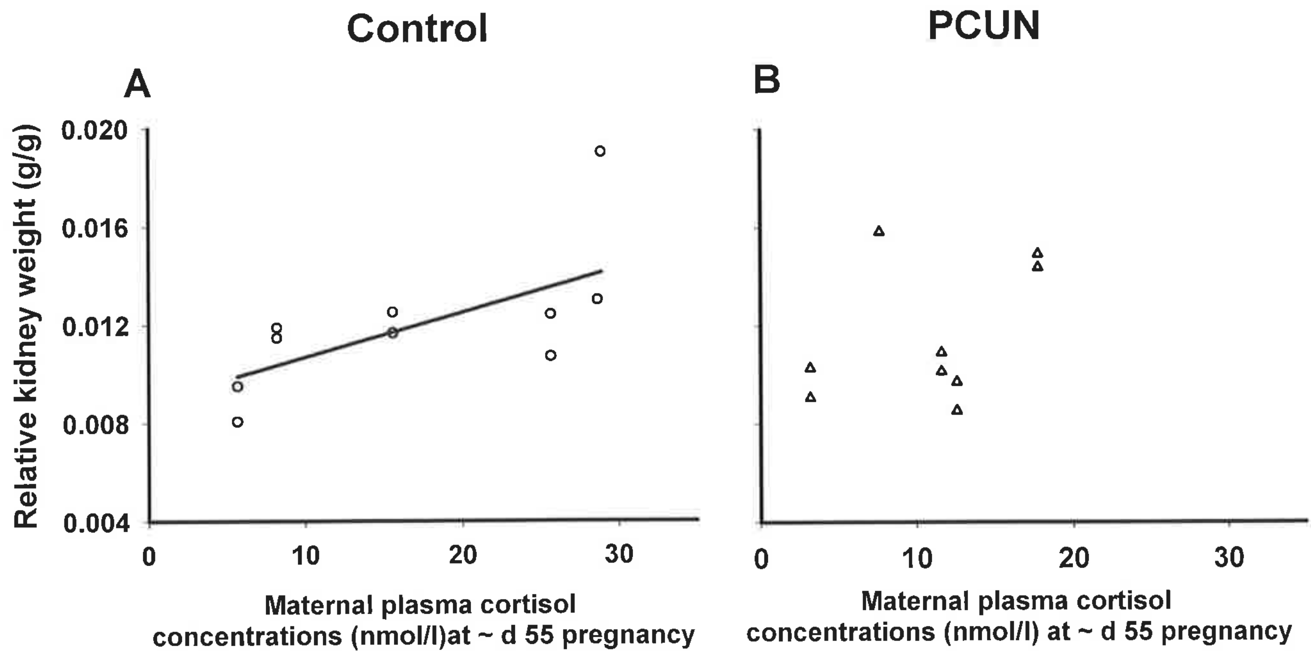

Figure 4.4 Relationship between relative kidney weight and maternal cortisol in early gestation in twin pregnancies

There was a significant relationship between relative kidney weight (y) and maternal plasma cortisol levels at PM (y) [$y = 0.0002x + 0.0089$, $r = 0.65$, $n = 11$, $P < 0.03$] in control twin pregnancies (open circles, A) but not PCUN twin pregnancies (open triangles, B).

4.4.3 PCUN AND KIDNEY IGF-1, IGF-1R, IGF-2, AND IGF-2R MRNA EXPRESSION

The expression of IGF-1 was higher in twin ($P < 0.002$, Figure 5B) compared to singleton (Figure 5A) fetuses independent of the level of maternal nutrition during the periconceptional period and was significantly higher in kidneys from PCUN compared to control fetuses independent of fetal number ($P < 0.008$, Figure 5A and B). In contrast, IGF-1R mRNA expression was lower in twin ($P < 0.0001$, Figure 5D) compared to singleton fetuses (Figure 5C) and expression was significantly lower in kidneys from PCUN compared to control fetuses ($P < 0.04$, Figure 5C and D). There was a significant relationship between relative kidney weight (y) and fetal kidney IGF-1 (x) mRNA expression at d 53 – 56 gestation in control singleton but not control twin or PCUN fetuses (Figure 7A).

Renal IGF-2 mRNA expression was significantly higher ($P < 0.0001$, Figure 6B), however and IGF-2R expression was significantly lower ($P < 0.008$, Figure 6D) in twin compared to singleton (Figure 6A and 6C) fetuses. There was no effect of PCUN on the expression of either kidney IGF-2 or IGF-2R mRNA at ~d 55 gestation in either singleton or twin fetuses.

4.4.4 PCUN, FETAL KIDNEY AND ADRENAL DEVELOPMENT

There was no relationship between relative fetal kidney and adrenal weights at~ d 55 pregnancy in singleton or twin pregnancies in either the control or PCUN nutritional groups. There was, however, a significant relationship between relative kidney weight (x) and fetal adrenal IGF-1 mRNA expression (y) in control (Figure 7C) but not PCUN singletons or twin fetuses. There was also a direct

relationship between fetal kidney IGF-1 mRNA (y) and adrenal IGF-1 mRNA (x) expression in both control (Figure 7B) and PCUN ($y = 0.46x + 0.0151$, $r = 0.66$, $n = 9$, $P < 0.04$) singletons but not twin fetuses.

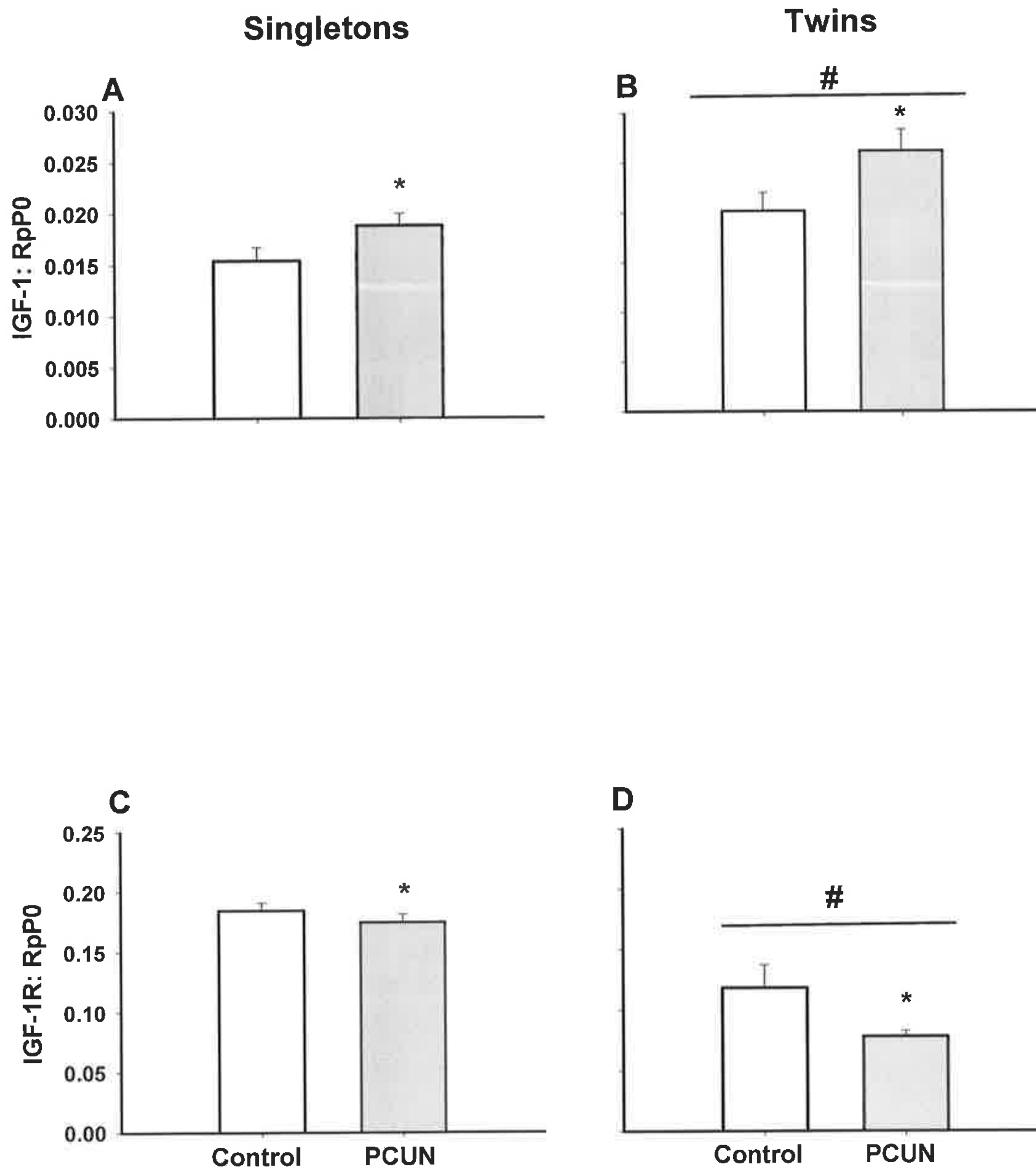

Figure 4.5 Kidney expression of IGF-1 and 1R at ~ d 55 gestation

Kidney IGF-1 expression in either singleton (A) or twin (B) fetuses. Kidney IGF-1R expression in either singleton (C) or twin (D) fetuses.

* denotes a difference ($P < 0.04$) between control and PCUN fetuses.

# denotes a difference ($P < 0.002$) between singleton and twin fetuses.

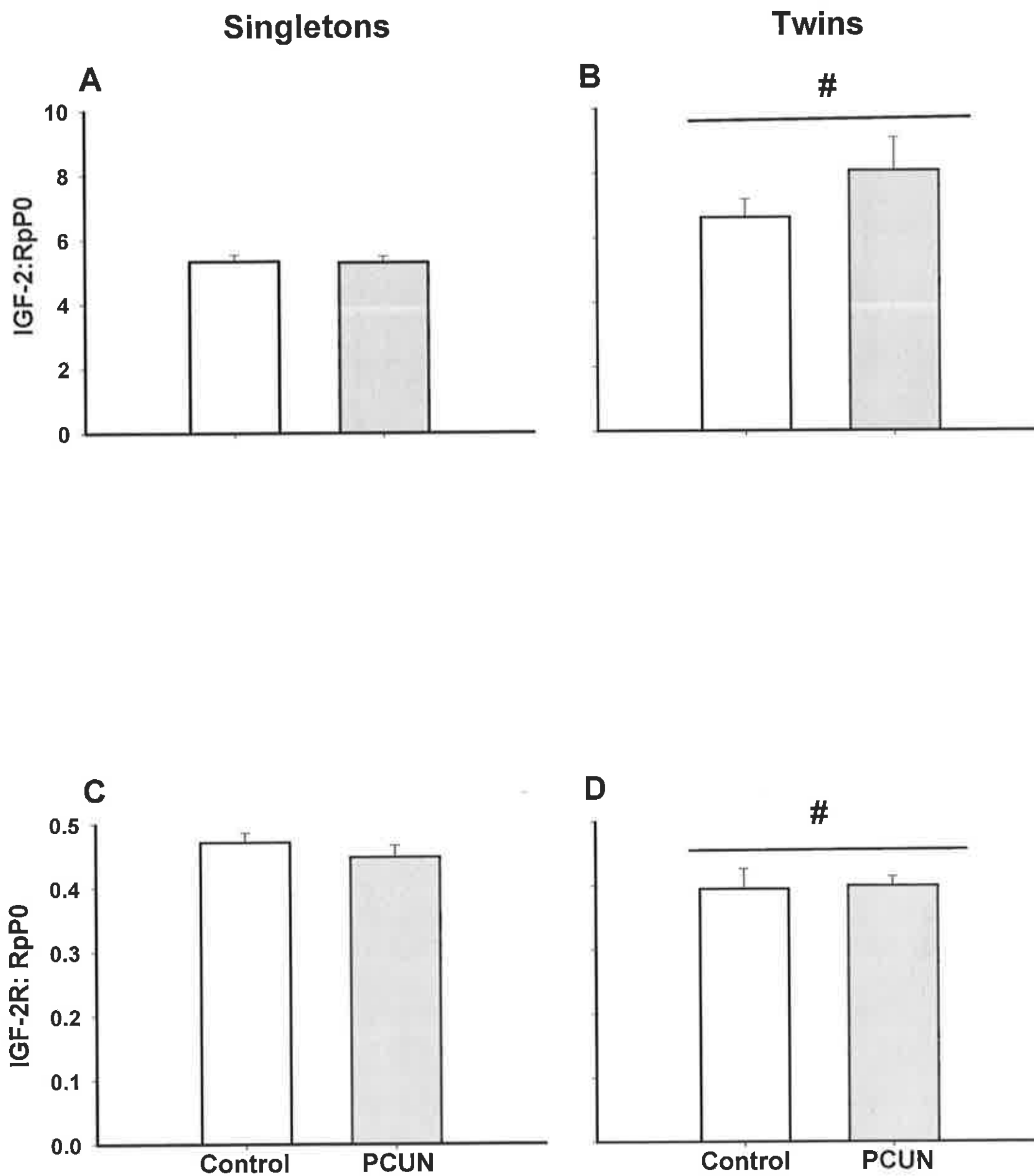

Figure 4.6 Kidney IGF-2 and 2R expression at ~ d 55 gestation

Kidney IGF-2 expression in either singleton (A) or twin (B) fetuses. Kidney IGF-2R expression in either singleton (C) or twin (D) fetuses.

# denotes a difference ($P < 0.008$) between singleton and twin fetuses.

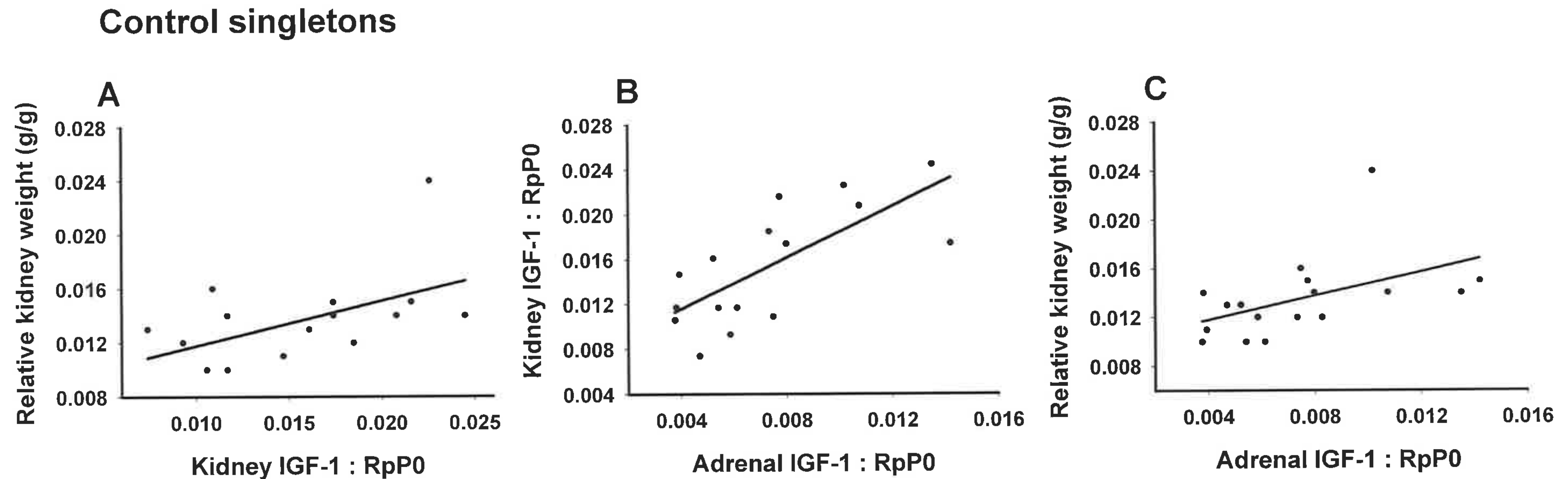

Figure 4.7 Control singletons: relationships between relative kidney weight or IGF-1 expression and adrenal IGF expression

There was a significant relationship (A) between relative kidney weight (y) and fetal kidney IGF-1 mRNA expression (x) at d 53 – 56 gestation [$y = 0.33x + 0.0084$, $r = 0.52$, $n = 16$, $P < 0.05$] in control singleton fetuses (closed circles). There was a significant relationship (B) between fetal kidney IGF-1 (y) and adrenal IGF-1 (x) mRNA expression at d 53 – 56 gestation [$y = 1.15x + 0.0070$, $r = 0.72$, $n = 16$, $P < 0.002$] in control singleton fetuses. There was a significant relationship (C) between relative kidney weight (y) and fetal adrenal IGF-1 mRNA expression (x) at d 53 – 56 gestation [$y = 0.49x + 0.0098$, $r = 0.48$, $n = 17$, $P = 0.05$] in control singleton fetuses.

