Table of Contents

ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS vi
LIST OF TABLES xi
LIST OF FIGURES xii
ABBREVIATIONS xiv
1. INTRODUCTION 1
1.1 Normal fetal growth and development 2
1.1.1 Fetal nutrition 2
1.1.2 Regulation of fetal growth by maternal nutrition 3
1.1.3 Placental regulation of fetal growth 4
1.1.4 Genetic and epigenetic determinants of fetal growth 7
1.1.5 Endocrine regulation of fetal growth and development 8
1.2 Altered fetal growth and development – causes, adaptations and consequences 16
1.2.1 The Developmental Origins of Health and Disease hypothesis 16
1.2.2 Maternal nutrition and the developmental origins of health and disease 19
1.2.3 Twinning and the developmental origins of health and disease 25
1.3 Aims of this thesis 28
2. MATERIALS AND METHODS 29
2.1 Animals 29
2.2 Nutrition 29
2.3 Surgery 30
2.3.1 Catheters 30
2.3.2 Animal preparation and anaesthesia 30

2.3.3 Catheterisation 31
2.3.4 Closure 32
2.3.5 Postoperative care 32
2.4 Experimental Procedures 33
2.4.1 Sample collection 33
2.4.2 Uterine blood flow measurement 33
2.4.3 Glucose and arginine challenges 34
2.4.4 Maternal fast and refeed 34
2.4.5 CRH/AVP challenge 34
2.4.6 Metyrapone challenge 35
2.4.7 Postmortem 35
2.5 Development of a method for estimation of blood volume parameters in pregnant sheep 35
2.5.1 Introduction 35
2.5.2 Methods 37
2.5.3 Results 39
2.5.4 Discussion 41
2.6 Biochemical Assays 43
2.6.1 Metabolite assays 43
2.6.2 Hormone Assays 43
2.7 Data Analysis 46

3. EFFECTS OF PERICONCEPTIONAL UNDERNUTRITION ON MATERNAL CARDIOVASCULAR ADAPTATION TO PREGNANCY 47
3.1 Introduction 48
3.2 Methods 50
3.2.1 Experimental design 50
3.2.2 Data analysis 50
3.3 Results 51
3.3.1 Maternal Weight 51
3.3.2 Blood volume 51
3.3.3 Uterine Blood Flow 52
3.3.4 Morphometric data 53
3.4 Discussion 55

4. EFFECTS OF PERICONCEPTIONAL UNDERNUTRITION ON OVINE SINGLETON PREGNANCY 59
4.1 Introduction 59
4.2 Methods 61
4.2.1 Experimental design 61
4.2.2 Data analysis 61
4.3 Results 62
4.3.1 Maternal weight 62
4.3.2 Fetal growth 64
4.3.3 Maternal metabolism 67
4.3.4 Fetal metabolism 68
4.3.5 Fetal glucose and arginine challenges 72
4.3.6 Maternal HPA axis 73
4.3.7 Fetal HPA axis 74
4.4 Discussion 77
4.4.1 Preconception undernutrition 78
4.4.2 Postconception undernutrition 81
4.4.3 Pre- and postconception undernutrition 82
4.4.4 Conclusions 84
5. EFFECTS OF TWINNING AND PERICONCEPTIONAL UNDERNUTRITION ON MATERNAL METABOLISM, FETAL GROWTH AND GLUCOSE-INSULIN AXIS FUNCTION IN OVINE PREGNANCY 85
5.1 Introduction 85
5.2 Methods 87
5.2.1 Experimental design 87
5.2.2 Data analysis 88
5.3 Results 89
5.3.1 Fetal growth 89
5.3.2 Metabolism 94
5.3.3 Fetal glucose-insulin axis 97
5.4 Discussion 100
5.4.1 Maternal metabolism 100
5.4.2 Placenta 101
5.4.3 Fetal growth 102

5.4.4 Fetal pancreatic function 103

5.4.5 Conclusions 104

6. **EFFECTS OF TWINNING AND PERICONCEPTIONAL UNDERNUTRITION ON HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION IN OVINE PREGNANCY** 106

6.1 Introduction 106

6.2 Methods 108

6.2.1 Experimental design 108

6.2.2 Data analysis 108

6.3 Results 109

6.3.1 Maternal HPA axis 110

6.3.2 Fetal HPA axis 113

6.4 Discussion 118

6.4.1 Maternal HPA axis 119

6.4.2 Fetal HPA axis 120

6.4.3 Conclusions 124

7. **C-TYPE NATRIURETIC PEPTIDE FORMS IN THE OVINE FETAL AND MATERNAL CIRCULATIONS** 125

7.1 Introduction 125

7.2 Methods 125

7.2.1 Experimental design 125

7.2.2 Statistics 126

7.3 Results 130

7.3.1 Fetal and maternal plasma CNP and NTproCNP 130

7.3.2 Response to fasting 131

7.3.3 Effect of twinning 132

7.3.4 Source of CNP forms in maternal plasma 133

7.4 Discussion 135

8. **CONCLUSIONS** 140

8.1 Periconceptional undernutrition 140

8.2 Twinning 140

8.3 Summary 143

9. **APPENDICES** 144

9.1 Appendix 1 – Contributions 144

9.2 Appendix 2 – Publications arising from this thesis 145
9.2.1 Scientific meeting abstracts 145
9.2.2 Journal publications 146
10. REFERENCES 147

List of Tables

Table 3-1: Blood volume parameters in different nutritional groups at 65 and 120 d gestation, and in non-pregnant sheep **51**

Table 3-2: Uterine blood flow at day 114 and 131 of gestation in different nutritional groups **52**

Table 3-3: Placental, uterine and fetal weights at 120 and 132 d **53**

Table 4-1: Maternal weights and food intake **63**

Table 4-2: Fetal morphometric data at days 110 and 132 **66**

Table 4-3: Maternal and fetal plasma metabolite levels **69**

Table 4-4: Maternal and fetal plasma insulin and IGF-1 levels **70**

Table 4-5: Maternal ACTH and steroid levels **73**

Table 4-6: Fetal ACTH and steroid levels **74**

Table 5-1: Maternal body weights **89**

Table 5-2: Fetal morphometric data at 111 and 132 d **91**

Table 5-3: Fetal organ weights at 132 d **92**

Table 5-4: Placenta measurements at 132 d **93**

Table 5-5: Maternal and fetal plasma metabolite and hormone levels **96**

Table 6-1: Maternal weights **109**

Table 6-2: Maternal ACTH and steroid levels at 114-121 d and 128-131 d **110**

Table 6-3: Fetal ACTH and steroid levels at 114-121 d and 128-131 d **113**

Table 7-1: Fetal and maternal plasma glucose, urea, insulin, and IGF-1 levels before, during and after maternal fasting **128**

Table 7-2: Fetal and maternal plasma CNP forms, glucose, insulin and IGF-1 levels in singleton and twin pregnancies **129**

List of Figures

Figure 1-1: Steroid synthesis **9**

Figure 2-1: Timeline of experimental protocol **33**

Figure 2-2: FD250S concentrations over 180 min in one sheep following an intravenous bolus of 40 mg in 10 mL saline. A: Exponential fall in plasma concentrations B: Semilogarithmic plot allowing extrapolation back to time 0 **40**

Figure 2-3: Bland-Altman plot of BV results obtained using FD250S and radiolabelled red cells **41**

Figure 3-1: Correlations between maternal cardiovascular parameters and feto-placental morphometric measurements **54**

Figure 4-1: Ewe weights from -71 to 132 d **63**

Figure 4-2: Fetal chest girth growth curves in singletons **65**

Figure 4-3: Maternal glucose (A) and insulin (B), and fetal glucose (C) and insulin (D) levels during fasting and refeeding **71**

Figure 4-4: Fetal glucose (A) and insulin (B) responses to an intravenous glucose tolerance test; arginine (C) and insulin (D) responses to an intravenous arginine challenge **72**

Figure 4-5: ACTH (A) and cortisol (B) responses to CRH/AVP challenge **75**

Figure 4-6: ACTH (A) and 11deoxycortisol (B) responses to metyrapone challenge **76**

Figure 5-1: Fetal chest girth growth curves **94**

Figure 5-2: Fetal glucose (A) and insulin (B) responses to an intravenous glucose tolerance test; arginine (C) and insulin (D) responses to an intravenous arginine challenge **98**

Figure 5-3: Maternal glucose (A) and insulin (B), and fetal glucose (C) and insulin (D) levels during the fast and refeed periods **99**

Figure 6-1: Maternal plasma ACTH (A), cortisol (B), cortisone (C) and progesterone (D) levels during the fast and refeed periods **112**

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Figure 6-2: Fetal plasma ACTH (A), cortisol (B), cortisone (C) and DHEA (D) levels during the fast and refeed periods 115
Figure 6-3: ACTH (A) and cortisol (B) responses to CRH/AVP challenge 116
Figure 6-4: ACTH (A) and 11deoxycortisol (B) responses to metyrapone challenge 118
Figure 7-1: Circulating maternal and fetal concentrations of (A) NTproCNP and (B) CNP during gestation 127
Figure 7-2: Plasma concentrations of (A) CNP and (B) NTproCNP drawn simultaneously from the maternal artery and utero-ovarian vein from six pregnant ewes with singleton pregnancies 130
Figure 7-3: Relationship between placental weight and maternal NTproCNP levels at 131 d 131

Abbreviations

ACTH	adrenocorticotropin hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ARU	animal research unit
AUC	area under the curve
AVP	arginine vasopressin
BMI	body mass index
BV	blood volume
C	control
C_0	concentration at time 0
CBG	corticosteroid-binding globulin
cHct	central haematocrit
cm	centimetre
CNP	C-type natriuretic peptide
COMT	catechol-O-methyltransferase
cpm	counts per minute
Cr	chromium
CRH	corticotropin-releasing hormone
CV	coefficient of variation
CVD	cardiovascular disease
d	days
D	dilution of solution
DHEA	dehydroepiandrosterone
DOHaD	Developmental Origins of Health and Disease
FD250S	250 kDa fluorescein isothiocyanate dextran
FFA	free fatty acids
g	gram
GH	growth hormone
GLUT	glucose transporter
GR	glucocorticoid receptor
h	hours
Hct	haematocrit
HPLC	high performance liquid chromatography
HPA	hypothalamic-pituitary adrenal
HSD	hydroxysteroid dehydrogenase
I	amount injected
ID	internal diameter
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
in	inch
kDa	kiloDalton
kg	kilogram
L	litre
MA	maternal artery
mg	milligram
min	minutes
mm	millimetre
mL	millilitre
mmol	millimole
mol	mole
MR	mineralocorticoid receptor
mTorr	milliTorr
mTOR	mammalian target of rapamycin

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ng	nanogram
nm	nanometre
NTproCNP	N-terminal proCNP
OD	outer diameter
PBS	phosphate buffered saline
PEG	polyethylene glycol
pg	picogram
pmol	picomole
PL	placental lactogen
pmol	picomole
POMC	pro-opiomelanocortin
PPAR	peroxisomal proliferator-activated receptor
PV	plasma volume
R_I	rate of infusion
RCV	red cell volume
RIA	radioimmunoassay
rpm	revolutions per minute
SARGG	sheep anti-rabbit gamma globulin
sec	seconds
SEM	standard error of the mean
tHct	total body haematocrit
T_3	L-triiodothyronine
T_4	L-thyroxine
UBF	uterine blood flow
UN -60-0	undernourished from 60 d before until mating
UN -2-30	undernourished from 2 d before until 30 d after mating
UN -60-30	undernourished from 60 d before until 30 d after mating
UOV	utero-ovarian vein
UV	ultraviolet
V	volt
Vd	volume of distribution
V_i	volume injected
VEGF	vascular endothelial growth factor
βHBA	β-hydroxybutyrate
µg	microgram
µL	microlitre
µm	micrometre

1. Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis postulates a link between conditions in early life and the risks of a number of chronic diseases in adulthood (Gillman, 2005). Due to a large body of human epidemiological data that relates size at birth to later disease risk, it is now widely accepted that such a link exists, and that the developmental plasticity of the fetus and child is the basis of this link. Further human and animal research has shown that maternal nutrition is an important influence on both size at birth and aspects of physiology that relate to disease risk. However, not only has it become clear that maternal nutrition can affect fetal and postnatal physiology without altering size at birth, but the link between size at birth and adult disease is not consistent in the case of multiple pregnancies. Hence, in order to understand the biochemical and physiological mechanisms underlying the developmental origins of disease, it is necessary to look past the physical phenotype of the fetus and to study biochemical and physiological changes in the mother, placenta and fetus resulting from altered prenatal environments.

As it has become clear that maternal nutrition in pregnancy contributes significantly to the prenatal environment and hence the risk of adult pathology, so it has become necessary to design experiments that can guide eventual clinical applications. The studies described in this thesis build upon a long history of basic and applied fetal physiology research at the Liggins Institute. They were designed to distinguish between the effects of pre- and postconception maternal undernutrition in sheep, and to compare singleton and twin pregnancies with a view to understanding the underlying mechanisms linking the prenatal environment with postnatal physiology.

This introduction will summarise some of the literature regarding both the normal control of fetal growth and development, and the causes of and adaptations to abnormal growth and development, with a focus on the human and sheep literature around maternal undernutrition and twinning. Discussion of the consequences of abnormal development will be limited to those that relate to adult physiology and pathology.

1.1 Normal fetal growth and development

1.1.1 Fetal nutrition

In contrast to postnatal life in which genetics is the main determinant of potential growth, in prenatal life, fetal growth is mainly regulated by fetal nutrition (Harding and Johnston, 1995). However, fetal nutrition is itself determined by a number of different factors.

1.1.1.1 Supply line and maternal constraint

Normal fetal growth and development are primarily dependent on an adequate supply of oxygen and nutrients, both of which require an intact maternal ‘supply line’ (Harding, 2001). Adequate fetal nutrition requires appropriate maternal intake, digestion and absorption of food, maternal metabolic and endocrine function to provide circulating levels of nutrients, systemic and utero-placental circulation, placental function and umbilical blood flow. These non-genetic limitations on fetal growth underlie the concept of maternal constraint. This concept was originally demonstrated by cross-breeding large Shire horses and small Shetland ponies to show that birth size was dependent on maternal size (Walton and Hammond, 1938). It has been repeated with embryo transfer in mice, thereby eliminating any influence of genotype, achieving similar results (Cowley *et al.*, 1989). The constraint of fetal growth by these factors can be an appropriate mechanism to ensure safe pregnancy and labour, but may also be inappropriate when pathological conditions limit supply line function. Smoking, for example, decreases fetal oxygenation and uterine blood flow, resulting in fetal growth restriction (Lambers and Clark, 1996). Thus, adequate maternal intake by itself does not guarantee adequate fetal nutrition and growth.

1.1.1.2 Fetal substrates and species differences

Much of the research in fetal physiology has been performed in sheep because it is possible to catheterise the fetus without causing premature labour. Normal gestational length for sheep is around 145 days compared with 280 days in the human. Although both the human and sheep use glucose, lactate and amino acids as the main metabolic substrates for the fetus, the sheep uses proportionately more lactate (Fowden, 1994). The human fetus also uses free fatty acids that readily cross the placenta, but this occurs to a much

lesser extent in the sheep (Elphick *et al.*, 1979). Although the fetus becomes capable of gluconeogenesis late in gestation, maternal supply remains the predominant source of glucose throughout gestation (Dalinghaus *et al.*, 1991).

Maternal glucose in the sheep is synthesised in the liver from gluconeogenic substrates produced from fermentation in the rumen (Bergman *et al.*, 1966), and therefore glucose concentrations fall quickly with undernutrition, resulting in fetal hypoglycaemia. The effect of undernutrition on maternal glucose levels in the human is smaller, as glucose is derived directly from the diet and also synthesised in the liver.

1.1.2 Regulation of fetal growth by maternal nutrition

Because of the many possible limitations in the supply line, there is a relatively poor correlation between maternal nutritional intake and birthweight, but animal studies have been able to illustrate the relationship and to distinguish the effects of nutrition at different stages of pregnancy. Sheep studies show that undernutrition from mating to 35 d gestation reduces fetal size at 35 d (Parr and Williams, 1982), and undernutrition from mating to 60 d reduces birthweight (Vincent *et al.*, 1985). The impact of mid gestation undernutrition has been more thoroughly assessed, although with varying protocols. Undernutrition from 28 – 80 d results in lower placental weights at 80 d but greater placental and fetal weights at 145 d (Dandrea *et al.*, 2001), while undernutrition from 30 to 96 d increases placental weight at 96 and 140 d, but does not change fetal weight (McCrabb *et al.*, 1991). Several other studies of maternal undernutrition show no change in either placental or fetal weights, but do show differences in placental phenotype (Luther *et al.*, 2005).

Late-gestation undernutrition in sheep is consistently associated with a reduction in fetal weight, especially in multiple pregnancies (Luther *et al.*, 2005), and direct measurements of fetal growth trajectory *in utero* in sheep have shown that fetal growth slows within three days of a decrease in maternal nutrition (Mellor and Matheson, 1979). The use of growth catheters has also shown that the fetuses of sheep undernourished from 60 d before until 30 d after mating grow slower in late gestation despite normal maternal nutrition after 30 d and normal fetal and placental size at birth (Oliver *et al.*, 2005). These slow-growing fetuses maintain their growth trajectory during a 10 d period of maternal undernutrition in late gestation, whereas fast growing fetuses slow their growth (Harding, 1997b). These observations demonstrate that fetal growth trajectory may be set

in early gestation, and that different growth trajectories may result in similar body sizes at birth.

The effects of overnutrition in sheep differ depending on whether the ewe is an adolescent or adult. Adolescent sheep on a high nutritional plane synthesise maternal tissue at the expense of placental and fetal growth (Wallace *et al.*, 1996), whereas overnutrition in adult sheep results in a normal size placenta and fetus despite high maternal adiposity and a shortened gestation (Wallace *et al.*, 2005).

Clarification of the relationship between maternal diet and birth size in humans has required large meta-analyses, and these show that chronic undernutrition increases the risk of having small babies (Kramer, 1987), and that protein/energy supplementation can increase birth size (Kramer and Kakuma, 2003). Furthermore, high carbohydrate and low protein diets in pregnancy are associated with decreased placental size and small, thin babies (Godfrey *et al.*, 1996a; Godfrey *et al.*, 1997).

The various effects of undernutrition at specific stages of pregnancy in humans have been demonstrated by the epidemiological studies of the Dutch Hunger Winter. Occurring at the end of World War II in the western Netherlands, this was a period of five months of severe undernutrition due to a German embargo of food transports. Adult rations dropped to 580 kilocalories per day, but the famine was abruptly relieved by the Allied liberation of the western Netherlands in May 1945. Exposure to the famine in late gestation resulted in decreased birthweight, crown to heel length and head circumference of the offspring, with a similar, but weaker, pattern following second trimester exposure (Stein *et al.*, 2004). There were no effects of first trimester exposure, but periconceptional exposure may have resulted in a small decrease in crown to heel length and head circumference.

1.1.3 Placental regulation of fetal growth

The placenta serves the functions of protecting the fetus from the maternal immune system, oxygenating fetal blood, transferring nutrients, and producing hormones and metabolites that contribute to maternal adaptation to pregnancy and fetal growth and development. Placental weight is positively correlated with fetal weight in normal human (Lurie *et al.*, 1999) and sheep pregnancies (Mellor and Murray, 1981), and experimental reduction of placental size in sheep by carunclectomy reduces fetal size (Owens *et al.*, 1986). Maternal hyperthermia in sheep also causes a reduction in placental size that is followed by fetal growth restriction (Galan *et al.*, 1999).

1.1.3.1 Placental growth and species differences

The placenta grows quickly relative to the fetus in early gestation but slower in later gestation. In the human it continues to grow slowly in late gestation, while the sheep placenta reaches peak size at around 100 d gestation before undergoing a structural remodelling that results in decreased weight but increased umbilical and uterine blood flow, and increased nutrient transfer to the fetus (Schneider, 1996). Ruminants have a non-invasive cotyledonary placenta, which in sheep consists of between 75 and 125 placentomes. The placentomes may be classified A, B, C or D based on phenotype. Type A placentomes are inverted with a small fetal component, and type D placentomes are everted with a large fetal component; types B and C are intermediate between these extremes (Vatnick *et al.*, 1991). Everted placentome phenotypes have a greater number of materno-fetal interfaces and greater materno-fetal absorptive surface (Krebs *et al.*, 1997), suggesting that placental efficiency may be increased. In sheep the embryo enters the uterus on day 4, develops into a blastocyst by day 6, but adherence to the endometrium does not occur until day 16 (Spencer *et al.*, 2004). Human embryos enter the uterus on day 2 to 3 with implantation occurring at day 6 to 7 (Norwitz *et al.*, 2001). Differences in placental function include greater permeability of the human placenta to fatty acids (Dutta-Roy, 2000; Saleh *et al.*, 1989) and ketones (Paterson *et al.*, 1967), with the fetal brain able to metabolise ketones (Adam *et al.*, 1975) to spare glucose for other tissues. In the sheep, however, ketones cross the placenta in only small amounts (Miodovnik *et al.*, 1982) and are metabolised in the placenta to maintain fetal lactate levels (Harding and Gluckman, 2001).

1.1.3.2 Nutrient transfer

Despite being less than a quarter the size of the fetus in late gestation, the placenta still accounts for more than half of the glucose consumption from the uterine circulation in late-gestation sheep pregnancy (Sparks *et al.*, 1983). Fetal nutrition, therefore, is partly limited by placental consumption of nutrients, but also by placental transfer capacity. The sheep placenta also uses glucose and amino acids to produce lactate, which is preferentially supplied to the fetus (Sparks *et al.*, 1983) where it is an important oxidative fuel. This process helps to confine nutrients in the fetal circulation as the placenta is less

permeable to lactate than glucose (Owens, 1991). Reduction in placental size is correlated with reductions in uterine and umbilical blood flows (Owens *et al.*, 1986), which are limiting factors for transfer of flow-limited nutrients such as oxygen (Bell *et al.*, 1999). Placentas limited in size by carunclectomy fail to increase uterine and umbilical blood flows with increasing gestational age, as occurs in control sheep (Owens *et al.*, 1986) and there is subsequent fetal hypoxaemia (Owens *et al.*, 1987b) and hypoglycaemia (Owens *et al.*, 1987a). In addition to the limitations imposed by uterine blood flow, transport of nutrients that require specific transport proteins is limited by the exchange surface area of the trophoblastic membrane and the levels of the transporter proteins.

Glucose crosses the placenta by facilitated diffusion using glucose transporters (GLUT). The predominant transporters in the placenta are the insulin-independent GLUT-1 and GLUT-3, the levels of which are regulated by maternal and fetal glucose levels (Das *et al.*, 1998). The expression of GLUT-1 peaks around 120 d gestation, while GLUT-3 continues to increase until term (Currie *et al.*, 1997), suggesting distinct roles during development. The placentas of heat-exposed ewes demonstrate decreased efficiency of glucose transport (Thureen *et al.*, 1992), suggesting a decrease in the number or activity of glucose transporters, while placentas following carunclectomy have increased clearance of glucose analogues per unit mass (Owens *et al.*, 1987c) suggesting increased glucose transport.

Amino acids are transported against concentration gradients by a variety of energy-dependent transporters in the placenta, with at least nine different systems identified in the human (Regnault *et al.*, 2005). Fetal concentrations are greater than maternal concentrations for many amino acids (Cetin, 2003). The active transport of amino acids requires transport proteins on both surfaces of the trophoblast cell as there must be uptake from the maternal circulation across the microvillous membrane, transport through the trophoblast cytoplasm, and finally transport across the basal membrane to the fetal circulation (Regnault *et al.*, 2005). The capacity of amino acid transport, therefore, is limited by trophoblast surface area and concentration of transport proteins, both of which increase with increasing gestational age (Mahendran *et al.*, 1994; Teasdale and Jean-Jacques, 1985). The maternal amino acid profile also influences placental amino acid transport capacity (Jozwik *et al.*, 2001).

In humans, levels of the system A transporters, which transport short-chain amino acids like alanine, serine, proline and glycine, are related to morphometric measurements at birth (Harrington *et al.*, 1999). Decreased levels of system A (Glazier *et al.*, 1997) and

taurine transporters (Norberg *et al.*, 1998) are also associated with growth restriction. In rats, placental system A transporter levels are reduced in intrauterine growth restriction due to protein restriction (Jansson *et al.*, 2006), while in guinea pigs they are reduced by uterine artery ligation causing growth restriction (Jansson and Persson, 1990). In the heat-stressed sheep model of placental and fetal growth restriction, system L transport is altered with the uterine uptake, utero-placental utilisation, placental-fetal flux, maternal-fetal flux and oxidation of leucine all decreased in the growth restricted fetus (Ross *et al.*, 1996). This suggests that reduced placental transport of amino acids may contribute to growth restriction. One mechanism relating to amino acid transport is demonstrated by knockout of the insulin-like growth factor 2 (IGF-2) gene in the labyrinthine placenta in mice. This results in a smaller placenta but increased efficiency of the system A transport system, possibly as a compensatory mechanism (Constancia *et al.*, 2002).

The placenta is also involved in amino acid metabolism such as the glycine-serine and glutamine-glutamate placenta-hepatic shuttles (Christensen, 1992) which act to shuttle nitrogen and carbon between the fetal liver and the placenta.

1.1.3.3 Placental endocrinology

The placenta further influences fetal growth by regulating a variety of hormones. It produces placental lactogen (PL) and growth hormone (GH), which contribute to maternal insulin resistance that increases availability of nutrients for placental transfer to the fetus (Bauer *et al.*, 1998). It limits fetal exposure to maternal glucocorticoids by the enzyme 11ß hydroxysteroid dehydrogenase type 2 (11ßHSD-2), which forms a functional barrier between the circulations by converting cortisol into inactive cortisone. Cortisol is known to slow fetal growth (Fowden *et al.*, 1996), and it has been shown that blocking 11ßHSD-2 (Lindsay *et al.*, 1996) causes fetal growth restriction. The placenta may also regulate fetal plasma levels of insulin-like growth factor 1 (IGF-1) (Iwamoto *et al.*, 1992) by taking it up from the fetal circulation (Bassett *et al.*, 1990).

1.1.4 Genetic and epigenetic determinants of fetal growth

1.1.4.1 Genetic polymorphisms

Although genetic factors may be thought of as providing a permissive rather than regulatory role in fetal growth, there are clear effects of genetic variants on birth size. Knock-out mice missing IGF-1 or IGF-2 are significantly smaller at birth (Liu *et al.*,

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1993). Also IGF-2, IGF-2 receptor (Kaku *et al.*, 2007) and catechol-O-methyltransferase (COMT) gene polymorphisms (Sata *et al.*, 2006) are associated with birth weight variation in humans.

1.1.4.2 Imprinted genes

Epigenetic modifications also influence fetal and placental development, and may be a link between early environment and later physiology and pathology. Imprinting is an epigenetic process whereby one allele of a gene is inactivated by DNA methylation or histone acetylation (Reik and Walter, 2001). Imprinted genes account for only 0.1-0.5% of the genome (Fowden *et al.*, 2006a), but are known to code for proteins that have an important influence on early development. Paternally expressed genes appear to stimulate fetal growth while maternally expressed genes limit growth (Fowden *et al.*, 2006a). DNA methylation patterns are reprogrammed during germ cell development and in the preimplantation embryo (Reik *et al.*, 2001) and are known to be affected by factors such as maternal diet (Lillycrop *et al.*, 2005), grooming behaviour (Weaver *et al.*, 2004) and uterine blood flow (Pham *et al.*, 2003). Examples of imprinted genes are the IGF-2 gene, which has important roles in controlling the nutrient supply for fetal growth by effects on placental growth and structure (Han and Carter, 2000), and the glucocorticoid receptor (GR) gene (Lillycrop *et al.*, 2005).

1.1.5 Endocrine regulation of fetal growth and development

1.1.5.1 Glucocorticoids

All steroid hormones are derived from pregnenolone via metabolic pathways involving a number of enzymes (Figure 1-1). In humans and sheep, the predominant glucocorticoid is cortisol, but there are important biochemical differences in adrenal development between humans and sheep. In humans there is a fetal zone of the adrenal cortex that lacks 3ß-hydroxysteroid dehydrogenase, and that provides oestrogen precursors (mainly dehydroepiandrosterone sulphate) in response to adrenocorticotropin (ACTH) and human chorionic gonadotrophin (Seron-Ferre *et al.*, 1978). This zone increases during gestation, regresses after birth and is not present in the adult. The remainder of the adrenal cortex is known as the definitive zone, which produces cortisol in response to ACTH. In sheep, the fetal adrenal is similar to the adult adrenal in that there is no distinct fetal zone as in humans (Wood, 2001).

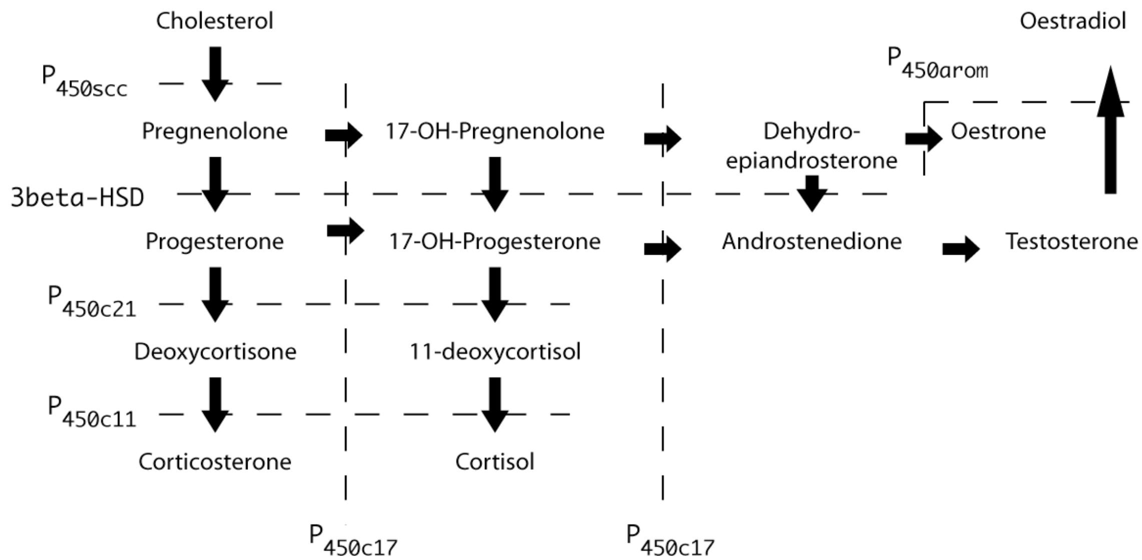

Figure 1-1: Steroid synthesis

Adapted from Fetal Growth and Development, 2001, Harding, R and Bocking, AD, p190
3beta-HSD – 3ß hydroxysteroid dehydrogenase
scc – side-chain cleavage
arom - aromatase

Sheep studies have shown that the plasma levels of cortisol are low and predominantly of maternal origin until late gestation (Hennessy *et al.*, 1982). The levels begin to increase gradually from around 120 d gestation reflecting increases in corticosteroid-binding globulin (CBG) and adrenal hypertrophy, as well as increased ACTH levels and increased adrenal response to ACTH (Challis and Brooks, 1989). The adrenal steroidogenic response to ACTH in *in vitro* studies alters over gestation with peaks around day 50 and in late gestation, with decreased responsiveness in the interim (Wintour *et al.*, 1975). Approaching parturition, there is a decrease in sensitivity of the hypothalamic and pituitary negative feedback system, which may be achieved at the level of the pituitary through increased levels of CBG (Challis *et al.*, 1995) and local expression of 11β-HSD enzymes. At the level of the hypothalamus, GR levels decline in late pregnancy (Matthews and Challis, 1996). These changes lead to a marked increase in both ACTH and cortisol levels. There is very good evidence that this fetal cortisol surge is necessary to induce parturition in sheep, as fetal hypophysectomy or adrenalectomy leads

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to prolonged gestation (Liggins *et al.*, 1967). It is believed that the cortisol surge causes induction of placental enzymes 17α-hydroxylase and 17, 20 lyase ($P450_{c17}$ enzymes) leading to decreased progesterone and increased oestrogen and prostaglandins, all of which lead to the onset of labour (Challis *et al.*, 2000). Cortisol levels have been shown to increase prematurely before preterm delivery (Bassett and Thorburn, 1969).

The steroid receptors are intracellular ligand-dependent transcription factors, which act on specific DNA sequences (Funder, 1997). The effects of glucocorticoids are mediated by the glucorticoid receptor and the mineralocorticoid receptor (MR). The GR shows high affinity for dexamethasone, moderate affinity for cortisol and corticosterone, and low affinity for aldosterone and the sex steroids. The MR binds corticosterone, aldosterone and cortisol with high affinity (Funder, 1997). The specificity of activation of the MR by aldosterone is provided by the presence of 11β-HSD2, which converts cortisol to inactive cortisone.

Cortisol has important metabolic effects on carbohydrate, protein and fat metabolism, and on maturation and growth. The late-gestation cortisol surge is responsible for the functional maturation of many organs, including the lungs (Liggins, 1969) and the gut (Sangild *et al.*, 1995). The change from growth to maturation reduces fetal growth rate immediately before birth, and absence of this cortisol surge due to adrenalectomy increases body weight at term (Fowden *et al.*, 1996). In humans prednisone administration during human pregnancy is known to reduce birth weight (Reinisch *et al.*, 1978), and sheep studies demonstrate that maternal administration of synthetic glucocorticoids results in decreased birth weights (Newnham and Moss, 2001).

Given the crucial role of cortisol in parturition and fetal growth, it is not surprising that the placenta maintains a separation between the maternal and fetal hypothalamic-pituitary-adrenal (HPA) axes. Fetal glucocorticoid levels are about ten-fold lower than maternal levels, with maternal cortisol levels accounting for around 40% of the variance of fetal cortisol (Gitau *et al.*, 1998). Separation between the maternal and fetal axes is provided by the presence of 11ßHSD-2 in the placenta, which deactivates natural active glucocorticoids, cortisol and corticosterone, to their inactive 11-keto forms (Benediktsson *et al.*, 1997). The enzyme is also present in the fetal membranes (Stewart *et al.*, 1995) and in other body tissues, serving the function of protecting mineralocorticoid receptors from occupation by glucocorticoids, thereby providing specificity. In normal human pregnancies placental 11ß-HSD2 mRNA expression increases more than fifty-fold,

peaking at term, is lower at equivalent gestational ages in pregnancies complicated by growth restriction (McTernan *et al.*, 2001). Carbenoxolone, which inhibits 11ßHSD-2 and presumably increases fetal cortisol exposure, results in decreased birthweight in rats (Lindsay *et al.*, 1996), as does maternal administration of synthetic glucocorticoids which are not inactivated by 11ßHSD-2 (Nyirenda *et al.*, 1998).

In the human, the placenta also produces corticotropin-releasing hormone (CRH) and ACTH (Petraglia *et al.*, 1987) and these may influence the activity of the maternal and fetal HPA axes. The secretion of ACTH from the syncytiotrophoblast layer is stimulated by CRH but not affected by glucocorticoids (Petraglia *et al.*, 1987).

1.1.5.2 Insulin

Insulin is detectable in the plasma of human fetuses at approximately 84 days gestation and in sheep at around 60 days, although it is detectable in the pancreas significantly earlier (Fowden and Hill, 2001). Secreted from the islets of Langerhans in the pancreas, insulin has an important role in the storage of excess energy substrates and in supporting growth. The effects of insulin binding on the cell include increased cell membrane permeability to glucose, amino acids, potassium and phosphate ions (Saltiel and Kahn, 2001).

The complex process of glucose uptake into cells is controlled by the sodium-dependent glucose co-transporters (SGLT family) and the facilitative sodium-independent glucose transporters (GLUT family) (Wood and Trayhurn, 2003). The most comprehensively studied are the class I facilitative transporters that contain GLUT1-4. GLUT-2 is expressed in the pancreatic β cells suggesting a role in the glucose-sensing mechanism (Newgard and McGarry, 1995). Insulin increases glucose uptake in skeletal muscle, cardiac muscle and adipose tissue cells by causing the translocation of the GLUT-4 glucose transporter to the cell surface (Saltiel and Kahn, 2001), with these tissues being responsible for the control of the postprandial glucose levels. Insulin promotes fetal growth mainly through increasing fat deposition and increasing the uptake and utilisation of glucose by tissues (Fowden, 1995). In addition, the uptake into cells of many amino acids is promoted by insulin along with the translation of mRNA causing increased protein formation (Umpleby and Russell-Jones, 1996).

Raised blood glucose levels play the predominant role in stimulating insulin secretion, but some amino acids also stimulate insulin release (Hertelendy *et al.*, 1970). In

the sheep fetus, insulin release occurs more rapidly following intravenous arginine than following glucose (Fowden, 1980), probably due to release from different compartments in the ß cell (Levin *et al.*, 1972) and by different mechanisms (Fowden, 1980). These responses increase between mid and late gestation (Aldoretta *et al.*, 1998). The role of the endocrine pancreas *in utero* is to match the rate of glucose utilisation to the rate of placental supply (Fowden and Hill, 2001), whereas in postnatal life it is to control the fluctuations in glucose levels that occur with intermittent feeding. Therefore, there is a maturational change that must take place in late gestation or early neonatal life. In the rat this is thought to occur around 2 to 4 weeks of age when there is a wave of ß cell apoptosis and neogenesis (Scaglia *et al.*, 1997). This remodelling is thought to involve replacement of immature ß cells that are more responsive to amino acids with mature ß cells that are more responsive to glucose (Hill and Duvillie, 2000). In the horse, the insulin response to glucose increases closer to term and with increasing fetal cortisol levels, whereas the insulin response to arginine does not change with increasing fetal cortisol levels (Fowden *et al.*, 2005). Furthermore, in sheep there is an increase in insulin response to glucose in late gestation (Houghton *et al.*, 1989) and an increased first phase insulin response in newborn lambs compared with the late-gestation fetus (Philipps *et al.*, 1979). These observations suggest that pancreatic remodelling may be occurring *in utero* in late gestation in larger mammals.

The role of insulin in fetal growth is suggested by the correlation between cord blood levels of insulin and birthweight in humans (Godfrey *et al.*, 1996b). Furthermore, experiments in sheep demonstrate a decrease in fetal growth rate following pancreatectomy, which is remedied by giving insulin (Fowden *et al.*, 1989). However, giving larger doses does not result in greater growth suggesting a permissive role in fetal growth; a situation seen also in rats (Cooke and Nicoll, 1984). Babies of diabetic mothers are larger than normal, although they are exposed to both hyperglycaemia and hyperinsulinaemia, and it is this combination of nutrients and hormone that results in their typical obesity (Tyrala, 1996).

1.1.5.3 Growth hormone

Growth hormone (GH) is a single chain 191 amino acid protein that is produced and released by the somatotrophic cells of the anterior pituitary. In human pregnancy a variant form of GH (GH-V) is produced by the placenta and secreted into the maternal

circulation (Alsat *et al.*, 1997). GH-V differs from GH by 13 amino acids and has high somatogenic and low lactogenic activities, and is secreted continuously rather than in a pulsatile manner. It gradually replaces pituitary GH during pregnancy. The sheep placenta also produces GH (Lacroix *et al.*, 2002).

GH is present in the circulation by 12 weeks in human fetuses (Wollmann, 2000), but both the roles and the control of the hormones of the somatotrophic axis are different in the fetus compared with postnatal life. GH concentrations are higher in the sheep fetus than lambs and adult sheep (Bassett *et al.*, 1970). GH is released in a pulsatile fashion in the sheep fetus, with average concentrations increasing from a gestational age of 100 days to 140 days, and a decline prior to delivery which continues postpartum (Bassett and Gluckman, 1986). GH secretion is stimulated by GH-releasing hormone and ghrelin, and inhibited by somatostatin and IGF-1 (Muller *et al.*, 1999). Somatostatin is secreted from both the hypothalamus and peripheral tissues including the gastrointestinal system and pancreas (Muller *et al.*, 1999).

The actual role of GH in fetal growth is unclear. GH receptors are expressed at low levels in fetal tissues but may be induced by the late fetal cortisol surge (Breier *et al.*, 1994a). Hypophysectomy of the fetal sheep with thyroxine replacement leads to reduced IGF-1 concentrations (Mesiano *et al.*, 1989) suggesting a possible role for GH in IGF-1 regulation. Congenital GH deficiency in humans results in a degree of prenatal growth failure (Gluckman *et al.*, 1992). In treatment studies where growth restricted fetal sheep were infused with GH, there was no improvement in fetal growth, although IGF-1 levels were restored to normal levels (Bauer *et al.*, 2003).

1.1.5.4 Insulin-like growth factors (IGFs)

IGF-1 and -2 are polypeptides with significant sequence homology with insulin (Daughaday and Rotwein, 1989), although IGFs retain the C-chain, which is cleaved in insulin. IGFs circulate bound to insulin-like growth factor binding proteins (IGFBPs) (Le Roith *et al.*, 2001). Approximately 90% of the IGFs circulate bound in a ternary complex with a molecular weight of 150 kDa which comprises IGF-1 or IGF-2, IGFBP-3 and an acid-labile subunit (Butler and Le Roith, 2001). IGFBPs regulate the bioactivity of both IGF-1 and IGF-2 by protecting them from proteolysis and contributing to their metabolic clearance (Butler and Le Roith, 2001). The mitogenic actions of IGF-1 and -2 are mediated by the IGF-1 receptor (Baker *et al.*, 1993), which is very similar to the insulin

receptor and a member of the family of tyrosine kinase growth factor receptors, while the IGF-2 receptor is involved in clearance of IGF-2 (Le Roith *et al.*, 2001).

IGFs are produced in a wide range of tissues including the liver, lung, kidney and muscle, and can act in an autocrine, paracrine and/or endocrine manner (Le Roith *et al.*, 2001). In postnatal life IGF-1 is primarily under GH control, but in fetal life the levels are regulated by fetal nutrient supply. Late-gestation maternal undernutrition results in a decrease in fetal plasma IGF-1 levels, but IGF-2 levels do not change (Bauer *et al.*, 1995). Glucose plays an important role in the regulation of IGF-1 and -2, but its effect on IGF-1 is probably insulin-dependent, whereas the effect on IGF-2 is insulin-independent (Oliver *et al.*, 1996). The principal growth effect of IGF-1 is due to cell proliferation (Froesch *et al.*, 1985), but it also has insulin-like effects on glucose metabolism (Holt *et al.*, 2003).

IGF-2 is thought to be important in embryonic growth and has important influences on placental growth (Baker *et al.*, 1993), whereas IGF-1 is more important in late-gestation growth. Rats that are IGF-1 deficient are 40% smaller at birth (Liu *et al.*, 1993). A chronic low dose infusion of IGF-1 to fetal sheep alters placental transfer capacity and morphology without affecting fetal growth (Bloomfield *et al.*, 2002), whereas higher doses result in anabolic effects on feto-placental protein and carbohydrate metabolism (Harding *et al.*, 1994) and selective organ growth (Lok *et al.*, 1996).

