THE IMPACT OF THE PERICONCEPTIONAL AND PREIMPLANTATION ENVIRONMENT ON ADRENAL DEVELOPMENT AND STEROIDOGENESIS IN THE FETAL SHEEP

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A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

April 2010

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Addendum:

1. Section 2.1, page 7, last sentence: This statement applies to the sheep rather than the human.
2. Section 2.1, page 52, line 12: "...have found that offspring exposed..."
3. Section 2.1, page 53, line 23: "...which have investigated..."
4. Section 2.4.3, page 87, line 8: "...this suggests that there may be a specific effect..."
5. Section 2.4.5.3, page 91, lines 19-20: "...have been found to occur only after 112 days of gestation (Wallace, 1948) it may be possible that..."
6. Section 2.4.5.3, page 92, second last line: "...occurs plays a part..."
7. Section 3.1, page 95, second last line: "...which have investigated..."
8. Section 3.2.1, page 96, line 15: comment added "The same animals were used in Chapter 3 as in Chapter 2."
9. Section 3.2.7, page 100, line 5: "...5 minutes prior to CRH..."
10. Figure 3.4, page 107: added "# denotes a significant decrease in fetal P_aO_2."
11. Figure 3.13, page 120: "# denotes a significant increase in plasma cortisol concentration compared to pre-infusion values" deleted and replaced by: "Different alphabetical subscripts denote mean values, which are significantly different."
12. Section 3.4.4, page 128, last line: "...that twins had a greater ACTH and cortisol concentrations..."
13. Section 3.4.5, page 130, third last line: "...which suggests..."
14. Section 4.3.3, page 146, first line: "on the" deleted
15. Figure 5.4, page 189: "Fetal plasma ACTH concentration in singletons at 116 – 145 days of gestation"
16. Section 5.4.2.2.2, page 206, line 12: "...absence of serum. Unfortunately..."
17. Section 6, page 209, line 15: "It is also not known..."
18. Section 6.3, page 215, line 4: "...in Chapter 5 provides important..."

DECLARATION

This body of scientific work contains no material that has been accepted for the award of any other degree or diploma in any other University or Tertiary Institution. To the best of my knowledge and understanding, this thesis contains no material previously published or written by any other person, except myself and where due reference is made in the text.

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ACKNOWLEDGMENTS

The journey of a PhD can be very challenging with its highs and lows, but I feel that the entire experience has made me a stronger person and helped me define who I am today. It undoubtedly has been a long road and I am thankful to have come to the end. Along the path there was much support and help from many people and I won't attempt to list them all here, but I am certain you know who you are.

Firstly, I would like to thank my supervisor Professor Caroline McMillen. You have been a great inspiration and mentor during these years of joy, excitement, tribulation and frustration. I certainly learnt far beyond science. And it looks like we won the most interesting race against time and managed to have two deliveries in one week!

I am also grateful to Professor Jeff Schwartz and Doctor Catherine Coulter for their invaluable help and advice.

I would like to take this opportunity to give a big thank you to Professor Pat Buckley for helping me kick start my PhD.

Many thanks to all the members of the McMillen Laboratory, past and present. Working with big animal models is ambitious, labour intensive and simply not possible without a team and what a great team "Team McMillen" was and is.

I would like to thank the Laboratory Animal Services staff at the University of Adelaide Medical School for their excellent and professional care of the animals

used in these studies. I am also grateful to the members of the Turretfield Research Centre who played a crucial role in running the nutritional protocols and the care and maintenance of the animals.

To my wonderful family who are probably still in disbelief that my work has come to its completion. Yes it is true, it is done. I would like to thank them for their support and understanding, particularly by managing their constant urge to ask about the progress of this thesis along the various stages.

To my husband John, yes I will say it again, it is done. This thesis is dedicated to you. Now that this heavy weight has been lifted we can go on and celebrate life!

COMMONLY USED ABBREVIATIONS

A B C

ad libitum	to any desired extent
AC	abdominal circumference
ACTH	adrenocorticotrophic hormone
AI	artificial insemination
ANOVA	analysis of variance
ART	assisted reproductive technologies
ATP	adenosine triphosphate
AUC	area under the curve
AVP	arginine-vasopressin
11-βHSD-2	11beta-hydroxyl steroiddehydrogenase
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CR	crown rump
CRH	corticotropin-releasing hormone
CYP17	cytochrome P450 17alpha-hydroxylase

D E F G

DMD	differentially methylated domain
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ET	embryo transfer
GR	glucocorticoid receptor

GIFT	gamete intrafallopian transfer

H I J K

h	hour(s)
Hb	arterial haemoglobin content
HPA axis	hypothalamo-pituitary-adrenal axis
HS	human serum
ICR	imprinting control region
ICSI	intracytoplasmic sperm injection
IGFs	insulin-like growth factors
IGF1	insulin-like growth factor 1
IGF2	insulin-like growth factor 2
IGF1R	insulin-like growth factor type 1 receptor
IGF2R	insulin-like growth factor type 2 receptor
i.m.	intramuscular
i.v.	intravenous
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production

L M N O

LOS	Large Offspring Syndrome
LH	lateral hypothalamic area
MAP	mean arterial blood pressure
MC2R	melanocortin type 2 receptor (ACTH receptor)
ME	metabolisable energy
MER	metabolisable energy requirements

min	minute(s)
MOET	multiple ovulation embryo transfer
mRNA	messenger ribonucleic acid
NAC	non-amplification control
ncRNA	non-coding ribonucleic acid
NS	no serum
O_2 content	arterial oxygen content

P Q R S

PAT	perirenal adipose tissue
PCO_2	arterial partial pressure of carbon dioxide
PCUN	periconceptional undernutrition
PG	prostaglandin
PGF	prostaglandin F 2 alpha
PGHS-II	prostaglandin H synthase type II
PM	post mortem
PO_2	arterial partial pressure of oxygen
POMC	proopiomelanocortin
PVN	paraventricular nucleus
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SOF	synthetic oviductal fluid
SPSS	statistical package for social sciences
SSC	cytochrome P450 side chain cleavage
StAR	steroidogenic acute regulatory protein

T U V W X Y Z

TGF	transforming growth factor beta
ZIFT	zygote intra-fallopian transfer

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ABSTRACT

Experimental and clinical studies provide evidence that perturbations and manipulation of the *in vivo* and *ex vivo* nutritional environment during the periconceptional period alters the development of the fetal hypothalamo-pituitary-adrenal (HPA) axis and gestation length. In particular periconceptional maternal undernutrition results in an earlier prepartum activation of the fetal HPA axis and adrenal development whereas culturing embryos *in vitro* in the presence of human serum is associated with delayed parturition in the sheep. It is not clear, however, whether the effects resulting from periconceptional undernutrition are due to the impact of undernutrition acting on the development of both the oocyte and embryo or on just the early embryo. It is also not known how culturing embryos *in vitro* in the absence or presence of human serum impacts on the prepartum activation of the HPA axis and adrenal development. Lastly, the intra-adrenal molecular mechanisms by which changes in the *in vivo* or *in vitro* nutritional environment of the early embryo alter HPA development have not been fully investigated.

This thesis provides evidence for the first time which suggests that periconceptional undernutrition may differentially target components of the fetal HPA axis depending on exposure to undernutrition during specific periconceptional time periods. Specifically, periconceptional undernutrition alters fetal adrenal growth and development whilst undernutrition during the preimplantation period alone is sufficient to alter the development of the fetal anterior pituitary in late gestation.

A further novel finding of this thesis is that when embryos were cultured *in vitro* in a defined medium fetal plasma ACTH concentration significantly increased in singletons whereas relative adrenal weight and adrenal CYP17 mRNA expression significantly increased in both singleton and twins in late gestation. This suggests that this embryo culture system affects adrenal growth and development, independent of fetal number and importantly, that there is an early activation of the HPA axis in the singleton fetus in late gestation. Interestingly, addition of serum to the *in vitro* culture media reverses the effects of culturing embryos *in vitro* in the absence of serum and the mechanism(s) by which restoration of fetal adrenal development occurs may involve the intra-adrenal IGF system.

In summary, alteration of the development of the fetal HPA axis appears to be dependent on specific periconceptional time windows of poor nutritional exposure and type of culture media to which an embryo is exposed to.

CHAPTER 1: LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 INTRODUCTION

The findings from a large number of clinical and experimental studies have shown that perturbations and manipulations of the intrauterine environment impacts on fetal development, gestation length and later adult life. Attempts have been made:

- to characterise types and severity of perturbations and their impact on the programmed development of the metabolic, cardiovascular and endocrine systems of the fetus and adult; and

- to identify critical ontogenic windows, during which intrauterine perturbations have a permanent effect on adult health.

In particular, evidence from animal models has suggested that perturbation of the early nutritional environment of the oocyte and/or embryo affects the development of the fetal hypothalamus-pituitary-adrenal (HPA) axis in late gestation, the timing of birth and health outcomes in later life (McMillen & Robinson, 2005).

This review will begin by giving a brief overview of the HPA axis, the development this axis in fetal life and the role of the fetal HPA axis in the timing of parturition. A detailed description of the ontogeny of the fetal HPA axis in the sheep and the factors which regulate fetal adrenal growth and steroidogenesis will then follow. The effects of intra-uterine perturbations, especially variations in maternal, fetal and embryonic nutrition during critical ontogenic windows, on feto-placental and adrenal growth and the development of the HPA axis will then be addressed and areas, which require further investigation, will be highlighted.

1.2 HYPOTHALAMUS-PITUITARY-ADRENAL AXIS – AN OVERVIEW

Cortisol is the main adrenal glucocorticoid hormone in humans and sheep and regulation of the HPA axis plays a central role in the response of the individual to stress. The initiation of a stress response, following disruption of physiological homeostasis, involves the activation of the HPA axis, to ultimately lead to restoration of homeostasis. Cortisol specifically regulates glucose, protein and lipid homeostasis (Beck & McGarrry, 1962; Swartz & Dluhy, 1978), and anti-inflammatory and immune responses (Nunez, 1988; Buckbinder & Robinson, 2002; Perretti & D'Acquisto, 2009) to stress.

Hypothalamic functions are regulated by afferents from the brainstem (Lowry, 2002) and higher centres such as the amygdala and the hippocampus (Feldman *et al.*, 1995). The parvicellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, when stimulated, secrete corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the hypophyseal portal system, which drains into the pituitary. CRH and AVP bind to CRH-R_1 and V_{1b} receptors on pituitary corticotrophic cells, respectively, which then regulate the synthesis and secretion of adrenocorticotropic hormone (ACTH) into the peripheral circulation. Circulating ACTH exerts its effects on the adrenal, via the melanocortin type 2 receptor (MC2R), resulting in an increase in cortisol synthesis and secretion.

The adrenal cortex is composed of three different zones: *zona glomerulosa*, *zona fasciculata* and *zona reticularis*. The outer *zona glomerulosa* primarily synthesises aldosterone, a mineralocorticoid which regulates sodium reabsorption in the renal tubules, salivary glands and gut. The z*ona fasciculata* and *zona reticularis* are the two inner zones, which are capable of synthesising cortisol. The *zona fasciculata,*

however, is the primary site for cortisol synthesis. Cortisol, like most steroids, is a derivative of cholesterol. The biosynthetic pathways for cortisol and aldosterone are summarized in Fig. 1.1. The steroidogenic acute regulatory (StAR) protein is an essential component of this process as it regulates the cholesterol transport from the outer to the inner mitochondrial membrane where the first step of the biosynthetic pathway occurs (Stocco, 2001).

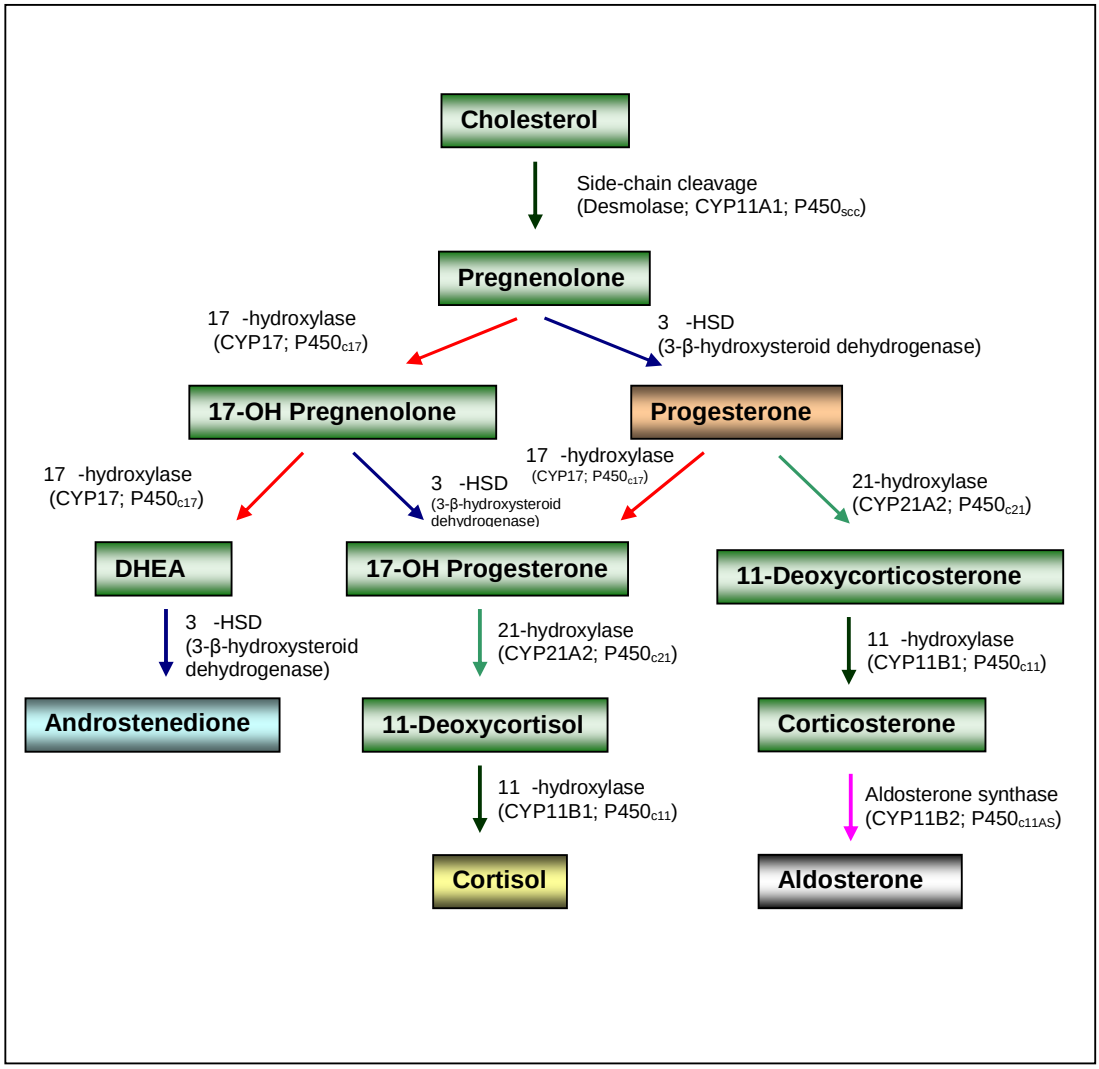


Figure 1.1: Major Steps in Adrenal Hormone Synthesis

The HPA axis is under negative feedback control as cortisol acts via glucocorticoid receptors (GR) to inhibit the stimulation of ACTH synthesis and secretion at the hypothalamus and pituitary (Fig. 1.2).

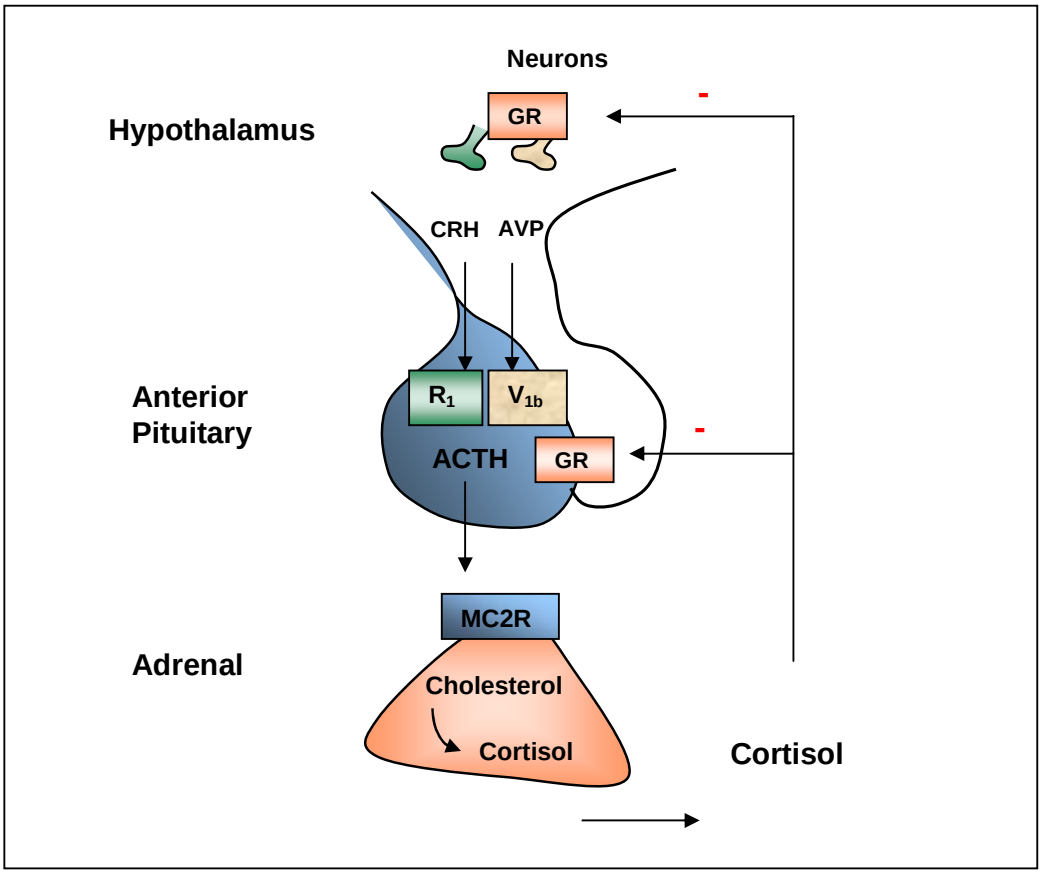


Figure 1.2: The negative feedback of the Hypothalamus-Pituitary- Adrenal Axis

The HPA axis is functional before birth in many species and has a range of specific functions in prenatal life. In human and sheep, a prepartum surge of cortisol in the fetus is essential for the maturation of lung, liver, kidney and gut in preparation for extrauterine life (Liggins, 1994). This surge is also critical for the initiation of labour in the sheep (for review see Whittle *et al.*, 2001). The majority of research on the development of the HPA axis *in utero* has been conducted

extensively in the fetal sheep. Therefore, the sheep is a well-established model for the study of the development of the HPA axis and its regulation before birth.

1.3 FETAL HPA AND PARTURITION

The role of the HPA axis in parturition has been extensively reviewed (Challis *et al.*, 2001; Schwartz & McMillen, 2001; Whittle *et al.*, 2001; Mastorakos & Ilias, 2003; Challis *et al.*, 2005). It is known that adrenalectomy, hypophysectomy, surgical disconnection of the hypothalamus from the pituitary, and bilateral lesions of the paraventricular nucleus in the sheep fetus, each abolish the prepartum cortisol surge and delay parturition (Liggins *et al.*, 1967; Drost & Holm, 1968; Liggins & Kennedy, 1968; Robinson *et al.*, 1977; Antolovich *et al.*, 1991; McDonald & Nathanielsz, 1991; Ozolins *et al.*, 1992). ACTH and cortisol administration to a fetus with ablations of the relevant components of the HPA axis result in restoration of circulating cortisol concentrations and parturition (Van Rensburg, 1967; Wintour *et al.*, 1980; Poore *et al.*, 1998). Similarly, administration of either ACTH or a glucocorticoid (cortisol, or the synthetic glucocorticoid dexamethasone) to an intact fetus *in utero* results in premature parturition (Liggins, 1968; Liggins *et al.*, 1973). Therefore, it is reasonable to argue that each level of the fetal HPA axis is essential for the normal timing of parturition.

The fetal cortisol surge induces prostaglandin H synthase type II (PGHS-II) expression in the fetal placenta resulting in an increased production of prostaglandin (PG), in particular PGE2 (Whittle *et al.*, 2000). It has been hypothesised that prostaglandin and/or cortisol act to induce placental CYP17 (Whittle *et al.*, 2001). Increasing the expression of CYP17 potentially directs steroidogenesis towards androgen and away from progestagen production leading

ultimately to a decrease in placental progesterone and a rise in estrogens, which are involved in the stimulation of myometrial contractions in the sheep (Fig. 1.3).

Figure 1.3: Fetal-Placental Interactions before and during parturition in sheep (adapted from Whittle et al., 2001)

In humans, the HPA axis is also activated in the fetus during late gestation and there is evidence suggesting that cortisol plays a similarly important role in the initiation of labour and timing of birth by stimulating PG synthesis (Whittle *et al.*, 2001; Smith, 2007). The difference between the sheep and the human, however, lies in the cellular pathways that result in the stimulation of PG synthesis in the fetal membranes and placenta. Placental production of CRH increases exponentially with gestation in the human (McLean *et al.*, 1995) and the level of CRH binding protein falls, increasing the bioavailability of CRH towards term (Linton *et al.*, 1990; Linton *et al.*, 1993). Placental CRH is released into the maternal circulation as well as into the fetal circulation (Nodwell *et al.*, 1999). CRH apparently acts to stimulate the further synthesis and secretion of cortisol. In a feed forward manner, cortisol then indirectly stimulates CRH production by the fetal membranes and placenta (Jones *et al.*, 1989). Evidence suggests that cortisol inhibits chorion trophoblast prostaglandin dehydrogenase (PGDH) expression and synthesis (Patel *et al.*, 1999a; Patel *et al.*, 1999b), which is responsible for the metabolism of PGs, whilst stimulating amnion and chorion PGHS-II expression and activity (Zakar *et al.*, 1995; Economopoulos *et al.*, 1996; Zakar *et al.*, 1996) directly resulting in an overall increase in membrane PG synthesis. Similarly, CRH also stimulates PGHS-II and inhibits PGDH synthesis and activity (McKeown & Challis, 2003; Gao *et al.*, 2007). These two pathways ultimately result in an increase in uterine contractility (Figure 1.4).

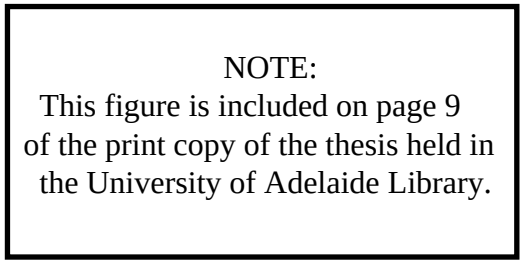


Figure 1.4: Fetal-Placental Interaction before and during parturition in humans (adapted from Whittle et al., 2001)

Clearly, there are a number of similarities in the regulation of events during labour between the sheep and human. In both species, there is an activation of the fetal HPA axis in late gestation resulting in a cortisol surge. In both species cortisol plays a key role in the modulation of PG production by directly or indirectly

stimulating intrauterine PGHS-II expression. In both species, PGs mediate the event of labour including myometrial contractility, cervical ripening and uteroplacental flow.

There are, however, also differences between the two species. In the sheep the increased concentrations of cortisol exert their effect on maternal uterine tissues through the mediation of estradiol whilst in humans cortisol exerts its effect on fetal, but possibly maternal tissues via CRH. One of the main differences between these two species is the source of androgen precursors for the increase of intrauterine estrogen synthesis at the end of gestation. In the sheep, placental pregnenolone is converted by CYP17 to estrogen thereby decreasing progesterone production. The human placental syncytiotrophoblast does not express CYP17 and as a result relies on maternal and fetal androgen precursor for estrogen synthesis and therefore a decrease in intrauterine progesterone concentrations does not occur. Regardless of these differences, many facets of the labour events in these two species are similar and this means that the sheep is a valuable animal model for the study of labour and timing of birth. Given the importance of the fetal HPA axis in parturition and in the fetal response to acute and chronic stress, it is therefore important to review the key stages of the development of this axis throughout gestation.

1.4 Ontogeny of the HPA in the Sheep Fetus

1.4.1 The Fetal Hypothalamus and Pituitary Gland

In fetal sheep, where the normal length of gestation is 150 ± 3 days, the neurohormones, CRH and AVP, have been localised in hypothalamic neurons as

early as 48 days of gestation (Watabe *et al.*, 1991) and 42 days of gestation (Levidiotis *et al.*, 1987), respectively. Fetal hypothalamic CRH mRNA and peptide are present by at least 60 days of gestation, gradually increase until 120 days of gestation and then markedly increase during the last 20 days of gestation with the highest abundance present at term (Matthews & Challis, 1995; Saoud & Wood, 1996; Keller-Wood *et al.*, 2006). In contrast, hypothalamic AVP mRNA and peptide remains unchanged throughout gestation but significantly increase after birth (Saoud & Wood, 1996; Challis *et al.*, 2001; Keller-Wood *et al.*, 2006). The AVP peptide in the pituitary is present in higher concentrations than the CRH peptide at 70, 100 and 130 days of gestation (Currie & Brooks, 1992) and at around 145 days of gestation, hypothalamic AVP mRNA is more highly expressed than CRH mRNA in the hypothalamus (Keller-Wood *et al.*, 2006).

The hypothalamic-hypophyseal portal system is present from at least 45 days of gestation in the sheep fetus (Levidiotis *et al.*, 1989). Corticotrophs have been visualised as early as 38 days of gestation in the fetal anterior pituitary (Mulvogue *et al.*, 1986) and there is evidence that corticotrophs of the fetal pituitary respond to CRH and AVP at 63 days of gestation *in vitro* (Durand *et al.*, 1986) and *in vivo* at around 100 days of gestation (Norman *et al.*, 1985; Rose *et al.*, 1985; MacIsaac *et al.*, 1989).

One interesting aspect to corticotroph maturation is the change in CRH responsiveness that occurs in late gestation. In contrast to the increase in CRH mRNA expression in late gestation, a number of studies have shown that the ACTH response to CRH declines by 140 days of gestation whilst the response to AVP increases both *in vivo* and *in vitro* (Norman *et al.*, 1985; Hargrave & Rose,

1986; Ozolins *et al.*, 1990; Carr *et al.*, 1995; Fora *et al.*, 1996). CRH R_1 mRNA and protein are present at 100 days of gestation and significantly decrease by 137 – 139 days of gestation in the fetal anterior pituitary (Green *et al.*, 2000). The number of CRH binding sites peaks at around 125 -130 days of gestation and then also progressively decrease as term approaches (Lu *et al.*, 1991). Young and colleagues (2003) have shown that CRH binding was decreased at 140 days of gestation, which was due to a decrease in the percentage of cells binding CRH and in the number of CRH receptors per cell (Young *et al.*, 2003a). All of these finding could contribute to the observed decrease in responsiveness of corticotrophs to CRH in late gestation. It has been suggested that the increase in CRH mRNA and protein may initiate the HPA activation in late gestation but play a lesser role in the sustaining the function of the axis near term (Whittle *et al.*, 2001). Similarly, V_{1b} mRNA expression is present also around 100 days of gestation and progressively decreases by 140 days of gestation whilst the V_{1b} protein levels peak at 120 days of gestation in the fetal pituitary (Young *et al.*, 2003b). These finding fail to explain the increased responsiveness to AVP observed in late gestation and the mechanism by which this occurs remains to be elucidated.

1.4.2 The Fetal Adrenal Gland

The ontogenic profile of circulating ACTH in the developing ovine fetus is characterized as a progressive rise from 110 days of gestation onwards (Norman *et al.*, 1985). Early studies suggested that this “surge” in ACTH concentration was preceded by the surge of cortisol (Rees *et al.*, 1975). With the improvement of available techniques, however, it was later shown that the rise in ACTH precedes the rise in cortisol concentration in late gestation (Matwijiw *et al.*, 1989).

The *zona glomerulosa* of the fetal adrenal is well defined by 53 days of gestation whilst the establishment of the *zona fasciculata* is evident by 100 days of gestation (Boshier & Holloway, 1989). Interestingly, adrenal cells secrete large amounts of corticosteroids upon stimulation by ACTH, which is mediated by cAMP, as early as 40 days of gestation (Wintour *et al.*, 1975). Responsiveness of cultured adrenal cells to ACTH stimulation decreases in mid-gestation but increases again towards term.

There is evidence suggesting that the fetal adrenal undergoes three main developmental stages throughout gestation. Firstly, between 40 and 90 days of gestation there is a rapid growth phase, primarily due to cellular hyperplasia, which occurs at the same time as the adrenal cortex and medulla form as separate components (Boshier & Holloway, 1989). During this phase, the genes for CYP17 and SCC are strongly expressed (Tangalakis *et al.*, 1989) and adrenal cortical cells that are positive for immunoreactive CYP17 are clearly present (cited in Matthews *et al.*, 1995). This is an indication that steroidogenic enzymes are present and active prior to the completion of adrenal zonation. At between 90-120 days of gestation the adrenal is relatively inactive and therefore this has been

characterised as a period of adrenal quiescence. During this period, the adrenal grows more slowly than at any other time in gestation (Boshier & Holloway, 1989). Concurrently, there is minimal CYP17 and SCC gene expression (Tangalakis *et al.*, 1989), the number of adrenal cortical cells that are positive for immunoreactive CYP17 are decreased (Matthews *et al.*, 1995) and basal fetal plasma cortisol concentrations are low (Norman *et al.*, 1985). Lastly, between 130 days of gestation and 2 days after birth there is an additional phase of rapid adrenal growth and differentiation, where cellular hypertrophy precedes cellular hyperplasia (Boshier & Holloway, 1989). During this period, CYP17, SCC and 21-hydroxylase gene expression (Phillips *et al.*, 1996a) as well as the number of adrenal cortical cells that are positive for immunoreactive CYP17 (cited in Matthews *et al.*, 1995) are increased and there is a concomitant rise in fetal plasma cortisol followed by a surge at 2-3 days before birth (Norman *et al.*, 1985).

The adrenal also exhibits enhanced responsiveness to ACTH in late gestation (Saez *et al.*, 1984). This may be explained by an increased expression of MC2R. Although a number of earlier studies found that MC2R expression did not increase in late gestation and that exogenous ACTH did not alter its expression (Simmonds *et al.*, 2001; Carter *et al.*, 2002), subsequent studies have demonstrated that adrenal MC2R expression increases as gestation progresses (Maia *et al.*, 2002; Wang *et al.*, 2004). Recent evidence suggests that not only does the MC2R play a role (Su *et al.*, 2005; Valego *et al.*, 2005; Carey *et al.*, 2006) but that the MC2R is essential in enhancing fetal adrenal responsiveness in late gestation (Su & Rose, 2008). An elegant study by Su and Rose (2008) using small interfering RNA treatment to knockdown MC2R demonstrated that the ability of ACTH to upregulate MC2R expression is attenuated in fetal adrenal cells at 135 -138 days

of gestation. They further showed that the cAMP responses to ACTH are also blocked by small interfering RNA treatment.

1.5 Regulation of Adrenal Growth and Steroidogenesis

The adrenal of the hypophysectomised fetal sheep is hypoplastic and in this model exogenous ACTH induces adrenal growth at 99-122 days of gestation (Boshier *et al.*, 1981) and adrenal growth and maturation at 135 days of gestation (Robinson *et al.*, 1983). Clearly, ACTH has mitogenic and differentiation actions at the fetal adrenal. Insulin-like growth factors (IGFs) and transforming growth factor beta 1 (TGF 1) are also intra-adrenal factors that have also been linked to the regulation of adrenal growth and steroidogenesis.

1.5.1 The Insulin-Like Growth Factors

Throughout fetal development, IGFs act as potent mitogenic and differentiation-promoting factors (Sara & Hall, 1990; Dupont *et al.*, 2003). The IGF system consists of IGF1 and IGF2 polypeptides, cell surface receptors that mediate their effect, including the insulin-like growth factor-1 receptor (IGF1R) and the insulin-like growth factor-2 receptor (IGF2R), as well as a family of IGF-binding proteins (IGF-BPs). The IGF peptides are structurally very similar to insulin (Rinderknecht & Humbel, 1978; Dull *et al.*, 1984; Daughaday & Rotwein, 1989). Both, IGF1 and IGF2, bind to the IGF1R, which is a trans-membrane tyrosine kinase (Gilmour *et al.*, 1988). When the IGF peptides bind to the extracellular subunit of the IGF1R, a conformational change occurs, which enables adenosine triphosphate (ATP) to bind to the receptor resulting in autophosphorylation (Wei *et al.*, 1995; Hubbard, 1997). The phosphorylated receptor then further phosphorylates substrate kinase

thereby initiating a cascade of enzyme reactions, which ultimately lead to cellular proliferation and differentiation (LeRoith & Roberts, 2003). Alternatively, only IGF2 binds to the IGF2R, which has a fundamentally different function to IGF1R (Kiess *et al.*, 1994). The IGF2R is a mannose-6-phosphate receptor that is involved in endocytosis, trafficking and lysosomal activity and acts to increase IGF2 clearance within tissues (Hassan, 2003).

1.5.2 Epigenetic Regulation of IGF Gene Expression

One particularity about two of the IGF genes, namely *Igf2* and *Igf2r*, is that they belong to the family of parentally imprinted genes. Genomic imprinting is a phenomenon whereby genes are expressed in a parent-of-origin specific manner, resulting in only one of the parental copies of a gene being expressed. Specifically, the *Igf2* gene is expressed from the paternal allele, whereas the *Igf2r* gene is expressed from the maternal allele. A number of recent reviews have discussed mechanisms of imprinting including the regulation of *Igf2* and *Igf2r* expression (Wrzeska & Rejduch, 2004; Fowden *et al.*, 2006; Wan & Bartolomei, 2008). At present there are two imprinting regulation models that are accepted and these are “the insulator model of regulation” and “the non-coding RNA model of regulation” (Wan & Bartolomei, 2008). The *Igf2* imprinted gene appears to be an example of “the insulator model” whilst the *Igf2r* gene is an example of “the non-coding RNA model”.

1.5.2.1 Epigenenetic regulation of Igf2 gene expression

There are a number of key factors that play a role in “the insulator model of regulation”. Firstly, imprinted genes exist as cluster over megabase regions, which contain a non-coding RNA (ncRNA) and an imprinting control region (ICR), which is often also referred to as differentially methylated domain (DMD). Usually, the ncRNA are expressed by the opposite parental alleles as protein-coding genes. This is the case in the regulation of *Igf2* expression for which the ncRNA is *H19.* In the maternal allele, CTCF, a protein with “insulator” mediation activity, binds to the DMD forming a complex, which acts as an “insulator”. In this context an insulator is defined as an element that blocks enhancer and promoter interaction thereby preventing transcription. It is not known, however, how “insulators” operate. Fundamentally, this complex blocks enhancers, which are shared by *Igf2* and *H19*, from accessing the *Igf2* promoter but allows access to the *H19* promoter resulting in the expression of this ncRNA. Conversely, the DMD of the paternal allele is methylated leading to secondary methylation of the H19 promoter, which as a consequence silences the paternal H19 gene (Figure 1.5). The mechanism of this secondary methylation is presently not known.

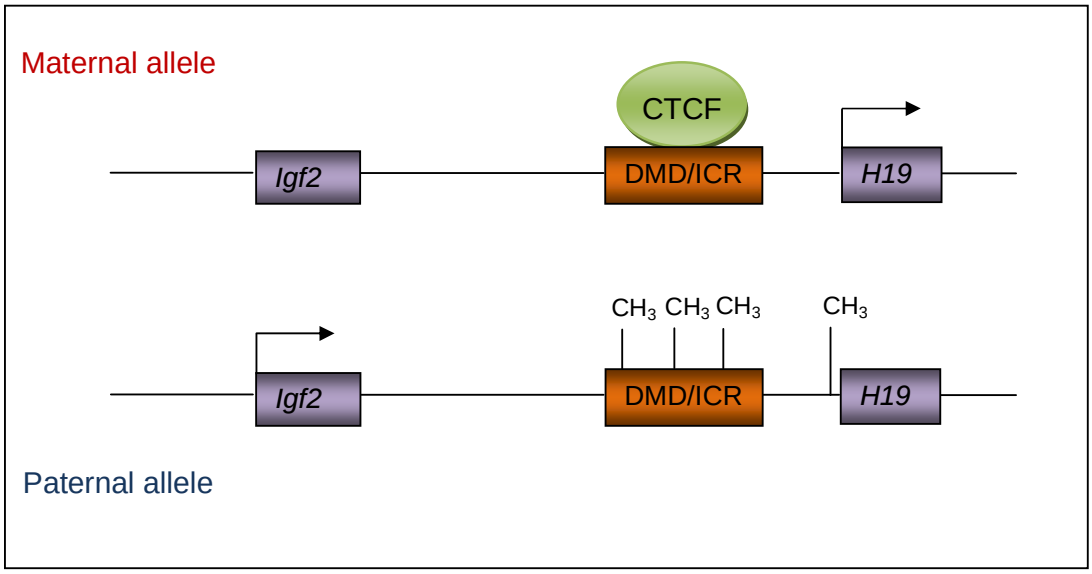


Figure 1.5: Regulation of imprinting at the H19/Igf2 locus (from Wan & Bartolomei, 2008)

1.5.2.2 Epigenetic regulation of Igf2r gene expression

Igf2r is part of an imprinted cluster that contains two maternally expressed genes, *Slc22a2* and *Slc22a3*, several non-imprinted genes, *Slc22a1*, *Mas* and *Plg*, and a gene called *Air*, which codes for a non-coding RNA, which is located within the *Igf2r* gene. Region 1 of the *Igf2r* gene contains the *Igf2r* promoter whilst region 2 contains the promoter for the *Air* ncRNA and is located in the second intron of the *Igf2r* gene. Principally, in the maternal allele, the ICR, which serves as the promoter of *Air*, is methylated. This prevents the transcription of the Air ncRNA and thereby allowing the transcription of the *Igf2r* gene. In the paternal allele, the ICR is unmethylated, which allows for the expression of the *Air* and results in the repression of *Igf2r* (Figure 1.6). Surprisingly, evidence suggests that the Air ncRNA also represses the other two non-overlapping maternally expressed genes *Slc22a2* and *Slc22a3* (not depicted on Figure 1.6) (Sleutels *et al.*, 2002). The

ability of a non-coding RNA to repress imprinted genes encompasses the concept of 'the non-coding model of regulation'. The proposed mechanisms by which Air ncRNA may repress imprinted genes are potentially complex and as such are beyond the scope of this review and will not be discussed further.

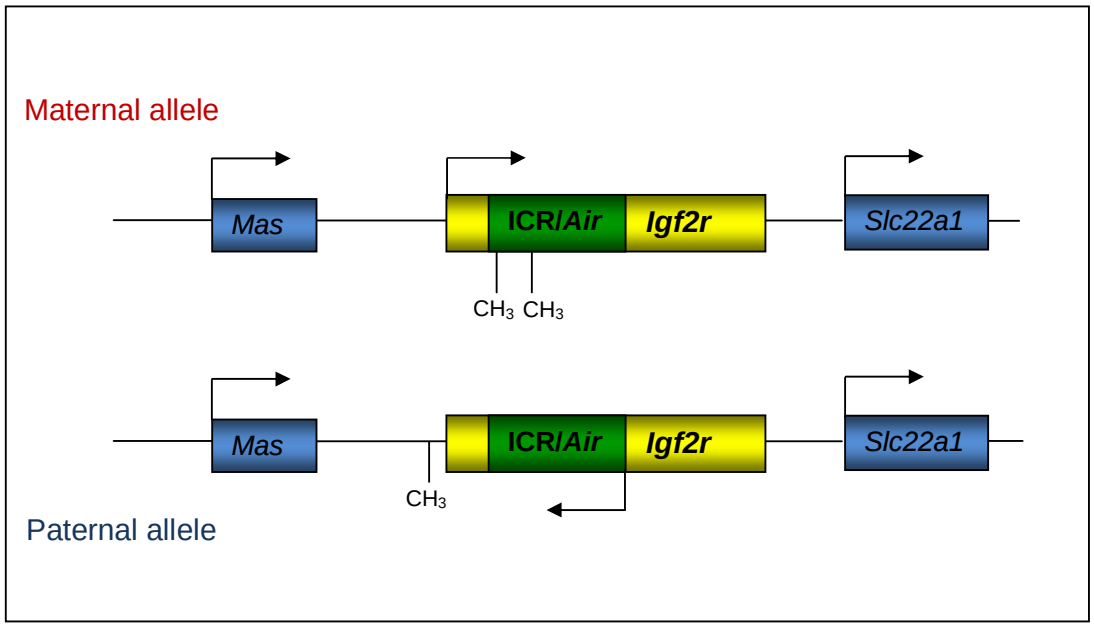


Figure 1.6: Regulation of imprinting at the Igf2r/Air locus (from Wan & Bartolomei, 2008)

1.5.3 Insulin-like Growth Factors and the Fetal Adrenal Gland

In the fetus, the liver is the main source of plasma IGFs present in the circulation (O'Dell & Day, 1998). IGFs, however, have also been localised to most fetal tissues, including the adrenal where its abundance is second only to that present in the liver, and it has been suggested IGFs may act locally in an organ-specific manner as well as systemically (Han *et al.*, 1988; Han *et al.*, 1992). Circulating IGF1 levels progressively rise from 50-80 days of gestation to 7 days post birth, and circulating IGF1 levels in adults are higher than in the fetus (Gluckman &

Butler, 1983). Conversely, circulating IGF2 levels in the sheep fetus are at a relatively high level throughout gestation until several days before birth. They then fall, and by around 12 hours post birth they reach levels that are maintained throughout the remainder of life (Gluckman & Butler, 1983). All components of the IGF system are expressed in the human (Ilvesmaki *et al.*, 1993; Rainey *et al.*, 2002) and in the fetal sheep adrenal (Han *et al.*, 1992). In both human and sheep, adrenal IGF2 is expressed at higher levels than IGF1 mRNA (Han *et al.*, 1988; Han *et al.*, 1992) and in sheep, IGF1R and IGF2R are present as early as 55 days of gestation (MacLaughlin *et al.*, 2007).

One possibility is that the trophic actions of ACTH on the adrenal are mediated by IGFs. At present, there are few studies, which have investigated the relationship between ACTH and IGF in the regulation of growth and steroidogenesis in the fetal adrenal and they have resulted in conflicting data. Previous studies performed have shown that ACTH stimulates IGF2 expression in cultured human fetal adrenal cells (Voutilainen & Miller, 1987, 1988; Mesiano *et al.*, 1993) and that IGF1 and IGF1 with and without the addition of ACTH act to stimulate CYP17 mRNA expression and cortisol production in these cells (Mesiano *et al.*, 1997). Another *in vitro* study found that IGF1 increases the steroidogenic responsiveness to ACTH in ovine fetal adrenal cells (Naaman *et al.*, 1989).

In vivo, the disconnection of the hypothalamus from the pituitary in the sheep fetus results in a smaller adrenal, but paradoxically there is no decrease in adrenal IGF2 mRNA expression in the fetal sheep (Phillips *et al.*, 1996a). When endogenous ACTH was increased, by administration of metyrapone (a blocker of 11β-hydroxylase) to the fetus, it was found that adrenals of treated animals were larger

than the adrenals of control animals in both rhesus monkey and sheep (Coulter *et al.*, 1996; Warnes *et al.*, 2004). Interestingly, in these models, adrenal IGF2 mRNA expression was increased in the fetal rhesus monkey (Coulter *et al.*, 1996) but not in the fetal sheep adrenal (Warnes *et al.*, 2004). It has also been shown that exogenous ACTH or cortisol, administered at 120-123 days of gestation, results in a decrease, rather than an increase, in IGF2 mRNA expression in the fetal sheep adrenal (Lu *et al.*, 1994). In addition, a stress stimulus, such as acute hypoxaemia, results in an increase in ACTH and cortisol output but inhibits IGF2 mRNA expression in the ovine adrenal (Braems *et al.*, 1998). Therefore, the interaction between ACTH and IGF2 is potentially complex with evidence supporting both excitatory (*in vitro*) and inhibitory (*in vivo*) effect of ACTH on *Igf2* gene and mRNA expression.

The IGF2R also mediates the level of IGF2 present in tissues and circulation through its actions to clear IGF2 and enhance intracellular degradation of IGF2. To date, the specific contribution of the IGF2R in adrenal growth and steroidogenesis in the sheep fetus has not been investigated.

1.5.4 Transforming Growth Factor Beta

It has recently been identified that the IGF2R plays a role in the activation of the latent form of the transforming growth factor beta 1 (TGFβ1). TGFβ1 is interesting in that it has been implicated in having an inhibitory role in the regulation of adrenal growth and steroid production.

There are a total of five TGF isoforms of which three, TGF 1, TGF 2 and TGF 3, are found during mammalian development (Roberts & Sporn, 1990). The receptors

for transforming growth factors are TGF receptors, T RI and T RII, which are ubiquitously expressed in mammalian cells (Massague, 2008). The interactions between these receptors and their ligands are unusual. In spite of having a ligand-binding domain T RI can only bind TGF that is already bound to T RII, that is, T RI requires a TGF : T RII complex in order to be recruited (Massague, 2008).

TGF 1 is a cytokine that has multiple biological functions and is present in a biologically inactive form. This latent TGF 1 (LTGF 1) is the result of a complex formation between TGF 1 and latency-associated peptide (LAP) (Miyazono *et al.*, 1988). In order to become biologically active, TGF 1 must be released from this complex. There are several pathways of LTGF 1 activation and one of which involves IGF2R. The model of LTGF 1 activation via IGF2R that has been proposed involves the IGF2R being associated with the urokinase receptor on the cell surface. This complex then allows simultaneous binding of urokinase, plasminogen and LTGF 1, which results in the urokinase-mediated conversion of plasminogen to plasmin. Subsequently, plasmin mediates the release of active TGF 1 from LTGF 1 (Godar *et al.*, 1999).

1.5.5 Transforming Growth Factor Beta and the Fetal Adrenal

In vitro, TGF inhibits basal and ACTH stimulated cortisol secretion (Feige *et al.*, 1986; Hotta & Baird, 1986; Le Roy *et al.*, 2000) whilst dramatically reducing steroidogenic acute regulatory protein (StAR) (Le Roy *et al.*, 2000), 3β-hydroxysteroid dehydrogenase (3βHSD) (Le Roy *et al.*, 2000) and CYP17 mRNA and protein (Perrin *et al.*, 1991; Le Roy *et al.*, 2000) in cultured adult bovine cells. TGF exerted a similar inhibitory effect on steroid production (Stankovic *et al.*,

1994) and CYP17 mRNA levels (Lebrethon *et al.*, 1994) in cultured human fetal adrenal cells. In ovine fetal adrenal cell culture, the actions of ACTH to induce cortisol production and increase CYP17 enzyme activity were attenuated by TGF whilst incubation of ovine fetal adrenals with TGFβ1 for 5 days resulted in a reduction of the activity of the cholesterol side-chain cleavage enzyme (Naaman-Reperant *et al.*, 1996). *In vivo,* at around 100 days of gestation, a time of adrenal quiescence, TGFβ1 mRNA is highly expressed whilst CYP17 mRNA expression is low in the ovine fetal adrenal (Coulter *et al.*, 2003). At 140 – 144 days of gestation, during the time of increased steroidogenic activity, fetal adrenal TGFβ1 mRNA is significantly decreased whilst CYP17 mRNA is significantly increased when compared to the expression levels at around 100 days of gestation (Coulter *et al.*, 2003). Taken together, these data suggest that TGFβ1 may play a role in the inhibition of adrenal steroidogenesis in the fetus.

1.6 Effects of Embryo/Fetal Number on Fetal Growth and Development

It is well documented in the literature that human twin pregnancy is associated with an increased risk of low birth weight (Zahalkova, 1978; Bleker *et al.*, 1979; Lung *et al.*, 2009), premature delivery (Bleker *et al.*, 1979; Keith, 1994; Kiely & Kiely, 2001; Blondel *et al.*, 2002) and perinatal mortality (Zahalkova, 1978; Kiely, 1990; Glinianaia *et al.*, 2000). In Australia, the rate of twin pregnancy has increased to 1.7% and premature birth occurs in around 50% of twin pregnancies (Davies, 2005).

There is evidence that suggests that down regulation of twin growth occurs early in gestation (Wilson, 1974; Leveno *et al.*, 1979; Taylor *et al.*, 1998). These findings are supported by a further study investigating growth restriction in multifetal human pregnancies reduced to twins (Sebire *et al.*, 1997). After reducing the number of embryos to two during the first trimester the remaining twins had a significantly lower birth weight when compared to the birth weights of the non-reduced twins (Sebire *et al.*, 1997). In addition, IVF singletons with a vanished co-twin have an increased rate of low birth weight when compared to singletons (Pinborg *et al.*, 2007). In the sheep the reduction of twin pregnancies to singleton pregnancies by unilateral fetectomy (removal of a fetus) during early gestation leads to altered fetal and placental growth trajectories. Interestingly, fetal weights in fetectomised ewes tend to be greater than that of twin-pregnant ewes and similar to that of single-pregnant ewes in late gestation (Vatnick *et al.*, 1991). The weights of fetectomised ewe placentas, however, were an intermediate between singleton and twin placentas and this was due to hypertrophic placental growth (Vatnick *et al.*, 1991). A study by MacLaughlin and colleagues (2005) demonstrated that the growth trajectories of the fetus and placenta are set as early as ~55 days of gestation (MacLaughlin *et al.*, 2005). Taken together these data suggest that the growth trajectory of the fetus and placenta are in part determined by the fetal number and are set in early gestation.