4.5 DISCUSSION

The objective of this study was to investigate whether the plane of nutrition of the ewe before conception and during early embryo development is a determinant of the growth trajectory of the fetal kidney during early fetal development. I have demonstrated for the first time that there are relationships between change in maternal weight during the periconceptional period and early gestation, maternal cortisol and fetal kidney weight at ~d 55 gestation. Furthermore periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. In addition, I have shown that periconceptional undernutrition and fetal number separately influence the level of expression of IGF-1 and IGF-2 mRNA in the fetal kidney.

4.5.1 PCUN, MATERNAL WEIGHT, MATERNAL WEIGHT CHANGE AND FETAL KIDNEY WEIGHT

A novel finding of the current study was the impact of maternal weight change during the periconceptional period and early pregnancy on the growth trajectory of the fetal kidney. In control singleton fetuses there was no relationship between relative kidney weight at ~ d 55 pregnancy and maternal weight change during the periconceptional period, but there was an inverse relationship between relative kidney weight and total maternal weight gain between the start of the feeding regime and ~ d 55 pregnancy. In contrast to singletons, there was an inverse relationship between fetal relative kidney weight and maternal weight gain during the periconceptional period in control twin fetuses. As in singleton fetuses there was an inverse relationship between fetal kidney weight and maternal

weight gain between the start of the feeding regime and ~ d 55 pregnancy. When maternal weight change during the periconceptional period was controlled for in the analysis, then this relationship was no longer significant, indicating that the relative growth of the fetal kidney during the first 55 days of pregnancy is in large part determined by the effect of maternal weight gain during the periconceptional period.

Periconceptional undernutrition had a marked impact on the influence of maternal weight change during the periconceptional period and early pregnancy on relative kidney weight at ~ d 55 pregnancy, and this effect was similar in singleton and twin fetuses. Following periconceptional nutrient restriction, a greater weight loss during the periconceptional period was associated with an increase in relative kidney weight in the fetus. Interestingly, this relationship was dependent upon the change in maternal weight gain which occurred in the PCUN group between d 10 and ~ d 55. Given that the loss of maternal weight during the periconceptional period and the subsequent maternal weight gain after d 10 of pregnancy were closely interdependent, it is difficult to separate the independent influences of maternal weight loss during the periconceptional period and maternal weight gain after d 10 of gestation on fetal kidney growth.

4.5.2 PCUN, FETAL NUMBER, MATERNAL CORTISOL CONCENTRATIONS AND FETAL KIDNEY WEIGHT

In control twin pregnancies there was an inverse relationship between maternal plasma cortisol levels at ~ d 55 pregnancy and maternal weight at ~ d 55 pregnancy or total weight gain before ~ d 55, however, these relationships were

altered after exposure to periconceptional undernutrition. Following periconceptional undernutrition there was an emergence of a positive relationship between maternal plasma cortisol and maternal weight at ~ d 55 pregnancy.

One possible mechanism through which maternal weight change during the periconceptional period and early pregnancy may influence the growth trajectory of the developing fetal kidney may be through exposure to maternal glucocorticoids during a critical window of development. It has been recently shown that exposure to excess maternal glucocorticoids during early pregnancy can permanently affect the structural development of the fetal kidney (Wintour *et al.*, 2003b). We found that there was a positive relationship between relative kidney weight and maternal plasma cortisol at ~ d 55 gestation in control twin, but not singleton pregnancies such that higher maternal plasma cortisol levels were associated with larger fetal kidneys. In control twin pregnancies it seems that the effect of maternal weight gain during the periconceptional period and early pregnancy on the growth trajectory of the fetal kidney in early pregnancy may act through maternal cortisol, because when the effects of cortisol are controlled for in the analysis, then these relationships are no longer significant.

The renin-angiotensin system (RAS) system is functional in the developing kidney between d 40 and 50 pregnancy in the sheep, and it has been hypothesized that an accelerated development of the intrarenal RAS system following administration of glucocorticoids at ~ d 27 pregnancy may be responsible for premature completion of nephrogenesis (Moritz & Wintour, 1999; Wintour *et al.*, 2003b). Administration of glucorticoids at ~d 27 pregnancy

resulted in an up regulation of the Angiotensin Type 1 (AT1) and AT2 receptors in the kidney of twin pregnancies at d 130 pregnancy (Moritz *et al.*, 2002b). Thus, it may be possible that in control twin pregnancies maternal weight loss during early pregnancy is associated with an increase in maternal plasma cortisol that alters kidney development through a programming of the intrarenal RAS system in twin fetuses specifically. In contrast to the control twin pregnancies the relationship between relative kidney weight and maternal weight change in PCUN twin pregnancies was not dependent on maternal cortisol. It is possible that the maternal weight gain experienced after the periconceptional undernturirion period results in an enhanced nutritional signal to the twin fetus which overrides the influence of maternal cortisol on kidney development. Interestingly, it has been recently shown that ewes of a low body condition score – a measure of maternal undernutrition – around the time of conception produced lambs that had lower nephron numbers compared to those of control lambs at six months of age (Gopalakrishnan *et al.*, 2005).

4.5.3 PCUN, FETAL KIDNEY WEIGHT AND RENAL IGF MRNA EXPRESSION

It has previously been shown that the relative kidney weights in twin sheep fetuses tended to be increased ($P < 0.07$) compared to singletons at d 133 pregnancy (Gardner *et al.*, 2004a), however, it appears that any accelerated growth of the fetal kidney in the twin fetus is not evident as early as ~ d 55 pregnancy. Interestingly, in the present study IGF-1 and 2 mRNA expression were each higher in kidneys from twin compared to singleton fetuses at ~ d 55 gestation and this may in part explain the potential increase in kidney growth in twins reported in later pregnancy. There is evidence that an increased fetal

exposure to cortisol increases IGF-1 expression in the kidney and liver during late gestation (Li *et al.*, 1998a; Li *et al.*, 1998b). One possibility is that transplacental cortisol exposure is enhanced in twin pregnancies which may in turn act to increase kidney IGF-1 mRNA expression in early pregnancy.

There is a positive relationship between relative kidney weight and renal IGF-1 mRNA expression in control singleton fetuses, but not in control twin or PCUN fetuses. This relationship may reflect a nutritional related stimulation of renal IGF-1 mRNA expression in the singleton pregnancy which is altered by factors present in the twin pregnancy. Again, one possibility may be that increased fetal exposure to maternal cortisol in twins may increase the level of renal IGF-1 mRNA expression and alter the direct relationship between relative kidney weight and the prevailing level of IGF-1mRNA expression.

Kidney IGF-1, but not IGF-2 mRNA was increased at d55 pregnancy after periconceptional undernutrition. It has also been previously shown that maternal nutrient restriction during early to mid-gestation decreased placental 11-βHSD-2 mRNA expression and function by 50% (Whorwood *et al.*, 2001; McMullen *et al.*, 2004) and decreased renal 11-βHSD-2 mRNA expression and increased AT1 receptor mRNA expression in the kidneys of lambs exposed to nutrient restriction in utero during early to mid-pregnancy (Whorwood *et al.*, 2001). We would therefore speculate that the increase in IGF-1 mRNA expression in both PCUN singleton and twin fetuses may be a consequence of an increase in transplacental exposure to maternal cortisol due to a decrease in placental and renal 11-βHSD-2 activity, which could in turn act to stimulate IGF-1 expression.

In the present study we have also reported that there was a concomitant decrease in IGF-1R and 2R mRNA expression in twin fetuses, and also a decrease in IGF-1R mRNA expression in PCUN fetuses. The down regulation in the expression of the renal IGF receptors may be a result of the increase in intrarenal IGF-1 and 2 expression.

4.5.4 PCUN, FETAL KIDNEY AND ADRENAL GROWTH

In the present study the relationship between fetal relative kidney weight and relative adrenal weight was investigated to determine whether the development of the fetal adrenal and kidney were interdependent. There was no relationship between fetal kidney and adrenal weights in either the control or PCUN groups in singletons or twins, however, there was a direct relationship between kidney and adrenal IGF-1 mRNA expression in control and PCUN singleton, but not twin fetuses. This indicates, at least in singleton fetuses, that kidney and adrenal IGF-1 mRNA expression may be up regulated by a common signal. There was also a direct relationship between relative kidney weight and adrenal IGF-1 mRNA expression in control but not PCUN singletons or twin fetuses, indicating that in control singletons that there is factor that influences the expression of IGF-1 in both the adrenal and kidney and stimulates growth in the control singleton.

4.5.5 SUMMARY

In summary, fetal kidney growth in early pregnancy in control singletons is directly related to intrarenal IGF-1 mRNA expression. It also appears that expression of

IGF-1 mRNA in the fetal kidney and adrenal may be stimulated by a common factor in control pregnancies. Renal IGF-1 mRNA expression is higher in twins than singletons and in contrast to singletons, there is no direct relationship between kidney weight and IGF1 mRNA expression in control twin pregnancies. We hypothesise that the increase in kidney IGF-1 mRNA expression may be a consequence of an increase in transplacental maternal cortisol stimulation, possibly a down regulation of the renal 11-βHSD-2 expression.

In PCUN fetuses, renal IGF-1 mRNA expression was higher than in control fetuses and, we postulate that this may be due to stimulation by cortisol as a result of a decrease in renal 11-βHSD-2 activity, which may be amplified by the "refeeding" phenomenon in PCUN pregnancies. This increase in maternal nutrient intake after a period of restriction may stimulate IGF-1 mRNA expression. Whilst kidney and adrenal IGF-1 expression are related in PCUN singletons, there is no relationship between kidney weight and f IGF-1 mRNA expression in PCUN singletons.

In conclusion, the present study has demonstrated that there are relationships between change in maternal weight during the periconceptional period and early gestation, maternal cortisol and the growth of the developing fetal kidney at ~d 55 gestation and that periconceptional undernutrition has a differential effect on these relationships in singleton and twin pregnancies. In addition, we have shown that periconceptional undernutrition and fetal number separately influence the level of expression of IGF-1 and IGF-2 mRNA in the fetal kidney. These findings highlight the importance of the periconceptional environment in setting

the growth trajectories of the fetal kidney for the programmed development of the renal and cardiovascular systems of the fetus and adult.

Chapter 5:

Impact of embryo transfer and *in vitro* culture in the presence or absence of serum on fetal and placental development during late gestation in the fetal sheep: Large Offspring Syndrome revisited

"Cry havoc and let slip the dogs of war"

- **Anthony, Julius Caesar**
- **William Shakespeare**

5. Impact of embryo transfer and *in vitro* culture in the presence or absence of serum on fetal and placental development during late gestation in the fetal sheep: Large Offspring Syndrome revisited

5.1 SUMMARY

Superovulation, artificial insemination, embryo transfer and *in vitro* embryo culture are used in a range of assisted reproductive technologies, and it has been demonstrated that varying the composition of the culture media can result in a change in pre and postnatal development. Culture of sheep embryos in media containing serum is associated with fetal overgrowth which is phenotypic of the Large Offspring Syndrome. It is not known how the combination of superovulation, artificial insemination and embryo transfer alone impacts feto-placental development in late gestation of the sheep. There have been no studies, however, examining the differential impact of superovulation, artificial insemination and embryo transfer alone or combined with *in vitro* embryo culture on singleton and twin pregnancies. I have therefore tested the hypothesis that superovulation, artificial insemination and embryo transfer with or without *in vitro* embryo culture in the presence or absence of human serum differentially alters the growth of the placenta, fetus and fetal organs during late gestation when compared to naturally conceived controls and that these effects are different in singleton and twin pregnancies. Embryos were collected 24h after artificial insemination of superovulated donor ewes, which were subsequently transferred to one of 3 treatment groups: - to intermediate ewes until day 7 [Embryo transfer, ET group (singletons = 6, Twins = 18)]; -to an *in vitro* culture of synthetic

oviductal fluid either without [ET+IVC+No Serum(NS) (singletons = 7, Twins = 16)] or with human serum [ET+IVC+Human Serum (HS) (singleton = 8, Twins = 4)] until day 6. Embryos were then transferred to final recipient ewes. In addition, naturally mated (NM) ewes were used as controls in this experiment [singletons = 9, twins = 16]. At 144/145d gestation, ewes were killed, and fetoplacental parameters were measured. The fetal weight, CRL and abdominal circumference were significantly larger in IVCHS singleton fetuses ($P < 0.03$). A novel finding in this study was lower fetal weights of twin fetuses in the ET and IVCNS groups compared to NM control twin fetuses ($P = 0.001$). In addition, placental weights were lighter in twin fetuses in the ET, IVCNS and IVCHS treatment groups ($P = 0.005$) and this is partially due to a failure to initiate compensatory growth of placentomes in twin pregnancies. In ET, IVCNS and IVCHS placentae there was a shift in the proportion of the population of ovine placentomes resulting in more everted types (types C and D) in both singleton and twin pregnancies compared to NM controls. The relative heart weights in all treatment groups were significantly higher in singleton but not twin fetuses ($P < 0.002$). In singleton fetuses, the relative weight of the brain and the pituitary was lower in the IVCHS group compared to the NM, ET and IVCNS groups ($P < 0.05$). I have demonstrated that the use of superovulation, artificial insemination and embryo transfer with or without *in vitro* embryo culture in the absence or presence of serum results in altered fetal growth that is explained by changes in the growth of the placenta in both singleton and twin pregnancies. These findings highlight the sensitivity of the embryo during the periconceptional period to an interaction between exposure to an *ex vivo* environment and fetal number in setting the growth trajectories of feto-placental development.

5.2 INTRODUCTION

With the advent of artificial reproductive technologies (ARTs) over the past four decades the ability to increase the reproductive efficiencies of subfertile individuals has been increased. *In vitro* production of embryos may include *in vitro* maturation of oocytes, *in vitro* fertilization, and *in vitro* culture of embryos (IVC) from the zygote to the blastocyst stage. *In vitro* culture is an essential and nascent step in most ARTs such as cloning, intracytoplasmic sperm injection and zygote intrafollopian transfer (McEvoy *et al.*, 2001). It has been demonstrated that *in vitro* culture in the human, mouse and domestic ruminant species during zygote and early embryo development is associated with altered fetal and postnatal development (Bowman & McLaren, 1970; Walker *et al.*, 1996a; Hansen *et al.*, 2005). The future health effects of exposing the preimplantation embryo to the *ex vivo* environment of *in vitro* culture have not been fully elucidated.

Between 1996 and 2002 there was a 120% increase in the number of live babies born in the US which were conceived by the use of ARTs (Stroup *et al.*, 2002). *In vitro* fertilization and intracytoplasmic sperm injection both utilize the process of IVC, and these ARTs have now been shown to be associated with increased pregnancy loss, low birth weight, babies being born small or very small for gestational age, preterm birth, intrauterine growth restriction and perinatal mortality (Doyle *et al.*, 1992; Tan *et al.*, 1992; McFaul *et al.*, 1993; Wang *et al.*, 1994; van Wagtendonk-de Leeuw *et al.*, 2000; Koivurova *et al.*, 2002a; Koivurova *et al.*, 2002b). Being small for gestational age after conception via ARTs has

been attributed to the higher rate of multi-fetal pregnancies (1990), however, a recent study has shown that singletons conceived by ARTs are also at an increased risk of low birth weight at term compared to spontaneously conceived singleton babies (Schieve *et al.*, 2002).

It has been observed in the mouse that culture of embryos from the pre-morula to the blastocyst stage of development results in a decrease in implantation rates and fetal weight on d 17 of gestation after transfer (Biggers *et al.*, 1965; Bowman & McLaren, 1970). Further investigation into the effects of culture conditions on fetal development determined that protein free culture conditions increased embryo and fetal viability, and culture conditions containing sera were associated with a decrease in fetal weight (Khosla *et al.*, 2001a) and an increase in fetal death (Khosla *et al.*, 2001a) compared to *in vivo* derived control fetuses.