1.1.5.5 Thyroid hormones

The thyroid hormones, L-thyroxine (T_4) and 3,5,3'-L-triiodothyronine (T_3), affect tissue accretion and differentiation, and are mostly bound to thyroxin-binding globulin and albumin in the circulation, which restricts the biologically active levels. Deficiency in fetal sheep due to thyroidectomy leads to reduced bodyweight and crown-rump length (Fowden and Silver, 1995) and reduction in skeletal muscle cell number (Finkelstein *et al.*, 1991). These changes may be due to the influence of thyroid hormones on the somatotrophic axis (Forhead *et al.*, 2002). The main metabolic action of T_4 is to stimulate oxygen utilisation by fetal tissues, and fetal oxygen and glucose consumption correlates with replacement T_4 levels in thyroidectomised sheep fetuses (Fowden and Silver, 1995). In humans, the concentrations of thyroid-stimulating hormone are higher and those of T_4 are lower in hypoxic growth-retarded fetuses, suggesting possible nutritional regulation (Thorpe-Beeston and Nicolaides, 1993).

1.1.5.6 Placental lactogen

Placental lactogen (PL) is a GH homologue, that is secreted by the placenta into the maternal and fetal compartments, and which can bind to fetal GH receptors (Breier *et al.*, 1994b). It is thought to simulate fetal growth by directing maternal glucose to the fetus by increasing maternal insulin resistance (Ryan and Enns, 1988), and may increase maternal appetite (Min *et al.*, 1996). Fasting of pregnant sheep results in a decreased number of PL receptors in fetal and maternal liver, whereas glucose infusion increases the number of receptors in the fetal liver, suggesting regulation by glucose or insulin (Freemark *et al.*, 1992). Circulating fetal plasma PL levels are also influenced by maternal fasting (Oliver *et al.*, 1992).

1.1.5.7 C- type natriuretic peptide

C-type natriuretic peptide (CNP) is synthesised within a broad range of tissues including the vascular endothelium (Heublein *et al.*, 1992), brain (Minamino *et al.*, 1993), reproductive and skeletal tissues (Yasoda *et al.*, 1998) , where the hormone serves to regulate cell proliferation and hypertrophy (Furuya *et al.*, 1991). In keeping with its probable paracrine action, circulating concentrations of the bioactive forms, chiefly CNP-22, are barely detectable in health (Hunt *et al.*, 1994). However, the aminoterminal fragment of proCNP (NTproCNP) circulates at levels 10-50 fold higher than those of CNP (Prickett *et al.*, 2001). Plasma NTproCNP has been found to be strongly correlated with skeletal growth and markers of bone formation in children and lambs, consistent with the activity of the CNP signalling pathway in growth plate tissues (Prickett *et al.*, 2005) and the crucial role of the hormone in endochondral bone growth (Chusho *et al.*, 2001).

Recent studies in lambs have shown that reduced nutrition impacts on CNP synthesis and is similar to the effects of glucocorticoid synthesis (Prickett *et al.*, 2007). However, little is know about the importance of CNP in fetal growth and development.

1.2 Altered fetal growth and development – causes, adaptations and consequences

1.2.1 The Developmental Origins of Health and Disease hypothesis

The period from conception to birth is a time of rapid cell replication and differentiation, and functional organ maturation. It is also a time when the development of organs and endocrine axes are sensitive to the intrauterine environment. Permanent changes in metabolic and endocrine function following particular prenatal environments are thought to be related to the risk of metabolic diseases in adulthood, such as type 2 diabetes and cardiovascular disease (CVD) (Barker, 2004b). The theories relating fetal and early postnatal development to the risk of adulthood pathology have variously been known as the Barker hypothesis, the Fetal Origins of Adult Disease hypothesis, and the Developmental Origins of Health and Disease (DOHaD) hypothesis, which is the term I will use in this thesis.

1.2.1.1 Epidemiology

To investigate the paradox that ischaemic heart disease was more common in low socioeconomic areas despite its increasing prevalence being associated with increasing affluence, Barker and Osmond studied the relationship between infant mortality, childhood nutrition and ischaemic heart disease in England and Wales (Barker and Osmond, 1986). The study found a strong geographical relationship between ischaemic heart disease mortality rates around 1970 and infant mortality rates fifty years earlier. It also demonstrated similar relationships with mortality from bronchitis, stomach cancer, and rheumatic heart disease. Forsdahl had earlier demonstrated that a poor standard of living in childhood was a risk factor for cardiovascular disease after noting similar relationships in Norway (Forsdahl, 1977), and postulated nutritional deficit as a mechanism. Barker also proposed that prenatal and early postnatal nutrition was the link between childhood poverty and adult disease based on several premises (Barker and Osmond, 1986). Firstly, poor nutrition was a contributing factor to most of the major causes of neonatal mortality, which were respiratory tract infections, diarrhoea, and other infectious diseases. Secondly, variations in neonatal mortality rates depended on

variations in poverty (Woolf, 1996) and its possible effects on maternal nutrition. Thirdly, there was evidence that serum cholesterol levels differ according to type of feeding as an infant (Darmady *et al.*, 1972).

Further epidemiological studies published several years later demonstrated that systolic blood pressure in adulthood was inversely related to birth weight (Barker *et al.*, 1989a), which was used as an easily measurable marker of fetal growth and the intrauterine environment. Blood pressure at age 36 fell by 2.57mmHg for men and 1.83mmHg for women from the lowest to the highest birth weight group. Given that hypertension is a well-known risk factor for CVD, this was a possible mechanism linking early-life events with adulthood disease. Studies that linked birth weight and CVD also collected data on weight at one year of age (Barker *et al.*, 1989b). This produced a combination of results showing that men with the lowest birth weights and the lowest weights at one year had the highest CVD mortality rates, but those who gained weight in the first year had incrementally decreasing mortality rates. However, those who were light at birth never caught up with those who were heavier, either in weight or decreasing mortality rates.

The weight of a newborn baby is not an independent variable in itself, nor is it a specific marker of any particular prenatal factor. Babies with the same weight may have taken different growth trajectories to achieve that weight and may have different body compositions and physiques. Use of body proportions was an attempt to more specifically mark a particular pattern of intrauterine growth. Thinness, as measured by ponderal index (birthweight/length^3), and stunting at birth were associated with later CVD (Barker *et al.*, 1993b). Further studies in men born in Helsinki found that thinness at birth had a much stronger association with CVD than birth weight (Forsen *et al.*, 1997).

Just as birth weight is a crude, non-specific measure for a number of changes *in utero*, CVD mortality does not differentiate between many different risk factors. Well-known risk factors for CVD are hypertension, diabetes mellitus, dyslipidaemia, smoking and obesity. Independent of the length of gestation, low birth weight and thin babies, and also those with a small head circumference are at greater risk of developing a combination of hypertension, type 2 diabetes, and hyperlipidaemia (Barker *et al.*, 1993a).

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1.2.1.2 Biological basis of DOHaD

The basis of the link between prenatal conditions and long-term function is developmental plasticity - a limited but critical period of development of an organ during which time its structure or function may be permanently changed (Bateson *et al.*, 2004). This phenomenon has also been known as programming (Barker, 2004a). A classic example of developmental plasticity is sex determination by the presence or absence of testosterone in early development (Viger *et al.*, 2005). A number of stimuli have been proposed that may contribute to the developmental origins of disease including fetal glucocorticoid exposure (Seckl, 2001), maternal nutrition (Harding, 2001), multiple pregnancies (Jefferies *et al.*, 2003), and maternal postnatal behaviour (Meaney, 2001). This thesis will focus on maternal undernutrition and twinning as possible stimuli, and the fetal glucose-insulin and HPA axes as possible intermediary mechanisms, as long-term change in these axes could theoretically result in adult pathology.

Altered glucose-insulin axis function in the form of impaired insulin secretion and insulin resistance is the basis of type 2 diabetes, which is a known risk factor for vascular disease in adulthood. Insulin resistance is defined as a reduction in the biological response to insulin (Chisholm *et al.*, 1997) and is often present before the onset of type 2 diabetes. Type 2 diabetes does not develop until the pancreatic β cells can no longer secrete enough insulin to maintain euglycaemia. The 'thrifty phenotype' hypothesis proposes that poor intrauterine and childhood nutrition results in poor development of the pancreatic ß cells and insulin resistance. This would not be disadvantageous in a thin person in a low nutrition environment, but would result in type 2 diabetes in an obese person in a high nutrition environment (Hales and Barker, 2001). Aspects of ß cell development that may be affected by the prenatal environment include vascularity and innervation of the islets, islet morphology, hormone synthesis and stimulus-secretion coupling (Fowden and Hill, 2001). Possible mechanisms of insulin resistance that may be affected by prenatal environment include altered adipocyte sensitivity to cortisol and adipocyte GLUT-4 levels (Budge *et al.*, 2005), and altered insulin signalling pathways in muscle and fat (Fernandez-Twinn *et al.*, 2005).

Activity of the HPA axis during fetal life plays an important role in length of gestation (Challis and Brooks, 1989) and the development of the glucose-insulin axis (Breant *et al.*, 2006). However, permanent alterations in HPA axis function can also have important influences on adult metabolism. Cortisol has a wide range of metabolic and

endocrine actions, and the known symptoms of cortisol excess in adulthood – dyslipidaemia, hypertension and insulin resistance – also form the triumvirate of the metabolic syndrome. Human studies have shown birthweight associations with fasting cortisol levels (Phillips *et al.*, 1998), adrenal responsiveness to ACTH (Reynolds *et al.*, 2001), and pituitary response to stimulation tests (Ward *et al.*, 2004). Higher cortisol levels in adulthood are associated also with raised blood pressure, impaired glucose tolerance, insulin resistance, and raised serum triglyceride levels (Phillips *et al.*, 1998).

Other mechanisms under investigation that link maternal diet and postnatal pathology include altered nephrogenesis (Wintour *et al.*, 2003) and cardiomyocyte proliferation (Corstius *et al.*, 2005).

1.2.2 Maternal nutrition and the developmental origins of health and disease

Maternal nutrition can affect fetal and postnatal physiology, in addition to influencing fetal growth and birth size as discussed earlier. These effects depend on the type, timing and duration of the nutritional insult.

1.2.2.1 Animal studies

A low protein diet throughout pregnancy in rats results in increased blood pressure (Langley and Jackson, 1994) and impaired glucose tolerance (Dahri *et al.*, 1991) in the offspring. A 35% overall reduction in intake in rats in late pregnancy causes fetal ß cell hypertrophy and increased insulin response to glucose *in vitro*, but not if the undernutrition occurred in early pregnancy (Alvarez *et al.*, 1997). In guinea pigs, a two day fast in late gestation changes fetal hypothalamic GR expression (Lingas *et al.*, 1999), and moderate undernutrition throughout pregnancy results in insulin resistance (Kind *et al.*, 2003).

In contrast to studies in small mammals with short gestations, studies in sheep allow investigation of discrete episodes of undernutrition in early, mid or late gestation. In addition to decreasing birthweight in sheep (Luther *et al.*, 2005), late-gestation maternal undernutrition alters fetal somatotrophic axis regulation (Bauer *et al.*, 1995) and pituitary and adrenal responses to hypoglycaemia (Edwards *et al.*, 2001). In the offspring, there are alterations in HPA axis function following 10 d of late-gestation undernutrition (Bloomfield *et al.*, 2003a).

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It is possible that undernutrition throughout pregnancy or in mid to late gestation may limit substrates for organ development and therefore lead to long-term changes. However, there is an increasing body of literature demonstrating that nutrition in early pregnancy also has long term effects, which, due to the small nutrient requirements at that time, cannot be due to limitation of fetal substrate supply for tissue synthesis. In sheep, undernutrition by 50% over the first 30 d of pregnancy results in offspring with cardiovascular dysfunction (Gardner *et al.*, 2004b) and increased cortisol levels in female offspring (Gardner *et al.*, 2006). Undernutrition by 15% for the first 70 d produces offspring with increased blood pressure and increased ACTH and cortisol responses to CRH/AVP (Hawkins *et al.*, 2000a). Undernutrition by 50% until 95 d alters aspects of cardiovascular control and adipocyte function in adult sheep (Gopalakrishnan *et al.*, 2004).

There are also several sheep undernutrition studies that extend across the period of conception. In ewes that were fed at 70% of controls from 45 d before until 7 d after mating, a direct relationship existed between weight change over the periconceptional period and fetal weight at 53-56 d gestation in control ewes, but not in undernourished ewes (MacLaughlin *et al.*, 2005). There was also loss of the relationship between adrenal IGF-2, IGF-2 receptor expression and adrenal weight (MacLaughlin *et al.*, 2007). Undernutrition from 60 d before until 7 d after mating had no effect on tests of HPA axis function (Edwards and McMillen, 2002) or leptin levels (Edwards *et al.*, 2005) in singleton fetuses in late gestation.

However, previous studies from Professor Harding's group at the Liggins Institute have used an approach to undernutrition in which feed intake is individually adjusted so the sheep lose 10-15% bodyweight (Harding, 1997a). This approach guarantees that the individual sheep is undernourished, whereas the studies described above used a recommended intake that does not take into account individual variations in metabolic rate. The studies from the Liggins Institute found that fetuses of ewes undernourished from 60 d before until 30 d after mating have a normal birthweight but a slower growth trajectory prior to parturition (Oliver *et al.*, 2005). They also have a greater insulin response to glucose (Oliver *et al.*, 2005) and an increased ACTH response to metyrapone (Bloomfield *et al.*, 2004) which suggests altered cortisol feedback mechanisms.

Fetal sheep studies also provide the ability to link patterns of fetal growth with postnatal physiology. The offspring of sheep undernourished for 20 d in late gestation have reduced size at birth, but display catch-up growth in the postnatal period (Oliver *et al.*, 2001a). They also have altered body composition as adults, but no change in HPA axis

function. However, 10 d of late gestation undernutrition results in normal birth size but increased ACTH response in the offspring to a CRH/AVP challenge and an insulin challenge (Bloomfield *et al.*, 2003a). This suggests that the changes in HPA axis function are linked to growth trajectory rather than birth size.

These animal studies demonstrate the developmental plasticity of important endocrine axes and the biological plausibility of a link between prenatal maternal nutrition and adult physiology.

1.2.2.2 Human evidence

In addition to the effects on birth size previously discussed, the famine of the Dutch Hunger Winter had a variety of long-term effects on adult physiology and pathology. Exposure to the famine in early gestation *in utero* resulted in increased BMI in women in adulthood (Ravelli *et al.*, 1999), decreased insulin secretion (de Rooij *et al.*, 2006a), altered coagulation profile (Roseboom *et al.*, 2000c) an atherogenic lipid profile (Roseboom *et al.*, 2000a) and increased incidence of cardiovascular disease (Roseboom *et al.*, 2000b). Mid gestation exposure resulted in increased rates of obstructive airway disease (Lopuhaa *et al.*, 2000), while mid and late gestation exposure resulted in decreased glucose tolerance (Ravelli *et al.*, 1998). Although there was no relationship between exposure to famine and adult blood pressure (Roseboom *et al.*, 1999), an increasing ratio of protein to carbohydrate in the maternal diet in the third trimester resulted in decreasing blood pressure in the offspring (Roseboom *et al.*, 2001b).

These results demonstrate that in humans the timing and nature of a prenatal nutritional insult determine its long-term effects.

1.2.2.3 Possible mechanisms

The effects of periconceptional undernutrition on the late-gestation fetus may be mediated through changes in the mother, placenta and/or fetus.

1.2.2.3.1 Maternal

Appropriate maternal cardiovascular adaptations are important for the success of pregnancy. In normal human pregnancies blood volume increases by approximately 45%, beginning to rise at 6-8 weeks, and reaching maximum at around 30 weeks (Bocking, 1994). In sheep the increase is only approximately 7% (Metcalfe and Parer, 1966).

Evidence that maternal cardiovascular adaptations to pregnancy are associated with changes in fetal growth comes from a small study in which echocardiographic parameters of left atrial diameter and inferior vena cava diameter were lower in early gestation in women who subsequently had growth-retarded babies (Duvekot *et al.*, 1995). Those women also had lower serum sodium concentrations, and smaller falls in serum creatinine and urea in early gestation compared to the women who subsequently had babies of normal weight. The authors concluded that fetal growth restriction is preceded by defective volume adaptation in early pregnancy. Further support for this idea is provided by an earlier study that found a correlation of 0.54 between birth weight and the rise in maternal plasma volume during pregnancy (Gibson, 1973).

The effect of maternal nutrition on blood volume adaptation has also been demonstrated in sheep. Undernutrition from 28 to 80 d gestation resulted in lowered angiotensinogen levels and plasma volumes during the time of restriction, but the differences disappeared after the restriction ended (Dandrea *et al.*, 2002). In rats receiving only 50% of dietary requirements during pregnancy, plasma renin activity was increased but sodium and water retention were significantly lower than controls (Leizea *et al.*, 1999).

Maternal endocrine adaptations to pregnancy include the development of insulin resistance, which may result from increasing levels of progesterone, cortisol and PL in pregnancy (Ryan and Enns, 1988). This insulin resistance increases the availability of nutrients for the placenta and fetus. Ewes undernourished from 60 d before until 30 d after mating had higher plasma glucose, urea, insulin and prolactin levels in late gestation than controls (Oliver *et al.*, 2005) suggesting increased insulin resistance. However, a recent study has shown that ewes undernourished around conception are less insulin resistant than controls in mid gestation, but that there is no difference in late gestation (Jaquiery *et al.*, 2005).

It has also been suggested that the effects of maternal undernutrition on fetal development could be due to stress-induced elevation of maternal plasma cortisol levels that subsequently cross the placenta to the fetus. However, cortisol levels are actually profoundly depressed during chronic undernutrition during pregnancy in sheep (Jaquiery *et al.*, 2006), making this explanation less likely.

1.2.2.3.2 Placental

In addition to the changes in placental size following altered maternal nutrition discussed earlier, there may be changes in structure and function that are not reflected in

morphology. The placenta of guinea pigs undernourished throughout pregnancy have reduced exchange surface area and increased barrier thickness for diffusion (Roberts *et al.*, 2001), while those from guinea pigs undernourished by 40% in mid gestation had decreased villous surface area (Dwyer *et al.*, 1992). Periconceptional undernutrition from 45 d before until 7 d after mating in sheep does not change placental histology and structure at 53-56 d gestation (MacLaughlin *et al.*, 2005), but undernutrition over the first half of pregnancy changes placental morphology (Kalache *et al.*, 2001). One theory linking placental structural changes and long-term development proposes that increased resistance to blood flow in the umbilical artery subjects the fetal heart to increased workload and permanent remodelling (Thornburg and Louey, 2005).

Undernutrition is also known to affect specific placental transport proteins. GLUT-1 levels in the sheep placenta are highest when there is mid gestation undernutrition followed by normal intake in late gestation (Dandrea *et al.*, 2001), while in rats late gestation undernutrition decreased placental GLUT-3 expression, but did not affect GLUT-1 or -4 (Lesage *et al.*, 2002). Amino acid transport across the sheep placenta is affected by maternal amino acid profile (Jozwik *et al.*, 2001).

The recognition that maternal diet can affect placental 11βHSD-2 activity, has resulted in a theory that the effects of a maternal nutritional insult may be mediated through fetal exposure to excess glucocorticoids (Langley-Evans *et al.*, 1995). It has subsequently been demonstrated in sheep that the 11ßHSD-2 enzyme expression in the placenta and fetal weight are both decreased following maternal glucocorticoid administration (Kerzner *et al.*, 2002). Chronic nutrient restriction by 30% from 26 d gestation onwards in sheep reduces placental 11ßHSD-2 activity in late gestation, but there is no effect if the nutritional restriction is limited to early, mid or late gestation (McMullen *et al.*, 2004). Such decreases in enzyme activity could potentially expose the fetus to excess glucocorticoids and their subsequent effects. It is not yet known how periconceptional undernutrition affects placental 11ßHSD-2 activity.

1.2.2.3.3 Fetal

Prior to initiation of placental blood flow, the oviductal and uterine fluid provides nutrition for the developing embryo. Both the levels of nutrients and maternal hormones may directly affect embryonic development at this time, with possible long-term effects.

Epigenetic modification of the fetal genome has been proposed as a mechanism linking maternal nutrition with altered fetal and postnatal development. The effects of

environment can be demonstrated by altered methylation status following *in vivo* growth of mouse embryos in an amino-acid deficient culture medium (Doherty *et al.*, 2000). Direct evidence of the effects of maternal diet on fetal epigenetic status has been obtained in the agouti mouse in which supplementation of the maternal diet with methyl groups alters fetal development in the direction of the leaner pseudo-agouti phenotype (Wolff *et al.*, 1998). In rats, a low protein diet throughout pregnancy resulted in decreased methylation of the GR and peroxisomal proliferator-activated receptor (PPAR) genes in the offspring, but the changes could be prevented by folate supplementation (Lillycrop *et al.*, 2005). Although the effect of maternal nutrition on altered epigenetic modification may be directly due to a deficiency of methyl donors (Waterland and Jirtle, 2004), the methylation status of some imprinted genes can be affected by glucocorticoid levels (Thomassin *et al.*, 2001), which in turn may be affected by maternal nutrition (Jaquiery *et al.*, 2006).

The altered metabolic and endocrine milieu of the fallopian tube and uterus resulting from altered maternal nutrition can also affect early embryonic development. The altered fetal and placental growth following *in vitro* fertilisation in cows (Bertolini *et al.*, 2002) and sheep (Holm *et al.*, 1996) illustrates the effects of early environment. Undernutrition in pigs alters maternal steroid and prostaglandin levels, and results in lower cleavage rates of the embryos (Mburu *et al.*, 1998), while a low protein diet in rats in the preimplantation period alters allocation of the blastocyst cells to the inner cell mass and trophectoderm (Kwong *et al.*, 2000). The long-term fetal effects of altered periconceptional hormonal milieu have been demonstrated in sheep by increased fetal and placental growth following a short period of progesterone administration around conception (Kleemann *et al.*, 2001).

One theory linking altered maternal nutrition and fetal development, as described earlier, is that the nutritional insult results in exposure of the fetus to excess glucocorticoids, whether through high maternal levels and/or a deficient placental barrier. Although maternal administration of synthetic glucocorticoids that cross the placenta has been used to model fetal glucocorticoid exposure, they are not a perfect analogy for fetal hypercortisolaemia. Not only do they alter placental endocrinology as previously discussed, but effects differ between different synthetic glucocorticoids (Derks *et al.*, 1997) and between maternal and fetal administration (Sloboda *et al.*, 2002).

Birthweights in humans and rats are decreased following antenatal maternal steroid administration (Reinisch *et al.*, 1978), and offspring of pregnant rats treated with

dexamethasone in the last trimester of pregnancy also have raised blood pressure (Levitt *et al.*, 1996) and altered glucose-insulin axis function in adulthood (Nyirenda *et al.*, 1998). The link between maternal diet, glucocorticoid exposure and offspring physiology has been shown by a rat study in which the hypertension in the offspring induced by a maternal low-protein diet was dependent on an intact maternal adrenal gland (Gardner *et al.*, 1997). If the maternal adrenal was removed, blood pressure of the offspring was similar to that of controls, whereas maternal corticosterone replacement restored the hypertension of the offspring. Two other sets of evidence support the idea that it is fetal overexposure to maternal glucocorticoids that is crucial to the programming of the HPA axis. The first is that inhibition of 11βHSD by carbenoxolone results in reduced birth weight and elevated offspring blood pressure (Lindsay *et al.*, 1996). The second is that blockage of maternal glucocorticoid synthesis by metyrapone eliminates the effects of a low-protein diet on birth weight and blood pressure (Langley-Evans, 1997).

The actual mechanisms by which excess fetal glucocorticoid exposure affects long-term physiology are not yet clear, but may involve altered fetal GR expression (Levitt *et al.*, 1996) and glucose-insulin axis function (Breant *et al.*, 2006).

1.2.3 Twinning and the developmental origins of health and disease

Human twins are born earlier and are lighter for their gestational age than singletons (Kiely, 1998). There is a similar growth discrepancy in twin pregnancies in sheep (Bloomfield *et al.*, 2007), but gestation length does not appear to be shortened (Ozturk and Aktas, 1996).

1.2.3.1 Causes of growth restriction

The altered growth and development of twin fetuses may result from several factors. Firstly, it is possible that fetal growth rate is set in early gestation in twin fetuses. In contrast to the late-gestation limitation of growth that results in growth-restricted singletons, twin growth appears to be slowed as early as 16 weeks gestation in humans (Farina *et al.*, 1999; Leveno *et al.*, 1979). However, there are clearly both early and late gestation effects on growth also, as human triplets reduced to twins at 8-11 weeks are heavier at birth than the non-reduced triplets (Boulot *et al.*, 2000), but not as heavy as non-reduced twins (Sebire *et al.*, 1997). The probability of twinning in sheep is affected by

maternal nutrition and weight at the time of conception (Kenyon *et al.*, 2004), but it is not known if there are differences in hormone and metabolite levels that may affect very early blastocyst development.

Secondly, the placentas of twin fetuses are smaller than singletons, although the total placental mass in a twin pregnancy is higher. This may limit growth by limiting nutrient transfer capacity.

Thirdly, maternal provision of nutrients may be lower per fetus in twin than in singleton pregnancies. Maternal metabolic rate is higher in twin pregnancies in humans (Shinagawa *et al.*, 2005) and they are more vulnerable to the accelerated starvation of late pregnancy (Casele *et al.*, 1996).

It is important to understand that the disparity in weight between twins of a pair probably arises for different reasons than the lower average weight of twins overall. Weight disparity within a pair must arise from feto-placental mechanisms including such factors as mix of fetal sexes (vom Saal, 1989), whereas the lower average weight in twins will have some component of maternal factors that limit nutrient availability.

1.2.3.2 Adaptations to twinning

There are a number of maternal adaptations in twin pregnancies to optimise fetal growth despite these limitations. In twin pregnancies, the mother typically gains more weight, which is related to birth size (Lantz *et al.*, 1996) and there is a greater increase in BV than in singleton pregnancies (Hytten, 1985). In addition, the fetal to placental weight ratio is higher in twin sheep pregnancies (Symonds *et al.*, 2000), suggesting improved nutrient transfer efficiency and/or fetal growth at the expense of placental growth.

1.2.3.3 Consequences of twinning

Despite the strong evidence linking smaller birth size with increased risk of long-term pathology, these relationships are much less clear when the smaller birth size is due to twinning. It is important to distinguish between the overall lower birth weight associated with being a twin, and the variation within a twin cohort or within a twin pair. Overall, human twins do not have increased long-term mortality compared with the general population (Christensen *et al.*, 1995; Vagero and Leon, 1994), but there is little literature on similar comparisons for chronic diseases such as high blood pressure and

insulin resistance in adulthood. There is limited evidence that the prevalence of type 2 diabetes is higher in adult twins (Poulsen *et al.*, 1999), but that particular study was not designed to compare twins and singletons. In prepubescent children, however, twinning was associated with insulin resistance (Jefferies *et al.*, 2004) and increased blood pressure (Jefferies *et al.*, 2003) independent of birth weight, weight disparity within a pair, and prematurity. Within a cohort of twins there are inverse associations between birthweight and blood pressure in childhood (Dwyer *et al.*, 1999) and adulthood (Iliadou *et al.*, 2004; Loos *et al.*, 2001). Cortisol responses to stress are also inversely related to birthweight (Wust *et al.*, 2005). Studies that have analysed the relationships between the heavy and light twins within a twin pair show that abnormal glucose tolerance (Bo *et al.*, 2000; Poulsen *et al.*, 1997) and high blood pressure (Poulter *et al.*, 1999) are more common in the lighter twin, but other studies do not find these relationships (Baird *et al.*, 2001). More complex analyses of the between-twin pair and within-twin pair associations with blood pressure suggest that shared maternal factors and individual feto-placental factors both impact on the inverse association between birth weight and blood pressure (Morley and Dwyer, 2005).

Thus it is not clear if the reduction in birth weight that comes from being a twin is related to long-term pathology, but it appears that the growth discrepancy between twins in a pair and the variation across the normal distribution of birthweight are related to postnatal pathology, as in singletons. It has been suggested that the lack of a clear association between twinning and long-term pathology is due to a fundamentally different physiology of development in twin compared with singleton pregnancies, so that the causes of low birthweight are different (Phillips *et al.*, 2001).

Sheep studies are beginning to demonstrate that this is the case. For example, twin sheep fetuses have been shown to have altered HPA axis function in late gestation (Edwards and McMillen, 2002; Gardner *et al.*, 2004a) and in postnatal life (Bloomfield *et al.*, 2007), with decreased relative adrenal weight and expression of IGF-1, IGF-1 receptor, IGF-2, IGF-2 receptor and $P450_{c17}$ at 53-56 d gestation (MacLaughlin *et al.*, 2007). They also have decreased expression of mRNA for receptors of the cortisol, GH and IGF-1 and -2 compared with singletons at 110 d following maternal undernutrition (Brennan *et al.*, 2005).

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1.3 Aims of this thesis

This thesis describes studies designed to further our understanding of the timing and nature of prenatal insults that may result in long-term changes in physiology, by studying periconceptionally undernourished singleton and twin sheep pregnancies.

The aim of the first experiment, described in chapter 3, was to describe the effects of singleton pregnancy on blood volume parameters and uterine blood flow in sheep, and to test the hypothesis that the effects of periconceptional undernutrition on the late-gestation sheep fetus are mediated through altered maternal BV and uterine blood flow. The study described in chapter 4 was designed to test the hypothesis that pre- and postconception maternal undernutrition in singleton sheep pregnancies would have different effects on fetal growth, physiology and endocrine status in late gestation, and in particular on the fetal glucose-insulin and HPA axes.

The aim of the experiment described in chapters 5 and 6 was to test the hypotheses that the late-gestation growth, physiology and endocrine status of twin fetuses is different to that of singletons, and that there is an interaction between twinning and periconceptional undernutrition in their effects on the late-gestation fetus. Chapter 5 describes the effects of twinning and periconceptional undernutrition on maternal and fetal growth, metabolism and glucose-insulin axis function, while chapter 6 focuses on the maternal and fetal HPA axes.

The aim of the experiment described in chapter 7 was to test the hypothesis that the regulation of plasma CNP and NTproCNP levels is independent in the mother and fetus during pregnancy, and is affected by nutrition. This experiment was performed in collaboration with Professor Eric Espiner's endocrine research group in Christchurch.

2. Materials and Methods

2.1 Animals

Ethical approval for the study was obtained from the University of Auckland Animal Ethics Committee. Romney ewes from the Ngapouri Research Farm were housed in a purpose-built facility in which both individual and group pens were available. The pens contained a water trough and feed bucket, and all sheep were within sight of other sheep. After acclimatisation to a concentrate feed consisting of 65% lucerne, 30% barley and limestone, molasses and trace elements (CamTech, Cambridge, New Zealand), the ewes were randomly allocated to one of four nutritional groups at 61 d before mating. A fortnight before mating with Dorset rams, the oestrous cycle of all ewes was synchronised (Wheaton *et al.*, 1993). Pregnancy and fetal number was established by ultrasound scanning at 55 d gestation. At 105 d the ewes were transported to the Animal Research Unit (ARU) at the Faculty of Medical and Health Science in Auckland, where they were housed in the sheep laboratory, which was climate-controlled with a temperature of 16ºC and a light cycle from 0530 to 1900 hours.

2.2 Nutrition

The four nutritional groups were:

Control
Undernutrition from 60 d before until mating (UN -60-0)
Undernutrition from 2 d before mating until 30 d after mating (UN -2-30)
Undernutrition from 60 d before until 30 d after mating (UN -60-30)

Control ewes were fed concentrate feeds at 3-4% of body weight per day. Nutritional management in the undernourished groups consisted of a 2 d fast followed by individually adjusted intake of concentrate feeds to achieve and then maintain a maternal body weight reduction of 10-15%. Ewes were housed in individual pens during undernutrition and group pens at other times, and weighed twice weekly. Following the period of undernutrition all ewes were fed maintenance feeds.

2.3 Surgery

2.3.1 Catheters

Vascular catheters were made by joining lengths of vinyl tubing (Critchley Electrical Products Pty Ltd., Australia) with a length of vinyl tubing of a slightly wider bore. For the thin-walled utero-ovarian vein catheters, however, silastic tubing (Silclear, New Milton, UK) was used for the intravascular portion. Cyclohexanone (Scientific Supplies, Auckland) was used to join vinyl catheters together, and xylene used to join silastic and vinyl. Two rings of vinyl tubing (#sv110: Internal diameter (ID) 0.025 in, outer diameter (OD) 0.05 in) were secured with cyclohexanone over the outer tubing above the junction to form a cuff around which the catheter could be secured with ties.

Fetal femoral artery and vein: A 0.15 m length of #sv55 vinyl tubing (ID 0.025 in, OD 0.05 in) was inserted into a 1 m length of #sv74 tubing (ID 0.040 in, OD 0.10 in).

Utero-ovarian vein: A 30 cm length of silastic tubing (ID 0.025 in, OD 0.050 in) was secured to #sv55 vinyl tubing.

Maternal artery and vein: Two rings of #sv110 were placed either 40 cm (for femoral catheters) or 20 cm (for carotid artery and jugular vein catheters) from the end of a 1.5 m length of #sv74 vinyl tubing.

Amniotic catheters: A 1.5 m length of #sv110 vinyl tubing had four holes cut in the distal 4 cm of the tube. At surgery, this end was tied into a perforated plastic ball.

Growth catheters: Two rings of #sv74 vinyl tubing were secured to a 1.5 m length of polyethylene tubing (ID 0.040 in, OD 0.060 in) with Sico-met primer and glue (Adhesives Australia Ltd, Auckland). When the glue was dry, a 2 m length of either red or green nylon fishing line was inserted into the tubing.

2.3.2 Animal preparation and anaesthesia

After the ewes had acclimatised to the laboratory for 5 d, surgery was performed between day 110 and 112 under aseptic conditions. Ewes were starved for 24 h before surgery, but had free access to water. Anaesthesia was induced with alfaxalone (Alfaxan-

CD RTU, 0.35 ml/kg, Jurox Pty Ltd, Rutherford, NSW, Australia) and maintained with halothane or isoflurane following intubation. Antibiotics were given to ewes before surgery (5 mL Streptopen, Pitman-Moore, Wellington, New Zealand). The ewe's abdomen was shorn and sterilised with iodine and chlorhexidine washes, and then covered with sterile drapes. A midline incision was made, and the presence of single or twin fetuses confirmed. In the case of twins, the twin in the right horn was designated 'twin A', and left horn as 'twin B'. The catheters were flushed with sterile 0.9% saline and introduced into the maternal abdomen via a trocar in the ewe's flank.

2.3.3 Catheterisation

Catheterisation was performed through a single uterine incision for each fetus.

2.3.3.1 Fetal femoral vessels:

Each hind limb was exteriorised and the hock-toe and hind limb lengths measured. The catheters were trimmed to hock-toe length, and inserted into the tarsal artery and vein. When fully advanced, they were secured with silk ties to the vessel, and the skin closed with 2-0 silk. If insertion into the tarsal vessel failed, the catheter was trimmed and inserted into the femoral vessel at the femoral triangle.

2.3.3.2 Chest girth growth catheters:

The front half of the fetus was exteriorised and the biparietal diameter measured. Incisions through the skin were made over the spine below the level of the scapula, and over the lower aspect of the sternum. The coloured nylon thread from each growth catheter was inserted subcutaneously around each half of the fetal chest using a large blunt needle. The thread was sutured to the sternum and the outer polyethylene tubing tied to the spine using 2-0 silk ties around the cuffs. If there were twins, the sutures of twin A were left short and those of twin B left long for identification at postmortem. The tubing was filled with saline to provide lubrication, the nylon line trimmed and the catheter plugged. The distance between the end of the plug and the end of the coloured line was measured twice daily and the mean recorded.

2.3.3.3 Amniotic catheters

The vinyl tubing of amniotic catheter was tied into the perforated plastic ball, which was then placed near the head of the fetus.

2.3.3.4 Utero-ovarian vein catheters:

After closure of the uterus, the utero-ovarian vein catheter was inserted 24 cm in a vein draining the pregnant uterine horn. The tip lay in the main utero-ovarian vein. The catheter was secured to the vein and to the tip of the horn to minimise the possibility of kinking.

2.3.3.5 Maternal artery and venous catheters:

After closure of the abdomen, catheters were introduced into the carotid artery and jugular vein, and tarsal artery and vein. The tarsal catheters were tunnelled subcutaneously from the upper thigh to above the knee, and inserted into the vessels.

2.3.4 Closure

Lost amniotic fluid was replaced with warm sterile normal saline. Gentamicin (80 mg, Roussel, Auckland, New Zealand) was also added to the amniotic fluid before the uterus was closed in two layers with 2-0 silk. The maternal abdomen was closed with umbilical tape to the peritoneum and aponeurosis, and 2-0 silk to the skin. Ten mL of bupivacaine was injected subcutaneously along the wound for analgesia. All catheters were flushed with heparinised saline and capped. The trocar wound on the flank where the catheters emerged was closed with a purse string suture.

2.3.5 Postoperative care

Catheters were flushed with heparinised saline daily for three days following surgery, and thereafter twice weekly. Fetal and maternal blood gases were checked on a Bayer Rapidlab 845 blood gas analyser (Bayer AG, Leverkusen, Germany), and blood glucose and lactate levels measured with a YSI 2300 analyser (Yellow Springs Instruments, Dayton, OH, USA). Growth catheters were measured twice daily and the results averaged. Ewes were fed a measured amount of concentrate feed in the morning with the aim of having a small residual the next day, and had free access to water.

2.4 Experimental Procedures

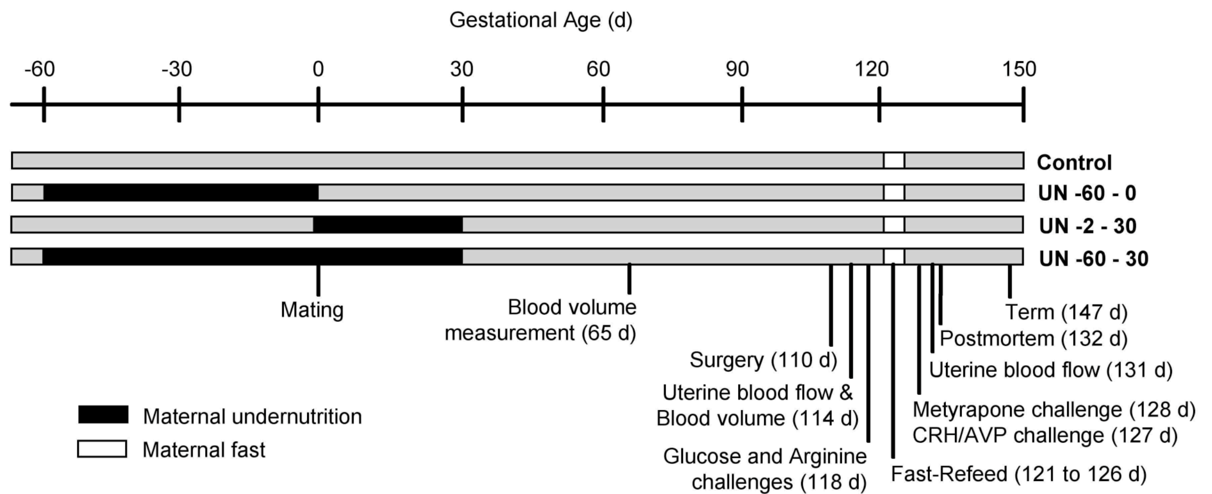

Figure 2-1: Timeline of experimental protocol

2.4.1 Sample collection

From 61 d before mating until transport to the ARU at 105 d, maternal blood samples were taken at regular intervals by jugular puncture and collected into a sodium heparin tube (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ, USA). In the ARU post-surgery, blood and amniotic fluid samples were collected into sterile syringes that had been flushed with heparinised saline, and placed on ice. Blood samples for CNP were drawn into chilled tubes containing EDTA (Vacutainer, Becton and Dickinson). Whole blood taken for antipyrine analysis was frozen immediately in frozen gel blocks. Other samples were centrifuged at 4ºC and 3000 rpm for 15 min, and the plasma stored at -80ºC until assay.

2.4.2 Uterine blood flow measurement

Uterine blood flow measurements were performed at 114 and 131 d. 120 mg antipyrine was dissolved in 20 mL sterile saline and infused into a fetal vein at 4 $mL.hr^{-1}$ following a 5 mL bolus. After 90 min to reach steady state, sets of blood samples were collected simultaneously from the maternal artery and the utero-ovarian vein every 15 min for 1 h, and immediately frozen. Antipyrine concentration was measured by high-performance liquid chromatography. Uterine blood flow was calculated from steady-state

antipyrine concentrations as previously described (Bloomfield *et al.*, 2002; Meschia *et al.*, 1967).

$$\text{Uterine blood flow} = R_I/([UOV]–[MA])$$

where R_I is the rate of infusion of antipyrine (which approximates to the rate of uptake by the uterine circulation) and [UOV] and [MA] are the concentrations of antipyrine in the utero-ovarian vein and maternal artery respectively.

2.4.3 Glucose and arginine challenges

At 118 d fetal glucose and arginine challenges were performed. Ewes were fasted overnight prior to the challenges. After a baseline blood sample at 0800, 1.5 g glucose (3 mL 50% dextrose) was given to a fetal vein, and arterial blood samples were taken at 2, 5, 10, 15, 30, 45 and 60 min. Four hours after the conclusion of the glucose challenges, by which time glucose and insulin had returned to baseline, an arginine challenge was performed. Arginine (Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile normal saline at a concentration of 100 $mg.mL^{-1}$, and passed through a 0.2 µm filter. After a baseline blood sample, 3 mL (300 mg arginine) was given to a fetal vein and arterial blood samples were taken at 2, 5, 10, 15, 30, 45 and 60 min. Challenges were performed simultaneously in twin fetuses. Ewes were allowed free access to water throughout the experiment and refed at the conclusion of the arginine challenge.

2.4.4 Maternal fast and refeed

Ewes were fasted from 121 to 124 d with free access to water, and maternal and fetal blood samples were taken daily. At 124 d ewes were refed and also given an intravenous glucose infusion of 25 g over 8 h, aiming to restore maternal and hence fetal blood glucose as rapidly as possible in a ‘square wave’ pattern. Further blood samples were collected 2, 4, 6 and 8 h after the start of the glucose infusion, and at 24 and 48 h of refeeding.

2.4.5 CRH/AVP challenge

At 127 d at 0900 a fetal pituitary stimulation challenge was performed using 2.0 µg of bovine corticotropin-releasing hormone (CRH) (Sigma Chemical Co, St Louis, MO, USA) in 0.2 mL sterile saline and 0.4 µg arginine vasopressin (AVP) (Sigma Chemical Co) in 0.2 mL sterile saline. After baseline fetal blood samples at -15 and 0 min, the combined solution was given to a fetal vein, and arterial samples taken at 15, 30, 45, 60, 120 and 240 min.

2.4.6 Metyrapone challenge

At 128 d at 0900 a metyrapone challenge was performed on the fetuses. Metyrapone (Novartis Pharma, Basel, Switzerland) was dissolved in sterile normal saline at a concentration of 20 $mg.mL^{-1}$, and passed through a 0.2 μm filter. After baseline blood samples at -30 and 0 min, 3 mL of solution (60 mg metyrapone) was given to a fetal vein and arterial blood samples were taken at 30, 60 and 120 min.