Fetal number and fetal sex also play an important role in the development of the fetal HPA axis in late gestation in the sheep. Fetal plasma ACTH concentrations are higher in singleton fetuses when compared to twin fetuses and the prepartum surge in cortisol occurred earlier in singletons (Edwards & McMillen, 2002a; Rumball *et al.*, 2008c). The prepartum activation of the fetal HPA axis also occurs

earlier in male than female fetuses (Edwards *et al.*, 2002). These findings suggest that there is a delayed activation of HPA axis in ovine twin fetuses, which appears initially to be counter-intuitive as twin pregnancies in often result in preterm delivery which would suggest a precocious rather than a delayed activation of the fetal HPA axis in twins. It has been also demonstrated, however, that ovine adrenal responsiveness to an intravenous bolus of synthetic ACTH is blunted in twins when compared to singleton at ~ 133 days of gestation (Gardner *et al.*, 2004). Furthermore, fetal adrenal weight, expressed as either an absolute weight or relative to body weight, and adrenal CYP17 mRNA are each significantly lower in twins when compared to singletons as early as 55 days of gestation (MacLaughlin *et al.*, 2007). These studies demonstrate that firstly, the growth trajectory and development of the fetal adrenal in the twin is different from that of the singleton sheep fetus and secondly, that these differences are established in early gestation.

It has been suggested that, therefore, the delay in the prepartum activation of the pituitary-adrenal axis in the twin fetus is programmed early in pregnancy and that the blunted adrenocortical responsiveness and the delayed activation of the HPA axis in twin fetal sheep may serve to ultimately protect the twin fetus from preterm delivery (MacLaughlin & McMillen, 2007).

1.7 Intra-Uterine Perturbation and The Fetal HPA Axis

Developmental programming has been defined as “either the induction, deletion or impaired development of a somatic structure or the ‘setting’ of a physiological system by an early stimulus operating during a critical window resulting in long

term consequences for function" (McMillen & Robinson, 2005). The role of excess glucocorticoids, either from endogenous overproduction or exogenous administration to mother or fetus, in the programming of physiological systems has been extensively reviewed (Seckl & Meaney, 2004; Moritz *et al.*, 2005; Sloboda *et al.*, 2005; Drake *et al.*, 2007; Seckl & Holmes, 2007; Seckl, 2008) and a range of experimental studies have shown that excess glucocorticoids during critical developmental windows result in hypertension, glucose intolerance and insulin resistance (Levitt *et al.*, 1996; Dodic *et al.*, 1998; Nyirenda *et al.*, 1998; McMillen & Robinson, 2005). There is also evidence that there may be programming of the fetal HPA axis itself by environmental and nutritional perturbations leading to the setting of a new 'set point' of the axis resulting in altered basal and/or stress induced stress response.

The impact of these perturbations is dependent on the type, severity and duration of the insult and there appear to be a number of critical windows during development, which are vital in the programming of the subsequent reactivity of the HPA axis. Most importantly, any perturbation of this system can result in the disturbance of the normal timing of labour and parturition.

There are a plethora of environmental and nutritional perturbations during development that have been interrogated in a range of animal models including acute and chronic hypoxia, low protein diet and global or single nutrient restriction, including maternal undernutrition. This review will briefly discuss the effects of acute and chronic hypoxemia on the HPA axis and primarily focus on the effects of maternal undernutrition on the HPA axis during a number of critical ontogenic windows in the sheep.

1.7.1 Effects of Acute Hypoxia on the Fetal HPA Axis

A number of studies have shown that hypoxia in late gestation results in the activation of the HPA axis. In fetal sheep, an increase in plasma ACTH and cortisol has been measured in response to acute or short-term hypoxemia (up to 24 hours) in late gestation (Jones *et al.*, 1977; Towell *et al.*, 1987; Jackson *et al.*, 1989; Hooper *et al.*, 1990). A subsequent study involving the disconnection of the fetal hypothalamus and pituitary has shown that a functional hypothalamo-pituitary connection is vital for the generation of this increased plasma ACTH and cortisol to acute hypoxemia (Ozolins *et al.*, 1992). The response of the fetal HPA axis to such intra-uterine stressor presumably facilitates fetal adaptation to sub-optimal conditions. However, sustained elevated glucocorticoid levels for an extended period of time, which may result from chronic or long-term hypoxemia, may have deleterious effects.

1.7.2 Effects of Chronic Hypoxia on the Fetal HPA Axis

It is technically more difficult to investigate the fetal HPA responses to the impact of chronic intrauterine stress, such as chronic hypoxia, however, a number of animal models have been developed to investigate the impact of chronic hypoxia on the fetal HPA axis.

One of the sheep models used to investigate chronic hypoxia and fetal development is the exposure of pregnant sheep and her fetus to high altitude (3820m). In a study by Harvey and colleagues (1993), pregnant ewes were exposed for 90 days to high altitude and subsequently were returned to sea level where their P_aO_2 was maintained at approximately 60 mmHg by N_2 infusion through a tracheal catheter. The fetal P_aO_2 was significantly lower in the

hypoxemic when compared to the normoxemic group, however, there was no significant difference in fetal plasma ACTH and cortisol concentrations between the two groups at ~ 136 days of gestation (Harvey *et al.*, 1993). Remarkably, however, after intra-fetal ACTH administration, there was only a significant increase in fetal plasma cortisol concentration in response to the challenge in the normoxemic, but not the hypoxemic, group (Harvey *et al.*, 1993). The authors suggested that this blunted adrenal response may play a role in the prevention of preterm delivery in the chronically hypoxic sheep.

Other sheep models that have been used to investigate chronic hypoxia include single umbilical artery ligation (SUAL) and placental restriction. In comparison to the 'high altitude' sheep model, where oxygen availability to the fetus is reduced, these two models may limit overall fetal substrate supply, including oxygen and glucose, to the fetus. Additionally, the average fetal P_aO_2 of the hypoxemic group of the 'high altitude' sheep model is 21mmHg whereas the average fetal P_aO_2 in placentally restricted and SUAL groups is 15mmHg and 16mmHg, respectively. This suggests that hypoxemia in the placentally restricted and SUAL models are more severe when compared to the 'high altitude' model.

Single umbilical artery ligation (SUAL) is a technique where one umbilical artery is isolated and ligated close to the fetal abdomen leading to a partial infarction of the placenta. A recent study showed that fetal P_aO_2 was significantly reduced in SUAL fetuses when compared to control fetuses throughout late gestation (Supramaniam *et al.*, 2006). Fetal plasma cortisol concentrations are higher in SUAL fetuses in late gestation and on average these fetuses delivered 6 days before the age when control fetuses underwent postmortem (Supramaniam *et al.*,

2006). Clearly, this model also results in an early activation of the HPA axis and preterm parturition.

Restriction of placental growth from conception can be achieved by uterine carunclectomy, whereby most endometrial caruncles are surgically removed from the uterus of the non-pregnant ewe prior to mating. This results in limited placental growth leading to limited substrate supply, including oxygen and glucose, to the fetus (Phillips *et al.*, 1996b). Relative adrenal weights but not absolute adrenal weights are significantly increased in fetuses from placentally restricted fetuses when compared to control fetuses (Ross *et al.*, 2000b), indicating adrenal sparing. Whilst fetal plasma ACTH concentrations do not differ between the placentally restricted and control groups, fetal plasma cortisol concentrations are significantly higher in the placentally restricted group when compared to the control group in late gestation (Phillips *et al.*, 1996b). This suggests that there is a precocious activation of the HPA axis in placentally restricted fetuses.

1.7.3 Effects of Maternal Undernutrition during early and late Gestation and the Fetal HPA Axis

There are a limited number of studies that have investigated the impact of maternal undernutrition during gestation on the development of the HPA axis in the fetal sheep. It is clear, however, that the effect of maternal undernutrition is dependent on the gestational window during which the ewe was subjected to undernutrition and/or the severity of nutrient restriction.

In a study by Edwards and colleagues (2001) a 50% reduction of maternal nutrient intake for the last 30 days of gestation resulted in a decrease in fetal plasma

glucose concentrations in the undernourished (UN) group when compared to the control group. Maternal undernutrition had no effect, however, on fetal plasma ACTH and cortisol concentration in late gestation but between 135 – 145 days of gestation fetal arterial blood pressure was increased in the UN group and mean arterial blood pressure was positively correlated with fetal plasma concentration of cortisol (Edwards *et al.*, 2001).

A study, which investigated the impact of a 15% reduction of maternal nutrient intake for the first 70 days of gestation in singleton fetuses, showed that there was also no difference in basal ACTH and cortisol fetal plasma concentrations at 114 – 115 and 126 – 127 days of gestation in the control and undernourished (UN) groups (Hawkins *et al.*, 1999). In this study, fetal plasma cortisol concentrations significantly increased between 114 – 115 and 126 – 127 days of gestation in the control but not in the UN group. Interestingly, the ACTH and cortisol responses to CRH/AVP challenges at 113 – 116 days of gestation were blunted in the UN group when compared to the control group (Hawkins *et al.*, 1999) whereas by 125 – 127 days of gestation only the cortisol response to CRH/AVP challenge was decreased in the UN group (Hawkins *et al.*, 1999; Hawkins *et al.*, 2000a). Fetal ACTH and cortisol responses to an endogenous stress stimulus such as hypoxemia were also blunted at 126 – 129 days of gestation in UN group when compared to the control group (Hawkins *et al.*, 2000b). Postnatally, adrenal weights were significantly increased and the ACTH and cortisol response to CRH/AVP challenge was significantly greater in the UN group when compared to the control group at ~ 85 days (Hawkins *et al.*, 2000a). These data suggest that mild maternal undernutrition during the first 70 days of gestation reduces pituitary and adrenal responsiveness to endogenous and exogenous stimuli in the fetus and an

enhanced adrenocortical response after birth. These studies further suggest that mild maternal undernutrition early in gestation not only alters the development of the HPA axis but that these changes are permanent, which is reflected in the increased HPA activity postnatally.

1.7.4 Effects of Maternal Undernutrition during the Periconceptional Period

Whilst nutritional perturbations during gestation can alter the function of the fetal HPA axis (Hawkins *et al.*, 1999; Hawkins *et al.*, 2000a; Edwards *et al.*, 2001), maternal undernutrition during the periconceptional period can also influence the development of this axis (Edwards & McMillen, 2002a; Bloomfield *et al.*, 2003; MacLaughlin *et al.*, 2007; Connor *et al.*, 2009a). The term "periconceptional" is defined as the period around conception and is often used generally to cover reasonably broad windows of early development. Over the past decade two main periconceptional models, investigating the impact of periconceptional maternal undernutrition on the development of the fetal cardiovascular system and the HPA axis, have emerged. These two models have been reviewed by MacLaughlin and McMillen (2007) and coined the "Adelaide" and "Auckland" models (MacLaughlin & McMillen, 2007). It is important to consider the differences between these two periconceptional models when reviewing their outcomes.

There are three main differences between these models including nutritional regime, the length of exposure to and timing of maternal nutrient restriction. The "Adelaide" model restricts maternal intake to 70% of the control level. In contrast the "Auckland" model restricts maternal intake to up to 28% of the control level to achieve a maternal weight loss of ~ 10 – 15% (MacLaughlin & McMillen, 2007). Thus, the "Adelaide" model uses more moderate nutrient restriction whilst the

"Auckland" model uses a relatively more severe nutrient restriction. Control animals are subjected to a maintenance diet in the "Adelaide" model whilst the control animals of the "Auckland" model are fed *ad libitum*. One direct consequence of the difference in feeding regime is that the weight difference between the control animals fed *ad libitum* and the undernourished group is ~ 20kg in the "Auckland" model (MacLaughlin & McMillen, 2007). In contrast, the weight difference between the control and undernourished groups is 2 – 5kg in the "Adelaide" model (Edwards & McMillen, 2002a; Edwards & McMillen, 2002b; Edwards *et al.*, 2005; MacLaughlin *et al.*, 2005). The period during which ewes are subjected to maternal undernutrition start at 60 days before mating and is continued until 7 days after in the "Adelaide" model whereas in the "Auckland" model maternal undernutrition is extended to 30 days after mating. This is an important difference as maternal undernutrition in the "Adelaide" model consists of the preconception and preimplantation periods encompassing oocyte development and maturation, follicular development and recruitment and embryo/blastocyst development including cleavage, blastulation, gastrulation and maternal zygotic transition (Figure 1.7). In contrast, the "Auckland" model encompasses the post implantation period as well as the periods of preconception and preimplantation development of the embryo (Figure 1.7). This extension of maternal undernutrition into the post implantation period may affect maternal histotroph and uterine milk production, development of the uterine glands, and the process of early placentation (MacLaughlin & McMillen, 2007).

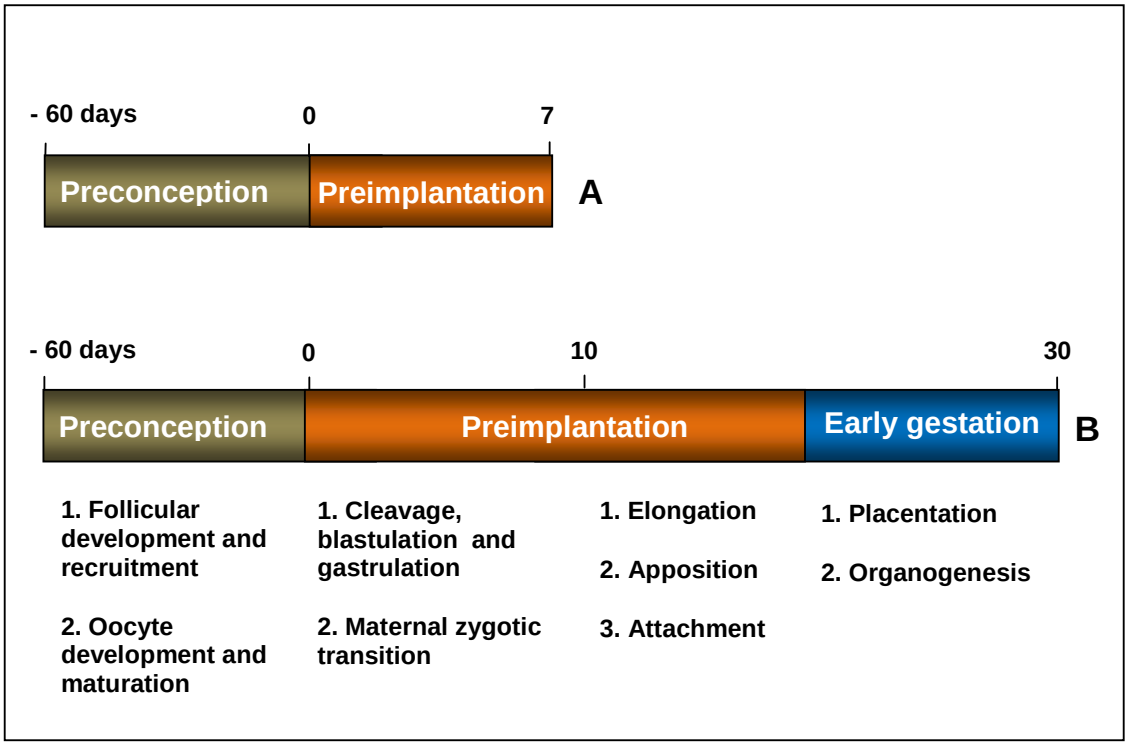


Figure 1.7: Schematic representation depicting the stages of development of the oocyte, embryo and placenta during the periods of exposure to maternal undernutrition in the (A) "Adelaide" model and (B) "Auckland"

1.7.4.1 Effects of maternal undernutrition extending from the periconceptional period into early gestation

In the following section, maternal undernutrition during the periconceptional and early gestational periods refers to the "Auckland" model as described above and the specific effects of maternal undernutrition extending from the periconceptional period in early gestation on fetal development will be reviewed.

1.7.4.1.1 Fetal growth and metabolism

At 131 days of gestation, fetal and placental weights were not different nor were the weights of lambs at term but between ~ 125 days of gestation and birth the fetal growth rate was reduced in singletons from ewes subjected to nutrient restriction when compared to the control group (Oliver et al., 2005). The reduction in IGF1 response to an undernutrition challenge was significantly greater in fetuses from the undernourished group when compared to the control group (Gallaher et al., 1998) and the insulin response to glucose challenge was greater in fetuses from nutritionally restricted ewes when compared to control group in late gestation (Oliver et al., 2001). The authors hypothesised that fetuses from ewes subjected the maternal undernutrition around the time of conception and early gestation "reprograms" IGF1 regulation in the developing sheep fetus in late gestation and that increased insulin responsiveness in these animals may be due to an early maturation of pancreatic cells of the developing fetus (Gallaher et al., 1998; Oliver et al., 2001). This in turn may explain the altered growth trajectory observed of the fetus from ewes that were undernourished. When lambs were subjected to a glucose challenge after overnight fasting the insulin response was similar in both nutritional groups. Interestingly, at 10 months of age, lambs from the undernourished group had a reduced insulin response, suggesting impaired glucose tolerance, when compared to the control group, this was particularly evident in females and singletons (Todd et al., 2009).

These data suggest that maternal undernutrition before conception and during early gestation alters growth and metabolism in the sheep fetus and these changes are permanent as reflected by the altered metabolism in the adult offspring.

1.7.4.1.2 Fetal HPA axis

In ewes that were subjected to a severely restricted diet to reduce their body weight by ~ 15% between 60 days before and 30 days after conception and, which carried singletons, the prepartum fetal cortisol surge occurred earlier and 50% of fetuses delivered prematurely (Bloomfield *et al.*, 2003; Bloomfield *et al.*, 2004; Kumarasamy *et al.*, 2005). At 131 days of gestation, the expression of adrenal CYP17 mRNA was significantly increased in singletons from nutrient restricted ewes when compared to the control group (Connor *et al.*, 2009a). This may in part explain the early activation of the fetal adrenal in singletons from nutritionally restricted ewes. Regardless of timing of the prepartum fetal cortisol surge, the surge preceded the rise in fetal and maternal prostaglandin concentrations, which is required for normal labour and delivery (Kumarasamy *et al.*, 2005). In these animals, fetal plasma ACTH concentrations in response to CRH/AVP challenge were significantly decreased in the UN group when compared to the control group (Rumball *et al.*, 2008c) whilst the cortisol response to ACTH challenge was not different between the nutritional groups at 127 days of gestation (Bloomfield *et al.*, 2004). This suggests that undernutrition before conception and during early gestation affects the responsiveness of the pituitary but not the adrenal. When the fetuses were subjected to a metyrapone challenge, which causes inhibition of cortisol synthesis by inactivation of the steroidogenic enzyme 11-deoxycortisol, the ACTH response was not different between the nutrition groups, however, there was a greater 11-deoxycortisol response in singletons from the UN group when compared to the control group at 128 days of gestation.

In summary, evidence from the literature suggests that maternal undernutrition prior to conception and for the first 30 days of gestation accelerates the maturation

of the HPA axis in singleton fetuses and that adrenal function in these animals is altered.

During this period of undernutrition maternal plasma ACTH and cortisol concentrations were decreased in undernourished ewes and the expression of MC2R, StAR and CYP17 mRNA were down regulated in the maternal adrenal (Jaquiery *et al.*, 2006) clearly indicating suppression of maternal HPA activity leading the authors to hypothesise that fetal exposure to excess maternal glucocorticoids is “unlikely” to explain the effects of maternal undernutrition. At 50 and 85 days of gestation, however, placental 11 -hydroxysteroid dehydrogenase-2, the enzyme that inactivates cortisol to cortisone, is significantly lower in ewes subjected to nutrient restriction when compared to the control group (Jaquiery *et al.*, 2006; Connor *et al.*, 2009b) and although fetal plasma cortisol and cortisone were significantly lower in fetuses from the undernourished group, the cortisol:cortisone ratio of these animals was significantly greater than the control group (Connor *et al.*, 2009b). This suggests that there may be a greater exposure of the fetus to cortisol and the possibility of fetal exposure to excess glucocorticoid resulting in altered neuroendocrine, metabolic and cardiovascular development cannot be discounted.

1.7.4.2 Effects of maternal undernutrition during the periconceptional period alone

In the following section maternal undernutrition during the periconceptional period alone, as discussed in the “Adelaide” model and its specific effects on fetal development will be reviewed.

1.7.4.2.1 Placental and fetal growth

A study by MacLaughlin and colleagues (2005) demonstrated that in ewes carrying control singletons, the maternal weight increase during the periconceptional period is accompanied by an increase in placental and fetal growth at ~ 55 days of gestation (MacLaughlin *et al.*, 2005). Interestingly, this direct positive relationship between maternal weight and placental growth is absent in undernourished ewes carrying singletons and is reversed in twin-carrying undernourished ewes, therefore in twin pregnancy the more weight the ewe lost during the periconceptional period the larger the placenta and fetuses grew (MacLaughlin *et al.*, 2005). In addition, the ponderal index, was significantly lower in twins from nutrient restricted ewes when compared to the control group (MacLaughlin *et al.*, 2005).The authors hypothesised that the presence of multiple embryos, concepti or copora lutea results in a pregnancy that is more vulnerable to the effects of moderate maternal nutrient restriction during the periconceptional period and that the periconceptional environment is important in setting the placental and fetal growth trajectories with implication in the programming of endocrine systems.

1.7.4.2.2 Fetal HPA axis

There were elevated fetal plasma ACTH concentrations between 115 days of gestation and 146 days of gestation and the cortisol response to a bolus dose of corticotrophin-releasing hormone was significantly greater at 139 – 144 days of gestation in twin fetuses of ewes undernourished during the periconceptional period (PCUN) when compared to the twin control group (Edwards & McMillen, 2002a). The increased circulating ACTH concentration and adrenal responsiveness to ACTH in the fetal PCUN twin occurred in the absence of either

an increase in adrenal MC2R or adrenal steroidogenic enzyme mRNA expression (Edwards *et al.*, 2002). These observations, in contrast to the findings of the "Auckland" model, indicate that moderate nutrient restriction during the periconceptional period alone has an impact on the development of the HPA axis in the fetal twin only. It may be that a more severe level of nutrient restriction or the extension of undernutrition into early gestation is required before the effects on the fetal HPA axis in singleton fetuses are recruited. It is not clear, however, whether the effects resulting from periconceptional undernutrition are due to the impact of maternal undernutrition on oocyte development or on embryo development during the preimplantation period or whether the impact occurs on both the oocyte and embryo.

Interestingly, the impact of periconceptional nutritional restriction occurs at a time when the nutrient demands of the embryo are minimal, but it is also a period during which, the entire genome of the embryo undergoes demethylation and re-establishment of methylation. Methylation in this context represents an epigenetic mechanism, that is, it results in a change in gene expression, which is not a result of a change in the DNA sequence (Jaenisch & Bird, 2003). DNA methylation can be affected by nutrition as dietary methyl donors and cofactors are involved in mammalian 1-carbon metabolism/methylation cycle (Waterland & Jirtle, 2004).

Therefore, nutritional perturbations may influence DNA methylation patterns during the preimplantation period resulting in permanently altered gene expression later in life and it is reasonable to speculate that if there is a change in availability of methyl donors and cofactors, due to nutrient availability, then nutritional regimes during early developmental windows may have an impact on the regulation of

gene expression within specific cell lineages of the embryo that may be permanent and heritable. Imprinted genes are particularly susceptible because of their epigenetic lability. Thus, one possibility is that it is at the level of epigenetic regulation of gene expression at which the early nutritional perturbation has an effect on the subsequent development of the fetal HPA axis. As highlighted above the *Igf2* and *Igf2r* genes are parentally imprinted genes, but to date there have been no studies on the effects of perturbations of periconceptional level of nutrition on the expression of these genes in the late gestation fetal adrenal.

In Chapter 2, I have described the impact of embryo number and/or moderate maternal undernutrition during the periconceptional period (-60 days to +6 days after conception) or during the preimplantation period alone (0 to 6 days after conception) on fetal and placental growth during late pregnancy.

In Chapter 3, I have tested the hypothesis that the impact of embryo number will result in a delayed activation and decreased responsiveness of the HPA axis whilst the impact of moderate maternal undernutrition during the periconceptional period (-60 days to +6 days after conception) or during the preimplantation period alone (0 to 6 days after conception) will each result in an early activation and increased responsiveness of the HPA axis in twins compared to singletons during late pregnancy.

In Chapter 4, I have tested the hypothesis that maternal undernutrition during the periconceptional period (-60 days to +6 days after conception) or during the preimplantation period alone (0 to 6 days after conception) will each result in an increase in IGF2R and CYP17 mRNA expression and a decrease in IGF2 and TGF 1 mRNA expression in the fetal adrenal in late pregnancy.

1.8 CULTURING EMBRYOS *IN VITRO*: EFFECTS OF MANIPULATIONS OF THE EARLY EMBRYONIC ENVIRONMENT ON DEVELOPMENT

Although the first successful embryo transfer occurred in rabbits in 1890 (Heape, 1890), it took 60 years before a live calf was produced from an embryo transfer (Betteridge, 2000) and another 30 years before Steptoe and Edwards announced the first successful human pregnancy and birth from in *vitro* fertilisation (Steptoe & Edwards, 1978). Today, Assisted Reproductive Technologies (ART) are used to overcome poor fertility and reproductive challenges in the human, to manage and conserve wildlife and to increase the number of offspring from "superior" females and to reduce the generation intervals in farm species (Boerjan *et al.*, 2000; Pukazhenthi & Wildt, 2004).

The main technologies used to assist reproduction in humans include *in vitro* fertilisation (IVF) and its highly specialised variant intracyoplasmic sperm injection (ICSI), gamete intrafallopian transfer (GIFT) and its variant zygote intrafallopian transfer (ZIFT). In the US, the most commonly used ART procedures in 2006 were IVF (~ 37.5%) and ICSI (~ 62.2) whilst GIFT and ZIFT were each used only

marginally (< 0.01 and 0.02 %, respectively) (Braude & Rowell, 2003). Each of these techniques requires superovulation, sperm preparation and assisted fertilisation (Rowell & Braude, 2003) but most importantly the processes of culturing embryo *in vitro* and embryo transfer are an essential steps in IVF, ICSI and ZIFT. Interestingly, the success rates are higher for day 5 human embryo transfers than for day 3 transfers (Linacre, 2007).

Assisted reproductive technologies used for ruminant species are artificial insemination (AI), multiple ovulation embryo transfer (MOET) and *in vitro* embryo production (IVP) (Cognie *et al.*, 2003; Mapletoft & Hasler, 2005; Lonergan, 2007). IVP is a process that involves three components namely oocyte maturation, oocyte fertilization and *in vitro* culture of the embryo (Lonergan, 2007).

There is accumulating evidence demonstrating that *in vitro* culture of the human or ruminant embryo during zygote or later embryo development is associated with altered fetal and postnatal development (Young *et al.*, 1998; Thompson & Peterson, 2000; Walker *et al.*, 2000; Farin *et al.*, 2001; Farin *et al.*, 2004; Bower & Hansen, 2005; Hansen *et al.*, 2005; Shiota & Yamada, 2009). However, the effects of exposing the preimplantation embryo to the *ex vivo* environment of *in vitro* culture on the development of the HPA axis have not been investigated.

1.8.1 Effects of in vitro Culture and Embryo Transfer on Human Development

In 2004, an estimated 2.5% of all births in Australia were the result of ART treatments (Linacre, 2007). Between 1989 and 2004, there was a 74% increase in live births resulting from ART treatments in New Zealand, whilst the number of live births occurring in the US as a result of ART treatments increased by ~ 250%

between 1996 – 2006 (Linacre, 2007; CDC, 2008). These ART treatments, which include culturing embryos *in vitro* and embryo transfer, result in an increased prevalence of obstetric problems including vaginal bleeding, placental abruption, placenta previa and caesarean births (Tan *et al.*, 1992; Koivurova *et al.*, 2002; Schieve *et al.*, 2007). In terms of pregnancy outcomes, it has been demonstrated that ART treatment is linked to an increase in pregnancy loss, multiple pregnancies, premature birth, low and very low birth weight, perinatal mortality and congenital malformation (Tan *et al.*, 1992; McFaul *et al.*, 1993; Wang *et al.*, 1994; Buckett *et al.*, 2007; Schieve *et al.*, 2007; Alukal & Lamb, 2008). Initially low birth weight and premature birth has been suggested to be a result of the increased incidence of multi-fetal pregnancies but subsequent evidence has clearly demonstrated that singleton pregnancies from ART treatments have an equal, if not greater risk for low birth weight and premature delivery when compared to singletons from non-assisted pregnancies (Schieve *et al.*, 2002; Helmerhorst *et al.*, 2004). Postnatally, there is evidence suggesting that children conceived from ART treatments have an increased risk of developing neurological impairments such as cerebral palsy (Stromberg *et al.*, 2002) and that growth rate is significantly reduced for the first three years of life when compared to naturally conceived children (Koivurova *et al.*, 2003).

1.8.2 Effects of in vitro Culture and Embryo Transfer on Ovine and Bovine Development

The most commonly used ART in ruminants is artificial insemination, however, the use of embryo technologies, such as culturing embryos *in vitro* and/or embryo transfer, are rising. It has been speculated that embryo technologies may become as dominant as AI because of their potential genetic improvement, including

increasing the efficiency of quantitative genetic selection and simplification in cross-breeding, in farm species (Hansen & Block, 2004). At present, however, the use of embryo technologies is limited because of the high cost and problems, such as the development of abnormal fetuses and offspring, arising from these technologies. In particular, during the nineties attention of scientific enquires was drawn to unusually large offspring as a result of ART and embryo culture, which was first reported by Willadsen and colleagues (1991) who noted that, although of normal appearance, some offspring were very large (Willadsen *et al.*, 1991). Culturing ovine and bovine embryos in various *in vitro* systems also resulted in offspring exhibiting the prominent feature of being abnormally large at birth (Walker *et al.*, 1996a; Young *et al.*, 1998). Before the end of the decade the term "Large Offspring Syndrome" (LOS) had been formulated to describe abnormal characteristics arising from the exposure of an ovine and bovine embryo to an *ex vivo* environment during early development (Walker *et al.*, 1996a; Young *et al.*, 1998).

One of the protein sources used in embryo culture systems is serum, which consists of hormones, proteins and growth factors (van Wagtendonk-de Leeuw *et al.*, 2000). Culturing systems using serum as a protein source results in an increase in embryo growth and blastulation rates but a decrease in ICM cell numbers of the blastocyst (Walker *et al.*, 1992a; Walker *et al.*, 1998). Interestingly, culturing embryos *in vitro* in the presence of serum reliably results in a proportion of offspring exhibiting characteristics of LOS (Thompson *et al.*, 1995; Sinclair *et al.*, 1999). LOS is characterised by significantly increased prenatal losses, enhanced fetal weight and weight at birth, organomegaly (abnormal enlargement of organs), including heart, liver and kidney, increased skeletal growth, physical

deformities, an increase in the mortality rate of the offspring and a delay in the timing of parturition (Willadsen *et al.*, 1991; Walker *et al.*, 1992b; Keefer *et al.*, 1994; Farin & Farin, 1995; Holm *et al.*, 1996; Young *et al.*, 1998; Sinclair *et al.*, 1999). Interestingly, the increase in gestation length fails to explain the enhanced fetal weight and weight at birth (Walker *et al.*, 1996a). Clearly, in ruminants the exposure of a preimplantation embryo to an *ex vivo* environment can have a profound effect on the developing fetus.

A study by Young and colleagues in 2001 interrogated a potential mechanism to attempt to explain fetal overgrowth observed in LOS offspring. In this study it was demonstrated that ovine fetuses, which exhibited LOS, had a significantly lower IGF2R mRNA expression in the liver, heart and kidneys of fetal sheep when compared to control animals at 125 days of gestation (Young *et al.*, 2001). It was shown that the observed decrease in IGF2R mRNA expression was due to an epigenetic change - the loss of methylation in the *Igf2r* gene (Young *et al.*, 2001). The authors argued that the decrease in mRNA expression of IGF2R, the clearance receptor for IGF2, would therefore contribute to an increase in bioavailability of IGF2 in fetal organs, which could explain the observed increase in organ size. Young and colleagues (2001), however, did not investigate whether there is an association of culturing embryos *in vitro*, fetal adrenal growth and the expression of the intra-adrenal IGF system.

As highlighted above culturing embryos *in vitro* in the presence of serum can result in LOS, which is associated with delayed parturition and a decrease in the parentally imprinted *Igf2r* gene. Thus, one possibility is that it is at the level of epigenetic regulation of adrenal *Igf2r* expression in embryos cultured *in vitro* in the

presence of serum which may alter the subsequent development of the fetal HPA axis. To date, however, there have been no studies on the effects of culturing embryo *in vitro* in the presence of serum on the growth of the fetal adrenal, the concentrations of ACTH and cortisol and the expression of the adrenal *Igf2* and *Igf2r* genes in the late gestation.

In Chapter 5, I have, therefore, tested the hypothesis that embryo transfer with *in vitro* culture in the presence of human serum results in a delayed activation of the HPA axis, a decrease in adrenal IGF2R and CYP17 mRNA expression and an increase in adrenal IGF2 and TGF 1 mRNA expression in the fetal adrenal in late gestation when compared to naturally conceived controls.

1.9 AIMS

The activation of the HPA axis is central for the normal timing of parturition and the maturation of fetal organs to ensure a successful transition from intra- to extra-uterine life. It is evident from experimental and clinical studies that nutritional perturbations and manipulations of the early environment of an embryo impact on fetal development and gestation length. It is not clear whether the effects resulting from periconceptional undernutrition are due to the impact of undernutrition during oocyte and embryo development or the impact of undernutrition during the development of the preimplantation embryo alone. Furthermore, the molecular mechanisms by which perturbations and manipulations of the early embryonic environment impact on the development and activation of the HPA axis have not been investigated.

In summary, the evidence presented in this thesis suggests that nutritional perturbations and manipulation of the early embryonic environment result in an alteration in adrenal development and in altered prepartum activation of the fetal HPA axis.

This thesis, therefore, investigates the effects of moderate maternal undernutrition during the periconceptional period and during the preimplantation period alone and of culturing embryos *in vitro* on the growth and development of the adrenal in late gestation in singleton and twin fetuses in the sheep.

The aims of this thesis are:

Chapter 2

1. **To determine the effects of fetal number and/or maternal undernutrition during the periconceptional period and during the preimplantation period alone on fetal and placental growth in late gestation**

- Ewes were fed either a maintenance (Control) diet, a reduced periconceptional diet of 70% of maintenance from 60 days before until 6 days after mating (PCUN), or a reduced preimplantation diet of 70% of maintenance for the first 6 days of gestation (PIUN). From day 8 of gestation until postmortem at ~ 137 days of gestation all ewes were fed a maintenance diet, therefore three nutritional treatment groups were generated (Control, PCUN and PIUN). In this chapter we determined the effects of fetal number, PCUN and PIUN on:

  - placental weight;
  - fetal growth; and
  - fetal organ weight;

  in singleton and twin pregnancies at 136 – 138 days of gestation.

Chapter 3

2. **To determine the effects of fetal number and/or maternal undernutrition during the periconceptional period and during the preimplantation period alone on the development of the HPA axis in late gestation**

- Ewes were fed either a maintenance (Control) diet, a reduced periconceptional diet of 70% of maintenance from 60 days before until 6 days after mating (PCUN), or a reduced preimplantation diet of 70% of maintenance for the first 6 days of gestation (PIUN). From day 8 of gestation until postmortem at ~ 137 days of gestation all ewes were fed a maintenance diet, therefore three nutritional treatment groups were generated (Control, PCUN and PIUN). Fetal plasma was collected between 119 and 138 days of gestation for the measurement of glucose, ACTH and cortisol concentrations. The ACTH and cortisol responses to CRH were measured between 130 and 132 days of gestation. In this chapter we determined the effects of fetal number, PCUN and PIUN on:

    - fetal plasma concentrations of glucose, ACTH and cortisol; and
    - fetal ACTH and cortisol concentrations in response to CRH;

  in singleton and twin pregnancies.

Chapter 4

3. To determine the effects of fetal number and/or maternal undernutrition during the periconceptional period and during the preimplantation period alone on adrenal growth, the expression of IGFs, CYP17 and TGF 1 mRNA and the epigenetic state of *Igf2* and *Igf2r* in late gestation

- Ewes were fed either a maintenance (Control) diet, a reduced periconceptional diet of 70% of maintenance from 60 days before until 6 days after mating (PCUN), or a reduced preimplantation diet of 70% of

maintenance for the first 6 days of gestation (PIUN). From day 8 of gestation until postmortem at ~ 137 days of gestation all ewes were fed a maintenance diet, therefore three nutritional treatment groups were generated (Control, PCUN and PIUN). In this chapter we determined the effects of fetal number, PCUN and PIUN on:

- Fetal adrenal weight; and
- adrenal expression of IGF1, IG2, IGF1R, IGF2R, CYP17 and TGF 1 mRNA
- methylation state of adrenal *Igf2* and *Igf2r* genes

in singleton and twin pregnancies at 136 – 138 days of gestation.

Chapter 5

4. To determine the effect of embryo number and/or culturing embryos *in vitro* on the development of the HPA axis including adrenal growth and adrenal expression of IGFs, CYP17 and TGF 1 mRNA in late gestation

- Ovine embryos were either transferred to an intermediate recipient ewe until day 6 of pregnancy (ET group) or cultured in 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) for 6 days. On day 6, embryos were collected from the intermediate recipients of the ET group, and embryos from the ET, IVCNS and IVCHS groups were each transferred to synchronized final recipient ewes. A group of ewes were naturally mated (NM) and served as control group, therefore, thus generating four treatment

groups (NM, ET, IVCNS and IVCHS). In this chapter we determined the effects of embryo number and embryo culture on:

- Fetal and adrenal weights;
- fetal plasma concentrations of ACTH and cortisol; and
- adrenal expression of IGF1, IG2, IGF1R, IGF2R, CYP17 and TGF 1 mRNA

in singleton and twin pregnancies at 144 - 145 days of gestation.

CHAPTER 2: IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON PLACENTAL AND FETAL GROWTH IN THE SHEEP DURING LATE GESTATION

2. IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON PLACENTAL AND FETAL GROWTH IN THE SHEEP DURING LATE GESTATION

2.1 INTRODUCTION

Perturbations during critical developmental widows during gestation have an impact on fetal development and offspring health. A range of epidemiological, clinical and experimental studies show that undernutrition before and immediately after conception alters fetal and adult health outcomes. The circumstances of the Dutch Winter Famine, which emerged and ended suddenly during 1944-45 and lasted for a period of 5 months when Germany imposed a food embargo, presented a unique opportunity to investigate the effect of malnutrition of the pregnant woman on the health outcomes of the offspring. There have been a number of studies, which have investigated the effects of malnutrition experienced by pregnant women during the Dutch Winter Famine in the first trimester alone. These studies have found that offspring exposed during the first trimester alone, not only had an increased prevalence of coronary heart disease in adult life (Roseboom *et al.*, 2000) but also had an earlier onset of coronary heart disease (Painter *et al.*, 2006). It was further shown, that women who were conceived during the famine had an almost five times increased risk of breast cancer (Roseboom *et al.*, 2006) and a higher body mass index later in later life (Ravelli *et al.*, 1999).

As summarised in Chapter 1, previous studies in the sheep have shown that undernutrition during the periconceptional period results in altered fetal and placental growth trajectories and there is evidence demonstrating that exposure to

undernutrition in this early period may reprogram development of the cardiovascular and endocrine systems (Edwards & McMillen, 2002a; Edwards & McMillen, 2002b; Bloomfield *et al.*, 2004; MacLaughlin *et al.*, 2005; Oliver *et al.*, 2005; MacLaughlin *et al.*, 2007). A 30% reduction in maternal nutrient from 60 days before until 7 days after mating resulted in an increase in arterial blood pressure in the ovine fetus in late gestation (Edwards & McMillen, 2002b). In another study using the same feeding regime, Edwards and McMillen (2002) showed that the activation of the fetal hypothalamus-pituitary-adrenal (HPA) axis in late gestation occurred earlier in twins (but not singletons) from the periconceptional undernutrition group when compared to the control group (Edwards & McMillen, 2002a). When maternal nutrient intake was restricted more severely for 60 days before until 30 days after mating a precocious activation of the HPA axis was observed in singleton fetuses and there was a higher probability of preterm delivery (Bloomfield *et al.*, 2004).

In summary, human and animal studies clearly indicate that the periconceptional period is an ontogenic window during which changes in maternal nutrition may have long-term consequences. It is not clear, however, whether the observed effects resulting from periconceptional undernutrition are due to the effects of poor maternal nutrition during oocyte and embryo development or during early embryonic development alone. Events during the preimplantation period, such as genome wide demethylation followed by *de novo* methylation of the entire embryo, may render this period particularly susceptible to changes in nutritional regime. To date, there are no studies, which have investigated the effects of moderate maternal undernutrition during the preimplantation period alone on fetal and placental growth and development in the sheep during late gestation.

In this study we have, therefore, investigated the effects of maternal undernutrition during the whole periconceptional period, from 60 days before until 6 days after mating, and maternal undernutrition during the early preimplantation period alone, that is, for the first 6 days of gestation, on fetal and placental growth at 136 – 138 days of gestation.

2.2 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.

2.2.1 Nutritional Management

Sixty-three South Australian Merino ewes were used in this study. Ewes were fed a diet, which consisted of lucerne chaff and pellets containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime, and molasses (Johnsons & Sons Pty. Ltd., Kapunda, South Australia, Australia). Eighty percent of the total energy requirements were obtained from the lucerne chaff and twenty percent of the energy requirements form the pellet mixture. The lucerne chaff provided 8.3MJ;kg metabolisable energy, 193 g/kg of crude protein and contained 85% dry matter and the pellets provided 8.0 MJ/kg metabolisable energy, 110g/kg of crude protein and contained 90% dry matter. All ewes received 100% of nutritional requirements to provide sufficient energy for the maintenance of a non-pregnant ewe as defined by the Agricultural and Food Research Council in 1993 (Council, 1993). At the end of an acclimatization period, ewes were randomly assigned to one of three feeding regimes:

Control (C) (n = 21): The Control ewes received 100% of the metabolisable energy requirements (MER), from around 60 days prior mating until 6 days after mating (Table 2.1). The Preconception period is defined as 60 days before mating whilst the early Preimplantation period is defined as the first 6 days of pregnancy. The sum of these two periods is defined as the Periconceptional period.

Table 2.1: Feeding duration in Control animals

	Length of time for nutritional regime (days)	
	Mean ± SEM	**Range**
Preconception period 100% MER	84.5 ± 7.0	46 - 131
Preimplantation period 100% MER	6 ± 0.0	6
Periconceptional period 100% / 100 % MER	94.4 ± 10.4	52 - 137

Periconceptional Undernourished (PCUN) (n = 21): The PCUN ewes received 70% of the control allowance from approximately 60 days prior mating until 6 days after mating (Table 2.2). All of the dietary components were reduced by an equal amount in the restricted diet.

Table 2.2: Feeding duration in PCUN animals

	Length of time for nutritional regime (days)	
	Mean ± SEM	**Range**
Preconception period 70% MER	76.8 ± 4.0	51 - 125
Preimplantation period 70% MER	6 ± 0.0	6
Periconceptional period 70% / 70% MER	82.8 ± 4.0	57 - 131

Preimplantation Undernourished (PIUN) (n = 21): The PIUN ewes were maintained on the 70% diet from mating until 6 days after mating only (Table 2.3). All of the dietary components were reduced by an equal amount in the restricted diet.

Table 2.3: Feeding duration in PIUN animals

	Length of time for nutritional regime (days)	
	Mean ± SEM	**Range**
Preconception period 100% MER	80.3 ± 7.7	51 - 125
Preimplantation period 70% MER	6 ± 0.0	6
Periconceptional period 100% / 70% MER	86.3 ± 7.7	57 - 131

From day 7 of pregnancy, all ewes were fed a control diet (100% of requirements) until postmortem at day 136 - 138 of pregnancy.

2.2.2 Mating and Pregnancy

Ewes were released in a group every evening with rams of proven fertility that were fitted with harnesses and marker crayons. Ewes were individually penned the following morning 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 day 0. Ewes were weighed approximately every week after commencing the feeding regime until postmortem at day 136 – 138 of pregnancy. Pregnancy and fetal number were estimated by ultrasound between 40 and 80 days of gestation and 48 of the total 63 ewes were pregnant (Figure 2.1). The number of fetuses carried by each ewe was confirmed at post-mortem (Figure 2.2).

Figure 2.1: Summary of ewe numbers in each protocol

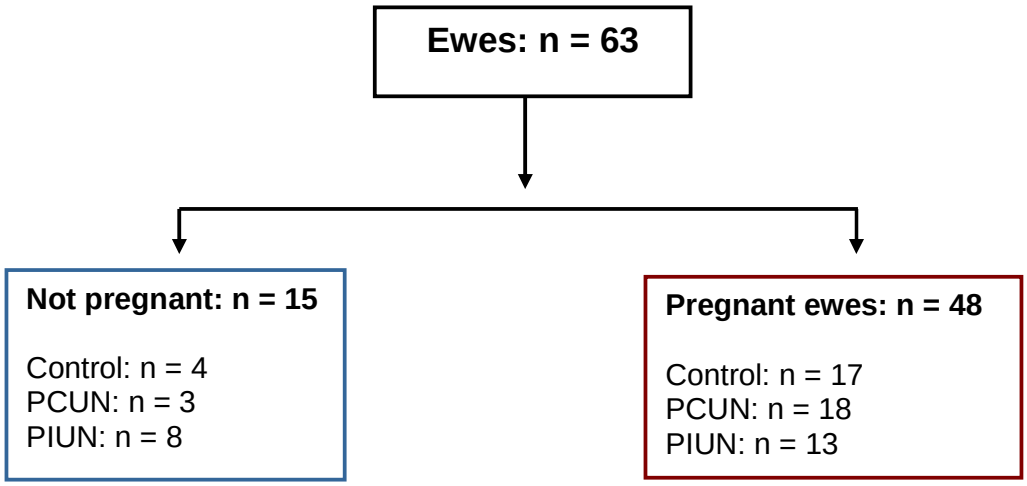
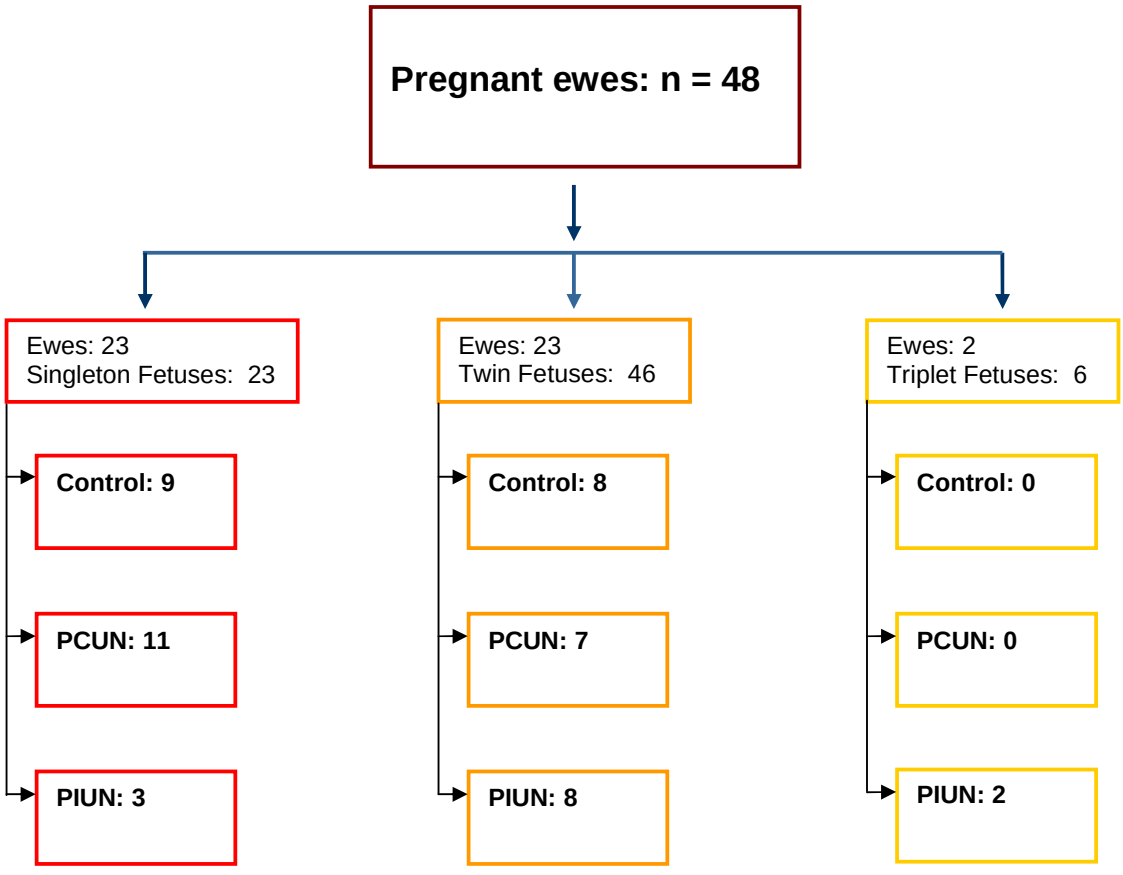


Figure 2.2: Summary of Pregnancy Outcomes



2.2.3 Animals and Surgery

Pregnant ewes (C: n = 17; PCUN: n = 18; PIUN: n = 13) (Figure 2) were transferred to the Medical School Animal House between 90 and 100 days of gestation (term = 150 ± 3 days of gestation). Ewes were fasted for 24 hours prior to surgery. Surgery was performed under general anaesthesia and aseptic conditions between 105 and 110 dg as previously described (Edwards & McMillen, 2002b). In brief, vascular catheters were inserted in a fetal carotid artery and jugular vein, a maternal jugular vein and the amniotic cavity. Vascular catheters were only inserted into one fetus in twin pregnancies. All catheters were filled with heparinized saline, and the fetal catheters exteriorized through an incision made in the ewes' flank. All ewes and fetal sheep received a 2 ml intramuscular injection of antibiotics (procaine penicillin 250 mg/ml, dihydrostreptomycin 250 mg/ml, and procaine hydrochloride 20 mg/ml; Penstrep Illium; Troy Laboratories, Smith-field, NSW, Australia) at the time of surgery.

2.2.4 Post Surgery

The ewes were housed in individual pens in animal holding rooms with a 12 hour light/dark cycle and fed once daily at 1100 h with water provided *ad libitum*. Animals were allowed to recover from surgery for at least 4 days before experimentation.