In vitro culture of ruminant embryos in media containing undefined sources of protein and growth factors such as serum or co-culture with support cells have resulted in offspring having a number of abnormalities including an increase in birth weight (Walker *et al.*, 1992b). This phenotype has been described as the "Large Offspring Syndrome" (LOS) (Young *et al.*, 1998) and is characterized by an increase in fetal size during early and late gestation, which varies between different treatments, but can be up to five fold greater than mean control birth weights. Other characteristics include decreased pregnancy rates, altered cell allocation during blastulation, decreased embryonic and fetal survival, organomegaly, altered fetal and neonatal blood chemistry and plasma nutrient concentrations, an increased incidence of physical deformities, increased rates of

abortion and dystoica, increased gestation length, and a high neonatal mortality (Willadsen *et al.*, 1991; Walker *et al.*, 1992b; Hasler *et al.*, 1995; Thompson *et al.*, 1995; Holm *et al.*, 1996; Walker *et al.*, 1996a; Sinclair *et al.*, 1997; Walker *et al.*, 1998; Young *et al.*, 1998; Sinclair *et al.*, 1999; Sangild *et al.*, 2000; Bertolini & Anderson, 2002; Bertolini *et al.*, 2002; Miles *et al.*, 2004). The increase in gestation length does not fully explain the enhanced fetal growth (Walker *et al.*, 1992b).

Sinclair and co-authors have shown that co-culture and culture of ovine embryos with serum leads to fetal overgrowth during late gestation, however, these authors did not find an impact of *in vitro* embryo culture on placental growth (Sinclair *et al.*, 1999). These authors speculated that the increase in conceptus size is "driven by the fetus and not by the placenta" (Young *et al.*, 1998). In contrast, other studies have shown an association between aberrant fetal and placental growth (Bertolini & Anderson, 2002), which may be due to augmentation of blastocyst development during culture. Culture conditions known to enhance fetal growth have also been shown to alter the allocation of cells between the ICM and trophectoderm with an increase in the ratio of trophectoderm: ICM cells (Walker *et al.*, 1996a; Walker *et al.*, 1998; McEvoy *et al.*, 2001). There is evidence in the cow that *in vitro* culture causes an increase in placental size and altered placentome number and placental morphology and morphometry (Bertolini *et al.*, 2002; Bertolini *et al.*, 2004; Miles *et al.*, 2004, 2005).

One possible explanation for the variation between outcomes following *in vitro*

embryo culture is the range of culture conditions used to produce the experimental offspring. Undefined *in vitro* culture systems (co-culture and culture with serum as a protein source) reliably alter embryo, fetal and placental development (Walker *et al.*, 1992a; Walker *et al.*, 1996a; Walker *et al.*, 1998; Young *et al.*, 1998). Both of these culture systems include the use of serum as a protein source, however, it has been shown that human serum is the optimal serum supplement for positively influencing embryo growth and blastulation in the sheep (Walker *et al.*, 1992a; Walker *et al.*, 1992b; Walker *et al.*, 1998). Defined *in vitro* culture systems also alter embryo development and the growth trajectory of the placenta (Eckert *et al.*, 1998; Miles *et al.*, 2005). A significant problem in evaluating the extent to which *in vitro* embryo culture and ARTs cause differences in fetal and placental development compared to naturally conceived offspring is that many experimental protocols use either multiple ovulation and embryo transfer (MOET) or artificially insemination (AI) to generate control animals (Young *et al.*, 1998). It has been shown that both MOET and AI fetuses and pregnancies develop differently than those derived from spontaneously conceived *in vivo*, and therefore they may not be appropriate as the sole controls in such studies (van Wagtendonk-de Leeuw *et al.*, 2000).

The literature describing the effects of *in vitro* culture on twin pregnancies is also sparse. It has been reported in the sheep that there was no difference in fetal or placental measurements in *in vitro* derived twin pregnancies when compared to controls (Walker *et al.*, 1992b; Holm *et al.*, 1996), however, twin fetuses resulting from IVC and microinjection were larger than *in vivo* derived fetuses (Walker *et al.*, 1992b). In the cow, there was no difference in fetal growth parameters when

twins derived by IVC were compared to controls, but there was an effect of IVC on placentome size with a trend towards more everted placentomes (Bertolini *et al.*, 2002).

I have therefore tested the hypothesis that superovulation, artificial insemination and embryo transfer with or without *in vitro* culture in the presence or absence of human serum differentially alters the growth of the placenta, fetus and fetal organs during late gestation when compared to naturally conceived controls and that these effects are different in singleton and twin pregnancies.

5.3 MATERIALS AND METHODS

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee.

5.3.1 EXPERIMENTAL DESIGN

In six replicates conducted from June to December 2002, zygotes were *in vivo* produced and either transferred to an intermediate recipient for *in vivo* culture (ET group) until d 6 or cultured in a defined synthetic oviductal fluid (SOF) medium in the absence *(in vitro* culture no serum, IVCNS) or presence of human serum (*in vitro* culture human serum, IVCHS) until d 6. On d 6 of development embryos were collected from the intermediate recipients of the ET group. Embryos from the ET, IVCNS and IVCHS groups were transferred to synchronized final recipients and allowed to proceed through pregnancy to d

144/145 when post mortem was performed (Figure 1). A group of ewes (n = 17) were synchronized (as per below), naturally mated and allowed to proceed through pregnancy until d 144/145 to serve as control animals for this experiment.

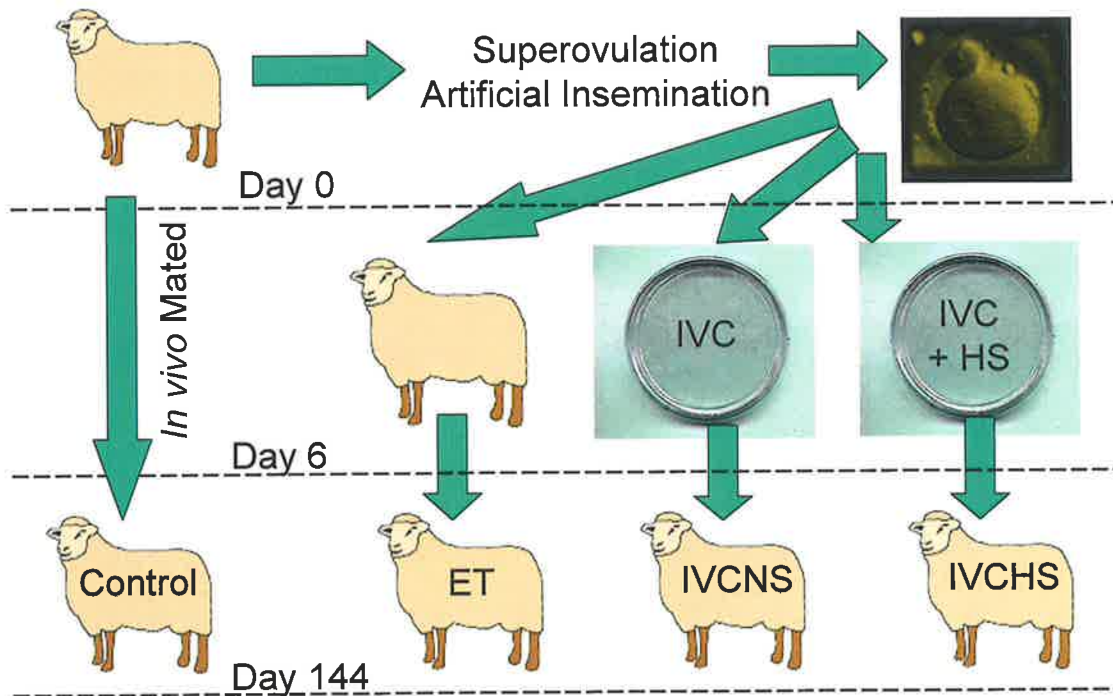

Figure 5.1 Schematic representation of ET and *in vitro* generation of the three experimental culture groups

5.3.2 Animals and Management

One hundred and one South Australian Merino ewes, paddocked under normal husbandry practices, were moved to covered yards four weeks prior to the commencement of the experiments (May 2002) for feeding and environmental adjustment at Turretfield Research Centre. All ewes were 4 – 5 years of age and of uniform frame size, body weight (56.4 ± 0.6 kg) and body condition score (3.0 ± 0.1). Ewes were randomly selected and grouped into either donor (n = 31), intermediate (n = 5) or final recipients (n = 65). Ewes selected as donors and intermediate recipients were housed indoors in single pens and exposed to natural photoperiod. Ewes selected as final recipients were divided into replicate groups and penned in covered yards as a group. Ewes were either fed individually in pens (donor and intermediate recipients) or as a group (final recipients). During this 4 week period, ewes were acclimatized to a pelleted diet containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime and molasses (Johnsons & Sons Pty Ltd, Kapunda, South Australia, Australia). The pellets provided 9.5 MJ/kg DM of metabolizable energy and 120 g/kg of crude protein and contained 90.6% dry matter. All ewes received 100% of nutritional requirements (7.6 MJ/ day for the maintenance of a 64 kg non-pregnant ewe) as defined by the Agricultural and Food Research Council (1993). Donor ewes were supplemented with 350 g of peas/day for 14 days prior to ovulation to increase ovulation rates, which increased the metabolizable energy of the diet to 10.6 MJ/kg DM and 151 g/kg of crude protein.

After embryos were transferred to final recipients, these ewes were housed indoors and individually penned and fed. Ewes were weighed approximately

every four weeks to verify that ewes were maintaining body weight on a maintenance diet. Pregnancy was diagnosed and fetal number estimated by ultrasound at d 45 of pregnancy and confirmed at post mortem. Final recipient ewes were transported from Turretfield Research Centre to the University of Adelaide Medical School at ~ d 100 of gestation. The feed allowance per singleton or twin pregnancy was increased by 15 % every 10 days from d 115 until post mortem.

5.3.3 SYNCHRONIZATION, SUPEROVULATION AND ARTIFICIAL INSEMINATION

5.3.3.1 Donor ewes

Donor ewes were treated following routine synchronization and superovulation procedure (Walker *et al.*, 1986; Holm *et al.*, 1996; Kakar *et al.*, 2005; Kelly *et al.*, 2005b). In brief, ewes were treated with progestagen pessaries (45 mg flugestone acetate; Intervet, Paris, France) for 12 days. Ewes were also administered 9.5 mL of follicle stimulating hormone (FSH; 190 mg NIH-FSH-P1 standard, Follotropin, Bioniche Inc., Belleville, ON, Canada) as two daily injections (i.m.), approximately 12 h apart (2.5, 2.0, 1.5, 1.5, 1.0 and 1.0 mL), commencing 48 h before removal of pessary. In addition, each ewe received 500 IU of equine chorionic gonadotropin (eCG, Pregnecol, Bioniche Inc.) at the time of the first FSH treatment. At 27 h after pessary removal synthetic gonadotrophin releasing hormone (GnRH, Fertagyl; 30 μg; Intervet, Paris, France) was administered i.m. Fresh semen was collected (using an artificial vagina) from two rams of proven fertility and diluted (1:4) with phosphate buffered saline (PBS) + 10% (v:v) heat inactivated sheep serum. At 18 h after GnRH treatment ewes

were inseminated by laparoscopy with approximately 20 X 10^6 spermatozoa. The same two sires were used for all replicates and breeding of naturally mated ewes.

5.3.3.2 Intermediate and final recipient ewes

Recipient ewes were synchronized following routine procedures (Kleemann *et al.*, 2001). Briefly, five intermediate and sixty-five final recipient ewes were treated with progestagen pessaries for 12 days and each ewe was injected with 400 IU eCG (Pregnecol, Bioniche Inc.) at the time of pessary removal. At this time ewes were introduced to vasectomized rams, wearing marking harnesses. Ewes that were marked were determined to have ovulated and used as recipients. Treatment of recipients was scheduled so that their ovulatory cycle were synchronous (± 12 h) with those of donor animals.

5.3.4 EMBRYO COLLECTION: DONOR EWES

The collection of zygotes from donor ewes followed routine procedures (Walker *et al.*, 1986; Kleemann *et al.*, 2001). In brief, approximately 12 – 17 h after the expected median time of ovulation, zygotes were collected. A Tom Cat catheter (Sherwood Medical, St. Louis, MO, USA) was placed into the fimbriated end of the oviduct. Each oviduct was flushed via a puncture in the uterine wall distal to the uterotubular junction with 10 ml of phosphate buffered saline (PBS; Flow Laboratories, North Ryde, NSW, Australia) supplemented with 10% heat inactivated sheep serum. Zygotes were recovered from flushings within 5 min of collection, washed three times in Hepes-buffered synthetic oviduct fluid supplemented with amino acids at defined concentrations reflecting oviductal

fluid concentrations (IVCNS medium) and then held at 38.5 °C in IVCNS medium until allocation to treatment groups.

5.3.5 EMBRYO CULTURE

After zygote collection from donor ewes during each replicate of the experiment, embryos were randomly assigned in equal numbers to either ET, IVCNS, or IVCHS groups.

5.3.5.1 Preparation of amino acid stocks

Preparation of amino acid stocks were prepared as previously described by Walker and co-workers and the concentrations of amino acids used in this study were those determined in sheep oviductal fluid (Walker *et al.*, 1992b). Solutions of individual amino acids, with the exception of arginine, cystine, lysine, and tyrosine, were prepared in synthetic oviductal fluid. Arginine and lysine were mixed in 0.1 N HCL, and cystine and tyrosine were mixed in 0.1 N NaOH. The concentration of cystine in oviductal fluid was not available, but cystine was included in medium at a concentration of 50 μM. Stock solutions were prepared freshly for every replicate. All amino acids were of the L-form (Sigma Chemical Co., St Louis, MO, USA).

5.3.5.2 Preparation of in vitro culture medium

Synthetic oviductal fluid medium was prepared as previously described by Walker and co-workers (Walker *et al.*, 1996b). All chemicals were analytical grade and were obtained from Mallinckrodt Specialty Chemical Co. (Paris, KY, USA) with

the exception of pyruvic acid, lactic acid, and penicillin (Sigma Chemical Co.). Medium was prepared freshly for each replicate of the experiment and equilibrated for 24 h in appropriate atmosphere prior to culture.

IVCNS medium was a mixure of SOF and amino acid stocks. Between 6.7 and 47.8 μl of each amino acid stock was added to 20 mL of SOF to give the required final concentrations. The concentration of each amino acid was validated by using the Waters Amino Acid Analyser (Waters, Millford, MA, USA).

IVCHS medium was a mixture of IVCNS medium and 20% human serum (HS). Human serum was prepared and collected freshly for each replicate. Whole venous blood (10 mL) was collected from a 24-h-fasted 23-year-old male (SMM) subject. The sample was immediately centrifuged (2000 X g for 20 min), plasma was allowed to clot and after clotting the serum was harvested by compression of the clot. Serum was then heat inactivated at 56.0 °C for 30 min, filtered and stored at 4.0 °C. IVCNS (16 mL) was mixed with HS (4 mL) to give a final concentration of HS of 20%. The volume of each stock was proportionately reduced with the addition of serum to the IVCNS medium.

5.3.6 *IN VIVO* CULTURE

Zygotes were transferred to intermediate recipients according to routine procedures (Kleemann *et al.*, 2001). Zygotes selected for the *in vivo* culture group were transferred by mid-ventral laparotomy to a single intermediate

recipient per replicate in groups of 7 to 22 embryos. These embryos were transferred to an oviduct ipsilateral to an ovary with at least one CL. On d 6 of development, embryos were collected by mid-ventral laparotomy. Embryos were recovered from flushings within 5 min of collection, washed three times in IVCNS medium and then held at 38.5 °C for up to 1h in IVCNS medium until transfer to final recipients.

5.3.7 *In vitro* CULTURE: DEFINED NO SERUM (IVCNS)

Zygotes allocated to the IVCNS group were washed three times in IVCNS medium, transferred to wells of a culture dish (Nunc Inc., Naperville, IL, USA) containing 600 μl of IVCNS medium covered with 300 μl equilibrated sterile mineral oil (Sigma Chemical Co.) and cultured for 6 days. *In vitro* culture occurred in an atmosphere of 5% CO_2: 5%O_2: 90% N_2 at 38.5 °C in groups of 12 –15 embryos. Medium was changed every 48 h.