2.4.7 Postmortem

On completion of the studies the ewes were killed with an overdose of pentobarbitone (25 mL of 300 $mg.mL^{-1}$). The uterus was removed and placed in a container that had been tared on the scales with two incontinence sheets. For singleton pregnancies, the fetus and uterus were dried with the sheets, which were weighed with the amniotic fluid. The following measurements were taken of the fetus: weight, forelimb and hindlimb lengths, chest girth, abdominal girth, crown-rump length, and biparietal diameter. In addition, the lengths of growth catheters from sternum attachment to spine were measured. The fetal organs were then rapidly dissected out and weighed, with tissues collected for histology, molecular biology and *in situ* studies. The placenta was dissected out and individual placentomes weighed and categorised according to Vatnick (Vatnick *et al.*, 1991). The uterus and membranes were separated and weighed.

2.5 Development of a method for estimation of blood volume parameters in pregnant sheep

2.5.1 Introduction

Measurement of blood volume (BV) parameters is rarely undertaken in a clinical setting except in specific situations such as investigation of polycythemia. This is despite the importance of appropriate intravascular volume management in surgical and unwell medical patients, plasma volume changes in heart failure (Kalra *et al.*, 2002) and the association between inadequate blood volume adaptation in pregnancy and both fetal growth restriction (Duvekot *et al.*, 1995) and pre-eclampsia (Silver *et al.*, 1998). Techniques such as echocardiography, clinical examination, urine output, central venous pressure and pulmonary arterial wedge pressure measurement provide surrogate estimates of intravascular filling and end-organ perfusion, but may be subjective, highly invasive or limited to intensive care units. However, more direct measurements of blood volume

parameters have been beset by inaccuracy, cost and complexity that have limited their application.

Several techniques exist to measure these parameters, most of which measure the dilution of a substance that is limited to the space of interest. The gold standard for estimating red cell volume (RCV) involves measuring the dilution of ^{51}Cr-labelled autologous red blood cells (International Committee for Standardization in Haematology, 1980), but the equivalent method for plasma volume (PV), measuring the dilution of ^{125}I-labelled albumin, actually measures an 'albumin space', which may overestimate PV (Hunyor *et al.*, 1983; Massey *et al.*, 2004). Albumin-labelling dyes such as Evans Blue and indocyanine green are prone to additional problems of dye extravasation, incomplete binding, and assay difficulties if plasma is turbid, as in pregnancy (Brown *et al.*, 1992). Several methods have been developed to estimate PV by measuring the dilution of large inert molecules. A proprietary fluorescein-labelled hydroxyethyl starch was found to estimate PV more accurately than radiolabelled albumin, with the overestimate of PV by albumin exaggerated in intensive care patients (Massey *et al.*, 2004). PV and BV were estimated in yellowfin tuna using a commercially available fluorescein-labelled dextran, and did not differ significantly from BV measured using radiolabelled red cells (Brill *et al.*, 1998). However PV estimated in pregnant women using a 70 kDa dextran was approximately 50 ml greater than results obtained using ^{125}I-labelled albumin (van Kreel *et al.*, 1998).

We wished to assess the blood volume parameters of pregnant sheep in response to changes in nutrition before and during pregnancy. A simple, non-radioactive method was required to allow assessment both in a laboratory and in the field. We chose to use a commercially available 250 kDa fluorescein-labelled dextran. Dextran is a polymer of anhydroglucose and relatively biologically inert. Dextrans labelled with fluorescein via thiocarbamoyl binding are available commercially in a variety of molecular weights. The carbamoyl-dextran linkage has been shown to be stable *in vivo* in rabbits, with no free fluorescein detectable (Schroder *et al.*, 1976). The elimination of dextran is dependent on its molecular weight, with larger molecules having longer half-lives (Mehvar and Shepard, 1992). Larger molecules (> 20 kDa) are predominantly eliminated from the plasma via the liver, whereas smaller molecules (< 20 kDa) have a significant renal elimination (Mehvar *et al.*, 1994).

2.5.2 Methods

2.5.2.1 Fluorescein isothiocyanate-dextran (FITC-dextran) preparation

250 kDa FITC-dextran (FD250S) was obtained from Sigma (St Louis, MO, USA). The FD250S was dissolved in sterile 0.9% saline at a concentration of 4 $mg.mL^{-1}$, passed through a 0.22 μm filter, then 11 mL aliquots placed in foil-wrapped glass bottles which were stored at -80°C until use. Exposure to light was minimised during preparation. Separate aliquots of 1 mL were also frozen for use in making the standard curve for the assay.

2.5.2.2 Sheep experiments using FD250S

The frozen FD250S solution was allowed to thaw overnight in the refrigerator, and then passed through a 0.22 μm filter into a sterile syringe and the level marked. After a baseline arterial blood sample of 15 mL, the FD250S solution was given over 20 sec into a jugular venous catheter, the syringe flushed with saline until clear, and 4 mL arterial blood samples taken every 10 min for 1 h. Blood samples were stored on ice during the experiment, then centrifuged at 2500 g for 10 min at 4°C and plasma stored at -80°C. The weight of the empty syringe was subtracted from the weight when filled to the mark with water to determine the exact amount of solution injected (I). In some experiments maternal and fetal blood, and amniotic fluid were also sampled up to 12 h after injection. Haematocrit was measured in duplicate on the baseline and 10 min samples using capillary tubes in a microfuge (12000 rpm for 5 min), with the mean of these results used in the analysis.

In six pregnant ewes at 120 d gestation, two trials using 40 mg and one trial using 170 mg of FD250S were performed 3 h apart on the same day to estimate repeatability of the method and compare results obtained using different doses. Samples of fetal blood and amniotic fluid were taken during the experiment to check for movement of FD250S across the placenta. Assessment of blood volume parameters using radiolabelled red cells was also done on this day.

2.5.2.3 Assay of FD250S

Plasma FD250S concentrations were measured by fluorimetry in 96 well microplates using quadruplicate 50 μL aliquots of plasma in 200 μL borate buffer (6.18 g boric acid (Serva Feinbiochemica, Heidelberg, Germany) in 500 mL water (0.2 $mol.L^{-1}$) and titrated to pH 8.8 with sodium hydroxide). Excitation wavelength was 485 nm and

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emission wavelength was 535 nm. A standard curve was included on each plate. The standard curve was prepared in pooled adult sheep plasma using the same solution of FD250S as used in the experiment. This 4 $mg.mL^{-1}$ solution was diluted to concentrations from 0 to 20 $\mu g.mL^{-1}$ (or up to 200 $\mu g.mL^{-1}$ if larger doses of FD250S were used) and quadruplicate 50 μL aliquots pipetted into 200 μL borate buffer.

The net fluorescence of each sample was obtained by subtracting the plasma fluorescence prior to FD250S injection from the sample fluorescence. The concentrations derived from the standard curve were then plotted on a semilogarithmic graph and extrapolated back to give concentration at time 0 (C_0), and a volume of distribution (Vd) obtained by dividing the amount injected (I) by this concentration. This Vd is the estimate of PV.

$$\mathbf{Vd = I/C_0 = PV} \qquad \mathbf{(1)}$$

The measured haematocrit is more precisely a central blood haematocrit (cHct). We have used factor of 0.91 to reflect the ratio (k) of total body haematocrit (tHct) to central haematocrit as is used in humans (Gregersen and Rawson, 1959).

$$\mathbf{k = tHct/cHct = 0.91} \qquad \mathbf{(2)}$$

An estimate of BV can then be obtained by the following equation.

$$\mathbf{BV = PV/(1-tHct)} \qquad \mathbf{(3)}$$

Red cell volume may also be estimated from PV.

$$\mathbf{RCV = (tHct \times PV)/(1 - tHct)} \qquad \mathbf{(4)}$$

2.5.2.4 Blood volume estimation using radiolabelled red cells

10 mL of blood was taken from each sheep on the morning of the blood volume estimation, mixed with 3 mL of Anticoagulant-Citrate-Dextrose solution, and labelled with ^{51}Cr by incubating with sodium radiochromate (Amersham Health, Amersham, United Kingdom) for 25 min. The red cells were washed twice with saline and resuspended in 5 mL of saline. This was given intravenously into a jugular vein over 10 sec, and the syringe repetitively filled with saline and injected until the saline was clear. A 10 mL blood sample was collected from the carotid arterial catheter into a heparinised tube 20 min later and, after haemolysis with Digitonin (Sigma, St Louis, MO, USA), counted for

20 min on a scintillation counter (Searle, Des Plaines, Ill, USA), along with the original syringe.

RCV was calculated using the method recommended by the International Committee for Standardization in Haematology (International Committee for Standardization in Haematology, 1980).

$$\mathbf{RCV = (S \times D \times V_i \times Hct) / B} \qquad \textbf{(5)}$$

Where S = concentration of radioactivity in diluted radiolabelled red cell standard (cpm.mL^{-1})

D = dilution of diluted standard solution (final volume divided by volume of red cell suspension put into it)

V_i = volume of labeled red cell suspension injected (mL)

B = concentration of radioactivity in blood sample drawn (cpm.mL^{-1})

Using the tHct, an estimate of BV can be derived.

$$\mathbf{BV = RCV/tHct} \qquad \textbf{(6)}$$

The error in the measurement of RCV by radiolabelled red cells has been estimated at 3.4% (Fairbanks *et al.*, 1996).

The ratio of total body haematocrit to central haematocrit (k) in pregnant sheep was also estimated from the measured RCV as follows:

$$\mathbf{tHct = RCV/(RCV + PV)} \qquad \textbf{(7)}$$

$$\mathbf{k = tHct/cHct}$$

2.5.2.5 Statistics

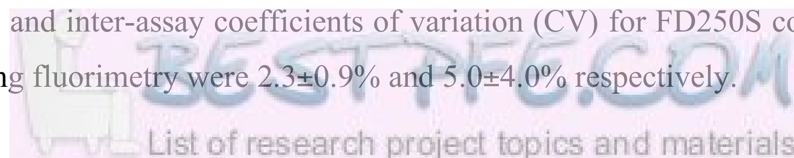
Results from different methods were compared using paired *t*-tests. Values are mean ± SD.

2.5.3 Results

The ewe weights ranged from 59 to 69 kg with a median of 65 kg.

2.5.3.1 Assay

The intra- and inter-assay coefficients of variation (CV) for FD250S concentration measurement using fluorimetry were 2.3±0.9% and 5.0±4.0% respectively.



2.5.3.2 Pharmacokinetics

The elimination of FD250S in pregnant ewes followed an exponential decay curve with a half-life of approximately 30 min, and formed a straight line on a semilogarithmic graph (Figure 2-2) (mean r^2 = 0.997±0.002, n=18). There was no increase in fluorescence of fetal plasma or amniotic fluid within 12 h following a maternal FD250S dose.

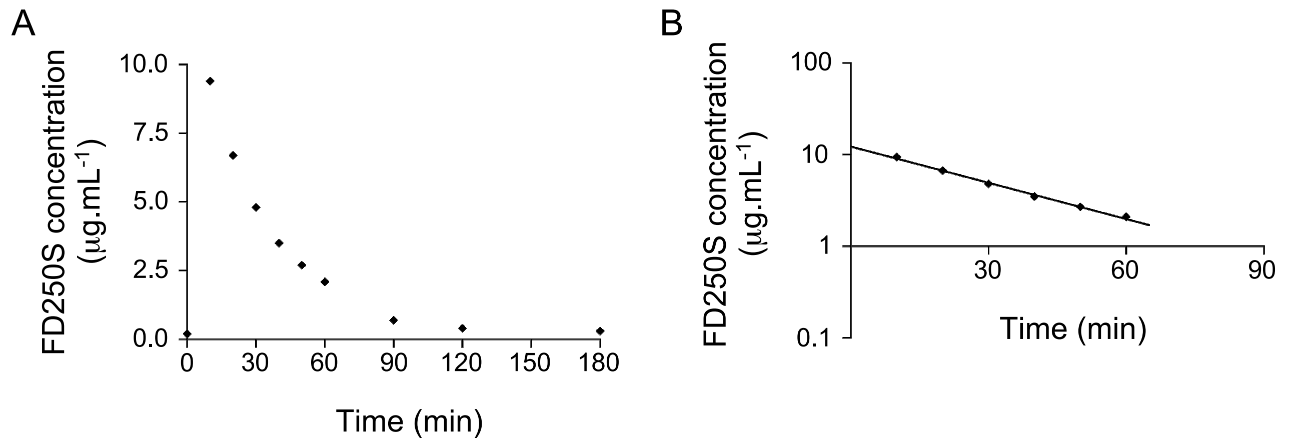


Figure 2-2: FD250S concentrations over 180 min in one sheep following an intravenous bolus of 40 mg in 10 mL saline. A: Exponential fall in plasma concentrations B: Semilogarithmic plot allowing extrapolation back to time 0

2.5.3.3 Blood volume parameters

The mean cHct for the pregnant ewes was 0.27±0.03. The mean PV of these ewes measured using the FD250S method was 3607±296 mL (56±4 $mL.kg^{-1}$), mean BV was 4790±390 mL (75±4 $mL.kg^{-1}$), and mean RCV was 1183±170 mL (18±2 $mL.kg^{-1}$).

In six pregnant ewes, where measurements using 40 mg and 170 mg of FD250S were performed on the same day, the mean difference in PV was 14±144 mL (p=0.82). In the same six ewes, in which two trials using 40 mg FD250S were performed 3 h apart on the same day, the mean CV of PV estimation was 3.8±2.3%.

The value for k derived from our six pregnant ewes using equations 2 and 7 is 0.96±0.06.

2.5.3.4 Validation

The BV results in ewes obtained using FD250S were lower than those obtained using radiolabelled red cells, with a difference of 302±449 mL (p=0.01), or 6±9% (Figure

2-3). The difference between FD250S-derived RCV and that measured by radiolabelled cells was 46±121 mL (p=0.13) or 4±10%.

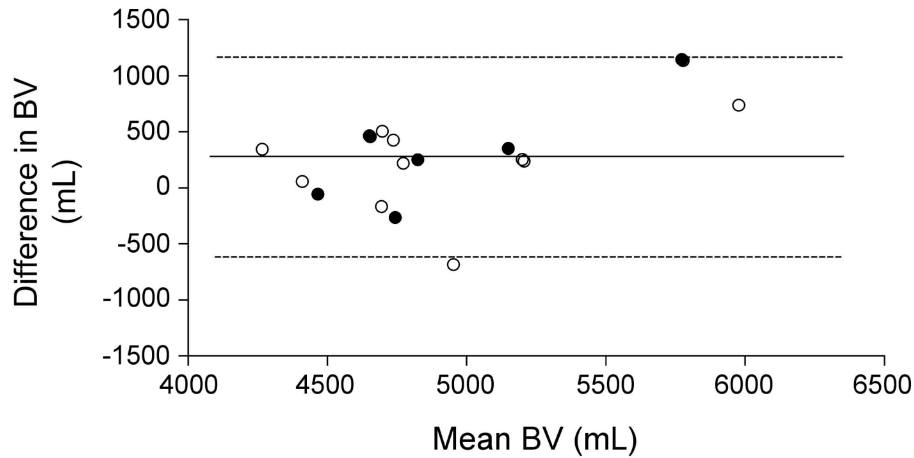


Figure 2-3: Bland-Altman plot of BV results obtained using FD250S and radiolabelled red cells

○ = 40 mg FD250S, ● = 170 mg FD250S. Top and bottom dotted lines represent mean ± 2SD. Difference in BV is calculated by subtracting BV obtained using the dextran method from BV obtained from the radiolabelled red cell method. Mean BV is the mean of the results from the two methods.

In order to test if a shorter FD250S protocol was possible, we compared results obtained using fewer time points with those using all points. The higher dose of FD250S (170 mg, approximately 2.5 $mg.kg^{-1}$) sampled up to 30 min gives results that are highly correlated with the full method (r^2 = 0.95) and accurate (-1.0±2.0%). The results using just a 10 min sample are also highly correlated with the full method (r^2 = 0.98), but significantly overestimate PV by 6.8±1.1%. The method using the lower dose cannot be significantly shortened without losing accuracy.

2.5.4 Discussion

This study has demonstrated that the measurement of the dilution of a 250 kDa fluorescein-labelled dextran is a reliable method for estimating blood volume parameters in pregnant sheep. The CV of 3.8% of repeated measures using the dextran method is comparable to the estimated 3.4% error of the radiolabelled red cell method (Fairbanks *et al.*, 1996). Although the BV results from the dextran method are 6% lower than those from radiolabelled red cells, the consistency of the results suggest that it is a reliable

method. This method may be further simplified by reducing the number of samples taken and could be adapted for use in small animal models by using just one sample, which gives a consistent, though overestimated, volume compared with the results obtained using all points.

Some of the variation in the results may be accounted for by errors in the measurement of RCV by radiolabelled red cells that, due to the low haematocrit of sheep, will have a proportionally greater effect on calculated blood volume than errors in measurement of PV. Measurement of haematocrit itself is prone to error, due both to variable trapping of plasma in the red cell column and difficulties in accurately reading the spun tube when using the microfuge method. In addition, an adjustment factor, k, is used to take into account the differences between (measured) central haematocrit (cHct) and whole body haematocrit (tHct). This difference is thought to arise from the larger proportion of blood in the capillaries that is plasma, due to limited passage of red cells through smaller vessels, resulting in a tHct lower than the cHct measured from blood in the large vessels (Chaplin *et al.*, 1953). In this study we have used a value for k of 0.91, taken from the human literature (Chaplin *et al.*, 1953). This is a commonly used value, although it appears that k may be slightly higher in pregnancy by approximately 1.5% (Muldowney and Flanagan, 1964). The value for k derived from our six pregnant ewes using equations 2 and 7 is 0.96 ± 0.06, which is within the range of published values. Furthermore, since pregnant ewes have a low haematocrit, this difference of 0.05 in k would change calculated BV only by approximately 90 mL or 1.8%. Despite these errors that may be incurred in calculating BV or RCV from PV and Hct, we found no significant differences between RCV derived from the dextran method and that measured using radiolabelled red cells.

This study is the first demonstration of this method in pregnancy and in sheep. The method has several advantages for a research situation. It is non-radioactive and simple, thereby allowing use on a research farm; it uses an inexpensive commercially available chemical that costs around US$20 per study in materials; and it has a simple, rapid assay. It may also be used to perform repeated measurements in a short space of time because of the short half-life of FITC-dextran in the sheep circulation. In this study repeated measurements of PV were made 3 h apart with no significant differences between the results.

The finding of an average BV in pregnant sheep of 75 $mL.kg^{-1}$ is similar to other values in the literature: 67.5 $mL.kg^{-1}$ in pregnant sheep using radiolabelled red cell

(Lotgering *et al.*, 1983); 74 $mL.kg^{-1}$ in pregnant sheep using Evans Blue (Metcalfe and Parer, 1966), and 63.5 $mL.kg^{-1}$ in non-pregnant sheep using radiolabelled albumin and red cells (Gillett and Halmagyi, 1966).

As the sheep is the primary animal used in the study of fetal physiology, there is a wide area of research that may find this technique useful. Although both fluorescein and dextran have been widely used in humans, recent concerns about adverse reactions to contaminated batches of dextran (Wills *et al.*, 2005) will limit possible clinical applications of this technique for the time being. However it could readily be translated to clinical use if concerns about adverse reactions to dextrans are allayed.

2.6 Biochemical Assays

2.6.1 Metabolite assays

Metabolite concentrations were measured on a Hitachi 902 autoanalyser (Hitachi Australia, North Ryde, NSW, Australia): glucose by enzymatic colorimetric assay (Roche, Basel, Switzerland); urea by kinetic UV assay (Roche); lactate and free fatty acids (FFA) by enzymatic colorimetric assays (Randox Laboratories Ltd, Ardmore, Crumlin, UK) and β-hydroxybutyrate (βHBA) by kinetic UV assay (Randox).

2.6.2 Hormone Assays

2.6.2.1 Insulin

Plasma insulin concentrations were measured by specific radioimmunoassay (RIA) established and validated for maternal and fetal sheep plasma. The first antibody, guinea pig anti-ovine insulin (GC4), was diluted in phosphate buffered saline (PBS) (pH 6.2) to a concentration of 1:80000, and combined with the samples, controls, and standards (ovine insulin, Sigma Chemical, St. Louis, MO, batch # I9254). After 24 h incubation, 100 µL of tracer (^{125}I-rhIns, Eli Lilly, Indianapolis, IN, USA) was added and incubated for a further 24 h. The second antibody-PEG complex was then added (0.5% sheep anti-guinea pig gamma globulin, 0.1% normal guinea pig serum, and 8% polyethylene glycol (PEG) 6000 in 0.01 $mol.L^{-1}$ PBS), and after 3 h further incubation at room temperature, the tubes were centrifuged at 3300 rpm at 4°C for 45 min. The supernatant was tipped off and the pellet counted for 2 min on the Cobra gamma counter (Packard Instruments Co., Meridien, CT, USA), and the concentration calculated from the standard curve. The minimal detectable

concentration was 0.03 $ng.mL^{-1}$ plasma and the inter- and intra-assay CV values were 11.1% and 11.0%, respectively.

2.6.2.2 IGF-1

Plasma IGF-1 was measured using an IGFBP-blocked RIA (Blum and Breier, 1994; Vickers *et al.*, 1999). Plasma samples were diluted 1:100 in acidic phosphate buffer (pH 2.8) to eliminate binding protein interference. The first antibody, rabbit-anti-IGF-1 (#878/4), was diluted in 200 $mmol.L^{-1}$ phosphate buffer (pH 7.8) together with excess IGF-2 (250 $ng.mL^{-1}$), and combined with the samples, standards (rh-IGF-1, Genentech, San Francisco, CA, USA) and controls. After incubation for 1 h at room temperature, 100 μL of tracer (^{125}I-IGF-1) was added and the mixture incubated for 48 h at 4°C. The second antibody-PEG complex was then added (2% #3 sheep anti-rabbit gamma globulin, 0.01% normal rabbit serum and 8% PEG 6000 in 0.01 $mol.L^{-1}$ PBS) and, after a further 1 h incubation at room temperature, the tubes were centrifuged at 3300 rpm at 4°C for 30 min. The supernatant was tipped off and the pellet counted for 1 min on the Cobra gamma counter. The results were calculated from the standard curve. The detection limit was 0.7 $ng.mL^{-1}$ and the inter- and intra-assay CV values were 17.5% and 10.0% respectively.

2.6.2.3 ACTH

ACTH was measured with a commercial RIA (Diasorin, Stillwater, MO, USA). Samples were thawed on ice and 100 μL of samples, standards or controls added to 200 μL of ACTH antiserum in all tubes except total count tubes and non-specific binding tubes. After addition of 200 μL of ^{125}I-ACTH to all tubes and incubating for 24 h, 500 μL of the precipitating complex was added to all except the total count tubes. After further incubation for 25 min, the tubes were centrifuged at 300 rpm for 20 min at 20°C. The supernatant was aspirated and the pellet counted, with the results calculated from the standard curve. The assay had inter- and intra-assay CV values of 16.1% and 15.2% respectively.

2.6.2.4 Steroids

Steroids were measured using mass spectrometry. The internal standards were: cortisol-d2 for cortisol; corticosterone-d8 for cortisone, dehydroepiandrosterone (DHEA), and progesterone; and 11deoxycortisol-d3 for 11deoxycortisol. 100 μL of internal standard (20 $ng.mL^{-1}$ in water) was added to 200 μL plasma. Steroids were extracted using 1 mL of ethyl acetate. After removal of the organic supernatant, samples were dried,

resuspended in 100 µL of mobile phase (80% methanol and 20% water), and transferred to high performance liquid chromatography (HPLC) injector vials. 25 µL was injected on to an HPLC mass spectrometer system consisting of a Surveyor MS pump and autosampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrapole mass spectrometer, all controlled by Finnigan Xcaliber software (Thermo Electron Corporation, San Jose, CA, USA). The mobile phase was isocratic, flowing at 600 $\mu L.min^{-1}$ through a Luna 3µC18(2) 100A 250 x 4.6 column at 35ºC (Phenomenex, Auckland, New Zealand). Retention times were: cortisol 6.05 min; cortisone 5.7 min; DHEA 9.3 min; progesterone 11.4 min; 11deoxycortisol 6.6 min. Ionisation was in positive mode and Q2 had 1.2 mTorr of argon for all steroids. The mass transitions followed, for internal standard and steroid respectively, were: cortisol-d2 365.3 → 121.2 at 28 V and cortisol 363.3 → 122.2 at 28 V; corticosterone-d8 355.3 → 125.2 at 24 V and cortisone 361.1 → 163.0 at 28 V, DHEA 271.2 → 197.0 at 18 V, and progesterone 315.1 → 109.0 at 26 V; 11deoxycortisol-d2 349.2 → 109.1 at 26 V and 11deoxycortisol 347.2 → 109.1 at 26 V. Mean inter- and intra-assay CV values were: cortisol 11.2% and 7.1%; cortisone 20.4% and 10.3%; DHEA 28.5% and 19.6%; progesterone 11.7% and 7.6%; 11deoxycortisol 16.8% and 10.7%.

2.6.2.5 CNP and NTproCNP

CNP and NTproCNP analyses were performed by Dr Tim Prickett at the Department of Medicine, Christchurch School of Medicine, Christchurch, New Zealand.

Plasma CNP was assayed by RIA as previously described (Yandle *et al.*, 1993) using a commercial antiserum (Phoenix Pharmaceuticals Inc., Belmont, CA, USA). Antiserum to CNP-22 was diluted $1:10^4$ with assay buffer prior to assay. The sample, standards and control were combined with 100 µL each of antiserum and radiolabelled CNP, incubated for 22 h at 4°C, and bound and free label separated by a solid-phase second antibody method. Intra- and inter-assay CVs were 3.6% and 8.3% respectively.

NTproCNP was assayed by RIA as previously described (Prickett *et al.*, 2001) using the primary rabbit antiserum (J39) raised against NTproCNP(1-15) (100 µL 1:6000 diluted antiserum per assay tube) Peptide standards were made from synthetic human proCNP(1-19). Intra- and inter-assay CVs were 4.9% and 6.4% respectively.

2.6.2.6 Antipyrine

Antipyrine was measured using reverse-phase HPLC. The system consisted of a Waters 2695 separation module, a Waters 2475 Multi λ Fluorescence Detector (Waters, Milford, MA, USA), and a Phenomenex Luna 3u c18(2)100A column (Phenomenex, Torrance, CA, USA). Blood samples were thawed on ice, 500 μL mixed with 500 μL water and 50 μL phenacetin (Sigma) internal standard (10 $\mu g.mL^{-1}$), and extracted with 4 mL dichloromethane and N-pentane (1:1). The tubes were then centrifuged at 3740 rpm for 15 min and placed at -80°C for 10 min to freeze the aqueous layer. The organic layer was decanted into 4 mL tubes and evaporated under a vacuum (Hetovac VR1, Heto-Holten A/S, Allerød, Denmark) for 2 h until dry. The samples were reconstituted with 200 μL of mobile phase (70:32 mixture of 6.7 $mmol.L^{-1}$ phosphate buffer, pH 7.2 and acetonitrile), centrifuged at 14000 rpm for 5 min and the supernatant transferred to limited volume inserts in HPLC vials. The flow rate was 1 $mL.min^{-1}$ under isocratic conditions. Retention times for antipyrine and phenacetin were 3.2 and 6.0 min respectively. A standard curve was prepared by spiking blank blood samples with antipyrine and carrying these samples through the entire procedure. Data were captured and analysed using Empower Pro software (Waters). The ratio of area under the curve (AUC) of antipyrine to phenacetin was compared with the standard curve to determine concentrations. The inter- and intra-assay CV values were 2.7% and 5.7% respectively.

2.7 Data Analysis

Data were entered into Excel spreadsheets (Microsoft Corp., Seattle, WA, USA) and transferred to JMP 5.1 (SAS Institute, Cary, NC, USA) for statistical analysis. Results are presented as mean±SEM or median (range) as appropriate. Where data were log-transformed to approximate normality, the geometric mean and upper SEM were used. Where data were not normally distributed and could not be transformed to a normal distribution, the Wilcoxon nonparametric test was used.

Where the experiment consisted of the four nutritional groups in singleton pregnancies, they were compared using ANOVA or ANCOVA as appropriate, with the Tukey-Kramer *post hoc* test used to correct for multiple comparisons. Repeated samples over time were analysed by repeated measures ANOVA. Growth data measurements were normalised to 114 d, and considered acceptable if the surgery to postmortem increment in catheter measurements was within ±2 cm of the actual increment in measurements

between surgery and postmortem. Fetal growth was compared over pre-fasting, fasting and post-fasting periods using linear regression with nutritional group as an independent variable. Changes in growth rate with maternal fasting were tested using multiple linear regression with a variable delineating pre-fasting from fasting, and the group by time period interactions being the variables of interest (Harding, 1997b).

Where the experiment consisted of singleton and twin pregnancies from the control and UN -60-30 groups, data were compared using a two-way ANOVA with nutritional group, single/twin, and their interaction as independent variables. If fetal data were being compared, the ewe number was nested within nutritional group and single/twins to account for the non-independence of twin pairs. Repeated samples over time were analysed by a similar repeated measures ANOVA. Growth rates were compared as described above, except that twinning, nutritional group, and their interaction were independent variables, and ewe number was nested within groups to allow for the non-independence of twins. Twins were divided into 'heavy' or 'light' based on postmortem weight.

Sex differences were investigated within singleton and twin pregnancies using a two-way ANOVA with sex, nutritional group and their interaction as independent variables. In twin pregnancies the effect of the sex mix of the pair, designated as mixed, male or female, was evaluated using ANOVA in male and female twin fetuses separately.

To estimate fetal weight at earlier time points, the measured change in fetal girth using the growth catheters was used to estimate chest girth at a particular gestational age by extrapolation backwards from measured chest girth at postmortem. The ratio of fetal weight to chest girth was measured at postmortem and then estimated at a particular gestational age by extrapolating backwards at a rate of 0.2 $g.mm^{-1}.day^{-1}$. This figure was derived from a large data pool (n=116) of fetal postmortems performed in our laboratory after similar experiments in chronically catheterised fetuses, where the slope of a least squares line through the fetal weight to chest girth ratios versus gestational age was 0.20±0.02 $g.mm^{-1}.day^{-1}$ (r^2=0.4, p<0.0001). Fetal weight was then estimated as a product of the estimated chest girth and the estimated fetal weight to chest girth ratio.

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3. Effects of periconceptional undernutrition on maternal cardiovascular adaptation to pregnancy

3.1 Introduction

Appropriate cardiovascular adaptations in the mother are essential for successful pregnancy. In women, these adaptations include an increase in blood volume of approximately 45%, beginning at 6-8 weeks and reaching a maximum at around 30 weeks gestation (Bocking, 1994). The increase in plasma volume is greater than that of red cell volume, so that there is an accompanying decrease in haematocrit. Some evidence suggests that the size of the increase in blood volume is related to the size of the feto-placental unit (Hytten and Paintin, 1963), with greater increases in multiple pregnancies (Rovinsky and Jaffin, 1965). Uterine blood flow also increases, particularly in late gestation, from 50 $mL.min^{-1}$ in week 10 to over 1300 $mL.min^{-1}$ at the end of gestation (Lunell *et al.*, 1982). These cardiovascular adaptations meet the increasing oxygen and nutrient needs of a growing fetus and placenta, but also persist through the postpartum period, with some parameters remaining altered at 12 weeks after delivery (Capeless and Clapp, 1991).

Despite the importance of sheep in studies of the physiology of pregnancy and the fetus, the normal cardiovascular adaptations to pregnancy in this species are not well described. An early study by Barcroft (Barcroft *et al.*, 1939) demonstrated that blood volume increased by 24% and plasma volume by 40% in pregnant ewes by full term, so that the haematocrit decreased 24%. However, a later study found that blood volume was only 7% higher and plasma volume 6% higher in pregnant ewes in the last month of gestation compared with postpartum values, with no change in haematocrit (Metcalfe and Parer, 1966). In both studies, increases in blood volume were approximately in proportion to increases in maternal weight so that volume per kilogram was unchanged. These early studies used only small numbers of animals and estimated blood volume using Evans Blue, a method that is prone to error in pregnancy (Brown *et al.*, 1992). Furthermore, values in

pregnancy were compared with postpartum values as a baseline, a time the cardiovascular parameters may still be affected (Capeless and Clapp, 1991). The changes in uterine blood flow in sheep are better described than those of blood volume, with a substantial increase in the last month of gestation in sheep (Daniel *et al.*, 1989; Lang *et al.*, 2003).

Inadequate cardiovascular adaptation is associated with abnormal pregnancy and impaired fetal growth. However the causal relationships between these abnormalities are difficult to establish. Inadequate blood volume expansion in human pregnancy has been associated with fetal growth restriction (Duvekot *et al.*, 1995) and pre-eclampsia (Silver *et al.*, 1998). Reduced uterine blood flow is also observed in association with intrauterine growth restriction (Nylund *et al.*, 1983) and pregnancy-induced hypertension (Lunell *et al.*, 1982).

In experimental animals, reduction of uterine blood flow by uterine artery ligation (Lafeber *et al.*, 1984; Wigglesworth, 1974) or utero-placental embolisation (Bauer *et al.*, 2003; Clapp *et al.*, 1980) causes fetal growth restriction and altered maturation of many fetal physiological and endocrine systems. However, in carunclectomised sheep the relationship between uterine blood flow and fetal weight is less clear (Owens *et al.*, 1986). Prevention of expansion of maternal blood volume and uterine blood flow in pregnant sheep in the last month of gestation by haemorrhage and diuretics did not alter fetal size at delivery (Daniel *et al.*, 1989), suggesting that there is not a clear causal relationship between these cardiovascular parameters and fetal growth. Similarly, in pigs uterine blood flow is correlated with litter size, and is maintained despite marked reductions in blood volume caused by weight loss in nutritionally restricted animals (Hard and Anderson, 1982).

Periconceptional undernutrition in sheep has been shown to alter fetal growth trajectory (Harding, 1997a), as well as feto-placental metabolism (Oliver *et al.*, 2005) and the maturation of the fetal glucose-insulin (Oliver *et al.*, 2001b) and hypothalamo-pituitary-adrenal axes (Bloomfield *et al.*, 2004; Hawkins *et al.*, 2000b; McMillen *et al.*, 2004), leading to preterm birth (Bloomfield *et al.*, 2003b). The mechanisms underlying these changes remain obscure.

We hypothesised that one possible mechanism was undernutrition-induced impairment of maternal cardiovascular adaptation to pregnancy, leading to reduced maternal blood volume and/or uterine blood flow. We examined the effects of defined periods of periconceptional undernutrition on maternal cardiovascular adaptation to pregnancy. Specifically, we measured maternal blood volume parameters in mid as well

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as late pregnancy, and studied the relationships between maternal blood volume, uterine blood flow and feto-placental size in late gestation.

3.2 Methods

3.2.1 Experimental design

Non-pregnant ewes were randomly allocated to control, UN -60-0, UN -2-30 or UN -60-30 nutritional groups (Figure 2-1). At 64 d gestation singleton-bearing ewes were put into metabolic cages and had bilateral jugular catheters inserted after infiltration with 2% xylocaine. The catheters were secured and placed on the back under a mesh stocking. Ewes were fasted overnight with free access to water, and the following morning at 0900 a blood volume estimation was performed using 40 mg of FD250S. After the completion of the experiment, the catheters were removed and the ewe returned to its pen and fed.

The ewes underwent surgery at the ARU at 111±1 d for placement of venous and arterial catheters and allowed to recover for at least 3 d. At 114 and 131 d, experiments to measure uterine blood flow were performed, and a further blood volume estimation was done on day 120 at 0900, after an overnight fast. The ewes were euthanised at 132 d and the fetus and placenta were weighed and measured.

3.2.2 Data analysis

Ewe weight at surgery (111±1 d) was used in the analyses of parameters in late gestation. Uterine blood flow data were log-transformed to approximate a normal distribution for use in parametric analyses. Blood volume and uterine blood flow data were compared between groups and over time using a two-way ANOVA with sheep number nested within nutritional group to allow for the non-independence of repeat measures within the same animal, and using the Tukey-Kramer method to correct for multiple comparisons. Pregnant and non-pregnant control sheep were compared using *t*-tests. Morphometric data were compared using ANOVA with Tukey-Kramer correction for multiple comparisons. Values in tables are least square means ± SEM. Lines were fitted on graphs using the least squares method.

3.3 Results

3.3.1 Maternal Weight

There were significant differences (p<0.05) in ewe weights between nutritional groups at 65 d, ranging from a mean of 70.0 ± 0.5 kg in the control group to 61.8 ± 0.5 kg in the UN -60-30 group (Table 3-1). There was an overall decrease in weight of 2.0 kg (p<0.01) from 65 to 110 d, presumably due to pre-surgical fasting, with the controls still being significantly heavier than the UN -2–30 and UN -60–30 groups at 110 d (p<0.05) (Table 3-1).

Gestational Age	Nutritional Group	N	Maternal weight † (kg)	Blood volume ($mL.kg^{-1}$)	Plasma volume † ($mL.kg^{-1}$)	Red cell volume † ($mL.kg^{-1}$)	Haematocrit
Day 65	Control	14	70.9±2.0^{a}	67.3±3.2	46.4±2.3	21.0±1.3	0.33±0.01
	UN -60-0	9	67.8±1.9^{b}	70.0±3.8	48.5±2.3	21.5±1.7	0.32±0.01
	UN -2-30	12	64.3±2.2^{c}	65.4±2.6	45.8±1.9	19.7±1.2	0.32±0.01
	UN -60-30	14	61.3±1.6^{d}	71.3±2.5	50.2±1.9	21.0±1.3	0.31±0.02
Day 120	Control	11	66.4±1.6^{ab}	69.4±2.6	51.4±2.5	18.0±0.9	0.28±0.02
	UN -60-0	7	64.0±0.9^{bc}	71.3±6.1	53.5±4.4	17.8±1.9	0.28±0.01
	UN -2-30	6	62.4±3.2^{cd}	67.7±3.7	50.1±3.0	17.6±1.4	0.29±0.02
	UN -60-30	8	62.0±2.0^{cd}	68.1±3.4	51.0±2.4	17.1±1.4	0.27±0.01
Non-pregnant	Control	7	67.0±4.4	63.6±3.7	47.5±3.4	16.1±0.8*	0.27±0.02*

Table 3-1: Blood volume parameters in different nutritional groups at 65 and 120 d gestation, and in non-pregnant sheep

Maternal weights are from 65 and 111 d. Data are mean ± SEM. Results at the same gestational age not connected by the same letter are significantly different, p<0.05.
† p<0.01 for time effect
** p<0.05 for comparison with pregnant controls at 65 d*

3.3.2 Blood volume

Blood volume estimations were performed on 7 non-pregnant ewes, 49 pregnant ewes at 65 d and 32 at 120 d, with 22 undergoing both studies. At 65 d, red cell volume was 31% greater in control pregnant ewes than in nonpregnant ewes (p<0.05) but there were no differences in plasma volume or overall blood volume (Table 3-1).

For all pregnant ewes combined, between 65 and 120 d red cell volume decreased (20.8±0.4 vs 17.4±0.6 $mL.kg^{-1}$, p<0.01) and plasma volume increased (46.8±0.8 vs 50.8±1.2 $mL.kg^{-1}$, p=0.02), so that overall blood volume did not change (67.6±1.1 vs 68.2±1.5 $mL.kg^{-1}$, p=0.76). In ewes that had blood volume estimations performed at both time points, results were correlated (r^2=0.23, p=0.02).

There were no differences in blood volume parameters between nutritional groups at either time point (Table 3-1).

3.3.3 Uterine Blood Flow

Uterine blood flow (UBF) measurements were performed on 31 ewes at 114 d and 17 ewes at 131 d, with 17 undergoing both studies. UBF increased by 20% between 114 and 131 d (1769±88 vs 2130±204 $mL.min^{-1}$, p<0.01). In sheep that had both studies performed the results were strongly correlated (r^2=0.86, p<0.01), and there was a similar increase over time of 26%. UBF was 18% higher in undernourished groups than in controls (Table 3-2).

Nutritional Group	Day 114		Day 131†	
	N	UBF	N	UBF
Control [a]	6	1604±77	3	1715±217
UN -60 – 0 [b]	9	1760±179	5	2310±386
UN -2 – 30 [b]	7	1717±196	5	2076±561
UN -60 – 30 [b]	9	1726±194	4	1783±182

Table 3-2: Uterine blood flow (UBF; $mL.min^{-1}$) at day 114 and 131 of gestation in different nutritional groups

Data are geometric mean ± SEM.
Different letters indicate significant difference between groups, p < 0.05.
† *p < 0.01 for time effect*

3.3.4 Morphometric data

There were no significant differences among nutritional groups in placental, uterine, or fetal weight (Table 3-3).

Nutritional group	120 d		132 d			
	N	Estimated fetal weight	N	Measured fetal weight	Placental weight	Uterine weight
Control	8	2933±116	7	4393±166	552±28	802±58
UN -60 – 0	8	2652±127	8	3851±174	628±25	744±29
UN -2 – 30	11	2775±155	7	4445±145	542±15	747±48
UN -60 – 30	8	2947±120	7	4317±128	594±38	802±43

Table 3-3: Placental, uterine and fetal weights (g) at 120 and 132 d

Data are mean ± SEM. There are no significant differences among groups.

Maternal blood volume at 120 d was correlated with estimated fetal weight at 120 d (r^2=0.20, p<0.02) (Figure 3-1A), and with UBF at 114 d (r^2=0.22, p=0.05) (Figure 3-1B). UBF was correlated with estimated fetal weight at 114 d (r^2=0.29, p<0.01), and with measured fetal weight (r^2=0.29, p=0.03) (Figure 3-1C), utero-placental weight (r^2=0.39, p<0.01) (Figure 3-1D) and placental weight at 132 d (r^2=0.36, p=0.01).