2.2.5 Maternal Health, Fetal Outcome and Postmortem

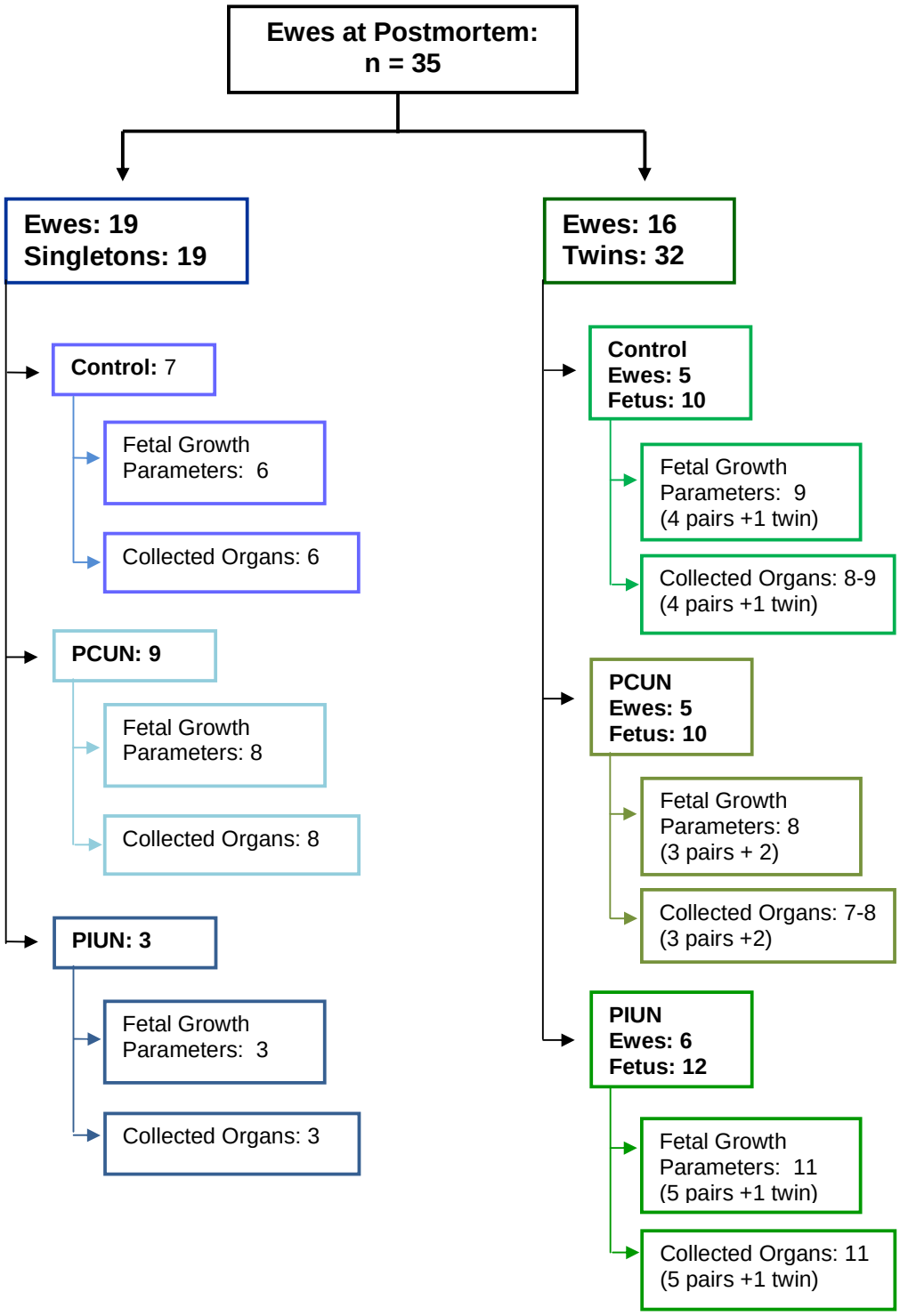
Three ewes in the Control group displayed irregular eating patterns followed by cessation of food intake and one ewe in the PCUN group exhibited hypoproteinaemia. Following veterinary consultation regarding these animals' health, the ewes were euthanased. One singleton fetus in the Control group and

one twin fetus of the PIUN group died shortly after surgery and these ewes were subsequently euthanased. Two ewes in the PIUN group carried triplets and these animals were excluded from the study.

A total of 13 fetal sheep delivered or died prior to post-mortem at 136 – 138 days of gestation and within 48 hours of fetal delivery or death, ewes and fetuses (if necessary) were killed with an overdose of pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia).

All other ewes (n = 35) were killed with an overdose of sodium pentobarbitone between 136 and 138 days of gestation, and the utero-placental unit was delivered by hysterotomy. Fetuses were immediately weighed and killed by decapitation. Fetal organs were then collected, weighed and samples were snap frozen in liquid nitrogen (Figure 2.3). Samples were then stored at -70° Celsius for further molecular analysis.

Figure 2.3: Summary of animal numbers at postmortem



2.2.6 Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM).

2.2.6.1 Ewe weights

The effects of periconceptional and preimplantation undernutrition on ewe weight and the change in ewe weight were determined using a one way Analysis of Variance (ANOVA) using the Statistical Package for Social Scientists (SPSS) for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A two way repeated measures ANOVA (mixed model) was used to determine the effects of time and nutritional treatment on maternal weight change in the periconceptional period. The Bonferroni post-hoc test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P<0.05$) was taken to be significant.

2.2.6.2 Mating and pregnancy outcomes

The effects of periconceptional and preimplantation undernutrition on pregnancy rates and on the proportion of singleton and twin pregnancies were compared using a Chi Squared Test. The effects of periconceptional and preimplantation nutrition on fetal survival rates were also compared using a Chi Square Test.

2.2.6.3 Placentome analysis

The effects of maternal nutritional treatment and fetal number on total placentome number, mean placentome weight, individual fetal weight, total fetal weight (twins), placental weight per fetus and placental weight per pregnancy were determined by a two way ANOVA using SPSS. Repeated measures ANOVA (mixed model) was

used to determine the effects of nutritional treatment and fetal number on mean placentome weight, total placentome weight and the total placentome number of each type of placentome. The Bonferroni post-hoc test was used post-ANOVA to identify significant differences between mean values. Relationships between fetal weights and placental weights were assessed by linear regression using Sigma Plot 10.0 (SPSS Inc., Chicago, IL, USA). A probability level of 5% ($P<0.05$) was taken to be significant.

2.2.6.4 Fetal growth measures and organ weights

The effects of maternal nutritional treatment and fetal number on growth measures (fetal weight, crown rump length, abdominal circumference and ponderal index) and fetal organ weights, expressed as absolute weights and relative to body weight, were determined using a two way ANOVA. When there was an interaction between the effects of nutritional treatment and fetal number, data from singletons and twins were split and the effects of nutritional treatment determined using a one way ANOVA. When there was a significant effect of nutritional treatment in the absence of any interaction between the effects of treatment and fetal number, fetal organ weight data are generally pooled for presentation. The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P<0.05$) was taken to be significant.

2.3 RESULTS

2.3.1 Ewe Weights

The weights of the non-pregnant ewes assigned to control, periconceptional or preimplantation nutrition treatment groups were not different before the start of the feeding regime (Figure 2.4).

Ewes in the PCUN group lost significantly more weight between 60 days before mating and mating or 6 days after mating when compared to the Control and PIUN groups (Figure 2.5 and Figure 2.6).

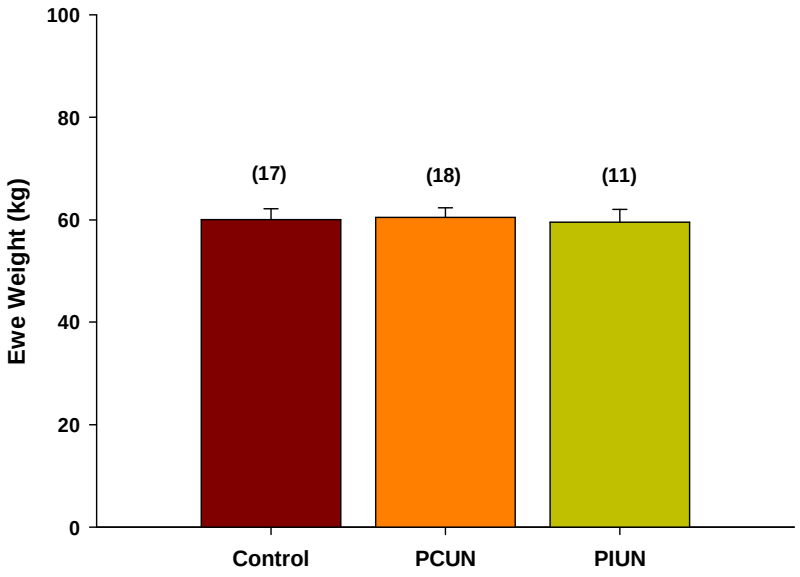


Figure 2.4: Non-pregnant ewe weight prior the start of the nutritional regime

There was no significant difference in ewe weights (mean ± SEM) before the beginning of the nutritional regime.

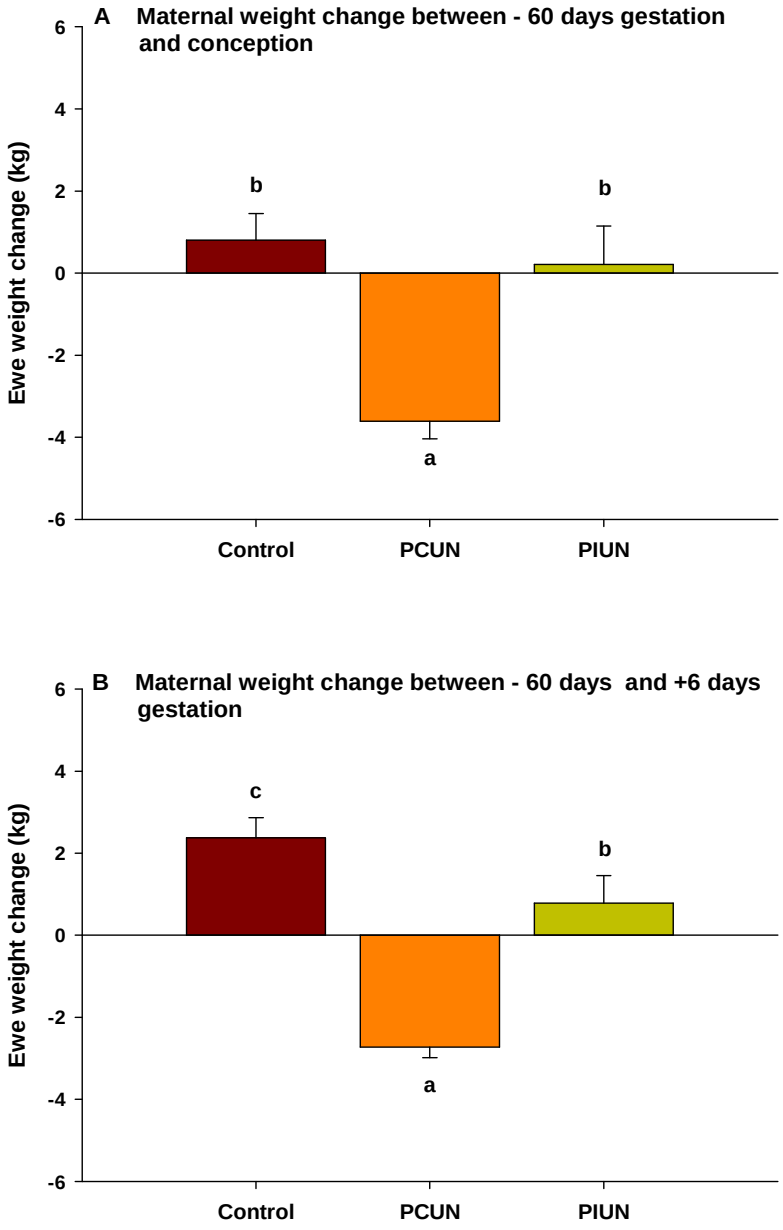


Figure 2.5: Change in maternal weight during the periconceptional period

There was a significant difference in weight change between 60 days before mating and mating (A) and 6 days after mating (B) in PCUN (n = 18) ewes when compared to the Control (n = 17) and PIUN (n = 11) groups. There was a significant weight change in the PIUN group compared to the Control group when the 6 days preimplantation period was included (B). Different alphabetic subscripts denote significant differences between mean values.

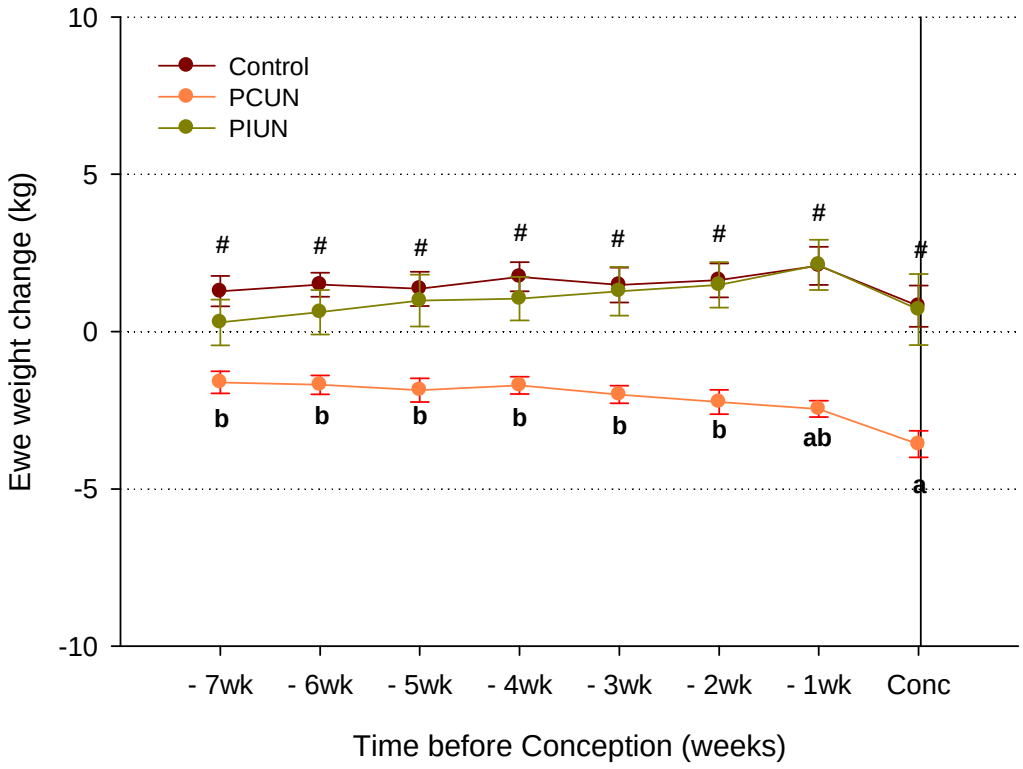


Figure 2.6: The effect of maternal nutritional treatment during the preconceptional period on the change in weight in non-pregnant in ewes

There was a significant weight change in the ewes in the PCUN group (n = 18) during the 7 weeks before conception, as denoted by the different alphabetic subscripts which denote significant differences between mean values at different time points before conception. There was no significant change in ewe weight during the preconceptional period in the Control (n = 17) and PIUN (n = 11) groups. The change of weight was significantly greater in the PCUN group when compared to both Control and PIUN group, as denoted by #.

2.3.2 Mating Outcomes

Overall, there was a 76.2% pregnancy success rate in ewes (n = 48) used in this study. There was no difference in the pregnancy rates of ewes in the different nutrition groups, which were 81% in the Control group, 85.7% in the PCUN group and 61.9% in the PIUN group. There was also no difference in the proportion of singleton (Control 52.9%, PCUN 61.7% and PIUN 27.3%) or twin (Control 47.1%, PCUN 38.9% and PIUN 72.7%) pregnancies between the nutrition groups.

2.3.3 Pregnancy Outcomes and Fetal Survival

The fetal outcomes of the ewes carrying singletons (n = 22) and twins (n = 18) are shown in Table 2.4. There was no effect of either fetal number or maternal undernutrition on the percentage of fetal sheep which survived to post-mortem at 136 – 138 days of gestation

2.3.4 Placental Growth

There was no effect of periconceptional and preimplantation undernutrition on fetal weight, placental weight, total placentome number and mean placentome weight per fetus or per pregnancy at 136 – 138 days of gestation (Table 2.5). The total placental weight ($P < 0.0001$) and placentome number ($P < 0.03$) per fetus was significantly smaller whilst the mean placentome weight per fetus was significantly greater ($P < 0.03$) in twins when compared to singletons. There was no effect of fetal number on fetal weights (Table 2.5). There was a significant increase in the total fetal weight ($P < 0.03$), total placental weight ($P < 0.0001$), total placentome number ($P < 0.0001$) and mean placentome weight ($P < 0.0001$) per pregnancy in twin compared to singleton pregnancies (Table 2.5).

Table 2.4: Maternal nutritional treatment and fetal survival

		Healthy ewes carrying life fetuses	Initial fetal number	Number of fetal sheep which died or delivered before 136-138 days of gestation				Fetal sheep which survived to 136 -138 days of gestation	
				Dead not delivered (clinical cause)	Dead not delivered (no known cause)	Dead just before or during delivery	Alive at delivery	Number	**%** of each group
Singletons Ewes (n = 22) Fetuses (n = 22)	Control	8	8	1	0	0	1	6	**75.0**
	PCUN	11	11	0	1	2	0	8	**72.7**
	PIUN	3	3	0	0	0	0	3	**100**
Twins Ewes (n = 18) Fetuses (n = 36)	Control	5	10	0	1	0	0	9	**90.0**
	PCUN	6	12	0	4	0	0	8	**66.7**
	PIUN	7	14	1	2	0	0	11	**78.6**
Total		40	56	2	8	2	1	45	**80.4**

Table 2.5: Effect of maternal nutritional treatment on feto-placental growth

Data expressed per fetus			Placentome number	Mean placentome weight (g)	Placental weight (g)	Fetal Weight (kg)	Data expressed per pregnancy	Placentome number	Mean placentome weight (g)	Placental weight (g)	Fetal Weight (kg)
Singletons	**Control**	**n = 6**	63.3 ± 7.2	6.4 ± 0.4	397.6 ± 42.7	4.5 ± 0.3	**n = 6**	63.3 ± 7.2	6.4 ± 0.4	397.6 ± 42.7	4.5 ± 0.3
	PCUN	**n = 8**	71.5 ± 2.6	6.6 ± 0.6	461.8 ± 36.4	4.8 ± 0.2	**n = 8**	71.5 ± 2.6	6.6 ± 0.6	461.8 ± 36.4	4.8 ± 0.2
	PIUN	**n = 3**	72.7 ± 14.5	5.9 ± 0.9	402.8 ± 24.9	4.3 ± 0.0	**n = 3**	72.7 ± 14.5	5.9 ± 0.9	402.8 ± 24.9	4.3 ± 0.0
Twins	**Control**	**n = 9**	44.2 ± 3.5**	7.7 ± 0.9*	332.5 ± 34.4*	4.2 ± 0.2	**n = 4**	90.3 ± 6.4**	7.6 ± 1.4*	667.7 ± 80.1**	8.4 ± 0.8**
	PCUN	**n = 8**	46.1 ± 3.9**	8.1 ± 0.7*	370.9 ± 34.5*	4.3 ± 0.2	**n = 3**	97.7 ± 10.2**	8.4 ± 1.0*	818.9 ± 39.1**	8.5 ± 0.7**
	PIUN	**n = 11**	43.7 ± 3.2**	8.0 ± 0.6*	339.4 ± 23.3*	4.5 ± 0.2	**n = 5**	90.2 ± 4.9**	7.6 ± 0.6*	683.9 ± 54.8**	9.0 ± 0.5**

Values are mean ± SEM, *denotes a significant difference between singletons and twins ($P < 0.03$), **denotes a significant difference between singletons and twins ($P < 0.0001$).

2.3.5 Relationship between Fetal and Placental Growth

In the Control and PCUN group, there was a direct and significant relationship between fetal weight and placental weight at 136 – 138 day of gestation. This relationship was not present, however, in the PIUN group (Table 2.6 and Figure 2.7).

Table 2.6: The relationships between individual fetal weight (y) and placental weight (x)

	Relationship between individual Fetal (y) and Placental Weight (x)
Control	y = 0.0046x + 2.70 (r = 0.68, n = 15, P < 0.005)
PCUN	y = 0.0039x + 2.96 (r = 0.60, n = 16, P < 0.02)
PIUN	y = 0.0025x + 3.53 (r = 0.31, n = 14, NS)

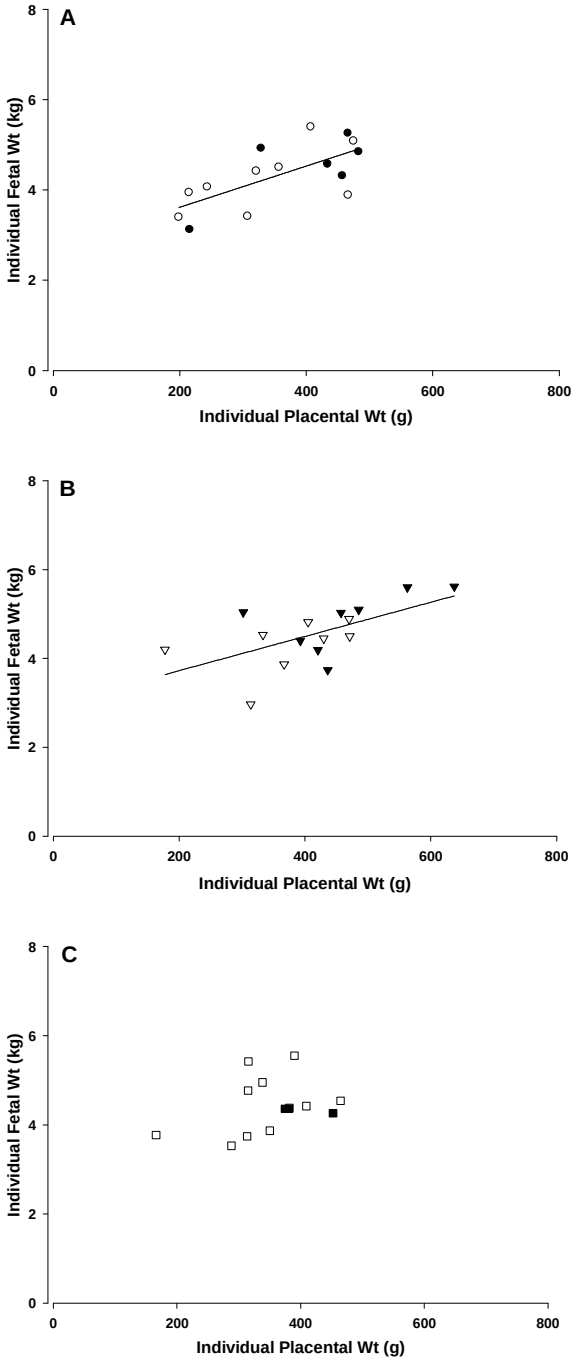


Figure 2.7: Relationship between fetal and placental weights

There was a positive relationship between fetal weight and placental weight at 136 – 138 days of gestation in the Control group (singletons closed circles, twins open circles, A) and PCUN group (singletons closed triangles, twins open triangles, B). This relationship was absent in the PIUN group (singletons closed squares, twins open squares, C).

2.3.6 Fetal Growth Measures

There was no significant effect of either maternal nutritional treatment or fetal number on fetal weight, crown rump length, abdominal circumference or ponderal index at 136 – 138 days of gestation (Table 2.7).

Table 2.7: Effect of maternal nutritional treatment on fetal growth

Growth parameters			Fetal weight (kg)	Crown-rump length (cm)	Abdominal circumference (cm)	Ponderal index (g/cm^3)
Singletons	Control	n = 6	4.5 ± 0.3	58.2 ± 1.6	35.9 ± 2.8	0.023 ± 0.001
	PCUN	n = 8	4.8 ± 0.2	60.4 ± 0.9	38.7 ± 0.7	0.022 ± 0.001
	PIUN	n = 3	4.3 ± 0.0	58.4 ± 0.3	38.4 ± 1.0	0.022 ± 0.001
Twins	Control	n = 9	4.2 ± 0.2	56.5 ± 1.6	37.6 ± 1.4	0.024 ± 0.001
	PCUN	n = 8	4.3 ± 0.2	57.6 ± 0.8	38.0 ± 0.4	0.022 ± 0.003
	PIUN	n = 11	4.5 ± 0.2	57.9 ± 1.1	38.1 ± 0.9	0.023 ± 0.001

Values are mean ± SEM

2.3.7 Fetal Organ Weights

There was no effect of periconceptional and preimplantation undernutrition on fetal perirenal adipose tissue (PAT) and organ weights, expressed as absolute weights, at 136 – 138 days of gestation (Table 2.7). There was, however, a significant decrease in the absolute pituitary ($P < 0.03$) and spleen ($P < 0.04$) weights in twin compared to singleton fetuses (Table 2.8).

2.3.7.1 Fetal organs derived from the ectoderm lineage

Whilst, there was no effect of maternal nutritional treatment on fetal brain weight, expressed as a proportion of fetal body weight, the relative brain weight was significantly increased in twin compared to singleton fetuses ($P < 0.04$, Figure 2.8). There was no effect of either maternal nutritional treatment or fetal number on relative fetal pituitary weight, at 136 – 138 days of gestation (Figure 2.9).

2.3.7.2 Fetal organs derived from the endoderm lineage

There was a significant interaction between the effects of maternal nutritional treatment and fetal number on the fetal lung weight relative to body weight ($P < 0.03$). In singleton pregnancies, there was no effect of periconceptional or preimplantation nutrition on relative lung weight whereas in twin pregnancies, relative lung weight in the PIUN group was significantly lower ($P < 0.02$) when compared to the PCUN group only (Figure 2.10). There was no effect of either maternal nutritional treatment or fetal number on fetal liver weight, expressed relative to body weight, at 136 – 138 days of gestation (Figure 2.11).

Table 2.8: Effect of maternal nutritional treatment on absolute fetal organ weights

Fetal Organs		Brain (g)	Pituitary (g)	Lung (g)	Liver (g)	Spleen (g)	PAT(g)	Heart (g)	Kidneys (g)
Singletons	**Control** (n = 6)	56.2 ± 1.7	0.123 ± 0.015	118.3 ± 9.8	110.8 ± 10.0	7.1 ± 0.9	17.8 ± 2.5	28.8 ± 2.0	25.1 ± 1.6
	PCUN (n = 8)	56.9 ± 2.2	0.130 ± 0.012	134.4 ± 5.2	133.9 ± 8.9	8.1 ± 0.5	18.5 ± 1.0	31.9 ± 1.5	26.8 ± 1.5
	PIUN (n = 3)	54.3 ± 2.0	0.137 ± 0.000	129.9 ± 8.3	144.8 ± 11.1	9.3 ± 0.6	18.1 ± 2.0	29.8 ± 1.4	23.3 ± 1.8
	Singletons Mean±SEM	**56.2 ± 1.2**	**0.129 ± 0.008**	**127.91 ± 4.6**	**122.4 ± 6.1**	**7.9 ± 0.5**	**18.2 ± 1.0**	**30.5 ± 1.0**	**25.6 ± 1.0**
Twins	**Control** (n = 8 - 9)	57.6 ± 1.2	0.107 ± 0.009	124.0 ± 5.1	107.5 ± 7.3	7.4 ± 0.4	21.5 ± 2.0	28.4 ± 0.7	20.5 ± 1.1
	PCUN (n = 7– 8)	59.2 ± 1.4	0.113 ± 0.011	135.2 ± 6.9	109.2 ± 4.3	6.8 ± 0.4	19.6 ± 1.3	28.1 ± 1.5	23.8 ± 0.9
	PIUN (n = 11)	56.9 ± 2.0	0.101 ± 0.009	114.3 ± 6.7	105.7 ± 5.7	7.1 ± 0.4	21.6 ± 2.0	28.9 ± 1.3	24.0 ± 1.4
	Twins Mean±SEM	**57.7 ± 1.0**	**0.106 ± 0.006***	**123.4 ± 3.9**	**107.3 ± 3.4**	**7.1 ± 0.2***	**21.0 ± 1.0**	**28.5 ± 0.7**	**22.9 ± 0.8**

Values are mean ± SEM *denotes a significant difference between singletons and twins ($P < 0.04$)

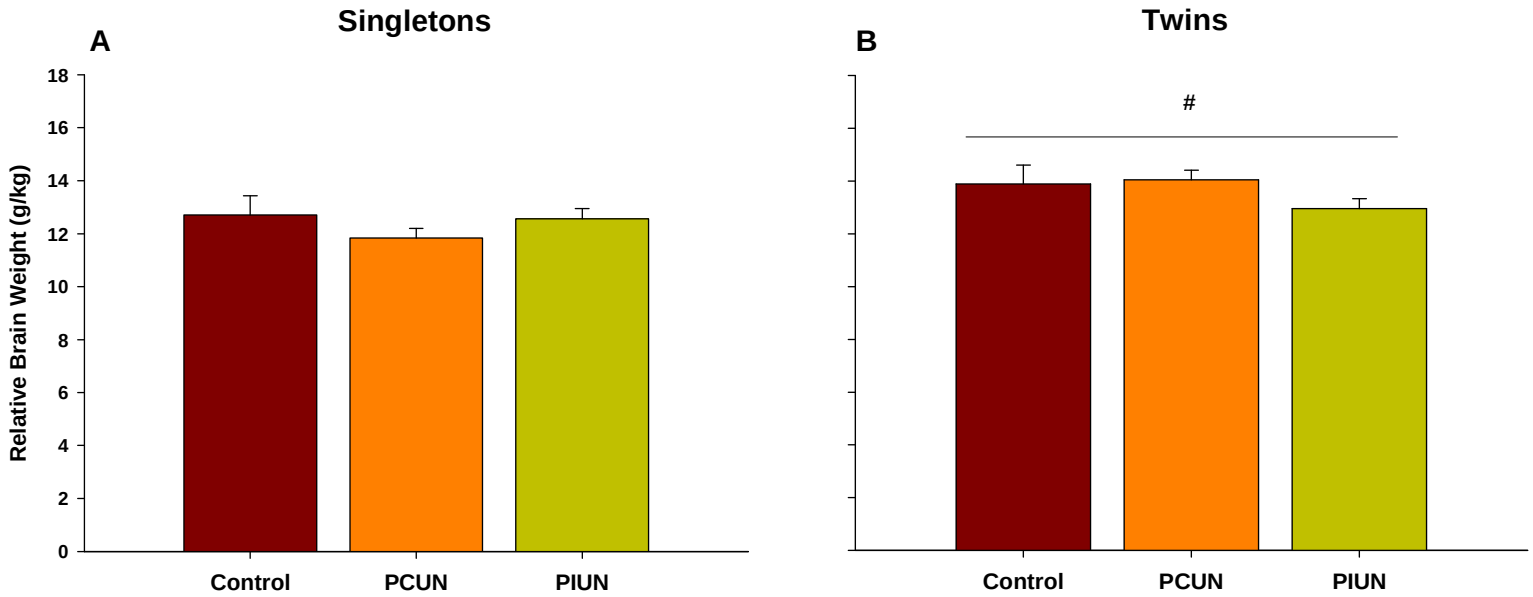


Figure 2.8: Relative brain weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

The relative brain weight was significantly higher in twin (B) compared to singleton (A) fetuses independent of nutritional treatment, as denoted by #.

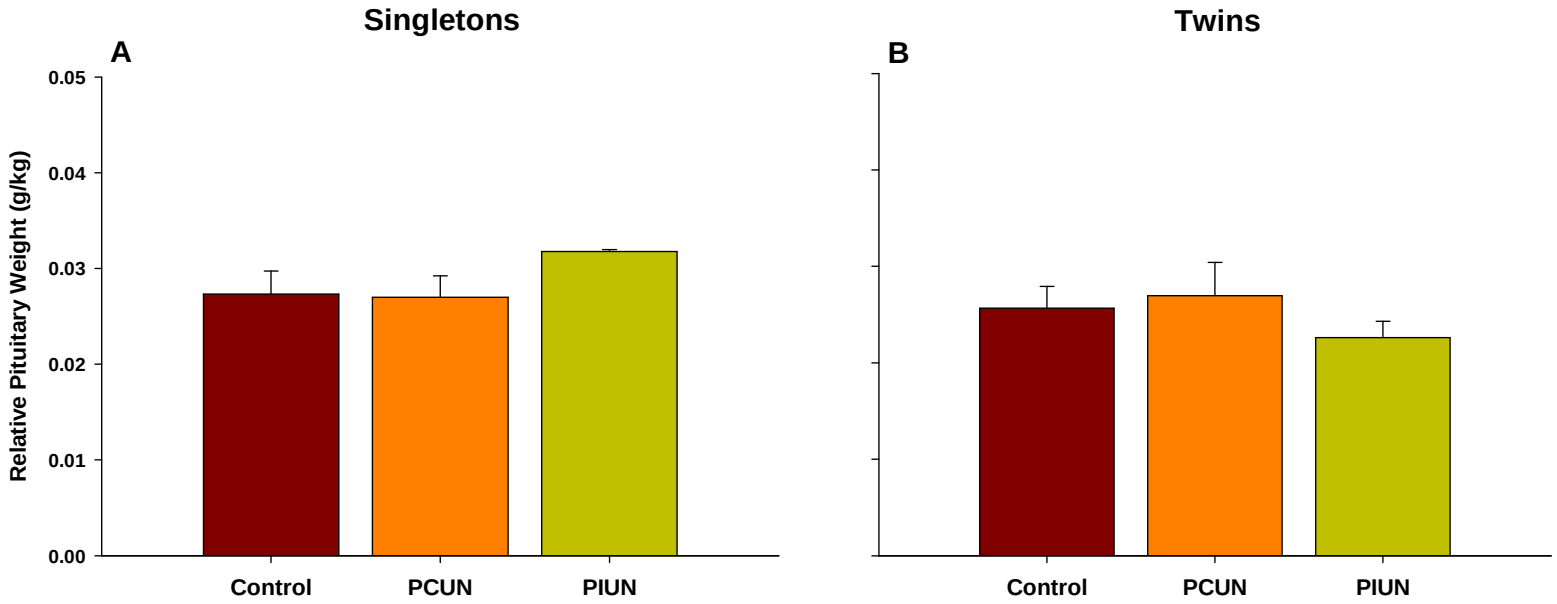


Figure 2.9: Relative pituitary weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of either maternal nutritional treatment or fetal number on the relative pituitary weight.

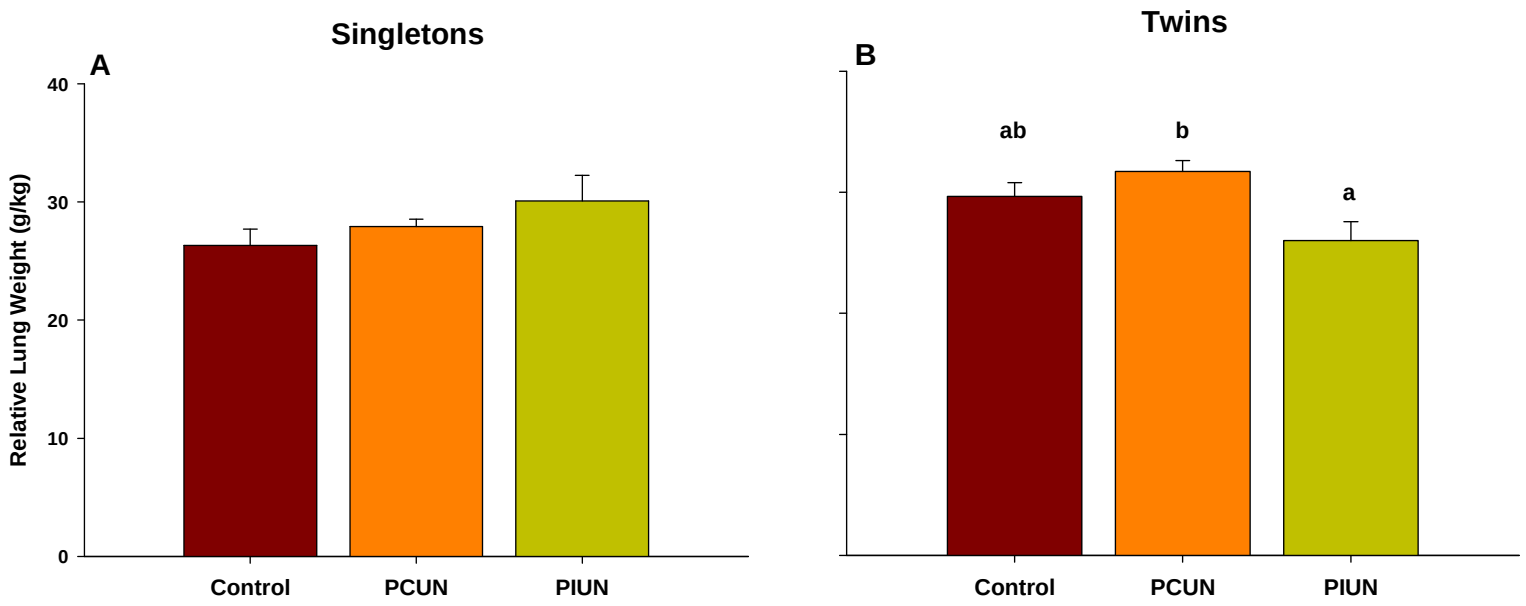


Figure 2.10: Relative lung weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of periconceptional or preimplantation undernutrition on singleton fetuses (A), however, the relative lung weight in twin fetuses (B) was significantly lower in the PIUN group compared to the PCUN group only, as denoted by the different alphabetic subscripts.

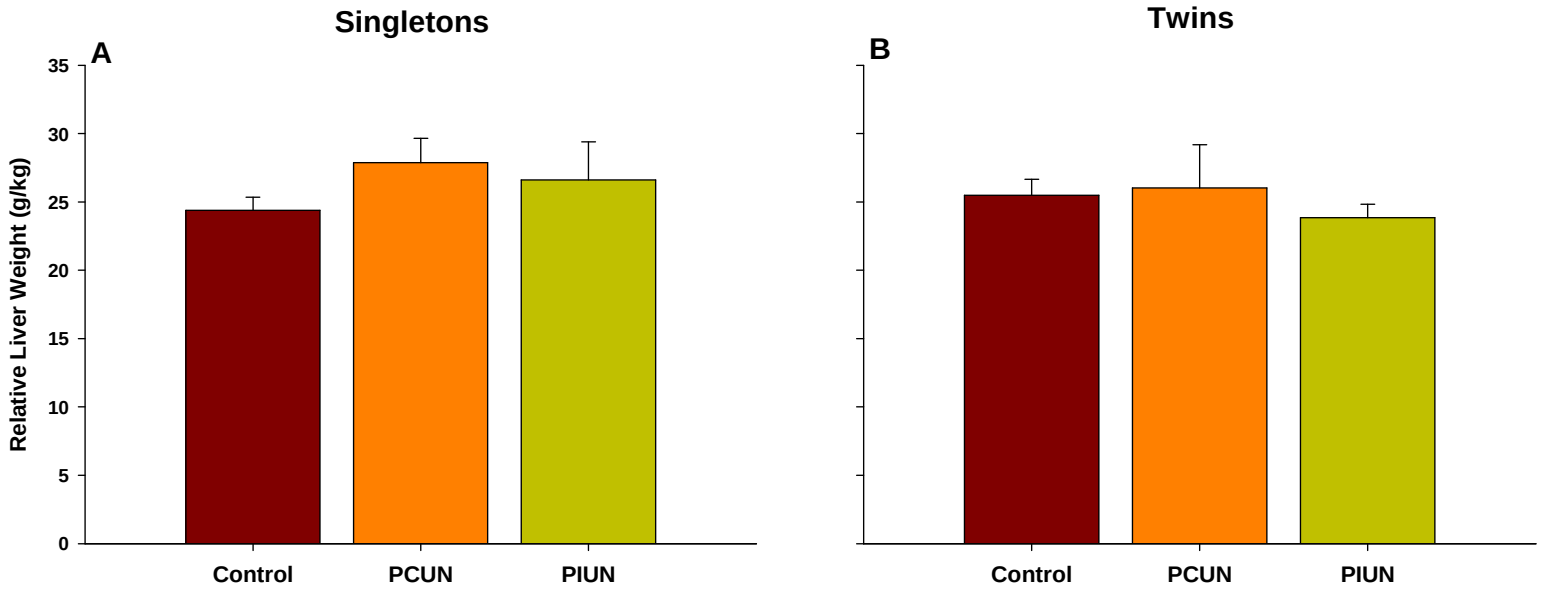


Figure 2.11: Relative liver weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of either maternal nutritional treatment or fetal number on the relative liver weight.

2.3.7.3 Fetal organs derived from the mesoderm lineage

There was a significant interaction between the effects of maternal nutritional treatment and fetal number on the fetal spleen weight relative to body weight ($P < 0.03$). In singleton pregnancies, the relative spleen weight in the PIUN group was significantly higher ($P < 0.05$) when compared to the Control and PCUN group whereas in twin pregnancies, there was no effect of periconceptional or preimplantation undernutrition on relative spleen weight (Figure 2.12).

There was no effect of maternal nutritional treatment on the relative weight of the perirenal adipose tissue. There was, however, a significant increase ($P < 0.01$) in the relative weight of the perirenal adipose tissue twin fetal sheep when compared to singletons (Figure 2.13).

There was no effect of either maternal nutritional treatment or fetal number on fetal heart weight and fetal kidney weight, expressed as relative to body weight, at 136 – 138 days of gestation (Figure 2.14 and Figure 2.15).

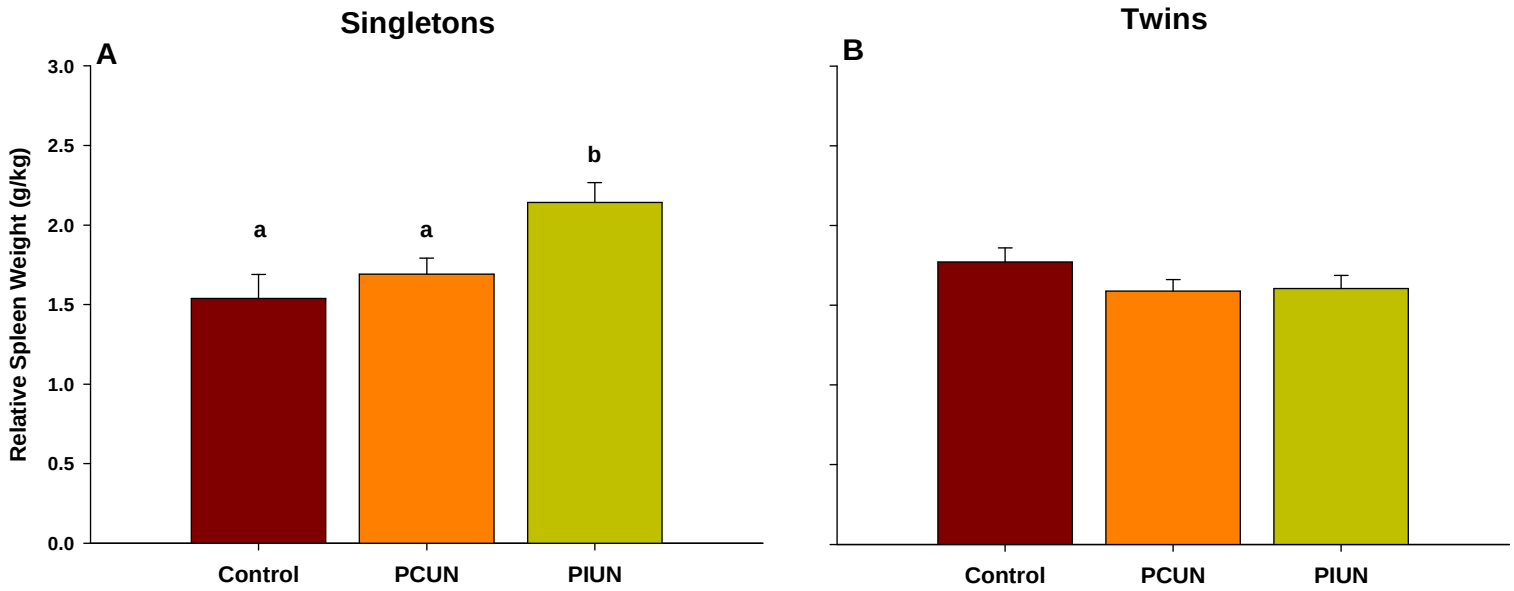


Figure 2.12: Relative spleen weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

The relative spleen weight in singletons fetuses (A) was significantly higher in the PIUN group compared to both the Control and PCUN groups, as denoted by the different alphabetic subscripts. There was no effect of periconceptional or preimplantation undernutrition on twin fetuses (B).

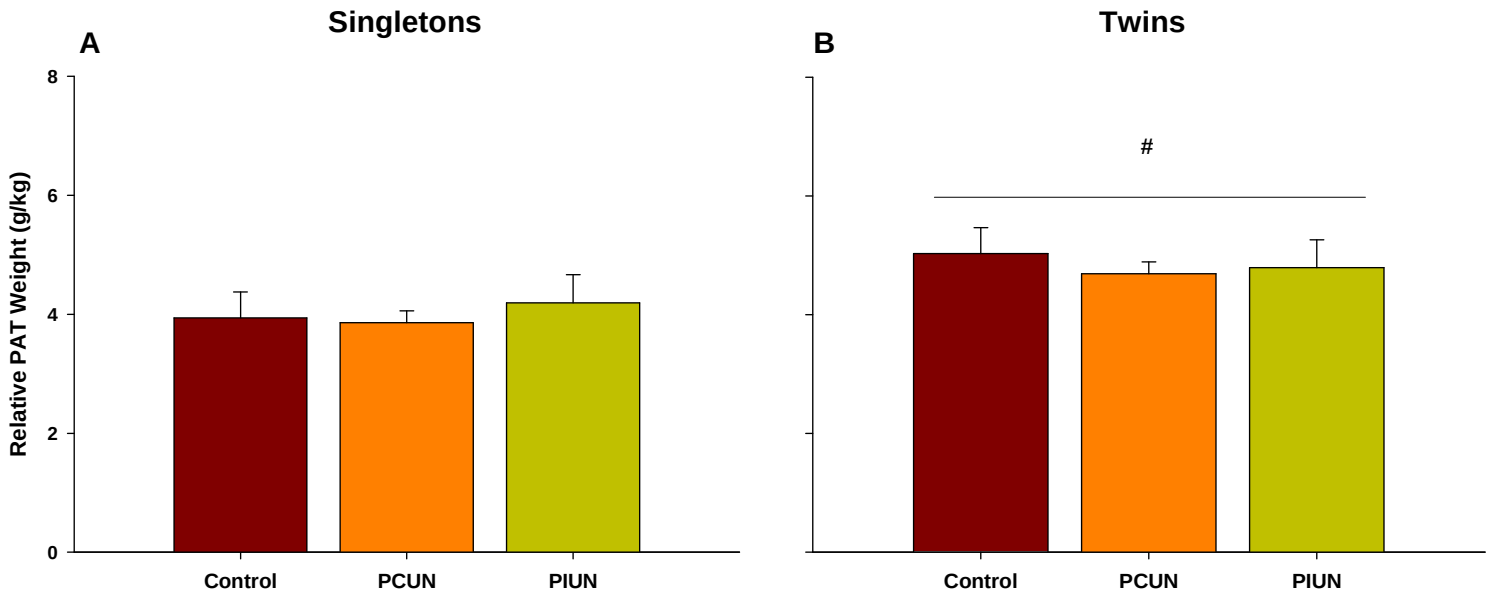


Figure 2.13: Relative perirenal adipose tissue (PAT) weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

The relative PAT weight was significantly higher in twin (B) compared to singleton (A) fetuses independent of nutritional treatment, as denoted by #.

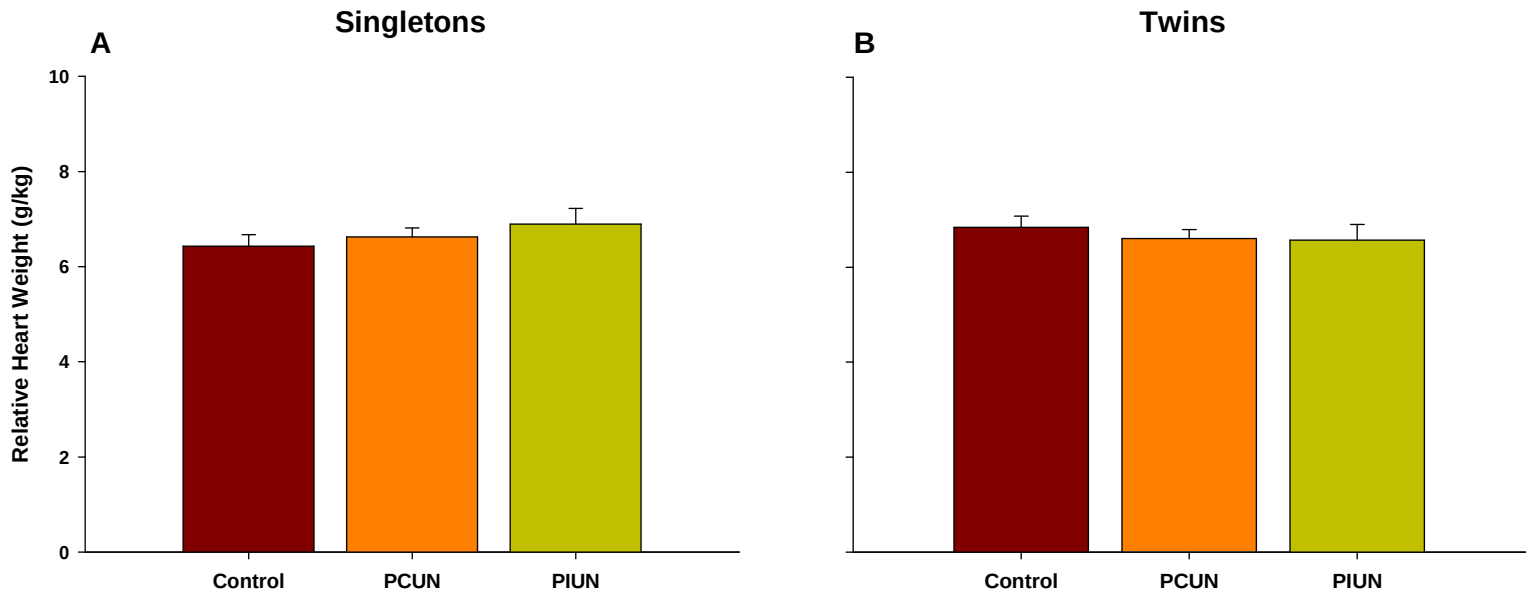


Figure 2.14: Relative heart weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of either maternal nutritional treatment or fetal number on the relative heart weight.