5.3.8 *In vitro* CULTURE: UNDEFINED HUMAN SERUM (IVCHS)

Zygotes allocated to the IVCHS group were washed three times in IVCHS medium, transferred to wells of a culture dish (Nunc Inc.) containing 600 μl of IVCHS medium covered with 300 μl equilibrated sterile mineral oil (Sigma Chemical Co.) and cultured for 6 days. *In vitro* culture occurred in an atmosphere of 5% CO_2: 5%O_2: 90% N_2 at 38.5 °C in groups of 12 –15 embryos. Medium was changed every 48 h.

5.3.9 EMBRYO TRANSFER TO FINAL RECIPIENTS

At d 6 of development, embryos from all three experimental groups were developmentally categorized. Embryos produced *in vivo* and *in vitro* were transferred laparoscopically on d 6 – 6.5 to the tip of a uterine horn ipsilateral to an ovary with at least one CL. Embryos were transferred in pairs (except for five instances where a single embryo was transferred, and this occurred for at least one transfer in each of the three groups) to produce a mixture of singleton and twin pregnancies. The developmental stages of the embryos transferred were equally balanced over the three treatment groups. If too few recipients were available during a given replicate of the experiment an equal proportion of embryos from the ET, IVCNS and IVCHS groups was transferred to final recipients.

All surgeries during the production of *in vivo* and *in vitro* embryos were conducted under general anaesthesia being induced by 10% (w/v) thiopentane sodium (Pentothal; Boehringer Ingelheim, Aratmon, NSW, Australia) and was maintained by a mixture of halothane and oxygen (May and Baker, Dageham, England, UK).

After transfer, ewes were allowed to proceed through pregnancy until post mortem.

5.3.10 COLLECTION OF TISSUES

Final recipient ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) on either d 144 or 145 of pregnancy

(term = 150 ± 3 days' gestation), and the utero-placental unit was delivered by hysterotomy. Fetuses were immediately weighed, growth parameters measured, dissected and individual organs were weighed. The placenta was immediately dissected and the placentomes were individually typed, weighed and counted. In addition, feto-placental fluids were collected and weighed.

5.3.11 STATISTICAL ANALYSIS

Data are presented as the mean ± SEM. The effects of *in vivo* (ET) and *in vitro* (IVCNS and IVCHS) culture during early embryo development on fetal weight, crown rump length, abdominal circumference, ponderal index, placental weight, total placentome number per fetus or pregnancy, mean placentome weight, ovine placental placentome distribution, the ratio of fetal : placental weight, volume of uteroplacental fluid, and the relative weights of the adrenal, kidney, heart, brain, pituitary, liver and spleen were determined using a 2 way Analysis of Variance (ANOVA) using the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). The relationship between fetal and placental weight was assessed by linear regression using Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA). A probability level of 5% ($P<0.05$) was taken to be significant.

5.4 Results

5.4.1 EMBRYO DEVELOPMENT AND SURVIVAL

5.4.1.1 Embryo transfer (ET) group

The recovery rate of embryos from intermediate recipients in the ET group was 68% and of those recovered, the blastulation rate was 51%. A total of 43 embryos (15 morulae and 26 blastocysts) in the ET group (*in vivo* culture) were transferred to 23 final recipients resulting in a pregnancy rate of 78%, embryo survival rate to d 45 of pregnancy of 53%, and embryo survival to ~ d 145 of 53% (Table 1). Fetal outcomes of the ET group were 6 singleton and 12 twin fetuses (Table 2).

5.4.1.2 In vitro culture no serum (IVCNS) group

The blastulation rate of the IVCNS group was 62%. A total of 42 embryos (8 morulae and 34 blastocysts) were transferred to 21 recipients in the IVCNS group resulting in a pregnancy rate of 91%, embryo survival rate to d 45 of pregnancy of 61% and embryo survival to ~ d 145 of 58% (Table 1). Fetal outcomes of the IVCNS group were 7 singleton and 16 twin fetuses (Table 2).

5.4.1.3 In vitro culture with human serum (IVCHS) group

The blastulation rate of the IVCNS group was 77%. A total of 39 embryos (2 morulae and 37 blastocysts) were transferred to 21 recipients in the IVCNS group resulting in a pregnancy rate of 76%, embryo survival rate to d 45 of pregnancy of

51% and embryo survival to ~ d 145 of 38% (Table 1). Fetal outcomes of the IVCNS group were 8 singleton, 4 twin fetuses (Table 2) and 3 triplet fetuses. It is likely that the triplet pregnancy resulted from a monozygotic twin pregnancy arising from one of the two embryos transferred to this ewe. We have thus excluded these fetuses from further analyses.

5.4.1.4 Natural mated control (NM) group

Fetal outcomes of the NM group were 9 singleton and 16 twin fetuses (Table 2).

Table 5.1 Rates of development of *in vivo* and *in vitro* cultured embryos

	Pregnancy Rate (%)	Embryo survival (%) to d ~ 45	Fetal survival (%) to d ~ d 145
ET	78	53	53
IVCNS	91	61	58
IVCHS	76	51	38

Pregnancy rate is defined as at least one of the embryos transferred establishes a pregnancy confirmed by ultrasound at d 45 of gestation.

Embryo survival is defined as the number of embryos per treatment group that established a pregnancy by d 45 of pregnancy.

Fetal survival is defined as the number of embryos per treatment group that established a pregnancy by d 45 of pregnancy and survived to ~ d 145 of pregnancy

Table 5.2 The number of viable fetuses present at ~ d 145 gestation in each of the culture and control groups.

	Singletons	Twins
NM	9	16
ET	6	12
IVCNS	7	16
IVCHS	8	4

5.4.2 FETAL GROWTH

There was a statistical interaction between the effects of fetal number and treatment group on fetal weight (P < 0.004) and crown rump length (P = 0.098). The data were therefore split into singleton and twin fetuses for further analysis.

5.4.2.1 Singletons

Fetal weight and abdominal circumference were each significantly greater in the IVCHS group compared to the NM, ET and IVCNS groups (P < 0.03, Figure 2A and 3C). Crown rump length (CRL) was also significantly higher in the IVCHS group compared to the NM group (P < 0.03, Figure 3A), but there was no difference in CRL between the NM, ET and IVCNS groups. The ponderal index of singleton fetuses was significantly lower in all treatment groups (ET, IVCNS and IVCHS) compared to the NM control group (P = 0.001, Figure 3E).

5.4.2.2 Twins

The weights of individual twin fetuses in the ET and IVCNS groups were significantly lower (P = 0.001, Figure 2B) compared to the weight of individual twin fetuses in the NM and IVCHS groups. The combined weight of twin fetuses in the ET group was also significantly lower than in the NM group (P = 0.05, Figure 2C). The total fetal weight per pregnancy was greater in twin compared to singleton pregnancies in all treatment groups (P <0.006).

The crown rump length of IVCHS twin fetuses was significantly longer compared to ET twin fetuses (P = 0.05, Figure 3B). Whilst the abdominal circumference

was lower in twins compared to singleton fetuses (P=0.008, Figure 3D), there was no effect of embryo treatment on abdominal circumference in twin pregnancies. The ponderal index of twin fetuses in all three treatment groups (ET, IVCNS and IVCHS) was significantly lower ($P < 0.001$) compared to NM control fetuses (Figure 3F).

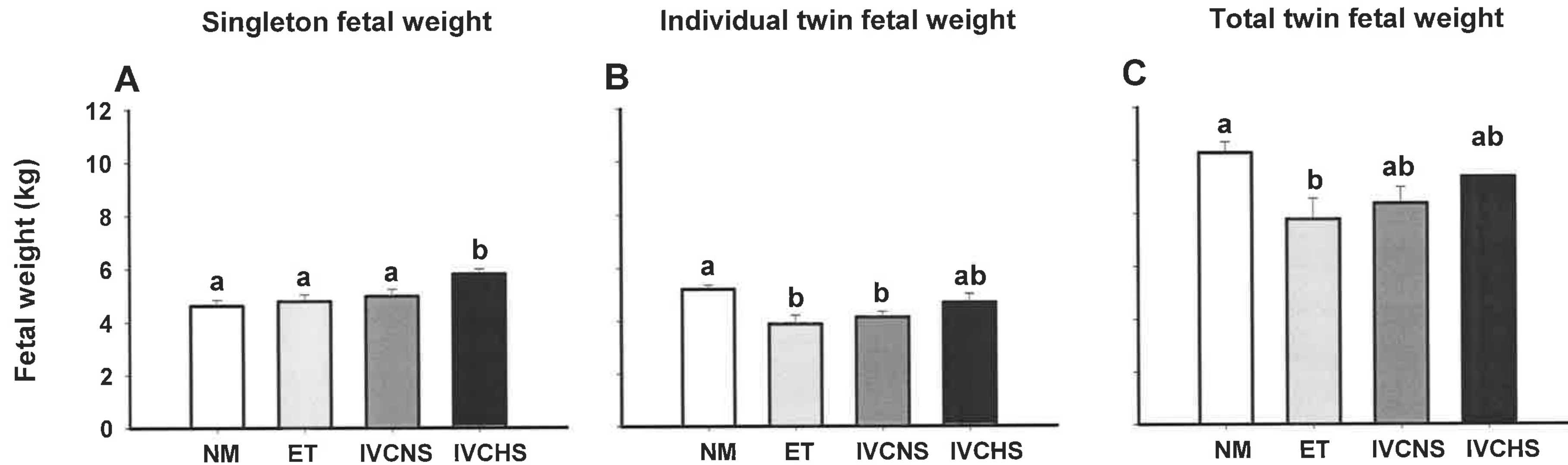

Figure 5.2 Individual fetal weight and total fetal weight per pregnancy in singleton and twin pregnancies.

There was a significant increase in fetal weight in singleton fetuses in the IVCHS (black bars, n = 8) group (A) compared to the NM (open bars, n = 9), ET (light grey bars, n = 6) and IVCNS (dark grey bars, n = 7) groups. There was a significant decrease in individual fetal weight of twin pregnancies in the ET (n = 12) and IVCNS (n = 16) groups compared to the NM controls (n = 16) but no difference when compared to the IVCHS group (n = 4) (B). There was a significant decrease in total fetal weight per pregnancy in the ET (n = 6) group compared to the NM group (n = 8) but not to the IVCNS (n = 8) or IVCHS groups (n = 2) (C). Different superscripts indicate significant differences between mean values within the singleton and twin groups.

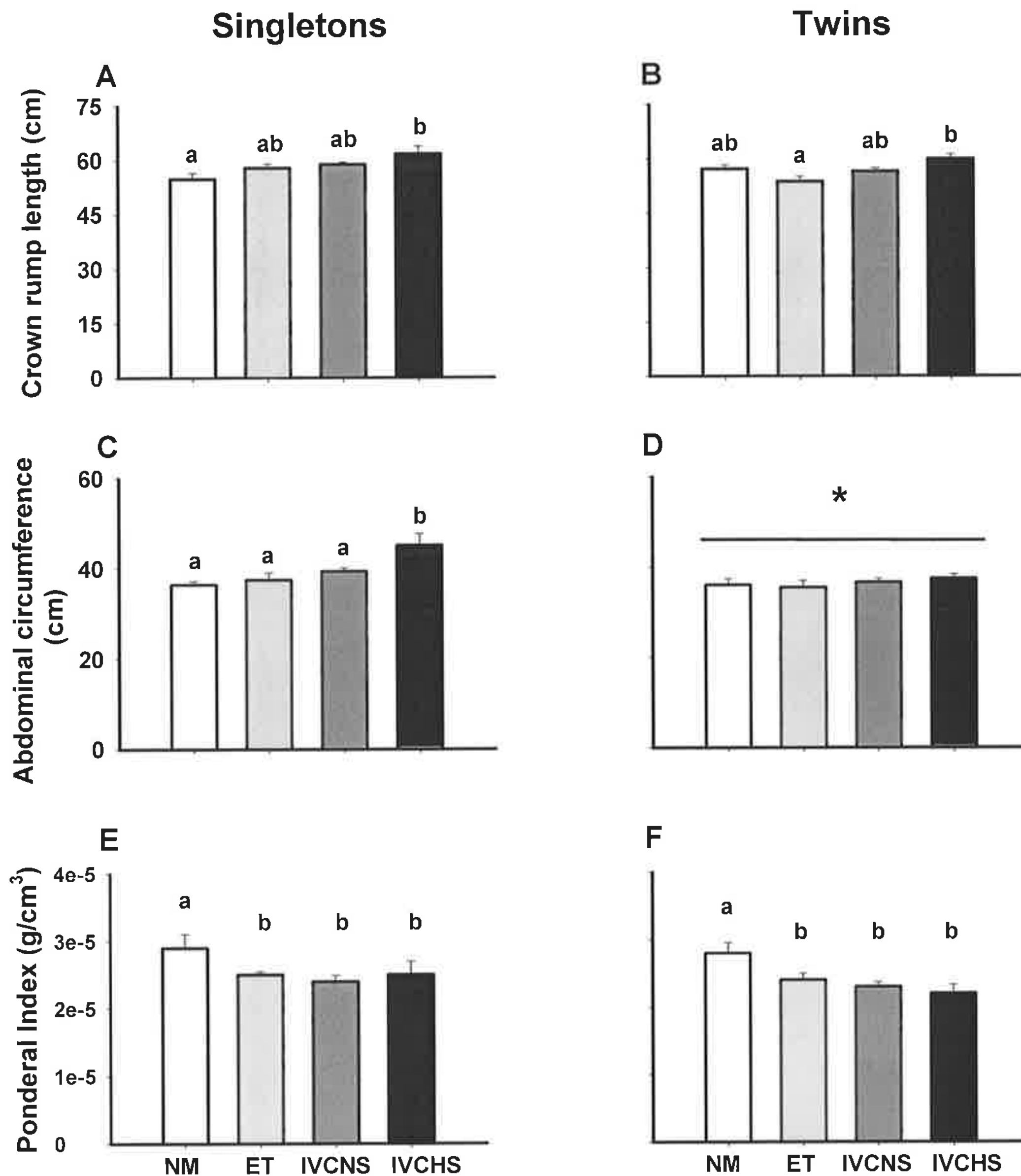

Figure 5.3 Crown rump length, abdominal circumference and ponderal index in singleton and twin pregnancies.

There was a significant increase in crown rump length in IVCHS (n = 8) compared to NM singleton fetuses (n = 9) but not compared to the ET (n = 6) or IVCNS groups (n = 7) (A) and a significant increase in crown rump length of IVCHS (n = 4) compared to ET (n = 12) in twin fetuses (B) but not compared to the NM (n = 16) or IVCNS (n = 16) groups. There was a significant increase in abdominal circumference of IVCHS (n = 8) compared to NM (n = 5), ET (n = 6) and IVCNS (n = 7) singleton fetuses (C). There was no effect of culture on abdominal circumference in twin fetuses, however, the abdominal circumference of twin fetuses were lower than singleton fetuses independent of experimental treatment (D). Ponderal index was significantly reduced in all culture groups compared to NM control fetuses in both singleton (E) and twin (F) pregnancies.

5.4.3 Placental growth

There was a statistical interaction between the effects of fetal number and treatment group on both individual and total placental weight per pregnancy, total placentome number per fetus and pregnancy and mean placentome weight per fetus and pregnancy . The data were therefore split into singleton and twin groups for further analysis.

5.4.3.1 Singletons

There was no effect of either *in vivo* (ET) or *in vitro* culture (IVCNS and IVCHS) on placental weight (Figure 4A), the ratio of fetal : placental weight (Figure 5A), total placentome number (Figure 6A) or mean placentome weight (Figure 6D) at ~ d 145 gestation compared to NM singleton pregnancies. There was, however, an effect of *in vivo* and *in vitro* embryo culture on the distribution of the type of ovine placentomes. When the ET, IVCNS and IVCHS groups were combined, there was a significant shift of the percent of weight of placentomes which was present in the more everted types (B, C and D; $P < 0.0001$, Figure 7D) compared to the distribution of the percent weight of placentomes in the NM singleton group (Figure 7A), which had an equal distribution of the percentage of weight of placentomes in all four types of placentomes (A, B, D and C). The percent number of placentomes that were type A was significantly greater in the placentae of NM ($P < 0.03$) than ET, IVCNS and IVCHS singletons (Table 3).