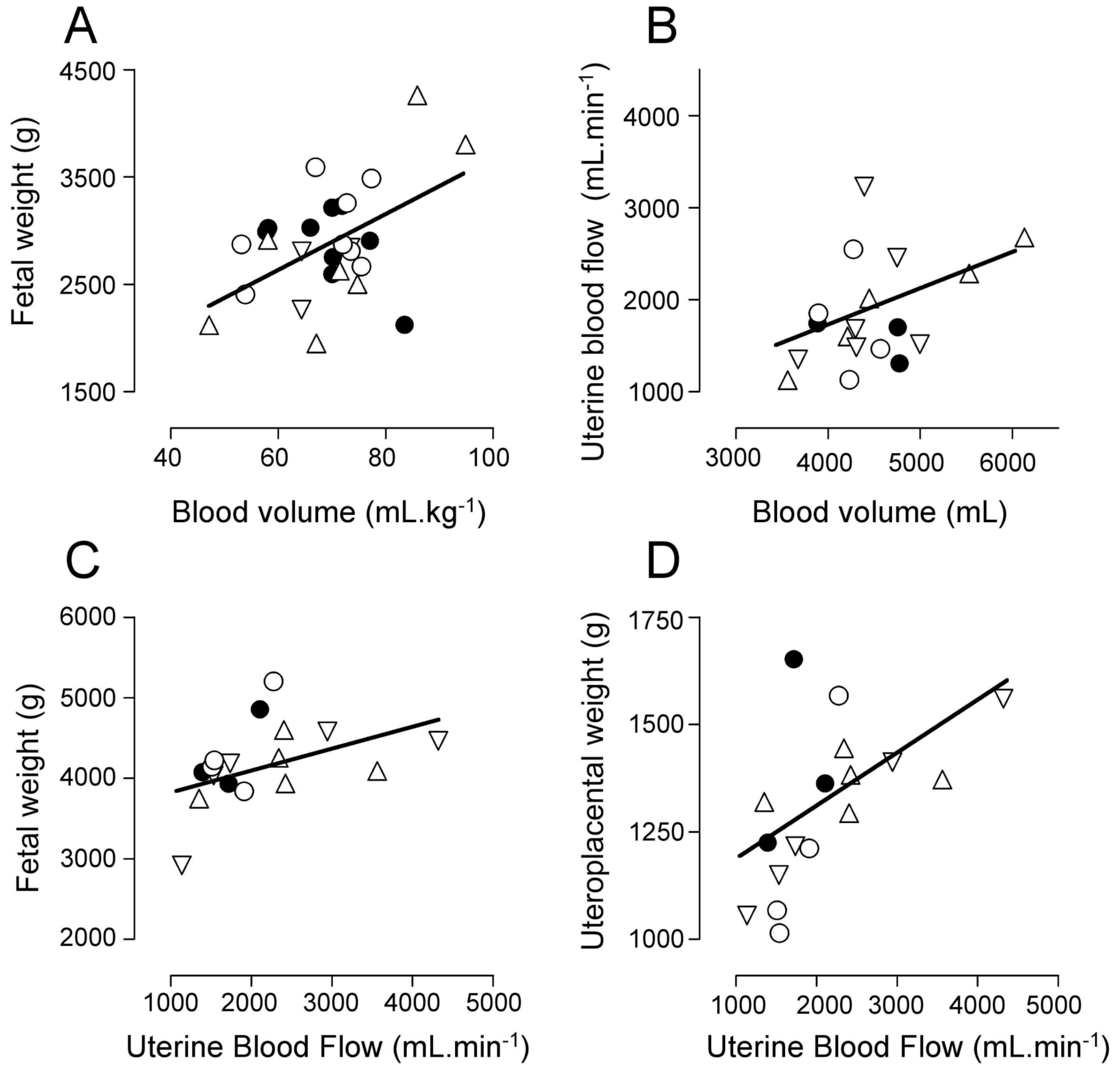

Figure 3-1: Correlations between maternal cardiovascular parameters and feto-placental morphometric measurements
A: blood volume at 120 d and estimated fetal weight at 120 d (r^2=0.20, p=0.02, n=27)
B: blood volume at 120 d and uterine blood flow at 114 d (r^2=0.22, p=0.05, n=18)
C: uterine blood flow at 131 d and fetal weight at 132 d (r^2=0.29, p=0.03, n=17)
D: uterine blood flow at 131 d and utero-placental weight at 132 d (r^2=0.39, p<0.01, n=17)

Control =●, UN -60-0 = △, UN -2-30 = ▽, UN -60-30 = ○.

3.4 Discussion

Appropriate cardiovascular adaptations in the mother are essential for a successful human pregnancy. Despite the extensive use of experiments in sheep to examine aspects of the physiology of pregnancy and the fetus, the extent and role of cardiovascular adaptations to pregnancy in this species have received little attention. This study shows that expansion of maternal blood volume is not an important component of the physiology of pregnancy in sheep, but that blood volume and uterine blood flow are nevertheless related to fetal and placental growth in late gestation. Intriguingly, maternal undernutrition early or even before pregnancy increases uterine blood flow in late gestation, suggesting that some aspects of maternal physiology in late pregnancy are influenced by events around the time of conception.

Maternal blood volume in pregnant sheep in mid gestation (65 d) was not different from that of non-pregnant controls in this study. This is the time when placental size is close to maximal in sheep (Alexander, 1964; Ehrhardt and Bell, 1995), and when substantial volume expansion has already occurred in human pregnancy (Pritchard, 1965). Furthermore, there was no significant change in blood volume between this time and late gestation (120 d) so that expansion of blood volume does not appear to be an important component of maternal adaptation to pregnancy in sheep. These findings are remarkably consistent with those of an earlier study (Metcalfe and Parer, 1966) (68.9 $mL.kg^{-1}$ in control sheep in this study compared to 74 $mL.kg^{-1}$ found by Metcalfe *et al*), allowing for the errors in measurement and the larger volume of distribution for the Evans blue method used earlier (Gregersen and Rawson, 1959). There was also no change in blood volume per unit maternal weight during pregnancy in both studies, although if the weights of the fetus and amniotic fluid, which are outside the maternal circulation, are subtracted, then there is a small rise of 12% in maternal blood volume with pregnancy in our study. However, this study would not have detected the small increase in blood volume over the last 1-3 weeks of pregnancy, nor the decrease over the last few days before delivery that have been reported elsewhere (Barcroft *et al.*, 1939; Daniel *et al.*, 1989). Blood volume was estimated in late gestation at 120 d because this was before there were likely to be changes related to the onset of parturition, since premature delivery has been observed in our previous studies of periconceptional undernutrition (Bloomfield *et al.*, 2003b).

Despite the lack of change in maternal blood volume, there was a 31% increase in red cell volume by mid gestation, with a return to basal levels in late gestation. This

finding is quite different from previous studies, which have reported either no change in haematocrit (Metcalfe and Parer, 1966) or a decrease in red cell volume between 50 and 70 days (Barcroft *et al.*, 1939). The reasons for this disparity could include methodological, breed or dietary differences. The present study used much larger numbers of animals than have previous researchers, and used a more reliable method of blood volume estimation in pregnant sheep. In addition, all sheep were of consistent breed, were housed individually under identical conditions and received the same specially formulated complete diet. Under these circumstances of high quality nutrition, the ewes may have been able to increase their red cell volume to an extent that was prevented by nutritional limitation in previous studies.

The reason for the increase in red cell volume by mid gestation is not clear. Red cell volume is normally controlled by erythropoietin to meet oxygen-carrying demands. Although placental size is already close to maximal by 60 d gestation, oxygen demand by the feto-placental unit continues to rise steeply in late gestation when maternal red cell volume has fallen again, making oxygen demand by the conceptus an unlikely explanation for our findings. Growth hormone and IGF-1 have been shown to stimulate erythropoiesis in a variety of situations including children with short stature (Vihervuori *et al.*, 1996), adults with GH deficiency (Christ *et al.*, 1997) and in animal studies (Kurtz *et al.*, 1988). Placental lactogen also binds to the GH receptor (Breier *et al.*, 1994b) and may also be involved in erythropoiesis in pregnancy (Jepson and Lowenstein, 1968). In sheep, placental lactogen is produced from 16 d gestation until the end of pregnancy (Martal and Djiane, 1977), and ovine placental growth hormone is produced between 30 and 60 d of gestation (Lacroix *et al.*, 1999), a period of high placental growth rate (Ehrhardt and Bell, 1995). These hormones may account for the increased erythropoiesis we have observed in early pregnancy.

Plasma volume, however, is controlled by the renin-angiotensin system to maintain cardiac filling and end-organ perfusion. The minimal change we have observed in sheep is similar to observations in the rabbit and cow (Prince, 1982), suggesting that a large increase in maternal plasma volume is not an integral aspect of pregnancy across species. The mechanisms of the pregnancy-induced plasma volume increase may be a combination of the increasing placental arteriovenous shunt stimulating the renin-angiotensin system, and the direct effect of oestrogen on this system (Longo, 1983; Magness *et al.*, 1993). In sheep, however, oestrogen remains low until late gestation (Challis and Patrick, 1981) which may account for the lack of increase of plasma volume observed in this study.

Although maternal blood volume did not change with pregnancy in this study, there was a moderately strong relationship between blood volume and fetal weight in late gestation. Blood volume was also related to uterine blood flow, which itself was related to fetal and placental weight. The causal relationships among these variables are difficult to distinguish. Several studies in sheep of restricted uterine blood flow have demonstrated the relationships between uterine blood flow and placental and fetal weight (Lang *et al.*, 2003; Owens *et al.*, 1986) but have not examined the effects on blood volume. When blood volume was restricted over the last 25-30 d of gestation in pregnant sheep, uterine blood flow was reduced only over the last 10 d (Daniel *et al.*, 1989) and mean fetal weight was not affected, suggesting that blood volume has only an indirect relationship to uterine blood flow. The data from this study could suggest that blood volume during pregnancy reflects conditions that influence fetal size.

We hypothesised that altered maternal cardiovascular adaptation to pregnancy might contribute to the mechanisms by which periconceptional undernutrition alters many aspects of feto-placental development in late gestation. Although different periods of undernutrition around the time of conception had no effects on maternal blood volume parameters, they did result in a greater uterine blood flow in late gestation. However, despite this increased uterine blood flow, placental and fetal weights were not altered at 132 d. Once again this argues against a causal relationship between cardiovascular parameters and feto-placental growth. However these findings raise the intriguing possibility that the increase in uterine blood flow is a compensatory change to maintain nutrient supply to the feto-placental unit, perhaps in the face of reduced maternal nutrient availability or impaired placental transport capacity. These possibilities were not examined in detail in the present study, but others have reported no changes in placental histology at 55 d after mild maternal undernutrition from 45 d before mating until 7 d after (MacLaughlin *et al.*, 2005).

Previous studies of periconceptional undernutrition in sheep have used nutritional regimes that span both the pre- and postconception periods (Bloomfield *et al.*, 2004; MacLaughlin *et al.*, 2005). This study was the first to examine the effects of those periods separately, and found that undernutrition confined to the 60 d before mating had similar effects on uterine blood flow in late gestation to a period of undernutrition only in the 30 d after mating, and to a more prolonged period of undernutrition both before and after mating. These data suggest that the mechanisms involved in regulating uterine blood flow in late gestation are influenced by nutritional signals very early in or even before

pregnancy. The nature of such signals is unknown, but they may be linked to changes in other maternal adaptations such as altered insulin sensitivity (Jaquiery *et al.*, 2005) or HPA axis function (Jaquiery *et al.*, 2006).

In conclusion, this study demonstrates that the maternal blood volume adaptations that are essential to healthy human pregnancy are not seen in pregnant sheep. However, in sheep maternal red cell volume increases in mid gestation, and both blood volume and uterine blood flow are related to fetal size in late gestation. Periconceptional undernutrition does not alter maternal blood volume in pregnancy, but does increase uterine blood flow in late gestation, perhaps as a compensatory mechanism to maintain feto-placental growth. Maternal nutritional status before and in very early pregnancy may alter the mother's cardiovascular responses in later gestation.

4. Effects of periconceptional undernutrition on ovine singleton pregnancy

4.1 Introduction

The link between altered fetal development and the risk of adult metabolic diseases (Barker, 1998) is thought to be partly mediated by permanent changes in the fetal glucose-insulin axis and HPA axis, and may be evidenced by changes in fetal growth patterns. Maternal nutrition has long been recognised as a possible underlying mechanism by which events before birth can influence postnatal physiology and disease risk in adult life (Campbell *et al.*, 1996). Aspects of maternal diet that influence fetal development include macronutrient balance (Godfrey *et al.*, 1996a), micronutrient intake (Rao *et al.*, 2001), and overall caloric intake (Stein *et al.*, 2004). The effects of undernutrition at various stages of pregnancy have been extensively evaluated in humans (Kyle and Pichard, 2006), sheep (Luther *et al.*, 2005), rats (Langley-Evans, 2001) and guinea pigs (Lingas *et al.*, 1999), and it is clear that different timings of undernutrition have different effects on fetal growth and the glucose-insulin and HPA axes.

The particular importance of periconceptional nutrition has been emphasised by the Dutch famine cohorts that showed exposure to famine in early compared with late gestation is associated with impaired insulin secretion (de Rooij *et al.*, 2006a), obesity (Ravelli *et al.*, 1999) and coronary heart disease (Roseboom *et al.*, 2000b). In rats, a low protein diet in the preimplantation period results in offspring with hypertension, and changes in birthweight and postnatal growth (Kwong *et al.*, 2000). Sheep experiments, however, allow direct assessment of fetal growth, metabolism and endocrinology during late gestation. Studies of periconceptional undernutrition in sheep from 60 d before until 30 d after mating have demonstrated altered late-gestation fetal growth trajectory (Oliver *et al.*, 2005) and response to maternal undernutrition (Harding, 1997a), insulin response to a glucose bolus (Oliver *et al.*, 2001b), and pituitary ACTH response to decreased negative feedback by cortisol (Bloomfield *et al.*, 2004). At least some of these endocrine effects persist after birth (Todd *et al.*, 2007). Undernutrition in sheep from 45 d before until 7 d

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after mating alters the relationships between maternal weight change and utero-placental weight at day 55 (MacLaughlin *et al.*, 2005), and fetal adrenal weight and IGF-2 and $P450_{c17}$ expression (MacLaughlin *et al.*, 2007). Undernutrition in sheep from mating to 30 d results in offspring with altered pituitary and adrenal responses to CRH/AVP (Gardner *et al.*, 2006), and with altered cardiovascular function following angiotensin II infusion (Gardner *et al.*, 2004b).

However, an important gap in the literature concerning the effects of periconceptional undernutrition on the fetus and offspring is the lack of examination of the pre- and postconception periods separately with the same undernutrition protocol. There is also little literature on maternal adaptation to pregnancy and maternal late-gestation HPA axis function following periconceptional undernutrition. One hypothesis linking maternal undernutrition to postnatal pathology in the offspring is that the fetus is exposed to excess maternal glucocorticoids as a result of either higher maternal levels or a less effective placental barrier. However, there is no direct evidence of these effects after periconceptional undernutrition.

The aims of the study described in this chapter were to determine the effects of periconceptional undernutrition of varying timing and duration on fetal growth, metabolism and endocrine status in late gestation, and in particular on the fetal glucose-insulin and HPA axes. We have also studied the maternal HPA axis in late gestation. The preconception period of undernutrition from 60 d before mating (UN -60-0 group) allowed the ewes to have a decreased body weight but be on a stable nutritional plane for the month before mating, and undergo weight gain during the pre-implantation period. Undernutrition from 2 d before mating until 30 d after (UN -2-30 group) resulted in ewes with a normal bodyweight but an acute decrease in nutrition at the time of conception, and weight loss during the pre-implantation period. Undernutrition through both of these periods (UN -60-30 group) resulted in ewes with low body weight on a stable nutritional plane at the time of mating, but restricted weight gain until post-implantation. We hypothesised that the nutritional groups would demonstrate different fetal growth and endocrine function depending on the timing and duration of the nutritional insult.

4.2 Methods

4.2.1 Experimental design

Non-pregnant ewes were randomly allocated to control, UN -60-0, UN -2-30 or UN -60-30 nutritional groups. Pregnancy and fetal number was established at 55 d by ultrasound, and only singleton pregnancies were used in this experiment. The ewes underwent surgery at 111±1 d for placement of maternal and fetal vascular catheters and fetal growth catheters, and allowed to recover for at least 3 d.

Baseline maternal and fetal blood samples were taken in the morning before feeding at 114, 117, 121, 127 and 131 d.

At 118 d, after an overnight fast, a glucose challenge was performed on the fetuses. Four hours after the conclusion of the glucose challenges, an arginine challenge was performed.

Ewes were fasted from 121 to 124 d, and maternal and fetal blood samples were taken daily. At 124 d ewes were refed and also given an intravenous glucose infusion of 25 g over 8 h, aiming to restore maternal and hence fetal blood glucose as rapidly as possible in a ‘square wave’ pattern. Further blood samples were collected 2, 4, 6 and 8 h after the start of the glucose infusion, and at 24 and 48 h of refeeding.

At 127 d at 0900 a pituitary stimulation challenge was performed using CRH and AVP, and at 128 d at 0900 a metyrapone challenge was performed.

The sheep were euthanised with an overdose of pentobarbitone at 132 d and a postmortem performed. The fetus and placenta were measured, dissected and weighed.

4.2.2 Data analysis

Metabolite and hormone data were averaged over the preconception period (-60 to 0 d), postconception (0 to 30 d), mid gestation (40 to 97 d), late gestation when in the laboratory (114 to 121 d), and prior to postmortem (128 to 131 d) for display in tables. Growth data were divided into prefasting (114 to 121 d), fasting (122 to 124 d) and refeeding (125 to 131 d) periods. Food intake was compared on days it was unrestricted between 114 and 131 d.

Changes in growth rate with maternal fasting were assessed using multiple linear regression with a variable delineating pre-fasting from fasting, with the group by time period interactions being the variables of interest (Harding, 1997a).

Data were compared between nutritional groups using ANOVA and the Tukey-Kramer method to correct for multiple comparisons. Changes of metabolite and hormone levels between time periods were investigated by matched pairs analysis within groups. Where data were not normally distributed and could not be transformed to a normal distribution, nonparametric tests were used.

For the fetal glucose and arginine challenges, AUC was calculated from baseline. Estimated fetal weight was also included as a covariate in the analyses of the glucose and arginine challenge data to account for the variable dose of glucose per kg fetal body weight. The illustrated AUCs are the least square means from the ANOVA analysis.

For the fetal CRH/AVP and metyrapone challenges AUC was calculated from baseline, taken as the average of the two baseline samples, and the data log-transformed to approximate normality where required. AUC histograms use geometric mean and the upper standard error if the data were log-transformed.

The effects of fetal sex were analysed using a two-way ANOVA with sex, nutritional group and the interaction as independent variables.

Data are mean ± standard error of mean (SEM) in both graphs and tables.

4.3 Results

Fifty-three singleton-bearing ewes (17 controls, 12 UN -60-0, 11 UN -2-30 and 13 UN -60-30) entered the experiment, 41 (10 controls, 10 UN -60-0, 9 UN -2-30 and 12 UN -60-30) completed the fast-refeed protocol, 36 (9 controls, 8 UN -60-0, 8 UN -2-30 and 11 UN -60-30) completed the HPA axis challenges, and postmortem data were available for 34 (9 controls, 8 UN -60-0, 7 UN -2-30 and 10 UN -60-30). Not all samples were available from all animals, due to catheter failures and some fetal losses.

4.3.1 Maternal weight

The mean weight of ewes at -71 d was 64.3±0.7 kg. Control ewes gained weight gradually during the experiment and were 6±2 % heavier at 110 d than at -71 d ($p<0.01$) (Figure 4-1). The UN -60-0 group lost 9.0±1.3% of weight during undernutrition and then recovered to be a similar weight at 110 d to that at -71 d. The UN -2-30 group were a similar weight to controls at mating, lost 15.0±0.5 % of weight with undernutrition, and had recovered to be a similar weight at 110 d to that at -71 d. The UN -60-30 group lost 15.1±0.3 % of weight with undernutrition, and were still 4±1 % lighter at 110 d than at -71

d ($p<0.01$). This group had the highest food intake from 114-131 d (Table 4-1). There was a brief period of weight loss from 60 d gestation in all groups due to shearing.

	-71 d	-2 d	30 d	110 d	Average daily food intake
Control	64.2±1.2	65.5±1.5[a]	68.4±1.5[a]	68.0±1.4[√√]	2.01±0.05[b]
UN -60-0	64.2±1.3	54.0±1.2[b]	60.6±1.2[b]	65.2±1.3	2.18±0.06[ab]
UN -2-30	63.7±1.8	66.8±2.1[a]	56.7±1.9[bc]	63.8±2.2	2.00±0.05[b]
UN -60-30	65.0±1.5	55.2±1.3[b]	54.6±1.2[c]	62.5±1.6[√√]	2.23±0.04[a]

Table 4-1: Maternal weights and food intake

Data are mean ± SEM in kg. Food intake is for the period from 114 to 131 d gestation. Different letters indicate significant differences between groups at the same time point, $p<0.05$.
[√√] $p<0.01$ for comparison between weights at -71 d and 110 d in the same group.

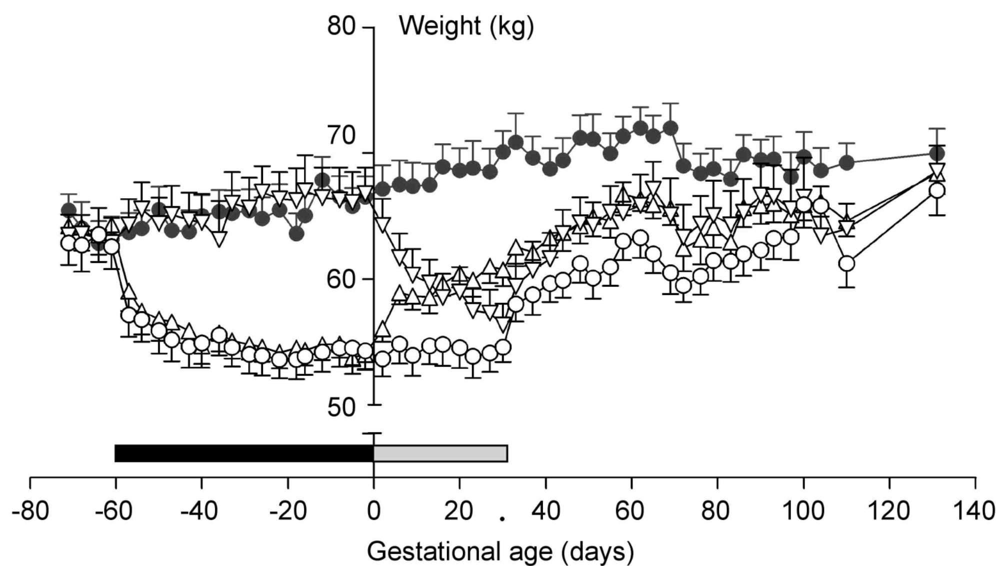

Figure 4-1: Ewe weights from -71 to 132 d

Black bar represents preconception undernutrition, and grey bar represents postconception undernutrition. Control = ●, UN -60-0 = △, UN -2-30 = ▽, UN -60-30 = ○. Data are mean ± SEM.

4.3.2 Fetal growth

The control fetuses slowed their growth by 37% with maternal fasting, although this change did not reach statistical significance (2.98±0.16 to 1.88±0.47 $mm.day^{-1}$, p=0.09) (Figure 4-2). The growth rate after maternal refeeding was 17% slower than prior to the fast (2.46±0.15 $mm.day^{-1}$, p=0.08 for time period comparison) although this difference also did not reach statistical significance.

The UN -60-0 fetuses were a similar size to controls at surgery, but grew significantly more slowly from 114 to 121 d ($p<0.05$) (Figure 4-2), and slowed their growth by 78% with maternal fasting (2.75±0.10 to 0.60±0.35 $mm.day^{-1}$, $p<0.01$). After maternal refeeding, their growth rate was 6% slower than prior to the fast (2.60±0.18 $mm.day^{-1}$, $p<0.01$ for time period comparison) and similar to controls. At postmortem they showed a trend to being lighter than controls ($p<0.10$), and had shorter hindlimbs, smaller hearts and a smaller fetal to placental weight ratio (Table 4-2).

The UN -2-30 fetuses grew at a rate similar to controls but did not slow their growth significantly with maternal fasting (3.23±0.25 to 1.57±0.33 $mm.day^{-1}$, p=0.25). After maternal refeeding these fetuses continued to grow at a rate similar to the pre-fasting period (3.03±0.31 $mm.day^{-1}$, p=0.25 for time period comparison), which was significantly faster than controls ($p<0.05$). They achieved a size similar to controls at postmortem.

The UN -60-30 fetuses were a similar size to controls at surgery and grew at a similar rate, but slowed their growth by 56% with maternal fasting (2.90±0.16 to 1.27±0.38 mm day^{-1}, p=0.02). After maternal refeeding, their growth rate was slower than prior to the fast (2.72±0.20 $mm.day^{-1}$, p=0.01 for time period comparison) and similar to that of controls. At postmortem they were a similar size to controls.

There were no significant effects of fetal sex on growth rates or fetal measurements.

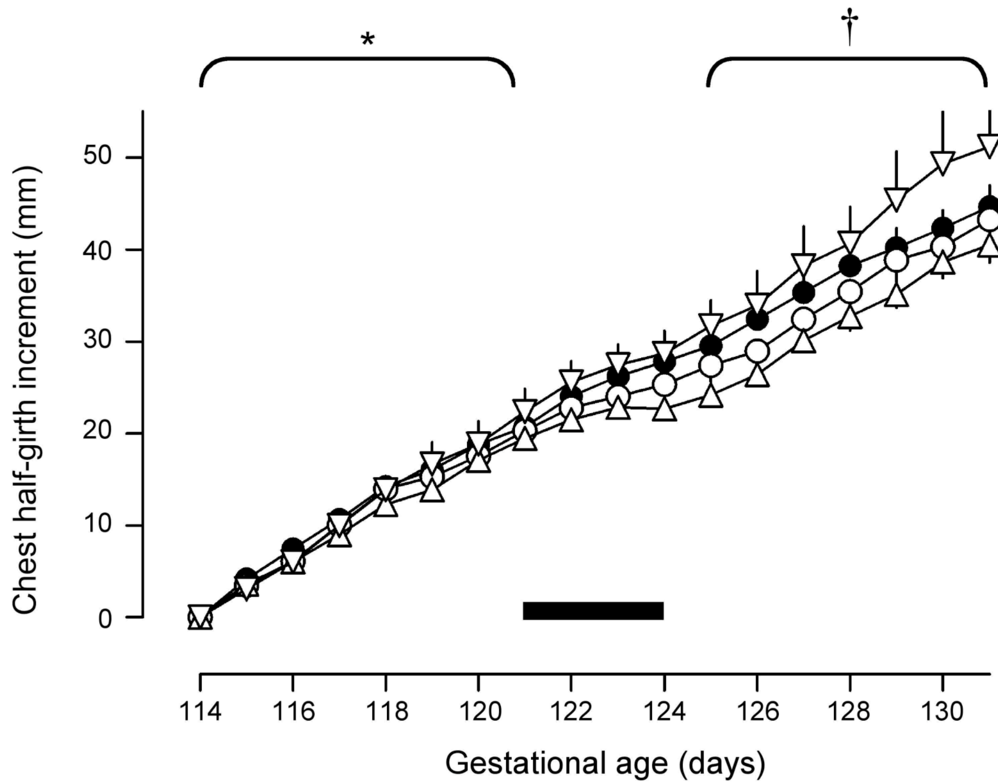

Figure 4-2: Fetal chest girth growth curves in singletons

Control = ●, UN -60-0 = △, UN -2-30 = ▽, UN -60-30 = ○. Ewes fasted from 121 to 124 d, represented by black bar. Data are mean ± SEM.
** p<0.05 difference in growth rates, control > UN -60-0*
† p<0.05 difference in growth rates, UN -2-30 > all other groups

	Control	UN -60-0	UN -2-30	UN -60-30
Surgery day 111±1	(n=18)	(n=12)	(n=11)	(n=14)
Chest girth (cm)	27.0±0.4	26.2±0.6	25.4±0.6	26.1±0.7
Hindlimb length (cm)	25.9±0.4	25.1±0.3	25.5±0.5	25.5±0.3
Post mortem day 132	(n=9)	(n=8)	(n=7)	(n=10)
Fetal weight (g)	4391±147[A]	3851±173[B]	4445±145[A]	4332±141[AB]
Chest girth (cm)	34.6±0.6[ab]	32.8±0.5[b]	34.8±0.5[ab]	35.5±0.8[a]
Crown-rump length (cm)	46.9±1.0	45.1±0.7	46.0±1.0	46.4±0.6
Ponderal index ($g.cm^{-3}.10^3$)	43.1±2.2	41.8±1.2	45.9±2.4	43.6±1.7
Hindlimb length (cm)	33.7±0.5[a]	31.3±0.8[b]	33.4±0.5[ab]	33.5±0.5[ab]
Brain weight (g)	45.6±1.5	46.9±2.1	48.3±1.3	47.8±1.3
Liver weight (g)	149±7	144±5	140±5	167±19
Kidney weight (g)	15.2±1.1	13.8±0.5	16.3±2.0	16.9±1.6
Adrenal weight (g)	0.27±0.02	0.25±0.04	0.26±0.02	0.28±0.03
Spleen weight (g)	10.5±1.6	8.7±0.6	9.3±0.5	12.9±2.4
Heart weight (g)	34.8±1.8[A]	29.3±1.5[B]	34.8±1.7[A]	33.3±0.9[AB]
Lung weight (g)	119±4	114±4	127±5	120±8
Chest thymus weight (g)	5.9±0.8	5.3±0.6	5.7±1.0	4.8±0.4
Neck thymus weight (g)	11.2±1.1	10.1±0.6	11.6±1.5	9.2±0.5
Thyroid weight (g)	1.20±0.10	0.93±0.09	1.03±0.10	1.02±0.07
Pancreas weight (g)	3.3±0.2	3.1±0.2	4.1±0.3	3.7±0.3
Membranes weight (g)	311±18	424±51	385±59	326±44
Amniotic fluid weight (g)	980±134	1286±348	949±162	1356±261
Placentome number	81.6±3.3	81.5±5.0	83.3±5.9	78.0±7.4
Placenta weight (g)	536±29	628±25	542±15	601±41
% A placentomes by weight	22±8	25±4	18±7	11±3
% B placentomes by weight	49±9	36±6	57±11	41±9
% C placentomes by weight	16±5	22±6	14±5	22±5
% D placentomes by weight	14±9	17±6	11±9	26±10
Fetal : Placental weight ratio	8.4±0.5[a]	6.2±0.3[b]	8.2±0.4[a]	7.4±0.4[ab]

Table 4-2: Fetal morphometric data at days 110 and 132

Different lower case letters indicate significant differences between groups, $p<0.05$.
Different upper case letters indicate significant differences between groups, $p<0.10$

4.3.3 Maternal metabolism

Control sheep maintained stable plasma glucose, urea (Table 4-3), insulin and IGF-1 levels (Table 4-4) from -61 d until 30 d. Plasma ßHBA levels were higher and plasma FFA levels were lower in the -60-0 d period compared to the -61 d baselines. Plasma glucose levels remained stable over the 45-97 d, 114-121 d and 128-131 d periods, but plasma insulin, IGF-1 and ßHBA levels increased over these periods and urea decreased. Plasma FFA levels were higher at 45-97 d, but had decreased again by 114-121 d.

During preconception undernutrition, both UN -60-0 and UN -60-30 group ewes had lower plasma glucose and insulin levels than at the -61 d baseline, although the decrease in insulin in the UN -60-30 group was not significant. There were no changes in plasma urea, ßHBA, FFA and IGF-1 levels. Following refeeding at mating, the UN -60-0 group then had increased plasma glucose and urea levels at 0-30 d, and decreased plasma FFA levels, but no change in plasma insulin levels. Metabolite and hormone levels remained stable through the 45-97 d period, but plasma insulin and ßHBA levels were then higher and FFA levels lower at 114-121 d. Urea was lower at 128-131 d than 114-121 d. The UN -60-30 group had similar changes after refeeding at 30 d although the increase in glucose was not significant and insulin levels tended to be lower than controls in the 45-97 d period. The UN -60-30 ewes also had an increase in plasma IGF-1 levels at 128-131 d compared to 114-121 d.

The UN -2-30 group had similar plasma metabolite and hormone levels to controls during the -60-0 d period. During postconception undernutrition plasma glucose, urea and insulin levels decreased, and plasma ßHBA and FFA levels increased. Following refeeding at 30 d, plasma glucose, urea and insulin levels increased, and plasma ßHBA and FFA levels decreased. Glucose remained stable through the remainder of gestation, but insulin and ßHBA were higher at 114-121 d compared to 45-97 d, before both decreased again in the 128-131 d period. Plasma FFA levels were lower at 114-121 d compared to the 45-97 d. Plasma IGF-1 levels were higher at 128-131 d compared to 114-121 d.

4.3.3.1 Fast-refeed experiment

Maternal plasma glucose (Figure 4-3A), insulin (Figure 4-3B), and IGF-1 (63.4±4.5 to 37.9±3.3 ng.mL^{-1}, p<0.01) levels decreased with fasting from 121 to 124 d, while urea (4.78±0.27 to 7.53±0.27 mmol.L^{-1}, p<0.01), FFA (0.16±0.03 to 1.96±0.05 mmol.L^{-1}, p<0.01) and ketone (0.52±0.03 to 1.62±0.08 mmol.L^{-1}, p<0.01) levels increased.

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Plasma glucose and insulin levels were lower in the UN -60-30 than in the UN -2-30 group during fasting, but there were no other differences in metabolite and hormone levels between groups. All maternal metabolite and hormone levels returned to baseline within 48 hours after refeeding.

4.3.4 Fetal metabolism

4.3.4.1 Routine samples

There were no differences between nutritional groups in fetal plasma lactate, urea (

Table 4-3), insulin, or IGF-1 (Table 4-4) levels at 114-121 d or 128-131 d. There was a trend for the UN -60-30 fetuses to have lower plasma glucose levels than the UN -60-0 fetuses ($p<0.10$). Fetal plasma lactate levels were higher in the 128-131 d period in all groups, while urea levels were lower, although the change was not significant in the UN -60-0 and UN -2-30 fetuses. Fetal plasma glucose levels were lower and IGF-1 levels were higher in the 128-131 d period in the UN -2-30 fetuses, but not in the other groups.

4.3.4.2 Fast-refeed

Fetal plasma glucose (Figure 4-3C), insulin (Figure 4-3D) and IGF-1 (69.9±4.1 to 47.6±4.9 $ng.mL^{-1}$, $p<0.01$) levels decreased with maternal fasting from 121-124 d, while urea levels increased (5.50±0.25 to 8.72±0.26 $mmol.L^{-1}$, $p<0.01$). Plasma glucose levels were lower in the UN -60-30 group during fasting than all other groups, but there were no other differences between metabolite and hormone levels between groups. All fetal plasma metabolite and hormone levels returned to baseline within 48 hours.

		Gestational age (d)	**Control**	**UN -60-0**	**UN -2-30**	**UN -60-30**
Glucose (mmol.L^{-1})	Maternal	-61	3.99±0.13	3.76±0.10	3.84±0.17	4.08±0.26
		-60 to 0	3.70±0.10^{AB}	3.38±0.04**^{B}	3.77±0.14^{A}	3.49±0.08*^{AB}
		0 to 30	3.60±0.07	3.62±0.11**	3.43±0.10**	3.42±0.07
		45 to 97	3.73±0.12	3.51±0.08	3.64±0.12**	3.52±0.06
		114 to 121	3.67±0.06	3.66±0.07*	3.66±0.09	3.53±0.05
		128 to 131	3.69±0.08	3.53±0.07	3.63±0.08	3.52±0.04
	Fetal	114 to 121	1.02±0.04^{AB}	1.08±0.05^{A}	1.05±0.05^{AB}	0.91±0.05^{B}
		128 to 131	1.03±0.07	1.02±0.09	0.98±0.03*	0.93±0.06
Urea (mmol.L^{-1})	Maternal	-61	6.54±0.44	6.28±0.57	5.50±0.44	5.78±0.46
		-60 to 0	7.00±0.27^{a}	5.40±0.28^{b}	6.97±0.57^{a}	5.35±0.27^{b}
		0 to 30	7.32±0.20^{a}	6.05±0.24*^{b}	4.75±0.25*^{c}	5.52±0.23^{bc}
		45 to 97	5.92±0.18**	5.73±0.17	5.40±0.40*	5.63±0.20
		114 to 121	5.03±0.28**	5.13±0.28	4.33±0.61*	5.13±0.38
		128 to 131	4.18±0.30*	3.91±0.53*	3.92±0.71	4.11±0.31*
	Fetal	114 to 121	5.65±0.25	5.65±0.26	5.21±0.62	5.76±0.35
		128 to 131	4.81±0.23**	4.53±0.42	4.50±0.67	4.62±0.21**
Lactate (mmol.L^{-1})	Fetal	114 to 121	1.33±0.05	1.27±0.04	1.45±0.07	1.46±0.07
		128 to 131	3.02±0.69**	2.07±0.22**	1.84±0.10**	2.24±0.24**
ßHBA (mmol.L^{-1})	Maternal	-61	0.14±0.01	0.18±0.04	0.20±0.09	0.18±0.02
		-60 to 0	0.20±0.01**	0.19±0.01	0.19±0.02	0.20±0.01
		0 to 30	0.19±0.01^{b}	0.20±0.01^{b}	0.39±0.03**^{a}	0.21±0.01^{b}
		45 to 97	0.24±0.02*	0.20±0.02	0.23±0.02**	0.21±0.01
		114 to 121	0.45±0.02**^{AB}	0.41±0.05**^{B}	0.55±0.05**^{A}	0.44±0.04**^{AB}
		128 to 131	0.49±0.03	0.47±0.05	0.36±0.04*	0.42±0.03
FFA (mmol.L^{-1})	Maternal	-61	0.52±0.07	0.38±0.10	0.42±0.09	0.58±0.12
		-60 to 0	0.35±0.04*^{b}	0.53±0.04^{ab}	0.37±0.07^{ab}	0.56±0.09^{a}
		0 to 30	0.31±0.06^{b}	0.25±0.07*^{b}	1.23±0.07**^{a}	0.51±0.07^{b}
		45 to 97	0.58±0.07**	0.35±0.06	0.43±0.08**	0.40±0.07*
		114 to 121	0.22±0.03**	0.20±0.04**	0.22±0.06*	0.19±0.03**
		128 to 131	0.15±0.04	0.20±0.06	0.26±0.04	0.17±0.03

Table 4-3: Maternal and fetal plasma metabolite levels

Day -61 was prior to the start of experiment. Days -60 to 0 and 0 to 30 were the periods of undernutrition. Days 40 to 97 were after undernutrition and prior to surgery. Days 114 to 121 were after surgery, and days 128 to 131 prior to postmortem. Data are mean ± SEM.
** $p<0.05$, ** $p<0.01$ for comparison with previous time period within the same group. Different uppercase letters $p<0.10$, different lowercase letters $p<0.05$, indicate significant differences between groups in this time period.*

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		Gestational age (d)	**Control**	**UN -60-0**	**UN -2-30**	**UN -60-30**
Insulin (ng.mL^{-1})	Maternal	-61	0.17±0.03	0.21±0.03	0.18±0.02	0.19±0.05
		-60 to 0	0.18±0.02	0.14±0.01*	0.20±0.05	0.13±0.02
		0 to 30	0.17±0.02	0.15±0.02	0.12±0.03*	0.13±0.02
		45 to 97	0.18±0.03^{A}	0.16±0.02^{AB}	0.18±0.03*^{AB}	0.11±0.02^{B}
		114 to 121	0.32±0.03*^{ab}	0.34±0.04*^{ab}	0.44±0.05**^{a}	0.25±0.02**^{b}
		128 to 131	0.44±0.05**	0.34±0.04	0.34±0.04**	0.35±0.04*
	Fetal	114 to 121	0.19±0.02	0.20±0.02	0.21±0.03	0.21±0.02
		128 to 131	0.23±0.03	0.22±0.03	0.25±0.03	0.22±0.03
IGF-1 (ng.mL^{-1})	Maternal	-61	43.5±3.1	39.9±2.7	42.1±5.9	41.7±2.4
		-60 to 0	40.8±1.8	44.9±2.3	42.9±3.8	40.8±1.9
		0 to 30	45.6±2.4	46.7±1.6	41.3±1.3	41.8±2.1
		45 to 97	46.5±2.1	45.9±1.6	47.4±1.9	44.4±2.0
		114 to 121	69.7±9.8*	53.3±5.3	61.0±9.6	46.6±5.0
		128 to 131	91.8±14.0**	58.1±5.3	86.3±14.8*	64.7±6.9**
	Fetal	114 to 121	70.4±5.8	55.9±4.2	76.4±9.4	62.4±6.1
		128 to 131	71.2±5.2	54.4±9.7	94.4±13.2*	77.5±14.3

Table 4-4: Maternal and fetal plasma insulin and IGF-1 levels

Day -61 was prior to the start of experiment. Days -60 to 0 and 0 to 30 were the periods of undernutrition. Days 40 to 97 were after undernutrition and prior to surgery. Days 114 to 121 were after surgery, and days 128 to 131 prior to postmortem. Data are mean ± SEM.
** p<0.05, ** p<0.01 for comparison with previous time period within the same group. Different uppercase letters p<0.10, different lowercase letters p<0.05, indicate significant differences between groups in this time period.*

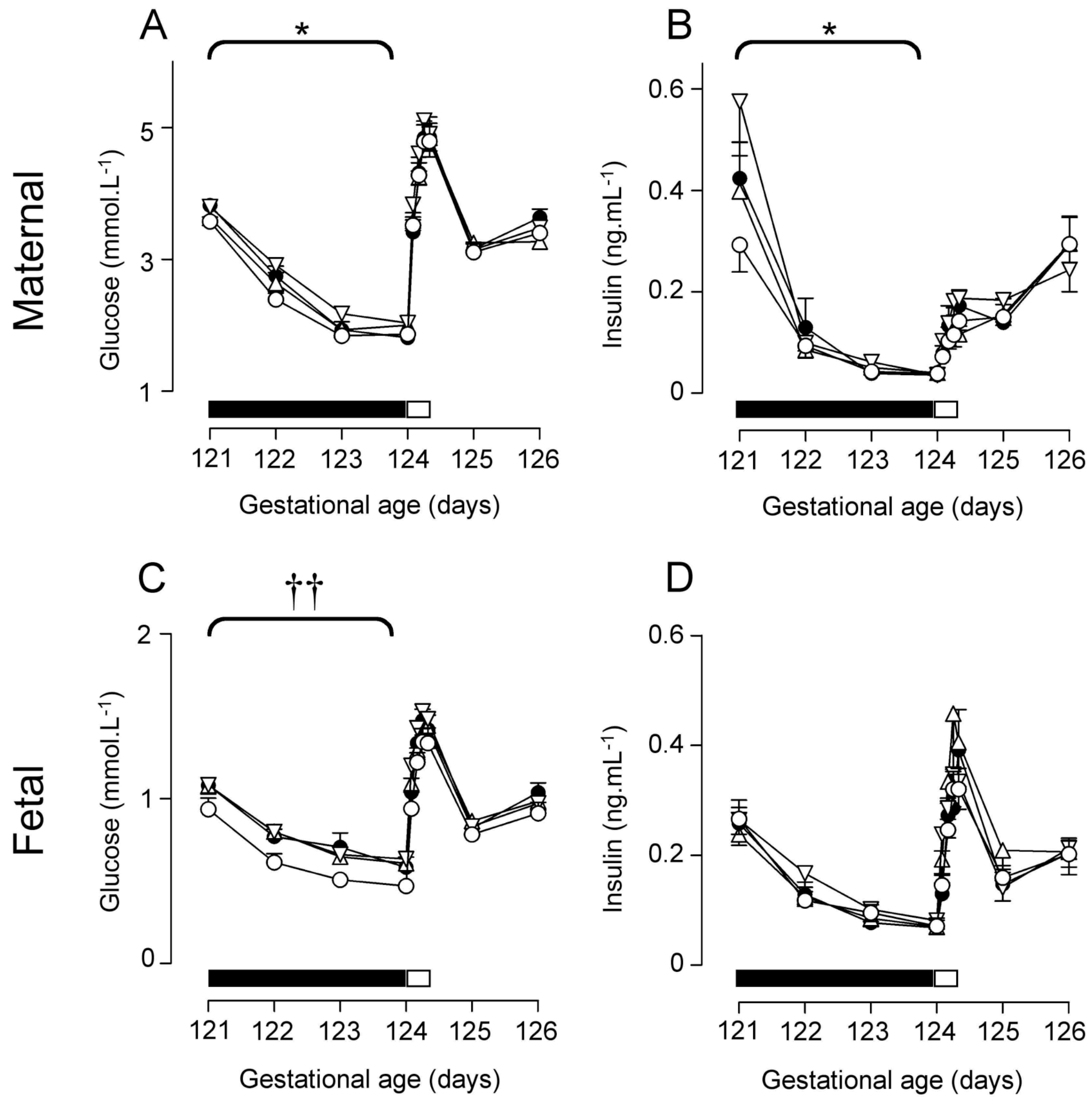

Figure 4-3: Maternal glucose (A) and insulin (B), and fetal glucose (C) and insulin (D) levels during fasting and refeeding

Fasting period (121 to 124 d) indicated by black bar, glucose infusion and initiation of refeeding by open bar. Control = ● (n=10), UN -60-0 = △ (n=10), UN -2-30 = ▽ (n=9), UN -60-30 = ○ (n=12). Data are mean ± SEM.
** p<0.05 for nutrition effect: UN -60-30 < UN -2-30*
†† p<0.01 for nutrition effect: UN -60-30 < all other groups

4.3.5 Fetal glucose and arginine challenges

Fetal plasma insulin levels peaked 15 min after an intravenous dose of glucose. There were no effects of nutritional group on the AUC of glucose and insulin in the glucose challenge, or on glucose or insulin peak levels (Figure 4-4A and B). Following the arginine bolus, fetal plasma insulin levels peaked at 2 min. There was no effect of nutritional group on arginine AUC and insulin AUC, or on peak plasma levels of arginine or insulin in the arginine challenge (Figure 4-4C and D). There was no effect of fetal sex on any of these parameters.