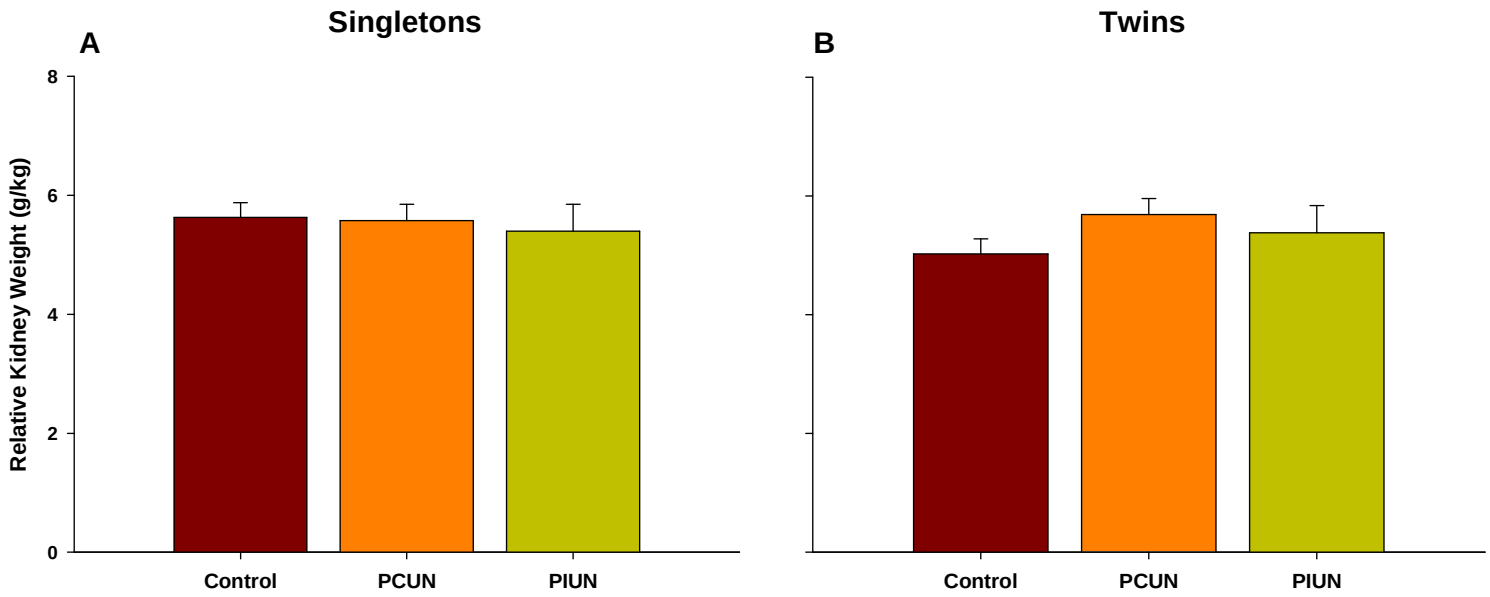


Figure 2.15: Relative kidney weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of either maternal nutritional treatment or fetal number on the relative kidney weight.

2.4 DISCUSSION

The objective of this study was to investigate whether the plane of nutrition of the ewe around the time of conception and particularly during the early preimplantation period alone is a determinant of the growth trajectory of the placenta and fetus in singleton and twin pregnancies in late gestation. This study, demonstrated for the first time that the development of the lung and spleen is differentially affected by undernutrition experienced during the preimplantation period alone in singleton and twin fetuses. These results are relevant in the context of the series of epidemiologic and clinical studies that show that restriction of maternal nutrient intake during various developmental windows is associated with changes in gestation length, altered fetal growth trajectories and specific adverse health outcomes in later life (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000; Roseboom *et al.*, 2006).

2.4.1 Non-pregnant and Pregnant Ewe Weights

There was no significant difference at the beginning of the feeding regime between the body weight of the ewes allocated to the Control, PCUN and PIUN groups. Ewes subjected to nutrient restriction for 60 days prior to mating (PCUN group) demonstrated a significant weight change when compared to the Control and PIUN groups. Whilst ewes in the Control and PIUN groups each had a small increase in body weight at the end of the preconception period, ewes from the PCUN group lost ~ 3.5kg during this period. By day 6 of gestation the mean body weight change of ewes of all nutritional groups differed significantly from each other. Control ewes gained ~ 2.5kg, PCUN ewes lost ~ 3.0kg, and the PIUN ewes did not gain as much (~ 1kg) as the Control group. The level of nutrient restriction

in the present study therefore resulted in relatively modest effects on the weight of the non-pregnant ewes and is consistent with previous studies employing this nutritional regime (Edwards & McMillen, 2002a; Edwards & McMillen, 2002b; MacLaughlin *et al.*, 2005; MacLaughlin *et al.*, 2007). Secondly, this study establishes a nutritional regime, which includes nutrient restriction during the early preimplantation period only, and demonstrates a significant differential impact on the change in ewe weight by the end of the feeding regime.

2.4.2 Pregnancy Success Rates and Outcomes and Fetal Survival

As expected, the overall pregnancy success rate was ~ 75%. Previous studies have shown that a 50% reduction of maintenance diet during the periconceptional period lead to in an increased rate of ova wastage (Rhind *et al.*, 1989), a delay in embryo development and an increase in embryo mortality (Abecia *et al.*, 1997) often leading to failure of implantation and establishment of pregnancy. In fact, the plane of nutrition has been reported to affect ovine and bovine pregnancy rates (O'Callaghan & Boland, 1999; Boland *et al.*, 2001). In this study, maternal nutrient restriction during the periconceptional and preimplantation period did not affect the pregnancy success rate. It is, therefore, possible that the level of nutrient restriction, to 70% of maintenance diet, was not sufficiently severe to have an effect on pregnancy success rate.

In this study, the overall fetal survival rate was ~ 80% and a small number of fetal sheep died prior to delivery with or without cause, during delivery or were delivered prematurely and this was independent of nutritional regime. Previous studies have shown that severe nutrient restriction to achieve a ewe body weight reduction of ~ 15% between 60 days before and 30 days after conception resulted

in premature delivery in 50% of singleton pregnancies (Bloomfield *et al.*, 2003; Bloomfield *et al.*, 2004; Kumarasamy *et al.*, 2005). This suggests that length of gestation is affected by maternal nutrient restriction around the time of conception. More moderate maternal nutrient restriction to 70% of maintenance diet between 60 days prior and 7 days after conception, however, did not result in premature delivery of either singleton or twins (Edwards & McMillen, 2002a), which is consistent with the results of the present study. It appears that the effect of nutritional perturbation during the periconceptional period on gestation length requires either a more severe level of maternal nutrient restriction and/or a period of undernutrition, which extends beyond implantation into early gestation.

2.4.3 Placental Growth

Although there is a contradiction between the findings of different studies, there is strong evidence showing that maternal undernutrition during early to mid-gestation gestation, which is a period of maximal placental growth, impacts on placental growth and development in ruminants. Previous studies have shown that, 15 – 50% maternal nutrient restriction during early to mid-gestation results in an increase in placental weight in singletons (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. In contrast, other studies investigating severe to moderate maternal undernutrition during the same period found that placental weight was decreased in a response to the restricted plane of nutrition (Holst *et al.*, 1992; Heasman *et al.*, 1999; McMullen *et al.*, 2005). An interesting study by McCrabb (1992) showed that ewe weights at mating influence the final impact of maternal nutrient restriction during early to mid-gestation on placental growth (Kelly, 1992; McCrabb *et al.*, 1992). This has led to the hypothesis that there is an

interaction between ewe weight and body condition at mating and maternal nutrient restriction such that the placenta has the capacity to undergo compensatory growth in response to either a low maternal weight or a reduction in her nutrition. This may be to ensure that there is an enhanced capacity of the placenta to transfer nutrients to the fetus, thus the level of maternal nutrition at conception results in development of a larger placenta as a result of the prediction of continuing poor maternal nutrition. Although maternal nutrient restriction during early and mid-gestation lead to significant altered placental growth and development, maternal nutrient restriction during the periconceptional period has no effect on placental growth parameters in early and late gestation (MacLaughlin *et al.*, 2005; Rumball *et al.*, 2008a), which is in agreement with the findings of this study. In the present study, the total placental weight, total placentome number and the mean placentome number per fetus or per pregnancy at 136 – 138 days of gestation were not affected by maternal undernutrition during the periconceptional or preimplantation period. Interestingly, MacLaughlin and colleagues (2005) demonstrated that in control singletons the maternal weight increase during the periconceptional period is accompanied by an increase in placental and fetal growth at ~ 55 days of gestation (MacLaughlin *et al.*, 2005) and that the positive relationship between maternal weight change and placental weight is absent in ewes carrying singleton fetuses and is reversed in ewes carrying twins subjected to moderate periconceptional undernutrition (MacLaughlin *et al.*, 2005). This latter study shows that the periconceptional environment is important in setting placental and fetal growth trajectories. In the present study, relationships between change in maternal weight and placental weight were no longer present at 136 – 138 days of gestation, which suggests that early gestation, a time of maximum placental growth, is a critical window during which the effects of maternal periconceptional

undernutrition are more evident. It is commonly accepted that placental size is a major determinant of subsequent fetal growth trajectory and that impaired placental growth or function as a result of a perturbation compromises fetal growth (Redmer *et al.*, 2004). The finding of a positive relationship between placental weight and fetal weight in the Control and PCUN group is, therefore, not surprising. Intriguingly, this relationship is lost in the PIUN group, that is, the size of the fetus was independent of the size of the placenta in the PIUN group. This suggests that there may be a specific effect of exposure to the maternal metabolic response to undernutrition imposed during the first 6 days of embryo development, which disrupts the structural and functional development of the placenta to ablate the normal feto-placental growth relationship.

It is established that the twin placenta and fetus develop differently from singletons. The specific differences lie in the nature of embryonic implantation and attachment, in the number of placentomes and placentome weight between singletons and twins. The lower number of placentomes and the associated higher mean placentome weight meet the future requirements of the developing twin (Wallace, 1948; Alexander, 1964, 1978; Vatnick *et al.*, 1991). The results of the present study support the concept of differential development of the twin placenta. Specifically, the total placental weight and placentome number per fetus was significantly smaller and the mean placentome weight per fetus was significantly greater in twins when compared to singletons. There was, however, a significant increase in the total placental weight, total placentome number and mean placentome weight per pregnancy in twin compared to singleton pregnancies.

2.4.4 Fetal Growth

Both, placental size and maternal nutrition are key factors that determine fetal growth (Mellor, 1983). In this study there was no significant effect of maternal nutrient restriction during the periconceptional or preimplantation periods on fetal weight, crown rump length, abdominal circumference or ponderal index at 136 – 138 days of gestation. This is in accordance with previous studies of the effects of periconceptional undernutrition on fetal growth (Edwards & McMillen, 2002a; Oliver *et al.*, 2005; Rumball *et al.*, 2008b). Interestingly, a study interrogating the impact of periconceptional undernutrition on fetal growth at ~ 55 days of gestation found that ponderal index was significantly lower in fetal PCUN twin sheep (MacLaughlin *et al.*, 2005) whilst Oliver and colleagues (2005) showed there was a significant decrease in chest girth increments in fetuses from undernourished ewes during late gestation (Oliver *et al.*, 2005). Thus periconceptional nutrient restriction may influence aspects of the fetal phenotype, particularly in twin pregnancy.

2.4.5 Fetal Organ Weights

2.4.5.1 Fetal organs derived from the ectoderm lineage

There was no effect of periconceptional or preimplantation undernutrition on brain weight expressed as absolute or relative to fetal weight at 136 – 138 days of gestation. Similarly, ewes subjected to a reduction of 30% feed intake between 26 and 135 days of gestation had fetuses with a significantly smaller brain weight at 90 days but not at 135 days of gestation (Osgerby *et al.*, 2002). In contrast, when the whole preimplantation and placentation period were included in the period of maternal nutrient restriction (first 90 days of gestation), relative brain weight was significantly increased in fetuses from the undernourished group when compared

to the control group at 130 days of gestation (Luther *et al.*, 2007), which is indicative of brain sparing. As previously suggested early gestation appears to be the critical window during which manipulation of the maternal diet modulates brain growth relative to body growth (Symonds *et al.*, 2007) and it appears that the exposure to undernutrition beyond the preimplantation period is essential to result in a change in brain growth. Exposure to undernutrition in the periconceptional and preimplantation periods alone are not sufficient to affect fetal brain growth. Twin fetuses in this study had a significantly increased relative brain weight, which highlights that "brain sparing" is a feature of a twin pregnancy

2.4.5.2 Fetal organs derived from the endoderm lineage

The mature fetal lungs are vital to the survival of the newborn *ex utero*. During fetal life, however, the fetus is entirely reliant on the placenta for gas exchange. It is interesting to note, that the fetal lungs were one of only two fetal organs that were affected by maternal undernutrition around the time of conception. Although there was no effect of periconceptional and preimplantation undernutrition on fetal lung weight at 136 – 138 days of gestation in singletons, there was a significant reduction in the relative weight of the fetal lung in the PIUN group when compared to the PCUN group only in twin pregnancies. In comparison, a study by McMullen and colleagues (2005) demonstrated that maternal nutrient restriction during mid-gestation resulted in a significant decrease in lung weight at 90 days and 135 days of gestation in singletons whilst Harding (1997) showed that a 20% reduction in maternal nutrition from 105 to 115 days of gestation resulted in smaller fetal lungs in late gestation (Harding, 1997; McMullen *et al.*, 2005). This suggests that maternal nutrient restriction during various gestational windows affect lung growth

and that lungs of twin fetuses are more vulnerable to the effects of maternal nutrient restriction during the periconceptional and preimplantation periods.

The reduction of maternal nutrient intake by 30% between 26 and 135 days of gestation had no effect on fetal liver growth between 45 and 135 days of gestation (Osgerby *et al.*, 2002) and only a more severe reduction in maternal nutrition between 28 and 78 days of gestation resulted in a relative increase in fetal liver weight at 78 days of gestation in the sheep (Vonnahme *et al.*, 2003). This suggests that the severity of maternal nutrient restriction is important in determining whether growth of the fetal liver is affected. This study shows that there was no effect of either fetal number or nutrient treatment around the time of conception on liver weight expressed as absolute or relative to fetal weight suggesting that either the periconceptional window is not critical for liver growth or that the liver is a more "robust" organ where moderate reduction of 30% of maternal nutrient is insufficiently severe to impact on liver growth.

2.4.5.3 Fetal organs derived from the mesoderm lineage

The spleen is a lymphoid tissue and is involved most notably in immunity and in hematopoiesis, the formation of blood cells. In this study, the relative spleen weight was significantly greater in singleton fetuses from ewes subjected to preimplantation nutrient restriction when compared to the Control and PCUN groups at 136 – 138 days of gestation. This effect was absent in the twin group. In contrast, maternal nutrient restriction for 7 days during mid-gestation had no effect on absolute or relative spleen weight from singleton fetuses at 90 days and 135 days of gestation (McMullen *et al.*, 2005) whilst ~ 120 days of maternal nutrient restriction during early and mid-gestation resulted in a significant reduction of

absolute spleen weight in singletons at 130 days of gestation (Luther *et al.*, 2007). Luther and colleagues (2007), however, did not comment on relative spleen weight and in this study absolute spleen weight was concomitantly reduced with fetal weight indicating that this represents an effect of intrauterine growth restriction.

It is clear from the evidence in the literature that maternal nutrient restriction during a variety of developmental windows results in an increase in perirenal adipose tissue in late gestation in the sheep (Bispham *et al.*, 2005; Brennan *et al.*, 2005; Edwards *et al.*, 2005). Edwards and colleagues (2005) found, however, that maternal undernutrition during the periconceptional period alone has no effect on fetal perirenal adipose tissue, which is in agreement with the findings of this study (Edwards *et al.*, 2005). This suggests the fetal fat deposition is not compromised by nutritional perturbations during the periconceptional period. Interestingly, twin fetal sheep had significantly greater perirenal adipose tissue weight than singletons at 136 – 138 days of gestation, independently of the level of maternal nutrition in the periconceptional period. In contrast, previous studies have reported that there was no significant difference in the relative weight of perirenal adipose tissue between singletons and twins at 110 days of gestation (Brennan *et al.*, 2005). Since differences in growth trajectories between singleton and twin fetuses have been found to occur only after 112 days of gestation (Wallace, 1948) it may be possible that the impact of fetal undernutrition on perirenal adipose tissue deposition is more significant in late gestation.

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 133 days of gestation (Gardner *et al.*, 2004), however, the present study did not find any evidence for

accelerated growth of the fetal kidney in the twin fetus by 136 – 138 days of gestation. After a 50% reduction of maternal nutrition during various developmental windows in early and mid-gestation Brennan and colleagues (2005) found that kidney growth was not affected at 110 days of gestation (Brennan *et al.*, 2005). Here we report that neither periconceptional nor preimplantation nutrient restriction affects kidney growth at 136 – 138 days of gestation. This suggests that, at least, kidney growth is not vulnerable to nutritional insults during the periconceptional and preimplantation periods. In this study, however, the cellular morphology and function of the kidney was not investigated.

Similar to the fetal kidney, fetal heart weight was not affected by either fetal number or periconceptional and preimplantation nutrient restriction at 136 – 138 days of gestation in the sheep. In comparison, evidence from the literature has shown that a reduction of maternal feed intake of 15% and 50% over a period of 60 – 70 days during early to mid-gestation has no effect on absolute or relative heart weight (Hawkins *et al.*, 2000c; Dong *et al.*, 2008). Interestingly, 50% maternal nutrient restriction during this gestational period results in a significant increase of the left and right ventricle indicative of bilateral ventricular hypertrophy (Vonnahme *et al.*, 2003). In contrast, ewes subjected to a reduction of 30% feed intake for an extended period (from 26 to 135 days of gestation) had fetuses with a significantly smaller heart weight at 135 day of gestation (Osgerby *et al.*, 2002). These results, therefore, suggest that the severity of nutrient restriction, the length of exposure to maternal undernutrition and developmental window during which the nutritional insult occurs plays a part in determining effect on the growth of the heart and that detailed measurements of the fetal heart may provide more

information on the specific effect of maternal nutrient restriction on heart growth and development.

2.4.6 Summary

The present study demonstrates that there is a differential effect of fetal number on fetal and placental growth at 136 – 138 days of gestation. The total fetal and placental weight per pregnancy was not affected by periconceptional and preimplantation undernutrition, however, total fetal and placental weights were greater in twins when compared to singleton fetuses.

Whilst the brain (derived from the ectodermal lineage) was affected by fetal number, the lung (endodermal origin) was affected by preimplantation nutrition restriction. Furthermore, perirenal fat and the spleen (mesodermal lineage) were affected either by the conditions of a twin pregnancy (fat) or by an interaction between fetal number and maternal periconceptional/preimplantation undernutrition (spleen). Therefore these studies highlight that organs derived from the different germ layers may have a differential susceptibility to the timing of maternal undernutrition experienced during, before and immediately after conception and the relative undernutrition experienced by a twin fetus in late gestation.

CHAPTER 3: IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON THE FETAL HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN THE SHEEP DURING LATE GESTATION

3. IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON THE FETAL HYPOTHALAMO-PITUITARY ADRENAL AXIS IN THE SHEEP DURING LATE GESTATION

3.1 INTRODUCTION

As discussed in Chapters 1 and 2, there are a number of experimental studies demonstrating that manipulations of the environment during the periconceptional period plays an important role in the development of endocrine and physiological systems, including neuroendocrine, cardiovascular and metabolic systems, and may result in permanent consequences for each of these systems after birth. Previous studies in the sheep have also highlighted the importance of both embryo number and the periconceptional environment in determining the timing and magnitude of the increase in fetal plasma ACTH and cortisol concentrations during late gestation (Edwards & McMillen, 2002a; Bloomfield *et al.*, 2003; McMillen *et al.*, 2004). Specifically, there is evidence demonstrating that the activation of the HPA axis in late gestation is delayed and adrenal responsiveness is blunted in twins when compared to singletons (Edwards & McMillen, 2002a; Gardner *et al.*, 2004). Interestingly, in twins from ewes subjected to 30% nutrient restriction during the periconceptional period, the activation of the HPA axis occurred earlier when compared to twins from the control group (Edwards & McMillen, 2002a). It is not clear, however, whether the observed effects resulting from periconceptional undernutrition are due to the effects of poor maternal nutrient during oocyte and embryo development or during early embryonic development alone. To date, there are no studies, which have investigated the effects of moderate maternal undernutrition during the preimplantation period alone on the development of the

fetal HPA axis in the sheep during late gestation. In this study we have, therefore, investigated the effects of maternal undernutrition during the whole periconceptional period, from 60 days before until 6 days after mating, and maternal undernutrition during the preimplantation period alone, that is, for the first 6 days of gestation, on the development of the fetal HPA axis in late gestation.

3.2 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.

3.2.1 Nutritional Management and Mating

The same animals were used in Chapter 3 as in Chapter 2. As discussed in the preceding chapter, 63 South Australian Merino ewes were used in this study and the feeding and mating protocols were as previously reported (Chapter 2). Briefly, sixty days prior to mating ewes were randomly assigned to one of three feeding regimes: Control (C, n = 21), Periconceptional Undernutrition (PCUN, n = 21) and Preimplantation Undernutrition (PIUN, n = 21). The Control ewes received 100% of the nutritional requirements from around 60 days prior mating until 6 days after mating. The PCUN ewes received 70% of the control allowance from approximately 60 days prior mating until 6 days after mating. The PIUN ewes were maintained on the 70% diet from mating until 6 days after mating only.

From 7 days after mating, all ewes were fed a control diet (100% of requirements) until postmortem (PM) at 136 – 138 days of gestation. Ewes were weighed approximately every week after commencing the feeding regime until day 6 of

pregnancy. As discussed in Chapter 2, pregnancy was diagnosed and fetal number estimated by ultrasound at around 60 days of pregnancy. The number of fetuses carried by each ewe was confirmed at PM.

3.2.2 Animals and Surgery

Pregnant ewes (C: n = 17; PCUN: n = 18; PIUN: n = 13) were transferred to the Medical School Animal House between 90 and 100 days of gestation (term = 150 ± 3 days of gestation). Surgery was performed under aseptic conditions between 105 and 110 days of gestation with general anaesthesia initially induced by an intravenous injection of sodium thiopentone (1.25g; Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained with inhalational 2.5 – 4% halothane (Fluothane, ICI, Melbourne, Vic, Australia) in oxygen. At surgery, catheters were implanted in a fetal carotid artery and jugular vein, a maternal jugular vein and the amniotic cavity, as previously described in Chapter 2. All ewes and fetal sheep received a 2ml intramuscular injection of antibiotics (procaine penicillin 250mg/ml, dihydrostreptomycin 250mg/ml, and procaine hydrochloride 20mg/ml; Penstrep Illium; Troy Laboratories, Smith-field, NSW, Australia) at the time of surgery.

3.2.3 Post Surgery

The ewes were housed in individual pens in animal holding rooms with a 12 hour light/dark cycle and fed once daily at 1100 h with water provided *ad libitum*. Animals were allowed to recover from surgery for at least 4 days before experimentation.

3.2.4 Blood Gas Analysis

Fetal arterial blood samples (0.5ml) were collected every day for 4 days after surgery and then 3 times per week thereafter for the analysis of blood gases (Figure 3.1). The measurement of arterial PO_2, PCO_2, pH, oxygen saturation, and hemoglobin (ABL 520 blood gas analyzer; Radiometer, Copenhagen, Denmark) were taken immediately after sampling.

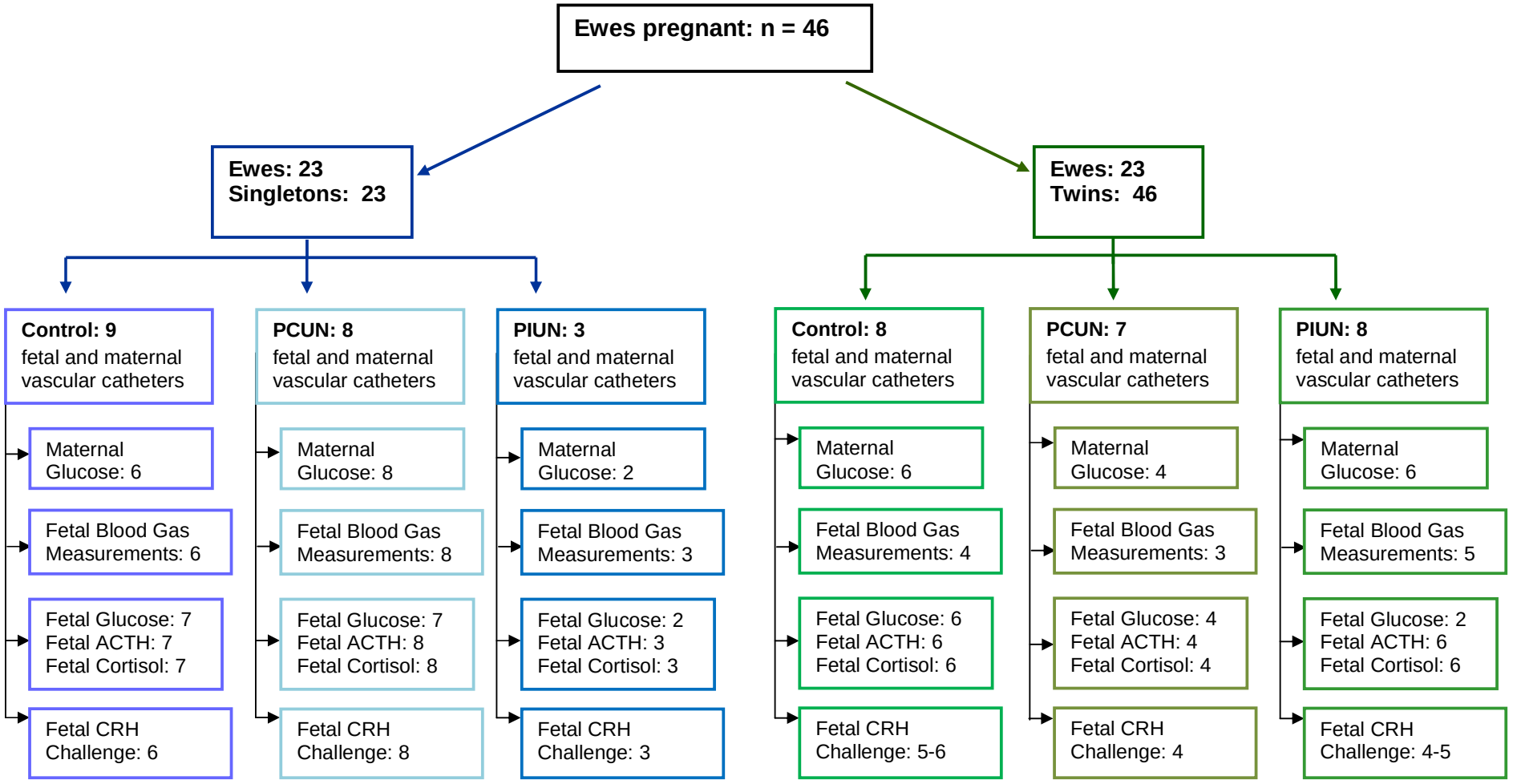
3.2.5 Blood Collection for Radioimmunoassays

Fetal arterial blood samples (3.5ml) and maternal venous blood samples (5ml) were collected in chilled tubes 3 times per week at 0800 – 1100 h. Blood collected for glucose or cortisol assay was collected in heparinised tubes (125IU, Sarstedt Australia, Inglefarm, SA, Australia). Blood samples used for ACTH assay were collected in tubes coated in ethylenediaminetetraacetic acid (EDTA), Sarstedt Australia, Inglefarm, SA, Australia) containing aprotinin (1000KIU.ml^{-1}, Sigma Chemicals, St Louis, MO, USA). All blood samples were centrifuged at 1500 g for 10 min and plasma was separated into aliquots and stored at -20°C for subsequent hormone and metabolite assays.

3.2.6 Plasma Glucose Determination

Maternal and fetal plasma glucose concentrations were determined by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase and measuring the formation of NADH photometrically at 340nm (KONELAB automated analysis system, Thermo Scientific, Waltham, USA), which was previously validated for sheep plasma (Edwards et al., 2001). The intraassay and interassay coefficients of variation were both < 5%.

Figure 3.1: Summary of animal numbers in each part of the experimental protocol



3.2.7 Corticotropin Releasing Hormone Challenge

At 130 – 132 days of gestation a bolus dose of corticotrophin-releasing hormone (CRH) (1 g bolus in 1ml saline: Peninsula Laboratories Inc, San Carlos, CA, USA) was injected into the fetal jugular vein in 32 fetal sheep to determine the plasma cortisol response to increased fetal ACTH concentrations (Figure 3.1). Fetal arterial blood samples (2.5ml) were collected at 30 and 5 minutes prior to CRH administration, and at 10, 20, 40, 60, 120, 240 minutes after CRH administration. The fetal blood samples were transferred to heparinised tubes (1.5ml) and EDTA coated tubes containing aprotinin (1ml). All blood samples were centrifuged immediately after collection at 1500 g for 10 min, and plasma was separated into aliquots and stored at -20°C for subsequent ACTH and cortisol radioimmunoassay.

3.2.8 ACTH and Cortisol Radioimmunoassay

Immunoreactive ACTH concentrations in fetal sheep plasma were measured using a DiaSorin ACTH radioimmunoassay kit (DiaSorin S.p.A., Vercelli, Italy) previously validated for fetal sheep plasma (Warnes *et al.*, 2004). The sensitivity of the assay was 4.5pg/ml, and the intraassay and interassay coefficients of variation were <10% and 13.9%, respectively.

Cortisol was extracted from fetal plasma in duplicates using dichloromethane (Bocking *et al.*, 1986) and measured using an assay previously validated for use in sheep plasma (Warnes *et al.*, 2004). The efficiency of recovery of ^{125}I-cortisol from fetal plasma using this extraction procedure was greater than 90%. Samples were then reconstituted in assay buffer (Tris hydrochloride, BSA, and sodium azide). Standards were serially diluted in assay buffer from a stock (1000nmol/liter)

solution (range, 0.78–100nmol/liter). Anti-cortisol/antisera (100 l; 1:15 dilution; 1:15 dilution; Orion Diagnostica, Turku, Finland) was added followed by ^{125}I-labeled cortisol (100 l; Amersham Pharmacia Biotech, Little Chalfont, UK). Tubes were vortexed and incubated at 37°C for 1 h before the addition of goat antirabbit serum (initial dilution 1:30; 100 l) and polyethylene glycol (1ml, 20%; BDH Laboratory Supplies, Poole, UK). Tubes were vortexed before centrifugation at 3700 g and 4°C for 30 min. The supernatant was aspirated and the precipitate counted on a gamma counter (Packard, Downers Grove, IL). The sensitivity of the assay was 0.2nmol/liter. The intra- and interassay coefficients of variation were less than 5% and 13.8% respectively

3.2.9 Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Data were log transformed where required, in order to normalise data variance for parametric analysis.

3.2.9.1 Fetal blood gas characteristics

Arterial oxygen content (O_2 content) per 100ml blood (ml/dl) was calculated for each fetus (O_2 content = (P_aO_2 × 0.003) + [Hb] × (S_aO_2/100) × 1.39). The effects of maternal undernutrition and fetal number on the gestational age profile of the fetal arterial blood gas variables, including P_aO_2, P_aCO_2, pH, hemoglobin, O_2 saturation and O_2 content, were determined using a multifactorial ANOVA with repeated measures using STATA 10 for Windows (StataCorpLp, Colege Station, TX, USA). Specified factors for the ANOVA included maternal nutritional treatment (Control, PCUN or PIUN), fetal number (singletons or twins) and time windows (119 – 122,

123 – 126, 127 – 130, 131 – 134 and 135 – 138 days of gestation) as specified factors and repeated measures. When there was an interaction between the effects any of the three specified factors, data were split accordingly and the effects of maternal nutritional treatment, fetal number and/or time were determined using a two way repeated ANOVA. The Duncan's Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

3.2.9.2 Plasma glucose, ACTH and cortisol concentrations

Maternal and fetal plasma concentrations of glucose between 119 and 138 days of gestation were averaged to obtain a mean value for glucose concentrations during this period in late gestation. The effects of maternal nutritional treatment and fetal number on mean maternal and fetal plasma glucose concentration were determined using a two way ANOVA. The effects of maternal nutritional treatment and fetal number on the gestational age profile of the fetal plasma ACTH and cortisol concentrations were determined using a multifactorial ANOVA with repeated measures. Specified factors for the ANOVA included maternal nutritional treatment (Control, PCUN or PIUN), fetal number (singletons or twins) and time windows (119 – 122, 123 – 126, 127 – 130, 131 – 134 and 135 – 138 days of gestation) as specified factors. When there was an interaction between the effects any of the three specified factors, data were split accordingly and the effects of maternal nutritional treatment, fetal number and/or time were determined using a two way repeated ANOVA. Duncans's Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

3.2.9.3 Plasma ACTH and cortisol responses to CRH

The effects of maternal nutritional treatment and fetal number on the plasma ACTH and cortisol responses to CRH were compared using a multifactorial ANOVA with repeated measures. Specified factors for the ANOVA included maternal nutritional treatment (Control, PCUN or PIUN), fetal number (singletons or twins) and time relative to CRH administration (-30, -5, 10, 20, 40, 60, 120, 240 min). Additionally, the post-infusion area under the curve (AUC) for ACTH and cortisol was calculated using the trapezoidal rule. The effects of maternal nutritional treatment and fetal number on ACTH and cortisol AUC were determined using a two way ANOVA. When there was an interaction between the effects of the specified factors, data were split accordingly and the effects of maternal nutritional treatment, fetal number and/or time were determined using a two way repeated ANOVA. The Duncan's Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

3.3 RESULTS

3.3.1 Maternal and Fetal Glucose Concentration

Maternal plasma glucose concentrations in late gestation were not different between the Control, PCUN and PIUN groups or singleton or twin bearing ewes (Figure 3.2). There was also no effect of maternal nutritional treatment or fetal number ($P = 0.08$) on the fetal plasma glucose concentrations in late gestation (Figure 3.3).

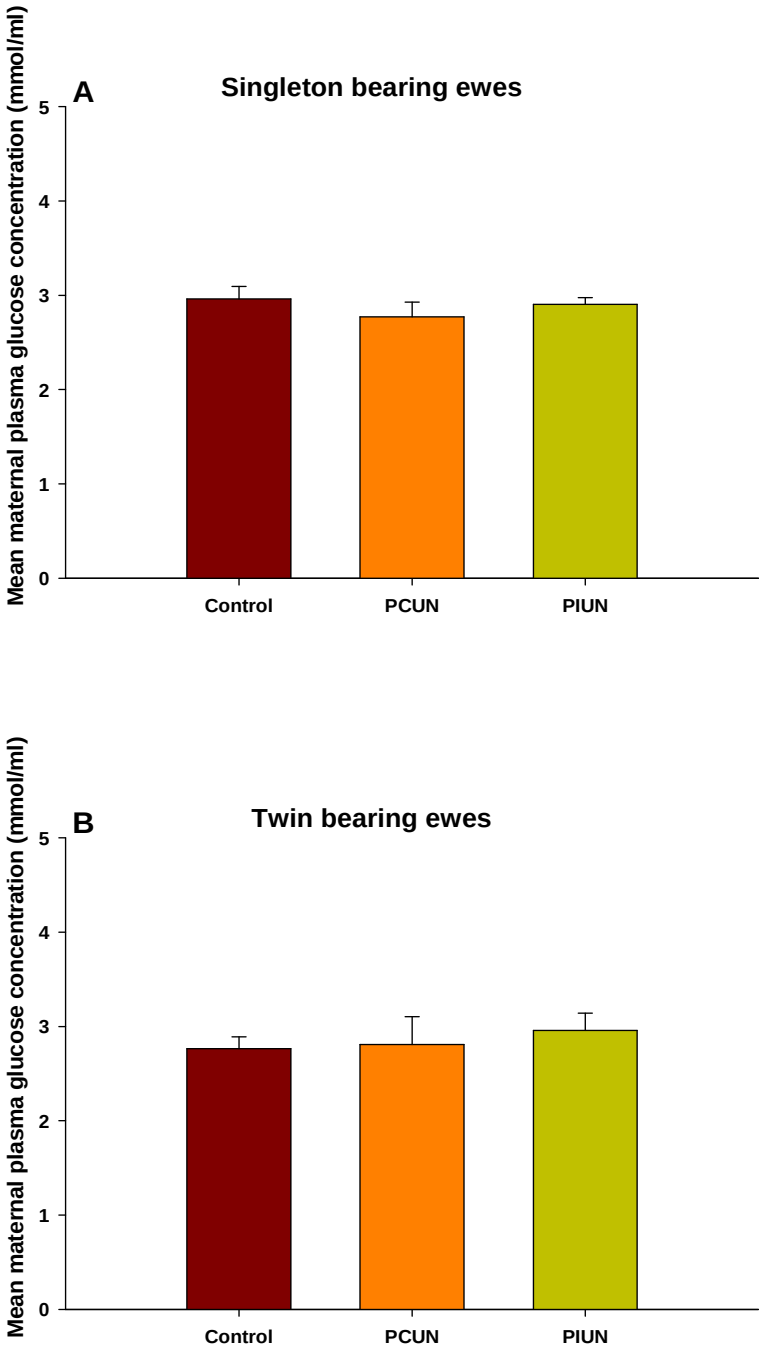


Figure 3.2: Maternal plasma glucose concentration in singleton bearing (A) and twin bearing (B) ewes

There was no effect of maternal nutritional treatment on mean maternal plasma glucose concentration.

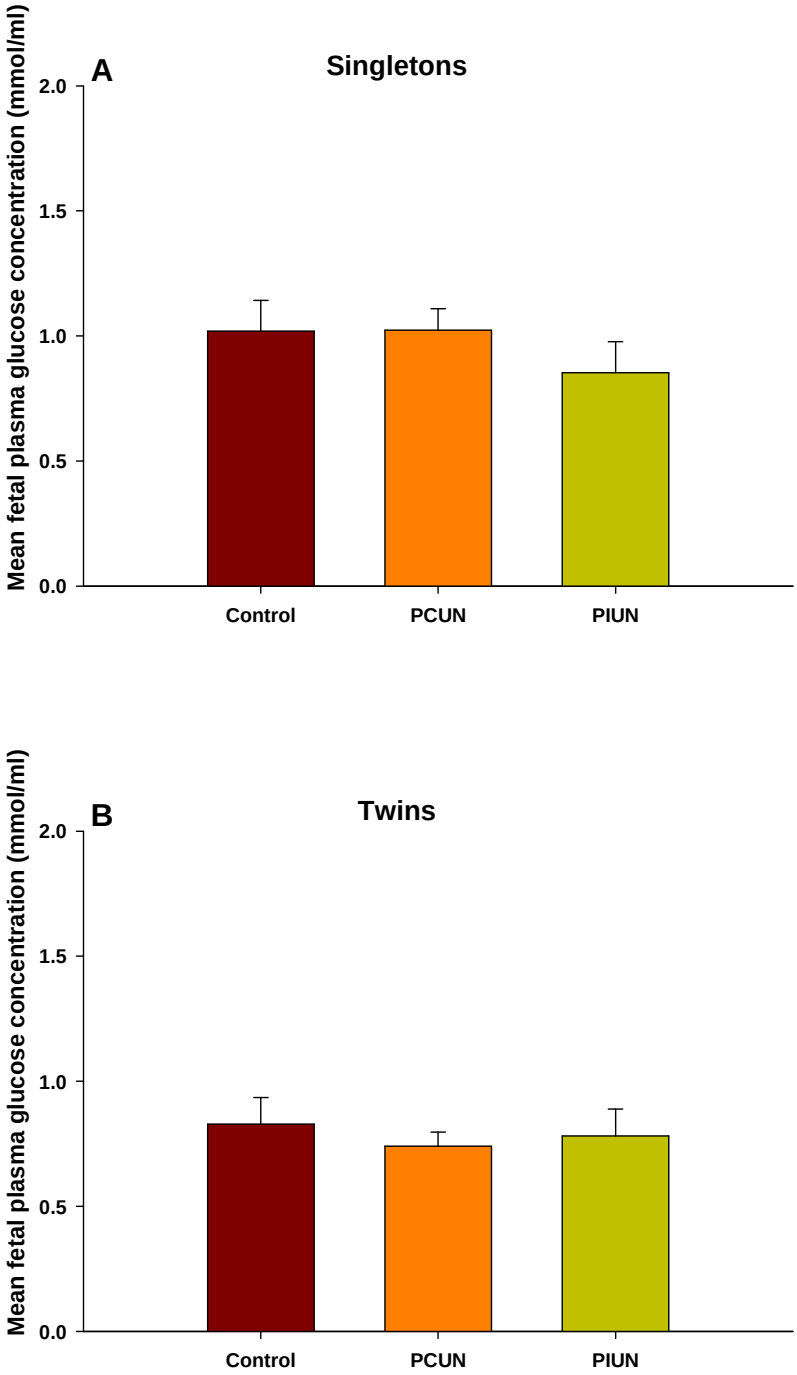


Figure 3.3: Fetal plasma glucose concentration in singletons (A) and twins (B)

There was no effect of maternal nutritional treatment or fetal number (P = 0.08) on fetal plasma glucose concentration.

3.3.2 Fetal Arterial Blood Gas Characteristics

3.3.2.1 P_aO_2, oxygen saturation and oxygen content

There was an interaction between the effects of nutritional treatment and fetal number on fetal P_aO_2 and oxygen content. The data were therefore split into the three nutritional treatment groups for further analysis.

Fetal P_aO_2 was significantly lower ($P < 0.04$) at 135 – 138 days of gestation when compared to either 127 – 130 or 119 – 122 days of gestation in the Control group whilst in the PCUN group fetal P_aO_2 was significantly lower ($P < 0.0005$) after 131 days of gestation when compared to before 131 days of gestation (Figure 3.4).

There was no effect of maternal nutritional treatment or fetal number on oxygen saturation. Oxygen saturation, however, significantly decreased ($P < 0.0001$) with gestational age in all treatment groups (Figure 3.5).

In the Control group, fetal oxygen content was significantly lower in twins when compared to singletons ($P < 0.05$) throughout gestation (Figures 3.6).

In the PCUN group there was no effect of fetal number on either fetal P_aO_2 or oxygen content (Figure 3.4 and 3.6).

In the PIUN group, fetal P_aO_2 and oxygen content were significantly decreased ($P < 0.015$ and $P < 0.014$) in twin fetuses when compared to singleton fetus throughout gestation (Figures 3.4 and 3.6).

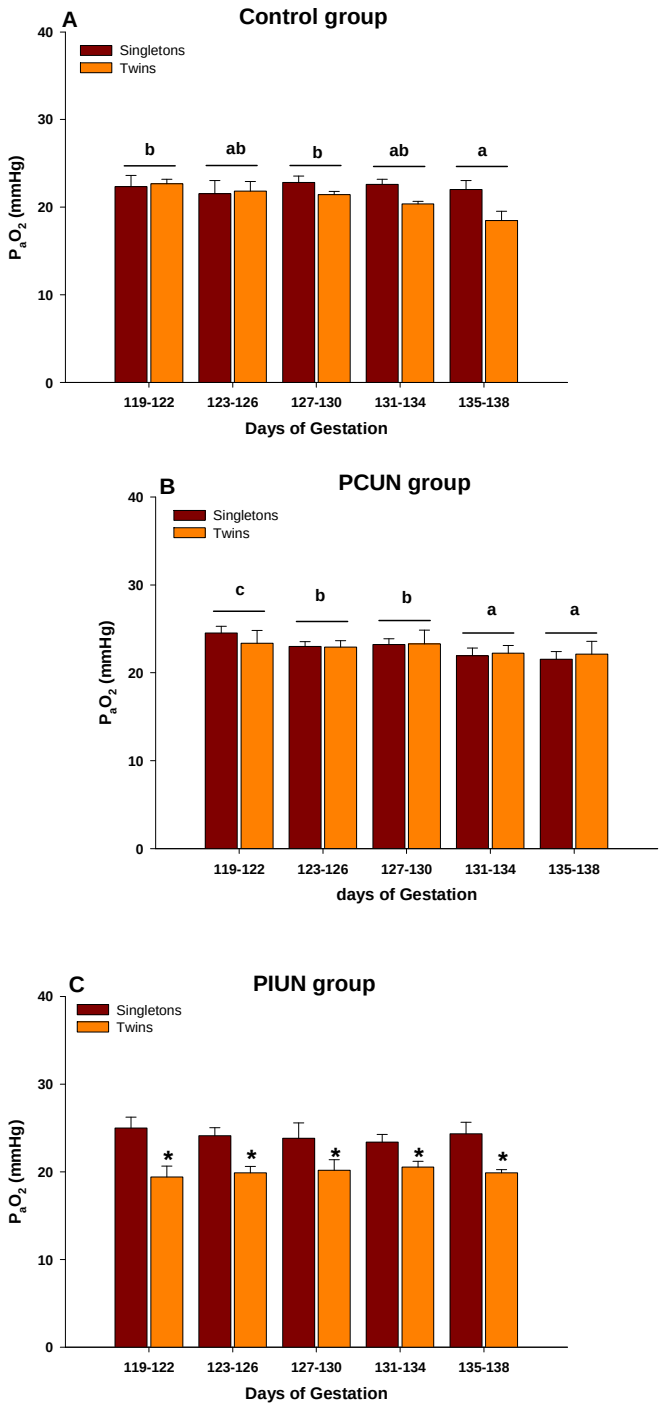


Figure 3.4: P_aO_2 in Control (A), PCUN (B) and PIUN (C) fetuses

There was a significant effect of gestational age on P_aO_2 in Control (A) and PCUN (B) but not PIUN (C). In twins, the fetal P_aO_2 was significantly lower in the PIUN group (C) only. Different alphabetical subscripts denote mean values which are significantly different and # denotes a significant decrease in fetal P_aO_2.

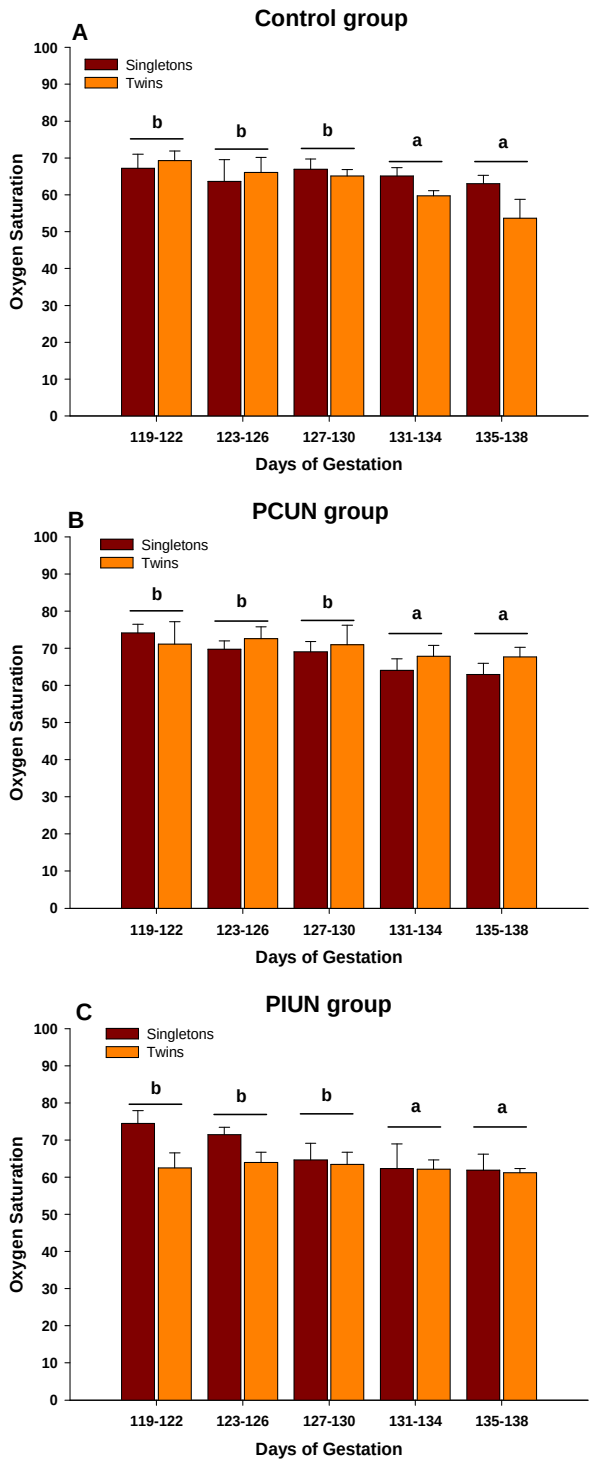


Figure 3.5: Oxygen Saturation in Control (A), PCUN (B) and PIUN (C) fetuses

Fetal oxygen saturation significantly decreased with gestational age in all nutritional groups. Different alphabetical subscripts denote mean values which are significantly different.