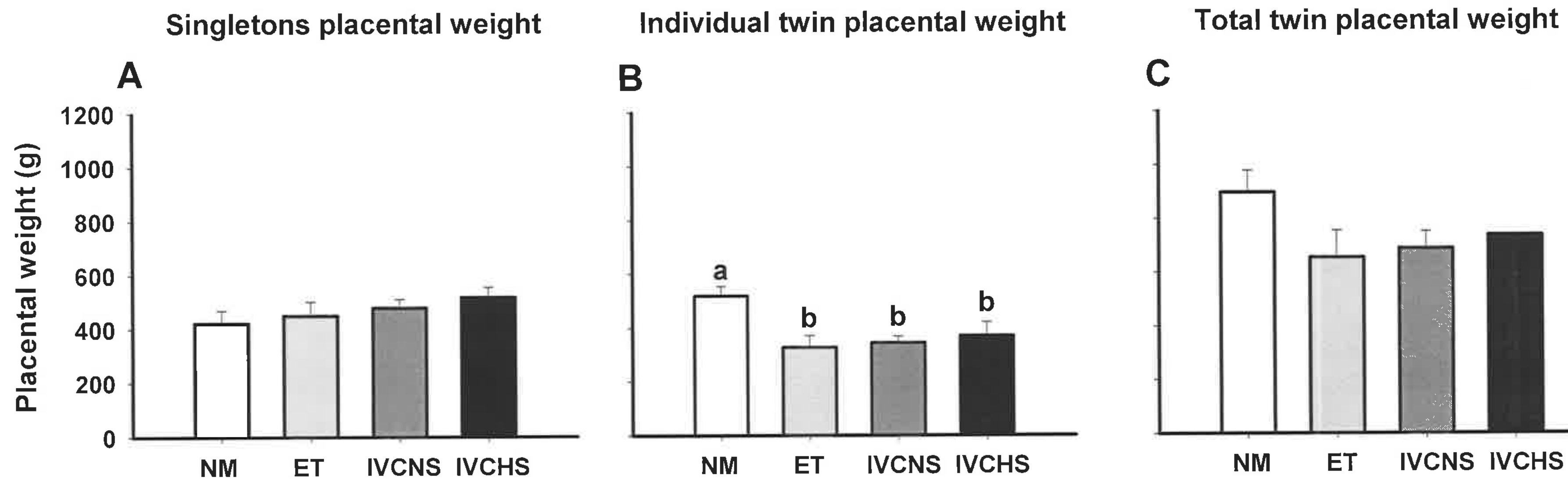

Figure 5. 4 Individual and total placental weights per pregnancy in singletons and twins.

There was no effect of *in vivo* or *in vitro* (ET, IVCNS and IVCHS) culture on placental weight in singleton pregnancies compared to the NM group (A). Individual placental weight of twin pregnancies was significantly lower in the ET (n = 12), IVCNS (n = 16) and IVCHS (n = 4) treatment groups compared to the NM group (n = 8) (B). There was no effect of *in vivo* or *in vitro* culture on total placental weight per pregnancy in pregnancies of twins in the ET (n = 6), IVCNS (n = 8) or IVCHS (n = 2) group compared to the NM group (n = 8) (C).

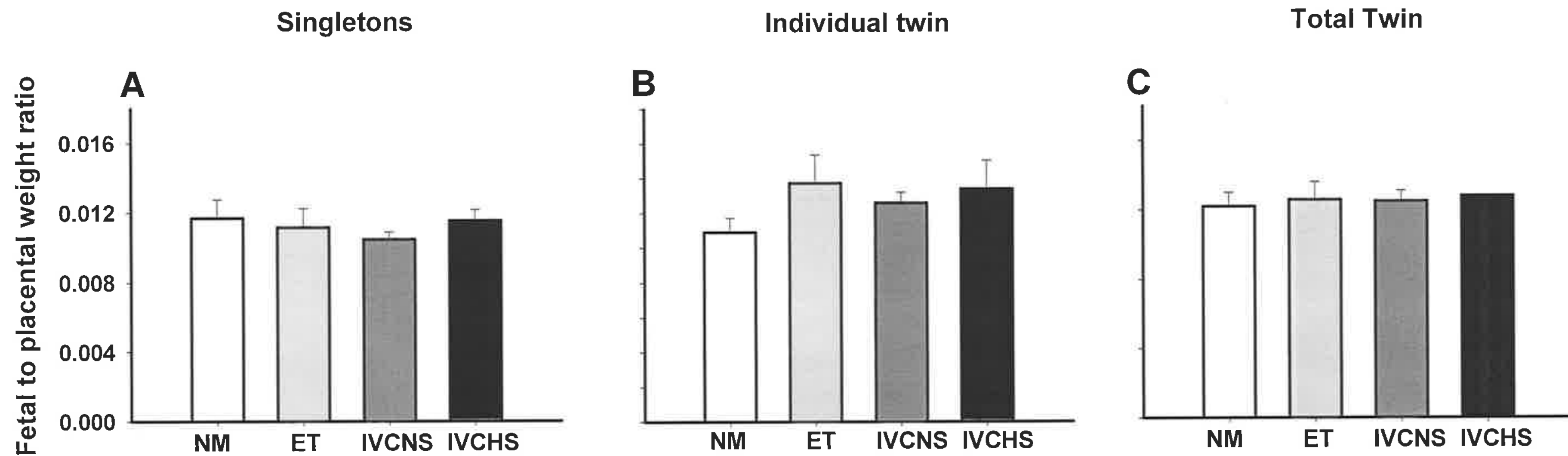

Figure 5.5 The ratio of fetal : placental weight in singleton and twin pregnancies

There was no effect of *in vivo* or *in vitro* culture (ET, IVCNS and IVCHS) on either the ratio of fetal : placental weight in singleton pregnancies (A), the ratio of the individual fetal weight to placental weight (B) or the ratio of the total fetal weight to placental weight ratio (C) in twin pregnancies.

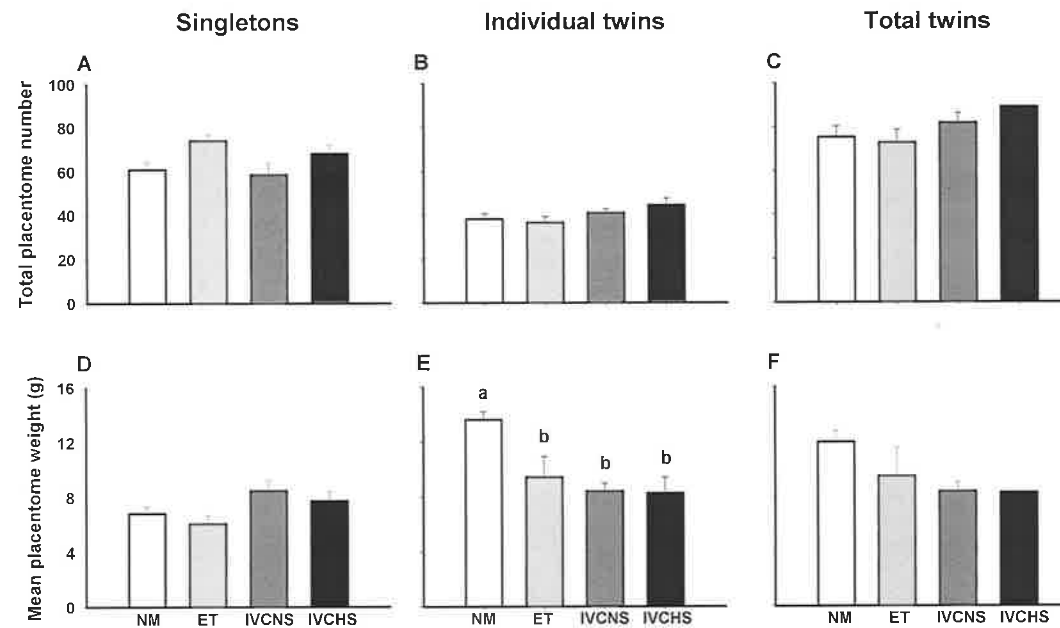

Figure 5.6 Total placentome number and mean placentome weight per pregnancy and fetus

There was no effect of *in vivo* or *in vitro* culture (ET, IVCNS, and IVCHS) on either total number of placentomes per pregnancy in singleton pregnancies (A) or per fetus (B) or per pregnancy (C) in twin pregnancies compared to NM control pregnancies. There was no effect of *in vivo* or *in vitro* culture on the mean placentome weight in singleton pregnancies (D) compared to NM controls. The mean placentome weight per fetus in twin pregnancies was significantly lower in the ET (n = 12), IVCNS (n = 16) and IVCHS (n = 4) groups (E) compared to the NM group (n = 8), however, there was no effect of *in vivo* or *in vitro* culture on mean placentome weight per pregnancy in twin pregnancies compared to NM controls (F).

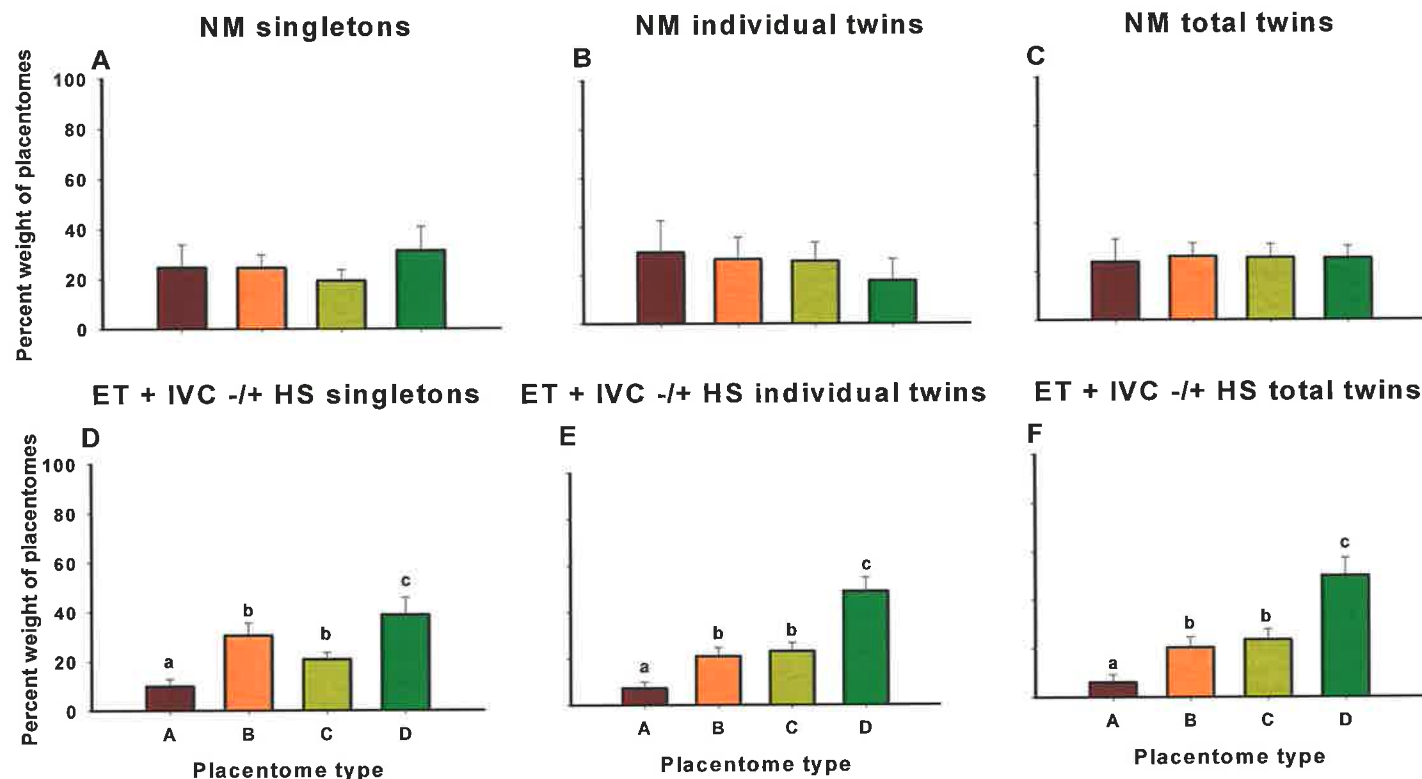

Figure 5.7 Distribution of placentomes in the four ovine placentome types.
There was an equal distribution of the percent of weight of the placenta in the four ovine placentome types in NM singleton pregnancies (n = 9, A), individual placentas of twin pregnancies (n = 8, B) and per pregnancy in twin pregnancies (n = 8, C). There was a significant shift in the percent of weight of the placenta in the four ovine placentome types to more everted types in the three culture groups (ET, IVCNS, and IVCHS) in singleton pregnancies (D), individual placentas in twin pregnancies (E) and per pregnancy in twin pregnancies (F).

Table 5. 3 The percentage of the total number of placentomes represented in the four types of ovine placentomes (A, B, C and D)

	Singletons				Individual twin				Total twin			
	NM	*ET*	*IVCNS*	*IVCHS*	*NM*	*ET*	*IVCNS*	*IVC HS*	*NM*	*ET*	*IVCNS*	*IVCHS*
%Number of Type A placentomes	27.5 ± 9.3*	22.6 ± 9.0	11.0 ± 5.6	14.6 ± 4.5	34.9 ± 12.8*	7.5 ± 3.0	11.5 ± 5.3	4.0 ± 1.7	29.9 ± 9.0	5.2 ± 2.4	11.6 ± 6.9	3.9
%Number of Type B placentomes	26.3 ± 5.9	38.4 ± 4.7	23.4 ± 7.0	35.6 ± 8.8	25.8 ± 7.5	23.9 ± 6.6	20.8 ± 4.6	27.7 ± 14.0	27.0 ± 4.6	22.5 ± 9.2	20.0 ± 4.9	29.5
%Number of Type C placentomes	19.0 ± 4.1	17.5 ± 4.1	31.3 ± 5.7	17.0 ± 3.0	24.6 ± 8.6	23.0 ± 4.9	24.4 ± 5.4	34.0 ± 10.7	23.9 ± 6.1	22.6 ± 6.5	24.6 ± 7.4	35.5
%Number of Type D placentomes	27.1 ± 9.0	21.3 ± 9.4	34.1 ± 8.4	32.9 ± 13.7	14.5 ± 7.3	45.5 ± 10.9	43.4 ± 8.0	34.8 ± 15.5	20.9 ± 5.1	49.8 ± 15.7	43.6 ± 10.7	31.5

* denotes a significant difference ($P < 0.03$) between NM singleton (n = 9) and individual twin (n = 8) and the ET (singletons: n = 6, individual twin: n = 11, total twin: n = 5), IVCNS (singletons: n = 7, individual twin: n = 16, total twin: n = 8) and IVCHS groups (singletons: n = 8, individual twin: n = 4, total twin: n = 2). NM total twin (n = 8)

5.4.3.2 Twins

The weights of the placenta for each individual fetus were significantly lower in the ET, IVCNS and IVCHS groups than in the NM group (P = 0.005, Figure 4B). There was, however, no significant effect of ET or *in vitro* culture (IVCNS and IVCHS) on the total weight of the placenta when compared to the NM group (Figure 4C). The total weight of the placenta in twin pregnancies was significantly greater than those of singletons (P < 0.01). There was no effect of either ET or *in vitro* culture on either the ratio of individual fetal weight or combined fetal weight to the placental weight (Figure 5B and C) or total placentome number per fetus or pregnancy (Figure 6B and C) when compared to the NM group. Total placentome number per pregnancy was significantly greater in twin compared to singleton pregnancies (P < 0.0001).