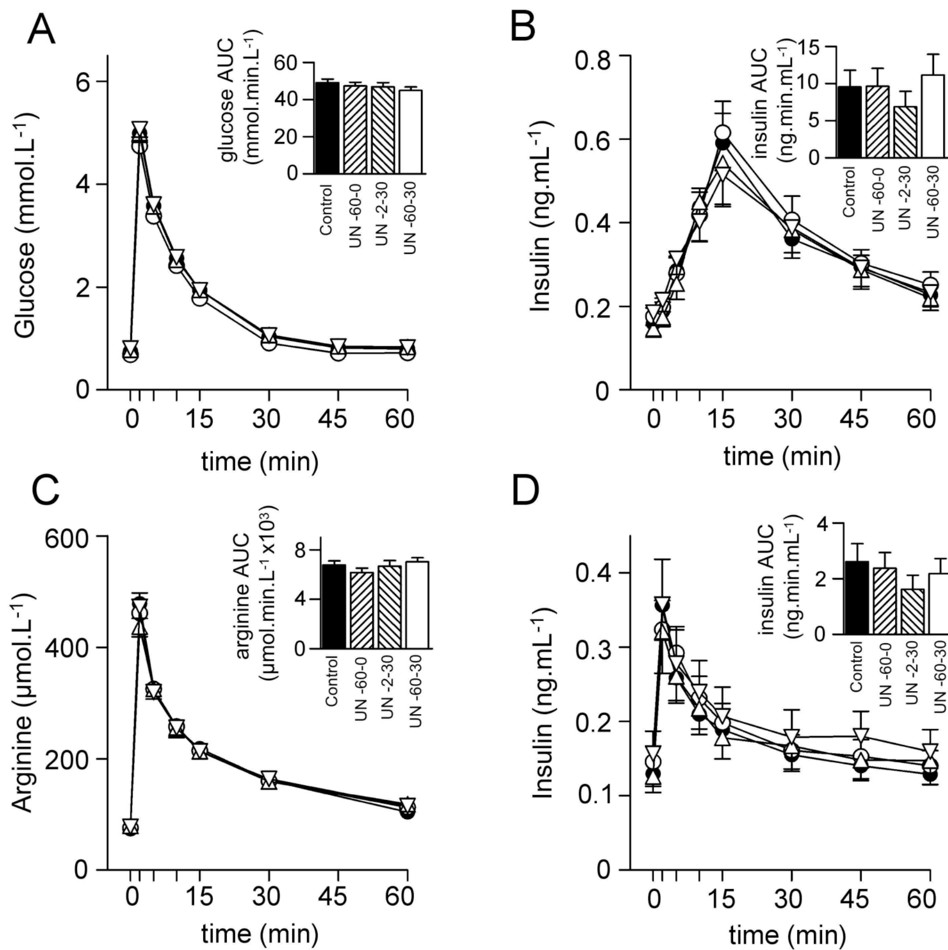

Figure 4-4: Fetal glucose (A) and insulin (B) responses to an intravenous glucose tolerance test; arginine (C) and insulin (D) responses to an intravenous arginine challenge

Areas under the curve shown as inset histograms. Control = ● (n=16), UN -60-0 = △ (n=12), UN -2-30 = ▽ (n=11), UN -60-30= O (n=13). Data are mean ± SEM.

4.3.6 Maternal HPA axis

There were no effects of nutritional group on maternal plasma ACTH, cortisol, cortisone and progesterone levels, or on the ratios of cortisol to cortisone and cortisol to ACTH at 114-121 d or 128-131 d (Table 4-5). Maternal plasma ACTH levels increased between 114-121 d and 128-131 d in the UN -60-30 ewes, while cortisol decreased in the UN -60-0 ewes over the same period. The cortisol to ACTH ratio and the cortisol to cortisone ratio decreased in the UN -60-30 and UN -60-0 ewes. Plasma progesterone levels did not change from 114-121 d to 128-131 d.

		Control	UN -60-0	UN -2-30	UN -60-30
n	114-121	11	10	9	12
	128-131	9	8	8	10
ACTH	114-121	33.2±5.8	26.8±3.2	37.8±5.9	25.7±13.4
($pg.mL^{-1}$)	128-131	33.9±10.8	31.8±1.8	36.9±8.0	34.1±3.1*
Cortisol	114-121	9.4±2.4	6.7±1.3	7.9±1.5	7.1±1.6
($ng.mL^{-1}$)	128-131	4.3±0.9	2.4±0.4*	7.2±1.2	5.6±2.6
Cortisone	114-121	2.1±0.2	1.6±0.1	2.1±0.3	1.6±0.2
($ng.mL^{-1}$)	128-131	1.7±0.1	1.3±0.2	2.1±0.2	1.6±0.5
Cortisol:ACTH	114-121	258±86	237±70	192±66	224±75
ratio	128-131	143±60	67±14*	204±66	94±41*
Cortisol:cortisone	114-121	3.7±0.5	3.8±0.50	3.5±0.6	3.8±0.5
ratio	128-131	3.1±0.6	1.9±0.2*	3.4±0.6	2.6±0.4*
Progesterone	114-121	9.0±0.4	8.7±0.8	10.4±1.2	10.0±0.6
($ng.mL^{-1}$)	128-131	9.0±0.7	10.6±1.3	10.7±1.0	10.5±1.2

Table 4-5: Maternal ACTH and steroid levels

Average maternal ACTH and steroid levels from 114–121 d and 128-131 d. Data are mean ± SEM.
** $p<0.05$ for comparison between time period within the same group.*

4.3.7 Fetal HPA axis

4.3.7.1 Baselines

There were no effects of nutritional group or fetal sex on fetal plasma levels of ACTH, cortisol, cortisone and DHEA, or on the ratios of cortisol to cortisone and cortisol to ACTH at 114-121 d or 128-131 d (Table 4-6). Fetal plasma cortisol levels increased from 114-121 d to 128-131 d, although the change was not significant in the UN -60-0 fetuses. Plasma DHEA levels decreased in the UN -60-0 fetuses over the same period. The ratios of cortisol to ACTH and cortisol to cortisone increased in all groups.

		Control	UN -60-0	UN -2-30	UN -60-30
n	114-121	11	10	9	12
	128-131	9	8	8	10
ACTH	114-121	20.7±4.7	20.9±2.4	18.6±2.5	17.2±1.1
($pg.mL^{-1}$)	128-131	19.6±2.5	30.4±7.8	21.7±4.8	15.8±2.0
Cortisol	114-121	0.96±0.31	1.30±0.77	0.73±0.17	1.00±0.20
($ng.mL^{-1}$)	128-131	4.94±1.89*	4.98±1.88	2.33±0.54**	3.40±1.05*
Cortisone	114-121	1.37±0.39	1.26±0.25	1.29±0.27	1.03±0.20
($ng.mL^{-1}$)	128-131	3.50±1.51	3.65±1.47	2.09±0.40*	1.54±0.21
Cortisol:ACTH	114-121	39±11	31±11	33±8	47±10
ratio	128-131	156±52*	154±27**	107±40**	151±44**
Cortisol:cortisone	114-121	0.74±0.07	0.67±0.17	0.56±0.07	0.97±0.16
ratio	128-131	1.82±0.35*	1.22±0.25*	1.63±0.30**	1.76±0.36*
DHEA	114-121	0.76±0.13	0.55±0.05	0.76±0.15	0.53±0.08
($ng.mL^{-1}$)	128-131	0.73±0.18	0.43±0.04*	0.64±0.11	0.62±0.10

Table 4-6: Fetal ACTH and steroid levels

Average fetal ACTH and steroid levels from 114-121 d and 128-131 d. Data are mean ± SEM.
** $p<0.05$, ** $p<0.01$ for comparison between time periods within the same group.*

4.3.7.2 CRH/AVP challenge

ACTH and cortisol levels rose rapidly following intravenous CRH/AVP in all animals. Nutritional group had no significant effect on ACTH AUC (Figure 4-5A) or cortisol AUC (Figure 4-5B), or the ratio of cortisol AUC to ACTH AUC (control: 82±21, UN -60-0: 75±21, UN -2-30: 69±14, UN -60-30: 92±28). There was no effect of fetal sex on these parameters.

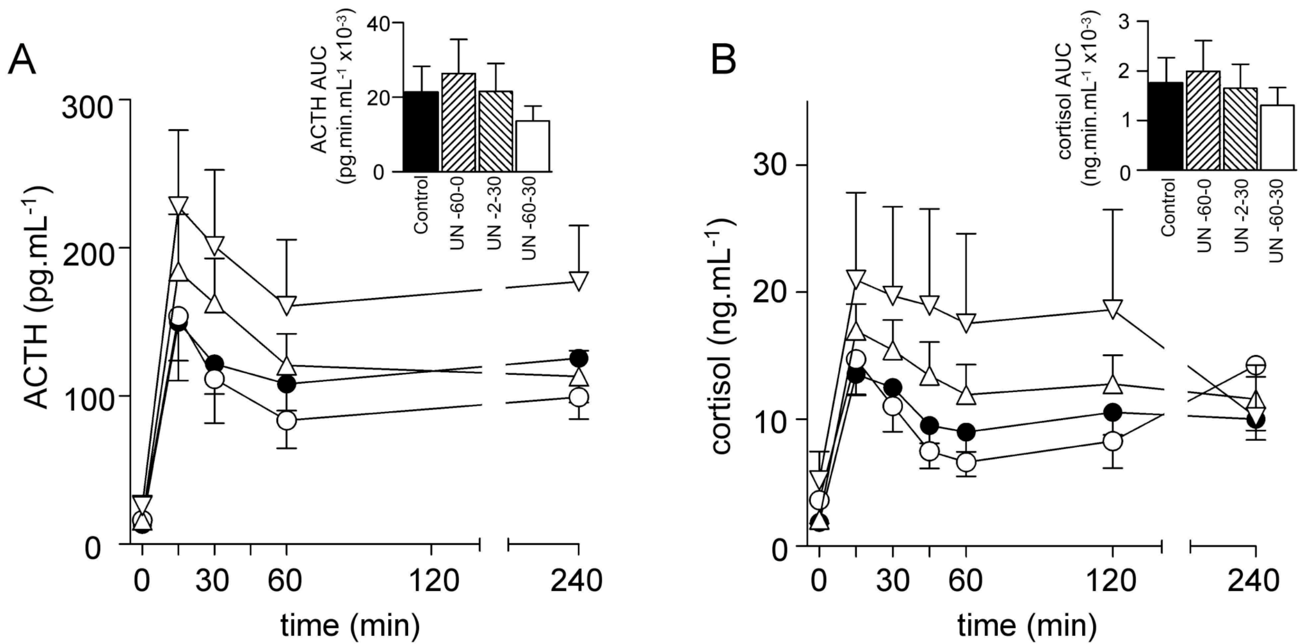

Figure 4-5: ACTH (A) and cortisol (B) responses to CRH/AVP challenge

Areas under the curve are shown as inset histograms. Control = ● (n=9), UN -60-0 = △ (n=9), UN -2-30 = ▽ (n=8), UN -60-30 = ○ (n=11). Data are mean ± SEM.

4.3.7.3 Metyrapone challenge

The metyrapone challenge resulted in decreased fetal plasma cortisol levels at 30 min in all groups ($p<0.01$ for time effect), with no difference between groups in the cortisol trough level ($p=0.52$) (control: 2.9±0.5 to 0.9±0.2, UN -60-0: 2.4±1.2 to 1.4±0.7, UN -2-30: 6.1±3.5 to 2.0±0.7, UN -60-30: 3.2±0.7 to 1.8±0.6 ng.mL^{-1}). Plasma ACTH (Figure 4-6A) and 11deoxycortisol levels (Figure 4-6B) increased in all groups. Nutritional group had no effect on ACTH AUC, 11deoxycortisol AUC, or the ratio of 11-deoxycortisol AUC to ACTH AUC (control: 185±156, UN -60-0: 113±58, UN -2-30: 112±67, UN -60-30: 215±139).

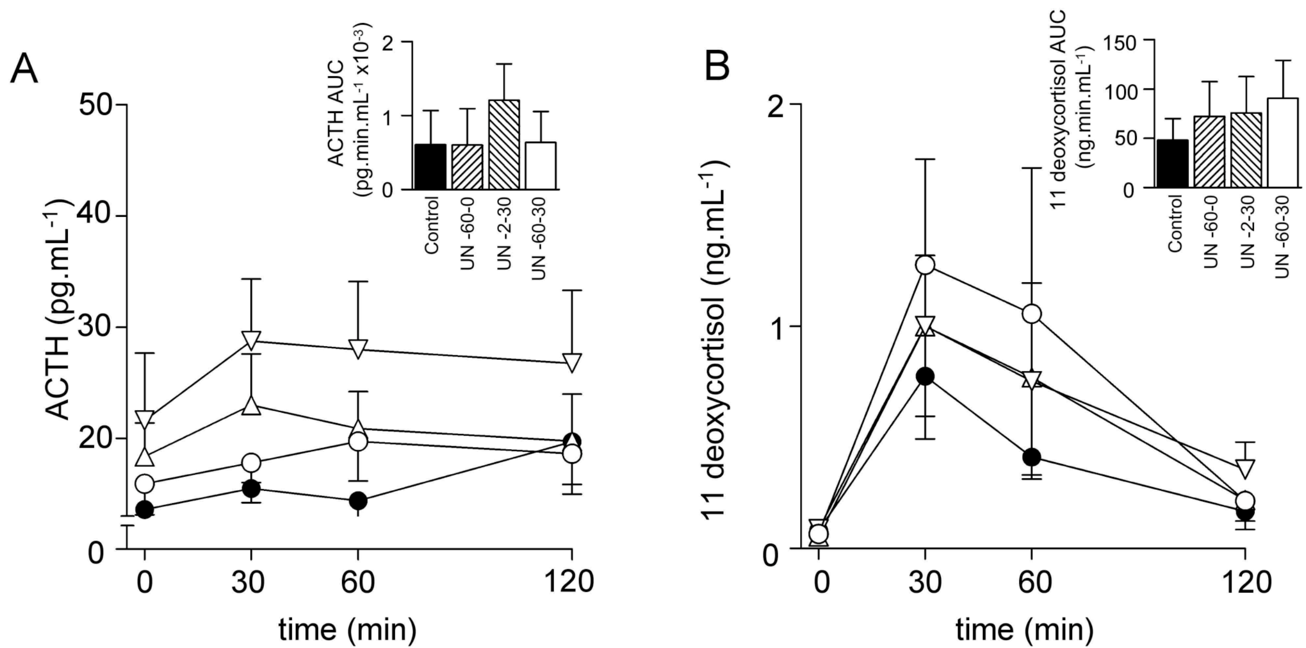

Figure 4-6: ACTH (A) and 11deoxycortisol (B) responses to metyrapone challenge

Areas under the curve are shown as inset histograms. Control = ● (n=9), UN -60-0 = △ (n=8), UN -2-30 = ▽ (n=8), UN -60-30= ○ (n=11). Data are mean ± SEM.

4.4 Discussion

The aim of this experiment was to distinguish between effects of pre- and postconception undernutrition on fetal growth, metabolism and endocrinology in late gestation. We hypothesised that the different effects on uterine environment, early embryo development, placental growth and maternal adaptation to pregnancy would result in different fetal outcomes. We found that preconception undernutrition resulted in slow fetal growth in late gestation and small size at 132 d compared with controls, and with a low fetal to placental weight ratio. In contrast, postconception undernutrition resulted in rapid fetal growth in late gestation and a fetal size at 132 d similar to controls. Fetuses undernourished throughout both periods grew at a similar rate to controls, but slowed their growth with maternal fasting. Thus, maternal nutrition prior to conception and in very early pregnancy results in different patterns of fetal growth in late gestation, but does not necessarily change fetal size near parturition. Our results reinforce the important concept that similar offspring sizes may result from different growth trajectories (Oliver *et al.*, 2005). Pre- and postconception undernutrition did not alter the function of the fetal glucose-insulin or HPA axes in late gestation.

The periconceptional undernutrition protocols used in this study may alter fetal development by the effects on conditions around conception and in early development, and/or by the different patterns of nutrient provision to the feto-placental unit through pregnancy, and/or by altered maternal and fetal endocrinology. Nutritional intake of ewes around conception resulting from the undernutrition regimens in this experiment will have different effects on environment for the early embryo, although the study did not address these effects directly. However, a variety of conditions around conception are known to influence fetal development. Undernutrition in the post-ovulation period in pigs alters the cleavage rate of embryos (Mburu *et al.*, 1998) and a low protein diet in rats decreases cell proliferation in the pre-implantation embryo resulting in lower cell numbers in the inner cell mass and trophectoderm (Kwong *et al.*, 2000). Altered maternal hormone status can also affect early development. For example, elevated progesterone levels around implantation can produce larger offspring in sheep (Kleemann *et al.*, 2001). Possible underlying mechanisms include effects on imprinted genes, which can influence feto-placental growth and function (Constancia *et al.*, 2002) and which undergo epigenetic modification in early development. These modifications may be vulnerable to altered glucocorticoid levels (Thomassin *et al.*, 2001) and dietary intake (Lillycrop *et al.*, 2005).

There were no significant differences in maternal glucocorticoid levels between nutritional groups in late gestation, suggesting that if different fetal exposures to glucocorticoids contribute to the differences between groups, the mechanism must lie in changes in the placental 11ßHSD-2 barrier or in the fetal rather than maternal HPA axis. Chronic nutrient restriction by 30% from 26 d gestation in sheep reduces placental 11ßHSD-2 activity in late gestation, but there is no effect if the nutritional restriction is limited to early, mid or late gestation (McMullen *et al.*, 2004). To our knowledge there are no studies investigating late-gestation placental 11ßHSD-2 activity following periconceptional undernutrition in the sheep.

4.4.1 Preconception undernutrition

The UN -60-0 regimen resulted in a small, slow-growing fetus in late gestation with a relatively large placenta. Human studies have tended to use body mass index (BMI) as an indicator of maternal nutrition. BMI, however, reflects a combination of genetic, environmental and dietary influences. Nevertheless, human studies have shown that a low maternal preconception BMI is associated with low offspring birth weight (Ronnenberg *et al.*, 2003) and small placentas (Thame *et al.*, 1997), and that risk of preterm delivery is highest for the combination of a low preconception BMI and poor weight gain during pregnancy (Schieve *et al.*, 2000). To our knowledge there are no studies in sheep that have investigated the effects of a low plane of nutrition prior to conception on late-gestation fetal growth trajectory. However, when ewes were fed at 70% of controls from 45 d before until 7 d after mating, a direct relationship existed between weight change over the periconceptional period and fetal weight at 53-56 d gestation in control ewes, but not in the undernourished ewes (MacLaughlin *et al.*, 2005). The correlation was no longer significant when placental weight was taken into account. The authors suggest that periconceptional undernutrition disrupts the relationship between maternal periconceptional weight change and feto-placental growth, but the biological basis of the difference in relationships between nutritional groups is unclear. In rats, a study of the offspring of dams that were protein-restricted before, but fed normally after mating, demonstrated increases in fetus, placenta and organ weights at birth, and elevated glucose and cholesterol levels in later life (Joshi *et al.*, 2003).

Some studies have suggested that the effects of poor maternal nutrition during pregnancy on fetal growth and postnatal physiology are due to a lack of substrates for tissue accretion during periods of organ growth (Barker *et al.*, 1995). The effect of

preconception nutrition on late-gestation fetal growth suggests that the mechanisms are more complex than that. This is demonstrated by a study that showed that growth response to a late-gestation nutritional insult is dependent on prior growth rate (Harding, 1997b). In that study slow-growing fetuses maintained their growth rate during 10 d of maternal undernutrition, whereas fast-growing fetuses did not. In the present study, however, the slow-growing UN -60-0 fetuses did slow their growth with fasting. The 10 d undernutrition period in the previous studies was designed to maintain maternal preprandial blood glucose concentrations of 1.4-1.6 mmol.L^{-1}, a decrease of around 40% from baselines of around 2.4 mmol.L^{-1}. This required 30-70 g of concentrate feed per day compared to the 950-1150 g.day^{-1} before the fast. Differences in the fetal response to a nutritional insult depend not only on the severity and time course of the insult (Mellor and Murray, 1982) and their own metabolic compensations, but also on the maternal and placental responses. Maternal blood glucose concentrations in the present study took 2 d to reach a stable trough with maternal fasting, approximately 50% below starting levels, demonstrating that this is a more severe insult than the undernutrition used in the previous studies. The fetus responds to limited nutrition by decreasing glucose uptake but increasing amino acid metabolism over the first 48 hours, while the placenta more gradually increases consumption of ketones from the mother (Harding and Gluckman, 2001). It has been postulated that slow-growing fetuses following periconceptional undernutrition may have reduced substrate demands or altered endocrine or paracrine systems to better cope with further episodes of nutritional limitation (Harding, 1997a), but the opposite appears to be the case in the UN -60-0 fetuses. This may be due to differences in the maternal and placental provision of nutrients during fasting, or in the fetal metabolic and endocrine responses.

As maternal weight, food intake and plasma metabolite and hormone levels in the UN -60-0 group were similar to those of controls in late gestation, and late-gestation uterine blood flow is increased in sheep undernourished around conception (Rumball *et al.*, 2007a), it is unlikely that a limitation in nutrient supply to the utero-placental circulation is responsible for the slower fetal growth in late gestation in the UN -60-0 group. However, periconceptionally undernourished ewes at 65 d gestation do demonstrate inadequate development of the normal insulin resistance associated with pregnancy (Jaquiery *et al.*, 2005), suggesting possible limitations on utero-placental nutrient provision in mid gestation. The UN -60-0 ewes were certainly not undernourished during pregnancy, and may have increased food intake during the first half of gestation as

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suggested by their rapid weight gain, although food intake was not directly measured. Research in adolescent sheep suggests that high nutrient intake in pregnancy results in a small placenta and fetus (Redmer *et al.*, 2004). However, it is not known whether this is due to the weight gain seen in adolescent sheep, suggesting that nutrient partitioning favours the mother, or to the different endocrine milieu of adolescence. Increased intake in normal adult ewes does not have this effect on feto-placental growth (Wallace *et al.*, 2005).

It is certainly possible that altered placental function may be contributing to the altered fetal growth. The UN -60-0 fetuses were smaller at postmortem with a relatively large placenta, and so had a significantly lower fetal to placental weight ratio than all other groups. Placental growth in sheep is maximal from 40 to 80 d gestation (Ehrhardt and Bell, 1995), whereas the fetus continues to grow throughout pregnancy. Thus the relationship between placental and fetal weight in studies of manipulated nutrition varies depending on the timing of insult during pregnancy (Heasman *et al.*, 1999). For example, maternal nutrient restriction in sheep through mid and late gestation results in large placentas and small fetuses, whereas restriction in mid gestation followed by normal nutrition results in large placenta and a large fetus (Faichney and White, 1987). In the UN -60-0 group, placental growth may be occurring at the expense of fetal growth, or impaired placental function may be limiting fetal growth.

Impaired placental remodelling may be one explanation for impaired placental function. Although there were no histological changes in placentomes at 55 d in sheep undernourished from 45 d before until 7 d after mating (MacLaughlin *et al.*, 2005), the sheep placenta does not reach peak size around 100 d gestation. It then undergoes a structural remodelling in the last trimester that results in decreased weight but increased umbilical and uterine blood flow, and increased nutrient transfer to the fetus (Schneider, 1996). The stimulus for the remodelling is not known, but in sheep undernourished by 50% between 28 and 77 d gestation, the placentas were smaller than those of controls at 80 d but larger at 140 d, primarily due to a gain in weight of the fetal component rather than a loss as occurred in the controls (Heasman *et al.*, 1999). Impaired nutrient transfer capacity is another possible contributing factor to poor placental transfer of nutrients. Placental GLUT-1 levels are altered by undernutrition in rats (Lesage *et al.*, 2002) and sheep (Dandrea *et al.*, 2001), and placental amino acid transport is affected by the maternal amino acid profile (Jozwik *et al.*, 2001).

If the cause for the slow late-gestation growth and small size of the UN -60-0 fetuses does indeed lie in effects of early environment on fetal development, it does not appear to be through effects on fetal glucose-insulin or HPA axis function in late gestation. Neither direct assessment by challenges, nor responses to a late-gestation maternal fast showed any differences between fetuses in this group and controls.

4.4.2 Postconception undernutrition

The UN -2-30 regimen resulted in fetuses that grew faster than those of other groups in late gestation and were similar size to controls at postmortem. Several sheep studies have investigated undernutrition from mating. In ewes undernourished by 50% from mating to 30 d gestation, offspring birthweight and postnatal growth were not affected, but cardiovascular function in later life was affected (Gardner *et al.*, 2004b). The fetuses of ewes undernourished by 15% from mating to 70 d gestation were not different in size from controls at 125-127 d gestation (Hawkins *et al.*, 1999). However, to our knowledge there are no other studies looking at fetal growth trajectory in late gestation following early gestation undernutrition. In rats, a low protein diet in the preimplantation period changes birthweight and postnatal growth (Kwong *et al.*, 2000). In humans, however, the Dutch famine cohorts that suffered exposure to famine in the first trimester of pregnancy did not have altered size at birth (Stein *et al.*, 2004). Poor maternal weight gain in the first and second, or second and third trimesters results in decreased birth weights, but poor weight gain in the first trimester alone, or the first and third trimesters results in normal birth weights (Abrams and Selvin, 1995). Our UN -2-30 group demonstrates a similar pattern to the latter cohort with high maternal weight gain in the mid gestation, and faster fetal growth in late gestation that is maintained through a maternal fast, such that fetus size was similar to controls at 132 d.

The more rapid fetal growth in the 126-131 d period in the UN -2-30 group did not appear to be due to differences in maternal food intake or increased maternal glucose levels, although, as we have previously shown increased uterine blood flow following postconception undernutrition (Rumball *et al.*, 2007a), there may be greater utero-placental nutrient supply. There were no differences in placental size or phenotype, but again it is possible that placental metabolism and transfer capacity may be different from controls. Although there were also no differences in fetal glucose-insulin and HPA axis challenge responses, the fetuses in this group did decrease their plasma glucose levels and increase their IGF-1 levels over the period of rapid growth. Furthermore, the ability to

maintain growth rate through a maternal fast suggested that the maternal and placental provision of metabolic and anabolic substrates is at least adequate. Placental function has been shown to improve following restriction by carunclectomy (Owens *et al.*, 1987c), but there are no data on placental function following periconceptional undernutrition.

In contrast to the similar HPA function found in the UN -2-30 and control fetuses, previous sheep studies in which ewes were undernourished by 15% from mating to 70 d gestation have found that the fetuses of undernourished ewes have blunted pituitary and adrenal responses in late gestation (Hawkins *et al.*, 1999; Hawkins *et al.*, 2000b). The different results between the studies may be due to the longer period of undernutrition in the previous studies impacting on fetal HPA axis development, or the severity of undernutrition experienced by the ewe. At the end of undernutrition at 70 d in the previous study the undernourished ewes had in fact gained a small amount of weight, in contrast to the 15% weight loss experience by ewes in our study.

4.4.3 Pre- and postconception undernutrition

In contrast with the growth patterns of fetuses following periods of pre or postconception undernutrition, the UN -60-30 regimen resulted in fetuses that grew at a similar rate to controls and were of similar size at surgery and postmortem. However, the UN -60-30 fetuses did slow their growth with fasting. The ewes of this group had not recovered their initial weight by late gestation, and their increased food intake may reflect an attempt to recover body weight while meeting the increased feto-placental demands of late gestation. When fasting prevented this compensatory strategy and exposed their lack of nutritional reserves, we observed lower maternal plasma glucose and insulin levels. The subsequently lower fetal plasma glucose levels may have accounted for the greater slowing of growth compared to control fetuses.

It is intriguing to speculate that the lack of an effect on fetal growth of undernutrition through both the pre- and postconception periods may reflect the opposing effects of the two different periods; preconception undernutrition decreasing but postconception undernutrition increasing fetal growth rate in late gestation. Such a speculation would need to invoke two different, and as yet unknown mechanisms. Alternatively, it is also possible that it is the consistency of nutritional intake through the mating period that distinguishes the control and UN -60-30 fetuses from the UN -60-0 and UN -2-30 fetuses. Changes from a low to high or high to low plane of nutrition may result in altered plasma metabolite and hormone levels, which in turn may alter oviductal and

uterine environment. The onset of and relief from undernutrition in sheep, for example, produces acute changes in plasma glucocorticoid levels (Jaquiery *et al.*, 2006) which in turn could alter early embryonic development.

A previous study of periconceptional undernutrition from 60 d before until 30 d after mating found that the fetuses of undernourished ewes grew more slowly than controls in the period prior to parturition (126 to 145d), but were the same weight at birth (Oliver *et al.*, 2005). In addition, the undernourished ewes delivered earlier than controls (Bloomfield *et al.*, 2003b). Fetal growth slows prior to delivery and it may be that the observations in the present study up to 131 d precede the differences in fetal growth rate observed in the earlier study. We also do not know if the UN -60-30 ewes in this study would have gone on to deliver early, or whether there were other unexplained differences in the physiological effects on the fetus of the undernutrition protocols used in the two studies.

This possibility is also suggested by the HPA and glucose-insulin axis tests. The UN -60-30 undernutrition regimen used in the present study was similar to that used in previous studies which found that fetuses of undernourished ewes had an increased fetal insulin response to a glucose challenge (Oliver *et al.*, 2001b) and an increased 11-deoxycortisol response to metyrapone (Bloomfield *et al.*, 2004). These results suggested that periconceptional undernutrition accelerated fetal pancreatic and HPA axis maturation, consistent with the tendency to premature delivery. However, the UN -60-30 fetuses in the present study did not demonstrate any differences compared to controls in responses to glucose, arginine, CRH/AVP or metyrapone challenge. There are several possible reasons for the different results. Firstly, the dose of metyrapone used in the present study was higher than in the earlier studies, which would have increased the stimulus to ACTH production in all groups and may have obscured the differences that were present at a lower dose. Secondly, as the present study was spread over three breeding seasons it is possible that flock or seasonal differences may have increased the variability of the data and obscured any differences. Thirdly, there were significant differences in the maternal starting weights and weight changes between the two sets of studies. In the present study the mean weight of control sheep increased from 64 to 68 kg during the study period, whereas in the earlier studies the change was from 56 to 70 kg. It is possible that the higher starting weight and limited weight gain in sheep in the present study has produced different effects on the placenta and fetus, as it has been shown that the weight change in the periconceptional period relates to feto-placental growth in early pregnancy

(MacLaughlin *et al.*, 2005), and in humans the degree of weight gain relates to the risk of preterm delivery (Schieve *et al.*, 1999). In the previous studies, the greater contrast of weight gain in the control sheep and weight loss in the undernourished sheep may have been crucial to producing the physiological and endocrine effects observed in the fetuses.

4.4.4 Conclusions

Our study demonstrates that pre- and postconception maternal undernutrition produce different patterns of fetal and placental growth but do not cause differences in the fetal glucose-insulin and HPA axes. The findings of this study have several important implications. Firstly, fetal size is a poor surrogate for fetal development. Just as the trajectory of postnatal growth modifies the risks of adult diseases associated with low birth weight (Osmond and Barker, 2000), fetal growth trajectory may similarly represent an important risk factor. Secondly, preconception undernutrition alone results in altered fetal development suggesting that the fetal effects are not due solely to limitation of substrates for tissue accretion, nor to excess fetal glucocorticoid exposure at the time of undernutrition. Thirdly, these results suggest that the effects of a low nutrition plane prior to conception should be studied in humans in order to understand the importance of preconception diet and to guide advice for women of childbearing potential.

5. Effects of twinning and periconceptional undernutrition on maternal metabolism, fetal growth and glucose-insulin axis function in ovine pregnancy

5.1 Introduction

Both human and animal studies have demonstrated an association between reduced size at birth and altered postnatal physiology (Lithell *et al.*, 1996; Oliver *et al.*, 2002) and the risks of metabolic and cardiovascular disorders in later life (Barker, 2004b). Most of the experimental studies have involved multitocous species, or mixtures of twin and singleton pregnancies, without careful examination of any possible differences between these.

Twins commonly have reduced size at birth compared to singletons. However the relationship between size at birth and postnatal physiology and disease risk is much less clear in twins, with some studies reporting reduced glucose tolerance (Poulsen *et al.*, 1997), greater increases in blood pressure in infancy (Levine *et al.*, 1994), and increased HPA axis responsiveness (Wust *et al.*, 2005) with reducing birthweight in twins, whereas others report no increased incidence of hypertension (Williams and Poulton, 1999) and coronary heart disease (Vagero and Leon, 1994) in twins, despite lower birth weights. There are many possible reasons for this lack of clarity and its challenge to the Fetal Origins of Adult Disease hypothesis, including small sample sizes and use of selected populations (Morley and Dwyer, 2005; Phillips *et al.*, 2001). However, it is also possible that the physiology of twin pregnancy is fundamentally different from that of singleton pregnancy, so that both the causes of low birthweight and the mechanisms underlying the associations with postnatal disease risk are also different. This is supported by human and animal studies in which the associations between birthweight and altered postnatal physiology in twins are stronger for the within twin-pair coefficient for birthweight rather than the between twin-pair coefficient (Bloomfield *et al.*, 2007; Dwyer *et al.*, 1999).

Studying differences between twin and singleton pregnancies in fetal growth patterns and physiology may help explain why these associations are less clear for twins, and may suggest mechanisms underlying these relationships.

Twinning is a periconceptional event, and twins develop in a different environment to singletons from conception at least until weaning. Throughout gestation twin fetuses compete for maternal nutrients, have smaller placentas than singletons, and restricted physical space. After delivery they compete for milk supply and parental attention. Maternal metabolic and nutritional status before conception can influence the likelihood of a twin conception (Kenyon *et al.*, 2004), but differences in maternal metabolic status later in pregnancy between twin and singleton pregnancies have received little attention.

Reduced birth size and ponderal index in singletons, which correlates with the risk of adult metabolic disease, is most often due to growth limitation in late gestation. However the growth of twins may be different from that of singletons from very early in pregnancy. When measured by ultrasound scanning, growth of twins lags behind that of singletons from around 16-20 weeks gestation (Farina *et al.*, 1999; Leveno *et al.*, 1979), although some data suggest that growth discrepancy (Iffy *et al.*, 1983) and growth disparity within twin pairs (Xu *et al.*, 1995) may occur earlier. It is not known to what extent these differing trajectories are determined in early gestation, or are in response to the differing levels of nutrients available in later gestation. Human triplets reduced to twins at 8-11 weeks are heavier at birth than the non-reduced triplets (Boulot *et al.*, 2000), but not as heavy as non-reduced twins (Sebire *et al.*, 1997) suggesting both early and late gestation effects.

Our group has previously reported the effects of another periconceptional influence, that of maternal undernutrition, on fetal growth and physiology in sheep. All of these studies were done in singleton pregnancies. Mild undernutrition of the ewe before and for the first month of pregnancy resulted in an altered maternal metabolic and endocrine environment throughout pregnancy (Oliver *et al.*, 2005). Fetal growth trajectory was slowed with altered growth responses to a nutritional challenge in late gestation (Harding, 1997a) but size at birth was unaffected (Oliver *et al.*, 2005). Fetal metabolic and endocrine maturation was also perturbed (Bloomfield *et al.*, 2004), including that of the glucose-insulin axis (Oliver *et al.*, 2001b). At least some of these effects persist after birth (Todd *et al.*, 2007).

Thus both twinning and periconceptional undernutrition result in altered fetal growth in late gestation, and are initiated at a similar time in pregnancy. If the physiology

of twin pregnancy is primarily determined in the periconceptional period, we hypothesised that a twin pregnancy may have similar features in late gestation to those of periconceptional undernutrition. Specifically, we hypothesised that, when compared to well-nourished singleton pregnancy, both twinning and periconceptional undernutrition would result in similar alterations in maternal metabolism in late gestation, fetal growth trajectory in response to a late gestation nutritional insult, and maturation of the fetal glucose-insulin axis. Conversely, if the physiology of twin pregnancy is also determined by additional influences in late gestation, there may be an interaction between the effects of twinning and those of periconceptional undernutrition. We therefore compared maternal metabolism, fetal growth and glucose-insulin axis function between singleton and twin pregnancies in ewes that were either well-nourished or exposed to periconceptional undernutrition.

5.2 Methods

5.2.1 Experimental design

Non-pregnant ewes were randomly allocated to control or UN -60-30 nutritional groups. Pregnancy and fetal number was established at 55 d by ultrasound. The ewes underwent surgery at 111±1 d for placement of maternal and fetal vascular catheters and fetal growth catheters and allowed to recover for at least 3 d. Both fetuses were catheterised in the case of twins. Twenty-eight singleton-bearing and 28 twin-bearing ewes entered this experiment.

Baseline maternal and fetal blood samples were taken in the morning before feeding on days 114, 117, 121, 127 and 131.

On day 118, after an overnight fast, a glucose challenge was performed on the fetuses. Four h after the conclusion of the glucose challenges, an arginine challenge was performed. Challenges were performed simultaneously in twin fetuses.

Ewes were fasted from day 121 to 124, and maternal and fetal blood samples were taken daily. On day 124 ewes were refed and also given an intravenous glucose infusion of 25 g over 8 h, aiming to restore maternal and hence fetal blood glucose as rapidly as possible in a ‘square wave’ pattern. Further blood samples were collected 2, 4, 6 and 8 h after the start of the glucose infusion, and at 24 and 48 h of refeeding.

The sheep were euthanised with an overdose of pentobarbitone on day 132 and a postmortem performed. The fetus and placenta were measured, dissected and weighed.

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For twins, placentomes were assigned to the relevant twin, and placental mass is reported per twin rather than total placental mass for the pregnancy.

5.2.2 Data analysis

Maternal food intake from 114 d was compared on days that it was unrestricted. Metabolite and hormone data were averaged over the periconceptional period (-60 to 30 d), mid gestation (40 to 97 d), and late gestation when in the laboratory (114 to 121 d) for display in tables.

Maternal metabolite and hormone data were compared using a two-way ANOVA with twinning, nutritional group, and the interaction between these terms as independent variables, and the Tukey-Kramer correction for multiple comparisons. A similar two-way repeated measures ANOVA was used for comparing the fasting and refeeding periods. Maternal to fetal glucose gradients were calculated as the difference between maternal and fetal plasma glucose levels, using data from all samples excluding the challenges and fasting-refeeding period.

For the fetal glucose and arginine challenges, AUC was calculated from baseline. Fetal data were compared using a two-way ANOVA with twinning, nutritional group, and their interaction as independent variables, and with sheep number nested within groups to allow for the non-independence of twins. The Tukey-Kramer method was used to correct for multiple comparisons. Estimated fetal weight was also included as a covariate in the analyses of the glucose and arginine challenge data to account for the variable dose of glucose per kg fetal body weight. The illustrated AUCs are the least square means from the multivariate analysis.

Fetal growth was compared over pre-fasting, fasting and post-fasting periods using linear regression with twinning, nutritional group, and their interaction as independent variables, and with sheep number nested within groups to allow for the non-independence of twins. Changes in growth rate with maternal fasting were tested for using multiple linear regression with a variable delineating pre-fasting from fasting, with the group by time period interactions being the variables of interest (Harding, 1997b).

Metabolite and hormone concentrations were compared in heavy and light twins using a two-way ANOVA with heavy/light, nutritional group, and their interaction as independent variables. Growth rates were compared using multiple linear regression with heavy/light, nutrition group, and their interaction as independent variables.

5.3 Results

Twenty-eight singleton-bearing ewes (15 control, 13 UN -60-30) entered the experiment, 22 (10 control, 12 UN -60-30) completed the fast-refeed protocol, and postmortem data were available for 19 (9 control, 10 UN -60-30). Twenty-eight twin-bearing ewes (13 control, 15 UN -60-30) entered the experiment, 22 (12 control, 10 UN -60-30) completed the fast-refeed protocol, and postmortem data were available for 19 (11 control, 8 UN -60-30). Not all samples were available from all animals because of catheter failures and some fetal losses.

The average weight loss due to periconceptional undernutrition was 14.8±1.2% in singleton and 16.3±2.5% in twin-bearing ewes. There was no weight difference between singleton and twin-bearing ewes at day 110, but UN -60-30 ewes were still lighter than controls (Table 5-1).

	Singleton-bearing ewes		**Twin-bearing ewes**	
	Control (n=15)	**UN -60-30** (n=13)	**Control** (n=13)	**UN -60-30** (n=15)
Day -71	64.1±1.3	65.0±1.5	62.9±1.9	66.0±1.5
Day -2	65.9±1.5	55.2±1.3**	64.1±1.8	55.6±1.1**
Day 110	68.5±1.5	62.5±1.6*	67.5±1.1	66.8±1.6*

Table 5-1: Maternal body weights (kg)

Data are mean ± SEM.
*** p<0.01, * p<0.05 for nutrition effect*

5.3.1 Fetal growth

At surgery, twin fetuses had smaller chest girths than singletons (Table 5-2). They then grew more slowly than singletons from 114 to 121 d (average girth increment 2.51±0.07 vs 2.95±0.12 mm.day^{-1}, p<0.01), and slowed their growth by 76% with maternal fasting (to 0.59±0.19 mm.day^{-1}) compared with 46% for singletons (to 1.58±0.31 mm.day^{-1}) (p<0.01 for single/twin by time effect) (Figure 5-1). Growth rate increased with refeeding, but twin fetuses still grew more slowly than singletons (2.05±0.10 vs 2.48±0.14 mm.day^{-1}, p<0.01). Twins had smaller chest girths, were significantly lighter and shorter and had smaller limbs than singletons at postmortem on day 132 (Table 5-2). Organ

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weights were less in twins, except for brain and pancreas weights (Table 5-3). Placentome number and total weight per fetus were also lower in twins than singletons with D placentomes contributing a smaller proportion of placental weight in twins (Table 5-4).