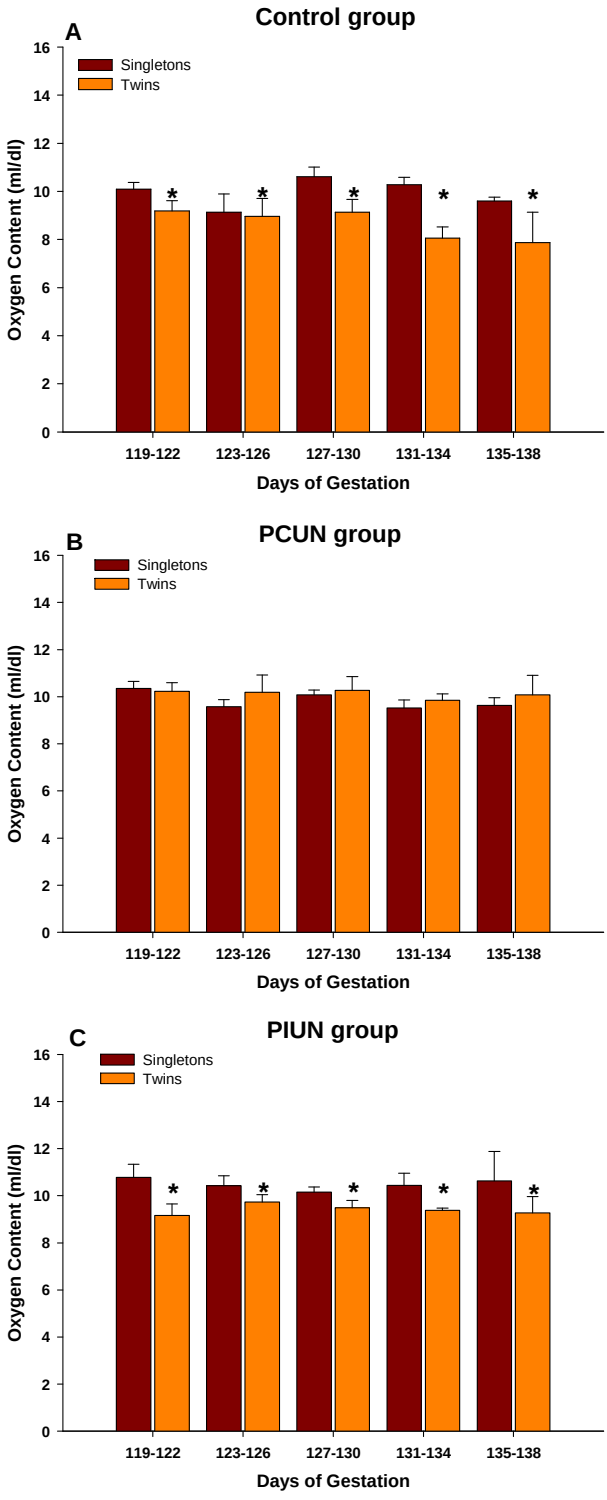


Figure 3.6: Oxygen Content in Control (A), PCUN (B) and PIUN (C) fetuses

Fetal oxygen content was significantly decreased in twins when compared to singletons in Control (A) and PIUN (B) groups.

3.3.2.2 P_aCO_2

There was a statistical interaction between the effects of maternal nutritional treatment, fetal number and gestational age ($P < 0.009$) on fetal P_aCO_2. The data were therefore split into singleton and twin fetuses for further analysis.

In singletons there was a further statistical interaction between the effects of maternal nutritional treatment and gestational age ($P < 0.05$). The singleton data were then analysed by splitting into nutritional treatment groups. In Controls, fetal P_aCO_2 significantly increased ($P < 0.0006$) after 131 days of gestation when compared to before 127 days of gestation whilst in the PCUN group fetal P_aCO_2 was significantly higher ($P < 0.02$) after 123 days of gestation when compared to before 123 days of gestation (Figure 3.7). There was a no effect of gestational age on fetal P_aCO_2 in the PIUN group (Figure 3.7).

In twin pregnancies, fetal P_aCO_2 was significantly higher ($P < 0.04$) in the PIUN group when compared to the Control group (Figure 3.8).

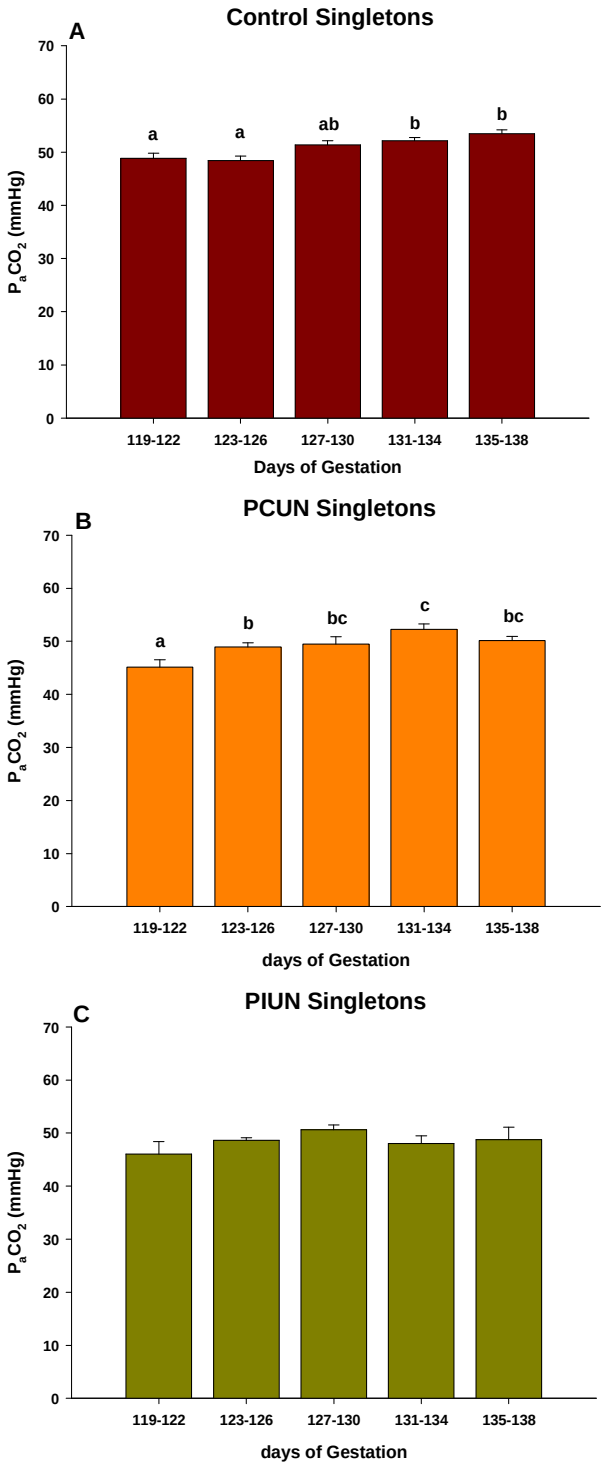


Figure 3.7: P_aCO_2 in singleton fetuses

Fetal P_aCO_2 significantly increased with gestational age in Control (A) and PCUN (B) but not in PIUN (C) singletons. Different alphabetic subscripts denote mean values which are different.

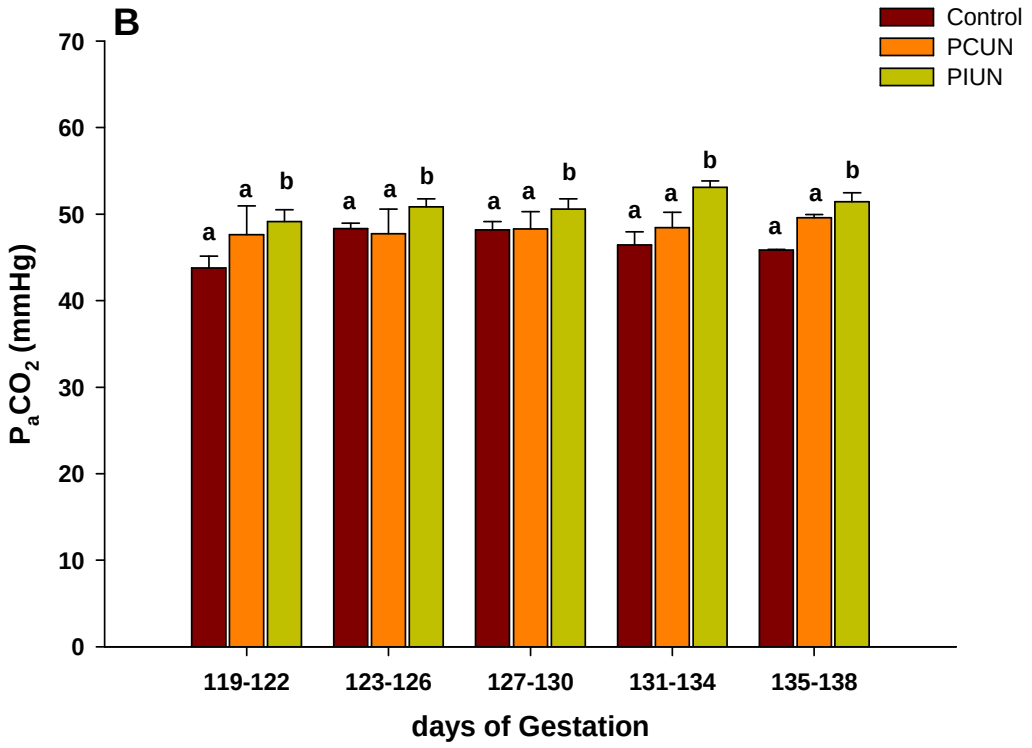


Figure 3.8: P_aCO_2 in twin fetuses

In twins, the fetal P_aCO_2 was significantly higher in the PIUN group compared to the Control group. Different alphabetical subscripts denote mean values which are significantly different.

3.3.2.3 Hemoglobin and pH

There was no effect of maternal nutritional treatment or fetal number on hemoglobin and pH (Table 3.1).

Table 3.1: Effect of maternal nutritional treatment on fetal hemoglobin and pH

	Days of gestation	Singletons			Twins		
		Control	PCUN	PIUN	Control	PCUN	PIUN
Hemoglobin (g/dl)	119-122	10.9 ± 0.6	10.0 ± 0.2	10.3 ± 0.2	9.5 ± 0.4	10.4 ± 0.6	10.7 ± 0.6
	123-126	10.4 ± 0 .4	9.8 ± 0.2	10.5 ± 0.7	9.7 ± 0.6	10.1 ± 1.0	10.9 ± 0.7
	127-130	11.4 ± 0.5	10.5 ± 0.3	11.4 ± 1.0	10.0 ± 0.6	10.4 ± 0.8	10.7 ± 0.5
	131-134	11.4 ± 0.6	10.7 ± 0.3	12.4 ± 2.0	9.7 ± 0.8	10.4 ± 0.6	10.7 ± 0.6
	135-137	10.9 ± 0.3	11.0 ± 0.4	12.6 ± 2.3	10.4 ± 1.0	10.7 ± 1.0	10.8 ± 0.6
pH	119-122	7.38 ± 0.01	7.39 ± 0.00	7.39 ± 0.01	7.39 ± 0.01	7.43 ± 0.05	7.37 ± 0.02
	123-126	7.39 ± 0.01	7.38 ± 0.01	7.39 ± 0.01	7.38 ± 0.00	7.39 ± 0.01	7.4 ± 0.1
	127-130	7.34 ± 0.00	7.37 ± 0.01	7.37 ± 0.0	7.38 ± 0.00	7.38 ± 0.01	7.37 ± 0.01
	131-134	7.37 ± 0.00	7.37 ± 0.01	7.38 ± 0.01	7.36 ± 0.01	7.38 ± 0.00	7.38 ± 0.01
	135-137	7.37 ± 0.00	7.37 ± 0.00	7.37. ± 01	7.37 ± 0.00	7.39 ± 0.0	7.38 ± 0.00

3.3.3 Plasma ACTH and Cortisol Concentrations during late Gestation

There was a significant interaction between the effects of maternal nutritional treatment, fetal number and gestational age on fetal plasma ACTH and cortisol concentration. The data were therefore split on the basis of fetal number for further analysis.

In singleton fetuses in all nutritional groups, plasma ACTH and cortisol concentrations were significantly increased ($P < 0.01$ and $P < 0.02$, respectively) at 135 – 138 days of gestation when compared to either 123 – 126 or 127 – 130 days of gestation. There was no effect of maternal undernutrition during the periconceptional or preimplantation periods on plasma ACTH or cortisol concentrations in singletons during late gestation (Figure 3.9 and Figure 3.10).

There was no effect of either nutritional treatment or gestational age on plasma ACTH concentration in twins (Figure 3.9). There was, however, a significant increase ($P < 0.05$) in fetal plasma cortisol concentrations at 136 – 138 days of gestation when compared to either 123 – 126 or 127 – 130 days of gestation in twins in all nutritional groups (Figure 3.10).

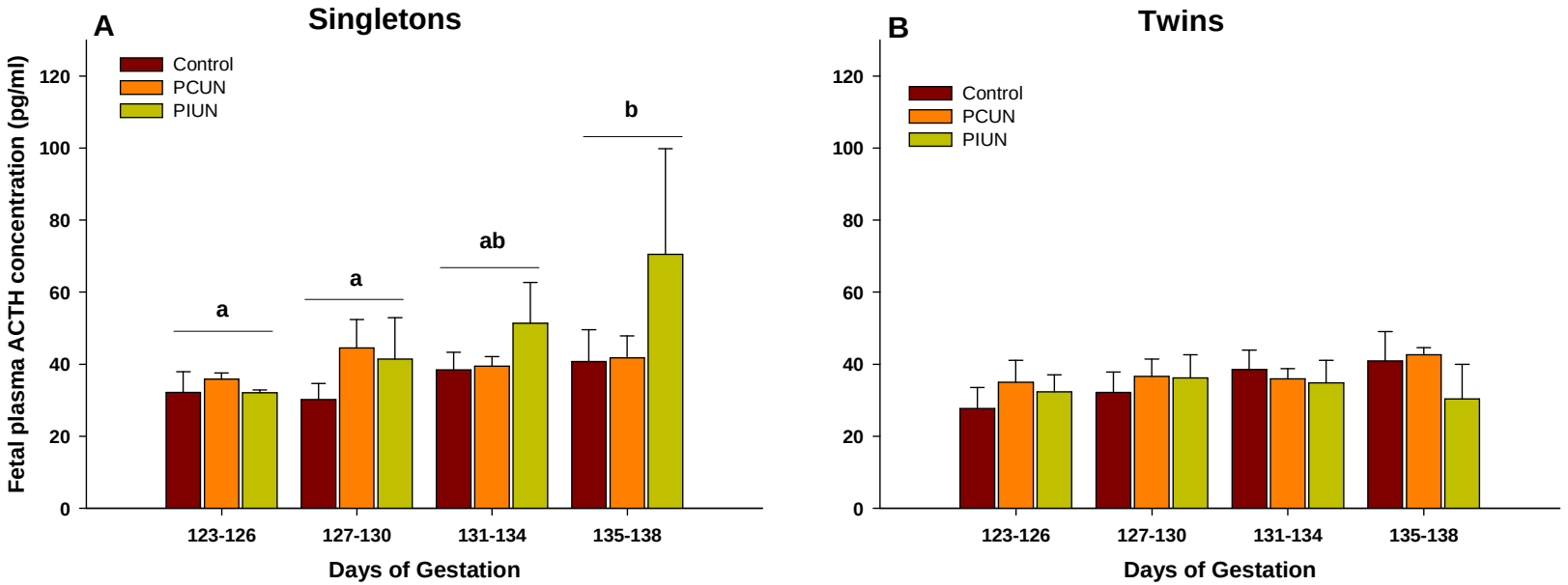


Figure 3.9: Fetal plasma ACTH concentration in singletons (A) and twins (B) during late gestation

There was no effect of maternal nutritional treatment on fetal plasma ACTH concentration in singletons (A) and twins (B). Fetal plasma ACTH concentration, however, significantly increase with gestational age in singletons. Different alphabetical subscripts denote mean values which are significantly different.

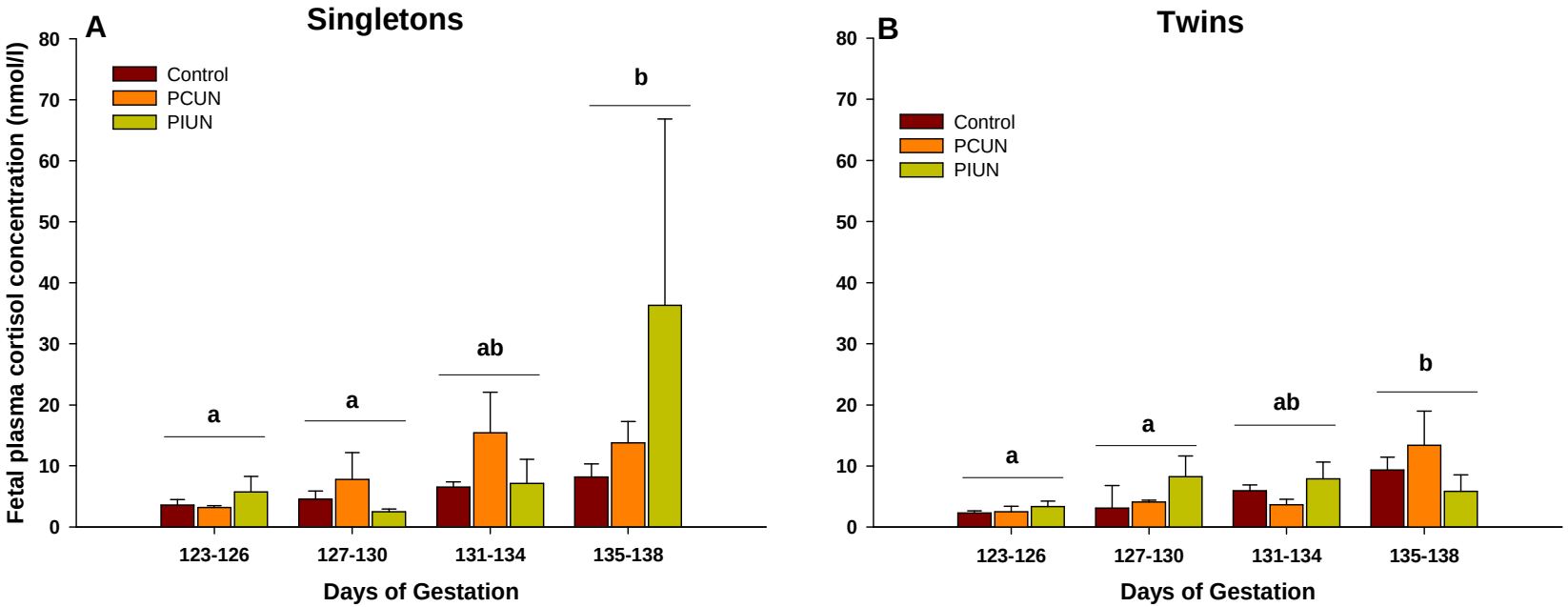


Figure 3.10: Fetal plasma cortisol concentration in singletons (A) and twins (B)

There was no effect of maternal nutritional treatment on fetal plasma cortisol concentration in either singleton (A) or twin (B) fetuses. Fetal plasma ACTH concentration, however, significantly increase with gestational age in both singleton and twin fetuses. Different alphabetical subscripts denote mean values which are significantly different.

3.3.4 CRH Challenge

3.3.4.1 ACTH response to CRH

Fetal plasma ACTH concentrations increased ($P < 0.0001$) in all nutritional treatment groups between 10 and 240 minutes after CRH injection, compared to the pre-injection values (Figure 3.11). There was no significant effect, however, of periconceptional or preimplantation undernutrition on the fetal ACTH response to CRH. The "area under the curve" (AUC) of the ACTH response to CRH was significantly smaller ($P < 0.05$) in twin fetuses when compared to singletons and this was independent of nutritional treatment (Figure 3.12).

3.3.4.2 Cortisol response to CRH

There was an interaction ($P = 0.05$) between the effects of fetal number and time on the cortisol response to CRH. The data were therefore split on the basis of fetal number for further analysis. There was a significant increase ($P < 0.0001$) in plasma cortisol concentrations in response to CRH between 10 and 240 minutes after CRH injection in both singleton and twin fetuses at 130 – 132 days of gestation (Figure 3.13). There was no significant effect, however, of periconceptional or preimplantation undernutrition on the fetal cortisol response to CRH.

There was an interaction ($P = 0.082$) between the effects of maternal nutritional treatment and fetal number on the cortisol response to CRH when expressed as the AUC. The data were therefore split on the basis of nutritional groups for further analysis. In the Control group, twin fetuses had a significantly smaller ($P < 0.05$) cortisol AUC when compared to singletons (Figure 3.14). There was no effect of fetal number, however, on cortisol AUC in the PCUN and PIUN groups.

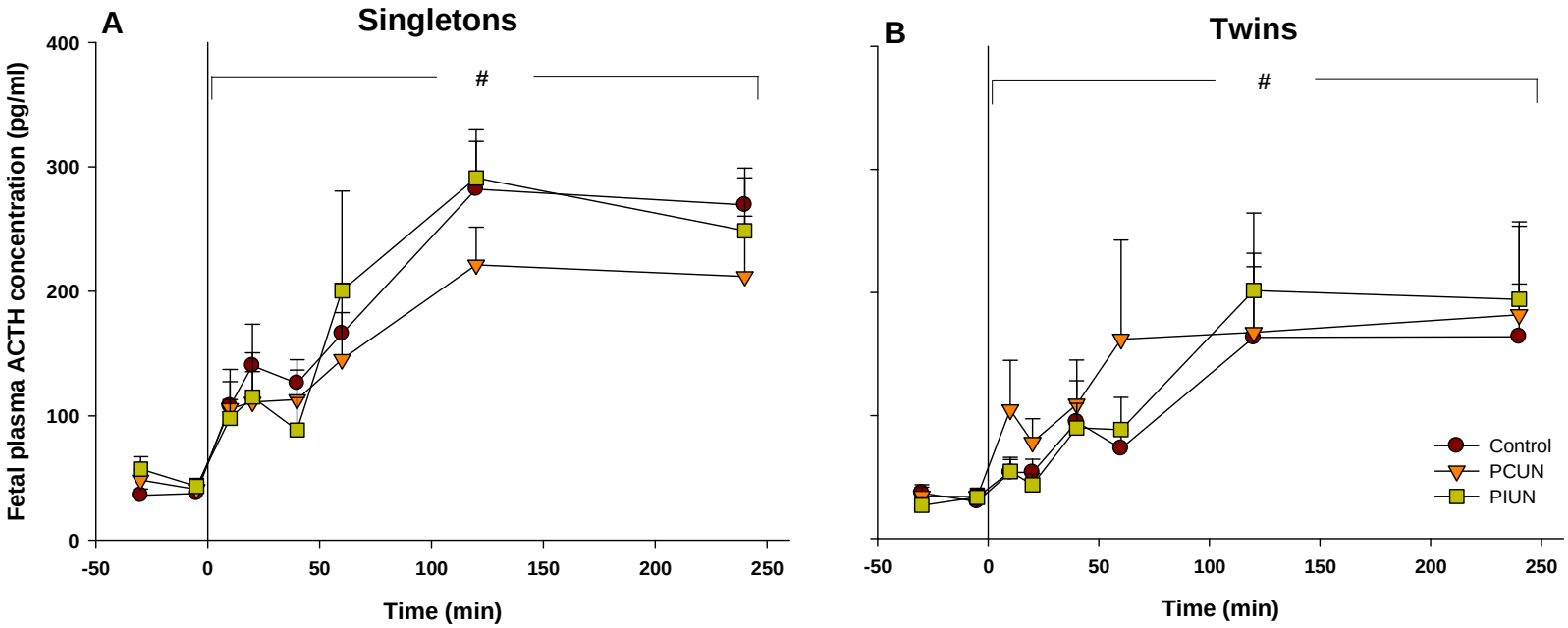


Figure 3.11: Fetal plasma ACTH concentration in response to CRH challenge in singletons (A) and twins (B)

There was a significant effect of time in singleton (A) and twin (B) fetuses in all nutritional groups in response to a 1 µg bolus dose of CRH injected at time point 0. # denotes a significant increase in plasma ACTH concentration compared to pre-infusion values.

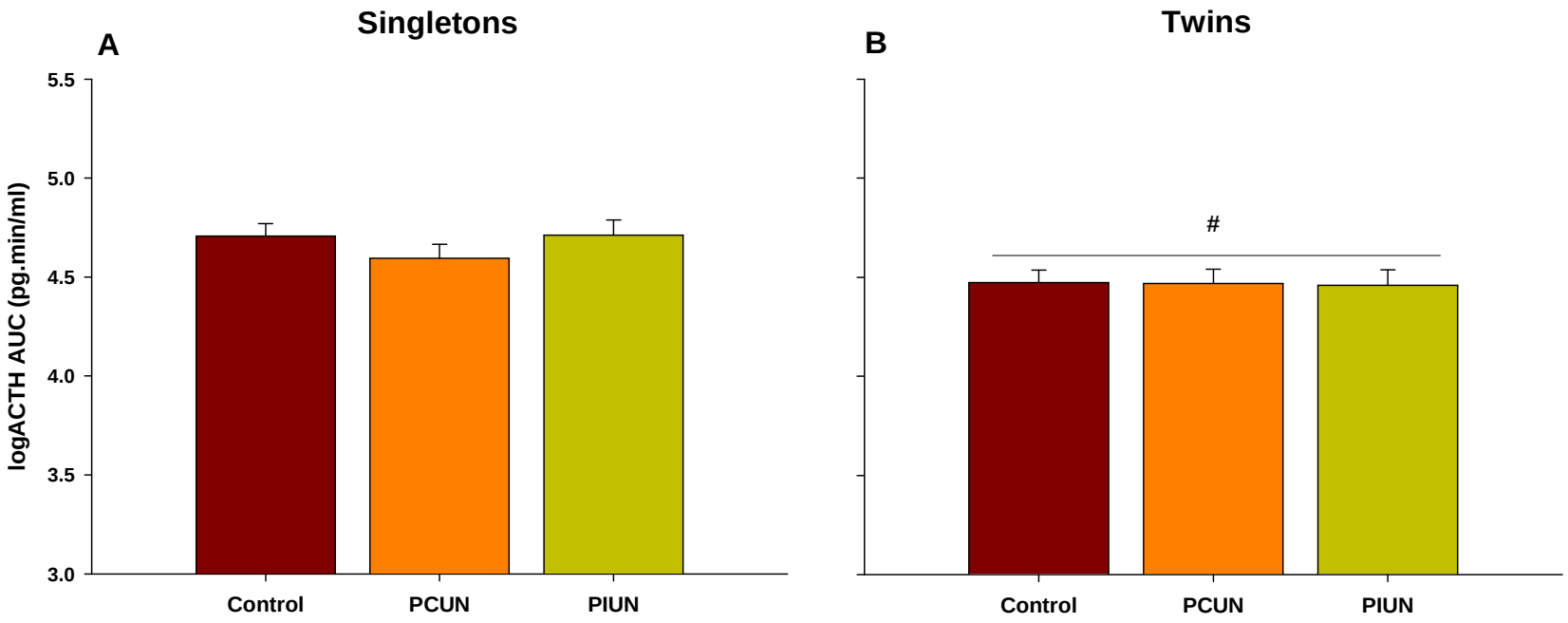


Figure 3.12: ACTH Area Under the Curve (AUC) in singleton (A) and twins (B)

The post-infusion ACTH AUC was significantly lower in twins (B) when compared to singletons (A) independent of nutritional treatment, as denoted by #.

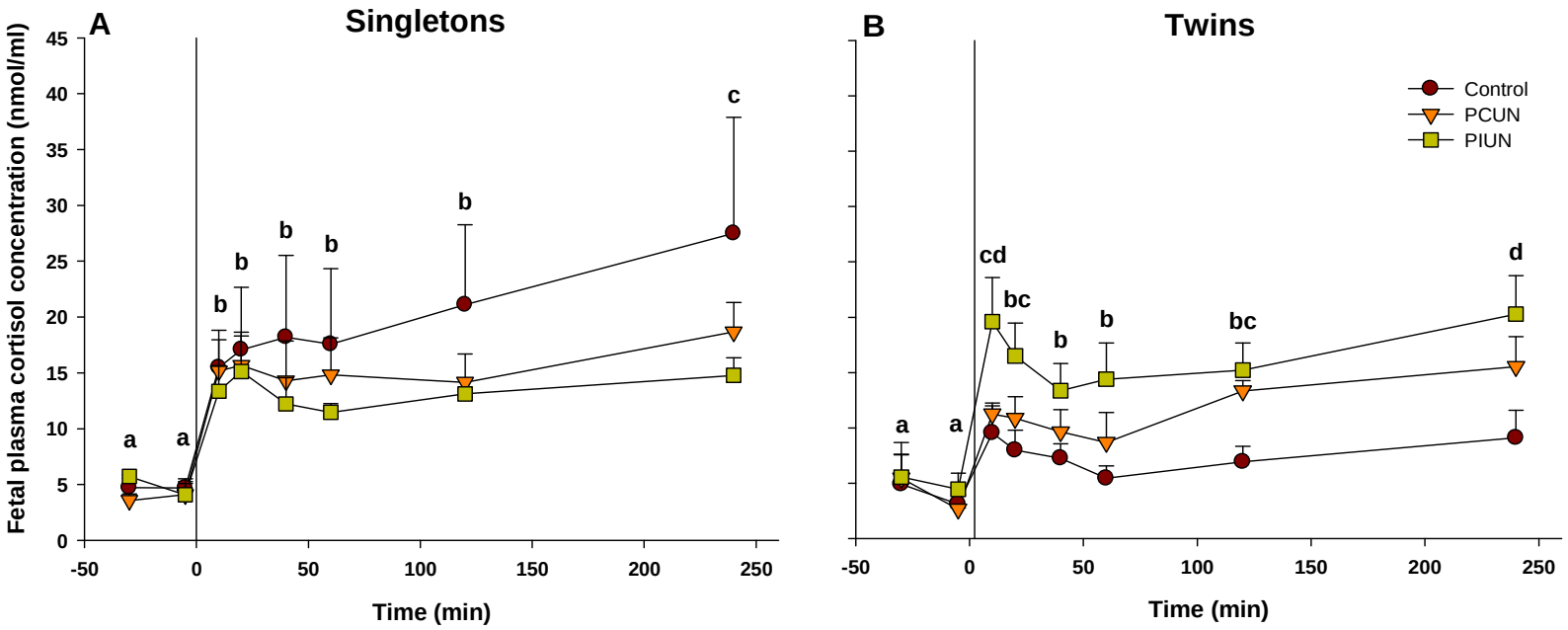


Figure 3.13: Fetal plasma cortisol concentration in response to CRH challenge in singletons (A) and twins (B)

There was a significant effect of time in all nutritional groups in singletons (A) and twin (B) fetuses in response to a 1 µg bolus dose of CRH injected at time point 0. Different alphabetical subscripts denote mean values, which are significantly different.

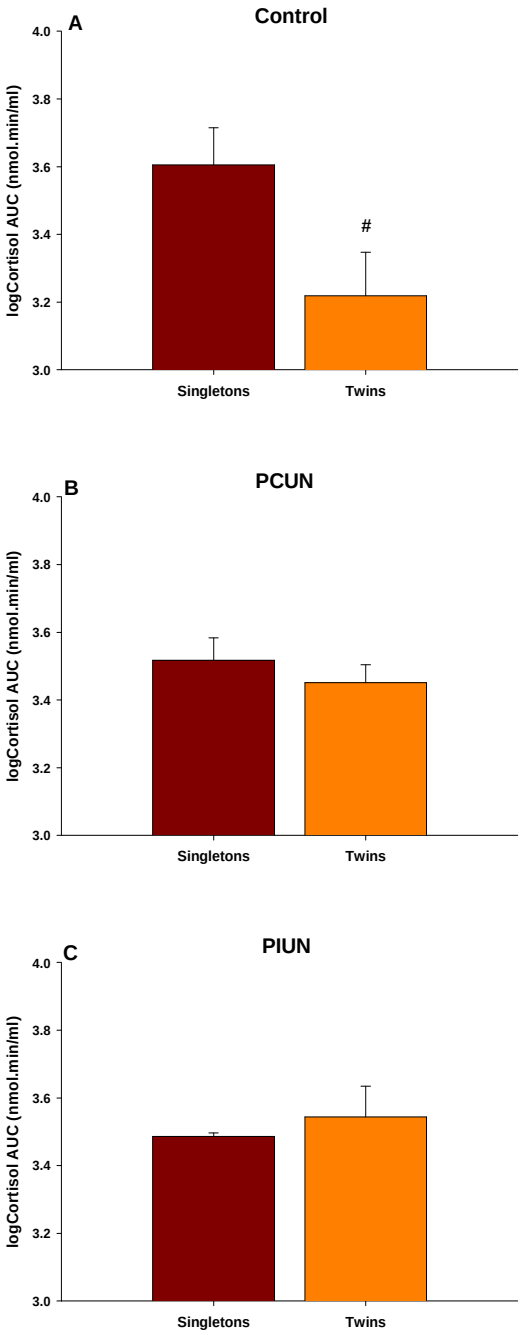


Figure 3.14: Cortisol Area Under the Curve (AUC) in Control (A) and PCUN (B) and PIUN (C)

The post-infusion cortisol AUC was significantly lower in twins when compared to singletons in the Control group (A), as denoted by #. There was no effect of fetal number on the cortisol AUC in the PCUN (B) and PIUN (C) groups.

3.4 DISCUSSION

The objective of this study was to investigate whether fetal number and the plane of nutrition of the ewe around the time of conception and particularly during the preimplantation period alone influences the timing of the activation of the HPA axis and the responsiveness of the HPA axis to an exogenous stimulus in late gestation. This study agrees with previous findings that there is a delay in the activation of the HPA axis and a decrease in adrenocortical responsiveness in twin compared to singleton sheep (Edwards & McMillen, 2002a; Gardner *et al.*, 2004). As previously speculated, the observed diminished adrenal responsiveness in the twin sheep fetus during late gestation may be an adaptive response designed to counter the impact of the potential exposure of a twin fetus to increased intrauterine stress, thus reduce the possibility of preterm delivery (Gardner *et al.*, 2004; McMillen *et al.*, 2004).

3.4.1 Maternal and Fetal Glucose Concentration

Previous studies have reported that ewes carrying twins have a lower plasma glucose concentration when compared to singleton-bearing ewes in late (Edwards & McMillen, 2002a; Rumball *et al.*, 2008b) but not early and mid-gestation (Rumball *et al.*, 2008b). Similarly, Edwards and McMillen (2002) and Rumball and colleagues (2008) found that fetal plasma glucose concentrations were significantly lower in twins when compared to singletons in late gestation (Edwards & McMillen, 2002a; Rumball *et al.*, 2008b). One speculation is that the observed lower maternal plasma glucose concentrations are the result of a greater nutritional demand of the twin pregnancies from the mother when compared to singleton pregnancies and that the lower fetal plasma glucose concentrations in

twins are due to the requirement of resource sharing. In contrast, in the present study the effect of fetal number on maternal glucose plasma concentrations was absent and, although there was a trend for fetal number to decrease fetal plasma glucose concentrations in late gestation, this failed to reach significance.

There is evidence that maternal nutrient restriction during gestation affects maternal and fetal plasma glucose concentrations. The reduction of maternal nutrient intake by 50% between 28 and 78 days of gestation resulted in significantly lower maternal and fetal plasma glucose concentrations at 78 days of gestation (Vonnahme *et al.*, 2003) and 50% nutrient restriction between 115 and 147 days of gestation also lead to lower maternal and fetal plasma in late gestation (Edwards & McMillen, 2002a; Yuen *et al.*, 2002). In ewes that were subjected to a severely restricted diet to reduce their body weight by ~ 15% between 60 days before and 30 days after conception maternal plasma glucose concentrations were significantly lower during the undernutrition period only whereas fetal plasma glucose concentrations where significantly lower in singletons from nutrient restricted ewes when compared to control singletons between 114 and 124 days of gestation (Rumball *et al.*, 2008b). When maternal intake was restricted to 70% between 60 days before and for the first 7 days after conception maternal and fetal plasma glucose concentrations was not affected in late gestation (Edwards & McMillen, 2002a). These latter findings agree with the present study where undernutrition during either the periconceptional or preimplantation period had no effect on maternal and fetal plasma concentrations of glucose between 119 and 138 days of gestation. This suggests that maternal undernutrition during the periconceptional and preimplantation period have no effect on maternal glucose concentration in later gestation and that it may be that

a more severe level of nutrient restriction or the extension of undernutrition into early gestation is required before an effect on fetal plasma glucose concentration in singleton fetuses is observed.

3.4.2 Fetal Arterial Blood Gas Characteristics

The fetal blood gas measures across late gestation in fetuses in all nutritional treatment groups were within the previously reported range for normal healthy fetuses (Char & Creasy, 1977; Robinson *et al.*, 1979; Simonetta *et al.*, 1997; Edwards *et al.*, 1999; Gardner *et al.*, 2002).

A previous study by Edwards and McMillen (2002) found that mean gestational arterial PO_2 was lower in twin compared to singleton fetuses. In contrast, in this study arterial PO_2 was not different between twin and singleton fetuses in the Control group in late gestation (Edwards & McMillen, 2002b).

Edwards and McMillen (2002) have reported that maternal undernutrition during the periconceptional period had no effect on arterial PO_2 in either singletons or twins whilst arterial PCO_2 was significantly lower in PCUN singletons when compared to controls (Edwards & McMillen, 2002b). In the current study, however, periconceptional undernutrition did not result in a change in either arterial PO_2 or PCO_2 in singleton or twin fetuses.

Interestingly, fetal arterial PO_2 was significantly lower in the twin when compared to singleton fetuses in the PIUN group whilst fetal arterial PCO_2 was significantly increased in PIUN twin fetuses when compared to Control and PCUN twins. In addition, maternal undernutrition during the early preimplantation period had no effect on fetal pH. Thus PIUN twin fetuses have a lower arterial PO_2 and a higher

arterial PCO_2 without being acidotic in late gestation. In Control and PCUN fetuses, there was a positive relationship between fetal and placental weight suggesting that as the placenta increases in size so does the fetus (see Chapter 2). In PIUN fetuses, however, this relationship was not present (see Chapter 2). Given the absence of a relationship between fetal and placental weight, it seems likely that low arterial PO_2 and a high arterial PCO_2 in PIUN twins is caused by a change in placental gas exchange capacity.

This study has therefore demonstrated that maternal undernutrition during the early preimplantation period alone, a time when nutrient demand of the embryo is minimal, has an effect on fetal arterial PO_2 and PCO_2 in twins in late gestation.

3.4.3 Plasma ACTH and Cortisol Concentrations during late Gestation

The activation of the HPA axis in late gestation and the subsequent prepartum surge of cortisol in the fetus is essential for the maturation of lung, liver, kidney and gut in preparation for extrauterine life (Liggins, 1994), the initiation of labour and timing of birth in the sheep (for review see Whittle *et al.*, 2001). In the current study there was a significant increase in fetal ACTH and cortisol concentrations between 123 – 130 and 135 – 138 days gestation in singletons in all nutritional groups. This is consistent with the well characterised prepartum surge in plasma ACTH and cortisol concentrations in singleton fetal sheep (Norman *et al.*, 1985; Challis & Brooks, 1989; Ozolins *et al.*, 1991; Phillips *et al.*, 1996a; Edwards & McMillen, 2002a). In twins, this increase in fetal plasma ACTH concentration was absent. It has been previously shown that there is a delay in the prepartum activation of the HPA axis in the twin fetal sheep (Edwards & McMillen, 2002a). It has been speculated that the delayed activation of the HPA axis in the twin may

serve to protect the fetus from preterm delivery (Edwards & McMillen, 2002a; McMillen *et al.*, 2004). It is therefore possible that in the twin the gestational window during which the activation of the HPA axis occurs is after 136 – 138 days of gestation. Intriguingly, fetal plasma cortisol concentrations in twins were significantly higher at 135 -138 days of gestation when compared to either 123 – 126 or 127 – 130 days of gestation and this pattern was similar to that in singleton pregnancies. The absence of a rise in ACTH concentrations may suggest that there is a decreased responsiveness of the fetal pituitary to CRH or AVP in late gestation or that there is a factor inhibiting ACTH secretion. There are a number of possible explanations for the increase in cortisol in the absence of an increase in ACTH in twin fetuses:

1. The action of a pituitary-derived factor, other than ACTH that is capable of stimulating adrenal steroidogenesis independently of or in synergy with ACTH. One such possible pituitary-derived candidate is N-proopiomelanocortin (N-POMC) (1-77), which is a cleavage product of proopiomelanocortin (POMC). A 48 h intra-fetal infusion of N-POMC (1-77) in late gestation results in larger adrenals when compared to saline infused control animals (Ross *et al.*, 2000a).

2. The increase in fetal plasma cortisol may be due to an increase in adrenal responsiveness to lower fetal plasma ACTH concentrations. This may be the case if adrenal expression of MC2R is upregulated in fetal twin sheep. Alternatively enhanced post receptor signalling may also lead to an increased adrenal sensitivity to lower concentrations of fetal ACTH in the twin.

3. The action of an intra-adrenal factor, which is upregulated in the adrenal and which acts independently or in synergy with ACTH to stimulate cortisol synthesis in the twin adrenal.

4. The removal of an inhibitory intra-adrenal factor, which limits adrenal steroidogenesis in the twin fetus in late gestation.

In the present study there was no effect of PCUN or PIUN on fetal plasma ACTH and cortisol concentrations in either singletons or twins in late gestation. Although Edwards and McMillen (2002) reported that periconceptional nutrient restriction had no effect on basal fetal ACTH and cortisol concentrations in singletons, they found that undernutrition during the periconceptional period increased plasma ACTH concentrations in twins (Edwards & McMillen, 2002a). One explanation for the different findings in these two studies may be that different antisera to ACTH were used in the ACTH radioimmunoassays in these two studies, which could result in different molecular weight forms of ACTH being measured in each study. In the study of Edwards and McMillen (2002) basal fetal plasma ACTH concentrations ranged from 50 – 110 pg/ml and were measured using an ICN Biomedicals radioimmunoassay, whereas in the present study, plasma ACTH concentrations ranged from 30 – 65 pg/ml and were measured using a DiaSorin radioimmunoassay. It has been shown that ACTH in fetal sheep plasma is present in a range of molecular weight forms, not all of which are equally effective at increasing adrenal cortisol output (Ozolins *et al.*, 1991). One possibility is that PCUN results in an increase in the output of higher molecular weight ACTH containing peptides from the fetal pituitary and that these are measured in the

ACTH assay used by Edwards and McMillen (2002) but not in the DiaSorin assay used in the present study.

3.4.4 ACTH and Cortisol response to CRH

In the current study a bolus dose of CRH resulted in the characteristic increase in fetal plasma ACTH and cortisol concentration in the late gestation fetal sheep. There was a significantly lower ACTH response to CRH in twins when compared to singletons independent of nutritional group. Furthermore, the plasma cortisol response to CRH was significantly lower in control twins when compared to control singletons. It is likely that the decreased cortisol response is a function of the decreased ACTH response to CRH in the twin fetus. Alternatively, it may be the result of blunted or delayed adrenocortical responsiveness in twins in late gestation. This is supported by evidence provided by Gardner and colleagues (2004) who showed that there was a significant reduction in fetal cortisol concentration in response to an intravenous bolus of synthetic ACTH in twins when compared to singleton at ~ 133 days of gestation (Gardner *et al.*, 2004). The current data suggest that the responsiveness of the pituitary to CRH is either delayed or blunted in twin fetuses in late gestation. The decreased responsiveness of the fetal pituitary may be explained by 1) the presence of more CRH non-responsive corticotrophs, 2) decreased sensitivity of pituitary corticotrophs to CRH 3) lower expression of the ACTH precursor, proopiomelanocortin (POMC) and/or activity of enzymes which cleave ACTH from POMC or 4) the presence of an intra-pituitary inhibitory factor which limits the effects of CRH stimulation.

It should be noted that in contrast to the present study, Rumball and colleagues (2008) showed that twins had greater ACTH and cortisol concentrations in

response to a bolus of CRH/AVP at 127 - 128 days of gestation when compared to singletons (Rumball *et al.*, 2008c). The differences in findings between these studies may be explained by AVP acting as a greater corticotrophic agent in the PCUN.

Interestingly, in the current study in contrast to the Control group, the cortisol response to CRH was not ablated in the PCUN and PIUN twin compared to singletons fetuses. This suggests an increased activation in the HPA axis of the PCUN and PIUN groups and this observation may be explained by:

1. Pituitary derived factors other than ACTH present in the PCUN and PIUN twin fetal sheep, which result in an enhanced cortisol response to CRH in these groups.

2. An increased expression of adrenal MC2R receptor and/or increased post receptor signalling leading to an increased adrenal cortisol response to ACTH in the PCUN and PIUN twin fetuses.

3. An increased expression of an intra-adrenal stimulatory factor or a decreased expression of an intra-adrenal inhibitory factor, which acts independently or in synergy with ACTH leading to a maintained cortisol response to CRH in the PCUN and PIUN twin fetuses.

It is not clear at this stage, which of these possible mechanisms explains the findings in this current study. It does however appear that both periconceptional and preimplantation undernutrition each act to alter the adrenal cortisol response

to CRH in the absence of a change in the ACTH response. In Chapter 4, I have investigated the effects of PCUN and PIUN on the intra-adrenal steroidogenic pathway and on adrenal IGF expression.

3.4.5 Summary

The present study confirms that the development of the fetal HPA axis differs in singleton and twin fetuses as it was found that the surge in ACTH plasma concentration is delayed in twin fetuses and that the ACTH response to exogenous CRH is blunted in twins when compared to singletons independent of periconceptional nutritional treatment. In addition, the cortisol response to CRH is blunted in control twins compared to singletons, and importantly, this blunting does not occur in the PCUN and PIUN twin groups. The results of the present study are relevant in the context that they provide evidence which suggests that nutritional perturbations during the first week of gestation alone can alter the development and responsiveness of the HPA axis in the twin fetus in late gestation.

CHAPTER 4: IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON FETAL ADRENAL GROWTH AND ADRENAL GROWTH FACTOR AND STEROIDOGENIC ENZYME EXPRESSION IN THE SHEEP DURING LATE GESTATION

4. IMPACT OF MATERNAL UNDERNUTRITION DURING THE PERICONCEPTIONAL AND PREIMPLANTATION PERIOD ON FETAL ADRENAL GROWTH AND GROWTH FACTOR AND STEROIDOGENIC ENZYME EXPRESSION IN THE SHEEP DURING LATE GESTATION

4.1 INTRODUCTION

As discussed in previous Chapters, it is clear that both embryo number and the periconceptional environment have a long term impact on the development on the HPA axis. Previous studies have shown that activation of the HPA axis in late gestation is delayed and adrenal responsiveness to ACTH is blunted in twins when compared to singletons (Edwards & McMillen, 2002a; Gardner *et al.*, 2004). The results from Chapter 3 confirm that that development of the fetal HPA axis differs in singleton and twin fetuses: the gestational surge in plasma ACTH concentrations is delayed and the ACTH response to CRH is blunted in twins when compared to singletons independent of nutritional treatment. Additionally, Edwards and McMillen (2002) demonstrated that moderate nutrient restriction during the periconceptional period led to a precocious activation of the HPA axis in twins (Edwards & McMillen, 2002a) and Chapter 3 provides evidence that there is a differential cortisol response to the stimulation of the HPA axis in the PCUN and PIUN twins when compared to control twins. In Chapter 3 I showed that the cortisol response to an intra-fetal CRH bolus is blunted in control twins but that the cortisol response does not differ between PCUN and PIUN singletons and twin fetuses. These findings suggest that not only does maternal undernutrition during the periconceptional period have an impact on the development of the HPA axis but undernutrition during the first week of pregnancy is sufficient to alter the development and responsiveness of the HPA axis in the twin fetus in late

gestation. It is not clear, however, how fetal number or exposure of the oocyte and/or early embryo to maternal undernutrition leads to alterations in the development of the HPA axis. One possibility is that adaptations to a twin pregnancy and/or to periconceptional and preimplantation undernutrition results in epigenetic modifications to genes which play a key role in adrenal growth and steroidogenesis. As discussed in Chapter 1, IGF2 and IGF2R are parentally imprinted, each known to be epigenetically regulated, and are implicated in the regulation of adrenal growth and steroidogenesis in the fetal sheep (MacLaughlin *et al.*, 2007; Ross *et al.*, 2007).

Therefore, this study investigated the effects of fetal number and maternal undernutrition during the periconceptional period, from 60 days before until 6 days after mating, and maternal undernutrition during the preimplantation period alone, that is, for the first 6 days of gestation, on adrenal growth, steroidogenic capacity and the epigenetic state of adrenal *Igf2* and *Igf2r* at 136 – 138 days of gestation in the fetal sheep.

4.2 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.

4.2.1 Nutritional Management and Mating

As discussed in the Chapter 2, 63 South Australian Merino ewes were used in this study and the feeding and mating protocols were as reported in Chapters 2 and 3. Briefly, sixty days prior mating ewes were randomly assigned to one of three

feeding regimes: Control (C, n = 21), Periconceptional Undernutrition (PCUN, n = 21) and Preimplantation Undernutrition (PIUN, n = 21). The Control ewes received 100% of the nutritional requirements from around 60 days prior mating until 6 days after mating. The PCUN ewes received 70% of the control allowance from approximately 60 days prior mating until 6 days after mating. The PIUN ewes were maintained on the 70% diet from mating until 6 days after mating only.

From 7 days after mating (7 days of gestation), all ewes were fed a control diet (100% of requirements) until postmortem (PM) at 136 -138 days of gestation. Ewes were weighed approximately every week after commencing the feeding regime until day 6 of pregnancy. Pregnancy was diagnosed and fetal number estimated by ultrasound at around 60 days of pregnancy. The number of fetuses carried by each ewe was confirmed at PM.

4.2.2 Animal Surgery and Blood Sampling

Implantation of maternal and fetal vascular catheters and blood sampling were performed as described in Chapter 3.