The mean placentome weight per individual fetus in twin pregnancies was significantly lower in the ET and *in vitro* culture groups (P = 0.007) compared to the NM group (Figure 6E). Whilst there was no effect of ET or *in vitro* culture on the mean placentome weight per twin pregnancy (Figure 6F), the mean placentome weight per pregnancy was significantly greater in twins than in singleton pregnancies (P = 0.004). When the ET, IVCNS and IVCHS groups were combined, there was a significant shift of the percent of weight of placentomes to the more everted types (B, C and D; P < 0.0001) per fetus and per pregnancy (Figure 7E and F) compared to the placentome distribution in either individual or total placentas of the NM group (Figure 7B and C), which were composed of an equal distribution of the percentage of weight of placentomes in all four types of placentomes (A, B, D and C). The percent number of

placentomes that were type A was significantly greater in the individual placentas of the NM group ($P < 0.03$) than ET, IVCNS and IVCHS groups (Table 3).

5.4.4 FETAL AND PLACENTAL ABNORMALITIES

It was noted during the collection of tissues of the ET, IVCNS and IVCHS pregnancies that there were a number of feto-placental malformations. There were craniofacial, organ and skeletal malformations in all culture groups (n = 10). The development of the chorioallantois and placentomes were highly affected by ET and *in vitro* culture during early embryo development. In many instances there was a thickening of the chorioallantoic membranes (n = 8), increased and abnormal chorioallantoic vascular development (n = 9) and bifurcation of the umbilical vessels (n = 4). In twin pregnancies, there were three pregnancies where the chorioallantois of the two placentae were conjoined, and placentomes were observed to be turgid and edematous in 9 pregnancies. ET and *in vitro* culture generated pregnancies (n = 5) where there was a high number of type C and D placentomes in the body of the uterus that were flat and extremely large (the largest was 56 g). The volume of utero-placental fluid was significantly larger ($P < 0.05$) in the IVCHS group compared to the ET group (2841 ± 559 vs. 1921 ± 241 g).

5.4.5 RELATIONSHIP BETWEEN FETAL AND PLACENTAL GROWTH

There were direct and significant relationships between either individual fetal (y) and placental weight (x) (Figure 8A) or total fetal (y) and placental weight (x) per

pregnancy (Figure 8B). These relationships were significant in all treatment groups per fetus and per pregnancy (Table 4).

Table 5.4 The relationships between individual fetal (y) and individual placental (x) weight or total fetal (y) and total placental (x) weight per pregnancy

	Individual placental weight	Total placental weight
Treatment Group		
NM	$y = 0.0035x + 3.37$ ($r = 0.64$, $n = 17$, $P < 0.006$)	$y = 0.0090x + 1.47$ ($r = 0.89$, $n = 16$, $P < 0.001$)
ET	$y = 0.0055x + 2.15$ ($r = 0.78$, $n = 18$, $P < 0.001$)	$y = 0.0087x + 1.49$ ($r = 0.89$, $n = 12$, $P < 0.001$)
IVCNS	$y = 0.0062x + 2.00$ ($r = 0.81$, $n = 23$, $P < 0.001$)	$y = 0.0115x + 0.01$ ($r = 0.92$, $n = 15$, $P < 0.001$)
IVCHS	$y = 0.0053x + 2.94$ ($r = 0.85$, $n = 12$, $P < 0.001$)	$y = 0.0095x + 1.19$ ($r = 0.83$, $n = 10$, $P < 0.003$)

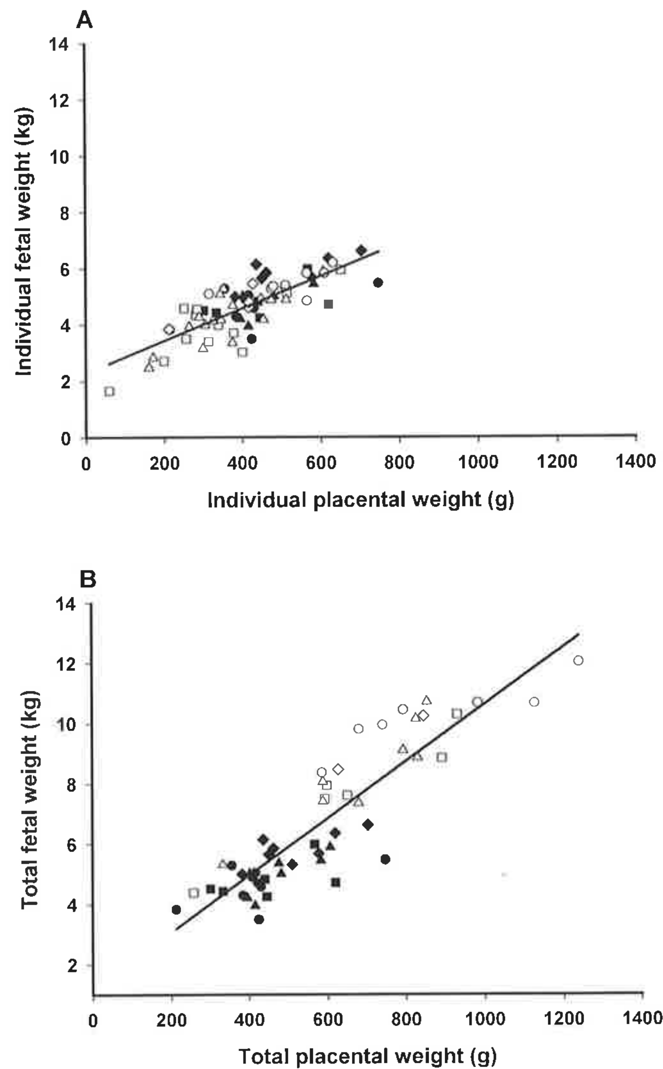

Figure 5.8 Relationship between fetal and placental weights

There was a significant positive relationship between individual fetal (y) and placental (x) weights [y = 0.0057x + 2.29, r = 0.79, n = 70, P = 0.0001, A] in both singleton and twin pregnancies (NM singletons = closed circle, NM twins = open circle, ET singletons = closed squares, ET twins = open squares, IVCNS singletons = closed triangles, IVCNS twins = open triangles, IVCHS singletons = closed diamonds and IVCHS twins = open diamonds). There was a direct relationship between total fetal (x) and placental (y) weights [y = 0.0094x + 1.18, r = 0.89, n = 53, P = 0.0001] in both singleton and twin pregnancies (B).

5.4.6 Fetal organ growth and development

5.4.6.1 Singletons

Relative heart weight was significantly higher ($P < 0.002$) in the ET, IVCNS and IVCHS compared to NM control fetuses (Figure 9A). The relative brain ($P < 0.01$, Figure 10A) and pituitary ($P < 0.05$, Figure 10C) weights were significantly lower in the IVCHS group compared to the NM, ET and IVCNS groups. There was no effect of ET or *in vitro* culture (IVCNS and IVCHS) on relative adrenal, kidney, liver (Figure 11A) or spleen weights (Figure 11C) in singleton fetuses compared to NM controls.

5.4.6.2 Twins

There was no effect of ET or *in vitro* culture (IVCNS and IVCHS) on relative heart weight compared to control NM fetuses (Figure 9B). The relative brain weight of ETfetuses was significantly higher ($P < 0.03$) compared to NM fetuses, but was not different from IVCNS or IVCHS fetuses (Figure 10B). The relative pituitary weight was significantly lower ($P < 0.05$) in IVCHS fetuses compared to NM, ET and IVCNS fetuses (Figure 10D). The relative liver weight was significantly lower ($P < 0.02$) in twin compared to singleton fetuses independent of experimental treatment (Figure 11B). The relative spleen weight in IVCHS fetuses was significantly lower ($P < 0.03$) compared to NM fetuses, but was not different from ET and IVCNS fetuses (Figure 11D). There was no effect of ET or *in vitro* culture (IVCNS and IVCHS) on relative adrenal or kidney weights in twin fetuses compared to NM control fetuses.

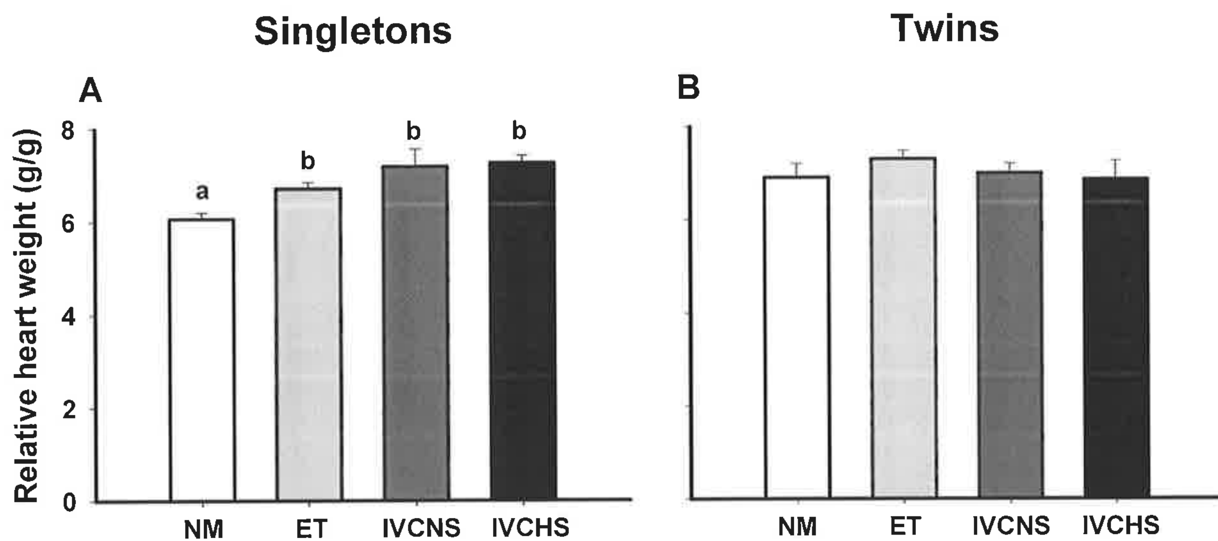

Figure 5.9 Relative heart weights in NM, ET, IVCNS and IVCHS in singleton (A) and twin (B) fetuses.

The relative heart weights of ET (n = 6), IVCNS (n = 6) and IVCHS (n = 8) fetuses were significantly higher than NM control fetuses (n = 7). There was no effect of *in vivo* or *in vitro* culture on relative heart weight in twin fetuses (B).

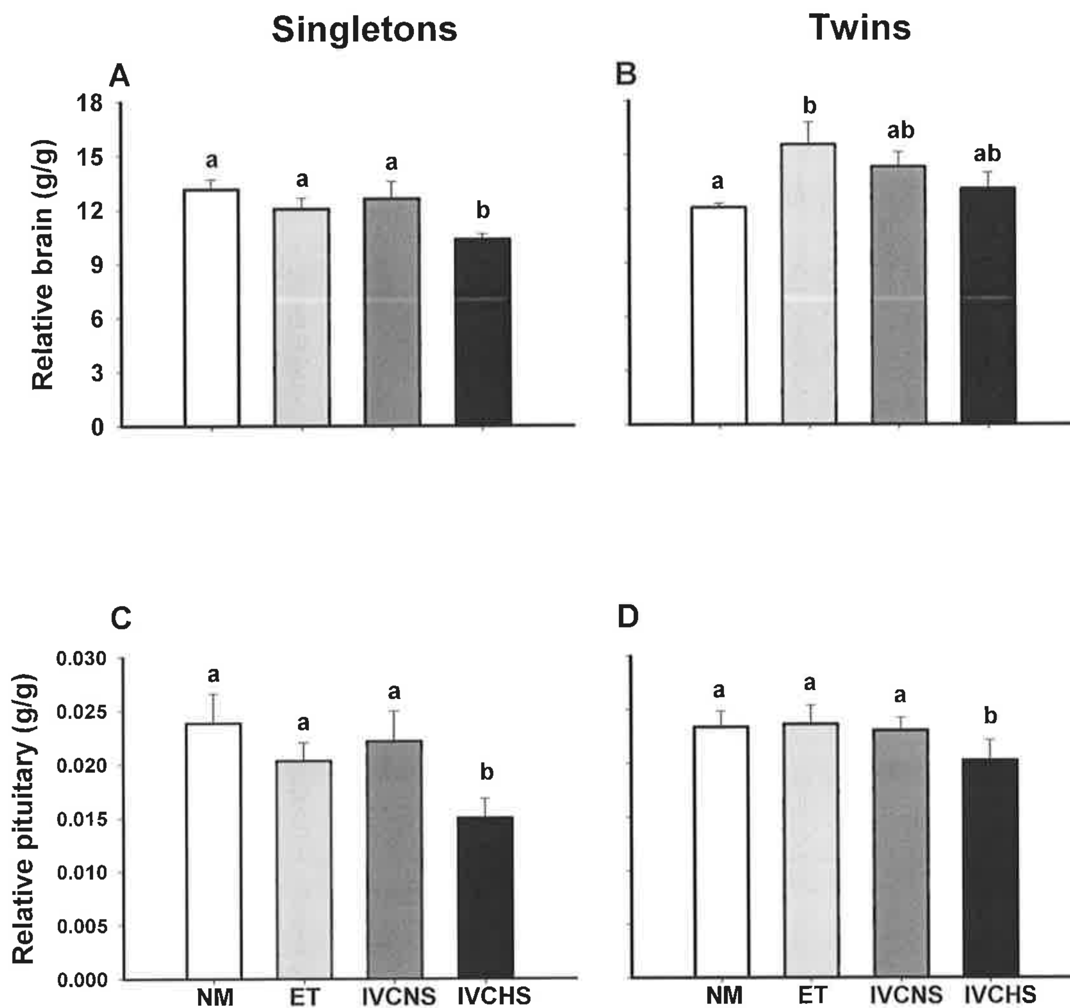

Figure 5.10 Relative brain and pituitary weights in singleton and twin fetuses

Relative brain (A and B) and pituitary (C and D) weights in singleton and twin fetuses. The relative brain (A) and pituitary (C) weights of singleton fetuses were significantly lower in the IVCHS (n = 7 – 8) group compared to NM (n = 7), ET (n = 6) and IVCNS (n = 5 – 7) groups. The relative brain weight in twin fetuses in the ET (n = 11) group was significantly higher compared to NM fetuses (n = 14) but not different to IVCNS (n = 12) or IVCHS (n = 4) fetuses (B). In twin fetuses, the relative pituitary weight was significantly lower in the IVCHS group (n = 4) compared to the NM (n = 13), ET (n = 11) and IVCNS (n = 16) groups (D).

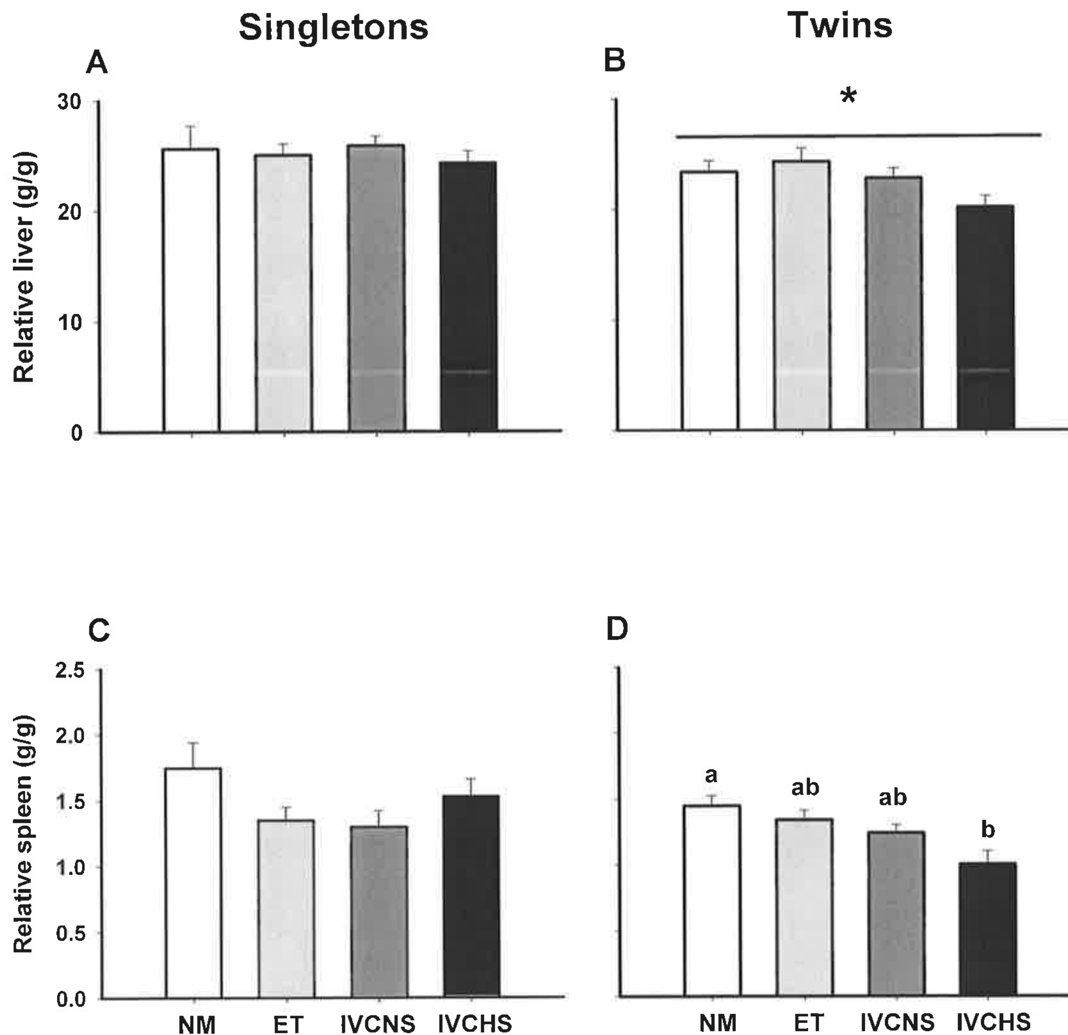

Figure 5.11 Relative liver and spleen weights in singleton and twin fetuses

Relative liver (A and B) and spleen (C and D) weights in singleton and twin fetuses. Relative liver weight was significantly lower in twin (B) compared to singleton (A) fetuses independent of experimental treatment. There was no effect of *in vivo* or *in vitro* culture (ET, IVCNS and IVCHS) on singleton fetuses (C), however, the relative spleen weight in twin fetuses (D) was significantly lower in the IVCHS group (n = 4) compared to the NM group (n = 15) but not different from the ET (n = 11) or IVCNS (n = 16) groups.