There was no effect of nutrition on chest girth or limb length at surgery (Table 5-2) or growth rates (UN -60-30 vs control: pre-fast 2.64±0.10 vs 2.66±0.08 $mm.day^{-1}$, p= 0.41, fast 1.02±0.24 vs 0.82±0.24 $mm.day^{-1}$, p=0.41, post-fast 2.27±0.14 vs 2.16±0.10 $mm.day^{-1}$, p=0.59). There was also no effect of nutrition on fetal size at postmortem (Table 5-2), but the fetuses of UN -60-30 ewes had increased liver weight but decreased spleen and chest thymus weights (Table 5-3). There was no difference in placental weight, but D placentomes contributed a greater proportion of placental weight in UN -60-30 sheep (Table 5-4).

Within twin pairs, the heavier fetus had a larger chest girth at surgery (Table 5-2), and grew faster from 114 to 121 d (2.64±0.11 vs 2.37±0.09 $mm.day^{-1}$, $p<0.01$). Although there was no difference in growth rates during the fast between heavy and light fetuses, there was an interaction between heavy/light and nutritional group such that the light fetus in control sheep grew more slowly during the fast than the light fetus in UN -60-30 sheep (0.23±0.31 vs 1.08±0.57 $mm.day^{-1}$, $p<0.05$). After refeeding, heavy fetuses continued to have a higher growth rate than light fetuses (2.38±0.15 vs 1.69±0.13 $mm.day^{-1}$, $p<0.01$) (Figure 5-1). Heavier fetuses had significantly heavier livers, spleens and hearts, but there were no significant differences in other morphometric measurements or placental size at postmortem.

There was no effect of fetal sex or the sex mix of twin pairs on fetal growth.

	Singletons		Twins					
	Control	UN -60-30	Control			UN -60-30		
			Overall	Heavy	Light	Overall	Heavy	Light
Surgery 111±1 d	(n=15)	(n=13)	(n=26)			(n=30)		
Chest girth (cm)	27.0±0.5	26.2±0.8	25.3±0.4†	26.3±0.5$^{\int}$	24.4±0.6	26.3±0.3†	26.5±0.4$^{\int}$	26.0±0.5
Hindlimb length (cm)	25.9±0.5	25.5±0.3	25.2±0.3	25.2±0.4	25.2±0.4	25.6±0.2	25.6±0.4	25.6±0.3
Post mortem 132 d	(n=9)	(n=11)	(n=20)			(n=16)		
Fetal weight (g)	4391±147	4317±128	3610±130††	3867±185$^{\int\int}$	3352±149	3665±86††	3844±113$^{\int\int}$	3487±97
Chest girth (cm)	34.6±0.6	35.3±0.7	32.0±0.5††	32.7±0.7	31.3±0.7	32.5±0.4††	32.5±0.6	32.5±0.6
Crown-rump length (cm)	46.9±1.0	46.4±0.6	42.8±0.7††	43.3±1.2	42.2±0.7	44.6±0.8††	44.8±0.9	44.4±1.3
Ponderal index ($g.cm^{-3}.10^{3}$)	43.1±2.2	43.5±1.6	46.4±1.4	48.2±2.3	44.6±1.6	42.0±1.6	43.2±1.9	40.8±2.7
Hindlimb length (cm)	33.7±0.5	33.3±0.5	31.1±0.4††	31.7±0.6	30.5±0.4	32.4±0.4††	32.8±0.7	32.1±0.4

Table 5-2: Fetal morphometric data at 111 and 132 d

Data are mean ± SEM.
†† *$p < 0.01$,* † *$p<0.05$ for twin effect*
$^{\int\int}$ *$p<0.01$,* $^{\int}$ *$p<0.05$ for heavy/light effect*

	Singletons		Twins					
	Control (n=9)	UN -60-30 (n=11)	Control (n=20)			UN -60-30 (n=16)		
			Overall	Heavy	Light	Overall	Heavy	Light
Brain	45.6±1.5	47.3±1.3	46.9±1.1	48.4±1.7	45.2±1.4	46.6±1.0	47.0±1.7	46.2±0.9
Liver	149±7	170±17*	100±4††	110±5$^{\int\int}$	88±5	106±5.5††*	116±8$^{\int\int}$	96±6
Kidney	15.2±1.1	16.5±1.5	12.7±0.7††	13.5±1.2	11.9±0.7	13.0±0.9††	13.5±1.4	12.6±1.2
Adrenal	0.27±0.02	0.29±0.03	0.21±0.02†	0.24±0.02	0.19±0.02	0.26±0.02†	0.26±0.02	0.27±0.03
Spleen	10.5±1.6	12.7±2.1**	5.4±0.4††	6.3±0.5$^{\int\int}$	4.4±0.6	7.3±0.6††**	8.2±0.8$^{\int\int}$	6.5±0.7
Heart	34.8±1.8	32.8±0.9	25.1±1.7††	28.0±1.4$^{\int}$	22.2±2.8	27.6±0.9††	28.8±1.5$^{\int}$	26.4±1.1
Lung	119±4	118±7	96±6††	108±6	85±10	100±5††	102±9	97±5
Chest thymus	5.9±0.8	4.5±0.5**	3.6±0.3††	4.2±0.4	3.1±0.5	3.2±0.3††**	3.5±0.4	3.0±0.4
Neck thymus	11.2±1.1	9.2±0.5	7.8±0.6†	8.9±0.7	6.5±0.9	8.4±1.1†	9.4±2.2	7.3±0.7
Thyroid	1.20±0.10	1.00±0.07	0.86±0.07††	0.91±0.10	0.82±0.10	0.80±0.06††	0.77±0.11	0.84±0.03
Pancreas	3.3±0.2	3.7±0.3	3.2±0.2	3.5±0.2	2.9±0.4	3.3±0.2	3.6±0.4	3.0±0.3

Table 5-3: Fetal organ weights (g) at 132 d

Data are mean ± SEM.
*** $p<0.01$, * $p<0.05$ for nutrition effect*
†† $p < 0.01$, † $p<0.05$ for twin effect
$^{\int\int}$ $p<0.01$, $^{\int}$ $p<0.05$ for heavy/light effect

	Singletons		Twins					
	Control (n=9)	**UN -60-30** (n=11)	**Control** (n=20)			**UN -60-30** (n=16)		
			Overall	Heavy	Light	Overall	Heavy	Light
Placentome number	81.6±3.3	77.3±6.6	52.9±2.8††	56.7±3.9	49.1±3.8	58.5±3.1††	53.9±2.7	63.1±5.4
Placenta weight (g)	536±29	594±38	382±23††	406±30	359±33	382±18††	373±28	391±25
% A placentomes by weight	22±8	10±3	25±6	25±8	25±10	22±6	25±9	21±10
% B placentomes by weight	49±9	37±9	47±6	53±8	42±9	44±7	43±10	42±10
% C placentomes by weight	16±5	20±5	19±5	18±6	20±9	14±4	11±4	22±10
% D placentomes by weight	14±9	32±11**	9±3†	4±3	14±5	20±7†**	21±10	15±6
Fetal : Placental weight ratio	8.4±0.5	7.5±0.4	9.9±0.5†	9.8±0.6	9.9±0.9	9.9±0.5†	10.7±0.8	9.1±0.5

Table 5-4: Placenta measurements at 132 d

Data are mean ± SEM. Placental weights in twins refer to placental weight per twin.
*** $p<0.01$ for nutrition effect*
†† $p < 0.01$, † $p < 0.05$ for twin effect

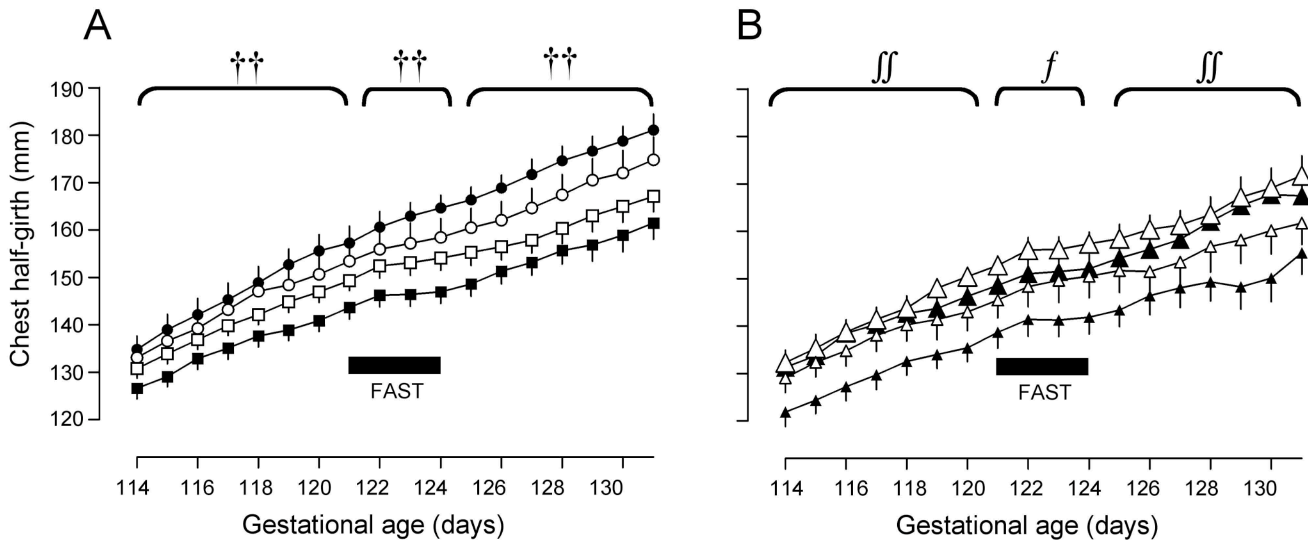

Figure 5-1: Fetal chest girth growth curves

A: Fetal growth curves in singleton and twins
Singleton control = ●, singleton UN -60-30 = ○, twin control = ■, twin UN -60-30 = □.
†† p<0.01 for twin effect

B: Fetal growth curves in heavy and light twins
Heavy control = ▲, light control = ▴, heavy UN -60-30 = △, light UN -60-30 = ▵.
∫∫ p<0.01, ∫ p<0.05 for heavy/light effect
f p<0.05 for heavy/light x nutrition interaction

5.3.2 Metabolism

5.3.2.1 Maternal

Plasma metabolite and hormone levels were not different in twin and singleton-bearing control ewes in early and mid gestation, but twin-bearing ewes had lower plasma glucose and insulin levels and higher plasma FFA and ketone levels than singleton-bearing ewes in late gestation (Table 5-5). Maternal food intake from 114 d was 14% less in twin than singleton-bearing ewes (1802±76 vs 2099±68 $g.day^{-1}$, p<0.01).

During the undernutrition period UN -60-30 ewes had lower plasma glucose, insulin and urea levels than controls, and higher plasma FFA and ketone levels. Decreased plasma insulin levels persisted into late gestation, accompanied by lower IGF-1 levels, but other plasma metabolite levels were not different from controls through the rest of

gestation (Table 5-5). Food intake from day 114 was 7% greater in UN -60-30 ewes than controls (2024±74 vs 1884±78 g.day^{-1}, $p<0.01$).

5.3.2.2 Fetal

Twin fetuses had lower plasma glucose, insulin and urea levels than singletons (Table 5-5) in late gestation, but there were no significant differences in plasma lactate (data not shown) or IGF-1 levels.

There were no significant differences between heavy and light twins in plasma glucose (0.83±0.03 vs 0.77±0.02 mmol.L^{-1}, p=0.13), insulin (0.15±0.02 vs 0.15±0.02 ng.mL^{-1}, p=0.84), IGF-1 levels (73.0±3.4 vs 69.4±3.2 ng.mL^{-1}, p=0.32), urea (5.23±0.20 vs 5.30±0.19 mmol.L^{-1}, p=0.81) or lactate (1.32±0.07 vs 1.36±0.08 mmol.L^{-1}, p=0.81) levels. Twinning reduced the maternal to fetal glucose gradient (2.46±0.03 vs 2.63±0.03 mmol.L^{-1}, $p<0.01$).

UN -60-30 fetuses had lower plasma glucose and insulin levels in singletons, and higher plasma urea levels in twins compared to controls. There were no differences in other plasma metabolite and hormone levels between nutritional groups (Table 5-5). There was no effect of nutritional group on the maternal to fetal glucose gradient (2.52±0.03 vs 2.52±0.03 mmol.L^{-1}, p=0.66).

There was no effect of fetal sex or sex mix of twin pairs on metabolite levels.

			Singletons		Twins	
		Gestational age (d)	Control (n=10)	UN -60-30 (n=12)	Control (n=12)	UN -60-30 (n=10)
Glucose (mmol.L^{-1})	Maternal	-61	4.02±0.14	4.08±0.26	3.99±0.14	3.80±0.09
		-60 to 30	3.71±0.10	3.48±0.07**	3.78±0.05	3.53±0.06**
		40 to 97	3.74±0.10	3.55±0.07	3.65±0.07	3.65±0.05
		114 to 121	3.69±0.06	3.53±0.05	3.33±0.07††	3.31±0.07††
	Fetal	114 to 121	1.03±0.04	0.91±0.05*	0.80±0.02††	0.81±0.03††
Insulin (ng.mL^{-1})	Maternal	-61	0.19±0.03	0.19±0.05	0.14±0.03	0.15±0.02
		-60 to 30	0.18±0.02	0.13±0.01**	0.18±0.02	0.11±0.02**
		40 to 97	0.20±0.04	0.11±0.02*	0.19±0.02	0.14±0.03*
		114 to 121	0.33±0.03	0.25±0.02*	0.17±0.02††	0.16±0.01*††
	Fetal	114 to 121	0.20±0.02	0.21±0.02	0.16±0.02††	0.14±0.02††
IGF-1 (ng.mL^{-1})	Maternal	-61	44.3±3.6	41.7±2.4	40.3±3.6	34.7±2.1
		-60 to 30	44.2±2.1	41.3±2.0	43.1±3.2	42.6±1.8
		40 to 97	46.5±2.2	44.4±2.0	47.6±4.9	45.2±1.5
		114 to 121	77.6±9.9	46.6±5.0*	70.1±7.1	56.9±8.0*
	Fetal	114 to 121	71.5±6.8	62.4±6.1	68.7±3.5	74.2±3.0
ßHBA (mmol.L^{-1})	Maternal	-61	0.15±0.01	0.18±0.02	0.16±0.02	0.19±0.02
		-60 to 30	0.19±0.01	0.21±0.01**	0.17±0.01	0.25±0.01**
		40 to 97	0.22±0.02	0.22±0.01	0.24±0.03	0.25±0.02
		114 to 121	0.45±0.02	0.44±0.04	0.73±0.11††	0.65±0.06††
FFA (mmol.L^{-1})	Maternal	-61	0.54±0.08	0.58±0.12	0.4 ±0.13	0.46±0.09
		-60 to 30	0.35±0.05	0.56±0.06**	0.25±0.02	0.62±0.07**
		40 to 97	0.56±0.06	0.37±0.06	0.43±0.06	0.44±0.07
		114 to 121	0.20±0.03	0.19±0.03	0.46±0.08††	0.37±0.03††
Urea (mmol.L^{-1})	Maternal	-61	8.39±0.50	9.34±0.48	8.92±0.61	7.21±0.64
		-60 to 30	7.46±0.29	5.55±0.17**	7.17±0.27	5.40±0.28**
		40 to 97	5.85±0.15	5.57±0.19	5.57±0.23	5.72±0.20
		114 to 121	5.08±0.33	5.13±0.38	4.40±0.35	4.60±0.21
	Fetal	114 to 121	5.71±0.29	5.76±0.35	5.14±0.21††	5.42±0.16**††

Table 5-5: Maternal and fetal plasma metabolite and hormone levels

Day -61 was prior to the start of experiment. Days -60 to 30 were the period of undernutrition. Day 40 to 97 was after undernutrition and prior to surgery. Day 114 to 121 was after surgery. Data are mean ± SEM.
†† $p < 0.01$ for twin effect
** $p<0.05$, ** $p < 0.01$ for nutrition effect*

5.3.3 Fetal glucose-insulin axis

Twinning had no effect on the glucose AUC in response to a glucose challenge (Figure 5-2A), but twin controls had a greater insulin response than singleton controls (Figure 5-2B). There was no effect of twinning on the arginine AUC (Figure 5-2C), but twins had a significantly lesser insulin response to arginine compared with singletons (Figure 5-2D). There were no significant differences in responses to the challenges between heavy and light twins.

Periconceptional undernutrition resulted in a decreased glucose AUC (Figure 5-2A), and abolished the effect of twinning on insulin response to glucose (Figure 5-2B). There was no nutritional effect on arginine AUC (Figure 5-2C) or insulin response to arginine (Figure 5-2D).

There was no effect of fetal sex or sex mix of twin pairs on responses to glucose or arginine challenges.

5.3.3.1 Maternal fast and refeed

Maternal plasma glucose (Figure 5-3A) and insulin levels (Figure 5-3B) decreased further and faster in response to fasting in twin than in singleton-bearing ewes, and returned to their previously lower levels on refeeding. Maternal plasma IGF-1 levels also decreased with fasting, but there was no difference between twin and singleton-bearing ewes (twin-bearing: 77.4±7.0 to 44.2±4.9 $ng.mL^{-1}$, singleton-bearing: 67.5±7.0 to 41.3±5.4 $ng.mL^{-1}$, $p<0.01$ for time effect).

Fetal plasma glucose (Figure 5-3C) and insulin levels (Figure 5-3D) decreased further in twin fetuses than in singletons in response to maternal fasting and returned to lower levels with refeeding. Fetal IGF-1 levels also decreased with fasting, but there was no difference between twin and singletons fetuses (twins: 78.7 to 50.5 $ng.mL^{-1}$, singletons: 69.6±5.7 to 43.1±5.7 $ng.mL^{-1}$, $p<0.01$ for time effect).

Maternal plasma glucose levels showed a trend to being lower in the fasting period in UN -60-30 than control singleton-bearing ewes ($p=0.07$) (Figure 5-3A), but there was no nutritional effect on maternal insulin levels (Figure 5-3B). Maternal IGF-1 levels dropped to a similar level in both UN -60-30 and control groups (UN -60-30: 60.4±6.1 to 45.5±6.0 $ng.mL^{-1}$, control: 84.6±7.0 to 40.2±4.1 $ng.mL^{-1}$, $p=0.03$ for group by time interaction).

Fetal plasma glucose levels dropped further in response to maternal fasting in UN - 60-30 than in control singleton fetuses, and were lower through refeeding (Figure 5-3C). Periconceptional undernutrition also resulted in lower plasma insulin levels during refeeding in both twins and singletons (Figure 5-3D). There was no difference in IGF-1 levels between nutritional groups.

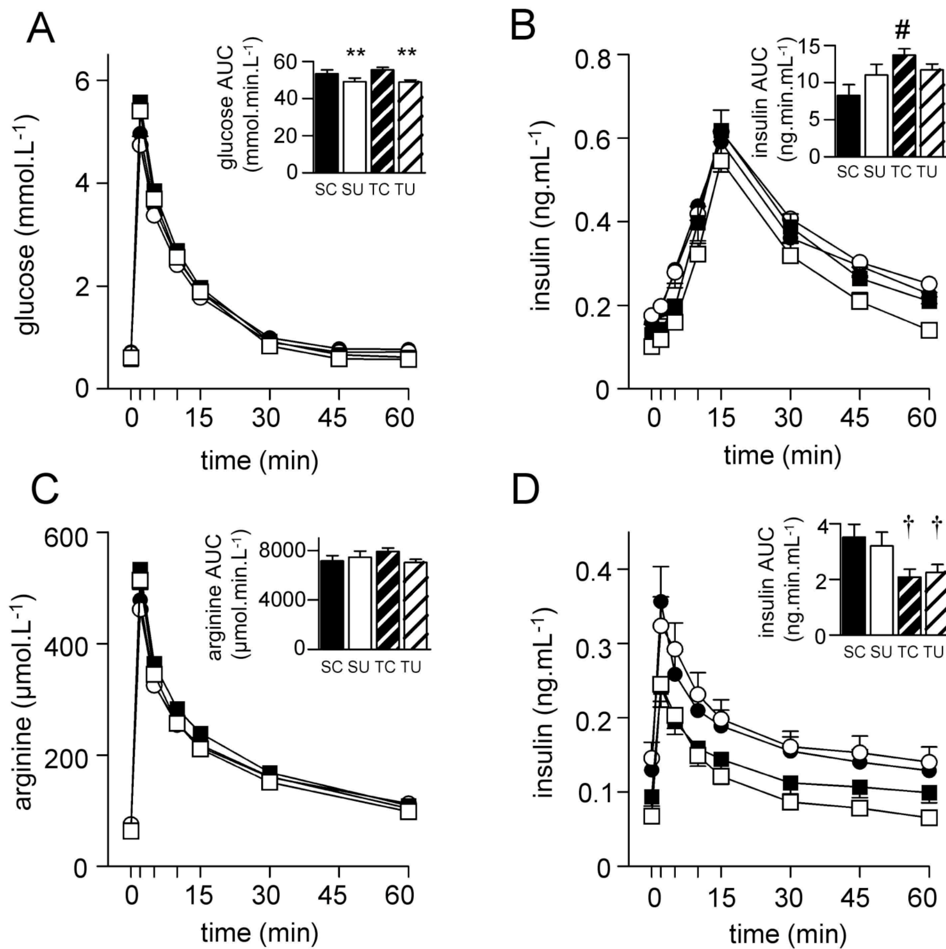

Figure 5-2: Fetal glucose (A) and insulin (B) responses to an intravenous glucose tolerance test; arginine (C) and insulin (D) responses to an intravenous arginine challenge

Areas under the curve shown as inset histograms are the least squares means from the multivariate analysis. Singleton control (SC) = ● (n = 15); singleton UN -60-30 (SU) = ○ (n = 13); twin control (TC) = ■ (n = 26); twin UN -60-30 (TU) = □ (n = 30). Data are mean ± SEM.

† $p < 0.05$ for twin effect

*** $p < 0.01$ for nutrition effect*

 # $p < 0.05$ for twin x nutrition interaction

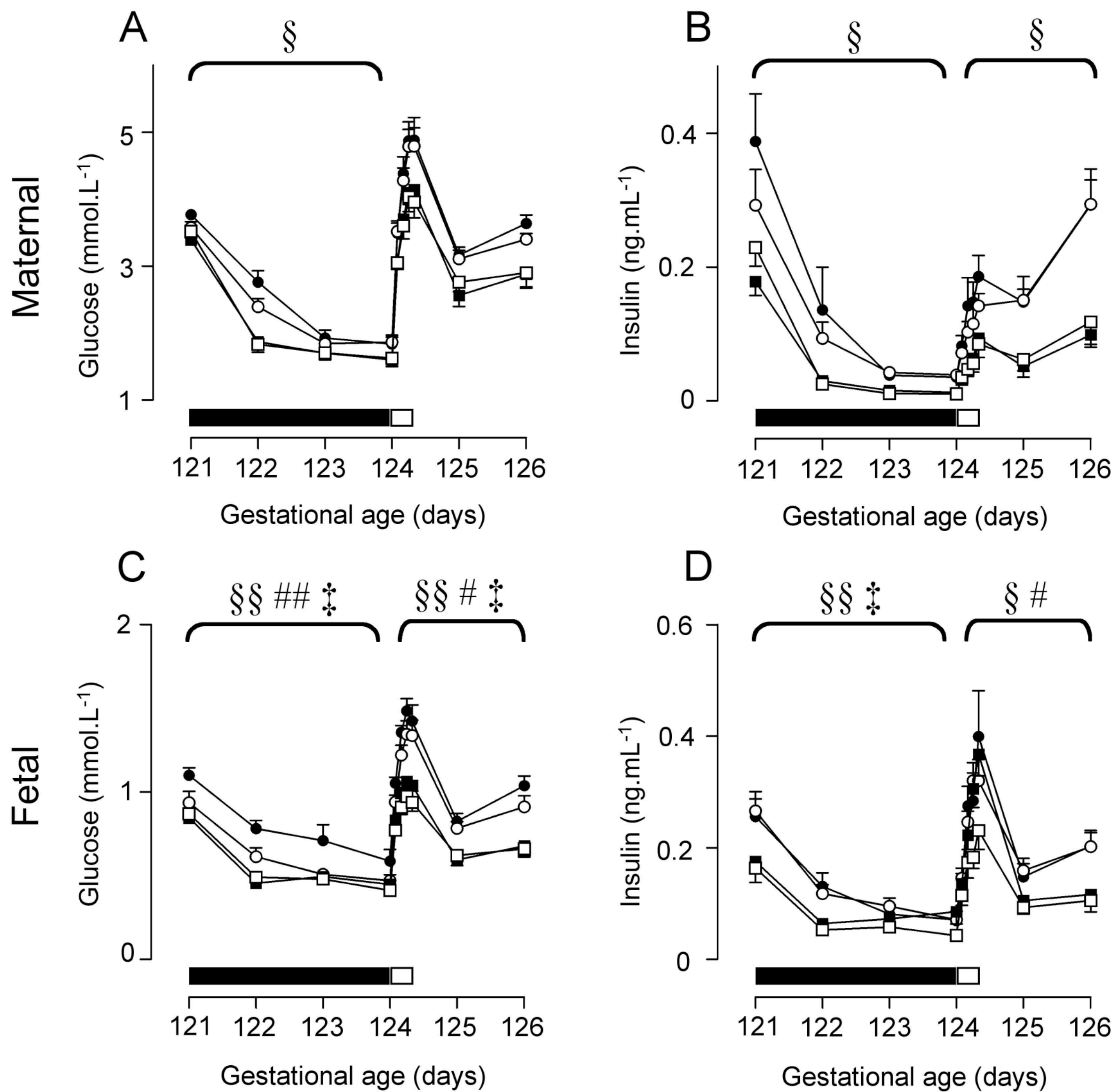

Figure 5-3: Maternal glucose (A) and insulin (B), and fetal glucose (C) and insulin (D) levels during the fast and refeed periods

Fasting period (days 121 to 124) indicated by black bar, glucose infusion indicated by open bar. Data are mean ± SEM. Singleton control = ● (n=10), singleton UN -60-30 = ○ (n=12), twin control = ■ (n=12), twin UN -60-30 = □ (n=10).
§ $p<0.05$, §§ $p<0.01$ for twin x time interaction
 # $p<0.05$, ## $p<0.01$ for twin x nutrition interaction
‡ $p<0.05$ for twin x nutrition x time interaction

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5.4 Discussion

We hypothesised that if the physiology of twinning is largely determined in the periconceptional period, then twinning and periconceptional undernutrition might have similar effects on maternal metabolism, fetal growth and fetal glucose-insulin axis function in late gestation. We found that twinning led to profound changes in late gestation on fetal growth and glucose-insulin axis function, and on maternal metabolism. However, periconceptional undernutrition had different effects on the fetal glucose-insulin axis and on placental morphology from twinning, and had fewer effects on maternal metabolism and fetal growth. Furthermore, there was an interaction between twinning and periconceptional undernutrition on aspects of fetal metabolism, growth and glucose-insulin axis responses. Thus the physiology of twin pregnancy is quite different from that of singleton pregnancy, and is likely to be determined by a combination of factors acting in both early and late gestation.

5.4.1 Maternal metabolism

We have shown that twinning affects maternal nutrient intake and metabolism in late-gestation pregnant sheep. The decreased food intake of twin-bearing ewes in late gestation may be due to physical restriction of the rumen by the larger mass of twin fetuses. Although we do not have any direct measures of body composition, twin-bearing ewes would almost certainly also have had reduced tissue stores, since their total weights were similar to those of singleton-bearing ewes despite carrying greater conceptus weights. The combination of reduced food intake, reduced tissue stores and the increased metabolic demand imposed by two fetuses (Shinagawa *et al.*, 2005) could together account for the lower plasma glucose and insulin levels and higher plasma FFA and ketone levels observed in these ewes, and also the more rapid fall in plasma glucose levels with fasting. The smaller increase in maternal plasma glucose levels in response to glucose infusion at the end of fasting may also reflect the greater fetal metabolic demand in twin-bearing ewes.

Although human studies have not shown any difference in food intake between twin and singleton pregnancies (Morley *et al.*, 2006), they have suggested that twin pregnancies are more vulnerable to 'accelerated starvation', with rapid development of ketonaemia during fasting (Casele *et al.*, 1996). This is due in part to the greater metabolic demand of twin fetuses, as evidenced by the correlation between maternal glucose disposal rate and conceptus mass (Marconi *et al.*, 1993). It is less clear whether decreased maternal

tissue stores also contribute to the 'accelerated starvation' in human twin pregnancies. Mobilisation of fat stores is a important contributor to maternal nutrition in late pregnancy in both humans and sheep, thus preserving glucose and amino acids for the fetus (Butte, 2000; Vernon *et al.*, 1981). However, in contrast to our sheep, women carrying twins tend to have a substantially greater weight gain than those carrying singletons.

During undernutrition UN -60-30 ewes had the expected change in metabolic profile, with decreased plasma glucose, insulin and urea levels, and increased FFA and ketone levels. Except for plasma insulin levels, these recovered and were not different in late gestation between UN -60-30 and control ewes. This is despite persistently reduced weight and increased food intake in UN -60-30 ewes. The increased food intake may reflect an attempt to recover tissue stores lost during the period of undernutrition, and at the same time meet the additional metabolic demands of the fetus. When this compensatory strategy was prevented during maternal fasting, we observed a more rapid decrease in glucose levels in UN -60-30 ewes, similar to that observed in twin-bearing ewes. However glucose infusion at the end of the fasting period resulted in maternal plasma glucose profiles that were similar in UN -60-30 and control ewes, suggesting that fetal metabolic demand was not affected by periconceptional undernutrition.

5.4.2 Placenta

Effects of twinning and periconceptional undernutrition on the placenta, a major consumer of energy and the mediator of nutrient transfer to the fetus, may also affect both maternal and fetal metabolism. In the twins in our study, the placenta of each fetus weighed about a third less than that of a singleton, so that overall there was about third more placental mass in the twin pregnancies. There were also fewer everted placentomes (types C and D), a morphology that is speculated to improve oxygen exchange efficiency (Penninga and Longo, 1998), although it may decrease glucose delivery (Fowden *et al.*, 2006b). In this study the maternal to fetal glucose gradient was lower in twins, suggesting either reduced placental glucose consumption, consistent with the reduced placental mass, or increased efficiency of placental glucose transfer. Similar changes have been demonstrated when placental size is restricted by carunclectomy, with reduced utero-placental glucose consumption (Owens *et al.*, 1987a) and increased placental clearance of glucose analogues per unit placental mass (Owens *et al.*, 1987c). The placentas of twin fetal sheep at 55 d gestation have increased fetal capillary density and volume (MacLaughlin *et al.*, 2005), and thus the relative reduction in placental size in twin

pregnancy may be associated with compensatory changes in placental structure and function to order to ensure adequate substrate supply to the fetus.

Periconceptional undernutrition resulted in changes in placental morphology that were in the opposite direction from those in twins, with a greater proportion of placentomes with an everted phenotype compared with controls. There was no change in the maternal to fetal glucose gradient following periconceptional undernutrition. These findings are consistent with other studies on early gestation undernutrition (Fowden *et al.*, 2006b; Steyn *et al.*, 2001) and have also been reported following dexamethasone exposure in early gestation (Laraya *et al.*, 2000) and prolonged hypoxia secondary to altitude (Penninga and Longo, 1998). Everted placentome phenotypes have been demonstrated to have a greater number of materno-fetal interfaces and greater materno-fetal absorptive surface (Krebs *et al.*, 1997), suggesting that placental efficiency may be increased. However, there were no histological changes in placentomes at 55 d gestation following 30% maternal undernutrition from 45 d before until 7 d after mating (MacLaughlin *et al.*, 2005); at this gestation placentomes cannot be categorised into different phenotypic subtypes. To our knowledge there are no direct measurements of placental transfer capacity or efficiency in sheep following periconceptional undernutrition. However, changes in placental function following altered maternal nutrition in rats, including brief manipulations only in early pregnancy, have been clearly demonstrated (Ericsson *et al.*, 2007; Jansson *et al.*, 2006). Jansson suggests that the placenta may act as a ‘nutrient sensor’, detecting prevailing nutrient conditions on the fetal supply line (Jansson and Powell, 2006) and regulating nutrient transport accordingly, perhaps via mammalian target of rapamycin (mTOR) (Roos *et al.*, 2007). Further work is necessary to determine whether similar changes occur in higher mammals such as the sheep.

5.4.3 Fetal growth

Twin fetuses grew more slowly than singletons in late gestation, and were smaller and lighter at post mortem. Within a twin pair, the heavy fetus grew faster than the light fetus but was already larger at surgery, suggesting that at least some effects of twinning on fetal growth trajectory are established earlier in gestation. This is consistent with previous studies in sheep suggesting that fetal growth trajectory is determined in early pregnancy, (Bloomfield *et al.*, 2006; Harding, 1997a), and with human data comparing fetal growth between reduced and non-reduced twin pregnancies (Boulot *et al.*, 2000; Sebire *et al.*, 1997). The greater impact of maternal fasting on fetal growth in twins is similar to that

found in an earlier study in which the fetuses of twin-bearing ewes undernourished from 112 d gestation slowed their growth more than singleton fetuses (Mellor and Murray, 1982). This suggests that growth rate in late gestation in twins may be more limited by nutrient supply than it is in singleton pregnancies, and this limitation may be partly explained by the altered metabolism in twin-bearing ewes in late gestation.

Our finding that periconceptional undernutrition had no effect on late-gestation fetal growth rate contrasts with a previous study showing that singleton fetuses grew more slowly in late gestation after periconceptional undernutrition (Oliver *et al.*, 2005). However in that study growth rate was measured in the period immediately prior to delivery (126 to 145 d). Since fetal growth slows close to delivery, any differences between groups may only become apparent at this time, and may not have been detected during the earlier period of study (114 to 131 d) in our experiment. However, our finding that the lighter twins of UN -60-30 ewes maintained their growth rate during maternal fasting better than lighter twins of control ewes is consistent with previous observations in periconceptionally undernourished singletons that fetal growth continued in the face of late-gestation maternal undernutrition, whereas it slowed in control fetuses (Harding, 1997a). We have previously speculated that this reflects some adaptation in feto-placental function induced by periconceptional undernutrition that allows continued fetal growth in the face of limited nutrient supply (Bloomfield *et al.*, 2006). It is not clear why such changes were not seen in singletons in the current study, although the short period of complete fast (3 d) in the current study compared with 10 d of severe undernutrition in the previous study may have limited our ability to detect a difference. Further evidence of the effects of periconceptional undernutrition on fetal development were seen in different patterns of organ growth with smaller spleens and chest thymus, but larger livers, as has been observed previously (Oliver *et al.*, 2005).

5.4.4 Fetal pancreatic function

The greater insulin response to glucose, but lesser response to arginine, observed in twin fetuses demonstrates altered pancreatic function by 118 d gestation. This pattern of response may indicate earlier pancreatic maturation in twins, as pancreatic maturation in late gestation is thought to involve apoptosis of amino acid-sensitive fetal β cell and their replacement by glucose-sensitive adult β cells (Fowden *et al.*, 2005; Scaglia *et al.*, 1997). If this were the case, it would contrast with the recently reported delay in the maturation of the HPA axis in twin sheep fetuses (McMillen *et al.*, 2004). A greater insulin response to

glucose challenge could also reflect reduced fetal insulin sensitivity. Twin pre-pubertal children have decreased insulin sensitivity compared with singletons, independent of prematurity and birth weight (Jefferies *et al.*, 2004), and our data raise the possibility that these differences may arise before birth. Alternatively, it is possible that the observed changes are a consequence of fetal development in a low glucose environment. However, sheep fetuses made chronically hypoglycaemic by maternal insulin infusions in late gestation had decreased rather than increased insulin responses to glucose (Limesand and Hay, 2003), as did fetuses with growth restriction induced by maternal hyperthermia (Limesand *et al.*, 2006). Therefore, the increased insulin response to glucose in twins is not readily explained by the expected effects of chronic hypoglycaemia and fetal growth restriction.

In contrast to twinning, periconceptional undernutrition decreased the glucose AUC in response to a glucose challenge, but did not affect responses to arginine challenge. A reduced glucose AUC suggests faster glucose disposal, but this is unlikely to be due to greater insulin sensitivity, since the insulin response was not reduced. Indeed, this group has previously shown in singletons that periconceptional undernutrition results in increased fetal insulin secretion in response to a glucose challenge (Oliver *et al.*, 2001b), and a similar pattern is present in singletons in this study, although it does not reach statistical significance. Thus our data suggest that periconceptional undernutrition results in increased glucose disposal by insulin-independent mechanisms, perhaps in part by tissues where glucose uptake is not insulin-dependent such as the placenta and fetal brain. Furthermore, the finding that periconceptional undernutrition abolishes the greater insulin response to glucose challenge seen in twins suggests a potential interaction between events in early and late gestation on fetal pancreatic maturation that requires further exploration.

5.4.5 Conclusions

This study demonstrates that the effects of twinning on the late-gestation sheep fetus have some similarities to the effects of periconceptional undernutrition, but also several differences, and that there is an interaction between the two. It seems likely that the physiology of twin pregnancy is the result of factors operating in both early and late gestation. Furthermore, the maternal metabolic and endocrine environment, fetal and placental growth, and fetal glucose-insulin axis function are all different in twin pregnancy from those in singleton pregnancies, making it essential that twins and singletons are addressed separately in any study of fetal physiology or its postnatal consequences.

Further exploration of these differences between twin and singleton pregnancy may help to explain the observed inconsistent relationships between birth weight and later disease risk in twins.

6. Effects of twinning and periconceptional undernutrition on hypothalamic-pituitary-adrenal axis function in ovine pregnancy

6.1 Introduction

Appropriate maturation and activation of the fetal HPA axis is integral to the appropriate timing of parturition in the sheep (Challis *et al.*, 2001). Removal of the pituitary, and therefore ACTH, causes prolonged gestation in sheep (Liggins *et al.*, 1967), while administration of ACTH or cortisol induces preterm labour (Liggins, 1968). Both before and after birth, plasma cortisol levels are regulated by ACTH under negative feedback control at the level of the pituitary and hypothalamus. However, in the period leading up to parturition this negative feedback is inhibited, the fetal pituitary becomes less sensitive to CRH (Lu *et al.*, 1991), and fetal plasma ACTH and cortisol levels rise concomitantly. Observed differences in HPA axis function in late gestation may therefore relate to altered proximity to parturition, or to permanent changes in the axis, which may or may not be manifestations of the same underlying process.

We have previously shown in singleton fetal sheep that periconceptional undernutrition increases pituitary ACTH production in response to decreased negative feedback by cortisol (Bloomfield *et al.*, 2004), and leads to a precocious rise in plasma ACTH and cortisol levels and preterm delivery (Bloomfield *et al.*, 2003b). Others have shown that periconceptional undernutrition also results in increased basal activity of the HPA axis in postnatal life (Gardner *et al.*, 2006).

Twinning is also a periconceptional event. However, in contrast to the effects of periconceptional undernutrition, in sheep there is evidence of reduced responsiveness of the HPA axis at adrenal level in twins in late gestation (Edwards and McMillen, 2002; Gardner *et al.*, 2004a), and gestation may be prolonged (Oliver MH, unpublished data; (Ozturk and Aktas, 1996), although there are no data regarding the activity of the central parts of the HPA axis. Furthermore, the prepartum cortisol surge appears to happen asynchronously in twin pairs (Schwartz and Rose, 1998) and is related to changes in adrenal sensitivity to ACTH (Block *et al.*, 1999). Thus, in twins, one fetus may be born

without experiencing a substantial cortisol surge. The factors determining which fetus generates the prepartum cortisol surge in twins are not known. In contrast to reduced adrenal sensitivity in twin fetal sheep before birth, we have shown that after birth there is increased responsiveness of the central HPA axis in twins compared to singletons (Bloomfield *et al.*, 2007). This responsiveness is strongly associated with the within-twin coefficient for birthweight, rather than the between-twin coefficient, suggesting an effect of factors related to the growth of individual fetuses rather than to their shared maternal environment.

Thus, twinning and periconceptional undernutrition both appear to increase activity of the HPA axis after birth in sheep, but before birth they have opposite effects, with twinning decreasing but periconceptional undernutrition increasing HPA axis activity. The similarity of effects after birth may be related to the decreased fetal growth trajectory seen both in twins (Rumball *et al.*, 2007b) and following periconceptional undernutrition (Oliver *et al.*, 2005), since there is an association between reduced birth weights, increased HPA axis responsiveness (Jones *et al.*, 2006; Phillips and Jones, 2006) and risk of metabolic diseases in later life in human singletons (Barker *et al.*, 1989a). However, the literature on humans is inconsistent regarding the relationship between birth weight and disease risk in twins (Phillips, 2004), with one explanation being that biology of growth is substantially different in twins from that of singletons (Phillips *et al.*, 2001).

Understanding how these two periconceptional events affect function of the higher aspects of the HPA axis might help explain the apparent dichotomy between fetal and postnatal HPA axis activity, and thereby also provide insights into the mechanism whereby preterm birth is avoided in twin-bearing ewes. We therefore investigated the higher centres of the HPA axis in late gestation in singleton and twin fetuses of ewes that had either been well fed throughout pregnancy or been exposed to periconceptional undernutrition. We studied HPA axis responses to an acute maternal nutritional deprivation, to central stimulation of the fetal HPA axis with CRH and AVP, and also the negative feedback response to decreased cortisol production by the adrenal gland.

In addition, as exposure of the fetus to inappropriate levels of maternal glucocorticoid has been proposed as a common mechanism underlying the relationship between undernutrition, reduced size at birth and increased risk of disease (Seckl and Meaney, 2004), and we have previously demonstrated that periconceptional undernutrition profoundly down-regulates the maternal HPA axis (Bloomfield *et al.*, 2004; Jaquiery *et al.*,

2006), we also studied the effect of twinning and periconceptional undernutrition on basal maternal HPA axis activity and on the maternal HPA axis response to the physiological stimulus of fasting.

6.2 Methods

6.2.1 Experimental design

Non-pregnant ewes were randomly allocated to control or UN -60-30 nutritional groups. Pregnancy and fetal number were established at 55 d gestation by ultrasound. The ewes underwent surgery at 111±1 d for placement of venous and arterial catheters and allowed to recover for at least 3 d. Both fetuses were catheterised in the case of twins.

Baseline maternal and fetal blood samples were taken in the morning before feeding at 114, 117, 121, 127 and 131 d.

Ewes were fasted from 121 to 124 d, and maternal and fetal blood samples were taken at the start and end of the fast. At 124 d ewes were refed and also given an intravenous glucose infusion of 25 g over 8 h, aiming to restore maternal and hence fetal blood glucose as rapidly as possible in a 'square wave' pattern. Blood samples were collected at 8 h after the start of the glucose infusion (fetus only), and at 24 h of refeeding.

At 127 d at 0900 a pituitary stimulation challenge was performed using CRH and AVP, and at 128 d at 0900 a metyrapone challenge was performed.

The sheep were euthanised with an overdose of pentobarbitone at 132 d and a postmortem performed.

6.2.2 Data analysis

Maternal metabolite and hormone data were compared using a two-way ANOVA with twinning, nutritional group, and the interaction between these terms as independent variables, and the Tukey-Kramer correction for multiple comparisons. A similar two-way repeated measures ANOVA was used for comparing the fasting and refeeding periods.

For the fetal CRH/AVP and metyrapone challenges, AUC was calculated from baseline. Fetal data were compared using a two-way ANOVA with twinning, nutritional group, and their interaction as independent variables, and with sheep number nested within groups to allow for the non-independence of twins. The Tukey-Kramer method was used to correct for multiple comparisons.