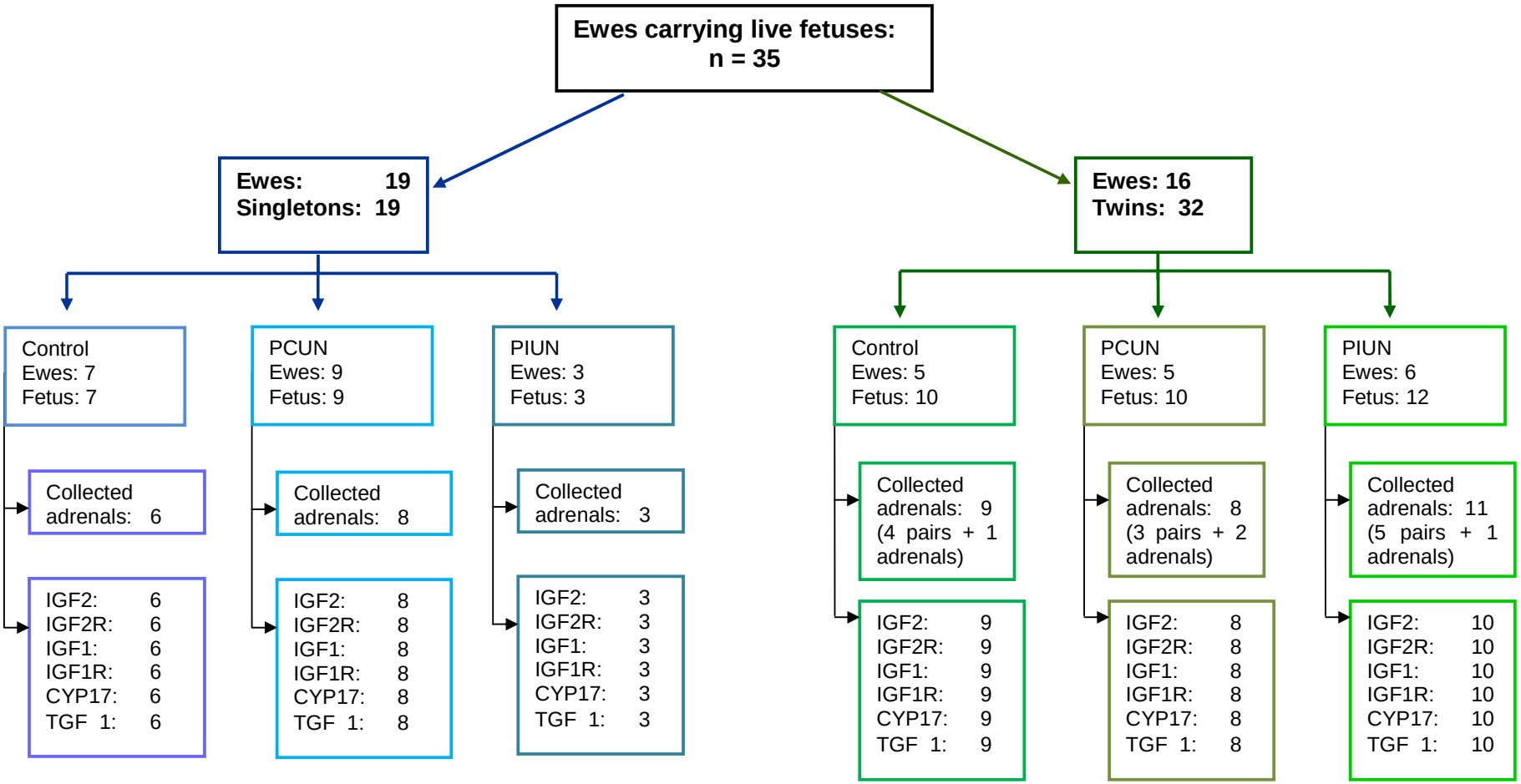
4.2.3 Collection of Adrenal Tissues

Ewes (n = 35, Figure 4.1) were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) between 136 and 138 days of gestation, and the utero-placental unit was delivered by hysterotomy. Fetuses were immediately weighed and killed by decapitation. Fetal adrenals were then collected, weighed and samples were snap frozen in liquid nitrogen. Samples were then stored at -70° Celsius for further molecular analysis.

4.2.4 Isolation of RNA and Reverse Transcription PCR

Total RNA was isolated from fetal adrenals (Figure 4.1) using Trizol® reagent (Invitrogen Life Technologies®, Carlsbad, CA) and treated with DNase 1 (Ambion, Austin, Texas, USA) to minimise genomic DNA contamination. Each sample was purified using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland) and RNA was quantified by spectrophotometric measurements at 260nm and 280nm. RNA integrity was confirmed by agarose gel electrophoresis. 3 g of total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen Life Technologies®, Carlsbad, CA) and random oligohexamers (100 M) for priming. Non amplification controls (NACs) containing no Superscript III reverse transcriptase for each sample were also synthesised.

Figure 4.1: Summary of animal numbers in each experimental group



4.2.5 Quantitative Real Time PCR

The relative abundance of IGF1, IGF2, IGF1R and IGF2R, CYP17 and TGF 1 mRNA transcripts in the fetal adrenal were measured by quantitative real time 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 4.1), water (2µl), and 50ng/µl cDNA (1µl) to give a total volume of 10µl. Each amplicon was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls for each primer set containing no cDNA were included on each plate. To ascertain that synthesised cDNA was free from genomic contamination non-amplification controls from each sample were also included.

Three replicates of cDNA from each sample of fetal adrenal were performed for each gene on each plate, and each plate was repeated twice. Amplification efficiencies were determined, from the slope of a plot of Ct (defined as the cycle number at which the fluorescence generated within a reaction crosses a set threshold line) 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, the acidic ribosomal protein P0 (RpP0), was calculated using Q-Gene analysis software (Muller *et al.*, 2002).

Table 4.1: Primer sequences for qRT PCR

Accession Number	Primer Name	5'/3'	Sequence
DQ152962	IGF-1 Fwd	5'→3'	TTG GTG GAT GCT CTC CAG TTC
	IGF-1 Rev	5'→3'	AGC AGC ACT CAT CCA CGA TTC
AY162434	IGF-1R Fwd	5'→3'	AAG AAC CAT GCC TGC AGA AGG
	IGF-1R Rev	5'→3'	GGA TTC TCA GGT TCT GGC CAT T
M89789	IGF-2 Fwd	5'→3'	GCT TCT TGC CTT CTT GGC CTT
	IGF-2 Rev	5'→3'	TCG GTT TAT GCG GCT GGA T
AF327649	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
NM_001009483	CYP17 Fwd	5'→3'	CCC CCA CAA GGC TAT CAT TGA
	CYP17 Rev	5'→3'	CTG CTG CCA CTC CTT CTC ATT
M36271	TGFβ1 Fwd	5'→3'	CTG CTG AGG CTC AAG TTA AAA GTG
	TGFβ1 Rev	5'→3'	CAG CCG GTT GCT GAG GTA G
BT021080	RpP0 Fwd	5'→3'	CAA CCC TGA AGT GCT TGA CAT
	RpP0 Rev	5'→3'	AGG CAG ATG GAT CAG CCA

4.2.6 Methylation Analysis

DNA methylation within the DMRs of *Igf2/H19* and *Igf2r* were analysed by combined bisulphite restriction assay (COBRA) in the laboratory of Dr. Catherine Suter (Victor Chang Medical Research Institute, New South Wales). Approximately 2µg of DNA from individual adrenals was subjected to bisulphite conversion (Epitect, Qiagen, Basel, Switzerland). PCR was performed on 100ng of bisulphite-converted DNA using primers and conditions that amplified methylated and unmethylated templates with no bias. For *Igf2/H19* the amplicon contained 19 individual CpG sites including the proximal CTCF binding site (Genebank Accession AJ566210, 2917 – 3209). For *Igf2r*, 24 individual CpG sites within a 148bp fragment derived from intron 2 on the *Igf2r* gene (Genebank Accession AY182033, 1828 – 1976) were investigated. Primers are shown in Table 4.2. COBRA was performed using restriction endonucleases that cleave only those amplicons derived from methylated templates. *Igf2/H19* and *Igf2r* amplicons were digested 20U of *Hinf*I and *Mlu*I (New England Biolabs, Ipswich, MA, USA) respectively, for 2 hours at 37°C. Digests were resolved on a 2.5% high resolution agarose gel and the intensity of uncut and cut fragments quantified using a Fujifilm FLA-5100 (Minatoku, Tokyo, Japan). Percentage of methylation was calculated by measuring the ratio of cut to uncut PCR product.

4.2.7 Statistical analysis

Data are presented as the mean ± SEM. The effects of maternal nutritional treatment and fetal number on the fetal adrenal weight, expressed as an absolute weight or relative to body weight, the relative expression of IGF1, IGF1R, IGF2, IGF2R, CYP17 and TGF 1 mRNA and the percentage of methylation for *Igf2/H19* and *Igf2r* in the fetal adrenal were determined using a two way Analysis of

Variance (ANOVA) using STATA 10 for Windows (StataCorpLp, Colege Station, TX, USA). When there was an interaction between the effects of maternal nutritional treatment and fetal number, data from singletons and twins were split and the effects of maternal nutritional treatment determined using a one way ANOVA. When there was a significant effect of nutritional treatment in the absence of any interaction between the effects of treatment and fetal number, adrenal weight and relative gene expression data are generally pooled for presentation. The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. Relationships between IGF2R and CYP17 were assessed by linear regression using Sigma Plot 10.0 (SPSS Inc., Chicago, IL, USA). A probability level of 5% ($P < 0.05$) was taken to be significant.

Table 4.2: Primer sequences for the *Igf2/H19* locus and *Igf2r* locus

DMR	**Acc. Number**	**Primer sequence (5'→3')**
Igf2/H19	AJ566210	**F:** ATTTTAAATAGGGTTGAGAGGTTGT
		R: AAACACAAAAAATCCCTCATTATC
Igf2r	AY182033	**F:** GTTAGATTTAGTTAYGTTTTGTAG
		R: RCAAAATCTACAAAACCC

4.3 RESULTS

4.3.1 Fetal Adrenal Weight

Total fetal adrenal weight was significantly greater ($P < 0.02$) in the PCUN, but not PIUN group compared to the Control group, in both singletons and twin pregnancies at 136 – 138 days of gestation (Figure 4.2). There was no effect of maternal nutritional treatment, however, when adrenal weight was expressed relative to fetal weight. Relative adrenal weight was significantly greater ($P < 0.05$) in twin compared to singleton fetuses (Figure 4.3).

4.3.2 Adrenal IGF1 and IGF1R mRNA expression

There was no effect of either maternal nutritional treatment or fetal number on adrenal IGF1 and IGF1R mRNA expression at 136 – 138 days of gestation (Figure 4.4).

4.3.3 Adrenal IGF2 mRNA expression and DMR methylation

When analysing the adrenal gene expression data from twin fetuses, it was noted that there was a substantial variation in the adrenal expression of IGF2 mRNA in fetal twin sheep (range of 3.60 – 12.36). The coefficient of variation for adrenal IGF2 mRNA expression in each treatment group was up to 7 fold higher in twins when compared to singleton fetuses. The data were therefore split into singleton and twin groups for further analysis. In singleton pregnancies, the fetal adrenal IGF2 mRNA expression in the PCUN group was significantly lower ($P < 0.03$) when compared to the Control and PIUN groups whereas there was no effect of maternal nutritional treatment on adrenal IGF2 mRNA expression in twin fetuses at 136 – 138 days of gestation (Figure 4.5).

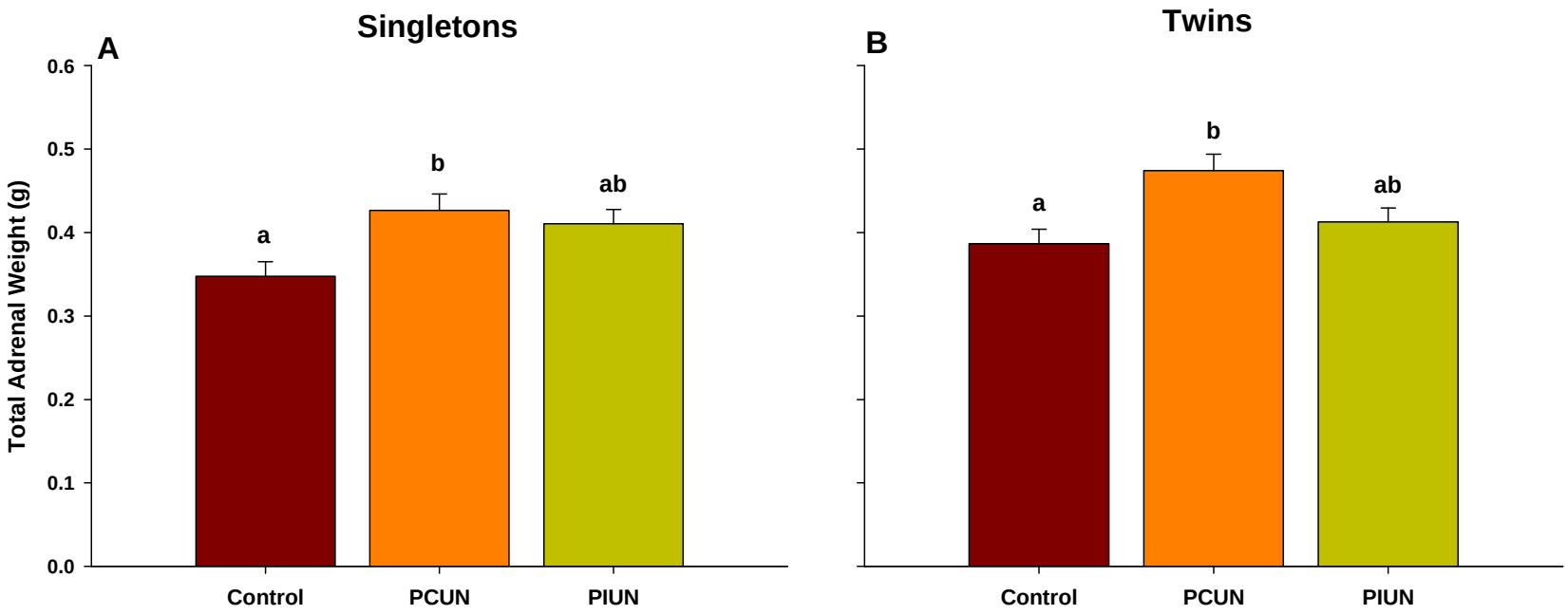


Figure 4.2: Absolute fetal adrenal weights in the Control, PCUN and PIUN singleton (A) and twin (B) fetuses

There was a significant increase (P<0.02) in absolute adrenal weight in the PCUN group when compared to the Control group but not the PIUN group. Different superscripts denote mean values which are significantly different to each other.

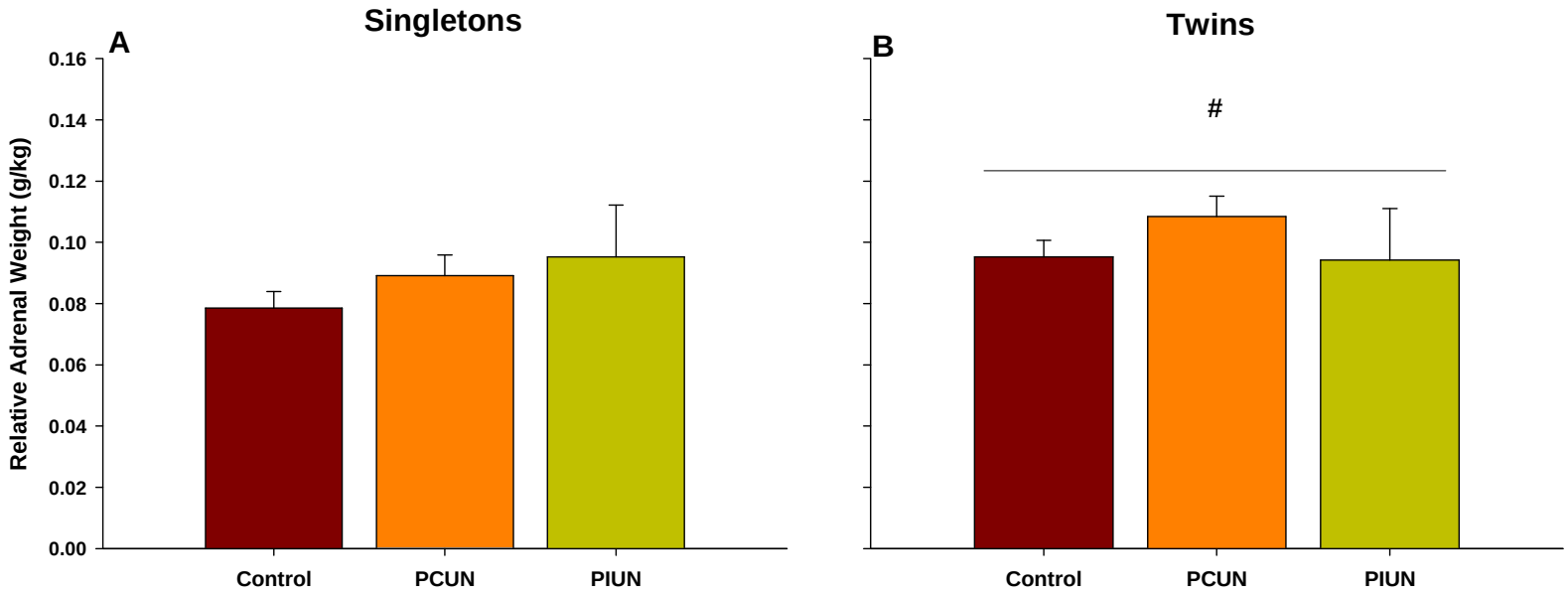


Figure 4.3: Relative adrenal weights in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

The relative adrenal weight was significantly higher in twin (B) compared to singleton (A) fetuses independent of nutritional treatment, as denoted by #.

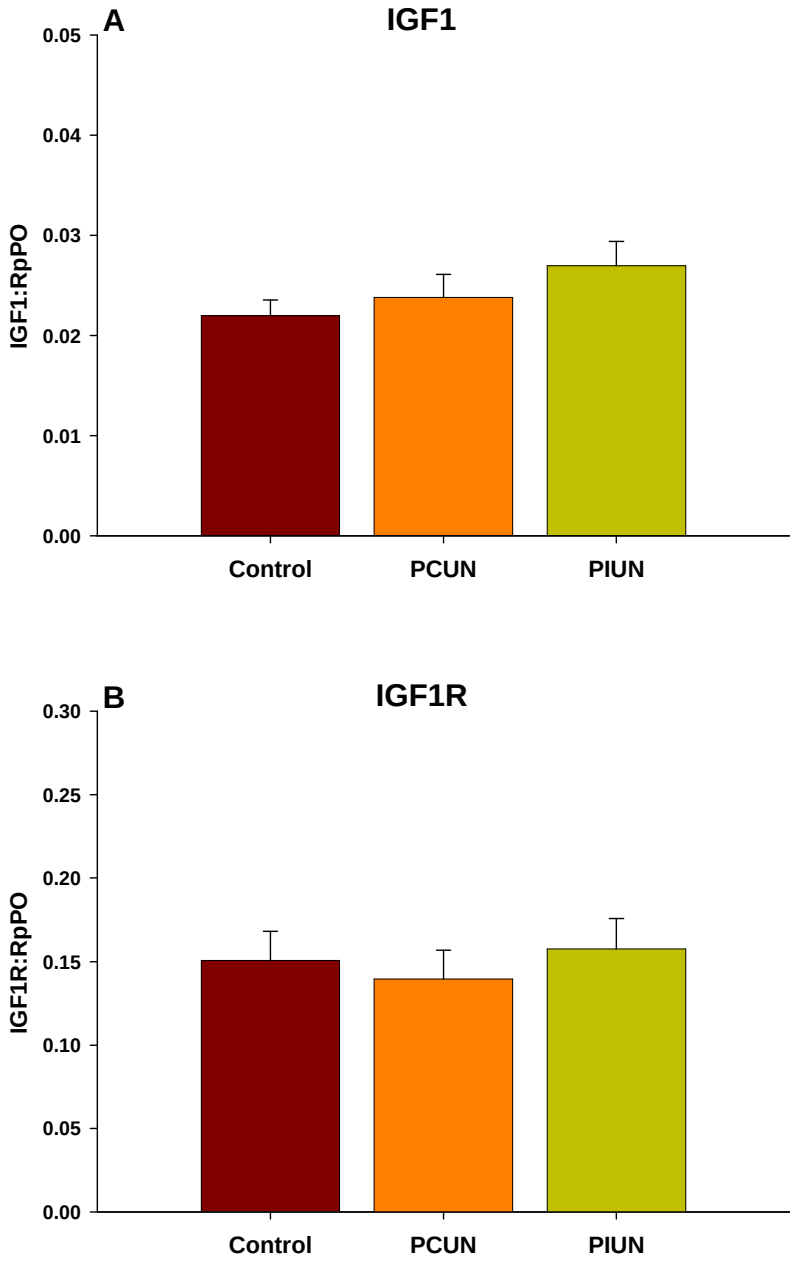


Figure 4.4: IGF1 and IGF-1R mRNA expression in the fetal adrenal (twin and singleton data combined)

There was no effect of maternal nutritional treatment or fetal number on adrenal IGF1 and IGF1R mRNA expression (Control: n = 15, PCUN: n = 16, PIUN: n = 13).

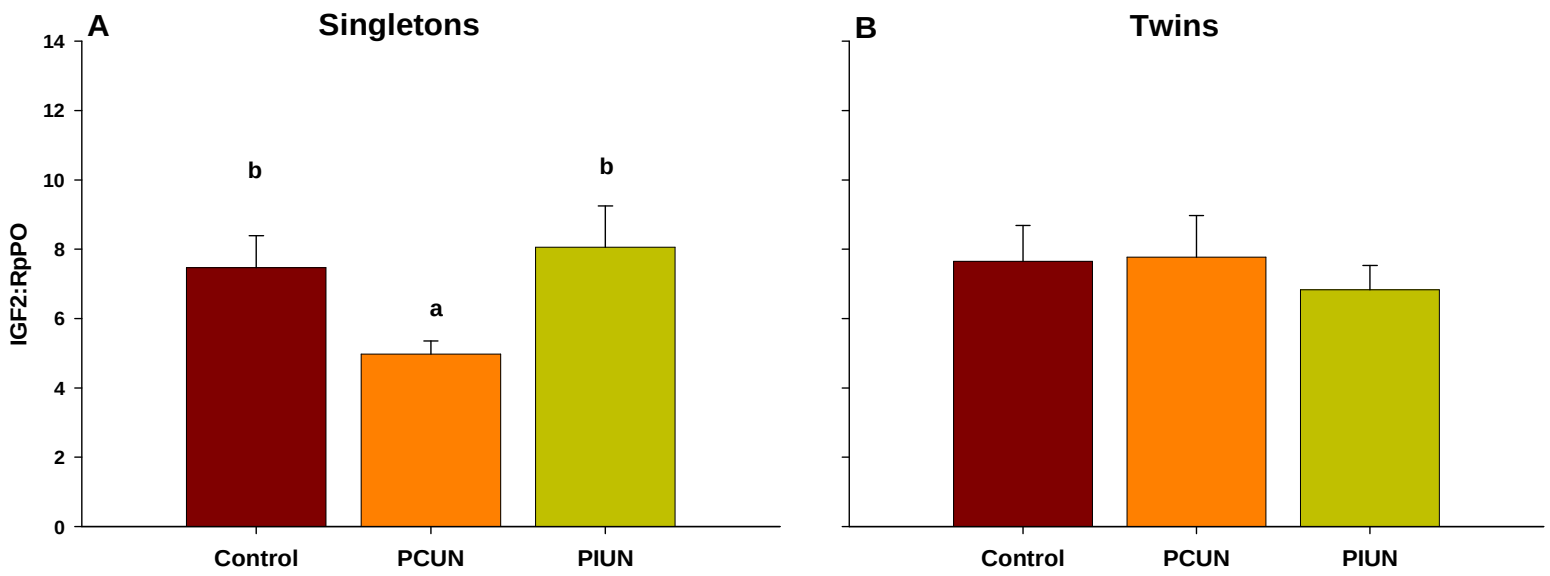


Figure 4.5: IGF2 mRNA expression in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

In singletons (A), there was a significant decrease in IGF2 mRNA expression in the PCUN group when compared to the Control and PIUN groups. In twins (B), there was no effect of maternal nutritional treatment or fetal number on fetal adrenal IGF2 mRNA expression. Different superscripts denote mean values which are significantly different to each other.

There was no effect of either maternal nutritional treatment or fetal number on the methylation status in the adrenal *Igf2/H19* DMR at 136 – 138 days of gestation (Figure 4.6).

4.3.4 Adrenal IGF2R mRNA expression and DMR methylation

The expression of IGF2R mRNA in the fetal adrenal was significantly increased ($P < 0.03$) in the PCUN group when compared to the Control and PIUN groups, and this effect occurred independent of fetal number (Figure 4.7). There was no separate effect of fetal number on the expression of adrenal IGF2R mRNA.

There was no effect of maternal nutritional treatment on the methylation status in the *Igf2r* DMR, however, there was significantly less methylation in the adrenal *Igf2r* DMR in twins when compared to singleton fetuses in all treatment groups (Figure 4.8).

4.3.5 Adrenal CYP17 mRNA expression

There was also a substantial variation in the adrenal expression of CYP17 mRNA in twin fetal sheep (range of 0.438 – 3.686). The data were therefore split into singleton and twin fetuses for further analysis. In singleton pregnancies, adrenal CYP17 mRNA expression in the PCUN group was significantly higher ($P < 0.03$) when compared to the Control and PIUN groups whereas there was no effect of nutritional treatment on adrenal CYP17 mRNA expression in twin fetuses at 136 – 138 days of gestation (Figure 4.9).

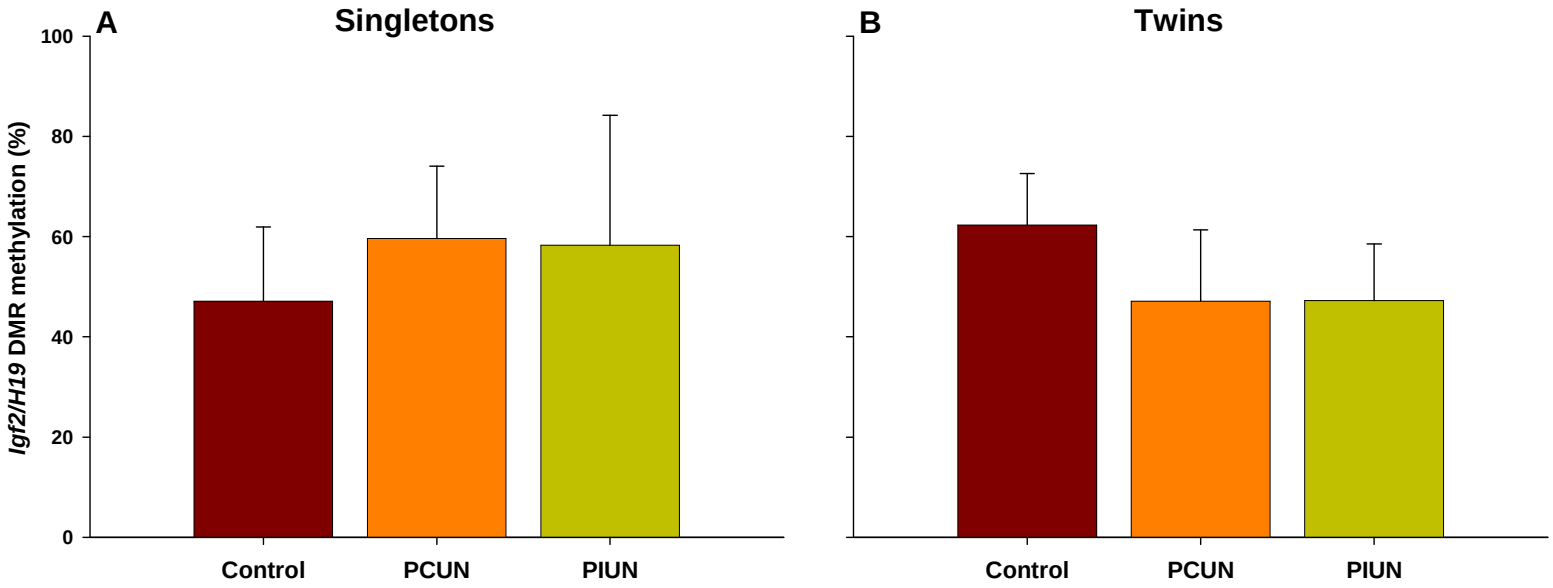


Figure 4.6: *Igf2/H19* DMR methylation in the fetal adrenal in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

There was no effect of maternal undernutrition or fetal number on adrenal *Igf2/H19* DMR methylation. (Control S: n = 6; Control T: n = 8; PCUN S: n = 7; PCUN T: n = 8; PIUN S: n = 3; PIUN T: n = 9)

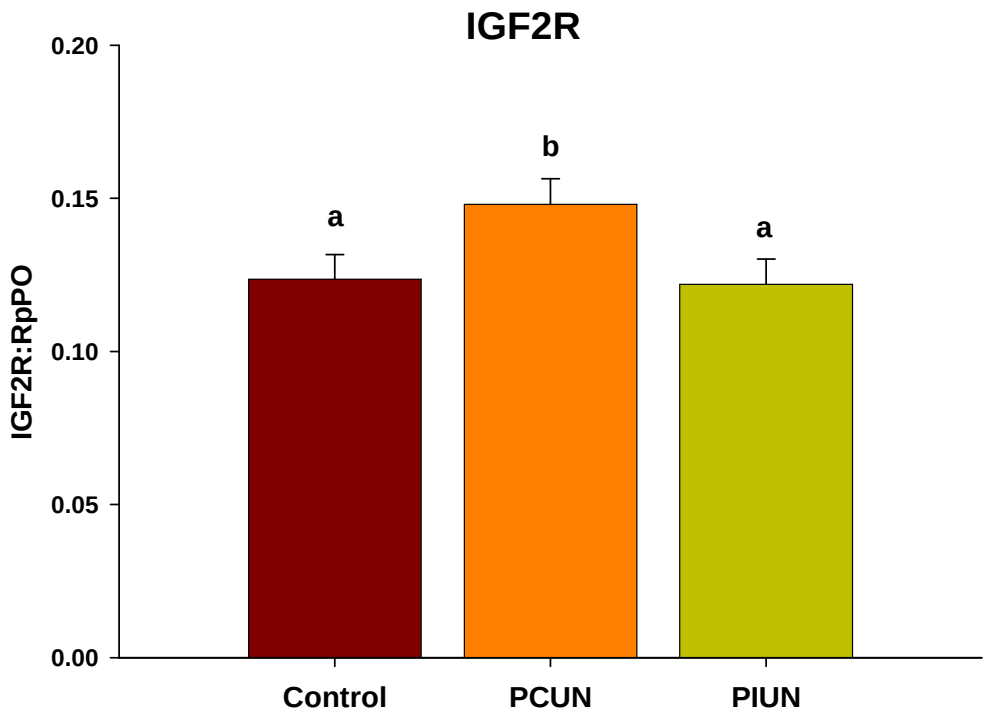


Figure 4.7: IGF-2R mRNA expression in the fetal adrenal (twin and singleton data combined)

There was a significant increase in IGF2R mRNA expression in the PCUN group (n = 16) when compared to the Control (n =15) and PIUN (n =13) groups. Different superscripts denote mean values which are significantly different to each other.

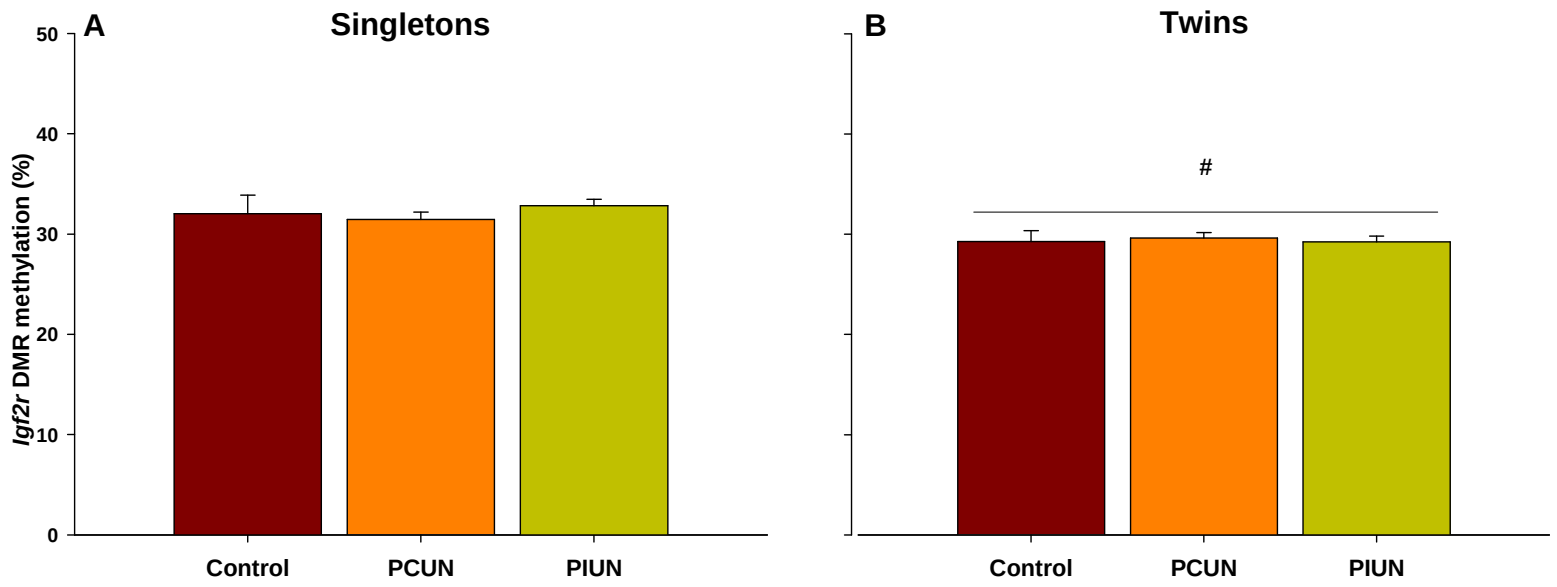


Figure 4.8: *Igf2r* DMR methylation in the fetal adrenal in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

The *Igf2r* DMR methylation was significantly lower in twin (B) compared to singleton (A) fetuses independent of nutritional treatment, as denoted by #. (Control S: n = 5; Control T: n = 9; PCUN S: n = 6; PCUN T: n = 8; PIUN S: n = 3; PIUN T: n = 10)

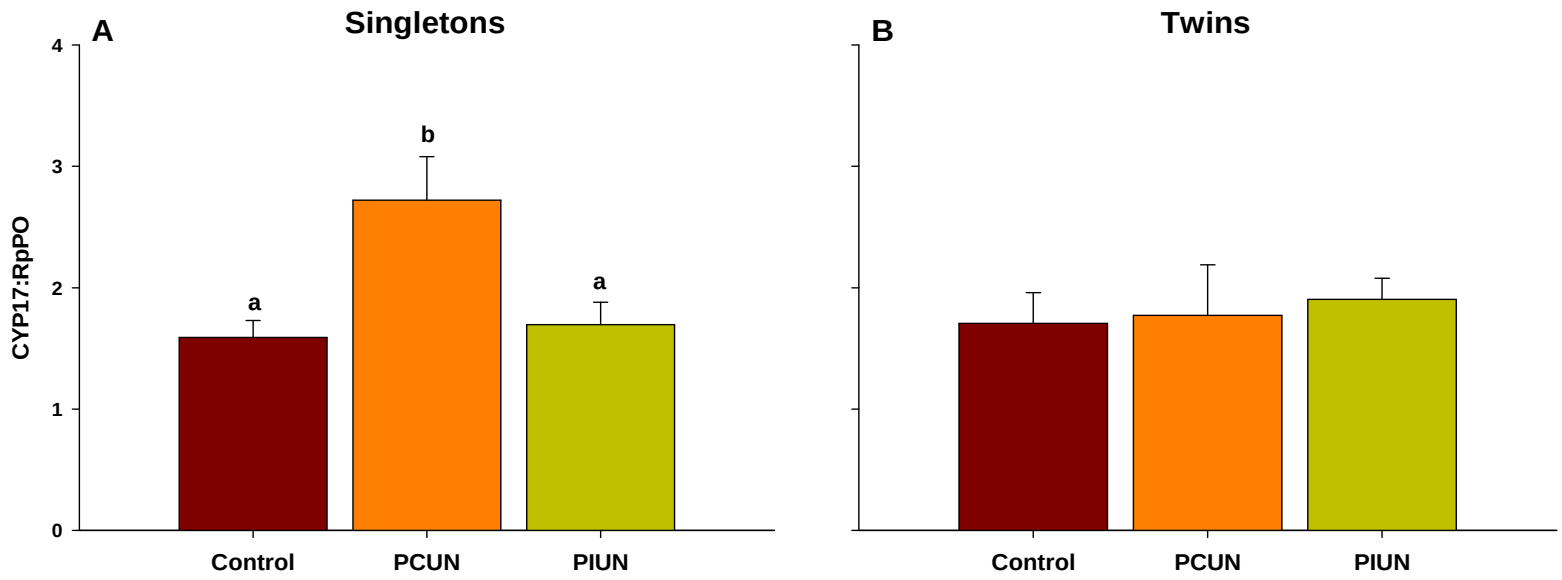


Figure 4.9: CYP17 mRNA expression in Control, PCUN and PIUN in singleton (A) and twin (B) fetuses

In singletons (A), there was a significant increase in CYP17 mRNA expression in the PCUN group when compared to the Control group only. In twins (B), there was no effect of maternal nutritional treatment or fetal number on fetal adrenal CYP17 mRNA expression. Different superscripts denote mean values which are significantly different to each other.

4.3.6 Relationship between IGF2R and CYP17 mRNA expression

In the Control and PCUN groups there was a direct and significant relationship between fetal adrenal IGF2R and CYP17 mRNA at 136 – 138 day of gestation. This relationship was not present, however, in the PIUN group (Table 4.2 and Figure 4.10).

Table 4.2: The relationships between fetal adrenal CYP17 (y) and IGF2R (x) mRNA

	Relationship between CYP17 (y) and IGF2R (x) mRNA expression
Control	y = 13.9x - 0.06 (r = 0.71, n = 15, P < 0.004)
PCUN	y = 18.9x - 0.55 (r = 0.56, n = 15, P < 0.04)
PIUN	y = 1.8x + 0.87 (r = 0.05, n = 14, NS)

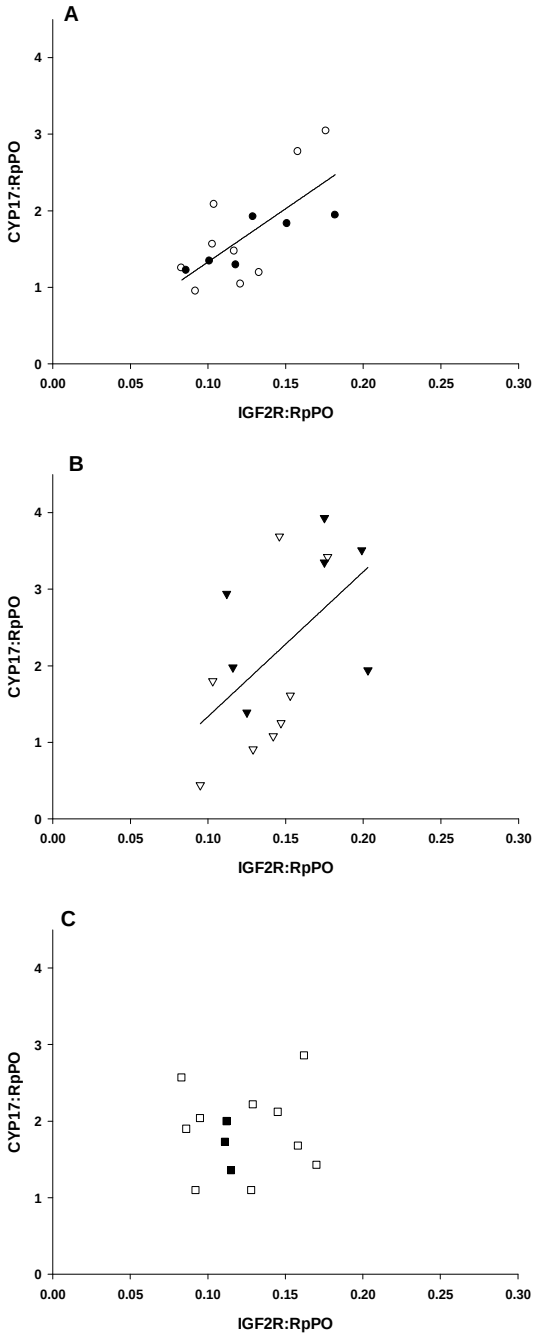


Figure 4.10: Relationship between fetal adrenal IGF2R and CYP17 mRNA

There was a positive relationship between fetal adrenal IGF2R and CYP17 mRNA in the Control group (singletons closed circles, twins open circles, A) and PCUN group (singletons closed triangles, twins open triangles, B). This relationship was absent in the PIUN group (singletons closed squares, twins open squares, C).

4.3.7 Adrenal TGF 1 mRNA expression

There was no effect of either maternal nutritional treatment or fetal number on adrenal TGF 1 mRNA expression at 136 – 138 days of gestation (Figure 4.11).

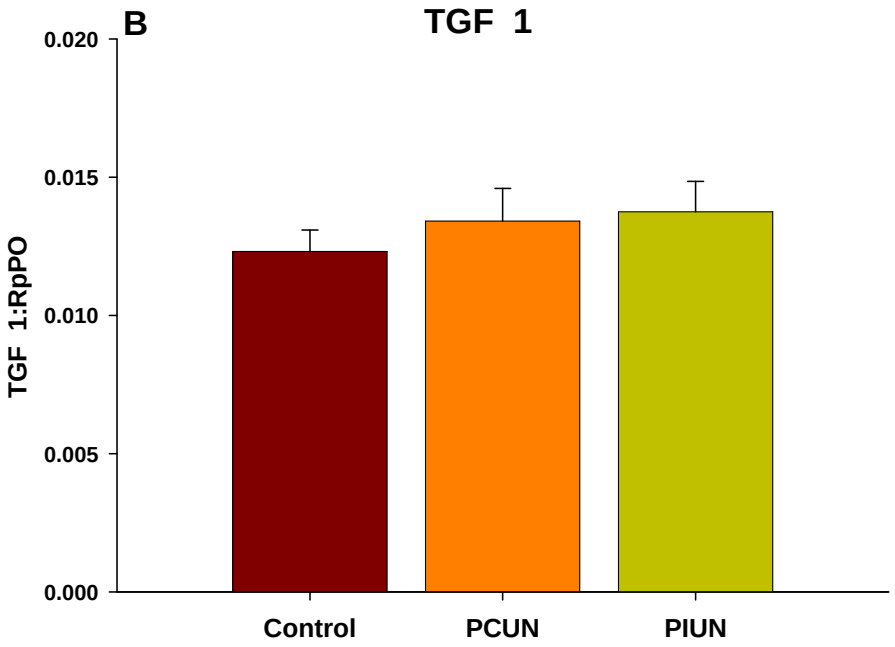


Figure 4.11: TGF 1 mRNA expression in the fetal adrenal (singleton and twins combined)

There was no effect of maternal nutritional treatment or fetal number on adrenal TGF 1 mRNA (Control: n = 15, PCUN: n = 16, PIUN: n = 13).

4.4 DISCUSSION

The objective of this study was to investigate possible mechanisms that may explain the effects the maternal plane of nutrition during the periconceptional and preimplantation periods and fetal number on the basal and stimulated fetal plasma ACTH and cortisol concentrations in late gestation as reported in Chapter 3. Specifically, the current study aimed to determine whether maternal undernutrition during different periconceptional windows and fetal number resulted in changes in fetal adrenal growth and in the expression of IGFs, CYP17 and TGF 1 mRNA expression in the fetal adrenal at 136 – 138 days of gestation. Furthermore, the effects of fetal number and maternal undernutrition on the methylation status of the *Igf2* and *Igf2r* genes were investigated.

4.4.1 Adrenal Growth, IGFs and CYP17 expression in Singletons and Twins

As reported in Chapter 3, the rise in plasma ACTH concentration in late gestation was absent or delayed in twins when compared to singletons, although there was an increase in fetal cortisol concentration in both singletons and twins in late gestation. The present study shows that the relative weight of the fetal adrenal is greater in twin compared to singleton fetuses independent of nutritional treatment (Table 4.3).

Table 4.3: Summary of results presented in Chapters 3 and 4, (yellow highlight represents an effect of fetal number).

Singleton Control Fetus	Twin vs Singleton Control Fetus
Adrenal weight	T > S
↑ plasma ACTH concentration with gestational age	No increase after 134 days of gestation (T) vs increase after 134 days of gestation (S)
↑ plasma cortisol concentration with gestational age	T = S
ACTH response to CRH Cortisol response to CRH	T < S T < S
Adrenal mRNA expression of: IGF1 and IGF1R IGF2 and IGF2R CYP17 TGF 1	 T = S T = S T = S T = S
Epigenetic state of *Igf2* and *Igf2r*: *Igf2/H19* methylation *Igf2r* methylation	 T = S T < S

Factors which are known to promote adrenal growth include ACTH and the IGFs. An endogenous or exogenous increase in fetal ACTH concentration results in larger adrenals when compared to control animals in the late gestation fetal sheep (Liggins, 1968; Boshier *et al.*, 1981; Lye *et al.*, 1983; Phillips *et al.*, 1996a; Warnes *et al.*, 2004). In addition, it has been reported that a 10 day intra-fetal infusion of IGF1 results in larger fetal adrenals in late gestation (Lok *et al.*, 1996; Coulter *et al.*, 2002; Ross *et al.*, 2007). Thus, these findings demonstrate that circulating IGFs are capable of stimulating adrenal growth in the absence of an increase in ACTH.

At present, there are few studies, which have investigated the relationship between ACTH and IGFs in the regulation of growth in the fetal adrenal and they have resulted in conflicting data. Previous studies have shown that ACTH stimulates IGF2 expression in cultured human fetal adrenal cells *in vitro* (Voutilainen & Miller, 1987, 1988; Mesiano *et al.*, 1993). In contrast, *in vivo*, an exogenous or endogenous increase in fetal ACTH concentration, results in a decrease, rather than an increase, in IGF2 mRNA expression in the fetal sheep adrenal (Lu *et al.*, 1994; Braems *et al.*, 1998). Therefore, the interaction between ACTH and IGF2 is potentially complex with evidence supporting both excitatory (*in vitro*) and inhibitory (*in vivo*) effect of ACTH on *Igf2* gene and mRNA expression.

In the current study, it is interesting that the adrenal is relatively larger in the twin fetus in the absence of an increase in circulating ACTH concentration when compared to singletons (Chapter 3). The radioimmunoassay used to measure ACTH in Chapter 3, measures predominantly the ACTH (1-39) peptide, rather than ACTH present in the larger molecular weight pro ACTH containing peptides

derived from the multihormone precursor Proopiomelanocortin (POMC). It may be that there is another pituitary derived factor, which promotes adrenal growth that may explain the relatively increased growth of the adrenal in the twin fetus. A study by Ross and colleagues (2000) investigated the effects of intra-fetal infusion of N-POMC (1-77), a cleavage product of POMC, on adrenal growth in the fetal sheep. A 48 h intra-fetal infusion of N-POMC (1-77) between 136 and 138 days of gestation resulted in larger adrenals when compared to saline infused control animals (Ross *et al.*, 2000a). This strongly supports a role for a POMC derived peptide other than ACTH in the stimulation of fetal adrenal growth. It may be that the relative degree of hypoxaemia experienced by the twin fetus in late gestation (Chapter 3) results in the stimulation of the secretion of a peptide such as N-POMC (1 – 77), in addition to ACTH, which stimulates adrenal growth.

An alternative possibility is that intra-adrenal IGFs may act to stimulate adrenal growth in the twin fetus in late gestation. It is the case, however, that there was no difference in adrenal IGF1 and 1R or in IGF2 and 2R mRNA expression between the twin and singleton fetal sheep (Table 4.3). Intriguingly, adrenal *Igf2r* methylation was significantly lower in twins when compared to singletons at 136 – 138 days of gestation (Table 4.3). In contrast to the findings of the present study, adrenal weight and the adrenal mRNA expression of IGF1 and 2 and IGF1R and 2R have shown to be significantly lower in twin compared to singleton fetuses at ~ 55 days of gestation (MacLaughlin *et al.*, 2007). It may be that the lower level of methylation of the adrenal *Igf2r* gene is present in twin fetuses from early pregnancy and that this explains the lower adrenal IGF2R mRNA expression in the twin compared to the singleton fetus at the earlier gestational age. It is further possible that epigenetic changes in the adrenal *Igf2r* gene play a greater role in

determining the level of IGF2R mRNA expression in early gestation but that other factors are able to overcome the effects of the relatively small change in *Igf2r* methylation to determine the level of IGF2R mRNA expression in the twin fetal adrenal in late gestation. The present study has found no evidence that the level of IGF2 mRNA expression is different between singleton and twin fetuses in late gestation or that the epigenetic state of the adrenal *Igf2/H19* gene is altered in the twin fetal sheep. This suggests that the main influence of embryo or fetal number on adrenal growth and development in normally nourished ewes is expressed through epigenetic regulation of adrenal IGF2R, rather than IGF2 expression.

Given that MacLaughlin and colleagues (2007) found that there was a direct relationship between adrenal weight and IGF1 mRNA and between CYP17 and IGF2 mRNA, lower expression of IGF2R at 55 days of gestation would be expected to counteract the decrease in IGF2 mRNA expression measured in the fetal adrenal at this early gestational age (MacLaughlin *et al.*, 2007). One possibility is that in early gestation the intra-adrenal IGFs play a stimulatory role in fetal adrenal growth and that in the face of low levels of adrenal IGF1 and 2 mRNA expression, the decrease in the expression of the IGF2R clearance receptor is important in maintaining adrenal growth and responsiveness in the twin fetus at this early stage. It does not appear, however, that the increased adrenal growth in the twin fetus in late gestation is a consequence of increased expression of IGFs or their receptors in the adrenal. It is possible that any pituitary or POMC derived peptides act to stimulate adrenal growth by an intra-adrenal pathway, other than through a change in IGF expression.

4.4.2 Periconceptional and Preimplantation Undernutrition and Adrenal Growth, IGFs, CYP17 and TGF 1 expression

It is clear from the results of the current and previous studies that there is a differential effect of periconceptional undernutrition in singleton and twin fetuses and therefore these will be discussed separately. The results from Chapters 3 and 4 are firstly summarised in Table 4.4.

Table 4.4: Summary of all results (Chapter 3 and 4), green highlight represents nutritional treatment effect.