5.5 Discussion

The objective of this study was to evaluate the combined effect of superovulation, artificial insemination and embryo transfer with or without *in vitro* culture in the absence or presence of human serum on fetal and placental development during late gestation in the sheep. It was also the purpose of this experiment to determine if ET or *in vitro* culture had a similar effect on fetal and placental growth in singleton and twin pregnancies. Whilst the results presented in this chapter confirm and are consistent with many of the findings of previous studies showing enhanced fetal development in singleton fetuses derived from *in vitro* culture, a novel finding of the present study was that fetal and placental growth are differentially affected in twin pregnancies. In addition the data reported indicate that placental growth determines fetal growth in each of the treatment groups.

5.5.1 IMPACT OF ET AND *IN VITRO* CULTURE ON FETAL GROWTH

Fetal weight, crown rump length and abdominal circumference were all greater in singleton fetuses derived after *in vitro* embryo culture in the presence of human serum. These results confirm numerous studies reporting enhanced fetal growth and size after *in vitro* embryo culture (Thompson *et al.*, 1995; Walker *et al.*, 1996a; Young *et al.*, 1998; Sinclair *et al.*, 1999) and that the use of human serum in culture systems generates fetuses exhibiting characteristics of the Large Offspring Syndrome. These results in conjunction with previous reports indicate that the primary factor resulting in an augmentation of fetal growth after embryo

culture is the presence of serum in the culture media, at least in singleton pregnancies.

The results in twin pregnancies were quite different. Twin fetuses of the ET and IVCNS groups were significantly smaller than fetuses resulting from spontaneously conceived control fetuses, and this differential effect of embryo culture on singleton and twin pregnancies has not been reported in the literature. These results are in contrast with those previously reported which have shown that neither defined or undefined *in vitro* culture had an effect on the weight of twin fetuses (Walker *et al.*, 1992a; Holm *et al.*, 1996). Fetal growth has previously been reported to be increased in twin pregnancies after IVC and microinjection. The differences in the results may be due to the use of 'control animals' generated in previous studies by multiple ovulation and embryo transfer (MOET). It has been demonstrated that MOET also causes alterations in fetal growth (van Wagtendonk-de Leeuw *et al.*, 2000). Thus, the use of MOET to generate a control group may result in different outcomes than when naturally conceived fetuses are used as controls, as in the current study.

Fetal weight was not different in twins in the IVCHS group compared to NM controls, indicating that the presence of human serum during culture in part restored the fetal growth trajectory in this treatment group, possibly counteracting the variables negatively affecting fetal growth in twin fetuses of the ET and IVCNS groups. Interestingly, the CRL of IVCHS fetuses was also significantly longer than in the ET group.

The ponderal index, a measure of soft tissue growth compared to skeletal growth, was significantly lower in all treatment groups in both singleton and twin fetuses. This is a very interesting finding, because one of the characteristic phenotypes of LOS is greater skeletal growth (Young *et al.*, 1998) and lower ponderal index values would indicate that fetuses were thinner for their length than NM fetuses. It may be the case that ET and *in vitro* embryo culture program the fetal growth trajectory and positively influences skeletal growth over the growth of the soft tissues.

5.5.2 Impact of ET and *in vitro* culture on placental growth

There was no effect of ET or *in vitro* culture on placental development in singleton pregnancies and this is consistent with previous findings of LOS in the sheep (Young *et al.*, 1998; Sinclair *et al.*, 1999). Sinclair and co-workers concluded that fetal "overgrowth appears to be driven by the fetus and not the placenta" (Sinclair *et al.*, 1999). I have shown, however, that ET and *in vitro* culture in twin pregnancies results in a lower placental weight. These findings are in contrast to those in the cow, which have shown that *in vitro* culture resulted in an increase in placental weight in late gestation (Bertolini *et al.*, 2002; Bertolini *et al.*, 2004; Miles *et al.*, 2004, 2005).

The decrease in placental weight in twin pregnancies appears to be due to a lack of normal compensatory growth that occurs in these pregnancies. In naturally conceived twin pregnancies there are significantly more placentomes per pregnancy but fewer per fetus compared to singleton pregnancies (Alexander, 1978). There was no effect of ET or *in vitro* culture on total placentome number

per fetus in twin pregnancies. In order to maximize placental transport with fewer placentomes compared to singletons, twin placentae undergo compensatory growth that results in a heavier mean placentome weight compared to singleton placentae (Vatnick *et al.*, 1991), and in Chapter 2 I have shown that this compensatory growth is set in early gestation. Interestingly, in twin pregnancies, the placenta in ET and *in vitro* derived pregnancies do not appear to undergo extensive compensatory growth resulting in significantly lighter mean placentomes when compared to the placentomes in the NM twin placenta.

Increasing the number of everted placentomes (types C and D) per placenta is another compensatory mechanism by which the placenta can adapt and increase its surface area of exchange (Alexander, 1978). It is hypothesized that placentomes of type C and D have an increased proportion of fetal tissue and thus increased surface area of exchange between the feta and maternal circulations (Steyn *et al.*, 2001), however, this hypothesis has recently been challenged by Fowden and co-workers (Fowden *et al.*, 2006). Ward and co-authors have determined that in the number of everted placentomes peaks between d 125 – 135 of pregnancy and that cortisol reduces the number of everted placentomes closer to term (Ward *et al.*, 2006). I have demonstrated that at ~ d 145 gestation in placentae of the NM group there is an equal distribution between the four types (A-D) of ovine placentomes. Interestingly, I have shown that ET and *in vitro* culture causes a shift in the population of everted placentomes in both singleton and twin pregnancies, increasing the number and proportion of placental weight represented by type B, C and D types. These findings are consistent with reports in the cow that *in vitro* embryo culture alters

the gross morphology of placentomes leading to a population that are increased in diameter, indicating an increase in eversion (Bertolini *et al.*, 2002; Farin *et al.*, 2006).

Whilst enhanced fetal growth of IVCHS singleton fetuses was not accompanied by an increase in placental weight, it is interesting to speculate as to whether the placentae of these fetuses are functionally more efficient in delivering nutrients to the fetus and possibly facilitating the mechanism by which fetal overgrowth occurs. *In vitro* embryo culture has been shown in the cow to increase the functional ability to transfer nutrients to the fetus by increasing glucose, which may be due to an increase in surface area of exchange at the materno-placental interface (Bertolini *et al.*, 2004). In contrast to this hypothesis I found no difference between the NM group and the three experimental groups in the ratio of fetal : placental weight signifying that the placental function *of* these pregnancies may not be significantly altered by *ET or in vitro* culture conditions. There was, however, a direct relationship between fetal weight and placental weight such that larger placentae facilitate the growth of a larger fetus, and this occurred in all treatment groups.

Based on the data showing a decrease in fetal weight of twins in the ET and IVCNS groups associated with decreased placental growth and with the strong relationship between fetal and placental weight, I hypothesize that the enhanced fetal growth characteristic of the Large Offspring Syndrome is driven by placental growth.

Placental development, specifically the placental and umbilical vasculature, may be more sensitive to the deleterious effects of culture systems than what has been reported. In this study there were a number of malformations, enhanced vascularization, and bifurcation of umbilical vessels. This may in part explain the incidence of polyhdramnios, hydramnios and hydrallantois in *in vitro* culture produced pregnancies in the absence of other observable characteristics of LOS.

5.5.3 IMPACT OF ET AND *IN VITRO* CULTURE ON CARDIOVASCULAR AND FETAL ORGAN GROWTH

The present study also confirms reports of sporadic organomegaly (Young *et al.*, 1998; Sinclair *et al.*, 1999) in ET and *in vitro* derived fetuses specifically in the heart, brain, pituitary and spleen, which may be due to an effect of culture on the development of the cardiovascular system. The fetal relative heart weights were increased in singleton but not twin fetuses of the ET, IVCNS and IVCHS groups. The relative brain weight, however, was lighter in singleton IVCHS fetuses than fetuses of the NM, ET and IVCNS groups, but in twin fetuses the relative brain weight was heavier in the IVCNS group compared to control NM fetuses. In both singleton and twin fetuses the relative weight of the pituitary gland was decreased in all treatment groups. The relative weight of the spleen is also reduced in twin fetuses in the IVCHS group.

The organs and tissues that were predominantly affected by ET and *in vitro* culture are all associated with the cardiovascular system. Sinclair and co-workers previously showed that the growth coefficient of the heart was significantly greater in fetuses derived from embryos culture *in vitro* in undefined

media (Sinclair *et al.*, 1999). Whilst there are conflicting reports as to whether the increased fetal growth after *in vitro* embryo culture persists in adult life, it has been shown that the elevated size and growth coefficient of the heart is present in postnatal life (McEvoy *et al.*, 1998). It is not clear why ET and *in vitro* embryo culture only influences heart growth in singleton fetuses, however, in humans, *in vitro* embryo culture results in a significant increase in the number of cardiovascular malformations that include ventricular septal defects and hyperplasia of the left ventricle (Anthony *et al.*, 2002). In this context, it is very interesting that just superovulation, artificial insemination and embryo transfer can result in an increase in the growth of the fetal heart.

The lower relative brain and pituitary weights in the *in vitro* embryo culture groups are also interesting in the context that *in vitro* embryo culture in humans has been associated with increased prevalence of neurological sequelae (Stromberg *et al.*, 2002). A lower relative brain or pituitary weight would indicate the opposite of brain "sparing" ie, brain "starving". Brain sparing associated with intrauterine growth restriction is hypothesized to be caused by preferential blood flow that favours directing cardiac output to the superior circulation, thus allowing brain growth at expense of the peripheral circulation (McMillen & Robinson, 2005). It is tempting to speculate that ET and *in vitro* embryo culture result in an altered development of a cardiovascular system that shunts cardiac blood flow to the peripheral circulation, away from the brain, resulting in lower relative brain and pituitary weights and an increase in growth of the peripheral organ systems and tissues. Abnormal development and vascularization of the chorioallantois and the observation of edematous placentomal tissue indicates high back pressure in

these tissues, which may cause increased cardiac work load and thus result in hypertrophy of the fetal heart in these fetuses. The failure of normal development of the chorioallantois in conjunction with the back pressure in the placentomes of *in vitro* derived fetuses may also be part of the mechanism leading to polyhydramnios. The spleen is relatively lighter in IVCHS twin fetuses compared to fetuses in the NM, ET and IVCNS groups. A reduced cardiac output to the peripheral circulation may also decrease the volume of blood in the spleen which may lead to the observed decrease in relative weight.

5.5.4 SUMMARY

The reason and mechanisms underlying the differential effects *of* ET and *in vitro* culture on singleton and twin pregnancies are not clear. In the present study it has been shown that twin pregnancies have significantly larger placentae, total number of placentomes and smaller abdominal circumference, which are characteristic of normal twin pregnancies. The decreased abdominal circumference is explained by the significantly lower relative liver weights in twin fetuses independent of experimental treatment. It has been previously shown that the development of the twin fetus, specifically the growth of cardiovascular system and pituitary—adrenal axis, is different to that of the singleton (Edwards & McMillen, 2002a, b; Gardner *et al.*, 2004a) and in Chapter 2 it was shown that growth trajectory of the twin is set early in gestation.

The decrease in embryo and fetal growth of human and murine embryos contrasts with the increase in embryonic and fetal growth more commonly reported after *in vitro* culture of the ovine or bovine embryo (Kruip 2000). Low

birth weights in humans after conception via ARTs has been attributed to the higher rate of multi-fetal pregnancies and the mouse as a litter bearing species. Sheep fetuses derived from ET and IVCNS also have a lower fetal weight, similar to that present in the human and mouse when multiple embryos are transferred and result in a twin pregnancy. Thus, the sheep may be a good model for studying the effects of *in vitro* embryo culture and embryo transfer on fetal, placental and neonatal development.

I have demonstrated that only superovulation, artificial insemination and embryo transfer can affect the placental, fetal and cardiovascular development. Previous studies in the cow have shown that use of MOET alone can lead to increased birth weight, gestation length and the incidence of physiological deformities (van Wagtendonk-de Leeuw *et al.*, 2000). Superovulation increases circulating oestrogen and progesterone concentrations resulting in altered oviductal and uterine development that is not temporally synchronized with the developing embryo (Boerjan *et al.*, 2000), which may mimic the effects of transferring an embryo to an asynchronous envinroment. Thus, superovulation and embryo transfer may be the basis of the altered development observed in LOS offspring and the addition of *in vitro* culture may magnify these physiological alterations.

The *ex vivo* environment of *in vitro* embryo culture systems has been defined to reflect the physiological environment of the oviduct and uterus, however, the developmental cues and communication that occurs *in vivo* between the developing embryo and maternal reproductive tract is lacking in culture systems. The absence of proper homeostatic and temporally correct cues may be

responsible for altering the growth trajectory and gene expression of the developing embryos. *In vitro* culture also has an effect on embryo growth rates so that blastulation occurs earlier *in vitro* than *in vivo* and increases blastulation rates (Walker *et al.*, 1992a; Walker *et al.*, 1992b; Watson *et al.*, 1994a; Walker *et al.*, 1996b) reflecting the findings in this study. Increased embryo growth rates and earlier blastulation as a result of *in vitro* culture and subsequent transfer to a chronologically synchronous recipient is one possible perturbing factor that causes an alteration in fetal and placental development. IVC embryos may be chronologically synchronous with the recipient, however, if the embryo is developmentally advanced the communication between conceptus and maternal reproductive system may be impaired resulting in altered growth trajectory. It has been demonstrated in the cow that more developmentally advanced blastocysts have an increased production of interferon-tau (Kubisch *et al.*, 2004), which has been demonstrated to affect the gene expression and uterine milk secretion of uterine glandular tissue (Spencer & Bazer, 2004a, b). The developmental competency of IVC embryos has been shown to be similar to *in vivo* derived embryos up to d 14 of development with an increase in embryo and fetal wastage between the beginning of implantation and ~ d 50 of gestation. We have shown that the highest rate of conceptus loss was in early gestation, and there was little fetal wastage from mid to late gestation. The high rate of conceptus loss during this critical window of development would implicate a failure in maternal recognition of pregnancy, implantation or placentation. It has been shown that cell allocation is altered in *in vitro* derived embryos in preference to the trophectoderm, which develops into the placenta and fetal membranes and is the site of interferon-tau production. Alteration of normal trophectodermal

development may be one mechanism of programming the LOS through enhancing or restricting maternal recognition of pregnancy, influencing uterine gland development, gene expression and secretions and subsequently affecting implantation and placentation. I have demonstrated that placental growth is driving fetal growth in the ET and *in vitro* treatment groups and this may be programmed by alterations in trophectodermal development.