Hormone concentrations were compared in heavy and light twins using a two-way ANOVA with heavy/light, nutritional group, and their interaction as independent variables.

Sex differences were investigated within singleton and twin pregnancies using a two-way ANOVA with sex, nutritional group and their interaction as independent variables. In twin pregnancies the effect of the sex mix of the pair, designated as mixed, male or female, was evaluated using ANOVA in male and female twin fetuses separately.

Data are presented as mean ± SEM. Geometric mean and SEM are used where appropriate.

6.3 Results

23 singleton-bearing ewes (11 control, 12 UN -60-30) entered the experiment, and 20 (9 control, 11 UN -60-30) completed the HPA challenges and had a postmortem. Twenty twin-bearing ewes (11 control, 9 UN -60-30) entered the experiment, 17 (9 control, 8 UN -60-30) completed the HPA challenges, and 16 (8 control, 8 UN -60-30) had a postmortem.

The average weight loss due to undernutrition was 15.1±0.4% in singleton-bearing ewes and 17.3±0.5% in twin-bearing ewes (Table 6-1). There were no significant weight differences between nutritional groups or singleton and twin-bearing ewes by day 110.

	Singleton-bearing ewes		Twin-bearing ewes	
	Control (n=11)	UN -60-30 (n=12)	Control (n=11)	UN -60-30 (n=9)
Day -71	64.5±1.6	65.5±1.6	62.7±2.1	68.7±1.5
Day -2	65.7±1.7^{a}	55.5±1.4^{b}	64.3±1.9^{a}	57.2±1.4^{b}
Day 110	68.4±1.6	62.8±1.7	67.7±1.0	67.9±2.4

Table 6-1: Maternal weights

Data are mean ± SEM in kg.
Different letters indicate significant differences between groups, p<0.05

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6.3.1 Maternal HPA axis

6.3.1.1 Baselines

There were no differences due to twinning or nutrition in baseline maternal plasma ACTH, cortisol or cortisone levels. Twin-bearing ewes, however, had 42 % higher baseline plasma progesterone levels than singleton-bearing ewes (Table 6-2). Plasma progesterone levels increased over the duration of the experiment, but remained higher in twin-bearing ewes. Maternal plasma ACTH, cortisol and cortisone levels did not change significantly between the start and end of the experiment, but both the cortisol to cortisone and cortisol to ACTH ratios declined (Table 6-2).

		Singleton-bearing ewes		Twin-bearing ewes	
		Control	UN -60-30	Control	UN -60-30
n	114-121	11	12	11	9
	128-131	9	10	9	8
ACTH ($pg.mL^{-1}$)	114-121	33.3±5	25.7±3.4	30.0±2.6	28.3±7.1
	128-131	33.9±11	34.1±3.1	38.3±2.2	31.0±6.6
Cortisol ($ng.mL^{-1}$)	114-121	10.0±2.6	7.1±1.6	7.5±1.1	6.4±0.7
	128-131	4.3±0.9	5.7±2.6	7.7±2.7	4.7±1.1
Cortisone ($ng.mL^{-1}$)	114-121	2.1±0.2	1.6±0.2	2.1±0.2	2.0±0.2
	128-131	1.7±0.4	1.6±0.5	2.1±0.5	1.5±0.3
Cortisol : ACTH ratio	114-121	258±85	224±75	238±59	269±85
	128-131$^{\surd\surd}$	143±60	94±41	142±45	141±60
Cortisol: cortisone ratio	114-121	3.8±0.5	3.8±0.5	3.3±0.3	3.0±0.2
	128-131$^{\surd}$	3.1±0.6	2.6±0.4	2.4±0.6	2.4±0.3
Progesterone ($ng.mL^{-1}$)	114-121	9.1±0.4^{b}	10.0±0.6^{b}	15.0±1.1^{a}	11.8±1.1^{ab}
	128-131$^{\surd}$	9.0±0.7^{b}	10.5±1.2^{b}	19.2±2.7^{a}	14.0±2.0^{a}

Table 6-2: Maternal ACTH and steroid levels at 114-121 d and 128-131 d

Data are means across time periods and are mean ± SEM.
Different letters indicate significant differences between groups, p<0.05
$^{\surd}$ *p<0.05,* $^{\surd\surd}$ *p<0.01 for time effect*

6.3.1.2 Fast and refeed

The 72-hour fast halved maternal plasma glucose levels, from 3.32±0.05 to 1.60±0.07 mmol.L^{-1} in twin-bearing ewes, and from 3.61±0.04 to 1.84±0.07 mmol.L^{-1} in singleton-bearing ewes ($p<0.01$ for time effect for twins and singletons).

Maternal plasma ACTH levels did not change with fasting or refeeding, but cortisol and cortisone levels increased with fasting, then decreased with refeeding (Figure 6-1). Twinning and nutritional group did not affect these changes. Plasma progesterone levels also increased with fasting, and decreased with refeeding in singleton-bearing ewes, but continued to increase in twin-bearing ewes (Figure 6-1). Progesterone levels in twin-bearing ewes remained elevated at day 131 compared to day 121 (16.6±1.7 vs 14.5±1.2 ng.mL^{-1}, $p=0.02$), whereas they had returned to baseline in singleton-bearing ewes (9.83±0.74 vs 10.07±0.54 ng.mL^{-1}, $p=0.45$).

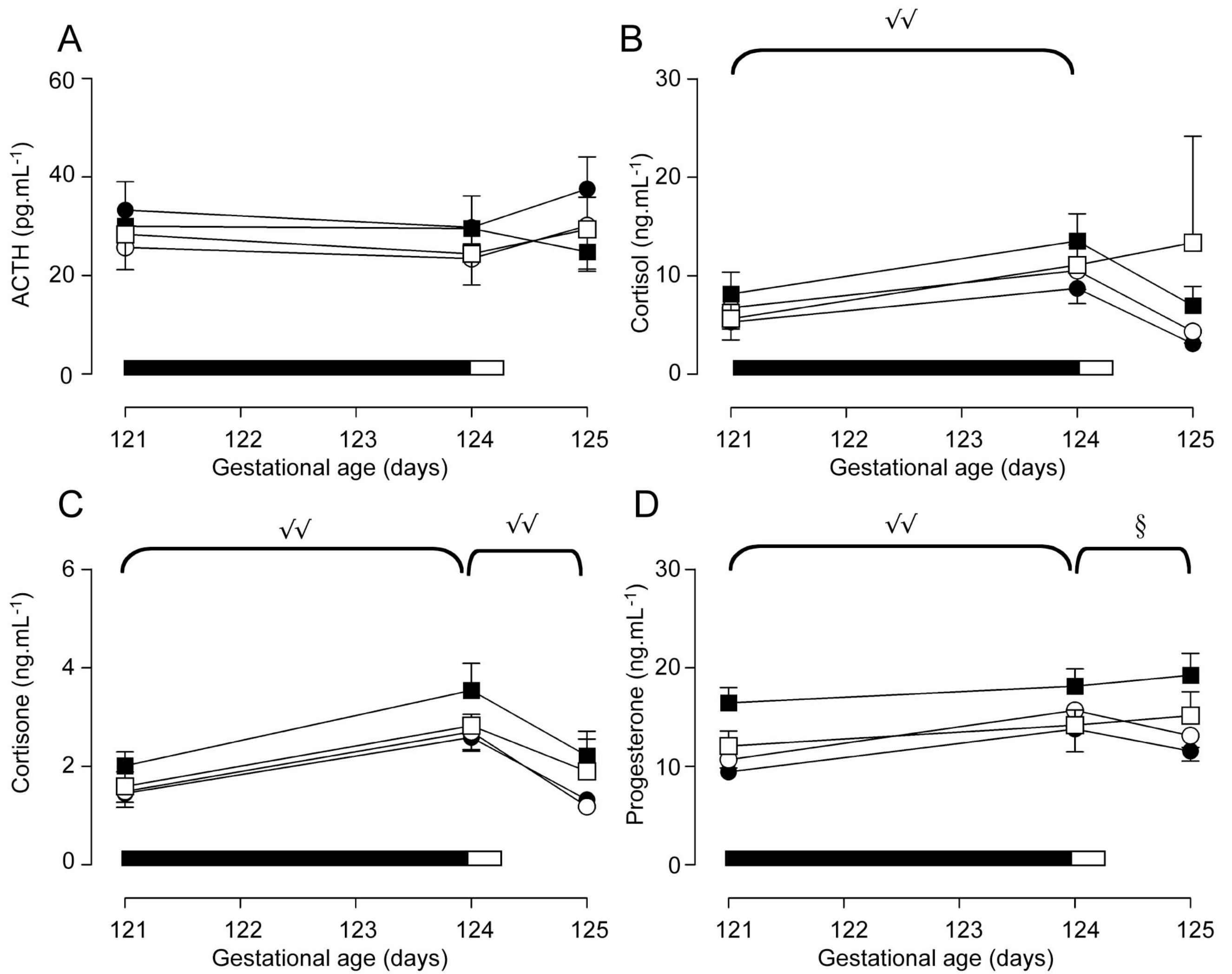

Figure 6-1: Maternal plasma ACTH (A), cortisol (B), cortisone (C) and progesterone (D) levels during the fast and refeed periods

Maternal fasting period indicated by black bar, and glucose infusion indicated by open bar. Data are mean ± SEM. Singleton control = ● (n=11), singleton UN -60-30 = ○ (n=12), twin control = ■ (n=11), twin UN -60-30 = □ (n=8).
√√ $p<0.01$ for overall time effect
§ $p<0.05$ for twinning x time interaction

6.3.2 Fetal HPA axis

6.3.2.1 Baseline

Twin fetuses had similar plasma ACTH levels to singleton fetuses, but lower cortisol, cortisone and DHEA levels (Table 6-3).

UN -60-30 singletons had similar plasma ACTH and cortisol levels to control singletons, but lower cortisone and DHEA levels (Table 6-3). However, there were no differences in plasma ACTH or steroid levels between nutritional groups in twins.

Fetal plasma ACTH and DHEA levels did not change between 114-121 d and 128-131 d, but cortisol, cortisone, the cortisol to ACTH ratio, and the cortisol to cortisone ratio all increased. There was no difference in cortisol levels between twin and singleton fetuses at 128-131 d, but cortisone levels remained higher in control singletons than in UN -60-30 singletons and all twins (Table 6-3).

There was no effect of fetal sex or sex mix of a twin pair on baseline values, and no differences between heavy and light twins in a pair.

		Singletons		**Twins**	
		Control	**UN -60-30**	**Control**	**UN -60-30**
n	114-121	11	12	22	18
	128-131	9	10	18	16
ACTH	114-121	20.7±4.7	17.2±1.1	19.0±1.2	20.7±1.5
($pg.mL^{-1}$)	128-131	19.6±2.5	15.8±2.0	20.7±2.2	20.6±2.9
Cortisol	114-121	1.00±0.34^{a}	1.00±0.20^{a}	0.54±0.05^{b}	0.55±0.07^{b}
($ng.mL^{-1}$)	128-131$^{√√}$	4.94±1.89	3.40±1.05	2.95±0.31	3.57±1.30
Cortisone	114-121	1.44±0.42^{a}	1.03±0.20^{b}	0.76±0.06^{c}	0.81±0.08^{c}
($ng.mL^{-1}$)	128-131$^{√√}$	3.50±1.51^{a}	1.54±0.21^{b}	1.93±0.25^{b}	2.27±0.56^{b}
Cortisol:ACTH ratio	114-121	39.4±11.5^{a}	47.6±10.1^{a}	27.3±3.5^{b}	24.0±3.1^{b}
	128-131$^{√√}$	156±52	152±45	140±20	117±22
Cortisol:cortisone	114-121	0.72±0.08^{ab}	0.97±0.16^{a}	0.71±0.05^{b}	0.67±0.05^{b}
ratio	128-131$^{√√}$	1.82±0.35	1.76±0.36	1.45±0.20	1.29±0.12
DHEA	114-121	0.77±0.14^{a}	0.53±0.08^{b}	0.42±0.04^{bc}	0.40±0.03^{c}
($ng.mL^{-1}$)	128-131	0.73±0.18^{a}	0.62±0.10^{b}	0.45±0.04^{c}	0.37±0.04^{d}

Table 6-3: Fetal ACTH and steroid levels at 114-121 d and 128-131 d

Data are means across the time periods, and are mean ± SEM.
Different letters indicate significant differences between groups, $p<0.05$
$^{√√}$ *$p<0.01$ for time effect*

6.3.2.2 Maternal fast and refeed

The 72-hour fast halved fetal plasma glucose levels, from 0.79±0.02 to 0.43±0.01 $mmol.L^{-1}$ in twins, and from 0.97±0.04 to 0.53±0.02 $mmol.L^{-1}$ in singletons.

Fetal plasma ACTH levels increased with fasting, and decreased quickly in response to maternal refeeding, with no additional effect of twinning or nutritional group (Figure 6-2A). Fetal plasma cortisol (Figure 6-2B) and cortisone (Figure 6-2C) levels increased in response to the fasting, with levels increasing more in twins than singletons, and decreased quickly in response to refeeding. Plasma DHEA levels (Figure 6-2D) decreased in response to fasting, but less in twins than singletons. On refeeding, DHEA levels increased in singletons, but continued to decrease in twins.

UN -60-30 singletons had lower baseline plasma glucose levels than control singletons (0.91±0.03 vs 1.03±0.03, $p<0.05$) and these dropped to lower levels with fasting (0.47±0.02 vs 0.58±0.02 $mmol.L^{-1}$, $p<0.05$).

Nutritional group did not affect the changes in fetal plasma ACTH, cortisol or cortisone levels in response to fast and refeeding. However, plasma DHEA levels decreased further in control than UN -60-30 fetuses during the fasting period (Figure 6-2D).

There was no effect of sex or sex mix of a twin pair on fetal responses to maternal fasting and refeeding.

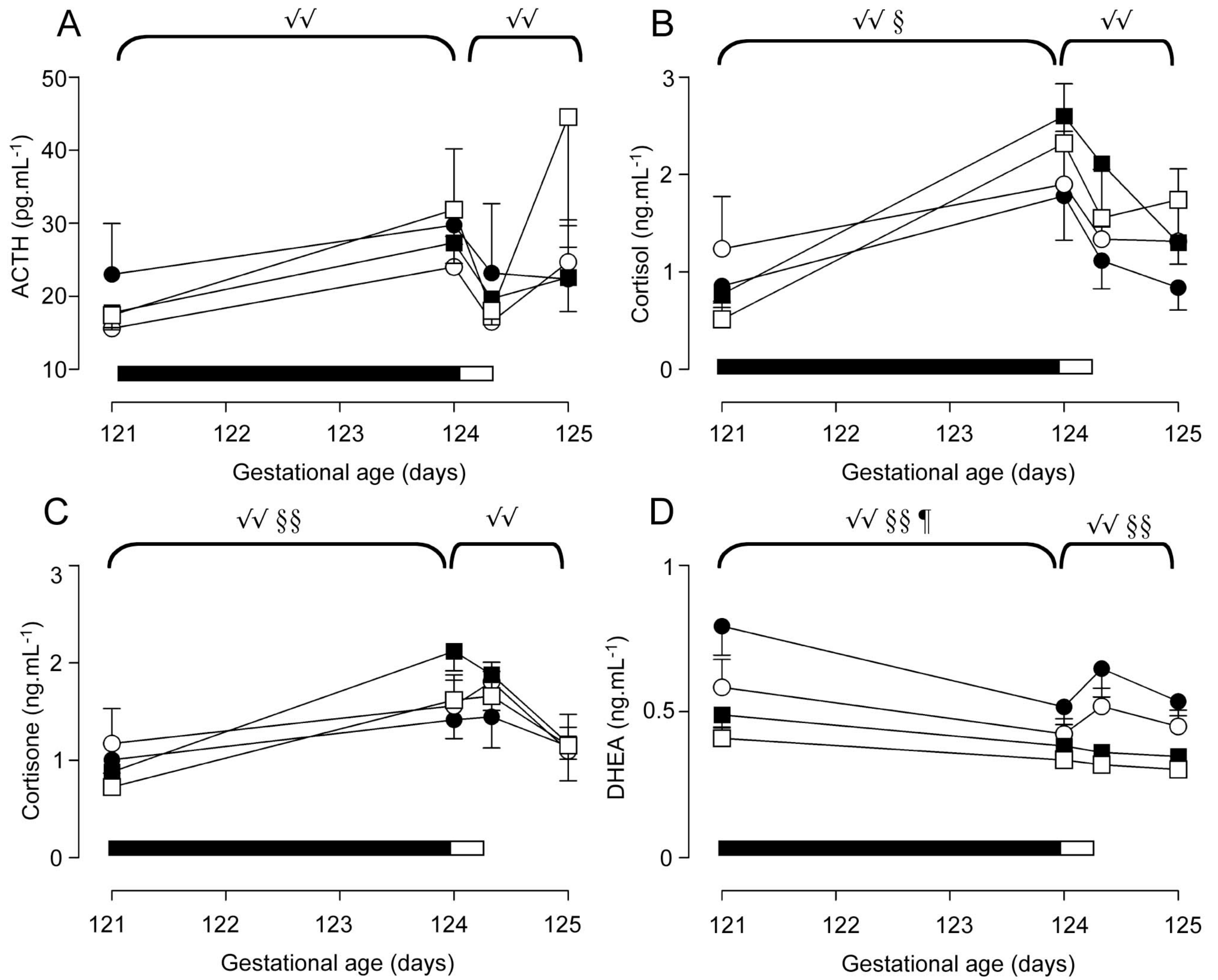

Figure 6-2: Fetal plasma ACTH (A), cortisol (B), cortisone (C) and DHEA (D) levels during the fast and refeed periods

Maternal fasting period indicated by black bar, and glucose infusion indicated by open bar. Data are mean ± SEM. Singleton control = ● (n=11), singleton UN -60-30 = ○ (n=12), twin control = ■ (n=22), twin UN -60-30 = □ (n=16).
√√ p<0.01 for overall time effect
§ p<0.05, §§ p<0.01 for twinning x time interaction
¶ p<0.05 for nutrition x time interaction

6.3.2.3 Fetal CRH/AVP challenge

There were no differences between groups in maternal plasma ACTH and cortisol levels at baseline. Fetal plasma ACTH levels rose rapidly following intravenous CRH/AVP in all animals. Twins had a greater ACTH (Figure 6-3A) and cortisol response (Figure 6-3B) than singletons.

In contrast, UN -60-30 fetuses had a decreased ACTH response to the CRH/AVP challenge in both twins and singletons (Figure 6-3A). There was also a decreased subsequent cortisol response (Figure 6-3B).

Twin fetuses had a smaller cortisol AUC to ACTH AUC ratio than singletons (68.7±7.8 vs 87.1±16.4, p<0.05), but in UN -60-30 fetuses the ratio was not different from that in controls (77.1±13.6 vs 73.6±8.4, p=0.82).

There was no effect of fetal sex or sex mix of a twin pair on responses to the CRH/AVP, and no differences between heavy and light twins within a pair.

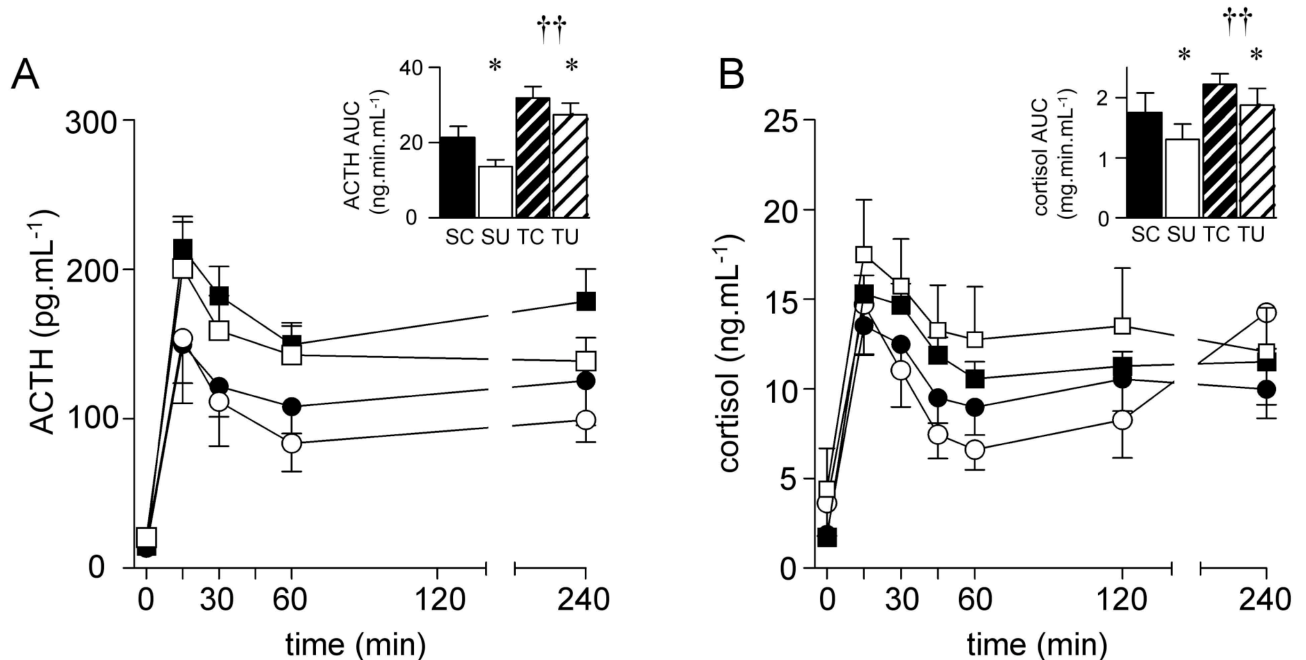

Figure 6-3: ACTH (A) and cortisol (B) responses to CRH/AVP challenge

Areas under the curve are shown as inset histograms. Singleton control (SC) = ● (n=9), singleton UN -60-30 (SU) = ○ (n=11), twin control (TC) = ■ (n=18), twin UN -60-30 (TU) = □ (n=16). Data are mean ± SEM.
†† p<0.01 for twinning effect
** p<0.05 for nutrition effect*

6.3.2.4 Metyrapone challenge

There were no differences between groups in maternal ACTH or cortisol levels at baseline. The metyrapone challenge resulted in decreased fetal plasma cortisol levels at 30 min in all groups, with no difference between groups in the cortisol trough ($p=0.26$) (twin control: 3.23±0.45 to 0.97±0.15, twin UN -60-30: 4.06±1.22 to 1.69±0.73, singleton control: 2.91±0.51 to 0.90±0.23, singleton UN -60-30: 3.18±0.68 to 1.77±0.57 $ng.mL^{-1}$, $p<0.01$ for time effect). Twinning resulted in a greater ACTH response to this fall than singletons (Figure 6-4A), and a subsequent greater 11-deoxycortisol response (Figure 6-4B), but did not affect the ratio of 11-deoxycortisol AUC to ACTH AUC (118±22 vs 200±92, $p=0.14$).

ACTH response to the fall in cortisol in the UN -60-30 fetuses was not different from that of controls, but there was a greater 11-deoxycortisol response (Figure 6-4B), although the ratio of 11-deoxycortisol AUC to ACTH AUC was similar (166±48 vs 123±34, $p=0.44$).

There was no effect of fetal sex or sex mix of a twin pair on responses to the metyrapone challenge, and no differences between heavy and light twins within a pair.

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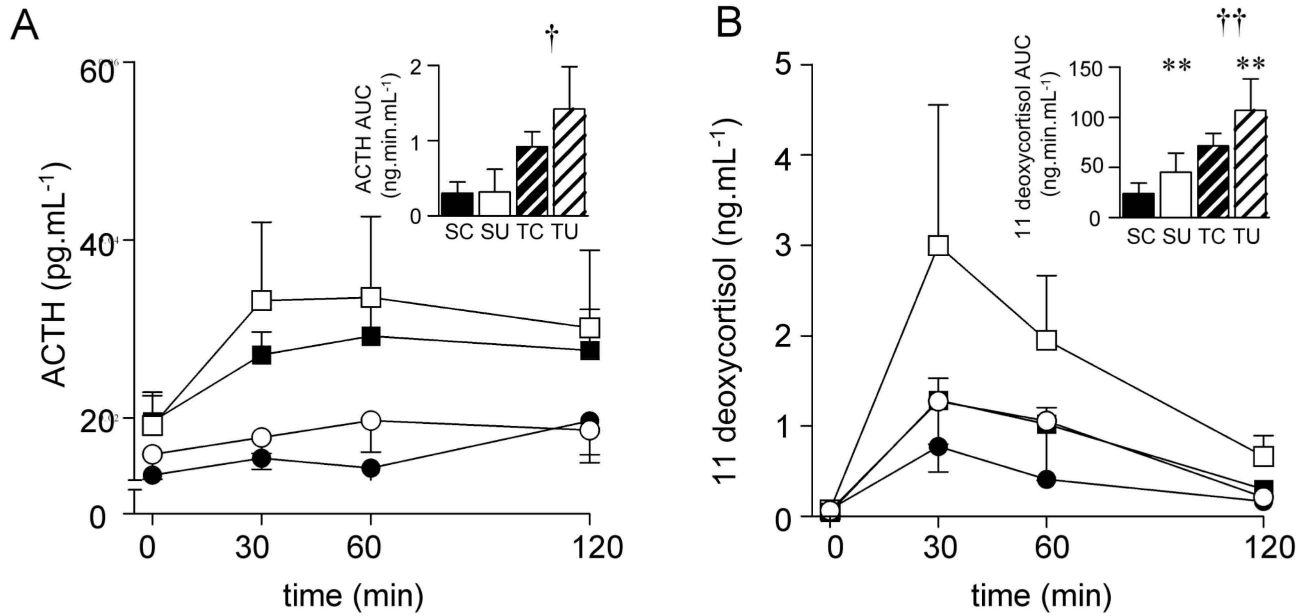

Figure 6-4: ACTH (A) and 11deoxycortisol (B) responses to metyrapone challenge

Areas under the curve are shown as inset histograms. Singleton control (SC) = ● (n=9), singleton UN -60-30 (SU) = ○ (n=11), twin control (TC) = ■ (n=18), twin UN -60-30 (TU) = □ (n=16). Data are mean ± SEM.
† p<0.05, †† p<0.01 for twinning effect
*** p<0.01 for nutrition effect*

6.4 Discussion

We hypothesised that twinning and periconceptional undernutrition would have opposite effects on maturation of the higher centres of the fetal sheep HPA axis in late gestation, consistent with the different length of gestation after these two periconceptional events, and the previously reported adrenal suppression in late-gestation twin fetuses (Edwards and McMillen, 2002). We found that, in late gestation, twinning suppressed baseline fetal steroid levels and decreased adrenal sensitivity to ACTH stimulation, but increased fetal pituitary ACTH response both to direct stimulation and also to decreased circulating cortisol concentrations, and thus decreased negative feedback by cortisol. In contrast, periconceptional undernutrition decreased pituitary ACTH response to direct stimulation, but increased adrenal 11-deoxycortisol response to decreased negative feedback by cortisol. Thus, although both twinning and periconceptional undernutrition

are associated with similar changes in HPA axis function after birth, they are associated with very different fetal HPA axis function before birth. Understanding these differences may help to elucidate the mechanisms by which events before birth have long-term effects on adult HPA axis function, and also shed light on the differences in the timing of the onset of parturition in twins of different species.

6.4.1 Maternal HPA axis

The maternal HPA axis is usually up-regulated by pregnancy, with elevated cortisol levels due to decreased pituitary sensitivity to negative feedback by cortisol (Keller-Wood and Wood, 2001). It is thought that this is due, at least in part, to the anti-mineralocorticoid actions of progesterone (Rupprecht *et al.*, 1993; Wambach and Higgins, 1979), leading to decreased negative feedback by cortisol at the higher centres of the HPA axis. The response of all ewes to the acute nutritional fast, with an increased cortisol response in the absence of an ACTH response, would be consistent with this decreased cortisol negative feedback at the hypothalamus and pituitary.

The acute rise in circulating progesterone concentrations with late-gestation fasting seen in this study, and the sustained elevation in twin-bearing ewes are novel observations. Differences in progesterone levels may be due to altered production or clearance. Placental production of progesterone is related to placental mass and is therefore higher in twin-bearing ewes (Butler *et al.*, 1981). Metabolic clearance of progesterone is via the liver and has been shown to be inversely related to feed intake in non-pregnant sheep (Parr *et al.*, 1993a), probably due to altered hepatic portal blood flow (Parr *et al.*, 1993b). Thus a decrease in clearance may have contributed to the rise in progesterone levels that we observed with maternal fasting. Similarly, decreased clearance may contribute to the continued rise in progesterone levels that we observed in twin-bearing ewes after refeeding, since we have previously shown that feed intake is lower in twin-bearing than singleton-bearing ewes in late-gestation (Rumball *et al.*, 2007b).

It is also possible that the rise in progesterone with fasting was due to a fasting-induced decrease in placental $P450_{c17}$ activity, such that placental steroidogenesis resulted in increased progesterone and decreased oestrogen production. This would be supported by the changes in fetal plasma levels of DHEA. DHEA is an oestrogen precursor which in sheep is thought to originate from the placenta or maternal adrenal glands, rather than from the fetal adrenal glands (Liggins *et al.*, 1985). Maternal levels of DHEA begin to rise from around 20 d prior to delivery, but fetal levels do not change approaching parturition

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(Nathanielsz *et al.*, 1982). The prolonged rise in progesterone in twin-bearing ewes and prolonged decrease in DHEA in twin fetuses following refeeding may result from a slower recovery in placental $P450_{c17}$ activity compared with singleton pregnancies.

6.4.2 Fetal HPA axis

The prepartum cortisol surge in fetal sheep begins around 128 d gestation in singleton pregnancies and 135 d in twin pregnancies (Edwards and McMillen, 2002). The lower glucocorticoid levels and the lower ratio of cortisol to ACTH in twin fetuses compared with singletons at 114 -121 d in this study are therefore unlikely to relate to differences in timing of the cortisol surge. Our results are in contrast to a previous study (Edwards and McMillen, 2002) that found lower ACTH levels in twin fetuses at 115 - 122 d, but no differences in cortisol levels, although in that study only one fetus of a twin pair was catheterised. A pattern of low cortisol but similar ACTH levels in twin sheep fetuses, similar to that of this study, along with decreased adrenal sensitivity to ACTH, has also been reported at 133 d (Gardner *et al.*, 2004a).

There are a number of possible explanations for decreased production and/or increased metabolism of glucocorticoids in late-gestation twin fetuses. Although inadequate adrenal development in twins could be one explanation, as they do have smaller adrenals at 53-56 d (MacLaughlin *et al.*, 2007) and 132 d gestation (Rumball *et al.*, 2007b), the increased cortisol response to maternal fasting in twin fetuses at 121-124 d demonstrates that the axis is fully functional and able to respond to a hypoglycaemic stress. The decreased ratio of cortisol to ACTH suggests that the adrenals of twin fetuses are less sensitive to ACTH stimulation, as has been observed previously (Gardner *et al.*, 2004a), which may be due to altered levels of the ACTH receptor, steroidogenic acute regulatory protein or adrenocortical steroidogenic enzyme activity. Although twin fetal adrenals at 53-56 d gestation have been shown to have decreased mRNA levels of $P450_{c17}$, the rate-limiting enzyme in cortisol biosynthesis, and also of IGF-1, IGF -2 and the IGF receptors (MacLaughlin *et al.*, 2007), which are thought to be important in stimulating growth of the fetal adrenal gland (Coulter *et al.*, 2002), we are not aware of any published data comparing the levels of these factors in late-gestation singleton and twin fetuses.

An alternative explanation for the differences in circulating cortisol concentrations is either altered production at extra-adrenal sites or altered trans-placental transfer from the mother. Both bioactive cortisol and inactive cortisone can be metabolised in the liver, but the interconversion of these by the 11ßHSD enzymes is thought to play an important role

in controlling local cortisol levels (Tomlinson and Stewart, 2001), although the influence on systemic levels in not known. 11ßHSD-1 is present in the fetal liver by 85 d, and functions predominantly to convert cortisone to cortisol (Tomlinson and Stewart, 2001), whereas 11ßHSD-2, which converts cortisol to cortisone, is undetectable in the liver but present in the kidney (Langlois *et al.*, 1995). Although it is not known whether hepatic 11ßHSD-1 activity is different in twin and singleton fetuses, hepatic size is reduced in twins (Rumball *et al.*, 2007b) and metabolic activity of the liver may also be altered. The liver also produces CBG, levels of which influence total cortisol levels. It has been demonstrated that circulating levels of CBG in the late-gestation sheep fetus respond to both glucocorticoid (Berdusco *et al.*, 1993) and ACTH infusions (Challis *et al.*, 1985), although it is not known whether levels are different in twins compared with singletons.

Differences in circulating maternal levels of glucocorticoid between twin- and singleton-bearing ewes are not responsible for the different fetal levels seen in our study, although differences in the placental 11ßHSD-2 barrier could influence the amount of maternal cortisol that crosses to the fetus. At 53-56 d gestation there is greater expression of 11ßHSD-2 mRNA in the placenta of twin fetuses than singletons, although there is no difference in protein expression and enzyme activity levels were not measured (MacLaughlin *et al.*, 2007). We are not aware of any data on placental 11ßHSD-2 activity in singletons and twins in late gestation.

This is the first study to assess higher centres of the HPA axis in both fetuses of a twin pair. The assessment took place at 127-8 d gestation, a time when the prepartum cortisol surge may just be starting (Edwards and McMillen, 2002; Norman *et al.*, 1985) and when CRH has its greatest effect on pituitary function (Whittle *et al.*, 2001). We found that central HPA axis sensitivity to stimulation is greater in twins than in singletons, whether assessed by the corticotrophic response to CRH/AVP or by the negative feedback response to a fall in circulating cortisol concentrations. It is not clear whether this represents a permanent increase in pituitary responsiveness to stimulation in twins, or a delay in the normal prepartum decrease in sensitivity of pituitary ACTH production to hypothalamic CRH stimulation that accompanies the cortisol surge (Lu *et al.*, 1991; Norman *et al.*, 1985), which would be consistent with a longer gestation in twin-bearing ewes (Ozturk and Aktas, 1996).

Basal ACTH secretion is regulated differently from stimulated ACTH secretion (Wood, 1991), with basal secretion probably still under cortisol negative feedback control,

whereas stimulated secretion is not. The greater ACTH response in twin fetuses to direct stimulation by CRH/AVP may be due in part to changes in receptor density, the relative proportions of the corticotroph subpopulations in the pituitary that do, or do not, express the corticotropin-releasing hormone receptor (Farrand *et al.*, 2006), or ACTH production from pro-opiomelanocortin (POMC). Farrand *et al* demonstrated that placental restriction in sheep, induced by removal of the majority of placental attachment sites prior to pregnancy, increased the proportion of corticotrophs expressing POMC, ACTH and the CRH type 1 receptor, but it is not known whether twinning also has an effect on these cells.

The greater ACTH response following decreased cortisol concentrations in the metyrapone challenge in twin fetuses could result from several factors. The similar decline in cortisol, which signifies successful inhibition of 11ß-hydroxylase by metyrapone, in all groups suggests that differences in cortisol response to metyrapone are not responsible. It also implies that the fetal adrenal is supplying similar proportions of circulating cortisol in the different groups. The difference, therefore, may lie in altered cortisol negative feedback sensitivity. The normal feedback relationship between ACTH and cortisol is known to be suspended in late gestation as both increase simultaneously (Wood, 1987). Negative feedback takes place at the hippocampus, hypothalamus and pituitary through the glucocorticoid and mineralocorticoid receptors (Keller-Wood *et al.*, 2006; Rose *et al.*, 1985), the occupancy of which is thought to be protected by local activity of the 11ßHSD-2 isozyme. Decreased pituitary expression of GR in late gestation may be a mechanism for the altered feedback (Keller-Wood *et al.*, 2006). GR levels in the higher centres of the HPA axis are known to be affected by antenatal dexamethasone (Dean *et al.*, 2001) and prenatal undernutrition (Lingas *et al.*, 1999) in guinea pigs and prenatal stress in rats (Welberg and Seckl, 2001), but the effect of twinning is not known. A further possible mechanism for the diminished feedback in late gestation would be changes in 11ßHSD activity in the higher centres of the HPA axis. 11ßHSD-2 expression in the brain is much more limited than 11ßHSD-1, at least in the rat (Robson *et al.*, 1998; Roland *et al.*, 1995), although dehydrogenase activity of 11ßHSD-1 may be significant (Yang *et al.*, 1995). 11ßHSD-1 deficient mice demonstrate exaggerated responses to stress, suggesting diminished glucocorticoid feedback (Harris *et al.*, 2001). The greater ACTH response to metyrapone in twins, therefore, may be due to increased sensitivity of

the negative feedback system, which in turn may relate to delayed parturition or permanent alteration of the axis.

However, the fact that we have previously demonstrated an increased pituitary response to CRH/AVP in post-pubertal twin sheep compared with singletons, and that HPA axis activity was related to birthweight within twin pairs, suggests that the changes in HPA axis function in twin fetuses are not due simply to a delay in the prepartum cortisol surge (Bloomfield *et al.*, 2007). Indeed, increased sensitivity of the HPA axis to insulin-induced hypoglycaemia, similar to our findings in twins, is reported to be associated with advanced rather than delayed maturation in sheep (Edwards *et al.*, 2001) and horses (Silver and Fowden, 1995). Furthermore, we have also demonstrated changes in the glucose-insulin axis in twin fetal sheep consistent with advanced pancreatic maturation (Rumball *et al.*, 2007b).

We therefore suggest that our data are consistent with the hypothesis that twinning may have long-term effects on postnatal HPA axis physiology, particularly at the level of the pituitary. However we cannot exclude the possibility that the observed changes are reflective of delayed prepartum cortisol surge as well as more permanent alterations in HPA axis function.

In contrast to the effects of twinning, fetuses of UN -60-30 ewes had pituitary resistance to CRH/AVP stimulation, but the increased 11-deoxycortisol response to decreased cortisol negative feedback was a similar response to that of twins. The 11-deoxycortisol response to metyrapone may be due to greater pituitary ACTH response to loss of negative cortisol feedback, as has been previously demonstrated (Bloomfield *et al.*, 2004) and/or greater adrenal response to ACTH. Others have reported no effect of periconceptional undernutrition from 60 days before until 7 days after mating on ACTH levels in late gestation in singleton fetuses (Edwards and McMillen, 2002). We have previously demonstrated increased basal cortisol concentrations at 127-8 d gestation in singleton fetuses following a similar periconceptional undernutrition regimen, no change in cortisol response to an ACTH challenge, an increased ACTH and 11-deoxycortisol response to metyrapone (Bloomfield *et al.*, 2004), an earlier prepartum cortisol rise and preterm birth (Bloomfield *et al.*, 2003b). The pituitary resistance to direct stimulation could be due to decreased CRH receptor levels in the pituitary, as occurs as parturition approaches (Lu *et al.*, 1991), altered corticotroph populations, or altered POMC processing, since this also changes approaching term (Holloway *et al.*, 2000). Whether

these changes relate to ontogeny, permanent changes in HPA axis function or both is not clear from this experiment, although increased basal cortisol levels and increased ACTH response to CRH stimulation have been found in lambs whose mothers were undernourished from mating until 30 d gestation (Chadio *et al.*, 2007).

6.4.3 Conclusions

This study demonstrates that twinning and periconceptional undernutrition have profound but very different effects on fetal HPA axis function in late gestation. Further research into the mechanisms underlying these differences may provide insights into the different effects of these periconceptional events on gestation length. In sheep, gestation length is decreased by periconceptional undernutrition but increased by twinning. However, in human pregnancy there is increasing evidence that poor nutrition around the time of conception (Rayco-Solon *et al.*, 2005) and twinning (Luke *et al.*, 1996) both decrease gestation length. Therefore, by understanding the different effects of these periconceptional events on fetal HPA axis function, insights may be gained into the onset of parturition in human preterm birth.

The changes in fetal HPA axis function that we describe may reflect altered maturation of the axis before birth, but may also indicate altered long-term function of the HPA axis, particularly at the level of the pituitary. It is also possible that both are manifestations of the same underlying alterations in the HPA axis. Antenatal glucocorticoids, for example, affect both gestation length and postnatal HPA axis function in guinea pigs (Dean *et al.*, 2001).

Despite evidence of long-term alterations in physiology as a result of twinning and lower birth weights, evidence linking twinning to increased rates of cardiovascular disease or its risk factors in later life in human studies remains conflicting (Phillips *et al.*, 2001). The substantially different fetal development of twins compared to both control and undernourished singletons, as we have shown in the glucose-insulin and the HPA axis, may be responsible for some of this conflict. These findings emphasise that the underlying causes of altered fetal development are more important in the relationship with adult health and disease than the gross effects on fetal growth.

7. C-type natriuretic peptide forms in the ovine fetal and maternal circulations

7.1 Introduction

Little is known of the part played by CNP in fetal and maternal health. However *in vitro* studies show that CNP is strongly expressed in placenta (Cameron *et al.*, 1996) and uterine tissues (Stepan *et al.*, 2000a). In the mouse embryo, CNP expression is also evident in brain and spinal cord (Cameron *et al.*, 1996) and is reported to increase in embryo truncal tissues during the course of gestation (Stepan *et al.*, 2001). In the human, the extremely high levels of NTproCNP in fetal plasma (Prickett *et al.*, 2004), and the evidence of a small fetal-maternal CNP gradient (Prickett *et al.*, 2004; Stepan *et al.*, 2000b) suggest that the fetal and maternal production of CNP may be independent. Whether CNP synthesis in pregnancy is subject to acute regulation and separately regulated in the fetus is not known.

This study illustrates the CNP forms in the ovine maternal and fetal circulation and shows that the utero-placental tissues are a major contributor to maternal levels. Furthermore, reciprocal changes in fetal and maternal circulating CNP levels in response to acute maternal undernutrition provide the first evidence that CNP synthesis is regulated independently in the mother and fetus in late gestation.

7.2 Methods

7.2.1 Experimental design

Thirty-nine ewes, 21 with singleton and 18 with twin pregnancies, underwent surgery at 111±1 d for insertion of maternal and fetal catheters. Ewes were fasted for 3 d, beginning after the morning blood sample at 121 d, and refed after sampling at 124 d. Paired arterial blood samples were drawn for measurement of CNP forms, glucose, urea, insulin and IGF-1 from both mother and fetus between 0800 and 0900 h before (114, 117 and 121 d), during (122, 123 and 124 d) and after fasting (125, 126, 128 and 131 d). To examine possible utero-placental contributions to maternal circulating levels, paired blood samples were also drawn for measurement of CNP forms from the maternal arterial and

utero-ovarian venous catheters before (114 and 121 d), at the end of (124 d) and after fasting (125, 126 and 131 d). The ewes were euthanised at 132 d with an overdose of pentobarbitone and placental and fetal weights measured.

7.2.2 Statistics

Data are presented as mean ± SEM. Student's *t*-test was used to analyse differences in analyte levels between mother and fetus. Repeated measures ANOVA with Bonferroni *post hoc* analysis was used to assess changes in levels over time, with group (single vs twin) included as a covariate where appropriate. Relationships between variables were explored using simple linear regression. Statistical significance was assumed when $p<0.05$.