	Control (C)	PCUN vs C	PIUN vs C	PCUN vs PIUN
Singletons	Adrenal weight	PCUN > Control	PIUN = Control	PCUN = PIUN
	↑ plasma ACTH concentration with gestational age	PCUN = Control	PIUN = Control	PCUN = PIUN
	↑ plasma cortisol concentration with gestational age	PCUN = Control	PIUN = Control	PCUN = PIUN
	Adrenal mRNA expression of:			
	IGF1 and IGF1R	PCUN = Control	PIUN = Control	PCUN = PIUN
	IGF2	PCUN < Control	PIUN = Control	PCUN < PIUN
	IGF2R	PCUN > Control	PIUN = Control	PCUN > PIUN
	CYP17	PCUN > Control	PIUN = Control	PCUN > PIUN
	TGF 1	PCUN = Control	PIUN = Control	PCUN = PIUN
	Epigenetic state of *Igf2* and *Igf2r*:			
	Igf2/H19 methylation	PCUN = Control	PIUN = Control	PCUN = PIUN
	Igf2r methylation	PCUN = Control	PIUN = Control	PCUN = PIUN
Twins	Adrenal weight	PCUN > Control	PIUN = Control	PCUN = PIUN
	↑ plasma ACTH concentration with gestational age	PCUN = Control	PIUN = Control	PCUN = PIUN
	↑ plasma cortisol concentration with gestational age	PCUN = Control	PIUN = Control	PCUN = PIUN
	Adrenal mRNA expression of:			
	IGF1 and IGF1R	PCUN = Control	PIUN = Control	PCUN = PIUN
	IGF2	PCUN = Control	PIUN = Control	PCUN = PIUN
	IGF2R	PCUN > Control	PIUN = Control	PCUN > PIUN
	CYP17	PCUN = Control	PIUN = Control	PCUN = PIUN
	TGF 1	PCUN = Control	PIUN = Control	PCUN = PIUN
	Epigenetic state of *Igf2* and *Igf2r*:			
	Igf2/H19 methylation	PCUN = Control	PIUN = Control	PCUN = PIUN
	Igf2r methylation	PCUN = Control	PIUN = Control	PCUN = PIUN

4.4.2.1 Singletons

Maternal nutrient restriction during the periconceptional period does not affect either the surge of ACTH and cortisol or the pituitary and adrenal responsiveness to exogenous CRH stimulation in the singleton fetus (Chapter 3). In the PCUN singleton, the absolute adrenal weight, but not relative adrenal weight, is greater when compared to control animals at 136 – 138 days of gestation (Table 4.4). Interestingly, there is also an increase in adrenal CYP17 mRNA expression in the absence of an increase in basal fetal ACTH and cortisol concentration in the PCUN singletons when compared to controls (Table 4.4). A previous study has shown that neither adrenal weight nor CYP17 expression is increased in the PCUN group at 144 – 145 days of gestation (Edwards & McMillen, 2002a). This suggests that the prepartum increase in ACTH in the week after 138 days of gestation in the sheep maximally stimulates adrenal growth and steroidogenesis in both the PCUN and control fetus.

One possibility is that the increase in adrenal growth and CYP17 expression in the PCUN singleton fetus is a consequence of the actions of a common pituitary or adrenal derived trophic factor. Interestingly, there was also a decrease in IGF2 mRNA expression in the adrenal in the PCUN fetus. There is evidence that IGF2 may be stimulatory to adrenal growth and steroidogenesis in early gestation (MacLaughlin *et al.*, 2007), whereas it is less clear whether IGF2 acts to stimulate or inhibit adrenal growth and steroidogenesis in late gestation. Whilst infusion of intra-fetal IGFs does result in an increase in fetal adrenal weight it does not result in a parallel increase in cortisol output (Ross *et al.*, 2007). This suggests that whilst IGFs stimulate growth they may inhibit steroidogenesis. Furthermore, a study by Ross and colleagues (2000) has previously shown that in growth

restricted fetal sheep, an increase in fetal adrenal weight and increase in fetal plasma cortisol concentrations are associated with a lower level of adrenal IGF2 expression (Ross *et al.*, 2000b). It is interesting in the present study, that there is a positive, rather than negative relationship between adrenal CYP17 and IGF2R mRNA expression at 136-138 days gestation. This contrasts with the presence of an inverse relationship between adrenal CYP17 and IGF2R mRNA expression in the singleton fetal sheep at ~ 55 days of gestation (MacLaughlin *et al.*, 2007). This suggests that there may be a change in the relationship between IGF2 expression in the fetal adrenal and adrenal growth and steroidogenesis between early and late gestation.

There are number of possible mechanisms that may explain the increases in adrenal weight and CYP17 mRNA expression and the decrease in IGF2 mRNA expression in PCUN singletons. One possibility is that the increase in adrenal growth and CYP17 mRNA expression and the decrease in IGF2 mRNA expression in the PCUN singleton fetus are the result of the direct actions of a common pituitary or adrenal derived trophic factor and that these intra-adrenal changes are independent of each other (Figure 4.12).

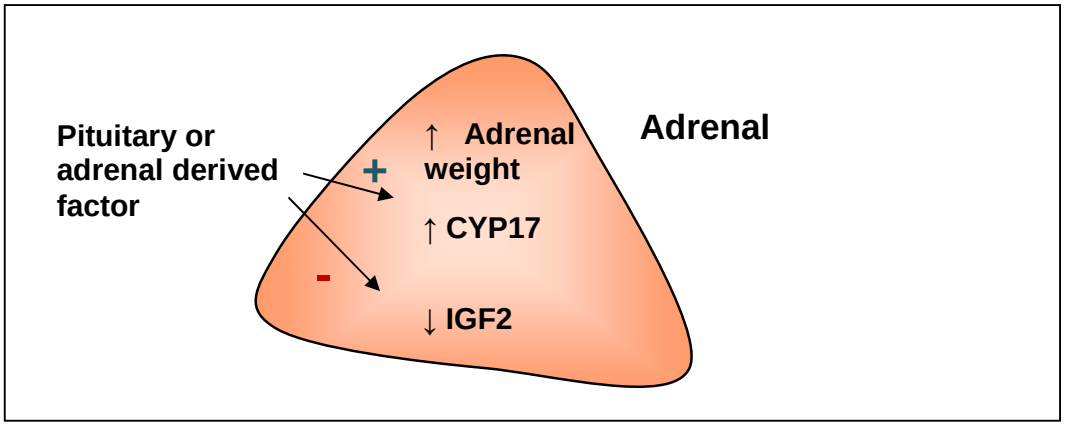


Figure 4.12: Schematic representation of the direct actions of a pituitary or adrenal derived factor.

An alternative possibility is that the increase in adrenal growth and CYP17 mRNA expression and the decrease in IGF2 mRNA expression are the result of either direct or indirect actions of a common pituitary or adrenal derived trophic factor and that these intra-adrenal changes may accordingly influence or depend on each other. Based on evidence presented above there are two possible scenarios considered here: a) IGF2 stimulates adrenal growth and inhibits steroidogenesis and b) IGF2 inhibits adrenal growth and steroidogenesis.

It is possible that in late gestation IGF2, although retaining its stimulatory effect on adrenal growth, acts to inhibit steroidogenesis. It is then possible that periconceptional undernutrition results in the secretion of an adrenal or pituitary derived factor with an adrenal growth stimulatory capacity leading to an increase in adrenal weight and CYP17 mRNA expression. There are several pathways by which this could be achieved including:

1. Factor stimulates adrenal growth and CYP17 mRNA expression whilst inhibiting IGF2 expression **directly**. A decrease in IGF2 could additionally remove its inhibitory effect on CYP17 mRNA expression (Figure 4.13 – A).

2. Factor stimulates adrenal growth and inhibits IGF2 mRNA expression **directly** whilst stimulating CYP17 mRNA expression **indirectly** by decreasing IGF2 and therefore removing its inhibitory action (Figure 4.13 – B).

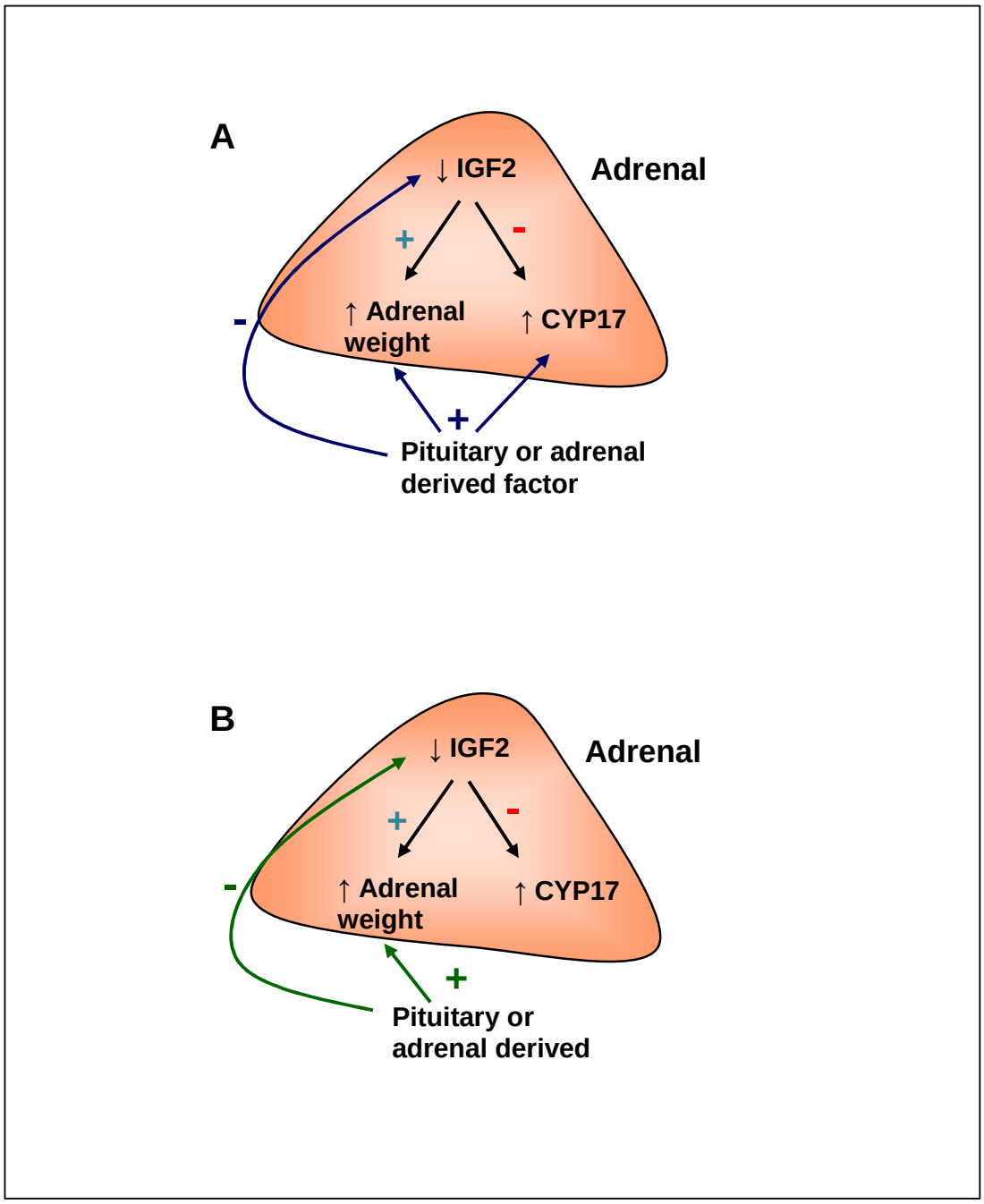


Figure 4.13: Schematic representation of possible direct or indirect actions of a pituitary or adrenal derived factor on adrenal growth, CYP17 and IGF2 mRNA expression, where IGF2 is stimulatory for adrenal growth and inhibitory for CYP17.

Alternatively, the actions of IGF2 may be inhibitory on both adrenal growth and steroidogenesis in late gestation. It is then possible that another adrenal or pituitary derived factor with the ability of overriding the actions of IGF2 is responsible for increasing adrenal weight and CYP17 mRNA expression in late gestation. There are several pathways by which this could be achieved including:

1. Factor stimulates adrenal growth and CYP17 mRNA expression whilst inhibiting IGF2 expression **directly**. A decrease in IGF2 could additionally remove its inhibitory effect on adrenal growth and CYP17 mRNA expression (Figure 4.14 – A).

2. Factor stimulates adrenal growth and inhibits IGF2 mRNA expression **directly** whilst stimulating CYP17 mRNA expression **indirectly** by decreasing IGF2 and therefore removing its inhibitory action. A decrease in IGF2 could additionally remove its inhibitory effect on adrenal growth (Figure 4.14 – B).

At this stage, it is not clear which of these mechanisms explain the effects of periconceptional undernutrition on adrenal growth, CYP17 and IGF2 mRNA expression in singleton fetuses in late gestation. Independent of which mechanism may be at work, the increase in IGF2R mRNA may enhance the clearance of adrenal IGF2 in these animals. In addition, it does not appear that the decrease in IGF2 mRNA expression or the increase in IGF2R mRNA expression, are a result of a change in the methylation status of the DMR in the *Igf2/H19* locus or the *Igf2r* gene.

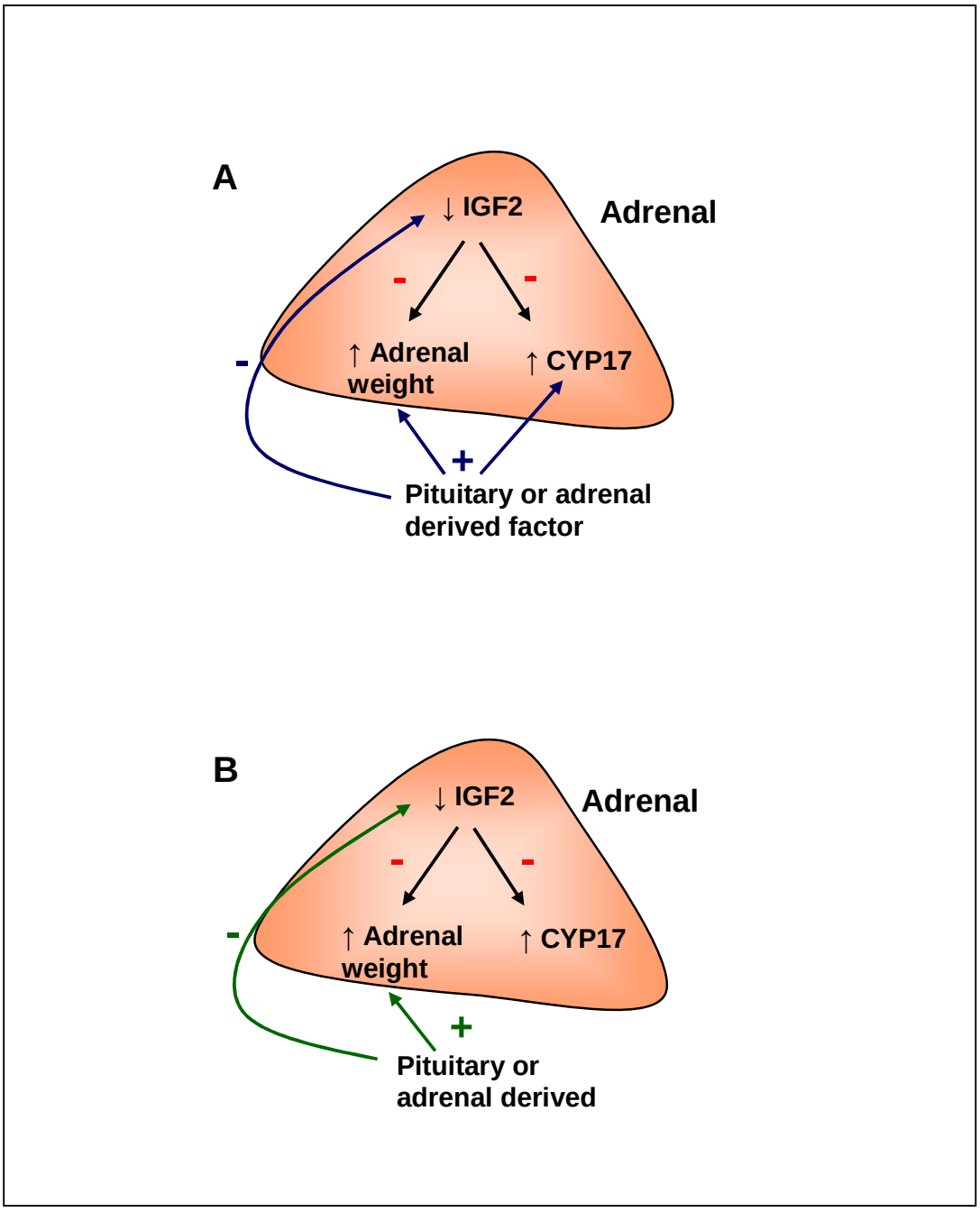


Figure 4.14: Schematic representation of possible direct or indirect actions of a pituitary or adrenal derived factor on adrenal growth, CYP17 and IGF2 mRNA expression, where IGF2 is inhibitory for both adrenal growth and CYP17.

As discussed in Chapter 1, it has been suggested that TGF 1 is an inhibitor of steroidogenesis (Naaman-Reperant *et al.*, 1996; Coulter *et al.*, 2003) and thus a possible candidate through which IGF2 would mediate its inhibitory effect. This then would lead to a decrease in TGF 1 mRNA expression or protein. There was, however, no change in TGF 1 mRNA expression suggesting that at least at the level of gene expression, IGF2 acts independently of TGF 1 at 136 – 138 days of gestation.

There was no effect of maternal nutrient restriction during the preimplantation period on the basal and exogenously stimulated ACTH and cortisol responses (Chapter 3) and the expression of IGFs, CYP17 and TGF 1 mRNA expression in singletons when compared to control animals at 136- 138 days of gestation. Thus, it appears that an exposure of the oocyte or the oocyte and early embryo to maternal undernutrition is required to result in a change in adrenal growth and steroidogenic capacity in the late gestation singleton fetus.

4.4.2.2 Twins

As reported in Chapter 3, pituitary and adrenal responsiveness to exogenous CRH stimulation was blunted in control twins compared to control singletons in late gestation (Table 4.3). Similarly, pituitary responsiveness to CRH stimulation was blunted in PCUN twins compared to PCUN singleton. In contrast to control twins, the cortisol response to CRH was not blunted in the PCUN twin compared to PCUN singletons (Chapter 3). In the PCUN twin there is a significant increase in the absolute adrenal weight, but not relative adrenal weight, and adrenal IGF2R mRNA expression when compared to control animals at 136 – 138 days of gestation (Table 4.4).

The blunted cortisol response to CRH stimulation in control twins when compared to control singletons suggests that potential maximal cortisol production is not reached, and therefore, it may be possible to increase the production of cortisol if necessary. Undernutrition during the periconceptional period appears to result in adaptive changes leading to a cortisol response to CRH that is not blunted in the absence of a change in adrenal CYP17 mRNA expression in PCUN twins in late gestation. This suggests that the PCUN twin adrenal is more responsive than Control twin adrenals and that the increased cortisol output is not mediated through an increase in CYP17 mRNA expression.

Similarly to the previous section the increase in adrenal growth and adrenal IGF2R mRNA expression in PCUN twins may be the result of the **direct** actions of a common pituitary or adrenal derived trophic factor. Additionally, this **direct** action of a pituitary or adrenal derived trophic factor may result in the concomitant absence of a blunted cortisol response to CRH in the PCUN twin (Figure 4.15).

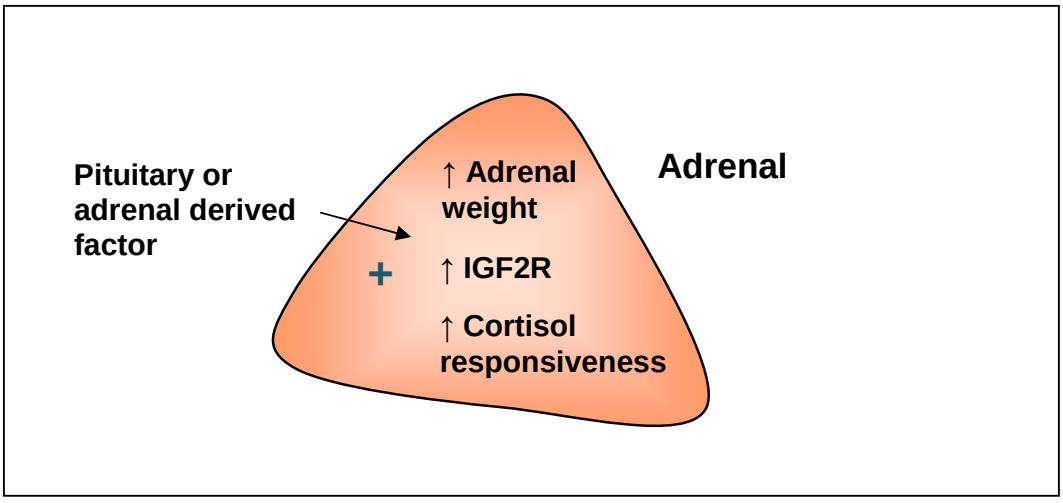


Figure 4.15: Schematic representation of the direct actions of a pituitary or adrenal derived factor.

Alternatively, if IGF2 is an inhibitor of steroidogenesis in late gestation as suggested by evidence discussed in the previous section, then it is possible that the **indirect** actions of a pituitary or adrenal derived factor results in the absence of a blunted cortisol response to CRH by **direct** stimulation of adrenal IGF2R mRNA expression in the PCUN twins (Figure 4.16). That is, it is possible that an increase in adrenal IGF2R mRNA expression leads to enhanced IGF2 clearance resulting in a reduction or removal of the steroidogenic inhibitory actions of IGF2 at the level of proteins. Consequently, the production of cortisol is increased by other means than increasing adrenal CYP17 mRNA expression.

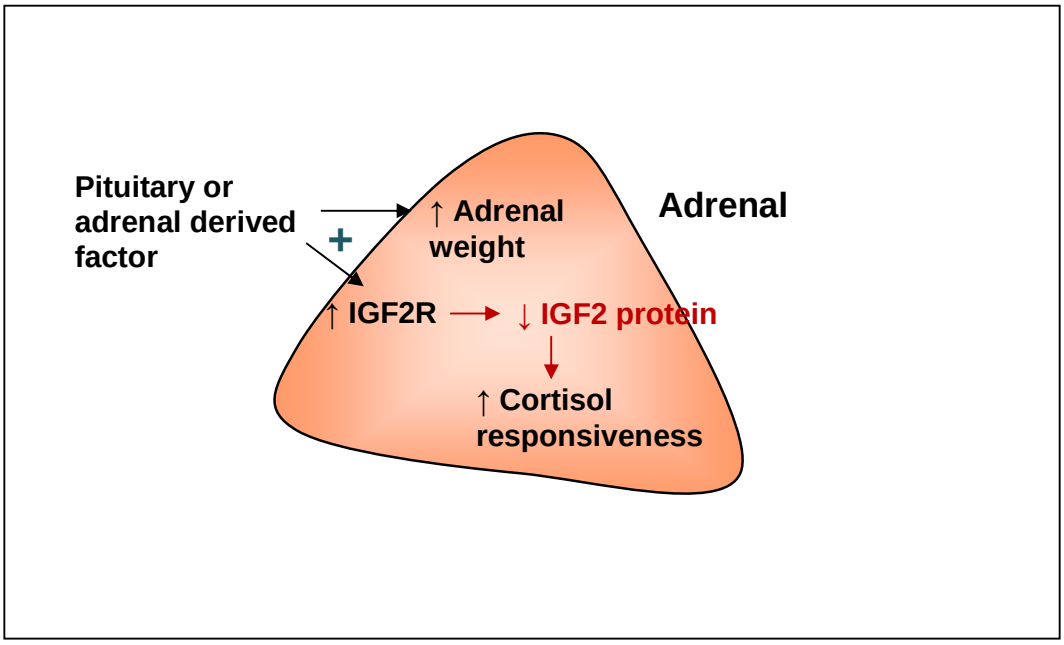


Figure 4.16: Schematic representation of the direct and indirect actions of a pituitary or adrenal derived factor.

It is interesting to note that the cortisol response to CRH in PCUN twins is not different to the response in PCUN singletons and that the cortisol response to CRH does not differ between PCUN and Control twin and singletons. This may be because potential maximal cortisol output has been reached in singletons but not twins and therefore there is still an opportunity for the cortisol response in the PCUN twin to increase to that measured in the PCUN singletons. If this is the case then it is possible that the initial increase in adrenal IGF2R mRNA expression to enhance IGF2 clearance would not result in a further increase in cortisol production in the PCUN singleton.

Similarly to the PCUN twins, the cortisol response to CRH was not blunted in the PIUN twin compared to PIUN singletons (Chapter 3). In contrast to the PCUN twin, however, there was no difference between absolute adrenal weight and adrenal IGF2R mRNA expression when compared to control animals at 136 – 138 days of gestation (Table 4.4). This suggests that the exposure of an embryo during the early preimplantation alone to maternal undernutrition is sufficient to result in increased adrenal responsiveness in late gestation. It is not clear, however, whether the mechanisms by which these adaptations occur are different to the ones resulting from the exposure of the oocyte and early embryo to maternal undernutrition or whether the molecular changes which are present in the adrenal in the PCUN treatment group are not responsible for cortisol responsiveness in late gestation.

4.4.3 Summary

The present study demonstrates that there is a differential effect of fetal number on adrenal growth at 136 – 138 days of gestation and that this effect does not appear to be mediated by changes in the mRNA expression of the IGF system. There is evidence, however, that there is a lower level of methylation of the *Igfr2* gene in the adrenal of twin fetuses and I speculate that this may play a role in the protection of adrenal growth in the twin fetus in early gestation.

Maternal undernutrition during the periconceptional period also has a differential action on adrenal growth and development in singleton and twin fetuses. The present study provides evidence, which suggests that the effects of periconceptional undernutrition on the development and responsiveness of the fetal adrenal in later gestation involves IGF2 and 2R. In this study, however, it appears that PCUN related changes in adrenal IGF2 and 2R mRNA expression are not the result of epigenetic changes in the methylation status of the IGF genes.

Furthermore, the results of the current study suggest that the PCUN related changes in adrenal development and responsiveness in the twin fetus in late gestation are identical to those recruited in the adrenal of the singleton fetus.

CHAPTER 5: IMPACT OF *IN VITRO* EMBRYO CULTURE ON FETAL ADRENAL GROWTH, STEROIDOGENIC AND GROWTH FACTOR GENE EXPRESSION IN THE SHEEP DURING LATE GESTATION

5. IMPACT OF *IN VITRO* EMBRYO CULTURE ON FETAL ADRENAL GROWTH, STEROIDOGENIC AND GROWTH FACTOR GENE EXPRESSION IN THE SHEEP DURING LATE GESTATION

5.1 INTRODUCTION

In the sheep, it is well established that prepartum activation of the hypothalamo-pituitary-adrenal (HPA) axis and an increase in fetal plasma cortisol concentrations are essential for the normal timing of parturition and the successful transition from intrauterine to extrauterine life (term = 150 ± 3 days of gestation) (Challis *et al.*, 2005). After a period of relative 'quiescence' between ~80 and 120 days of gestation, there is a prepartum increase in adrenal growth, adrenal steroidogenic enzyme expression and in the basal and ACTH stimulated level of cortisol synthesis and secretion (Ozolins *et al.*, 1990; Tangalakis *et al.*, 1990; Kerr *et al.*, 1992; Myers *et al.*, 1992; McFarlane *et al.*, 1995; Phillips *et al.*, 1996a). It has been reported that there is a significant increase in the mRNA expression of the key steroidogenic enzyme, cytochrome P450 17α hydroxylase (CYP17) in the fetal adrenal during late gestation (Myers *et al.*, 1992; Phillips *et al.*, 1996a), which is preceded by a decrease in the expression of the putative inhibitor of adrenal steroidogenesis, transforming growth factor beta 1 (TGFβ1) (Coulter *et al.*, 2003).

Recent reports highlight the importance of both embryo number and the periconceptional environment in determining the timing and magnitude of the increase in fetal plasma ACTH and cortisol concentrations during late gestation (Edwards & McMillen, 2002a; Bloomfield *et al.*, 2003; McMillen *et al.*, 2004). We have previously reported that the prepartum activation of the fetal HPA axis is delayed in twin compared to singleton sheep fetuses and that this difference in timing has its origin in early gestation (Edwards & McMillen, 2002a; MacLaughlin

et al., 2007). As early as ~55 days of gestation, fetal adrenal weight is lower in twin compared to singleton fetuses, as is the adrenal mRNA expression of CYP17 and insulin-like growth factor 1 and 2 (IGF1 and 2) (MacLaughlin *et al.*, 2007). In late gestation, fetal plasma ACTH concentrations are lower, the prepartum cortisol surge occurs later and adrenocortical responsiveness to ACTH is blunted in twin in comparison to singleton fetuses (Edwards & McMillen, 2002a; Gardner *et al.*, 2004). Interestingly, a 30% reduction in maternal nutrition during the periconceptional period (from 60 days before until 7 days after conception) resulted in an earlier activation of the HPA axis in twin fetuses, whilst a more severe restriction of maternal nutrition imposed during the periconceptional and early gestation period (up to 30 days of pregnancy) resulted in a premature activation of adrenal steroidogenesis in singleton pregnancies (Edwards & McMillen, 2002a; Bloomfield *et al.*, 2003). Thus the nutritional environment of the early embryo impacts on the subsequent development of the fetal adrenal and on the timing of adrenal activation in the prepartum period. Whilst *in vivo* nutrition during the periconceptional period has been implicated in the subsequent development of the fetal adrenal, there are also data, which suggest that *ex vivo* nutrition of the embryo may also impact on adrenal development and the timing of delivery in a number of species.

In humans, there is a 2-fold increase in the risk of preterm delivery, low birth weight, very low birth weight and the occurrence of being small for gestational age in singletons conceived after assisted reproductive technologies (ART) (Van Voorhis, 2006). In contrast in the sheep, one common consequence of *in vitro* embryo culture (IVC) in the presence of human serum is the ‘large offspring syndrome’ (LOS). LOS is often characterised by increased prenatal losses, fetal

overgrowth, organomegaly (enlarged heart, liver and kidney) and a delay in the timing of parturition (Walker *et al.*, 1996c; Young *et al.*, 1998). It has been demonstrated that there is a decrease in the expression of the IGF2R in the liver, heart and kidneys of fetal sheep which show the characteristics of this syndrome at 125 days of gestation (Young *et al.*, 2001). IGF2R is a clearance receptor and acts to decrease the bioavailability of IGF2, and it has been argued that a decrease in IGF2R expression would therefore contribute to an increase in the bioavailability of IGF2 in fetal organs (Young *et al.*, 2001). Whilst there is evidence that IVC results in an altered timing of delivery in the sheep, it is not known, whether IVC alters the timing of the activation of the fetal HPA axis, fetal adrenal growth and/or steroidogenic capacity.

This study, therefore, investigated whether IVC, in the absence or presence of human serum alters the timing of the activation of the HPA axis in late gestation. Additionally, the current study aimed to determine whether IVC, in the absence or presence of human serum resulted in changes in fetal adrenal growth and in the expression of CYP17, TGF 1, IGF1, IGF2, IGF1R and IGF2R mRNA during the phase of prepartum steroidogenic activation of the adrenal at ~145 days of gestation.

5.2 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.

5.2.1 Experimental Design

A total of 54 South Australian Merino ewes were used in this study. The methods of synchronisation, superovulation and artificial insemination of ewes, embryo collection and the culturing of embryos *in vitro* have been previously described in detail by MacLaughlin (PhD thesis by MacLaughlin, 2006). In brief, ewes were subjected to superovulation followed by artificial insemination for the production of zygotes. Embryos were collected and either transferred to an intermediate recipient until day 6 of pregnancy (ET group) or cultured in 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) for 6 days. On day 6, embryos were collected from the intermediate recipients of the ET group, and embryos from the ET, IVCNS and IVCHS groups were transferred to synchronized final recipients. A group of ewes were synchronized and naturally mated to serve as control or 'naturally mated' animals (NM) for this experiment. Pregnancies were allowed to proceed through pregnancy until 144 – 145 days of gestation (term = 150 ± 3 days) when post mortem was performed (Figure 5.1).

5.2.2 Animals and Management

Four weeks prior to the commencement of experiments, ewes that were paddocked under normal husbandry practices were moved to covered yards for feeding and environmental adjustment at Turretfield Research Centre, Rosedale, South Australia. All ewes were 4 – 5 years of age and of uniform frame size and body weight (56.4 ± 0.6 kg). Ewes were randomly selected and grouped into either donors, intermediate or final recipients. Ewes were either fed individually in pens (donor and intermediate recipients) or as a group (final recipients). During this four 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).

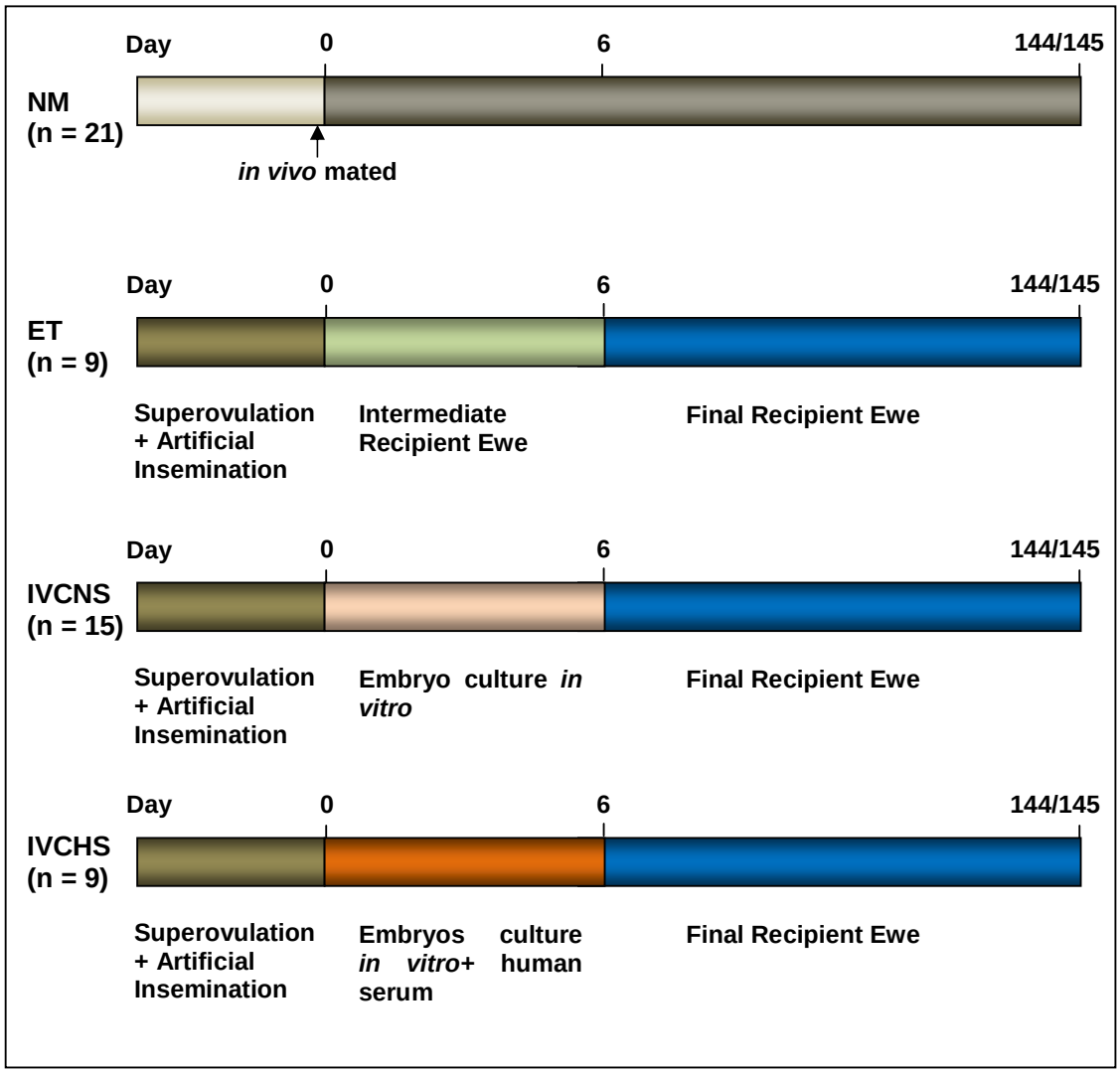


Figure 5.1: Schematic representation of the experimental treatment groups

The pellets provided 9.5 MJ/kg DM of metabolisable 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 (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 metabolisable energy of the diet to 10.6 MJ/kg DM and 151 g/kg of crude protein. Following embryos transfer, final recipient ewes were housed indoors and individually penned and fed.

Pregnancy was diagnosed and fetal number estimated by ultrasound at 45 days of pregnancy and confirmed at post mortem (Figure 5.2). Final recipient ewes were transported from Turretfield Research Centre to the University of Adelaide Medical School at ~100 days of gestation.

5.2.3 Animals and Surgery

Pregnant ewes (NM: n = 11; ET: n = 9; IVCNS: n = 15; IVCHS: n = 9) were transferred to the Medical School Animal House between 90 and 100 days of gestation (term = 150 ± 3 days of gestation). Surgery was performed under aseptic conditions between 110 and 120 days of gestation with general anaesthesia initially induced by an intravenous injection of sodium thiopentone (1.25g; Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained with inhalational 2.5 – 4% halothane (Fluothane, ICI, Melbourne, Vic, Australia) in oxygen. At surgery, catheters were implanted in a fetal carotid artery and jugular vein, a maternal jugular vein and the amniotic cavity, as previously described in Chapter 2. All ewes and fetal sheep received a 2 ml intramuscular injection of antibiotics (procaine penicillin 250 mg/ml, dihydrostreptomycin 250 mg/ml, and procaine hydrochloride 20 mg/ml; Penstrep Illium; Troy Laboratories, Smith-field, NSW, Australia) at the time of surgery.

5.2.4 Blood Collection for Radioimmunoassays

Fetal arterial blood samples (3.5ml) and maternal venous blood samples (5ml) were collected in chilled tubes 3 times per week at 0800 – 1100 h. Blood for cortisol assay was collected in heparinised tubes (125IU, Sarstedt Australia, Inglefarm, SA, Australia). Blood samples used for ACTH assay were collected in tubes coated in ethylenediaminetetraacetic acid (EDTA), Sarstedt Australia, Inglefarm, SA, Australia) containing aprotinin (1000KIU.ml^{-1}, Sigma Chemicals, St Louis, MO, USA). All blood samples were centrifuged at 1500 g for 10 min and plasma was separated into aliquots and stored at -20°C for subsequent hormone and metabolite assays.

5.2.5 ACTH and Cortisol Radioimmunoassay

Immunoreactive ACTH concentrations in fetal sheep plasma were measured by radioimmunoassay using a kit from ICN Biomedicals, Inc. (Seven Hills, Australia), previously validated for fetal sheep plasma (McMillen *et al.*, 1990). The sensitivity of the assay was 0.9pg/ml, and the rabbit antihuman ACTH-(1–39) had a cross-reactivity of less than 0.1% with b-endorphin,aMSH,a-lipotropin, and b-lipotropin. The interassay coefficient of variation was 11.2%, and the intraassay coefficient of variation was less than 10%.

Cortisol was extracted from fetal plasma using dichloromethane as previously described (Bocking *et al.*, 1986). The efficiency of recovery of ^{125}I-cortisol from fetal plasma using this extraction procedure was greater than 90%. Cortisol concentrations were then measured by radioimmunoassay using an Orion Diagnostica kit (Orion Diagnostica, Turku, Finland) previously validated for fetal sheep plasma (Edwards *et al.*, 2001). The sensitivity of the assay was

0.078nmol/litre and the cross-reactivity of the rabbit anti-cortisol was < 1% with pregnenolone, aldosterone, progesterone and oestradiol. The interassay coefficient of variation was 11.2% and the intraassay coefficient of variation was < 10%.

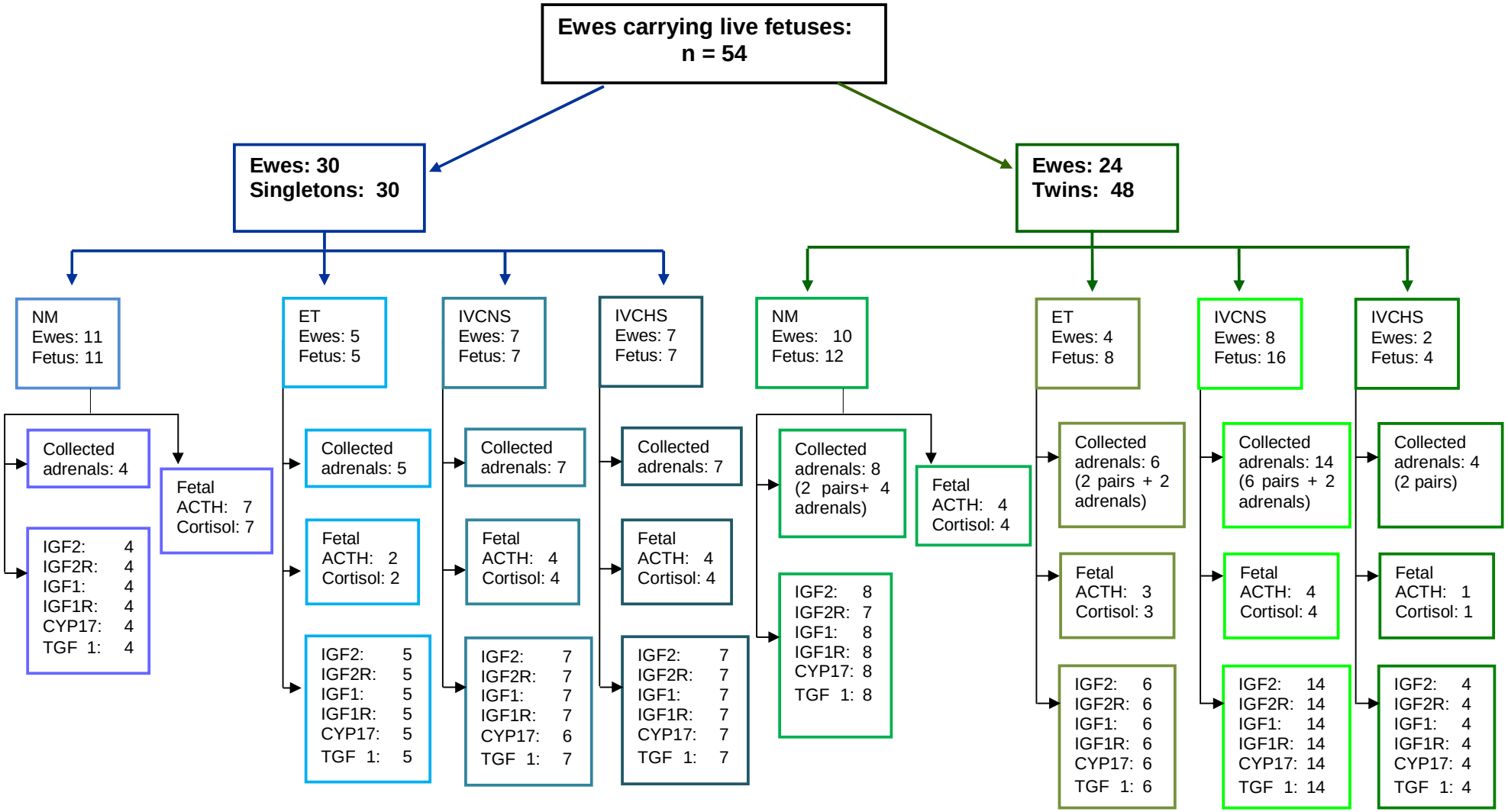
5.2.6 Collection of Fetal Adrenals

Final recipient ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty. Ltd., Peakhurst, NSW, Australia) on either 144 or 145 days of gestation, and the utero-placental unit was delivered by hysterotomy. Fetuses were immediately weighed and dissected. Fetal adrenals were weighed and snap frozen in liquid nitrogen and stored at -70° Celsius for further molecular analysis (Figure 5.2).

5.2.7 Isolation of RNA and Reverse Transcription PCR

Total RNA was isolated from fetal adrenals (singletons: NM, n = 4; ET, n = 5; IVCNS, n = 7; IVCHS, n = 7; twins: NM, n = 8; ET, n = 6; IVCNS, n = 14; IVCHS, n = 4) using Trizol® reagent (Invitrogen Life Technologies®, Carlsbad, CA) and treated with DNase 1 (Ambion, Austin, Texas, USA) to minimise genomic DNA contamination. Each sample was purified using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland) and RNA was quantified by spectrophotometric measurements at 260nm and 280nm. RNA integrity was confirmed by agarose gel electrophoresis. 3 g of total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen Life Technologies®, Carlsbad, CA) and random oligohexamers (100 M) for priming. Non-amplification controls (NACs) containing no Superscript III reverse transcriptase for each sample were also synthesised.

Figure 5.2: Summary of animal numbers in each experimental group



5.2.8 Quantitative Real Time PCR

The relative abundance of IGF1, IGF2, IGF1R and IGF2R, CYP17 and TGF 1 mRNA transcripts in the fetal adrenal were measured by quantitative real time 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 (as delineated in Chapter 4), water (2µl), and 50ng/µl cDNA (1µl) to give a total volume of 10µl. Each amplicon was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls for each primer set containing no cDNA were included on each plate. To ascertain that synthesised cDNA was free from genomic contamination NAC from each sample was also included.

Three replicates of cDNA from each sample of fetal adrenal were performed for each gene on each plate, and each plate was repeated twice. Amplification efficiencies were determined, from the slope of a plot of Ct (defined as the cycle number at which the fluorescence generated within a reaction crosses a set threshold line) 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, the acidic ribosomal protein P0 (RpP0), was calculated using Q-Gene analysis software (Muller *et al.*, 2002).

5.2.9 Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Data were log transformed where required, in order to normalise data variance for parametric analysis.

5.2.9.1 Fetal growth parameters and organ weights

The effects of embryo transfer (ET) and *in vitro* embryo culture (IVCNS and IVCHS) and of embryo number on fetal weight and fetal adrenal weight expressed as absolute weights and relative to body weight, were determined using a two way Analysis of Variance (ANOVA) using the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). When there was an interaction between the effects of embryo treatment and embryo number, data from singletons and twins were split and the effects of embryo treatment determined using a one way ANOVA. When there was a significant effect of embryo treatment in the absence of any interaction between the effects of embryo treatment and fetal number data were generally pooled for presentation. The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

5.2.9.2 Plasma ACTH and Cortisol Concentrations

The effects of embryo treatment and of embryo number on the gestational age profile of the fetal plasma ACTH and cortisol concentrations were determined using a multifactorial ANOVA with repeated measures using STATA 10 for Windows (StataCorpLp, Colege Station, TX, USA). Specified factors for the ANOVA included embryo treatment (NM, ET, IVCNS or IVCHS), embryo number

(singletons or twins) and time windows (116 - 120, 121 – 125, 126 – 130, 131 – 135, 136 – 140 and 141 – 145 days of gestation) as specified factors. When there was an interaction between the effects any of the three specified factors, data were split accordingly and the effects of embryo treatment, embryo number and/or time were determined using a two way repeated ANOVA. When there was a significant effect of embryo treatment in the absence of any interaction between the effects of embryo treatment and embryo number data were generally pooled for presentation. Bonferroni's Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

5.2.9.3 Fetal adrenal gene expression

The effects of embryo treatment and of embryo number on the relative expression of IGF1, IGF1R, IGF2, IGF2R, CYP17 and TGF 1 mRNA in the fetal adrenal was determined using a two way ANOVA. When there was an interaction between the effects of embryo treatment and embryo number, data from singletons and twins were split and the effects of embryo treatment determined using a one way ANOVA. When there was a significant effect of embryo treatment in the absence of any interaction between the effects of treatment and embryo number, mRNA expression data were pooled for presentation. The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($P < 0.05$) was taken to be significant.

5.3 RESULTS

5.3.1 Fetal Growth

There was a significant interaction between the effects of fetal number and embryo treatment on fetal weight ($P < 0.03$). In singleton pregnancies, fetal weight was significantly greater ($P < 0.03$) in the IVCHS group compared with the NM, ET and IVCNS groups whereas in twin pregnancies, fetal weight in the IVCNS group was significantly lower ($P < 0.02$) when compared to the NM group (Table 5.1).

5.3.2 Fetal Adrenal Growth and Development

There was no effect of either fetal number or embryo treatment on absolute adrenal weight (Table 5.1). The relative adrenal weight was greater ($P < 0.03$), however, in the IVCNS group when compared to the NM, ET and IVCHS groups and this effect was independent of fetal number (Figure 5.3).

5.3.3 Plasma ACTH and Cortisol concentrations during late gestation

There was a statistical interaction between the effects of embryo treatment and fetal number ($p < 0.001$) on fetal plasma ACTH concentration. There was also a statistical interaction between fetal number and gestational age ($P < 0.034$) on fetal plasma cortisol concentration. The data were therefore split into singleton and twin fetuses for further analysis.