The underlying cellular and physiological mechanisms that program the embryo for altered fetal and placental development are not fully elucidated. It has been shown in the mouse that *in vitro* culture has the ability to alter gene expression (Khosla *et al.*, 2001a; Khosla *et al.*, 2001b). In particular, it has been postulated that culture conditions could result in the epigenetic deregulation through gene silencing or induction of developmentally important genes, which play a key role in regulating fetal and placental growth (Young & Fairburn, 2000; Khosla *et al.*, 2001b; Wrenzycki *et al.*, 2004). The critical window for possible programming by epigenetic changes has been hypothesized to be the 8 to 16 cell stage, when the embryo is moving from the oviduct to the uterus and when the activation of the embryonic genome occurs (Young *et al.*, 1998; Young & Fairburn, 2000). Insulin-like growth factor 2 and its receptor are two such genes of interest (Walker *et al.*, 1996a). Young and colleagues have reported there was a significant decrease in the expression of IGF-2R, resulting in an in crease in the bioavailability of IGF-2 and over growth of hearts in LOS fetuses (Young *et al.*, 2001).

In summary the present study has demonstrated that superovulation, artificial insemination with or without *in vitro* embryo culture in the absence or presence of

human serum differentially alters fetal and placental development in singleton and twin pregnancies. A novel finding of this study is that twin fetal weight in the ET and IVCNS groups is lower than naturally conceived controls indicating that the process of superovulation, artificial insemination and manipulation by embryo transfer is sufficient to alter the growth trajectory of the fetus. I have shown that IVCHS results in an increase in fetal weight and CRL as previously reported and whilst this is not the case in twins, there is evidence that the presence of human serum during the period of embryo culture counteracts the deleterious mechanisms resulting in lower fetal weights in the ET and IVCNS groups. In addition, I have shown that placental weight is lower in ET and *in vitro* derived twin but not singleton pregnancies, and I have suggested that this phenomenon may be attributed to the failure to initiate normal compensatory placental growth by twin placentomes. Furthermore ET and *in vitro* embryo culture caused a shift in the population of ovine placentomes to those that are more everted in both singleton and twin pregnancies. I hypothesize that the altered growth trajectories, enhanced in singleton IVCHS and restricted in twin ET and IVCNS fetuses, are therefore placental, rather than fetal in origin. I suggest that the cardiovascular system (fetal and placental) are programmed by ET and *in vitro* culture and that the characteristic organomegaly of the Large Offspring Syndrome is mediated by alterations in cardiac output and peripheral vascular development. Whilst the mechanisms underlying the cause of programming of the Large Offspring Syndrome during early embryo development and the subsequent physiological adaptations are largely unknown, these findings highlight the sensitivity of the embryo during the periconceptional period to an interaction between exposure to

an *ex vivo* environment and fetal number in setting the growth trajectories of feto-placental development.

Chapter 6:

Summary and conclusions

"An investment in knowledge pays the best interest"

- Benjamin Franklin

6. Summary and Conclusions

Maternal undernutrition during the periconceptional period of development has been demonstrated to "program" altered cardiovascular development and an increased activation of the pituitary-adrenal axis in the late gestation fetal sheep. *Ex vivo* nutrition in culture systems used to produce embryos *in vitro* has also been demonstrated to affect fetal and cardiovascular development in the late gestation sheep fetus. It was the purpose of this thesis to investigate the origins of the physiological adaptations programmed in the embryo during the periconceptional period that may subsequently manifest into pathophysiology of the cardiovascular and neuroendocrine systems in later life.

Maternal nutrient restriction during the periconceptional period resulted in a decrease in maternal weight and body condition, which were associated with a disruption of the normal relationship between maternal nutrient status and early feto-placental development. In control ewes carrying singleton fetuses the more weight a ewe gained the larger her placenta and fetus were at d 50 gestation. Maternal nutrient restriction during the periconceptional period disrupted the relationship between maternal weight gain and placental weight, however, the relationship between fetal and placental weight was maintained. In twin pregnancies there was no relationship between measures of maternal body weight and placental or fetal growth, but, nutrient restriction during the periconceptional period resulted in an emergence of an inverse relationship between maternal weight loss and feto-placental growth. Thus in these pregnancies, the more maternal weight lost, the larger the placenta and hence

the fetus. It is intriguing that there is no relationship between fetal and placental weight in control twin pregnancies, however, it is well established that the placentas of twin pregnancies undergo compensatory growth in order to maximize the materno-fetal exchange surface, to result in an increase in total placental mass and in mean placentome weight. The results of Chapter 2 indicate that the mechanisms of normal compensatory growth in twin pregnancies are active early in gestation during a period of development when the nutrient demands of the fetus are minimal, resulting in a placenta that is well developed to handle the demands of the increased nutrient demands of the fetus during mid to late gestation.

The 'over abundant' growth of the twin placenta early in gestation suggests that the presence of twins possibly as early as the blastocyst stage of development, as signalled by the presence of either 2 corpora lutea or 2 embryos, induces a predictive utero-placental growth response in preparation for the increased demands of twin fetuses in late gestation. The question arises, then, how does the maternal reproductive tract and the individual concepti recognise that there are two concepti present in the maternal reproductive tract. Endocrine and paracrine communication between the embryo and maternal epithelium lining the oviduct and uterus commences very early in embryo development. I suggest that the dialogue between twin embryos and the maternal uterine epithelium begins with ovulation and involves the mechanism underlying maternal recognition of pregnancy. It has been demonstrated that the level of ovarian progesterone production is directly related to the number of corpora lutea, and thus two ovulations begins preparation for the establishment of a twin pregnancy (Brien *et*

al., 1987; Chagas e Siliva *et al.*, 2003). The embryo signals its presence during maternal recognition of pregnancy in the ruminant via the secretion of interferon-tau. There is evidence that progesterone and interferon-tau act in concert to alter and prepare the uterine LE and GE for pregnancy, specifically to prevent luteolysis and upregulate gene expression of the glandular epithelium to produce nutriment and factors essential for implantation and attachment (Spencer & Bazer, 2004b; Spencer *et al.*, 2004a). Plasma progesterone concentrations increase with maturation of the CL, and experiments investigating asynchronous embryo transfer have shown that higher levels of progesterone at a developmentally inappropriate time alters uterine secretions and subsequently either signals the embryo to enhance development that persists into mid to late gestation or creates an environment that is too advanced for embryo survival (Sinclair *et al.*, 1998a). Increased interferon-tau production by two embryos may thus alter LE and GE gene expression to produce increased uterine nutrition and/or factors required for twin implantation and placentation. Studies transferring blastocysts at different developmental stages to recipients have demonstrated that embryos which are developmentally more advanced, produce more interferon-tau and have a higher rate of establishing pregnancies (Kubisch *et al.*, 2004). Thus, it is possible that double the amount of interferon-tau production by two embryos may enhance embryonic survival but may also alter the normal "servomechanism" of uterine preparation for pregnancy to recognize the presence of two concepti.

It is also interesting that maternal nutrient restriction during the periconceptional period causes an emergence of an inverse relationship between feto-placental

growth and maternal weight loss in twin pregnancies such that the more weight a ewe loses during early gestation the larger the placenta and thus the larger fetal mass produced. This phenomenon may indicate another form of utero-placental predicative response of placental compensatory growth. I had hypothesized that maternal nutrient restriction during the periconceptional period would cause an increase in maternal progesterone plasma concentrations around the time of conception due to release of endogenous progesterone in adipose tissue stores and a decrease in liver clearance, however, results in Chapter 3 indicate that there was no effect of nutrient restriction on circulating progesterone concentrations. It is possible that there is a local secretion of progesterone and due to hepatic clearance the increase was not observed in our investigation. An increase of plasma progesterone concentrations coupled with progesterone levels of a double ovulation may be a mechanism that programs the trophectoderm and LE for an increased growth trajectory of the placenta, possibly for times of "famine". When nutrient levels were restored after d 7 of development, the enhanced predictive growth trajectory of the placenta was able to maximize fetal growth.

Evidence from Chapter 5 indicates that manipulation of the periconceptional environment either *in utero* by increased progesterone, resulting from superovulation or through the action of *ex vivo* factors inherent in *in vitro* embryo culture systems program the embryo for altered fetal and placental development. A number of laboratories are currently investigating the possibility that the programming of the LOS phenotype is due to altered gene expression and more specifically an epigenetic change of imprinted genes in placental or fetal tissues.

An alteration in the methylation of these genes would lead to either an over or under expression of genes intimately involved in regulating fetal and placental growth (Young & Fairburn, 2000; Wrenzycki *et al.*, 2004). One such candidate gene of interest is IGF-2 and its receptor, and it has been shown in the sheep that there is an increase in the bioavailability of IGF-2 fetal exhibiting the LOS, however, the perturbing factors that alter gene methylation have not been fully elucidated.

Normal embryo development is dependent upon the homeostatic regulation of the embryonic environment, which involves continual cross talk between the embryo and maternal reproductive tract in order that reciprocal developmental cues are exchanged to maintain developmental synchrony and developmental processes such as maternal recognition of pregnancy, embryo growth and implantation (Spencer & Bazer, 2004b; Spencer *et al.*, 2004b). The static nature of *in vitro* culture systems causes an accumulation of deleterious metabolic waste products and does not provide any developmental cues to the developing embryo, which may explain the developmental acceleration of embryo growth, altered embryo metabolism and alteration of cell allocation in the blastocyst (Walker *et al.*, 1992b; Hasler *et al.*, 1995; Thompson *et al.*, 1995; Van Soom *et al.*, 1996; Walker *et al.*, 1996b). In addition, altered gene expression observed in *in vitro* derived embryos may result from a lack of "developmental guidance" from maternal endocrine signals (Walker *et al.*, 1998; Boerjan *et al.*, 2000; Niemann & Wrenzycki, 2000).

Acceleration of embryonic growth results in an advanced stage of development

that is inappropriate for chronological age and subsequent transfer to a chronologically synchronized recipient, thus possibly resulting in an unintentional asynchrony between the maternal reproductive tract and conceptus. The use of multiple ovulation and embryo transfer procedures has also resulted in alterations in fetal development, which has been proposed to be due to the increased level of progesterone produced by multiple CLs (van Wagtendonk-de Leeuw *et al.*, 2000), causing accelerated development of the reproductive tract compared to appropriate chronological development, resulting in asynchrony and temporally inappropriate signalling to the zygote and early embryo in the oviduct and uterus (Boerjan *et al.*, 2000). Interestingly, it has also been shown that progesterone administration during early embryo development can result in an artificial asynchrony between conceptus and an endocrine induced advancement in the maternal development of the uterine epithelium, which has been shown to enhance fetal and placental growth in mid-gestation (Kleemann *et al.*, 2001).

Thus, the work presented in this thesis demonstrates that relatively subtle changes in the level of maternal nutrition during the periconceptional period as well as *ex vivo* manipulation of the embryo result in potential altered embryo, fetal and placental development, which may be the result of developmentally inappropriate materno-embryonic communication.

An important finding of this thesis is that changes in fetal growth and development after either *in vitro* culture or periconceptional undernutrition were explained by changes in placental weight in both singleton and twin pregnancies. I have demonstrated that in late gestation the placenta is the major determining

factor of fetal growth in pregnancies derived from natural mating, ET and IVC and that growth trajectories of the fetus and the placenta are each set early in gestation. Compensatory placental growth in twin pregnancies is essential for normal maintenance of fetal development in late gestation. In twin pregnancies the effects of superovulation, artificial insemination and embryo transfer are sufficient with or without *in vitro* culture to cause a failure in the initiation of compensatory placental growth, which results in placentae of twin pregnancies being similar in weight to those in the singleton pregnancy group. One explanation of these results is that replacement of two embryos in the absence of high maternal progesterone concentrations derived from two CLs, results in the development of a placenta which is appropriate to sustain a singleton but not a twin pregnancy. Thus the placental weight of a twin pregnancy established in these circumstances is programmed to be the same as when only one of the two replaced embryos survive to result in a singleton pregnancy.

Separately, culture of embryos in the presence of serum independently results in enhanced placental growth when either one or two embryos are transferred and result in a pregnancy. In singleton pregnancies, this enhanced growth supports the growth of a fetus which is larger than that present in control pregnancies, whereas in twin pregnancies, this enhanced growth restores the growth of the fetus to that present in the normal twin pregnancy. Thus, I must disagree with the findings of Sinclair and colleagues that the fetal "overgrowth appears to be driven by the fetus and not by the placenta" (Sinclair *et al.*, 1997; Sinclair *et al.*, 1998b; Sinclair *et al.*, 1999). In fact, I postulate that the Large Offspring Syndrome arises as a consequence of an early reprogramming of placental development

through epigenetic changes in the expression of imprinted genes which regulate placental growth and development. Specifically, I hypothesize that the impact of changes in IGF-2 and IGF-2R expression in tissues from fetuses generated following the exposure of the embryo to an *ex vivo* environment are a consequence of the altered fetal nutrient environment and consequently are not present in early gestation when the nutrient demands of the fetus are minimal.

Alterations to the periconceptional environment have also been demonstrated to augment cardiovascular and neuroendocrine development in the sheep. Results reported in this thesis indicate that "programming" during embryo development selectively alters the growth trajectory of these systems. In this thesis, I have shown that the delayed prepartum activation of the HPA axis in late gestation in twin pregnancies may be programmed from early gestation (Chapter 3). The lower expression of growth factors and steroidogenic capability and resulting lower weight of the adrenal glands in twin fetuses during early gestation foreshadows the delayed activation of the HPA axis and blunted adrenal responsiveness in late gestation (Edwards & McMillen, 2002a; Gardner *et al.*, 2004a). Interestingly, the relationship between measures of the HPA axis activity (fetal plasma ACTH/cortisol) and cardiovascular system (mean arterial blood pressure) in twin fetuses during late gestation (Edwards & McMillen, 2002b; Gardner *et al.*, 2004a) is mirrored during early gestation by a relationship between relative heart and adrenal growth (Chapter 3).

In Chapter 4 it was demonstrated that maternal weight change not only affected the growth trajectory of the fetal kidney but also altered the gene expression of

intrarenal growth factors. Impaired renal growth and development has been associated with the development of increased blood pressure in postnatal life (Moritz & Wintour, 1999; Wintour *et al.*, 2003a; Wintour *et al.*, 2003b). During early gestation nephrogenesis occurs and within critical windows of development the fetal kidney is acutely sensitive to glucocorticoid exposure. The results in Chapter 4 indicate that change in maternal weight loss around the time of conception in twin fetuses results in a relatively larger kidney during early gestation, however, when the effects of maternal cortisol are controlled for in the analysis, then this effect is ablated. The expression of placental 11-βHSD-2 has been found to be increased in twin pregnancies (Chapter 3), thus how the effects of maternal cortisol selectively enhance the development of twin renal growth is not clear. It is possible that renal 11-βHSD-2 expression is differentially regulated and allows cortisol induced growth, and it would be interesting to determine whether intrarenal expression of 11-βHSD-2 expression is altered by either the periconceptional undernutrition or the presence of multi-fetal pregnancy. It is clear that further work is required to investigate intrarenal mechanisms of growth during early gestation, including the intrarenal renin-angiotensin system, which can alter renal nephrogenesis and may be activated by periconceptional undernutrition. Whilst nephrogenesis is not complete at ~ d 55 of gestation, determining how periconceptional undernutrition might alter the morphology of the developing kidney would be interesting.

The results of this thesis therefore highlight the complex interactions between the periconceptional environment (*in vivo* or *ex vivo*) and embryo or fetal number on the programming fetal and placental development. Maternal undernutrition

during the periconceptional period and superovulation, artificial insemination and embryo transfer with or without *in vitro* culture in the absence or presence of serum alters fetal development, and I have demonstrated that these changes in fetal growth can be explained by changes in placental growth trajectory. Furthermore, a novel finding of this study is that perturbations of the periconceptional environment affect feto-placental development differently in singleton and twin pregnancies.

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