7.3 Results

Not all samples were available from all animals, due to a combination of catheter failure and some fetal losses, but complete data to the end of the experiment were obtained from 13 singleton and 10 twin-bearing ewes. Mean fetal weight at 132 d was 4514±125 g for singletons and 3687±100 g for twins ($p<0.01$). Mean total placental weight was 582±29 g for singleton-bearing and 808±32 g for twin-bearing ewes ($p<0.01$).

7.3.1 Fetal and maternal plasma CNP and NTproCNP

Maternal plasma concentrations of NTproCNP (273±10 pmol.L^{-1}, n=39, day 114) and CNP (40±4 pmol.L^{-1}, n=39) were higher than fetal levels (NTproCNP, 221±7 pmol.L^{-1}, n=56; CNP, 3.8±0.2 pmol.L^{-1}, n=43) prior to fasting (both $p<0.001$), with the mean maternal:fetal ratio 1.3±0.1 for NTproCNP and 11.8±1.0 for CNP. In fetal plasma, the ratio of NTproCNP:CNP was also much higher than in maternal plasma (57±3 vs 8.8±0.6, $p<0.001$) consistent with enhanced degradation of CNP in fetal tissues or plasma. Over the course of the 18-day study, fetal levels of NTproCNP declined significantly ($p<0.001$, Figure 7-1A).

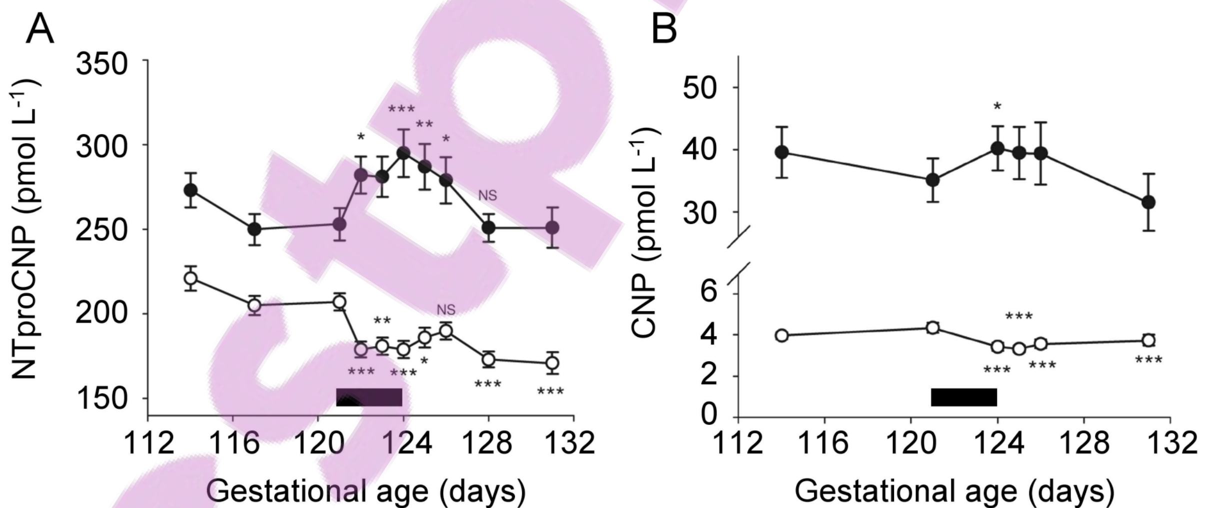

Figure 7-1: Circulating maternal and fetal concentrations of (A) NTproCNP and (B) CNP during gestation

*Ewes were fasted during 121–124 d represented by black bar. Results are expressed as means ± SEM. Maternal = ● (n=39), fetal =○ (n=57). Significant differences from baseline values (121 d) are indicated by asterisks, * p < 0.05, ** p < 0.01, *** p < 0.001.*

7.3.2 Response to fasting

Within 24 h of food withdrawal, there was a rapid fall of fetal plasma NTproCNP levels (207±5 to 179±5 $pmol.L^{-1}$, $p<0.001$). Levels remained low for the duration of the fasting period and then increased within 24 h of refeeding (Figure 7-1A). Similar proportional and directional changes ($p<0.001$) were observed in fetal plasma CNP levels (Figure 7-1B).

Coincident with these changes in the fetus were reciprocal changes in maternal levels. Maternal plasma NTproCNP levels increased from 253±10 to 282±11 $pmol.L^{-1}$ within 24 h of food withdrawal ($p<0.05$), rising to a peak of 295±14 $pmol.L^{-1}$ ($p<0.001$) at the end of the fast (124 d) (Figure 7-1A). Levels then fell progressively after refeeding to attain basal levels by 128 d. Changes in maternal CNP levels were similar in direction and proportion (35±3 $pmol.L^{-1}$ before to 40±4 $pmol.L^{-1}$ at end of the fast, $p<0.05$, Figure 7-1B).

Maternal and fetal plasma levels of glucose, insulin and IGF-1 all decreased with fasting, while urea levels increased (Table 7-1). All levels returned towards baseline after refeeding.

		114 d	121 d [1]	124 d [2]	131 d
Glucose ($mmol.L^{-1}$)	Maternal**	3.34±0.08	3.61±0.05	1.76±0.07††	3.49±0.07
	Fetal**	0.86±0.03	0.96±0.02	0.49±0.02††	0.90±0.03
Urea ($mmol.L^{-1}$)	Maternal**	5.00±0.16	4.48±0.19	7.69±0.32††	4.39±0.29
	Fetal**	5.73±0.14	5.24±0.16	9.04±0.26††	4.87±0.23
Insulin ($ng.mL^{-1}$)	Maternal**	0.19±0.03	0.30±0.04	0.03±0.00††	0.23±0.03
	Fetal**	0.13±0.01	0.20±0.02	0.07±0.01††	0.15±0.01
IGF-1 ($ng.mL^{-1}$)	Maternal*	49.8±4.5	67.7±6.3	57.2±4.6†	82.8±8.0
	Fetal*	62.4±2.8	73.8±3.6	52.8±5.2††	76.2±5.1

Table 7-1: Fetal and maternal plasma glucose, urea, insulin, and IGF-1 levels before, during and after maternal fasting

[1] *beginning of fasting period,* [2] *end of fasting period*
** $p< 0.01$, * $p< 0.05$ *for changes over time*
†† $p< 0.01$, † $p< 0.05$ *for post hoc comparison of concentrations at 121 and 124 d.*
Values are mean ± SEM for 50 fetuses and 35 ewes

7.3.3 Effect of twinning

Maternal plasma levels of both NTproCNP and CNP were higher in twin than in singleton pregnancies (Table 7-2). The reverse was true in the fetus, where mean NTproCNP and CNP levels were lower in twins than in singletons, although this difference did not reach statistical significance for NTproCNP (p=0.1 and p=0.027 NTproCNP and CNP respectively) (Table 7-2). Maternal and fetal plasma levels of glucose and insulin were higher in singleton than twin pregnancies throughout the experimental period (Table 7-2).

		Single		**Twin**	
		Day 114	Day 131	Day 114	Day 131
CNP ($pmol.L^{-1}$)	Maternal**	26.4±2.3	25.2±3.5	54.9±7.1	41.1±9.8
	Fetal*	4.22±0.39	4.22±0.48	3.55±0.21	3.43±0.28
NTproCNP ($pmol.L^{-1}$)	Maternal**	245±10	238±14	306±15	269±22
	Fetal	233±16	178±10	214±6	166±9
Glucose ($mmol.L^{-1}$)	Maternal**	3.58±0.05	3.64±0.06	2.98±0.13	3.29±0.12
	Fetal**	1.02±0.03	1.02±0.05	0.74±0.03	0.81±0.03
Insulin ($ng.mL^{-1}$)	Maternal**	0.26±0.03	0.32±0.04	0.08±0.02	0.12±0.02
	Fetal**	0.18±0.02	0.19±0.02	0.10±0.02	0.13±0.02
IGF-1 ($ng.mL^{-1}$)	Maternal†	47.8±6.0	75.8±10.3	52.6±6.8	91.2±12.7
	Fetal	68.8±4.4	82.7±8.2	58.1±3.4	71.9±6.5

Table 7-2: Fetal and maternal plasma CNP forms, glucose, insulin and IGF-1 levels in singleton and twin pregnancies

** $p < 0.05$, ** $p < 0.01$ for singles vs twins*
† $p < 0.05$ for group by time interaction
Values are mean ± SEM for 20 singleton fetuses and their mothers, and 30 twin fetuses and their 15 mothers.

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7.3.4 Source of CNP forms in maternal plasma

Mean plasma levels of CNP in the utero-ovarian vein were at least 3-4 fold higher than the corresponding maternal arterial levels (Figure 7-2). During the 18 d study period, plasma levels of CNP in the utero-ovarian vein declined significantly ($p<0.003$).

Mean plasma levels of NTproCNP were also slightly higher, though not statistically significant, in the utero-ovarian vein than in the maternal artery, presumably reflecting the longer half life of the amino terminal (bio-inactive) fragment in the circulation.

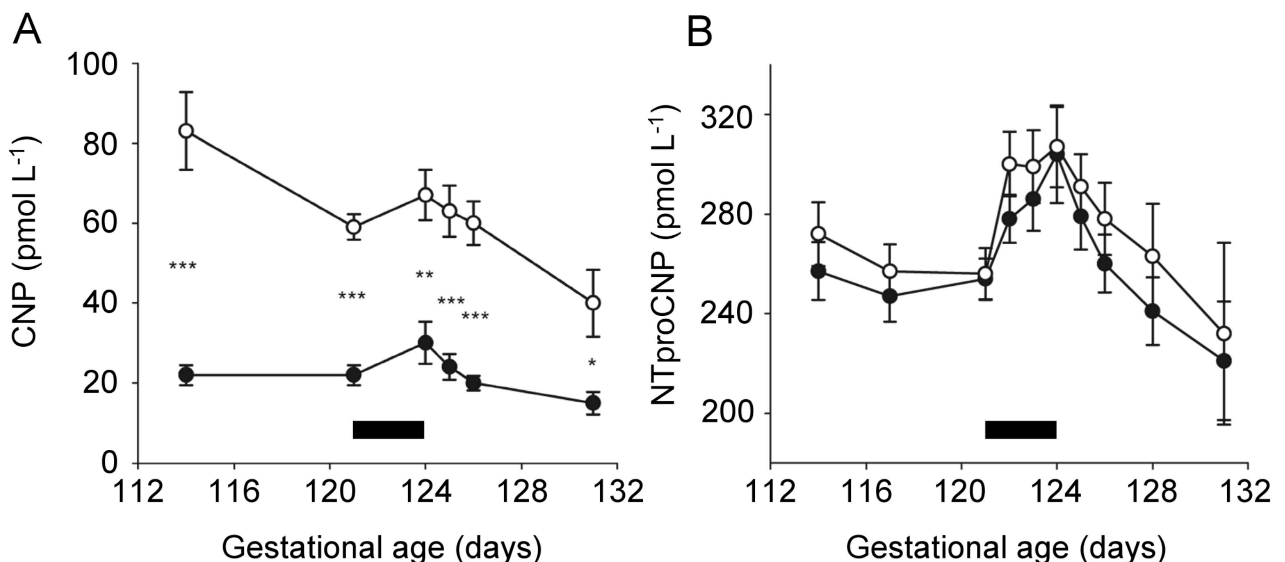

Figure 7-2: Plasma concentrations of (A) CNP and (B) NTproCNP drawn simultaneously from the maternal artery and utero-ovarian vein from six pregnant ewes with singleton pregnancies

*(Note that the lower arterial CNP concentrations in this figure, compared to those of ewes in figure 7-1, reflect the lower CNP levels in singleton pregnancies). Results are expressed as means ± SEM. Maternal artery = ●, utero-ovarian vein =○. Significant differences between arterial and venous values are indicated by asterisks, * p<0.05, ** p<0.01, *** p<0.001.*

There were significant correlations between placental weight and maternal NTproCNP levels at 131 d (Figure 7-3), and between placental weight and maternal CNP (r^2=0.18, p<0.05). Fetal CNP and NTproCNP levels were not related to fetal or placental weight.

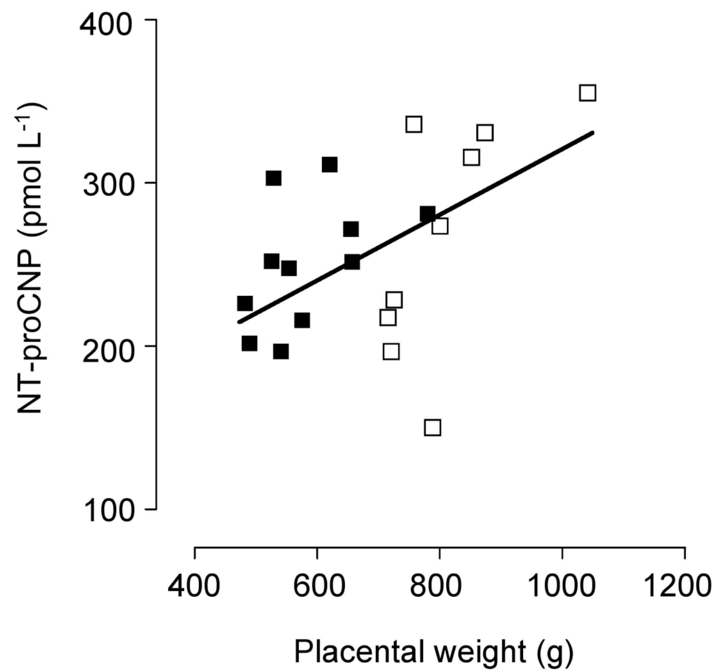

Figure 7-3: Relationship between placental weight and maternal NTproCNP levels at 131 d

Singleton = ■ (n=12), twin-bearing = □ (n=9). r^2*=0.26, p=0.01*

7.4 Discussion

These studies in healthy chronically cannulated pregnant ewes reveal a number of new findings pertaining to the circulating forms and regulation of CNP in ovine pregnancy. First, significantly higher concentrations of both CNP (11.8 fold) and NTproCNP (1.3 fold) in the maternal plasma than in fetal plasma constitute strong evidence that synthesis and/or metabolism in mother and fetus are independent. Second, the striking and reciprocal responses in CNP levels to acute maternal undernutrition suggest that the hormone is independently regulated in the two circulations. Third, the findings of a high CNP gradient across the gravid uterus, higher maternal levels of both CNP and NTproCNP in twin than in single pregnancy, and a correlation between placental weight and maternal NTproCNP levels strongly suggest that utero-placental tissues are a major contributor to maternal levels in late gestation. In addition, we confirm previous original observations made in humans (Prickett *et al.*, 2004) showing that the ratio of NTproCNP to CNP is greatly increased in fetal plasma, consistent with high rates of CNP degradation in the fetus. Taken together these findings suggest a potential role for CNP in fetal welfare that deserves further investigation.

Previous reports of circulating CNP forms in mother and fetus are sparse, and to our knowledge none have studied plasma levels contemporaneously over an extended time period, as described here in healthy sheep. In late gestation (114-131 d) maternal plasma CNP levels were some 50 fold higher than those previously reported in non-pregnant adult ewes (20 – 40 $pmol.L^{-1}$ vs 0.6 $pmol.L^{-1}$ (Charles *et al.*, 2006)), and NTproCNP levels 10 fold higher than non-pregnant levels. In contrast, in samples drawn at the same time from the fetus, plasma CNP levels were 3 – 4 $pmol.L^{-1}$, similar to levels observed at term in the human fetus (Prickett *et al.*, 2004). The 10-fold lower levels of CNP in the fetus than in the mother suggest that maternal transfer of bioactive forms to the fetus is minimal, consistent with the very limited transplacental passage of other peptide hormones in ruminants (Schroder, 1995). However the plasma NTproCNP levels were high in the fetus (200–230 $pmol.L^{-1}$) and were similar to those observed in the human fetus at term (Prickett *et al.*, 2004). Furthermore, the ratio of NTproCNP to CNP was some 6-fold higher in the fetal than in the maternal circulation. Assuming that the clearance rate of NTproCNP does not differ in the two circulations, these findings suggest that both synthesis and degradation of CNP are enhanced in the ovine fetus. A similar (5-fold) difference in the ratio of NTproCNP to CNP has been reported in human mothers and fetuses (Prickett *et*

al., 2004), and might suggest that limiting circulating bioactive CNP concentration in the fetus may carry advantages, for example in preventing skeletal tissue overgrowth prior to birth in mammals. Clearly further work is required to localize sources of CNP synthesis and degradation in the fetus. For example, comparative studies of CNP degradation rates in maternal and fetal circulations and the contribution of the clearance receptor (NPR-C) and other degradative enzymes need to be assessed. Hydrolysis by neprilysin (EC 3.4.24.11, neutral endopeptidase), the main CNP-catabolising enzyme (Potter *et al.*, 2006) warrants particular attention in future studies. The 2-fold higher concentration of neprilysin activity in human fetal than maternal plasma (Walther *et al.*, 2004), widespread peripheral expression of neprilysin in rodent embryo (including skeletal) tissues (Facchinetti *et al.*, 2003), and raised neprilysin immunoreactivity in bone tissues of the newborn when compared to the adult (Ruchon *et al.*, 2000), could be relevant to our findings on fetal CNP which is an excellent substrate for neprilysin (Kenny *et al.*, 1993).

Additional evidence that fetal and maternal CNP are regulated separately in the two circulations is provided by our observations during maternal fasting. Food restriction to 25% of normal in 4-week-old lambs rapidly and reversibly reduces CNP synthesis, reflected in proportionate changes in circulating levels of both CNP and NTproCNP (Prickett *et al.*, 2007). The present study shows that similar responses are evoked by nutritional deficiency in the fetus. The fall in fetal NTproCNP levels was equally if not more prompt than that observed in postnatal lambs. For reasons of limited blood volume, CNP sampling was less intensive, but the fall in plasma levels was proportionate to that seen for NTproCNP, suggesting that CNP synthesis is also acutely sensitive to nutritional status in the fetus. In postnatal lambs, plasma NTproCNP concentration is highly correlated with linear growth rate and markers of bone formation (Prickett *et al.*, 2005). Catabolic interventions such as glucocorticoid administration promptly reduce circulating CNP forms and alkaline phosphatase, and reduce skeletal growth velocity. Whether the fasting-induced reduction in CNP synthesis observed here in fetal lambs represents diminished cell proliferation within CNP producing tissues such as growth plate chondrocytes (Chusho *et al.*, 2001) requires further investigation of the main source(s) of circulating CNP levels in the fetus.

Changes in plasma glucose, insulin and IGF-1 levels were similar in direction and time course to the changes in fetal plasma CNP levels during fasting and refeeding. However insulin suppresses CNP expression in vitro (Igaki *et al.*, 1996), and large

increases in plasma IGF-1 do not affect CNP levels in growing lambs (Prickett *et al.*, 2007), making it unlikely that these hormones have any direct role in the fetal CNP response to maternal fasting.

In contrast to the fall in fetal plasma levels of NTproCNP and CNP during maternal fasting, maternal levels rose, with a time course and magnitude of peak response that reciprocated those observed in the fetus. However the fall in maternal plasma glucose, insulin and IGF-1 levels were broadly similar to those seen in the fetus. These unexpected findings, while confirming the independence of CNP synthesis within fetal and maternal circulations, strongly suggest separate regulatory systems, and presumably separate functions, for CNP in mother and fetus.

The mechanisms underlying these differential responses were not explored in this study, but the close temporal relationships raise the possibility that the maternal rise could be in response to the abrupt fall in fetal CNP synthesis or action. Previous findings of raised plasma CNP forms in the presence of loss-of-function mutations of the CNP receptor (NPR-B) suggest the possibility of feedback inhibition in this system (Olney *et al.*, 2006). However the fact that fetal plasma NTproCNP levels fell over the time course of our study without any apparent effect on maternal levels makes this explanation unlikely.

Reduced renal clearance of the hormone is another possible explanation. Plasma NTproCNP but not CNP levels increase as renal function declines in adult humans and dogs (Tim Prickett, unpublished observations). However there is no evidence that fasting for 3 days would induce acute but reversible renal impairment in the ewes in our study. The small transient rise in plasma urea concentrations observed with fasting is consistent with the expected increase in protein oxidation over this period, and was not of sufficient magnitude to suggest impaired renal function. Moreover, the proportionate increase in both maternal CNP and NTproCNP concentration makes it unlikely that changes in renal function underlie the maternal response.

Another intriguing possibility is that an increase in maternal CNP synthesis represents a homeostatic (compensatory) response of the utero-placental unit to nutrient deprivation. Both the nitric oxide (NO) and CNP signalling pathways within the uterine vascular wall play an important part in maintaining the greatly increased uterine blood flow during late gestation (Itoh *et al.*, 1998). Fasting may lead to a fall in maternal plasma arginine and citrulline concentrations (Jane Harding, unpublished observations), leading to

reduced NO production (Wu and Meininger, 2002) and a compensatory increase in CNP synthesis (Zhang *et al.*, 1999) and activity (Madhani *et al.*, 2003). Furthermore, vascular endothelial growth factor (VEGF) is important in maintaining NO production (Ahmed *et al.*, 1997; Ni *et al.*, 1997) and placental VEGF expression is reduced during short term (5 days) fasting in sheep in mid gestation (McMullen *et al.*, 2005). Since VEGF inhibits CNP synthesis at concentrations within the physiological range (Doi *et al.*, 1996), fasting-induced reduction in VEGF production (McMullen *et al.*, 2005) or activity (Ahmad and Ahmed, 2005; Nagamatsu *et al.*, 2004; Reynolds *et al.*, 2005) in placental tissues would also lead to an increase in maternal CNP concentrations. This is consistent with a previous report that in women with pre-eclampsia, where nutrient supply to the fetus is also threatened, plasma NTproCNP levels were increased 35% in maternal plasma, and reduced 17.5% in the fetus when compared to values observed in healthy normotensives at term (Prickett *et al.*, 2004). Further studies are required to determine the role of CNP in utero-placental physiology and evaluate its potential as a possible marker of nutrient supply to the feto-placental unit.

Our finding of consistently higher CNP levels in plasma from the utero-ovarian vein than from the simultaneously sampled maternal artery suggests that the utero-placental tissues make a major contribution to systemic plasma levels of CNP in pregnant sheep. Plasma NTproCNP levels were also higher in the utero-ovarian vein than the maternal artery, but the difference was smaller and not statistically significant, in keeping with the much longer half life of this bio-inactive form when compared to CNP. Our study was not designed to determine which tissues contributed to the higher levels in the utero-ovarian vein. These may include placental tissues (Cameron *et al.*, 1996), myometrium (Stepan *et al.*, 2002) and the wall of the uterine arteries themselves (Itoh *et al.*, 1998). However our findings that maternal plasma levels of both CNP and NTproCNP were higher in ewes carrying twins than in those carrying singletons, and the correlation between maternal NTproCNP levels and placental weight, both suggest that at least some is likely to be of placental origin.

This study shows that circulating CNP levels in pregnant ewes are more than 10 fold higher than those of healthy pregnant women (mean 1.8±0.2 pmol.L^{-1}) at term. Anatomical differences in placentation (modified epitheliochorial in sheep, hemochorial in humans) may perhaps contribute to this difference, although the additional intervening tissue layers would reduce the access of any CNP produced by the fetal cotyledonary

tissues to maternal circulation in the sheep, potentially resulting in lower rather than higher maternal plasma levels in this species. Another possible explanation is the marked difference in maternal plasma VEGF concentration (an inhibitor of CNP synthesis) and which increases progressively during pregnancy in the human but not ovine pregnancy (Vonnahme *et al.*, 2005). However mediated, sustained plasma CNP concentrations ranging from 20-60 $pmol.L^{-1}$ may have important haemodynamic effects (Drewett *et al.*, 1995) over and above the paracrine vasodilator actions within the uterine vasculature and now need to be re-evaluated.

Whether circulating levels of CNP forms in the fetus reflect skeletal growth rates, as shown in postnatal lambs and children (Prickett *et al.*, 2005) has not yet been studied. Transcripts for components of the CNP signalling pathway have been identified in fetal long bones of rodents, and the fetal mouse tibia is highly responsive to exogenous CNP *ex vivo* (Yasoda *et al.*, 1998). However genetic manipulations (Chusho *et al.*, 2001; Tamura *et al.*, 2004; Yasoda *et al.*, 2004) and spontaneous mutations (Bartels *et al.*, 2004) appear to affect postnatal growth but not size at birth. This is consistent with our finding of no significant correlation between fetal weight and plasma CNP forms at 131 d gestation. Despite this, our study shows that fetal levels of NTproCNP fall from mid to late gestation at the time when fetal growth velocity also falls. The lower plasma levels of NTproCNP in twin than in singleton fetuses are also consistent with the hypothesis that CNP may contribute to fetal skeletal growth in both species. However further work on the concentration of hormone, including the presence of proCNP(1-103) in skeletal and other tissues, during different phases of fetal growth are required to clarify role for CNP in growth regulation before birth.

In summary, this is the first report of circulating CNP forms in normal pregnant ewes and their fetuses in late gestation. We have shown that plasma NTproCNP and CNP levels are acutely and independently regulated by nutrient supply in both mother and fetus. Our data suggest that rates of CNP synthesis and degradation are high in the fetus, while the utero-placental tissues are a major source of CNP in the mother. CNP may have a role in maintaining fetal welfare, and provide a possible marker of utero-placental nutrient supply.

8. Conclusions

The studies described in this thesis were designed to investigate the effect of events around conception on maternal cardiovascular adaptation to pregnancy and fetal growth, physiology and endocrinology. We were interested specifically in periconceptional undernutrition, twinning and their interaction.

8.1 Periconceptional undernutrition

This is the first study to compare pre- and post-conception undernutrition in the sheep within the same experimental protocol. It has previously been demonstrated that poor weight gain in early pregnancy does not affect birth size in humans (Abrams and Selvin, 1995). However, low body mass index at conception reduces birth weight (Ronnenberg *et al.*, 2003), and poor nutrition in early gestation results in adult pathology (Roseboom *et al.*, 2001a). Furthermore, undernutrition in early pregnancy in sheep affects postnatal cardiovascular (Gardner *et al.*, 2004b) and HPA axis function (Gardner *et al.*, 2006). In the sheep, maternal undernutrition from 60 d before until 30 d after mating has been shown to affect late-gestation fetal growth (Harding, 1997a), metabolism and glucose-insulin axis (Oliver *et al.*, 2001b) and HPA axis function (Bloomfield *et al.*, 2004). However, there have been no previous studies investigating the effects of pre and postconception undernutrition separately on maternal adaptation to pregnancy and late-gestation fetal growth, physiology and endocrinology.

The first two experimental chapters in this thesis report experiments designed to test the hypothesis that pre and/or postconception undernutrition in sheep have different effects on the mother and fetus. The distinction between nutrition before and after conception is of both academic and practical interest. If preconception nutrition alone had important effects, this would suggest that maternal nutrition affects the oocyte and/or very early embryonic development, perhaps due to effects on ovarian follicular recruitment and maturation or on the oviductal nutritional or hormonal environment. The public health strategies required to optimise pregnancy outcome would be of a different magnitude and nature to educate women of childbearing potential, rather than just those who are pregnant.

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If postconception undernutrition alone had important effects, then the underlying mechanisms are more likely to relate to the processes of blastocyst maturation, implantation, maternal adaptation to pregnancy and placental growth and function. In this case, the public health message would emphasise the need for awareness of possible conception and the significance of the immediate postconception period for the baby's development. Maternal nutrition around conception is already known to have effects on epigenetic modifications in the fetal genome (Waterland and Jirtle, 2004), early embryo development (Kwong *et al.*, 2000), and late-gestation feto-placental growth (Harding, 1997a) and endocrinology (Oliver *et al.*, 2001b). However the specific timing of these effects, their interrelationships, and the mechanisms linking prenatal environment and postnatal pathology are unknown.

One mechanism by which periconceptional undernutrition may affect feto-placental development is through impaired maternal cardiovascular adaptation to pregnancy. Inadequate increases in blood volume and uterine blood flow are associated with fetal growth restriction in human pregnancy, although causal relationships are not well established (Duvekot *et al.*, 1995; Nylund *et al.*, 1983). Maternal nutrition is known to affect blood volume in pregnant sheep (Dandrea *et al.*, 2002), but the effects of periconceptional undernutrition on maternal cardiovascular adaptations to pregnancy have not previously been investigated.

Although we demonstrated that blood volume adaptation to pregnancy in sheep is less pronounced than in humans, the increase in late-gestation uterine blood flow in undernourished groups signifies long-term structural or physiological changes in the mother and/or placenta due to periconceptional undernutrition. It is possible that the increase in uterine blood flow is a compensatory strategy to maintain fetal growth in late gestation following decreased maternal nutrition around conception, but the period of undernutrition did not appear to be critical for induction of this strategy.

In contrast, the effects of pre and post-conception undernutrition on fetal and placental growth were different. Preconception undernutrition resulted in a relatively large placenta and small fetus that grew slowly in late gestation, whereas postconception undernutrition resulted in a normal size placenta and a fetus that grew more rapidly in late gestation. Undernutrition both pre- and postconception resulted in normal placental and fetal size but altered fetal growth response to a nutritional challenge. These different effects suggest different underlying mechanisms, and may help to explain why there are

such variable relationships reported between maternal nutrition, fetal growth and size at birth. Further studies would be needed to distinguish the effects of maternal nutritional status at conception from those of changes in maternal nutrition immediately after conception. More detailed studies of the effects of the different periods of undernutrition on placental structure, nutrient transfer capacity and endocrine function may also help clarify some of the mechanisms involved.

This study assessed also the regulation of the recently discovered C-type natriuretic peptide (CNP) in pregnancy. The plasma levels of CNP are associated with postnatal skeletal growth in humans and sheep (Prickett *et al.*, 2005), and are responsive to nutrition in lambs (Prickett *et al.*, 2007). We demonstrated that the placenta is a source of CNP in pregnancy, and that the maternal and fetal plasma levels are acutely and independently regulated by nutrition. This raises the possibility that CNP may be one of the mediators of the link between nutrition and fetal growth, and highlights it as an area for further research.

Altered glucose-insulin axis function is the basis of type 2 diabetes and an important component of adult metabolic disease. Poor maternal nutrition around conception is known to affect glucose-insulin axis function in both the fetal sheep (Oliver *et al.*, 2001b) and the adult human (de Rooij *et al.*, 2006b). There were no significant effects of pre and/or postconception undernutrition on glucose-insulin axis function in the experiment on singleton fetuses detailed in chapter 4. However, the twin experiments demonstrated that maternal undernutrition through both the pre- and postconception periods resulted in a decreased glucose area under the curve following a glucose bolus, although there was no difference in insulin response. The fact that this effect was found in the twin but not the singleton experiment was probably due to the greater statistical power of the twin study due to larger numbers of fetuses and fewer groups, since a similar trend is evident in the singleton experiment, but it did not reach statistical significance. This finding may indicate increased glucose disposal by insulin-independent tissues such as the placenta or brain. Assessment of the activity of the different glucose transporters in different tissues may help clarify this.

Increased activity of the HPA axis in adult humans is related to reduced birth size, and is postulated to underlie the increased incidence of multiple adulthood pathologies (Phillips *et al.*, 1998). Maternal nutrition around conception is known to affect HPA axis function in fetal (Bloomfield *et al.*, 2004) and postnatal sheep (Gardner *et al.*, 2006),

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which in turn may affect development of the glucose-insulin axis (Breant *et al.*, 2006). In the singleton experiment described in chapter 4, pre and/or postconception undernutrition did not have significant effects on fetal HPA axis function. However, the more powerful twin experiment demonstrated that undernutrition through both the pre- and postconception periods resulted in fetuses with decreased pituitary ACTH response to stimulation by CRH/AVP, and an increased adrenal response to decreased cortisol negative feedback. Once again, the pattern of changes was similar in the singleton experiment, but the differences were not statistically significant. In previous studies, undernutrition through both pre and postconception in sheep resulted in premature delivery (Bloomfield *et al.*, 2003b). The changes we have observed may therefore reflect precocious activation of the HPA axis in preparation for early delivery, consistent with that earlier study. If this were the case, the observed changes in HPA axis function would be expected to disappear after birth. Alternatively, they may reflect permanent changes in the activity of the axis and therefore have implications for later health.

Once mechanism by which maternal nutrition during pregnancy may lead to changes in fetal and postnatal physiology is by altered fetal exposure to maternal glucocorticoids *in utero*. However, we did not identify any differences in maternal late-gestation HPA axis function resulting from periconceptional undernutrition that may have contributed to the observed changes in fetal HPA axis function. Nevertheless, it is still possible that differences in placental 11ßHSD-2 activity between groups could have resulted in different levels of fetal exposure to glucocorticoids, and analysis of the placentomes collected at postmortem is currently underway to address this issue. In addition, measurement of glucocorticoid and mineralocorticoid receptor expression at different levels of the HPA axis is being undertaken in an attempt to localise the site of the observed differences in negative feedback by cortisol, and activity of the steroidogenic enzymes is being measured in the adrenal to explore the basis of the differences in adrenal sensitivity between nutritional groups.

8.2 Twinning

The experiments described in chapters 5 and 6 were designed to test the hypothesis that the biology of fetal development is different in twin and singleton fetuses. Given the well-established relationship between low birth weight and adult physiology and pathology (Barker, 2004b), it may be expected that twins would have a higher incidence of adult pathology, as on average they are born lighter than singletons. However, the literature is

very inconsistent. Twins do not appear to have increased mortality compared with the rest of the population (Christensen *et al.*, 1995; Vagero and Leon, 1994), but there is evidence of decreased insulin sensitivity (Jefferies *et al.*, 2004) and increased blood pressure (Jefferies *et al.*, 2003) in childhood. We explored one possible explanation for the inconsistency of the relationship between the low birth weight in twins and adult pathology, which is that their biology of fetal development is fundamentally different from that of singletons. Most sheep studies have used a mixture of twins and singletons, and although some have investigated twin endocrinology in late gestation, very few have studied both twins of a pair, and none in as much detail as our study.

We found that twinning resulted in decreased maternal food intake and lower plasma glucose and insulin levels in late gestation, but higher plasma FFA and ketone levels. Furthermore, twin-bearing ewes were not heavier than singleton-bearing ewes, despite the greater conceptus weight. Therefore it is likely that maternal provision of nutrients to the uteroplacental circulation is relatively less for twin fetuses than singletons, again emphasising the importance of studying maternal physiological adaptations during pregnancy in seeking to understand changes in fetal physiology. Twin fetuses also had a greater fetal to placental weight ratio than singletons, again suggesting that more detailed studies of placental function may help elucidate some of the underlying mechanisms.

Twins grew more slowly in late gestation than singletons and slowed their growth more with maternal fasting. This suggests that growth rate in late gestation in twins may be more limited by maternal nutrient supply than it is in singleton pregnancies. Interestingly, the light twin of a pair from periconceptionally undernourished ewes maintained its growth in the face of a maternal fast better than the light twin of a control pair. This may reflect a feto-placental adaptation to periconceptional undernutrition that allows maintenance of growth in the face of poor nutrition. Such adaptations have previously been postulated to explain the maintenance of growth rate observed in the singleton fetuses of periconceptionally undernourished ewes in the face of 10 d of maternal undernutrition in late gestation (Harding, 1997a). This suggests that fundamental aspects of fetal metabolism and growth are established in the periconceptional period, but the physiological basis of these adaptations is not known.

Twin fetuses had greater insulin responses to glucose than singletons, but smaller insulin responses to arginine. These changes in glucose-insulin axis function are consistent with accelerated pancreatic maturation as, compared to fetal ß cells, adult-type ß

cells are thought to be more responsive to glucose than amino acids (Hill and Duvillie, 2000). However, twin fetuses had suppressed baseline HPA axis function compared to singletons in late gestation, increased ACTH response to stimulation by CRH/AVP and to decreased cortisol negative feedback, and decreased adrenal sensitivity to ACTH stimulation. These findings are consistent with delayed rather than accelerated maturation. Although twin pregnancies in humans are more likely to deliver prematurely than singleton pregnancies (Kiely, 1998), twin pregnancies in sheep do not appear to be shortened, and in fact may be prolonged (Ozturk and Aktas, 1996). If the apparent delay in HPA axis maturation that we observed in twins does relate to length of gestation, this would suggest that different endocrine systems are maturing at different rates. However it is also possible that the observed changes may represent permanent alterations in HPA axis function in twins, as has been suggested previously (Bloomfield *et al.*, 2007).

This study was designed also to assess the interaction between twinning and periconceptional undernutrition. The effects of periconceptional undernutrition on the HPA axis were similar in both twin and singleton fetuses, but periconceptional undernutrition appeared to abolish the difference in insulin response to glucose observed between control twins and singletons. Previous studies have demonstrated that the fetuses of periconceptionally undernourished ewes have increased insulin response to glucose in late gestation (Oliver *et al.*, 2001b), and although the pattern of change was similar in this study, it was not statistically significant. Thus it appears that the periconceptional events of twinning and undernutrition have distinct and different effects on the fetal HPA axis, but have interacting and perhaps similar effects on the fetal glucose-insulin axis. Ongoing studies of the fetal pancreas and muscle at tissue level, particularly of the glucose transporters and insulin signalling pathways, will help clarify the extent of these potential similarities.

Much of the original epidemiological research in the area of developmental origins of health and disease correlated birth weight with adult physiology and pathology. More recent studies in sheep have demonstrated that similar birth weights may result from different growth trajectories, and that the effects of maternal nutrition on fetal endocrine function can occur independently of effects on birthweight. Our studies provide several examples demonstrating that fetal size is poorly reflective of fetal development. Firstly, fetuses of ewes undernourished only postconception were similar size to controls at postmortem, but were growing more rapidly in late gestation to achieve that size. This

demonstrates that different growth trajectories may result in the same body size. Secondly, the singleton fetuses of ewes undernourished through both pre and postconception were similar size to control singleton fetuses at postmortem, but had significant differences in fetal physiology and endocrinology. Thirdly, the singleton fetuses of ewes undernourished only preconception were a similar weight at postmortem to heavy twin fetuses, but had much larger placentas and smaller fetal to placental weight ratios. These findings confirm that birthweight is a poor reflection of fetal growth and development.

8.3 Summary

These studies demonstrate that the periconceptional events of maternal undernutrition and twinning have substantial effects on maternal adaptation to pregnancy and late-gestation fetal growth, physiology and endocrinology. They show firstly, that fetal weight does not reflect variations in fetal growth, or differences in fetal endocrinology and physiology. Secondly, nutrition before and after conception alters fetal and placental development in different ways. This stresses the need for human research that focuses specifically on the nutrition of women around the time of conception. Thirdly, the biology of twin pregnancy and fetal development is fundamentally different from that of singleton pregnancy, perhaps explaining the inconsistent relationships between birth weight and later disease risk in twins. These findings emphasise the complexity of the relationships between maternal nutrition, fetal growth and late-gestation physiology, and the importance of the periconceptional period in the developmental origins of health and disease.

9. Appendices

9.1 Appendix 1 – Contributions

The author of this thesis performed all of the work except for the following:

The nutritional manipulation, management and sampling of the sheep prior to arrival at the Animal Research Unit took place at Ngapouri Research Farm, under the oversight of Dr Mark Oliver. The author of the thesis was intermittently involved in the ewe management and blood sampling during this period.

Dr Anne Jaquiery assisted with the blood volume assessments at 65 d gestation.

Dr Michael Rutland at the Nuclear Medicine Department of Auckland City Hospital analysed the radioactive blood samples used in the validation of the method for assessing blood volume.

Toni Mitchell, Dr Alex Buckley, Dr Sanne Husted, Dr Mark Green and members of the Fetal Growth research group assisted with some of the sheep surgery, maternal and fetal experimental work and postmortems.

Chris Keven and Eric Thorstensen assisted with some of the insulin and IGF-1 radioimmunoassays.

Eric Thorstensen, Sonia Alix, Sulee Kuy and Pierre van Zijl performed the steroid and autoanalyser assays.

Dr Tim Prickett performed the CNP and NTproCNP assays.

9.2 Appendix 2 – Publications arising from this thesis

9.2.1 Scientific meeting abstracts

Rumball CWH, Van Zijl P, Bloomfield FH, Harding JE. Assessment of blood volume parameters in periconceptionally undernourished ewes. The 19th National Workshop on Fetal and Neonatal Physiology, Adelaide, A14, 2005.

Rumball CWH, Van Zijl P, Bloomfield FH, Harding JE. Blood volume parameters at day 65 of pregnancy in periconceptionally undernourished ewes. Perinatal Society of New Zealand Annual Scientific Meeting, Auckland, p14, 2005.

Bloomfield FH, Rumball C, Oliver MH, Jaquiery AL, Harding JE. Periconceptional undernutrition and twin size both affect growth and metabolic responses of twin fetal sheep to an acute maternal fast in late gestation. Pediatric Research P3-032, 58 :1107, 2005.

Bloomfield FH, Rumball C, Oliver MH, Jaquiery AL, Harding JE. Effect of periconceptional undernutrition on insulin responses to glucose and arginine stimulation in late gestation twin fetal sheep. Pediatric Research P3-033, 58: 1108, 2005.

Rumball CWH, Thorstensen E, Bloomfield FH, Harding JE. Twin fetal sheep have decreased insulin response to arginine but not glucose compared with singletons. Perinatal Society of Australia and New Zealand 10th Annual Congress, Perth, FC13.1, p132, 2006.

Rumball CWH, Van Zijl P, Bloomfield FH, Harding JE. Effects of twinning and periconceptional undernutrition on the HPA axis of the late gestation sheep fetus. Early Human Development 82: 554, 2006.

Rumball CWH, Bloomfield FH, Harding JE. Effects of twinning and periconceptional undernutrition on growth and glucose-insulin axis function of the late-gestation sheep fetus. 33rd Annual Meeting of the Fetal and Neonatal Physiological Society, Cambridge, P26, p139, 2006.

Bloomfield FH, Oliver MH, Rumball CWH, Jaquiery AL, Challis JR, Harding JE. Programming influences of periconceptional undernutrition. Proceedings of the Sixth International Congress of Neuroendocrinology, Pittsburgh, S81, p69, 2006.

9.2.2 Journal publications

Prickett TCR, Rumball CWH, Buckley AJ, Bloomfield FH, Yandle TG, Harding JE, Espiner EA. C-type natriuretic peptide forms in the ovine fetal and maternal circulations: evidence for independent regulation and reciprocal response to undernutrition. Endocrinology 2007;148(8):4015-4022.

Rumball CWH, Bloomfield FH, Harding JE. Cardiovascular adaptations to pregnancy in sheep and effects of periconceptional undernutrition. Placenta 2007; In submission.

Rumball CWH, Harding JE, Oliver MH, Husted S, Bloomfield FH. Effects of twinning and periconceptional undernutrition on maternal metabolism, fetal growth and glucose-insulin axis function in ovine pregnancies. J Physiol 2007; In submission.

Rumball CWH, Oliver MH, Harding JE, Bloomfield FH. Effects of periconceptional undernutrition on ovine singleton pregnancy. 2007; In preparation.

Rumball CWH, Oliver MH, Thorstensen E, Jaquiery AL, Husted S, Harding JE, Bloomfield FH. Effects of twinning and periconceptional undernutrition on late-gestation hypothalamic-pituitary-adrenal function in ovine pregnancy. Endocrinology 2007; In submission.

Rumball CWH, Van Zijl PL, Rutland MD, Bloomfield FH, Harding JE. A method for assessment of blood volume parameters in pregnant sheep using fluorescein-labelled dextran. Placenta 2007; In submission.

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