Table 5.1: Effect of embryo transfer and culturing embryos *in vitro* on fetal and adrenal gland weights at 144/5 days of gestation

	Singletons				Twins			
	NM	**ET**	**IVCNS**	**IVCHS**	**NM**	**ET**	**IVCNS**	**IVCHS**
Fetal Weight (kg)	4.46 ± 0.40[a]	4.89 ± 0.28[a]	4.98 ± 0.26[a]	5.90 ± 0.20[b]	5.31 ± 0.20[b]	4.58 ± 0.35[ab]	4.08 ± 0.23[a]	4.68 ± 0.33[ab]
Adrenal Weight (g)	0.46 ± 0.02	0.45 ± 0.02	0.52 ± 0.03	0.52 ± 0.04	0.53 ± 0.02	0.46 ± 0.04	0.50 ± 0.03	0.46 ± 0.02

Different alphabetic subscripts denote significant differences between mean values, where within rows a < b, $P < 0.03$

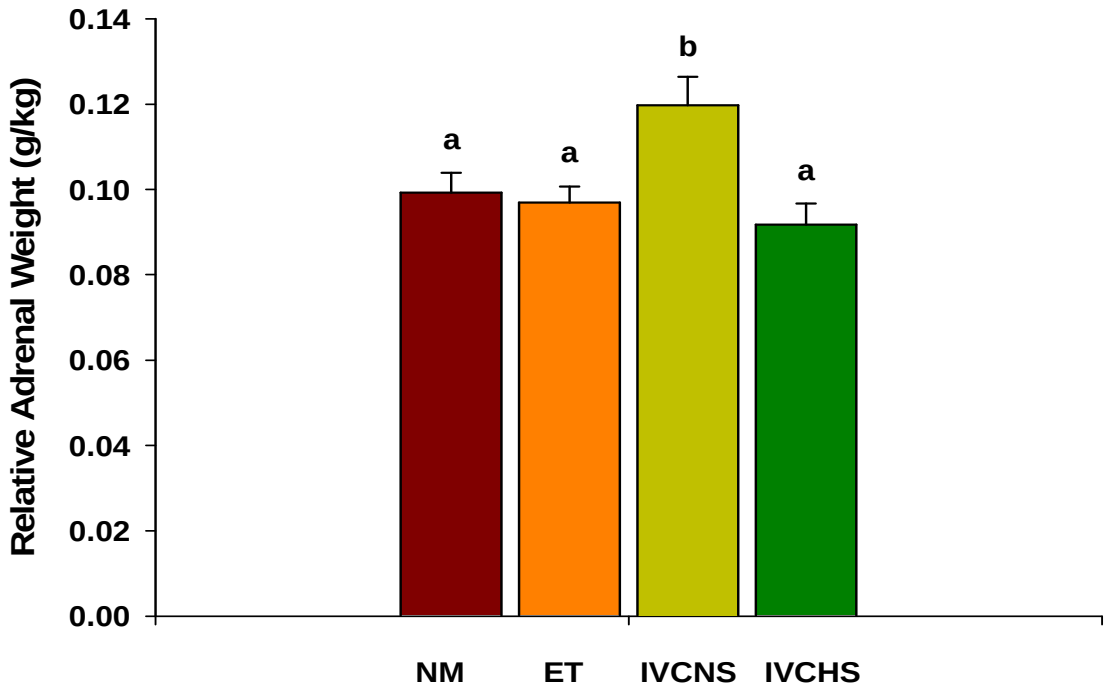


Figure 5.3: Relative fetal adrenal weights in the NM, ET, IVCNS and IVCHS groups

There was a significant increase ($P < 0.03$) in relative adrenal weight in IVCNS group (n = 21) when compared to the ET (n = 24), the IVCHS (n = 20) and the NM group (n = 12). Different superscripts denote mean values which are significantly different to each other.

5.3.3.1 Plasma ACTH concentrations

In singleton fetuses, fetal plasma ACTH concentrations were significantly higher ($P < 0.0040$) in the ET, IVCNS and IVCHS groups when compared to NM groups (Figure 5.4). The fetal plasma ACTH concentrations were also significantly lower in the IVCHS when compared to the IVCNS group ($P < 0.0040$) (Figure 5.4).

In twins, there was a further statistical interaction between the effects of embryo treatment and gestational age ($P < 0.05$). The twin data were then analysed by splitting into embryo treatment groups. In the NM group, fetal plasma ACTH concentrations significantly increased by 43.7 pg/ml ($P < 0.0001$) between 116 - 120 and 141 – 145 days of gestation. There was no effect of gestational age on fetal plasma ACTH concentrations in the ET, IVCNS and IVCHS groups.

5.3.3.2 Plasma cortisol concentrations

In singleton fetuses in all embryo treatment groups, plasma cortisol concentrations were significantly increased ($P < 0.00001$) after 136 days of gestation when compared to either 116 – 120 or 121 – 125 days of gestation (Figure 5.5).

In twin fetuses, there was no effect of embryo treatment on fetal plasma cortisol concentrations, however, plasma cortisol concentrations increased significantly ($P < 0.00001$) after 136 days of gestation compared to before 136 days of gestation (Figure 5.5).

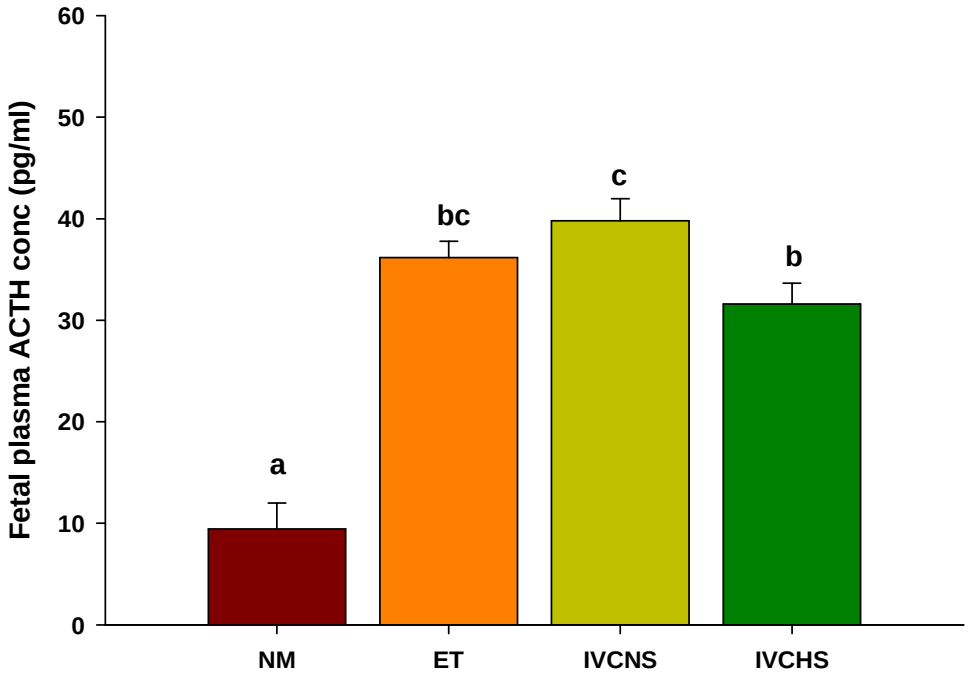


Figure 5.4: Fetal plasma ACTH concentration in singletons at 116 – 145 days of gestation

Fetal plasma ACTH concentrations were significantly higher in ET, IVCNS and IVCHS groups when compared to the NM group in singletons (NM: n = 7, ET: n = 7, IVCNS: n = 4, IVCHS: n = 4). Different alphabetical subscripts denote mean values, which are significantly different.

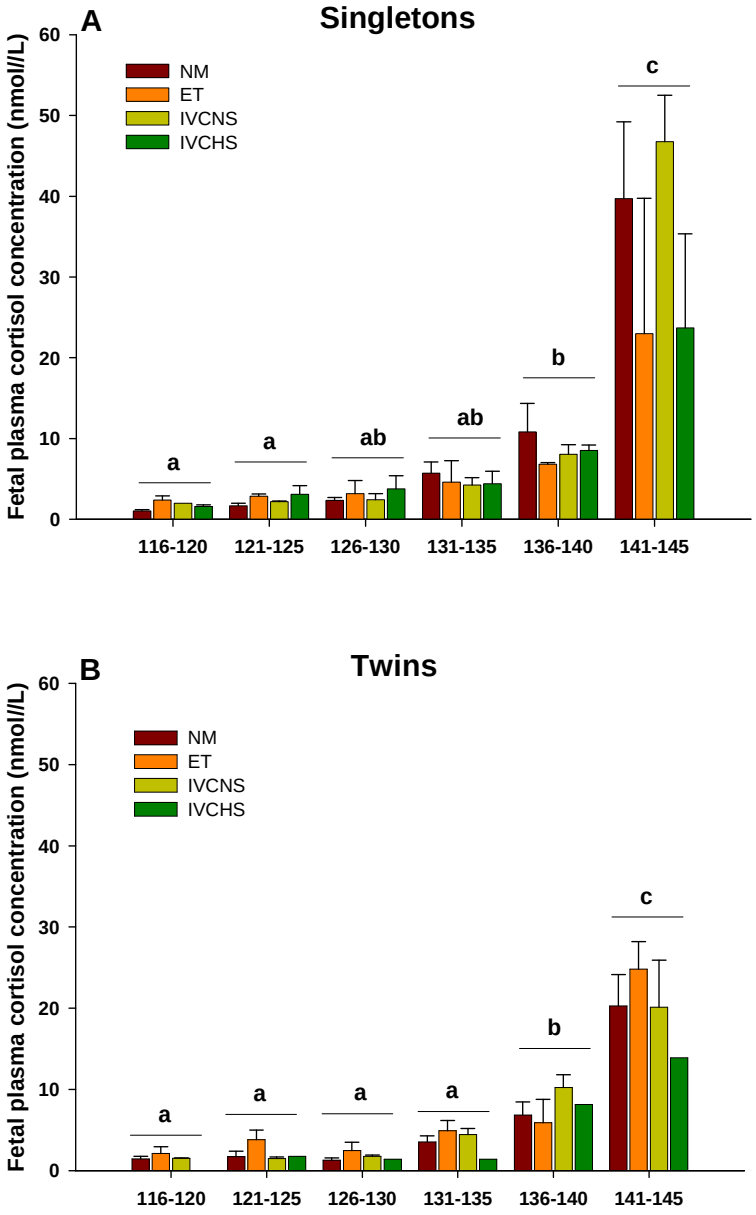


Figure 5.5: Fetal plasma cortisol concentration in singletons (A) and twins (B)

There was no effect of *in vivo* or *in vitro* (ET, IVCNS and IVCHS) culture on fetal plasma cortisol concentration in either singleton (A) or twin (B) fetuses. There was a significant effect of gestational age on fetal plasma cortisol concentration in both singleton and twin fetuses. Different alphabetical subscripts denote mean values which are significantly different.

5.3.4 Adrenal IGF1 and IGF1R mRNA expression

There was no effect of embryo treatment on the expression of either IGF1 or IGF1R mRNA in fetal adrenal tissue. The expression of IGF1 ($P < 0.02$, Table 5.2) and IGF1R mRNA ($P < 0.03$, Table 5.2) were each significantly higher, however, in adrenals from twin fetal sheep when compared to singletons at 144 - 145 days of gestation.

Table 5.2: Effect of fetal number on the relative expression of IGF1 and IGF1R mRNA in the fetal adrenal

	Singletons	**Twins**
IGF1:RpPO	0.021 ± 0.003	0.032 ± 0.003#
IGF1R:RpPO	0.24 ± 0.02	0.35 ± 0.03#

# denotes difference between singletons and twin, $P < 0.03$

5.3.5 Adrenal IGF2 and IGF2R mRNA expression

There was no effect of fetal number or treatment on adrenal IGF2 mRNA expression at 144 – 145 days of gestation (Figure 5.6). In both singleton and twin fetuses, however, the expression of IGF2R mRNA in the fetal adrenal was significantly decreased ($P < 0.02$) in the IVCHS group compared to NM, ET and IVCNS groups at 144 – 145 days of gestation (Figure 5.6).

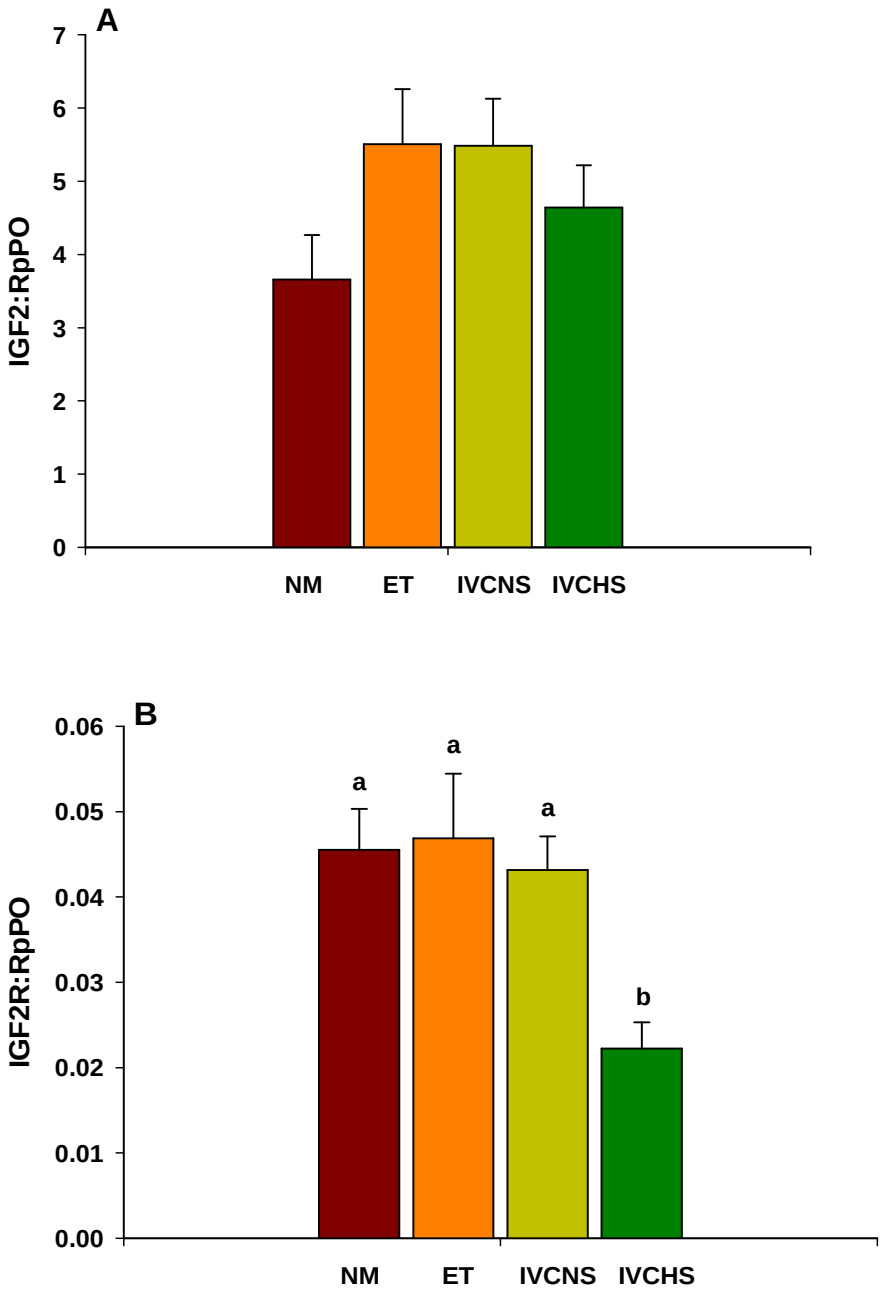


Figure 5.6: IGF2 and IGF2R mRNA expression in the fetal adrenal

There was no effect of either embryo treatment or fetal number on IGF2 mRNA expression (A). There was, however, a significant decrease ($P < 0.02$) in IGF2R mRNA expression in the IVCHS group (n = 11) when compared to the NM (n = 11), ET (n = 11) and IVCNS (n = 21) groups (B).

5.3.6 Adrenal CYP17 and TGF 1 mRNA expression

There was no effect of fetal number on the expression of CYP17 mRNA in the fetal adrenal. In singleton and twin fetuses, the expression of CYP17 mRNA was higher ($P < 0.05$, Figure 5.7) in the IVCNS group when compared to the NM group, but not when compared to ET or IVCHS groups at 144 – 145 days of gestation. Whilst, there was no effect of embryo treatment on adrenal TGF 1 mRNA expression, the expression of TGF 1 mRNA was significantly increased in adrenals from twin compared to singleton fetuses ($P < 0.02$, Figure 5.8).

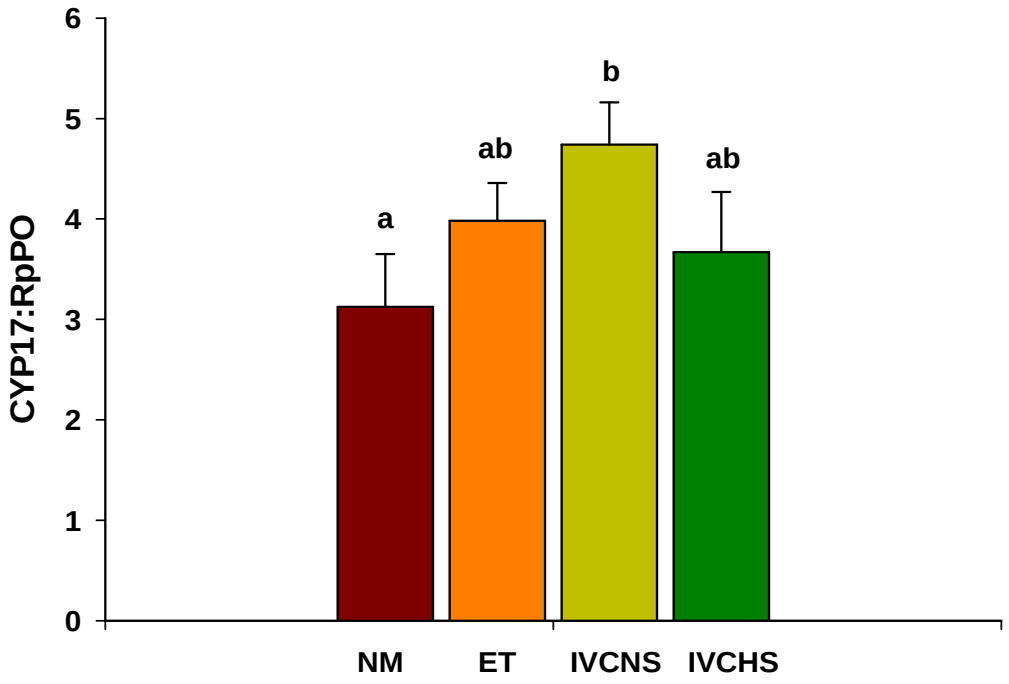


Figure 5.7: CYP17 mRNA expression in the fetal adrenal

There was a significant increase ($P < 0.05$) in adrenal CYP17 mRNA expression in the IVCNS group (n = 20) when compared to the NM group (n = 12).

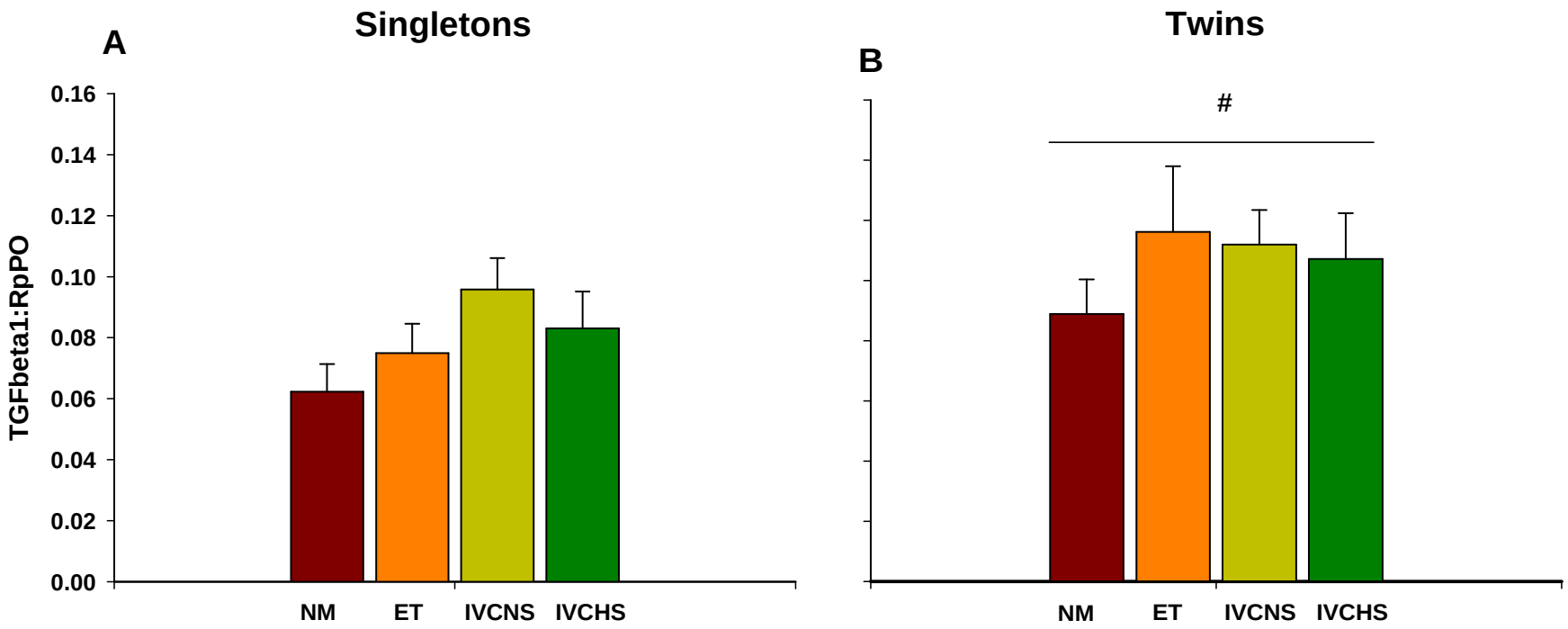


Figure 5.8: TGF 1 mRNA expression in fetal adrenal tissue

There was no effect of embryo treatment on the expression of TGF 1 mRNA expression. TGF 1 mRNA expression was significantly increased ($P < 0.02$) in twins (B) when compared to singletons (A), # denotes a difference between singleton and twin fetuses.

5.4 DISCUSSION

The objective of this study was to investigate whether manipulations of the periconceptional environment, specifically culturing embryos *in vitro* in the absence or presence of serum during the preimplantation period, and embryo/fetal number influences the timing of the activation of the HPA axis in late gestation. Additionally, the current study aimed to determine whether culturing embryos *in vitro* in the absence or presence of serum and embryo number resulted in changes in fetal adrenal growth and in the expression of adrenal IGFs, CYP17 and TGF 1 mRNA expression in the fetus at 144 - 145 days of gestation.

5.4.1 Adrenal Growth, IGFs, CYP17 and TGFβ1 expression in Singletons and Twins

In the present study, it has been found that adrenal IGF1 and IGF1R mRNA expression were significantly higher in twins when compared to singleton fetuses at 144 – 145 days of gestation, independent of embryo transfer and culture treatment during the periconceptional period. Interestingly, despite this difference neither absolute nor relative adrenal weights differ between singleton and twin fetuses at this gestational age. In contrast to the findings in the present study, MacLaughlin and colleagues (2007) have shown that relative adrenal weight and adrenal IGF1 and IGF1R mRNA expression are significantly lower in twin fetuses compared to singletons at ~ 55 days of gestation (MacLaughlin *et al.*, 2007). In this latter study, there was a positive relationship between fetal adrenal weight and adrenal IGF1 mRNA in twins but not singletons at this age (MacLaughlin *et al.*, 2007). The authors of this study concluded that in twins adrenal growth appears to be predominantly related to IGF1 mRNA expression in early gestation.

Table 5.3: Summary of results where yellow highlight represents embryo/fetal number effect

	Twin vs Singletons Fetus
Fetal growth	Interaction; split by number
Adrenal growth	T = S
Gestational fetal plasma ACTH concentration (time period)	Interaction; split by number
Gestational fetal plasma cortisol concentration (time period)	Interaction; split by number
Adrenal mRNA expression of: IGF1 and IGF1R IGF2 and IGF2R CYP17 TGF 1	 T > S T = S T = S T > S

Intriguingly, however, as shown in Chapter 4, relative adrenal weight is significantly greater in twins compared to singletons at 136-138 days gestation and this occurred in the absence of difference in the expression of adrenal IGF1 and IGF1R mRNA (Chapter 4). This suggests that in late gestation adrenal growth in the twin fetus is not mediated directly by IGF1 and IGF1R but by an alternative pituitary or adrenal derived factor as discussed in the previous Chapter.

In summary, whilst adrenal growth may be mediated by IGF1 and IGF1R in the twin fetus at 55 days of gestation, an alternative pituitary or adrenal derived factor appears to be the main driver of adrenal growth in the twin fetus at 136 – 138 days of gestation and the involvement of IGF1 and IGF1R in adrenal growth is also relatively minor in the twin fetus at 144 – 145 days of gestation. This suggests that the role of IGF1 and IGF1R in adrenal growth changes over time and at this stage, it is not clear what role IGF1 and IGF1R play in adrenal development at 144 – 145 days of gestation.

In the current study, the expression of TGFβ1 is higher in the fetal adrenal of the twins when compared to singleton at 144 – 145 days of gestation, independent of embryo transfer and culture treatment during the periconceptional period. Interestingly, there was no significant difference in adrenal CYP17 mRNA expression between twin and singleton fetuses at this age.

TGFβ1 has been proposed to be a steroidogenic inhibitor. *In vitro*, TGF attenuates the action of ACTH to increase CYP17 enzyme activity and cortisol production in fetal adrenal cells in sheep (Naaman-Reperant *et al.*, 1996). Furthermore, incubation of fetal ovine adrenal cells with TGFβ1 for 5 days results in the reduced activity of the cholesterol side-chain cleavage enzyme (Naaman-

Reperant *et al.*, 1996). *In vivo*, TGFβ1 is highly expressed in the ovine fetal adrenal at around 100 days of gestation when CYP17 expression is low (Coulter *et al.*, 2003). At 140 – 144 days of gestation, during the time of increased steroidogenic activity, fetal adrenal TGFβ1 mRNA is significantly decreased whilst CYP17 mRNA is significantly increased when compared to the expression levels at around 100 days of gestation (Coulter *et al.*, 2003). This strongly suggests that TGFβ1 acts *in vivo* as an inhibitor of steroidogenesis.

Furthermore, previous studies have shown that the well-characterised prepartum HPA activity is delayed in twin fetuses where ACTH and cortisol concentrations were significantly lower in twin when compared to singleton fetal sheep between 116 and 146 days of gestation and that this delay has its origin in early gestation (Edwards & McMillen, 2002a; MacLaughlin *et al.*, 2007). As previously speculated, the observed delayed activation of the HPA axis in the twin sheep fetus during late gestation may be an adaptive response designed to protect the fetus from the possibility of preterm delivery (McMillen *et al.*, 2004) and although activation of the HPA axis is delayed in twins, twin fetuses do not have extended length of gestation and are born at the same gestational age as singletons. This may suggest that the delay in the activation of the fetal HPA axis in late gestation is transient such that the activation is maximal in both singleton and twin fetuses after 144 – 145 days.

In light of these data, there are therefore a number of possible explanations for the observed increase in the expression of TGFβ1 mRNA in twin fetuses at 144 – 145 days of gestation. These include:

1. TGFβ1 inhibits steroidogenesis by regulating the expression or activity of steroidogenic enzymes other than CYP17

2. Whilst TGFβ1 may inhibit steroidogenesis other intra-adrenal factors may act to ensure the timely delivery of the twin fetus

5.4.2 Embryo transfer and culturing embryos in the Absence or Presence of Serum and the Activity of the HPA Axis, Adrenal Growth, IGFs and CYP17

It is clear from the results of the current study that there is a differential effect of fetal number and culturing embryos *in vitro* in the absence and presence of serum on the growth and development of the fetal adrenal. These results are firstly summarised in Table 5.4.

Table 5.4: Results summary, green highlight represents embryo treatment effects

	NM	ET vs NM	IVCNS vs NM	IVCHS vs NM
Singletons	Fetal growth (After split by fetal number)	ET = NM	IVCNS = NM	IVCHS > NM (HS > ET) (HS > NS)
	Adrenal growth	ET = NM	IVCNS > NM (NS = ET) (NS = HS)	IVCHS = NM
	Gestational fetal plasma ACTH concentrations (After split by fetal number)	n/a*	IVCNS > NM	IVCHS > NM (IVCNS > IVCHS)
	↑ plasma cortisol concentration after 135 days of gestation (After split by fetal number)	n/a*	IVCNS = NM	IVCHS = NM
	Adrenal mRNA expression of: IGF1 and IGF1R IGF2 IGF2R CYP17 TGF 1	 ET = NM ET = NM ET = NM ET = NM ET = NM	 IVCNS = NM IVCNS = NM IVCNS = NM IVCNS > NM IVCNS = NM	 IVCHS = NM IVCHS = NM IVCHS < NM IVCHS = NM IVCHS = NM
Twins	Fetal growth (After split by fetal number)	ET = NM	IVCNS < NM (NS = ET) (NS = HS)	IVCHS = NM
	Adrenal growth	ET = NM	IVCNS > NM (NS = ET) (NS = HS)	IVCHS = NM
	↑ plasma ACTH concentration after 135 days of gestation (After split by fetal number) (Further interaction; split by embryo treatment)	No increase in ACTH with gestational age	No increase in ACTH with gestational age	n/a#
	↑ plasma cortisol concentration after 135 days of gestation (After split by fetal number)	ET = NM	IVCNS = NM	n/a#
	Adrenal mRNA expression of: IGF1 and IGF1R IGF2 IGF2R CYP17 TGF 1	 ET = NM ET = NM ET = NM ET = NM ET = NM	 IVCNS = NM IVCNS = NM IVCNS = NM IVCNS > NM IVCNS = NM	 IVCHS = NM IVCHS = NM IVCHS < NM IVCHS = NM IVCHS = NM

* denotes ET: n = 2 and # denotes HS: n = 1

5.4.2.1 The effect of embryo transfer and culturing embryos in the absence of human serum

5.4.2.1.1 Singletons

In the present study, there was no significant difference in the absolute and relative adrenal weight or in the expression of adrenal IGF1, IGF1R, IGF2, IGF2R, TGF 1 and CYP17 mRNA between the ET and NM groups. This suggests that embryo transfer *per se* does not impact on adrenal growth and the levels of IGF, TGF 1 and CYP17 mRNA expression in the singleton fetus in late gestation. Unfortunately, there were only two singletons providing hormone data for the ET group and thus, these data were excluded from analysis.

A novel finding of the present study is that culturing embryos *in vitro* in the absence of serum resulted in an increase in fetal plasma ACTH concentration in late gestation, greater relative adrenal weight and an increase in the mRNA expression of adrenal CYP17 when compared to NM in singleton pregnancies at 144 – 145 days of gestation. This suggests that the process of culturing embryos *in vitro* in a defined medium results in an earlier activation of the HPA axis when compared to the NM group in late gestation. This is relevant in the context of the finding that Assisted Reproductive Technologies, such as *in vitro* fertilisation, utilise the process of culturing embryos *in vitro* and have been associated with preterm births in humans. A two-fold increase in the risk of preterm delivery was noted in IVF singletons compared with spontaneously conceived singletons (Jackson *et al.*, 2004).

One possible mechanism that could account for an increase in adrenal growth and the expression of adrenal CYP17 mRNA in IVCNS singletons is the increase in

fetal plasma ACTH concentrations observed in these animals. As discussed in Chapter 4, the ability of ACTH to stimulate growth and steroidogenesis in the late gestational sheep has been extensively investigated (Liggins, 1968; Boshier *et al.*, 1981; Lye *et al.*, 1983; Phillips *et al.*, 1996a; Warnes *et al.*, 2004). It is possible however that the increase in ACTH concentration is not the only factor stimulating adrenal growth and CYP17 mRNA expression in IVCNS singletons. Additional mechanisms, which may contribute to an increase in adrenal size and CYP17 mRNA expression include an increase adrenal sensitivity through an increase in the expression of the ACTH receptors.

Interestingly, whilst adrenal CYP17 expression was higher in the IVCNS group there was no difference in fetal plasma cortisol concentration between the IVCNS and NM fetuses in singleton pregnancies. This may be because the fetal adrenal in both NM pregnancies are maximally stimulated at this gestational age.

5.4.2.1.2 Twins

As reported in singletons, in this study embryo transfer alone does not affect adrenal growth and the expression of adrenal IGF1, IGF1R, IGF2, IGF2R, TGF 1 and CYP17 mRNA in twins in late gestation suggesting that embryo transfer alone does not impact on adrenal growth and the levels of IGF, TGF 1 and CYP17 mRNA expression in the twin fetus in late gestation.

Similarly to the singleton fetus, culturing embryos *in vitro* in the absence of serum resulted in an increase in relative adrenal weight and an increase in the mRNA expression of adrenal CYP17 in twins when compared to the NM group at 144 – 145 days of gestation. Unlike in singletons, however, these changes occurred in

the absence of an increase in fetal plasma ACTH concentrations in late gestation. Furthermore, the fetal plasma cortisol concentrations in IVCNS twins are not different to the NM group in late gestation. These observations may be explained by:

1. Pituitary derived factors other than ACTH are present in the IVCNS twin fetal sheep, which stimulate adrenal growth, CYP17 mRNA expression and maintains fetal cortisol production.

2. An increased expression of adrenal MC2R receptor and/or increased post receptor signalling leading to an enhanced adrenal responsiveness to ACTH in the IVCNS twin fetuses.

3. There is an increased expression of an intra-adrenal stimulatory factor or a decreased expression of an intra-adrenal inhibitory factor, which acts independently or in synergy with ACTH leading to an increase in adrenal growth, CYP17 mRNA expression and maintaining cortisol production in the IVCNS twin fetuses.

It is not clear at this stage, which of these possible mechanisms explains the findings in this current study. It does however appear that culturing embryos *in vitro* in the absence of serum act to alter the adrenal growth and the expression of CYP17 mRNA whilst maintaining fetal plasma cortisol concentration in late gestation in the absence of a rise in fetal plasma ACTH concentration.

5.4.2.2 The effect of culturing embryos in the presence of human serum

5.4.2.2.1 Singletons

In the present study, culturing embryos *in vitro* in the presence of human serum resulted in a significant increase in fetal weight, no change in absolute or relative adrenal weight and a significant decrease in adrenal IGF2R mRNA expression in singletons at 144 – 145 days of gestation when compared to singletons derived after natural mating, embryo transfer or after culture in the absence of serum. Interestingly, fetal plasma ACTH concentrations of IVCHS singletons, although significantly increased when compared to singletons from the NM group, were significantly reduced when compared to singletons from the IVCNS group in late gestation.

Historically, *in vitro* culture of the sheep or cow embryo in media containing serum can result in offspring exhibiting the Large Offspring Syndrome (LOS), which includes fetal overgrowth, organomegaly and prolonged gestation (Walker *et al.*, 1996b; Young *et al.*, 1998; Farin *et al.*, 2004). In the current study, the increased fetal weight observed in singleton derived from embryos cultured *in vitro* in the presence of serum when compared to NM singletons confirms these previous findings. A study by Young and colleagues (2001) reported that there was a decrease in the expression of the *Igf2r* gene and protein in a range of tissues, including liver, kidney, heart and muscle at 125 days of gestation in fetuses which showed the large offspring phenotype (Young *et al.*, 2001). The authors of this study concluded that a decrease in IGF2R can lead to an increase in IGF2 bioavailability, which may explain organomegaly. In the current study, it was found that the expression of adrenal IGF2R mRNA was significantly decreased in IVCHS singletons and intriguingly, unlike the result found in the IVCNS group, the relative

adrenal weights in this group was not higher than in the NM group. This suggests that culturing embryos in the presence of serum protects adrenal from overgrowth. Alternatively, as LOS is a possible but not certain consequence of culturing embryos *in vitro* in the presence of serum, the IVCHS group of the current study may consist of a mixed fetal population where some fetuses may exhibit LOS phenotype whilst other do not within this group, and thus the effects of IVCHS on adrenal growth may have been masked.

One interesting aspect of this study is that the effects of culturing embryos *in vitro* in the absence of serum appear to be either reduced or disappear when serum is added to the culture media. In IVCHS singletons, fetal plasma ACTH concentrations are significantly reduced whilst relative adrenal weight and the expression of CYP17 mRNA are restored when compared to IVCNS singletons in late gestation. An additional difference between these two groups is the significantly lower expression of adrenal IGF2R mRNA in IVCHS singletons, which may be involved in the explanation of this phenomenon. There are, however, a number of possible mechanisms including:

1. It may simply be that the presence of serum influences the development of the pituitary and results in lowering fetal plasma ACTH concentrations in this group. It is possible that at this level fetal plasma ACTH concentrations are not sufficient to cause an increase in relative adrenal weight and adrenal CYP17 mRNA expression. The effects of IVCHS on IGF2R in this instance may therefore be a separate phenomenon that is not involved in the restoration of adrenal growth and adrenal CYP17 mRNA expression in the IVCHS group.

2. An alternative possibility is that the decrease in IGF2R expression in the IVCHS group is involved in the restoration of adrenal growth and CYP17 mRNA expression to levels comparable to the NM group. As discussed in Chapter 4 IGF2 is a potential inhibitor of adrenal growth and steroidogenesis in late gestation. It is then possible that decrease in IGF2R mRNA could lead to an increase in IGF2 protein, which results in the restoration of adrenal growth and adrenal CYP17 mRNA expression.

5.4.2.2.2 Twins

Culturing embryos *in vitro* in the presence of serum also resulted in a significant decrease in adrenal IGF2R mRNA expression in twins at 144 – 145 days of gestation when compared to twins derived after natural mating, embryo transfer or after culture in the absence of serum. Unfortunately, there was only one twin providing hormone data for the IVCHS group and thus, these data were excluded.

As discussed in the above section, the effects of culturing embryos *in vitro* in the absence of serum appear to be either reduced or ablated when serum is added to the culture media in singletons. This also appears to be the case in twin fetuses. In IVCHS twins, fetal weight, relative adrenal weight and the expression of CYP17 mRNA are restored when compared to IVCNS twins in late gestation. If IGF2 is and inhibitor of adrenal growth and steroidogenesis in late gestation as discussed in Chapter 4, it is possible that that decrease in IGF2R mRNA can lead to an increase in IGF2 protein, which results in the inhibition of adrenal growth and adrenal CYP17 mRNA expression leading to normal adrenal growth and adrenal CYP17 mRNA expression in the IVCHS twin.

5.4.3 Summary

The present study demonstrates that there is a differential effect of fetal number on the expression of adrenal IGF1, IGF1R and TGF1 mRNA expression at 144 - 145 days of gestation. Their role in adrenal growth and development in the fetal twin in late gestation, however, remains to be elucidated.

A novel finding of the current study was that when embryos were cultured in a defined medium, there was a significant increase in relative fetal adrenal weight and the expression of adrenal CYP17 mRNA in both singleton and twin fetuses at 144 – 145 days and an increase in fetal plasma ACTH concentration throughout late gestation in singletons when compared to the NM group. This suggests that there is an early activation of the HPA axis in these animals in late gestation.

Furthermore, the results of the current study provides evidence which suggests that the addition of serum to the *in vitro* culture media restore restores/ablates the effects of culturing embryos *in vitro* in the absence of serum and that the mechanism by which the restoration of fetal adrenal development occurs may involve the intra-adrenal IGF system.

CHAPTER 6: SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

The activation of the fetal HPA axis is essential in the normal timing of parturition and the maturation of fetal organs. Experimental and clinical studies provide evidence that embryo number as well as *in vivo* and *ex vivo* nutritional perturbations and manipulations of the early embryonic environment impact on fetal development and length of gestation. In particular, it has been demonstrated that the activation of the HPA axis in late gestation is delayed and adrenal responsiveness to ACTH is blunted in twins when compared to singletons in the sheep. Previous studies further provide evidence that suggests that periconceptional maternal undernutrition alters the timing of the prepartum activation of the fetal HPA axis and adrenal development whereas culturing embryos *in vitro* in the presence of serum is associated with fetal overgrowth and delayed parturition in the sheep. It is unclear, however, whether the effects resulting from periconceptional undernutrition are due to the impact of undernutrition during the periconceptional period or undernutrition during the preimplantation period alone. It is also not known how culturing embryos *in vitro* in the absence or presence of serum impacts on the prepartum activation of the HPA axis and adrenal development. Lastly, molecular mechanisms by which embryo number and perturbations and manipulations of the early embryonic environment impact on the growth and development of the fetal adrenal in the sheep have not been investigated.

It was the main purpose of this thesis to investigate:

1. The potential role of the components of the IGF system in the growth and development of the fetal adrenal in singletons and twins

2. The impact of manipulations of the *in vivo* nutritional environment during critical developmental windows including the periconceptional and preimplantation periods on the development of the fetal HPA axis and fetal adrenal growth and whether the fetal adrenal IGF system plays a key role in such manipulation

3. The impact of manipulation of the *ex vivo* nutritional environment during the preimplantation period on the development of the fetal HPA axis and fetal adrenal growth and whether the fetal adrenal IGF system plays a key role in such manipulation

6.1 The Role of IGFs in the Development of the Fetal Adrenal and the Singleton/Twin Story

A novel and intriguing conclusion of this thesis is that the role of intra-adrenal IGF1, IGF1R, IGF2 and IGF2R may change over the period of gestation in singleton and twin fetuses. MacLaughlin and colleagues (2007) have demonstrated that adrenal growth and development is suppressed as early as 55 days of gestation in the twin compared to the singleton fetus (MacLaughlin *et al.*, 2007). This latter study provides evidence, which suggests that IGF2 stimulates adrenal growth in the singleton fetus and that when levels of IGF2 mRNA expression is significantly decreased, as occurs in twin fetuses, adrenal growth appears to be predominantly related to IGF1 expression (MacLaughlin *et al.*, 2007). Given that MacLaughlin and colleagues (2007) found that there was a

direct relationship between CYP17 and IGF2 mRNA in singletons and an inverse relationship between CYP17 and IGF2R mRNA in twins, IGF2 would be expected to be a steroidogenic stimulator at this gestational age (MacLaughlin *et al.*, 2007). Thus, during early gestation IGF1 appears to be the main driver of adrenal growth in the twins. Conversely, IGF2 appears to play a stimulatory role in fetal adrenal growth in singletons whilst having a stimulatory role in adrenal steroidogenesis in both singleton and twin fetuses.

As described in Chapter 4 adrenal weight is significantly greater in twins when compared to singletons by late gestation i.e. at 136-138 days of gestation. However, by this age there was no difference in the expression of IG1 or IGF1 mRNA expression in adrenals of singleton and twin fetuses. Conversely, the findings presented in Chapter 5 show that although adrenal IGF1 and IGF1R expression was significantly higher in twins when compared to singletons, there was no difference in relative or absolute adrenal weight between singletons and twins at 144 – 145 days of gestation. This suggests that in late gestation IGF1 plays a relatively minor role in promoting adrenal growth and that an alternative pituitary or adrenal factor may have taken on this role at this stage of development.

We would therefore conclude based on the work of this thesis that the role of IGF1 and IGF1R in fetal adrenal growth changes over gestation.

Intriguingly, however, is that there is some evidence presented in this thesis, which suggests that there may also be a change in the nature of the action of IGF2 in the fetal sheep adrenal across gestation. As discussed above, there is strong evidence suggesting that IGF2 stimulates adrenal growth and steroidogenesis in

early gestation. Interestingly, there is a direct relationship between adrenal CYP17 and IGF2R mRNA expression in the fetal sheep at 136 – 138 days of gestation (Chapter 4), which contrasts with the inverse relationship between adrenal CYP17 and IGF2R found at 55 days of gestation (MacLaughlin *et al.*, 2007). Furthermore, a previous study by Ross and colleagues (2000) found that in growth restricted fetal sheep, an increase in fetal adrenal weight and increase in fetal plasma cortisol concentrations were associated with a lower level of adrenal IGF2 expression (Ross *et al.*, 2000b). I speculate that these data together with the mechanisms proposed for a role of IGF2 in the control of fetal adrenal growth presented in Chapters 4 and 5 suggest that IGF2 inhibits adrenal growth and steroidogenesis in late gestation.

As discussed in Chapter 4, adrenal *Igf2r* methylation was significantly lower in the absence of a decrease in IGF2R mRNA expression in twins when compared to singletons at 136 – 138 days of gestation. This suggests that, although a decrease in methylation is present in late gestation an alternate factor is able to override the relatively small but significant change in IGF2R methylation. Specifically, I hypothesize that embryo or fetal number results in epigenetic changes in the fetal adrenal *Igf2r* gene leading to the suppression of IGF2R mRNA expression for the purpose of maximising the stimulatory role of IGF2 in early gestation. As gestation progresses, however, the role of IGF2 shifts from stimulatory to inhibitory, and other factors are able to overcome the effects of the relatively small change in *Igf2r* methylation to determine the level of IGF2R protein expressed in the twin fetal adrenal in late gestation.

To shed some light on the role of *Igf2r* methylation it would be certainly interesting to direct future research to investigate the effects of embryo number on the epigenetic status of the *Igf2r* gene by measuring the level of methylation of its DMR region in singleton and twin fetuses at 55 days of gestation. Also, the question still remains: is intra-adrenal IGF2 inhibitory or stimulatory in the regulation of adrenal growth in late gestation? In order to address this question it would be important to first measure the levels of IGF2 protein within the fetal sheep adrenal in singleton and twin fetal sheep during the period 55d – 138d gestation. It may also be possible in the fetal sheep model to infuse a pharmacological blocker of IGF2 directly into the fetal renal and hence adrenal vein during late gestation, in order to test whether this results in an increase in fetal adrenal growth during this period. Furthermore it would be important to understand whether other pituitary POMC derived peptides, including POMC 1-77 act to stimulate or inhibit IGF2 and IGF2R expression.

6.2 *IN VIVO* NUTRITIONAL ENVIRONMENT – PERTURBATION DURING DIFFERENT CRITICAL WINDOWS IMPACTS SPECIFIC ORGAN SYSTEMS

As discussed in the preceding literature review, maternal undernutrition during the periconceptional period impacts on the development of the fetal HPA axis in the late gestation sheep. For the first time, the findings of this thesis suggest that periconceptional undernutrition may differentially target components of the fetal HPA axis depending on exposure to undernutrition during specific periconceptional time periods.

In Chapter 3 it was demonstrated that in contrast to the Control group, the cortisol response to CRH was not ablated in the PCUN and PIUN twin compared to singletons fetuses. To investigate potential mechanisms explaining this phenomenon adrenal weight and the expression of fetal adrenal IGF1, IGF2, their receptors and CYP17 mRNA were measured as described in Chapter 4. Intriguingly, it was found that there was an increase in CYP17 and a decrease in IGF2 adrenal mRNA expression in singletons and a significant increase in adrenal weight and adrenal IGF2R mRNA expression in both singleton and twin fetuses from ewes exposed to undernutrition during the whole periconceptional period. This effect was absent in fetuses from ewes exposed to undernutrition during the preimplantation period only.

These data firstly highlight that exposure to maternal undernutrition during the periconceptional and preimplantation periods result in an increased adrenal response to ACTH in twin fetuses to levels similar to that present in the singleton fetus. Secondly, the fetal adrenal in late gestation appears to be a key target of nutritional perturbation during the periconceptional period however maternal undernutrition during the preimplantation period alone appears not to adversely affect adrenal growth and development in the late gestation sheep.

Fascinatingly the preimplantation window of adversity does appear to impact on the development of the pituitary as evidenced by some recent findings showing that maternal undernutrition during either the periconceptional period or the preimplantation period results in a significant decrease in the expression of the glucocorticoid receptor in the fetal anterior pituitary at 136 – 138 days of gestation (Zhang, unpublished data).

In summary, the fetal HPA axis appears to be readily programmable by perturbation and manipulation of the *in vivo* nutritional environment during the periconceptional period. Importantly, exposure to maternal undernutrition during different developmental windows may have differential actions on the fetal pituitary and adrenal. Specifically, alteration of fetal adrenal growth and development requires exposure to poor maternal nutrition during both pre and post conception periods whilst maternal undernutrition during the first week of gestation is sufficient to alter the development of the fetal anterior pituitary in late gestation. Further work is required to determine whether maternal undernutrition during the preimplantation period alone is sufficient to cause a change in GR expression within either the fetal hippocampus or hypothalamus.

6.3 *Ex vivo* Environment – the Impact of Exposure of the Embryo to Culture Media containing Serum

An important finding of this thesis is that when embryos were cultured in a defined media the development of the fetal HPA axis was altered and that the addition of serum to the *in vitro* culture media reduces/ablates these effects in the late gestation sheep. Specifically, the study in Chapter 5 provides important evidence that there is an early activation of the fetal HPA axis in the singleton fetus which had been exposed to defined culture media as an embryo.

As discussed in the previous Chapters, there is substantial evidence in the literature that culturing embryos *in vitro* in the presence of serum is associated with prolonged length of gestation, the Large Offspring Syndrome and changes in the expression of the imprinted *Igf2r* gene. Specifically, Young and colleagues

(2001) postulated that organ overgrowth, which is a characteristic of LOS, reported in their study is associated with a decrease in IGF2R mRNA expression and that the change of the level of IGF2R mRNA expression is the result of epigenetic regulation (Young *et al.*, 2001). In Chapter 5 it was reported that unlike the results found in the IVCNS group, the relative adrenal weights in the IVCHS group were not higher than in the NM group, which suggests that culturing embryos in the presence of serum protects the fetal adrenal from overgrowth. Further evidence of the potential protective nature of serum addition to the culture medium is found in Chapter 5: fetal plasma ACTH concentrations were significantly reduced in singletons and CYP17 mRNA expression restored in both singleton and twins in the IVCHS when compared to the IVCNS group.

Although adrenal overgrowth was absent in the IVCHS group, there was a significantly lower expression of adrenal IGF2R mRNA in IVCHS when compared to the NM and IVCNS groups. In contrast to Young and colleagues' (2001) findings in a range of other fetal tissues, it may be that the role of IGF2R is protective in the fetal adrenal such that adrenal overgrowth is prevented. This again suggests that the role of IGF2 is inhibitory to fetal adrenal growth and development in late gestation. Unfortunately the studies reported in Chapter 5 were not able to establish whether *Igf2r* gene expression was epigenetically regulated as there was a limitation of the availability of adrenal tissue for these experiments.

6.4 CONCLUSION

In conclusion, the results of this thesis therefore highlight the complex interactions between the periconceptional *in vivo* or *ex viv*o environment and embryo or fetal number on the programming of the development of fetal HPA axis and in particular the fetal adrenal. Maternal undernutrition during the periconceptional period may differentially target components of the fetal HPA axis depending on exposure to undernutrition during specific periconceptional time periods. Furthermore, culturing embryos *in vitro* in the absence of serum results in an early activation of the HPA axis and alters adrenal development whilst *in vitro* culture in the presence of serum reduces/ablates these effects in the late gestation fetal sheep.

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